

Isolation of novel quorum-sensing active bacteria from microbial mats in Shark Bay Australia

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Keywords: Shark Bay, microbial mats, quorum sensing, AHL biosensors

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ABSTRACT

Quorum sensing is a potent system of genetic control allowing phenotypes to be coordinated across localized communities. In this study, quorum sensing systems in Shark Bay microbial mats were delineated using a targeted approach analyzing whole mat extractions as well as the creation of an isolate library. A library of 165 isolates from different mat types were screened using the AHL biosensor *E. coli* MT102. Based on sequence identity 30 unique isolates belonging to *Proteobacteria*, *Actinobacteria* and *Firmicutes* were found to activate the AHL biosensor, suggesting AHLs or analogous compounds were potentially present. Several of the isolates have not been shown previously to produce signal molecules, particularly the members of the *Actinobacteria* and *Firmicutes* phyla including *Virgibacillus*, *Halobacillus*, *Microbacterium* and *Brevibacterium*. These active isolates were further screened using thin-layer chromatography (TLC) providing putative identities of AHL

molecules present within the mat communities. Nine isolates were capable of producing several spots of varying sizes after TLC separation, suggesting the presence of multiple signalling molecules. This study is the first to delineate AHL-based signalling in the microbial mats of Shark Bay, and suggests quorum sensing may play a role in the ecophysiological coordination of complex phenotypes across microbial mat communities.

Introduction

Microbial communities in the environment can often cooperate, aggregate, and form biofilms for a variety of purposes including anti-predation, resource allocation, syntrophic interactions, and resistance to environmental stresses. One such mechanism communities often deploy is quorum sensing, a population dependent process of microbial communication which allows co-ordination of phenotypes across localized communities. Biofilms are essentially microorganisms embedded in a matrix of exopolymeric substances that includes polysaccharides, extracellular DNA, polypeptides, and a range of other molecules (Stoodley *et al.*, 2002). Occasionally biofilms can accrete soil and other minerals to create lithified biofilm systems such as microbial mats and stromatolites (Reid *et al.*, 2000).

Microbial mats and stromatolites are thought to be representative of some of the earliest forms of life dating back as far as 3.7 billion years (Dodd *et al.*, 2017; Proemse *et al.*, 2017). These complex communities can play host to a wide range of microbial life, with high rates of chemical cycling occurring within the mat structure (Visscher and Stolz, 2005; Pace *et al.*, 2018). Microbial mats have been found in a wide range of environments around the world from Antarctic lakes (Jungblut *et al.*, 2016), hot springs (Dobretsov *et al.*, 2010), coastal systems (Bolhuis and Stal, 2011), to hypersaline marine settings such as Shark Bay, Western Australia (Ruvindy *et al.*, 2016).

Shark Bay is a shallow bay which receives little freshwater from rainfall and also experiences a high rate of evaporation, which results in increased salinity, nearly double that of sea water of at least 68 g/L (Arp *et al.*, 2001), which can pose a challenge for microorganisms (Goh *et al.*, 2006; Burns *et al.*, 2009). As such a number of studies have previously focused on identifying microbial community composition employing both culture-dependent and culture-independent techniques (Leuko *et al.*, 2007; Leuko *et al.*, 2009; Ruvindy *et al.*, 2016; Allen *et al.*, 2009). In addition, given the extreme conditions these ecosystems are subjected to, other studies have focused on common adaptations of mat communities to hypersaline conditions (Goh *et al.*, 2010; Goh *et al.*, 2011; Wong *et al.*, 2016). Such adaptations include use of organic and inorganic solutes, adapted protein structures, as well as biofilm formation to prevent desiccation (Charlesworth and Burns, 2016).

High throughput amplicon sequencing of community DNA has revealed that Shark Bay mat ecosystems are dominated by *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Planctomycetes*, *Bacteroidetes*, *Firmicutes*, halophilic archaea, and a range of unclassified bacteria and archaea (Wong *et al.*, 2015, Wong *et al.*, 2017). At a functional level, these mats are characterised by a range of metabolic activities that drive multiple biogeochemical cycles, including the carbon cycle in particular the Wood–Ljungdahl pathway, methanogenesis and photosynthesis as well as other cycles such as sulphur metabolism, and these are all intrinsically intertwined (Visscher and Stolz, 2005; Wong *et al.*, 2015; Wong *et al.*, 2017; Wong *et al.*, 2018). It has even been suggested quorum sensing may be responsible for coordination of metabolic activities in these ecosystems indicative of tightly coupled microbial interactions (Decho *et al.*, 2010). Additionally, quorum sensing may play a role in the formation of biofilms in these communities aiding in the structure and integrity of microbial mat systems (Reid *et al.*, 2000; Decho *et al.*, 2009). There are a range of potential quorum sensing systems employed by microorganisms, including auto-inducing peptides

(Sturme *et al.*, 2002), furanasoyl borate diesters (Rajamani *et al.*, 2007) quinolones (Diggle *et al.*, 2006) and pyrones (Brachmann *et al.*, 2013), however perhaps the best studied are the n-acyl homoserine lactones (AHLs). Initially thought to be confined to *Proteobacteria* (Manefield and Whiteley, 2007), AHL production has been found in a range of groups that are also present within the Shark Bay microbial mats including *Cyanobacteria* (Sharif *et al.*, 2008), *Actinobacteria* (Ma *et al.*, 2016), *Firmicutes* (Romero *et al.*, 2010) and Archaea (Zhang *et al.*, 2012). Biofilm formation has also been linked to these groups, with some controlled via quorum sensing (Hammer and Bassler, 2003; Llamas *et al.*, 2005).

However, quorum sensing in these complex mat environments has received little study, except one study describing AHLs in the marine stromatolites of the Bahamas (Decho *et al.*, 2009), which primarily focused on a meta-metabolomic approach directly extracting signalling molecules from stromatolites (Decho *et al.*, 2009). A key finding was that signalling molecules present within stromatolites were prone to breakdown by the increase of pH during the day time, due to the diel cycle of photosynthetic organisms present in these systems fixing carbon (Yates *et al.*, 2002; Decho *et al.*, 2009). Other studies have suggested the presence of AHL inhibitory molecules, such as diketopiperazines, being produced by mat dwelling organisms, including *Cyanobacteria*, *Proteobacteria* and even some haloarchaea (Dobretsov *et al.*, 2010; Abed *et al.*, 2013). Intriguingly some microorganisms possess the capacity to both produce signals as well as the enzymes which can quench these systems (Romero *et al.*, 2010; Chan *et al.*, 2011).

The majority of research in the literature has previously focused on quorum sensing in individual isolates, typically pathogens (Miller and Bassler, 2001) rather than studying the potential ecological role these signals may have in the environment. This is likely due to difficulties in detecting quorum sensing in metagenomics datasets given poor sequence conservation of the luxI/R system, and novel luxI type proteins (Williamson and Borlee,

2005), as well as difficulties in extracting sufficient signalling molecules to be detected by biosensors. Difficulties in extracting signalling molecules directly from the environment can include high turnover rates of signal molecules due to both chemical (e.g. pH) and biological challenges such as lactonases (Yates *et al.*, 2002; Horswill *et al.*, 2007; Kalia *et al.*, 2011). However, recent studies have addressed quorum sensing in environments including sponges, corals, and mangrove rhizospheres, by isolating a range of organisms and screening for AHL activity (Ma *et al.*, 2016; Zimmer *et al.*, 2014; Bose *et al.*, 2017). The present study adopted a similar approach, whereby putative AHL signal molecules were extracted for the first time directly from Shark Bay mats, as well as a library of microbial isolates produced and characterised for both AHL-like activity and the ability to form biofilms.

Materials and Methods

Sample site and collection. Sampling of both pustular and smooth microbial mats occurred at two sites in Shark Bay Western Australia: Nilemah (26°27'336''S, 114°05.762''E) and Garden Point (26°21'14.7''S, 113°52'51''E) during the day on the 7th of July 2016. Sampling has been described previously (Pagès *et al.*, 2014; Wong *et al.*, 2015), and at the time of sampling water temperature was 14.7 °C, salinity 75 g/L and pH 8.48 at Nilemah, with temperatures of 13.9 °C, salinity 41 g/L, and pH 8.22 at Garden Point. The four different mat types analysed were designated as Nilemah Smooth (NS), Nilemah Pustular (NP), Garden Point Smooth (GS), and Garden Point Pustular (GP). Images of samples analysed are shown in Supplementary Figure 1. Smooth mats are characterised by smooth and flat surfaces, and distinct pigmented layering can be observed in a vertical cross section. Pustular mats are characterised by surfaces of mucilage-containing pustules, with a examples of white bivalve shells sometimes trapped between sediment. Samples were stored at 4°C during transit and processed immediately upon sample return.

Microbial mat enrichments. To facilitate the isolation of microorganisms associated with these mats, enrichments were performed using select media that has been modified to mimic conditions in Shark Bay, (Allen et al, 2009). Upon sample return, vertical sections of mats (i.e. top of the mat to bottom, Supplementary Figure 1) approximately 4 cm by 4 cm were removed using a sterile scalpel. Each section was then vortexed in 7.5 mL of sterile saline (7.5 %) solution and used to inoculate 20 mL of three different types of growth medium: Marine Broth (MB)(Bin Saidin *et al.*, 2017) containing 19.45 g NaCl, HP-LB (Allen *et al.*, 2009) containing 15 g NaCl/L, and DSM-97 containing 150 g NaCl/L (Allen *et al.*, 2008). These growth media were selected based on previous isolation attempts from Shark Bay microbialite communities (Allen et al., 2009), whereby salinity levels of the various media employed in the current study were adjusted to mimic as close as possible the salinity levels present in Hamelin Pool (in the case of media DSM-97 a higher concentration of NaCl was used in an attempt to isolate any more haloextreme microorganisms). The resulting enrichments were then incubated at either 30 °C for the HP-LB and MB treatments or 37 °C for DSM-97 enrichments. Culture conditions were aerobic and in normal lab light. All enrichments were subjected to shaking at 200 RPM.

Following four-week incubations of these enrichments, serial dilutions (10^{-1} , 10^{-2} , and 10^{-3}) were performed in sterile saline (7.5 %) solution. Resulting dilutions were spread onto respective growth medium agar plates (1.5% agar) and incubated at 30°C (MB, HP-LB) or 37°C (DSM-97) until colonies were observed. These conditions have been used previously to isolate microorganisms from the Shark Bay mats (Allen *et al.*, 2008; Allen *et al.*, 2009). Colonies were sub-cultured continuously until axenic colonies were obtained. Purity of isolates was confirmed by phase contrast microscopy of single colonies stained by methylene

blue. Initial checks of isolates to ascertain whether they were single or mixed morphologies was undertaken by phase contrast microscopy of single colonies stained by methylene blue, after which 16S rRNA gene sequencing was performed to confirm purity.

AHL screening of Shark Bay microbial mat isolates. After axenic cultures were obtained, each unique colony morphology - based on colour, shape and size of individual colony - was given an alphanumeric code for ease of identification corresponding to location, mat type, and growth medium e.g. NSMM1 applies to an isolate from Nilemah smooth mats in Marine Broth. To obtain sufficient biomass mat isolates were grown for 7 days to facilitate the detection of (any) signal molecules. Each of these unique isolates were inoculated in 25 mL of growth medium and depending on media type were grown at 30° C or 37°C at 200 rpm for one week. The cultures were then centrifuged at 5,500 rpm and the supernatant harvested for ethyl acetate extraction.

Ethyl acetate extractions of Shark Bay microbial mat isolates. In order to extract signalling molecules, ethyl acetate extractions of duplicate cultures were performed on isolates as described previously (Shaw *et al.*, 1997). The cell free supernatant was subjected to an equal volume of ethyl acetate and shaken vigorously, and phases allowed to separate. The ethyl acetate was then filtered through Whatman No. 1 filter paper, to remove any cell debris, and this process was repeated three times for every extraction. Any signal molecules are expected in the flow-through. The resulting ethyl acetate was air-dried in a fume hood. The same extraction procedure was also followed for 25 mL of each uninoculated media as a control. Dried extracts were redissolved in 200 µL of HPLC grade methanol with 0.1% formic acid and stored at -80° C.

Ethyl acetate extractions of Shark Bay microbial mats. In addition to analysing mat isolates for AHL-activity, direct extractions of microbial mats using ethyl acetate was also undertaken. Replicate vertical sections of approximately 15 g were taken from smooth and pustular mats from both sites in Shark Bay (i.e. Garden Point and Nilemah), and vortexed in 50 mL of acidified ethyl acetate (0.1 % acetic acid). Tubes were shaken at room temperature at 100 rpm for 48 h. The ethyl acetate was then filtered and collected for air-drying, and this process was then repeated on the filtrate two further times. The three volumes of ethyl acetate were pooled and evaporated before being redissolved in 200 μ L of methanol with 0.1% formic acid before being stored at -80° C.

Microtitre-based screening of extracts for putative signal molecules. Extracts from isolate supernatant, growth medium controls, and direct mat extractions were screened using the AHL biosensor *E. coli* MT102 harbouring a plasmid containing a luxR protein to activate Green Fluorescence Protein (GFP) production (Charlesworth *et al.*, 2015). An overnight culture of the biosensor was grown in LB medium before being diluted one in ten in AB minimal media (Clark and Maaløe, 1967). Controls of the screen included diluted biosensor alone to detect background fluorescence, a 1:10 dilution of LB:AB medium, and an acidified methanol control to ensure no fluorescence was generated by the methanol used to dissolve extracts. In triplicate, 10 μ L of each extract (tests or controls) were added to a microtitre plate well and allowed to evaporate. Following evaporation, 200 μ L of diluted *E. coli* MT102 biosensor was added to each well and incubated at 30° C and 150 rpm shaking for 4 h. Following incubation, fluorescence was determined using a CLARIOstar plate reader (BMG LABTECH). Data was analysed for any statistical significance using a paired T-test.

Thin-layer Chromatography. Isolate extracts that were found active by the AHL biosensor well plate assay were subjected to analysis by thin-layer chromatography to determine type and polarity of putative signalling molecules. Two microlitres of associated standards, N-decanoyl-L-homoserine lactone (C10), N-octanoyl-L-homoserine Lactone (C8), N-oxo-hexanoyl-L-homoserine lactone (oxo-C6) and N-hexanoyl-L-homoserine lactone (C6), and 20 μ L of each extract were spotted on C18 reverse phase silica sheets before being run in 60:40 HPLC grade methanol to MilliQ water. The silica sheets were then allowed to dry before being overlaid with *E. coli* MT102 in LB agar according to established methods (Charlesworth *et al.*, 2015). The plates were incubated at 30° C overnight and GFP fluorescence visualised using a GE Typhoon Phosphorimager with relative image light levels adjusted in the Image Quant software package supplied by the manufacturer.

Identification of AHL-active isolates using 16S rRNA gene sequencing and phylogenetic analysis. Utilizing 16S rRNA gene sequencing, the cultures were verified as axenic and putative identities were obtained for the AHL-active mat isolates. A 16S rRNA gene colony PCR was performed using both universal archaeal 16S rRNA gene primers (21F/985R; Goh *et al.*, 2006) and universal bacterial primers (27F/1492R; Lane 1991, Zimmer *et al.*, 2014). DNA was extracted from single colonies by vortexing a colony in 50 μ L sterile MilliQ water. Positive controls used were *Escherichia coli* for the bacterial control and *Haloferax mucosum* for the archaeal positive control. the negative control was sterile MilliQ water. PCR conditions were as follows: 95 °C for 3 min, 30 cycles of 94 °C for 30s, 55 °C for 20 s and 72 °C for 3 min. This was followed by 6 min at 72 °C then held at 4 °C. The resulting products were separated on 1% agarose TAE gels at 80V for 30 min. The PCR products that were clearly defined bands of the expected size (ca. 1400 bp) were then subjected to ExoSAP-IT (Thermo Fisher Scientific, VIC, Australia) purification procedures according to manufacturer instructions, before submission to the Ramaciotti Centre for Gene Function Analysis

(Kensington, NSW, Australia) for Sanger Sequencing. The resulting sequences were trimmed and cleaned using FinchTV (Geospiza. FinchTV 1.4. 0. Geospiza, Inc. Seattle, Washington) software then analysed using BLAST – Basic Local Alignment Search Tool - (Altschul *et al.*, 1990) to identify their closest relatives in NCBI GenBank. The 16S rRNA gene sequences generated were then aligned using MAFFT (Kato and Standley, 2013), and alignment gaps were removed with UGENE (Okonechnikov *et al.*, 2012). Maximum likelihood phylogenetic trees were constructed with IQ-TREE v. 1.6.1, with a total of 1000 bootstrap replicates. The tree was subsequently visualised with iTOL (Hoang *et al.*, 2018; Letunic and Bork, 2016). Isolate 16S rRNA gene sequences have been deposited in Genbank under accession numbers MH974483 to MH974512

Biofilm production in Shark Bay mat isolates. To test whether the mat isolates were capable of producing biofilms (a process described earlier as often mediated by quorum sensing), 200 μ L of inoculated growth medium (OD 0.2) of either MB, HP-LB, or DSM-97, were added in triplicate to 96 well plates and allowed to grow for 7 days shaking at 200 rpm at the temperatures described above to facilitate any biofilm formation. Following incubation, supernatant was discarded and plates washed with saline solution (7.5%) before drying in a 65 ° C oven. After drying, 150 μ L of 0.1% crystal violet solution was added for 15 min and allowed to stain. Following staining, excess crystal violet was thoroughly rinsed with water to remove any unbound crystal violet, before being allowed to dry. After plates were completely dried 150 μ L of 95% ethanol was added to each well, mixed, and allowed to resolubilise for 30 min. Absorbance was measured at 570 nm on a CLARIOstar plate reader (BMG LABtech).

RESULTS

Putative AHL activity in whole microbial mat extractions. In order to ascertain if any signalling molecules were present within the Shark Bay mats, samples (pustular and smooth

mats) from both Garden Point and Nilemah were directly subjected to ethyl acetate extractions. The resulting extracts were then tested for AHL activity using the *E. coli* MT102 biosensor. The fluorescence as a result of GFP production was determined as a measure of AHL activity, shown in Figure 1. Pustular mat samples from both locations were able to induce significant AHL activity ($P > 0.05$) when compared to the methanol control. Extracts from smooth mat samples from both locations were unable to induce any GFP production significantly compared to no extract controls.

Detection of AHL activity in microbial isolates enriched from Shark Bay mats. After enrichment, a total of 165 isolates were cultivated on either MB, HP-LB, or DSM-97 media. Of these, 68 isolates were obtained from Nilemah (50 from smooth mats, 18 from pustular mats) and 97 isolates from Garden Point (40 from smooth mats, 57 from pustular mats). In order to produce axenic cultures, original enrichments were plated on to respective growth plates (15% agar) and streaked consistently across several weeks until single morphologies were present. This was based on colony morphology including the size, colour, and shape of individual colonies. It was not the purpose of this study to compare media types in terms of microbial isolation from the mats, and thus no analyses at this level were undertaken.

After producing axenic cultures, these isolates were subjected to ethyl acetate extractions as described earlier before being screened with the *E. coli* MT102 biosensor for signal molecule production. After comparing activity levels to culture-free medium as a baseline, 30 isolates were found to activate the AHL biosensor, and these activity levels are indicated in Figure 2. A paired t-test confirmed these values to be significantly ($p < 0.05$) higher than the media control. While a range of activities were observed, to avoid any over-interpretation of the data, no quantitative comparisons between strains can be given due to the broad detection range of the LuxR biosensor. In addition, due to the fact that different culture media can result in different background fluorescence, biosensor assay results between media types can

also not be quantitatively compared (Charlesworth *et al.*, 2015). Thus, results of the different isolates were grouped together based on media type to facilitate accurate evaluation of a given isolate against media-alone controls. To identify the AHL-active bacteria from the mats, these isolates were subjected to 16S rRNA gene sequencing.

Identification of Shark Bay mat isolates. In order to facilitate identification of the putative microbial mat AHL producers, the 16S rRNA gene PCR product obtained was sequenced. The sequences obtained were used for a BLAST search in GenBank in order to assess their taxonomic affiliation with each other and known sequences. The closest related sequences are shown in Table 1. All isolate sequences were affiliated with the three phyla of *Actinobacteria*, *Firmicutes*, or *Proteobacteria*. Despite attempts to grow on high salinity media, no archaea could be isolated in the present study. Several of the genera identified in this study have not been shown previously to produce signal molecules, particularly the members of the *Actinobacteria* and *Firmicutes* phyla including *Virgibacillus*, *Halobacillus*, *Microbacterium* and *Brevibacterium*. These sequences were then used to construct a phylogenetic tree shown in Figure 3 that illustrates the relationship of isolate sequences to each other and relevant reference sequences. Groups branched into three primary phyla: the *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. As there does not appear to be any significant clustering that can be attributed to either mat type or specific location in Shark Bay (i.e. Nilemah or Garden Point), as above further discussion will group the isolates from the Shark Bay microbial mats together.

At the genus level isolates from the present study were less than 0.1% of the total community as seen in community sequencing of the mats (Wong *et al.*, 2015). However, at the phylum level, *Alphaproteobacteria* was the most represented phyla the isolates with putative signalling capability fall under, and this is consistent with this group being the most represented in recent molecular analyses of Shark Bay mats (Wong *et al.*, 2015). From this

study, *Alphaproteobacteria* was the most abundant taxonomic group at 9% and 24% of smooth and pustular mat datasets, respectively (Wong *et al.*, 2015).

Thin-layer chromatography of Shark Bay mat isolates. Extracts of mat isolates which induced positive results in the well based assay (Figure 2), were selected for TLC in order to ascertain if multiple signals were present as well as determine potential identities of the signalling molecules. The silica sheets were applied in a 60:40 methanol to water ratio before being overlaid with *E. coli* MT102 and GFP fluorescence visualised. These plates contained extracts and AHL standards, and the respective retardation factor (R_f) values of each area of activity is shown in Table 2. Several isolates including GSBM7 (*Proteobacteria*), NSMM8 (*Firmicutes*), GPBM1(*Proteobacteria*) and NPH2 (*Proteobacteria*) possessed R_f values of approximately 0.57. This value corresponds to that of the C8 AHL standard also separated on the same TLC sheet, indicating an AHL of this length is likely produced by these isolates. A representative TLC showing putative signal molecules in some of the isolates is shown in Figure 4. Nine isolates were capable of producing several spots of varying sizes after TLC separation - specifically GPBM1 (*Proteobacteria*), GSBM7 (*Proteobacteria*), NSTM4 (*Proteobacteria*), GSBM10 (*Actinobacteria*), GSBM8 (*Firmicutes*), NSBM3 (*Firmicutes*), NPH2 (*Proteobacteria*), GPTH4 (*Proteobacteria*) and GPMH5 (*Firmicutes*) - suggesting the presence of multiple signalling molecules. Isolate GSBM10 (affiliated with *Actinobacteria*) contained as many as four different signal molecules (Table 2). Any confirmation of AHL identities would require further analytical chemistry techniques such as MS and NMR, which would be the grounds for future work.

Biofilm production in Shark Bay microbial mat isolates. As biofilm production is both important for structure and integrity of microbial mats and often mediated by quorum sensing, the isolates determined to have AHL activity were further examined for biofilm forming capacity, under the same growth media and conditions as described above. This was

achieved via the crystal violet assay which can semi-quantitate any biofilms formed in 96-well plates. The higher absorbances indicate stronger biofilm formation. These results are shown in Figure 5, with each isolate compared to the respective medium control. The most significant biofilm formation relative to media control was found in the DSM-97 isolates (putatively identified as *Halomonas* spp.; Table 1), with strong biofilm formation uniform across these isolates compared to the media control. In the Marine Broth isolates, several microorganisms such as two *Bacillus* sp. (NSTM7 and NSMM12) and a *Pseudoxanthobacter* sp. (NSBM1) appeared to have little to no biofilm formation compared to the media control (Figure 5), whereas other isolates - for example GSTM1 (*Stappia* sp.), GPTM4 (an uncultured proteobacterium), GPTM5 (a *Halomonas* sp.), and NSMM1 (a *Cellulomonas* sp.) - formed significantly stronger biofilms (Figure 5). The HP-LB isolates, in particular GSTH4, NPH2, and GSNH2 (*Virgibacillus* sp., *Pseudovibrio* sp., *Virgibacillus* sp., respectively) generally produced strong biofilms with the exception of GPTH4 (a *Halomonas* sp.), which produced no measurable biofilm in this assay.

Discussion

Although quorum sensing has been well documented in clinical strains and some environmental isolates, rarely have quorum sensing systems been studied in their ecological context. To gain an understanding into microbial communication in the mat systems of Shark Bay, this study combined culture-based approaches with whole mat metabolite extractions for the first time. Complementing this work, during the course of the present investigation, metagenomes from the smooth mats in Shark Bay were analysed in detail, with some sequences present that corresponded to known AHL synthase genes, LuxR receptors, as well as potential quorum quenching systems (Wong et al., 2018).

Previous work analysing the metagenomes of Shark Bay mats (Wong *et al.*, 2018) has revealed the presence of several AHL related genes including AHL synthases in two separate proteobacterial (Alpha- and Deltaproteobacteria) metagenomically assembled genomes (MAGs). However, no such AHL synthase or other AHL mimicry synthases were detected outside of these two MAGs, and this is in contrast to the present study which found a higher number of proteobacterial isolates producing AHL or AHL like molecules, and similar putative AHL-like activity occurring in the Actinobacteria and Firmicutes phyla. No AHL synthases were found in the metagenomically assembled genomes of Firmicutes and Actinobacteria (Wong *et al.*, 2018). This is likely due to poor conservation of AHL synthases and receptor sequences (Schuster *et al.*, 2004) as well as potentially novel synthases of AHLs or AHL like mimics in phyla such as Actinobacteria and Firmicutes (Romero *et al.*, 2010; Bose *et al.*, 2017). Other AHL-related genes such as those for encoding AHL acylases and lactonases were also detected suggesting these could act to degrade signals produced (Wong *et al.*, 2018), as well as potentially interfering with any direct AHL mat extractions as may have been the case in the present study, particularly in the smooth mat samples (Figure 1). Furthermore, Firmicutes comprises only 2.33% and 0.56% of smooth and pustular mats respectively, whereas Actinobacteria only make up 0.78% and 1.54% of both mats (Wong *et al.*, 2015). Their lower abundance compared to Proteobacteria may contribute to AHL synthases not detected by cultivation-independent methods.

While these recent metagenomic analyses has provided new insight into potential AHL-related systems in the Shark Bay mats, there are still shortcomings to pure metagenomic approaches for evaluating quorum sensing within a given environment. One of the key drawbacks is poor conservation of luxI AHL synthase sequences (Gould *et al.*, 2004). Despite studies indicating AHL activity in phylogenetic groups such as archaea (Zhang *et al.*, 2012), *Actinobacteria* (Bose *et al.*, 2017), *Firmicutes* (Ma *et al.*, 2016) and *Cyanobacteria*

(Sharif *et al.*, 2008), genes encoding AHL synthases were not detected in the Shark Bay metagenomes from these phyla (Wong *et al.*, 2018). This may be due to the presence of novel synthases (Belin *et al.*, 2012; Zhang *et al.*, 2012; Guan *et al.*, 2007) or a lack of conservation within the AHL synthase family (Gould *et al.*, 2004). This suggests as new genes encoding AHL synthases are added to databases, metagenomic screening for AHL potential will improve. Similarly, metagenomic techniques will not be able to identify novel agonistic activity that may occur from AHL agonists such as the diketopiperazines (Holden *et al.*, 1999), indoles (Guan *et al.*, 2007), and other novel agonists. Thus, it is important to perform both cultivation and chemical analysis of AHL active mat isolates as undertaken in the present study, to better inform and characterise the AHL-signalling potential in the Shark Bay microbial mat communities.

Putative AHL activity in whole microbial mat extractions. Direct extraction of mat samples for putative signal molecules has yielded success previously, with a variety of AHLs being detected from marine mats in the Bahamas (Decho *et al.*, 2009). While this study was able to detect AHLs, they were predominantly found in night time samples, as the photosynthetic cycle driving cyanobacterial dominated mats can lead to sharp changes in pH (Decho *et al.*, 2009). It was found that the alkaline conditions of daytime samples led to rapid degradation of AHLs, and thus prompt extraction procedures were required to detect AHLs (Yates *et al.*, 2002; Decho *et al.*, 2009). Given some similarities exist between the Bahaman and Shark Bay mat environment, it is reasonable to assume similar physiochemical conditions could also impair AHL extraction in the Shark Bay mats. The direct mat extractions in the present study were able to detect some AHL activity in the pustular mats from both locations in Shark Bay, although activity in the Garden Point samples was diminished (Figure 1), potentially due to the various difficulties encountered when extracting from the environment including pH conditions and any related enzymatic signal molecule

degradation. It is unclear at this stage whether differences observed between pustular mats at different Shark Bay locations is significant and/or ecologically relevant, and further work is needed to clarify. Interestingly the pH conditions detected during sampling in Shark Bay in the present study (pH 8.22 and 8.48 for Garden Point and Nilemah, respectively), are both considered alkaline enough to speed the degradation of AHLs as found in the Bahaman marine stromatolites where pH >8.2 caused shorter chain AHLs to rapidly diminish (Decho *et al.*, 2009).

It is likely that the chemical conditions of the mats as well the remote location of samples added to difficulties in extracting sufficiently high levels of AHLs to activate the AHL biosensor in the present study. One potential explanation for the lower AHL activity within the direct mat extractions could be the production of AHL quenching enzymes such as acylases and lactonases, and this is consistent with the genes encoding these enzymes detected in Shark Bay smooth mat metagenomes described recently (Wong *et al.*, 2018). Future work focusing on identifying the differences between the mat communities at the metagenomic level - such as the presence of these AHL-quenching genes in the pustular mat community - could help to ascertain whether such signal molecule degrading processes contributes to any differences between ecosystem function between the mat types. Potentially, if these genes encoded active enzymes, they could degrade exogenous AHLs prior to extraction. Ethyl acetate extractions of the mat could also potentially extract AHL inhibitory molecules that could further mask any potential AHL activity.

Putative AHL activity of isolates from Shark Bay microbial mats. Due to the difficulties described above in identifying signal molecule activity in direct mat extractions, other techniques were employed to characterise the extent of signalling in Shark Bay mat microbial communities. Recent studies successively screened isolate libraries for AHL activity from a range of environments including mangroves (Ma *et al.*, 2016) and coral systems (Zimmer *et*

al., 2014). In order to screen for AHLs in the microbial mats from Shark Bay in the present study, the first step was creating an isolate library of these mats. This isolation effort was undertaken across a range of growth media with differing levels of salt to allow for different halophilic organisms to grow, based on previous isolation studies from this environment (Allen *et al.*, 2009). Approximately 165 unique isolates based on colony morphology were produced across the varying media types before being screened for AHL activity. This resulted in 30 isolates capable of activating the AHL biosensor, belonging to three groups previously known to produce AHLs, the *Proteobacteria*, *Actinobacteria*, and *Firmicutes* groups.

In comparison with previous microbial diversity studies in Shark Bay, at the genus level the isolation of *Halomonas* sp., *Virgibacillus* sp., *Bacillus* sp., *Idiomarina* sp., and *Halobacillus* sp. in the present study was consistent with isolates obtained from a previous cultivation study on the Shark Bay mats (Allen *et al.*, 2009). At the phylum level, the phyla the majority of isolates with putative signalling capability in the present fall under is the *Alphaproteobacteria* (Table 1), the most abundant phylum using non-culturing approaches as seen from high-throughput sequencing of total mat DNA (Wong *et al.*, 2015, Wong *et al.*, 2018). However of interest is that the isolates identified in the present study were only detected in low abundance at the genus level (less than 0.1%) in recent cultivation-independent analyses of total community DNA from the Shark Bay mats (Wong *et al.*, 2015, Wong *et al.*, 2018). While these isolates obtained in the present study could indeed be at low abundance, they may have important roles in pioneering biofilm construction through quorum sensing. This study also demonstrates that cultivation-dependent analyses can compensate for the shortcomings of metagenomics, in which genes may not be detected if they are novel (thus absent from the databases) or low in abundance.

In addition to the putative signalling isolates in the present study being not necessarily dominant community members, it is possible that isolates were favoured to grow under the growth conditions employed. However, the primary focus of this study was not to identify dominant isolates but rather any isolates with the potential for microbial communication. Which shows that AHL signalling does occur in this diverse environment as well as creating the potential for future experiments between isolates from this environment. What this does demonstrate is the importance of combining traditional microbial methods such as isolation with metagenomic studies, as a more complete picture of microbial communication in these systems can be obtained. The relative importance and exact ecological roles of these individual isolates in terms of regulating phenotypes via signalling in these microbial mats remain to be determined and should be the subject of future investigation.

One particularly interesting result of the isolate driven approach is the number of *Bacillus* *Spp.* and actinobacterial isolates capable of inducing biosensors. Only a handful of studies have demonstrated the presence of AHL activity within both *Firmicutes* and *Actinobacteria* groups (Bose *et al.*, 2017; Ma *et al.*, 2016) despite both groups being common in a range of environments, including the Shark Bay microbialite communities (Ruvindy *et al.*, 2016; Wong *et al.*, 2015). Work in this present study describes nine new *Firmicutes* and three *Actinobacteria* isolates and presents a significant finding in countering the traditional dogma of AHL active bacterial groups being limited to Gram-negative bacteria. Previous culture-based studies of environmental samples have also found *Actinobacteria* and *Firmicutes* isolates that appear to produce AHLs (Ma *et al.*, 2016; Romero *et al.*, 2010; Zimmer *et al.*, 2014; Bose *et al.*, 2017; Chan *et al.*, 2011), although it was suggested these may be the result of horizontal gene transfer (Ma *et al.*, 2016). However, the findings in the present study on putative AHLs in the *Actinobacteria* and *Firmicutes* are preliminary, and future chemical

identification of any signalling molecules would be required to definitively ascribe AHL production to these taxonomic groups.

The production of AHLs within these groups is currently not as well understood as the more well-studied *Proteobacteria* (Polkade *et al.*, 2016; Chan *et al.*, 2011; Ma *et al.*, 2016). As relatively few isolates from the *Firmicutes* and *Actinobacteria* groups have been identified as AHL producing previously, it is also likely that the genes responsible for signal production and sensing in these environments may be novel. This may help explain the lack of quorum sensing related genes identified from these groups in the bioinformatic screens of Shark Bay metagenomic data recently described, as no quorum sensing related genes were identified in a reconstructed *Actinobacteria* MAG (Wong *et al.*, 2018). The finding of phylogenetically diverse signal molecule producers (Figure 3) in the Shark Bay mats suggests that AHL production may be more widespread within these groups than first thought and potentially not the result of horizontal gene transfer. This finding highlights the need for culture-based approaches in both discovering novel AHL producers as well as potential agonistic mimics.

TLC analysis of putative signal molecules from Shark Bay mat isolates. In order to determine putative identities of the agonistic molecules in the mat microorganisms isolated in the present study, thin-layer chromatography (TLC) was performed in conjunction with the *E. coli* AHL biosensor. This TLC analysis revealed considerable new data on the number of potential type of signal(s) employed by the isolates. Ten of the isolates screened were capable of producing more than one unique signal with one actinobacterial isolate (GSBM10) capable of producing at least four separate signals (Table 2). This is the first evidence of an *Actinobacteria* producing four putatively unique signals, with *Actinobacteria* isolates only previously shown to produce two signals, oxo-C10 and oxo-C12 AHLs (Bose *et al.*, 2017). In addition, at present only oxo-C8 AHLs have been identified within a single *Firmicutes* isolate an *Exiguobacterium* sp. (Biswa and Doble, 2013), whereas several *Firmicutes* isolates in the

present study (NSMM8, GSBM8, NSBM3 and GPMH5) produced multiple signals. This demonstrates that multiple signalling molecules likely remain to be discovered within this phylum.

Interestingly, the *Firmicutes* isolates GPMH5 and NSMM8 both produce signals approximating C8 AHLs, as did a range of other mat isolates from both *Actinobacteria* (GSBM10) and *Proteobacteria* (GPBM1, GSBM7, NPH2 and GPTH4). This shows the potential for multiple unrelated isolates to produce similar signals which could be explained by horizontal gene transfer (HGT) as in other environments (Rajput and Kumar, 2017), although genomic analysis would be required to confirm this hypothesis. This ability to produce more than one unique signal could allow for multiple phenotypes regulated by quorum sensing within the same organism, a situation found in several other bacteria such as *Pseudomonas aeruginosa* (Erickson *et al.*, 2002). In particular, it could be that different signal molecules are produced by the same mat organism at different growth stages or different times during a diel cycle, potentially regulating different phenotypes. Such potential tight coupling and regulation of microbial activities/phenotypes in the Shark Bay mats may be vital to ecosystem function.

This ability of some Shark Bay mat isolates to produce putatively the same molecules allows for potential cross-talk scenarios to occur within the microbial mats. This could even occur potentially across phyla, with a *Bacillus* isolate (NSMM8) and a *Neisotobacter* isolate (GSBM7) producing a signal at the same R_f value as that of the C8 AHL standard (Table 2). The finding here of isolates outside the *Proteobacteria* phylum producing these type of molecules is supported by a recent study of mangrove isolates, that also detected C8 AHLs in the *Firmicutes* phyla (Ma *et al.*, 2016). This evidence for the potential for cross-talk could lead to syntrophic interactions important in maintaining microbial mat metabolisms (Decho *et al.*, 2010). In general the signal molecules from the Shark Bay mat isolates had similar

polarities to shorter chain AHLs such as C8 and oxo-C6 AHLs, which would be more readily degraded under alkaline pH conditions (Yates *et al.*, 2002). In an earlier study on the Bahaman stromatolites, while a range of between C4 to C14 AHLs were detected, the shorter chain AHLs were significantly degraded in day time samples when alkalinity increased (Decho *et al.*, 2009), and this was the same time period in which the samples from Shark Bay in the present study were collected. This may help explain the difficulty in extracting signal molecules directly from the mats described earlier.

Potential role of quorum sensing in Shark Bay microbial mats. Results from this study showed evidence for the presence of quorum sensing systems in Shark Bay microbial mat communities, however the precise role quorum sensing may play in these mats is still yet to be determined. A phenotype commonly linked to quorum sensing and also important in the formation and maintenance of microbial mats is biofilm formation (Decho *et al.*, 2005; Reid *et al.*, 2000), and the AHL active isolates in this study were thus further screened for this phenotype. The majority of isolates (26/30) in the present study were found to produce biofilms of varying strengths (Figure 5), with stronger biofilm formation occurring in the more halophilic isolates particularly a *Halomonas sp.* (*Proteobacteria*) (e.g. isolate NSMD1). This could be the result of an adaptation to halophilic conditions, as biofilm formation is considered an adaptive response to prevent desiccation (Poli *et al.*, 2011), a common selective pressure in halophilic environments such as the Shark Bay mats. In addition, several *Virgibacillus* (*Firmicutes*) isolates including *Virgibacillus pantothenicus*, have previously been reported to produce biofilms of varying strengths, although the mechanism by which their biofilms are regulated has yet to be reported (Sarkar *et al.*, 2011). Little is currently known about the *Nesiotobacter* genera (*Proteobacteria*) with regards to biofilm formation, although *Nesiotobacter exalbescens* has been suggested to be involved in hydrocarbon degradation and biofouling as a part of a mixed species biofilm (Al-Awadhi *et al.*, 2012).

The capacity of several of the Shark Bay mat isolates to produce strong biofilms could also aid the formation of these microbial mats. *Halomonas* sp. have been demonstrated to produce biofilms in response to exogenous AHL signals (Llamas *et al.*, 2005), a scenario that could potentially be occurring in the *Halomonas* isolates in the present study that both demonstrated AHL activity and strong biofilm formation. Some *Halomonas* isolates have been reported to act as pathogens that affect scallop crops and the production of exopolymeric substances were key to this pathogenicity (Rojas *et al.*, 2009). This study also suggested interference with quorum sensing of this scallop *Halomonas* isolate as a method of control of its pathogenicity (Rojas *et al.*, 2009). For those isolates that did not appear to form strong biofilms in the present study, it is unclear at this stage whether these isolates could not adhere to the microtitre plate substratum, or whether they do not inherently have the capacity to form biofilms. Although the present study could not conclusively link quorum sensing to biofilm formation in the mat isolates without further transcriptomic or other evidence, it is reasonable to suggest some biofilm formation within the mat communities is likely to be regulated by AHL-based quorum sensing.

As alluded to earlier, in addition to quorum sensing regulating important individual phenotypes such as biofilm formation, there is the potential for microbial communication in mat systems to tightly couple and regulate differing microbial metabolisms. For example syntrophic interactions and co-operation within microbial mats between *Cyanobacteria* and sulfate-reducing bacteria has been hypothesized to be regulated by quorum sensing (Decho *et al.*, 2010). The isolates cultured in this study could be further utilized to understand the microbial mat ecosystem via the use of synthetic microbial communities which could lead to greater understanding of interactions and communication complexity within the microbial mat ecosystem (De Roy *et al.*, 2014; Baumgartner *et al.*, 2006).

Conclusions. This study describes evidence of quorum sensing within the complex environment of the Shark Bay microbial mat systems for the first time. By demonstrating the presence of AHL active isolates within a defined environment this can help to better understand the ecological role quorum sensing has; firstly, by demonstrating it exists within a given environment such as microbial mats and hypothesize on potential roles in the ecosystem, and secondly by establishing a culture collection that allows for later experiments to potentially replicate interactions which would occur within the original ecosystem. This demonstrates the effectiveness of combining isolate screening with more global analysis techniques such as metagenomic analyses of total community DNA, as one approach alone can miss important details. Future work can target potential novel producers of AHL or AHL mimics isolated in the present study where no current genetic or chemical understanding is known. With such diverse groups detected via AHL biosensors, these mat ecosystems also have the potential to reveal important information about cross-phylum - or in the case of the archaea cross-domain - communication systems. As quorum sensing has been demonstrated in these microbial mats, further investigation is warranted to identify any potential role of microbial communication in the formation and maintenance of modern microbial mat ecosystems. In addition, future screens of Shark Bay mat systems for quorum sensing inhibitory systems should be undertaken to fully elucidate the complex role of quorum sensing in these environments.

Acknowledgements. This work was supported by the Australian Research Council and the US National Science Foundation (NSF EAR 1052974).

Competing Financial Interests. No competing financial interests exist. The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- Abed RMM, Dobretsov S, Al-Fori M, Gunasekera SP, Sudesh K, Paul VJ. (2013). Quorum-sensing inhibitory compounds from extremophilic microorganisms isolated from a hypersaline cyanobacterial mat. *J Ind Microbiol Biotechnol* **40**: 759–772.
- Al-Awadhi H, Al-Mailem D, Dashti N, Khanafer M, Radwan S. (2012). Indigenous hydrocarbon-utilizing bacterioflora in oil-polluted habitats in Kuwait, two decades after the greatest man-made oil spill. *Arch Microbiol* **194**: 689–705.
- Allen MA, Goh F, Leuko S, Echigo A, Mizuki T, Usami R, *et al.* (2008). *Haloferax elongans* sp. nov. and *Haloferax mucosum* sp. nov., isolated from microbial mats from Hamelin Pool, Shark Bay, Australia. *Int J Syst Evol Microbiol* **58**: 798–802.
- Allen MA, Goh F, Burns BP, Neilan BA. (2009). Bacterial, archaeal and eukaryotic diversity of smooth and pustular microbial mat communities in the hypersaline lagoon of Shark Bay. *Geobiology* **7**: 82–96.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic Local Alignment Search Tool. *J Mol Biol* **215**: 403–410.
- Arp G, Reimer A, Reitner J. (2001). Photosynthesis-induced biofilm calcification and calcium concentrations in Phanerozoic oceans. *Science (80-)* **292**: 1701–1704.
- Baumgartner LK, Reid RP, Dupraz C, Decho AW, Buckley DH, Spear JR, *et al.* (2006). Sulfate reducing bacteria in microbial mats: Changing paradigms, new discoveries. *Sediment Geol* **185**: 131–145.
- Belin P, Moutiez M, Lautru S, Seguin J, Pernodet J-L, Gondry M. (2012). The nonribosomal synthesis of diketopiperazines in tRNA-dependent cyclodipeptide synthase pathways. *Nat*

Bin Saidin J, Abd Wahid ME, Le Pennec G. (2017). Characterization of the in vitro production of N-acyl homoserine lactones by cultivable bacteria inhabiting the sponge *Suberites domuncula*. *J Mar Biol Assoc United Kingdom* **97**: 119–127.

Biswa P, Doble M. (2013). Production of acylated homoserine lactone by Gram-positive bacteria isolated from marine water. *FEMS Microbiol Lett* **343**: 34–41.

Bolhuis H, Stal LJ. (2011). Analysis of bacterial and archaeal diversity in coastal microbial mats using massive parallel 16S rRNA gene tag sequencing. *ISME J* **5**: 1701–1712.

Bose U, Ortori CA, Sarmad S, Barrett DA, Hewavitharana AK, Hodson MP, *et al.* (2017). Production of N-acyl homoserine lactones by the sponge-associated marine actinobacteria *Salinispora arenicola* and *Salinispora pacifica*. *FEMS Microbiol Lett* **364**: 1–7.

Brachmann AO, Brameyer S, Kresovic D, Hitkova I, Kopp Y, Manske C, *et al.* (2013). Pyrones as bacterial signaling molecules. *Nat Chem Biol* **9**: 573–578.

Burns BP, Anitori R, Butterworth P, Henneberger R, Goh F, Allen M a., *et al.* (2009). Modern analogues and the early history of microbial life. *Precambrian Res* **173**: 10–18.

Chan K-G, Atkinson S, Mathee K, Sam C-K, Chhabra SR, Cámara M, *et al.* (2011). Characterization of N-acylhomoserine lactone-degrading bacteria associated with the *Zingiber officinale* (ginger) rhizosphere: co-existence of quorum quenching and quorum sensing in *Acinetobacter* and *Burkholderia*. *BMC Microbiol* **11**: 51.

Charlesworth J, Burns BP. (2016). An overview of extremophilic adaptations and biotechnological applications in diverse environments. *AIMS Microbiol* **2**: 1–11.

Charlesworth J, Kimyon O, Manefield M, Burns BP. (2015). Detection and characterization

of N -acyl- L -homoserine lactones using GFP-based biosensors in conjunction with thin-layer chromatography. *J Microbiol Methods* **118**: 164–167.

De Roy K, Marzorati M, Van den Abbeele P, Van de Wiele T, Boon N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ Microbiol* **16**: 1472–1481.

Decho AW, Norman RS, Visscher PT. (2010). Quorum sensing in natural environments: emerging views from microbial mats. *Trends Microbiol* **18**: 73–80.

Decho AW, Visscher PT, Ferry J, Kawaguchi T, He L, Przekop KM, *et al.* (2009). Autoinducers extracted from microbial mats reveal a surprising diversity of N-acylhomoserine lactones (AHLs) and abundance changes that may relate to diel pH. *Environ Microbiol* **11**: 409–20.

Diggle SP, Cornelis P, Williams P, Cámara M. (2006). 4-quinolone signalling in *Pseudomonas aeruginosa*: old molecules, new perspectives. *Int J Med Microbiol* **296**: 83–91.

Dobretsov S, Abed RMM, Maskari SMS, Sabahi JN, Victor R. (2010). Cyanobacterial mats from hot springs produce antimicrobial compounds and quorum-sensing inhibitors under natural conditions. *J Appl Phycol* **23**: 983–993.

Erickson DL, Endersby R, Kirkham A, Vollman DD, Rabin HR, Mitchell I, *et al.* (2002). *Pseudomonas aeruginosa* Quorum-Sensing Systems May Control Virulence Factor Expression in the Lungs of Patients with Cystic Fibrosis *Pseudomonas aeruginosa* Quorum-Sensing Systems May Control Virulence Factor Expression in the Lungs of Patients with Cystic. *Infect Immun* **70**: 1783–1790.

Goh F, Leuko S, Allen M, Bowman JP, Kamekura M, Neilan B, *et al.* (2006). *Halococcus hamelinensis* sp. nov., a novel halophilic archaeon isolated from stromatolites in Shark Bay,

Australia. *Int J Syst Evol Microbiol* **56**: 1323–9.

Goh F, Barrow KD, Neilan BA, and Burns BP. (2010) Identification and regulation of novel compatible solutes from hypersaline stromatolite-associated cyanobacteria. *Archives of Microbiology* **192**: 1031–1038.

Goh F, Jeon YJ, Barrow K, Neilan B, Burns BP. (2011). Osmoadaptive strategies of the archaeon *Halococcus hamelinensis* isolated from a hypersaline stromatolite environment. *Astrobiology* **11**: 529–36.

Gould T, Schweizer HP, Churchill ME. (2004). Structure of the *Pseudomonas aeruginosa* acyl-homoserinelactone synthase LasI. *Mol Microbiol* **53**: 1135–1146.

Guan C, Ju J, Borlee BR, Williamson LL, Shen B, Raffa KF, *et al.* (2007). Signal mimics derived from a metagenomic analysis of the gypsy moth gut microbiota. *Appl Environ Microbiol* **73**: 3669–76.

Hammer BK, Bassler BL. (2003). Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol* **50**: 101–104.

Hoang DT, Chernomor O, Von Haeseler A, Minh BQ, Vinh LS. (2018). UFBoot2: Improving the ultrafast bootstrap approximation. *Mol Biol Evol* **35**: 518–522.

Holden MT, Ram Chhabra S, de Nys R, Stead P, Bainton NJ, Hill PJ, *et al.* (1999). Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Mol Microbiol* **33**: 1254–66.

Horswill AR, Stoodley P, Stewart PS, Parsek MR. (2007). The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. *Anal Bioanal Chem* **387**: 371–80.

Jungblut AD, Hawes I, Mackey TJ, Krusor M, Doran PT, Sumner DY, *et al.* (2016).

Microbial mat communities along an oxygen gradient in a perennially ice-covered Antarctic lake. *Appl Environ Microbiol* **82**: 620–630.

Kalia VC, Raju SC, Purohit HJ. (2011). Genomic analysis reveals versatile organisms for quorum quenching enzymes: acyl-homoserine lactone-acylase and -lactonase. *Open Microbiol J* **5**: 1–13.

Katoh K, Standley DM. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol* **30**: 772–780.

Letunic I, Bork P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res* **44**: W242–W245.

Leuko S, Allen MA, Goh F, Burns BP, Walter, MR, and Neilan BA. (2007). Analysis of intergenic spacer region length polymorphisms to investigate the halophilic archaeal diversity of stromatolites and microbial mat. *Extremophiles* **11**: 203-10.

Leuko S, Raftery M, Burns BP, Walter MR, and Neilan BA. (2009) Global protein-level responses of *Halobacterium salinarum* NRC-1 to prolonged changes in external sodium chloride concentrations. *J Proteome Res* **8**: 2218-25.

Llamas I, Quesada E, Martínez-Cánovas MJ, Gronquist M, Eberhard A, González JE. (2005). Quorum sensing in halophilic bacteria: detection of N-acyl-homoserine lactones in the exopolysaccharide-producing species of Halomonas. *Extremophiles* **9**: 333–41.

Ma ZP, Lao YM, Jin H, Lin GH, Cai ZH, Zhou J. (2016). Diverse Profiles of AI-1 Type Quorum Sensing Molecules in Cultivable Bacteria from the Mangrove (*Kandelia obovata*) Rhizosphere Environment. *Front Microbiol* **7**: 1–14.

Manefield M, Whiteley AS. (2007). Acylated homoserine lactones in the environment:

chameleons of bioactivity. *Philos Trans R Soc Lond B Biol Sci* **362**: 1235–40.

Miller M, Bassler B. (2001). Quorum sensing in bacteria. *Annu Rev Microbiol* **55**: 165–199.

Okonechnikov K, Golosova O, Fursov M, Varlamov A, Vaskin Y, Efremov I, *et al.* (2012).

Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics* **28**: 1166–1167.

Poli A, Di Donato P, Abbamondi GR, Nicolaus B. (2011). Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by archaea. *Archaea* **2011**: 1–13.

Polkade A V., Mantri SS, Patwekar UJ, Jangid K. (2016). Quorum sensing: An under-explored phenomenon in the phylum Actinobacteria. *Front Microbiol* **7**: 1–13.

Proemse BC, Eberhard RS, Sharples C, Bowman JP, Richards K, Comfort M, *et al.* (2017). Stromatolites on the rise in peat-bound karstic wetlands. *Sci Rep* **7**: 1–8.

Rajamani S, Zhu J, Pei D, Sayre R. (2007). A LuxP-FRET-based reporter for the detection and quantification of AI-2 bacterial quorum-sensing signal compounds. *Biochemistry* **46**: 3990–7.

Rajput A, Kumar M. (2017). In silico analyses of conservational, functional and phylogenetic distribution of the LuxI and LuxR homologs in Gram-positive bacteria. *Sci Rep* **7**. e-pub ahead of print, doi: 10.1038/s41598-017-07241-5.

Reid R, Visscher P, Decho A, Stolz J. (2000). The role of microbes in accretion, lamination and early lithification of modern marine stromatolites. *Nature* **406**: 989–992.

Rojas R, Miranda CD, Amaro AM. (2009). Pathogenicity of a highly exopolysaccharide-producing halomonas strain causing epizootics in larval cultures of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). *Microb Ecol* **57**: 129–139.

Romero M, Avendaño-Herrera R, Magariños B, Cámara M, Otero A. (2010).

Acylhomoserine lactone production and degradation by the fish pathogen *Tenacibaculum maritimum*, a member of the Cytophaga-Flavobacterium-Bacteroides (CFB) group. *FEMS Microbiol Lett* **304**: 131–139.

Ruvindy R, White RA, Neilan BA, Burns BP. (2016). Unravelling core microbial metabolisms in the hypersaline microbial mats of Shark Bay using high-throughput metagenomics. *ISME J* **10**: 183–196.

Sarkar S, Roy D, Mukherjee J. (2011). Enhanced protease production in a polymethylmethacrylate conico-cylindrical flask by two biofilm-forming bacteria. *Bioresour Technol* **102**: 1849–1855.

Schuster M, Urbanowski ML, Greenberg EP. (2004). Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci* **101**: 15833–15839.

Sharif DI, Gallon J, Smith CJ, Dudley E. (2008). Quorum sensing in Cyanobacteria: N-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium *Gloeotheca* PCC6909. *ISME J* **2**: 1171–82.

Stoodley P, Sauer K, Davies DG, Costerton JW. (2002). Biofilms as complex differentiated communities. *Annu Rev Microbiol* **56**: 187–209.

Sturme MHJ, Kleerebezem M, Nakayama J, Akkermans ADL, Vaughn EE, de Vos WM. (2002). Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie Van Leeuwenhoek* **81**: 233–43.

Visscher PT, Stolz JF. (2005). Microbial mats as bioreactors: Populations, processes, and products. *Palaeogeogr Palaeoclimatol Palaeoecol* **219**: 87–100.

Williamson L, Borlee B. (2005). Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. *Appl Environ Microbiol* **71**: 6335–6344.

Wong HL, Smith DL, Visscher PT, Burns BP. (2015). Niche differentiation of bacterial communities at a millimeter scale in Shark Bay microbial mats. *Sci Rep* **5**: 1–17.

Wong HL, Ahmed-Cox A, and Burns BP. (2016) Molecular ecology of hypersaline microbial mats: current insights and new directions. *Microorganisms* **4**: 6, doi:10.3390/microorganisms4010006.

Wong HL, Visscher PT, White RA, Smith DL, Patterson MM, Burns BP. (2017). Dynamics of archaea at fine spatial scales in Shark Bay mat microbiomes. *Sci Rep* **7**. e-pub ahead of print, doi: 10.1038/srep46160.

Wong HL, White RA, Visscher PT, Charlesworth JC, Vázquez-Campos X, Burns BP. (2018). Disentangling the drivers of functional complexity at the metagenomic level in Shark Bay microbial mat microbiomes. *ISME J*. e-pub ahead of print, doi: 10.1038/s41396-018-0208-8.

Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, *et al.* (2002). N-Acylhomoserine Lactones Undergo Lactonolysis in a pH-, Temperature-, and Acyl Chain Length-Dependent Manner during Growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun* **70**: 5635–5646.

Zhang G, Zhang F, Ding G, Li J, Guo X, Zhu J, *et al.* (2012). Acyl homoserine lactone-based quorum sensing in a methanogenic archaeon. *ISME J* **6**: 1336–1344.

Zimmer BL, May AL, Bhedi CD, Dearth SP, Prevatte CW, Pratte Z, *et al.* (2014). Quorum sensing signal production and microbial interactions in a polymicrobial disease of corals and the coral surface mucopolysaccharide layer. *PLoS One* **9**. e-pub ahead of print, doi: 10.1371/journal.pone.0108541.

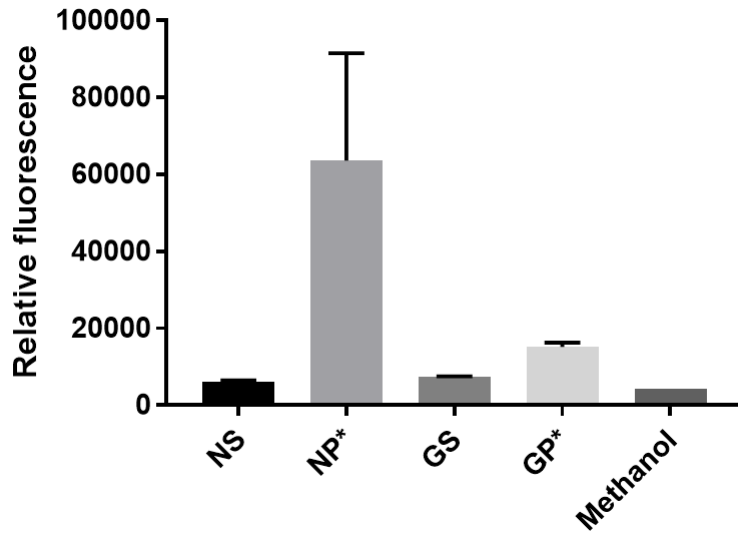


Figure 1. Examination of potential AHL activity from ethyl acetate extractions of whole Shark Bay mat samples screened with AHL biosensor *E. coli* MT102. Activity was measured in terms of GFP fluorescence induced. NP: Nilemah Pustular; NS: Nilemah Smooth; GS: Garden Point Smooth; GP: Garden Point Pustular. Error bars represent standard deviation of the mean of triplicates. An * asterisk denotes extracts with a ($P > 0.05$) and two standard deviations higher compared to the methanol control.

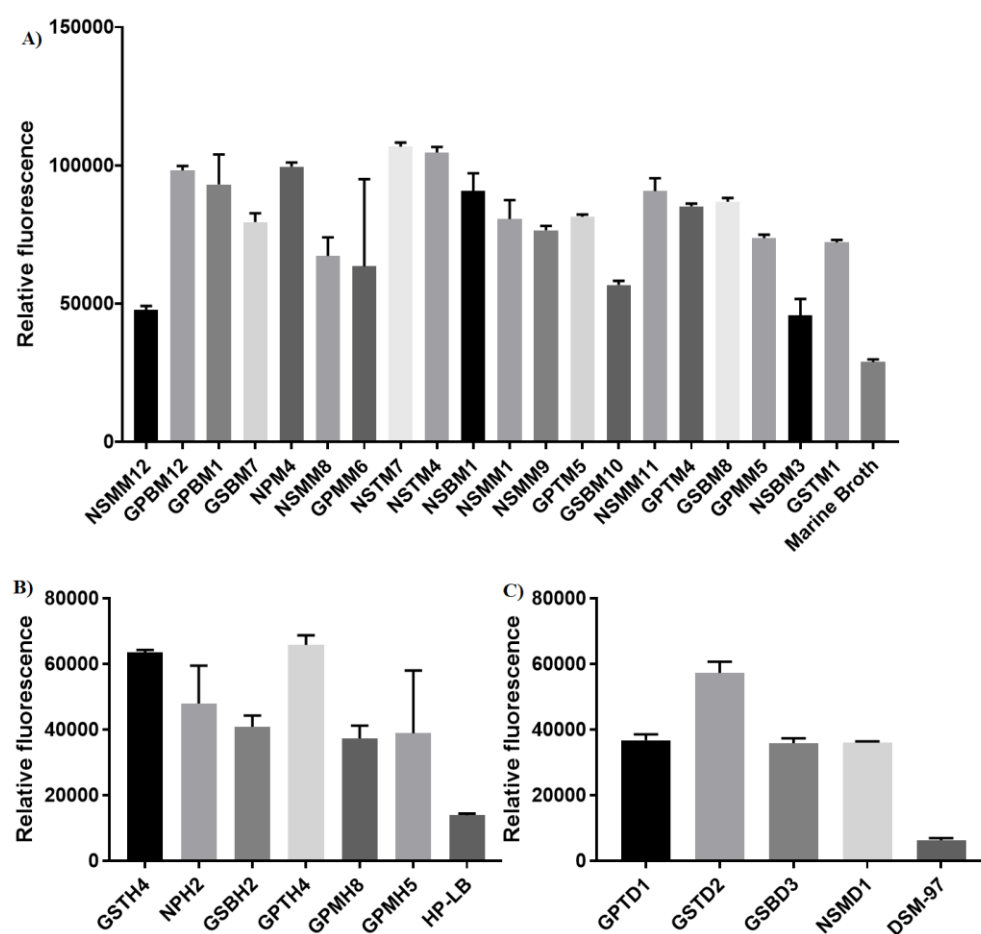


Figure 2. Measurement of AHL activity using the AHL biosensor *E. coli* MT102 in late phase extracts of isolates from Shark Bay microbial mats. The isolates are grouped according to growth medium controls, A) Marine broth isolates, B) HP-LB isolates and C) DSM-97 isolates. All isolates were compared to medium control and error bars represent the standard deviation of the mean of triplicates.



Figure 3. Phylogenetic tree of 16S rDNA sequences from Shark Bay microbial mat isolates displaying AHL activity. Branches named by identification code and in brackets the top match from NCBI database e.g. NSMM12 (Bacterium BA3B) constructed with a total of 1000 bootstrap replicates using the maximum parsimony method. Groups branch into three primary phyla, firmicutes, actinobacteria and proteobacteria and these are indicated. Reference sequences are included and a key to identify the origin of the sequences is at the bottom of the image. The label “^T” indicates the type strain of the species.

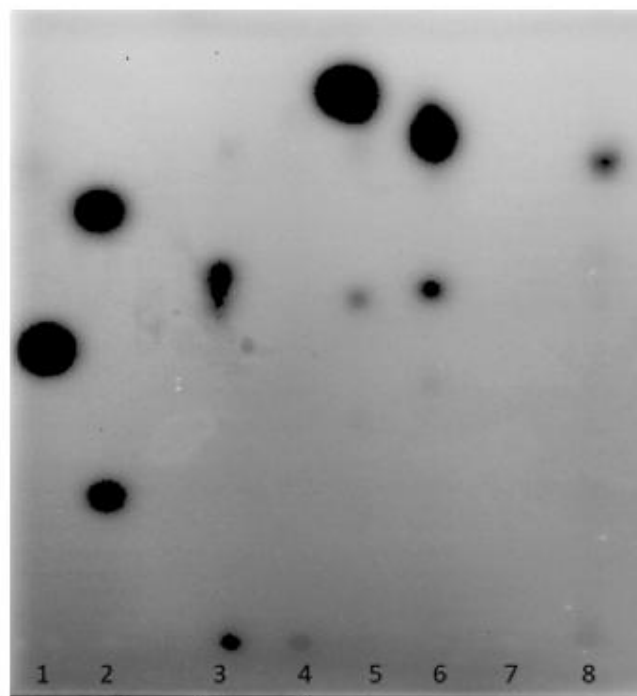


Figure 4. Representative results of signal molecule detection and separation in Shark Bay microbial isolates by thin-layer chromatography. Supernatant extracts were overlaid with the *E. coli* MT102 biosensor. Lane 1: Oxo-Hexanoyl homoserine lactone (oxo-C6) and Octanoyl homoserine lactone (C8); Lane 2: Hexanoyl homoserine lactone (C6) and Decanoyl homoserine lactone (C10) ; Lane 3: NSMM8; Lane 4: GPM12; Lane 5: GPBM1; Lane 6: GSBM7; Lane 7: NPM4; Lane 8: NSMM12.

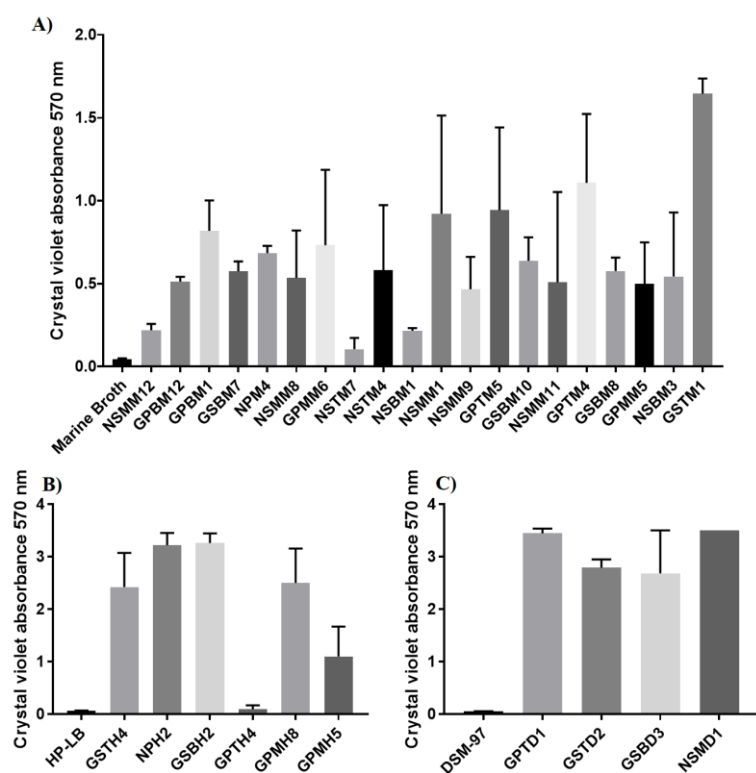


Figure 5. Measurement of biofilm formation in Shark Bay microbial isolates testing positive for signal production using the *E. coli* MT102 biosensor. Biofilm formation was detected using crystal violet and isolates were compared to their respective media control shown on the far left of the figure. A) Marine Broth isolates; B) HP-LB isolates; C) DSM-97 isolates. Error bars represent the standard deviation of the mean of triplicates.

Table 1. Identification of AHLactive isolates from Shark Bay microbial mat communities

Isolate ID	Class	Closest match by Genbank identity	Identity %	Accession #
NSMM12	Firmicutes	Bacterium BA3B 16S ribosomal RNA gene, partial sequence	99%	DQ235550.1
GPBM12*	Proteobacteria	Uncultured alpha proteobacterium clone BBD_216_35 16S ribosomal RNA gene, partial sequence	99%	DQ446088.2
GPBM1*	Proteobacteria	<i>Nesiotobacter</i> sp.	99%	GU213180.1

		H02E 16S ribosomal RNA gene, partial sequence		
GSBM7*	Proteobacteria	<i>Nesiotobacter exalbescens</i> strain WB1-6 16S ribosomal RNA gene, partial sequence	99%	KU996359.1
NPM4*	Proteobacteria	<i>Nesiotobacter exalbescens</i> strain LA33B 16S ribosomal RNA gene, partial sequence	99%	NR_04_1917.1
NSMM8*	Firmicutes	<i>Bacillus firmus</i> partial 16S rRNA gene, isolate SR1-59	99%	LN995458.1
GPMM6*	Actinobacteria	<i>Microbacterium</i> sp. strain H200-55 16S ribosomal RNA gene, partial sequence	99%	MG754435.1
NSTM7	Firmicutes	<i>Bacillus aquimaris</i> strain M2 16S ribosomal RNA gene, partial sequence		
	99%	KY928099.1		
NSTM4*	Proteobacteria	<i>Halomonas</i> sp. S74-1 16S ribosomal RNA gene, partial sequence	99%	EU868848.1
NSBM1	Proteobacteria	<i>Pseudoxanthobacter</i> sp. strain A8 16S ribosomal RNA gene, partial sequence	94%	KX066401.1
NSMM1*	Actinobacteria	<i>Cellulomonas</i> sp. C1-25c1 16S ribosomal RNA gene, partial sequence	98%	JX517235.1

NSMM9*	Proteobacteria	<i>Donghicola</i> sp. strain PrRy144 16S ribosomal RNA gene, partial sequence	99%	MF948953.1
GPTM5*	Proteobacteria	<i>Halomonas</i> sp. 5b partial 16S rRNA gene, strain 5b	99%	LT629933.1
GSBM10*	Actinobacteria	<i>Brevibacterium</i> linens strain B1 16S ribosomal RNA gene, partial sequence	99%	MG757546.1
NSMM11*	Proteobacteria	<i>Idiomarina</i> sp. strain E525-7 16S ribosomal RNA gene, partial sequence	99%	MF975615.1
GPTM4*	Proteobacteria	Uncultured bacterium clone SHFH710 16S ribosomal RNA gene, partial sequence	95%	FJ203620.1
GSBM8*	Firmicutes	<i>Bacillus licheniformis</i> strain DH12 16S ribosomal RNA gene, partial sequence	99%	KF646684.1
GPM5*	Actinobacteria	<i>Chryseoglobus</i> sp. strain IHBB 9314 16S ribosomal RNA gene, partial sequence	99%	KU921548.1
NSBM3*	Firmicutes	<i>Virgibacillus marismortui</i> gene for 16S ribosomal RNA, partial sequence, strain: BMT2	99%	LC260007.1
GSTM1*	Proteobacteria	<i>Stappia</i> sp. MBTDCMFRIMab33 16S ribosomal RNA gene, partial	99%	KR004806.1

		sequence		
GSTH4*	Firmicutes	<i>Virgibacillus</i> sp. Bac333R 16S ribosomal RNA gene, partial sequence	99%	KP795875.1
NPH2*	Proteobacteria	<i>Pseudovibrio</i> sp. SPO723 16S ribosomal RNA gene, partial sequence	100%	DQ660386.1
GSBH2*	Firmicutes	<i>Virgibacillus salarius</i> gene for 16S ribosomal RNA, partial sequence, strain: BAFBRB9	99%	LC260003.1
GPTH4	Proteobacteria	<i>Halomonas venusta</i> strain Z8 16S ribosomal RNA gene, partial sequence	100%	MF575810.1
GPMH8*	Firmicutes	<i>Virgibacillus</i> <i>marismortui</i> gene for 16S ribosomal RNA, partial sequence, strain: BMT2	99%	LC260007.1
GPMH5*	Firmicutes	<i>Halobacillus</i> sp. CZB19 16S ribosomal RNA gene, partial sequence	99%	KR347336.1
GPTD1*	Proteobacteria	<i>Halomonas</i> sp. GX18B9-2 16S ribosomal RNA gene, partial sequence	99%	KF201601.1
GSTD2*	Proteobacteria	<i>Halomonas</i> sp. strain G20 16S ribosomal RNA gene, partial sequence	99%	KY501243.1
GSBD3*	Proteobacteria	<i>Halomonas</i> sp. strain YM21 16S ribosomal	100%	KX225425.1

		RNA gene, partial sequence		
NSMD1*	Proteobacteria	<i>Halomonas salina</i> partial 16S rRNA gene, isolate salina	99%	AM945688.1

*Indicates isolates shown to form biofilms in the present study

Table 2. Thin layer chromatography of AHLactive Shark Bay microbial mat isolates

Mat isolate ID	Phylum	Closest genbank identity	Rf value
NSBM12(NSMM12)	Firmicutes	Bacterium BA3B	0.62
GPBM1	Proteobacteria	<i>Nesiotobacter</i> sp.	0.57, 0.88
GSBM7	Proteobacteria	<i>Nesiotobacter</i> sp.	0.57, 0.80
NSMM8	Firmicutes	<i>Bacillus</i> sp.	0.57, 0.76
GPM6	Actinobacteria	<i>Microbacterium</i> sp.	0.89
NSTM4	Proteobacteria	<i>Halomonas</i> sp.	0.82 0.65
NSMM1	Actinobacteria	<i>Cellulomonas</i> sp.	0.82
NSMM9	Proteobacteria	<i>Donghicola</i> sp.	0.89
GSBM10	Actinobacteria	<i>Brevibacterium</i> sp.	0.3, 0.5, 0.72, 0.79
GPTM4	Proteobacteria	Uncultured bacterium clone	0.72
GSBM8	Firmicutes	<i>Bacillus</i> sp.	0.625, 0.84
NSBM3	Firmicutes	<i>Virgibacillus</i> sp.	0.625, 0.84
NPH2	Proteobacteria	<i>Pseudovibrio</i> sp.	0.38, 0.57 0.79
GPTH4	Proteobacteria	<i>Halomonas</i> sp.	0.57, 0.79
GPMH8	Firmicutes	<i>Virgibacillus</i> sp.	0.83
GPMH5	Firmicutes	<i>Halobacillus</i> sp.	0.57, 0.79
GPTD1	Proteobacteria	<i>Halomonas</i> sp.	0.91

GSTD2	Proteobacteria	<i>Halomonas sp.</i>	0.91
OxoC6 AHL	AHL standard		0.81–0.83
C6 AHL	AHL standard		0.69–0.75
C8 AHL	AHL standard		0.5–0.58
C10 AHL	AHL standard		0.26–0.33
C4 AHL	AHL standard		0.90
OxoC12 AHL	AHL standard		0.34