Description of the microbiota in epidermal mucus and skin of sharks and rays

Ana Maria Galeano [1] , **Martha J Vives** [2] , **Susana Caballero** Corresp. [1]

[1] Laboratorio de Ecología Molecular de Vertebrados Acuáticos, LEMVA, Biological Sciences Department, Universidad de los Andes, Bogota, Colombia
[2] Centro de Investigaciones Microbiológicas, CIMIC, Biological Sciences Department, Universidad de los Andes, Bogota, Colombia

Corresponding Author: Susana Caballero
Email address: sj.caballero26@uniandes.edu.co

Skin mucus in fish is considered as the first barrier between the organism and the environment, partly because it can prevent microorganisms from colonizing the skin. During copulation in sharks, the male bites the female generating wounds, which have high chances of becoming infected by opportunistic bacteria from the water or the mouth. The role of skin mucus for protection against pathogens is not well understood. Describing the microbial component of epithelial mucus may allow future understanding of this first line of defense in sharks. In this study we analyze mucus and tissue samples obtained from 19 individuals of three shark species, the nurse shark (*Ginglymostoma cirratum),* the lemon shark (*Negaprion brevirostris)* and the stingray (*Dasyatis americana).* We also collected water samples from the area where the animals were found. Total DNA was extracted from all samples, and the bacterial 16s gene was amplified and sequenced by Next Generation Sequencing technology. Sequences were analysed and a summary of the bacterial diversity in the epithelial mucus of these elamosbranch species is presented. We found potentially pathogenic bacteria in water samples such as *Pasteurella* spp., *Haemophilus* spp. and *Halomonas* spp. but these were not found in the tissue or mucus samples of any species. We found some bacterial groups such as *Flavobacterium, Pseudoalteromonas, Lactobacillus* and *Bacillus* that could play a role protecting the animals from pathogenic infection. Future studies are needed to describe the metagenome and the functional role of these bacteria and its potential role as beneficial symbionts in ray and shark mucus and tissue.

Description of the microbiota in epidermal mucus and skin of sharks and rays

Ana María Galeano Mora [1], Martha Josefina Vives [2], Susana Caballero [1]
[1] Laboratorio de Ecología Molecular de Vertebrados Acuáticos, LEMVA, Universidad de los Andes, Carrera 1 No. 18A-10, Bogota, Colombia
[2] Centro de Investigaciones Microbiológicas, CIMIC, Universidad de los Andes, Carrera 1 No. 18A-10, Bogota, Colombia

Corresponding Author:
Susana Caballero [1]

Email address: sj.caballero26@uniandes.edu.co

Abstract

Skin mucus in fish is considered as the first barrier between the organism and the environment, partly because it can prevent microorganisms from colonizing the skin. During copulation in sharks, the male bites the female generating wounds, which have high chances of becoming infected by opportunistic bacteria from the water or the mouth. The role of skin mucus for protection against pathogens is not well understood. Describing the microbial component of epithelial mucus may allow future understanding of this first line of defense in sharks. In this study we analyze mucus and tissue samples obtained from 19 individuals of three shark species, the nurse shark (*Ginglymostoma cirratum),* the lemon shark (*Negaprion brevirostris)* and the stingray (*Dasyatis americana).* We also collected water samples from the area where the animals were found. Total DNA was extracted from all samples, and the bacterial 16s gene was amplified and sequenced by Next Generation Sequencing technology. Sequences were analysed and a summary of the bacterial diversity in the epithelial mucus of these elamosbranch species is presented. We found potentially pathogenic bacteria in water samples such as *Pasteurella* spp., *Haemophilus* spp. and *Halomonas* spp. but these were not found in the tissue or mucus samples of any species. We found some bacterial groups such as *Flavobacterium, Pseudoalteromonas, Lactobacillus* and *Bacillus* that could play a role protecting the animals from pathogenic infection. Future studies are needed to describe the metagenome and the functional role of these bacteria and its potential role as beneficial symbionts in ray and shark mucus and tissue.

Keywords: microbiota, shark, ray, skin, mucus, Ion Torrent

Introduction

Fish immune system includes, as a first barrier, the mucosal immune system. This system protects fish physically, chemically and biologically from threats or pathogens found in its habitat (Subramanian, MacKinnon & Ross, 2007; Subramanian, Ross & Mackinnon, 2008; Raj et al., 2011). The mucosal immune system is subdivided in three subgroups that correspond to the place where the mucus is secreted: the gut, the gills and the skin (Salinas, Zhang & Sunyer, 2011). Skin mucus is considered as the first barrier of protection because it can prevent microorganisms from colonizing the skin (Cone, 2009). Some studies suggest that this mucus is constantly renewed, therefore, it reduces the pathogenic load found on the surface of the fish (Nagashima et al., 2003). Additionally, it is secreted in higher quantities as a response to threats (Mittal & Datta Munshi, 1974; Gostin, Neagu & Vulpe, 2011; Rai et al., 2012). Furthermore, this viscose substance consists of molecules that may help in healing and protecting the skin (Cameron & Endean, 1973; Al-Hassan et al., 1985), including secretion of antimicrobial and regenerative substances (Hansen & Olafsen, 1999).

The epithelial mucus is sometimes considered to be an ideal surface for bacterial adhesion (Hansen & Olafsen, 1999). In fact, accumulation of microorganisms appears to take place during the lifetime of the individual (Hansen & Olafsen, 1999), leading to the establishment of the microbiota in the skin of the fish. However, it is also recognized that the mucus has a concentration of molecules that prevent the adhesion of pathogenic bacteria (Crouse‐Eisnor, Cone & Odense, 1985). For that reason, the role or the relationship between the mucus and environmental bacteria is not clear (Luer, 2012). It has been suggested that bacteria found in this layer may have three possible roles (Salminen et al., 2010): a) bacteria may stimulate mucus and antimicrobial compound production, b) bacteria may activate and help modulate the immune response in the fish, and c) the interaction between different types of bacteria may actively exclude or compete with potentially pathogenic bacteria (Salminen et al., 2010).

The mucus layer in sharks and rays has been poorly studied. However, it is known that mucus from stingray skin appear to accelerate healing processes of their wounds, and that bacteria found in the mucus have antibacterial activity against human pathogens (Luer et al., 2014). Reproductive behavior in this group is characterized by aggressiveness during the courtship and copulation (Pratt & Carrier, 2001; Carrier, Pratt & Martin, 2015). In sharks, the male bites the female in her dorsal or pectoral fins generating wounds in those areas (Pratt & Carrier, 2001). In some species polyandry is very common (Saville et al., 2002; Carrier et al., 2003). This behavior drives competition between males and avoidance in the females (Klimley, 1980; Gordon, 1993; Pratt & Carrier, 2001). There are also morphological characteristics related to this trait. Sexual dimorphism occur in shark species in which the males have modifications in their teeth so they can grab easily to the female in order to remain close to her while mating. Females have thicker dermal denticles (tooth-like structures that provide hydrodynamics and protection) than males as a protection against these bites (Carrier, Musick & Heithaus, 2012). In the case of rays, the females prick the male with their caudal spine (Pratt & Carrier, 2001). It has been shown in some stingray species that when many males are involved in the mating process, sometimes a few of them are found dead (Gilad et al., 2008). In spite of these apparently aggressive behaviors, copulation is necessary and the wounds provoked in the process have a high chance of becoming infected (Daly-Engel et al., 2010) due to opportunistic bacteria in the water and in the oral cavity of these animals. Due to the high concentration of pathogenic microorganisms found in the

aquatic environment (Magnadottir, 2010), it is important to determine the microbiota component of the epithelial mucus, the skin tissue and to understand if the bacteria found in these are similar or different from that in the water surrounding the animals. In this way, it will be possible to start understanding the role of mucus in the protection against pathogens. In this study we present a summary of the bacterial diversity in the epithelial mucus from three elasmobranch species, the nurse shark (*Ginglymostoma cirratum),* the lemon shark (*Negaprion brevirostris)* and the stingray (*Dasyatis americana)*. We also hypothesize about the possible role of some of the bacteria found in the mucus and in the skin.

Materials and Methods

Sample collection

Mucus and skin tissue samples were obtained from 19 healthy individuals; 14 of them were obtained from animals captured in Bimini, Bahamas: four corresponded to juvenile nurse sharks (*Ginglymostoma cirratum)*, six to juvenile lemon sharks (*Negaprion brevirostris)* and four to adult stingrays (*Dasyatis americana)*. Samples from an additional five adult nurse sharks were collected at Oceanario from Islas del Rosario (CEINER), in the Colombian Caribbean. For each individual, a sample of skin tissue and mucus was obtained, following sampling protocols approved by the Animal Care Committee of Universidad de los Andes (CICUAL) (Bogotá, Colombia). The skin tissue sample was taken from the posterior part of the dorsal fin, and the mucus from the skin surface. A water sample was also collected from the place where each individual was captured. Therefore, three samples were associated with each individual, for a total of 57 samples. The individuals were captured and raised slightly to the surface of the water, thus the samples could be taken away from the water, but the animal could continue breathing. Skin samples were preserved in ethanol 90%. All samples were maintained at 4º C until processing.

DNA Extraction and PCR amplification

DNA was extracted from all samples collected. The Tissue and Cells DNA Isolation Kit (MoBio Laboratories, Inc) was used, following the manufacturer instructions. The primers 515f and 806r were used in order to amplify the region V3-V4 from the bacterial 16s rRNA gene. PCR amplification conditions were as follows: an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturing at 94°C for 45 seconds, annealing for 45 seconds at 50°C and extension for 45 seconds at 72°C, followed by a final extension of 20 minutes at 72°C. Successful amplification was The was confirmed on a 1% agarose gel.

Ion torrent library preparation, quantification and sequencing

From the 57 samples, 32 were used to construct libraries. Samples were chosen depending on their final DNA concentration, once the PCR products were cleaned using magnetic beads. Two libraries, each with 16 barcodes, were prepared using the protocol Ion Xpress™ Plus gDNA Fragment Library Preparation (Life Technologies). Libraries were quantifyed with the Qubit kit. Template preparation was made following the Ion PGM™ Template OT2 200 Kit (Life Technologies) protocols. Libraries were prepared for sequencing using the protocol Ion PGM™ Sequencing 200 Kit v2 (Life Technologies). Libraries were loaded on one Ion 318 chip and sequenced in the Ion Torrent PGM (Life Technologies).

Bioinformatic analyses

Sequences were separated by barcodes directly by the Ion Torrent PGM and saved by the ion reporter in different files; analyses of sequence quality were made with FastQC (Andrews, 2014). File format was changed from BAM to FASTQ. Trimmomatic (Bolger, Lohse & Usadel, 2014) was used to edit sequences with low quality. Sequences with low quality (less than 20 reads) and shorter than 50 bp were removed. Afterwards, all files were combined. Demultiplexing was done by comparing the mapping file of the chip with the files containing the sequences. The resulting files were analyzed with QIIME (Caporaso et al., 2010) to assign a taxonomic identity (OTU) for each sequence. Finally, R was used (R Development Core Team, 2010) with the package ggplot2 to evaluate the diversity in each sample (Wickham, 2009).

Multidimentional scaling analysis

A multidimentional scaling analysis was made to compare the relationship between OTU genera diversity in the samples. Horn dissimilarity index was used to build the pairwise distance matrix. For the visualization of these data, a Principal Component Analysis (PCoA) was constructed, using the commands vegdist and cmdscale from the package VEGAN (Oksanen et al., 2011) from R programming language (R Development Core Team, 2010). The distance between each point (sample) explains how similar samples are regarding OTU assignment.

Results

The 32 samples used to build the libraries included a mucus or tissue sample for each of the individuals sampled and only four of the 19 samples of water. The other samples had low concentration of bacterial DNA that could not be used for NGS sequencing analysis. After sequencing and elimination of error sequences, a total 219,162 reads were obtained from the Ion Torrent PGM. After Trimmomatic edition and QIIME analysis, 3,639 sequences were assigned taxonomically; these sequences belonged to 15 individuals and 4 samples of water. From those total reads, 404 did not correspond to bacterial alignments and another 53 where only identified to the level of Bacteria kingdom; those 457 sequences were discarded. Hereby, 3,182 sequences were used in computational analysis. Thirty-six phyla and candidate phyla were identified in all or in some of the samples (Figure 1). One hundred and thirteen orders, 224 families, 168 genus and 55 species were identified.

The distribution of phyla diversity in the samples is shown in Figure 2. Most phyla were found in every sample and with a similar distribution in all of them. Some samples contained more reads than others; in general, mucus and tissue samples had a higher number of reads than water samples. Thirteen phyla were identified across all or most of the individuals sampled (Acidobacteria, Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Cyanobacteria, Firmicutes, GN02, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes and Verrucomicrobia). Twenty-tree bacterial phyla were identified overall. The percentage in which each phylum was identified from the total reads (sequences) obtained and analyzed is shown in Table 1. Only a few sequences were assigned to the species level. Most of them were assigned to genus or to higher taxonomic levels (Figure 2).

Several reported bacterial fish pathogens, symbionts and commensals were found in the mucus, tissue and water samples (Table 1 and Supplementary Material 1). It is interesting to note that some fish pathogens were only found in the water and not in the mucus/tissue samples, such as *Pasteurella* spp., *Haemophilus* spp. and *Halomonas* spp.

The PCoA graph (Figure 3) showed few groupings type of sample or by species, suggesting no correlation between the type of sample or the species sampled with the bacteria identified in them.

Discussion

Microorganisms found in the skin surface of fish may be part of the first barrier of defense against pathogens. Nevertheless, depending on the characteristics and populations of these animals, the role of the whole microbiome may vary. Within the twelve bacterial genera found only in water samples, three have been described as pathogens for fish, including *Pasteurella* spp., *Haemophilus* spp. and *Halomonas* spp (Bullock, 1961; Hawke et al., 1987; Austin, 2005).

There is a species found only in water samples too, *Acinetobacter johnsonii*, which has been described as fish pathogen (Kozińska et al., 2014). Other sequenced bacteria present in the results of water samples, such as *Moraxella sp*., are opportunistic bacteria and have been found in other animals, for example in mammals (Whitman, 2015). Some of the 143 genera found only in the elasmobranch samples may also play a role as pathogens; *Alteromonas* (Boone & Bryant, 1980), *Mycobacterium*, *Nocardia, Rhodococcus, Shewanella*, *Staphylococcus* and *Chryseobacterium* have been reported as pathogens for various fish species (Hansen & Olafsen, 1999; Austin, 2005). *Syntrophobacter* is another genera present in mucus and skin samples and considered as possible pathogen for fish, due to the fact that bacteria that belong to this group, degrade propionate, a corticoid used in healing skin (Schulze et al., 2006). However, many species of *Flavobacterium, Pseudoalteromonas, Lactobacillus* and *Bacillus*, also found only in elasmobranch samples, are considered symbionts of marine fish (Anand et al., 2011; Luer et al., 2014). Some species of *Flavobacterium* have been studied as commensal to fish, and have shown antimicrobial activity against *Vibrio* sp., fish pathogens (Lal & Tabacchioni, 2009). *Bacillus polymyxa*, found in mucus and skin samples in this study, has been isolated from fish guts and some strains of this species synthesizes antibiotics (Olmos, 2014). Similarly, *Bacillus subtilis* has been suggested as a probiotic involved in the optimization of fish feeding (Merrifield & Rodiles, 2015). Finally, various bacteria sequenced from mucus and skin samples are considered normal flora of fish gills or skin (i.e. Xanthomonadales, Caulobacteriales (Sugita et al., 1996) and *Plesiomonas shigelloides)*.

Three genera found in mucus and tissue samples (*Streptococcus*, *Pseudomonas* and *Vibrio*) are sometimes reported as pathogens and sometimes reported as symbionts. For example, *S. parauberis* produces streptococcosis in some fish (Austin, 2005; Nho et al., 2009; Abrahamian & Goldstein, 2011), but other *Streptococcus* spp. inhibit the growth of pathogenic bacteria (Hansen & Olafsen, 1999). Similarly, *Pseudomonas putrefaciens* acts as a pathogen for fish (Abrahamian & Goldstein, 2011), but *P. fluorescens* inhibits growth of pathogens (Subramanian, Ross & MacKinnon, 2008) and has been isolated from healthy salmon eggs and mucus (Cipriano & Dove, 2011; Akinyemi et al., 2016). Finally, *Vibrio* have been reported several times as an important pathogen for marine life because of its high survival and capacity of acclimation in its host, as they hydrolyze urea and use it as source of carbon and nitrogen (Hansen & Olafsen, 1999). Many species have been described as infectious for *Negaprion brevirostris*, specially when they are physically injured (Grimes et al., 1984a; Grimes, Gruber & May, 1985); others are associated to mortality of sharks in captivity (Grimes et al., 1984b), and others to infections

caused by hooks (Borucinska et al., 2002). There are some species that, depending on the strain, are pathogenic or not, such as *V. alginolyticus* and *V. parahemoliticus* (Austin & Austin, 2007; Abrahamian & Goldstein, 2011). Other species, such as *Vibrio alginolyticus* and *V. fluviales,* are considered pathogenic for fish (Zorrilla et al., 2003); *Vibrio fortis* has been reported as a sea horse pathogen (Wang et al., 2016); *Vibrio shilonii* has been found to cause coral bleaching (Kushmaro et al., 2001).

There are various bacteria identified in the mucus samples that are considered in other studies as symbionts or pathogens for other animals or humans. For example some species of *Bacteroides* have been described as human pathogens in periodontal disease and *Prevotella copri*, found in mucus and skin samples, has been identified as pathogen in intestinal inflammation. Additionally, species from *Helcoccocus* have also been described as pathogens for humans (Chow & Clarridge, 2014). Many species of Chlamydiae are reported as pathogens for birds and mammals (Whitman, 2015) and *Enterococcus cecorum* has been reported as a pathogen in chicken (Jung & Rautenschlein, 2014).

As examples of symbiosis of species of bacteria (found in samples of this study) with humans or other animals, it is worth mentioning *Lactobacillus zeae,* which has been found to be protective biota for nematodes (Zhou et al., 2014); *Butyrivibrio* and *Selenomonas* are found in the gastrointestinal tract of ruminants*; Faecalibacterium prausnitzii, Peptoniphilus, Ruminococcus, Megamonas* (Chow & Clarridge, 2014) and *Butyricimonas* (Wexler, 2007) are normal important bacteria in the human gut microbiota. Other species sequenced from mucus samples were *Sulcia muelleri* (Moran, Tran & Gerardo, 2005), *Baumannia cicadellinicola* (Cottret et al., 2010) and *Carsonella ruddii* (Thao et al., 2000), which have been described in symbiotic association with insects. A very interesting case is *Janthinobacterium lividum,* which has been found in the skin of some amphibians and appears to prevent infection by *Batrachochytrium dendrobatidis* (Brucker et al., 2008). These are startling examples that may have relation with the findings of this study; however, there should be deeper investigations to identify the pathogenicity or symbiosis properties specifically in elasmobranch or fish.

According to this information, the role of the mucus and the bacteria associated to it may depend on numerous variables, including the virulence and pathogenicity of each microorganism (Hansen & Olafsen, 1999). Opportunistic bacteria can acquire virulence determinants with environmental changes by diverse ways, for example by i) Increasing their numbers by exploiting the higher production of mucus (glycoproteins) induced by presence of toxic substances in the water (Hansen & Olafsen, 1999), by ii) shifting from a non-infectious state to an infectious one through an activation caused by a physical or chemical change in the environment (Hansen & Olafsen, 1999) or by iii) Reaching the dermal layer to infect the host taking advantage of some reduction of the defensive mucus layer, caused by the presence of abrasive substances in the surroundings of the fish (Benhamed et al., 2014). These three opportunities for the bacteria to infect the hosts not only benefit these microorganisms but also affect the host by reducing their physiological condition (Austin, 2005), and may explain the finding of the reported bacterial pathogens on the skin of healthy animals.

The genera considered as fish pathogens found in the water samples but absent in the elasmobranch samples present an interesting question. We suggest that there may be specific

antimicrobial activity in the skin environment, or partial control against infections that exists in low concentration in the mucus, but this may also be a result of the low number of samples and replicates analyzed (Rakers et al., 2010).

In this study, the bacterial diversity in the mucus and tissue included a wide range of phyla and genus that have been described as pathogens, non-pathogens and some that have scarcely been studied in relationship to potential or confirmed hosts. As general observations, samples from mucus and tissue usually produced a higher number of sequences than water samples, and this is probably due to the low concentration of bacteria in the water environment. Similarly, samples taken from shark species produced a higher number of reads in sequencing than those from the ray species. In general, there are few sequences taxonomically identified compared to the total. This may be the result of the shorter length of the sequences, the kits used in sequencing (to make libraries of short sequences) and the low concentration of DNA found in the original samples.

The simultaneous presence of pathogens and possible symbionts varied between samples; however, the role of each genus or species should be verified for each of the host species considered in this analysis. According to these results, we suggest that the role of the epithelial microbiota may be considered as a first line of defense against infectious organisms but it could also be a potential threat for the injured host. This could depend on the whole combination of bacteria and their interaction between them in each host. As mentioned before, each fish may accumulate a specific community of microorganisms in its life span depending on the environments it inhabits during its development and growth (Hansen & Olafsen, 1999).

The little grouping in the PCoA graph suggests that the bacterial composition in these species of elasmobranch does not depend on the host species, or the type of sample. Instead, it could be related to the life span and habitat characteristics of each individual but it would be necessary to perform a larger sampling effort from each host species and geographic locations to confirm this lack of association. This study represents the first contribution to describe shark and ray skin and mucus microbiomes. Next steps to further understand the role of bacterial communities and skin and mucus of elamobranchs require functional metagenomics and metabolomics analyses to unveil the role of these bacteria.

Conclusions

We found high diversity of both pathogenic and non-pathogenic bacteria in samples of water, skin and mucus obtained from the three species studied. However, the number of sequences generated was higher for the mucus and skin samples, particularly of the two shark species. There was no particular bacterial composition found exclusively in one species or in one type of sample. Unfortunately, a high number of reads were not taxonomically identified, which could be the result of the type of libraries constructed, due to the length of the sequences analyzed or due to low DNA concentration in some of the samples obtained. Lastly, we suggest that some of the non-pathogenic bacteria identified in the mucus and skin samples of the sharks and ray species studied could have a protective function against pathogenic bacteria and other microorganisms found in the water.

Acknowledgements

We want to give a special thank you to all the people involved in samples collection, particularly to the Bimini Shark Laboratory, to D. Cardeñosa, and to R. Vieira and his team at Oceanario Islas del Rosario (Ceiner). Financial support for this project was provided by Proyecto de Ciencias Básicas, Vicerrectoria de Investigaciones, Universidad de los Andes. We thank E. Salguero and A. P. Jimenez for their help with sequencing, and A. Reyes and S. Movilla with bioinformatic analyses.

344
345 References
346
347 Abrahamian FM., Goldstein EJC. 2011. Microbiology of animal bite wound infections. *Clinical*
348 *microbiology reviews* 24:231–46. DOI: 10.1128/CMR.00041-10.
349 Akinyemi AA., Ekelemu JK., Oyelakin OO., Oloyede AR., Green BM. 2016. Molecular
350 characterization of bacteria associated with the African catfish *Clarias gariepinus* (Burchell,
351 1822) from Yew-Mata station on Yewa river by 16s gene sequencing method. *Global*
352 *Journal of Bio-Science and Biotechnology* 5:295–300.
353 Al-Hassan JM., Thomson M., Criddle KR., Summers B., Criddle RS. 1985. Catfish epidermal
354 secretions in response to threat or injury. *Marine Biology* 88:117–123.
355 Anand P., Chellaram TC., Kumaran S., Shanthini F. 2011. Screening for antibiotic producing
356 marine bacteria against fish pathogens. *International Journal of Pharma and Bio Sciences*
357 2:B314–B325.
358 Andrews S. 2014. FastQC: A Quality Control Tool for High Throughput Sequence Data.
359 Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Accessed
360 Dec 11, 2014).
361 Austin B. 2005. Bacterial Pathogens of Marine Fish. In: Belkin S., Colwell R.R. (eds) Oceans
362 and Health: Pathogens in the Marine Environment. Springer, Boston, MA. pp: 391–413.

363 Austin B., Austin DA. 2007. *Bacterial Fish Pathogens: diseases of farmed and wild fish*.
364 Springer.

365 Benhamed S., Guardiola F a., Mars M., Esteban MÁ. 2014. Pathogen bacteria adhesion to skin
366 mucus of fishes. *Veterinary microbiology* 171:1–12. DOI: 10.1016/j.vetmic.2014.03.008.

367 Bolger A., Lohse M., Usadel B. 2014. Trimmomatic: a flexible trimmer for illumina sequence
368 data. Bioinformatics 30, 2114–2120. DOI: 10.1093/bioinformatics/btu170.
369 Boone DR., Bryant MP. 1980. Propionate-Degrading Bacterium, *Syntrophobacter wolinii* sp.
370 nov. gen. nov., from Methanogenic Ecosystems. *Applied and Environmental Microbiology*
371 40:626–632.
372 Borucinska J., Kohler N., Natanson L., Skomal G. 2002. Pathology associated with retained
373 fishing hooks in blue sharks, *Prionace glauca*, with implications for their conservation.
374 *Journal of Fish Diseases* 25:515–521. DOI: 10.1046/j.1365-2761.2002.00396.x.
375 Brucker RM., Harris RN., Schwantes CR., Gallaher TN., Flaherty DC., Lam BA., Minbiole KPC.
376 2008. Amphibian Chemical Defense: Antifungal Metabolites of the Microsymbiont
377 *Janthinobacterium lividum* on the *Salamander plethodon cinereus*. *Journal of Chemical*
378 *Ecology* 34:1422–1429. DOI: 10.1007/s10886-008-9555-7.
379 Bullock GL. 1961. A schematic outline for the presumptive identification of bacterial diseases of
380 fish. *The Progressive Fish-Culturist* 23:147–151. DOI: 10.1577/1548-
381 8659(1961)23[147:ASOFTP]2.0.CO;2.
382 Cameron AM., Endean R. 1973. Epidermal secretions and the evolution of venom glands in
383 fishes. *Toxicon* 11:401IN1407-406IN2410.
384 Caporaso J., Kuczynski J., Stombaugh J., Bittinger K., Bushman F., EK C., 2010. 2010. QIIME
385 allows analysis of high- throughput community sequencing data. *Nature Methods* 7, 335–
386 336. DOI: 10.1038/nmeth.f.303.
387 Carrier JC., Murru FL., Walsh MT., Pratt HL. 2003. Assessing reproductive potential and

388 gestation in nurse sharks (*Ginglymostoma cirratum*) using ultrasonography and endoscopy:
389 An example of bridging the gap between field research and captive studies. *Zoo Biology*
390 22:179–187. DOI: 10.1002/zoo.10088.
391 Carrier JC., Musick JA., Heithaus MR. 2012. *Biology of sharks and their relatives*. CRC press.
392 Carrier JC., Pratt HL., Martin LK. 2015. Group Reproductive Behaviors in Free-Living Nurse
393 Sharks, *Ginglymostoma cirratum.* Jeffrey C. Carrier, Harold L. Pratt, Jr. and Linda K (eds.)
394 Martin Source: 1994:646–656.
395 Chow S-K., Clarridge JE. 2014. Identification and clinical significance of *Helcococcus species*,
396 with Description of Helcococcus seattlensis sp. nov. from a Patient with Urosepsis. *Journal*
397 *of Clinical Microbiology* 52:854–858. DOI: 10.1128/JCM.03076-13.
398 Cipriano RC., Dove A. 2011. *Far from superficial: microbial diversity associated with the*
399 *dermal mucus of fish.* Health and diseases of aquatic organisms: bilateral perspectives. MSU
400 Press, East Lansing.
401 Cone RA. 2009. Barrier properties of mucus. *Advanced drug delivery reviews* 61:75–85. DOI:
402 10.1016/j.addr.2008.09.008.
403 Cottret L., Milreu PV., Acuña V., Marchetti-Spaccamela A., Stougie L., Charles H., Sagot MF.
404 2010. Graph-based analysis of the metabolic exchanges between two co-resident
405 intracellular symbionts, *Baumannia cicadellinicola* and *Sulcia muelleri*, with their insect
406 host, *Homalodisca coagulata*. *PLoS Computational Biology* 6.
407 Crouse-Eisnor RA., Cone DK., Odense PH. 1985. Studies on relations of bacteria with skin
408 surface of *Carassius auratus* and *Poecilia reticulata*. *Journal of fish biology* 27:395–402.
409 Daly-Engel T., Grubbs R., Feldheim K., Bowen B., Toonen R. 2010. Is multiple mating
410 beneficial or unavoidable? Low multiple paternity and genetic diversity in the shortspine
411 spurdog *Squalus mitsukurii*. *Marine Ecology Progress Series* 403:255–267. DOI:
412 10.3354/meps08417.
413 Gilad S., Meiri E., Yogev Y., Benjamin S., Lebanony D., Yerushalmi N., Benjamin H., Kushnir
414 M., Cholakh H., Melamed N. 2008. Serum microRNAs are promising novel biomarkers.
415 *PloS one* 3:e3148.
416 Gordon I. 1993. Pre-copulatory behaviour of captive sandtiger sharks, *Carcharias taurus*. In: *The*
417 *reproduction and development of sharks, skates, rays and ratfishes*. Springer, 159–164.
418 Gostin IN., Neagu AN., Vulpe V. 2011. SEM investigations regarding skin micro-morphology
419 and modifications induced by bacterial infections in *Cyprinus carpio* and *Salmo trutta fario.*
420 *International Journal of Energy and Environment* 5:274–281.
421 Grimes DJ., Colwell RR., Stemmler J., Hada H., Maneval D., Hetrick FM., May EB., Jones RT.,
422 Stoskopf M. 1984a. *Vibrio* species as agents of elasmobranch disease. *Helgoländer*
423 *Meeresuntersuchungen* 37:309–315. DOI: 10.1007/BF01989313.
424 Grimes DJ., Gruber SH., May EB. 1985. Experimental infection of lemon sharks, *Negaprion*
425 *brevirostris* (Poey), with *Vibrio* species. *Journal of Fish Diseases* 8:173–180. DOI:
426 10.1111/j.1365-2761.1985.tb01212.x.
427 Grimes DJ., Stemmler J., Hada H., May EB., Maneval D., Hetrick FM., Jones RT., Stoskopf M.,
428 Colwell RR. 1984b. *Vibrio* species associated with mortality of sharks held in captivity.
429 *Microbial ecology* 10:271–82. DOI: 10.1007/BF02010940.
430 Hansen GH., Olafsen J a. 1999. Bacterial interactions in early life stages of marine cold water
431 fish. *Microbial Ecology* 38:1–26. DOI: 10.1007/s002489900158.
432 Hawke JP., Plakas SM., Minton RV., McPhearson RM., Snider TG., Guarino AM. 1987. Fish
433 pasteurellosis of cultured striped bass (*Morone saxatilis*) in coastal Alabama. *Aquaculture*

65:193–204. DOI: 10.1016/0044-8486(87)90231-6.
Jung A., Rautenschlein S. 2014. Comprehensive report of an *Enterococcus cecorum* infection in a broiler flock in Northern Germany. *BMC Veterinary Research* 10:311. DOI: 10.1186/s12917-014-0311-7.
Klimley AP. 1980. Observations of courtship and copulation in the nurse shark, *Ginglymostoma cirratum*. *Copeia* 1980:878–882.
Kozińska A., Paździor E., Pękala A., Niemczuk W. 2014. *Acinetobacter johnsonii* and Acinetobacter lwoffii - the emerging fish pathogens. *Bulletin of the Veterinary Institute in Pulawy* 58:193–199. DOI: 10.2478/bvip-2014-0029.
Kushmaro A., Banin E., Loya Y., Stackebrandt E., Rosenberg E. 2001. *Vibrio shiloi* sp. nov., the causative agent of bleaching of the coral *Oculina patagonica*. *International Journal of Systematic and Evolutionary Microbiology* 51:1383–1388.
Lal S., Tabacchioni S. 2009. Ecology and biotechnological potential of *Paenibacillus polymyxa*: a minireview. *Indian Journal of Microbiology* 49:2–10. DOI: 10.1007/s12088-009-0008-y.
Luer C. 2012. *Novel Compounds from Shark and Stingrays Epidermal Mucus whit Antimicrobial Activity Against Wound Infection Pathogens*.
Luer C., Walsh C., Ritchie K., Edsberg L., Wyffels J., Luna V., Bodine A. 2014. Novel compounds from shark and stingray epidermal mucus with antimicrobial activity against wound infection pathogens. *Mote Marine Lab, Sarasota FL. In press*:1–39.
Magnadottir B. 2010. Immunological control of fish diseases. *Marine biotechnology* 12:361–379.
Merrifield D., Rodiles A. 2015. The fish microbiome and its interactions with mucosal tissues. In: *Mucosal Health in Aquaculture*. 273–289.
Mittal AK., Datta Munshi JS. 1974. On the regeneration and repair of superficial wounds in the skin of *Rita vita* (Ham.)(Bagridae, Pisces). *Cells Tissues Organs* 88:424–442.
Moran NA., Tran P., Gerardo NM. 2005. Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. *Applied and environmental microbiology* 71:8802–10. DOI: 10.1128/AEM.71.12.8802-8810.2005.
Nagashima Y., Kikuchi N., Shimakura K., Shiomi K. 2003. Purification and characterization of an antibacterial protein in the skin secretion of rockfish *Sebastes schlegeli*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 136:63–71. DOI: 10.1016/S1532-0456(03)00174-1.
Nho S-W., Shin G-W., Park S-B., Jang H-B., Cha I-S., Ha M-A., Kim Y-R., Park Y-K., Dalvi RS., Kang B-J., Joh S-J., Jung T-S. 2009. Phenotypic characteristics of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*). *FEMS Microbiology Letters* 293:20–27. DOI: 10.1111/j.1574-6968.2009.01491.x.
Oksanen J., Blanchet F., Kindt R., Legendre P., Minchin P., O'Hara R., et al. 2011. Vegan: Community Ecology Package . R package version 2.0-2. Available online at: http://CRAN.R-project.org/package=vegan.
Olmos J. 2014. *Bacillus subtilis:* A potential probiotic bacterium to formulate functional feeds for aquaculture. *Journal of Microbial & Biochemical Technology* 6:361–365. DOI: 10.4172/1948-5948.1000169.
Pratt HL., Carrier JC. 2001. A review of elasmobranch reproductive behavior with a case study on the nurse shark, *Ginglymostoma cirratum*. *Environmental Biology of Fishes* 60:157–188. DOI: 10.1023/A:1007656126281.
R Development Core Team. 2010. R: A Language for Statistical Computing . Vienna: R Foundation for Statistical Computing.

480 Rai AK., Srivastava N., Nigam AK., Kumari U., Mittal S., Mittal AK. 2012. Healing of
481 cutaneous wounds in a freshwater teleost, *Labeo rohita*: scanning electron microscopical
482 investigation. *Microscopy research and technique* 75:890–897.
483 Raj VS., Fournier G., Rakus K., Ronsmans M., Ouyang P., Michel B., Delforges C., Costes B.,
484 Farnir F., Leroy B., Wattiez R., Melard C., Mast J., Lieffrig F., Vanderplasschen A. 2011.
485 Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells.
486 *Veterinary Research* 42:1–10. DOI: 10.1186/1297-9716-42-92.
487 Rakers S., Gebret M., Uppalapati S., Meyer W., Maderson P., Sell A F., Kruse C., Paus R. 2010.
488 "Fish matters": the relevance of fish skin biology to investigative dermatology.
489 *Experimental Dermatology* 19:313–324. DOI: 10.1111/j.1600- 0625.2009.01059.x.
490 Salinas I., Zhang Y-A., Sunyer JO. 2011. Mucosal immunoglobulins and B cells of teleost fish.
491 *Developmental and comparative immunology* 35:1346–65. DOI: 10.1016/j.dci.2011.11.009.
492 Salminen S., Nybom S., Meriluoto J., Collado MC., Vesterlund S., El-Nezami H. 2010.
493 Interaction of probiotics and pathogens—benefits to human health? *Current Opinion in*
494 *Biotechnology* 21:157–167.
495 Saville KJ., Lindley AM., Maries EG., Carrier JC., Pratt HL. 2002. Multiple paternity in the
496 nurse shark, *Ginglymostoma Cirratum*. *Environmental Biology of Fishes* 63:347–351. DOI:
497 10.1023/A:1014369011709.
498 Schulze AD., Alabi AO., Tattersall-Sheldrake AR., Miller KM. 2006. Bacterial diversity in a
499 marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains.
500 *Aquaculture* 256:50–73. DOI: 10.1016/j.aquaculture.2006.02.008.
501 Subramanian S., MacKinnon SL., Ross NW. 2007. A comparative study on innate immune
502 parameters in the epidermal mucus of various fish species. *Comparative biochemistry and*
503 *physiology. Part B, Biochemistry & molecular biology* 148:256–63. DOI:
504 10.1016/j.cbpb.2007.06.003.
505 Subramanian S., Ross NW., Mackinnon SL. 2008. Comparison of the biochemical composition
506 of normal epidermal mucus and extruded slime of hagfish (*Myxine glutinosa*). *Fish &*
507 *shellfish immunology* 25:625–32. DOI: 10.1016/j.fsi.2008.08.012.
508 Subramanian S., Ross NW., MacKinnon SL. 2008. Comparison of antimicrobial activity in the
509 epidermal mucus extracts of fish. *Comparative biochemistry and physiology. Part B,*
510 *Biochemistry & molecular biology* 150:85–92. DOI: 10.1016/j.cbpb.2008.01.011.
511 Sugita H., Shibuya K., Shimooka H., Deguchi Y. 1996. Antibacterial abilities of intestinal
512 bacteria in freshwater cultured fish. *Aquaculture* 145:195–203. DOI: 10.1016/S0044-
513 8486(96)01319-1.
514 Thao ML., Moran NA., Abbot P., Brennan EB., Burckhardt DH., Baumann P. 2000.
515 Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Applied and*
516 *Environmental Microbiology* 66:2898–2905. DOI: 10.1128/AEM.66.7.2898-2905.2000.
517 Wang X., Zhang Y., Qin G., Luo W., Lin Q. 2016. A novel pathogenic bacteria (*Vibrio fortis*)
518 causing enteritis in cultured seahorses, *Hippocampus erectus* Perry, 1810. *Journal of fish*
519 *diseases* 39:765–9. DOI: 10.1111/jfd.12411.
520 Wexler HM. 2007. Bacteroides: the good, the bad, and the nitty-gritty. *Clinical Microbiology*
521 20:593–621.
522 Whitman WB. 2015. *Bergey's manual of systematics of Archaea and Bacteria.* New York: Wiley.
523 Wickham H. 2009. ggplot2: Elegant Graphics for Data Analysis. NewYork,NY: Springer.
524 Zhou M., Yu H., Yin X., Sabour PM., Chen W., Gong J. 2014. *Lactobacillus zeae* protects
525 *Caenorhabditis elegans* from enterotoxigenic *Escherichia coli*-caused death by inhibiting

enterotoxin gene expression of the pathogen. *PLoS ONE* 9.
Zorrilla I., Morinigo MA., Castro D., Balebona MC., Borrego JJ. 2003. Intraspecific characterization of Vibrio *alginolyticus* isolates recovered from cultured fish in Spain. *Journal of Applied Microbiology* 95:1106–1116. DOI: 10.1046/j.1365-2672.2003.02078.x.

572
573 Figure and Table legends
574 Figure 1. Summary of taxonomic assignments. Venn diagram showing the quantity of OTU's
575 shared between mucus and tissue elamosbranch samples and water or OTU´s unique to either
576 elasmobranch samples (purple) or water samples (blue).
577
578 Figure 2. Bacterial phyla composition of each sample. The first letter in the sample labels
579 corresponds to the type of sample: (M) Mucus, (T) tissue and (W) water, followed by the sample
580 number. Sample Size indicates the number of reads identified in each sample.
581
582 Fig 3. Principal Component Analysis (PCoA) of distances for all samples. The first letter of the
583 labels corresponds to the type of sample: (M) Mucus, (T) tissue, (W) water, followed by the
584 sample number. The letter after the dot corresponds to the host species, (G) for *Ginglymostoma*
585 *cirratum,* (N) for *Negaprion brevirostris* and (D) for *Dasyatis americana.*
586

Figure 1

Summary of taxonomic assignements

Venn Diagram showing the quantity of OTU´s shared between water and mucus and skin of Elasmobranch species

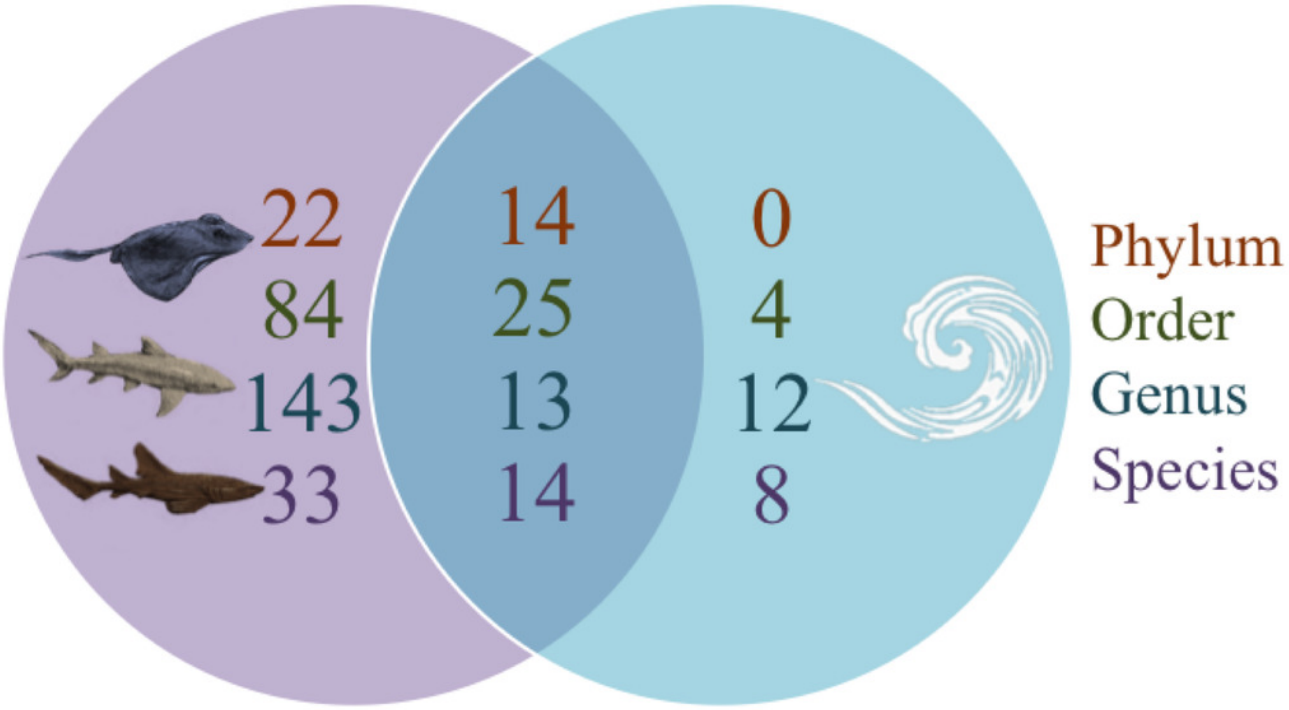

Figure 2

Bacterial Phyla composition of each sample

The first letter in the sample names corresponds to the type of sample

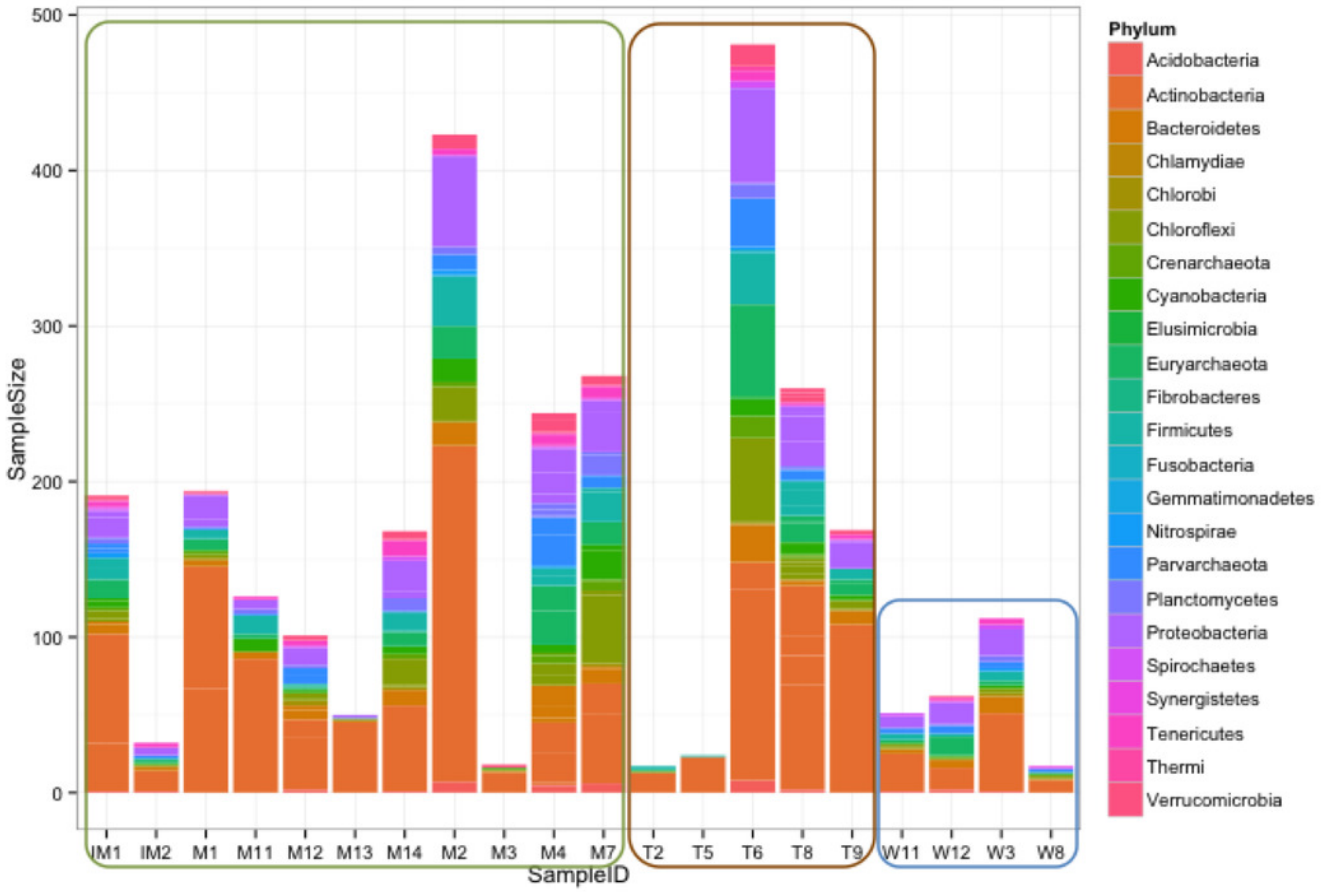

Figure 3

Principal Component analysis

distances for all samples.

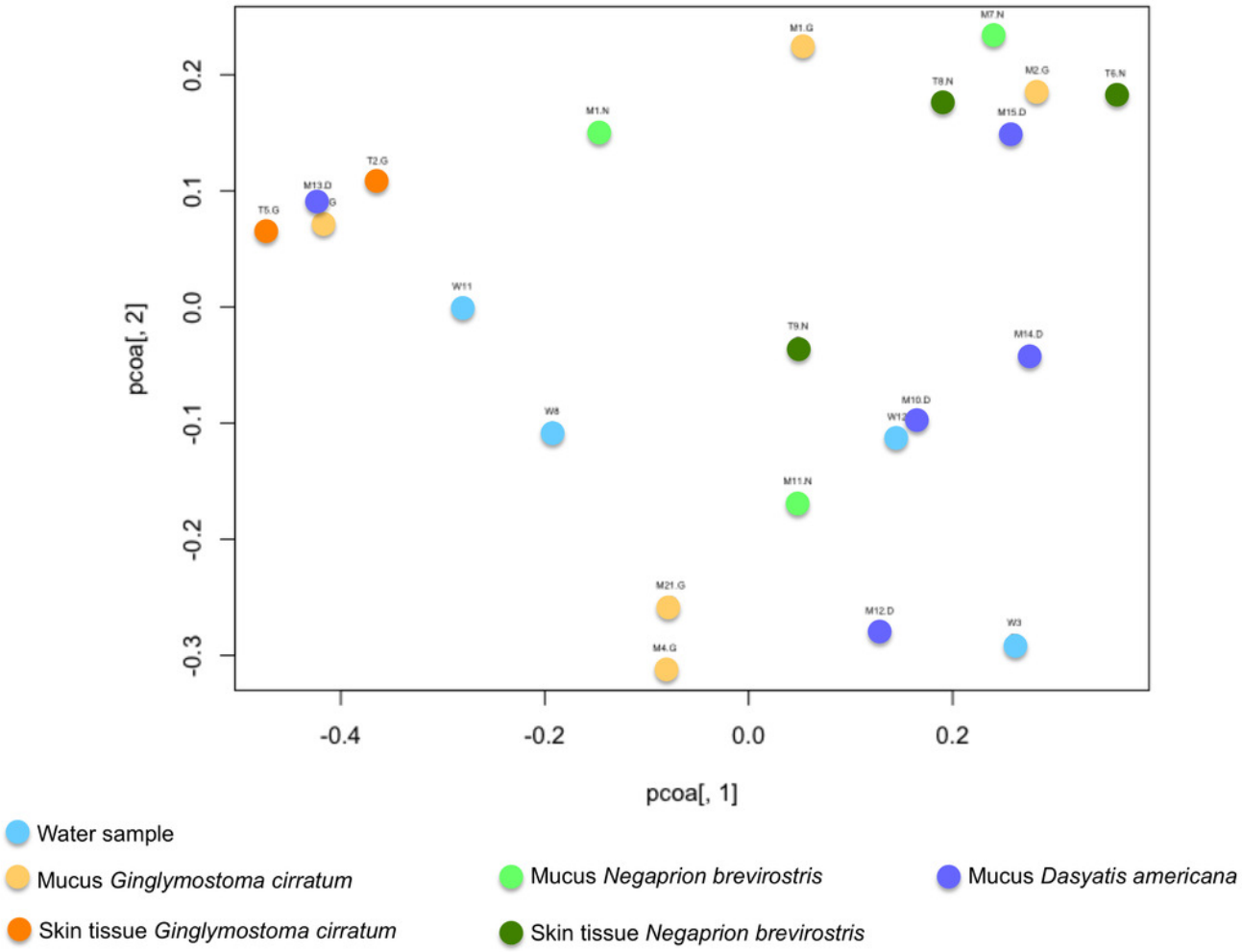

Table 1(on next page)

Read abundance of Phyla

Reads identified and assigned in 3182 reads obtained

1 Table 1. Read abundance of phyla identified in 3,182 reads obtained from 32 combined
2 samples analyzed, including a mucus or tissue sample for each of the sharks and rays
3 sampled and four water samples.

Phylum	% phylum found in the total number of analyzed reads
Acidobacteria	1,23
Actinobacteria	44,34
Bacteroidetes	4,92
Chlamydiae	0,06
Chlorobi	0,46
Chloroflexi	6,55
Crenarchaeota	1,49
Protista	3,09
Elusimicrobia	0,09
Euryarchaeota	7,11
Fibrobacteres	0,03
Firmicutes	6,28
Fusobacteria	0,06
Gemmatimonadetes	0,10
Nitrospirae	0,36
Parvarchaeota	4,05
Planctomycetes	2,16
Proteobacteria	12,20
Spirochaetes	0,76
Synergistetes	0,16
Tenericutes	1,92
Thermi	0,166
Verrucomicrobia	2,32

4