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Potential hazard to human and animal health from bacterial and fungal contaminants in small freshwater reservoirs †

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Abstract: In general, the assessment of microbiological quality in aquatic systems focuses on the presence of some bacterial groups or species. Although Fungi is not a mandatory microbiological parameter, recently the WHO advises its detection/quantification. Its concentration and diversity varies greatly among the various types of aquatic systems. Fungi are mesophilic, dependent on organic matter to growth, and their presence can be associated with pollution. Depending on their concentration and diversity, fungi may pose a risk to human and animal health. The objective of the present work was to evaluate the presence of some bacterial indicators (*Escherichia coli*, fecal enterococci, among others) and fungi (total, yeasts and molds) in freshwater reservoirs (water tanks) with different sources, sun exposures, anthropogenic and animal influences. Additionally, it was intended to assess the diversity of molds. For this, filamentous colonies were isolated, purified and morphologically identified (whenever possible to the genus). The three tanks differed in bacterial (presence of *Escherichia coli*, fecal enterococci, *Proteus* sp. and *Staphylococcus aureus*) and fungal (total and mold) presence. Regarding molds, 16 different taxa were identified and, depending on the water tank, *Penicillium*, *Aspergillus* and *Fusarium* genera and the Chytridiomycota phylum were the most representative. Some of the taxa isolated may pose a risk to human and animal health (*Trichophyton*, *Aspergillus fumigatus*, and some dematiaceous). The water reservoirs presented different fungal communities. Although preliminary, the results show that freshwater tanks can be a source of potentially pathogenic bacteria and fungi, to humans and animals that use them.

Keywords: freshwater tanks; molds; *E. coli*; enterococci, *Proteus*; *S. aureus*; dematiaceous; dermatophyte

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1. Introduction

Fresh water has high microbial diversity, as microorganisms play a fundamental role in the nutrient cycle and in the purification of aquatic ecosystems. However, they can also cause disorders and be pathogenic for humans, animals and other organisms. The classic parameters used are fecal indicator organisms (FIO) associated to pollution of fecal origin: fecal coliforms from the *Enterobacteriaceae* family, fecal enterococci and clostridia, although there are other organisms such as helminths or protozoa that can also pose health risks to humans [1,2]. Among these indicators, fecal coliforms are widely used, being associated with a high number of human intestinal infections, and involved or being participants in pathologies such as meningitis, urinary tract infections and nosocomial pneumonias [3]. However, recent studies pointed out that these organisms may not be sufficient as indicator organisms, and it

may be necessary to assess the presence of others such as filamentous fungi and yeasts. It is therefore important to know the presence of these organisms in their counts, distribution, diversity and behavior, in different aquatic environments [4,5]. Fungi are heterotrophic eukaryotes widely distributed in nature. They are present in soil, air, organic matter and water, especially in untreated water, reservoirs and distribution systems. Several authors have reported the presence of yeasts and molds in the aquatic environment, the latter being found in greater numbers [6,7]. Among many different taxa of fungi found in aquatic environments, several species are opportunistic or pathogenic, produce toxins and are allergenic. Most of these species belong to the phyla Ascomycota, Zygomycota and Chytridiomycota. The fungi with the most significant presence are *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, and *Curvularia*, and some of them increase the risk of diseases in humans and animals [7,8]. Although most of the time fungal infections or mycoses do not result in the death of patients, they can be a public health problem. The most common mycoses are caused by dermatophytes that affect the skin, hair and nails, and are very contagious [9,10]. Hyaline, dematiaceous and dimorphic fungi can affect various tissues and organs causing severe mycoses [11,12]. Systemic mycoses are difficult to treat and have an unpredictable prognosis, especially in immuno-incompetent populations.

The main objective of the present work was to evaluate the presence of FIO (*E. coli*, fecal enterococci), other bacterial species associated to humans and animals) and fungi (mainly molds) in freshwater reservoirs from three different sources, sun exposures, anthropogenic and animal influences.

2. Materials and Methods

2.1. Localization of water tanks

The present study was carried out between April and June 2022. All the tanks are located in the *Campus* of the University of Trás-os-Montes e Alto Douro (UTAD), in Vila Real (Latitude: 41.2885° N; Longitude: 7.7391° W; Altitude: 462m), and are close to each other (115 to 270 m apart). Tanks 1 and 2 are mostly fed by natural fountains and are nearby foot paths and pastures, while tank 3 is fed by rainwater, and in an inner courtyard with restricted access (Figure 2).



Figure 1. Visual aspect of the three tanks in the University *Campus* UTAD. Tanks 1 and 3 are oriented to the North and Northeast, and tank 2 to the East.

Tank 1 water was cloudy with cherry blossoms, and although surrounded by vegetation it had the cleanest water compared to the other tanks. In tank 2 the water was covered with macroalgae, filamentous microalgae and aquatic plants such as *Lemna* sp. Tank 3 had very turbid water, green in color due to the massive growth of microalgae, surrounded by vegetation and does not get direct sunlight.

2.2. Culture media

In the evaluation of the water samples microbiology the following culture media were used: a) for bacteria: Slanetz & Bartley Agar (Slanetz, Oxoid) for fecal enterococci, Chromogenic Coliform Agar (Chromo, Oxoid) for *Escherichia coli* and other fecal coliforms; Cysteine Lactose Electrolyte-Deficient Agar with Andrade indicator (C.L.E.D., Liofilchem) for bacteria that cause urinary infections (*S. aureus*, *E. coli* and *Proteus vulgaris*); b) for fungi: Yeast Malt agar (YMA, Liofilchem), Yeast Glucose Chloramphenicol Agar (YGCA, Himedia), Mycosel Agar (Mycosel, Liofilchem) for dermatophytes; and Potato Dextrose Agar (PDA, Liofilchem), for molds isolation and maintenance. All media were prepared according to the manufacturers' specifications.

2.3. Water sampling and microbiological analyses

For each tank, three independent water samples were collected using 500 mL sterilized plastic bottles. Each bottle was quickly submerged, approximately 30 cm deep, except for tank 3 that was shallower. The samples were taken in 20th April (tanks 1 and 2, air temperature 12 °C, water temperature 8 °C) and 9th May (tank 3, air temperature 21 °C; water temperature 10 °C), between 10:30 and 11:00 a.m. For the detection of bacteria, the membrane filtration technique was used, filtering 100 mL of water per filter (0.45 µm pore). For the quantification of fungi, in addition to the membrane filtration technique, the spread of a small volume (100 or 200 µL per plate) of the sample on the Petri dish surface was used. The media were incubated at 37 °C (bacteria and fungi) and 25 °C (fungi). After 24-48 hours of incubation (bacteria) or 2, 5 and 7 days (fungi) the colony forming units (UFC) were counted and expressed as UFC/100 mL or UFC/mL, respectively for the membrane and the spread techniques. In the case of fungi, all morphologically distinct colonies were quantified, isolated, purified and maintained (in PDA) until their identification.

3. Results and Discussion

3.1. Water samples bacterial load

In the Chromo medium, large numbers of total coliforms (uncountable, (> 300 CFU/100 mL), *E. coli*, and other colonies of yellow and white color that probably correspond to Actinobacteria and yeasts were detected. In Slanetz medium, only red colonies were detected, which indicate the presence of fecal enterococci. The maximum concentration of *E. coli* and fecal enterococci was obtained in tank 3, with respectively 191 and 282 CFU/100 mL of water (Table 1), values above the recommended guideline for recreational water (the European Union threshold 100 CFU/mL for *E. coli* and the WHO guideline 200 CFU/100 mL for fecal enterococci [10]). The simultaneous presence of *E. coli* and fecal enterococci may indicate recent fecal contamination, as fecal enterococci are able to survive in water longer than *E. coli* [13,14]. In C.L.E.D. medium, used in the clinic, the presence of *S. aureus*, *Proteus* spp., yeasts and Actinobacteria was detected.

It would be expected, due to its location and easy access for people and animals that tanks 1 and 2 would present higher values of FIO than tank 3, located in an interior courtyard. Additionally, tanks 1 and 2 collect water from fountains and rain, unlike tank 3 fed exclusively by rainwater. But, the results point out a higher bacterial load in tank 3 that can be explained by the many bird droppings observed on site, a lower volume of water, a lower rate of water renewal (high retention time).

S. aureus indicates human presence (the inner courtyard is surrounded by offices, a museum and a laboratory). In all the tree tanks *Proteus* was the most represented bacteria. This *Enterobacteriaceae* is a saprophytic mostly associated to animal organic

matter, and is present in mammalian gastrointestinal tract. Also, is often associated or responsible for infections in the urinary tract [15].

Table 1. Presumptive bacteria counting, at 37 °C, by membrane filtration (UFC/100 mL) on the differential media Slanetz and Chromo media, or by spread techniques (UFC/100 mL) on C.L.E.D. medium. Mean values (n=3) ± standard deviation).

Presumptive bacteria	Tank 1	Tank 2	Tank 3
<i>E. coli</i> (UFC/100 mL) ¹	4 ± 2	43 ± 6	133 ± 58
Fecal coliforms (UFC/100 mL) ²	> 300	> 300	> 300
Fecal enterococci (UFC/100 mL) ¹	8 ± 3	3 ± 1	167 ± 115
<i>S. aureus</i> (UFC/mL)	23 ± 15	3 ± 6	77 ± 45
<i>Proteus</i> sp. (UFC/mL)	2690 ± 279	260 ± 46	740 ± 426
Other ³ (UFC/mL)	0	0	27 ± 25

¹fecal indicator organisms (FIO)

²Other than *E. coli*

³such as Actinobacteria and yeasts

3.2. Water samples fungal load

Overall, the total fungi concentrations were low [0-100 UFC/100 mL] and [0-100 UFC/mL], depending on the incubation temperature and the quantification method, and highly variable within the sampling replicas. In general, highest fungal loads were obtained by the inoculum spreading method, compared to the membrane filtration method. Furthermore and in average, at 25 °C more colonies of yeasts and molds grew than at 37 °C. In addition, with YGCA, more fungi were recovered, both at 25 °C and 37 °C. At 37 °C, there was no growth of yeasts in any of the tanks and there was only growth of molds, at 37 °C, in tanks 1 and 3. In Mycosel, it showed more growth at 25 °C, while at 37 °C there was only growth of molds in tank 3.

In all media molds dominate over yeasts. Yeasts weren't recovered from tank 3. This pattern is generally observed in waters from different sources [16,17].

The analysis of the frequency of molds found in the ponds is shown in Figure 2. Clearly, the diversity of fungi and the prevalence of taxa varied among the three water reservoirs. Tank 1 presented higher diversity, than tank 3 and tank 2. Among the identified genera, the ones common to the three reservoirs were also the most frequently: *Penicillium* (14.3-28.6%), *Aspergillus* (9.5-21.4%) and *Fusarium* (3.6-33.3%). These results are in agreement with previous works in fresh water [10,18]. *Aspergillus*, the second most abundant genus in this work, was reported as the most frequent in other studies [19].

The genera *Penicillium* and *Aspergillus* were particularly isolated and include species that can be allergenic or cause human infections. These two genera can be found in environmental samples (soil, water, rhizosphere and air) and produce large amounts of spores [20].

The dematiaceous group had an expressive prevalence in water tanks (21, 24 and 15%, respectively for tanks 1, 2 and 3) with several genera identified: *Phialophora*, *Fonsecaea*, *Rhinochrysiella*, *Ulocladium*, and *Stachybotrys*. Some taxa were isolated only in one of the tanks: Chytridiomycota, *Basidiobolus* sp., *Scopulariopsis* sp. and Oomycota (tank 1), *Rhizopus* sp. (tank 2) and *Acremonium* sp. (tank 3). Dermatophytes were isolated only from tanks 1 (11%) and 3 (14%).

It is important to note that several of the identified taxa are of clinical interest: *Basidiobolus* sp. and several dermatophyte species are pathogens [21], and although *Aspergillus* spp. are opportunistic fungi, *A. fumigatus* is responsible for approximately 90% of diagnosed invasive aspergillosis [22].

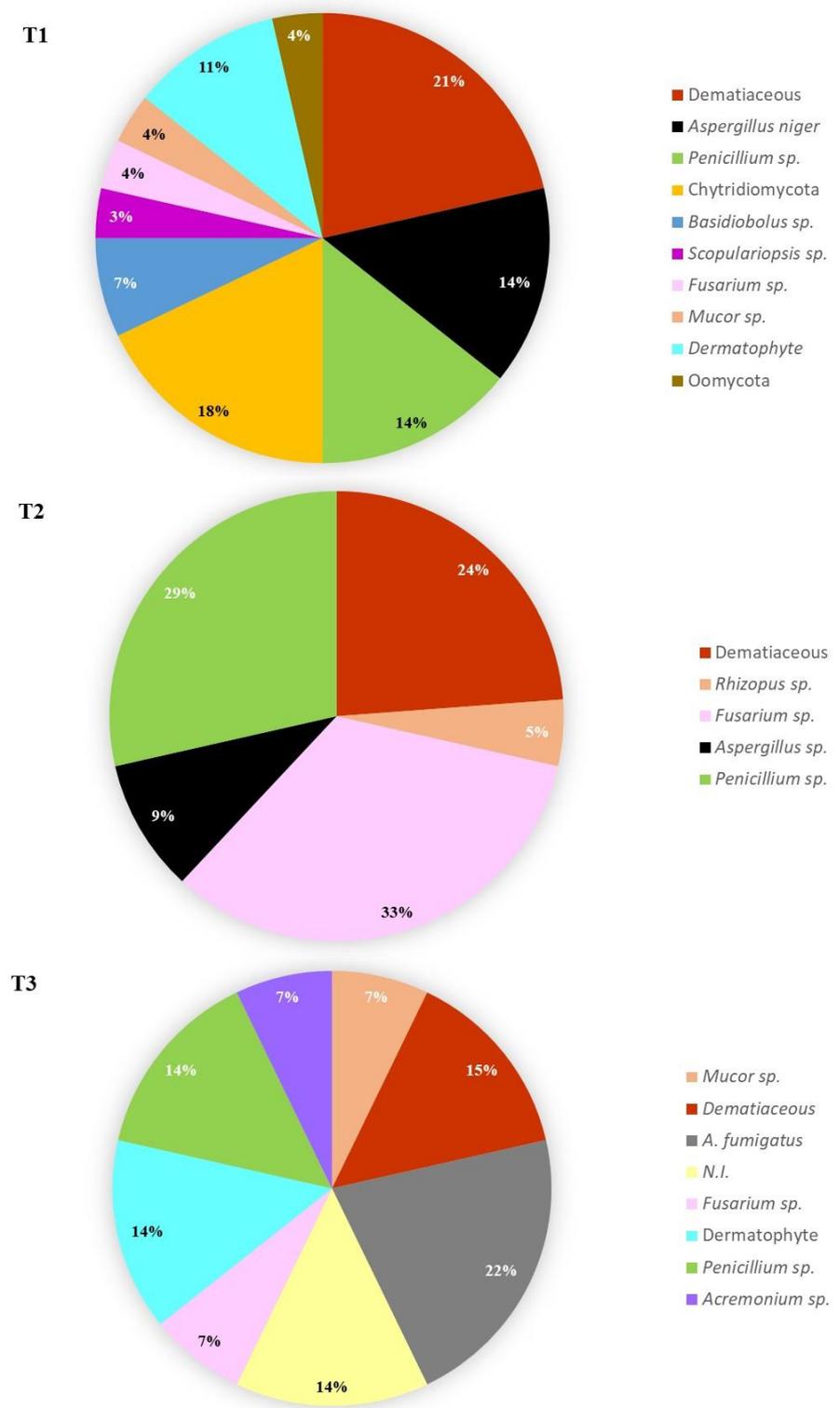


Figure 2. Fungal taxa frequency (%) at tanks (T) 1, 2 and 3. N.I. - Not identified.

4. Conclusions

Small freshwater constructions such as tanks are very frequent in green spaces managed by man, due to their useful, recreational, pleasant and aesthetic values. However, its microbiology is poorly understood. Our results point to the presence of

potential hazard microorganisms in freshwater tanks. The relationship between FIO/other bacteria and bacteria/fungi should be further studied to better understand their significance and potential risks in these largely ignored constructions.

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Data Availability Statement: The raw data are available upon request. Please contact the corresponding author.

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

References

1. Taylor, H. (2003). Surface waters. In *Handbook of Water and Wastewater Microbiology*. Mara, D., Horan, N., Eds.; Academic Press, 2003, pp. 611-626.
2. Ashbolt, N. J.; Grabow, W. O.; Snozzi, M. Indicators of microbial water quality. In *Water quality: Guidelines, Standards and Health: Assessment of risk and risk management for water-related infectious disease*. Fewtrell, L., Bartram, J., Eds.; IWA Publishing, London, 2001, pp 289-316.
3. Kator, H.; Rhodes, M. 8 - Detection, enumeration, and identification of environmental microorganisms of public health significance. In *Handbook of Water and Wastewater Microbiology*. Mara, D., Horan, Nigel, Eds.; Academic Press, 2003, pp. 113-144.
4. Hageskal, G.; Knutsen, A.K.; Gaustad, P.; de Hoog, G.S.; Skaar, I. Diversity and significance of mold species in Norwegian drinking water. *App Environ Microbiol* **2006**, *72*, 7586-7593.
5. Pereira, V.J.; Basílio, M.C.; Fernandes, D.; Domingues, M.; et al. Occurrence of filamentous fungi and yeasts in three different drinking water sources. *Water Res* **2009**, *43*, 3813-3819.
6. Hoffman, J. J.; Burton, M.J., Leck, A. Mycotic keratitis - A global threat from the filamentous fungi. *J Fungi* **2021**, *7*, 273.
7. Brandão, J.; Gangneux, J.P.; Arikian-Akdagli, S. et al. Mycosands: Fungal diversity and abundance in beach sand and recreational waters — Relevance to human health. *Sci Total Environ* **2021**, *781*, 146598.
8. Shearer, C.A.; Descals, E.; Kohlmeyer, B.; et al. Fungal biodiversity in aquatic habitats. *Biodivers Conserv* **2007**, *16*(1), 49-67.
9. Chakrabarti, A.; Shivaprakash, M.R. Microbiology of systemic fungal infections. *J Postgrad Med* **2005**, *51*, 16.
10. WHO. Guidelines on recreational water quality. Volume 1: coastal and fresh waters. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.
11. Ameen, M.; Arenas, R. (2009). Developments in the management of mycetomas. *Clin Exp Dermatol* **2005**, *34*, 1-7.
12. Revankar, S.G.; Sutton, D.A. Melanized Fungi in Human Disease. *Clin Microbiol Rev* **2012**, *25*, 720-720.
13. Boehm, A.B.; Sassoubre, L.M. Enterococci as Indicators of Environmental Fecal Contamination. In *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Gilmore, M.S., Clewell, D.B., Ike, Y., Shankar, N., Eds. Boston: Massachusetts Eye and Ear Infirmary; 2014, pp. 73-90.
14. WHO. Guidelines for drinking-water quality: fourth edition incorporating the first and second addenda. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO.
15. O'Hara, CM; Brenner, FW; Miller JM. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clin Microbiol Rev* **2000**; *13*: 534-546.
16. Arvanitidou, M.; Kanellou, K.; Vagiona, D.G. (2005). Diversity of *Salmonella* spp. and fungi in northern Greek rivers and their correlation to fecal pollution indicators. *Environ Res* **2005**, *99*, 278-284.
17. Hageskal, G.; Lima, N.; Skaar, I. The study of fungi in drinking water. *Mycol Res* **2009**, *113*(Pt 2): 165-72.
18. Gonçalves, A.B.; Paterson, R.R. M.; Lima, N. Survey and significance of filamentous fungi from tap water. *Int J Hyg Environ Health*, **2006**, *209*, 257-264.
19. Kelley, J.; Paterson, R.; Kinsey, G.; Pitchers, R., Rossmoore, H. Identification, significance and control of fungi in water distribution systems. In *Proceedings of the Water Technology Conference, Denver, CO, USA (9-12 November 1997)*.
20. Webster, J.; Weber, R. Introduction to fungi. Cambridge University Press, 2007.
21. Raugi, G.; Nguyen, T.U. 22 - Superficial Dermatophyte Infections of the Skin. In *Netter's Infectious Diseases*, Jong, E.C., Stevens, D.L., Eds.; Saunders. 2012, pp 102-109.
22. Araújo, R.; Pina-Vaz, C.; Rodrigues, A.G. Surveillance of airborne *Aspergillus* in a Portuguese University Hospital. *Mycoses* **2005**, *48*(s2), pp. 45.