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Microbiome as a Tool to Monitor Aquarium Systems

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Simple Summary

This study tracked bacterial communities in seven zoo aquarium systems during their first year to understand how they respond to environmental changes. Researchers collected water and surface samples monthly from one saltwater and six freshwater filtration systems, analyzing bacterial populations through both cultivation and DNA sequencing. Key findings showed that bacterial numbers grew steadily before stabilizing. Each aquarium developed its own unique bacterial community that changed frequently over time. Freshwater and saltwater systems had significantly different microbial compositions. Despite various disturbances resulting from maintenance activities and environmental conditions, the systems maintained their ability to process ammonia—a critical function for removing toxic waste. The research demonstrates that while aquarium microbiomes are dynamic and respond to external influences, their core nitrogen-processing capability remains stable. The authors suggest that advanced DNA sequencing methods could help develop better aquarium management protocols by monitoring these bacterial communities, ultimately benefiting fish health and welfare. This represents an important step toward understanding how to maintain stable, healthy aquatic environments in captive settings.

Abstract

The bacterial microbiome in aquaria plays an essential role in system stability by metabolizing toxic compounds like ammonia. This study monitored microbiome changes in seven zoo aquatic systems during their first year to assess responses to external influences. Over one year (October 2021–October 2022), water and swab samples were collected from one seawater tank and six filtration systems at regular intervals. Bacterial cultivation included total bacterial counts. Metagenomic analysis was performed on samples corresponding to environmental events using Oxford Nanopore sequencing. Taxonomical analysis at the phylum and genus levels used EPI2ME software. Diversity analyses and statistical tests were performed using R. Total bacterial counts increased steadily after inoculation and stabilized by the end of the collection period. Diversity analysis revealed significant differences within and between freshwater and saltwater tanks. Each aquarium exhibited a distinct bacterial community with frequent compositional changes. Despite environmental conditions and maintenance interventions and resulting disturbances that affected the microbiome, the overall nitrifying capacity remained unaffected. Nitrifying taxa emerged

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as potential indicators for environmental effects. Combined with investigations of ecological function, next-generation sequencing could facilitate the development of aquarium management protocols, ultimately improving fish welfare.

Keywords: nitrogen cycle; aquatic systems; environmental events; microbiome analysis

1. Introduction

The microbiome in aquaria comprises a myriad of bacteria, archaea, fungi, and viruses and plays a crucial role in maintaining animal health and welfare, as well as the stability of these complex artificial ecosystems [1]. Microbial communities contribute to nutrient cycling, waste degradation, and water quality maintenance, thereby influencing key parameters such as pH, ammonia levels, and dissolved oxygen concentrations [2,3]. Aquaria host complex biosystems, which require specific and well-designed biological and technical components to ensure the health of every organism. Essential to every aquarium is the management of the nitrogen cycle. The nitrogen cycle in aquaria is driven by two processes: nitrification and denitrification [4]. Coupling with an atmosphere containing 70% nitrogen gas via denitrification is an important feature. However, water volume and a low heterotrophic biomass do not necessarily pose a risk, even if fish excretion, lost feed, and decaying plants release toxic ammonia [5]. It is pivotal to establish a healthy ecosystem and to safeguard the longevity of organisms in an aquarium. Nitrification allows for the conversion of toxic ammonia into less toxic nitrate [6] via oxidation driven first by bacteria of the *Nitrosomonas* genus, members of which oxidize ammonia to less toxic nitrite, while *Nitrobacter* bacteria then complete the oxidation reaction, producing nitrate [7].

Aerobic denitrifiers, especially Proteobacteria, return nitrogen to the photoautotrophic biome of an artificial aquarium via denitrification, from nitrate to nitrogen. Imbalances or disruptions of the nitrogen cycle of an affected microbiome can have detrimental effects on fish health, leading to increased susceptibility to diseases, compromised growth, and behavioral abnormalities [8].

The microbiome composition is specific to a tank or system and depends mainly on the initially introduced microbiome and environmental conditions [9]. The factors that drive shifts in microbiome composition and function are poorly understood but essential for timely interventions to avoid stressful conditions, maintain fish health, and avoid disease outbreaks.

High-phylogenetic-resolution 16S ribosomal RNA Nanopore sequencing was used to monitor the microbiome dynamics of seven newly established aquaria life support systems (LSSs) in the Leipzig Zoo for one year. The differences in the microbial communities in salt and freshwater aquaria were analyzed, especially the development of nitrifying and denitrifying bacteria and their long-term dynamics, as well as their responses to environmental events.

2. Materials and Methods

2.1. Sample Collection

Water sampling targeted six life support systems (LSSs) (Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) and one great seawater tank (Table 1). These LSSs have different characteristics, such as water temperature range and representative macrofauna, as described in Table 1.

Table 1. The main characteristics and main inhabitants of all tanks sampled in the study.

LSS	Water Type	Connected Tanks	Main Inhabitants	Total Water Volume (m^3)	Basin Temperature Range (°C)	Other Characteristics
Koi	Freshwater	1	Koi carps	54	12.2–23.9	Located outside; visitors can touch water surface and fish
Panorama	Freshwater	9	1: South American fish 2: Central American fish 3: Madagascan cichlids 4: West African fish 5: South Asian fish 6: Southeast Asian fish 7: New Guinean fish 8: Amazonas fish	514	24–25.7	Biggest freshwater tank
Corals	Saltwater	3	1: Caribbean fish 2: Stony corals 3: Soft corals	1:5.7; 2:5.7; 3:5.7	21.6–24.9	Chalk reactor included; salinity ~33 g/L
Clownfish	Saltwater	2	1: Clown fish 2: Fish from pacific coasts	00:01	22.9–25.1	NA
Malawi	Freshwater	1	Cichlids from lake Malawi	ND	22.9–25.1	Imitating high water hardness from lake Malawi
North America	Freshwater	1	Minnows	ND	17.9–20.8	Smallest tank/system
Ring basin	Saltwater	1	Sphyrnidae (hammerhead sharks) and batomorphi (rays)	205	23.4–26.5	Old ground material and filter unit; filled with freshwater during construction period

LSS = life support system; NA = not applicable; ND = not determined.

Water samples for culture and genomic analysis were collected from each LSS from October 2021 to October 2022 (weekly from 7 October to 1 November 2021 and bi-weekly from December 2021 to October 2022).

A total of 2.5 L of water was withdrawn in sterile single-use plastic bottles (VWR International, Radnor, PA, USA) at the 4 mechanical filtration chambers, directly from the outlet of the aquarium pipe leading the water to the filter unit. Swabs were taken from four randomly selected filter substrate elements (Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) out of the biofiltration chamber. Due to the size of the filtration sets of the Ring basin tank, there was no easy access for sample collection; in this case, only water was taken directly from the tank. All events that disrupted the normal and daily settings of the aquaria were considered "disturbance events" and were recorded by management personnel. These included targeted biological alterations (inoculation with starting culture, removal of bigger fish, introduction of new fish, and addition of salt and trace elements), undertaken hygienic measures (filter mat exchange), technical issues (electricity failure), and external disruptions that affected the basins (altered human interactions, storms, and lowest and highest recorded temperature). The samples from that day were used for sequencing analysis of the microbiome. Ammonia levels were measured at the time of these sampling events.

2.2. Sample Processing

2.2.1. Total Bacterial Count

Samples were processed and cultured on the day of collection. A ten-fold serial dilution series was prepared by mixing 0.5 mL of sample with 4.5 mL of saline–tryptone solution (0.85% NaCl with 0.1% tryptone). Swab samples were first resuspended in 2 mL of water from the corresponding filter system. Then, 100 µL aliquots from undiluted samples and serial dilutions were spread-plated onto appropriate agar media. For freshwater samples, sheep's blood and casein soybean digest (CASO) agar were used. For saltwater samples, blood salt and marine salt agar (MSA) were used. Sheep blood and blood salt agar plates were incubated at 37 °C for 48 h, and CASO and MSA plates were incubated at 25 °C for the same time. Following incubation, colony-forming units (CFUs) were enumerated, and total bacterial counts were calculated according to the method described by Farmiloe et al. [10].

2.2.2. Concentration of Nucleic Acids from Water Samples

To optimize the DNA yield, water samples were concentrated using a tangential flow filtration (TFF) system under negative pressure produced by a Masterflex Easy-load™ pump (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the water was flowed through a Vivaflow™ 50 tangential flow cassette (Sartorius AG, Goettingen, Germany). The eluate (15 mL) was kept at −80 °C until DNA extraction.

2.2.3. DNA Extraction

A MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for DNA extraction and purification, following the protocol designed for biofluids/liquid samples. Either 500 µL of water or the tip of the swab, cut directly into the bead beating tube, was used as a template, following the manufacturer's protocol with slight modifications. A HulaMixer™ (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used instead of the Vortex with vortex adaptor mixing. Purification was performed using magnetic beads, which were washed twice with the supplied buffer and once with 80% ethanol. The samples were stored at −80 °C until further analysis.

2.3. Nanopore Sequencing

In contrast to Illumina 16S short reads, long reads of the full 16S gene obtained by Oxford Nanopore Technology (ONT) sequencing have been shown to yield high taxonomic resolution [11], as demonstrated, for example, by a study investigating mock microbial communities, which revealed the high specificity of ONT [12]. To assess the microbiome composition, metagenomic analysis of the samples was performed using Oxford Nanopore Technology, targeting the 16S ribosomal RNA. The samples were prepared for 16s sequencing using a Rapid sequencing amplicons-16s barcoding kit (SQK-16S024, Oxford Nanopore Technologies, Oxford, UK). Library preparation was carried out according to the manufacturer's protocol with minor changes; the 10 mM Tris-HCL pH 8.0 with 50 mM NaCl required for elution was replaced by nuclease-free water. From the obtained purified PCR products, 5 µL was pooled for preparation of the DNA library for sequencing. From this pool, 10 µL was used as the DNA library. Sequencing was performed on a MinION Mk1C sequencing device (MinKNOW v21.11.6), and Flow Cell R9 Version (FLO-MIN106D) was used (Oxford Nanopore Technologies, Oxford, UK). The priming kit (EXP-FLP002) was prepared following the same manufacturer's protocol. The sequencing run was adjusted for 24 h with the recommended minimum quality score value of 7.

2.4. Data Analysis

Fastq sequencing data was retrieved from the device and used for data analysis using the Fastq16s program (V2022.01.07) of EPI2ME Desktop Agent 3.5.6. (Oxford Nanopore Technologies, Oxford, UK). The data was analyzed at phylum and genus levels.

2.4.1. Data Processing for Downstream Analysis

EPI2ME-labs version v1.1.18 was used to assign the taxonomic ranks with each read in EPI2ME output csv files, following the analysis of EPI2ME 16S CSV Output tutorial. The output table was used in R software version 3.6.3 [13] and RStudio version 1.2.5033 (RStudio Team, 2019), using phyloseq [14] for data standardization, merging, and filtering; microeco [15] for creating taxonomic bar plots, alpha and beta diversity, differential abundance testing, and functional analysis; and vegan packages [16] for multivariate community analysis and group homogeneity tests.

Data standardization and filtering were performed by transforming counts to relative abundance, then multiplying by the median sample read depth [17]. The standardized data were merged at the genus level, then filtered by removing taxa that were only present in very low numbers in a small minority of samples. The cutoff was 10 counts for a taxon in at least 20% of samples or a total relative abundance of at least 1% of the total number of reads/samples. These filtered taxa were then used in the downstream analysis, except the analysis of alpha diversity, which was performed using standardized unmerged, unfiltered data.

2.4.2. Alpha and Beta Diversity Analysis

To study the diversity within each aquarium community, the observed features/taxa, the richness, and Shannon indices were used as measures for alpha diversity (within samples). Beta diversity (between samples) was examined using the Bray–Curtis dissimilarity measure and non-metric multidimensional scaling (NMDS) as the ordination method.

2.4.3. Multivariate Community Analysis and Group Homogeneity Test

Permutational multivariate analysis of variance (PERMANOVA) was performed to examine the significance of beta diversity across different LSS types and between salt- and freshwater. Homogeneity of dispersion tests were also performed to examine the homogeneity of each group regarding the taxonomic composition of their samples prior to the PERMANOVA test.

2.4.4. Differential Abundance and Indicator Taxon Analysis

The Linear discriminant analysis Effect Size (LEfSe) method was used as previously described [18] to determine the taxa most likely to explain differences among different LSSs and between the two water types. To complement LEfSe's group-level biomarker detection, Cohen's d was calculated for all pairwise comparisons of sample groups using abundance values collapsed at the taxonomic level of interest.

3. Results

3.1. Total Bacterial Count

The total bacterial count (TBC) showed a comparable trend in all LSSs; it increased sharply after starter culture inoculation into the aquaria and stabilized over time. Interruptions in microbial composition occurred in both water types, mainly after extreme environmental events, inoculation, the addition of new fish, and maintenance interventions (Figure 1 and Supplementary File S1, Figures S1–S5).

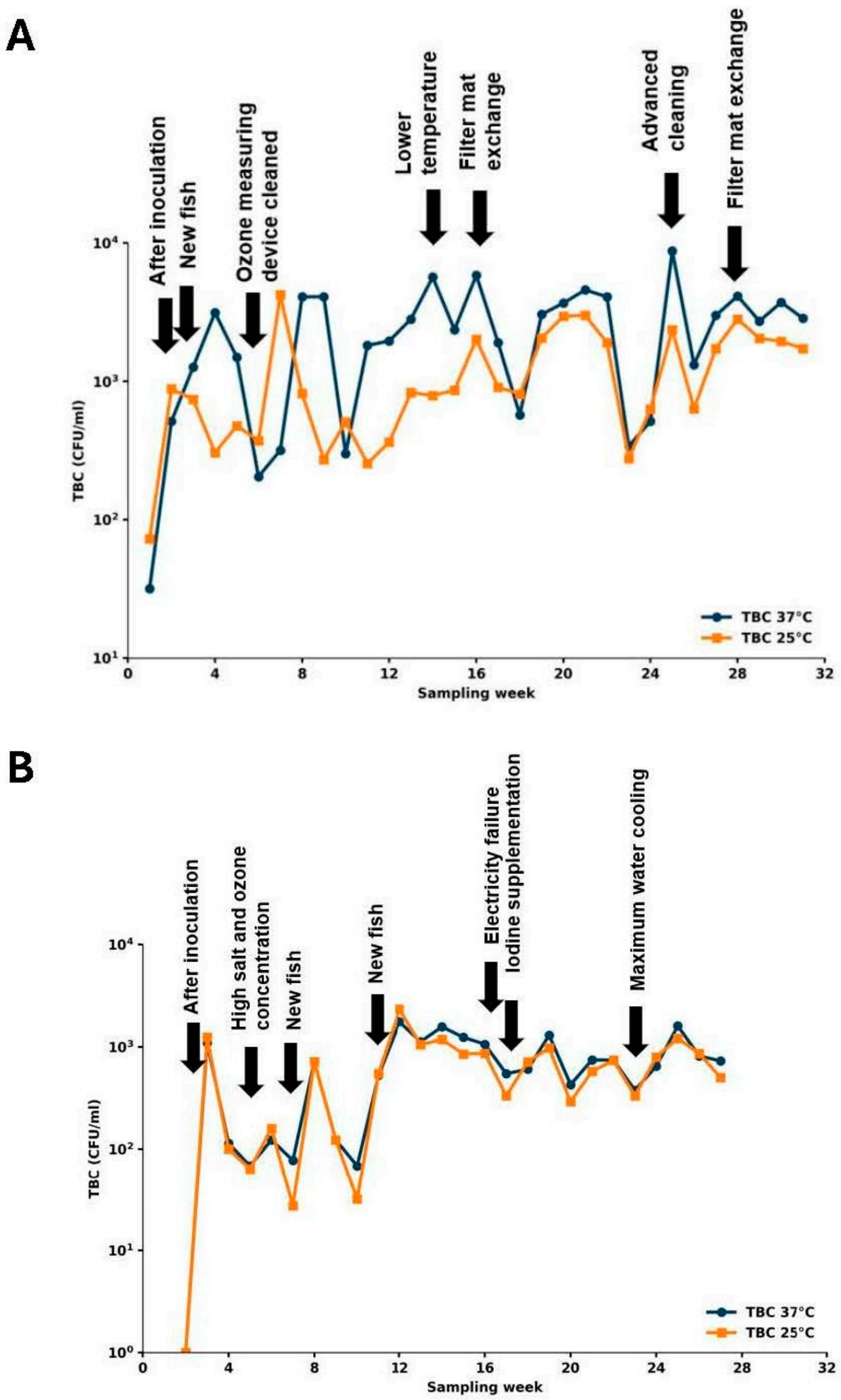

Figure 1. (**A**) Example of the total bacterial count of water samples obtained from the freshwater North America basin. (**B**) Example of the total bacterial count of water samples obtained from the saltwater Ring basin. Bacterial counts at 37 °C and 25 °C plotted throughout a one-year period. Specific environmental events are indicated by arrows.

TBC fluctuations in the freshwater basins differed at the two temperatures, and the overall TBC increased over time, with a higher TBC count at 37 °C (Figure 1 and Supplementary File S1, Figures S1–S3). Organic matter washed into the freshwater basin when cleaning devices, replacing the filter mat, or cleaning the basin appeared to cause differential prominent TBC spikes at 25 °C and 37 °C. In contrast, the TBC in the saltwater basin fluctuated, in general, in unison at 25 °C and 37 °C (Supplementary File S1, Figures S4 and S5).

3.2. Nanopore Sequencing

A total of 69 sequencing analyses coupled to single events were performed in the 7 LSSs: 12 for the Koi basin; 9 each for the Malawi, North America, Clownfish, and Corals basins; 10 for the Panorama basin; and 11 for the Ring basin.

The average number of reads varied considerably across basins, with standard deviations indicating substantial variability within each location. The data are summarized in Table 2.

Table 2. The average number of sequencing reads and standard deviations in samples obtained from all basins.

Basin	Average Reads	Standard Deviation
North America	33,119	31,546
Malawi	22,828	21,806
Panorama	145,185	65,224
Koi	144,253	231,170
Clownfish	12,166	6344
Corals	7191	9091
Ring	7320	15,123

3.3. Taxonomic Composition

In all LSSs, denitrifying Proteobacteria were observed as the most abundant phylum (>75%) (Figures 2 and 3). Polymeric organic matter processing *Bacteroidetes* and nitrifying *Nitrospirae* were the second most abundant phyla in salt- and freshwater LSSs, respectively. Additional denitrifying signature phyla *Firmicutes* and *Deinococcus thermus* were observed exclusively in samples from the saltwater LSSs and freshwater LSSs, respectively. Altogether, denitrifiers dominated all studied populations.

In-depth analysis of the bacterial taxa showed the effects of environmental events and maintenance procedures (Figure 3).

In the Koi LSS, denitrifying proteobacteria accounted for 89.7% at the lowest annual temperature and when the filter mat was changed. With the storm attack in the 15th week, the abundance of polymeric organic matter processing *Bacteroidetes* and nitrifying *Nitrospirae* increased, and the abundance of *Proteobacteria*, which also degrade complex organic matter [19], decreased to 74.2%. At the highest annual temperature, proteobacteria abundance dropped to 50.1%, while *Nitrospirae* abundance increased slightly.

After filter-mat changes, the addition of salt, and the addition of new fish in the Malawi, North America, and Panorama LSSs, the *Nitrospirae* phyla started to increase, reaching 32.5% and representing the second most abundant phyla. Also, with a continuous supply of fresh tap water in the Panorama LSS, the abundance of *Nitrospirae* reached 32.5%, with 45% *Proteobacteria* and 16.5% *Bacteroidetes.*

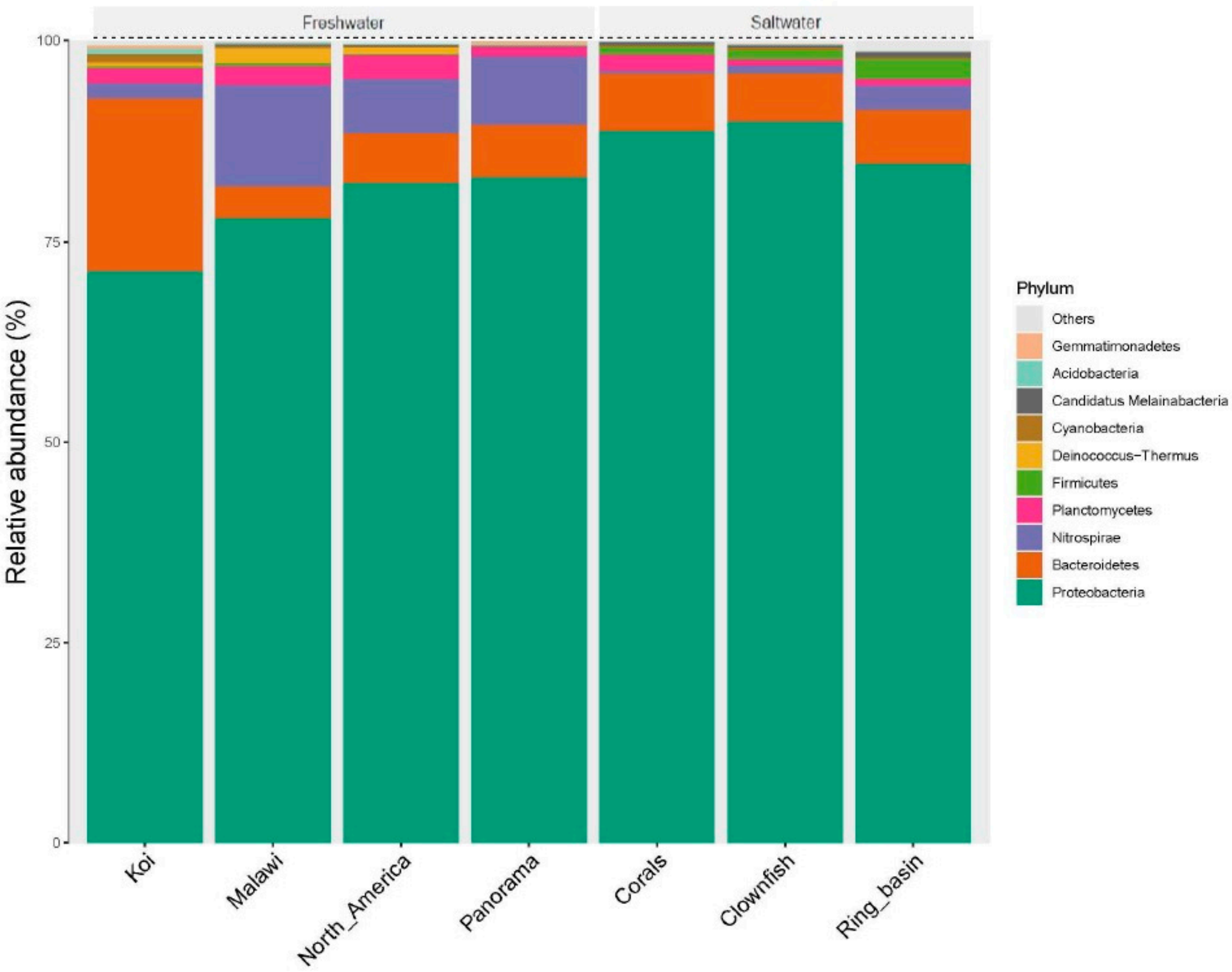

Figure 2. Composition of microbial communities at the phylum level showing the relative abundance of the ten most abundant phyla identified across both types of life support systems across the whole period: freshwater basins (first four columns) and saltwater basins (last three columns) during the sampling period. Less abundant phyla are collectively represented as “Others”.

In saltwater LSSs, the relative abundance of all phyla was affected by supplementation with iodine, filter-mat changes, and salt concentration. In the Ring basin LSS, *Proteobacteria* almost exclusively dominated (i.e., survived) when iodine was added in week 14, then decreased in the next week upon power failure, with a slight increase in *Nitrospirae* (3.7%) and the other phyla. *Proteobacteria* abundance continued to decrease to 80.1% in the next week, with increases in the other phyla found in week 14. At the coolest water temperature, the abundance of *Nitrospirae* remained almost the same, unlike that of *Bacteroidetes* and *Firmicutes*, which had notably decreased (4.6% and 3%) (Figure 4). In the Clownfish LSS, the lowest relative abundance of *Proteobacteria* was 75.8% after the second filter-mat change. In the Coral LSS, abundance of *Proteobacteria* decreased in correlation with falling salt concentration, i.e., the lowest abundance (72%) was found at the lowest salt concentration. Upon power failure, the abundance of *Proteobacteria* rose, and after one year, *Proteobacteria* again dominated the bacterial population, at 93.2%. These responsive dynamics appear to be directly related to intake of organic matter.

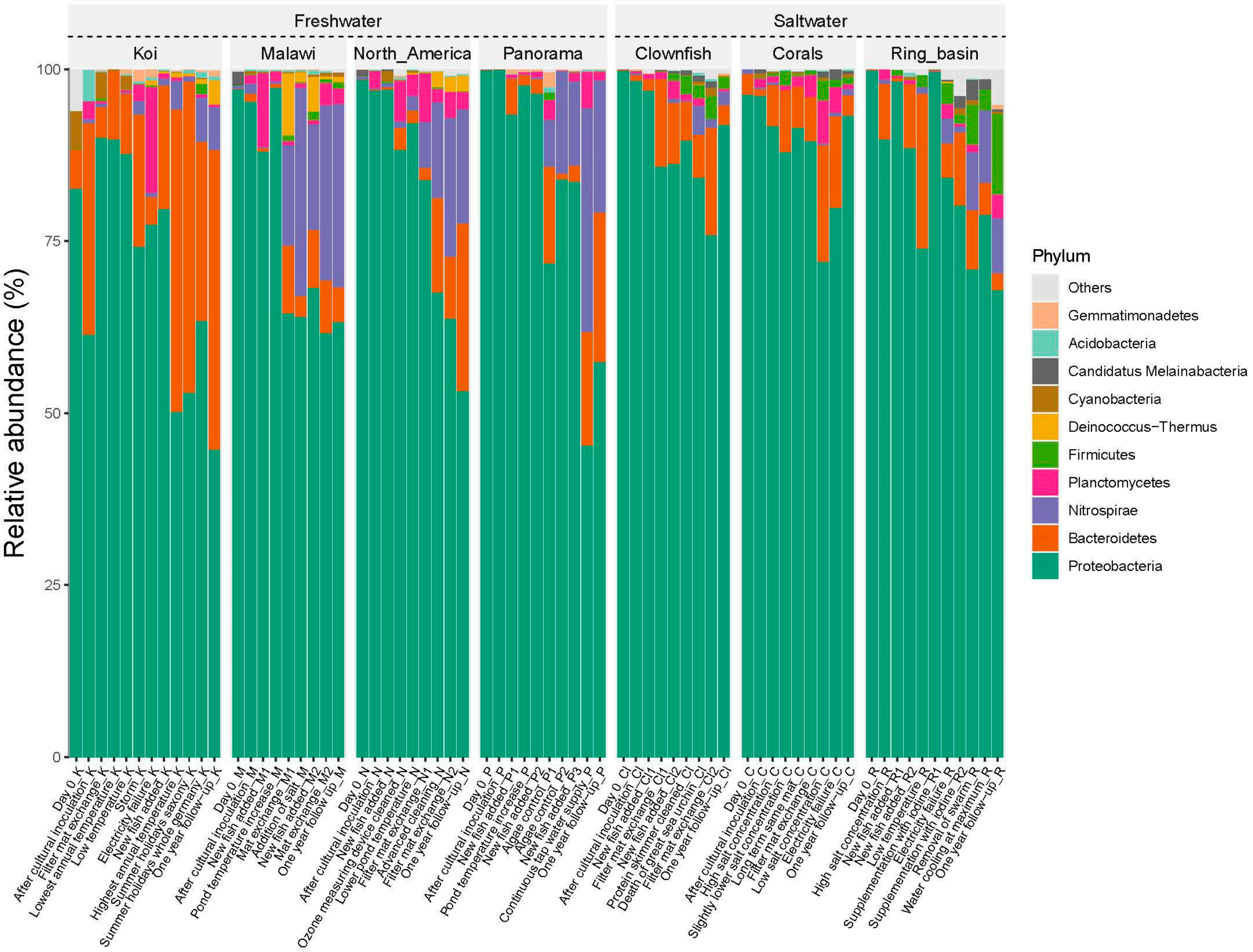

Figure 3. Relative abundances of the ten most prevalent microbial phyla in both types of life support systems—freshwater basins (four columns to the left) and saltwater basins (three columns to the right)—showing variations associated with different environmental conditions over a one-year sampling period. Phyla with lower relative abundances are grouped under "Others".

The abundance of the 50 top genera in all LSSs revealed that samples from the freshwater LSS clustered according to their content in two groups, while most samples from saltwater LSSs clustered together (Figure 4, top row 2). The relatively high abundance of some taxa characterized certain LSSs—namely, *Nitrospirae* in freshwater LSSs and *Marinicella* and *Ruegeria* in saltwater LSSs.

All of these observations appear to indicate very dynamic responsiveness to changing circumstances of the composition of the key nitrifying and denitrifying microbial populations in freshwater and saltwater.

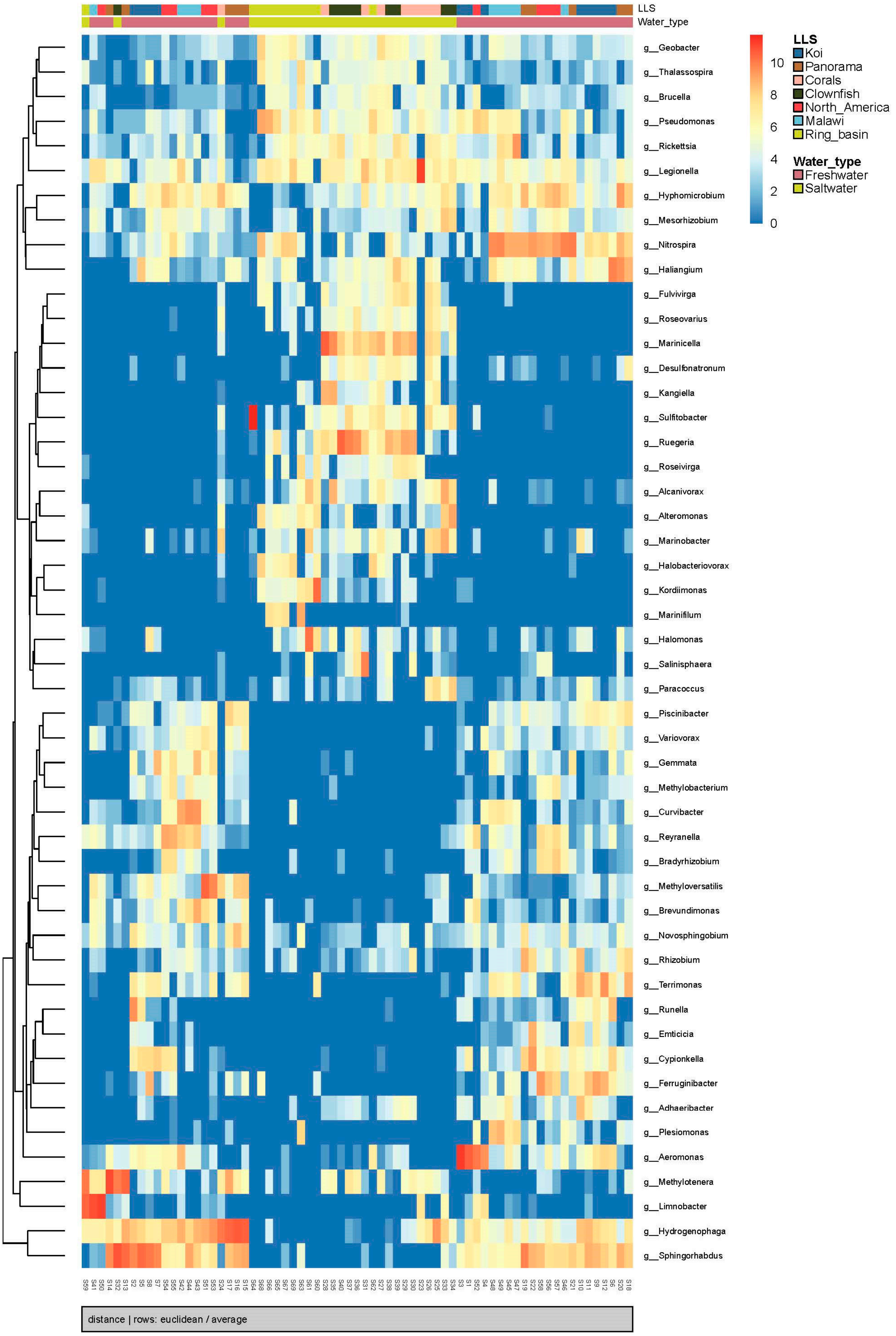

Figure 4. Heat map showing the 50 most abundant genera in the LSS microbiomes across the sampling period. Rows represent genera, and color intensity indicates relative abundance, with warmer colors representing higher abundance and cooler colors representing lower abundance. The top annotation rows denote LSS type (row 1) and water type (row 2). Data from all 69 NGS analyses are included.

3.4. Alpha Diversity

Within-sample diversity measurement revealed that samples from freshwater LSSs had a significantly higher number of observed taxa (Supplementary File S2, Figure S6) and yielded an insignificantly lower Shannon index than those from the saltwater LSSs.

Of all the saltwater LSSs, the Ring basin had the fewest observed taxa (mean = 110) and yielded a significantly different richness index compared to the samples obtained from the Coral and Clownfish LSSs (Supplementary File S2, Figure S7). Samples from the Coral LSS also had a higher diversity than samples from other saltwater LSSs based on the Shannon index (4.1). Among the freshwater LSSs, the richness of samples from the Malawi LSS was significantly higher than that of those from the Panorama LSS and significantly more diverse, i.e., showing a higher number of observed taxa (mean = 354 vs. 222) and a higher Shannon index (4 vs. 3). The samples from the other freshwater LSSs showed no significant differences in their richness and diversity measures. Therefore, population richness appears to be linked to the individual environments in each LSS.

3.5. Beta Diversity, Multivariate Community Analysis, and Group Homogeneity Tests

The non-metric multidimensional scaling (NMDS) of the distance between samples (Figure 5) showed distinct clustering of the samples from the same water type and a higher diversity in saltwater samples (Figure 5).

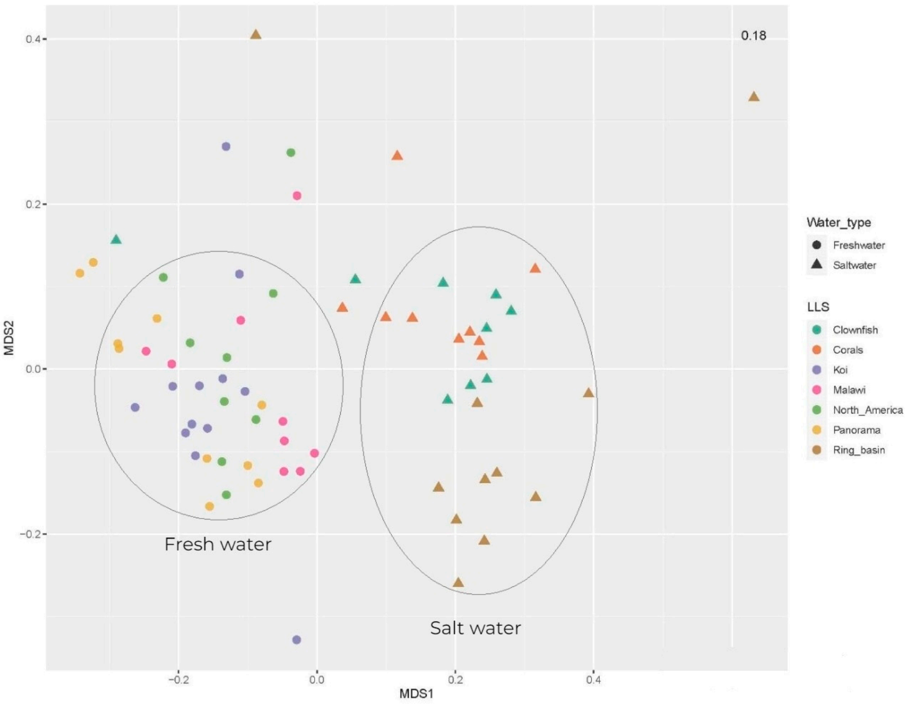

Figure 5. Non-metric multidimensional scaling (NMDS) ordination illustrating differences in collective microbial community structure among the samples obtained from all basins over the collection period based on Bray–Curtis dissimilarity of beta diversity.

This was confirmed by the Bray–Curtis dissimilarity measure for the differences between the two water types and between all compartments, with a statistically significant difference ($p = 0.001$) between the bacterial communities in the two water types (Supplementary File S2, Figure S8). Samples from the various LSSs were also significantly different ($p = 0.001$) from each other, with the Ring basin showing the highest diversity (Supplementary File S2, Figure S9).

All groups were homogeneously dispersed (F 0.7141, p value of 0.639; F 1.8976, p value of 0.179) for LLS and water-type groups. The PERMANOVA result additionally indicated statistically significant Bray–Curtis distance differences (p value 0.001) between the two water types and different LLS communities, with R2 0.17642 and 0.15774, respectively. About 17.64% of total variance was referred to water type, and 15.78% of variance was influenced by the LLS.

Homogeneous dispersion testing yielded F 0.7141 ($p = 0.639$) and F 1.8976 ($p = 0.179$) for LLS and water-type groups, respectively. The PERMANOVA result also indicated statistically significant Bray–Curtis distance differences ($p = 0.001$) between the two water types and different LLS communities, with R2 0.17642 and 0.15774, respectively. About 17.64% of total variance was referred to water type, and 15.78% of variance was influenced by the LLS. In essence, the microbiological compositions of fresh- and saltwater LSSs differ significantly.

3.6. Differential Abundance and Indicator Taxon Analysis

A linear discriminant analysis effect-size analysis (LEfSe) for freshwater and saltwater systems (Supplementary File S2, Figure S10) and for each LSS (Supplementary File S2, Figure S11) yielded a more fine-grained representation of the dominant genera.

In saltwater, *Ruegeria* (d = −0.96) and *Marinicella* (d = −0.98) were found to have the greatest abundance (Figure S13). The relative abundance of these taxa significantly differed between the two types of water (Figure S13). In freshwater, *Sphingorhabdus* (d = 0.82), *Hydrogenophaga* (d = 0.56), *Aeromonas* (d = 0.52), and *Nitrospirae* were the most abundant taxa, abundances significantly differing from saltwater (d = 0.25), and all showed an LDA score of 4–5 (Supplementary File S3).

In each LSS, there were taxa characterizing the local bacterial composition, with an LDA score close to 5 and significantly different abundances: *Aeromonas* (d = 0.83) in the Koi basin, *Curvibacter* (d = 1.33) in the Malawi basin, *Hydrogenophaga* (d = 2.02) and *Methyloversatilis* (d= 1.1) in the North America basin, *Sphingorhabdus* (d = 2.4) in the Panorama basin, *Methylotenera* (d = 2.58) and *Marinicella* (d = 1.33) in the Corals basin, *Ruegeria* (d = 1.36) in the Clownfish basin, and *Pseudomonas* (d = −1.38) in the Ring basin (Supplementary File S3). Notably, *Sulfitobacter* (d = 1.86) was found to be uniquely prominent in the seawater Ring basin. The differences in microbial populations between freshwater and saltwater at a more fine-grained level indicate adaptations to each LSS.

4. Discussion

Metagenomic analysis offers comprehensive resolution of the composition of unculturable complex microbial communities. Using ONT 16S analysis to study newly established freshwater and saltwater aquaria in Leipzig Zoo, an excellent resolution of Operational Taxonomic Units (OTUs) down to the species level (Figure 4) was obtained without the need for an additional computational approach to gain classification up to the species level, for instance when using Illumina 16S sequencing [20].

It is absolutely necessary to establish a stable nitrogen cycle in new aquaria to provide reliable nitrification and denitrification and maintain low nitrogen concentrations in the aquarium water. The microbial communities delivering this need to be resilient and able to response to external stimuli.

In this multivariate community analysis, samples from both fresh- and saltwater LSSs were collected over a one-year period and analyzed by bacterial cultivation and ONT. The results of 69 16S analyses across seven LSSs revealed that each aquarium had a unique composition at different time points throughout the one-year period (Figure 3) but also that fresh- and saltwater systems give rise to distinctly different bacterial communities.

TBC monitoring indicated fluctuations and steadily increasing values over time, with a higher TBC count at 37 °C, in general (Supplementary File S1, Figures S1–S3) in the freshwater basins. In the saltwater basin, TBC fluctuated, in general, almost in unison at 25 °C and 37 °C (Supplementary File S1, Figures S4 and S5); it would therefore seem that to monitor freshwater basins, both temperatures should be routinely used, whereas for saltwater basins, either of the two culture conditions appears sufficiently reliable to monitor TBC. Organic matter washed into the freshwater basin when cleaning devices, when replacing the filter mat, or when cleaning the basin appeared to cause prominent TBC spikes, indicating a dynamic response at the overall population level.

Diversity analysis of the microbiome (Figure 5 and Supplementary File S2) showed that the diversity of the analyzed saltwater systems was higher compared to freshwater systems, with some phyla dominating the community (Figure 3) in both freshwater and saltwater systems, confirming previous reports [21]. The *Sphingorhabdus* (*Alphaproteobacteria*) and *Hydrogenophaga* (*Betaproteobacteria*) genera [22,23], known for degrading organic matter and complex recalcitrant compounds, were more significantly associated with freshwater LSSs (Figure 4, rows 49 and 50). In contrast, the *Marinicella* and *Ruegeria* genera (both *Gammaproteobacteria*) emerged as indicator taxa with significantly high abundances in saltwater LSSs (Figure 4, rows 13 and 17). Therefore, denitrifying bacteria dominate the populations in freshwater and saltwater LSSs, indicating successful establishment of typical denitrifying populations as described for aquaculture RAS, aquaponics, and wastewater systems [20,24–27] and estuary water and octocorals [28,29], respectively.

In saltwater systems, polymeric organic matter processing *Bacteroidetes* followed as the second most abundant phylum, whereas nitrifying *Nitrospirae* was the second most abundant phylum in freshwater aquaria (Figure 4, row 10). These phyla and their lineages are usually included in the initial aquarium inoculate to help establish the nitrification process [20]. Denitrification seems to be supported by signature phyla *Firmicutes* in saltwater LSSs (range: 0–11.8%) and *Deinococcus thermus* (range: 0.03–0.25%) in freshwater LSSs, confirming the observation of a 22% share of *Deinococcus* in the aerobic denitrifying population in a freshwater RAS system culturing Prussian cap (*Carassius auratus gibelio*) [30].

Of all the studied LSSs, the Panorama freshwater LSS supports the highest fish species diversity (including predator fish species *Osteoglossum bicirrhosum*), which possibly results in higher biological activity and a concomitantly increased release of ammonia, as reflected by a high *Nitrospirae* abundance (up to 32.5%) in this LSS.

In conclusion, the dominance of denitrifying bacteria appears to indicate microbial community structures poised to efficiently denitrify as soon as nitrifying bacteria have detoxified ammonium generated by the intake and degradation of organic matter.

External factors potentially affect microbial composition [21,31,32] and can therefore be pivotal in shaping the water microbiome. In addition, with increasing temperature, the total ammonia nitrogen originating from leaching processes in deposited materials may increase [33]. An increased nitrogen concentration is counterproductive in typically low-nutrient aquarium environments.

In the Leipzig Zoo aquarium, the koi display is partially located outdoors and therefore directly exposed to environmental temperature changes. At the highest annual temperature, the abundance of *Bacteroidetes* increased, reaching around 40%, displacing *Proteobacteria*, which declined from >75% to 50%. Since both phyla include denitrifying bacteria [20,25,34], although protobacteria have been described as competitive under low nutrient/substrate conditions [35,36], the microbiome adaptation in favor of *Bacteroidetes* may reflect adaptation to an increased formation of ammonia at higher temperatures [33].

The Malawi, North American, and Panorama indoor LSSs responded to management interventions such as filter-mat changes, the addition of salt, and the introduction of new fish with a dynamic expansion of nitrifying *Nitrospirae* (increase to >40% of all phyla), which appears to be a consequence of the release of biological material and, thus, ammonia by these interventions.

Ammonium was detected in only 8/33 and 15/28 measurements across all freshwater and saltwater LSSs, respectively, indicating good control of the nitrogen cycle at the majority of time points in all LSSs. At the time points at which ammonium was detected, the overall average was 26.5 µg/L in freshwater LSSs and 40.00 µg/L in saltwater LSSs (Supplementary File S2, Figure S12), remaining below the safe maximum of 50 µg/L. Therefore, measured ammonia values indicate that the microflora in the pattern revealed by the microbiome analysis were resilient and able to balance out any perturbations of the nitrogen cycle in all the LSSs.

A more fine-grained representation of the dominant genera showed a prominent occurrence of *Sulfitobacter* in the saltwater LSSs for sharks and rays (Supplementary File S2, Figure S7). *Sulfitobacter* indicate active degradation of diatom-derived dimethylsulfoniopropionate (DMSP) and their detoxification by oxidizing sulfides to sulfite [37]. Therefore, in this case, the microbiome appears to have responded to a substantial diatom abundance in this LSS.

A significant spike in Legionella in the filter units of the Coral seawater LSS was observed. *Legionella* ssp. thrives in warm seawater [38]. It is quite remarkable that *Legionella* was found only in the filter of one of three warm saltwater LSSs in the absence of a particular *Legionella* spike in the water samples of the Coral LSS. This implies a low abundance of Legionella in seawater LSSs and the need for a more focused monitoring effort including microbiological culture for *Legionella* in the three seawater LSSs.

Significant microbiome diversity changes induced by 90% replacement of water have been observed in a saltwater LSSs [39]. It was concluded that the disequilibrium caused by mixing the microbial communities of old and added water significantly affected the microbial community diversity and structure of the studied estuary tank, but this appeared to have a minimal influence on metabolic and functional potential.

Although the abundance of denitrifying proteobacteria was the highest in all LSSs throughout the collection period, their abundance dropped below 50% in the sample obtained from the freshwater Panorama LSS at the point of continuous water replenishment. It is tempting to speculate that this could be a functional adaptation of the microbial community to a diluted concentration of organic matter. Likewise, the population shift towards nitrifying *Nitrospirae* (32%) may optimize the responsiveness of the system to any new incoming ammonium.

Microbiome studies are particularly important during the establishment phase of new aquaria to ensure that the required taxa are available. Long-term studies can also offer valuable insights into the ecosystem's health. In addition to the essential neutralization cycle of ammonium, beneficial taxa within the aquarium can influence fish pathogens [20,26]. A recent study even confirmed a direct link between the environmental water microbiome, zebrafish microbiomes, and fish health [40].

To the best of our knowledge, this is the first comprehensive descriptive analysis of bacterial taxa in freshwater and saltwater zoological aquaria. By characterizing each unique aquatic system and identifying possible impactful events for the aquarium microbiomes, the extensive ONT dataset baseline described here may contribute to the improvement of monitoring and management of various aquarium types. The study pinpointed characteristic taxa patterns involved in nitrogen and sulfur cycling and their association with the intake of organic matter.

The observations made in regards to community displacements within denitrifying bacteria and shifts between denitrifying and nitrifying bacteria should be verified in a more controlled aquarium model allowing for the controlled addition of ammonia or organic matter while monitoring the microbial community response using 16S ONT. Dynamic expansion of nitrifying *Nitrospireae* would be expected in response to the addition of ammonium and organic matter, whereas the dynamic expansion of denitrifying *Proteobacteria* and *Bacterioides* would be expected mainly in response to the addition of organic matter. An experimental setup with technical and biological replicates and shorter sampling intervals would help to substantiate the more fine-grained dynamics within the denitrifying populations in terms of response time and magnitude. It might be possible to determine thresholds of ammonium and organic matter intake that trigger these dynamic expansions. Likewise, the supposed link between diatom and DMSP degrading *Sulfitobacter* could be studied by inoculating saltwater models with diatoms to monitor the *Sulfitobacter* response.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci13020125/s1, Supplementary File S1: Figure S1. The TBC at 37 °C and 25 °C of the Malawi basin over the one-year period. Special environmental events were recorded. Figure S2. The TBC at 37 °C and 25 °C of the Koi basin over the one-year period. Special environmental events were recorded. Figure S3. The TBC at 37 °C and 25 °C of the Panorama basin over the one-year period. Special environmental events were recorded. Figure S4. The TBC at 37 °C and 25 °C of the Corals basin over the one-year period. Special environmental events were recorded. Figure S5. The TBC at 37 °C and 25 °C of the Clownfish basin over the one-year period. Special environmental events were recorded. Supplementary File S2: Figure S6. Box plots of alpha diversity using observed features measure (A) and Shannon measure (B) of fresh- and saltwater systems. Different letters indicate significantly different diversity measure values. Figure S7. Box plots of alpha diversity using Observed features measure (A) and Shannon measure (B) of the seven different LSS. Different letters indicate significantly different diversity measure values. Figure S8. Box plots of beta diversity distance of fresh- and saltwater systems using Bray-Curtis. *** indicates significance at $p = 0.001$. Figure S9. Box plots of beta diversity distance of the seven different LSS using Bray-Curtis. Box line = median. Figure S10. The top 20 differentially abundant genera in the two water types as revealed by the linear discriminant analysis effect size (LEfSe). Figure S11. The top 20 differentially abundant genera in the different LSS as revealed by the linear discriminant analysis effect size (LEfSe). Figure S12. Mean and SD of ammonia values > 0 of ammonia detected across all 4 freshwater LSS (8/33) and 3 saltwater LSS (15/28). Supplementary File S3: Figure S13. In freshwater *Sphingorhabdus*, *Hydrogenophaga*, *Aeromonas*, and *Nitrospirae* were the top abundant taxa and significantly different from saltwater. They showed the greatest effect size (LDA score 4–5). Figure S14. In each LSS, there were taxa characterizing the local bacterial composition, with LDA scores close to 5.

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