PacBio next-generation sequencing uncovers Apicomplexa diversity in different habitats

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Article

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Abstract

The phylum *Apicomplexa*comprises a large group of protozoan organisms that are obligatory intracellular parasites and can infect various vertebrate and invertebrate hosts, leading to several significant medical and veterinary pathologies, including toxoplasmosis, cryptosporidiosis, theileriosis and eimeriosis. However, information on their diversity and distribution in nature is still limited, particularly in rivers. To address this gap, the study employed next-generation high-throughput 18S rRNA amplicon sequencing based on PacBio technology to determine the diversity and composition of the microeukaryotic parasitic taxa group (i.e., *Apicomplexa*). Principal component analysis (PCA) and principal coordinate analysis (PCoA) indicated the habitat heterogeneity for the physicochemical parameters and the *Apicomplexa* community. These results were supported by PERMANOVA ($P < 0.001$), ANOSIM ($P < 0.001$), Cluster analysis, and Venn diagram. Dominant apicomplexan parasites in inlet samples were *Gregarina* (38.54%), *Cryptosporidium* (32.29%), and *Leidyana* (11.90%). Outlet samples had *Babesia*, *Cryptosporidium*, and *Theileria* as dominant groups. Surface water samples had *Toxoplasma*(16%) and a lower relative abundance of *Cryptosporidium* (8.33%). The next-generation high throughput sequencing covered a wide range of parasites in Egypt for the first time to our knowledge, which could be useful for legislation of the standards for drinking water and wastewater reuse.

Introduction

The microbial community present in rivers and wastewater treatment plants (WWTPs) consists of more than just prokaryotes, as it also encompasses eukaryotic organisms, such as protozoa, fungi, algae, and microscopic metazoans [1–3]. The *Apicomplexa* are a quintessentially parasitic microeukaryotic group, which includes many gut-associated taxa that can cause significant harm to both humans and animals (e.g., *Cryptosporidium parvum*, *Toxoplasma gondii*, etc.) [4–6]. The *Apicomplexa* is a diverse group of parasitic protists that comprises over 6,000 known and potentially many more unknown species. These parasites are obligate, meaning they can only survive by living inside a host, and can infect virtually all vertebrates and many invertebrates [6]. The *Apicomplexa* acquire nutrients by parasitizing their host. One of the essential roles of wastewater treatment methods is eliminating parasitic apicomplexan species, such as *Cryptosporidium* and *Toxoplasma* [7–9]. However, little is known about the removal of apicomplexan species in wastewater treatment plants (WWTPs) and their composition in different habitats.

Apicomplexans often have high host specificity, but some are generalists. These parasites can cause significant harm to their hosts, including humans and domestic animals, and are considered the most important eukaryotic parasites from a medical standpoint. Various *Eimeria* species, which are specialized to infect particular hosts, can be detected in multiple vertebrates, including poultry and rabbits. Theileriosis causes significant losses in cattle farming, while *Toxoplasma gondii* is responsible for causing toxoplasmosis and infects approximately 20% of the worldwide human population. *Toxoplasma gondii* is a widely prevalent protozoan parasite capable of infecting almost every mammalian and avian

species, with a high proportion of humans (15–70%) being seropositive [10]. A global study on enteric pathogens found that *Cryptosporidium* was the second most frequently identified pathogen among infants in developing countries [11]. In favorable conditions, Cryptosporidiosis can cause an epidemic of diarrhea, even in developed countries. *Cryptosporidium* species have the ability to cause watery diarrhea in humans and are known for causing gastrointestinal disease and morbidity, especially among patients infected with HIV.

Theileria parva and *Theileria annulata* are responsible for causing east coast fever (theileriosis) and bovine tropical theileriosis in cattle and water buffaloes, respectively, while several *Babesia* species cause babesiosis in various animals including cattle, dogs, horses, and rarely humans [11,12]. The diseases caused by these pathogens can lead to economic losses as they may cause low milk production, poor growth, and mortality in infected animals. Although developed countries have successfully implemented vector eradication programs, tropical and subtropical countries still suffer from economic losses due to these diseases. Alternative approaches are needed for effective control of piroplasmosis since vector eradication is not feasible in most tropical regions. Commercially available vaccines, containing live attenuated *Babesia bovis* and *B. bigemina*, have been widely used in the New World and Australia [12].

Previous studies identified very few species of Apicomplexa (e.g., *Cryptosporidium*, *Toxoplasma*, and *Isospora*) by using PCR [13], real-time PCR, or multiplex PCR [14–16]. Indeed, these methods could not identify several neglected apicomplexan species. So, we employed next-generation high throughput 18s rRNA amplicon sequencing based on PacBio technology in this study to reveal the diversity and composition of the *Apicomplexa* and its spatial variation between the different habitats (influent and effluent of WWTP and the Nile River water samples).

Results And Discussion

The total number of OTUs of microeukarytes was 52214. The total number of high-quality reads for microeukaryotes was 136127. The PCA showed that the ph-ch parameters tended to cluster together based on different habitats, particularly inlet versus surface water. However, some low-quality samples of the outlet showed similarities to the inlet samples, and the other high-quality samples showed similarities to the surface water samples (Fig. 1). The changes in environmental parameters during the wastewater treatment process and in the receiving environment were emphasized by the results of PERMANOVA ($P < 0.001$) and ANOSIM ($P < 0.001$). Significantly, the difference in ph-ch between inlet and surface water (ANOSIM; $R = 0.94$) was more prominent than that between inlet and outlet or outlet and surface water (ANOSIM; $R < 0.67$) (Table 1).

Table 1
PERMANOVA and ANOSIM to test the significance of the differences in physicochemical parameters between the habitats.

Group	PERMANOVA		ANOSIM	
	t	*P*	R	*P*
Inlet vs. Outlet	2.10	0.004	0.38	0.003
Inlet vs. Surface water	5.19	< 0.001	0.94	< 0.001
Outlet vs. Surface water	3.27	< 0.001	0.67	< 0.001

During the study period, the ph-ch parameters of the inlet wastewater samples obtained from Zenin WWTP were marked by the presence of organic constituents, demonstrated by COD, and the presence of abundant nutrients such as TKN, NH_3-N, NO_2-N, NO_3-N, TP, and TN. Their corresponding average concentrations were 270 mgO_2/L, 34.97 mg/L,18.5 mg/L,0.22 mg/L, 1.8 mg/L, 2.59 mg/L and 36.99 mg/L, respectively. The results of treated effluent indicated the average percentage of removal was more than 82% of COD, 74% of TP, and almost 60% of total nitrogen content. Biodegradation of organic matter in the activated sludge process takes place by the action of bacteria, protozoa, and metazoan [22]. Bacteria dominated all other biota groups in the activated sludge process, and influence the process of mineralization and degradation of organic and inorganic nutrients [22,23]. The microeukaroytes, such as free-living protozoa, feed on dispersed bacteria and suspended particles to purify and clean the wastewater [22].

Overall, the PCoA revealed that the *Apicomplexa* community exhibited a significant difference between different habitats. The inlet samples were clustered far from the outlet and surface water samples (Fig. 2). The results of Cluster Analysis (Fig. 3) supported those of PCoA (Fig. 2). The apicomplexan species represented a lower proportion (< 0.01%) in the microeukaryotic communities. Similar findings concerning microeukaryotic communities, which contains the parasitic *Apicomplexa*, were observed in different habitats in China [21,24] and in USA [25]. The Venn diagram revealed that no shared apicomplexan species were found between the different habitats. While 68 (89%) unique apicomplexan species were observed in the inlet samples, lower unique species were observed in the outlet (n = 4) and surface water samples (n = 7) (Fig. 4). The study's findings suggest that the physicochemical parameters of various habitats impacted the apicomplexan community. Further studies are needed to investigate the specific factors that drive the distribution of apicomplexan species in different aquatic environments.

The heatmap showed that the inlet samples comprised more apicomplexan genera than the outlet and surface samples. The significant decrease in parasite abundance from high levels in the raw sewage inlet to low levels in the effluent and the receiving surface water environment (Nile River water) indicated the successful removal of parasites during the wastewater treatment process (Fig. 5). The results are consistent with a previous study by Freudenthal et al. (2022), which highlights the importance of

wastewater treatment technology in reducing potential health risks associated with treated wastewater reuse. Effective removal and inactivation of pathogenic microorganisms are essential in ensuring the safety of treated wastewater reuse. Earlier studies have identified ciliates (protists) and rotifers (metazoans) as the most likely predators for small protists and bacteria in WWTPs [27,28]. Additionally, the network analysis results revealed associations between parasitic protists (e.g. *Dientamoeba*, *Entamoeba*, and *Giardia*) and their potential predators (ciliates and rotifers) may be explained by predation [26].

The taxonomic composition of the apicomplexan community revealed that the most dominant parasites in the inlet samples were *Gregarina* (relative abundance; 38.54%), *Cryptosporidium* (32.29%), and *Leidyana* (11.90%). *Babesia, Cryptosporidium*, and *Theileria* were the dominant taxonomic groups of *Apicomplexa* communities in the outlet samples, accounting for 33.33%, 25%, and 16.67%, respectively. In earlier research, conventional detection methods consistently found that wastewater is a potential hotspot for parasites [29–31]. Our study highlights the diversity and abundance of the frank parasitic *apicomplexans*, particularly in the Nile River using next-generation sequencing technology. In surface water samples, the harmful parasite (i.e., *Toxoplasma*) appeared in relative abundance around 16%, and the relative abundance of *Cryptosporidium* (8.33%) was lower compared to the other environments (Fig. 6). Loads of *Apicomplexa* in wastewater may be due to the population density, per capita water consumption, survival of the excreted stage (e.g., cysts or oocysts) in wastewater [32], habits, and presence of cats or other domestic animals in the area [32,33]. Maritz et al. (2019) identified different parasitic protists taxa such as *Entamoeba*, *Blastocystis*, and *Trichomonas* in sewage samples using 18S rRNA amplicon sequencing. While a wide parasitic protists range, including *Blastocystis*, *Entamoeba*, and *Trichomonas Dientamoeba*, *Guttulinopsis*, *Giardia*, and *Rosculus* were detected in WWTPs using shotgun metagenome [26]. Gad and co-workers used machine learning approaches, such as, SourceTracker and Fast Expectation-mAximization Microbial Source Tracking (FEAST) to track the *Apicomplexa* OTUs in the Changle River watershed. Their research revealed that a considerable portion of exogenous *Apicomplexa* from both the main channel and tributaries may have originated from untreated domestic and swine wastewater, as well as treated wastewater (i.e., effluents of WWTPs) [24]. In line with our findings, *Cryptosporidium* species were also detected in the Changle River (China), particularly in the river's tributary [24]. These parasitic microeukaryotes can persist in surface waters for extended periods, even up to several months [34]. Therefore, additional studies are warranted to assess the potential public health hazards associated with the presence of parasitic *Apicomplexa*, including *Cryptosporidium*, in the Nile River.

Conclusion

Our study utilized next-generation high throughput 18S rRNA amplicon Pacbio sequencing to biomonitor different habitats including the inlet and outlet of a WWTP and surface water. This novel approach enabled us to detect a wide range of eukaryotic parasites. Our findings revealed the presence of some apicomplexan species such as *Cryptosporidium*, *Toxoplasma*, and *Theileria* in the outlet (treated sewage samples) and surface water samples from the Nile River. These species may indicate insufficient

wastewater treatment and pose health threats to humans and animals when present in drinking water, water recreation areas, and aquatic food production systems. Furthermore, it can be deduced that water containing oocysts can act as an indirect source of human infection when used for purposes such as irrigation, washing, and processing of agricultural products. Our study has implications for the development of legislation concerning drinking water and wastewater reuse standards at the national level based on WHO recommendations. Further studies are required to explore the diversity and composition of microeukaryotic parasites in other habitats such as drinking water and swimming pools using amplicon sequencing and shotgun metagenomics technologies.

Material And Methods

Samples collections

A total of 34 samples were collected from three habitats: inlet (n = 8) and outlet (n = 8) of Zenin wastewater treatment plant and an urban location at the Nile River (n = 18). Three samples from each habitat have been screened using next-generation high-throughput 18S rRNA PacBio sequencing. To analyze the microeukaryotic community, water samples (~1000 mL) were filtered through 0.22 µm Sterivex-GP filters (Millipore, Bedford, MA, USA), and the filters were kept at −80°C until DNA extraction.

Physicochemical analysis

Environmental variables were determined according to the International Standard Methods for Water and Wastewater [17]. Briefly, a AD 360 DO meter (Adwa Instruments, Inc, Europe) was used to determine the temperature, dissolved oxygen (DO) and DO saturation of the water samples *in situ*. pH was measured using bench pH meter Jenway model 3510. Electric conductivity (EC) and total dissolved solids (TDS) were measured by 4510 Jenway conductivity meter. Chemical oxygen demand (COD) was measured according to dichromate method 5210-D using digestion for two hours at 150^{o}C on HANNA COD reactor and followed by colorimetric measurement using Lovibond spectrodirect after cooling. Total Kjeldahl Nitrogen (TKN) was measured using mercuric sulfate digestion method followed by titration method 4500-Norg, ammonia nitrogen (NH_3-N) was measured titrimetric according to method 4500-NH_3, nitrite nitrogen (NO_2-N) was detected according to colorimetric method 4500-B and nitrate nitrogen (NO_3-N) by modified sodium salicylate method according to Scheiner (1974). Total nitrogen was calculated as the sum of organic nitrogen, nitrite nitrogen, nitrate nitrogen, and ammonia. Total phosphorous (TP) was measured according to the method (4500-C).

DNA Extraction, 18S rRNA Gene Full-Length High-Throughput Sequencing

In this study, the collection of samples was subjected to extraction of environmental DNA using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, USA) was used to extract environmental DNA from the samples collected in this study. The amount of DNA was measured using the Qubit 2.0 fluorometer with Qubit dsDNA BR assay kit (Life Technologies, Grand Island, NY, USA). The 18s RNA full-length genes were

amplified using the primer pairs Euk-A and Euk-B [18]. PCR reactions were conducted in triplicate for each sample, with 25 μL reaction volume containing 5 μL TransStart FastPfu Buffer (5×), 2 μL dNTPs (2.5 mM), 0.5 μL TransStart FastPfu DNA Polymerase (2.5 units/μL, TransGen Biotech, Beijing, China), 0.4 μM of forward and reverse primers, and 10 ng of template DNA. PCR amplification involved an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30s, 55°C for 45s, and 72°C for 90s, and a final extension at 72°C for 10 min. PCR products were purified using the QIAquick@ Gel Extraction Kit (Qiagen, Santa Clarita, CA, USA), and sequencing libraries were prepared using the SMRTbellTM Template Prep Kit (Pacific Biosciences, Menlo Park, CA, USA) according to the manufacturer's instructions. Finally, the sequencing was conducted on a PacBio Sequel II platform (Creative-proteomics, NY, USA).

Sequence analysis

The 18S rRNA gene sequences of microeukaryotes were subjected to quality trimming using PacBio SMRT portal v2.3.0. Reads that did not meet the minimum pass of ≥3, minimum predicted accuracy of ≥90%, and length criteria of 1340-1640 bp were discarded. After quality control, an average of 15125 high-quality reads per sample remained, which were used for chimera-checking and clustering into operational taxonomic units (OTUs) at the 97% identity cutoff using UPARSE v7.0.1001 [19]. The representative sequence of each OTU was classified using the SILVA database v138 and RDP classifier at a confidence threshold of 80% [20]. To standardize the uneven sequencing effort, all samples were randomly subsampled to the smallest library sizes with 14000 reads for microeukaryotic communities.

Statistical analysis

To characterize the patterns of physicochemical parameters in different habitats, Principal Component Analysis (PCA) was used based on the Euclidean distance. For mapping the apicomplexan OTUs in different environments, Principal Coordinate Analysis (PCoA) was used based on the Bray-Curtis distance index. In order to show the relationship between the apicomplexan community (response group) and physicochemical parameters (explanatory group) in different habitats, Distance-based Redundancy Analysis (RDA) was employed. The significance of differences in physicochemical parameters among the habitats was tested using Permutational Multivariate Analysis of Variance (PERMANOVA) and Analysis of Similarity (ANOSIM) [21]. The statistical analyses and visualization were performed using PRIMER v.7.0.21 (Quest Research Limited, Auckland, New Zealand) and R v4.1.0 (https://www.r-project.org/).

Declarations

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Author Contributions:

Mahmoud Gad: Methodology, Formal analysis, Data curation, Writing-Original draft preparation, Visualization, Resources, Funding acquisition, Supervision, Project administration. **Mariam E. Fawzy**: Investigation, Methodology, Writing - Review & Editing**. Ahmad Z. Al-Herrawy**: Resources, Writing - Review & Editing**. Sayeda M. Abdo**: Investigation, Methodology, Software. **Noura Nabet:** Investigation, Methodology, Software. **Anyi Hu:** Conceptualization, Methodology, Software, Validation, Writing - Review & Editing

Additional Information: NA

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Data availability. The raw sequence data of 18S rRNA genes was deposited in the NCBI short reads archive database under BioProject number PRJNA952662**.**

Conflicts of Interest: The authors declare no conflict of interest.

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Figures

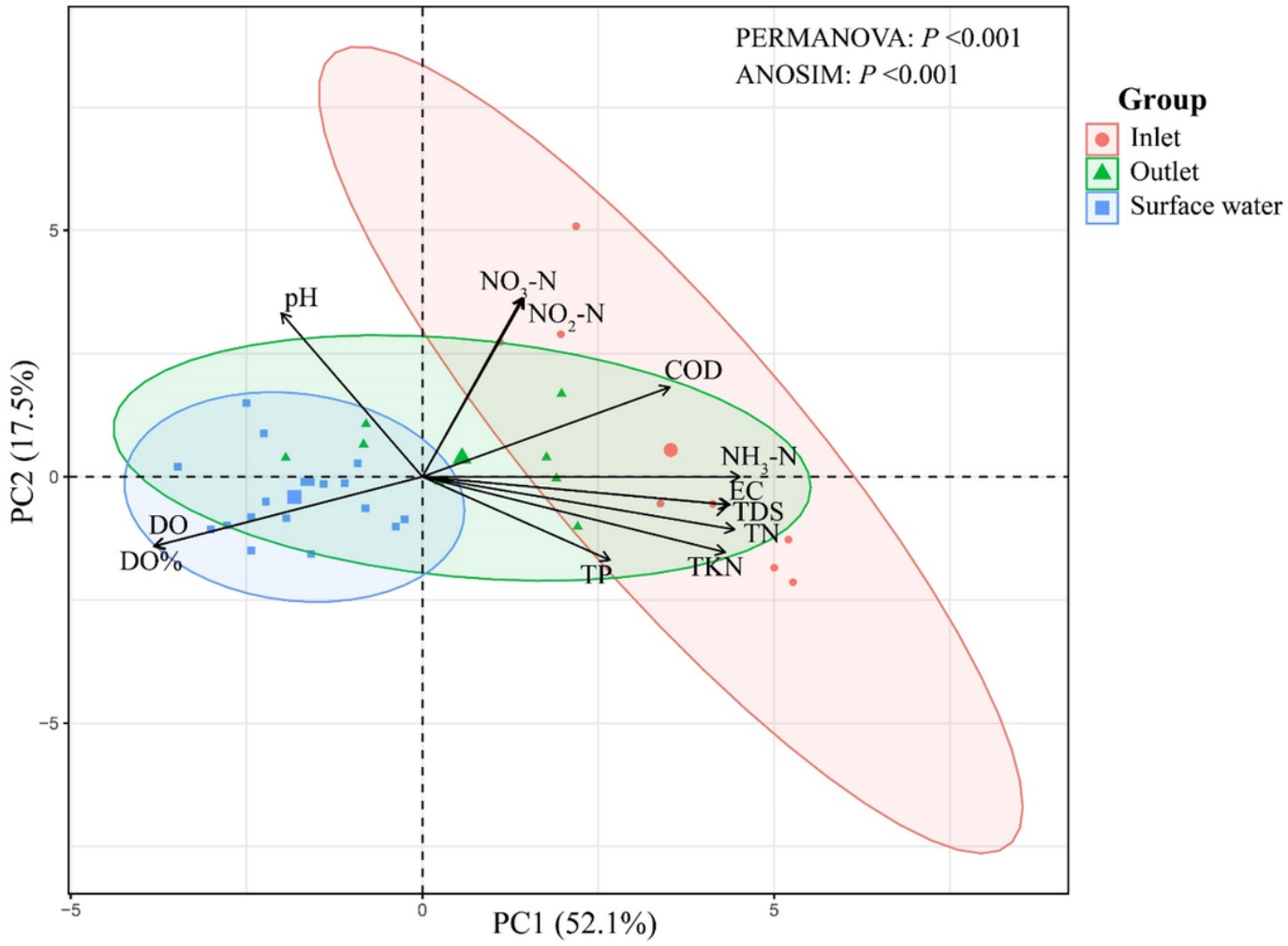

Figure 1

Principal component analysis (PCA) plot for spatial variation of physicochemical parameters between different habitats (principal components (PC1 and PC2) explained 69.6% of the total variation).

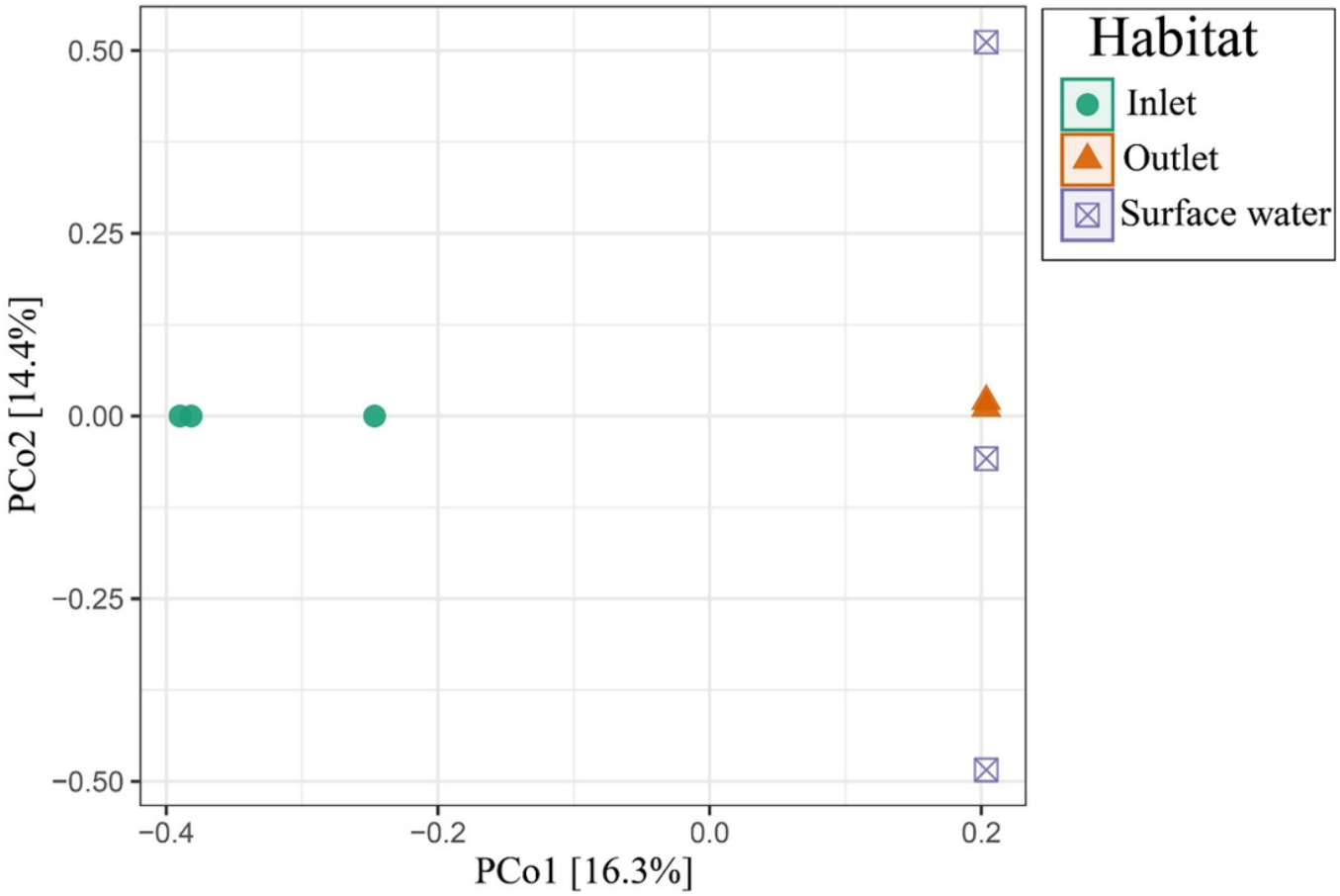

Figure 2

Principal Coordinate Analysis (PCoA) based on Bray Curtis similarity index showing the β-diversity patterns of the *Apicomplexa*community.

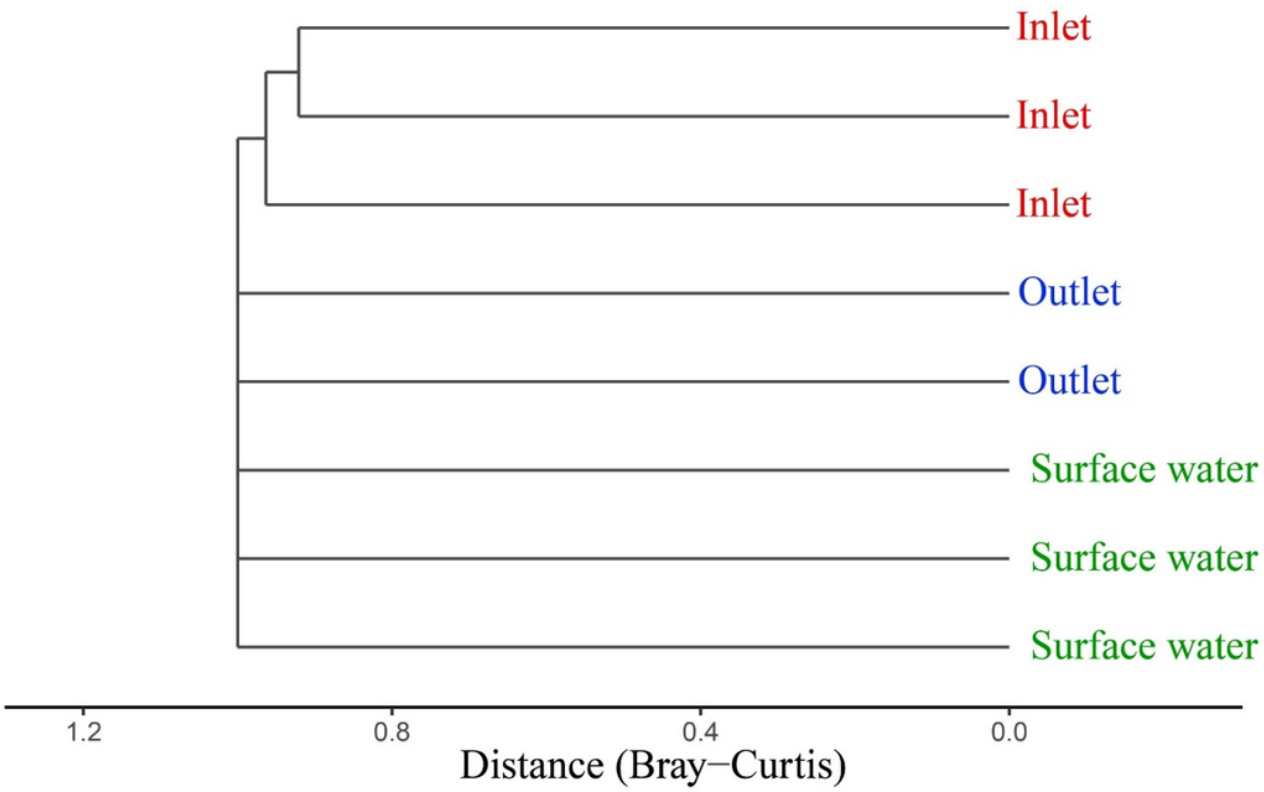

Figure 3

Cluster Analysis of *Apicomplexa* OTUs in different environments

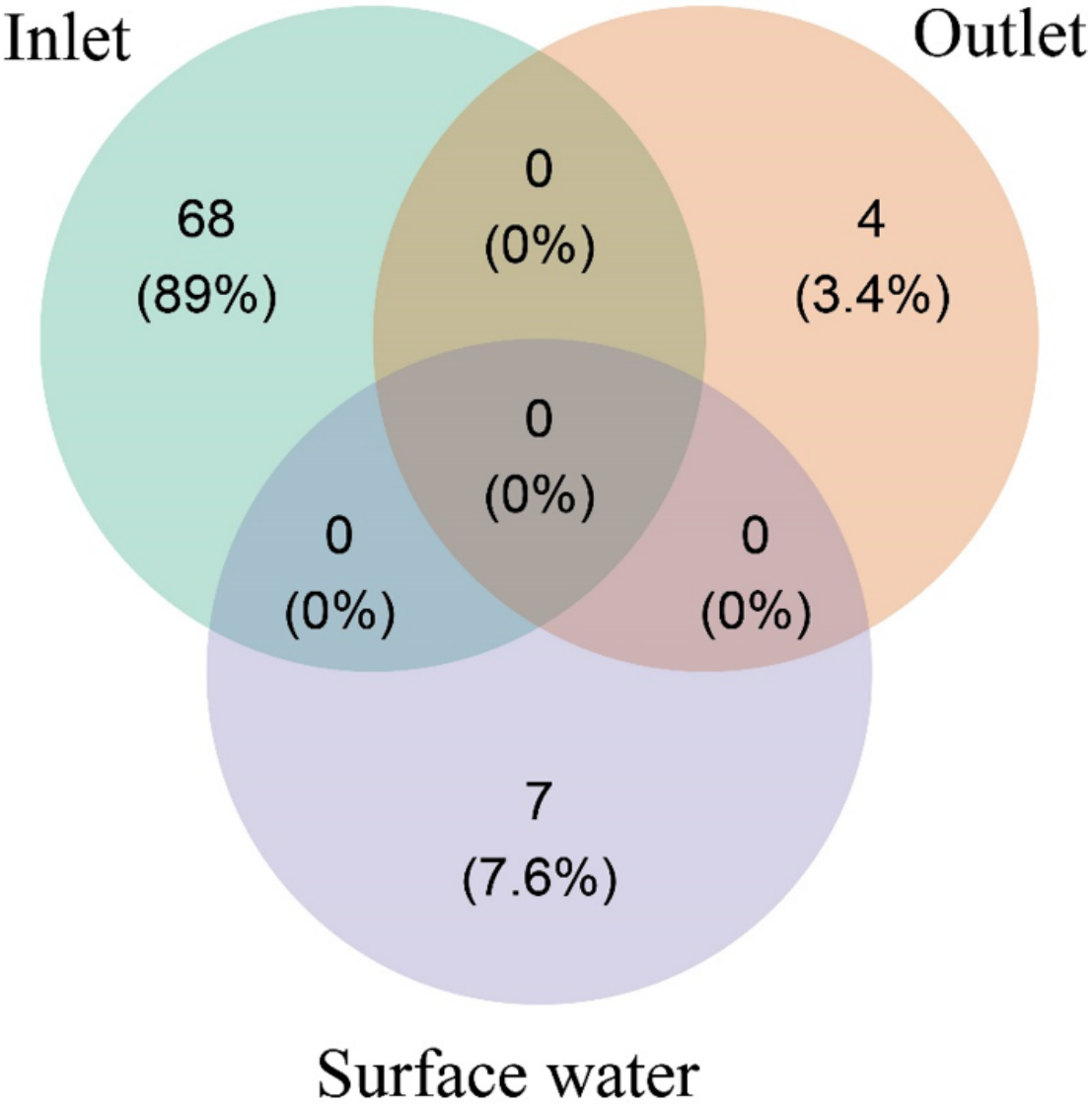

Figure 4

Venn shape shows the unique and shared *Apicomplexa* OTUs in different habitats

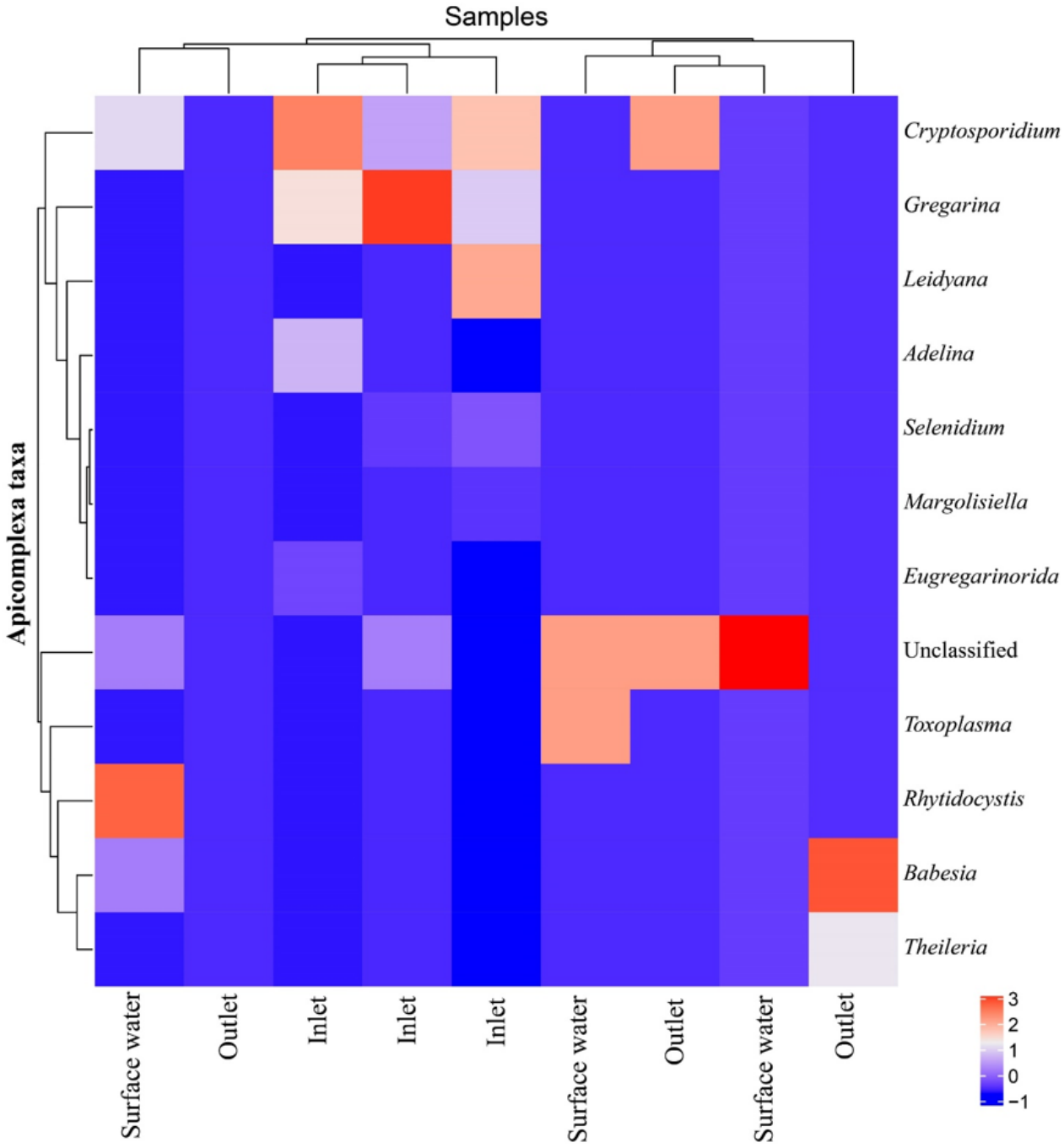

Figure 5

Heatmap showing the distribution of *Apicomplexa*taxa in different environments

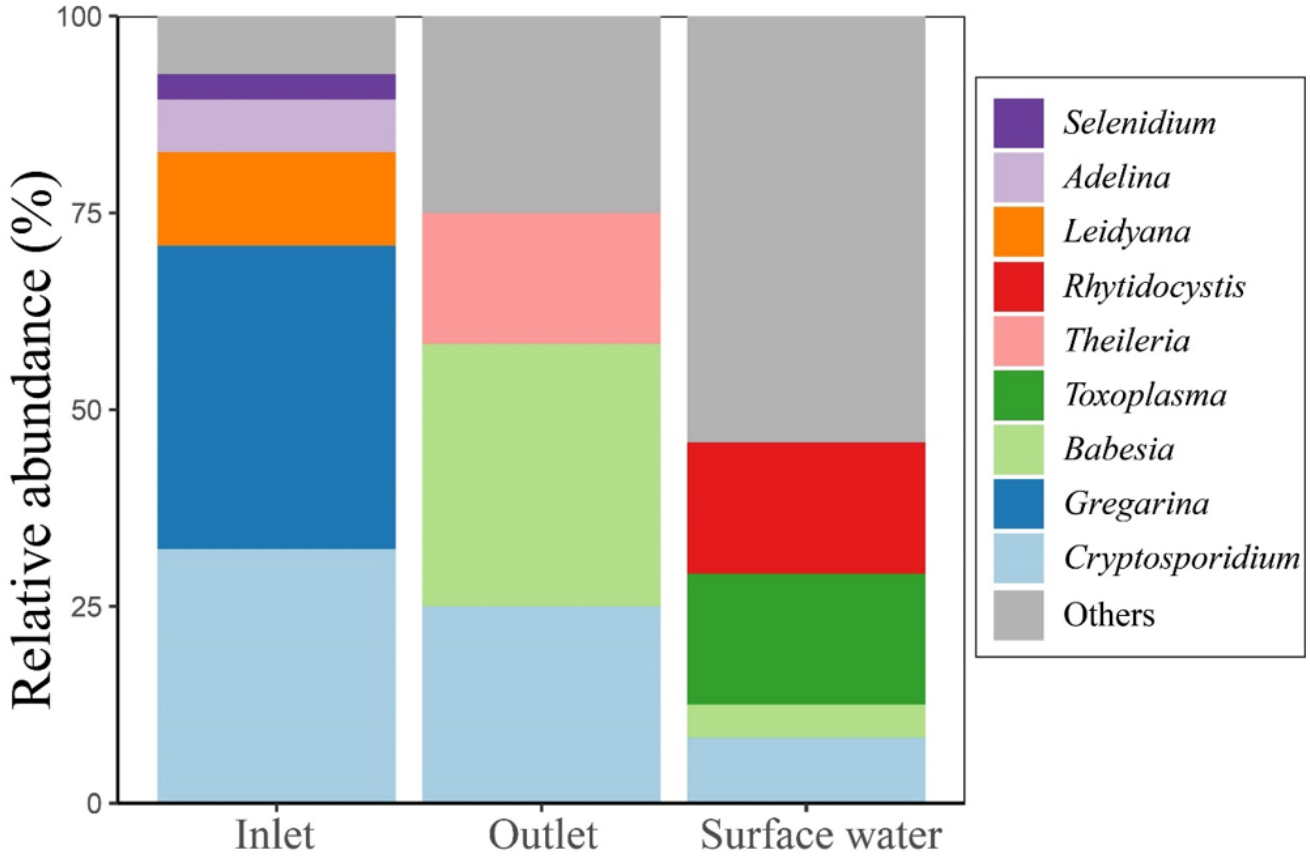

Figure 6

Taxonomic composition of the *Apicomplexa*community. Others refer to unclassified and less contributed taxa.