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Registration Welcome Notification for **Antonie van Leeuwenhoek**

Antonie van Leeuwenhoek (ANTO) <em@editorialmanager.com> to me Thu, 24 Feb 2022, 16:42

Dear Dr Indraningrat,

You have registered as a user on the Editorial Manager site for **Antonie** van Leeuwenhoek.

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When you registered, you created your own password. For security reasons, passwords are never sent by email. If you forget your password, please click this link:
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ANTO-D-22-00075 : Submission Confirmation

Antonie van Leeuwenhoek (ANTO) <em@editorialmanager.com> to me Thu, 24 Feb 2022, 17:23

Dear Dr Indraningrat,

Thank you for submitting your manuscript, *Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths*, to **Antonie** van Leeuwenhoek.

The submission id is: ANTO-D-22-00075

Please refer to this number in any future correspondence.

During the review process, you can keep track of the status of your manuscript by accessing the journal website.

Your username is: indra_ningrat
If you forgot your password, you can click the 'Send Login Details' link on the EM Login page at <https://www.editorialmanager.com/anto/>

Should you require any further assistance please feel free to e-mail the Editorial Office by clicking on "Contact Us" in the menu bar at the top of the screen.

Thank you very much.

With kind regards,
Springer Journals Editorial Office
Antonie van Leeuwenhoek

Gmail interface showing an email from Christopher Dunlap (em@editorialmanager.com) to me. The email subject is "Your Submission ANTO-D-22-00075". The email content includes a "Revision Due" calendar event for Friday, June 17, 2022, and an "Agenda" for Friday, June 17, 2022. The main text of the email reads:

Dear Dr Indraningrat,

We have received the reports from our advisors on your manuscript, "Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths", submitted to **Antonie** van Leeuwenhoek.

Based on the advice received, I have decided that your manuscript can be accepted for publication after you have carried out the revisions as suggested by the reviewer(s).

Attached, please find the reviewer's comments for your perusal. You are kindly requested to also check the website for possible reviewer attachment(s).

Please submit your revised manuscript online by using the Editorial Manager system.

Your username is: Indra_ningrat
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Please submit your revised manuscript as a Word file.

I am looking forward to receiving your revised manuscript before 17 Jun 2022.

In order to add the due date to your electronic calendar, please open the attached file

Thank you very much.

With kind regards,
Christopher Dunlap

Browser address bar: <https://mail.google.com/mail/u/0/#search/antonie/FMfcgzGqPpgGSHXzwrMqKZPHkdVNPpNx>

Gmail interface showing an email from Christopher Dunlap (em@editorialmanager.com) to me. The email subject is "Your Submission ANTO-D-22-00075R1 - [EMID:d68bcab4fa4c4458]". The email content reads:

Dear Dr Indraningrat,

We are pleased to inform you that your manuscript, "Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths", has been accepted for publication in **Antonie** van Leeuwenhoek.

You will be contacted by Author Services in due course with a link to complete the grant of rights. Please note that you will receive your proofs after the publishing agreement has been received through our system.

Please remember to quote the manuscript number, ANTO-D-22-00075R1, whenever inquiring about your manuscript.

With best regards,
Christopher Dunlap
Editor

Antonie van Leeuwenhoek

Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths

--Manuscript Draft--

Manuscript Number:	ANTO-D-22-00075R1	
Full Title:	Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths	
Article Type:	Original Article	
Section/Category:	Ecology, genomics and evolution of specialised metabolism	
Keywords:	sponges; prokaryotic community; depth; Antimicrobial activity	
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	FP7 People: Marie-Curie Actions (607786 (BluePharmTrain))	Dr. Detmer Siphema
Abstract:	<p>In this study, we used 16S rRNA gene amplicon sequencing to investigate prokaryotic community composition of the Caribbean sponges <i>Xestospongia muta</i> and <i>Agelas sventres</i> from three depth ranges: < 30 m (shallow), 30–60 m (upper mesophotic), and 60–90 m (lower mesophotic). The prokaryotic community in shallow samples of <i>X. muta</i> was enriched in Cyanobacteria, Chloroflexota, and Crenarchaeota compared to samples from mesophotic depths, while mesophotic samples of <i>X. muta</i> were enriched in Acidobacteriota. For <i>A. sventres</i>, relative abundance of Acidobacteriota, Chloroflexota, and Gammaproteobacteria was higher in shallow samples, while Proteobacteria and Crenarchaeota were enriched in mesophotic <i>A. sventres</i> samples. Antimicrobial activity was evaluated by screening crude extracts of sponges against a set of Gram-positive and Gram-negative bacteria, a yeast, and an oomycete. Antibacterial activities from crude extracts of shallow sponge individuals were generally</p>	

higher than observed from mesophotic individuals, that showed limited or no antibacterial activities. Conversely, the highest anti-oomycete activity was found from crude extracts of *X. muta* individuals from lower mesophotic depth, but without a clear pattern across the depth gradient. These results indicate that sponge-associated prokaryotic communities and the antimicrobial activity of sponges change within species across a depth gradient from shallow to mesophotic depth.
Keywords: sponges, prokaryotic community, depth, antimicrobial activity

Dear editors,

We would like to thank the reviewers for their time, efforts and feedbacks to improve our manuscript.

We have carefully addressed every comment given by the reviewers and have made some changes in the manuscript and supplementary files as suggested. Details for every response are available along with this letter.

We hope that the revised manuscript is now suitable for publication in *Antonie van Leeuwenhoek*.

With kind regards,

Anak Agung Gede Indraningrat

On behalf of all authors

Comments for the Author:

Reviewer #1: Revision of the manuscript "Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths" by Indraningrat and colleagues submitted to *Antonie van Leeuwenhoek*. It is an interesting study, especially because the effect of depth on the sponge microbiome has seldom been investigated, and thus it is more than welcomed. However, I have numerous questions, comments and suggestions.

Thank you for your very thorough reading of the manuscript and your helpful suggestions. This is highly appreciated.

Abstract

Lines 36-37: In "For *A. sventres*, relative abundance of Acidobacteria, Chloroflexi, and Gammaproteobacteria was higher in shallow samples, while Proteobacteria and Thaumarcheota were enriched in mesophotic *A. sventres* samples." Please standardize, Proteobacteria or the respective class for both. My option would be for the class as it is becoming more often to refer to the Proteobacteria classes, instead of the phylum.

*The reviewer is correct that the use of phylum level for all phyla except for Proteobacteria, for which we used a class-level designation throughout the manuscript is phylogenetically not entirely consistent. However, we have chosen to do so as Proteobacteria often represent a very large proportion of the bacterial community in sponges and hence the use of the classes of Proteobacteria in combination with phylum names for the other groups is often done to describe the major groups of bacteria in sponges. E.g., Pita et al., 2018 (Pita L, Rix, L, Slaby B, Franke A, Hentschel U. *Microbiome* 6, 2018), Thomas et al., 2016 (Thomas T, Moitinho-Silva L, Lurgi M, et al. *Nature Communications* 7, 2016), Dat et al., 2021 (Dat TTH, Steinert*

G, Cuc NTK, Smidt H, Sipkema D. Front Microbiol 12, 2021). Therefore, we have opted to follow this pragmatic use of taxonomic indicators.

Line 39: Congratulations for including an oomycete in the panel for the antimicrobial activity, because I have never seen before!

Thank you!

Introduction

Line 60: Remove "R.L." from "Simister, R. L.", please.

Done!

Lines 62 and 99: "Defence" and "Defense", please select the appropriate spelling and check throughout the ms.

We use the word defence following the UK spelling and have adapted throughout the manuscript content, except for references that originally use the word defense (US spelling)

Lines 63-65: I would safely say more than 7000 compounds. Please, have a look specially into the Figure 5 from Carroll et al., 2019. Natural Product Reports. Marine natural products. DOI: 10.1039/c8np00092arsc.li/npr

Indeed, the number of compounds is growing rapidly. Reviewer 2 also made a comment on this, even suggesting over 11,000 compounds. We have adopted his/her suggestion including the reference to MarinLit.

Lines 79-81: Please note that although the example is valid, the present study used sponge extracts for the antimicrobial tests. Thus, the origin of such activity, host of microorganisms, can not be determine.

Indeed, we agree that our study has focused on sponge extracts, but we consider it is still relevant to shows diversity of biosynthetic gene clusters of sponges from different depth as ultimately, they encode the machinery to produce many compounds with bioactivity although we cannot directly link biological activity to presence of certain gene clusters. We included the example because so few studies exist on specialized metabolite-encoding gene clusters from sponges beyond shallow water.

Lines 103-107: Plakortis angulospiculatus was transplanted from 10 to 75 and from 75 to 10, so it was a dual transplantation, right? If so, I believe an adjustment to the phrase needs to be done, as it is "the reciprocal transplantation of Plakortis angulospiculatus from 10 to 75 m".

The adjustment of phrase has been made.

Lines 111-115: Do the authors know the origin of those compounds, sponge or microorganisms? Also, the only activity of sventrin was feeding deterrence?

Based on these cited references, those compounds are reported from sponge and bromopyrrole alkaloids are commonly found in sponges, but the sponge also included its microbes and as such it is not known whether it is produced by the sponge or by its microbes, an issue common to many bioactive compounds isolated from marine invertebrates. Indeed, the only reported bioactivity of sventrin up to now is feeding deterrence.

Material and Methods

Lines 124-125: Just as a curiosity. Which was the range of sampling from the shallow species within the <30m? Actually, I noticed the depth details of each sample are in Table 1. Thus, the authors might want to cite this table here.

X. muta specimens were taken at average 27 m and A. sventres specimens were taken at average 20 m. The depth of sampled individuals can also be found at the bottom of figure 2. We think that a reference to the figure may therefore even be more insightful than to Table 1 at this point in the paper and now referred to it.

Lines 140-141: Concentrations were most likely checked with spectrophotometer and the quality with agarose gel, correct? If so, please clarify it.

It is correct. The sentence has been clarified

Lines 146-147: Are the authors sure that they used the degenerated primer pair? The primer pair sequencing provided does not have any degeneration as the original primer pair was described by Folmer et al., 1994 (LCO1490F and HCO2198R). Besides, the dgLCO1490F and dgHCO2198R were described by Meyer et al., 2005. Please see <https://www.spongebarcoding.org/primers.php>

Thank you for the correction. We used many primers sets to amplify COI amplicons including the non-degenerate one that was wrongfully included in the submitted manuscript. We used multiple primer sets because of difficulties to obtain amplicons from all samples. Ultimately, the degenerate primer pair turned out to be best and we have followed the PCR program as described by Meyer et al (2005), but referred to the wrong primers. We have fixed these mistakes in the manuscript.

Lines 151 and 171: Two distinct way to say the same thing: 1 uL DNA (10-20 ng) and 1 uL template DNA (10-20 ng/ul). I would opt for the former.

We have changed line 171 as suggested.

Line 158: Whether I am not wrong the fragment size of COI gene is 640 bp, how the fragment from A. sventres is 707 bp? Perhaps a fragment of the primer was left on the sequence.

Indeed, the standard fragment size of COI is approximately 640 bp, but the maximal length could reach approximately 710 bp. The final sequence did not contain primer sequences

since these sequences were trimmed. Difference of COI fragment length between X. muta and A. sventres happened after quality checking and trimming lower quality sequence fragments.

Lines 159-160: Which method was used for the phylogenetic inference? Please, provide the references for the maximum likelihood algorithm.

We have updated the text in the manuscript to provide more details: the phylogenetic tree was constructed in MEGA6 using the maximum likelihood algorithm with 500 bootstrap replicates. We applied the Nearest-Neighbor-Interchange (NNI) to optimise tree topology.

Lines 165-166: The EMP changed the name of the primer pair 515FY and 806RB to 515F-806R, please have a look into the site again.

We have updated the name of the primer pair following the newest information in the EMP website.

Line 177: Barcodes were added on both primers?

Yes

Line 185-189: Please consider to rephrase to "Specifically, raw data was analyzed using NG-Tax (Galaxy version 1.0, Ramiro-Garcia et al., 2016), which is an open sequencing platform for high-throughput 16S rRNA gene amplicon analysis and has been applied to detect prokaryotic composition from different niches (Wampach et al., 2018, Deng et al., 2021, Dat et al., 2018, Edwards et al., 2020), with forward and reverse paired-end reads being trimmed to 70 nucleotides". Why the fragments were so short? with the primer pair used, the length from each primer should be around at least 150 bp.

Indeed, the sequence product from each primer should have length of 150 bp. However, the best quality taxonomic assignments with NG-TAX are obtained with 70 nt per read (Ramiro-Garcia et al., 2016). As mentioned in the Methods, each 70 nt forward read was later concatenated with paired reverse reads to a total of 140 nt.

Lines 191-192: I would suggest to use the latest version of the Silva, which is more comprehensive than v. 128. Recent articles are shifting from OTUs to ASVs, the authors might want to do the same. Did mitochondria was not part of the dataset? Usually, chloroplast and mitochondria are removed, the first appears within cyanobacteria classification and the latter, within proteobacteria. Please, verify it.

Indeed, we agree with reviewer that the Silva database originally used was rather old. It was because the completion of the manuscript took a lot more time than projected for various reasons. Therefore, in the revised manuscript we have re-classified our data using the latest SILVA database v138 and have replaced the term OTUs to ASVs (which technically they already were) as suggested. With this new classification we found mitochondria which indeed appeared within Proteobacteria. We have updated the methods section (Line 192) that we removed Chloroplasts and Mitochondria.

Lines 203-204: I believe the "Benjamin-Hochberg method" needs a reference(s).

A reference about Benjamin-Hochberg method is added as suggested

Lines 207-208: Why only at phylum level? Most likely the differences between sponge species and among depth would be noticed at lower taxonomic level. Please, consider it.

*We show differences at the phylum level (Now Figure 2) to give a general overview on how similar or dissimilar prokaryotic community composition is among *X. muta*, *A. sventres* and seawater across different depths. Detail variation of ASVs among sponge specimens and seawater samples were visualised in the heat map (Figure 3). In addition, the beta diversity plot exploring the impact of depth was based on a distance matrix of differences in relative abundance of ASVs.*

Line 210: In "across all samples." is seawater included or only sponges?

"Across all samples" includes both sponge samples and seawater samples.

Line 214: Why "subOTU sequences" I am not familiar with the spongeEMP online server, because I tried to use it couple of times without success. Could the authors provide more details, please?

subOTU sequences refers to OTU sequences from the EMP database that had no mismatch or 1 mismatch with the most abundant OTUs (now ASVs) in this study. To do the analysis, we initially performed a local BLAST database (example on how to make database <http://georg.iba-science.de/makeblastdb.html>) by using sequences of sponge EMP as database and the most abundant ASVs from our study as the query. A sequence in the local database that matched with our OTU sequence was selected and uploaded to the spongeEMP online server to be checked whether it was part of the sponge-enriched cluster or not. We did not provide all this information in the Methods of this paper, but refer to Dat et al., 2018, where a more elaborate description is included in the Methods.

Line 222: I do not think that "tissues" is needed in "sponge holobiont tissues", as holobiont already includes the sponge + microorganisms.

It is right that "sponge holobiont tissue" may be a bit too much. We deleted the word "holobiont" and kept "tissues" as we believe that "sponge tissue" may be most common way to describe it.

Line 226: 427 g, the "g" should be italicized.

The letter g has been italicized

Line 227: Please a space is missing in "drynesswith".

A space is added

Line 229: Please change from "obtained crude extracts" to "the crude extracts obtained".

Done

Line 235: It sounds a bit strange "animals-related microbial species". Please, consider rephrase.

The sentence has been rephrased.

Lines 236-239: I would suggest rephrase this piece by removing the redundance, in this case the number of bacterial and fungal strains. Can be something like: "Briefly, the following growth media were used for the bacterial and yeast strains: liquid Lysogeny Broth medium (LB, Oxoid) for E. coli, Nutrient broth (Oxoid) for A. salmonicida and B. subtilis, Trypticase Soy Yeast Extract for S. simulans (DSMZ medium no. 92) and Universal Medium for Yeast (DSMZ medium no. 186) for C. oleophila until an optical density of 0.5 was reached, measured at 660 nm."

Suggestion was adapted.

Results

Lines 273-275: I think the authors need to change the order of the text or the figures. For instance, Figure 1 has nothing to do with permanova and/or PCoA graphs. The entire paragraph shows the results of Figure 2. Then, the next paragraph will describe Figure 1.

We followed the reviewer's suggestion. In the revised manuscript, we have swapped figure 1 and figure 2. Now each figure is in line with the text.

Line 273: "between sample types" or among sample types? Please, add "and" between A. sventres and seawater and remove the comma in "X. muta, A. sventres, seawater"

Done

Line 275: Please refer to Figure 2a, instead of Figs. 1-3.

Done

Lines 275-277: I believe the order of Suppl table 3 has to change, as the first comparison in the ms is between each sponge species and seawater and between sponge species. Note also that the title of the comparison is "Sample type (sponge pairs), but the comparisons include seawater as well.

Thanks for the suggestion, we have switched table A into table B. Now "sample type" comes before "depth".

Lines 278-279: In "contributed only 9 % to the variance of the prokaryotic community composition" where can I find the 9%? It is not in Figure 2. Also, please cite the correct figure.

The value 9% can be found in Table 2, in parameter depth and seawater under the column R². It is indeed not in a figure and we removed the reference to Fig. 2.

Line 280: Where one can find the "32, 18 and 66 %"? They certainly did not come from the PCoAs. Please, cite the correct figure.

This value can be found in Table 2 as well, in parameter depth and seawater under the column R². Also, here the reference to the figure was removed.

Line 281: Please add "respectively" after "p = 0.001, 0.009, and 0.003".

Done

Line 283: I do not see the need to say "category(ies)", I would suggest to remote them, as it was also not used previously.

The word "categories" has been removed as suggested

Lines 284-288: Please, provide the right figure. Standardize the way to refer to the depth, please. Also, how about *A. sventres*? It was the only one that showed significant difference between UM and Shallow. Carefully, check all the tables for the sponge species, it seems there is a space missing between genus and species.

A reference to Figure 1B has been added.

The way to refer depth has been standardized. Previously, in lines 284-288 we used the word "category" after each depth e.g. shallow, upper and lower mesophotic and also refers depth as "water layer". We have removed words "category, categories and "water layer" and have changed them into "depth" through out the manuscript.

*Indeed, there was a significant difference between UM and shallow water in *A. sventres* prokaryotic communities. This was not observed for *X. muta* and seawater.*

A space missing for some entries in tables between sponge genus and sponge species was found and has been corrected.

Lines 290-293: Note that the name of the phyla changed recently and because the Silva database used was kind of old, the names are how they used to be in the past (<https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/ijsem.0.005056>). Even though there are still controversy, there are some databanks that are using the new names, for instance Silva and GTDB. Was there any phylum present only in *A. sventres*?

Thanks for the information. Indeed, we previously used the older version of SILVA database. Therefore, in the revised manuscript, we have updated the total number of phyla and name

following the latest SILVA database. Even, with this current update we did not obtain a phylum that was exclusively present in A. sventres.

Lines 294-296: However, those five OTUs were not among the most abundant, right? Thus, how relevant do you think there really are for the sponge hosts?

Those 5 OTUs (now ASVs) were not the 5 most abundant OTUs, but present among the 100 most abundant OTUs (see Fig. 3). The fact that they were present among the most abundance OTUs could indicate their importance for the host. For example, the deltaproteobacterium OTU254 was found consistently in X. muta and A. sventres individuals, but not in seawater. Therefore, it is likely it is at least somehow adapted to living inside sponges.

Lines 302-304: I understand the authors' statement and the analyses, but I was wondering how significative it really was, once only in one replicate it was highly abundant and, in the rest, it was moderate to low abundant.

In this paragraph we only write about ASVs that are among the most abundant ASVs in the dataset. The ones varying with depth may not be the most dominant ones, but are among the 100 most abundant ASVs. It is not clear which ASVs we mention that “in one replicate it was highly abundant and, in the rest, it was moderate to low abundant” or is that only a theoretical question? Because the OTUs mentioned for X. muta are not ambiguous in this respect.

Lines 305 and 313. I did not expect that Thaumarchaeota would decrease its abundance with depth as it happened with X. muta, and in opposite to what occurred with A. sventres. Interesting.

Indeed. Not completely surprising though. For seawater we clearly expected to see an increase with depth, but the Thaumarchaeon (now it has changed into Crenarchaeotal ASVs in the new classification) most dominant in seawater is a different one than the ones in X. muta that are again different ones than the one in A. sventres. This is not a functional explanation, but may be linked to the explanation.

Lines 335-336: However, according to the definition ZOI >10 mm would be strong, but between 5 and 10 would be moderate. XM11 and XM12 were around 8, so they would be moderate and not strong.

Thanks for the correction. The word strong on line 337 has been changed to moderate

Discussion.

Line 368: Please, remove the name from the reference Simister et al., 2012.

The word “Rachel” has been removed from the cited reference Simister et al 2012

Lines 370-372: The first time I read the "this value reduced by 82% to 162 uMol" it was a bit confusing. I understood, however, I believe it would be easier to follow if the authors described as they did in the next line "reduced to 162 uMol (82% reduction)". Do the authors also measure the irradiance or the values presented are from the same region that the samples were collected?

We adapted the textual change on how to present the reduction of irradiance in line 372. No, we did not measure irradiance at the sampling site. Therefore, we used secondary data from the cited reference. The data measured by Vermeij and Bak are geographically close to our sampling site.

Line 378: The "bleached" meaning is just because of the pale colour, mostly like related with the low relative abundance of cyanobacteria or because *X. muta* showed signs of damage or disease?

Do the authors think that the amount of nutrients along the depth would also influence their results?

*The bleached *X. muta* observed in mesophotic is likely only due to the reduction of Cyanobacteria as showed similar morphology. The impact of nutrients could certainly be a factor affecting prokaryotic communities in sponges. However, we do not have data to support this statement.*

Line 380: Also observed for *Cliona* (doi:10.1007/s00338-016-1402-7)

*Thanks. A short sentence about *Cliona* and the suggested reference have been added.*

Lines 396-413: Have a look into these two articles: Robbins et al., 2021. ISMEJ. A genomic view of the microbiome of coral reef demosponges and Engelberts et al., 2020. ISMEJ. Characterization of a sponge microbiome using an integrative genome-centric approach. They might assist in putative functions for Chloroflexota, Acidobacteriota, Actinobacteriota, Thaumarchaeota and Proteobacteria.

Thanks for the suggested articles. We have added two short sentences to incorporate these 2 references to help explaining putative roles of important phyla in our study.

Lines 437-440: Not sure if I am convinced by this statement. *X. muta* has a diverse microbial community and it is difficult, to my mind, to pinpoint that the reduced levels of cyanobacteria might be related with decrease chemical defense, as there might be several other symbionts responsible for the deterrent compounds.

*We agree that *X. muta* has a diverse microbial composition and other prokaryote symbionts could play a role in synthesizing deterrent compounds. However, the Cyanobacteria was the phylum that was most clearly reduced at greater depth in *X. muta* (Figs. 2 and 3) and Cyanobacteria are known as potent producers of antimicrobials (e.g. Silva-Stenico et al., 2013. DOI: 10.2174/1389201014666131227114846). Therefore, we believe that Cyanobacteria are actually one of the prime candidates related to changes in biological activity, but present this as a soft point as no hard data is available.*

Lines 452-454: I fully agree, but it would be more reasonable if the producer of the anti-Saprolegnia was a culturable microorganisms. Ecologically, it would not be good and sustainable to collect the *X. muta* to produce the extract.

*Indeed, by no means we meant to suggest that collecting *X. muta* for its extracts to produce a compound against Saprolegnia would be a good idea. We also agree that it would be good if the producers of the bioactive compound would be a culturable bacterium so that the production of a compound can be more sustainable. We modified the text a bit to make sure that this is now reflected in the discussion*

Table 1: What is/are the difference(s) between average actual depth and depth category? I think I understood, but still, it is quite hard to get it. Perhaps change from "depth category" to "sequence depth" would help. Another question, what do the authors mean with average phylogenetic diversity (Shannon)? Whether it is Shannon, were the libraries normalized? what was the sequence depth in the normalized dataset?

Perhaps there is indeed too much information. We reduced to only show here "depth category" as the exact depth of individual samples is also included in Fig. 2. Depth category refers to the three zones we sampled: lower mesophotic, upper mesophotic and shallow.

Phylogenetic Diversity (PD) is an alpha diversity index which includes the sum of all branch lengths of the OTUs in the group. It is different from Shannon index and we now included the abbreviation "PD" to more clearly indicate which index it is as indeed the name "phylogenetic diversity" for an index is a bit unfortunate. The data were normalized when creating the OTU table using R package DESeq2 that is integrated in the microbiome package. The average sequence depth after normalization is 66977 reads

Suppl table S2: This table is confusing. For instance, in the third line, the comparison as far as I can tell is between *X. muta* from LM and UM against UM and Shallow, but the category line is seawater. As seawater was also collected, it is kind of difficult to be sure about the comparisons. Perhaps there might a clearer way to present the statistics.

Thanks for the advice. We have revised the initial table by breaking it down into four different tables. We hope it will get clearer.

Suppl tables S4 and 5. I would kindly ask the authors to put the samples in the order in all tables. I believe it would be easier for the readers to access the information. Also standardize the nomenclature used. In Supp table 4a, what "middle" means once in the third column it is upper mesophotic?

*Indeed, the order of ASVs in table S4 looks a bit random. In the revised manuscript, we have ordered them alphabetically based on phylum level from Bacteria to Archaea. The order of table has been arranged based on sample type starting from *X. muta*, *A. sventres* and seawater (for S4) and *X. muta* and *A. sventres* (for S5). Indeed, "middle" was the initial term we used when preparing the manuscript and should be UM.*

Suppl tables 5. I believe something might be wrong here. For *X. muta*, how anova and post hoc for *C. oleophila* can be 0.04 once none extract was active against the yeast?

Thanks for pointing this out. The value 0.04 refers to post hoc test for S. parasitica and not the one for C. oleophila, so that was a mistake that has been fixed now.

Reviewer #2: The review article titled "Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths" authored by Anak Agung Gede Indraningrat et al. investigates the effect of depth on the composition of microbial community associated with marine sponge holobionts. They employ 16S rRNA gene amplicon sequencing to query the prokaryotic profile, which is a well validated experimental method to map the relative abundance of microbiota in marine sponge holobionts. In addition, they explore the dependence of antimicrobial activity of overall sponge metabolome on depth of sponge collection by performing a disk diffusion assay of the sponge crude extracts against gram-positive and gram-negative bacteria, yeast, and oomycete. The authors have demonstrated dexterity in analyzing and reporting the sponge-associated microbiome data. The difference in the prokaryotic profile with changing depth has been discussed with validation from statistical measurements. Antimicrobial activity of crude sponge extracts with changing depth has also been studied. The motivation for the research has been presented in a convincing way and justified with appropriate results

While the authors have provided exciting new information to the scientific community with regards to the effect of depth on the microbial composition and antimicrobial activity in sponge holobionts, the reviewer is of the opinion that the discussions on the microbiome and metabolome were presented as two separate parts of the paper. The readers would benefit from a brief intellectual discussion regarding the correlation (if any) between the changes in the prokaryotic architecture and the metabolomic diversity. For example, does the reduction of cyanobacteria in *X. muta* with depth lead to the reduction in certain metabolites that were contributing to the antimicrobial activity? Similarly, for *Agelas*, is there any comment regarding the reduction of antimicrobial activity with depth?

Our prokaryotic data analysis was based on relative abundance of taxa which cannot be directly linked to quantitative data on antimicrobial activity without introducing a bias. Therefore, we have been cautious to not overinterpret our data and speculate much more beyond our data. We are aware that we do not have all data that would be desirable as GC-MS data and LC-MS data identifying specialized metabolites would be a great addition, but that would almost be a study on its own. In addition, then we would still miss information which metabolite causes which activity. As such, we consider this as the start of a new research project in which we identify interesting trends both for prokaryotic community composition and antimicrobial activity that should in the future be further complemented by metagenomic studies to identify biosynthetic gene clusters present and biochemical studies to identify the bioactive molecules present in the samples.

For a similar reason we did not elaborate in the discussion of changes of bioactivity of Agelas sventres with depth as a comparison between “shallow” and “upper mesophotic” did not yield significant differences in antimicrobial activity for any of the indicator organisms tested.

In addition to the above broad and general suggestion, the following are some minor issues that demand the authors attention:

- 1) Line 53: HMA is high microbial abundance instead of abundant.

Thanks for the correction.

- 2) Line 64: According to MarinLit, there are 11741 metabolites reported from the phylum Porifera.

<https://marinlit.rsc.org/compounds?taxonomy=Porifera%1F%1F%1F%1F%1F%1Ffalse>

Indeed, the number of compounds is growing rapidly. Reviewer 1 also made a comment on this, suggesting over 7,000 compounds. We have adopted your suggestion here including the reference to MarinLit.

- 3) Line 84: "...potential for bioprospecting for novel..." rephrase requested.

We changed the wording into “potential of bioprospecting for novel”

- 4) Line 254: Reference for the grouping into three categories required to provide support to the weak, moderate, and strong classification.

A reference about the grouping is added. In addition, we modified the grouping into four categories namely weak, moderate, strong and very strong following cited reference.

- 5) Line 272: Agelas is also a HMA sponge, any comment from the authors on the lesser number of OTUs observed in seawater.

Indeed, A. sventres is a HMA sponge and in our study, it has a lower number of OTUs compared to X. muta and seawater. However, richness is not the only parameter relevant to determine whether sponges are HMA or LMA. Another relevant type of data is phylum-level diversity. As we can see in figure 2, A. sventres has a substantial number of phyla represented at high relative abundance, which is different from LMA sponges that are typically dominated by one phylum; either Proteobacteria or Cyanobacteria.

- 6) Line 275-277: Does the prokaryotic community composition comparison between the two sponge genera (X.muta and Agelas) include sponges from all depth?

Yes, except that for A. sventres we did not have individuals from the lower mesophotic zone.

- 7) Line 345: "From the crude extracts....." rephrase requested.

The sentence is revised to “Among crude extracts from specimens from the upper mesophotic zone...”

8) Line 352: Paragraph need to be justified.

The paragraph has been justified

9) Fig. 2: The gridlines in the PCoA plot can be removed.

Gridlines have been removed from the PCoA

10) Fig. 2: The sample types in legend for X.muta and A.sventres have to in italics.

X. muta and A. sventres have been written in italics.

11) Fig. 4: The figure quality in the PDF was not good. Blurred and faded text.

We have improved the figure quality in the revised manuscript.

In conclusion, the article provides some preliminary information regarding the effect of depth on sponge-associated microbiota composition and the antimicrobial activity of crude sponge extracts. Considering the novel contribution regarding depth effect in *X. muta* and *Agelas*, the article can be considered for publication after addressing the minor issues as outlined above.

Dear Editor,

We are pleased to submit an original research article entitled “**Sponge holobionts shift their prokaryotic communities and antimicrobial activity from shallow to lower mesophotic depths**” for consideration for publication in *Antonie van Leeuwenhoek*. Our manuscript provides insights for marine ecology studies as well as future bioprospecting of marine sponges that suit with the scope of the journal. Sponges in shallow water have been intensively studied both for their prokaryotic community composition and their antimicrobial activities, however, a limited information is available on how depth influences prokaryotic community and antimicrobial activities in marine sponges.

In this manuscript, we report a significant difference in prokaryotic community composition between the mesophotic and shallow sponge specimens for the sponge species *Xestospongia muta* and *Agelas* sp. Specific ASVs assigned to the phyla Cyanobacteria, Chloroflexota, Acidobacteriota, Actinobacteriota, Proteobacteria and Crenarchaeota were significant contributors to the variance observed along the depth gradient. We also found that antibacterial activities were generally higher from shallow extracts, whereas there were limited or no antibacterial activities from mesophotic-water sponges. On the other hand, a strong anti-oomycete activity was found both in lower mesophotic and shallow extracts of *X. muta*, but without a clear pattern along the depth gradient.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose and this manuscript has been approved by all co-authors.

Best wishes, and trusting that this manuscript will be of interest to the readers of *Antonie van Leeuwenhoek*

Anak Agung Gede Indraningrat, Georg Steinert, Leontine. E. Becking, Benjamin Mueller , Jasper de Goeij, Hauke Smidt and Detmer Sipkema

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1 **Sponge holobionts shift their prokaryotic**
2 **communities and antimicrobial activity from shallow**
3 **to lower mesophotic depths**

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30 **Abstract**

31 In this study, we used 16S rRNA gene amplicon sequencing to investigate prokaryotic community composition
32 of the Caribbean sponges *Xestospongia muta* and *Agelas sventres* from three depth ranges: < 30 m (shallow), 30–
33 60 m (upper mesophotic), and 60–90 m (lower mesophotic). The prokaryotic community in shallow samples of
34 *X. muta* was enriched in Cyanobacteria, Chloroflexotai, and ThaumCrenarchaeota compared to samples from
35 mesophotic depths, while mesophotic samples of *X. muta* were enriched in Acidobacteriota. For *A. sventres*,
36 relative abundance of Acidobacteriota, Chloroflexotai, and Gammaproteobacteria was higher in shallow samples,
37 while Proteobacteria and ThaumCrenarchaeota were enriched in mesophotic *A. sventres* samples. Antimicrobial
38 activity was evaluated by screening crude extracts of sponges against a set of Gram-positive and Gram-negative
39 bacteria, a yeast, and an oomycete. Antibacterial activities from crude extracts of shallow sponge individuals were
40 generally higher than observed from mesophotic individuals, that showed limited or no antibacterial activities.
41 Conversely, the highest anti-oomycete activity was found from crude extracts of *X. muta* individuals from lower
42 mesophotic depth, but without a clear pattern across the depth gradient. These results indicate that sponge-
43 associated prokaryotic communities and the antimicrobial activity of sponges change within species across a depth
44 gradient from shallow to mesophotic depth.

45 Keywords: sponges, prokaryotic community, depth, antimicrobial activity

46

47 Introduction

48 Sponges (phylum Porifera) occupy a wide range of habitats from shallow-water to deep-sea ecosystems
49 and from tropical to polar regions (Downey et al. 2012; Van Soest et al. 2012). In these habitats, sponges fulfil
50 prominent ecosystem functions, such as seafloor structuring, involvement in various biogeochemical cycles, and
51 the provision of shelter for other marine fauna (Bell 2008; De Goeij et al. 2017; Beazley et al. 2013). Sponges are
52 commonly associated with a wide variety of microbial taxa (e.g., bacteria, archaea, eukaryotes) that live within
53 their tissues. Generally, they can be classified as either low microbial abundance (LMA) or high microbial
54 abundance (HMA) sponges (Hentschel et al. 2003). LMA sponges contain microbial communities similar in
55 concentration to the ambient seawater, whereas HMA sponges can host up to four orders of magnitude higher
56 microbial concentrations, which may constitute more than one-third of the sponge holobiont's biomass (Hentschel
57 et al. 2003). Moreover, these communities are generally distinct from those present in the surrounding seawater
58 and remain stable through space and time (Erwin et al. 2012; Pita et al. 2013; Hardoim and Costa 2014; Gantt et
59 al. 2017; Erwin et al. 2015; Enticknap et al. 2006), indicating their specificity for co-habitation with sponges
60 (Schmitt et al. 2012; Simister et al. 2012a; Thomas et al. 2016) Sponge-associated microbial communities are
61 considered to play important roles in nutrient cycles within the host tissue (Keren et al. 2017; Mohamed et al.
62 2010; Taylor et al. 2007; Zhang et al. 2015) as well as in defence mechanisms through the production of bioactive
63 secondary metabolites (Hentschel et al. 2012; Horn et al. 2016; Slaby et al. 2017). To date, more than 511,000 of
64 these sponge-derived compounds have been described and many of these compounds display unique features
65 which are potentially applicable to therapeutic uses (Marinlit).

66 However, ecological and biotechnological studies on sponges and their associated microbial
67 communities are typically performed in easily accessible, shallow-water (< 30 m) depth habitats, for example on
68 tropical coral reefs. Comparable information from deeper parts of those reefs, the so-called mesophotic zone (30–
69 150 m water depth), (Lesser et al. 2018; Kahng et al. 2010; Slattery et al. 2011; Lesser et al. 2009), or a depth
70 gradient are largely unavailable due to more challenging logistics (Morrow et al. 2016; Olson and Gao 2013;
71 Steinert et al. 2016). Mesophotic coral reefs are deeper reef communities whose community structure and
72 function change with increasing depth based on the availability of light and trophic resources (Lesser et al.
73 2018). In particular, primary producers, such as corals and macroalgae decrease in abundance with increasing
74 depth due to light limitation, while sponges were found to increase in abundance (Lesser et al. 2020; Lesser
75 et al. 2018). Mesophotic communities are commonly further divided in upper mesophotic (30–60 m) and
76 lower mesophotic zone (60–150 m) (Lesser et al. 2018).

77 From a biotechnological perspective, especially the functional diversity of secondary metabolism-
78 associated gene clusters in mesophotic habitats and beyond is largely underexplored and may harbour different
79 sources of novel compounds for therapeutic and industrial applications (Sipkema 2017). For example, unique
80 polyketide synthase and non-ribosomal peptide synthetase gene clusters of microbial origin were reported from
81 three deep-sea sponges *Inflatella pellicula*, *Poecillastra compressa*, and *Stelletta normani*, which may provide
82 hints to novel compounds (Borchert et al. 2016). Furthermore, the TARA Ocean study highlighted the sharp
83 increase of unknown functional genes as depth increased (Sunagawa et al. 2015), which further reinforces the
84 potential ~~of for~~ bioprospecting for novel compounds in mesophotic habitats and beyond (Sipkema 2017). Whether
85 similar patterns can be observed across a narrower depth gradient from shallow to mesophotic depths needs to be
86 explored. Differences in the environmental conditions between shallow water and the mesophotic zone relate to
87 light intensity, temperature, nutrient availability, predation, and human impact. These altered (a)biotic factors may
88 lead to differences in microbial community composition in marine invertebrate holobionts (i.e. host and
89 symbionts), including sponges (Morrow et al. 2016; Olson and Gao 2013; Slattery et al. 2016; Steinert et al. 2016).
90 Accordingly, these studies indicate that while sponges may maintain a stable core of associated bacteria from
91 shallow water to mesophotic depth, certain sponge-associated bacterial taxa vary with depth (Olson and Gao
92 2013). A study from the Pacific indicated a significant change of the prokaryotic community composition of the
93 sponge *Callyspongia* sp. From shallow to mesophotic habitats. However, the exact environmental factor(s)
94 responsible for such differences could not be determined (Steinert et al. 2016). Additionally, more comprehensive
95 studies on coral holobionts have indicated morphological adaptations and symbiont specializations with greater
96 depth, with species inhabiting the mesophotic zone harbouring a specific photosynthetic endosymbiont
97 (*Symbiodinium*) community to adapt to low light conditions (Bongaerts et al. 2015; Brazeau et al. 2013; Gonzalez-
98 Zapata et al. 2018; Lesser et al. 2010; Vermeij and Bak 2002).

99 Sponges commonly use secondary metabolites as chemical defen~~ce~~se to deter predation and fouling and
100 to compete for space with neighbouring benthic marine organisms (Pawlik et al. 1995; Loh and Pawlik 2014; Page
101 et al. 2005). In some cases, these compounds also serve as general antimicrobial substances (Webster 2007;
102 Newbold et al. 1999; Sarah et al. 2003). However, little is known about how the metabolomes of sponges change
103 over depth and first studies are rather opposing. While the transplantation of *Aplysina cavernicola* from 40 m to
104 7–15 m did not alter its metabolite profile (Thoms et al. 2003), the ~~clonal~~ transplantation of *Plakortis*
105 *angulospiculatus* from 10 to 75 m ~~and vice versa~~ resulted in a much stronger deterrent effect of shallow-adapted

106 individuals towards predation by the spongivorous pufferfish *Canthigaster rostrata* compared to deep-adapted
107 specimens (Slattery et al. 2016).

108 To further elucidate the effect of water depth on prokaryotic community composition as well as the
109 production and antimicrobial activity of secondary metabolites we studied two Caribbean HMA sponge species—
110 *Xestospongia muta* and *Agelas sventres*— that commonly occur across the entire shallow-to-lower mesophotic
111 depth gradient. Moreover, both species have been reported to produce bioactive compounds. In *X. muta*, multiple
112 secondary metabolites with predator-deterrent and antimicrobial activities were identified (Chanas and Pawlik
113 1997; Morinaka et al. 2007; Patil et al. 1992), whereas only one bioactive compound, the feeding-deterrent
114 compound sventrin, has been reported in *A. sventres* (Assmann et al. 2001). We sampled both species at three
115 depths: < 30 m (shallow), 30–60 m (upper mesophotic depth) and 60–90 m (lower mesophotic depth). For all
116 samples (1) the prokaryotic community composition was determined using Illumina MiSeq 16S rRNA gene
117 amplicon sequencing, (2) the antimicrobial activity of sponge tissue extracts was examined against six microbial
118 indicator strains—two Gram-positive and two Gram-negative bacterial strains, a yeast and an oomycete.

119 **Material and Methods**

120 **Sample collection and sponge tissue processing**

121 *Xestospongia muta* and *Agelas sventres* individuals were collected between 4 and 22 November, 2015
122 on the reef slope in front of the Substation (12°05'04.4"N 68°53'53.7"W) on the leeward side of Curaçao, Southern
123 Caribbean. Samples were collected from three different depths categories—along a shallow-mesophotic depth
124 gradient: < 30 m (shallow), 30–60 m (upper mesophotic), and 60–90 m lower mesophotic) (bottom of Fig. 24).
125 From each depth-category, five biological replicates (i.e. different individual sponges; $n = 5$) were collected for
126 each species. Shallow sponge individuals were collected by SCUBA diving, while upper and lower mesophotic
127 individuals were taken using a submarine, the “Curasub”. Three 1-L seawater samples were collected from each
128 depth category using a Niskin bottle to serve as background seawater prokaryotic community profile. Upon arrival
129 in the laboratory, sponges were cleaned from visible debris (e.g. mud, sand), rinsed three times using sterile
130 artificial seawater (ASW, 33 g L⁻¹ synthetic sea salt [Instant Ocean Reef Crystals, Aquarium Systems, Sarrebourg,
131 France]) and were cut into pieces of ~ 0.1 cm³. Three to four randomly chosen pieces of tissue from each individual
132 were preserved in a 15 mL Falcon tube (Sigma-Aldrich) containing 10 mL of RNAlater stabilization solution
133 (Thermo Fisher Scientific). Seawater samples were filtered through 0.2-µm pore size nitrocellulose filters (Sigma-
134 Aldrich). The preserved sponge tissues and filters were stored at -20 °C until further analysis.

135

136 **DNA extraction**

137 DNA was extracted from sponge samples (~ 200 mg biomass per sample) and seawater filters using the
138 Fast DNA Spin kit for soil (MP biomedical) following manufacturer's instructions with the slight modification
139 by conducting 2 times 45 s of bead beating cell lysis (Precellys 24 Bertin Instruments, Montigny-le-Bretonneux,
140 France). DNA concentrations were checked using a spectrophotometer (DeNovix DS-11, Wilmington, USA) and
141 ~~the quality of DNA were was~~ visualized on ~~a 100 mL of~~ 1 % agarose gel.

143 **Sponge identification**

144 Sponge specimens were identified by manually inspecting the type of spicules of each specimen.
145 Furthermore, molecular identification of sponge samples was conducted by amplifying cytochrome oxidase
146 subunit I (COI) encoding genes using primers dgLCO1490F (5'-GGT CAA CAA ATC ATA AAG AYFA TYFG
147 G-3') and dgHCO2198R (5'-TAA ACT TCA GGG TGA CCA AARA AAYF CA-3') (Meyer et al. 2005). PCR
148 amplification of the COI fragment was performed in a volume of 50 µL containing 28.75 µL nuclease free water,
149 10 µL 5x Green Gotaq Flexi buffer, 1 µL 10 mM dNTPs, 1 µL forward primer (10 µM), 1 µL reverse primer (10
150 µM), 3 µL MgCl₂ (25 mM), 4 µL Bovine Serum Albumin (BSA), 0.25 µL Gotaq HotStart DNA Polymerase (5
151 U/µL) and 1 µL DNA (10–20 ng), following the protocol as ~~previously described by Meyer et al~~ (Meyer et al.
152 2005). PCR products were visualised on a 1 % agarose gel, purified using the Thermo Scientific GeneJET PCR
153 Purification Kit and Sanger sequenced in both directions (GATC Biotech AG, Germany). The chromatograms of
154 forward and reverse COI sequences of each specimen were assembled and quality checked manually using
155 Geneious (Kearse et al. 2012) version 10.0.9. Additionally, six and four reference COI sequences of *X. muta* and
156 *A. sventres*, respectively, were retrieved from the Sponge Gene Tree server (Erpenbeck et al. 2008), along with
157 COI sequences of other sponge species as outgroups. All COI sequences were aligned using MEGA6 (Tamura et
158 al. 2013) with the MUSCLE algorithm resulting in a final sequence length of 644 nt and 707 nt for *X. muta* and
159 *A. sventres*, respectively. Subsequently, phylogenetic trees were generated in MEGA6 based on the COI sequences
160 by applying the maximum likelihood algorithm (Tamura et al. 2011) ~~with 500 bootstrap replicates and the Nearest-~~
161 ~~Neighbor-Interchange (NNI) to optimise tree topology.~~

163 **Prokaryotic community profiling using 16S rRNA gene amplicon sequencing**

164 Prokaryotic community composition was assessed by Illumina MiSeq amplicon sequencing of the V4
165 region of the 16S rRNA gene using a two-step amplification procedure. PCR was conducted using the 2nd version

166 of the EMP (Earth Microbiome Project) primer pair 515F~~Y~~ (5'GTGYCAGCMGCCGCGGTAA3') (Parada et al.
167 2016) and 806R~~B~~ (5'GGACTACNVGGGTWTCTAAT 3') (Apprill et al. 2015). Subsequently, Unitag 1 and
168 Unitag 2 were added to the forward and reverse primer, respectively, as previously described (Van Lingen et al.
169 2017). In the first step PCR, 25 μ L PCR reactions contained 16.55 μ L nuclease free water (Promega, Madison,
170 USA), 5 μ L of 5 \times HF buffer, 0.2 μ L of 2 U/ μ L Phusion hot start II high fidelity polymerase (Thermo Fisher
171 Scientific AG), 0.75 μ L of 10 μ M stock solutions of each primer, 0.75 μ L 10 mM dNTPs (Promega) and 1 μ L
172 ~~template~~-DNA (10–20 ng/ μ L). Amplification was performed at 98 $^{\circ}$ C for 3 min, followed by 25 cycles at 98 $^{\circ}$ C
173 for 25 s, 50 $^{\circ}$ C for 20 s, 72 $^{\circ}$ C for 20 s and a final extension of 7 min at 72 $^{\circ}$ C. PCR products were visualized on
174 a 1 % (w/v) agarose gel. Subsequently, 5 μ L of these first-step PCR products were used as template in the second
175 PCR reaction to incorporate 8 nt sample specific barcodes. The second step PCR was performed in triplicate for
176 each sample in 50 μ L PCR reactions which contained 31 μ L nuclease free water (Promega), 10 μ L of 5 \times HF
177 buffer, 0.5 μ L of 2 U/ μ L Phusion hot start II high fidelity polymerase (Thermo Fisher Scientific AG), 5 μ L
178 equimolar mixes of 10 μ M forward primer (barcode-linker-Unitag1) and reverse primer (barcode-linker-Unitag2),
179 1 μ L 10mM dNTPs (Promega) and 2.5 μ L of the first PCR product as template. The second step PCR was
180 performed for five cycles with the same amplification program as the first step PCR. The PCR products were
181 purified following a method as previously described (Dat et al. 2018), and the purified library was sequenced at
182 GATC Biotech AG (Germany) by Illumina Miseq sequencing.

183

184 **Raw sequence processing**

185 Raw sequence data was processed using a previously described protocol (Dat et al. 2018) with slight
186 modifications. Specifically, raw data was analyzed using NG-Tax (Galaxy version 1.0) (Ramiro-Garcia et al.
187 2016) with forward and reverse paired-end reads being trimmed to 70 nucleotides. NG-Tax is an open sequencing
188 platform for high-throughput 16S rRNA gene amplicon analysis and has been applied to detect prokaryotic
189 composition from different niches (Wampach et al. 2018; Deng et al. 2021; Dat et al. 2018; Edwards et al. 2020).
190 Subsequently, both reads were concatenated, resulting in sequences of 140 bp as an optimum accurate length that
191 was used for further sequence data processing (Poncheewin et al. 2020). Taxonomic assignment was done by
192 utilizing a customized version of the SILVA 1328 SSU database (Yilmaz et al. 2014), and ~~OTUASVs~~ classified
193 as Chloroplasts ~~and Mitochondria~~ were removed from the analysis.

194

195 **Prokaryotic community analysis**

196 Data analyses were performed in R version 3.5.0 (<https://www.r-project.org>) and Microsoft Excel.
197 Community 16S rRNA gene abundance data processing and analyses in R were performed using the following R
198 packages: phyloseq version 1.21.0 (McMurdie and Holmes 2013), microbiome version 0.99.90 (Lahti et al. 2017),
199 and ggplot2 version 2.2.1 (Wickham 2016). The NG-Tax generated phylogenetic OTUASV tree was processed
200 using the ape package version 4.1 (Paradis et al. 2004), and phylogenetic diversity was calculated using the picante
201 package version 1.6-2 (Kembel et al. 2010). Phylogenetic diversity of each group of samples was analyzed using
202 Kruskal-Wallis and Wilcoxon rank sum test to assess significance of potential differences among groups of
203 samples for the parameters “sample types” (i.e. *X. muta*, *A. sventres*, and seawater) and “depth category” (i.e.
204 shallow, upper mesophotic, lower mesophotic). The raw *p*-values were adjusted using the Benjamin-Hochberg
205 method (Benjamini and Hochberg 1995). The prokaryotic community composition was visualized by principal
206 coordinate analysis (PCoA) based on Hellinger transformed relative abundances of OTUASVs using Bray–Curtis
207 distances. The adonis and betadisper functions as implemented in vegan package version 2.5.2 (Kolde 2015) were
208 employed to estimate the variance and dispersion of beta diversity, by applying two factors: “sample type” and
209 “depth category”. ~~Community composition at phylum level was calculated based on average relative abundance~~
210 ~~among specimens.~~

211 A heatmap was generated in R using pheatmap version 1.0.8 (Kolde 2015) for the most abundant
212 OTUASVs ($\geq 0.25\%$, $n = 100$) based on average relative abundance across all samples. Subsequently, the most
213 abundant OTUASVs listed in the heatmap ($n = 100$) were used to identify OTUASVs that were significantly
214 enriched in the 3,569 sponge specimens (comprising 269 sponge species) from the sponge microbiome project
215 (Moitinho-Silva et al. 2017). Sequence comparison was done based on a method described previously (Dat et al.
216 2018). Briefly, sponge microbiome project subOTU sequences were selected based on having no more than one
217 nucleotide mismatch with sequences of the most abundant OTUASVs observed in this study. The selected
218 subOTU sequences were then uploaded to the spongeEMP online server (www.spongeemp.com) to identify
219 OTUASVs that were significantly enriched in sponges. Furthermore, the most abundant OTUASVs were checked
220 by a G-test using the script *group_significance.py* in QIIME version 1.9.1, and raw *p*-values were adjusted using
221 the Benjamin-Hochberg FDR correction for multiple comparisons.

222

223 Preparation of crude extract of sponge tissue and antimicrobial activity screening

224 Crude extracts of sponge holobiont-tissues were prepared based on ~~a previous method the method by~~
225 ~~Rohde et al.~~(Rohde et al. 2015) with a slight modification on the amount of starting tissue samples. Briefly, 0.3 g

226 of lyophilized sponge sample was transferred to a 35 mL glass tube (Kimax) and resuspended in 10 mL
227 methanol:ethyl acetate (1:1). The tube was incubated at room temperature (20 °C) and shaken at 150 rpm for 20
228 min, followed by 10 min of centrifugation at 427 g (Thermo Scientific Sorvall Legend XTR Centrifuge TX-1000,
229 Waltham, Massachusetts). Crude extracts were transferred into pre-weighed glass tubes and evaporated to dryness
230 with a speed-vac (Eppendorf Vacufuge Concentrator, Hamburg, Germany). Extraction of each sponge sample was
231 conducted three times, and ~~the obtained~~ crude extracts obtained from each of the extractions were pooled in the
232 same pre-weighed glass tube and stored at -20 °C until further use.

233 Six microbes were used as indicator strains to evaluate antimicrobial activity of sponge extracts, namely
234 the Gram-positive bacteria *Bacillus subtilis* DSM 402 and *Staphylococcus simulans* DSM 20037, Gram-negative
235 bacteria *Escherichia coli* K12MG1655 and *Aeromonas salmonicida* DSM 19634, the yeast *Candida oleophila*
236 DSM 70763, and the oomycete *Saprolegnia parasitica* CBS223.65. These indicators were selected because they
237 represent ~~human and/or environmental~~ causative agents of disease~~s~~ in animals ~~related microbial species~~. Briefly,
238 the following growth media were used for the ~~four bacterial strains and the yeast strains~~: liquid Lysogeny Broth
239 medium (LB, Oxoid) for *E. coli*, Nutrient broth (Oxoid) for *A. salmonicida* and *B. subtilis*, Trypticase Soy Yeast
240 Extract for *S. simulans* (DSMZ medium no. 92) and Universal Medium for Yeast (DSMZ medium no. 186) for
241 *C. oleophila*. Cultures were grown until an optical density of 0.5 was reached, measured at 660 nm. Subsequently,
242 200 µL of each active culture was spread with a sterile hockey stick on agar media with the same composition as
243 the corresponding liquid media. The oomycete *S. parasitica* was prepared by inoculating agar plugs of 1 x 1 cm
244 from a lawn of fresh *S. parasitica* culture plate on one-fifth strength of Potato Dextrose Agar (PDA, Oxoid) plates
245 supplemented with 1 % of Bacto agar (Oxoid).

246 Antimicrobial properties of each crude extract were examined using the disc diffusion assay (Rohde et
247 al. 2015) by adding 20 µL extract (0.5 mg per disc) to three 6 mm cellulose paper discs (Whatman). Paper discs
248 containing the crude extract were air-dried for 30 min. As negative control, triplicate discs containing 20 µl
249 methanol: ethyl acetate (1:1) were included. Paper discs containing sponge crude extracts were tested against
250 indicator strains on agar plates. Plates containing sponge extracts and indicator strains were incubated at 37 °C
251 for *E. coli* and *S. simulans*, at 30 °C for *A. salmonicida*, *B. subtilis* and at 20 °C for *C. oleophila* for 48 h. The
252 plates containing extracts and *S. parasitica* were incubated at 20 °C for 96 h. After incubation, the radius of the
253 zone of inhibition (ZOI) surrounding each disc was measured to the nearest mm using digital callipers (Perel,
254 Gavere, Belgium), and the average ZOI radius for each extract was calculated from triplicate discs. In addition,
255 to differentiate the level of inhibition from each crude extract, the recorded ZOI radius was grouped into ~~four~~ three

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256 categories: weak (0–5 mm), moderate (6–10 mm), ~~and~~ strong (≥ 10 –20 mm) ~~and very strong~~ (> 20 mm) (Davis
257 and Stout 1971). Analysis of variance (ANOVA) was done to compare average ZOIs formed by sponge crude
258 extracts against indicator strains. Subsequently Tukey Post-Hoc test was applied to assess significance in
259 ANOVA.

260

261 Results

262 Molecular sponge taxonomy

263 Genetic analysis of the COI gene sequences obtained from all sponge samples used for this study showed that all
264 COI sequences of suspected *Xestospongia muta* samples could be assigned to the same species (Supplementary
265 Fig. 1A). In contrast, four out of five of the suspected *Agelas sventres* samples from the lower mesophotic depth
266 ~~category~~ formed a separate clade from the other *A. sventres* samples and *A. sventres* reference COI gene
267 sequences. These four lower mesophotic samples may therefore either represent a new undescribed or another
268 known *Agelas* species for which no COI gene sequence is available (Supplementary Fig. 1B). Hence, all *Agelas*
269 samples from the lower mesophotic depth ~~category~~ were excluded from further analysis as taxonomy could not
270 be unequivocally established.

271

272 Impact of water depth on sponge-associated prokaryotic communities

273 Across 34 samples (25 sponge and 9 seawater samples), 2,277,222 high quality reads were clustered into 4,394
274 ~~Amplicon Sequence Variants~~ Operational Taxonomic Units (OTUs/ASVs) (Table 1, Supplementary Table 1). *X.*
275 *muta* samples yielded the highest number of observed ~~OTUs/ASVs~~ on average, followed by seawater and *A.*
276 *sventres* samples (Supplementary Fig. 2).

277 The prokaryotic community significantly differed ~~among between~~ sample types (*X. muta*, *A. sventres*
278 ~~and~~ seawater) (PERMANOVA, $p = 0.001$) and sample type contributed to 74 % of the variance of the prokaryotic
279 community composition (Figs. 1–3, Table 2, Supplementary Table 2). Post-hoc pairwise comparison showed
280 that both sponges significantly differed in prokaryotic community composition from seawater and from each other
281 ($p = 0.003$ for both comparisons; Supplementary Table 3). Depth ~~category~~ did not have a significant effect when
282 all sponge and water samples were analysed together and contributed only 9 % to the variance of the prokaryotic
283 community composition (Figs. 1–3, Table 2). However, depth ~~category~~ did show a significant effect when the
284 impact on prokaryotic community was analysed per sample type, contributing to 32, 18 and 66 % of the variance
285 for *X. muta*, *A. sventres*, and seawater, respectively ($p = 0.001$, 0.009, and 0.003 ~~respectively~~; Figs. 1–3, Table 2).

286 Post-hoc pairwise comparison showed that for *X. muta* the prokaryotic community significantly changed between
287 the lower mesophotic depth ~~category~~ and both the upper ~~mesophotic~~ and shallow depth ~~categories~~ ($p = 0.02$), but
288 not between the upper mesophotic and shallow depth ~~categories~~ (Supplementary Table 3, Fig. 1B). Within
289 seawater samples, there was a marked significant difference in the prokaryotic community composition between
290 the lower mesophotic ~~depth water layer~~ and the upper mesophotic and shallow ~~depth layers~~ ($p = 0.001$; Figs. 4
291 ~~3D~~; Supplementary Table 3). In turn, the upper mesophotic and shallow ~~depth water layer~~ did not show a
292 significantly different prokaryotic community composition.

293 At phylum level, in total ~~23-29~~ phyla (~~20-26~~ bacterial and 3 archaeal phyla) were identified (Fig. 42).
294 Some phyla were consistently found in all *A. sventres* and *X. muta* samples: Acidobacteri~~ota~~,
295 Actinobacteria/Actinobacteri~~ota~~, Chloroflex~~ota~~, Gemmatimonadetes/Gemmatimonad~~ota~~, Nitrospirae/Nitrospiro~~ta~~,
296 Proteobacteria (Alpha-, Gamma- and Delta-), Spirochaet~~otae~~, Dadabacteria, Myxococc~~ota~~ and
297 Thaumarchaeota/Crenarchae~~ota~~, Bacteroidetes/Bacteroid~~ota~~, Cyanobacteria, Entotheonellae~~ota~~ and
298 ~~AncK6~~Tectomicrobia were present in *X. muta* samples, but absent in *A. sventres* samples. Thermoplasm~~atota~~
299 Euryarchae~~ota~~, and Marinimicrobia were only observed in seawater samples.

300 At the OTUASV level, only 5 of the 100 most abundant OTUASVs were shared between *X. muta* and *A.*
301 *sventres*: OTUASV254 (*Albidovulum*, ~~AlphaDelta~~proteobacteria), OTUASV9 and OTUASV49 (~~AqS1~~,
302 uncultured Gammaproteobacteria), OTUASV147 (Sva0996, ~~Aetino~~Actinobacteri~~ota~~baacteria) and OTUASV75
303 (uncultured bacterium, PAUC34f) (Fig. 3). Furthermore, 20 (of the 100) OTUASVs were 100 % related to sponge-
304 enriched clusters in the sponge EMP database (Fig. 3). These belong to ~~Aetino~~Actinobacteri~~ota~~baacteria (Sva0996),
305 Acidobacteri~~ota~~ (PAUC26f, Subgroup 9, TK85), Chloroflex~~ota~~ (TK10, S085, SAR202, Caldilineaceae),
306 Cyanobacteria (*Candidatus Synechococcus spongiarum* group), Nitrospiro~~tae~~ (*Nitrospira*), Nitrospin~~otae~~
307 (MD2898-B26), Gemmatimonad~~otetes~~ (PAUC43f) and ~~Cren~~Thaumarchaeota (*Candidatus Nitrosopumilus*).

308 The impact of depth was evident as indicated by differences in relative abundance of a number of
309 predominant OTUASVs between the different depth zones. In shallow *X. muta*, the relative abundance of
310 OTUASV200 and OTUASV423 both belonging to Cyanobacteria (*Candidatus Synechococcus*
311 *spongiarum* group, Subsection I) was significantly higher than in deeper samples where OTUASV423 was
312 completely absent (Fig. 3, Supplementary Table 4). Additionally, a significant decrease in relative abundance of
313 OTUASV87 (~~Thaum~~Crenarchaeota, *Candidatus Nitrosopumilus*), OTUASV113 (Actinobacteri~~ota~~, Sva0996
314 marine group), OTUASV29 (Chloroflex~~ota~~, SAR202) and OTUASV81 (Chloroflex~~ota~~, TK10) was observed
315 from shallow to the lower mesophotic in *X. muta* (Fig. 3, Supplementary Table 4). In contrast, the acidobacteri~~otal~~

316 [OTUASV7](#) ([Vicinamibacteriales subgroup 6](#)) and [OTUASV28](#) (subgroup 11) had a significantly higher relative
317 abundance in the lower mesophotic compared to individuals from the upper mesophotic and the shallow waters.

318 In *A. sventres*, four [OTUASVs](#) had a significantly higher relative abundance in shallow than in specimens
319 from the upper mesophotic: [OTUASV503](#) (Acidobacteriota, PAUC26f), [OTUASV552](#), [OTUASV602](#)
320 ([Chloroflexotai](#), SAR202) and [OTUASV591](#) ([AqS1, uncultured](#) Gammaproteobacteria). In contrast, the relative
321 abundance of [OTUASV514](#) (Proteobacteria, *Endozoicomonas*) and [OTUASV527](#) ([ThaumCren](#)archaeota,
322 [Nitrosopumulaceae Marine group I](#)) was significantly higher in specimens from the upper mesophotic compared
323 to their shallow water counterparts (Fig. 3, Supplementary Table 4).

324 For the seawater [OTUASVs](#) assigned to Cyanobacteria, members of the genera *Prochlorococcus*
325 ([OTUASV809](#) and [OTUASV997](#)), ~~and~~ *Synechococcus* ([OTUASV-816](#)) and *Cyanobium* ([OTUASV-842](#)) were
326 present at significantly higher relative abundance in shallow and upper mesophotic samples than in the lower
327 mesophotic seawater samples (Supplementary Table 4). These cyanobacterial [OTUASVs](#) in seawater were
328 different from those observed in *X. muta*. The same trend was observed for [OTUASV812](#)
329 (~~ActinoActinobacteriota~~ *Actinobacteria*, *Candidatus Actinomarina*). On the other hand, a significantly increased relative
330 abundance in deep seawater samples was seen for [OTUASV681](#) ([ThaumCren](#)archaeota, *Candidatus*
331 *Nitrosopelagicus*) and [OTUASV808](#) (Gammaproteobacteria, *Acinetobacter*) as compared to middle and shallow
332 seawater samples.

333

334 **Antimicrobial activity of sponge tissue samples**

335 All 15 *X. muta* and 10 *A. sventres* crude extracts (i.e. from holobiont tissue samples) were screened for
336 antimicrobial activity against six different indicator strains (Fig. 4A). For *X. muta*, recorded antibacterial activity
337 came mainly from four (out of the five) shallow specimens, of which XM14 was the only crude extract that
338 inhibited three of the four bacterial strains: *Bacillus subtilis*, *Staphylococcus simulans*, and *Aeromonas*
339 *salmonicida* (Fig. 4A, Supplementary Table 5). Crude extracts from shallow specimens XM11, XM13 and XM15
340 produced small ZOI radii against *E. coli*. The only non-shallow *X. muta* specimen with antibacterial activity was
341 XM7 (upper mesophotic depth) that was found active against *S. simulans*, whereas none of the lower mesophotic
342 *X. muta* specimens showed antibacterial activity. All *X. muta* extracts were inactive against the yeast *C. oleophila*.
343 In contrast, inhibition of the oomycete *Saprolegnia parasitica* was most prominent for the lower mesophotic *X.*
344 *muta* specimens with two extracts with an intermediate ZOI radius (XM2 and XM4), whereas a large ZOI radius
345 was displayed by XM3 and XM5 extracts. In addition, two shallow crude extracts of *X. muta* (XM11 and XM12)

346 displayed a ~~moderate strong~~ inhibition against *S. parasitica*. Overall, the impact of depth was significant when
347 the average ZOI radii of shallow *X. muta* extracts against *E. coli* were compared with those produced by the upper
348 and lower mesophotic depth crude extracts (Tukey post hoc test, $p = 0.03$, Supplementary Table 5). Additionally,
349 the average ZOI radius of *X. muta* extracts against *S. parasitica* was significantly larger from specimens from the
350 lower mesophotic compared to those from the upper mesophotic depth ~~category~~ (Tukey post hoc test, $p = 0.04$;
351 Supplementary Table 5). For the remaining antimicrobial activities, sampling depth did not have significant
352 impact.

353 For *A. sventres*, all crude extracts from shallow specimens were active against *S. simulans* (Fig. 4B),
354 with weak activities shown for AS7 and AS8 and moderate inhibition for AS6, AS9, and AS10 (Supplementary
355 Table 5). ~~From the Among~~ crude extracts from specimens from the upper mesophotic zone, only AS4 (weak) and
356 AS5 (moderate) were active against *S. simulans*. For all bacteria tested, extracts from shallow *A. sventres*
357 individuals showed higher activity than extracts from the upper mesophotic (Fig. 4B). However, those differences
358 between depth ~~categories~~ were never found to be significant (Supplementary Table 5). No inhibition of the yeast
359 *C. albicans* or the oomycete *S. parasitica* was observed for *A. sventres* crude extracts.

360

361 Discussion

362 In this study, we analysed the prokaryotic community composition of two sponge species, *Xestospongia muta* and
363 *Agelas sventres*, over a depth range from near surface waters to the lower mesophotic zone (0–90 m). Both *X.*
364 *muta* and *A. sventres* as well as the seawater show a shift in prokaryotic communities from the shallow to those
365 of the upper and the lower mesophotic depth, respectively. In addition, we found changes in the activity of
366 metabolites produced by the sponge holobionts at different water depths.

367 A recent study of *X. muta* revealed differences in its prokaryotic community composition between 9 and
368 28 m (Villegas-Plazas et al. 2018). Interestingly, those differences were only present in autumn and not in spring.
369 Observed changes of prokaryotic composition were proposed to be triggered by differences in temperature, light,
370 nutrient, and turbidity between seasons (Villegas-Plazas et al. 2018). Although the depth range of the *X. muta*
371 specimens studied by Villegas-Plazas *et al.* were all in our shallow water ~~depthcategory~~, the potential
372 environmental factors identified in that study to potentially cause differences in prokaryotic community
373 composition have an extended gradient over larger depth. In our study, the recorded average temperature at
374 shallow and lower mesophotic depth were 27 °C and 23 °C, respectively, which were also reported from other
375 studies (Steinert et al. 2016; Lesser et al. 2010). Although temperature difference was evident, it is unlikely that

376 the observed 4 °C difference between depths would be crucial to influence prokaryotic community composition
377 (Steinert et al. 2016). For example, previous studies have shown that the elevated temperature within this range
378 did not alter bacterial composition in sponge (Webster et al. 2008; Simister et al. 2012b). Thus, it is likely that
379 other factors, such as irradiance, play a more pronounced effect to the observed difference of prokaryotic
380 composition. The average irradiance at 5 m water depth in the leeward side of Curaçao was in the range of 900
381 $\mu\text{Mol photons m}^{-2}\text{s}^{-1}$ and this value reduced ~~by 82 %~~ to 162 $\mu\text{Mol photons m}^{-2}\text{s}^{-1}$ (82 % reduction) at 30 m depth
382 and ~~was further exponentially~~ decreased at 50 m to approximately 25 $\mu\text{Mol photons m}^{-2}\text{s}^{-1}$ (93 % reduction;
383 (Vermeij and Bak 2002)). The irradiance at lower mesophotic depth at approximately 80 m was between 10 and
384 19 $\mu\text{Mol photon m}^{-2}\text{s}^{-1}$ (98–99 % reduction from 5 m depth; (Morrow et al. 2016)). Reduced light-levels with
385 increasing depth were reflected in the decreases in the relative abundances of photoautotrophic cyanobacteria in
386 both seawater planktonic and *X. muta*-associated prokaryotic communities (while cyanobacteria were absent in *A*
387 *svetres* both in shallow water and the mesophotic zone). Moreover, the morphological appearance of *X. muta*
388 individuals that were observed during the sampling campaign at mesophotic depth generally displayed a paler
389 colour ('bleached') compared to their shallow counterparts. Such conditions are likely related to a decline of
390 phototrophic symbionts as also observed in individuals of sponge *Petrosia ficiformis* between well lighted and
391 dark areas (Burgsdorf et al. 2014). ~~Also other environmental factors than light may be involved in sponge~~
392 ~~bleaching as observed by the bleaching of the sponge *Cliona varians forma incrustans* at high water temperatures~~
393 (Hill et al. 2016).

394 However, there was a marked difference between planktonic seawater cyanobacterial species and *X.*
395 *muta*-associated species. Cyanobacteria in seawater were dominated by the commonly found genera
396 *Prochlorococcus* and *Synechococcus* (Flombaum et al. 2013; Ma et al. 2009). In contrast, the most dominant
397 cyanobacterial OTUs (OTU200 and 423) found in *X. muta* were identified as "sponge-enriched" (and
398 different from the ones in seawater) and ~~was identified as *Candidatus Synechococcus spongiarum* group, could~~
399 ~~only be identified at class level (Subsection D)~~. Cyanobacteria are commonly found in association with HMA
400 sponges and contribute considerably to the sponge holobiont metabolism via photosynthesis. Additionally,
401 sponges efficiently eat cyanobacteria as part of the planktonic POM pool, specifically *Prochlorococcus* and
402 *Synechococcus* (Yahel et al. 2003; Pile et al. 1996; Morganti et al. 2017). Consequently, the relative abundance
403 of Cyanobacteria in sponge holobionts as well as in seawater is commonly reported to decline following a depth-
404 dependent reduction in light availability (Morrow et al. 2016; Lesser and Slattery 2013; Lesser et al. 2020).
405 However, the increase of inorganic nutrients and of non-cyanobacterial planktonic POM (e.g., heterotrophic

406 bacteria, prochlorophytes) may both serve to mitigate the loss of cyanobacteria along a depth gradient (Morrow
407 et al. 2016; Lesser and Slattery 2013; Lesser et al. 2020). In both cases, the host and the symbionts may shift to
408 increased rates of heterotrophy for compensating the decline in irradiance, due to elevated levels of POM and
409 inorganic nutrients, respectively (Morrow et al. 2016).

410 Similar to Cyanobacteria, ~~OTUASV~~s from Chloroflexota, Acidobacteriota,
411 ~~ActinoActinobacteriota~~ and Proteobacteria contributed significantly to differences
412 between prokaryotic assemblages across different water depths in both sponge species. Sponges are considered
413 as hot spots for Chloroflexota, which are especially prevalent in HMA sponges (Bayer et al. 2018; Schmitt et al.
414 2011). Among the most abundant Chloroflexota OTUASVs identified in both sponges, OTUASV81 (TK10) and
415 OTUASV552 (SAR202) in *X. muta* and *A. sventres*, respectively, were identified as members of sponge-enriched
416 clusters, and their relative abundances declined with depth. Although ecological functions of Chloroflexota in
417 sponges remain unclear, in shallow habitats they may be phototrophic given that some members of this phylum
418 possess Reaction Centre II to capture and utilize sunlight for energy (Nowicka and Kruk 2016; Ward et al. 2018).
419 Furthermore, a metagenomics-based analysis indicated that sponge-associated Chloroflexota genomes were
420 enriched in genes encoding glycosyl hydrolases acting on sialic acid and glycosaminoglycan suggesting their
421 involvement in the degradation of host-derived compounds (Robbins et al. 2021). In *X. muta*, the relative
422 abundance of the two most abundant acidobacteriota OTUASVs, OTUASV7 (Subgroup 6) and OTUASV28
423 (Subgroup 11), increased in deep specimens (Fig. 3). Also, in *A. sventres*, acidobacteriota OTUASV503
424 (PAUC26f) increased in relative abundance in specimens from the upper mesophotic depth. Acidobacteriota are
425 among the prevalent heterotrophic bacterial taxa in sponges (O'connor-Sánchez et al. 2014) and are often regarded
426 for their versatile metabolic capacities, such as nitrite and nitrate reduction, their ability to cope with disturbed or
427 food-limited environments, and their production of exopolysaccharide (EPS), part of the DOM pool (Kielak et al.
428 2016). It is also plausible that Acidobacteriota might be involved in the degradation of recalcitrant organic
429 substrates, which often accumulate in deep water habitats (Quaiser et al. 2008) or in sponge-derived
430 polysaccharides (Robbins et al. 2021). However, due to a low number of samples, the tendency of an increase in
431 the relative abundance of Acidobacteriota with increasing depth was not found to be significant.

432 ~~ActinoActinobacteriota~~ (Sva0996) were represented by members of sponge-enriched clusters
433 with higher relative abundance in shallow specimens of *X. muta* (OTUASV113). The role of Sva0996 in sponges
434 is unknown, however, previous studies suggested this taxon to be present at high nitrate concentrations and in
435 high primary productivity areas (Nelson et al. 2014; Fortunato et al. 2013; Seo et al. 2017). In addition, some

436 [Actinobacteriota in sponges harbour the tauABC gene encoding a taurine transporter to indicate their potential](#)
437 [role in sulphur metabolism](#) (Engelberts et al. 2020). The archaeal phylum of [ThaumCrenarchaeota](#) is mainly linked
438 to ammonia oxidation and has been reported as dominant phylum in sponges both from shallow and deep water
439 (Jackson et al. 2014; Dat et al. 2018; Zhang et al. 2014). No consistent trend was observed for the most dominant
440 [thaumarchaeotal-crenarchaeotal OTUASVs](#) for the different sample types. While [OTUASV87](#) (*Candidatus*
441 *Nitrosopumilus*) is most abundant in *X. muta* in shallow specimens, the relative abundance of [OTUASV527](#)
442 (Marine group I) in *A. sventres* and [OTUASV681](#) (*Candidatus Nitrosopelagicus*) in seawater was highest in
443 deeper samples. Despite the fact that these dominant [thaumarchaeotal-crenarchaeotal OTUASVs](#) do not belong to
444 sponge-enriched clusters, the observed trend in relative abundance may confirm that different members of this
445 phylum are specialized to adapt to distinctive ammonia concentrations and related physical factors (temperature,
446 light intensity, and dissolved oxygen) (Ijichi and Hamasaki 2017). Lastly, the most abundant
447 gammaproteobacterial [OTUASVs](#) that changed with depth in *A. sventres*, [OTUASV591](#) ([no further taxonomic](#)
448 [assignmentAqS1](#)) and [OTUASV514](#) (*Endozoicomonas*), do not affiliate with a sponge-enriched cluster. Some
449 possible roles assigned to members of the genus *Endozoicomonas* in sponges include antibiotic production, nitrate
450 reduction and production of bromopyrrole as a feeding deterrent compound (Neave et al. 2016).

451 Sponges, as many other sessile fauna in reef habitats, need to defend themselves against biofouling,
452 predatory organisms, and/or pathogenic bacteria (Webster 2007; Rohde et al. 2015), but this need may depend on
453 the specific predators/competitors present in a given ecosystem (Becerro and Paul 2004). Sponge holobionts
454 biosynthesize various antimicrobial and deterrent compounds (Helber et al. 2018). *Agelas* species are chemically
455 well-defended sponges by producing a group of brominated-pyrrole-containing alkaloids and are unpalatable for
456 a typical spongivorous fish such as the Bluehead wrasse, *Thalassoma bifasciatum* (Pawlik 2011; Chanas et al.
457 1997). [In addition, and extracts of A. sventres individuals showed generally more diverse and stronger antibacterial](#)
458 [activities than X. muta extracts.](#) *X. muta* is also dominated by brominated compounds and feeding frequencies by
459 parrot fishes—including *Sparisoma aurofrenarum*, *Scarus croicensis*, and *Scarus laeniopterus*—were found to
460 increase in bleached individuals, suggesting the reduced level of cyanobacterial symbionts to be responsible for
461 its decreased chemical defence (Dunlap and Pawlik 1998). However, no direct evidence is available at present on
462 the shifts in production and activity of specific metabolites from the same species over a depth gradient. In this
463 study, we observed a general trend that antimicrobial activity against the four bacterial indicator strains was higher
464 for extracts from shallow sponges than for specimens collected at upper and lower mesophotic habitats, but this
465 difference was generally not significant due to large intraspecific variation between biological replicates.

466 [However, it supports a recent study for a much larger depth gradient for the sponge *Geodia barretti* where the](#)
467 [dominant secondary metabolite, baretin, completely disappeared below a depth of 1000 m](#) (Steffen et al. 2022).
468 Moderate to strong inhibition of *S. parasitica* was observed solely from *X. muta* crude extracts (and not *A.*
469 *sventres*). Although no clear trend related to depth could be observed, the anti-*Saprolegnia* activity is an
470 interesting observation. *Saprolegnia* spp. are fungal-like oomycetes that are parasitic to fish and fish eggs and
471 resistant to a wide range of antifungals, making infections with *Saprolegnia* a serious threat in the aquaculture
472 industries (Earle and Hintz 2014; Hu et al. 2013). Malachite green is the chemical most used to prevent
473 *Saprolegnia* infections, but since the compound is toxic, also to other organisms, it has been banned world-wide
474 (Srivastava et al. 2004; Stammati et al. 2005). Therefore, development of novel anti-*Saprolegnia* drugs is urgent
475 (Earle and Hintz 2014; Takada et al. 2010), and anti-*Saprolegnia* metabolites from [ideally a cultivable bacterium](#)
476 [from](#) the sponge holobiont *X. muta* [would](#) be an interesting new lead.

477

478 **Conclusion**

479 We investigated the impact of depth on prokaryotic community composition and antimicrobial activity
480 associated with the tropical sponges *X. muta* and *A. sventres* from shallow water to mesophotic depth. For both
481 species, depth had a significant impact on the associated prokaryotes with respect to different relative abundances
482 of specific [OTUASVs](#) assigned to Cyanobacteria, Chloroflex[otai](#), Acidobacteri[otaa](#),
483 [ActinoActinobacteriotabacteria](#), Proteobacteria and [ThaumCren](#)archaeota. Clearly, we are just at the beginning to
484 uncover how depth and/or depth-associated environmental conditions can cause shifts in prokaryotic communities
485 and metabolite activity, but we show that these shifts occur. We hypothesize that changes in prokaryotic
486 communities within the same holobiont species may therefore also change their ecological function at different
487 depths, such as their role in chemical defence of their host. Additionally, crude extracts of shallow sponge
488 specimens showed stronger and more diverse antibacterial activities compared to extracts from mesophotic depths,
489 but these differences were not significant.

490

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497

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499 designed the experiments. Anak Agung Gede Indraningrat, Benjamin Mueller and Jasper M. de Goeij performed
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508

509 **Data availability.**

510 Illumina MiSeq raw sequence data can be accessed via the NCBI Sequence Read Archive (SRA) ID: SRP142603
511 with accession numbers SRX3998987–SRX3998883. The COI gene sequences can be accessed at GenBank under
512 the accession numbers: MH285785–MH285814. R markdown file, and the required files for 16S rRNA gene
513 analysis can be found at <https://github.com/mibwurrepo/Indraningrat-et-al.-SpongesDepthGradient2021>

514

515 **Competing of interests**

516 The authors declare no competing of interests

517

518 **Ethics approval.** This article does not contain any studies with human participants and/or animals performed by
519 any of the authors. The formal consent is not required in this study.

520

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763
764 **Tables**

765 **Table 1** Overview of sample data with sample identifiers (ID), sponge species, average actual depth, and depth
 766 category, average number of reads, average number of Θ TUASVs, and average phylogenetic diversity. All values
 767 are given with their corresponding standard deviation. A more detailed description of individual specimens is
 768 available in Supplementary Table 1. LM: lower mesophotic; UM: upper mesophotic.

Sample ID	Species/Sample type	Average depth (m) and Depth category	Average number of reads	Average number of Θ TUASVs	Average Phylogenetic Diversity (PD)
XM1-XM5	<i>X. muta</i>	78.20 ± 8.78 (LM)	53,595 ± 51,996	174 ± 18	17.54 ± 1.11
XM6-XM10	<i>X. muta</i>	50.20 ± 2.05 (UM)	54,253 ± 61,518	179 ± 10	18.28 ± 0.24
XM11-XM15	<i>X. muta</i>	27 ± 0 (shallow)	89,129 ± 69,868	163 ± 14	17.72 ± 0.57
AS1-AS5	<i>A. sventres</i>	53 ± 1 (UM)	63,389 ± 30,710	70 ± 6	11.94 ± 0.86
AS6-AS10	<i>A. sventres</i>	18 ± 8.21 (shallow)	74,756 ± 52,091	70 ± 6	12.38 ± 0.53
SW1-SW3	Seawater	87 ± 4.35 (LM)	93,275 ± 35,371	102 ± 50	12.74 ± 2.78
SW4-SW6	Seawater	46.67 ± 5.77 (UM)	48,825 ± 17,635	148 ± 14	15.36 ± 0.43
SW7-SW9	Seawater	20.33 ± 0.57 (shallow)	58,438 ± 8,580	123 ± 5	14.52 ± 0.30

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770 **Table 2** Multivariate analysis of prokaryotic community data after Hellinger transformation based on parameter
 771 sample type (sponge and seawater), depth (sponge and seawater) and depth for subsets *Xestospongia muta*, *Agelas*
 772 *sventres* and seawater. Df, degrees of freedom

Parameter	Θ TUASVs	Df	PERMANOVA		Betadisper	
			R^2	p -value	F	p -value
Sample types (sponges and sea water)	4,394	2	0.74	0.001	4.46	0.02
Depth (sponges and sea water)	4,394	2	0.09	0.17	1.02	0.4
Depth (<i>X. muta</i> only)	2,576	2	0.32	0.001	1.89	0.2
Depth (<i>A. sventres</i> only)	699	1	0.18	0.009	0.35	0.54
Depth (seawater only)	1,119	2	0.66	0.003	7.19	0.006

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774 Figure Legends

775 **Fig. 1** Principal Coordinate Analysis (PCoA) of prokaryotic community composition of (A) sponges and
 776 seawater samples using Bray-Curtis distance based on relative abundance of Θ TUASVs after Hellinger

777 transformation. Additionally, the PCoA was done separately for each sample type: (B) *Xestospongia muta*, (C)
778 *Agelas sventres* and (D) seawater.

779 **Fig. 2** Prokaryotic community composition of sponge specimens and seawater samples at the phylum level, based
780 on relative abundance of assigned 16S rRNA gene OTUs. Phyla with average relative abundance lower than 0.25
781 % in all samples (*Bdellovibrionota*, *SAR324*, *Planctomycetota*, *NB1-j*, *Deinococcota*, *Schekmanbacteria*,
782 *Nanoarchaeota*, *Desulfobacterota*, *Woesearchaeota*, *Deinococcus-Thermus*, *Planctomyces*) were coloured in
783 black. Sampling depth of each sponge specimen and seawater sample is indicated below each bar (lower
784 mesophotic – black; upper mesophotic – dark gray; shallow – light gray). Individual samples were labelled based
785 on sample type: XM (*Xestospongia muta*), AS (*Agelas sventres*), SW (seawater), followed by sample number.

786 **Fig. 3** Heatmap of OTUASVs with average relative abundance ≥ 0.25 % among all samples. OTUASVs were
787 grouped at phylum level. OTUASVs highlighted in red were identified as “sponge-enriched” in the sponge EMP
788 database. The letter in parentheses for OTUASV taxonomy indicates the lowest taxonomic rank that was obtained:
789 c (class), o (order), f (family), g (genus).

790 **Fig. 4** Average radius of the zone of inhibition and standard deviation of *Xestospongia muta* (A) and *Agelas*
791 *sventres* (B) crude extracts against indicator strains.

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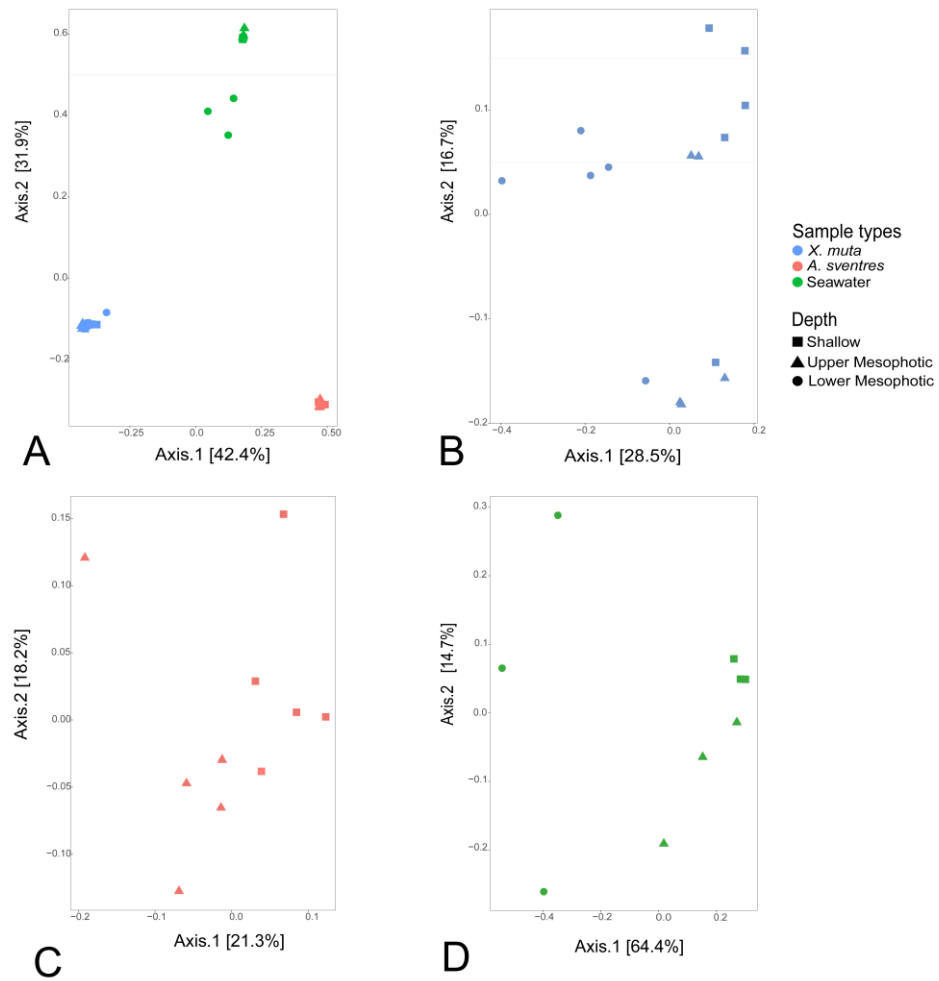
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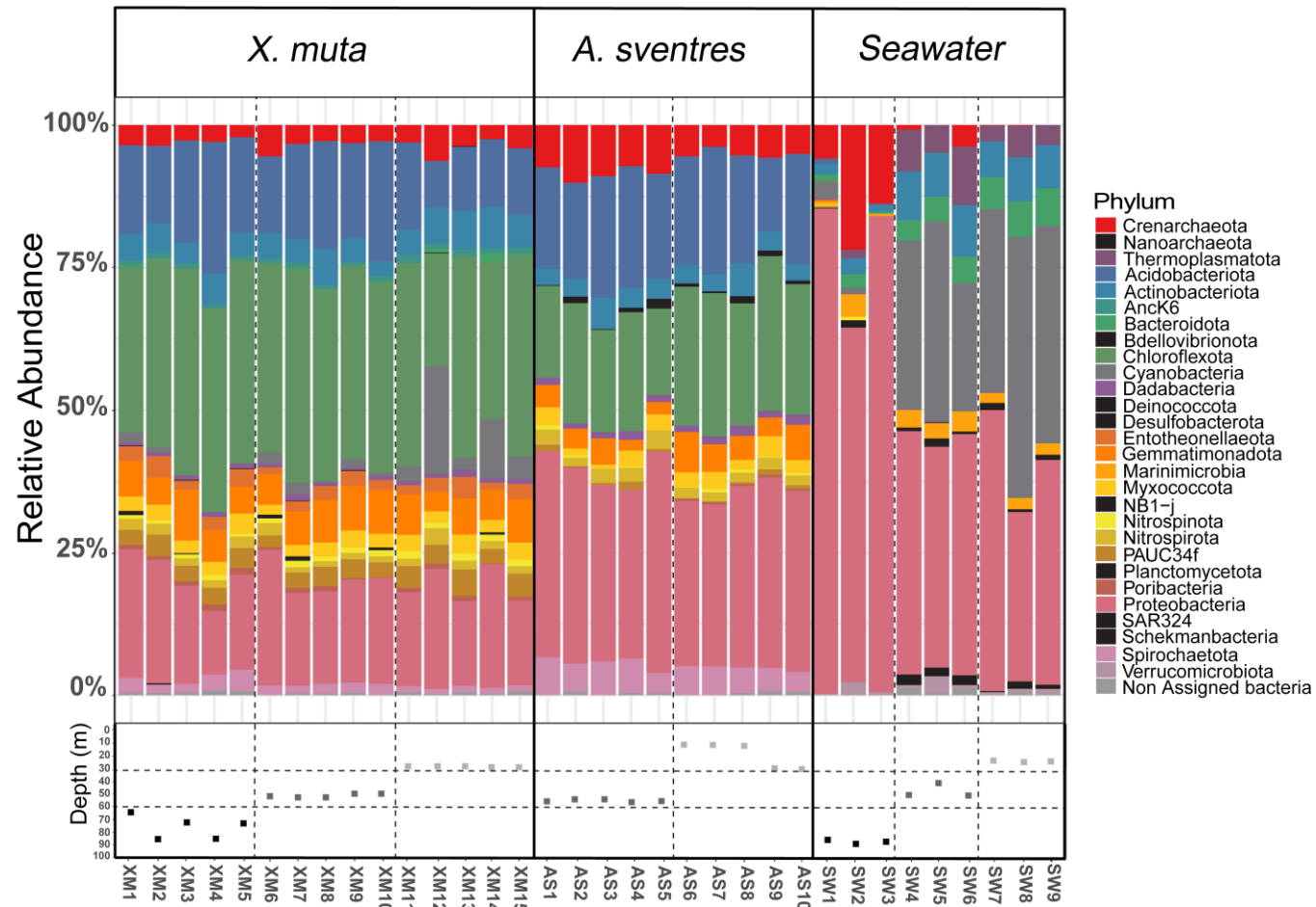
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Fig. 1





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Fig. 2

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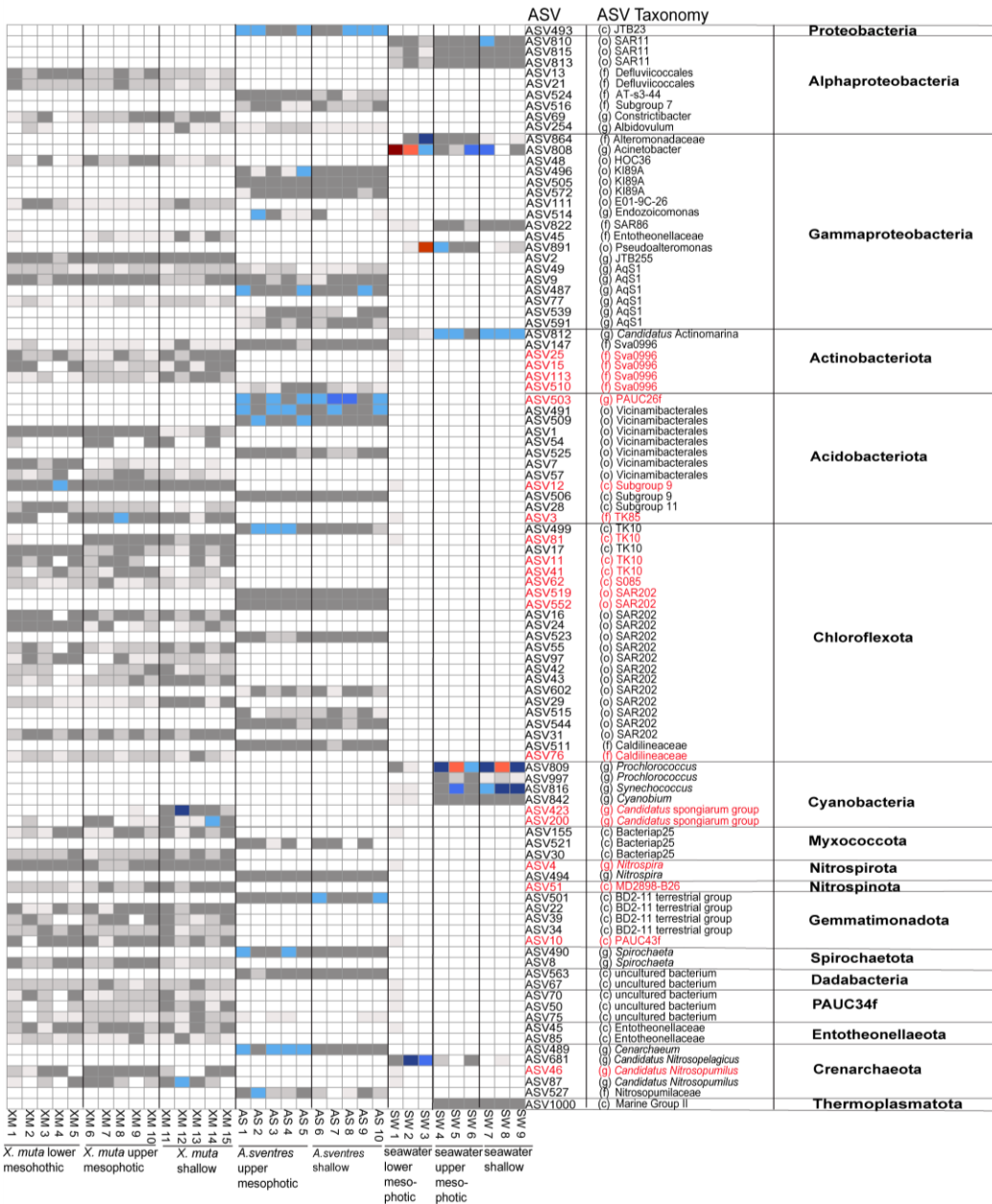
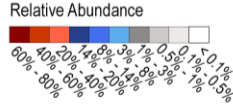


Fig. 3

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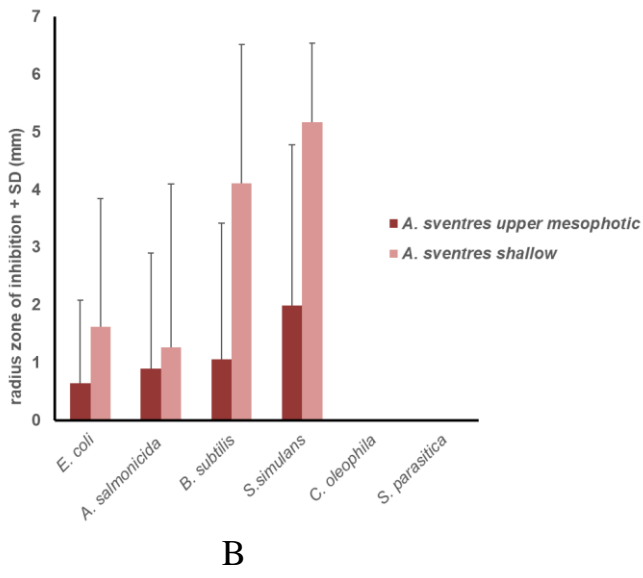
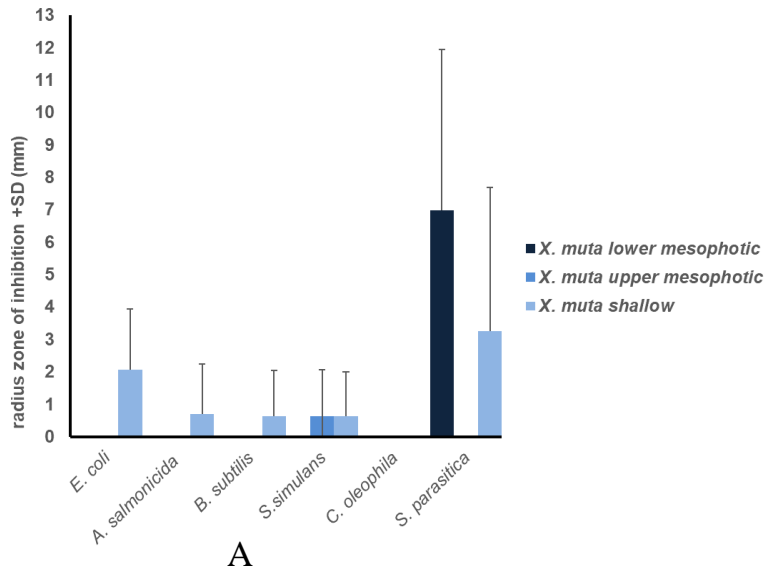


Fig. 4.

Supplementary Information

Supplementary Table 1. Detailed overview of each sponge specimen and seawater sample arranged from deep (LM: lower mesophotic; UM: upper mesophotic) to shallow, including information regarding 16S rRNA amplicon sequence data.

Core ID	Species	Actual Depth (m)	Depth Category	Number of Reads	Number of ASVOTUs	Phylogenetic Diversity
XM1	<i>X. muta</i>	66	LM	47230	200	19.15
XM2	<i>X. muta</i>	86	LM	27517	165	17.28
XM3	<i>X. muta</i>	72	LM	13666	180	18
XM4	<i>X. muta</i>	85	LM	35555	153	16.16
XM5	<i>X. muta</i>	82	LM	144005	170	17.11
XM6	<i>X. muta</i>	51	UM	21932	166	18.25
XM7	<i>X. muta</i>	52	UM	18902	186	18.63
XM8	<i>X. muta</i>	52	UM	25927	171	18.11
XM9	<i>X. muta</i>	48	UM	41288	188	18.02
XM10	<i>X. muta</i>	48	UM	163216	182	18.38
XM11	<i>X. muta</i>	27	Shallow	40656	160	17.9
XM12	<i>X. muta</i>	27	Shallow	60677	146	16.93
XM13	<i>X. muta</i>	27	Shallow	136515	165	17.75
XM14	<i>X. muta</i>	27	Shallow	186619	185	18.51
XM15	<i>X. muta</i>	27	Shallow	21179	159	17.52
AS1	<i>A. sventres</i>	54	UM	104359	64	10.88
AS2	<i>A. sventres</i>	52	UM	53094	71	13.06
AS3	<i>A. sventres</i>	52	UM	70372	64	11.47
AS4	<i>A. sventres</i>	54	UM	69361	76	11.77
AS5	<i>A. sventres</i>	53	UM	19759	74	12.54
AS6	<i>A. sventres</i>	12	Shallow	47923	68	12.06
AS7	<i>A. sventres</i>	12	Shallow	20605	63	11.69
AS8	<i>A. sventres</i>	12	Shallow	114551	67	12.34
AS9	<i>A. sventres</i>	27	Shallow	144184	75	13.03
AS10	<i>A. sventres</i>	27	Shallow	46515	77	12.76
SW1	seawater	84	LM	111117	77	13.18
SW2	seawater	92	LM	52537	160	15.28
SW3	seawater	85	LM	116171	70	9.76
SW4	seawater	50	UM	36066	137	14.9
SW5	seawater	40	UM	68949	143	15.43
SW6	seawater	50	UM	41461	164	15.76
SW7	seawater	20	Shallow	49916	125	14.48
SW8	seawater	21	Shallow	58323	117	14.25
SW9	seawater	20	Shallow	67075	126	14.85

Supplementary Table 2. Statistical analysis on differences in phylogenetic diversity (PD) of sponge and seawater samples tested using Kruskal Wallis and Wilcoxon rank sum test based on parameters(A) sample types and (B, C, D) depth. LM: lower mesophotic; UM: upper mesophotic for X. muta, A. sventres and seawater, respectively.

A. Sample type

<u>Sample type</u>	<u>A. sventres</u>	<u>Seawater</u>
<u>X. muta</u>	<u>0.003</u>	<u>0.003</u>
<u>A. sventres</u>	=	<u>0.003</u>

B. Depth (X. muta)

<u>Depth</u>	<u>UM</u>	<u>Shallow</u>
<u>LM</u>	<u>0.015</u>	<u>0.024</u>
<u>UM</u>	=	<u>0.174</u>

C. Depth (A. sventres)

<u>Depth</u>	<u>Shallow</u>
<u>UM</u>	<u>0.007</u>

D. Depth (seawater)

<u>Depth</u>	<u>UM</u>	<u>Shallow</u>
<u>LM</u>	<u>0.3</u>	<u>0.3</u>
<u>UM</u>	=	<u>0.3</u>

Supplementary Table 3. Pairwise comparison of [beta diversity](#) on sample types and subset of sample types based on depth-categories. LM: lower mesophotic; UM: upper mesophotic

A Sample type (sponge)						
	pairs	F.Model	R2	p.value	p.adjusted sig	
	<i>X. muta</i> vs <i>A. sventres</i>	66.00	0.74	0.00	0.003	
	<i>X. muta</i> vs Seawater	32.89	0.60	0.00	0.003	
	<i>A. sventres</i> vs Seawater	37.06	0.69	0.00	0.003	
B Depth (sponge and seawater)						
	pairs	F.Model	R2	p.value	p.adjusted sig	
	LM vs UM	2.14	0.10	0.08	0.23	
	LM vs shallow	2.51	0.12	0.04	0.12	
	UM vs shallow	0.27	0.01	0.87	1	
C Depth (<i>X. muta</i>)						
	pairs	F.Model	R2	p.value	p.adjusted sig	
	LM vs UM	2.78	0.26	0.01	0.02	
	LM vs Shallow	3.75	0.32	0.01	0.02	
	UM vs Shallow	1.82	0.19	0.06	0.17	
D Depth (<i>A. sventres</i>)						
	F.Model	R2	p.value	p.adjusted sig		
	UM vs Shallow	1.786981	0.1825876	0.007	0.007	
E Depth (Seawater)						
	pairs	F.Model	R2	p.value	p.adjusted sig	
	LM vs UM	4.924592	0.5518003	0.1	0.001	
	LM vs Shallow	7.989329	0.66637	0.1	0.001	
	UM vs Shallow	2.980473	0.4269729	0.1	0.7	

Supplementary Table 4. The most abundant Θ TUASVs (> 0.25% relative abundance) in each sample type (*X. muta*, *A. sventres* and seawater) that change with depth. The highest means of relative abundances of Θ TUASVs in each depth category are highlighted in bold. ~~Microbial-Taxonomy is provided~~-based on NG-TAX output (SILVA database 128) ~~from for~~ Phylum ~~to and~~ the lowest level at which it was classified (if applicable). Fold difference indicate changes of relative abundance between different depths.

LM: lower mesophotic; UM: upper mesophotic

A. *X. muta*

Θ TUASV	FDR_P	<i>X.muta</i> LM mean	<i>X.muta</i> UM mean	<i>X.muta</i> shallow mean	Taxonomy	Fold difference (shallow/UM)	Fold difference (shallow/LM)
Θ tuASV28	0.002	600.40	385.20	302.00	Acidobacteri ota <u>aa</u> , Subgroup 11	0.78	0.50
Θ tuASV7	0.004	1186.20	150.60	138.40	Acidobacteri ota <u>aa</u> , Subgroup 6 <u>Vicinamibacteriales</u>	0.92	0.12
Θ tuASV113	0.001	92.20	296.80	1081.60	Actino bacteriota <u>ba</u> acteria, Sva0996 marine <u>group</u> Cyanobacteria, <u>Candidatus</u> <u>Synechococcus spongiarum</u> <u>group</u> <u>Subsection</u> <u>I</u>	3.64	11.73
Θ tuASV423	0.001	0.00	19.00	3299.20	Cyanobacteria, <u>Candidatus</u> <u>Synechococcus spongiarum</u> <u>group</u> <u>Subsection</u> <u>I</u>	173.64	NA
Θ tuASV200	0.002	50.00	324.00	2868.00	<u>group</u> <u>Subsection</u> <u>I</u>	8.85	57.36
Θ tuASV29	0.001	280.40	139.20	737.00	Chloroflex otai <u>i</u> , SAR202	5.29	2.63
Θ tuASV81	0.001	36.40	1438.00	2331.80	Chloroflex otai <u>i</u> , TK10	1.62	64.06
Θ tuASV145	0.001	16.60	151.80	1322.60	Proteobacteria, <u>Entotheonellaceae</u> <u>Pseudohongiella</u>	8.71	79.67

<u>o</u> <u>tu</u> ASV87	0.03	382.20	372.00	1831.60	<u>Thaum</u> <u>Cren</u> archaeota, <i>Candidatus Nitrosopumilus</i>	4.92	4.79
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B. *A. sventres*

OTUASV	FDR_P	<i>A. sventres</i> UM mean	<i>A. sventres</i> shallow mean	Microbial Taxonomy	Fold differences (shallow/middleUM)
otu ASV503	0.0005	3058.60	4445	Acidobacteri ota , PAUC26f	1.45
otu ASV552	0.0005	975.80	2416.60	Chloroflex otai , SAR202	2.48
otu ASV602	0.0005	505.20	1231.40	Chloroflex otai , SAR202	2.44
otu ASV591	0.0005	537.00	1126.40	Proteobacteria, AqS1 Gammaproteobacteria	2.10
otu ASV514	0.02	1179.20	436.80	Proteobacteria, <i>Endozoicomonas</i>	0.37
otu ASV527	0.001	1322.8	559.6	ThaumCren archaeota, Marine Gre †Nitrosopumilaceae	0.42

C. seawater

OTUASV	FDR_P	seawater LM mean	seawater UM mean	seawater shallow mean	Taxonomy	Fold difference (shallow/UM)	Fold difference (shallow/LM)
otu <u>ASV</u> 812	0.001	553.67	2490.33	3401.00	Actinobacteria <u>Actinobacteriota</u> , <i>Candidatus Actinomarina</i>	1.37	6.14
otu <u>ASV</u> 809	0.001	617.67	7299.00	11181.67	Cyanobacteria, <i>Prochlorococcus</i>	1.53	18.10
otu <u>ASV</u> 816	0.001	193.67	3156.33	7847.33	Cyanobacteria, <i>Synechococcus</i>	2.49	40.52
otu <u>ASV</u> 842	0.001	91.67	1008.67	961.00	Cyanobacteria, <u>Cyanobium</u> Synechococcus	0.95	10.48
otu <u>ASV</u> 997	0.001	0.00	925.33	355.67	Cyanobacteria, <i>Prochlorococcus</i>	0.38	N/A
otu <u>ASV</u> 808	0.001	34672.33	2481.33	2004.33	Proteobacteria, <i>Acinetobacter</i>	0.81	0.06
otu <u>ASV</u> 681	0.002	7519.67	633.67	22.67	ThaumCren archaeota, <i>Candidatus Nitrosopelagicus</i>	0.036	0.003

Supplementary Table 5. Statistical test on the radius zone of inhibition (ZOI) of sponge crude extracts against microbial indicator strains. Panel A: Comparison of ZOI radii of sponge extracts of *X. muta* from different depths. Panel B: Comparison of ZOI radii of sponge extracts of *A. sventres* from different depths. In both tables, recorded ZOIs for each crude extract are provided, along with average inhibition and standard deviation per depth category. Analysis of variance (anova) was applied with p-values <0.05 being indicative of significant differences (highlighted in bold). Subsequently, only values found significant were tested using the Tukey post hoc test to determine which pair-wise comparison of sponge crude extracts gave a statistically significant result (highlighted in bold). Non-significant anova results were not tested in Tukey post hoc test (N/A, not applicable). ZOI radii were grouped into ~~three~~ four categories, namely weak (0 -5 mm), moderate (5-10 mm), strong (> 10-20 mm) and very strong (> 20 mm)~~strong (> 10 mm)~~. LM: lower mesophotic; UM: upper mesophotic

A. X. muta

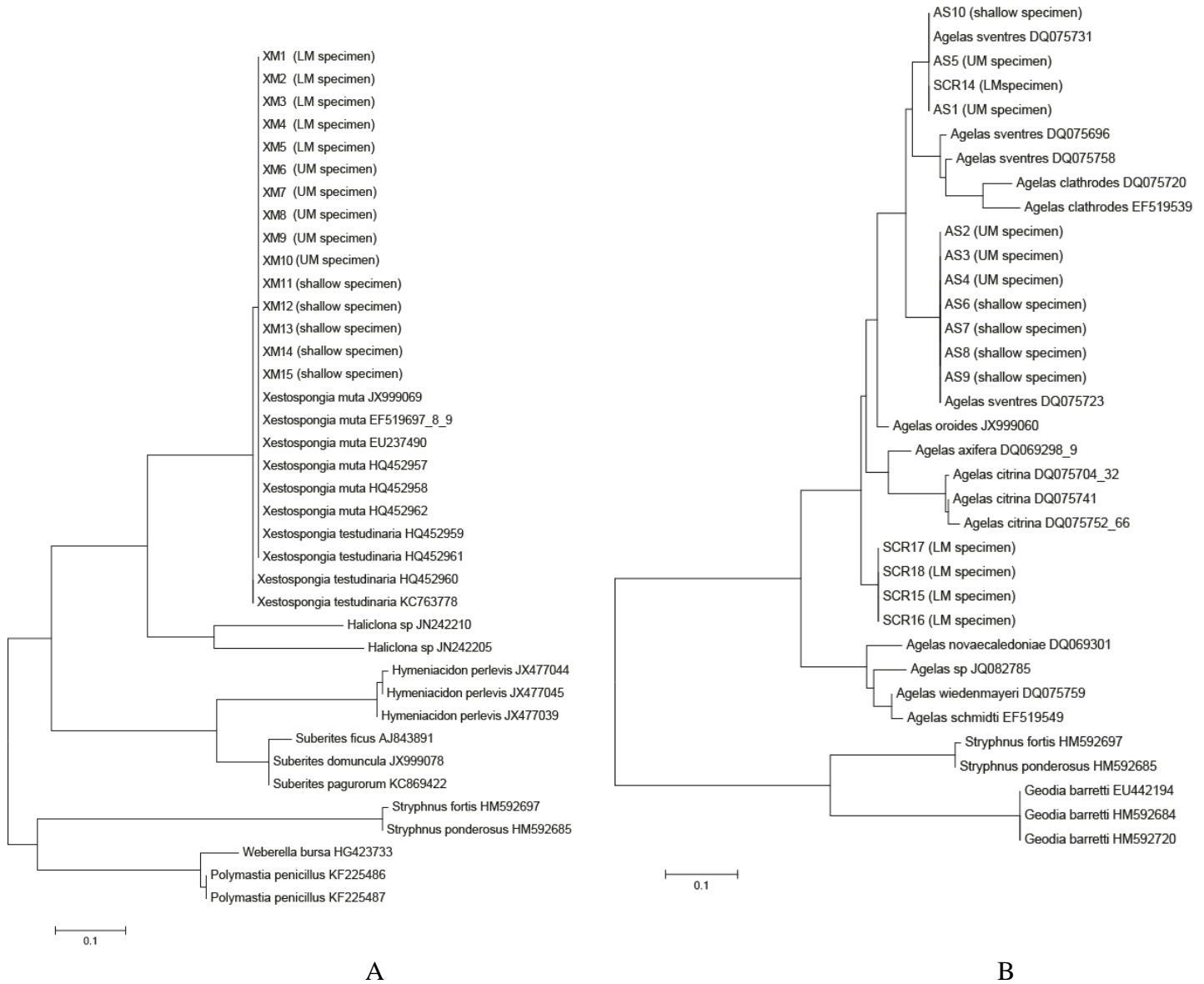
		radius zone of inhibition (mm)					
Sponge extracts		<i>E. coli</i>	<i>A. salmo- nicida</i>	<i>B. subtilis</i>	<i>S. simulans</i>	<i>C. oleo- phila</i>	<i>S. para- sitica</i>
LM	XM1	0	0	0	0	0	0
LM	XM2	0	0	0	0	0	5.55
LM	XM3	0	0	0	0	0	13.43
LM	XM4	0	0	0	0	0	6.51
LM	XM5	0	0	0	0	0	9.38
mean inhibition and standard deviation		0±0	0±0	0±0	0±0	0±0	6.97±4.96
UM	XM6	0	0	0	0	0	0
UM	XM7	0	0	0	3.2	0	0
UM	XM8	0	0	0	0	0	0
UM	XM9	0	0	0	0	0	0
UM	XM10	0	0	0	0	0	0
mean inhibition and standard deviation		0±0	0±0	0±0	0.64±1.43	0±0	0±0
shallow	XM11	3.72	0	0	0	0	8.14
shallow	XM12	0	0	0	0	0	8.09
shallow	XM13	3.27	0	0	0	0	0
shallow	XM14	0	3.47	3.14	3.1	0	0
shallow	XM15	3.28	0	0	0	0	0
mean inhibition and standard deviation		2.05±1.88	0.69±1.55	0.63±1.40	0.62±1.39	0±0	3.25±4.44
ANOVA (p-value)		0.02	0.40	0.40	0.60	N/A	0.04

Tukey	pvalue_LM_UM	1	1	1	1	N/A	0.04
Post hoc test	pvalue_LM_shallow	0.03	0.5	0.5	0.5	N/A	0.3
	pvalue_UM_shallow	0.03	0.5	0.5	0.5	N/A	0.4

B. *A. sventres*

		radius zone of inhibition (mm)					
Depths	Sponge extracts	<i>E. coli</i>	<i>A. salmonicida</i>	<i>B. subtilis</i>	<i>S.simulans</i>	<i>S. parasitica</i>	<i>C. oleophila</i>
UM	AS1	0	0	0	0	0	0
UM	AS2	0	0	0	0	0	0
UM	AS3	0	0	0	0	0	0
UM	AS4	0	0	0	4.07	0	0
UM	AS5	3.22	4.47	5.27	5.86	0	0
mean inhibition and standard deviation		0.64±1.44	0.89±2.00	1.05±2.36	1.99±2.79	0±0	0±0
shallow	AS6	4.15	0	5.59	6.86	0	0
shallow	AS7	0	0	0	3.15	0	0
shallow	AS8	0	0	4.01	4.68	0	0
shallow	AS9	3.96	6.32	5.93	5.5	0	0
shallow	AS10	0	0	5	5.63	0	0
mean inhibition and standard deviation		1.62±2.22	1.26±2.83	4.11±2.41	5.16±1.37	0±0	0±0
ANOVA (p-value)	pvalue_UM_shallow	0.4	0.8	0.08	0.05	N/A	N/A

Supplementary Figure 1. (A). Phylogenetic tree constructed with the maximum likelihood algorithm with 500 bootstrap replications and Nearest-Neighbor-Interchange (NNI) for ML Heuristic of *X. muta* specimens and (B) *A. sventres* specimens based on the COI gene. The number (0.1) below the reference bar indicates percentage distance of sequence. LM: lower mesophotic; UM: upper mesophotic



Supplementary Figure 2. Phylogenetic diversity (PD) of prokaryotic communities in *X. muta*, *A. sventres* and seawater at different depths.

