Identification and chemical characterization of *N* acyl-homoserine lactone quorum sensing signals across sponge species and time.

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Abstract

Marine sponges form symbiotic relationships with complex microbial communities, yet little is known about the mechanisms by which these microbes regulate their behavior through gene expression. Many bacterial communities regulate gene expression using chemical signaling termed quorum sensing. While a few previous studies have shown presence of *N*-acyl-homoserine lactones (AHLs) based quorum sensing in marine sponges, the chemical identity of AHL signals has been published for only two sponge species. In this study we screened for AHLs in extracts from 15 sponge species (109 specimens in total) from the Mediterranean and Red Sea, using a wide-range AHL-biosensor. This is the first time that AHL presence was examined over time in sponges. We detected the presence of AHL in 46% of the sponge species, and found that AHL signals differ for certain sponge species in time and across sponge individuals. Furthermore, for the Mediterranean sponge species *Sarcotragus fasciculatus*, we identified 14 different AHLs. The constant presence of specific AHLs molecules in all specimens, together with varying signaling molecules between the different specimens, make *S. fasciculatus* a good model to further investigate the function of quorum sensing in sponge-associated bacteria. This study extends the knowledge of AHL-based quorum sensing in marine sponges.

Introduction

Quorum sensing (QS) is a complex system that enables bacteria to coordinate their behavior, in effect acting as a multicellular organism (Fuqua, *et al.*, 1994). Through QS, bacteria regulate their gene expression in a population density-dependent manner. At low cell density, bacteria produce and release low levels of signaling compounds, which diffuse in and out of the bacterial cells. As bacterial density increases, especially for bacteria that are in enclosed niches, the chemical signals accumulate within the environment. Once the bacteria reach a critical level (quorum), they can detect and respond to the signals by regulating their gene expression (Parsek & Greenberg, 2000, Venturi & Subramoni, 2009). QS can regulate a variety of cellular processes such as cell division, production of virulence factors and secondary metabolites, plasmid transfer and biofilm formation (Fuqua, *et al.*, 1994, Venturi & Subramoni, 2009). Through the study of QS, it is possible to disentangle complicated regulatory networks in bacteria (Welsh & Blackwell, 2016). One of the best-understood microbial QS

systems is the one that uses *N*-acyl-homoserine lactones (AHLs) as chemical signals. Canonical AHL-QS systems produce and respond to AHLs using two proteins that mediate signal production and response, LuxI and LuxR homologue proteins respectively (Nealson, *et al.*, 1970). QS is considered not only a mechanism by which bacteria interact and regulate their own genetic expression, but it also involves inter-kingdom signaling through which plants and animals (hosts) can manipulate symbiont-symbiont and host-symbiont interactions (González & Venturi, 2013). In addition, QS plays an important role in the maintenance of coral reef ecosystem health, regulates the settlement of invertebrate larvae and has a strong impact on a variety of marine invertebrates including sponges (reviewed in (Hmelo, 2017)).

Sponges (phylum Porifera) form close associations with a wide variety of microorganisms (Laroche, *et al.*, 2007, Hentschel, *et al.*, 2012, Webster & Thomas, 2016), which can constitute up to 35% of sponge biomass (Vacelet & Donadey, 1977). Specific sponge species can harbor dense microbial communities, and are called high microbial abundance (HMA) sponges, while other species are almost devoid of microorganisms, and are termed low microbial abundance (LMA) sponges (Reiswig, 1974, Hentschel, *et al.*, 2003). The dense microbial population in the sponge tissue and the enclosed niche likely favor bacterial interactions through signaling molecules, such as AHLs. One AHL, namely *N*-3-oxododecanoyl-L-homoserine lactone, can to affect the expression of immune and apoptotic genes of the host sponge *Suberites domuncula*, possibly enabling the sponge to monitor and regulate bacterial populations (Gardères, *et al.*, 2014). Moreover, bacteria capable of performing QS were isolated from marine sponges and signaling molecules able to activate QS-biosensors were found in sponge extracts (Taylor, *et al.*, 2004, Mohamed, *et al.*, 2008, Zan, *et al.*, 2012, Britstein, *et al.*, 2015, Saurav, *et al.*, 2016). Molecules interfering with QS are also found in sponges, suggesting complex signaling interactions (e.g., (Costantino, *et al.*, 2017, Saurav, *et al.*, 2017)).

Using biosensor analysis, AHLs were detected in 77% of analyzed southeastern Australian sponges, providing initial evidence for a high frequency of AHL-QS in sponges; the chemical structure of these compounds was not analyzed (Taylor, *et al.*, 2004). More recently, C6-HSL, C7-HSL and OC12-HSL were identified in the Mediterranean Sea sponge *Suberites domuncula* (Gardères, et al., 2012); while

C8-HSL, C10-HSL and OH-C18-HSL were detected in the Red Sea sponge *Theonella swinhoei* (Britstein, *et al.*, 2015). Furthermore, a full AHL-QS system (designated TswIR) was identified in the microbiome of *T. swinhoei* by metagenomics (Britstein, *et al.*, 2015). The novel TswIR system was associated with an uncultured alphaproteobacterium of the Rhodobacterales order, termed Rhodobacterales bacterium TS309.

In this study we report on: (i) frequency of AHLs in 15 sponge species from the Mediterranean and Red Sea, identified using TLC analysis coupled with a wide-range AHL-biosensor, (ii) variation of AHL detection through time, for four selected sponge species, (iii) chemical identity of the AHLs in three specimens of *Sarcotragus fasciculatus* (for which AHLs were detected by TLC in 100% of tested specimens, n=20) and (iv) lack of connection between relative abundance of the sponge symbiont TS309 in the sponge *T. swinhoei* and presence of AHL signals in its host tissue. This study provides baseline information for future work in AHL quorum sensing in marine sponges.

Methods

Sample collection

We screened 15 sponge species, with a total of 109 specimens, for the presence of AHL signal molecules by TLC analysis coupled with a wide range AHL-biosensor. Eight sponge species were collected from the Red Sea (*Amphimedon chloros, Crella cyathophora, Diacarnus erythraenus, Negombata magnifica, Pione vastifica, Siphonochalina siphonella, Suberites clavatus,* and *Theonella swinhoei*) and seven from the Mediterranean Sea (*Axinella polypoides, Axinella verrucosa, Chondrosia reniformis, Cinachyrella levantinensis, Petrosia ficiformis, Sarcotragus fasciculatus, Sarcotragus* sp.). Sponge samples were collected in duplicate or triplicate by SCUBA diving. Samples were immediately frozen in liquid nitrogen or dry ice upon surfacing from the dives. Frozen tissues were freeze-dried using a lyophilizer (VirTis). To determine whether the signals were affected by season, a periodical screen was performed on four selected sponge species: *S. fasciculatus, Sarcotragus* sp., *S. siphonella,* and *T. swinhoei*. These were collected every four months for 24 months, with different specimens collected at each collection time. Sponge species and sample

locations are listed in Table S1. Voucher samples used for DNA extraction were conserved in 100% ethanol, and are stored at the Marine Microbiology Laboratory, University of Haifa, Israel. Chemical identification of the AHL molecules in the sponge extract was performed for three specimens of *S. fasciculatus* collected by SCUBA from Sdot Yam, Mediterranean Sea, Israel at depths of 3-6m. Sponges were immediately frozen in liquid nitrogen upon surfacing from the dives. Frozen tissues were freeze dried using a lyophilizer (VirTis), and extracted as described later.

AHL detection in sponge tissue extracts using *Agrobacterium tumefaciens* NT1(pZLR4) reporter system

Two grams of dried sponge tissue per sample was ground to powder in liquid nitrogen, and AHLs were extracted using a modified Bligh-Dyer procedure (Bligh & Dyer, 1959) and via solid-phase extraction (SPE), as described in (Zan, *et al.*, 2012). AHLs were detected using a thin-layer chromatography (TLC) overlay technique. This technique detects AHLs using bacterial biosensor that phenotypically responds when exposed to exogenous AHLs. The organic extract of each individual sponge was dissolved in 40 µL of ethyl acetate acidified with acetic acid (0.1%). Thirty microliters of these extracts were loaded onto C18 reversed-phase (RP)-TLC plates (Millipore) and developed in 70% methanol in water. The plates were dried and overlaid with 100 mL of soft AB agar (0.7%) supplemented with 0.5% glucose and 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and seeded with 10 mL of the AHL-biosensor *Agrobacterium tumefaciens* NT1(pZLR4) (Luo, *et al.*, 2001), OD_{600} of 0.1-0.5. C6- HSL and C8-HSL standards (Sigma-Aldrich, St. Louis, MO, USA) were also loaded on the TLC.

AHL extraction and identification in *S. fasciculatus* using LC-HRMS and LC-HRMS/MS analysis

Three samples of *S. fasciculatus* dried sponge tissue (450I- 5.2077g, 452I- 7.037g, 453I- 5.3870g) were extracted, separately, with methanol (MeOH, 2 X 200mL), MeOH and Chloroform ($CHCl_3$) in different ratios (2:1, 1:1, 1:2) and then with $CHCl_3$ (2 X 200mL). According to the specific procedure set up in our laboratory, the MeOH extracts were partitioned between H_2O and *n*-Butanol (BuOH); each *n*-BuOH layer was combined with $CHCl_3$ extracts and concentrated in rotary vacuum.

Extracts were analyzed on a Thermo LTQ Orbitrap XL high-resolution ESI mass spectrometer coupled to an Agilent model 1100 LC system. A 5 µm Kinetex C18 column (50×2.1 mm), maintained at 25 °C, was operated using a gradient elution of H_2O with 0.1% formic acid and acetonitrile (ACN), running at 200 mL min^{-1}. The gradient program was as follows: 10% ACN for 3 min, 10–90% ACN over 30 min, 90% ACN for 2 min. All the mass spectra were recorded in the positive-ion mode. Mass spectrometry parameters were a spray voltage of 5 kV, a capillary temperature of 230°C, a sheath gas rate of 12 units N_2 (ca. 120 mL min^{-1}), and an auxiliary gas rate of 5 units N_2 (ca. 50 mL min^{-1}). Data were collected in the surface induced dissociation (SID) mode at 40 eV. five microliters of a mixture of commercially available synthetic AHLs (C4-HSL, C6-HSL, OC6-HSL, C8-HSL, OC8-HSL, OH-C8-HSL, C10-HSL, OC10-HSL, OH-C10-HSL, C12-HSL, OC12-HSL, OH-C12-HSL, C14-HSL, OC14-HSL, OH-C14-HSL, C16-HSL, OC16-HSL, OH-C16-HSL, C18-HSL, OC18-HSL, and OH-C18-HSL) were used (10 mg mL^{-1} each); the extracted ion chromatogram was generated at *m/z* 102.0550, corresponding to the characteristic product ion deacylated homoserine lactone. AHLs in *S. fasciculatus* specimens I450, I451, I452 were identified on the basis of the comparison of their retention time and HRMS/MS spectra with those of the synthetic standards.

Theonella swinhoei microbial biodiversity analysis and Distribution of Rhodobacterales bacterium TS309 among periodically collected samples

Distribution and abundance of Rhodobacterales bacterium TS309, previously reported as a symbiont of *T. swinhoei* harboring QS genes (Britstein, *et al.*, 2015), were evaluated using the16S rRNA gene sequence of TS309 as query in a Blastn search (Camacho, *et al.*, 2009) against *T. swinhoei* 16S gene data (see supplementary materials for method). OTUs with identity of 97% or higher to the query sequence were used to determine the relative abundance of this bacterium among the periodically collected *T. swinhoei* samples. In each sample, the relative abundance of TS309 was calculated by dividing the reads sum of the relevant OTUs by the total number of reads in the sample. Bar plots were generated in R using the package ggplot2 (Wickham, 2009).

Results

Out of the 15 sponge species screened using the biosensor *A. tumefaciens* NT1 (pZLR4), seven species (46%) displayed a positive AHL result in at least one of the replicates, with four being AHL positive in all replicates (*Axinella verrucosa, Crella cyathophora, S. fasciculatus* and *Suberites clavatus*, Figure 1). No correlation was found between the TLC AHL result and the classification of the sponge species tested as HMA or LMA (Figure 1, and Table S1). Four sponge species were chosen for a periodical screen that was performed every four months for a period of 24 months, to test for potential changes in AHL profiles throughout the year. *S. fasciculatus* showed a constant presence of AHLs in all the specimens tested (n=20) throughout the study period (Figure 2A, Figure S1A). *S. siphonella*, by contrast, displayed a constant negative AHL result, based on 17 specimens analyzed during a period of two years (Figure 2B). *T. swinhoei* and *Sarcotragus* sp. showed variable presence of AHLs, also when collected during the same month, a year later (Figure 2, Figure S1B).

S. fasciculatus was the only species that showed a constant positive AHL result of the four sponge species that were collected periodically. Accordingly, we chose it for further elucidation of AHL identity. This was done for extracts from three different specimens of *S. fasciculatus*, using LC-HRMS/MS (Figure 3). The characteristic homoserine lactone product ion at *m/z* 102.05 is originated upon SID from the protonated precursor of each AHL. Once extracted from the chromatogram, it allowed us to trace the generating AHLs, whose retention times were compared against those of commercially available synthetic standards (details in Materials and Methods). Several unusual AHLs whose precursor masses and/or retention times had no correspondence with the standard AHLs were also detected by this method (Figure 3, Table 1). In particular the precursor ions at *m/z* 256.1907 and *m/z* 340.2846, corresponding to C10-HSL and C16-HSL in the standards mixture respectively, were extracted at shorter retention time in I450 and I451 chromatograms, suggesting the presence of methyl branched acyl chains (Thiel, *et al.*, 2009). In addition, the aforementioned putative methyl branched C9-HSL and C15-HSL showed an odd chain length (Gould, *et al.*, 2006), as for OC7:1-HSL and OC9-HSL that were revealed in all three sponge specimens at coherent retention time with their structures.

Interestingly, two putative AHLs having polyunsaturated acyl chains (Wagner-Döbler, *et al.*, 2005) were detected in all three crude extracts; they displayed $[M+H]^+$ pseudomolecular ion peaks at *m/z* 264.1230 and *m/z* 224.1281, which were indicative of the molecular formulas $C_{14}H_{16}O_4N$ and $C_{12}H_{18}O_3N$, respectively. As no standard was available, it can be only argued that the two molecules correspond to OC10:3-HSL and C8:2-HSL, respectively. The small amount of the compounds prevented the full confirmation of the structures. Nine of the total 14 different AHL molecules (C6:1-HSL, OC7:1-HSL, OC8:1-HSL, OHC8-HSL, OC10:1-HSL, OC9-HSL, OC10:3-HSL, OC16:1-HSL, C8:2-HSL) were present in all three sponge individuals (Table 1).

To explain the variability in AHL presence in different *T. swinhoei* specimens, we tested the presence of the previously identified AHL producer, Rhodobacterales bacterium TS309 (Britstein, *et al.*, 2015), in the same sponge individuals collected for AHL-analysis. Accordingly, we analyzed whether the relative abundance of TS309 corresponded to the presence of AHLs detected in the periodically sampled *T. swinhoei* (Figure 4). The median relative abundance of TS309 was 0.39% throughout the study time. There was no detectable difference in the relative abundance of TS309 in relation to the presence of AHLs in the sponge extract from the same specimen (Figure 4). It should be noted that our data provides the relative abundance of TS309 and not its absolute abundance.

Our next step was to investigate whether we could detect other changes in the microbial community of *T. swinhoei* that would correlate with the presence of AHLs in the sponge extract. *T. swinhoei* housed a highly diverse microbial community, with Bray Curtis dissimilarity confirming weak differences (R^2=0.29575) among samples in the different collection times (Permutational multivariate analysis of variance (PERMANOVA), $p = 0.001$). However, the separation according to presence of AHLs was not confirmed (PERMANOVA, $p = 0.064$). Details on this analysis are provided in Supplemental Material.

Data Deposition

All raw amplicon Illumina sequences were deposited in the NCBI Sequence Read Archive under project number PRJNA398158. NCBI Biosamples numbers are SAMN07502168, SAMN07502169, SAMN07502170, SAMN07502171, SAMN07502172, SAMN07502173.

Discussion

QS in bacteria regulates a wide range of important biogeochemical bacterial behaviors, thus, understanding how bacteria interact in high density environments is important to predict their impact on complex ecosystems (Hmelo, 2017). Marine sponges represent one of these complex marine environments. The results from this study provide the first base information for AHL QS in 15 sponge species. The high frequency of AHLs found in a previous study by Taylor et al. (2004) was attributed to the high density of bacteria within sponges (Taylor, *et al.*, 2004). Unexpectedly, in our study, AHLs were detected in both high and low microbial abundance sponges. Low microbial abundance sponges may include microniches of high bacterial abundance in which QS occurs (Mohamed, *et al.*, 2008). Although overall bacterial densities in LMA sponges, between 10^5–10^6 bacteria per gram of sponge wet weight (Hentschel, *et al.*, 2006), may be below the QS threshold, at micrometer scale, many niches, such as sponge tissue, can enable QS (Mohamed, *et al.*, 2008). The use of a biosensor allows high sensitivity detection of AHL signal molecules as the biosensor can detect signals down to 5nM (Zhu, *et al.*, 2003). Our results might underestimate the presence of AHL-QS, given that some AHLs, especially long-chain ones, are not readily detected by the biosensor used (Steindler & Venturi, 2007). Thus, we can conclude that at least 46% of the species analyzed, use AHL signaling.

Based on our TLC results, different sponge species show variable patterns in terms of AHLs; the microbiome associated to *S. siphonella* likely does not use AHL-QS signaling, while *T. swinhoei* and *Sarcotragus* sp. vary in terms of AHL presence based on the specimen collected. Even in *S. fasciculatus*, that showed positive AHL profiles in all specimens tested by TLC, we could detect variability in some of the AHL molecules, when examining the chemical nature of the signals in three different sponge specimens using LC-MS/MS (Figure 3). Symbionts in AHL-negative sponge species, e.g. *Amphimedon chloros, Pione vastifica* and *Diacarnus erythraenus* (Figure 1), may be utilizing

different bacteria-bacteria quorum sensing systems not involving AHLs, such as signal peptides and AI-2 LuxS QS systems (Luo, *et al.*, 2001, Zan, *et al.*, 2011); or may synthesize AHLs at low concentrations, below detection limit of the AHL-biosensor (Gould, *et al.*, 2006).

The variability in AHL presence observed for several sponge species (i.e. *A. polypoides*, *Sarcotragus* sp. and *T. swinhoei* (Figures 1 and 2)) may be explained by: (i) changes in the microbial community, and accordingly, different signal molecules produced, and/or (ii) QS regulation, i.e. LuxI homologues not constitutively expressed. We investigated the structure of symbiotic microbial communities in different specimens of *T. swinhoei* and among different collection times, and compared it to the presence or absence of AHLs in the sponge extracts of the same specimens (supplementary information, Figure S2). Collection time weakly influenced the microbial community structure (supplementary information, Figure S2), however, based on the PERMANOVA analysis, there was no significant separation of community structure according to the presence of AHLs in the sponge extract. In addition, no OTU was shown to be significantly enriched in AHL positive specimens using negative binomial modeling that was conducted in the R package DESeq2 (Love et al., 2014; McMurdie and Holmes, 2014). These findings lead us to speculate that in *T. swinhoei*, gene regulation on the production of AHL may be responsible for the presence/absence of AHLs detected in sponge extracts, rather than the presence of a specific OTU in the symbiotic microbial community. In a previous study we identified in the genome of the sponge symbiont Rhodobacterales bacterium TS309 of *T. swinhoei*, a potential inhibitor of AHL production, termed *tswR*-T; the latter may be involved in the absence of AHL signals in most *T. swinhoei* specimens analyzed (Britstein, *et al.*, 2015). Different is the case of *Sarcotragus* sp, where the AHL profile varied not only in presence but also in type of signal molecules (Figure S1B), this may derive from differences in the associated microbial communities, suggesting the presence of different AHL-QS systems. A congeneric species, *Sacrotragus spinosulus* from the Mediterranean Sea displayed a dynamic microbial community with constant dominant bacterial species and turnover of less abundant community members, in time and across host individuals. This can be observed in better detail for the sponge species *S. fasciculatus* where nine AHLs were found in all replicates, while five varied according to sponge specimen (Table

1 and Figure 3). AHL variability in different individuals of the same sponge species was also previously reported in *Suberites domuncula* (Gardères, *et al.*, 2014). AHL signaling is taxon specific, such that different taxa will use different AHL structures to communicate (Rajput, *et al.*, 2016). Molecules that were found in all three specimens of *S. fasciculatus* could be the product of a constant core microbial community, while variability in signal molecules between different individuals could be explained by variable microbial symbionts. It was previously shown that in *S. fasciculatus* (previously designated *Ircinia fasciculata*) there is a specific mix of generalist symbionts that is determined by factors that are specific to the host, such as host genetics and the environment, e.g. radiation and seawater temperature (Erwin, *et al.*, 2012).

This is the third study that tested chemical identity of AHLs in sponge extracts; the previous ones analyzed extracts from two different sponge species. The AHL signal molecules identified in *S. fasciculatus* (C6:1-HSL, OC7:1-HSL, OC8:1-HSL, OHC8-HSL, OC10:1-HSL, OC9-HSL, OC10:3-HSL, OC16:1-HSL, C8:2-HSL, and additional five molecules that were tentatively described, Table 1) are different from those previously identified in *T. swinhoei* (C8-HSL, C10-HSL and OHC18-HSL) (Britstein, *et al.*, 2015) and *S. domuncula* (C6-HSL, C7-HSL and OC12-HSL) (Gardères, *et al.*, 2012). Diverse AHL signal molecules found in different sponge species may represent cues that attract specific symbionts from the environment towards the sponge host.

Concluding Remarks

This study represents a wide screen of AHL presence in a large number of specimens from different marine sponge species. We confirm the previously described variability in AHLs according to sponge species and individuals and further provide first evidence for variability in AHLs in time. Unexpectedly, and differently from previously suggested, the presence of AHLs cannot be directly attributed to the high density of bacteria in sponges, as AHL presence did not relate to the HMA versus LMA nature of the sponge species. We speculate that different mechanisms can explain variability in AHL production in sponges, including microbial community structure and genetic regulation. Further investigation is required to understand the mechanisms regulating AHL production

in sponges and due to lack of cultivability of most sponge symbionts, the main challenge remains to understand what phenotypes QS regulates within the sponge holobiont. The here described consistent presence and high diversity of AHLs in *S. fasciculatus* makes this sponge species a potentially interesting model system for future studies on the sources and roles of AHLs in sponges.

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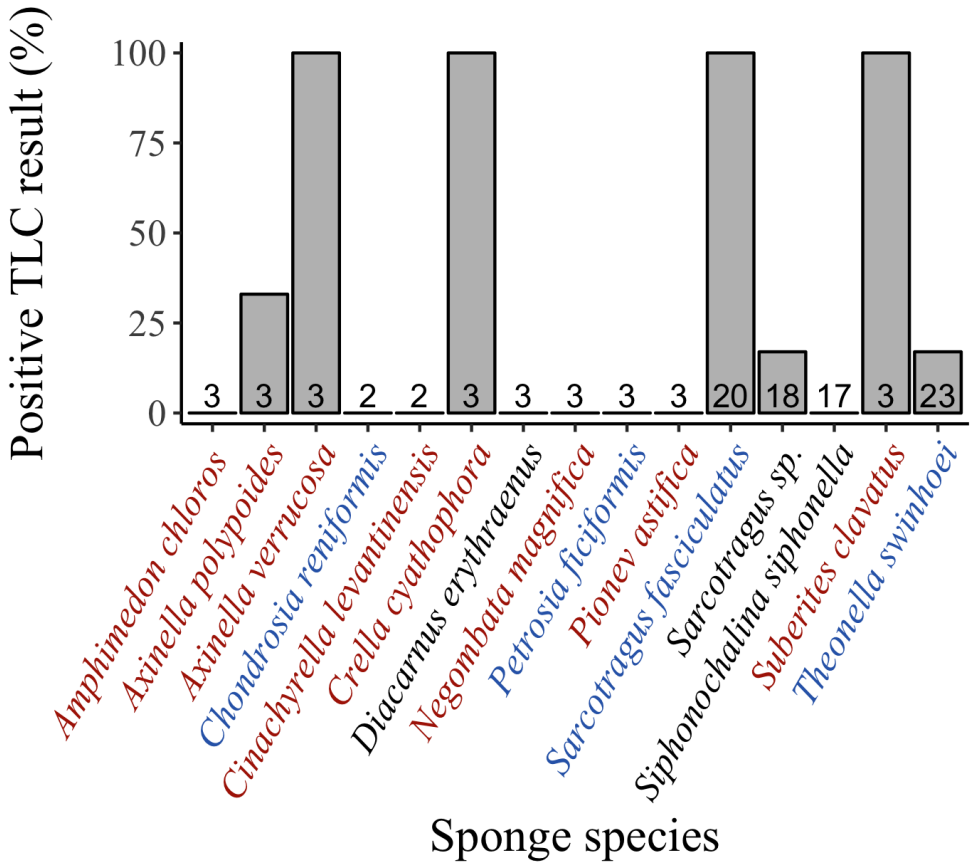

Figure 1: Positive AHL results based on TLC overlay assays with *A. tumefaciens* NT1(pZLR4) reporter strain of extracts from 15 sponge species. The number of replicates analyzed (n) is indicated by a number at the bottom of each column. Font colors of sponge species names refer to their high microbial abundance (HMA, blue), low microbial abundance (LMA, red) nature, or black for species with no information. The literature source for HMA and LMA definitions of the sponge species is available in Table S1.

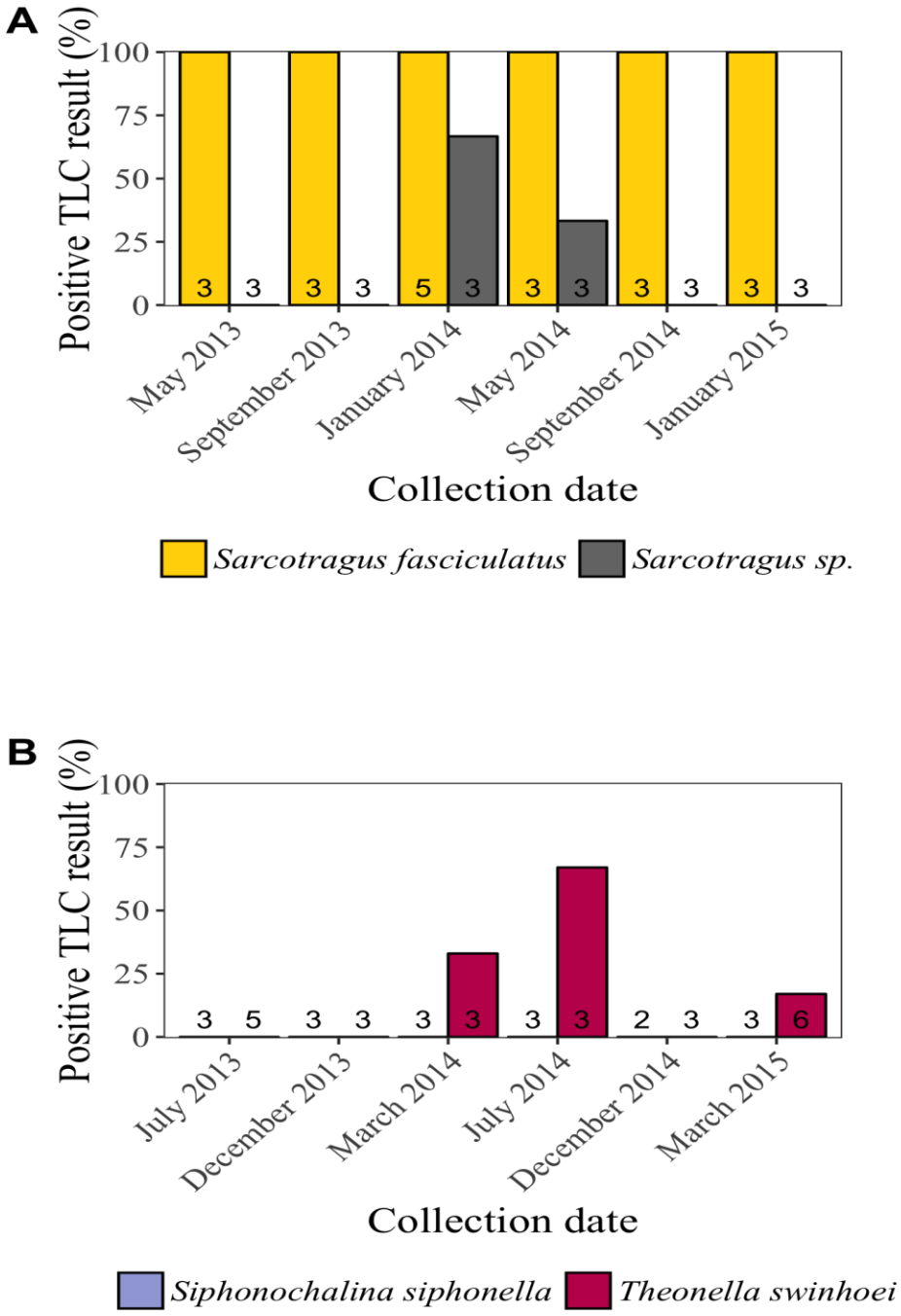

Figure 2: Periodical screen for AHLs of four sponge species, two from the Mediterranean Sea (A) and two from the Red Sea (B). Month of sponge collection is indicated. The number of replicates analyzed (n) is indicated by a number at the bottom of each column. Results are based on TLC overlay assays with *A. tumefaciens* NT1(pZLR4) reporter strain.

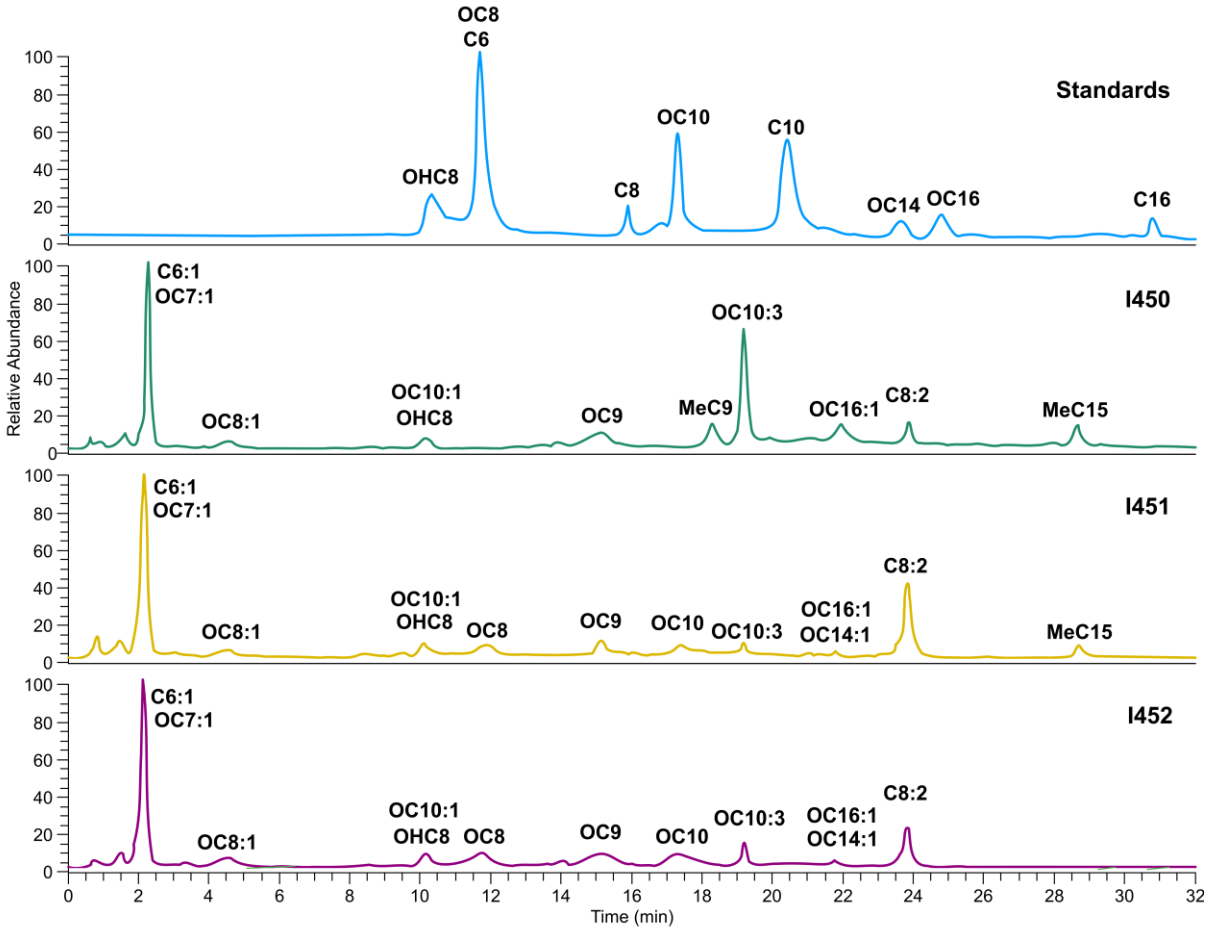

Figure 3: Chemical identification of AHLs in *Sarcotragus fasciculatus.* Extracted ion chromatograms from the LC-HRMS analysis at *m/z* 102.05, corresponding to deacylated homoserine lactone, of the standard mixture of synthetic AHLs (blue trace) and extracts from three specimens of *S. fasciculatus*: I450 (green trace), I451 (yellow trace), I451 (purple trace).

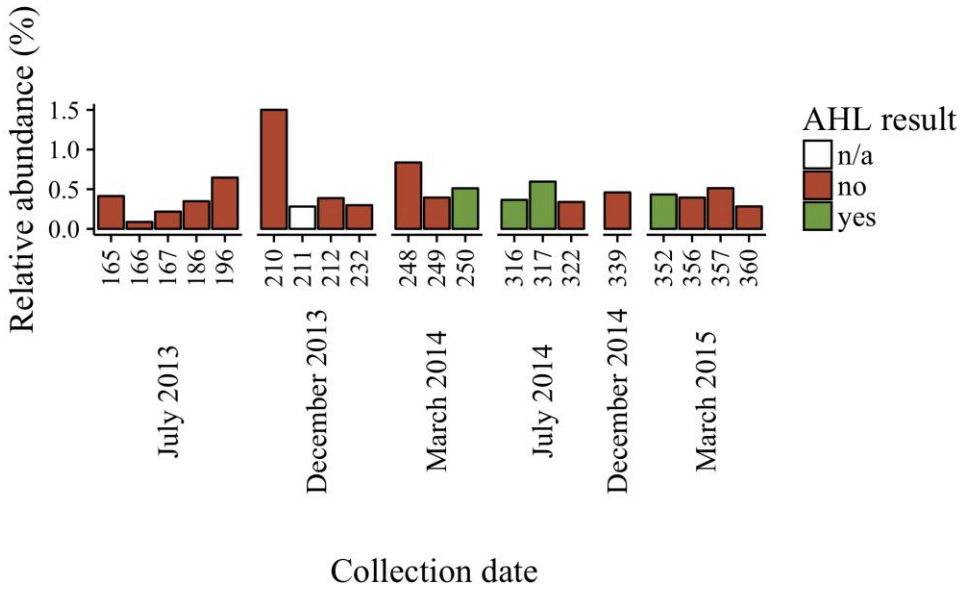

Figure 4: Relative abundance of OTUs with ≥97% identity to 16S rRNA of Rhodobacterales bacterium TS309 in *T. swinhoei* specimens for which also AHL presence was examined by TLC overlay assay with *A. tumefaciens* NT1(pZLR4) reporter. Numbers represent voucher-ID of each specimen. Samples are organized according to collection date.

Table 1: LC-MS detection of AHLs in three specimens of *S. fasciculatus*.

	I450	**I451**	**I452**
C6:1*	V	V	V
OC7:1*	V	V	V
C8:2*	V	V	V
OC8:1*	V	V	V
OHC8	V	V	V
OC10:1*	V	V	V
OC8	-	V	V
OC9*	V	V	V
OC10	-	V	V

MeC9*	V	-	-
OC10:3*	V	V	V
OC14:1*	-	V	V
OC16:1*	V	V	V
MeC15*	V	V	-

*** No standard available, tentatively described based on retention time and mass fragmentation, (see methods).**

AHLs present in all three specimens are highlighted in bold.