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Colonization Dynamics and Diversity of Holomycota in a Newly Built Urban Wastewater Treatment Plant: An Eight-Month Time-Series Analysis

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Article

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Abstract

High-throughput sequencing of eukaryotic 18S rDNA genes was conducted on 23 time-series samples collected over eight months from a newly constructed urban Seine-Morée-wastewater treatment plant (WWTP), generating 521,031 reads. Among these reads, 74,432 (14.2%) are affiliated with *Holomycota*, spanning nine phylogenetic groups. *Cryptomycota* dominated the fungal community (76.01%), followed by *Chytridiomycota* (11.66%), *Ascomycota* (5.65%), *Zoopagomycota* (3.02%), *Basidiomycota* (1.37%), and *Blastocladiomycota* (1.36%), while *Mucoromycota*, *Nucleariidae*/*Fonticula*, and *Microsporidia* each represented < 1% of total *Holomycota* reads. The fungal community was highly uneven, with 50 abundant operational taxonomic units (OTUs) (≥ 0.1% relative abundance) constituting 96.5% of all sequences, while the vast majority of taxa (298 OTUs) were rare (< 0.1%), forming a "rare biosphere" that contributed only by 3.5% of the total reads. Among the 50 abundant taxa, only fifteen highly dominant operational taxonomic units (OTUs), each with a relative abundance ≥ 1%, overwhelmingly structured the fungal community, and accounted for 85% of the total fungal reads. The cultivable fraction (82 OTUs) made up just 8.5% of fungal reads, with *Synchytrium cupulatum* (6%) emerging as the most prevalent species. Additionally, nineteen species affiliated with the human gut mycobiota (e.g., *Geotrichum, Candida, Saccharomyces, Penicillium*) accounted for 3.62% of fungal reads. Among the 348 identified OTUs, at least 32 are affiliated with taxa exhibiting potential pathogenicity toward humans, plants, or animals, highlighting the importance of monitoring fungal communities within wastewater ecosystems under the One-Global-Health framework. From an ecological perspective, early-stage fungal colonization may contribute to the stabilization and structuring of microbial communities within the WWTP. Hence, these communities contribute to ecosystem construction, stabilization, and functioning. Our findings underscore the predominant role of fungal “dark matter” (e.g., *Cryptomycota*) and reveal the substantial potential for novel taxonomic and functional discoveries within the wastewater mycobiome.

Originality-Significance Statement

This study is unique in its sequential monitoring of microbial colonization in a newly constructed wastewater treatment plant (WWTP). Unlike conventional approaches that introduce sludge from an existing WWTP for seeding, our research examines the natural colonization by microorganisms present in raw water. We employed high-throughput sequencing to provide a comprehensive view of the population structure, dynamics, and establishment kinetics of the wastewater microbiome over an 8-month period. In this study, we are focusing on the *Holomycota*, which encompasses *Fungi* (*Eumycota*), *Rozellomycota* (*Cryptomycota*), *Microsporidia*, and *Aphelida*. It contribute**s** to understanding eukaryotic evolution, particularly in the lineages affiliated with fungi and related groups. Such study is central to the One Health framework because it directly examines the interconnected health of humans, animals, and the environment through a single critical nexus: The urban wastewater. Wastewater represents a convergence point for human, animal (domestic, and wildlife), and environmental (soil and water) microbiomes and contaminants. Analysis of the mycobiome within this matrix provides a simultaneous and integrated snapshot of all three sectors. By characterizing fungal communities, the study functions as a predictive tool for zoonotic and environmental threats, enabling early detection of potential

pathogens and resistance traits that can circulate across ecosystems and populations, thereby supporting proactive risk mitigation. The results also have a direct policy relevance at key human-environment interfaces, particularly in practices such as wastewater reuse in agriculture or sludge application to soils, where management decisions directly affect food safety, ecosystem integrity, and public health. By relying on a unified wastewater-based epidemiology approach, this work demonstrates how integrated surveillance can generate actionable insights across One Health domains, effectively operationalizing the One Health concept rather than merely invoking it.

Introduction

On a global scale, the annual production of wastewater exceeds 330 km^3, encompassing sewage from households, hospitals, and chemical industries [1]. Wastewater treatment plants (WWTPs) play a vital role in ensuring environmental health and safety, as well as contributing to the attainment of sustainable urban development objectives. Approximately 60% of this wastewater undergoes treatment before being released into the environment, employing a range of biological processes [2]. The efficacy of these biological treatment processes is wholly dependent on the activity and diversity of microbial metabolism, adapted to the removal of pollutants [3]. Currently, most studies on microbial ecology focus on the taxonomic classification of microbial communities encompassing the three domains of life, *Archaea*, *Bacteria* and *Eukarya* [4,5]. Among the eukaryotic microorganisms found in WWTPs, the *Holomycota* represent a major monophyletic clade within the *Opisthokonta*, encompassing true fungi (*Fungi*), *Nucleariida*, *Rozellida (Cryptomycota)*, *Microsporidia*, and *Aphelida* [6]. *Holomycota* play key ecological roles in wastewater systems, contributing to the degradation of organic matter and promoting the stabilization of activated sludge [7]. Fungi play diverse ecological roles as saprophytes, parasites, mutualists, and symbionts, often adopting multiple trophic strategies. They are ubiquitous in the environment, with significant diversity found within urban wastewater treatment plants. Disease-causing fungi are a major threat to the health of humans, animals, and plants. This problem is getting worse, as many fungi are becoming drug-resistant and new, difficult-to-identify types are emerging. Making matters more complex, these pathogens can be found in wastewater treatment plants. When the treated water is used for farming or as a potential drinking water source, it can spread fungi and create new health risks [8]. To address this, we need to study profoundly the "wastewater mycobiota" all the fungi living in these systems. Understanding this fungal community is key to managing the spread of disease, an approach that aligns with the "One-Global-Health" view of a unified planetary health. Therefore, characterizing the wastewater mycobiota is critical, as these systems are hotspots for disseminating antibiotic-resistant fungi. A deeper understanding of these communities is key to developing targeted strategies for controlling phytopathogens. This, in turn, could reduce our reliance on agrochemicals, lower global infection rates, mitigate substantial crop losses and their economic impacts, and ultimately safeguard both public health and agricultural livelihoods. Reducing these losses is a key driver for achieving environmental protection and sustainable food systems that ensure adequate nourishment for all,

aligning with United Nations Sustainable Development Goal (SDG) target objectives, specifically aiming to "reduce food losses along production and supply chains, including post-harvest losses" by 2030 [9].

Previous studies have reported on the diversity and taxonomic characterization of fungal communities in WWTPs. However, these studies have several limitations, including: (i) for some studies, the use of culture-dependent methods, which significantly underestimate the diversity and richness of fungal microbiota. (ii) the inherent limitation of established WWTPs as sampling sites, where exogenous microbial influx complicates the resolution of the authentic resident mycobiota and, (iii) the focus on exploring fungal communities in only few samples from wastewater treatment plants. These samples were often collected at single time points or specific plant areas, without prior descriptions of fungal temporal dynamics and the mechanisms of ecosystem stability in relation to fungal diversity kinetics [10]

Our study aims to address these gaps by utilizing high-throughput sequencing technology to describe WWTP fungal diversity. Two key features define it: (i) the use of 23 time-series datasets to analyse fungal diversity and its temporal dynamics in a WWTP over eight months period. (ii) The collection of samples from a newly established WWTP that started without external seeding.

RESULTS

Physical and chemical characteristics of the WWTP. The physicochemical properties of the wastewater are summarized in Table 1. The influent wastewater pH ranged from 7.7 to 8.5. Both biological oxygen demand (BOD) and chemical oxygen demand (COD) were significantly reduced by the WWTP, with nearly 70% of BOD and COD removed by the 68th day, ultimately achieving a 98% reduction in COD by the end of the sampling period. The analysis of nitrogen evolution revealed three distinct stages. Initially, there was a high concentration of ammonia in the aerobic basin from the startup of the WWTP to day 40, a point at which NH_4 level falls rapidly ammonia levels gradually decreased, and nitrite began to appear. By day 133, complete oxidation of nitrites to nitrates was observed, and this nitrate concentration remained relatively stable until the end of the sampling period. Biological dephosphatation commenced on day 40 and was fully accomplished by day 140 (Fig. 1A).

Principal Component Analysis reduced five physicochemical parameters to two principal components explaining 97% of total variance. PC1 (60.9%) represents the primary nitrification gradient from ammonium to nitrate dominance over time. PC2 (36.9%) captures a transient nitrite peak during the intermediate transition phase (Fig. 1C). Hence, sample ordination revealed three distinct temporal phases: an initial ammonium-rich phase (days 18–40), a transitional nitrite accumulating phase (days 40–133), and a nitrate phase starting in parallel with the nitrite apparition and continuing until day 236 (Fig. 1A), with phosphorus fractions, following complementary patterns (Fig. 1B). The results demonstrate that the ecosysystem undergoes a predictable biogeochemical succession, consistent with recovery following a high-ammonium input event (Fig. 1A).

Hierarchically clustered heat map fingerprinting the top dominant 50 *Holomycota* OTUs across the 23 time-series samples illustrates strong temporal structuring of fungal OTUs in the wastewater samples, characterized by the dominance of a limited number of taxa and transient abundance peaks in response to changing environmental conditions, while the majority of OTUs remained consistently rare. Clustering of sampling days further demonstrated that microbial community composition evolved dynamically over time, with intermediate sampling periods exhibiting the highest abundance and co-occurrence of multiple OTUs (Fig. 1D). These synchronized peaks suggest that environmental drivers and metabolic products such as nitrogen and phosphorus availability, nitrite or nitrate production, or microbe-to-microbe interactions, may have shaped microbial dynamics. In contrast, a limited number of OTUs showed relatively stable abundances across the entire monitoring period. Overall, the heat map emphasizes the highly dynamic and non-linear nature of microbial temporal responses, reflecting ecological niche differentiation and succession processes within the studied aerobic basin. This analysis divided the time series samples into three distinct taxonomic groups, corresponding to three phases: Phase one (pink box, days 13 to 40), phase two (red box, days 68 and 82), phase three (green box, days 133 to 236) as depicted in Fig. 1D.

Overall molecular diversity analyses of fungal OTUs within the WWTP

Phyla abundances. Overall, 521,031 high-quality reads were derived from 18S rDNA sequences originating from 23 samples, averaging approximately 22,653 reads per sample. Within this pool of 521,031 18S rRNA gene sequences that underwent analysis, 74,432 were identified as fungi. Using a clustering approach based on 97% rDNA sequence similarity, we observed the presence of 1,423 OTUs affiliated with the eukaryotic domain, with 348 of these being classified as fungal OTUs (Table 2). Based on taxonomic affiliations using the Silva database-138 (https://www.arb-silva.de/), the analysis revealed that the 348 fungal OTUs were affiliated with nine distinct phyla. These phyla include *Cryptomycota, Chytridiomycota, Ascomycota, Zoopagomycota, Basidiomycota, Blastocladiomycota, Mucoromycota, Microsporidia*, and *the Nucleariidae* and *Fonticula group*. Notably, two prominent fungal phyla, *Cryptomycota* and *Chytridiomycota*, accounted for ~ 76% and 11.7% of the total fungal reads, respectively, contributing to a combined ~ 88% of the fungal reads (Fig. 2B). Following these dominant phyla, *Ascomycota, Zoopagomycota, Basidiomycota*, and *Blastocladiomycota* were identified with relative proportions of 5.03%, 3.02%, 1.37%, and 1.36% of the total fungal reads, respectively (Fig. 2B, Table 3a). The remaining three phyla *Mucoromycota*, Microsporidia, and the *Nucleariidae* and *Fonticula* group collectively represented less than 1% of the total fungal population (Fig. 2B, Table 3a). Temporal analysis revealed distinct succession patterns (Fig. 2A; Fig. 2D). *Ascomycota* were the most abundant fungal phyla at the onset of the WWTP operation, on day 13. *Cryptomycota* then exhibited a very high relative abundance compared to all eukaryotic phyla, associated with well-established phase 1 and phase 2 (from day 28 to day 82), appearing as a real population explosion. *Chytridiomycota* high abundance was associated with phase 2 and the early phase 3, spanning from day 68 to day 182. Based on Fig. 2A, fungal representatively (*Holomycota*) expressed as a percent of total *Eukarya* 18S rDNA reads fluctuates

dramatically across samples, ranging from dominant (~ 75%) in early samples to a minor component (~ 5%) in later samples, indicating significant temporal shifts in the eukaryotic community structure (Fig. 2A). However, when analysing fungal phyla abundance relative to each individual sample, *Cryptomycota* emerges as the overall most represented fungal phylum throughout the WWTP (Fig. 2C; Fig. 2D).

OTUs abundances. Although 348 operational taxonomic units (OTUs) were identified, a small number of dominant taxa primarily structured the community. Fifty high-abundance OTUs (≥ 0.1% of total reads) accounted for only 14.4% of the total OTUs but contributed by 96.5% of total fungal reads. Among these, just fifteen are predominant (with ≥ 1% of total reads) represented ~ 85% of fungal reads (Fig. 2E, Table 3d). The other, 35 OTUs, each comprising between ≥ 0.1% and < 1% of total fungal reads, collectively accounted for only 11.5% of the total reads. In contrast, the remaining majority of OTUs (298; 85.6% of the total OTUs) were detected at low abundances (< 0.1%), forming a rare biosphere that collectively contributed only by 3.5% of total fungal reads.

The affiliated fraction comprised 161 OTUs (46.4% of total OTUs), representing up to 14.20% of the fungal reads, could be assigned to a cultivated genus or species. Notably, members of the *Chytridiomycota, Ascomycota*, and *Basidiomycota* phyla accounted for 7.30%, 4.2%, and 1.15% respectively, of the assigned fungal reads (Table 3b). Among which 98 affiliated with cultivated species, where the predominant ones affiliate with *Ascomycota*, represented by 37 OTUs, followed by *Cryptomycota* (17 OTUs), *Chytridiomycota* (13 OTUs), *Basidiomycota* (11 OTUs), *Zoopagomycota* (8 OTUs), and *Mucoromycota* (6 OTUs). Additionally, *Nucleariidae* and *Fonticula* group, *Blastocladiomycota*, and *Microsporidia* are each represented by two OTUs respectively (Table 3c). In stark contrast, the cultivated fraction within *Cryptomycota*, the predominant fungal phylum, contributed only by 0.56% of the fungal reads (Table 3b). Within these groups, OTU_40 (*Synchytrium cupulatum*, *Chytridiomycota*) and OTU_130 (*Geotrichum*, *Ascomycota*) were particularly notable, together representing approximately 6.02% and 1.75% of the total fungal reads, respectively (Table 3d).

The unaffiliated fraction of fungi encompassed 187 OTUs representing 53.6% of the total fungal OTUs, and up to 85.80% of the fungal reads, highlighting the largely unexplored fungal diversity in the WWTP (Table 3b). This fraction of unaffiliated and multi-affiliated OTUs at the genus level, while confidently assigned to higher taxonomic ranks, likely represent a reservoir of fungal dark matter, reflecting lineages that remain poorly characterized. The large part was attributed to *Cryptomycota* with 75.45% of the total fungal reads.

The Core persistent OTUs over the 236 days. Five core OTUs showed persistence over the 236-day sampling period, including three affiliated with *Cryptomycota* and two affiliated with *Geotrichum* and *Saccharomyces*, respectively (Fig. 2F). Despite representing less than 2% of total OTU richness, these core taxa collectively accounted for approximately 56% of total fungal reads. This marked discrepancy between richness and abundance highlights a highly redundant fungal community structured around a small, persistent core. The core community was dominated by *Cryptomycota*-affiliated OTUs,

complemented by two cultivable *Ascomycota* taxa affiliated with *Geotrichum* and *Saccharomyces*. These five OTUs predominated mainly during the first 82 days of sampling.

Potential pathogenic fungal populations for humans, plants and animals within the WWTP.

Among the 348 identified OTUs, 127 were associated with potential pathogenicity in humans, plants, or animals, representing ~ 36.49% of the total fungal OTUs, and 11.72% of the total fungal reads. These OTUs span across seven fungal phyla. Notably, a high proportion of human pathogens was observed, comprising both yeasts and filamentous fungi. Table 5 provides a detailed view of the potential pathogenic fungi detected in this study.

The most abundant pathogenic genera and species, each representing more than 0.1% of total fungal reads, are comprised of 11 OTUs, accounting for approximately 10.5% of total fungal reads. These OTUs are primarily affiliated with ten genera: Geotrichum, *Penicillium*, Candida, Synchytrium, *Apiotrichum*, Saccharomyces, *Pseudorhizidium, Pichia, Cutaneotrichosporon, Lobulomyces* and *Mucor*.

Two distinct periods of fungal genera colonization were identified. The first period, spanning from day 13 to day 82, exhibited a diverse assemblage of genera.

The colonization kinetics reveal a clear ecological succession driven by a few quantitatively dominant fungi. *Geotrichum* functions as the primary resident generalist, maintaining high counts throughout the entire timeline and representing the most stable biomass. A dramatic shift occurs around Day 68, when *Synchytrium* explodes from virtual absence to become the overwhelming dominant organism. *Apiotrichum* and *Cutaneotrichosporon* emerge as secondary players of quantitative significance, with the latter peaking precisely during the transition phase and the former showing a resurgence in the late stages. *Saccharomyces* and *Penicillium* appear as early pioneers with initially high counts that rapidly decline, while *Lobulomyces* proves to be a late-stage specialist. In contrast, genera such as *Pseudorhizidium*, *Pichia*, and *Mucor* never achieve sufficient counts to be considered quantitatively important, appearing only as trace immigrants with counts in the single digits. (Fig. 3).

Cryptomycota are not currently recognized as direct pathogens of humans, animals, or plants; instead, their ecological role involves acting as microbial parasites that often infect protists and other microorganisms. Their abundance in the WWTP is highly significant, with 130 OTUs and 56573 reads. This accounts for 76% of the total fungal reads, approximately 11% of the total eukaryotic reads, and 37% of the total fungal OTUs (9% of the total eukaryotic OTUs) (Table 3a). Among the 15 most predominant OTUs, *Cryptomycota* contribute by 10 OTUs, representing 10% of the total eukaryotic reads (Table 3d). More research is needed to fully understand their ecological and medical significance.

Discussion

The fungal kingdom is estimated to encompass up to 3.8 million species, presenting an immense diversity of life forms, nutritional strategies and interactions with other organisms [11]. In the present

study, we are analysing fungal diversity and its population dynamics, within an aerobic basin of WWTP over a period of 236 days in a context where they are sequentially colonizing and building up a stable and efficiently functioning ecosystem. To have a holistic view of the process, we analysed both physicochemical parameters and microbial components (*Eukarya* and *Bacteria*) starting from the first inoculation of the basin with raw sewer microbes, over 236 days, from March thru October. As the microbes from the sewer first enter the basin, still diluted in the black water, they form the planktonic phase of the influent inoculum. They then begin constructing a robust food web comprised of microbial communities adapted to the available nutrients and environmental conditions. These microorganisms engage in various physical and metabolic interactions, modifying and optimizing the ecosystem to support their growth.

At the fungal phylum level, and in comparison to previous studies using fungal internal transcribed spacer (ITS) gene, [12] ; Zhang [13] and Assress [10] we identified more fungal phyla in our study. The study of Niu *et al.* identified seven fungal phyla in activated sludge from the WWTPs. The most abundant and significant phyla were *Ascomycota* (51.82% of total reads) and *Basidiomycota* (42.94% of total reads) and other minor phyla: *Blastocladiomycota, Chytridiomycota, Cryptomycota, Entomophthoromycota, Glomeromycota*, and unclassified fungi (1.02%). In the study of Zhang, they identified only four fungal phyla in activated sludge samples from 18 wastewater treatment plants (WWTPs) across China: *Ascomycota* (most dominant, 43.44% of total sequences) *Basidiomycota* (18.3%), *Mortierellomycota*, and *Chytridiomycota*. This study does not indicate the presence of *Cryptomycota* [13] .However, 35.44% of OTUs were unclassified, indicating that there may be more undiscovered fungal groups present in these environments.

In the study of Assress *et al*., 2019, when exploring the fungal communities in a mix of domestic, industrial, and hospital wastewater sources, the authors detected five fungal phyla: *Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota*, and *Zygomycota* [10].

In the study of Hirakata *et al.* [14], the fungal community structures of activated sludge and influent sewage, were analysed using V4 and V9 region-specific primers for 18S rRNA gene and reported the identification of six fungal phyla. The detected fungal phyla include *Ascomycota, Basidiomycota Chytridiomycota, Discicristoidea, Hyphochytriomycetes*, and *Cryptomycota*. In this study, the dominant fungal phyla varied depending on the sequencing region used (V4 vs. V9 amplicons). In activated sludge, *Cryptomycota* was most dominant. In influent sewage, *Ascomycota* and *Cryptomycota* were the major fungal groups. In a recent study on the Down-flow Hanging Sponge reactor, Stüer [15] et al., used V3-V4 primers sequencing of the Eukaryotic DNA, they showed that *Cryptomycota* represent 12.9% of total reads.

Our study highlights the predominance of early-diverging fungal lineages such as *Cryptomycota* and *Chytridiomycota* within fungal communities. Our results are on line with the various studies of Niu et al., [12] *et al*., Hirakata *et al*. [14], Stüer *et al* [15], and Matsunaga *et al*. [16].

In our previous publication, we demonstrated that exploration of OTU occurrence shows the persistence of 19 OTUs for eukaryotic populations [17]. These are affiliated with nine phylogenetic groups: *Holomycota, Holozoa, Rhizaria, Alveolata, Euglenozoa, Stramenopiles, Hyphochytriomycetes, Peronosporomycetes, Ichthyosporea, Heterolobosea* and unknown phylum. Fungi contribute by five predominant and persistent OTUs, accounting for 12.5% of the total eukaryotic reads. They are among the key players in the WWTP, particularly during the initial phase of microbiome establishment. Their interactions with prokaryotic and other eukaryotic components may play a pivotal role in the dynamics of microbiome establishment in the WWTP, influencing and shaping the overall structure of the wastewater ecosystem. They are likely involved in organic matter degradation, potential parasitic or symbiotic interactions. Further research is needed to determine their specific metabolic functions in wastewater treatment systems.

So far, for eukaryotic microorganisms, only molecular tools have detected unexplored taxa, including fungi. This so-called fungal dark matter being highly predominant is becoming associated with early diverging lineages such as *Chytridiomycota* and *Cryptomycota* at the base of the fungal tree, [18–20]. Currently, we lack important information on their ecological roles. Novel methods, such as single-cell genome sequencing, are poised to enhance our understanding of fungal evolution and the broader fungal kingdom.

Potential metabolic role of fungi within the WWTP. From the late 1950s to the mid-1960s, researchers began recognizing the potential of fungi in wastewater treatment. Later studies further highlighted their metabolic versatility in pollutant degradation and sludge transformation processes. Thanh and Simard reported treatment of wastewater by various yeast species [21]. After carrying out a number of surveys of receiving water bodies, trickling filter, activated sludge, and anaerobic digester for the various types of microorganisms found in the processes, and as they exhibited high degradation rates and strong capabilities for breaking down cellulose, hemicellulose, and lignin, Cooke advocated the use of fungi in wastewater treatment, [22]. Among the 15 most dominant OTUs (85% of total reads), only two, *Geotrichum* and *Synchytrium* are among the cultured genera. The remaining 13 OTUs are part of the dark matter for which we do not have representative species. *Geotrichum* is both present and metabolically active in many wastewater contexts, especially lipid- and phenolic-rich effluents (e.g., food/agro-industrial wastewaters), and it contributes to COD and color removal via lipolytic and ligninolytic systems [23]. *Geotrichum candidum* found in our study is known for its biotechnological importance, it is associated with industrial applications. Apart from its role as a starter in the dairy and brewing industries, this species has been administered as a probiotic nutritional supplement in fish. Strains of this species produce a plethora of biotechnologically important enzymes, including cellulases, β-glucanases, xylanases, lipases, proteases, and α-amylases, for complete review see [24]. WWTP fungal-diversity surveys by Assress [10], show the presence of *Synchytrium* in wastewater; but there is no evidence for its involvement in pollutant degradation, they might degrade plant debris, and serve as saprobes or microbial trophic links. Metatranscriptomics, enrichment culture and enzyme assays are needed in wastewater contexts. Recent work of Sugimori showed that *Rhodotorula* and *Cryptococcus* species, also found in our study, are commonly found in contaminated sites and are able to degrade a variety of

specific contaminants, such as petroleum, phenanthrene, and benzopyrene [25]. Due to these abilities, *Rhodotorula* species have been widely applied in various kinds of wastewater treatment processes for the removal of pollutants [26]. Santos *et al*. showed that *Cryptococcus* species play important roles in winery wastewater treatment processes by converting large-molecule organics into small, degradable lipids [27]. Nhi-Cong le et al., reported that *Trichosporon hyphae* are involved in the formation of biofilms and sludge flocs and contribute greatly to the degradation of respectively sec-hexylbenzene and caffeine in wastewater [28]. Felczak *et al.* reported the degradation of quinolone, a pollutant widely distributed in environment, [29]. Rot fungi possess an ability to degrade pharmaceuticals, pesticides, and dyes [30]. Among other species and genera found in our study, *Aspergillus*, and *Mucor*, are widely used in the elimination of dangerous xenobiotics [31] ; [32]; [33].

In general, we suggest that fungi existing in activated sludge might contribute greatly to the degradation of many types of pollutants and the formation of sludge flocs in wastewater treatment processes. Unfortunately, the majority of them are part of fungal dark matter; more investigation is needed to determine their metabolic potential in the wastewater treatment.

Fungal nitrification and denitrification: Expanding the nitrogen paradigm. Our study is broadening the traditional model of the nitrogen cycle to include fungi as central actors, not just bacteria and archaea. Recent studies increasingly challenge the classical view that nitrification and denitrification are processes governed almost exclusively by prokaryotes. Mounting evidence now demonstrates that fungi not only contribute but also can act as major drivers of nitrogen cycling, especially in organic-rich, oxygen-limited environments. Their metabolic flexibility enables them to couple carbon degradation with nitrogen transformations, positioning them as essential integrators of biogeochemical processes.

While ammonia oxidation is traditionally attributed to autotrophic bacteria and archaea, fungi have been shown to perform this process efficiently. They can oxidize urea and ammonia into nitrite and nitrate, with some studies reporting nitrification rates one to four orders of magnitude higher than those of their bacterial counterparts. Rajkumar *et al*. showed that two heterotrophic organisms *Fusarium* sp. and *Penicillium* isolated from the soil with pH 4.3, tested for their heterotrophic nitrifying ability in glucose peptone liquid medium, were found to produce significant quantities of nitrite and nitrate [34]. Filamentous species such as *Aspergillus flavus* and *Verticillium* sp. display exceptional nitrifying activity, typically producing more nitrate than nitrite, indicative of complete oxidation [35]; [36]; [37]; [38]; [39]. Other studies shows fungi such as *Paecilomyces variotii* can also remove both nitrogen and phosphorus, underscoring their biotechnological potential in biological nutrient removal [40].

Given the metabolic adaptability of fungi, it is likely that they represent a major component of this process in natural and engineered systems. Recent evidence highlights the emerging ecological role of *Rozellomycota* (*Cryptomycota*) in nitrogen cycling. Their abundance, particularly the LKM15 clade, shows significant negative correlations with ammonium and total nitrogen concentrations in aquatic and engineered systems, suggesting active participation in nitrogen removal processes [15].

In our study, during the early operational phase (day 13 thru day 82), canonical bacterial autotrophic nitrifiers (*Nitrosomonas, Nitrosospira, Nitrosopumilus*) were totally absent, while ammonium levels declined sharply, coinciding with the dominance of fungi and other heterotrophic taxa. The sharp decline in ammonium concentration observed in the aerobic basin during the period from day 40 thru 82 may be partly attributed to the metabolic activity of heterotrophic nitrifiers and denitrifiers, as well as to nitrogen assimilation by the expanding and diversifying microbiota detected during the early and intermediate stages. This is corroborated by the late emergence of autotrophic nitrifiers (e.g., *Nitrosomonas, Nitrosospira, Nitrospira*) [17]. These patterns suggest that heterotrophic bacteria, protists and fungi may be the primary ammonia oxidizers at this stage.

The involvement of fungi in denitrification cannot be excluded. Many studies show fungi carrying out denitrification a process once considered exclusive to bacteria, with the first experimental evidence provided by Shoun and Tanimoto [41] and then by Kobayashi [42] in *Fusarium oxysporum*, revealing mitochondrial reduction of nitrate and nitrite to gaseous nitrogen compounds, coupled to ATP synthesis. Since then, several taxa such as *Fusarium solani, Cylindrocarpon tonkinense, Gibberella fujikuroi*, and *Trichosporon cutaneum* were confirmed as true denitrifiers. Notably, *Fusarium solani* can both nitrify and denitrify, reflecting an integrated nitrogen metabolism [43,44]. Unlike bacterial denitrification, which often serves as an alternative respiratory pathway, fungal denitrification occurs efficiently under suboxic conditions. Recently, Zhong *et al*. (2022) reported that, at the ecosystem scale, fungi contribute substantially to both nitrification and denitrification. Along a 3,000 km grassland transect in China, fungi accounted for 25% of the total nitrification and 46% of the denitrification potential [45].

Collectively, these findings establish certain fungi as pivotal agents in the nitrogen cycle, performing both nitrification and denitrification when conditions inhibit autotrophic bacteria and archaea. This dual functionality, coupled with their metabolic versatility, exemplifies ecological redundancy where taxonomically distinct organisms sustain similar biogeochemical roles. Such redundancy fortifies the resilience and stability of nitrogen cycling amidst environmental stress, preserving ecosystem function even when dominant microbial populations decline. Consequently, the role of fungi in nutrient cycling affirms not only their ecological significance but also their considerable biotechnological promise for applications in wastewater treatment, soil remediation, and nature-based climate mitigation strategies.

Residual fungal components of the human gastrointestinal tract (HGT).

While bacteria dominate the gut numerically, a substantial fungal population is present. Crucially, the majority of these fungi are only identifiable via non-culture-based techniques, highlighting their elusiveness [46]. Although fungi make up a smaller portion of the HGT microbiome, they play a crucial role in health and disease. The HGT contains a small fungal population, over 400 fungal species 4 phyla and about 140 genera, associated with HGT. They are mainly affiliated with *Ascomycota*, *Basidiomycota*, and *Chytridiomycota* phyla, making up approximately 0.1% of the HGT microbiota under normal circumstances, and there is a stable relationship of antagonism, synergy, or symbiosis between, or among fungi, bacteria, and viruses in the human and animal gut under normal circumstances. [47] ; [48].

Recent sequencing analyses have unveiled a more diverse fungal community in the HGT than previously recognized. Lai et al, 2023 characterized four mycobiome enterotypes using ITS profiling of 3363 samples from 16 cohorts. They noticed the presence of four predominant genera: *Candida, Saccharomyces, Penicillium, and Aspergillus.* Among these, *Candida albicans, Candida tropicalis, Candida parapsilosis, and Candida glabrata, Malassezia spp, Cladosporium spp, Trichosporon spp*. are frequently observed species [49]. *Geotrichum*, a genus ubiquitous in air, soil, water, sewage, plants, and human faeces, was the most prevalent morphotype in Petri dish cultures [50]. A study on faecal samples from 111 healthy subjects using pyrosequencing showed *Penicillium*, *Aspergillus*, and *Candida* as the most abundant genera, with 22.3%, 22.2%, and 16.9%, respectively [51].

In our study, nineteen species affiliated with the HGT mycobiota (e.g., *Geotrichum, Candida, Saccharomyces, Penicillium*) accounted for 3.62% of fungal reads. This is in concordance with other studies reporting that *Geotrichum* and *Saccharomyces* are the most frequent yeast in human faecal samples [52]. While some fungi are transiently present in the gut after oral intake, others, such as *Candida* spp. (e.g., *Candida albicans*), *Saccharomyces* spp. (e.g., *Saccharomyces boulardii*), *Rhodotorula* spp., *Aspergillus* spp., *Penicillium* spp., *Trichosporon mycotoxinivorans*, *Alternaria* spp., *Cladosporium* spp., *Trichoderma* spp, and *Aureobasidium* spp., are part of the resident gut flora Hof [53]. *Saccharomyces* and *Penicillium* are found in food products like bread and meat [54]. In our study we recovered *Geotrichum, Saccharomyces, Candida*, *Penicillium*, and *Mucor* representing > 0.1% of total fungal reads. The remaining OTUs affiliate with *Yarrowia, Aspergillus, Acremonium, Rhodotorula, Cryptococcus, Malassezia, Basidiobolus, Mucor*, and *Rhizopus* with less than 0.1% of total fungal reads.

Geotrichum is represented by three OTUs among which the cultivated *G. candidum* and two other *Geotrichum* unaffiliated OTUs. *G. candidum* is widely used in the production of certain dairy products, including rind cheeses such as Camembert, and Saint-Nectaire. Evidence has shown that many fungal species are directly associated with food ingestion prior to sampling. Nevertheless, some filamentous fungi such as *Aspergillus*, *Penicillium and Rhizopus* were reported. Other rare several fungal genera are detected with a low relative abundance, such as *Pichia*, *Rhodotorula, Cryptococcus, Mucor* and *Yarrowia*. The presence of *Aspergillus, Rhodotorula, Penicillium, Candida, Synchytrium*, and *Mucor* was reported in all WWTPs studied by Assress et *al* [10].

Most importantly, *Geotrichum* and *Saccharomyces* were among the core OTU present permanently in the WWTP. *Geotrichum* and *Saccharomyces* are present in human stools due to their use in food production. It contributes to the flavor, texture, and surface development by breaking down proteins and lipids. *Saccharomyces cerevisiae* is a key yeast in bread baking (baker's yeast), where it ferments sugars to produce carbon dioxide, causing dough to rise. It is essential in beer and wine fermentation, converting sugars into alcohol and carbon dioxide. *Geotrichum* is also involved in fermentation processes for dairy products and some cured meats.

Pathogenic fungal species within the WWTP species may be harmful to human, plant and animal health.

In addition to their crucial role in food industry, bioremediation, and overall biotechnology, fungi are one of the main causes of human, animal and plant diseases, especially, in immunocompromised patients yousfi [55]. Some pathogenic fungi in WWTPs can be a serious threat to human, animal end environmental health since effluents from WWTPs are increasingly being considered not only for use in irrigation but also to produce quality water for urban and for drinking [7]. Unlike bacteria and viruses, fungi are not routinely monitored in wastewater or included in most water quality regulations. As a result, there is limited data on their prevalence and potential risks. This threat associated with the presence of pathogenic fungi in WWTPs is an issue of concern because fungi are not completely removed by conventional WWTPs and they are not included in regulatory frameworks [56]. Furthermore, they produce mycotoxins, which are considered toxic to humans [57]. Pathogenic fungi also have a significant impact on crop and plant life, influencing food security and ecosystem diversity [58]. More importantly, the emergence of drug resistant and less susceptible pathogenic fungi in the last decades has also become a great concern on the risks they may cause [59]. All these observations call for thorough and comprehensive investigation and characterization of fungal communities in WWTPs.

Fungal pathogenic diversity and abundance. Our study identified 348 OTUs, among which 162 were associated with potential pathogenicity in humans, plants, animals, protists or algae, including both yeasts and filamentous fungi. These accounted for ~ 45% of the total detected fungal OTUs, and ~ 22% of the total fungal reads. These OTUs were primarily affiliated with ten genera: *Synchytrium, Geotrichum, Saccharomyces, Apiotrichum, Candida, Pseudorhizidium, Penicillium, Cutaneotrichosporon, Lobulomyces*, and *Pichia*. The wide taxonomic range of these OTUs, spanning seven fungal phyla, underlines the diversity of pathogenic fungi in the studied WWTP plant environment.

Comparing our results with the literature, Fijalkowski *et al.* (2017) highlighted the presence of pathogenic fungi in sewage sludge, including *Fusarium, Neurospora, Aspergillus, Penicillium, Absidia, Saccharomyces, Candida, Mucor, Rhizopus, Cryptococcus*, and *Trichophyton* [60]. Zhang (2018) further identified opportunistic fungal pathogens such as *Candida, Rhodotorula, Fusarium*, and *Aspergillus*, posing potential health risks to wastewater workers [13]. Our study aligns with these findings but shows a different abundance profile, notably with lower representation of *Aspergillus* and *Cryptococcus* (< 0.1%). In a recent paper Ariyadasa *et al*., examined fungi (and other eukaryotes, viruses) in raw wastewater influent, noting that potentially pathogenic fungal taxa are among those detected [61]. *Geotrichum* sp., *Mucor* sp., and *Penicillium* sp., were recorded previously as cycloheximide-resistant fungi in activated sludge; therefore, these fungi may be harmful to wastewater treatment workers. [62].

Temporal shifts in fungal community composition. Our study identified mainly two distinct phases of fungal colonization in wastewater systems. The initial phase (days 13–82) featured a diverse fungal assemblage dominated by *Candida*, *Saccharomyces*, *Penicillium*, and *Geotrichum*, while the later phase (days 133–236) was characterized by a pronounced shift toward *Synchytrium*, *Apiotrichum*, *Saccharomyces*, and *Lobulomyces*, particularly at sampling points 224 and 236. These temporal shifts underscore dynamic structural changes, with *Geotrichum* and *Saccharomyces* emerging as persistent

cultivable taxa. Notably, *Candida* spp. a major nosocomial pathogen linked to systemic infections in immunocompromised populations [63], [64] represented only 2.62% of cultivated fungal reads, contrasting with its higher prevalence in prior studies [48]. Other detected opportunistic pathogens included *Saccharomyces cerevisiae* invasive infections in vulnerable hosts; [65] and *Geotrichum candidum* geotrichosis; [66]; [67].

Public health and ecological implications. The presence of pathogenic fungi such as *Candida, Geotrichum, Synchytrium*, and *Penicillium* in wastewater raises concerns for occupational exposures (e.g., wastewater workers) and ecosystem health. Of particular note is the dominance of *Cryptomycota*, a poorly understood group representing 74% of fungal reads, 11% of eukaryotic reads, and 37% of fungal OTUs. While not currently classified as a direct human health threat, *Cryptomycota's* role as microbial parasites could indirectly disrupt soil and aquatic ecosystems by altering microbial community structure, nutrient cycling, and organic matter degradation. Their high abundance warrants further investigation into their ecological and medical significance.

Agricultural and soil ecosystem risks. Several detected fungi, including *Geotrichum candidum, Synchytrium* spp., *Fusarium*, and *Penicillium* spp., are recognized phytopathogens associated with crop diseases (e.g., sour rot, fusariosis) and mycotoxin contamination. For instance, *Synchytrium endobioticum* causes potato black wart disease, while *Synchytrium aureum* infects horticultural crops [68]. These pathogens threaten agricultural productivity and soil health by outcompeting beneficial microbes, disrupting nutrient cycles, and reducing crop yields. Furthermore, persistent fungi such as *Aspergillus*, *Mucor*, and *Rhizopus* in wastewater-impacted soils may impair organic matter turnover and soil fertility.

Mitigation and future directions.

Although the 18S rDNA marker offers broad phylogenetic coverage and reduced primer bias across the *Holomycota*, its taxonomic resolution is intrinsically limited at lower ranks (i.e., genus and species levels) compared with the Internal Transcribed Spacer (ITS) region. As a result, while our approach effectively captured the overall diversity patterns and colonization dynamics of major lineages, it likely underestimated true species-level richness. Future investigations based on ITS1 or ITS2 amplicon sequencing would complement these results by enabling finer taxonomic discrimination and potentially uncovering cryptic species dynamics within the established communities.

Nevertheless, our findings highlight the need for targeted monitoring of fungal pathogens in wastewater systems to mitigate public health risks and ecological imbalances. The survival of fungal spores in wastewater sludge underscores the necessity of complete pathogen inactivation before agricultural and landscaping reuse. Air or water-dispersed spores also pose risks for long-distance dissemination of plant and animal diseases. Future studies should prioritize elucidating *Cryptomycota's* functional roles and refining sludge treatment protocols to limit environmental and agricultural contamination.

Concluding remarks

This study offers new insights into the WWTP fungal community structure and the role of early diverging fungal lineages in WWTPs. The fungal community is highly diverse, encompassing nine fungal phyla, with *Cryptomycota* and *Chytridiomycota* representing dominant phylogenetic groups. These fungi directly influence habitat physicochemical parameters and interact with other microbial communities, contributing to the establishment of stable microbial networks and supporting essential ecosystem services.

Our research highlights that WWTPs serve as a significant reservoir of potential fungal pathogens, posing substantial threats to human, animal, and plant health, thereby endangering entire ecosystems. Beyond fungi, municipal wastewater is also a potential source of emerging infectious diseases, including future epidemics or pandemics caused by viruses, bacteria, fungi, and parasites. Unlike bacteria and viruses, fungi are not routinely monitored in wastewater, despite their persistence and potential pathogenicity. To mitigate the risks associated with wastewater-borne microbial pathogens, it is imperative to integrate fungi into water quality regulations. Establishing regulatory limits for persistent fungal pathogens will enhance risk assessment and environmental safety. Surveillance of wastewater pathogens within the One Health framework is critical for early detection, prevention, and rapid response to emerging health threats. Additionally, comprehensive treatment and rigorous effluent evaluation are essential to minimize fungal dissemination. Effective monitoring of key fungal pathogens should incorporate a combination of molecular methods to improve detection accuracy and regulatory compliance.

Materials and methods

Study area. The Seine-Morée WWTP, located in Le Blanc-Mesnil (48° 57' 09.6" N 2° 27' 46.5" E, Seine-Saint-Denis, France), was started up, *de novo*, without external sludge inoculation. It has treatment capacity of 50.000 m^3/day. It treats wastewater from a residential area of 200,000 inhabitants in the northeastern of Paris and effluents from Roissy-Charles de Gaulle International Airport. This plant started de novo without any external sludge inoculation. It was filled up with potable water, and gradually supplied with raw wastewater. The plant performance data was made available by the wastewater facility. Water temperature, Biochemical oxygen demand (BOD), Chemical oxygen demand (COD), pH, gross flow rate discharge, total suspended solids (TSS), oxygen concentration in aerobic sludge, settling volume, dryness, total volatile suspended solids, Mohlman index, nitrogen (total Kjeldahl nitrogen (TKN), ammonia (N-NH4), nitrite (N-NO2 and nitrate N-NO3) and phosphorus concentrations were determined according to standard methods. Physicochemical parameters measurements were performed every day by the SIAAP (Syndicat Interdépartemental pour l'Assainissement de l'Agglomération Parisienne) laboratory at the entrance and the exit of the biological tank. The measurements were performed according to the French standards methods: TSS, according to NF EN 872; TKN, according to NF EN 25663; Ammonia, according to NF EN ISO 11732; nitrite and nitrate, according to NF EN ISO 13395; Orthophosphate and total P, according to NF EN ISO 6878. For the sequencing work, the sampling was done independently of physicochemical parameter measurements.

A summary of important plant chemical and operational parameters is shown in Table 1: pH, COD, BOD, nitrogen total (nitrogen Kjeldahl, ammonia, nitrite and nitrate) and phosphorus concentrations were determined using consensus methods. All physicochemical parameters for each sample time-point were presented in Table 1.

Sludge and water samples. In this study, twenty-three sludge samples were collected over 236 consecutive days from the Seine-Morée wastewater treatment plant. For each sampling event, 2 liters of mixed liquor were taken directly from the outlet of the aeration basin. The samples were then concentrated by centrifugation, and the resulting pellets were stored at -20°C for subsequent genomic analysis.

DNA extraction. It was performed according to Nucleo spin soil DNA kit (Macherey Nagel GmbH & Co. KG,Durën, Germany) recommendations. Metagenomics DNA was quantified by spectrophotometric method, using the WPA Biowave II UV/Visible spectrophotometer. (Biochrom, Cambridge, UK) and a TrayCell Fibre optic micro cell (Hellma GmBH & Co. KG, Müllheim, Germany).

PCR amplification and DNA sequencing. The eukaryotic diversity was assessed by PCR amplification of the V9 hypervariable region of the 18S rRNA gene using primers 1193F and 1379R (25 cycles, performed in triplicate). The resulting amplicons were sequenced on an Illumina MiSeq platform at the Genoscope Center (Évry, France).

Bioinformatic processing.

Sequence quality control and bioinformatics processing. For 18S rDNA Illumina reads, quality control began by removing adapters and primers on the whole reads and low quality nucleotides from both ends, and then we continued the next steps using the longest sequence without adapters or low quality bases. Reads shorter than 30 nucleotides after trimming and read pairs that come from the low-concentration spike-in library of Illumina PhiX Control were discarded. This policy allows submission of high quality data (without contamination) in order to interrogate databases and to improve subsequent analysis. Overlapping 18S rDNA paired end reads were merged with pear v0.9.11. (https://github.com/easyb uilde rs/easyb uild-easyc onfig s/pull/6653/files).

Dereplicated 18S and 16S rDNA reads were independently clustered with swarm 2.1.12 (https://bioweb.pasteur.fr/packages/pack@swarm @2.1.12), using a distance cutoff of 3%, and singletons OTUs were removed. Chimeric sequences were detected with VSEARCH (https://github.com/torognes/vsear ch), and removed for subsequent analyses.18S rDNA sequence analyses were continued using the FROGS pipeline "Find, Rapidly, OTUs with Galaxy Solution" (https://frogs.toulouse.inrae.fr/). Taxonomic affiliation of 18S rDNA reads was performed with BLAST 2.6 on the SILVA_132_18S database. A Biological Observation Matrix file (BIOM) comprising both abundance and taxonomy was generated and imported into R (version 3.5.2) for statistical analysis.

Statistical processing. R software (version 3.5.2) was used to examine BIOM files. 18S OTUs were analysed. Considering the dispersion in the total number of reads identified in each sample, OTUs abundances were scaled. The total number of reads counts in each individual sample divided the variable read count. Here, we focused our analysis on fungal OTUs.

Principal component and heatmap analyses. In order to get insights into the relationships between WWTP time series samples, centred, scaled, principal component analyses (PCOA) were implemented on physicochemical parameters datasets respectively on line according to [69] https://biit.cs.ut.ee/clustvis/. To get insight into the relationships between physicochemical and bacterial communities evolution, we implemented a heatmap analyses of the OTUs Abundance throughout the study..

Declarations

Data availability

Raw data for relative abundance of eukaryotic, fungal and bacterial communities at the different taxonomic levels will be made available and provided on reasonable request. Sequences reported in this study were deposited in EMBL databases (https: //www.ebi.ac.uk/) under accession numbers ERR4106944-ERR4106967.

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Tables

Tables are available in the Supplementary Files section.

Figures

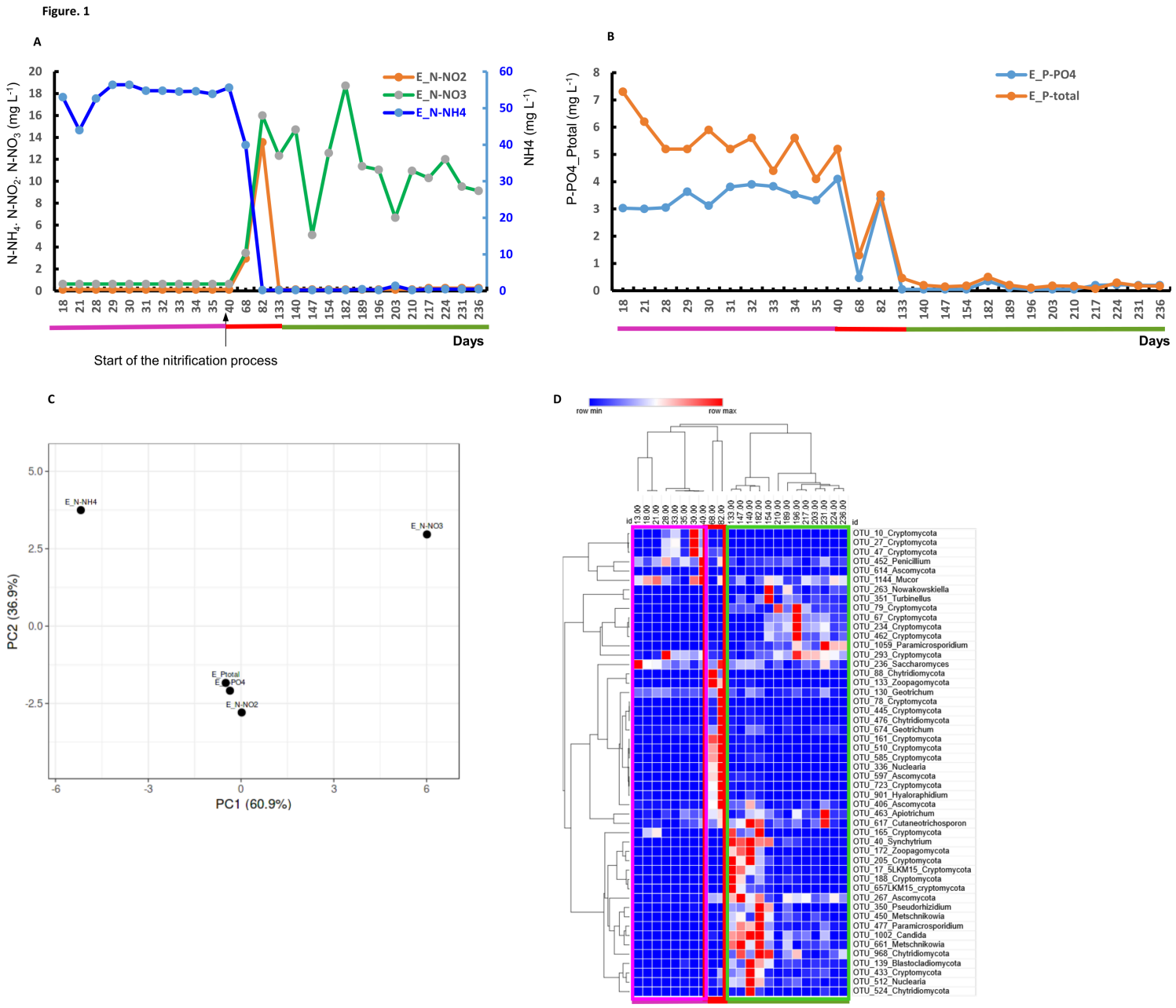


Figure 1

(A). Evolution of the physicochemical parameters (A) Ammonia, nitrite and nitrate; (B) total phosphate and inorganic within the aerobic basin SM WWTP through the 236 days of the study. Colored lines delimit the three periods of physicochemical parameter evolution: Pink represents the first period. Red. the intermediate period. and green represents the third period. (C) Principal component analysis (PCA) on the physicochemical parameters within the SM wastewater treatment plant. (D) Hierarchically clustered heat map of the top 50 fungal communities at the genus level (the phylum level is indicated when the genus was not identified) from each wastewater sample of the SM_WWTP

Figure 2.

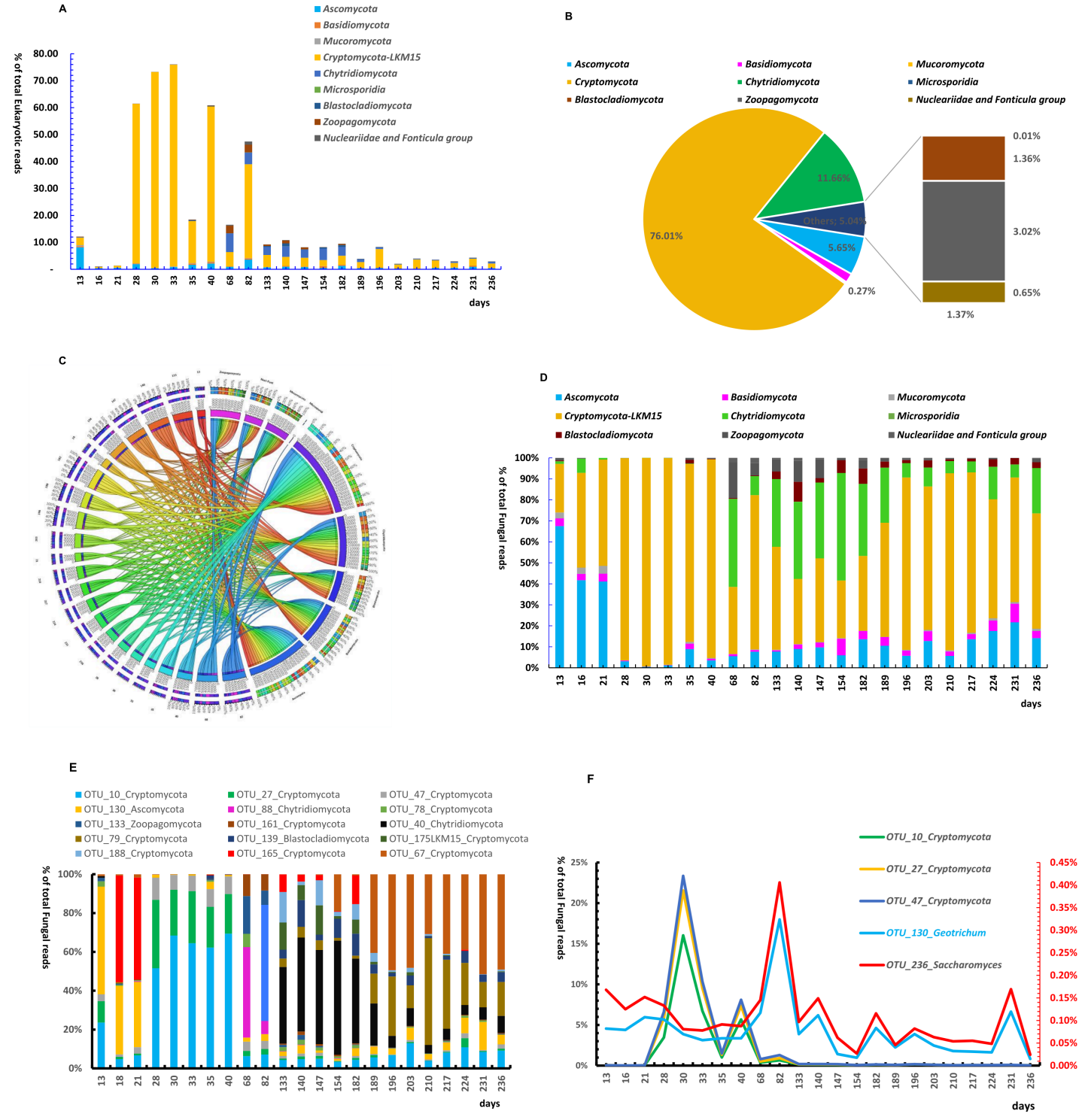


Figure 2

(A) Mean percentage of reads corresponding to the nine Holomycota phyla. calculated from the total eukaryotic reads identified within the wastewater treatment environment. (B) Relative read abundance of Holomycota communities. classified at the phylum level. (C) Circos plot illustrating the daily relative abundance of Holomycota communities at the phylum level over a 236-day sampling period. (D) Histogram representing the daily relative abundance kinetics of Holomycota communities at the phylum

level over a 236-day sampling period. (E) Abundance and colonization kinetics of the fifteen high abundance OTUs over a 236-day sampling period. (F) Abundance and colonization kinetics of the five persistent OTUs over a 236-day sampling period. (red scale correspond to the low abundance OTUs)

Figure. 3

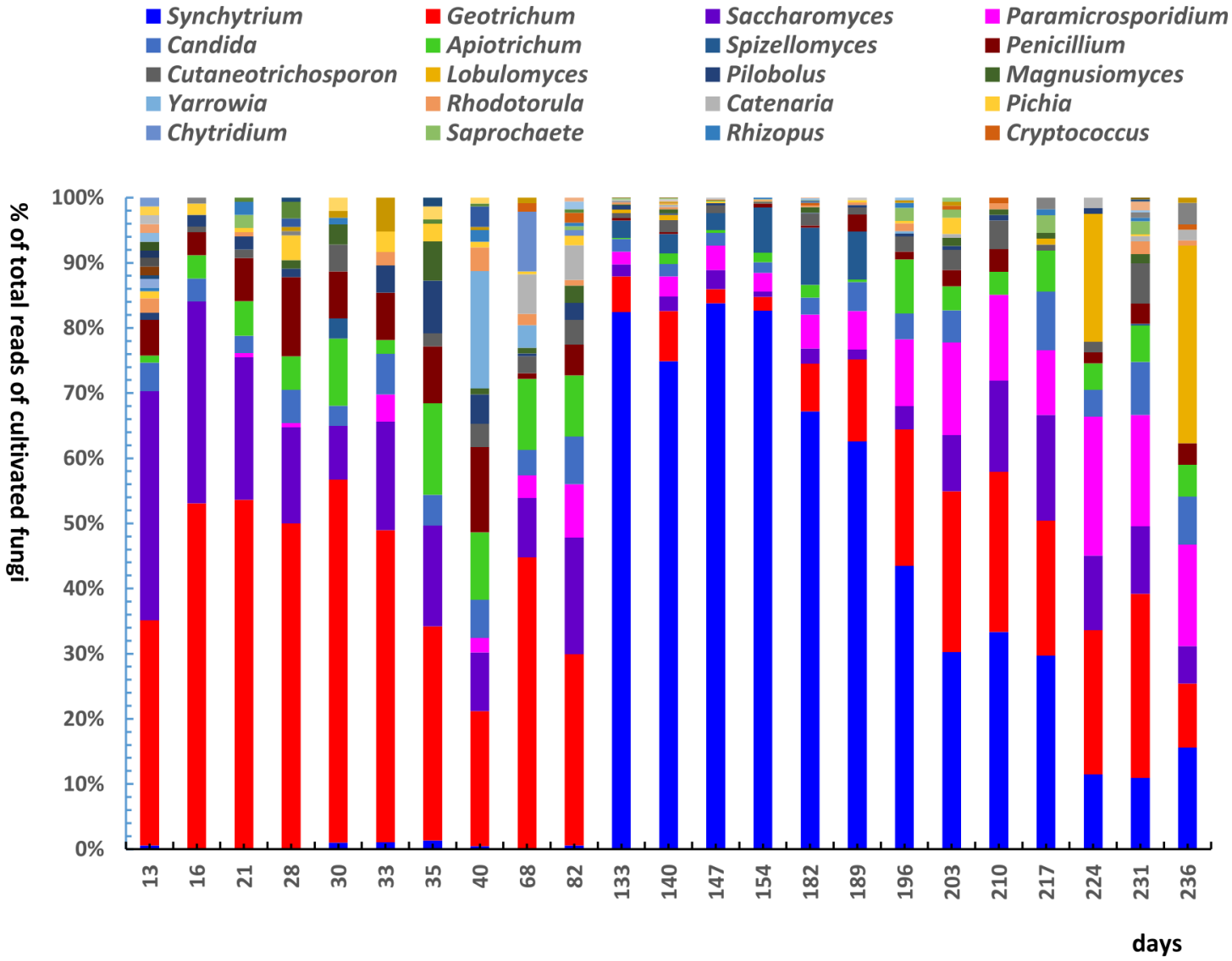


Figure 3

Colonization kinetics of the main potential pathogenic genera within the Holomycota throughout the 236-day study period in the WWTP

Supplementary Files

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- Tables.ppt