rRNA depletion for holobiont metatranscriptome profiling across demosponges

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Abstract

The pervasive association of demosponges with diverse bacterial communities has been a research topic since the late 1970s. Studies characterizing these bacterial communities have detected over 40 bacterial phyla associated with sponges and determined that sponge microbiomes differ from the bacterioplankton of the surrounding water and include sponges-specific bacterial associates. Despite the large body of information available on the diversity of the sponge microbiome, the molecular mechanisms used by sponges to interact with their bacterial symbionts and *vice versa* remain poorly understood. Yet, studying how sponges interact with their microbiomes is pivotal to understanding how these systems react to environmental changes and when these changes lead to a disruption of sponge-microbe associations. An important factor hampering the characterization of the sponge-microbiome molecular cross-talk mechanisms is the lack of broadly applicable molecular methods to, for instance, efficiently sequence meta-transcriptomes across a broad range of sponge species. To overcome this limitation, we implemented a hybrid-capture strategy capable of depleting both sponge and bacterial rRNA from total RNA extracts of highly divergent demosponges with microbiomes of different complexity. Our pan-demosponge rRNA depletion strategy allows for the efficient characterization of the metatranscriptome of diverse demosponge holobionts and the simultaneous quantification of gene expression in both the host and its microbiome.

Introduction

With almost 8,000 species, class Demospongiae is the most diverse sponge class comprising ~70% of all described sponge species. Demosponges occur at all depths in all oceans, and representatives of this sponge class can also inhabit lakes and rivers of all continents except Antarctica (Van Soest et al., 2012).

One of the most striking aspects of the biology of demosponges is their pervasive association with bacterial communities of varying diversity and composition (Hentschel, Piel, Degnan, & Taylor, 2012). First discovered and described in the late 1970s, the study of sponge-bacteria

associations has evolved into a new and vastly expanding field. To date, more than 40 bacterial phyla have been found in association with sponges, forming the sponge holobiont (Thomas et al., 2016). Importantly, these bacterial associates differ from the bacterioplankton of the surrounding water and sediments and include sponge-specific bacterial clades (Simister, Deines, Botté, Webster, & Taylor, 2012; Thomas et al., 2016). Despite the large body of information available on sponge microbiome diversity, the molecular mechanisms used by sponges to interact with their bacterial symbionts and *vice versa* remain largely unexplored (Hudspith et al., 2021; Webster & Thomas, 2016). This knowledge is needed, for example, to better appreciate how changes in the symbiotic communities in response to short-term or long-term environmental change affect the sponge host.

Particularly the lack of experimentally tractable sponge models has made the study of sponge-bacteria associations difficult (Pita, Fraune, & Hentschel, 2016). Besides, the lack of broadly applicable methods for functional genomics of demosponges has limited the study of sponge holobionts to a few species. Thus, generalizations about the biology of demosponge holobionts are based on the few species for which genomic or (meta-)transcriptomic resources are available (Britstein et al., 2020; Moitinho-Silva et al., 2017; Radax et al., 2012; Ryu et al., 2016; Srivastava et al., 2010). Consequently, developing molecular methods applicable to a wide taxonomic spectrum of demosponges to, for instance, simultaneously characterize the transcriptome of the sponge host and its microbiome - under control and experimental setups - is pivotal. Here, we present a pan-demosponge rRNA capturing strategy capable of depleting the rRNAs of the sponge host and its associated bacterial community. We successfully use this procedure to sequence the meta-transcriptomes of *Lendenfeldia chondrodes* and *Tethya wilhelma* and show that rRNA

depleted libraries can be used to determine bacterial differential gene expression. *Lendenfeldia chondrodes* and *T. wilhelma* belong to the demosponge subclasses Keratosa and Heteroscleromorpha, respectively. These subclasses diverged >700MYA (Dohrmann & Wörheide, 2017) and represent the phylogenetic diversity breadth of the Demospongiae.

Materials and Methods

Selection of 28S and 18S rRNA demosponge sequences for pan-demosponge depletion bait design

Full-length nuclear 18S, 5.8S, and 28S and mitochondrial 12S and 16S rRNA sequences from the demosponges *Amphimedon queenslandica, Tethya wilhelma, Lendenfeldia chondrodes,* and *Spongilla lacustris* (Accesion numbers OX176765-OX176794) were processed by siTOOLs Biotech (Germany) using a proprietary bait selection technology and resulted in a cocktail of 106 pan-demosponge ribosomal depletion baits. The resulting pan-demosponge bait set can be purchased directly from siTOOLs Biotech (Germany). Supplementary Table 1 presents in detail which sequences were used for each marker by species.

Bleaching experiments in *Lendenfeldia chondrodes*

Round explants (1cm in diameter) were exposed to shading in the same 360L marine aquarium (25 °C water temperature and a 12:12 h light cycle) where the parental sponges are kept. We used black plastic foil mounted on plexiglass panels to cover the aquarium space and shade the explants. Neither the plastic foil nor the plexiglass panels were in contact with the water or blocked the water (in-/out-)flow. Control and treatment (*i.e.*, shaded) explants were placed on (open) Petri dishes (14cm diameter; Carl Roth AG, Germany), separated to avoid contact (and fusion), and visually inspected every week. Petri

dishes were weekly cleaned from accumulating sand or fine sediment using a Pasteur pipette. This process took less than one hour and did not interfere with the shading treatment. Due to the symmetrical arrangement of the water inflow in the experimental tank and the presence of a single, central outflow, both control and treatment sponges were exposed to similar temperature, nutrients, water pH, and water currents, thus allowing to constrain these potentially confounding effects from the experimental setting. Whole explants were fixed in liquid nitrogen after 12-15 weeks of exposure to darkness, avoiding transferring other organisms found in the aquarium, like small amphipods or polychaetes. Two 1cm diameter control (unshaded) explants were fixed in liquid nitrogen the same day the shading treatment ended. Tissue samples were kept at -80 °C until DNA/RNA extraction.

Total RNA extraction in *Lendenfeldia chondrodes* and *Tethya wilhelma*

RNA from flash-frozen treatment (n=2) and control (n=2) *L. chondrodes* (see above) and one flash-frozen control *T. wilhelma* specimen was extracted using Trizol. Briefly, the frozen tissue was submerged in Trizol and homogenized using a Polytron PT1300D homogenizer. We vigorously mixed the homogenate for five minutes at room temperature and added 200 µl chloroform per ml homogenate for phase separation. We centrifuged the samples at maximum speed (21,000 rcf) for 15 minutes and transferred the upper phase to a new microfuge tube for precipitation with isopropanol. After centrifugation (21,000 rcf at 16 °C), we washed the pellet twice with ethanol 80%, air-dried it, and resuspended it in nuclease-free water. We assessed RNA yield and purity using a Nanodrop 100 and controlled the quality of the extracts using agarose gels (1%) before checking RNA integrity on a Bioanalyzer 2100.

qPCR-based abundance estimation of Cyanobacteria and Actinobacteria in control vs. bleached *Lendenfeldia chondrodes*

We used a qPCR-based relative quantification to determine the abundance of Cyanobacteria and Actinobacteria in control *vs.* bleached *L. chondrodes* sponges. We selected Actinobacteria because specific primers previously tested in sponges are readily available, and we expected this taxon to be insensitive to the shading treatment in contrast to the photosynthetic Cyanobacterium. Besides, OTUs belonging to these bacterial phyla occur in *L. chondrodes* (Vargas, Leiva, & Wörheide, 2021). For this, we extracted DNA from three control and three bleached *L. chondrodes* explants using the NucleoSpin® Tissue kit (Macherey-Nagel). We used 15uL qPCR reactions of the Kappa SYBR Fast Ready-Mix (Kappa Biosystems) to conduct, for each tissue sample, three qPCR reactions using (1) universal bacterial primers, (2) Cyanobacteria specific primers, and (3) Actinobacteria specific primers (Bayer, Kamke, & Hentschel, 2014). We used two technical replicates for all qPCR reactions, no-template negative controls, and a Rotor-Gene Q (Qiagen) RT-qPCR machine for cycling. We analyzed the raw fluorescence measurements obtained for the samples using the program LinReg (http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LinRegPCR.zip&description=LinRegPCR:%20analysis%20of%20quantitative%20PCR%20data&sub=LinRegPCR) to obtain Cq and efficiency values for each reaction. We used REST12 (Pfaffl, Horgan, & Dempfle, 2002) to estimate bacterial abundance fold changes and their associated p-values using the mean Cq and efficiency values derived from each reaction's two technical replicates.

Ribosomal RNA depletion in *Lendenfeldia chondrodes* and *Tethya wilhelma*

Ribosomal RNA depletion from total RNA samples of *L. chondrodes* and *T. wilhelma* was done by siTOOLs using the riboPOOL Buffer Set (Hybridization Buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl; Depletion Buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 M NaCl) and magnetic beads. One µg total RNA was incubated with 100 pmol pan-bacteria and pan-demosponge (see Supplementary Table 2 for details) riboPOOL probe mixtures roughly determined from the bacterial to sponge RNA ratio observed in total RNA bioanalyzer runs of each of the studied sponge species (Fig. 1). To prepare the riboPOOL mixture, pan-demosponge and pan-bacteria riboPOOLs were diluted in nuclease-free water to a final concentration of 100pmol/µl (100 µM) and mixed to the desired ratios to a final volume of 10 µl (e.g., 5µl of pan-demosponge riboPOOL with 5µl pan-bacteria riboPOOL for the 50:50 % mix). The hybridization was done by siTOOLs for each sample independently in 0.2 µl thin-wall PCR tubes, using one µg total RNA diluted in 14 µl ddH2O, 5µl Hybridisation Buffer and, one µl 100pmol/µl riboPOOL mix. For samples with enough RNA, a negative depletion ("no riboPOOL") control was performed replacing the riboPOOL mix with dd H_2O. The hybridization reaction was incubated at 68 °C for 10 minutes and slowly cooled down to 37 °C in a PCR machine with a ramping rate of 3 °C/min. The hybridized rRNA was captured using 540 µl siTOOLs riboDepletion beads washed once with 540 µl Depletion Buffer and resuspended in 480 µl Depletion Buffer. Eighty µl beads were added to each hybridization reaction and the mix was incubated for 15 minutes at 37 °C followed by five minutes at 50 °C. The capturing reaction was placed on a magnetic rack and the (clear) supernatant containing the depleted RNA was transferred to a new 1.5 mL tube. The rRNA-depleted samples were finally purified using the Machery Nagel RNA XS Clean-up Kit following the manufacturer's instructions and eluted in 10µl ddH_2O. The concentrated, rRNA-depleted

samples were quantified in a Biotek EPOCH Photometer with a Take3 Micro-Volume Plate and the depletion efficiency was controlled on a Bioanalyzer 2100 using the Agilent RNA 6000 Pico Kit and diluting the sample to 5ng/µl if necessary (Supplementary Table 3). The depleted RNA was transported in dry ice for library preparation at the Department of Earth and Environmental Sciences, Paleontology & Geobiology, Ludwig-Maximilians-Universität München.

Library preparation, sequencing, and bioinformatic analyses of rRNA depleted *Lendenfeldia chondrodes* and *Tethya wilhelma* samples

Lexogen's CORALL kit was used to construct total RNA (non-polyA-selected) Illumina-ready libraries using one nanogram of the depleted RNA samples, *i.e.*, two control and two bleached *L. chondrodes* samples and one *T. wilhelma*. After library preparation, we quality-controlled and estimated the length of the fragments in the libraries on a Bioanalyzer 2100 using Agilent's High Sensitivity DNA kit. We sequenced one equimolar pool of the four (i.e., two treatment + two control) *L. chondrodes* libraries and one *Tethya* library on an Illumina MiniSeq using one High Throughput 150 Cycles Kit for each species. We used the resulting data for *de novo* transcriptome assembly and annotation using TransPi (Rivera-Vicéns, Garcia-Escudero, Conci, Eitel, & Wörheide, 2021) and used *blast* to quantify the number of transcripts matching the Uniprot (trembl) Bacteria database. To compare the effect of rRNA depletion on the representation of bacterial transcripts in *T. wilhelma* and *L. chondrodes*, we used TransPi to assemble and annotate a similar amount of polyA-selected reads obtained from publicly available RNA-Seq datasets (*T. wilhelma*; SRR4255675) or previously sequenced polyA-selected libraries derived from bleached (n=2) and control (n=2) *L. chondrodes* (PRJEB24503). Within TransPi we evaluated transcriptome completeness using

BUSCO v4 for Metazoa and Bacteria (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015). To evaluate the ability of rRNA-depleted and polyA-selected libraries to detect transcripts from the bacterial symbionts, we used Salmon (Patro, Duggal, Love, Irizarry, & Kingsford, 2017) to map the metatranscriptomic and polyA-selected reads to the predicted genes of one cyanobacterium (Supplementary File 1) and one actinobacterium (Supplementary File 2) present in the microbiome of *L. chondrodes* (Vargas et al., 2021), and used the resulting count matrices in DESeq2 (Love, Huber, & Anders, 2014) as a proof-of-concept to assess bacterial differential gene expression in bleached *vs.* control *L. chondrodes* specimens.

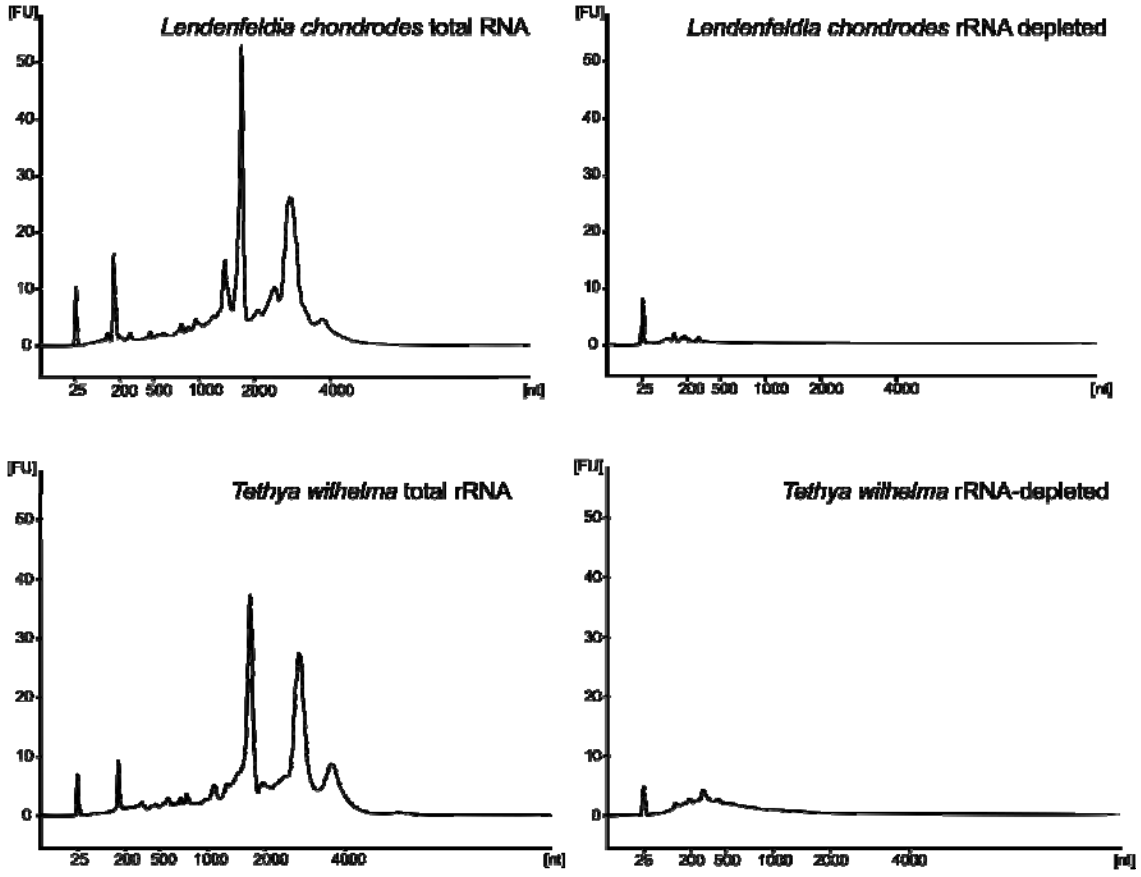

Figure 1. Bioanalyzer electropherograms of "no riboPOOL" input total RNA extracts and riboPOOL depleted control and bleached samples of *Lendenfeldia chondrodes* and samples of *Tethya wilhelma* showing effective rRNA depletion.

Results

Pan-demosponge rRNA depletion enrich bacterial transcripts in two phylogenetically distant demosponges with contrasting microbiomes

In both *L. chondrodes* and *T. wilhelma*, rRNA depletion using the newly designed pan-demosponge baits resulted in rRNA-free RNA extracts (Fig. 1). The assembled meta-transcriptomes for these two species contained 111,891 (with 31,797 >500bp) and 100,018 (33,141 >500bp) transcripts, respectively. For comparison, the polyA-selected transcriptomes for *L. chondrodes* and *T. wilhelma* contained 36,370 (16,754 >500bp) and 53,880 (20,509 >500bp) transcripts respectively. We also observed an increase in the total (bacterial + metazoan) number of contigs containing ORFs recovered from each species when using rRNA depletion. In this regard, the *L. chondrodes* and *T. wilhelma* transcriptomes assembled from rRNA-depleted RNA had 2.34 and 1.65 times more contig containing ORFs than their polyA-selected counterparts. The observed increase in transcripts (and contigs containing ORFs) was accompanied by a decrease in the transcriptome N50 and mean transcript length of the metatranscriptome of both species compared to their polyA counterparts (see Table 1). A BUSCO analysis revealed marked differences in the percentage of complete BUSCOs between the rRNA-depleted and polyA-selected transcriptomes in *L. chondrodes* and *T. wilhelma*. Here, the rRNA-depleted *L. chondrodes* and *T. wilhelma* metatranscriptomic assemblies had 73.6% and 70.8% complete metazoan BUSCOs, respectively, while their polyA-selected counterparts had 91.5% and 91.3% complete BUSCOs. This drop in the percentage of complete BUSCOs recovered in the rRNA-depleted assemblies roughly matches the increase in the fragmented BUSCOs observed in these datasets; fragmented BUSCOs accounted for 14.8% and 13.2% of the BUSCOs present in the metatranscriptomes of *T. wilhelma* and *L. chondrodes*, respectively.

Regarding bacterial transcriptome completeness, rRNA depletion had a different effect in the two species investigated in this study. In *L. chondrodes*, compared with the polyA-selected assembly, rRNA-depletion resulted in a considerable increase in the complete bacterial BUSCOs recovered. In this species, the number of complete bacterial BUSCOs found was 53.3%, contrasting with the 9.7% bacterial BUSCOs detected in the polyA-selected assemblies. This trend was also evident if the analysis was restricted to Cyanobacteria and Actinobacteria BUSCOs (Supplementary Table 4). However, in *T. wilhelma*, rRNA depletion did not result in a marked increase in the bacterial BUSCOs detected. In this species, we found 19.3% complete bacterial BUSCOs in the rRNA-depleted assembly and 9.7% complete bacterial BUSCOs in the polyA-selected *T. wilhelma* transcriptome. Table 1 presents a summary of the rRNA-depleted and polyA-selected (non-depleted) assemblies of *L. chondrodes* and *T. wilhelma*.

In line with the BUSCO results, blast searches against bacterial sequences deposited in the uniprot knowledge base (*i.e.*, swissprot + trembl) revealed a marked increase in the number of bacterial transcripts detected in rRNA-depleted *vs.* polyA-selected transcriptomes in *L. chondrodes* (Fig. 2). In *T. wilhelma*, the difference in the number of transcripts assigned to bacteria in the rRNA-depleted *vs.* polyA-selected transcriptome was less pronounced (Fig. 2).

Table 1: Description of *L. chondrodes* and *T. wilhelma* rRNA depleted and polyA-selected transcriptomes. Transcript N50 and mean length in base pairs. *Busco v5 yielded identical results.

	L. chondrodes		*T. wilhelma*	
	rRNA depleted	polyA selection	rRNA depleted	polyA selection
N transcripts	111,891	36,370	100,018	53,880
N50	932	2,044	1,155	2,101
Mean length	555	994	641	901
Total N ORFs (host+ symbiont)	49,185	21,019	43,261	26,212
Complete/Fragmented BUSCOs (Metazoa v4)	73.6%/13.2%	91.5%/1.4%	70.8%/14.8%	91.3%/1.8%
Complete/Fragmented BUSCOs (Bacteria v4)*	53.3%/8.1%	9.7%/4.0%	19.3%/7.3%	9.7%/2.4%

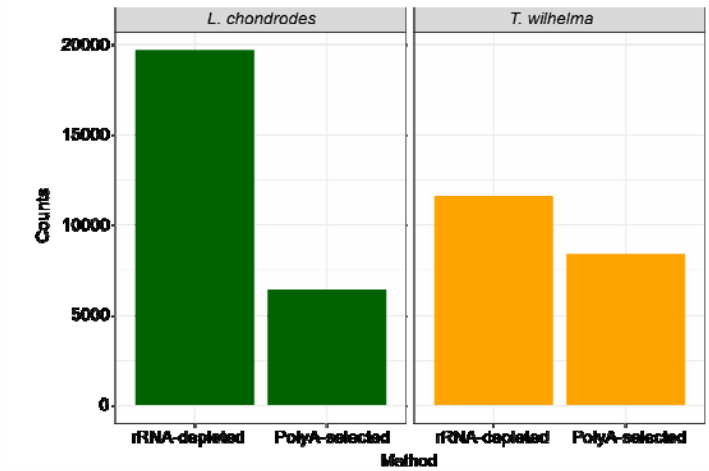

Figure 2: Number of transcripts in the assembled reference transcriptome matching bacterial sequences deposited in the uniprot knowledge base in rRNA-depleted vs. polyA-selected transcriptomes of the demosponges *Lendenfeldia chondrodes* and *Tethya wilhelma.*

rRNA-depletion enables differential gene expression analyses of the *L. chondrodes* holobiont

rRNA-depleted *L. chondrodes* libraries resulted in better transcript coverage and higher median transcript counts than polyA-selected equivalents. In rRNA-depleted libraries, 5,633 of 5,857 (>99%) bacterial transcripts had non-zero read counts in at least one of the four libraries sequenced, and 1,820 of 5,857 (32%) bacterial transcripts were detected in all sequenced libraries. In these libraries, the median count ranged between zero (in one of the bleached specimens) and 25 counts. In contrast, only 1,828 of 5,857 bacterial transcripts (32%) had non-zero counts in at least one of the four polyA-selected libraries analyzed, and only seven transcripts (0.001%) were detected in all four polyA-selected libraries analyzed. The median count for these libraries was zero independently of whether the libraries derived from bleached or control samples. In agreement with these results, rRNA-depleted libraries yielded broad count distributions that contrasted with those obtained via polyA-selection,

which had prominent peaks at low counts, indicating a general depletion of bacterial reads in these libraries (Fig. 3). The depletion of bacterial transcripts (*i.e.*, low count peak) in the sequenced transcriptomes was evident in bleached *L. chondrodes* independent of the method used for library preparation. However, compared to polyA-selected libraries, rRNA-depleted libraries obtained from bleached *L. chondrodes* recovered considerably more (4,918 *vs*. 167) bacterial transcripts than polyA-selected libraries from similar samples. Relative quantification assays performed via RT-qPCR (Table 2) revealed a significant negative 6 log2 fold change in the abundance of cyanobacteria in shaded relative to control sponges. In contrast, the abundance of symbiotic Actinobacteria remained unaffected by shading (Table 2).

rRNA-depleted libraries partially mirrored this microbiome change, yielding cyanobacterial transcript count distributions biased towards lower counts in bleached *L. chondrodes* specimens (Fig. 3). In contrast, rRNA-depleted libraries derived from bleached specimens had higher actinobacterial transcript counts (Fig. 3). PolyA-selected libraries generally failed to detect actinobacterial transcripts (Fig. 3). For Cyanobacteria, these libraries mirrored the decay in cyanobacterial abundance measured via qPCR. However, since most bacterial transcripts had very low counts in polyA-selected libraries, the count matrices obtained using this library preparation method did not fit the negative binomial distribution used by DESeq2 for differential gene expression analyses and could not be used to assess expression

Table 2: REST analysis of the relative abundance quantification of the cyanobacterium Candidatus *Synechococcus spongiarum* and a sponge-associated Actinobacteria in bleached *vs.* control sponge explants. We used 1000 iterations for the calculation of p-values.

Bacterial Group	Used as	Mean Reaction Efficiency*	Expression	Std. Error	95% C.I.	P(H1)	Result
Eubacteria	Reference	0.963	1.000				
Cyanobacteria	Target	0.8461	0.022	0.011 - 0.046	0.007 - 0.061	0.007	DOWN
Actinobacteria	Target	0.9614	0.589	0.254 - 1.339	0.165 - 2.351	0.300	

* For the REST calculations, the efficiency was calculated for each reaction using the program LINREG

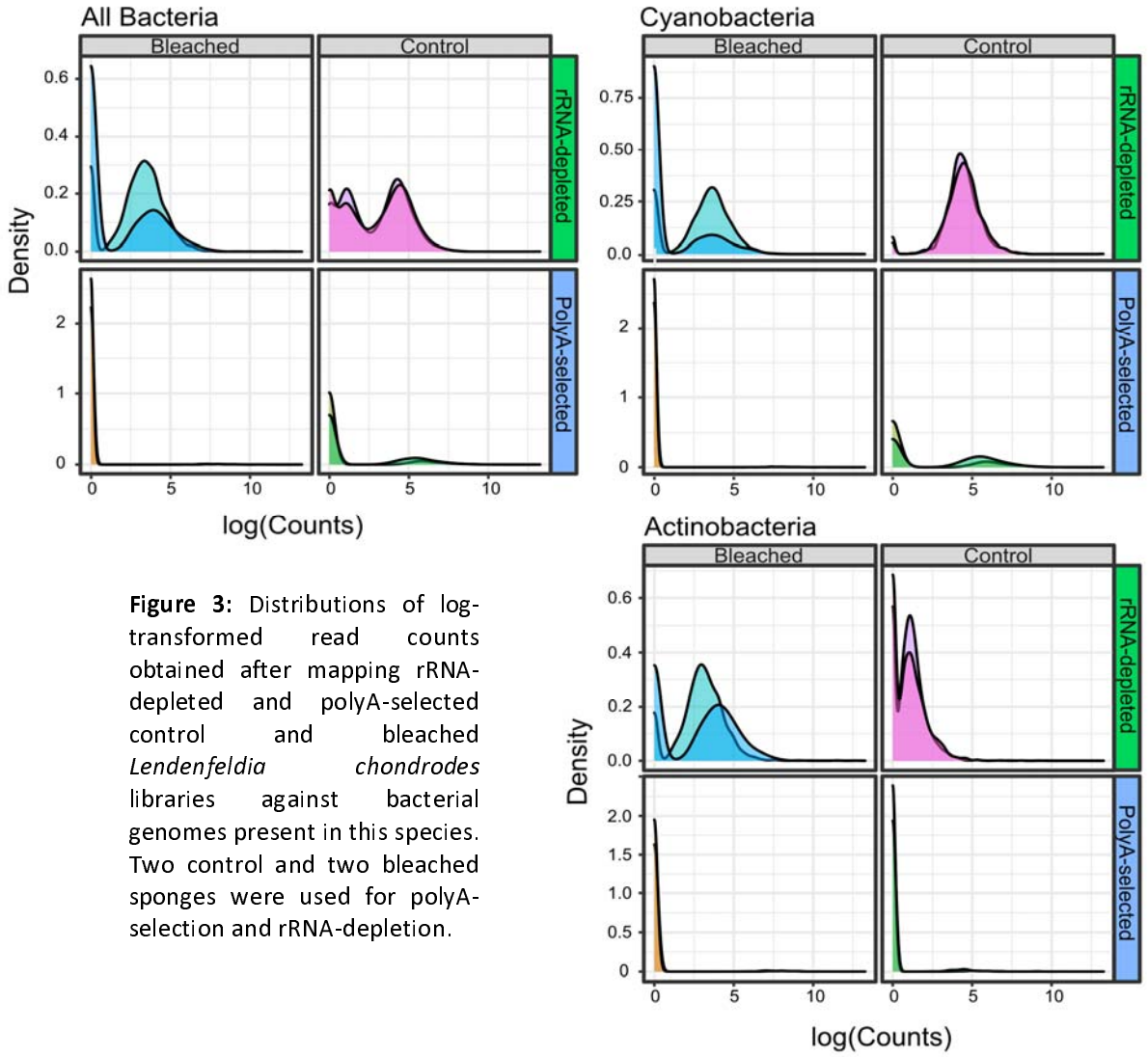

Figure 3: Distributions of log-transformed read counts obtained after mapping rRNA-depleted and polyA-selected control and bleached *Lendenfeldia chondrodes* libraries against bacterial genomes present in this species. Two control and two bleached sponges were used for polyA-selection and rRNA-depletion.

changes in the *L. chondrodes* microbiome. Contrary to the results obtained using polyA-selection, bacterial differential gene expression analyses of control *vs.* bleached using DESeq2 was possible for count matrices derived from rRNA-depleted sponges libraries. These analyses resulted in 464 differentially expressed transcripts with a log2 fold change (LFC) > 1 or < -1, and a Benjamini-Hochberg corrected p-value < 0.05 (Fig. 4). Of the 464 differentially expressed transcripts, 48 belonged to Actinobacteria and 416 to Cyanobacteria. Most Actinobacterial transcripts (*i.e.*, 47) were upregulated in bleached sponges. In contrast, all 416 differentially expressed cyanobacterial transcripts were downregulated in bleached sponges.

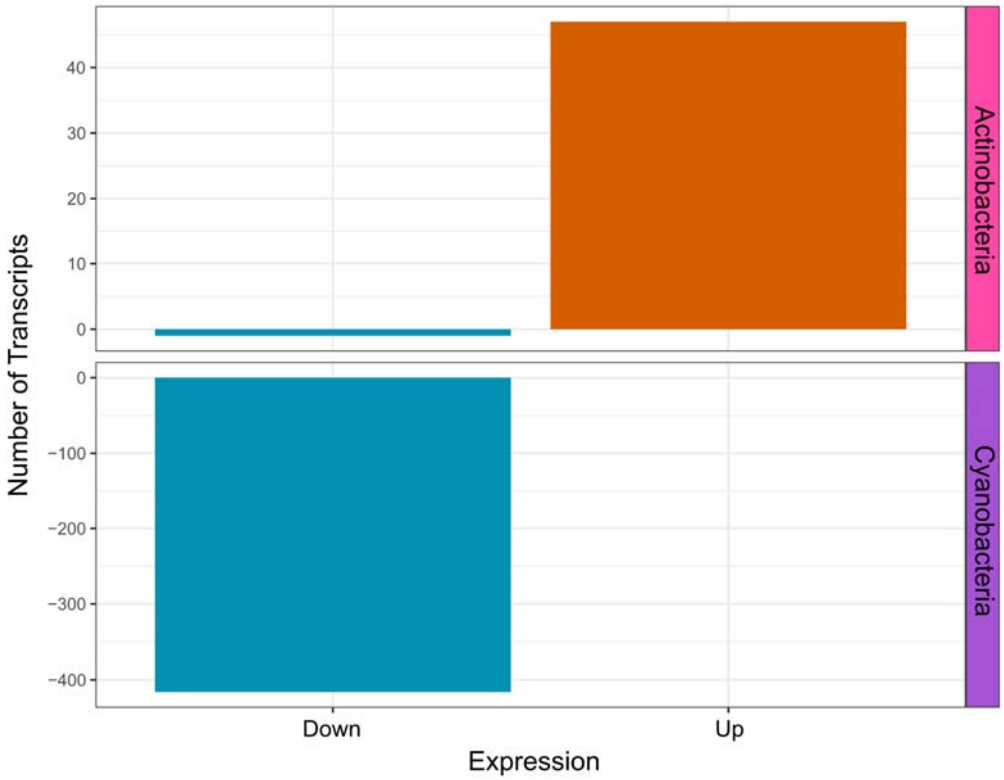

Figure 4: Number of up- or down-regulated transcripts by bacterial phylum in rRNA-depleted control *vs.* bleached *Lendenfeldia chondrodes*. Two control and two bleached sponges were used for rRNA-depletion.

Discussion

Sponges and their microbial communities are integrated systems with complex physiologies (Webster & Thomas, 2016) that, in some contexts, are better regarded as holobionts with physiological and metabolism different from those of their constituents. Thus, analytical methods to study how sponges respond to changes due to intrinsic (*e.g.*, ontogenetic changes) and extrinsic factors (*e.g.*, competition, predation by other organisms, or varying environmental conditions) must account for this complexity. Here, we designed a pan-demosponge rRNA-depletion bait set for metatranscriptome sequencing in Demospongiae, the most species-rich sponge class, and tested it in two not closely related demosponge species, namely *T. wilhelma* and *L. chondrodes*. Our rRNA-depletion strategy detected two to

five times more bacterial BUSCOs and recovered two times more protein-coding transcripts than polyA-selected assemblies of these species. Thus, our library preparation strategy seems, as intended and expected, to better capture the complex nature of sponge holobionts than polyA-based methods for producing RNASeq libraries.

A reduction in assembly N50 and contig mean length accompanied the increase in protein-coding transcripts, bacterial transcripts, and bacterial BUSCOs. This is expected given the shorter length of prokaryotic genes compared with eukaryotic genes (Xu et al., 2006). Thus, the drop in N50 and contig mean length observed in our metatranscriptomes likely reflects the effect of a larger bacterial gene pool detected in these assemblies on these two metrics. In addition, it is also likely that the metatranscriptomic assemblies are more fragmented than their polyA-selected counterparts. In this regard, the increased number of fragmented BUSCOs in both metatranscriptomic assemblies provides evidence for their fragmentation. Considering that rRNA-depleted RNA extracts include non-polyadenylated sponge RNA species (*e.g.*, lncRNAs) and substantially more bacterial mRNA transcripts not present in polyA-selected equivalents, the observed fragmentation of rRNA-depleted compared to polyA-selected assemblies at similar sequencing depths is not entirely unexpected. Using similar read lengths, a relation between transcriptome complexity, assembly quality, and sequencing depth exists (Francis et al., 2013; Wang et al., 2011), and comparisons between multiple non-model animals suggested that ca. $30x10^6$ pairs of reads could consistently generate high-quality metazoan assemblies (Francis et al., 2013). We followed this recommendation but obtained metatranscriptomic assemblies that substantially differed from their polyA-selected equivalents.

Sequencing depth recommendations available to date are based on the analysis of polyA-selected libraries. Our results using two sponge species with contrasting microbiomes

(Francis et al., 2017; Vargas et al., 2021) suggest that bacterial abundance might influence the quality of the obtained metatranscriptomes. In this regard, *T. wilhelma*'s metatranscriptomic assembly barely differed from its polyA-selected counterpart in the number of complete bacterial BUSCOs while *L. chondrodes*' assembly using rRNA depletion showed a substantial increase in the obtained number of complete bacterial BUSCOs compared to the polyA-selected assembly. *Tethya wilhelma*'s microbiome is simpler than that of *L. chondrodes* (Francis et al., 2017; Vargas et al., 2021), and the bacteria in *T. wilhelma* occur in lower abundances than in *L. chondrodes*, a species with abundant cyanobacterial symbionts. Assuming a baseline of $30x10^6$ read pairs for polyA-selected metazoan transcriptomes, increasing the number of reads *n* times with *n* equal the number of holobiont members seems adequate when sequencing rDNA-depleted total RNA. However, the optimal sequencing depth for rRNA-depleted libraries remains to be empirically determined and individually adjusted based on the complexity of the studied holobiont.

Cyanobacteria dominate the microbiome of *L. chondrodes* (Vargas et al., 2021), and exposing this species to shading causes it to bleach (Curdt, Schupp, & Rohde, 2022). As our RT-qPCR relative quantification results indicate, Cyanobacteria were significantly less abundant in shaded *vs.* control *L. chondrodes* sponges. On the contrary, Actinobacteria, a non-photosynthetic bacterial phylum in *L. chondrodes*' core microbiome (Vargas et al., 2021), did not change in abundance upon shading. As expected, differential gene expression analyses using rRNA-depleted libraries resulted in a depletion of cyanobacterial transcripts in the metatranscriptome of bleached *L. chondrodes* specimens. For Actinobacteria, however, differential gene expression analyses using rRNA-depleted libraries suggested an increase in the abundance of transcripts derived from this bacterial group. According to our qPCR

results Actinobacteria did not change in abundance. Thus, it is not clear to us whether the observed increase in actinobacterial transcript abundance reflect a true change in actinobacterial gene expression in bleached *vs.* control sponges or a better detectability of actinobacterial transcripts as a consequence of the decay of the cyanobacterial population. In this regard, besides increasing the sequencing depth for experiments using metatranscriptomic data, quantifying the abundance of different bacterial groups seems necessary to dissect whether the detected gene expression changes are real or, on the contrary, are artifacts caused by changes in bacterial abundance or composition in the studied sponges.

Here, we have presented an rRNA-depletion strategy for the simultaneous sequencing of the transcriptomes of demosponges and their microbial communities. We tested this strategy in two non-closely related demosponges with contrasting microbiomes and showed its capacity to recover more bacterial transcripts and capture the transcriptional changes caused by environmental perturbations (*i.e.*, shading). We consider our strategy an opportunity to standardize the generation of sponge metatranscriptomes, facilitating the metatranscriptomic profiling of sponge holobionts along their ontogeny, the study of their responses to environmental change, and between-species comparisons of these systems. We furthermore emphasize that - following individual taxon-specific adaptations - this approach can be expanded to cover other sponge classes and animal groups.

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Data Availability: The metatranscriptomic reads generated for this study are available under Bioproject PRJEB53671 PolyA-selected reads were taken from Bioproject PRJEB24503 and PRJNA288690. The metatranscriptomic assemblies generated with the corresponding annotations, the bacterial metagenomic assemblies used to map metatranscriptomic reads for differential gene expression analyses, and the scripts used are available in the project repository: https://gitlab.lrz.de/cbas/cbas_resources.

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