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ABSTRACT

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Subjects Biodiversity, Environmental Sciences, Microbiology, Bioinformatics, Genomics

Keywords *Lubomirskia baicalensis*, Primmorphs, Symbionts, Opportunistic pathogen.

INTRODUCTION

Sponges (phylum Porifera) are ancient multicellular animals that have existed for more than 635 million years and are unique compared to other Metazoa (*Love et al., 2009*). These organisms are involved in water filtration and recycling of particles, molecules, ion including carbon cycling, silicon cycling, oxygen depletion, nitrogen cycling, conversion of dissolved organic matter and partially can be support by metabolic exchange between symbiont and host (*Taylor et al., 2007; Fan et al., 2012; de Goeij et al., 2013*). Sponges are also good bioindicators of the state of the environment and play an important role in aquatic ecosystems (*Wilkinson & Fay, 1979; Webster, 2007; Wulff, 2007; Bell, 2008*). Numerous types of sponges are potential sources of biologically active molecules, which are extremely important for biomedicine a pharmacology due to their anticarcinogenic, antiviral and antibacterial properties (*Tsujii et al., 1988; Munro et al., 1999; Shakeri & Sahebkar, 2015*). Most types of sponges are marine, freshwater sponges are much less diverse. Freshwater sponges in the illuminated habitat zone are colored in green tones due to symbionts - unicellular green algae or Cyanobacteria (*Wilkinson, 1980, Alex et al., 2012, Chernogor et al., 2013, Raharinirina et al., 2017*).

Sponge symbionts can occupy more than 40-60% of the volume of sponges and influence host metabolism (*Bayer et al., 2007; Webster et al., 2012*). Sponges can maintain highly diverse, specific symbiont communities, despite the constant influx of water microorganisms in the process of filter-feeding, in the same time, they are strongly affected by waterborne viruses, bacteria, archaea and eukaryotic microorganisms (*Thomas et al, 2016, Moitinho-Silva et al, 2017*). Symbiont communities are characterized by specialists and generalists rather than opportunists' bacteria and core sponge microbiomes are usually stable (*Sagar et al., 2010; Miller et al., 2011; Fan et al., 2013; Fujise et al., 2014; Hester et al, 2015*). Sponge symbionts are species-specific and are divided into two clusters - the basic (core) microbiome, consisting of microorganisms found in most sponge species, and the variable microbiome, consisting of "narrow specialists", which differ in their relative numbers and are rarely found in other species (*Thomas et al, 2016, Moitinho-Silva et al, 2018*).

Currently, disease and mass mortality of sea sponges and corals to marine environments have been observed worldwide (*Webster et al., 2004; Olson et al., 2006; Hoegh-Guldberg et al., 2007; Pita et al., 2018*). Such diseases affect natural sponge populations and threaten all sponge-associated biodiversity (*Webster, 2007; Olson et al., 2006; Stabili et al., 2012*). Many researchers associate sea sponge diseases with changes in the composition of symbionts and the appearance of opportunistic infection resulting from changes in water temperature caused by global warming, light intensity, and salinity (*Webster et al., 2008; Luter et al., 2010; Sagar et al., 2010; Miller et al., 2011; Fan et al., 2013; Fujise et al., 2014*).

Freshwater sponges from Lake Baikal also are affected by disease and mortality. Lake Baikal is located in southeastern Siberia (53°30′N 108°0′E) and is the world's largest (23,000 km), deepest (1.643 m) and oldest (> 24 million years) freshwater body (*Kozhova & Izmest'eva, et al., 1998*). Lake has many features inherent to the ocean: abyssal depths, a huge mass of water, oxygen-rich water that stretches to the very bottom, internal waves and seiches, strong storms and high waves, upwelling et al. (*Kozhov 2013; Smirnov et al., 2014; Troitskaya et al., 2015*). Endemic freshwater sponges (Demosponges, Lubomirskiidae) dominate in Lake Baikal in the littoral zone at depths of 3 to 35 m. They cover near 50% of the available surfaces (*Pile et al., 1997*) and represent a complex consortium of many species of eukaryotes and prokaryotes (*Sand-Jensen & Pedersen, 1994; Bil et al., 1999*). These sponges are chlorophyll-containing freshwater algae (*Latyshev et al., 1992; Bil et al., 1999*).

The first appearance of anomalously colored pink *L. baicalensis* (Pallas, 1776) sponges were found in 2011. In subsequent years, the external signs of the disease have changed and now sponges are found throughout the lake's littoral with various symptoms of body lesions, such as discoloration, tissue necrosis and dirty purple bacterial covers on individual branches. The number of *L. baicalensis* sponges that are most susceptible to the disease has decreased significantly, annually are observed to 10–20% of diseased sponges that die during the winter period (*Timoshkin et al., 2016; Khanaev et al., 2018*). Currently, diseased and dying sponges have been observed in many areas of the lake (*Kravtsova et al., 2014; Khanaev et al., 2018; Belikov et al., 2019*). Researchers observed a large-scale disturbance in the spatial distribution and structure of phytocoenoses of the coastal zone of Lake Baikal (*Timoshkin et al., 2016*). Some authors described bacterial communities in diseased sponges, but pathogenic agents have not

been identified (*Kaluzhnaya et al., 2015; Kulakova et al., 2018*). The etiology and ecology of disease and mass death of sponges remain unknown.

The development of a model to investigate the transmission of pathogenic agents from diseased sponges require a detailed study of pathogen-host interactions in the environment. However, these experiments with sponges under natural conditions of Lake Baikal are difficult to reproduce. We used the cell culture of the Baikal sponge *L. baicalensis* primmorphs for experimental infection *in vitro* to identify changes in the microbiomes of diseased sponges using the 16S rRNA gene.

The purpose of this research was to use cell culture of primmorphs in vitro to study the microbiomes of healthy and diseased sponges during their mass mortality. Results of this study will allow us to assume cell culture of primmorphs can model sponge-microbe interactions *in vitro* and will expand understanding about symbiotic relationship of microorganisms with freshwater Baikal sponges during mass mortality.

MATERIAL AND METHODS

Sponge collection and cell culture of primmorphs

The endemic freshwater Baikal sponges *L. baicalensis* Pallas, 1776 (Demospongiae, Haplosclerida, Lubomirskiidae) were the object of this study (Figs. 1A–1B).

Figure 1 **Samples of the healthy sponge and primmorphs.** (A) The healthy freshwater Baikal sponge *L. baicalensis*, (B) cell culture of primmorphs of *L. baicalensis* obtained from the sponge. Scale bars are 5 mm. Canon EOS 200D digital camera.

Specimens were collected in the individual containers from Lake Baikal in the Olkhon Gate area (53° 02′ 21 N′′; 106° 57′37 E′′) at a depth of 12 m (water temperature 3–4 °C) by scuba divers in February 2015. The collected samples of sponges were immediately placed in containers with Baikal water and ice and transported to the laboratory. The sponges without visible symptoms of the disease and having a green color were selected (Figs. 1A-1B) The group of healthy samples included healthy sponge and primmorphs cultivated at 1 and 14 day (Table 1).

Table 1 **Samples of sponges *L. baicalensis* and cell culture of primmorphs**. Samples were collected from the strait Olkhon Vorota, Lake Baikal.

Samples of diseased and sick sponges were collected to obtain a bacterial suspension to infect healthy cell cultures of primmorphs. The group of diseased samples included diseased and sick sponges and experimentally infected primmorphs and primmorphs obtained from these diseased sponges (Table 1 and Figs. 2A–2B).

Figure 2 **Samples of diseased sponges.** (A) The diseased freshwater Baikal sponge *L. baicalensis*. (B) The sick freshwater Baikal sponge *L. baicalensis.* Canon EOS 200D digital camera.

The primmorphs were obtained by a method of mechanical dissociation of cells according a previously described technique (*Chernogor et al., 2011*). A clean sponge was squashed, and the cell suspension obtained was subsequently filtered through a sterile 200-, 100-, and 29-μm-mesh nylon to eliminate pieces of skeleton and spicules of the maternal sponge. The gel-like suspension was diluted 10-fold with Baikal water, placed in a refrigerator, and stored for 3 min at 3–6 °C until a dense precipitate formed, and then washed several times with sterile Baikal water until the complete elimination of the turbid uppermost layer. Natural Baikal drinking water (NBW) that was obtained at a depth of 500 m, passed through sterilizing filters and processed with ultraviolet light, and ozone (patent of the Russian Federation No. 2045478) was used as the growth medium. The suspension was placed into 200–500-ml cultural bottles (Nalge Nunc International, Rochester, NY, USA) and washed with NBW twice every 30 min for the first 2 h. Primmorphs were cultivated in NBW at 3–4°C and light intensity of 47 lux or 0.069 watt with 12 h mode of day and night change.

Experimental infection of primmorphs

Healthy cell cultures of primmorphs (2-4 mm in diameter) with green color were transferred to six 24-well plates (Nalge Nunc International, Rochester, NY, USA), 1-2 pieces per well. We

used to three 24-well plates for each experiment for infection of primmorphs with suspensions of microorganisms from diseased and sick sponges (Fig. 3).

Figure 3 **Experimental design study of the microbiomes of healthy, diseased sponges and primmorphs.**

Notes. 1 The suspensions of microorganisms from diseased sponge.

2 The suspensions of microorganisms from sick sponge.

The suspensions of microorganisms were obtained by squeezing 10 g samples of diseased and sick sponges. The cell suspensions were purified by filtering through sterile 100- and 29-μm mesh nylon to eliminate pieces of skeleton and spicules from the maternal sponge and were subsequently filtered through 10.0 μm filters (Millipore, Germany). Then, the cell suspensions were diluted 10 times with cold NBW and the cellular debris was removed by centrifugation at 1500 rpm for 3 minutes. Healthy primmorphs were infected with 25 μl of suspensions from the diseased and sick sponges. Primmorphs were cultivated in 2 ml of NBW at 3-6 °C with a 12 hour day and night cycle for 30 days. The observations were carried out during of the infection with daily descriptions also for fixing and DNA extraction for sequencing of microbiomes.

Microscopy studies

We observed changes of infected and diseased cell cultures of primmorphs every day for one month with an Axio Imager Z 2 microscope (Zeiss, Germany) equipped with fluorescence optics (self-regulating, blue HBO 100 filter, 358/493 nm excitation, 463/520 nm emission). Samples of cell cultures were stained with a NucBlue Live ReadyProbes reagent (Invitrogen, USA). All images were taken with a Canon EOS 200D digital camera. In addition, samples were prepared for scanning electron microscopy (SEM) analyses. Fixation for SEM was performed according to the following procedure: pre-fixation in 1% OsO4 − 10 min, washing in cacodylate buffer (30 mM, pH 7.9) − 10 min, fixation in 1.5% glutaraldehyde solution on cacodylate buffer (30 mM, pH 7.9) − 1.0 h, washing in cacodylate buffer (30 mM, pH 7.9) − 30 min, postfixation in 1% OsO4 solution on cacodylate buffer (30 mM, pH 7.9) − 2 h; washing in filtered Baikal water − 3×15 min at room temperature, and dehydration in a graded ethanol series. The specimens were

placed onto SEM stubs, dried to a critical point, and coated with liquid carbon dioxide (BalTec CPD 030) using a Cressington 308 UHR sputter coater before examination under a Sigma series scanning electron microscope (Zeiss, Germany) operated at 5.0 kV.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from the samples of sponge tissue (0.1 - 0.2 g) and primmorphs after bead beating using the TRIzol LS reagent (Invitrogen, USA) according to the manufacturer's protocols. Total DNA from three technical replicates for each sample was suspended in 18 µl of RNase-free water and stored at - 70 °C pending for further analysis. The universal bacterial primers 518F and 1064R (*Ghyselinck et al., 2013*) were used to amplify the V4–V6 hypervariable region of the bacterial 16S rRNA gene. The following program was used to amplify 16S rRNA genes using PCR: 3 min at 96 °C; 30 cycles at 94 °C for 20 s, 55 °C for 20 s and 72 °C for 1 min with a final 10-minute incubation at 55 °C. PCR products were quantified using the NanoDrop device, mixed equally and sequencing using the 454 GS Junior Sequencing System (Roche, Basel, Switzerland) and with GS FLX Titanium series reagents. The raw sequencing reads were submitted to the NCBI Sequence Read Archive under BioProject PRJNA 480187 (454 GS platform).

Processing of sequencing data

Bioinformatics and statistical analyses were performed using the QIIME2 2019.1 pipelines (*Bolyen et al., 2018*). First, reads were first demultiplexed then analyzed for quality to determine trimming parameters. The first 17 nucleotides for each read was trimmed and the total length of reads were truncated to 360 nucleotides due to the decrease in quality score observed after 360 nucleotides. Reads containing any ambiguities were removed as were reads exceeding the probabilistic estimated error of 2 nucleotides. After quality screening and trimming, the DADA2 pipeline was used to remove chimeric variants and to identify sub-OTUs (*Callahan et al., 2016*). Sub-OTUs are defined by analysis of polymorphic sites within amplicons and have shown a greater taxonomic sensitivity than OTUs clustered by a 3% dissimilarity threshold (*Callahan et al., 2016; Thompson et al., 2017*). Analysis of sub-OTUs in place of OTUs has proven effective in resolving

fine scale ecological temporal dynamics and community changes in the human microbiome, which is why we used sub-OTUs rather than OTUs in analyzing the sponges, and primmorphs microbiome (*Eren et al., 2014; Tikhonov et al., 2015*). The SILVA 132 database was used for taxonomic assignment. Reference sequences in the SILVA 132 database were first trimmed to the V4–V6 region with the 518F/1064R primers used in the PCR. Taxonomy assignments were performed using q2-feature-classifier (*Bokulich et al., 2018*). Sequences identified as mitochondria were removed from libraries prior to analysis. The relative abundance of mitochondrial reads across all libraries was <0.000001% which had a minimal effect on library size upon removal. Sub-OTU relative abundance values were calculated by transformation to library read depth. In total 8 libraries were analyzed. The alpha-diversity indices (Chao1, Shannon diversity index) were calculated using the QIIME software to establish the abundance and diversity of the sequences. Weighted Unifrac dissimilarity values were used for β-diversity measurements (*Lozupone & Knight, 2005*). Principal Coordinates Analysis (PCoA) (*Halko et al., 2010*) were used to visualize β-diversity, and the significance of grouping variable (stage of life and health) were assessed using pairwaise - PERMANOVA test (*Anderson 2001*). For interpretation of the microbial community, we used tidyr R package and ggplot2 R package to build a heat map selecting 20 most abundant sub-OTUs.

RESULTS

Microscopy studies cell cultures of primmorphs

Healthy cultures of primmorphs had a bright green color and bright red autofluorescence of chlorophyll in cells on the second and following days of cultivation (Figs. 4A−4B). We observed different cells of sponges, amoebocytes with nuclei and inclusion of green symbiotic algae were clearly visible. A completely different picture was observed in infected of primmorphs. All samples of primmorphs lost green color at infected with suspension of microbial cells from diseased and sick sponges at 3-4 days. We observed a misbalance in cells of sponges, chaotic arrangement of algae, destruction their cell walls and leakage of contents with increase number bacteria of different types at 7 days (Figs. 4C−4D). In the same time, in primmorphs infected with diseased sponge, there was full loss of green alga and tissues of sponge with the growth of

different bacteria for 21 days (Figs. 4E−4F). The loss of chlorophyll autofluorescence was observed in all experimental samples. Bleaching and death cell culture of primmorphs observed after 21 days. A similar developmental dynamic of infection has been observed at the primmorphs becomes infected with the suspension of cells from a sick sponge.

Figure 4 **Light and fluorescence images cell cultures of primmorphs of sponge *L. baicalensis.*** (A) Light microscopy, showing green microalgae located within amoebocytes in the healthy cell culture of primmorphs. Arrows show sponge amoebocytes within microalgae (B) Fluorescence microscopy, showing red autofluorescence of chlorophyll-containing intracellular of green algae in the healthy primmorphs. (C) The primmorphs infected with cellular suspension from the diseased sponge observed the death of green algae symbionts, sponge cell (indicated by arrow). Shown massive numbers, of different bacteria at 7 day (indicated by arrow). (D) Fluorescence microscopy, showing the death of microalgae (red color) in infected primmorphs from diseased sponge at 7 day. Bacteria shown blue color. (E) The primmorphs infected with cellular suspension from the diseased sponge for 21 days, shown residues of green algae in cell culture of primmorphs and huge biomass of bacteria (indicated by arrow). (F) Fluorescence microscopy showing death of green algae primmorphs infected suspension from the diseased sponge and massive of different bacteria for 21 day. Bacteria in infected primmorphs, shown blue color. Samples of primmorphs stained with the NucBlue Live ReadyProbes reagent for fluorescence microscopy. Scale bars: 10 μm.

We observed similar results in both diseased, sick sponges and primmorphs. The dirty scurf, fetid odor, and the formation of films were observed in all infected cultures, which was probably associated with the growth of different types of bacteria. We found that the same pattern of disease development was observed using SEM. The surface of epithelium was clean even and smooth in healthy primmorphs (Fig. 5A). At the same time, infected primmorphs had desquamated epithelium destroyed by different groups of bacteria (Fig. 5B). As a result, the surfaces of infected primmorphs became uneven and eroded loose by numerous microorganisms penetrating into the spongin leading to the degradation, necrosis and death of cells and tissues (Fig. 5C). We observed a huge amount of bacteria in infected cultures of primmorphs that formed a bio-cake after 30 day of cultivation (Fig. 5D).

Figure 5 **SEM images of cell cultures of primmorphs.** (A) The epithelial surface of healthy cultures were clean, flat and smooth. (B) The surface of the primmorphs infected with the cellular suspension from the diseased sponge. Observed melting of the epithelial cells of sponge, increased different bacteria at 7 day. (C) The primmorphs infected with cellular suspension from the diseased sponge, the death of green algae symbionts, sponge cells and massive growth of different bacteria for 21 day. (D) Bio-cake formed in infected cultures of primmorphs from diseased sponge at 30 day. Scale bars are 1 μm.

Moreover, there was a large increase biomass of different bacteria accompanied by the death of cells and tissues of primmorphs.

Samples description

We investigated the composition of microbiomes of two groups: healthy and diseased samples, sponges and cell culture of primmorphs. The healthy group included the sponge and the cell culture of primmorphs that were prepared from this sponge (Table 1). The group of diseased samples included diseased, sick sponges and experimentally infected cell culture of primmorphs. A total of 8 samples were obtained from two groups of healthy and diseased sponges and primmorphs sequenced to generate V4–V6 16S rRNA gene profile. The sum of reads for all libraries passing the quality control parameters for this study totaled 30,488 reads with a mean library depth of 3,811 reads/library. The number of reads ranged from 2155 in healthy primmorphs to 7564 in infected primmorphs for the 454 GS samples. The estimates of sampling depth using Michaelis-Menten fit to rarefaction curves show that the composition of microbiomes at the sub-OTU level is average underestimated by 8.2% (Table 2).

Table 2 **Summary of microbial communities in sponges and primmorphs.**

Notes.

The name of samples: SH (Healthy sponge); PH1 (Primmorphs for 1 day); PH14 (Primmorphs for 14 day); SD (Bleached sponge); PD (Primmorphs diseased); PID

(Primmorphs infected by diseased sponge); SS (Sick sponge); PIS (Primmorphs infected by sick sponge). Samples IDs are referred to Table 1.

Total number of sub-OTUs in healthy sponge and primmorphs ranged from 35 to 61, and that in the diseased ranged from 43 to 76. Microbial diversity within each group was calculated and compared between the two groups. The microbial richness estimator (Chao 1 index values) showed no significant difference (Fig. 6A).

Figure 6 **The alpha-diversity indexes (Chao1 and Shannon index) of the data distribution.** A) The distribution between the group's adult sponges and primmorphs. B) The distribution between the groups of healthy and diseased of sponges and primmorphs. Samples were referred to Table 1.

We did not find significant differences between diversity the adult sponges and primmorphs by using Shannon index. The alpha-diversity indices (Shannon index) have significant difference between healthy and diseased groups (Table 3, Fig. 6 B).

Table 3 **The alpha-diversity indices (Chao1, Shannon index)**. The alpha-diversity were calculated using the QIIME2 software to establish the abundance and diversity of the sequences.
Notes.
Samples IDs are referred to Table 1.

Microbial communities of samples of diseased sponges and infected primmorphs were more diverse. The Shannon index for healthy samples varied from 1.98 to 2.86 and for diseased samples − between 3.34 and 6.22 ($p < 0.05$) due to the high abundance of Chloroplasts in healthy samples. The variation in data distribution between the groups was analyzed using PERMANOVA, which indicated a significant difference (p-value <0.05) between healthy and diseased groups under weighted Unifrac. The beta - diversity results of PCoA indicated the data different distribution between the two groups of healthy and diseased (Fig. 7). The results showed that the group of healthy and diseased sponges and primmorphs different from each other, and it was found that these differences (p value <0.05) significant (Table 4).

Figure 7 **The beta-diversity results of PCoA indicating the data distribution between groups.** Samples of the healthy sponge and primmorphs are grouped into one cluster and differ significantly from the group of diseased. Samples were referred to Table 1.

Table 4 **Results pairwaise PERMANOVA test.**

Notes.

Indicates p-value <0.05.

The beta - diversity results of PCoA indicating the data distribution between two groups (Fig. 7). We not found differences in the distribution between healthy samples of the sponge and primmorphs. In addition, there were differences in the group of diseased and sick sponges and primmorphs. A significant difference (pseudo-F 13.8) between healthy and diseased groups samples was found but no difference between sponges and primmorphs was detected (Table 4). An analysis of the main coordinates (PCoA), based on the UniFrac weighted distance value, showed that communities of healthy sponges and primmorphs were grouped separately from communities of diseased sponges and infected primmorphs. Variations of beta-diversity in the group of diseased sponges and primmorphs were higher than in the group of healthy ($p < 0.05$), and beta-diversity in the group of healthy were more similar.

Composition of the microbial community

Abundance and significant difference between the two groups at the phylum level

The changes of the microbial community structure distribution and relative abundances of microbiota of sponges and cell cultures of primmorphs were analyzed at the phylum level (Fig. 8).

Figure 8 **Taxonomic profiles of the microbial communities at the phylum level.**
Relative abundance of reads assigned to phyla (to %). Samples were referred to Table 1.

Five bacterial phyla comprised > 96% of the community composition: *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Verrucomicrobia* and *Dependentiae*. The number of sequencing readings at the phylum level gave provided insights of a shift in microbial communities in healthy and diseased groups. Healthy group of sponge/primmorphs were mainly composed of *Cyanobacteria* (Chloroplast), but diseased group contained of *Bacteroidetes* and *Proteobacteria* (Fig. 8). The most significant differences were observed in the composition and structure of representatives of the taxonomic group *Cyanobacteria*/Chloroplasts in microbiomes of diseased and healthy *L. baicalensis* sponges and cell cultures of primmorphs. The phylum *Cyanobacteria* dominated with abundances at 75-89% in healthy sponges and cell cultures of primmorphs, while Chloroplast abundance decreased to 10-20% in diseased sponges and 0,7-22% in diseased and infected primmorphs. The most abundant bacteria at the phylum level were *Bacteroidetes*, with abundances of 48%, 72%, 65%, 55% and 41% in the diseased group of sponges and primmorphs (Fig.8). The other bacterial phyla were *Proteobacteria* with abundances 32%, 18%, 33%, 45% and 33%, respectively. We found that the *Alphaproteobacteria* group was dominant in healthy sponges and primmorphs by the phylum of Proteobacteria, whereas in the diseased group of sponges and primmorphs replaced by on *Gammaproteobacteria*. The report also demonstrated increased the *Dependentiae* (TM6) to 4% in primmorphs infected with sick sponge.

Abundance and significant difference between the two groups at the family level

The abundance of the most common microbial groups at the family level shown in Table 5.

Table 5 **Abundance and significant difference between the two groups at the phylum/family level.**

Notes.

(to %). Colored lines indicate a shift in microbial communities in healthy and diseased groups.

The composition of microbial communities depicted in a heatmap for 20 most abundant family (Fig. 9).

Figure 9 **Heatmap showing the family with significant differences of relative abundances amongst the two groups.** Sample ID are referred to Table 1. Heatmap based on the scale of 0-8 log.

The most abundant microorganism at the family level were Chloroplast, homologous to the Chlorophyta symbiont of *Lubomirskia* sp., which dominates in healthy sponges and primmorphs with abundances at 84%, 77%, 75%, respectively, whereas in diseased group in the sponge/primmorphs its abundance were less (0.7-9%, 0.7-22%) (Table 5). *Cyanobiaceae* were found in a healthy sponge (5%) and primmorphs (4-0.4%) and decreased with time cultivation while the *Pseudanabaenaceae* presented in diseased sponges (11%). Also, importantly the family *Flavobacteriaceae* was significantly more abundant with 13% to 62% in diseased sponges and primmorphs (Table 5). Other abundant family included *Crocinitomicaceae* (17% in PID and 18% in SS). We found increased relative abundances in *Sphingobacteriales* NS11-12 marine group in infected primmorphs (4% in PID, 26% in PIS), and uncultured eubacterium env. OPS 17 was found in diseased sponge only (12%). Representatives of the *Chitinophagaceae* family were mainly found in healthy group of sponges and primmorphs (5-18%) and diseased sponges (6-10%) but not found in infected primmorphs. *Alphaproteobacteria* showed a high abundance in healthy group of sponge and primmorphs. The family *Sphingomonadaceae* have been abundant (3-7%) in healthy group, but insignificantly in diseased group *Terasakiellaceae* are mainly present in diseased sponge. The family *Burkholderiaceae* was few (0.5-2%) in the healthy group but was highly abundant in diseased group (14%, 15%, 19%, 20%, and 26%, respectively). Representatives of the families *Moraxellaceae* (10-22%) and *Pseudomonadaceae* (0.3-3%) were found in diseased and infected primmorphs. In addition, *Nannocystaceae* were found up to 9% in diseased sponge and primmorphs. The *Vermiphilaceae* was found (4%) only in primmorphs infected from sick sponge.

DISCUSSION

This research focused on the use of cell culture of primmorphs *in vitro* for comparing of their microbiomes with healthy sponges with the goal to show the transfer of microorganisms from diseased sponges to healthy primmorphs. Our experimental results were show that use of cell culture of primmorphs is equivalents of healthy sponges and both the alpha and beta diversity indices had not differences between the groups of adult sponge and primmorphs. The microbial community of healthy sponges and primmorphs are grouped separately from the communities of diseased sponges and infected primmorphs, which confirms the suitability the cell culture of primmorphs as a model sponge system.

The microbial community of healthy group of sponge/primmorphs were mainly composed of the phylum *Cyanobacteria* (*Chlorophyt*a symbiont of *Lubomirskia* sp.) with minimal abundance of bacteria, which indicates on their healthy state. One of the distinctive features of Baikal sponges and another freshwater sponge is their ability to live in symbiosis with various zoochlorellae (*Bil et al., 1999*). We previously showed that the primary photosynthetic algae belonging to green algae of the order Chlorophyta dominate in healthy *L. baicalensis* sponges and cell cultures of primmorphs (*Chernogor et al., 2013*). These unicellular eukaryotes entering the complex symbiotic communities of Baikal sponges produce a significant amount of carbohydrates, chlorophyll, fatty acids and secondary metabolites (*Bil et al., 1999; Latyshev et al., 1992*). Known, symbiosis between algae and freshwater sponges provides mutual benefits of photosynthesis, as oxygen and nutrients passing from algae to sponge and carbon dioxide and phosphate from sponge to algae (*Wilkinson, 1980; Pita et al., 2018*).

We found the mass death of green symbionts (*Chlorophyta*) in diseased group and a shift in their microbial communities of sponges/primmorphs, associated with increase in the abundant of different phyla *Bacteroidetes* and *Proteobacteria* with dominated families *Flavobacteriaceae* and *Burkholderiaceae*. We observed increasing of relative abundance of *Flavobacteriaceae* in diseased sponges, especially in cultures of infected of primmorphs. It is likely, that these bacteria may be pathogen for healthy Baikal sponges. The members of the genus *Flavobacterium*, which belongs to the phylum *Bacteroidetes*, are typical bacteria of saline and freshwater ecosystems that can be opportunistic pathogens (*Chen et al., 2017; Kinnula et al., 2017*). Other researchers have shown that some species of *Flavobacterium* contain proteolytic and collagenolytic enzymes (*Nakayama et al., 2016*). In addition, *Flavobacteriaceae* and *Cryomorphaceae* associated with white band disease of corals (*Gignoux-Wolfsohn et al., 2015; Certner & Vollmer, 2017*). It is

known, that species included the *Cytophaga-Flavobacterium* group are associated with diseases in marine ecosystems (*Dobretsov et al., 2007; Romero et al., 2010; Certner & Vollmer, 2017*). These bacteria regulate a diverse array of activities include symbiosis, antibiotic production, motility, sporulation, virulence and formation of biofilms (*Fuqua et al., 2001; Miller & Bassler, 2001; Singh et al., 2017*).

We founded an increase in relative abundance the *Burkholderiaceae* family in diseased sponges/primmorphs. Interestingly, the families *Flavobacteriaceae* and *Burkholderiaceae* have highly abundant in diseased group of sponges/primmorphs in all samples. The family *Burkholderiaceae* is characterized by the presence of ecologically extremely diverse organisms and contains environmental saprophytic organisms, phytopathogens, opportunistic pathogens, including those for freshwater ecosystems (*Coenye*, 2014).

Such stressful changes in the composition of sponge microbiomes are possibly related to environmental factors at Lake Baikal, such as an increase in temperature, changes in the warming tendencies of surface water layers and an increase in vertical heat exchange. (*Smirnov et al., 2014; Troitskaya et al., 2015*). These changes can be a stress factor for aquatic organisms, which leads to eutrophication in the coastal zone Baikal (*Timoshkin et al., 2016; Bondarenko et al., 2019*).

A similar response to thermal stress in the bacterial biosphere and sponge-microbe associations was described by the authors in marine environments (Simister et al., 2012 a; 2012 b; Erwin et al., 2012). Different microorganisms, both beneficial and harmful, have developed their important roles of vital activity in sponges during evolution (*Thomas et al., 2010; Taylor et al., 2007*). Moreover, in obligate symbiotic systems, especially in sponges, the representatives of the microbial community are very specific and closely related, thus the death of even one species often does not compensate for the loss of the functionally equivalent species (*Thomas et al., 2010; Mao-Jones et al., 2010; Fan et al., 2012*).

In this way, disbalance revealed by us in the investigated microbial communities of sponges and model cell cultures of primmorphs may be due to several different opportunistic bacteria including colonizing pathogens of diseased tissue (*Price et al., 2017*). We found bio-cake layers containing different bacteria in model cultures of primmorphs infected from diseased sponges during the month of cultivation (Fig. 5D). Similar studies were carried out by other researchers that showed the presence of the biofouling organisms disrupts the functioning of

549 sponges due to non-optimal feeding conditions in an aquarium (*Alexander et al, 2015*). This
550 phenomenon is similar to joint coordinated interaction of many types of bacteria, which leads to
551 formation of layers of bio-cake on the artificial membranes (*Waheed et al., 2017*). It is likely,
552 that the distinguishing features of Baikal sponge disease was a shift in microbial composition
553 from commensal bacterial symbionts to opportunistic species.
554 The primmorphs system described here is a powerful new model system for studying
555 basic mechanisms of the development of sponge disease, which will be valuable in future
556 studies. The results our study will help to expand understanding about microbial relationship in
557 the development of disease and the death of Baikal sponges.
558

559 # CONCLUSIONS

560
561 We have developed a convenient experimental cell culture of primmorphs for investigating the
562 transmission of pathogenic agents in infected sponges and study pathogen-host interactions in the
563 environment for future research. Our experimental results were show that use of cell culture of
564 primmorphs is equivalents of healthy sponges, which confirms the suitability of primmorphs as a
565 model sponge system. Was found a shift in microbial communities of diseased sponges
566 associated with the mass death of green symbionts and increased abundances of several different
567 opportunistic colonizers. The results of this study will help broaden our understanding about
568 symbiotic relationships in freshwater sponges.
569

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Ethics statement

We confirm that the field studies did not involve endangered or protected species. For the described field studies in the water area of Lake Baikal, special permits were not required. Ethical restrictions do not apply to sponges and no permits were required to collect sponge samples.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

• Lubov Chernogor conceived and designed the experiments, contributed reagents/materials. performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
• Elizaveta Klimenko performed the experiments and bioinformatic analysis.
• Igor Khanaev contributed materials, performed the experiments.
• Sergey Feranchuk performed bioinformatic analysis in first redaction of manuscript.
• Sergei Belikov review & editing: reviewed drafts of the paper, visualization, analysis tools.

DNA Deposition

Raw sequencing data were submitted to the NCBI Sequence Read Archive under accession number PRJNA480187.

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Figure 1

Samples of the healthy sponge and primmorphs.

(A) The healthy freshwater Baikal sponge L. baicalensis, (B) cell culture of primmorphs of L. baicalensis obtained from the sponge. Scale bars are 5 mm. Canon EOS 200D digital camera.

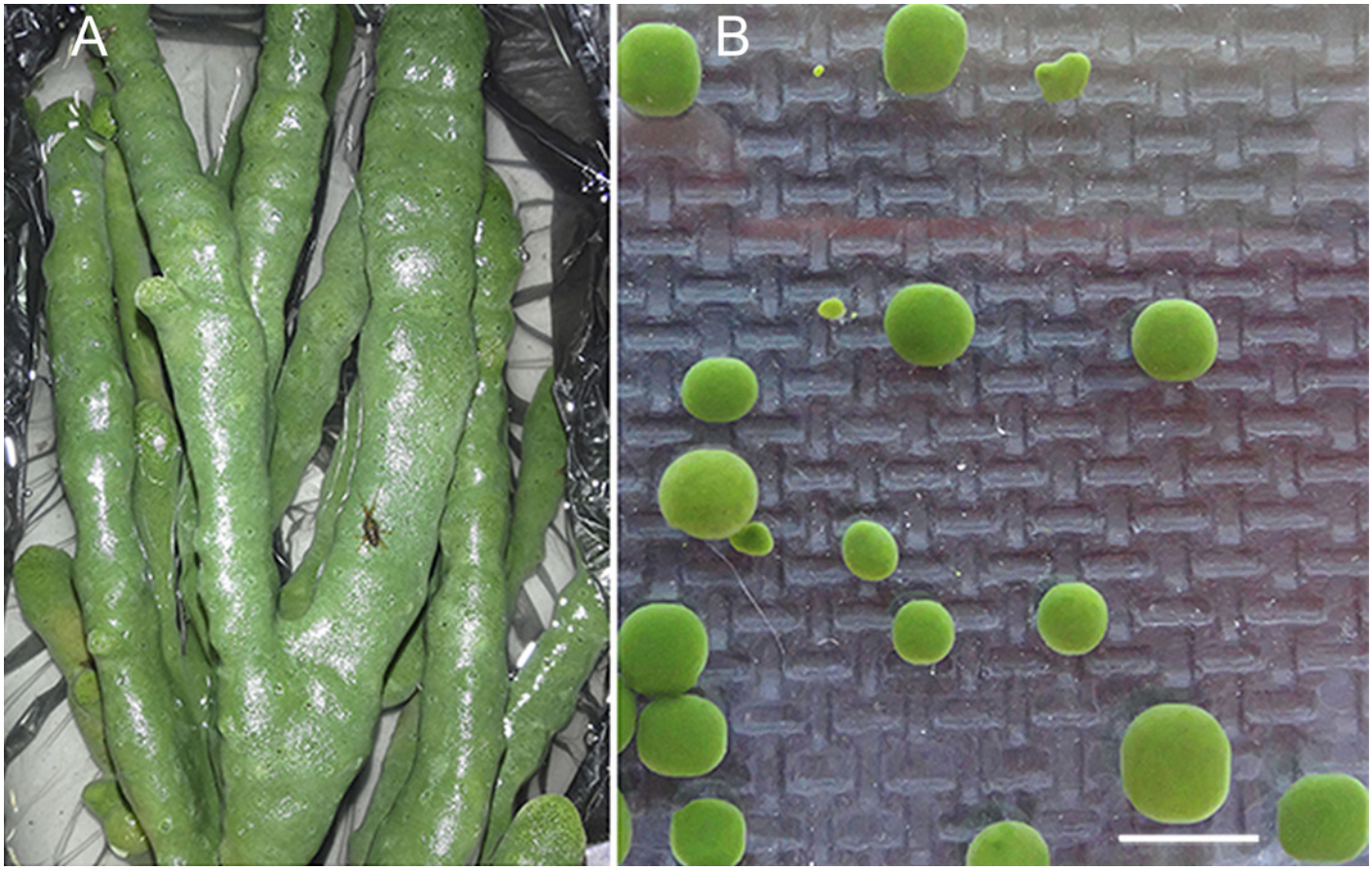

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Figure 2

Samples of diseased sponges.

(A) The diseased freshwater Baikal sponge *L. baicalensis*. (B) The sick freshwater Baikal sponge *L. baicalensis.* Canon EOS 200D digital camera.

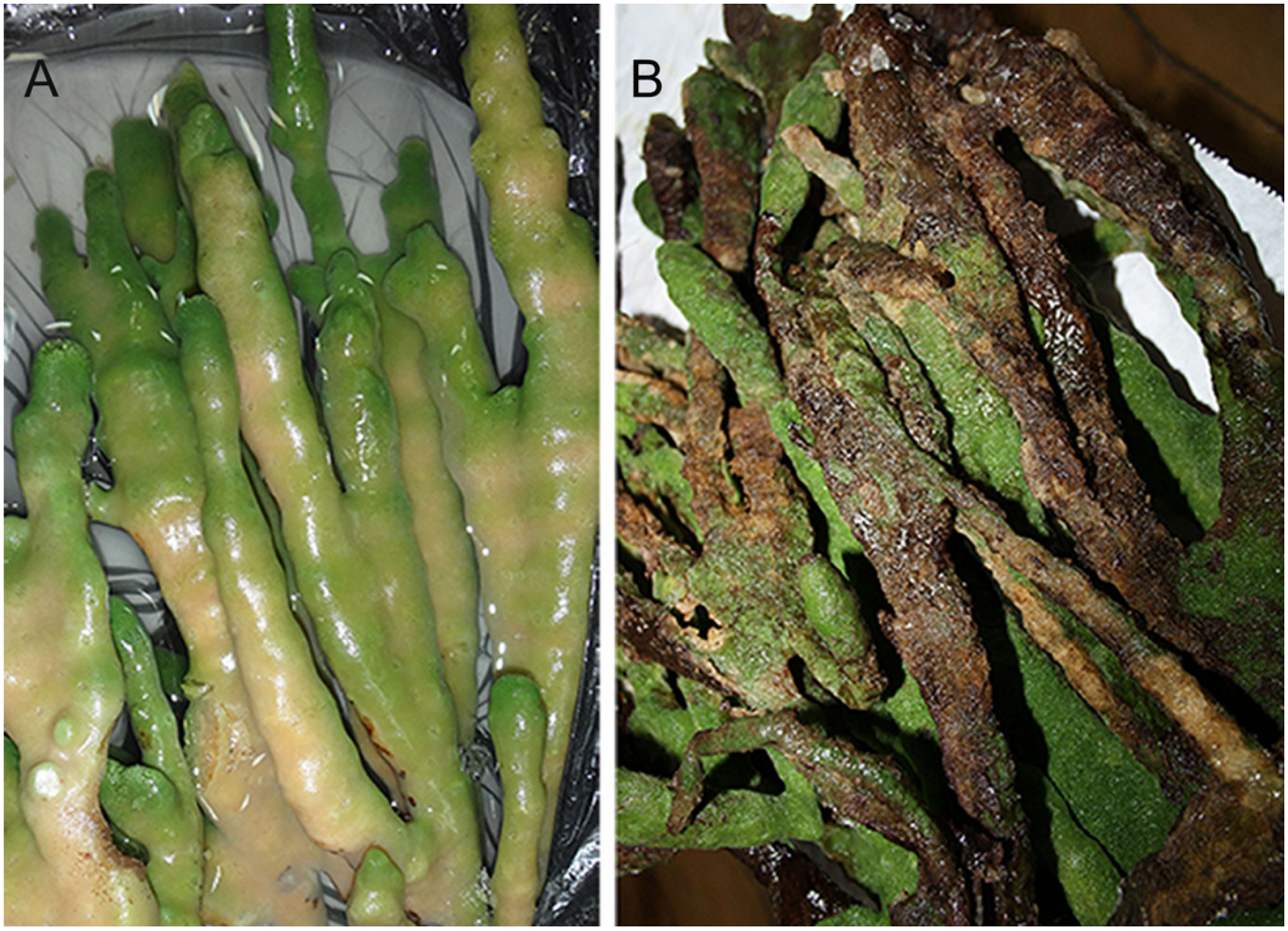

Figure 3

Experimental design study of the microbiomes of healthy, diseased sponges and primmorphs.

Notes. 1 The suspensions of microorganisms from diseased sponge. 2 The suspensions of microorganisms from sick sponge.

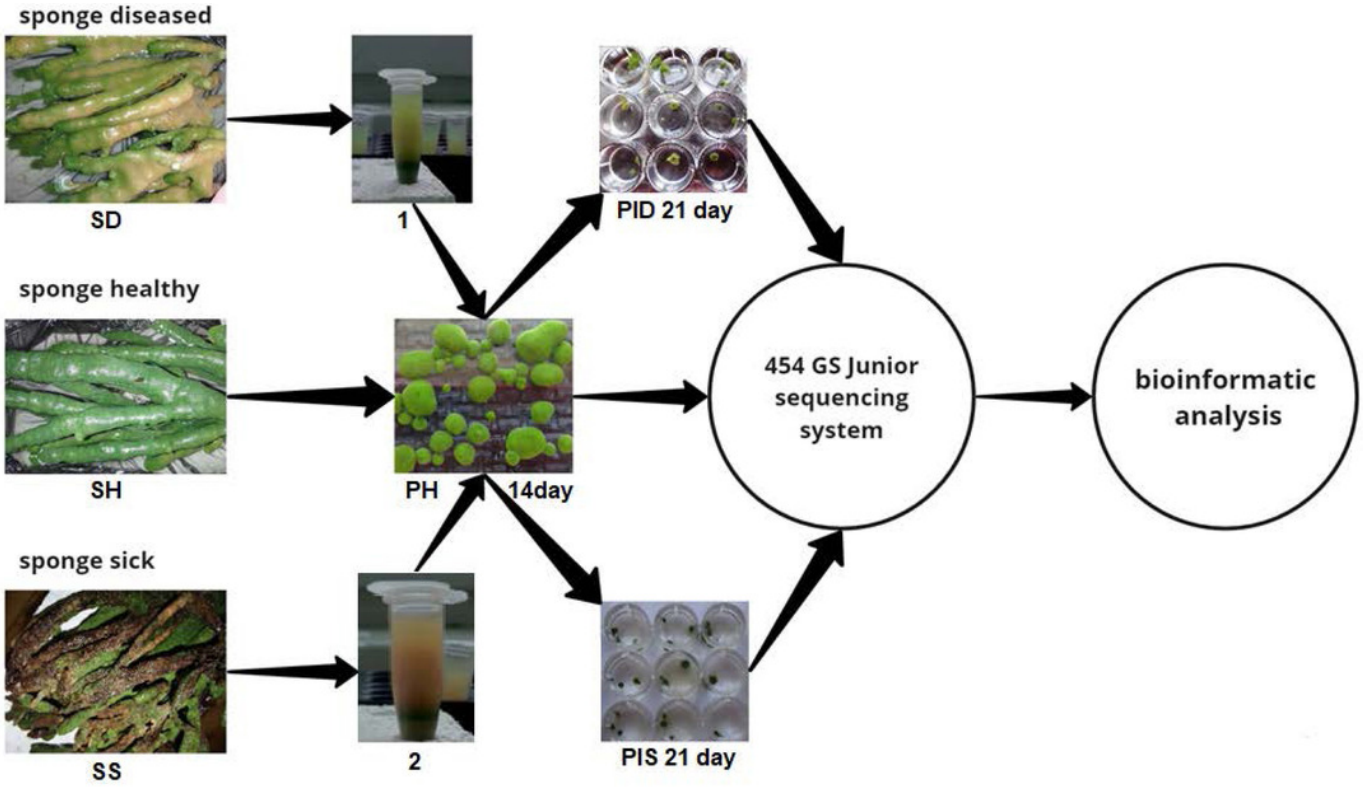

Figure 4

Light and fluorescence images cell cultures of primmorphs of sponge *L. baicalensis.*

(A) Light microscopy, showing green microalgae located within amoebocytes in the healthy cell culture of primmorphs. Arrows show sponge amoebocytes within microalgae (B) Fluorescence microscopy, showing red autofluorescence of chlorophyll-containing intracellular of green algae in the healthy primmorphs. (C) The primmorphs infected with cellular suspension from the diseased sponge observed the death of green algae symbionts, sponge cell (indicated by arrow). Shown massive numbers, of different bacteria at 7 day (indicated by arrow). (D) Fluorescence microscopy, showing the death of microalgae (red color) in infected primmorphs from diseased sponge at 7 day. Bacteria shown blue color. (E) The primmorphs infected with cellular suspension from the diseased sponge for 21 days, shown residues of green algae in cell culture of primmorphs and huge biomass of bacteria (indicated by arrow). (F) Fluorescence microscopy showing death of green algae primmorphs infected suspension from the diseased sponge and massive of different bacteria for 21 day. Bacteria in infected primmorphs, shown blue color. Samples of primmorphs stained with the NucBlue Live ReadyProbes reagent for fluorescence microscopy. Scale bars: 10 µm.

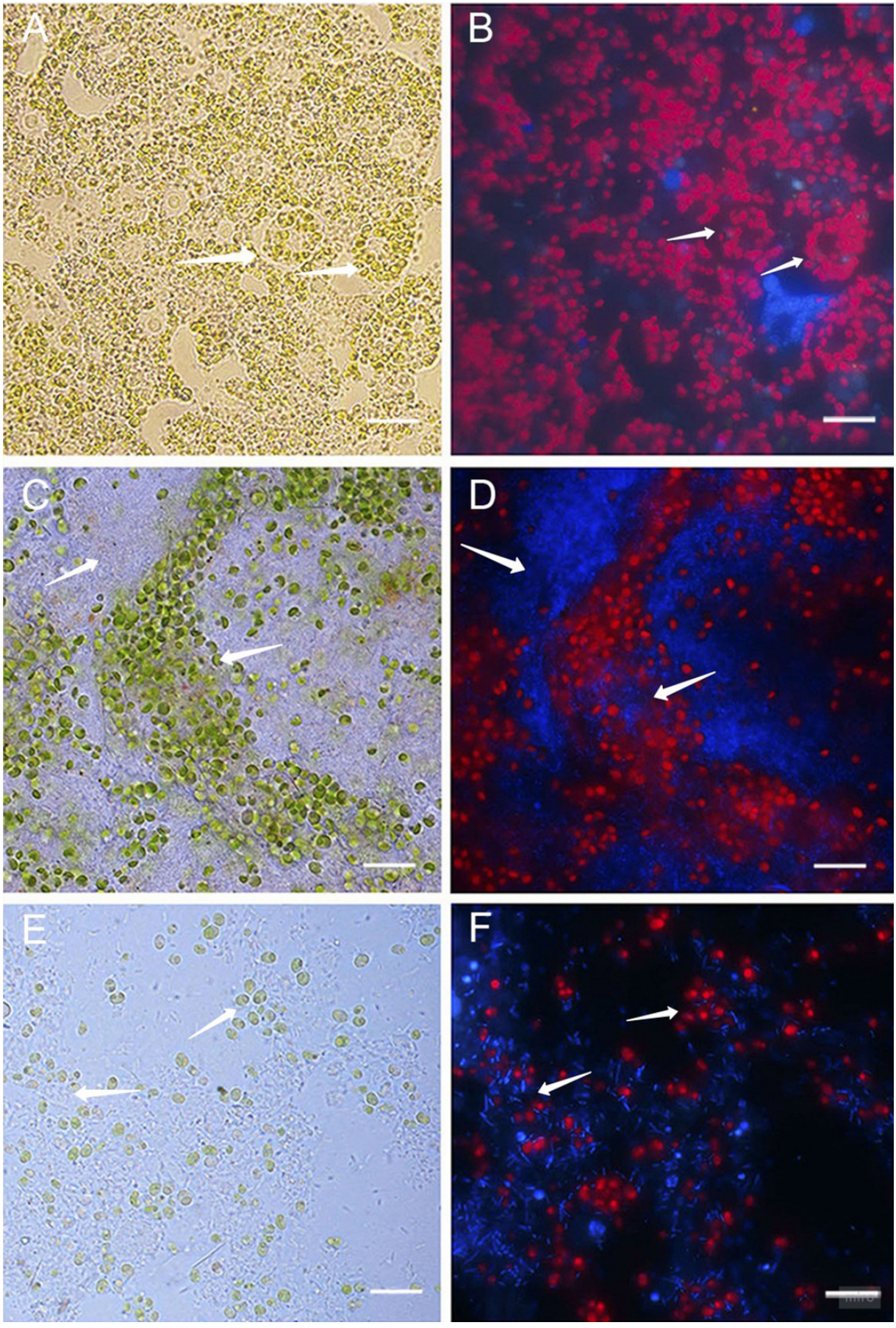

Figure 5

SEM images of cell cultures of primmorphs.

(A) The epithelial surface of healthy cultures were clean, flat and smooth. (B) The surface of the primmorphs infected with the cellular suspension from the diseased sponge. Observed melting of the epithelial cells of sponge, increased different bacteria at 7 day. (C) The primmorphs infected with cellular suspension from the diseased sponge, the death of green algae symbionts, sponge cells and massive growth of different bacteria for 21 day. (D) Bio-cake formed in infected cultures of primmorphs from diseased sponge at 30 day. Scale bars are 1 μm.

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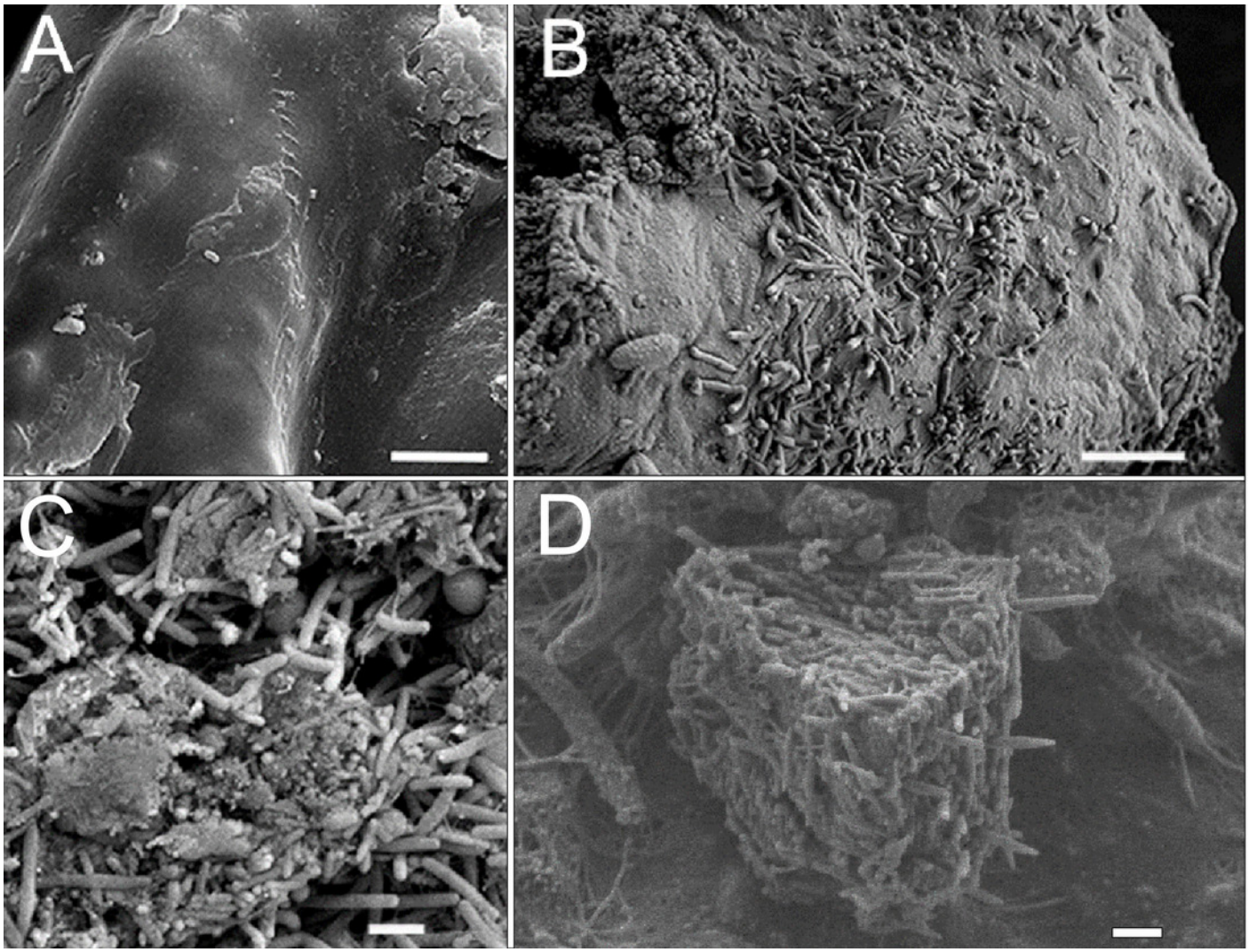

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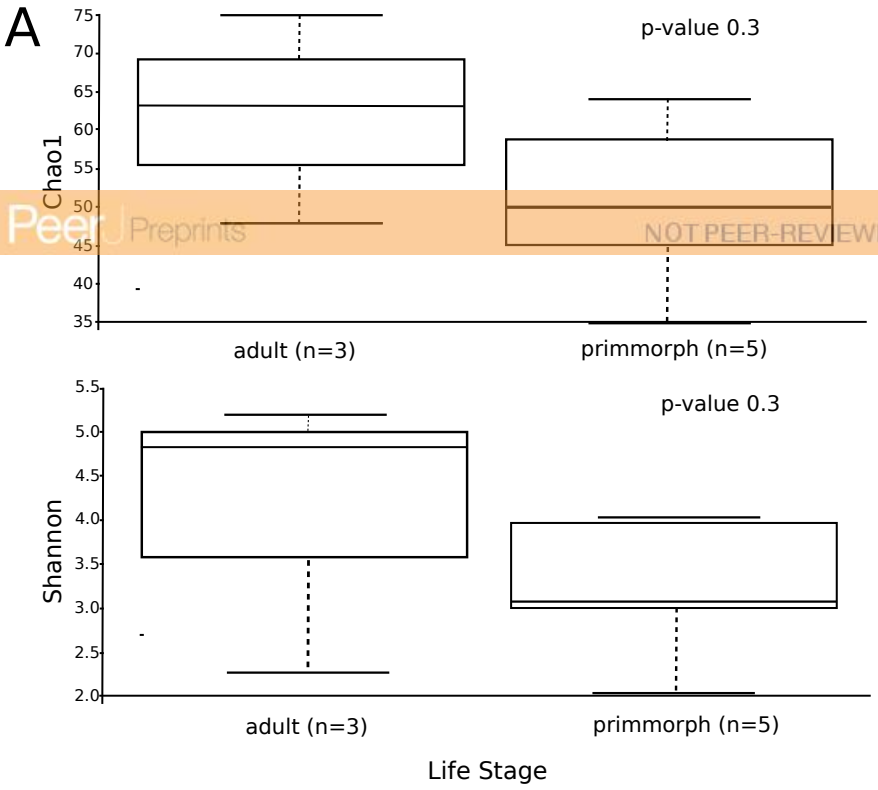
Figure 6(on next page)

The alpha-diversity indexes (Chao1 and Shannon index) of the data distribution.

A) The distribution between the group's adult sponges and primmorphs. B) The distribution between the groups of healthy and diseased of sponges and primmorphs. Samples were referred to Table 1.



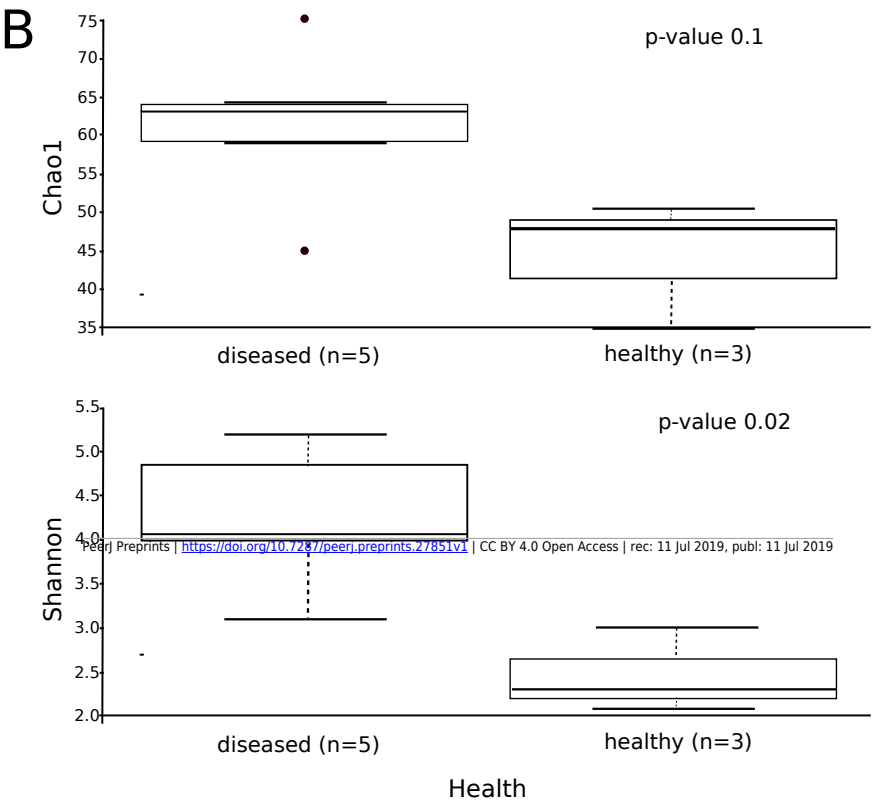


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Figure 7 (on next page)

The beta-diversity results of PCoA indicating the data distribution between groups.

Samples of the healthy sponge and primmorphs are grouped into one cluster and differ significantly from the group of diseased. Samples were referred to Table 1.

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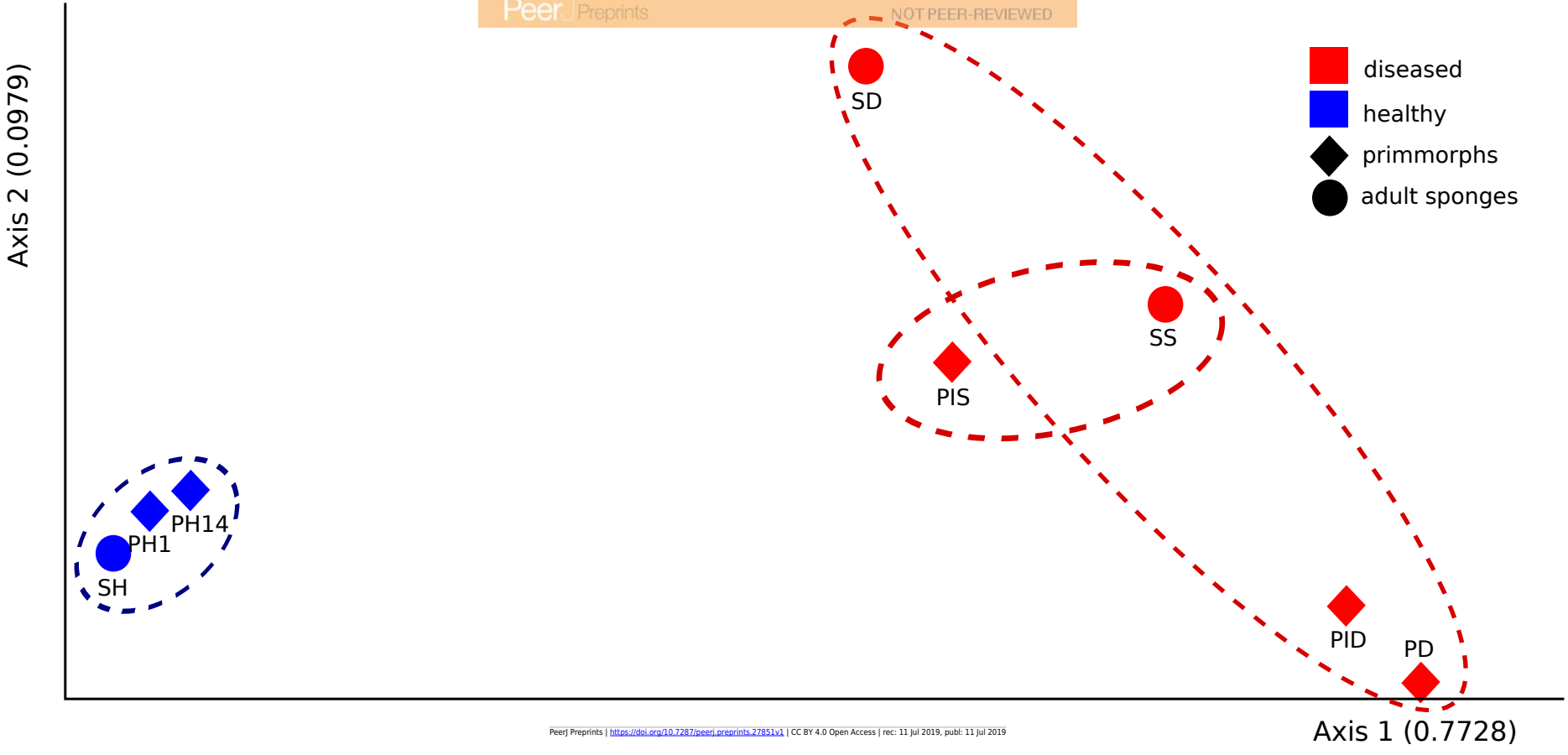

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Figure 8

Taxonomic profiles of the microbial communities at the phylum level.

Relative abundance of reads assigned to phyla (to %). Samples were referred to Table 1.

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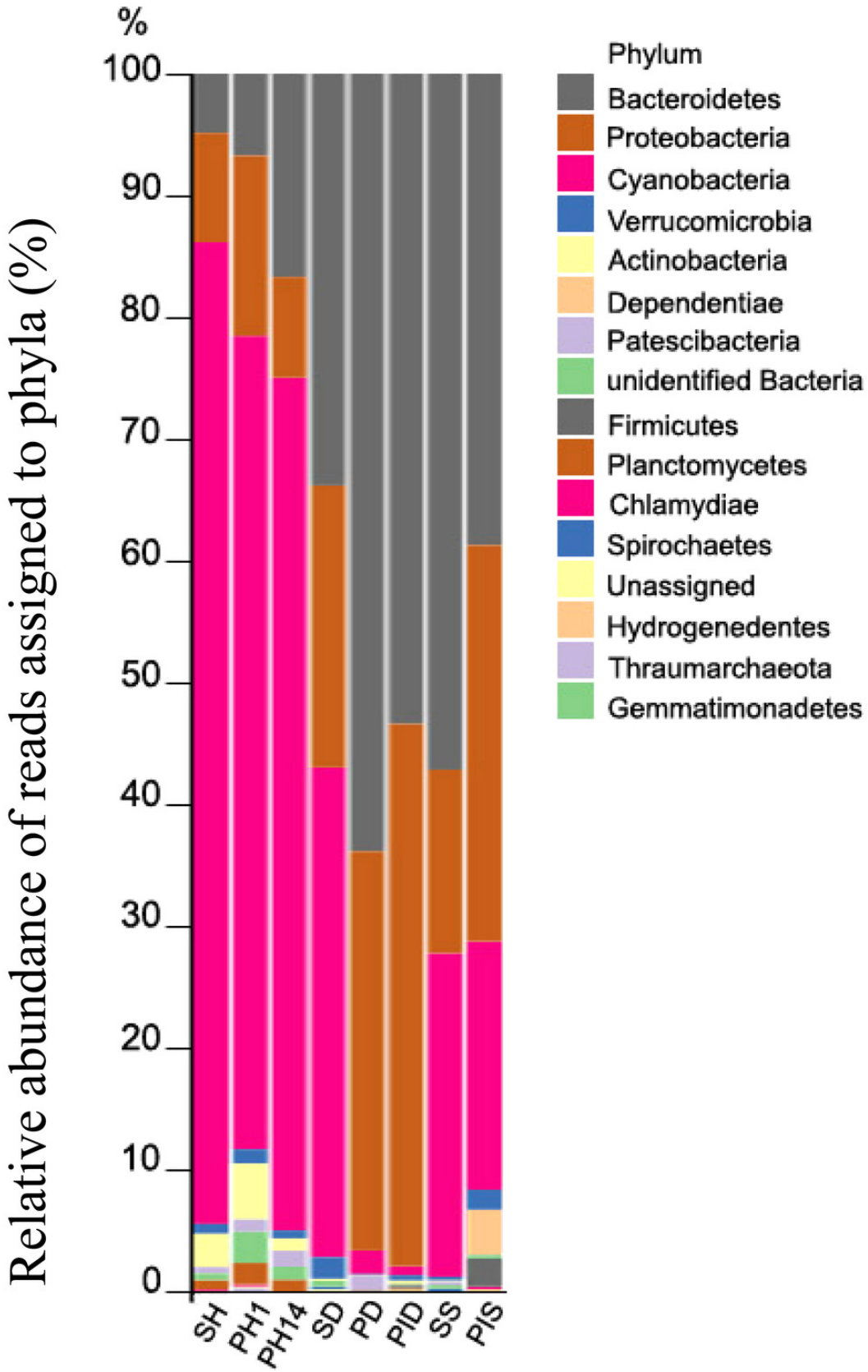
%
100
90
80
70
60
50
40
30
20
10
0
Relative abundance of reads assigned to phyla (%)
SH
PH1
PH14
SD
PD
PID
SS
PIS
Phylum
Bacteroidetes
Proteobacteria
Cyanobacteria
Verrucomicrobia
Actinobacteria
Dependentiae
Patescibacteria
unidentified Bacteria
Firmicutes
Planctomycetes
Chlamydiae
Spirochaetes
Unassigned
Hydrogenedentes
Thraumarchaeota
Gemmatimonadetes

Figure 9

Heatmap showing the family with significant differences of relative abundances amongst the two groups.

Sample ID are referred to Table 1. Heatmap based on the scale of 0-8 log.

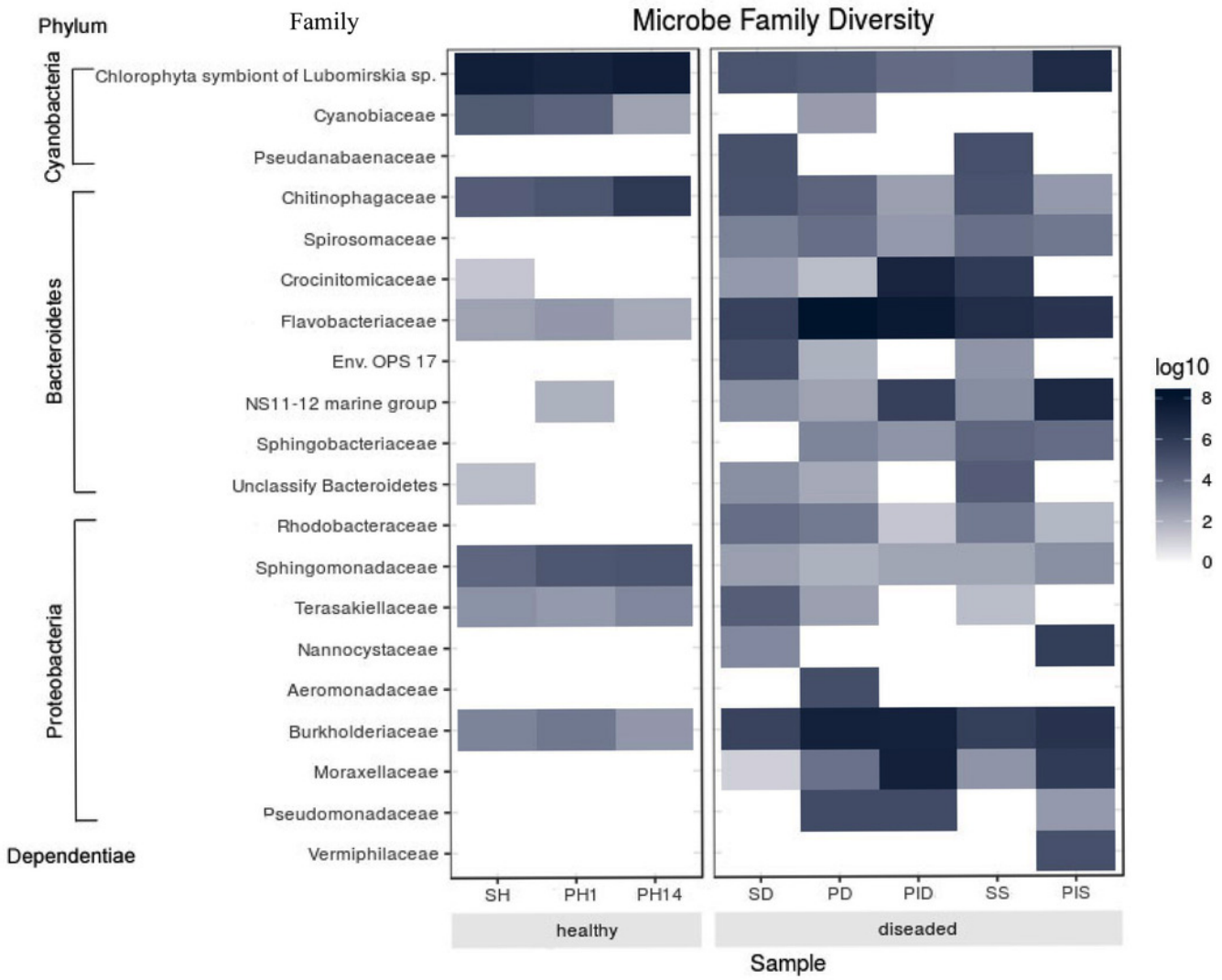

Table 1(on next page)

Samples of sponges *L. baicalensis* and cell culture of primmorphs.

Samples were collected from the strait Olkhon Vorota, Lake Baikal.

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Table 1 **Samples of the sponge *Lubomirskia baicalensis*.** Samples were collected from the strait Olkhon Vorota, Lake Baikal.

Coordinate	Samples ID	Description
53° 02′ 21 N′′ 106° 57′37 E′′	SH	Healthy sponge with bright green color
	PH1	Primmorphs cultivation for 1 day
	PH14	Primmorphs for14 day
	SD	Diseased sponge with pale green color
	PD	Primmorphs diseased
	PID	Primmorphs infected by diseased sponge suspension
	SS	Sick sponge with brown spots
	PIS	Primmorphs infected by sick sponge suspension

Table 2(on next page)

Summary of microbial communities in sponges and primmorphs.

Notes. The name of samples: SH (Healthy sponge); PH1 (Primmorphs for 1 day); PH14 (Primmorphs for 14 day); SD (Bleached sponge); PD (Primmorphs diseased); PID (Primmorphs infected by diseased sponge); SS (Sick sponge); PIS (Primmorphs infected by sick sponge). Samples IDs are referred to Table 1.

Table 2 **Summary of microbial communities in sponges and primmorphs.**

Sample analysis data	Healthy			Diseased				
	SH	**PH1**	**PH14**	**SD**	**PD**	**PID**	**SS**	**PIS**
Input	2511	2449	2879	3340	7296	8064	3314	4969
After clean	2284	2155	2648	2379	6273	7564	2940	4331
subOTU	48	50	35	75	62	41	65	57
Michaelis-Menten fit	53.81	53.81	38.37	80.13	66.18	47.4	68.06	62.05

Table 3(on next page)

The alpha-diversity indices (Chao1, Shannon index).

The alpha-diversity were calculated using the QIIME2 software to establish the abundance and diversity of the sequences. **Notes.** Samples IDs are referred to Table 1.

Table 3 **The alpha-diversity indices (Chao1, Shannon index).** The alpha-diversity were calculated using the QIIME2 software to establish the abundance and diversity of the sequences.

Samples	Chao1	Shannon
PH1	51,00	3,01
PH14	35,00	2,04
PH30	61,00	3,25
PD	64,00	4,16
PID	45,00	3,09
PIS	59,00	3,90
SH	48,00	2,30
SD	76,00	5,21
SS	65,00	4,88

Table 4(on next page)

Results pairwaise PERMANOVA test.

Notes. Indicates p-value <0.05.

Table 4: **PERMANOVA results.**

Partition variable	**pseudo-F**	**p-value**
Life Stage (Sponge/primmorphs)	0,4	0,6
Health (Healthy/diseased)	13,8	0,01*

Table 5(on next page)

Table 5 Abundance and significant difference between the two groups at the phylum/family level.

Notes. (to %). Colored lines indicate a shift in microbial communities in healthy and diseased groups.

Table 4 **The abundance of the most common microbial groups at the phylum/family levels (in %).**

Phylum		Family	Healthy			Diseased				
			SH	PH1	PH14	SD	PD	PID	SS	PIS
Cyanobacteria		Chlorophyta symbiont of *Lubomirskia* sp.	84.15	77.3	74.85	9.34	1.76	0.76	2.41	21.9
		Cyanobiaceae	5.04	4.43	0.41	0	0.2	0	0	0
		Pseudanabaenaceae	0	0	0	10.63	0	0	7.32	0
Bacteroidetes		Flavobacteriaceae	0.48	0.8	0.32	20.04	62.04	32.56	36.47	12.67
		Crocinitomicaceae	0.15	0	0	0.93	0.07	17.32	18.03	0
		NS11-12 marine group	0	0.34	0	1.36	0.16	4.13	0.88	25.55
		Chitinophagaceae	4.56	7.18	17.61	10.34	1.19	0.15	6.44	0.32
		Spirosomaceae	0	0	0	2	0.83	0.18	2.27	0.92
		Sphingobacteriaceae	0	0	0	0	0.41	0.2	3.1	1.36
		Env. OPS 17	0	0	0	11.91	0.1	0	0.7	0
		Unclassify Bacteroidetes	0.19	0	0	1.28	0.13	0	4.54	0
Proteobacteria	**α**	Rhodobacteraceae	0	0	0	3.71	0.57	0.04	1.62	0.12
		Sphingomonadaceae	3.34	7.01	5.31	0.78	0.1	0.12	0.42	0.45
		Terasakiellaceae	0.82	0.75	0.93	6.63	0.18	0	0.19	0
	β	Burkholderiaceae	1.26	2.18	0.57	19.33	25.65	19.79	14.92	14.26
	γ	Moraxellaceae	0	0	0	0.14	0.75	22.19	0.7	9.57
		Pseudomonadaceae	0	0	0	0	3.07	2.56	0	0.32
		Aeromonadaceae	0	0	0	0	2.8	0	0	0
	δ	Nannocystaceae	0	0	0	1.57	0	0	0	8.63
Dependentiae		Vermiphilaceae	0	0	0	0	0	0	0	3.92