Depth effect on the prokaryotic community assemblage associated with sponges from different rocky reefs

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Background. Marine sponges are considered harboring one of the richest microbial symbiont communities, which inhabit from shallow to mesophotic ecosystems providing a comfortable place to many symbiotic species. Although the assemblage of the associated bacterial communities can be affected by depth, marine sponges are also known to share a uniform microbial population from different regions. Implementing next-generation sequencing (NGS) approaches to characterize their microbial communities has notoriously increased precision and quantity of the taxonomic complexity studies associated with these organisms. This study assessed depth effect on the assemblage of bacterial communities associated with two marine sponges of the genera *Aplysina* (Nardo, 1834) and *Ircinia* (Nardo, 1833) in rocky reefs from Punta Arena de la Ventana (Gulf of California) and Pichilingue (La Paz Bay) in the coast of Baja California Sur, Mexico to estimate if the sponge bacterial communities are modeled by the environment (depth) or by the host (sponge species). **Methods**. Specimens of *Aplysina* sp were collected by scuba diving at two different depths, 10 m and 2 m, while *Ircinia* sp samples were collected at 2 m. The

DNA of sponge-associated bacteria was extracted from 1 cm^3 of tissue, purified and sent to the NGS Core at Argonne National Laboratory, IL, USA for amplicon sequencing. Primer trimmed pair-ended bacterial 16S rDNA gene sequences were merged using Ribosomal Database Project (RDP) Paired-end Reads Assembler. Chao1, Shannon and Simpson (alpha) biodiversity indices were estimated with the 'iNEXT' package. Permutational analysis of variance (PERMANOVA) was performed with the 'adonis' function from the package 'vegan', and Bray-Curtis distance estimations were calculated using the 'vegdist' function. **Results**. The analyses showed that seven phyla were the most abundant among all samples from *Aplysina* sp. Those phyla were: Proteobacteria, Acidobacteria, Cyanobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, and Planctomycetes. In *Ircinia* sp

the dominant phylum was Acidobacteria. Through a PERMANOVA analysis, the effect of depth and species on bacterial community structures was estimated, observing that depth was the main factor influencing the bacterial community. The analysis of similarities (ANOSIM) showed a significant difference between the bacterial communities from different depths. Finally, through an unsupervised bi-clustering analysis, outcomes of this approach were found also highlighting clustering of the sponge samples based on depth instead of species. The microbial diversity analysis strongly confirms and suggests that sponge bacterial communities are modeled mainly by the environment (depth) rather than host (sponge species).

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Abstract

Background. Marine sponges are considered harboring one of the richest microbial symbiont communities, which inhabit from shallow to mesophotic ecosystems providing a comfortable place to many symbiotic species. Although the assemblage of the associated bacterial communities can be affected by depth, marine sponges are also known to share a uniform microbial population from different regions. Implementing next-generation sequencing (NGS) approaches to characterize their microbial communities has notoriously increased precision and quantity of the taxonomic complexity studies associated with these organisms. This study assessed depth effect on the assemblage of bacterial communities associated with two marine sponges of the genera *Aplysina* (Nardo, 1834) and *Ircinia* (Nardo, 1833) in rocky reefs from Punta Arena de la Ventana (Gulf of California) and Pichilingue (La Paz Bay) in the coast of Baja California Sur, Mexico to estimate if the sponge bacterial communities are modeled by the environment (depth) or by the host (sponge species).

Methods. Specimens of *Aplysina* sp were collected by scuba diving at two different depths, 10 m and 2 m, while *Ircinia* sp samples were collected at 2 m. The DNA of sponge-associated bacteria was extracted from 1 cm^3 of tissue, purified and sent to the NGS Core at Argonne National Laboratory, IL, USA for amplicon sequencing. Primer trimmed pair-ended bacterial 16S rDNA gene sequences were merged using Ribosomal Database Project (RDP) Paired-end Reads Assembler. Chao1, Shannon and Simpson (alpha) biodiversity indices were estimated with the 'iNEXT' package. Permutational analysis of variance (PERMANOVA) was performed with the 'adonis' function from the package 'vegan', and Bray-Curtis distance estimations were calculated using the 'vegdist' function.

Results. The analyses showed that seven phyla were the most abundant among all samples from *Aplysina* sp. Those phyla were: Proteobacteria, Acidobacteria, Cyanobacteria, Chloroflexi, Actinobacteria, Bacteroidetes, and Planctomycetes. In *Ircinia* sp the dominant phylum was Acidobacteria. Through a PERMANOVA analysis, the effect of depth and species on bacterial community structures was estimated, observing that depth was the main factor influencing the bacterial community. The analysis of similarities (ANOSIM) showed a significant difference between the bacterial communities from different depths. Finally, through an unsupervised bi-clustering analysis, outcomes of this approach were found also highlighting clustering of the sponge samples based on depth instead of species. The microbial diversity analysis strongly

confirms and suggests that sponge bacterial communities are modeled mainly by the environment (depth) rather than host (sponge species).

Introduction

Marine sponges are considered harboring one of the richest microbial symbiont communities (Taylor et al., 2007; Simister et al., 2012) because they contain abundant associated microorganisms that reach up to 50% of their total weight (Hentschel et al., 2003; Usher et al., 2004). Sponge tissues provide a comfortable place to many symbiotic species, such as heterotrophic bacteria, facultative anaerobes, dinoflagellates, cyanobacteria, archaea, fungi, and even viruses (Webster & Hill, 2001; Schippers et al., 2012). Remarkably, several studies have already reported a wide range of microbial phylum composition from 15 to several tenths or even more associated with marine sponges (Taylor et al., 2007; Webster and Thomas, 2016; Villegas-Plazas et al., 2019). Implementing next-generation sequencing (NGS) approaches to characterize marine sponge microbial communities has notoriously increased precision and quantity of the taxonomic complexity associated to these marine organisms (Schmitt et al., 2011; Webster & Taylor, 2012; Reveillaud et al., 2014).

Moreover, the complex microbial communities of marine sponges have been unveiled through NGS coupled with microbial diversity analyses to highlight that sponge microbiomes are largely host-specific and often stable across temporal scales under specific environmental conditions (Morrow et al., 2015; Weigel & Erwin, 2015; Morrow, Fiore & Lesser, 2016; Cleary et al., 2019). Sponges can inhabit from shallow to mesophotic ecosystems – in deep water they are apparently less influenced by abiotic factors, such as temperature, light, salinity changes due to water drags or even by anthropogenic impact (Kahng, Copus & Wagner, 2014; Olson & Kellogg, 2010). Otherwise, in shallow ecosystems these abiotic factors might exert a direct influence on the sponges, thus, also on their associated microbial communities. Some studies have determined sponge associated microbial community changes at different water depths from shallow (0-30 m) to mesophotic areas (30-150 m) (Olson & Kellogg, 2010; Lesser, Slattery & Leichter, 2009; Kahng, Copus & Wagner, 2014). Though the specificity of the sponge microbiota appears more related with host phylogeny, differences in depth can be showing variance between microbial communities in shallow and deep reefs (Steinert et al., 2016)

However, to our knowledge no studies are available that evaluate whether among the same shallow water sponges (0-30 m) the community varies according to its range of distribution. Although changes in abiotic factors are not as evident, as it could occur in mesophotic zones (30-150 m), the depth gradient could influence the composition of the microbial community associated with these sponges (Olson & Gao, 2013; Steiner et al., 2016).

Aplysina species are often associated with shallow rocky reefs. This species belongs to the Verongiida order and are distributed along the East Pacific from Mexico to Panama (Caballero-George et al. 2010; Cruz-Barraza et al. 2012). With respect to species of the genus *Ircinia*, they are conspicuous and abundant in areas exposed to light in rocky-coral biotopes (Parra-Velandia & Zea, 2003) and more abundant in localities near sources of continental discharge with greater turbidity and load of organic material in suspension (Zea, 1994).

This study assessed the effect of depth on the assemblage of the bacterial communities associated with two marine sponges of the genera *Aplysina* (Nardo, 1834) and *Ircinia* (Nardo, 1833) – associated with rocky reefs from Punta Arena de la Ventana (Gulf of California) and Pichilingue (La Paz Bay) in the coast of Baja California Sur, Mexico – to estimate if the sponge bacterial communities are modeled by the environment (depth) or by the host (sponge species).

Materials & Methods

Sample collection and heterotrophic bacteria isolation

Aplysina sp specimens (n = 8) were collected in triplicate by scuba diving in Punta Arena, Baja California Sur, Mexico (24 ° 03 '24 "N and 109 ° 49 '23" W) at different water depths (2 m - 10 m). For this study, the depth of 2 m was considered shallow and 10 m as deep (Apl-S: 2m; Apl-D 10 m).

Three specimens in triplicate of *Ircinia* sp were collected in the Pichilingue locality inside La Paz Bay in Baja California Sur (24 ° 17 '33” N and 110 ° 19 '48” W) at 2 m depth (Fig. 1) (Permission Secretaría de Medio Ambiente y Recursos Naturales SEMARNAT-08-049b Positive

Ficta). The sponge samples were placed in sterile plastic bags and transferred to ice. In the laboratory, the epibiont organisms were removed and washed three times with sterile natural sea water, the outermost layer or pinacoderm was separated with a scalpel and mesohyl pieces were cut from different areas of the sponges, placed in tubes and frozen at -20 ºC. The identification of the sponges was carried out by Dr. Cristina Vega Juárez from the Bentos Laboratory of the Institute of Marine Sciences and Linmology of the Universidad Autónoma de México (UNAM), using dichotomous keys and bibliography for the East Pacific on sponge taxonomy (Goméz et al, 2002; Cruz-Barraza & Carballo-Cenizo, 2008; Carballo-Cenizo & Cruz-Barraza, 2010).

Total DNA extraction

Pieces of approximately 1 cm^3 were taken from each of the samples and their replicates to form a composite sample; they were finely fragmented with a scalpel; 500 µL of TE buffer (10 mM Tris-HC1 containing ethylenediaminetetraacetic acid (EDTA) 1 mM Na2, Thermo Fisher Scientific, Waltham, MA, USA) were added and sonicated for 10 min to detach the bacteria (Bransonic 3510). Then, they were centrifuged at 8000x g for 10 min, and the supernatant was placed in another 2 ml tube for DNA extraction. After that, the method of Phenol: Chloroform: isomilic was used (Sambrook & Russell, 2002; Caamal-Chan et al., 2019). The DNA was resuspended in 50 µl of TE buffer (pH 8.0). Total DNA was treated with RNase A (10 mg mL^{-1}, Promega, Madison, WI, USA) at 37 °C for 30 min. The integrity of the DNA was analyzed by agarose gel electrophoresis. Purity (λ 260 nm / 280 nm ratio) and quantity were evaluated with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were stored at -20 °C.

16S V4 rDNA sequencing

Microbial 16S rDNA gene V4 regions were amplified using primer 515F (5′-GTGC CAGCMGCCGCGGTAA-3′) and 806R (5′-GGAC TACHVGGG TWTCTAAT-3′) (Kozich et al., 2013). Amplicons of 16S rDNA were generated using Illumina MiSeq 500-cycle kit with Illumina MiSeq sequencing platform in the Next Generation Sequencing Core at Argonne National Laboratory, Argonne, IL, USA.

Sequence processing and microbial diversity analysis

Primer trimmed pair-end bacterial 16S rDNA gene sequences were merged using Ribosomal Database Project (RDP) Pair-end Reads Assembler. The assembled sequences with an expected maximum error adjusted Q score less than 25 over the entire sequence were eliminated. VSEARCH (v2.4.3, 64 bit) was used to remove chimeras de novo, followed by removing chimeras by reference using RDP 16S rDNA gene (Rognes et al., 2016). High quality and chimera-free sequences were then clustered at 97% sequence similarity by CD-HIT (4.6.1) and RPD Classifier with a confidence cutoff at 50% (Cole et al., 2014). These sequences resulted in the identification of unique operational taxonomic units (OTUs) and their abundance in each sample (Wang et al., 2007; Fu et al., 2012; Bonder et al., 2012; Chen et al., 2013). The operational taxonomic units (OTU) were analyzed with the R programming language, using custom scripts (www.r-project.org). To calculate the biodiversity indices of Chao1 and Shannon and Simpson (alpha) "iNEXT" was used (Hsieh, Ma & Chao, 2016). The data were normalized, the frequency of the best hits for each individual taxon for each metagenome was divided by the total number of hits per sample. Finally, the PERMANOVA statistical analysis was performed using the "adonis" function, the Bray-Curtis distance was calculated using the "vegdist" function, and the principal coordinates analysis with the "pcoa" function, all of them using the package "vegan" (Oksanen et al., 2014).

Results

Sequencing run metrics

From all the samples sequenced, 379 392 reads were generated; after processing, 85 818 low-quality reads and chimeras were removed to keep high-quality pair-end-assembled reads, of which 146 787 reads could be assigned to prokaryotic taxa. The sequencing effort was assessed by Good's coverage analysis with a mean value for all sample reads of 70.64% ± 0.055% and a completeness analysis (full coverage reached below 5000 reads) (*Fig. S1*). A total of 1 102 OTUs were obtained by similarity clustering at 99% nucleotide identity and 786 OTUs after singleton removal within each sample. The raw sequence reads are deposited in NCBI BioProject Database Accession number: PRJNA760541.
(https://dataview.ncbi.nlm.nih.gov/object/PRJNA760541?reviewer=ht1lj2o5cn5tkk782fcjqloktp)

Microbial communities associated with sponges

The resulting OTUs for this study showed that seven phyla were the most abundant among all samples from *Aplysina* sp (10 m depth), *Aplysina* sp and *Ircinia* sp (2 m depth) with a minimal presence of Archaea (0.56% - 1.65% of the total classified reads). Proteobacteria represented the most abundant phylum (85.84%) for *Aplysina* sp (10 m) and was among the most abundant for *Aplysina* sp and *Ircinia* sp (2 m) samples (31.42% and 31.35%, respectively) (Fig. 2a).

Acidobacteria represented the most abundant (47.11%) phylum for *Ircinia* sp samples and also was abundant (9.23%) for *Aplysina* sp (2 m) sample, in contradistinction to *Aplysina* (10 m) samples, where this phylum had a low relative abundance (Fig. 2a). Cyanobacteria was among the most abundant (4.69% - 21.73%) phylum for all samples, which was the second most abundant (21.73%) for *Aplysina* sp (2 m) sample (Fig. 2a). Chloroflexi was only abundant in *Aplysina* (2 m) samples (15.96%) (Fig. 2a). Bacteroides and Actinobacteria were among the most abundant phyla for *Aplysina* sp (2 m) (4.56%) and *Ircinia* sp samples (Fig. 2a). Planctomycetes was among the most abundant phylum for shallow *Aplysina* sp samples (4.44%) (Fig. 2a). Instead, Proteobacteria showed a higher abundance of 2- and 3-fold than *Aplysina* sp (10 m) for *Aplysina* sp (2 m) and *Ircinia* sp (Fig. 2a). Interestingly, 30.1% of the total OTUs were shared among all samples analyzed (Fig. 2b). *Aplysina* sp (2 m) samples showed the highest amount of specific OTUs (24.5%), followed by *Ircinia* sp 13.2% and *Aplysina* sp (10 m) 11.7% samples (Fig. 2b). Both *Aplysina* sp samples (2 and 10 m) showed the highest ratio of exclusively shared OTUs (10.4%) (Fig. 2b).

Microbial community diversity and depth effect on sponge species

To determine bacterial taxonomic diversity, richness, and evenness of the bacterial communities associated to sponges -both 2 and 10 m samples- alpha diversity indices were estimated by the OTUs rarefaction sampling curves (Fig. 3a) and through rarefaction (interpolation) and extrapolation (R/E). Sampling curve analysis for Chao1 (order q = 0) (Fig. 3b), Shannon (order q = 1) (Fig. 3c), and Simpson (order q = 2) (Fig. 3d) indices showed differences due to curve clustering of the samples analyzed (Fig. 3). Furthermore, the principal coordinates analysis (PCoA) and constrained correspondence analysis (CCA) were performed to determine the clustering of the *Aplysina* sp (10 m), *Aplysina* sp (2 m), and *Ircinia* sp samples of bacterial

communities. The clustering either for PCoA and CCA (Fig. 4a-b, respectively) showed two well-defined and discrete groups based on sample depth regardless of the species (*Aplysina* sp or *Ircinia* sp).

To estimate depth and species effect on the bacterial community structures for deep and shallow *Aplysina* sp and *Ircinia* sp samples, a permutational analysis of variance (PERMANOVA) was applied. The PERMANOVA analysis showed that depth was the main factor influencing the bacterial community structures in the sponge samples ($R_2 = 0.507$, $P = 0.008$), and sponge species did not have a significant effect ($R_2 = 0.184$, $P = 0.122$). Moreover, the PERMANOVA analysis for depth interaction species showed that depth ($R_2 = 0.507$, $P = 0.004$) was the main factor influencing bacterial community structures regardless of sponge species ($R_2 = 0.110$, $P = 0.110$). Furthermore, an analysis of similarities (ANOSIM) was performed to determine differences among the bacterial communities. The ANOSIM analysis showed a significant difference between depths in the bacterial communities (Fig. 5). Finally, an unsupervised bi-clustering analysis was performed based on sample correlation to estimate the degree of relationship among samples and support the beta diversity analyzed (PCoA and CCA). Interestingly, the outcome of this approach also highlights sponge sample clustering based on depth instead of sponge species (*Fig. S2, Fig. S3*).

Discussion

This study examined the associated prokaryotic communities with *Aplysina* and *Ircinia* sponges were examined for the first time from the Gulf of California. These sponges are very abundant in Baja California Sur, and were initially collected in order to evaluate their potential production of bioactive compounds and to isolate the heterotrophic bacteria associated with them. Differences were observed in the cultivable bacteria isolated between the sponges of the same genera and between genera (Gándara-Zamudio, 2011; Aguila-Ramírez, 2012; Ortíz-Aguirre, 2012), it was considered that these differences were probably related to the site and the depth at which they were collected, for this reason it was considered that the characterization of the communities prokaryotes from both sponges by sequencing the 16S gen, would allow us to know the diversity of bacteria and archaea associated with the sponges and thus be able to explain the differences found in the antimicrobial activity tests. *Ircinia* is only found in shallow areas without a depth

slope, unlike *Aplysina*, which is distributed in a rocky reef that ranges from 2 to 10 m deep. However, when analyzing the results of the sequencing, a similarity was found between the bacterial community of the sponges collected in the shallowest areas, therefore, the analysis was carried out focusing on the effect of depth on the bacterial diversity of these two genera of sponges.

A variation was found in relative abundance of the bacterial phyla associated with *Aplysina* sp at different depths. Archaea were present in a low abundance percentage. This low abundance could have been since specific primers for archaea were not used. For example, other authors (Chaib De Mares et al., 2017) have mentioned that when bacterial-specific primers were used, only 6% of the readings were classified as Archaea. On the other hand, when Archaea-specific primers were used, this proportion was 89%. The phylum Thaumarchaeota was the most abundant within archaea and was found in all samples. However, the 2-meter *Aplysina* samples were more abundant. This phylum – Thaumarchaeota – is known to comprise Nitrifying archaea, which was highly abundant in 14 investigated sponge species (Dat et al., 2018).

The most abundant phylum at both depths was Proteobacteria, which was evidenced that it was the predominant (85%) phylum for the deeper water samples (Fig. 2a). Proteobacteria have also been previously reported as a prominent group of sponge-associated microbial communities and highly abundant in the marine environment whether as planktonic or as symbiotic organisms (Li et al., 2006; Jasmin et al., 2015; Dat et al., 2018). This phylum has a direct role contributing to biogeochemical cycles through extracellular enzyme production besides performing some symbiotic functions in sponges, such as nitrogen fixation and secondary metabolite production for the chemical defense of the host (Stabili et al., 2014). Furthermore, the Cyanobacteria and Chloroflexi phyla showed a high proportional abundance in the *Aplysina* samples from the shallow zone. Those phyla have also been characterized with a remarkable transcriptional activity of genes directly involved in photosynthesis and carbon fixation and also responsible for converting ammonia into nitrate in marine sponges (Han, Li & Zhang, 2013; Bibi & Azhar, 2021). The high proportion of these bacteria in the shallowest sponges could be explained

because the light intensity required for photosynthesis is greater in this area (Erwin et al., 2012; Souza et al., 2017; Glasl et al., 2019; Fiore et al., 2020).

As reported by Hardoim et al. (2021), the prokaryotic communities associated with *A. caissara* and *A. fulva* were very similar among these two species and dominated by Chloroflexi, Proteobacteria, Crenarchaeota, and Acidobacteria. In contrast, in this study, Chloroflexi were only represented with a high relative abundance in 2-m *Aplysina* samples and Crenarchaeota did not represent an important component in any of the samples. However, these same authors (Hardoim et al., 2021) mentioned that community composition was largely different for *Aplysina* species. For instance, the most abundant phyla encountered in *A. fulva, A. cauliformis, A. archeri, A. cavenicola,* and *A. aerophoba* sampled in seven distinct sites were assigned to Proteobacteria, Chloroflexi, unclassified bacteria, Acidobacteria, and Actinobacteria (Thomas et al., 2016), or *A. fulva* collected in Brazil with the community dominated by Cyanobacteria, Proteobacteria, and Chloroflexi (Hardoim et al., 2009), which coincides with the phyla with the highest relative abundance in the 2-m *Aplysina* samples collected at Punta Arena BCS.

Global bacterial community structure for shallow samples has a similar proportion of Proteobacteria phylum (Fig. 2a). For *Ircinia* sp samples Acidobacteria and Proteobacteria were the most abundant phyla, which is consistent with the results of different studies (Mohamed 2007, Schmitt et al., 2007; Schmitt et al., 2008; Mohamed et al., 2008; Yang et al., 2008; Yang et al., 2011; Pita Galán, 2014; Hardoim et al., 2012; Pita, López-Legentil & Erwin, 2013; Engelberts et al., |2020) where they report that the core bacterial community associated with this genus is made up of seven phyla Proteobacteria, Acidobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria, Firmicutes and Nitrospira. Despite being a very abundant and diverse group, the Acidobacteria phylum is not as well studied as Proteobacteria, so very little information is available on the species belonging to this phylum in marine environments. The advance in the functional genome analysis, such as that reported by Engelberts et al., (2020). These authors analyzed specific genes involved in metabolic pathways and biogeochemical cycles and found that some species of Acidobacteria participate in denitrification, nitrification, ammonification, metabolism of the taurine, exopolysaccharide production and synthesis of B complex vitamins.

Acidobacteria have also been found in a high percentage in sponge species, such as *Xestospongia testudinaria* and *Luffariella variabilis*, but these bacteria had not been reported as predominant in sponges of the genus *Ircinia* (Webster et al., 2013) in most studies, Proteobacteria and Cyanobacteria are mentioned as the main components of the community associated with different species of *Ircinia* (Hardoim & Costa, 2014). It should be noted that this study would be the first report where the abundance of Acidobacteria is greater than that of proteobacteria for the genus *Ircinia*. For the shallow *Aplysina* samples, the most abundant were Proteobacteria, Cyanobacteria, Chloroflexi and Acidobacteria; deep *Aplysina* samples were dominated only by the Proteobacteria phylum. These prokaryotic taxa with a high relative abundance in this study are also abundant in other marine sponges (Moitinho-Silva et al., 2017; Dat et al., 2018).

In coral ecosystems the sponge-associated microbial community was observed to be more influenced by host identity (Steinert et al., 2016). This study found that sponges of different species collected in shallow waters (*Aplysina* 2 m and *Ircinia*) share more OTUs than among the same sponge at different depths (*Aplysina* 2 and 10 m) (Fig. 2b). Additionally, the *Aplysina* samples at 10 m and those at 2 m showed characteristic and discrete OTUs distributions (Fig. SF3). Environmental variability is an important factor in the sponge microbial community. A stable isotopic analysis in giant barrel sponge *Xestospongia muta* showed changes in the relationship $_{15}N$ / $_{13}C$ in sponges as depth increased (transition from dependency on photoautotrophy to heterotrophy), leading to a more stable microbial community along the depth gradient (Morrow et al., 2016).

A high proportion (~30%) of shared OTUs for *Aplysina* and *Ircinia* might be due to the ecophysiological similitudes among those species, since they are inhabiting similar reef areas, high diversity of organisms and undergoing the same selective pressures (Souza et al., 2017; Pearman et al., 2019; Turon et al., 2019). However, a higher proportion of shared OTUs for *Aplysina* samples was expected for both depths (10.4%) since those samples belong to the same species, and because other studies have found that host-specific prokaryotic communities are stable despite geographical and temporal differences (Erwin et al., 2015; Hardoim & Costa, 2014; Dut et al., 2019), to a greater extent in this case which is the same location and at the same time. Several phyla are stable in *Aplysina* samples collected at different depths, but their relative

abundance percentage differs markedly. In the deepest samples a clear predominance of Proteobacteria is observed, while in those at 2 m, the highest abundance percentage is divided mainly into three phyla, which include Proteobacteria, Cyanobacteria and Chloroflexi. Although this study does not have data on environmental parameters, other studies have found that the temperature difference between shallow areas of the reef and deep sites averaged 4^{o} C (from 3 m to 91 m deep), which was unlikely to affect sponge-microbial communities. Studies examining the effect of elevated temperatures found no change (at sub-lethal temperatures) in sponge bacterial communities during short term experiments (Webster et al., 2008; Simister et al., 2012; Steinert et al., 2016). Therefore, and due to the presence of bacteria of the phylum Cyanobacteria and Chloroflexi in greater abundance at 2 m, might imply that light intensity plays an important role in community changes, as other authors have suggested (Lesser et al., 2010).

Alpha diversity indices for richness and evenness estimated with rarefaction (interpolation) and extrapolation (R/E) sampling curves showed the clustering for *Aplysina* deep samples and overlapping both *Aplysina* and *Ircinia* shallow samples (Fig. 3). The beta diversity PCoA and CCA analyses showed a clustering directly related with depth instead of a relationship among sponge species supported with PERMANOVA and ANOSIM analyses (Fig. 3-4). The clustering analyses (PCoA and CCA) showed two well-defined groups, one corresponding to *Aplysina* bacteria collected in deeper areas and the other one that includes *Aplysina* and *Ircinia* bacteria from the shallow area. Interestingly, these findings are in accordance with the variation in the bacterial community structure assemblage, which is influenced directly by environmental factors, such as depth, temperature, and light intensity and not by the host sponge species (Maldonado & Young, 1998; Thoms et al., 2003; Olson & Gao, 2013; Morrow, Fiore & Lesser, 2016; Thomas et al., 2016; Pearman et al., 2019; Souza et al., 2019). These results differ from those reported in other studies. Gantt et al. (2019) found that bacterial communities exhibited a high degree of host specificity with greater intraspecific than interspecific similarity between locations and detected a significant effect of location on microbial diversity and composition within each host sponge species.

Despite the sample size differences among *Aplysina* for 2 m and 10 m depth, we were able to determine significant differences due to the characteristic prokaryotic structure assemblage for

every depth analyzed (Fig. 2a, Fig. 5, and SF3).The sponge microbiome characterization has the full potential to be applied as a tool to determine environmental perturbations, such as those derived from pollution, xenobiotics spills, red tides, and others derived from the global climate change that has been affecting in the last years.

Conclusions

The environment plays a fundamental role to provide the appropriate conditions to sustain and preserve life that inhabits it. Marine ecosystems are not the exception, and it is even more relevant that the environmental conditions play an essential role to influence directly into the biological diversity, especially for those organisms that are attached to the marine ground, such as corals, sponges, algae.

Therefore, the performed microbial diversity analysis strongly suggests that abundance of the phyla that make up the sponge bacterial communities is mainly influenced by the environment (depth) rather than host (sponge species).

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Figure 1

Geographic localization of sponge sample collecting. Pichilingue and Punta Arena locations in Baja California Sur.

Each station is indicated in red. White bar represents 100 km. Map data © Google, Data SIO, NOAA, U.S. Navy, NGA, GEBCO, Image Landsat/Copernicus. Data LDEO-Columbia, NFS, NOAA

Figure 2

Microbial assemblage of *Aplysina spp.* and *Ircinia spp.* at different depths.

a) Microbial diversity structure and b) Venn diagram for OTUs shared between *Aplysina spp*. and *Ircinia spp.* samples at different depths.

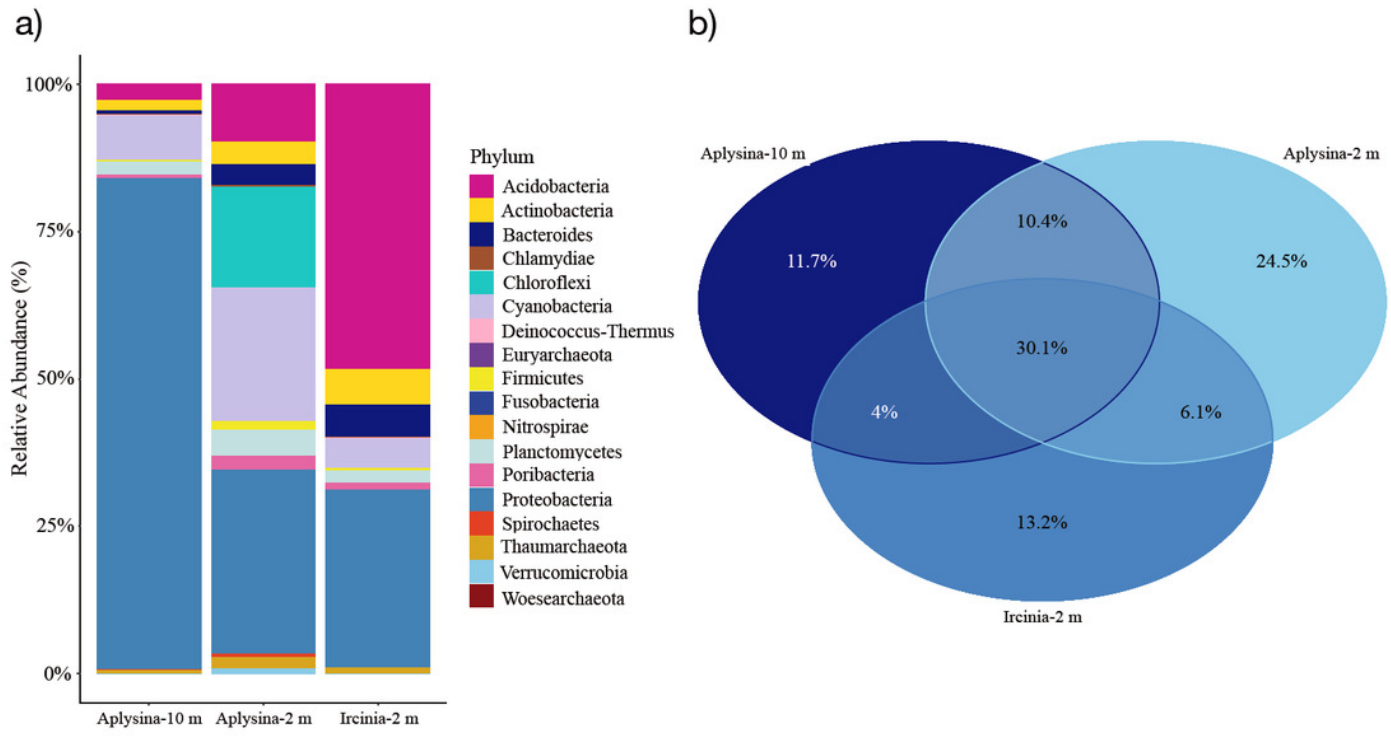

Figure 3

Rarefaction sampling curves and alpha diversity estimations for *Aplysina sp* and *Ircinia sp* samples at different depths

a) Rarefaction sampling curves, b) Chao1 index estimations (q = 0), c) Shannon index estimations (q = 1), d) Simpson index estimations through rarefaction (interpolation) and extrapolation (R/E) sampling curves

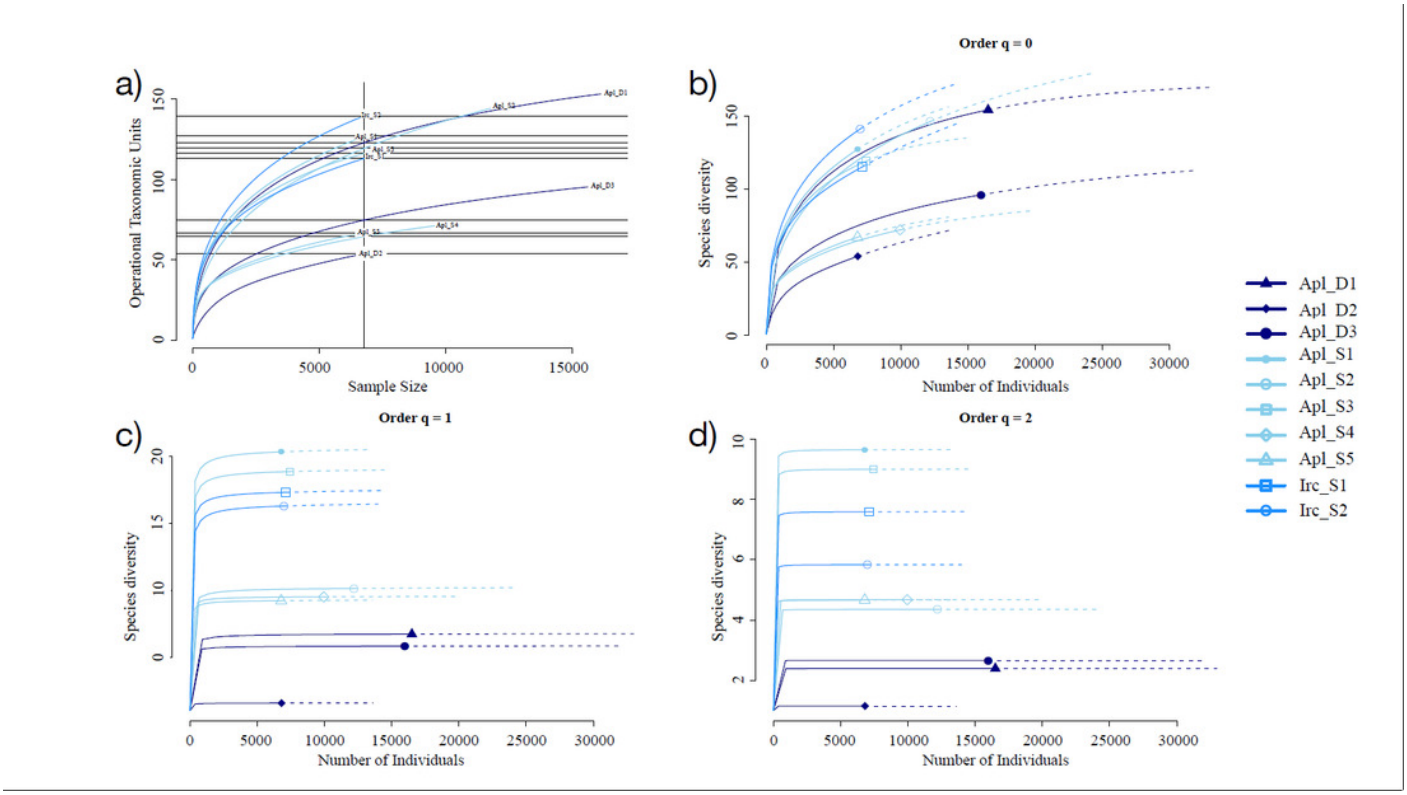

Figure 4

Sample clustering analysis of *Aplysina sp* and *Ircinia sp* samples at different depths

a) Principal coordinates analysis and b) canonical correspondence analysis for *Aplysina sp* and *Ircinia sp* samples at different depths

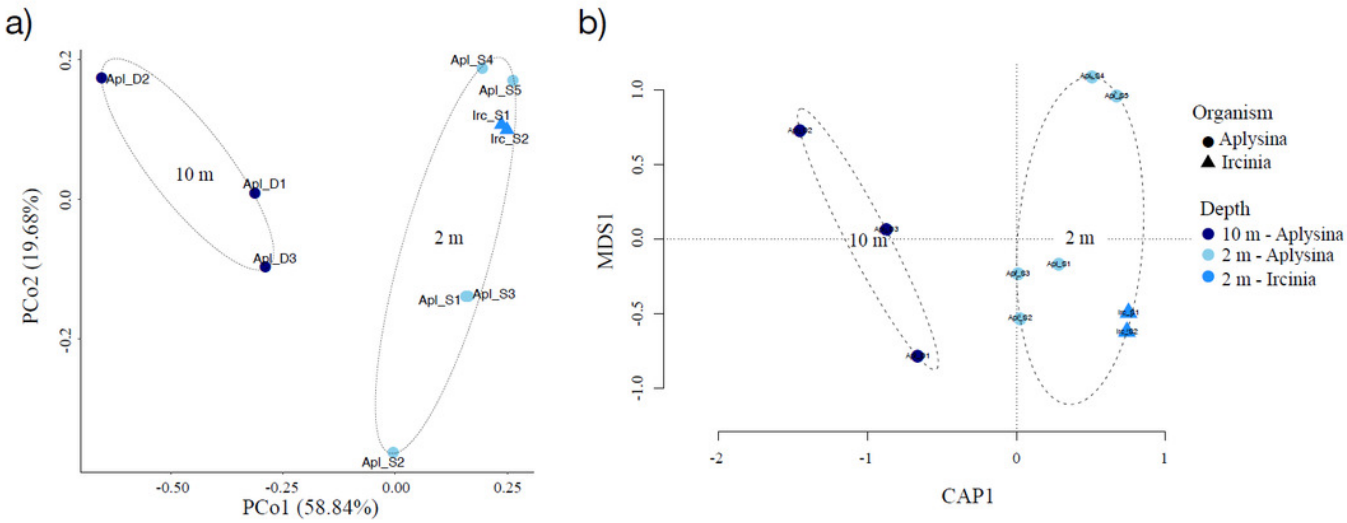

Figure 5

Analysis of similitude (ANOSIM) between sponge samples. ANOSIM analysis showed differences due to depth between *Aplysina sp* and *Ircinia sp* samples