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Microeukaryotic communities diversity with a special emphasis on protozoa taxa in an integrated wastewater treatment system

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Abstract

This study developed an integrated wastewater treatment system that combines an upflow anaerobic sludge blanket (UASB), downflow hanging non-woven fabric (DHNW), and anaerobic baffled reactor (ABR) to explore the effect of treatment stages on the diversity of microeukaryotic communities. This study aimed to bridge the knowledge gap regarding the influence of integrated system stages on microeukaryotic community diversity. Through 18S rRNA amplicon sequencing, we identified unique microeukaryotic communities across different stages, with the aerobic phase hosting 35.77% of unique amplicon sequence variants (ASVs). The results of principal component analysis (PCA) and non-multidimensional scale analysis (nMDS) demonstrated the significant influence of wastewater treatment on both environmental factors and the microeukaryotic communities. Ciliophora was notably abundant in the effluent (42.09%) and sludge (17.11%). The aerobic stage was dominated by Ochrophyta, a diverse group of algae instrumental in nutrient removal, such as nitrogen and phosphorus, through biological processes. A redundancy analysis (RDA) revealed a positive correlation between chemical and biochemical oxygen demand and Cryptomycotina, highlighting its potential as a bioindicator for treatment efficacy. The detection of protozoan species, such as *Acanthamoeba castellanii* and *Vermamoeba vermiformis*, in the outlet stage poses health risks, whereas *Cryptosporidium* sp. was found in both the inlet and aerobic stages but not in the outlet. Our study reveals the complex nature of microeukaryotic diversity in the wastewater treatment system and its implications for treatment performance and public health.

Keywords Integrated wastewater treatment system, Wastewater treatment, 18S rRNA amplicon sequencing, Treatment efficacy, Multivariate statistical models

Introduction

The escalating challenge of water quality necessitates a comprehensive approach to water resource management, where wastewater treatment plays a pivotal role in ensuring future water security [1]. On-site wastewater treatment systems (OWTS), particularly in rural areas and locales lacking centralized sewer systems, have emerged as practical and economical solutions for managing domestic wastewater [2, 3]. These systems are not just functional but also central to the broader ecological dynamics of wastewater treatment. Beyond their functional benefits, these systems play a crucial

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role in the larger ecological context of wastewater treatment, contributing to the sustenance of diverse microeukaryotic communities. Such communities, including bacterivorous protists, significantly reduce sludge production, improve sludge sedimentation, and enhance the quality of effluent water, thereby serving as vital biological indicators of the wastewater treatment process [4, 5]. Furthermore, specific fungal taxa, notably within the Ascomycota phylum, are recognized for their roles in facilitating denitrification and cellulose degradation, underscoring their ecological importance [6, 7].

The adoption of upflow anaerobic sludge blanket (UASB) reactors, especially in tropical climates, has gained traction due to their economic efficiency. These systems are lauded for their low operational costs, effective pollutant removal capabilities, reduced sludge production, and potential for methane generation as a source of bioenergy [8–11]. However, the UASB process also faces certain challenges, including susceptibility to the solids in the influent, reduced effectiveness at temperatures below 30 °C, and difficulties in nitrogen and phosphorus removal, as well as in pH management for high-strength wastewaters [5]. The integration of downflow hanging non-woven fabric (DHNW) system with UASB technology has shown a substantial improvement in treatment quality, achieving removal efficiencies of 90% for chemical oxygen demand (COD), 78% for biological oxygen demand (BOD), 95% for TSS, and 72% for TN, along with a significant reduction in coliform levels by 3 log¹⁰ [12]. A collaborative UASB/DHS (Downflow Hanging Sponge) and DHNW system approach in treating municipal wastewater demonstrated over 90% removal of crucial pollutants like COD and BOD, and an 80% reduction in total nitrogen (TN) [6]. In light of the limited research conducted on this subject, this study focused on investigating the microeukaryotic community structure, with particular attention to protozoa, in an integrated system utilizing UASB, DHNW, and anaerobic baffled reactor (ABR).

A key aspect of these processes involves microbial eukaryotes, with their roles ranging from predation to aiding in sludge sedimentation and effluent quality enhancement. For instance, eukaryotes like protozoans and metazoans are instrumental in reducing turbidity, BOD, and suspended solids, while also mitigating bacterial pathogen exposure risks [7]. Certain eukaryotic organisms, particularly fungi, play a pivotal role in the granulation process during sewage treatment [13], and are also instrumental in the breakdown of biomass components such as cellulose, hemicellulose, and lignin [14].

Fungi are recognized for their role in denitrification and possess the ability to thrive in varying oxygen concentrations, employing three distinct metabolic pathways for energy production: oxygen respiration, ammonia fermentation, and denitrification (nitrite

respiration) [10]. In addition, certain Rhizaria eukaryotes have been observed to accumulate nitrate in aerobic conditions and subsequently utilize it for denitrification under anoxic conditions [15]. The microbial ecosystem within wastewater treatment plants (WWTPs) encompasses not only bacteria but also a significant eukaryotic population, which includes fungi, protists, and microscopic metazoans, contributing to the overall functionality [16]. Despite their lower numbers compared to bacteria, protists significantly influence the community structure of both prokaryotic and eukaryotic organisms they prey upon [17, 18], thus affecting denitrification, nitrification, and flocculation processes [19]. Protists, representing a large portion of the eukaryotic biomass in WWTPs, are central to the microbial ecosystem of wastewater systems. They enhance effluent clarity by consuming free bacteria and small, unsettled flocs. Their population dynamics provide insights into the conditions of wastewater treatment and the age of sludge, with different types like amoeba, flagellates, and ciliates indicating varying sludge age conditions and operational health [20, 21].

The presence of protists in WWTPs is important not only for their role in regulating the microbial community but also because they include gut-associated taxa that can be harmful to humans and animals [16]. These parasites, such as *Cryptosporidium*, *Giardia* and *Entamoeba*, are pathogenic and their removal is a key function of WWTPs [22, 23]. For example, *Giardia lamblia* is a waterborne protozoan parasite that causes diarrhea and other gastrointestinal symptoms in humans [24]. *Entamoeba histolytica* is another parasitic protist that causes amoebic dysentery, a severe form of diarrhea, and can also invade other organs, such as the liver and lungs [25, 26]. Therefore, the removal of these parasites through efficient wastewater treatment processes is crucial for protecting public health.

The primary objective of this study is to elucidate the complexity and dynamics of eukaryotic community structures across various stages of an integrated wastewater treatment system, utilizing advanced high-throughput 18S rRNA amplicon sequencing techniques. Our investigation centers on evaluating how these diverse microbial communities contribute to the treatment system's efficiency, particularly focusing on their role in the removal of pathogenic or potentially pathogenic microorganisms from key eukaryotic taxonomic groups, such as Amoebozoa and Apicomplexa. By shedding light on the interaction between microbial dynamics and treatment performance, this research aims to enhance our understanding of microbial ecology within wastewater treatment processes and to identify potential bioindicators of the treatment performance.

Materials and methods

The integrated system design and sample collection

The treatment unit was installed and commissioned in the Zeinen wastewater treatment plant, Giza, Egypt (30°02′06.3″N 31°10′49.3″E). Our study utilized an integrated wastewater treatment system, incorporating two overlapping Upflow Anaerobic Sludge Blanket (UASB) reactors as the primary (anaerobic) treatment stage. This was followed by a downflow hanging non-woven fabric (DHNW) reactor for secondary (aerobic) treatment, and an Anaerobic Baffled Reactor (ABR) for the tertiary treatment phase. The compact treatment unit measures 2.0 m in length, 2.0 m in width, and 1.0 m in depth. The UASB reactors themselves have dimensions of 2.0 m in length, 1.25 m in width, and 1.0 m in depth, with a hydraulic retention time maintained at 5 h throughout the study period while; the flow rate was 0.5 m³/h. During the course of our investigation, the system underwent a modification, integrating a small unit with a newly added upper compartment. This compartment situated centrally within the modified reactor—referred to as the “piffled” reactor—houses the chlorine used for disinfecting the final effluent. Chlorine dosing was carefully managed to achieve a residual concentration of 0.5 mg/L, with a contact time of 30 min. A regulated dropper connects the chlorine bottle to the piffled reactor as shown in Fig. 1 and

its working conditions are documented in Additional file 1: Table S1 [27]. The operational parameters of the integrated system remained consistent from January to December 2021. Throughout this period, a total of 40 batches of samples were collected from January to December 2021 at various stages within the treatment system. Sample volumes of 1 L and 2 L were collected for microeukaryotic community and physicochemical analysis, respectively. One sample (three replicates) from each stage was processed for the analysis of microeukaryotic communities.

Physicochemical characterization

Physicochemical characterization of influent wastewater and different treatment stages were carried out according to the American Public Health Association [28]. Characterizations include chemical oxygen demand (COD), biochemical oxygen demand (BOD), total suspended solids (TSS), phosphates (TP), nitrates–nitrogen (NO₃-N), nitrites–nitrogen (NO₂-N), total Kjeldahl nitrogen (TKN), and ammonia–nitrogen (NH₄-N). Temperature, dissolved oxygen (DO), and DO saturation were determined in wastewater samples in situ using an AD 360 DO meter (Adwa Instruments, Inc., Europe). The pH was measured using a bench pH meter Thermo Scientific model Orion Start AIII.

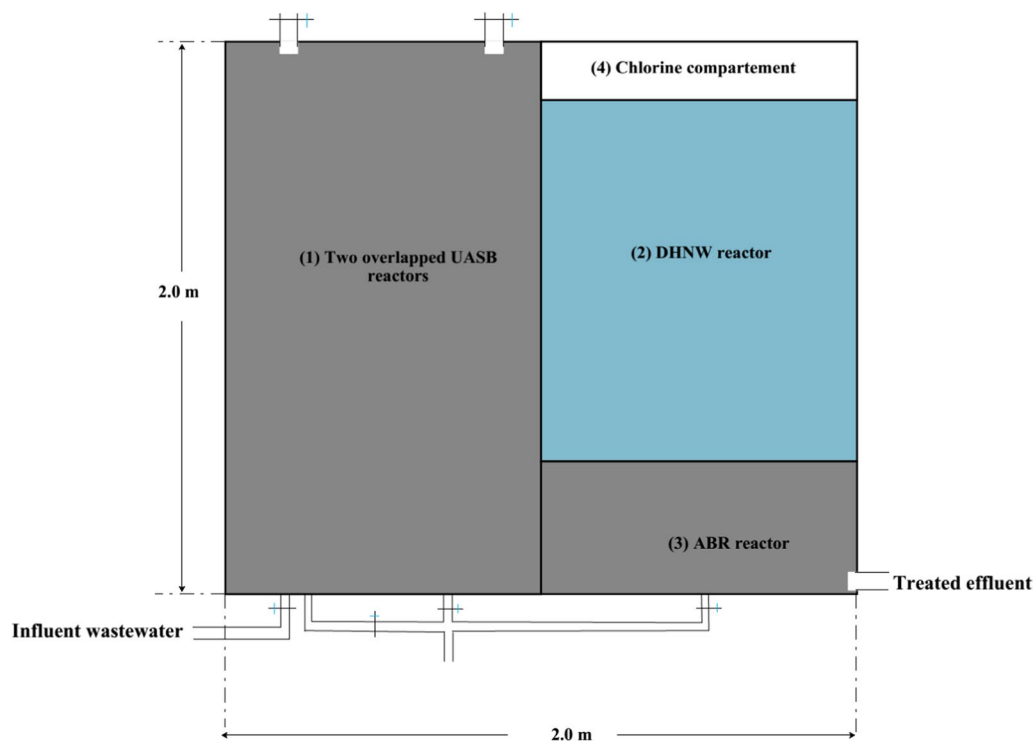


Fig. 1 Schematic diagram of the units for 100 persons where: **1** The two overlapped USAB reactors (Anaerobic stage), **2** The DHNW reactor (aerobic stage), **3** The ABR reactor, and **4** The chlorine compartment

18S rRNA high-throughput amplicon sequencing analysis

Wastewater samples (200 mL influent and 300–500 mL of other treatment stages) were filtered through a 0.2 μm -pore polycarbonate membrane (47 mm in diameter, Millipore, Billerica, MA, USA). The filtered membranes were stored at $-20\text{ }^{\circ}\text{C}$ until DNA extraction. Three replicates of each sample were mixed well before DNA extraction. DNA was directly extracted from filter membranes using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, USA). The hypervariable V4 region of eukaryotic 18S rRNA genes was amplified by using A-528F (5'-GCGGTAATTCAGCTCCA A-3') and B-706R (5'-AATCCRAGAATTCACCTCT-3') primer pair [29]. The PCR amplification cycles consisted of initial denaturation at $95\text{ }^{\circ}\text{C}$ for 5 min, followed by 25 cycles of $95\text{ }^{\circ}\text{C}$ for 30 s, $50\text{ }^{\circ}\text{C}$ for 30 s, and $72\text{ }^{\circ}\text{C}$ for 60 s, and a final extension at $72\text{ }^{\circ}\text{C}$ for 10 min. The purified PCR products were pooled in equal quantities and then sequenced on an Illumina platform (Illumina Inc., San Diego, CA, USA).

Sequence analysis

The raw paired-end reads underwent denoising and assembly using DADA2 v1.16.0 (<https://benjjneb.github.io/dada2/tutorial.html>) [30]. Amplicon sequence variants (ASVs) were generated at 100% sequence identity from the high-quality reads. The taxonomic classification of 18S rRNA reads was conducted using the RDP classifier and Protist Ribosomal Reference (PR2) database (version:

4.7.2) [31]. We randomly used 64,000 sequences per sample to normalize sequencing effects across the samples. The normalized final data sets retained 2681 microeukaryotic ASVs.

Statistical analysis

Principal component analysis (PCA) was employed to discern patterns within the physicochemical parameters, while non-metric multidimensional scaling (nMDS) facilitated the characterization of microeukaryotic community structures. Redundancy analysis (RDA) was instrumental in exploring the associations between microeukaryotic communities (response group) and physicochemical parameters (explanatory group). To assess the presence of statistically significant variations in physicochemical parameters across different treatment stages, permutational multivariate analysis of variance (PERMANOVA) and analysis of similarity (ANOSIM) tests were applied. Statistical analyses and visualization were performed R v4.0.2 (<http://www.R-project.org/>) and PRIMER v7.0.21.

Results and discussion

Performance of the compact unit

The PCA results revealed significant shifts in physicochemical parameters across different stages of treatment, as illustrated in Fig. 2. These findings were further corroborated by both PERMANOVA and ANOSIM analyses, with details presented in Table 1.

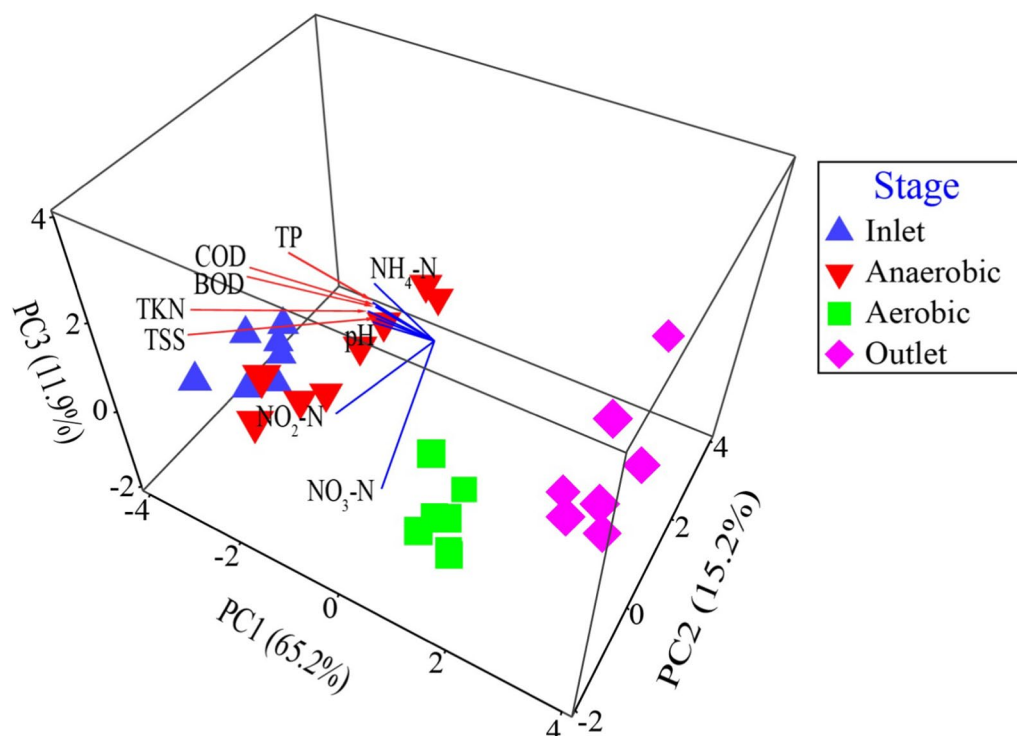


Fig. 2 PCA ordination for mapping the environmental factors in different stages of the treatment unit

Table 1 PERMANOVA and ANOSIM for significance test of wastewater characteristics in different treatment stages of the treatment unit

Groups	PERMANOVA		ANOSIM	
	t	P	R	P
Inlet versus anaerobic	3.45	0.0001	0.70	0.0002
Inlet versus aerobic	7.43	0.0003	1	0.0001
Inlet versus outlet	9.86	0.0001	1	0.0001
Anaerobic versus aerobic	4.11	0.0001	0.81	0.0001
Anaerobic versus outlet	5.79	0.0001	1	0.0001
Aerobic versus outlet	4.57	0.0001	0.84	0.0001

Pronounced differences were observed in multiple pairwise comparisons, including inlet versus outlet ($P=0.0001$), anaerobic versus aerobic ($P=0.0001$), anaerobic versus outlet ($P=0.0001$), and aerobic versus outlet ($P=0.0001$). The first three principal components, PC1, PC2, and PC3, explained 65.2%, 15.2%, and 11.9% of the total variance, cumulatively accounting for 92.4% of the total observed variability. The PCA vectors indicated that nutrients (e.g., $\text{NH}_4\text{-N}$, TP, and TKN) and organic pollution parameters (e.g., BOD and COD) were associated with sewage samples rather than the aerobic and outlet samples, suggesting that the integrated wastewater treatment system was working effectively.

The overlapped UASB reactors

The overlapped UASB reactors were used to remove the TSS efficiently. The pH value of the influent wastewater slightly decreased from 7.5 to 7.2. The influent COD_{tot}, BOD and TSS were reduced by 63.4%, 66.3%, and 70%, to be 141.3, 99.7, and 41.8 mg/l, respectively (Table 2). The decrease in the pH value and the reduction in COD, BOD, and TSS levels attributed to the degradation of organic loads in the anaerobic environment and production of fatty acids (acidogenesis and methanogenesis processes) [10, 32–35]. The FC count was reduced from 1.0×10^7 to 3.9×10^5 (two log units). The reduction in the TSS level leads to the reduction in the FC count in the primary treatment step [36].

The DHNW reactor

Further improvement of the overlapped UASB effluent was carried out using the DHNW reactor. Table 2 shows the performance of the DHNW reactor for COD, BOD, and TSS. The removal efficiencies of COD, BOD, and TSS were 57.6%, 71.7%, and 62.4% with residual concentration

of 60, 29, and 15.6 mg/l, respectively. The DHNW reactors can achieve COD, BOD and TSS removal beside nitrification/denitrification, the biological oxidation of ammonia to nitrite and nitrate [10, 11, 37]. The DHNW reactors are, therefore, worth consideration for onsite treatment. The packing material used in this study provide a high ratio (50%) [10, 11]. About 71.7% and 62.4% removal of BOD and ammonia were removed by the DHNW.

The concentrations of nutrients (TKN, ammonia, and TP) in raw wastewater as well as the treated effluents are presented in Table 2. The residual concentrations of TKN, ammonia, and TP were 16.3, 14.2, and 4.0 mg/l with removal percentage of 52.5%, 51.7%, and 20.5%, respectively.

The performance of the ABR reactor

The ABR provides the opportunity for settling of any residual particulate matters. Consequently, polishing of the DHNW effluent was achieved [38]. The packing material has a well-developed porous structure, where nitrifiers proliferate and contribute to nitrification. While, in deep zoon in the DHNW, denitrification process can take place. The pH value of the overlapped UASB reactors was reduced from 7.5 to 7.2 and then increased to 7.3 in the DHNW and decreased again to 7.2 in the ABR final treated effluent (Table 2). This is an indicator of nitrification changes the pH value as 2 mol of H^+ are released per mole of NH_4^+ oxidized to nitrite, and the decline of pH over time can be interpreted as time to build up the nitrifying community [39].

The integrated system performed overall removal rates of 90.8%, 93.4%, 95.7%, 56.8%, 49.7%, 32.57%, 28.75%, and 43.6% for COD, BOD, TSS, TKN, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, and TP, respectively. Such performance aligns with findings from other studies employing various treatment configurations for municipal wastewater. For instance, a UASB/DHS (downflow hanging sponge) and DHNW system demonstrated more than 90% removal of COD and BOD, and over 80% removal of total nitrogen (TN) [40]. An integrated system comprising a facultative pond (FP), a high-rate algal pond (HRAP), and a rock filter (RF) reported reductions of 92.09% for TSS, 89.39% for $\text{NH}_4\text{-N}$, 89.26% for BOD, and 82.24% for COD [23]. Centralized systems are commonly employed in urban wastewater management, wherein wastewater is transported through a collection network to a centralized WWTPs. In densely populated urban settings, such centralized WWTPs offer economic viability due to their large-scale processing capabilities. However, onsite wastewater treatment systems (OWTS) emerge as

Table 2 The main characteristics of raw wastewater and the treated effluents

Parameter	Raw wastewater	UASB Effluent	%R	DHNW	%R	ABR	%R	T%R
pH	7.5	7.2		7.3		7.2		
COD	386.4	141.3	63.4	60.0	57.6	30.9	48.7	92.0
BOD	296.3	99.7	66.3	29	71.7	13.8	49.8	95.4
TSS	139.8	41.8	70.0	15.6	62.4	4.2	74.1	97.0
TKN	38.8	34.5	11.0	16.3	52.5	13.0	19.6	66.6
Ammonia	31.7	29.3	7.3	14.2	51.7	11.0	19.5	65.3
Nitrates	0.058	0.021	64.2	0.114	- 535.2	0.034	69.8	41.4
Nitrites	0.005	0.003	39.8	0.003	- 8.4	0.004	- 34.1	17.4
TP	6.2	5.0	19.7	4.0	20.5	3.3	17.3	47.2
FC	1.0×10^7	3.9×10^5	96.1	7.2×10^4	81.5	1.5×10^3	79.9	99.99

suitable alternatives for rural regions, where deploying large-scale centralized systems faces financial and managerial challenges due to lower population densities and the spatial distribution of communities [41, 42].

Dynamics of microeukaryotic communities in the stages of an integrated wastewater treatment system

In this study, the nMDS ordination method showed a clear separation between microeukarotic communities in the integrated wastewater treatment unit stages except between anaerobic and aerobic (Fig. 3). This observation

is potentially attributed to the design of the Upflow Anaerobic Sludge Blanket (UASB) reactor, where the anaerobic stage precedes the aerobic stage, both characterized by brief detention times, possibly leading to the observed similarity in microeukaryotic communities between these stages. Bacteria and other eukaryotes play a vital role in complex microbial consortia, which are a part of biological wastewater treatment. The efficiency of the process depends on the microbial consortia’s capacity to adapt to the characteristics and conditions of the wastewater [43]. As a result, it is possible to assume that

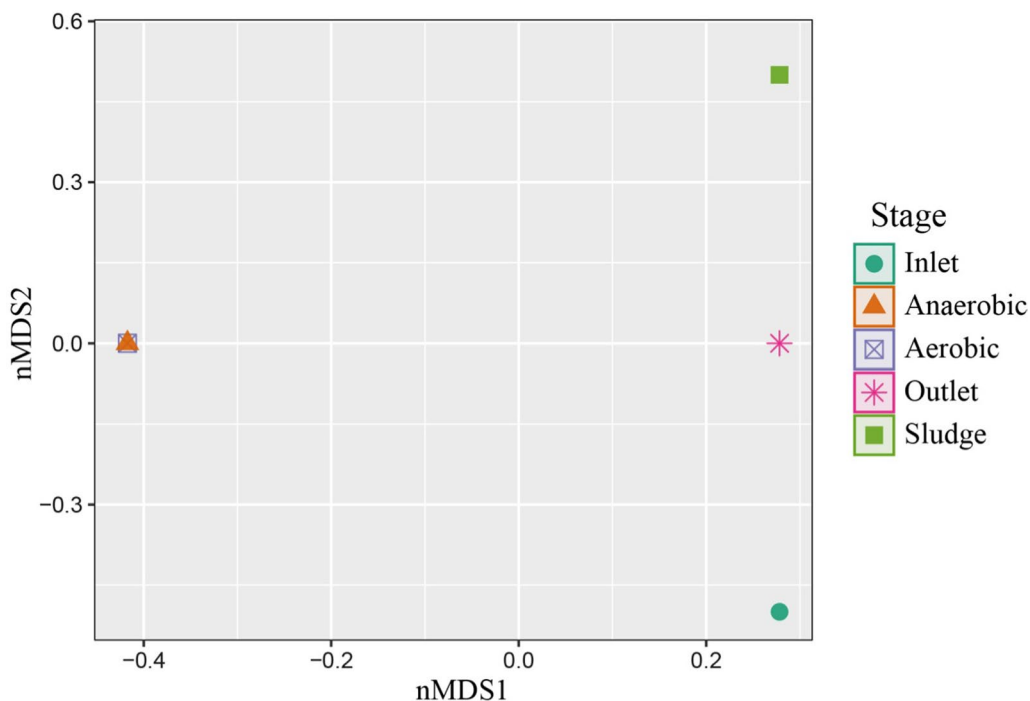


Fig. 3 nMDS ordination plot for a microeukaryotic community composition in the stages of the treatment unit

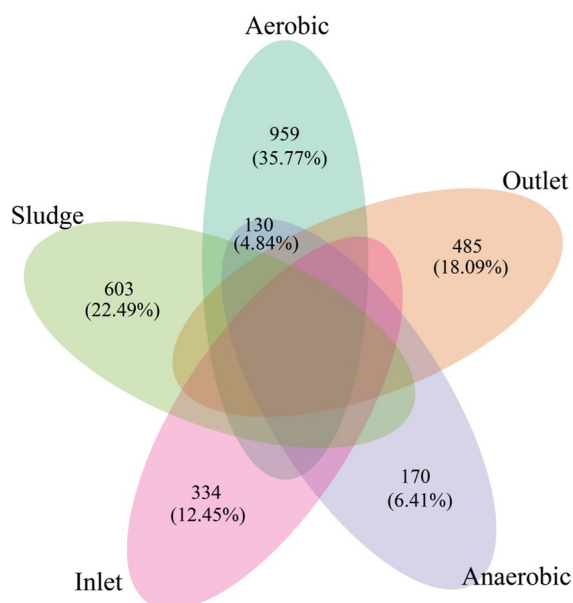


Fig. 4 Venn diagram showing the unique and shared ASVs of the microeukaryotic communities across different treatment stages

understanding microbial communities’ dynamics and behavior would help improve biological wastewater treatment processes.

Our analysis identified 2681 microeukaryotic ASVs, with their distribution scrutinized across different stages of the treatment system. The results showed that the majority of the microeukaryotic species were unique to each stage, with the highest number of unique ASVs observed in the aerobic stage (35.77%). On the other hand, only a small fraction of ASVs (4.84%) were shared between the aerobic and anaerobic stages. These findings suggest that each stage of the treatment system harbors a distinct microeukaryotic community, which may play a unique role in the overall wastewater treatment process (Fig. 4). The comparison of alpha diversity indices between different stages of the wastewater system reveals that the aerobic stage exhibits the highest alpha diversity values (Fig. 5). This is likely due to the presence of high levels of oxygen and nutrients, which can support the growth of a diverse range of aerobic microorganisms. In contrast, the anaerobic stage displays the lowest alpha

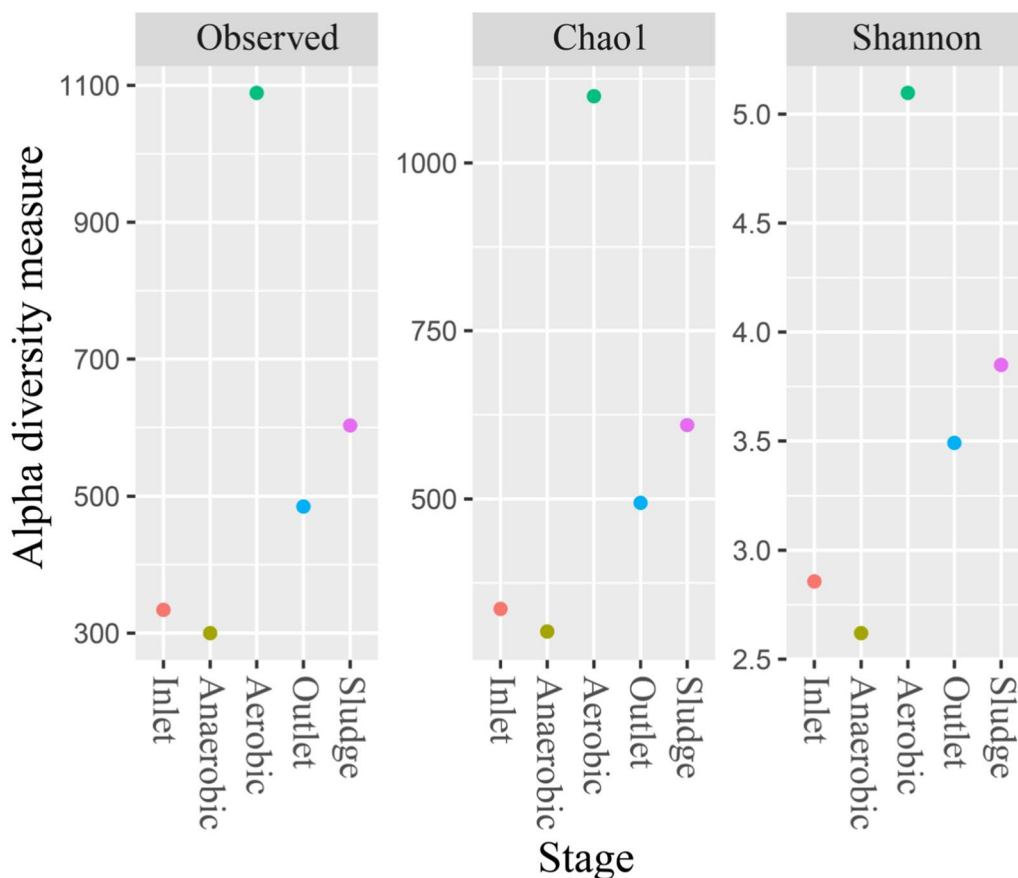


Fig. 5 Alpha diversity indices (Observed, Chao1, and Shannon) for microeukaryotes in different stages in the treatment unit

diversity values (Fig. 5), which is expected as anaerobic conditions can limit the growth of many aerobic microorganisms, resulting in lower diversity.

Microeukaryotes, including protozoa and fungi, play pivotal roles in wastewater treatment systems. Their activities directly influence the removal of organic and inorganic pollutants and the stability of microbial consortia, which are crucial for process performance. For instance, ciliates, a prominent group of protozoa observed in our study, are known to enhance the removal of suspended solids and pathogens, thereby improving water quality. Their predation on bacteria leads to a more efficient reduction of BOD and TSS, directly linking their presence to the treatment process's nature [20, 44]. Metazoa constitute a large proportion of microeukaryotes in the sludge of the aerobic stage and are typically associated with the treatment of wastewater containing high concentrations of organic matter [43]. For instance, Rotifera as a microscopic metazoan is from the most abundant taxa in the aerobic stage, comprising nearly 15% of the community. Rotifera plays a role in the breakdown of organic matter in wastewater by grazing on bacteria, thus controlling bacterial populations and aiding in the sedimentation process [45].

Figure 6 reveals interesting patterns in the relative abundance of different taxa across different treatment stages. For example, Opisthokonta (70.24–93.85%),

which includes fungi and animals, was the most abundant taxon in all treatment stages except for the effluent stage (28.25%), where Alveolata (42.27%), a group of unicellular organisms dominated. In addition, the aerobic (11.68%) and effluent (26.75%) stages had the highest relative abundance of Stramenopiles, a group of algae and diatoms, likely due to the high levels of oxygen and nutrients in this stage. Conversely, the anaerobic stage had a much lower relative abundance of Stramenopiles (<1%). Ochrophyta (26.42%), comprises a diverse group of algae, among the most abundant microeukaryotic taxa, particularly in the outlet stage (Additional file 1: Figure S1). These algae are known for their capability to remove nutrients, such as nitrogen and phosphorus, from wastewater through biological processes [46]. The presence of these diverse algal species in outlet and aerobic stages enhances wastewater treatment by increasing oxygen through photosynthesis. They are essential for the effective removal of pollutants from wastewater [22, 47]. Ciliophora, which are most abundant in the effluent (42.09%) and sludge (17.11%) (Additional file 1: Fig. S1), are known to play a significant role in the removal of suspended solids [48]. Oligohymenophorea is most abundant in the outlet stage, comprising over 34% of the community (Additional file 1: Fig. S2). This group includes a variety of ciliated protozoa, many of which are important indicators of water quality. Some species are known to be

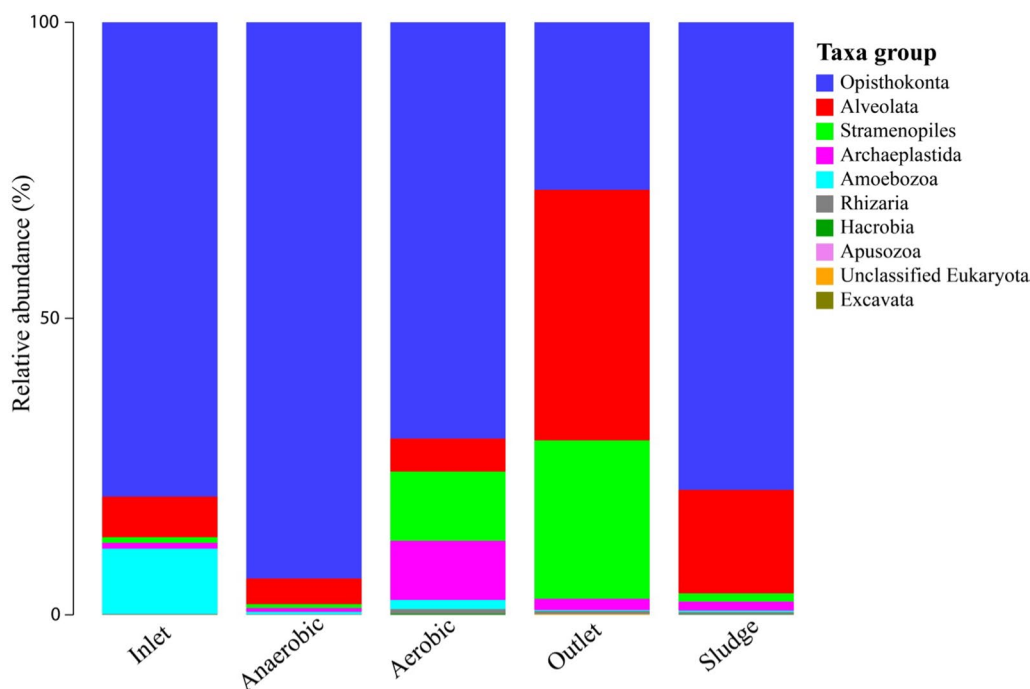


Fig. 6 bar plot of microeukaryotic community composition and their relative abundances. The top taxa groups (Rank 1) in all the treatment stages are shown. The remaining taxa groups are shown in the Additional file 1: Figs. S1, S2, S3

tolerant of pollutants and other environmental stressors, while others are highly sensitive and can only survive in pristine conditions [49].

Fungi, which represented the largest proportion of microeukaryotes in the anaerobic (93.12%) and inlet (78.40%) of the integrated wastewater treatment system (Additional file 1: Figure S1), are known for their ability to degrade a wide range of organic compounds in wastewater treatment systems [42]. In addition, some fungal species have been shown to remove heavy metals from wastewater through mechanisms such as biosorption, precipitation, and volatilization [50]. Cryptomycota was from the most abundant taxa in all stages (Additional file 1: Fig. S2). Cryptomycota is a recently discovered fungal phylum, and little is known about its ecology or physiology [51]. It has been found in a variety of environments, including soil, freshwater, and marine sediments, but its role in these ecosystems is still unclear. Chytridiomycota, particularly prevalent in the inlet stage and constituting over 32% of the community (Additional file 1: Fig. S2), are aquatic fungi known for their significant contributions to nutrient cycling and organic matter decomposition in freshwater environments [52]. Additional file 1: Figure S3 presents a detailed overview of various microeukaryotic taxa identified in this study, notably Cryptomycotina and Chytridiomycotina, previously documented in wastewater treatment research [53]. These taxa are instrumental in the breakdown of complex organic compounds, a critical process for reducing COD and enhancing the overall efficiency of organic matter removal from wastewater [6, 54].

Our RDA reveals a significant positive correlation between Cryptomycotina and key performance indicators such as COD and BOD (Fig. 7), underscoring the pivotal role microeukaryotic communities in enhancing

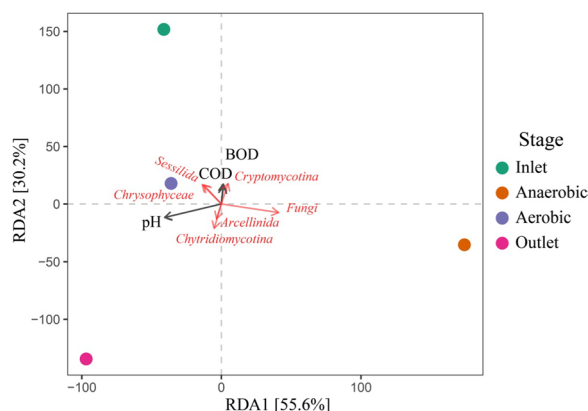


Fig. 7 RDA of explained the relationship between microeukaryotic communities and environmental factors in an integrated wastewater treatment pilot-scale system

wastewater treatment efficiency through the bioconversion of organic pollutants. This finding supports the broader scientific consensus on the utility of various microorganisms, including bacteria, fungi, and protozoa, as bioindicators of wastewater treatment efficacy. Research highlights the pivotal importance of microbial community dynamics, evidenced by alterations in their structure and composition, in providing essential insights into the operational effectiveness of WWTPs [55, 56]. The selective enrichment or depletion of microbial groups, prompted by the organic pollutant degradation process, results in shifts within the community structure, serving as an indicator of the treatment process's effectiveness [55, 57, 58]. Cryptomycotina, as observed in the current study, could serve as a bioindicator for treatment performance in the integrated treatment system. However, further studies are required to confirm this association and determine the usefulness of Cryptomycotina as a reliable and sensitive bioindicator of wastewater treatment performance.

Protozoa taxa across wastewater treatment stages: implications for public health and treatment efficacy

The heatmap provided the frequency of different species of Apicomplexa in different stages of the wastewater treatment process (Fig. 8). Apicomplexa is a diverse phylum of parasitic protists that includes many pathogenic species known to cause disease in animals and humans. One notable observation highlighted in our heatmap analysis is the detection of *Cryptosporidium* sp. genetic material in the inlet stage of our wastewater treatment system (Fig. 8). This discovery is particularly concerning as *Cryptosporidium* sp. is a well-documented waterborne pathogen responsible for causing gastrointestinal illnesses in humans. The presence of this pathogen's genetic material at the treatment plant's entry point suggests potential contamination of the incoming wastewater with *Cryptosporidium* genes, signaling the urgent need for robust screening and treatment measures. While the identification of genetic material indicates the pathogen's presence in the system, it's crucial to distinguish that this does not automatically imply the viability or infectivity of *Cryptosporidium* forms. Given the organism's known resilience to conventional disinfection methods and the challenges inherent in its removal from wastewater, this observation underscores the critical public health implications linked to *Cryptosporidium*.

The absence of *Cryptosporidium* in the outlet samples of the treatment system (Fig. 8) could potentially be attributed to the predominance of Ciliophora (e.g., *Vorticella*) (Additional file 1: Figs. S1 and S3), which are known to be effective in controlling such parasitic protists. This hypothesis is consistent with the findings from

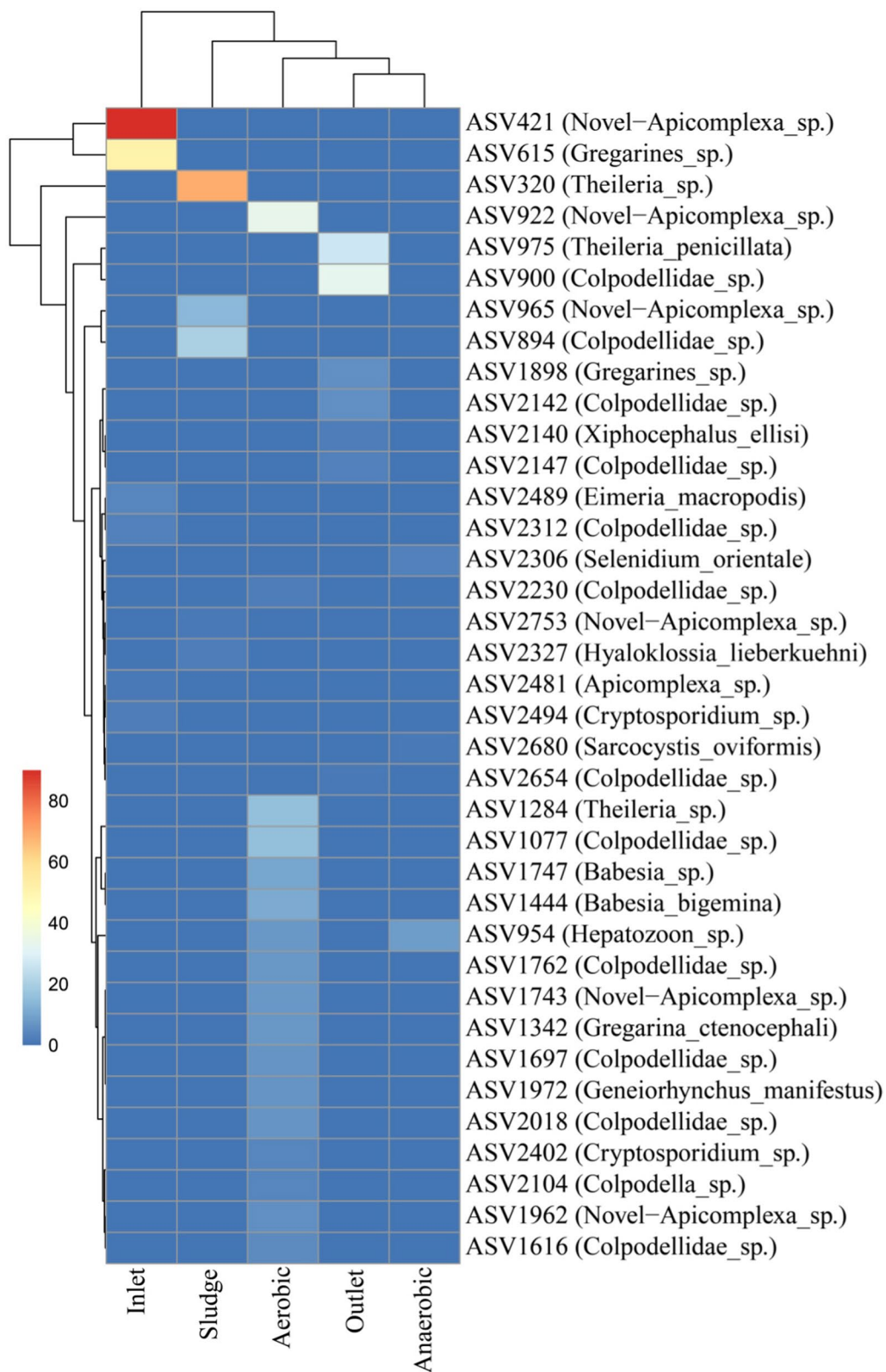


Fig. 8 Heatmap showing the parasitic Apicomplexa species in the treatment unit stages

network analysis, demonstrating associations between parasitic protists like *Entamoeba*, and *Giardia*, and their probable predators, specifically ciliates and rotifers [16]. These associations might be indicative of predation dynamics within the system, suggesting that these predators play a crucial role in mitigating the presence of harmful parasites in the treated water [16]. This underscores the importance of effective treatment processes to remove or inactivate pathogens before the treated water is discharged into the environment. Most studies on the prevalence of *Cryptosporidium* oocysts in wastewater have reported a prevalence of 6–100%. Activated sludge technology has been found to be ineffective for the removal of *Cryptosporidium* oocysts. Stabilization ponds and constructed wetlands are efficient for the reduction of *Cryptosporidium* from wastewater, especially when the retention time is longer than 20 days at suitable sunlight and temperature. High-rate filtration and chlorine disinfection are inefficient for the reduction of *Cryptosporidium* from effluents, whereas ultrafiltration and UV irradiation were found to be very efficient for the reduction of *Cryptosporidium* oocysts. Adequate tertiary treatment may result in high quality effluent with low risk of *Cryptosporidium* for unrestricted irrigation and other non-potable applications [59].

Additional notable genera of Apicomplexa, including *Babesia*, *Eimeria*, and *Theileria*, have been identified (Fig. 8). This observation corroborates the findings presented in the studies by Gad et al. (2023) and Ariyadasa et al. (2023), further substantiating the prevalence of these genera in wastewater. The presence of enteric parasites from the Apicomplexa group in wastewater can be attributed to factors such as population density, individual water usage, and the persistence of the excreted stage (such as cysts or oocysts) in the wastewater environment, the presence of domestic animals (e.g., cats) in the vicinity [60, 61]. Focusing on microbiological contaminants, it has been described that wastewater effluents may contain a significant concentration of several pathogens, including bacteria, viruses, protozoa, and helminths [57–59]. Among them, viruses and protozoa tend to be highly resistant to conventional disinfection treatments employed in WWTPs [62]. These microorganisms are responsible for waterborne diseases, associated to the fecal–oral route, mainly causing gastroenteritis alongside other pathologies depending on the etiological agent [63, 64]. Therefore, if WWTPs are not efficient in removing these contaminants, water reuse for irrigation may compromise water bodies and human health [65]. To address these concerns, we advocate for the adoption of culture-based methods or viability assays in future

studies to assess the actual infectivity of these microorganisms post-disinfection. Techniques such as propidium monoazide–quantitative PCR (PMA–qPCR) and reverse transcription quantitative PCR could differentiate between live and dead cells, providing a more accurate assessment of the disinfection stage’s effectiveness [66]. Another noteworthy finding is the high frequency of several unclassified Apicomplexa sp. (e.g., Novel-Apicomplexa-Class-sp) particularly in the inlet and aerobic stages (Fig. 8). Further investigation is warranted to determine the identity and potential pathogenicity of these species and potential risks associated with their presence in wastewater treatment systems and the effectiveness of treatment processes in removing them. The use of molecular techniques, such as shotgun metagenome, may provide more detailed information on the diversity and abundance of these parasites in wastewater.

The provided heatmap displays the abundance of different types of amoebae in different treatment stages of the integrated system (Fig. 9). Overall, *Vermamoeba vermiformis* was the most dominant type of amoeba found in both aerobic and anaerobic treatment stages. *V. vermiformis* was known to be a common amoeba found in various water sources, including wastewater treatment plants [67, 68]. It is a niche for various amoeba-resisting microorganisms such as bacteria and giant viruses, indicating its role in harboring and potentially aiding the survival and growth of pathogenic organisms [69]. The association between *V. vermiformis* and pathogenic bacteria like *Legionella pneumophila*, a bacterium known to cause Legionnaires’ disease. This association is especially notable in hospital water networks, which could pose a risk for nosocomial infections [70]. It has also been suggested that *V. vermiformis* can be used as a bioindicator of water quality in wastewater treatment plants [71]. The presence of this species in the outlet stage suggests that the treatment process was not effective in completely removing all microorganisms from the wastewater. Other amoebae identified in various stages of the integrated treatment system include *Acanthamoeba castellanii*, *Acanthamoeba jacobsi*, and *Arcella vulgaris*, as depicted in Fig. 9. These species have also been identified in other wastewater treatment plants [72]. *Acanthamoeba* species (e.g., *A. castellanii*) are known to cause severe keratitis and granulomatous amoebic encephalitis (GAE). They are found in various environments like lakes, swimming pools, tap water, heating, and air conditioning units, among others. The trophozoites and cysts of these species can enter the body through various means, including the eye, nasal passages, or broken skin, leading to severe infections in both healthy and immunocompromised individuals [73].

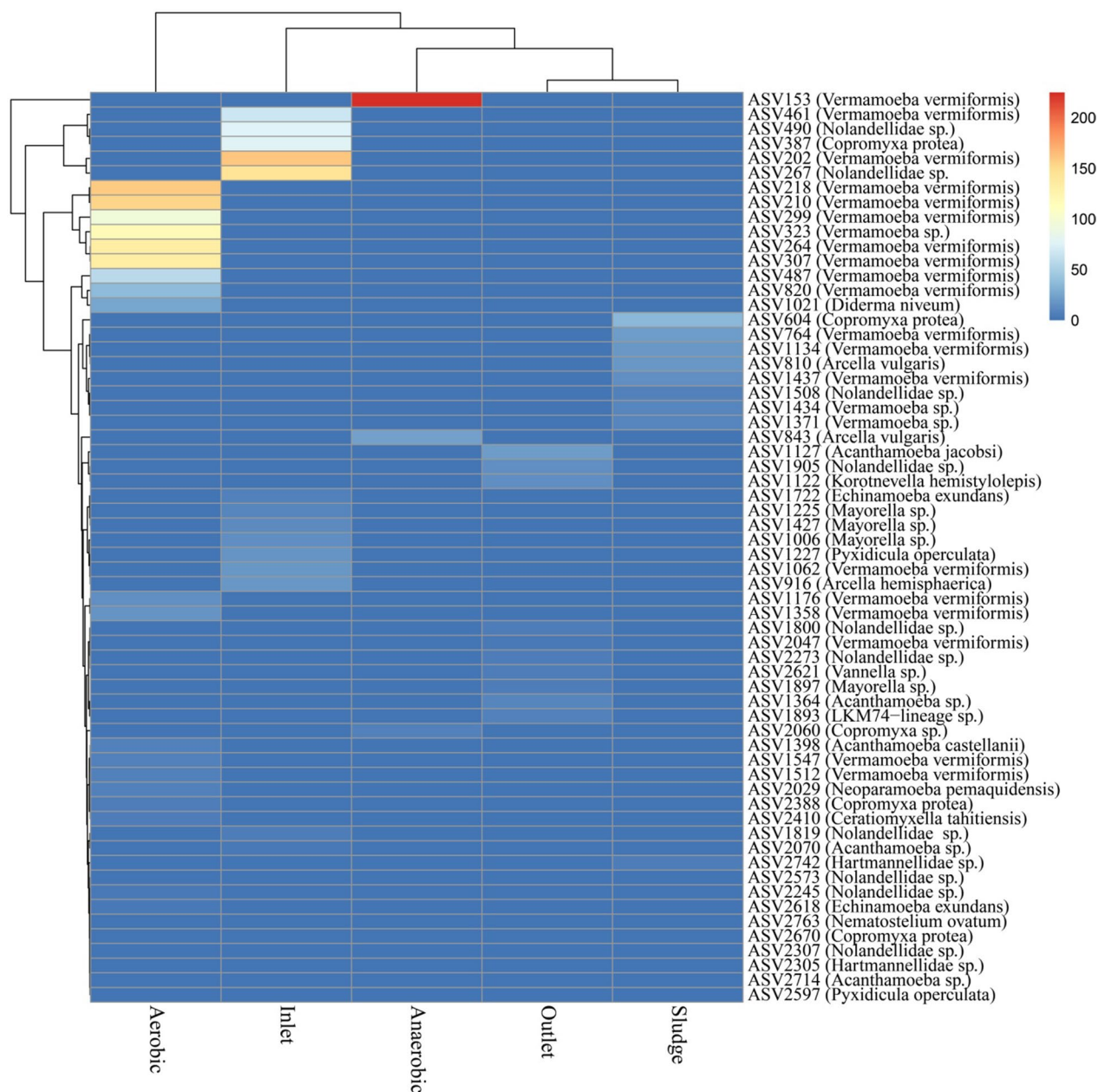


Fig. 9 Heatmap showing the lobose amoebae species in the treatment unit stages

Limitations of the study

One of the limitations identified in our study relates to the comprehensive assessment of wastewater disinfection efficiency and its impact on the presence and potential harmful effects of pathogenic microeukaryotic species. While our research highlighted the presence of certain protozoan species, such as *Acanthamoeba castellanii*, *V. vermiformis*, and *Cryptosporidium* sp., across various stages of the treatment process, we acknowledge that a more detailed examination of

the disinfection stage’s effectiveness is crucial for fully understanding the public health implications. Specifically, our study focused on the detection of microeukaryotic DNA but did not extend to evaluating the viability or infectivity of these organisms following the disinfection process. The reliance on molecular detection methods, without complementary assays to distinguish between live and dead cells, presents a gap in our assessment of the actual risk posed by these microorganisms. We recommend that future research should

incorporate both culture-based methods and viability assays, such as propidium monoazide–quantitative PCR (PMA–qPCR) and reverse transcription quantitative PCR to assess the viability of these microorganisms [74] and to provide a more accurate evaluation of disinfection effectiveness. This would offer valuable insights into the capability of the disinfection process to inactivate or remove potentially harmful microeukaryotic species, thereby enhancing our understanding of the implications for public health and the overall efficacy of wastewater treatment systems.

Conclusion

This study elucidates the dynamic interplay between wastewater treatment stages and microeukaryotic community composition, emphasizing the transformative impact of treatment processes on microbial ecosystems. Our findings reveal how distinct treatment stages uniquely influence the structure and function of microeukaryotic communities, underscoring the critical role of tailored treatment designs in fostering microbial diversity and operational efficacy. The identification of significant taxa, including Cryptomycotina (fungal group), in relation to their roles in enhancing treatment efficiency, presents a groundbreaking perspective on the utilization of microeukaryotic communities for bioaugmentation and process optimization. Our findings underscore the potential of these communities as sensitive bioindicators for the real-time assessment and management of wastewater treatment systems, offering a promising avenue for future research and application. Moreover, the detection of pathogenic/potentially pathogenic microorganisms such as *Cryptosporidium* sp. and *V. vermiformis* in the treatment process underscores the importance of integrating microeukaryotic community analysis into public health and environmental safety protocols. This aspect of our research highlights the crucial role of advanced microbial analysis in ensuring the efficacy of wastewater treatment processes in pathogen removal and environmental protection.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12302-024-00907-8>.

Additional file 1: Table S1. Operating conditions of the compact unit. **Figure S1.** Bar plot of microeukaryotic community composition and their relative abundances. Only the top 10 taxa groups (Rank 2) in all the treatment stages are shown. Others refer to the less contributed taxa groups. **Figure S2.** Alluvial bar plot of microeukaryotic community composition and their relative abundances. Only the top 10 taxa groups (Rank 3) in all the treatment stages. **Figure S3.** Heatmap of microeukaryotic community composition at rank 6 level and their relative abundances. The top 20 taxa groups (Rank 6) in all the treatment stages are shown. The presence of higher-level taxa in some cases refers to no classification at the genus level.

Author contributions

Mahmoud Gad: methodology, formal analysis, data curation, writing—original draft preparation, visualization, resources, funding acquisition. Mohammed Yosri: methodology, software, writing—review and editing. Mariam E. Fawzy: investigation, methodology, data curation, review and editing. Reda M. Moghazy: data curation, review and editing. Esmat M. S. Elfeky: methodology, software, review and editing. Mohamed A. Marouf: investigation, methodology, software, review and editing. Mohamad A. El-Khateeb: Resources, conceptualization, methodology, validation, supervision, project administration, review and editing.

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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 PRJNA953044.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest, financial or otherwise.

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