Evaluación de sistemas oxidativos basados en luz UV sobre la inactivación y susceptibilidad de hongos patógenos humanos aislados de plantas de tratamiento de la ciudad de Cali.

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ABBREVIATIONS AND SYMBOLS LIST

The abbreviations used in the document are as follows:

- **AOP**: Advanced oxidation processes
- **hv**: Ultraviolet or visible light
- **IFI**: Invasive fungal infection
- **(LEDs):** Light-emitting diodes
- **PMS:** peroxymonosulfate
- **PDS**: Peroxydisulfate
- **UVC**: Ultraviolet light of 254 nm
- **WHO**: World Health Organization
- **WWTP**: Wastewater treatment plant

SUMMARY, KEYWORDS, AND MANUSCRIPT STRUCTURE

SUMMARY

Aquatic systems, such as wastewater and drinking water, serve as reservoirs for pathogenic fungi, including species resistant to antifungals like azoles and echinocandins. In Cali, Colombia, wastewater and drinking water were evaluated, revealing 14 genera and 21 species of yeasts, with *Candida* present in all sampling sites. A 32.7% resistance to fluconazole was observed, influenced by physicochemical parameters and the presence of heavy metals, including Cr, Cd, Fe, Zn, and Pb. Tests with *C. albicans* showed that UVC/peroxide processes follow first-order kinetics, with UVC/H_2O_2 proving most effective, reducing susceptibility to fluconazole from 64 to 8 µg/mL. Yeasts exhibited low reactivation in darkness, indicating that using $^{\bullet}OH$ and $SO_4^{\bullet-}$ radicals is a cost-efficient method for treating resistant yeasts. It is advisable to investigate the spread of resistant communities in the Cauca River and to expand these treatments to hospital systems.

KEYWORDS

Photolysis, UVC/peroxides, antifungal resistance, Candida albicans, aquatic systems.

STRUCTURE OF THE THESIS MANUSCRIPT

The manuscript of this Doctoral thesis was divided into **five (5) chapters**, as follows:

Chapter 1 begins with an introduction that describes the context, the importance of the study, and the problem motivating the research (i.e., Chapter 1 presents the background and theoretical framework of this Thesis).

Chapter 2 presents a systematic literature review of all relevant topics supporting the research, which helped us identify the gaps in the state of the art that need to be addressed in the Doctoral Thesis.

Chapter 3 outlines the experimental plan, which includes the initial phase of the research, practical aspects of isolating and testing the antifungal susceptibility of fungi from aquatic systems in Cali, Colombia, and the application of advanced oxidation processes (AOPs) based on ultraviolet light for inactivating fungi.

Chapter 4 covers the achievement of the specific objectives 1 and 2 of the Doctoral Thesis, which were focused on the characterization of the water samples (influent and effluent) and the identification of the species and the susceptibility to antifungal agents of the isolates from municipal wastewater treatment plants of the city of Cali, Colombia.

- **Chapter 5** addresses the specific objectives 4 and 5 of the Doctoral Thesis, thus presenting the results about the inactivating capability of three oxidation processes based on UV light on a representative resistant-fungal strain, in addition to the determination of the routes of action of the tested AOPs and the modifications of the response to antifungal agents and morphological changes on the representative fungal strain.

CHAPTER 1:

BACKGROUND AND THEORETICAL FRAMEWORK OF THE THESIS

This chapter begins with an introduction, which describes the context, the relevance of the study, the problem that motivates the research of the thesis, provides an introduction to the water pollution and possible solutions (section 1.1), followed by an overview of the problem of fungi in aquatic systems (section 1.2) and the input of antifungals or fungicides into water systems (section 1.3). Then, the concept of advanced oxidation processes based on UV light (Section 1.4), some case studies (Section 1.5), and the thesis hypothesis and objectives (Section 1.6) are presented.

1.1. Introduction

Wastewater pollution, especially from industrial and pharmaceutical activities, poses serious risks to ecosystems and public health. The release of untreated or inadequately treated effluents into bodies of water can introduce toxic substances and pathogens, posing serious risks to human and animal health. [1-3]. Antifungal agents are recognized as emerging micropollutants that, after being consumed, metabolized, and excreted, reach aquatic systems through domestic, hospital wastewater, industrial wastewater, and agricultural runoff, negatively impacting water quality, allowing the adaptation of pathogenic microorganisms, generating molecular mutations, and promoting the evolution of fungal resistance, which affects the efficacy of antifungal treatments [4-6].

In recent years, the use of antifungals in clinical therapy, as well as in the agricultural and industrial sectors, has increased, accompanied by a rise in the number of species exhibiting intrinsic resistance, dose-dependent susceptibility, and acquired resistance to the pharmaceutical products. Fungi have various mechanisms to resist antifungal exposure, including molecular mutations of the antifungal target enzyme, alterations in enzymes related to ergosterol synthesis, and changes in the expulsion pumps: ATP-binding cassette (ABC) and major facilitators (MF) [7 - 9].

As part of the solution to this problem, a fungal degradation method is expected to be used. However, the methods to be used must be efficient and environmentally friendly [10]. Advanced oxidation processes (AOPs) have become in the last decades a good alternative to remove micropollutants by treating water at room temperature and normal pressure, based on the production of highly reactive radicals, especially hydroxyl radicals (HO•) through various chemical, photochemical, sonochemical or electrochemical reactions, thus allowing the effective decontamination of water [11]. Currently, multiple AOPs have been investigated, as evidenced by

the numerous fundamental and applied research papers published on the topic of elimination of organic pollutants [5] [12- 17]. However, to our best knowledge, there are no studies on the use of alternative disinfection methods for inactivating mold or yeast strains resistant to antifungal or fungicides in aquatic systems. Thereby, disinfection of water can be a barrier to limit the spread of antimicrobial-resistant fungi.

1.2. Fungal problems in aquatic systems

Fungi are ubiquitous eukaryotic organisms that, although they offer multiple benefits [18], are associated with approximately 1.5 million deaths and 1.7 billion surface infections per year [19]. The genera most involved in invasive fungal infections (IFIs) include *Candida, Cryptococcus, Aspergillus, Pneumocystis,* and *Fusarium*, with mortality rates ranging from 30% to 88%. For their treatment, there are three main groups of antifungals: polyenes, azoles, and echinocandins [20], each with specific applications depending on the etiological agent [21, 22]. The massive use of antifungals, both in medicine and in agricultural and hygiene products, has generated resistance in fungi, altering the epidemiology of IFIs [23, 24]. Examples include multidrug-resistant *Candida auris* [25, 26] and species such as *C. krusei* and *Aspergillus terreus*, which exhibit intrinsic resistance. Azole resistance in *Candida* and *Aspergillus* occurs by mechanisms such as genetic alterations in ergosterol synthesis and changes in drug efflux [27, 28]. In *Candida* spp., more than 140 mutations have been identified in the ERG11 gene, while in A. fumigatus, more than 30 mutations have been found in the CYP51A gene. Antifungal resistance has increased in the last two decades, affecting both environmental and hospital settings [29-31]. In South America, *C. albicans* and *C. parapsilosis* are prevalent in countries such as Brazil, Argentina, and Colombia. Due to this situation, continuous surveillance, proper diagnosis, and accurate identification of the causative agent are required to ensure timely and effective treatment [26]. Moreover, strategies to deal with Fungi in aqueous matrices are required.

1.3. Problems with the entry of antifungals into water systems.

Fungicides, biocides, and/or antifungals enter the environment due to medical, veterinary, and agricultural practices, which generate fungal resistance to these compounds. Pathogenic and environmental fungi develop resistance mechanisms or select resistant mutants, which impact their evolution, pathogenicity, and ecological habitat [1,8]. Various media contribute to the introduction of resistant microorganisms into the environment. Sewage carries antifungals that are not processed by the human body into sewers, treatment plants, and surface waters, where resistance is observed in filamentous fungi and yeasts. Rivers contaminated with urban and agricultural effluents harbor more resistant microorganisms, which are influenced by industrial waste containing heavy metals [16, 17,32].

Medical wastes and wastewater from hospitals and pharmacies also contribute to the increase in microbial resistance. The demand for antifungal agents for humans and animals is increasing, and discharges from pharmaceutical plants are linked to the development of multiple resistance in organisms. This has led to the implementation of management measures, such as

prudent use of these compounds, surveillance in human and veterinary health, education for farmers and prescribers, and restrictions on the use of growth promoters in animals [33- 34]

Household products such as paints, cosmetics, soaps, and shampoos contain antifungal substances that end up in sewage and wastewater [35]. In agriculture, fungicides are applied to high-value crops, but many of them reach the soil and groundwater [36-42]. In Colombia, a high use of azole fungicides has been reported, some of which are associated with resistance genes in *Aspergillus fumigatus*, such as TR34/L98H and TR46/Y121F/T289A [43, 44]. Studies in Bogota found resistant strains in floriculture soils, highlighting the need for agricultural surveillance and resistance monitoring in clinical isolates [45]. In animal production, antifungals are used to treat infections and in aquaculture, but if not properly managed, they can contaminate soil, leachates, wastewater, and surface water. This underlines the importance of implementing measures to prevent antimicrobial resistance in various sectors [38-40].

1.4. Advanced oxidation processes based on ultraviolet light (UV)

Light is an effective tool for disinfecting aquatic systems, highlighting the use of various technologies and wavelengths. UV radiation has been widely used due to its ability to inactivate microorganisms by physical and chemical mechanisms that directly affect their cellular and genetic structure. Low-pressure (LP) mercury vapor lamps emit monochromatic light at 254 nm, while medium-pressure (MP) lamps generate polychromatic light in the range of 200-600 nm. In addition, light-emitting diodes (LEDs) represent a modern technology that offers advantages such as the absence of mercury, instantaneous warm-up times, longer lifespan, and ease of integration into aquatic systems [46-48]. UVC radiation, with a range of 200-300 nm and an absorption peak at 254 nm, acts directly on the DNA of microorganisms, causing genomic damage by altering nucleotide base pairing [49, 50]. On the other hand, UVA (315-400 nm) inactivates microorganisms through the formation of reactive oxygen species (ROS) and indirect photooxidation reactions that damage DNA and proteins [51-54]. These biological effects include growth reduction, protein denaturation, pyrimidine dimer formation, and cell membrane destruction, ultimately leading to the death of microorganisms [55, 56].

Photolysis of water to generate hydroxyl radicals ($^{\bullet}OH$) (Eq. 1.1)

$$H_2O + hv\ (UVC) \rightarrow {}^{\bullet}OH + {}^{\bullet}H \qquad (1.1)$$

(Far UVC light [< 200 nm] breaks water molecules, forming hydroxyl radicals and hydrogen atoms).

The combination of UV irradiation with hydrogen peroxide (UV/H_2O_2) has proven to be an effective technique for removing contaminants in aquatic systems [57]. This process generates highly reactive hydroxyl radicals by breaking the -O-O- bond of H_2O_2 using 254 nm UV irradiation. The combination of UV irradiation with hydrogen peroxide (UV/H_2O_2) has proven to be an effective technique for removing contaminants in aquatic systems. This process generates highly reactive hydroxyl radicals by breaking the -O-O- bond of H_2O_2 using 254 nm UV irradiation [58-60] (Eq. 1.2).

Photolysis of H_2O_2 under UV light:

$$H_2O_2 + hv\ (UV) \rightarrow 2\ ^{\bullet}OH \quad (1.2)$$

(UV light [254 nm] breaks the O-O bond of hydrogen peroxide, generating two hydroxyl radicals).

Advanced oxidation processes employing sulfate radicals offer significant advantages over those based on hydroxyl radicals, such as their high oxidation potential, which ranges from 2.5 to 3.1 V depending on the activation method used. These radicals stand out for their high selectivity and efficiency in the removal of contaminants, in addition to being effective over a wide pH range and in various aqueous matrices. The main chemicals used to generate sulfate radicals are peroxydisulfate (PDS, $S_2O_8^{2-}$) and peroxymonosulfate (PMS, HSO_5^-). The O-O Bond cleavage in PDS and PMS requires high energy, which can be provided by UVC radiation of 254 nm. In processes involving PDS, oxidizing radicals such as $^{\bullet}OH$ are generated through the interaction of sulfate radicals with water or hydroxide anions, particularly at basic pH. Although PDS and PMS produce small amounts of sulfate radicals at room temperature, this process is more efficient at temperatures above 30°C [61, 62]. (Eq 1.3 -1.5).

Breakage of the O-O bonded by UV light:

$$S_2O_8^{2-} + UV \rightarrow 2\ SO_4^{\bullet-}\ (\text{sulfate radicals}) \quad (1.3)$$

Formation of hydroxyl radicals:

$$SO_4^{\bullet-} + H_2O \rightarrow {}^{\bullet}OH + HSO_4^- \quad (1.4)$$

Interaction with hydroxyl anions:

$$SO_4^{\bullet-} + {}^-OH \rightarrow {}^{\bullet}OH + SO_4^{2-} \quad (1.5)$$

1.5. Case studies of advanced oxidation processes based on UV light.

Regarding the efficacy of UV-based processes, studies have been conducted on different microorganisms (particularly on non-resistant fungi). Nourmoradi et al. investigated the inactivation of *Aspergillus* species in drinking water, observing that turbidity and the presence of ferric ions decrease the efficacy of the process [63]. Pereira et al. evaluated the inactivation of yeasts in various drinking water sources, concluding that species such as *Rhodosporidium* and *Rhodotorula* are more resistant. At the same time, UV fluences below 32 mJ cm^{-2} were able to inactivate 99% of other species, such as *Cryptococcus* and *Candida* [64]. Wen et al. analyzed photoreactivation and dark repair of fungal spores at different temperatures, determining that higher temperatures promote photoreactivation. In contrast, dark repair is low and not significantly affected by temperature [65].

The use of UV LEDs at 255 nm and 265 nm on *Aspergillus* species was investigated by Oliveira et al., who found that LEDs emitting at 265 nm were more effective, achieving significant reductions in fungal viability and causing DNA damage, mitochondrial dysfunction, heat shock

protein production, and inhibition of DNA repair. Additionally, pyrimidine dimer formation was observed to vary with both wavelength and exposure time. Overall, it highlights the efficacy of UV radiation to inactivate microorganisms. These variables affect its effectiveness, such as turbidity and temperature, and the advantages of modern technologies, such as UV LEDs, which offer an effective and sustainable solution for decontamination of aquatic systems [66].

Studies have shown that sunlight (which has some UV components) combined with H_2O_2 can reduce the concentration of this compound from 500 to 5 mg/L, achieving more effective disinfection than in dark conditions. Likewise, it has been demonstrated that low concentrations of H_2O_2, such as 10 mg/L, are sufficient to inactivate chlamydospores of *Fusarium equiseti* in water using a 60 L CPC photoreactor, which highlights the efficiency of this method in practical applications [67].

Research has analyzed the inactivation of fungal spores in groundwater using UV and UV combined with PMS (UV/PMS) [68]. The results show that spore size consistently influences their inactivity, with larger spores being less susceptible to treatment. While UV irradiation damages spore DNA without affecting the cell wall, UV/PMS treatment causes more severe damage, disrupting the cell membrane and cell wall, as well as releasing intracellular materials. It was observed that increasing the PMS dose improves inactivation efficiency, and that UV/PMS is more effective than UV alone [69]. Regarding spore resistance to UV irradiation, *P. polonicum* was found to be the least resistant, followed by *T. harzianum* and *A. niger* [70]. On the other hand, advanced disinfection processes using ultraviolet light-emitting diodes (UV-LEDs) in combination with H_2O_2, PS, and PMS have been evaluated for the inactivation of fungal spores in water. However, the results indicate that the addition of these compounds does not significantly improve the reduction of spore capturability. *A. niger* was shown to be the most UV-resistant species compared to *T. harzianum* and *P. polonicum* [71, 72].

Considering the background and the limited studies on the treatment of resistant fungi using UV-based systems, this thesis presents an opportunity for research, summarized in the hypothesis and aims of the work.

1.6. Hypothesis and aims of the thesis

1.6.1. Hypothesis

UV light-based oxidation processes, such as UVC/PMS, UVC/PDS, and UVC/H_2O_2, can effectively inactivate and modify the antifungal susceptibility response of pathogenic fungal strains resistant to antifungals isolated from aquatic systems in Cali.

1.6.2. General objective

To evaluate the inactivation, reactivation, and susceptibility changes of fungi isolated from wastewater treatment plants in the city of Cali, through oxidation processes based on UV light.

1.6.3. Specific objectives

- Characterize the water samples (influent and effluent), object of study, from the treatment plants of the city of Cali (physicochemical analysis, heavy metals).
- Identify the species and the susceptibility to antifungal agents of the isolated fungi from wastewater treatment plants of the city of Cali.
- Determine the inactivating capability of three oxidation processes based on UV light on a representative resistant fungal strain.
- To establish the routes of action of the tested AOPs, in addition to the modifications of the response to antifungal agents and morphological changes on the representative fungal strain.

1.7. References

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CHAPTER 2.

2. DISINFECTION PROCESSES ARE USED TO ELIMINATE FUNGI IN AQUATIC SYSTEMS

It provides a systematic review of the literature on all relevant topics supporting doctoral research. The first part of Chapter 2 includes a brief introduction, outlining the problem, justification, and objectives of the bibliographic review (Section 2.1), followed by the results and discussion (Section 2.2). Then, the conclusions and perspectives from the literature review are presented (Section 2.3); additionally, some final remarks on the information in Chapter 2 are given in Section 2.4. The methodology is outlined in Annex 1. It is important to note that the information **in this chapter** was published in the paper titled "**Alternative and Classical Processes for Disinfection of Water Polluted by Fungi: A Systematic Review" in Water 2024, 16(7), 936**; doi: **https://doi.org/10.3390/w16070936**).

2.1. Introduction

In this chapter, a literature review is presented about classical and alternative methods for disinfecting water contaminated with fungi, highlighting the importance of addressing antifungal resistance in the context of public health. Classical processes, such as chlorination, ozonation, and UV radiation, as well as advanced oxidation methods (AOPs), including photo-Fenton, photocatalysis, and combinations of UV with peroxides, are discussed. The efficiency of these methods, mechanisms of action, and gaps in the treatment of resistant fungi were evaluated. In addition, challenges such as the lack of information on the elimination of antifungal resistance genes and the need to optimize and scale up AOPs for mass applications are identified. The

potential of solar disinfection (SODIS) as a cost-effective solution in low-income countries is also highlighted. Additionally, it is worth noting that this bibliometric analysis investigated the inactivation of fungi in water, addressing concerns about water contamination by fungi and the development of antifungal resistance. Our revision highlighted key information that identifies a knowledge gap in the use of advanced oxidation processes based on ultraviolet light, leading to the research's problem statement (Table 2.1).

Table 2.1. Previous reviews about the inactivation of fungi by classical and alternative treatments

Main Topics about Fungi Inactivation in Water	Reference
Fungi treatment by classical systems and advanced disinfection processes are discussed. However, kinetics models are not presented.	[20]
Fungi inactivation in drinking water. The main focus is on classical and UV-based processes.	[21]
Disinfection strategies for addressing fungal pathogens in medical devices and surgical instruments (medicine field but no water systems).	[23]
Focus only on sulfate radical-based processes and bacteria more than fungi.	[22]
Treatment of mold spores in the food industry.	[24]
Systematic review of the different classical and alternative processes, and kinetic aspects of water disinfection and antifungal resistance; in addition to a bibliometric analysis on fungi inactivation.	This work

2.2. Results and discussion

A total of 7111 potentially relevant studies were identified through the database search for the years 1983–2022. There were 72 duplicates, and 7061 were excluded based on title and abstract selection. A total of 50 studies were included in the final review, which were conducted in countries from Asia, Africa, Europe, North America, South America, and Central America, as shown in Figure 2.1.

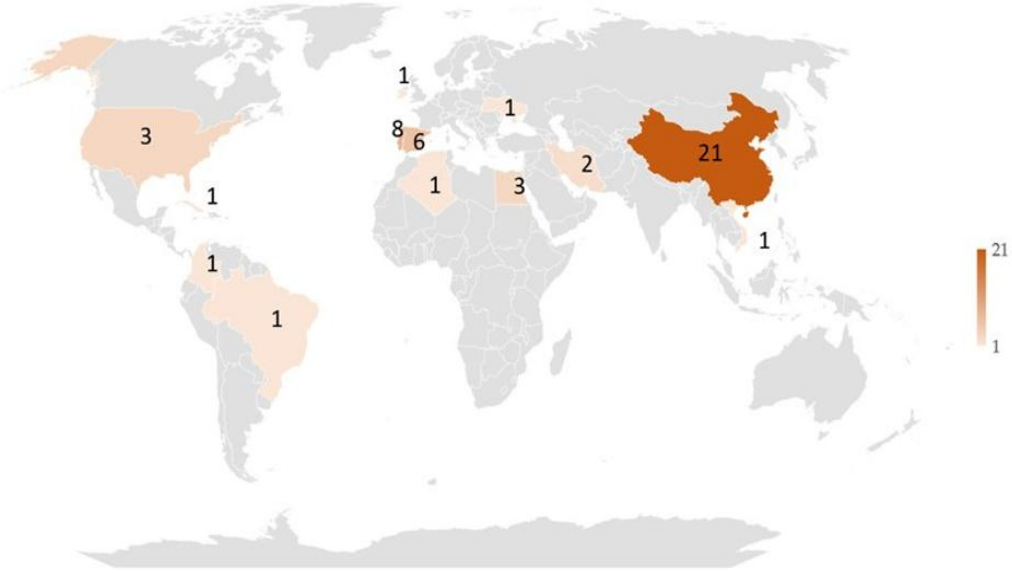

Figure 2.1. Worldwide distribution of studies about classical and alternative disinfection methods for fungi inactivation in aquatic systems. Articles per country: China (21), Iran (2), Spain (6), Portugal, and Colombia (1).

The most significant number of the considered studies came from China (~40.0%). It can be mentioned that studies on aquatic systems (drinking water, hot water systems, groundwater, wastewater, and synthetic wastewater) using classical disinfection methods (13 studies) were conducted in Asia (China, Iran), Europe (Portugal), North America (United States), and Central America (Cuba). Meanwhile, for alternative disinfection processes (37 studies), the study places were distributed in Asia (China, Iran, Vietnam), Europe (Spain, Portugal, Ireland, Ukraine), Africa (Algeria, Egypt), and South America (Brazil, Colombia).

Figure 2.2 shows the annual number of papers about fungal control in the field of disinfection from the first article published in 1983 until 31 December 2022. Figure 2.2 reveals that the number of publications in this field has increased significantly over the last 5 years. Meanwhile, the cumulative annual number of publications is growing slowly. This indicates that research in the field of disinfection is expanding, and it is of great interest due to the importance of controlling pathogenic fungi from a public health and environmental perspective.

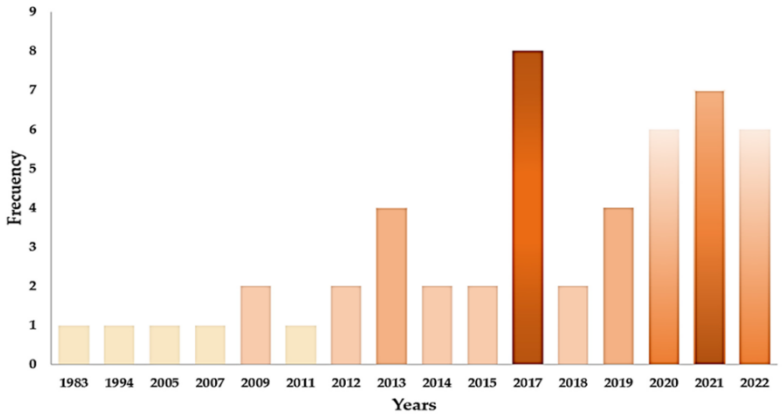

Figure 2.2. Annual distribution of the number of publications on disinfection processes for fungi inactivation.

2.2.1. Characteristics of the Included Studies

Characteristics of the Included Studies Chlorination and ultraviolet (UV) light are the most common classical disinfection methods in aquatic systems, accounting for 63.6% (14/22) of these tools used (Figure 2.3). Meanwhile, for alternative disinfection methods, advanced oxidation processes such as the combination of UVC light with peroxymonosulfate (PMS), peroxydisulfate (PDS), and hydrogen peroxide (H_2O_2) are the most studied techniques (Figure 2.3B). Studies from

Asian countries reported the use of both classical disinfection methods (45.8%) and alternative methods (54.1%). In European countries, works on advanced oxidation processes were higher (68.8%) than those on classical disinfection methods (31.2%).

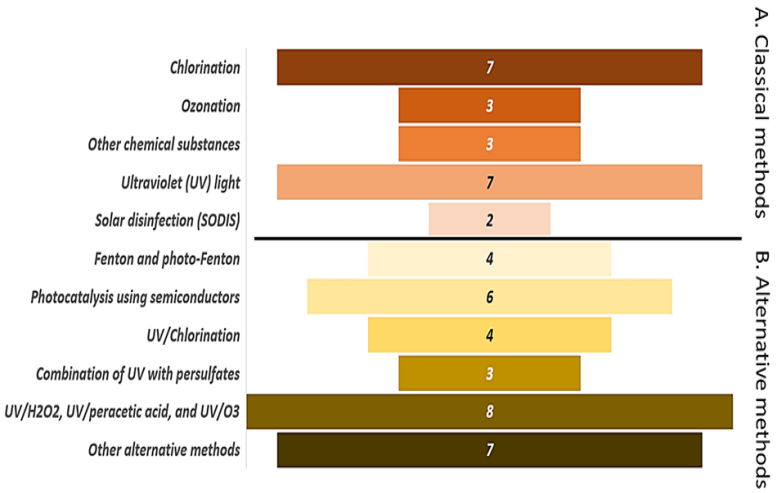

Figure 2.3. Disinfection methods for the treatment of fungi in aquatic systems. (A) Classical methods. (B) Alternative methods

In the Americas, papers from the United States and Cuba described the use of classical treatments (4/6, 66.7%), while works in Brazil and Colombia reported the application of alternative methods (2/6, 33.3%) such as photo-Fenton, TiO_2-photocatalysis, and plasma activation. In turn, studies from African countries only considered alternative disinfection methods. Regarding the fungal strains, the most studied are *A. niger* (16.7% classical vs. 16.4% alternative); *P. polonicum* (13.0% classical vs. 10.9% alternative); *T. harzianum* (9.3% classical vs. 10.9% alternative); and *A. flavus* (5.6% classical vs. 1.8% alternative). In the cases of *C. albicans* (14.5%) and *F. solan*i (9.1%), they were treated with conventional disinfection methods. Figure 2.4. illustrates the log removal rate of fungal species using various alternative and classical disinfection methods. The classical techniques show a higher number of analyzed species, with 77.8%; the fungi *Aspergillus fumigatu*s (4.6 log) and *Penicillium polonicum* (5.0 log) exhibit the highest inhibitions. On the other hand, in the alternative methods, Trichoderma sp. (4.5 log) and *Acremonium* sp. (4.0 log) are outstanding microorganisms.

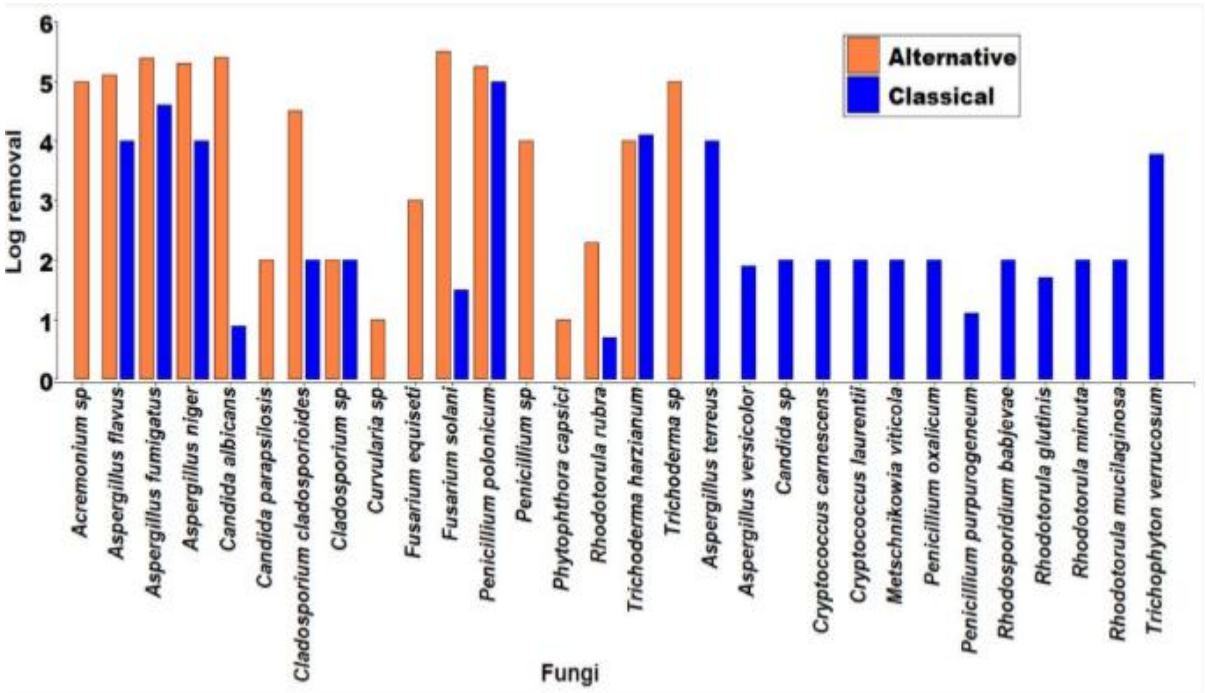

Figure 2.4. Fungal strains have been utilized in both classical and alternative methods for the disinfection of aquatic systems.

2.2.2. The Methods for Fungi Elimination in Aquatic Systems

Conventional and alternative methods (See detailed tables in the Annex 1), which encompass physical, chemical, and physicochemical systems, have been employed to disinfect aquatic systems contaminated with fungi. Some physical techniques transfer the fungi from one phase to another. For instance, coagulation removes both particles and microorganisms from the liquid phase to the solid phase [113]. Meanwhile, in chemical processes, strong oxidants are used, which can react with the basic components of the fungal cell or alter its metabolism, ultimately inactivating it. However, oxidative reactions do not specifically affect one substrate, and other organic and inorganic components in the water matrix may also be involved in the process [114-117]. Furthermore, only one method of treatment can have drawbacks (such as the tolerance of microorganisms to the process action, or unsafe/limited operational procedures), so the combination of two or more processes has been proposed, resulting in novel and alternative systems [9,17,118]. Below is a review of the utilization of classical processes (e.g., chlorination, ozonation, and ultraviolet irradiation (UV)) and alternative methods (e.g., UV/chlorine, UV/ozone, UV/persulfates, photo-Fenton, and heterogeneous photocatalysis) for the inactivation of fungi in aqueous matrices.

2.3. Conclusions and Outlooks

The use and research on classical and alternative methods of disinfection in aquatic systems (sewage, groundwater, and drinking water, among others) have become assertive actions for the

reduction in fungal loading and, consequently, the decrease in the risk for human and animal health, as well as the contribution to limiting the development of drug-resistant fungi.

From the revised works, it was evident that the structure/species of the fungi plays a significant role in disinfection kinetics, particularly in the application of classical methods. Furthermore, in classical treatments, such as chlorination or ozonation, the concentration of the oxidant is crucial for disinfection. The coupling of methodologies allows for improved disinfection rates. On the other hand, in the AOPs, fungi inactivation occurs, either through direct DNA damage or indirect damage caused by highly reactive radicals, which can lead to substantial damage to both external and internal cell components. Additionally, it is worth noting that solar disinfection techniques are gaining popularity and offer a viable alternative for low-income sites.

Research focused on advanced oxidation processes (UV/hydrogen peroxide, UV/ozone, photo-Fenton, or UV/persulfates) needs to be optimized and scaled up for applications toward the inactivation of fungi in wastewater or drinking water. In addition, advances in the synthesis and use of new materials for disinfection, in both homogeneous and heterogeneous systems (e.g., PMS/metal oxides, MTUF-Ag, graphene oxide/Fe_3O_4/Ag, $CoxNi^{1-}x/Fe_2O^4/SiO_2/TiO_2$), are being discussed. However, in-depth studies using these materials are needed to clearly understand their environmental impacts, reuse, and recovery from aqueous samples, as well as their feasibility for large-scale applications. Furthermore, the literature on the coupling/merging of diverse AOPs for inactivating fungi in water remains scarce. Thus, future workers should consider all these lacking topics. Furthermore, additional studies on SODIS for the inactivation of fungi in water are necessary. This method is promising for widespread application in low-income countries.

We should also note that several reports have established the application of a wide variety of methods for inactivating fungi, primarily focusing on commercial strains or those isolated from surface water. Further work should consider strains from hospital wastewater. Additionally, it is worth noting that in 2022, the World Health Organization (WHO) introduced the first list of priority fungal pathogens to guide research and development of public policies aimed at mitigating invasive fungal infections, particularly those caused by drug-resistant fungi. Several pathogenic fungi are considered potential environmental contaminants in aquatic systems, posing a risk to human health, particularly among immunocompromised populations.

From the elaboration of this review, it was found that despite the use of classical and alternative methods as effective disinfection processes for fungi inactivation, there is a lack of information on antifungal susceptibility after treatments, pathogenicity, and resistance genes in fungi circulating in water. It is worth noting that the key role of wastewater in the evolution and spread of antibiotic resistance is becoming increasingly evident. In addition, the ability of disinfection processes to inactivate antibiotic-resistant bacteria and eliminate their resistance genes has been demonstrated. However, this is not the case with fungal studies. It has only been possible to present the degree of inactivation, damage to the cell wall and membrane, attack on DNA, and the respiratory chain of fungal spores. This review has also demonstrated that, although resistant fungi can be found in aquatic systems, there are knowledge gaps concerning the inactivation of antifungal-resistant microorganisms in water that need to be addressed. Thereby, a future

research question would be: Are disinfection methods capable of eliminating anti-fungal-resistant pathogenic fungi and decreasing the genetic determinants of antifungal resistance?

2.4. Final remarks of Chapter 2

Antifungal resistance has profound public health implications, especially for immunocompromised individuals, such as patients with chronic diseases, neonates, older adults, and transplant recipients. Antifungal-resistant fungi, such as *Candida auris* and *Aspergillus fumigatus*, are associated with invasive infections that have high mortality rates (30-88%) and represent a therapeutic challenge due to the limited efficacy of available treatments. Additionally, resistance can arise from the overuse of antifungals in agriculture, industry, and medicine, which contributes to the spread of resistance genes in the environment, including aquatic ecosystems.

This increases the risk of exposure to resistant fungi in drinking water sources, hospitals, and wastewater, which can facilitate the transmission of infections that are difficult to treat. Therefore, it is crucial to implement responsible antifungal management strategies, improve disinfection systems in contaminated waters, and develop public policies to mitigate antifungal resistance and protect public health.

From the literature review, it is evident that both classical and alternative disinfection methods have proven effective in reducing the fungal load in aquatic systems, thereby reducing health risks and limiting the development of antifungal resistance. However, the structure and species of the fungi influence the efficacy of treatments, and there is still a lack of studies on resistant fungi, resistance genes, and their treatment in aqueous matrices. Moreover, it is recommended to enhance the disinfecting performance, integrate advanced oxidation processes, and conduct further studies on disinfection in low and middle-income regions (as Colombia), to explore the capacity of the easy operation and low-to-moderate cost processes to eliminate resistant fungi and evaluate the changes in the susceptibility to antifungals after treatments.

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CHAPTER 3.

3. METHODOLOGY

This chapter outlines the experimental plan conducted in the thesis, which consisted of four sections. The first section presents research activities related to physicochemical variables and the concentration of heavy metals in the aquatic systems of the city of Cali (section 3.1). The second section describes the methodology for isolating and testing the antifungal susceptibility of fungi isolated from aquatic systems in Cali (section 3.2). Then, the third section presents experimental components related to the application of advanced oxidation processes (AOP) based on ultraviolet light for the inactivation of a target relevant fungus (section 3.3). Finally, section 3.4 outlines the methodological aspects of data analysis.

3.1. First phase: Physicochemical variables, concentration of heavy metals in aquatic systems of the city of Cali

In this study, the concentration of heavy metals in aquatic systems was investigated in four water systems of the city of Cali, focusing on physicochemical parameters (Figure 3.1). Samples were taken from two types of water: drinking water (Meléndez River, drinking water treatment plant "Puerto Mallarino" in the Cauca River) and wastewater (South Channel of the Cauca River, "Cañaveralejo-PTAR" wastewater treatment plant) [1].

Physical-chemical parameters and heavy metal concentrations were analyzed. Samples were collected at a depth of approximately 0.5 m, as far from the shore as possible, taking care to avoid disturbing the bottom and staying clear of backwaters and stagnant areas. They were then immediately transported to the laboratory in a cooler for processing and analysis, with the process starting within six hours of collection. One liter of water samples (in triplicate) was divided into two equal 500 mL portions—one stabilized with nitric acid for heavy metal analysis, and the other used for physical-chemical parameter analysis. These samples were kept at 4 °C for no more than two days before testing. Standard procedures were used to determine the physical-chemical parameters and heavy metal concentrations. The parameters measured included total phosphorus, total dissolved solids, nitrate, total nitrogen, conductivity, and temperature. The heavy metals analyzed were iron, silver, copper, nickel, lead, mercury, cadmium, and zinc [2-5]

Environmental metadata measurements were taken at the time of sampling, along with the sample material. These data were incorporated into subsequent analyses and correlation models. The metadata included dates and times, latitude and longitude, ambient, sample, and storage temperatures, conductivity, pH, and relative humidity [2].

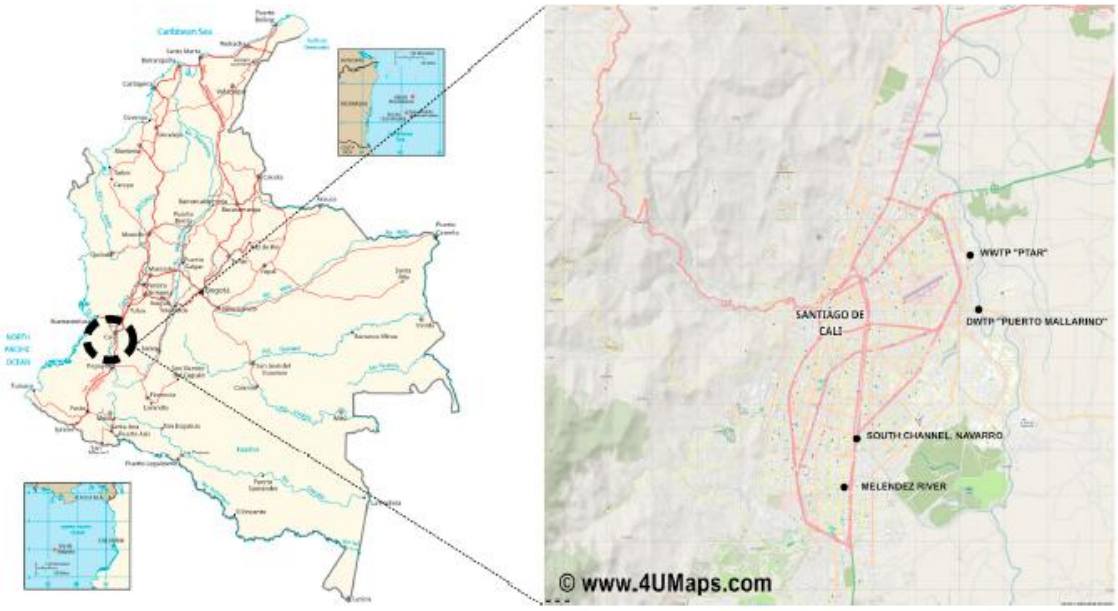

Figure 2.1. Sites of water sampling. Treatment plant “Puerto Mallarino”, Cauca River; WWTP: wastewater treatment plant “PTAR”; and Melendez River. The map on the right was modified from www.4UMaps.com (accessed October 21, 2022) under the license Data CC BY-SA by OpenStreetMap.

3.2. Second Phase. Isolation and antifungal susceptibility of fungi isolated from aquatic systems in Cali

Serial dilutions (10^{-1}–10^{-3}) were made with peptone water (Oxoid Ltd., Hampshire, United Kingdom). Equal volumes (200 µL) from each dilution were spread over the surface of Dicloran Rose Bengal Chloramphenicol (DRBC) (Merck, Darmstadt, Germany) and CHRomagar Candida™ agar plates (BBL International Inc., London, UK). All the plates were incubated for 48 h at 30 °C. Colony counts were performed on individual plates. Representative yeast colonies were selected and grouped by morphotype, isolated, and conserved using Sabouraud Dextrose Agar (Merck), immersed in sterile mineral oil, and cryopreserved in 30% glycerol (*v*/*v*). A macroscopic evaluation of colonies was performed with a stereoscope (Celestron Lab’s S10-60 Stereo, Celestron, LLC, Torrance, CA, USA), taking into account the shape, edge, surface, appearance, elevation, brightness, and consistency. All the yeasts were evaluated for sugar assimilation using the API 20C AUX system (BioMérieux, Marcy l’Etoile, France) according to the manufacturer’s instructions. Several carbohydrates were evaluated, including D-Glucose, Glycerol, 2-keto-Gluconate calcium, L-Arabinose, D-Xylose, Adonitol, Xylitol, D-Galactose, Inositol, D-Sorbitol, Methyl-D-Glucopyranoside, N-Acetyl-Glucosamine, D-Lactose (Bovine), D-Maltose, D-Sucrose, D-Trehalose, D-Melezitose and D-Raffinose [5]. The galleries were incubated

at 30 °C for 72 h in an airtight box containing a small volume of water to create a humid atmosphere. The observation of yeast growth was considered positive. The negative control contained no carbon source, and the positive control contained glucose. The carbohydrate assimilation profile obtained for each tested isolate was interpreted using ApiwebTM software (BioMérieux, reference: 40011) [1]

For DNA extraction, each yeast liquid culture was centrifuged for 2 min at 12,000× *g* and the supernatant were discarded. DNA was extracted using Kit GeneJet (Thermo Fisher, Waltham, MA, USA) and resuspended in 50 µL of TE (10 mM Tris, 1 mM EDTA, pH 7.4). DNA was visualized on a 1% (*w*/*v*) agarose gel, and its concentration and purity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The ITS1-5.8-S-ITS2 (Internal transcribed spacer, ITS) and D1/D2 domain of the large subunit of the ribosome (LSU) regions were amplified using the polymerase chain reaction (PCR) procedure in a final volume of 25 µL. A total of 5 µL of genomic DNA (approximately 1 ng/µL) was taken and resuspended in 20 µL of the PCR mixture comprising 0.4 µL primer Forward 20 pmol/µL, 0.4 µL primer Reverse 20 pmol/uL, 1 µL 1 mM dNTPs, 1.5 mL MgCl2 50 mM 0.2 µL sterile milli-Q H2O, 0.6 µL dimethyl sulfoxide (DMSO), 0.2 µL of Taq Polymerase (5 U/µL) (Bioline, London, United Kingdom), and 2.5 µL of 10X Buffer. We used ITS5/ITS4 primers for the ITS region and NL1/NL4 primers for the LSU region. PCR products were analyzed using 1.5% (*w*/*v*) agarose gel electrophoresis in 1X TAE buffer (tris base, acetic acid, EDTA, distilled water), and run at 100 V for 1 h. To visualize band migration, the gel was stained with SybrGreen (LONZA) and observed under UV light. A 100-bp or 1-kb ladder (Gibco BRL, Burlington, ON, Canada) was used to estimate amplicon size. Amplification products were purified and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) at CorpoGen company. Subsequently, the sequences were edited, assembled, and compared in Genbank and Mycobank databases using the Basic Local Alignment Search Tool (BLAST) algorithm. A 98.41% or 99.5% threshold was used for identification at the taxonomic level of species for ITS and LSU, respectively [6].

The antifungal susceptibility test was performed using the broth microdilution technique, following the M27-A3 guidelines from the Clinical and Laboratory Standards Institute (CLSI) [7]. Amphotericin B (AMPB, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in dimethyl sulfoxide and fluconazole (FCZ, Pfizer Central Research) was suspended in sterile water. Microtiter plates with 96 round-bottom wells were prepared with a range of final concentrations from 0.03 to 16 µg/mL for Amphotericin B and from 0.125 to 64 µg/mL for FCZ. Serial two-fold dilutions of the various drugs were prepared in RPMI 1640 medium (with L-glutamine, without bicarbonate; Sigma Chemical Co.) and buffered to pH 7.0 using 0.165 M [N-morpholino]propane-sulfonic acid solution (MOPS; Sigma Chemical Co.). The yeast suspension was made at a McFarland concentration of 0.5 (adjusted to final concentrations of approx. 2.5×10^3 CFU/mL). The inoculated plates were incubated for 24–48 h at 35 °C. Strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22,019 were used as controls to detect any abnormalities and deactivation of the antifungal. To determine the MIC, the concentration that produced a reduction in yeast growth of ≥50% compared to control growth without FCZ and total absence of growth for AMPB after 48 h of incubation was considered. Interpretations of the results were based on breakpoints provided

by M57S and M27M44S of CLSI [8,9]. Isolates were classified as sensitive (S), dose-dependent sensitive (DDS), and resistant (R).

Physico-chemical parameter analyses (temperature, pH, turbidity, and electrical conductivity) were determined in situ using a 350 multi-parameter probe (Merck, Germany). Water samples were transported on ice to the laboratory for analysis of salinity, solid particles, dissolved oxygen, total phosphorus, and nitrites, as described in the current National Drinking Water Quality Standard (GB5749). The concentrations of the seven heavy metals iron (Fe), copper (Cu), lead (Pb), cadmium (Cd), zinc (Zn), silver (Ag), and chromium (Cr) were determined using a flame atomic absorption spectrophotometer (Model ZEEnit 700P, Analytik Jena, Germany) [2,5].

3.3. Third phase: Application of advanced oxidation processes (AOP) based on ultraviolet light for the inactivation of a representative resistant fungus.

To achieve the specific objectives related to advanced oxidation processes based on UV light, the following procedure was applied: i. Selection of operating conditions, ii. evaluation of the ability of the systems to inactivate the fungal strains, iii. Identification of inactivation routes, iv. Evaluation of the antifungal activity, and v. Analysis of the damage caused by the different treatments on the fungal surface structures.

The operational conditions were selected based on information from the literature. It was decided to use a homemade aluminum reflector box equipped with a UVC lamp (OSRAM HNS®) of 8 W and maximum emission at 254 nm, whose volumetric photon flux was 2.19×10^{15} Einstein/Ls (Intensity 2.3 W cm^{-1}). Three different inorganic peroxides (H_2O_2, PDS, and PMS) at a concentration of 500 µmol L-1 were considered (this concentration was chosen based on previous works [10].

The aqueous solution of isolated target fungus to be treated consisted of 5 mL of the yeast inoculum with 45mL of sterile type 1 water (pH 7.2 ± 0.2) and constant agitation (200 rpm) at 25 ± 2 °C, according to protocol of Serna-Galvis et al. [17, 18], was left for 30 min in darkness and three serial dilutions were made in physiological saline (10^{-1} to 10^{-4}) to check the concentration of the inoculum (Time 0). Then, it was exposed to processes. Sampling was performed at 15, 30, 45, 60, and 80 s, and serial dilutions (10^{-1} to 10^{-4}) were performed. Each disinfection experiment was carried out in triplicate [10, 11]. After incubation time, CFU mL^{-1} were counted, and yeasts that survived the treatments were observed under a compound binocular microscope at 400X magnification. Moreover, several images were taken at various stages of the processes action to monitor changes in the yeast surface structures (morphology).

After the exposure to UVC and UVC light plus the three peroxides at a concentration of 500 µmol L^{-1}. Some surviving yeasts were taken and seeded in Sabouraud broth. They are left in incubation for 5 days. and then, reseeded in solid medium to confirm yeast regrowth [5-8]. For the treated yeasts that regrew in Sabouraud broths, the study of susceptibility to the reference antifungal was conducted to determine if there were changes in the original cut-off points. The

macrodilution technique, as standardized by the CLSI for the study of antifungal susceptibility (Documents M27-A3, M38-A, and M44-A), was used [12-16].

The different treatments were applied under the established conditions. Inactivation and reactivation percentages, as well as disinfection rate constants, were evaluated, along with changes in yeast susceptibility patterns resulting from different treatments involving UVC alone and UVC combined with the peroxides. Also, the inactivation pathways were investigated through control experiments and the use of radical-scavenger agents, such as ethanol [17]. More experimental details are provided in annex 4.

3.4. Data analysis

Correlation tests were performed to investigate the relationship between physicochemical parameters, heavy metal concentrations, and fluconazole susceptibility values for the different yeast species. Principal component analysis (PCA) was performed to determine patterns and relationships in the data. Data output was provided in the form of correlation biplots using the packages FactoMineR and ggrepel for R v4.1.1. The correlation significance level was set at $p < 0.05$. A comparison between the different sampling points for each variable was performed using Kolmogorov–Smirnov tests ($p < 0.05$).

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CHAPTER 4.

4. YEAST FROM WATER SYSTEMS OF THE CITY OF CALI, COLOMBIA

Chapter 4 is focused on the achievement of the specific objectives 1 and 2 of the doctoral thesis. The first part provides an introduction, briefly describing the problem and justification of the work (Section 4.1), followed by the results presented description (Section 4.2) and the corresponding discussion of the results (Section 4.3). Then, the conclusions (Section 4.4) and remarks of Chapter 4 (Section 4.5) are shown.

-The information presented in this Chapter was published in the article entitled "***Water Quality, Heavy Metals, and Antifungal Susceptibility to Fluconazole of Yeasts from Water Systems"***, Int. J. Environ. Res. Public Health 2023, 20, 3428. doi: https://doi.org/10.3390/ijerph20043428

4.1. Introduction

The influence of physicochemical parameters and heavy metals on the levels of yeasts revealed significant associations, suggesting that these factors may modulate antifungal resistance and the presence of yeasts in aquatic systems in Cali, Colombia, as well as their resistance to antifungals. This highlights their relevance for public and environmental health. Yeasts, especially of the genus *Candida*, were identified in different water sources, including drinking and wastewater treatment plants, as well as rivers. Herein, the susceptibility to fluconazole and amphotericin B was evaluated, finding a worrying resistance to fluconazole, while all yeasts were sensitive to amphotericin B. Additionally, it is noted that the city's aquatic systems discharge their contents into the Cauca River, which could spread resistant yeasts to other areas, thereby increasing the risk of invasive infections in humans and animals. The importance of including yeast counts in routine water quality analyses and updating microbiological regulations to address this issue was detailed. The need for further studies to assess the impact of resistant yeasts on the environment and human health, as well as to develop public policies that promote the sustainable management of aquatic ecosystems, is also emphasized. The objective of this part of the thesis was to evaluate the water quality, heavy metal concentrations, and antifungal susceptibility of yeasts present in aquatic systems in Cali, Colombia, including wastewater and drinking water.

4.2. Results

Yeast counts varied among samples when cultured on a DRBC medium. There were no significant differences in the number of yeasts at each sampling site during the dry and wet seasons. A higher concentration of yeasts was identified in the intake wastewater from the “PTAR” WWTP (2.4 x 10^5 CFUmL^{-1}), while the Melendez River had a lower concentration (<1.0 x 10^3 CFU mL^{-1}). The “Puerto Mallarino” DWTP and South Channel presented intermediate concentrations of yeasts (7.7 × 10^4 CFUmL^{-1} and 3.6 × 10^4 CFUmL^{-1}, respectively). In the present study, we recorded a total of 73 yeast isolates from all sources. Sixty-eight yeast isolates were identified, belonging to 14 genera and 21 species; five isolates were not identified. The genus *Candida* represented most of the yeast isolates. It was interesting that elevated levels of *Candida* species (8 × 10^3 CFUmL^{-1}) were also detected in the samples from the “PTAR” WWTP. Chromagar Candida medium showed colonies of three morphotypes: *C. albicans, C. tropicalis*, and *Pichia kudriavzevii* (syn. *C. krusei*). Among the yeast species identified, *Rhodotorula mucilaginosa* (13.7%), *Candida* sp. 1 (8.2%), and *C. tropicalis* (8.2%) were the most frequent (Figure 4.1)

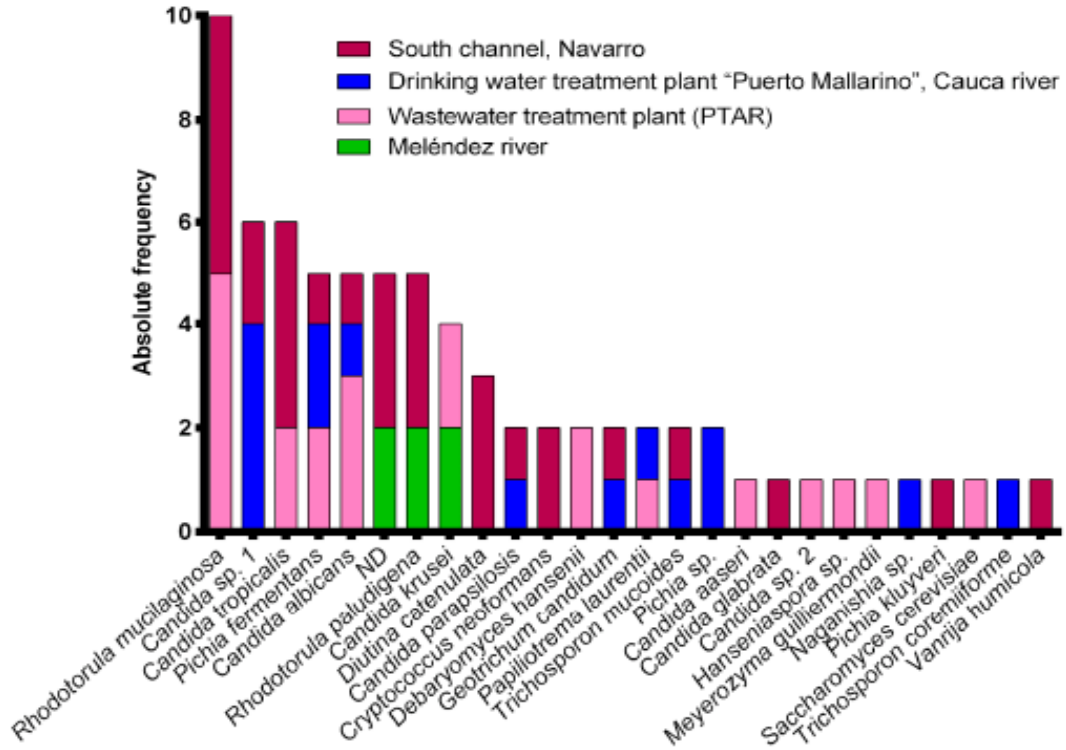

Figure 4.1. Absolute frequency of yeast species found in different aquatic systems in Cali, Colombia.
ND = not determined.

However, several isolates were not identifiable to the taxonomic level of species according to the criteria established by Vu et al. [29] using sequence analysis. In this sense, six strains marked as *Candida* sp. one were closely related to C. intermedia, while the strain Candida sp. P46 (*Candida* sp. 2) was closely associated with *C. pseudolambica*—the strain *Naganishia* sp. PM20 was related to *N. diffluens*, and two strains of the genus Pichia were associated with *P. fermentans*, with 94.87% of sequence identity compared to the type strain of the species.

The antifungal susceptibility of 49 yeast isolates was assessed after 24–48 hours of growth. The reading of the biomass appearance was carried out with the aid of an inverted mirror. The minimum inhibitory concentration (MIC) breakpoints for FCZ for *C. albicans*, *C. parapsilosis*, and *C. tropicalis* isolates with MIC ≤ 2 μg mL^{-1} were considered susceptible, those with MIC 4 μgmL^{-1} were considered susceptible dose-dependent (DDS), and those with MIC ≤ 8 μgmL^{-1} were considered resistant; *C. glabrata* isolates with MIC 32 μgmL^{-1} were considered DDS, while isolates with MIC 64 μgmL^{-1} were considered resistant. All *P. kudriavzevii (C. krusei*) and *Rh. mucilaginosa* isolates were considered resistant regardless of the MIC value. In the case of amphotericin B, yeasts with an epidemiological cutoff value MIC ≤ 2 μg mL^{-1} were considered susceptible. Yeast isolates with sensitivity to FCZ corresponded to 49%, followed by resistant isolates (32.7%), and dose-dependent sensitive isolates (18.4%). All yeast strains were sensitive to AMPB (Table 4.1).

Within each aquatic system, the ratios of susceptibilities varied for FCZ. In the South Channel, the ratio of sensitivity was higher (59.1%) compared to other susceptibilities, with the least resistance to FCZ (27.3%). On the other hand, the ratio of sensitivity from the “Puerto Mallarino” DWTP was 36.4% and the percentage of resistant yeasts was 36.4%. This resistance ratio was similar at the “PTAR” WWTP (35.7%). We found variations in sensitivity to FCZ among *C. tropicalis* isolates according to the site sampled; for example, at South Channel, there were sensitive and DDS strains, but at “PTAR” WWTP, we found sensitive and resistant strains (i.e., P4). A similar trend was observed for *C. albicans*. For example, strains CS55 (South Channel) and P1 (WWTP) showed an MIC of 64 and 8μg mL^{-1}, respectively. However, strains P13B (WWTP) and PM22 (DWTP) were DDS and sensitive, respectively, suggesting variability among populations of this species.

Table 4.1. Minimal inhibitory concentrations (MIC) of yeasts associated with water systems in Cali against Fluconazole (FCZ) and Amphotericin B (AMPB).

Strain	Species	Susceptibility to Fluconazole	FCZ MIC (µg/mL)	AMPB MIC (µg/mL)
CS11	*Rh. paludigena*	Sensitive	2	2
CS14	ND	Sensitive	2	2
CS15	*V. humicola*	Sensitive	0.5	0.25
CS16	*C. parapsilosis*	Sensitive	2	0.5
CS17	*D. catenulata*	Sensitive	0.5	1
CS18	*D. catenulata*	Sensitive	0.5	0.5
CS19	*C. tropicalis*	DDS	4	2
CS1A	*C. glabrata*	DDS	4	0.5
CS1B	*Rh. mucilaginosa*	Resistant *	1	2
CS21	*P. fermentans*	DDS	4	2
CS22	ND	Sensitive	0.25	2
CS23	*Cr. neoformans*	Sensitive	0.125	2
CS24A	*Cr. neoformans*	Sensitive	2	1
CS4	*T. mucoides*	Sensitive	2	2

Table 1. *Cont.*

Strain	Species	Susceptibility to Fluconazole	FCZ MIC (µg/mL)	AMPB MIC (µg/mL)
CS45	*P. kluyveri*	Sensitive	0.5	0.5
CS51A	*D. catenulata*	Resistant	8	2
CS51B	*C. tropicalis*	Resistant	16	2
CS55	*C. albicans*	Resistant	64	2
CS7	*C. tropicalis*	Sensitive	1	2
CS7B	*G. candidum*	Resistant	2	2
CS7C	ND	Resistant	64	2
CS9	*Candida* sp. 1	Sensitive	0.25	1
M2	ND	Sensitive	2	2
M12	ND	Resistant	64	2
P1	*C. albicans*	Resistant	8	1
P13B	*C. albicans*	DDS	4	1
P14	*C. aaseri*	Sensitive	0.5	0.5
P16A	*C. tropicalis*	Sensitive	2	2
P16B	*P. laurentii*	Sensitive	0.5	2
P20A	*D. hansenii*	DDS	4	2
P22	*P. kudriavzevii* (*C. krusei*)	Resistant *	4	1
P24A	*D. hansenii*	Resistant	64	0.5
P24B	*Rh. mucilaginosa*	Resistant	64	2
P3	*S. cerevisiae*	DDS	4	2
P3A	*Hanseniaspora pseudoguilliermondii*	Sensitive	1	2
P4	*C. tropicalis*	Resistant	64	0.5
P46	*Candida* sp. 2	Sensitive	0.25	2
P9A	*P. fermentans*	Sensitive	2	2
PM14	*G. candidum*	Sensitive	0.5	1
PM15	*Pichia* sp.	DDS	4	2
PM18	*T. coremiiforme*	Sensitive	2	0.5
PM19	*P. fermentans*	DDS	4	2
PM20	*Naganishia* sp.	Sensitive	0.125	2
PM22	*C. albicans*	Sensitive	0.5	2
PM24	*Candida* sp. 1	DDS	4	2
PM4A	*T. mucoides*	Resistant	8	2
PM4B	*P. laurentii*	Resistant	16	2
PM54	*Candida* sp. 1	Resistant	64	0.5
PM54A	*C. parapsilosis*	Resistant	16	2

* Due to intrinsic resistance, these strains were marked as resistant, despite the observed MIC. Yeast strains labeled with “CS” were isolated from the South Channel, Navarro; yeast strains labeled with “P” were isolated from the “PTAR” WWTP; yeast strains labeled with “PM” were isolated from the “Puerto Mallarino”

DWTP, Cauca River; and yeast strains labeled with “M” were isolated from the Melendez River. ND: not determined. DDS: dose-dependent Sensitive.

In the present study, PCA was used to establish associations between physico-chemical parameters, the concentrations of heavy metals, and yeast levels from different sampling sites. Table 4.2 shows the correlation between heavy metals and physico-chemical parameters of water samples and the MIC of FCZ-resistant yeasts. High correlation values ($R^2 > 0.5$, $p < 0.05$) were obtained for the ratio of iron to *T. mucoides* and *D. catenulate*; zinc to *C. parapsilosis* and *D. catenulata*; iron to *C. parapsilosis*; Cd to *C. albicans*, Rh. *mucilaginosa*, and Candida sp 1; phosphates to *D. hansenii*; nitrates to *Rh. mucilaginosa*; pH to *C. tropicalis* and *D. hansenii*; conductivity to C. parapsilosis; and temperature to *C. parapsilosis* and *C. albicans*. Figure 4.2 depicts the distribution of the physico-chemical parameters and heavy metal concentration variables formed by the first two axes, which explained 81.0% of the total variance. A positive association was observed between yeast levels with total dissolved solids and nitrate levels in the WWTP, and with conductivity in the South Channel. The correlation between the levels of antifungal-resistant yeasts and the presence of heavy metals varied between the sampling sites. Yeast levels of *C. albicans*, *C. parapsilosis*, *Candida* sp. 1, *P. fermentans*, *T. coremiiforme*, *P. laurentii*, and *G. candidum* were influenced by the presence of Pb in the “Puerto Mallarino” DWTP. Furthermore, *C. albicans*, *C. tropicalis*, *C. krusei*, *C. aaseri*, Candida sp. 2, *P. fermentans*, *S. cerevisiae*, P. *laurentii,* and *D. hansenii* were influenced by Cr in the “PTAR” WWTP. Finally, *C. parapsilosis*, *C. tropicalis*, C. *krusei*, *C. albicans*, *Candida* sp. 1, *P. fermentans*, *D. catenulata*, *Rh. mucilaginosa*, *Cr. neoformans*, *V. humicola*, and *G. candidum* were influenced by Zn and Cu in the South Channel. We also found significant correlations between specific heavy metals and some specific fluconazole-resistant yeast species.

Table 4.2. Correlations between physico-chemical parameters, metal concentrations, and MIC of fluconazole-resistant yeasts.

Heavy Metals/ Parameters	*Cp*	*Ct*	*Ca*	*Pk*	*Rhm*	*Tm*	*Gc*	*Dh*	*Pl*	*Dc*	*Csp1*
Fe	**0.469**	0.090	0.251	0.192	−0.816	0.543	0.352	−0.135	0.406	**0.816**	0.333
Ag	0.020	0.404	0.249	0.095	0.000	−0.089	−0.027	**0.456**	−0.188	**−0.816**	0.333
Zn	0.506	0.269	**0.470**	0.095	**−0.816**	−0.134	0.027	0.026	0.295	**0.816**	−0.333
Cu	**0.506**	0.000	0.249	0.190	**−0.816**	−0.134	0.188	−0.134	0.188	**−0.816**	**−0.666**
Cd	**−0.292**	−0.324	**0.805**	0.024	**0.816**	0.208	0.000	−0.194	−0.388	**−0.816**	**0.912**
Cr	−0.179	0.173	0.000	0.079	0.500	−0.198	−0.208	**0.505**	−0.148	**−1.000**	−0.182
Pb	−0.066	0.172	0.331	0.239	0.000	0.172	0.147	0.000	−0.147	0.000	0.333
Mg	0.303	0.224	0.359	0.190	**−0.816**	−0.044	0.080	0.080	0.134	0.000	−0.333
TP	0.060	**0.449**	0.304	−0.047	0.000	−0.269	−0.241	**0.510**	−0.134	**−0.816**	0.000
Turbidity	0.263	0.224	0.359	0.191	**−0.816**	0.000	0.080	0.080	0.080	0.000	0.000
TDS	0.183	−0.022	**−0.700**	−0.024	**−0.816**	−0.135	−0.027	−0.054	0.135	0.000	**−0.666**
Nitrate	−0.060	−0.045	−0.415	−0.429	**0.816**	**−0.674**	−0.403	0.134	−0.188	**−0.816**	**−1.000**
NT	0.101	0.314	0.304	0.000	**−0.816**	0.000	−0.134	0.241	0.026	0.000	0.333
pH	0.067	**0.522**	0.464	0.237	0.000	−0.124	0.029	**0.534**	0.029	0.000	−0.235
COND	**0.604**	0.074	0.402	0.342	**−0.816**	0.322	0.386	−0.178	0.326	0.000	−0.235
TEMP	**0.469**	0.372	**0.526**	0.289	**−0.816**	0.024	0.207	0.178	0.148	0.000	−0.235

Cp: *Candida parapsilosis*, Ct: *C. tropicalis*, Ca: *C. albicans*, Rhm: *Rhodotorula mucilaginosa*, Pk: *Pichia kudriavzevii* (*C. krusei*), Tm: *Trichosporon mucoides*, Gc: *Geotrichum candidum*, Dh: *Debaryomyces hansenii*, Pl: *Papiliotrema laurentii*, Dc: *Diutina catenulata*, Csp1: *Candida* sp. 1. TP = total phosphorus, TDS = total dissolved solids, NH4 = nitrate, TN = total nitrogen, COND = conductivity, TEMP = temperature. The values in bold present a statistically significant ($p < 0.05$) positive or negative correlation between variables.

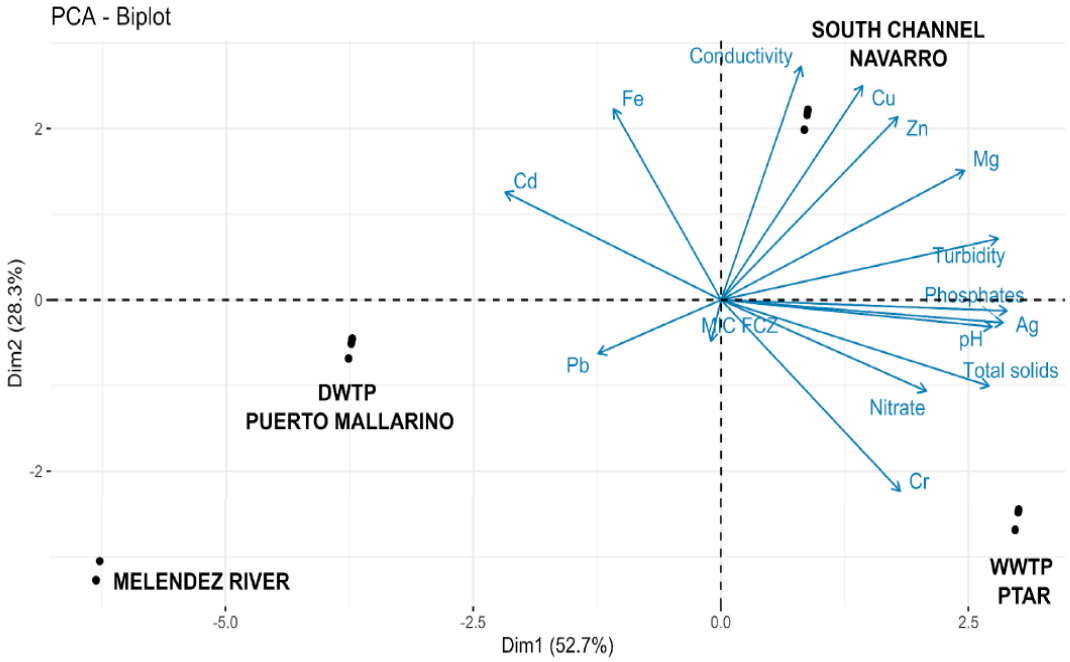

Figure 4.2. Principal component analysis correlation biplots of the interaction of physico-chemical parameters, heavy metal concentrations, yeast levels, and the association of the various sampling points (C = rainwater canal “South Channel”; PM = DWTP “Puerto Mallarino”; P = WWTP “PTAR”).

4.3. Discussion

Aquatic environments can become polluted due to various anthropogenic activities, including the production of agricultural, industrial, and medical waste, the expansion of urban communities without adequate sanitation infrastructure, and poor wastewater management [1, 10, 12]. One of the primary indicators of water quality is the presence of microorganisms, with the main concern being water intended for human consumption and recreational activities, as well as surface water, groundwater, and wastewater [32]. Some studies have indicated that the increased contamination of aquatic environments was significantly positively correlated with the relative abundance of yeasts; however, few studies have been conducted to assess the quality of aquatic ecosystems contaminated with yeasts [33,34]. In this study, yeasts were detected at all sampling

sites, including the intake of the “Puerto Mallarino” DWTP. At this plant, the order of the process carried out to treat the water is as follows: collection, sand removal, application of activated carbon, pre-chlorination, coagulation, flocculation, sedimentation, and filtration. In addition, there is post-chlorination and chemical stabilization with lime. However, the use of chlorine alone does not guarantee the absence of pathogenic fungi in drinking or recreational water. In fact, Ma et al. showed that there were non-significant changes in the fungal community structure observed before and after the initiation of treatment of a hospital hot water system treated with monochloramine in situ [35].

This DWTP adheres to the parameters established in Colombia, as outlined in Decree 1575 of 2007 [36] and Resolution 2115 of 2007 [37], which govern the physicochemical and microbiological quality control of drinking water. The physicochemical analysis includes parameters such as pH, color, turbidity, nitrate, fluoride, and residual chlorine, among others.

Microbiological standards include the absence of pathogenic microorganisms and fecal bacteria (*Escherichia coli* and *Enterococcus* spp.), as well as the determination of the presence of *Giardia* sp. and *Cryptosporidium* spp. It is worth noting that fungi are not included as potential water contaminants in these documents. The possible reasons for this may be the lack of knowledge of the fungal load in the water, the use of divergent culture methods, the heterogeneous mechanisms of pathogenicity of the fungi, and, consequently, the low number of reports connecting the presence of fungi in the tap water and the appearance of diseases in humans as stated by Kauffmann-Lacroix et al. [38].

Our results suggest that regulations about microbiological procedures should be updated, and yeast counting should be included in routine analyses. The number of yeasts was significantly higher in the “PTAR” WWTP than Melendez River. The variability in microbial counts observed in the present study can be attributed to the differing water quality levels of these environments, which receive varying amounts and types of pollutants, including wastewater, industrial effluents, and others. However,

the number of heterotrophic yeasts in the Melendez River (1.0×10^3 CFU mL^{-1}) was higher than that reported in other rivers around the world, such as Rio Doce, Brazil (from 10 to 466 CFU mL^{-1}) [9], some rivers in South Africa (Mooi River (from 0.5 to 9 CFU mL^{-1}) [11] and Eersterivier River catchment (1.0×10^2 CFU/mL^{-1})) [39], the River Danube in the area of Bratislava (100–210 CFU mL^{-1}) [40], and a lake in Patagonia, Argentina (22–141 CFU mL^{-1}) [41]. However, at the Tagus estuary, Portugal, a higher level of yeasts was reported (5.3–3272 CFU mL^{-1}) [42]. We highlight that the threshold of environmental quality in the eutrophic ecosystem was above 1.0 CFU mL^{-1}) [33]. An important aspect was the isolation of *C. albicans*, *C. krusei*, and *C. parapsilosis*, which are related to a high burden of fungal infections in the healthcare environment in Colombia [21]. The highest number of the genus *Candida* (8×10^3 CFU mL^{-1}) was counted at the “PTAR” WWTP. Our results were consistent with those of Assress et al. [12], who also found

that Candida spp. were the predominant species in WWTPs in Gauteng, South Africa. The results demonstrated the attractiveness of using yeasts as a microbiological indicator of organic pollution in aquatic ecosystems [12].

Regarding pigmented yeasts, we found only yeasts from the genus *Rhodotorula*, with high carotenoid production in almost all isolates [26]. *Rh. mucilaginosa* was found only at the "PTAR" WWTP and the South Channel in high numbers. These yeasts are associated with skin diseases, mucosal and invasive fungal infections, especially in immunosuppressed patients, and they exhibit unique metabolic activity in highly eutrophicated waters or severely contaminated municipal wastewater, indicating their link to human activity [43]. Studies on the risk of infection by different species of environmental yeasts have been limited in Colombia. For example, *Rhodotorula* spp. have been isolated from the nails of patients with superficial mycosis in Antioquia, Colombia [44]. Cr. neoformans is primarily associated with the droppings of various bird species, especially pigeons, but is also isolated from bark, tree trunk hollows, and decaying wood [45]. It has been reported in wastewater in previous studies [46]. Cr. neoformans causes opportunistic infections that typically affect the central nervous system of immunosuppressed patients, with high mortality rates if untreated. [47]. In Colombia, the main challenge in treating infections caused by this yeast is resistance to fluconazole [48]. *M. guilliermondii* is a globally distributed opportunistic pathogen that lives in various habitats. It exists on human skin and the surface microbiota of mucous membranes. It can cause serious fungal infections such as candidemia [49]. It is a yeast considered a promising species in the field of biotechnology, especially in the biocontrol of moldy bacteria during the storage of fruits and vegetables [50]. The presence of these yeasts is understandable, since city wastewater effluents containing antimicrobials, antimicrobial resistance-carrying microbes, and antimicrobial resistance genes are discharged at these two sites. Among the most reported opportunistic yeasts, we found *M. guilliermondii*, *C. lusitaniae*, *C. tropicalis*, *P. laurentii*, *Rh. glutinis*, and *Rh. mucilaginosa* in surface waters [11,39].

The main fungal pathogens in humans are *Candida* species, which develop resistance to triazoles and echinocandins. However, resistance to AMPB (Polyene) is extremely rare, despite its use for more than 60 years as a monotherapy, mainly for invasive fungal infections [51,52]. For this reason, it is interesting to know whether amphotericin B-resistant yeast strains can be found in the environment. The results obtained with the MIC assay showed that all strains of the different yeast species (including Candida species) were found to be sensitive to AMPB, with values at or below 2 μg/mL, confirming that resistance to this antifungal is rare in the assessed yeasts, and suggesting a low interaction of this antifungal with yeast communities in this location.

This was not the case for FCZ, where 32.7% of yeast species were resistant to FCZ. These results were consistent with results obtained in previous studies focusing on the isolation of antifungal-resistant environmental yeast. Brilhante et al. reported azole resistance in Candida spp. isolated from Catú Lake, Ceará, Brazil, in a high number [53]. Similarly, Medeiros et al. reported in 2008 that 50% of the yeasts isolated from water samples of the lakes and rivers of the Rio Doce,

Brazil, showed resistance to itraconazole and less resistance to FCZ [54]. However, an evaluation carried out in 2012 showed significant growth of yeast resistant to FCZ [9].

The yeasts isolated at the South Channel (Navarro) showed the highest percentage of resistance (37.5%) to FCZ, possibly associated with the high load of contaminants that this canal receives along the south of the city. Table 1 illustrates the pathogenic species *C. tropicalis, C. albicans, C. parapsilosis, D. catelunata, Rh. mucilaginosa, and D. hansenii,* showing the highest MIC values to FCZ (64 µg/mL). Similarly, antifungal resistance to FCZ has been reported in *Candida* species from tropical freshwater environments in Brazil and China [9,53–55]. The presence of environmental yeasts with high resistance to FCZ is probably because this azole is the most widely used antifungal in humans and animals, and it is among the most reported drugs in hospital and home wastewater [56,57]. In addition, there are fungicides routinely used in agriculture that share the action mechanism of azoles, which can generate cross-resistance in yeasts found in these aquatic environments [56–58].

In Asia, some studies reported a significant increase in resistant *C. tropicalis* isolated from poultry, and this has been associated with the extensive use of azoles in agriculture [56,59,60]. Brilhante et al. suggested that azole resistance in the *Candida* strains recovered from aquatic environments would be influenced by the activity of efflux pumps [53]. Several chemical compounds present in the environment, when interacting with microorganisms, would trigger the expression of genes that lead to these efflux pumps or other proteins involved in resistance to antifungals [56–58]. *Rhodotorula*, Candida, Pichia, and *Trichosporon* are yeasts typical of strongly eutrophic waters [61], and their presence indicates that aquatic systems are significantly polluted by industrial and municipal wastewater, where they can metabolize aromatic substances and heavy metals and have been considered as good indicators of pollution [1,8,33,62]. It is not surprising to find these yeasts in the environment; however, in a report by the WHO, which included a list of fungal priority pathogens, it recommended carrying out this type of study to provide epidemiological data that allow the establishment of local priorities about the dynamics of fungal pathogens and the prevalence of resistance to reduce the impact of drug resistance worldwide [63].

The yeast species compositions in the aquatic systems studied were dissimilar and were conditioned by the type of water, but also by the processing conditions. Yeast culture is an effective way to study their diversity, as many more yeast species, especially some possible new species, were recorded and isolated using the culture method. We found an association between yeast levels with certain physico-chemical parameters, such as total dissolved solids, levels of nitrites, and conductivity in the “PTAR” WWTP and South Channel. These results can be explained by the high load of waste that these two places receive, especially due to domestic discharges and agricultural activities that cause an increase in the concentration of organic matter and salts that come from fertilizers. Bafico demonstrated in his study that pollution from urban sources strongly influences the periphytic community structure and dynamics of Lake Nahuel Huapi (Patagonia, Argentina), with greater biomass and cell densities at highly contaminated sites

[41,64]. In addition, the highest number of fluconazole-resistant yeast isolates was detected at these two sites (31.3% and 37.5%, respectively).

The observed spatial variation could be attributed to site-specific anthropogenic activities, such as eutrophication, the influx of domestic and industrial waste, and run-off from agricultural settings. Effluents from the Melendez River, rainwater at the South Channel (Navarro), and the WWTP (PTAR) discharge into the Cauca River. In addition, the vicinity of the river presents different levels of human interactions (e.g., illegal mining, steel making, informal settlements, sewage and raw pharmaceuticals discharge, and poultry, agricultural, and industrial waste), which influence yeast counts and the species present, and the activation or acquisition of antifungal resistance mechanisms. The yeast count obtained during the dry and wet seasons did not present significant differences at each sampling site, with the reported number corresponding to the average of the two sampling moments.

Colombia, being a tropical country, experiences changes in climatic conditions that are conditioned by two phenomena, the “La Niña” phenomenon (there can be rain all year with some sunny days) and the “El Niño” phenomenon (there can be dry days all year with some rainy days). This means that temperature and relative humidity conditions each year are not very variable. Precipitation in January and May 2018 was 50–100 mm and 100–150 mm, respectively, with average temperatures for the two months between 20 and 28 °C [65]. The yeast count obtained during the dry and wet seasons did not present significant differences at each sampling site, with the reported number corresponding to the average of the two sampling moments. For this reason, the data from the two sampling moments were averaged. The results differed from those found by Steffen et al., who found significant differences in site-specific yeast concentrations in the dry summer months, when concentrations increased significantly during the rainy months [39], and from the results of Ortíz-Vera et al., who found greater differences in community composition (relative abundance of fungal phyla) in the dry than in the rainy season [66].

On the other hand, heavy metal pollution in aquatic systems is a global problem originating from increased industrialization and urbanization, since they are accumulative, toxic, and carcinogenic in water bodies and biota. As seen from the result of the PCA, there was a significantly positive correlation between levels of *Candida* spp., *P. fermentans*, among others, and heavy metals, especially Cr, Zn, Cu, and Pb. There is little open literature on the effect of metal levels on the diversity and structure of the fungal community in aquatic environments. Assress et al. found that the distribution of classes such as *Pezizomycetes, Lecanoromycetes, Agaricostilbomycetes, Schizosaccharomycetes*, and *Dothideomycetes* was influenced by Mg and Zn concentrations [12]. Meanwhile, members of classes *Eurotiomycetes, Exobasidiomycetes, Orbiliomycetes, Glomeromycetes, Saccharomycetes,* and L*eotiomycetes* were correlated with Ni and Mn levels, and Fe was the main environmental parameter influencing the fungal community belonging to classes *Agaricomycetes, Pucchinomycetes, Atractiellomycetes, Sordariomycetes,* and *Archaeorhizomycetes* in WWTPs located in Gauteng Province, South Africa.

Some studies have shown that the content of organic matter, calcium, and amorphous phase can increase the sensitivity to heavy metal contamination and the persistence of organic pollutants in different matrices (water, air, and soil) and interfere with or inhibit some enzymes and metabolic processes [67]. It is also established that the organic matter content is known to positively influence the capacity of the capacity of change, buffering capacity, and retention of heavy metals. For example, some physicochemical factors, such as pH and oxidation-reduction potential, can influence the increased toxicity and genotoxicity of some yeasts to heavy metals [68, 69].

It has been demonstrated that the presence of heavy metals in aquatic environments affects the structure of bacterial communities resistant to antibiotics and the persistence over long periods of genes resistant to antibiotics by forming stable complexes of antibiotics and metal ions [70]. Dickinson et al. demonstrated the co-selection of heavy metals and antibiotics in the environment [71]. The prevalence and persistence of resistant genes to antibiotics and heavy metals have been demonstrated in WWTPs [72]. High levels of antibiotic-resistant genes are reported to be related to high concentrations of Cr [73], Hg, and Zn [74]. Rajasekar et al. found that Cd, Pb, and Cu had a significant positive correlation with the sul2 and strB genes, conferring resistance to sulfonamides and streptomycin, respectively [75]. This would explain the results of our study, which found that *Rh. mucilaginosa*, *C. albicans*, and *Candida sp.* 1 were positively associated with the presence of Cd, and *D. catelunata* was associated with Fe.

Saccharomyces cerevisiae and some pathogenic yeasts, such as *Candida glabrata* and *Candida albicans,* have been reported to possess ATP-binding cassette (ABC) transporters, which have evolved to play critical roles in adapting to environmental challenges, including heavy metal stress and multidrug resistance (MDR). For example, Yap1, Yap2, and Yap8 transporters regulate the response to oxidative and heavy metal stress by inducing the expression of the yeast activator protein YCF1, the pleiotropic drug resistance elements PDR5, and the multidrug transporter involved in multidrug resistance and singlet oxygen species resistance (SNQ2) in response to a variety of toxic metals. Yeast ABC transporters play an important role in plasma membrane homeostasis, the site of action of antifungals, such as polyenes and azoles, and are involved in resistance to different drugs, which could explain the correlation between the presence of some heavy metals and the susceptibility to fluconazole of some yeasts isolated in this study [76,77].

Concerning the presence of heavy metals in soils, *Suillus luteus* uses different mechanisms, such as the exclusion and regulation of heavy metals through transporters located in the cell membrane and chelating agents, which are released to the outside of the cell wall, through storage, and the trapped ions are stored or transported in vesicles and expelled from the cells, in the same way that oxidizing agents relieve oxidative stress caused by reactive oxygen species through reducing agents such as cytochromes p450 (CYP450) [78]. CYP450 exists in a wide range of organisms, including bacteria, fungi, and mammals, catalyzing a variety of oxidation reactions that facilitate the detoxification of xenobiotics, such as heavy metals, the metabolism of drugs, including antifungals, and the biosynthesis of steroids, such as ergosterol [79]. One of these

antifungals, fluconazole, acts on ergosterol biosynthesis in fungal cells by inhibiting a CYP450-dependent fungal enzyme, lanosterol 14α-sterol demethylase. Resistance to fluconazole can occur through the increased expression of the ERG11 gene, which encodes the drug target enzyme sterol 14α-demethylase (Erg11p), or by mutations in Erg11p that result in a reduced affinity for fluconazole and an overexpression of efflux pumps that transport fluconazole out of the cell [80]. However, more extensive studies are needed to determine the influence of physicochemical parameters, heavy metals, and other contaminating compounds on yeasts resistant to Cd antifungals in water systems.

4.4. Conclusions of the Chapter

We identified opportunistic yeast pathogens, including strain-specific azole-resistant *C. albicans*, *C. parapsilosis, C. tropicalis,* and *Candida* sp. 1 (closely related to *C. intermedia*), in various aquatic environments, such as WWTPs and DWTPs. Melendez River was an exception. These findings are concerning since this drug is often used as a prophylactic treatment in HIV-positive patients. We highlighted the importance of conducting studies focused on isolating antifungal-resistant environmental yeasts to examine their role in the deterioration of aquatic environments.

The results of the correlation analysis showed a general spatial variation in the characteristics of wastewater samples. Yeast counts and some species are considered opportunistic pathogens, such as *C. albicans*, *Rh. mucilaginosa*, and *D. catelunata*, showed an association with parameters and chemical compounds indicative of the presence of sewage or contaminated water, which act as selection agents for pathogenic yeasts.

The results indicated that the aquatic systems of the city of Cali serve as a reservoir of FCZ-resistant yeasts and a potential source of invasive fungal infections, which is important for the “One Health” approach. Like other ecosystem services, these must be protected and managed sustainably.

4.5. Final remarks of Chapter

The part of the doctoral thesis examined water quality, the presence of heavy metals, and the antifungal susceptibility of yeasts in aquatic systems in Cali, Colombia. Water samples from four sources were analyzed: the Meléndez River, the drinking water treatment plant “Puerto Mallarino” (DWTP), the rainwater channel “South Channel,” and the wastewater treatment plant “PTAR.” Seventy-three yeast isolates were identified, with 68 belonging to 14 genera and 21 species, and the genus Candida being the most common. The susceptibility of yeasts to the antifungals fluconazole and amphotericin B was tested, revealing that 32.7% of the yeasts were resistant to fluconazole, while all were sensitive to amphotericin B.

The study revealed that yeast levels were higher in the wastewater treatment plant “PTAR” and lower in the Meléndez River. Additionally, a correlation was observed between yeast levels and physicochemical parameters, including dissolved solids, nitrates, and heavy metals (Cr, Zn, Cu, Pb). Some yeast species, such as *Candida albicans*, *Rhodotorula mucilaginosa,* and *Diutina catelunata*, showed specific associations with metals such as cadmium and iron. These fluconazole-resistant yeasts pose a potential risk to public health, particularly in immunocompromised patients, as fluconazole is widely used in medical treatments.

In terms of impact, our study highlights how Cali's aquatic systems act as reservoirs for antifungal-resistant yeasts, which could contribute to the spread of invasive fungal infections. This finding aligns with the One Health approach, which connects human, animal, and environmental health and promotes the sustainable management of aquatic ecosystems. This information is expected to drive future research and inform the implementation of measures to protect public health and water resources. In the same address, the fundamental research on strategies/processes to eliminate antifungal-resistant yeast is an imperative necessity. Thereby, in this doctoral thesis, the evaluation of UV-based advanced oxidation processes to treat a relevant antifungal-resistant yeast was carried out, and the results are presented in the subsequent chapter.

4.6. References

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CHAPTER 5.

5. ADVANCED OXIDATION PROCESSES BASED ON UVC LIGHT FOR THE INACTIVATION OF ANTIFUNGAL-RESISTANT STRAINS

Chapter 5 contains the results for the achievement of the specific objectives 4 and 5. It provides an introduction that briefly describes the problem and justification of this part of the doctoral research in Section 5.1, followed by the results description in Section 5.2. The results were discussed in Section 5.3. Then, the conclusions and the final remarks on the chapter are shown in Sections 5.4 and 5.5, respectively. We should mention that the information presented in this chapter gave rise to a paper entitled ***"Evaluation of ultraviolet light-based oxidative systems for the inactivation and susceptibility of a fluconazole-resistant Candida albicans strain",*** which was submitted to the journal: Water, Manuscript ID: water-3790774 (July 15, 2025, at 8 pm; and it is currently under review).

5.1. Introduction

The issue of fungal infections, especially those caused by *Candida albicans*, is a priority for the World Health Organization (WHO). These infections pose a significant threat to public health due to their high rates of illness and death, along with growing resistance to available antifungal medications like fluconazole. Although yeasts are found in various aquatic environments, their presence in water has received little research, possibly because their ingestion rarely results in clear clinical symptoms. However, these yeasts can be opportunistic and present a serious risk to immunocompromised patients. Every year, fungal pathogens cause millions of illnesses and deaths worldwide, affecting both people with weakened and normal immune systems. *Candida albicans* is especially concerned in aquatic environments contaminated with agricultural and human antifungals, as these substances can trigger genetic mechanisms that promote resistance. Recent studies have identified antifungal-resistant strains in freshwater samples, highlighting their high prevalence and resistance, particularly to fluconazole.

The rising use of antifungals in clinical treatment, agriculture, and industry has led to the development of both intrinsic and acquired resistance in various fungal species. This highlights the pressing need to develop fungal decontamination systems for aquatic and surface environments, particularly in developing countries. Currently, environmental control techniques rely on manual cleaning and chemical disinfection, whereas aquatic systems utilize strong oxidants that alter the structure and metabolism of fungi.

In this context, advanced oxidation processes (AOPs), which produce reactive oxygen species (ROS) such as hydroxyl radicals, have become a promising method for treating fungi in water. These processes, combining ultraviolet-C (UVC) light with inorganic peroxides, have proven effective in damaging the cellular components of microorganisms. Although UVC radiation alone

has a strong disinfecting effect, pairing it with oxidizing agents can significantly improve efficacy, reduce reactivation in the dark, and lower antifungal resistance. However, to date, no studies have been published on the inactivation of resistant strains of *Candida albicans* in water using the combination of UVC with different inorganic peroxides. This chapter aims to evaluate the effectiveness of these methods in inactivating, reactivating in the dark, and changing the susceptibility of a resistant strain of *Candida albicans* to fluconazole.

5.2. Results

5.2.1. Inactivation of fluconazole-resistant C. albicans with inorganic peroxides in the dark and UVC control.

Initially, the treatment of fluconazole-resistant C. albicans with UVC light alone and three different inorganic peroxides (i.e., PDS, PMS, and H_2O_2) at a concentration of 500 μmol L^{-1} was conducted. The progression of the C. albicans strain under these systems can be seen in Figure 5.1. After 60 seconds of treatment, the inactivation rate with UVC light reached 99.94%, with a decrease of 3.25 log units. The inactivation percentages using PDS, PMS, and H_2O_2 were 6.76%, 82.30%, and 85.83%, respectively, corresponding to decreases of 0.85, 1.04, and 1.08 log units. When comparing the three oxidants, a statistically significant difference was detected, with a p-value of <0.0001 (the significance level for correlation was set at $p < 0.05$). Data analysis was performed using Infostat version 2020.12.00 and OriginPro version 10.1.0.0, revealing that the effectiveness of microorganism inactivation followed the order: H_2O_2 > PMS 211 > PDS.

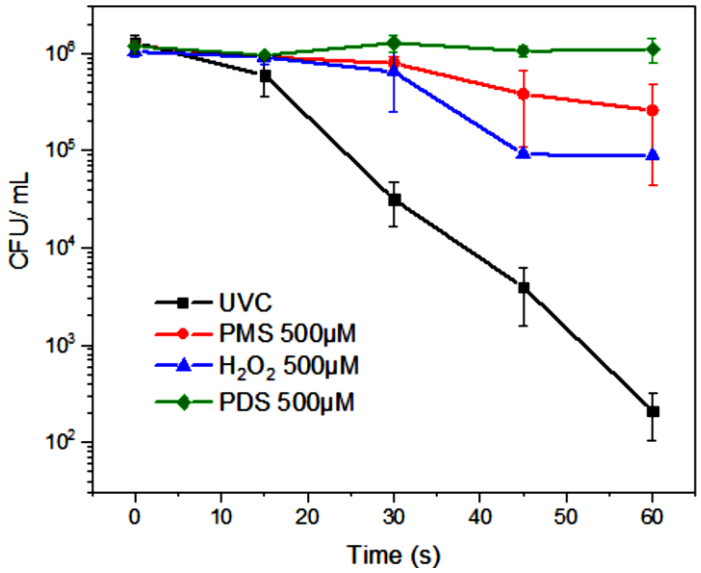
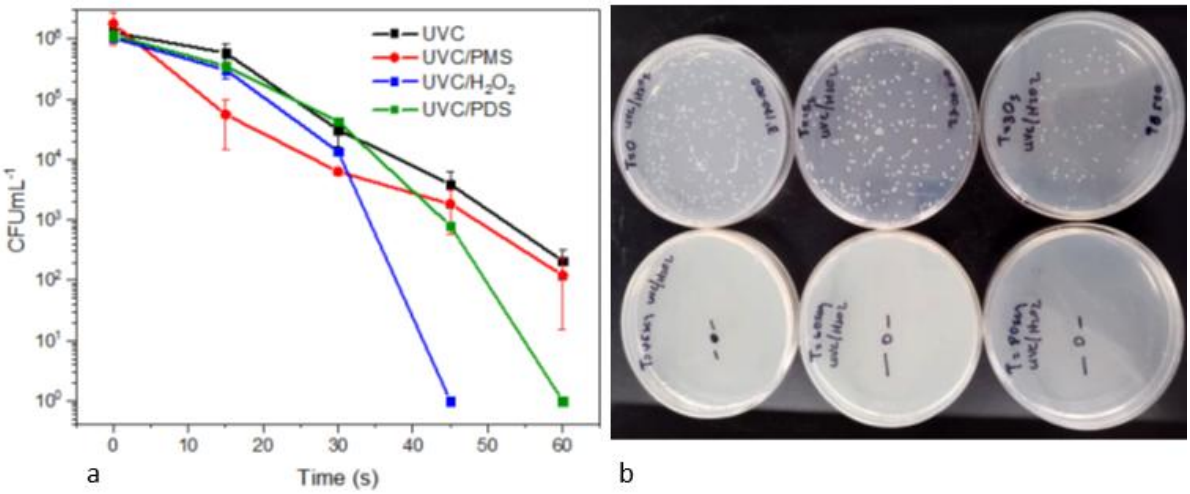


Figure 5.1. Inactivation of the fluconazole-resistant *C. albicans* by photolysis and direct oxidation with 214 peroxides. Experimental conditions: deionized water. V: 50 mL, P: 8W, [PMS]: [H2O2]: [PDS]: 500 215 μmol L-1, pH: 7.4. UVC lamp (254 nm).

5.2.2. Inactivation of fluconazole-resistant C. albicans by photooxidation with the different peroxides

To enhance the yeast inactivation, the UVC light was combined with the peroxides (generating photooxidation processes). Figure 5.2 illustrates the evolution of C. albicans yeast through photooxidation processes, specifically PDS, PMS, or H_2O_2, combined with UVC irradiation. After 60 s of photooxidation, the percentages of disinfection by UVC/PDS, UVC/PMS, and UVC/H_2O_2 were 99.58%, 99.90%, and 99.99%, respectively, and the corresponding inactivation



values were 2.39, 3.02, and 6.16 log units, respectively (see Figure 5.2a). Figure 5.2b shows the growth of resistant *C. albicans* yeast in the culture medium at different reaction times for the UVC/H_2O_2 treatment. No growth was observed after 45 s; these results evidenced the superior behavior of the UVC/H_2O_2 system under the tested conditions.

Figure 5.2. Evolution of fluconazole-resistant *C. albicans* under different photooxidation systems. a) Comparison of the photo-oxidative methods. Experimental conditions: deionized water. V: 231 50 mL, [PMS]: [H_2O_2]: [PDS]: 500 μmol L^{-1}, pH: 7.4. UVC lamp (254 nm). b) Growth of resistant *Candida albicans* in the culture media during treatment with UVC/H_2O_2 (time points: 0, 15, 30, 45, 60, and 80s).

5.2.3. *Kinetic results of the inactivation of fluconazole-resistant C. albicans by photooxidation with 236 different peroxides.*

The shape of the curves in Figure 5.2a indicates that the inactivation of the target microorganism can follow pseudo-first-order kinetics. It is important to remember that pseudo-first-order kinetics follow the mathematical expression shown in Equation 1, where $[N]_t$ is the microorganism population at any given time, $[N]_0$ is the initial fungal population, t is the time, and k is the pseudo-first-order rate constant (Eq 5.1).

$$Ln\frac{[N]_t}{[N]_0} = -kt \quad (5.1)$$

The fitting to such a kinetics of the disinfection tests presented in Figures 5.2 and 5.3a was assessed. Table 5.1 presents the kinetics results obtained during photolysis, oxidation, and photooxidation of fluconazole-resistant *C. albicans* using various oxidizing agents (PMS, PDS, and H_2O_2) with and without UVC radiation exposure.

Table 5.1. Pseudo-first-order kinetic results for photolysis, oxidation, and photo-oxidation of the fluconazole-resistant strain of *C. albicans*.

Treatment	k (s^{-1})	$t_{1/2}$ (s)	Equation	R^2
UVC	0.1728	4.011	y = -0.1728x + 0.8607	0.9957
PMS	0.0263	26.36	y = -0.0263x + 0.1167	0.9449
UVC/PMS	0.1510	4.590	y = -0.1510x + 0.595	0.9761
H_2O_2	0.0597	11.61	y = -0,0597x - 0,033	0.8619
UVC/H_2O_2	0.3164	2.191	y = -0,3164x - 1,1922	0,8815
PDS	0.0003	2310	y = -0.0003x -0.055	0.0038
UVC/PDS	0.2824	2.454	y = -0.2824x + 0.0635	0.9449

Conditions: deionized water. V: 50 mL, pH: 7.4. Lamp UVC (254 nm, P: 8W). [PMS]: [H_2O_2]: [PDS]: 500 µmol L^{-1}.

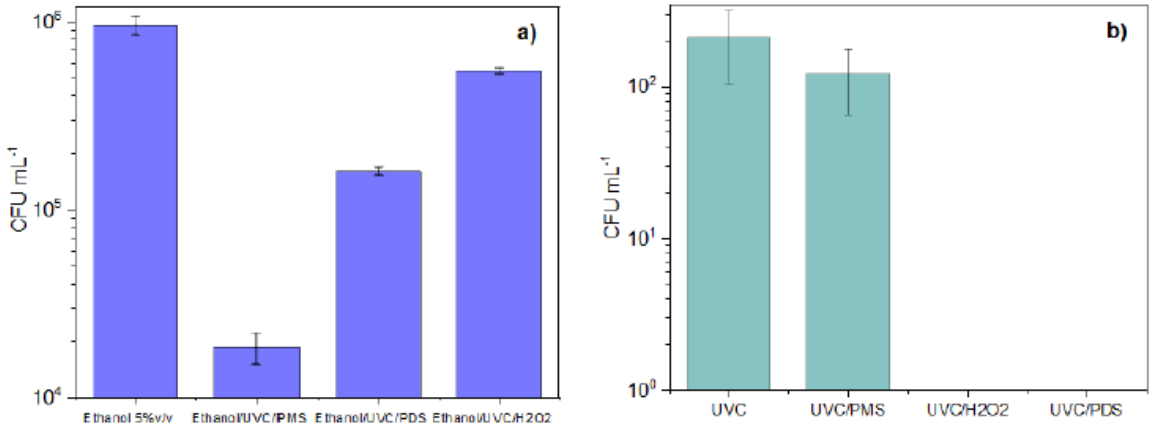
5.2.4. *Elucidation of the routes involved in the inactivation of fluconazole-resistant C. albicans by photooxidation processes*

The combination of UVC light with inorganic peroxides (i.e., photooxidation processes) can produce strong disinfecting species, such as the hydroxyl radical and/or the sulfate anion radical. To demonstrate the participation of these species in the inactivation of the target yeast by photooxidation systems, experiments using a well-known scavenger (i.e., ethanol, k with hydroxyl radical: 2.8 x10^9 s^{-1} and k with sulfate radical: 7.7 x10^7 s^{-1}, [39]) were performed. Firstly, the

interaction of the resistant *C. albicans* strain with 261 ethanol was evaluated. Then, the treatment of the microorganism in the presence of the scavenger by the three photooxidation processes.

Figure 5.3a shows the growing response of the resistant *C. albicans* strain during the treatments in the presence of ethanol. The alcohol did not induce a significant inactivating action on the microorganism (0.24 log reduction of the yeast population). Moreover, after 60 s of treatment, the inactivation percentages were: 99.95% (equivalent to 3.29 log reduction) for Ethanol/UVC/PMS, 9.19% (1.04 log reduction) for Ethanol/UVC/PDS, and 46.58% (0.33 log reduction) for Ethanol/UVC/H_2O_2. It can be noted that for all the photooxidation systems (Figure 3a), a greater growth of the strain (i.e., a lower disinfection) was observed in the presence of ethanol compared to its absence (Figure 10a), thus confirming that radical species take part in the action routes of the three photooxidation processes.

To demonstrate the relevant role of the peroxide precursor in generating radicals, the effect of inorganic peroxide concentration on the photooxidation systems was also investigated. Then, the concentrations of PDS, PMS, and H_2O_2 were changed from 500 μmol L^{-1} to 50 μmol L^{-1}. Figure



5.3b presents the remaining population of the resistant yeast by UVC/PDS, UVC/PMS, and UVC/H_2O_2 (with the oxidant concentration of 50 μmol L^{-1}), which was 99.99%, 99.64%, and 99.99%, and the corresponding inactivation of 5.00, 2.45, and 5.30 log units' reduction, respectively.

Figure 5.3. Inactivation kinetics of fluconazole-resistant *C. albicans* by diverse disinfection methods in the presence of ethanol.

Experimental conditions: deionized water. V: 50 mL, P: 8W, pH: 7.4. UVC lamp (254 nm). **a)** Remaining fungus population after 60 s of treatment in the presence of the scavenger during the photooxidation treatments *(Experimental conditions:* Ethanol (5%v/v). [PMS]: [H_2O_2]: [PDS]: 500 μmol L^{-1}. Treatment time:

60 s). **b)** Remaining fungus population after 60 s of treatment at low concentration of the peroxide by the photooxidation treatments (*Experimental conditions*: [PMS]: [H_2O_2]: [PDS]: 50 µmol L^{-1}).

5.2.5. Reactivation of C. albicans in darkness

To better understand if the surviving strains could recover or adapt under these specific conditions, the reactivation of the treated yeast was evaluated. It is essential to note that the detection limit for culturing microorganisms on plates is 100 CFU/mL. The reactivation in darkness (at the detection limits) was applied after exposures to the different treatments (i.e., UVC and UVC plus oxidants) at 30, 45, 60, 80, and 120 s (Table 2). At 80 s of action of the UVC-only system, no yeast regrowth was observed for this system. The combination of UVC with the inorganic peroxides showed no reactivation in the dark when the photooxidation treatment was applied for 60 s. We must note that the treatment with the best performance was the UVC/H_2O_2, which showed no reactivation when the microorganism was treated for 45 seconds (Table 5.2).

Table 5.2. Inhibition and reactivation of fluconazole-resistant *Candida albicans* strains in darkness.

Treatment	30s		45s		60s		80s		120s	
	I	R	I	R	I	R	I	R	I	R
UVC	+	+	+	+	+	+	-	+	-	-
UVC/H_2O_2	+	+	-	+	-	-	-	-	-	-
UVC/PMS	+	+	+	+	+	+	-	-	-	-
UVC/PDS	+	+	+	+	-	+	-	-	-	-

* I: Inhibition. R: Reactivation

**: + means achievement, - represents no achievement

5.2.6. Susceptibility to fluconazole of the treated C. albicans

For those yeast strains that recovered after the treatments (see the previous section), susceptibility tests to fluconazole were performed. Table 5.3 and Figure 5.4 summarize the changes observed in the fluconazole-resistant *C. albicans* strain (initially with a cutoff of 64 µg/mL) after photolysis and photooxidation with different peroxides. For the yeast treated with UVC alone, the cutoff decreased from 64 to 16 µg/mL; thus, this yeast strain remains resistant. In contrast, UVC/PMS and UVC/PDS treatments showed no significant changes in susceptibility to the antifungal drug. During UVC/H_2O_2 treatment, the yeast's cutoff value dropped from 64 to 8 µg/mL (Figure 5.4), which, according to CLSI, indicates a drug-sensitive yeast.

Table 5.3. Percentage inhibition of fluconazole-resistant *C. albicans* against different advanced oxidation processes.

Method	Control	64µg mL^{-1} (CFU mL^{-1})	% I*	32µg mL^{-1} (CFU mL^{-1})	% I*	16µg mL^{-1} (CFU mL^{-1})	% I*	8µg mL^{-1} (CFUmL^{-1})	% I*
Without treatment	6170	4043	34.47	5280	14.42	6360	-3.08	6233	-1.02

UVC	7253	112	98.46	17	99.77	1970	72.84	1907	73.71
UVC/H_2O_2	8013	0.33	100.00	2	99.98	2	99.98	382	95.23
UVC/PMS	3943	2036	48.36	2607	33.88	2290	41.92	1990	49.53
UVC/PDS	6103	4833	20.81	3967	35.00	3747	38.60	3713	39.16
C. parapsilosis ATCC 22019	6980	524	92.49	602	91.38	687	90.16	1580	77.36

* Percentage of inhibition. [H_2O_2], [PMS], [PDS]: 500 µmol L^{-1}

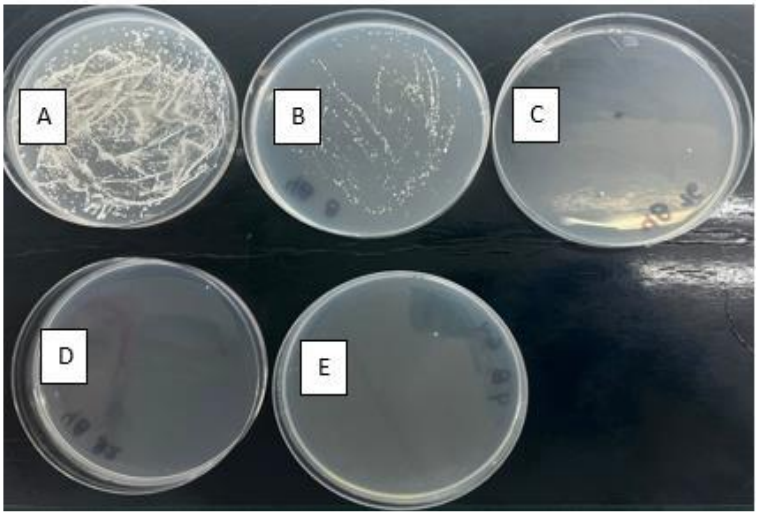


Figure 5.4. Susceptibility to fluconazole of the treated resistant *Candida albicans* treated by UVC/H_2O_2. **A:** without antifungal, **B:** 8 µg mL^{-1}, **C:**16 µg mL^{-1}, **D:** 32 µg mL^{-1}, and **E:** 64 µg mL^{-1}.

5.2.7. Changes in yeast morphology after the UVC/H_2O_2 treatment.

As shown in the previous subsections, under the tested conditions, the UVC/H_2O_2 system demonstrated the most effective disinfecting action against fluconazole-resistant *C. albicans* (see Figures 5.2a and 5.2b). Then, the physiological effects of this photo-oxidation process on the yeast were considered. Alterations in yeast morphology were observed using microscopy. Hence, the morphology of *C. albicans* was compared before and after treatment (Figure 5.5). When comparing the morphologies of yeasts without treatment (no UVC or oxidants) to those exposed to the UVC/H_2O_2 process, notable cellular clustering and visible damage to the cell wall and membrane were observed (see Figure 15.5b). The yeasts showed ruptures, swelling, and appeared translucent, increasing in size, with the formation of empty shells. Others had rough and wrinkled cell surfaces, indicating oxidative stress and structural damage (Figure 5.5b).

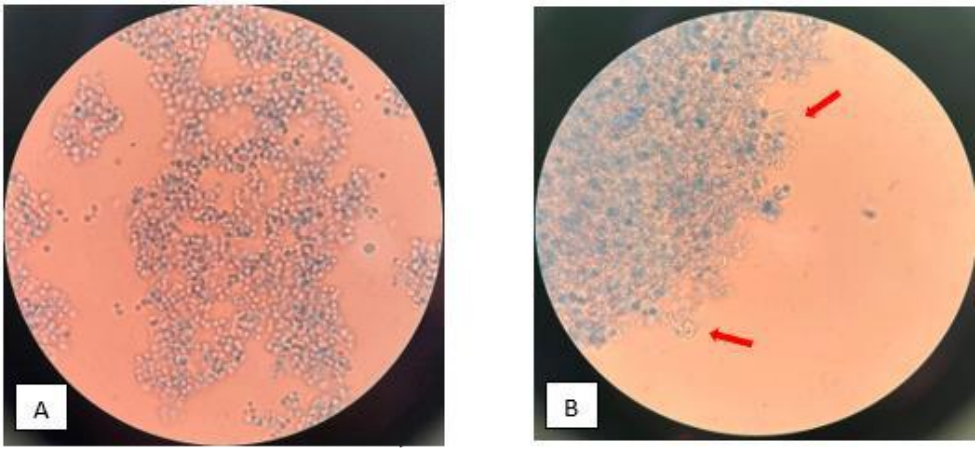


Figure 5.5. Changes in yeast morphology after the UVC/H_2O_2 treatment. **a)** Untreated fluconazole-resistant *C. albicans*. **b)** Fluconazole-resistant *C. albicans* treated with the UVC/H_2O_2 process

5.3. Discussion

5.3.1. Direct yeast inactivation by the inorganic peroxides

The results in Figure 5.1 revealed that among the three inorganic peroxides (acting alone), H_2O_2 had the highest inactivation percentage of 85.83%, corresponding to a 1.08 log loss of the initial yeast population. The disinfecting action of H_2O_2 toward our target yeast (Figure 5.1) is consistent with the previous work by Al-Salihi et al., who found that hydrogen peroxide shows complete antifungal activity against non-resistant *Candida albicans* [40]. Additionally, it is worth noting that H_2O_2 itself has demonstrated an antifungal effect against *Candida* spp. strains, inhibiting their growth at low concentrations (in the mmol L^{-1} range) [41].

The hydrogen peroxide can penetrate the yeast. Inside the yeast cell, H_2O_2 can generate reactive oxygen species (ROS), which can alter the internal balance, leading to oxidative stress and severe damage [42, 43]. It has also been shown to cause cellular damage, leading to the oxidation of cellular components and alterations in DNA and protein structure and function, which result in mutations and loss of enzymatic activity [42]. Unsaturated lipids in cell membranes can also undergo peroxidation due to oxidative stress caused by hydrogen peroxide, compromising the integrity of the cell membranes. Similarly, a previous study on the response of *Candida albicans* to oxidative stress induced by H_2O_2 reported changes in protein abundance and phosphorylation events. The ROS (coming from H_2O_2 inside the cell) can cause cell damage, in addition to alterations in antioxidant mechanisms, regulation of protein phosphorylation, and signaling pathways [44].

Concerning the other two peroxides (which were less efficient than H_2O_2 in inactivating the yeast, see Figure 5.1), at the experimental level, PMS showed a higher inactivation percentage (82.30%) than PDS (6.36%, Figure 5.1). To understand the differences among the three peroxides,

we can consider the electric charge on each of them. H_2O_2 is neutral, while PMS is negatively charged, and PDS is doubly charged. Due to the negative charges on PMS and PDS, they have limited penetration into the cell of yeast. For instance, electrostatic repulsion can occur between the persulfates and the lipid cell membrane of the microorganism, thereby making them less efficient than H_2O_2 for inactivating fungi.

5.3.2. Inactivation of the target microorganism by the UVC light alone

According to the results in Figure 5.1, UVC radiation demonstrated high effectiveness in inactivating our target *Candida albicans*. To interpret this result, we must consider that the cell wall and membrane of yeast can allow UVC light to enter, which facilitates damage to genetic material and other internal components. In addition, yeasts have a larger size compared to bacteria, increasing the surface area through which UVC radiation can enter. Although yeasts possess mechanisms to repair the damage, they may not be able to counteract the formation of pyrimidine dimers and other effects of UVC light when sufficiently high doses of radiation are applied [45].

The mechanism of the UVC action is based on the induction of photochemical reactions that damage their RNA and DNA through the formation of pyrimidine dimers (thymine and cytosine dimers) cyclobutane (CPDs) and pyrimidine (6-4)-pyrimidone photo products (6-4PPs), which block the function of DNA polymerase, the enzyme responsible for copying DNA during replication. These dimers, which are abnormal covalent bonds between adjacent bases in DNA, prevent cells from dividing correctly by altering the action of RNA polymerase, which transcribes DNA into messenger RNA. This affects the production of proteins essential for cell survival, preventing the yeast from reproducing and functioning normally, leading to cell death or inactivation as the yeast cannot repair the accumulated damage [46, 47]. Furthermore, UVC radiation can also cause damage to the yeast cell membrane, affecting lipids and proteins essential for its structure and function. In some cases, ROS are generated, which induce additional oxidative damage to lipids, proteins, and DNA, amplifying the germicidal effect [47, 48].

Our results, for UVC acting alone, observed in Figure 5.1, agree with data obtained by Pereira and collaborators, who also found that UVC light significantly reduced this type of microorganism. Additionally, we should note that some commercial devices, such as the MUVi-UVC, developed by Mobile UV Innovations Pty Ltd for the disinfection of mobile medical equipment, have confirmed the inactivation of *Candida auris*, a multidrug-resistant yeast, within 5 minutes of exposure, with an efficacy of 99.999%. Our results, combined with previous literature, suggest that UVC technology is a useful tool for certain fungal species [49-51]. However, some fungi species could develop resistance to the UVC action. Although *Candida* is not resistant to UVC radiation, it can activate DNA repair mechanisms, such as nucleotide excision repair, a mechanism that removes DNA segments containing lesions such as cyclobutane pyrimidine dimers and 6-4 photoproducts (6-4PPs) or make incisions on both sides of the lesion and replace the damaged segment with new nucleotides via DNA polymerase and DNA ligase [47,48]. Hence, stronger systems, e.g., photooxidations, should be applied. In such systems, the synergy between radical species and UVC light is an alternative to inhibit repair mechanisms.

5.3.3. *Yeast treatment by the photooxidation systems – disinfection, kinetics, and routes*

Figure 5.2 presents the results of the yeast inactivation under the three photooxidation systems (i.e., UVC/H_2O_2, UVC/PDS, and UVC/PMS). For these disinfection curves, kinetics fitting was performed, revealing that direct oxidation and photooxidation followed a pseudo-first-order pattern (Table 5.1). The adjustment was obtained from an improved linearization when the variations in the natural logarithm of the number of CFU mL^{-1}, regarding the initial population, were plotted against the treatment time (note that for the photooxidation systems, only the points after the lag phase were considered). Then, the values of the reaction rate constant (k) were determined from the slope of the linear form (Equation 5.2). To calculate the half-life time ($t_{1/2}$), we used Equation 5.2. In this study, k is linked to the speed of microorganism inactivation in the process, and $t_{1/2}$ indicates the time required to halve the yeast population by the tested treatments. Also, the coefficient of determination (R^2) reflects how well the model fits the experimental data obtained [52]

$$t_{1/2} = \frac{Ln(2)}{K} \tag{5.2}$$

Results in Table 5.1 revealed that the treatment with the greatest rate constant was UVC/H_2O_2 (k: 0.3164 s^{-1}), followed by UVC/PDS (k: 0.2824 s^{-1}), and UVC/PMS (k: 0.1510 s- Additionally, the data indicated that the control assay with only UVC light yielded a rate constant of 0.1728 s^{-1}, which is higher than the rate constants observed in the assays performed in darkness with the peroxides. It is relevant to note that combining UVC radiation with peroxides, specifically UVC/H_2O_2 and UVC/PDS, results in a significant increase in the inactivation kinetics constants (and consequently decreases the $t_{1/2}$ values) compared to oxidation by the peroxide or UVC acting independently. Moreover, we should mention that the UVC/H_2O_2 combination achieved the shortest half-life time, recorded at 2.191 s. The control assay with UVC light showed a half-life time of 4.011 s, which is lower than the peroxides under dark conditions, where the half-life time ranged from 11.61 to 2310 s.

To demonstrate the usefulness of the combination of UVC with the inorganic peroxides, the synergy was calculated (S = kUVC/peroxide/(kUVC + kperoxide)). Table 5.4 shows the synergy values for the different treatments (photooxidations). When the result of this operation is a value that exceeds 1.0 (which translates into a positive synergy), it can be concluded that the combination of the peroxide and UVC-based significantly increases the inactivation efficiency. On the contrary, values below 1.0 establish that the treatment shows antagonist effects. The photooxidation treatments with H_2O_2 showed good synergy, having an S value of 1.4. The UVC/PDS system exhibited synergistic behavior (S: 1.6). In the dark, PDS displayed slow kinetics; however, the application of UVC to PDS enhanced its performance, resulting in synergy. On the other hand, the combination of UVC with PMS was antagonistic (Table 5.4); indeed, this photooxidation system exhibited the worst performance for inactivating yeast (see Figure 5.3a).

Table 5.4. Synergy for the inactivation of a fluconazole-resistant strain of *C. albicans* by photooxidation treatments.

Treatment	UVC/H_2O_2	UVC/PDS	UVC/PMS
S value*	1.4	1.6	0.8

*Synergy, $S = k_{UVC/peroxide}/(k_{UVC} + k_{peroxide})$

The origin of the synergy in the photooxidation system can be linked to the formation of radical species (e.g., $HO^{\bullet}$ and $SO_4^{\bullet-}$) coming from the homolytic cleavage of the O-O bond on H_2O_2, PDS, and PMS. Then, to verify the participation of radical routes in the photooxidation processes, experiments using a radical scavenger (ethanol) and decreasing the peroxide concentration were performed (see Figure 5.3a). The addition of ethanol into the photooxidative treatments for *C. albicans* induced a drastic decrease in inactivation (high remaining fungus population), compared to those carried out in water without the scavenger (Figure 5.3).

The decrease of the photooxidative capacity toward the yeast in the presence of ethanol was from 6.16 to 0.33 log reduction for UVC/H_2O_2, from 2.39 to 1.04 log reduction for UVC/PDS, and from 3.02 to 3.29 log reduction for UVC/PMS. These results agree with those reported by Wang and collaborators, who established that the presence of alcohols demonstrates the generation of radicals in the processes [53]. Regarding the diminution of the peroxide concentration (Figure 5.3b), it can be noted that when the amount of PDS, PMS, and H_2O_2 was changed from 500 μmol L^{-1} to 50 μmol L^{-1} the inactivation efficiencies were decreased. Thus, if the concentration of the peroxide (i.e., the radical precursor) is lower, the production of radicals is diminished. Thereby, the results using the scavenger and decreasing the peroxide concentration support the relevant participation of radical species in the inactivation of fluconazole-resistant *C. albicans* by phototoxication processes.

After evidencing the participation of radicals on the yeast disinfection, it must be mentioned that the UVC irradiation combined with H_2O_2 had a remarkable ability to inhibit *C. albicans* yeasts; experimentally decreased 6.16 log of the yeast population, improving the values obtained with H_2O_2 alone (1.08 log reduction) and UVC alone (3.25 log reduction, Figure 1). The UV irradiation of wavelength at 254 nm breaks the chemical bond in H_2O_2 and generates $^{\bullet}OH$ (Equation 5.3) [54]. The UV/H_2O_2 process is an attractive option for producing a non-selective and highly reactive radical ($^{\bullet}OH$, E_0 = 1.8-2.7 V) [55], which is mainly responsible for the target yeast inactivation in this photooxidation system, in addition to the direct actions of H_2O_2 and UVC.

$$H_2O_2 + \ h\nu \rightarrow 2\ {}^{\bullet}OH \qquad (5.3)$$

Figure 5.2a also shows that photooxidation with peroxydisulfate (PDS) and peroxymonosulfate (PMS) in combination with UV radiation enabled the inactivation of yeast, attributed to the action of radicals (as demonstrated previously, Figure 10). It must be considered that the peroxydisulfate anion ($S_2O_8^{2-}$) acts as a strong oxidant in water treatment processes, especially when activated to generate highly reactive radicals. In a pH range of 7.0 to 7.4 (neutral or slightly alkaline conditions), activation of $S_2O_8^{2-}$ with UV light can generate both $SO_4^{\bullet-}$ radicals (Equation 5.4). Additionally, the sulfate radical can react with water to produce $^{\bullet}OH$ (Equation 5.5). Besides, the activation of PMS by UVC radiation (λ: 254 nm) generates radicals $SO_4^{\bullet-}$ and $^{\bullet}OH$ through homolytic cleavage of the peroxide bond in PMS (see Equation 5.6) [56]. Then, in the UVC/PMS process, both radicals could contribute to the yeast inactivation (Figures 5.3 and 5.4).

$$S_2O_8^{2-} \xrightarrow{h\nu} 2SO_4^{\bullet-} \quad (5.4)$$

$$SO_4^{\bullet-} + H_2O \rightarrow {}^{\bullet}OH + SO_4^{2-} \quad (5.5)$$

$$HSO_5^- \xrightarrow{h\nu} SO_4^{\bullet-} + {}^{\bullet}OH \quad (5.6)$$

It should be considered that the cell wall of *C. albicans* is mainly composed of β-glucans (β-1,3 and β-1,6), Chitin (β-1,4-N-acetylglucosamine), and mannoproteins (protein-bound mannans) [57]. Previous studies have shown that •OH can interact with mannans and glucans, causing their degradation, with degradation rates reaching between 82% and 91.5% in the absence of salicylate. Indeed, yeast mannans and glucans, such as laminarin, lichenan, curdlan, and CM-glucan, show high susceptibility to degradation by $^{\bullet}OH$ [58]. This suggests that the UVC/H_2O_2 process, through the generation of $^{\bullet}OH$, affects the structure of these polysaccharides, promoting their fragmentation and structural alteration.000000

In the case of the UVC/persulfate systems, we can consider that the generated sulfate and hydroxyl radicals can also interact with the organic compounds in the yeast cell structures, causing their degradation [56]. The sulfate radical is more selective than the hydroxyl radical [59], allowing for the oxidation of specific sites on yeast cell components, which explains the slower inactivation of the target microorganism by the UVC/persulfate system compared to the UVC/H_2O_2 system, as observed in Figure 5.3.

5.3.4. Extent of the photooxidation treatments – Reactivation and susceptibility to fluconazole of the treated yeast

To go beyond measuring the decrease in yeast population, the reactivation and susceptibility to fluconazole of the treated *C. albicans* were assessed (Tables 5.2 and 5.3). The results in this study showed that, after 80 s, there was no reactivation of the *C. albicans* strain subjected to the different photooxidation processes (Table 5.2). Interestingly, the UVC/H_2O_2

system limited yeast reactivation in the dark at shorter treatment times (45 and 60 s) compared to the UVC alone and the other photooxidation systems. The non-reactivation of the microorganism denotes that the action of the photooxidation process induced very strong and irreparable damage to the yeast cell.

Our results are consistent with those of Wen and co-workers, who have also evaluated the reactivation in darkness of fungal spores of *Trichoderma harzianum*, *Aspergillus niger*, and *Penicillium polonicum*, indicating that the repair mechanism in darkness is too weak. This reinforces the conclusion that dark repair of fungi does not occur significantly, which contrasts with photoreactivation, which does show higher levels of recovery under UVA light [60]. Additionally, the literature suggests that dark attenuation reduces photoreactivation, and a prolonged dark delay may be an effective strategy for reducing fungal photoactivation [60]. In the same address, another work reports that the dark reactivation of *Trichoderma harzianum, Aspergillus niger,* and *Penicillium polonicum* spores after 2-$\log_{10}$ inactivation by UV irradiation is negligible. In fact, the concentration of surviving fungi de decreased slightly in the dark. This suggests that DNA repair mechanisms, such as the nucleotide excision repair system, are almost blocked in the absence of light in fungi [61].

On the other hand, the application of UVC light alone and the UVC/persulfate system to *C. albicans* showed a reduction in resistance at two cut-offs (16 µg mL^{-1}), with the fungal strain remaining resistant. However, with the UVC/H_2O_2 system, the yeast transitioned from being resistant to sensitive to fluconazole, with a cut-off of 8 µg mL^{-1} (Table 5.3). Again, these results indicate that the UVC/H^2O^2 system performs best. It is important to consider that the action of this photooxidation process transforms the microorganism into a sensitive yeast. This highlights a beneficial aspect of disinfecting water loaded with antifungal-resistant microorganisms, as it can contribute to limiting the proliferation of resistance. This agrees with the studies conducted by Nowrozi et al., who evaluated the efficacy of UV on the sensitivity of 12 strains of *Candida spp* to the pharmaceuticals itraconazole, fluconazole, and amphotericin B, where the authors determined that after subjecting the strains to UV light, MICs decreased steadily for all drugs studied [62]. They similarly evaluated the effect of UV radiation on 12 clinical strains of *Rhizopus* spp. After irradiation with UV, the MICs decreased [63]. Contrary to the report by Lotfali et al., who found that prolonged exposure to UVC of fungi such as *Aspergillus* spp., *Verticillium* spp., and *Alternaria spp* increases resistance to Itraconazole, Voriconazole, and Fluconazole [64]. Exposure to UV light modifies the defense mechanisms of the fungus (e.g., changes in the expression of certain genes related to resistance and pathogenicity) [65]. In our case, the concomitant action of UVC and radicals (as in UVC/H_2O_2) could lead to significant alterations in the fungus' defense mechanisms, rendering it sensitive to fluconazole.

5.3.5. Morphology changes by the action of the UVC/H_2O_2 process

From the pictures in Figure 5.5, it can be observed that photooxidation with H_2O_2 caused damage to the surface structures of the yeast cell wall and cell membrane; the cells became clumped, increased in size, and appeared empty inside. These results are expected because, as shown in the previous sections, the UVC/H_2O_2 process involves the attack of hydroxyl radicals in addition to the direct action of H_2O_2 and the UVC light on the *C. albicans.*

To explain the morphological changes of the yeast (results in Figure 5.4), we should consider that the hydroxyl radical interacts with mannans and glucans (the main components of the outer part of the target yeast), causing their degradation. In fact, yeast mannans and glucans, such as laminarin, lichenan, curdlan, and CM-glucan, are highly susceptible to degradation by $^{\bullet}OH$ [58]. Consequently, the surface structures of the yeast cell are strongly affected by the UVC/H_2O_2 process. Additionally, it is essential to acknowledge that hydrogen peroxide can directly cause significant damage to Candida albicans cell wall components, including glucans. This damage occurs primarily through oxidation processes that affect the structure and function of cell wall polymers, weakening the cell wall and making the cell more susceptible to lysis.

H_2O_2 and $^{\bullet}OH$ can also oxidize amino acid residues in cell wall proteins, causing misfolding, loss of function, and fragmentation. Although lipids are not the major component of the *C. albicans* cell wall, ROS induces lipid peroxidation, which affects the cell wall structure. Cumulative damage to glucans, proteins, and other cell wall components can compromise their fluidity, permeability, and integrity, facilitating the entry of damaging agents [66]. Furthermore, when hydrogen peroxide penetrates the yeast cell, proteins with iron-sulfur groups are susceptible to oxidation, leading to the Fenton reaction and the production of $^{\bullet}OH$ radicals (Equation 5.7), which cause significant internal cellular damage [43]. Additionally, it is well-known that exposure to high concentrations of H_2O_2 can cause cell death in a manner analogous to apoptosis [42].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad (5.7)$$

5.4. Conclusions

The *Candida albicans* strain examined was susceptible to inactivation by the photolytic method using UVC light and by the direct action of peroxides, such as H_2O_2 and PMS. The combination of UVC and chemical oxidants (photooxidations) significantly enhanced the inactivation of *C. albicans*, mainly showing synergy in the cases of UVC/H_2O_2 and UVC/PDS. The photooxidations followed pseudo-first-order kinetics, with half-lives of less than 10 seconds. Experiments with ethanol as a scavenger confirmed the important role of radical species in the photooxidative processes. Tests of reactivation in darkness demonstrated that the UVC/H_2O_2 system caused significant damage to the yeast, indicating its potential for application and relevance in controlling *C. albicans* in aquatic systems. Surface morphological changes and yeast

clustering observed after UVC/H_2O_2 treatment indicated alterations in the yeast cell structure. Additionally, colonies subjected to the final stage of the photooxidative processes with H_2O_2 showed high susceptibility to fluconazole, being changed from resistant to sensitive. Our results suggest that the photooxidation methods can be tested for future applications in environmental water, wastewater, and hospital wastewater samples loaded with antifungal-resistant microorganisms. Finally, it would be important to determine the potential damage to the yeast genetic material (in further work) to corroborate the elimination of the genes involved in fluconazole resistance.

5.5. Final remarks of Chapter 5

The problem of infections caused by *Candida albicans*, a priority pathogen according to the WHO, is due to its antifungal resistance and high mortality rate in cases of invasive candidiasis. Systems based on ultraviolet-C (UVC) light combined with chemical oxidants such as hydrogen peroxide (H_2O_2), peroxodisulfate (PDS), and peroxomonosulfate (PMS) were evaluated for the inactivation of a fluconazole-resistant strain in aqueous media. The experiments were conducted over short periods (0-80 seconds) and analyzed for inactivation, reactivation in the dark, and changes in susceptibility to fluconazole. The results showed that the UVC/H_2O_2 combination was the most effective, achieving 99.99% inactivation in 60 seconds and a reduction of 6.16 logarithmic units in the yeast population. This system also had the highest inactivation rate constant (k: 0.3164 s^{-1}) and the shortest half-life ($t_{1/2}$: 2.191 s). In comparison, the UVC/PDS and UVC/PMS systems were less effective, with reductions of 2.39 and 3.02 log, respectively. The action of hydroxyl ($^{\bullet}OH$) and sulfate ($SO_4^{\bullet-}$) radicals generated in the photooxidation processes was confirmed by using ethanol as a radical scavenger and by the decrease in the concentration of oxidants.

In this context, advanced oxidation processes (AOPs), which generate reactive oxygen species (ROS) such as hydroxyl radicals, have emerged as a promising solution for treating fungi in water. These processes, which combine ultraviolet-C (UVC) light with inorganic peroxides, are effective in damaging cellular components of microorganisms. Although UVC radiation alone has a strong disinfecting effect, its combination with oxidizing agents can significantly improve efficacy, reducing reactivation in the dark and antifungal resistance. However, to date, no studies have been published on the inactivation of resistant strains of *Candida albicans* in aqueous media using the combination of UVC with different inorganic peroxides. This work aims to assess the efficacy of these processes in inactivating, reactivating in the dark, and altering the susceptibility to fluconazole of a resistant strain of *Candida albicans*.

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PRODUCTS OF THE THESIS

-One review (https://doi.org/10.3390/w16070936)

-Two research papers (https://doi.org/10.3390/ijerph20043428; Manuscript ID: water-3790774)

-Participation in an international event (22nd ISHAM congress 2025, held in Foz do Iguaçu, Brazil, from 20-24 May 2025) with work entitled “Evaluation of the inactivation, reactivation, and susceptibility of a *Candida albicans* strain by advanced oxidation processes based on UV light”.

- 4 undergraduate theses.

FUTURE WORK AND OUTLOOKS

From the development of the present thesis, it was evidenced that further work should be performed in the following topics:

1. Photolysis and photooxidation studies on real wastewater.

2. Studies with other types of fungi prioritized by the World Health Organization, such as *Aspergillus fumigatus, Cryptococcus neoformans, or Candida auris.*

3. Process optimization: Perform detailed life cycle analysis (LCA) and life cycle costs (LCC) to select the best process for pilot-scale applications.

4. Combination of oxidation systems: Explore combinations of processes such as electrochemistry and photo-Fenton (photo-electro-Fenton) for the removal of resistant fungi in wastewater.

5. Construction of a pilot reactor: Design and construct a pilot reactor for the treatment of real hospital wastewaters.

6. Studies of the impact at the genetic level of the fungi subjected to these photo-oxidation treatments.

7. Search for antifungals, fungi, and resistance genes in different water systems.

ANNEX 1 Chapter 2. (Methodoly)

Article: Alternative and Classical Processes for Disinfection of Water Polluted by Fungi: A Systematic Review

This review was conducted according to the PRISMA guide's identification, screening, choice, and inclusion phases (preferred reporting elements for systematic reviews and meta-analyses) [1]. Multiple approaches to compiling publications were considered. We addressed the objective of methods for the disinfection of fungi and antifungal-resistant genes present in aquatic systems. Data and information associated with these interesting topics from each retrieved article were extracted, further described, and analyzed, following the steps detailed below.

Search Strategy and Selection Criteria

Articles about disinfection methods for the elimination of fungi in water systems (artificial and natural) were consulted in search engines and electronic databases: PubMed, Science Direct, Scopus, WOS, Springer, Scielo, PLOS, Hinari, Redalyc, and Taylor. The articles were reviewed without restriction on the year of publication. A strategy was used that incorporated thesauri such as Medical Subject Headings (MeSH), AGROVOC, the descriptors in Health Sciences (Dec's), UNESCO, and Boolean operators AND, &) (OR) (NOT, -) (XOR). Different combinations of keywords were made: "Disinfection AND fungi AND Waters", "Disinfection, Elimination, Antifungal resistance", "Chlorination, "antifungal resistance genes", "resistant fungi in the environment", "water systems", "wastewater", "sewage", "wastewater", "fungi in water", "river pollution", "aquatic environment", resistance azole antifungals, advanced oxidation process, Ozonation, Fenton/solar Fenton oxidation, Heterogeneous photo-catalysis, Ultraviolet irradiation, Ionizing irradiation. Mendeley® (**https://www.mendeley.com/**) was used as the reference manager system.

Eligibility Criteria

Each article included in this systematic review met the following inclusion criteria: (1) The search strategy was restricted to original studies or abstracts published in scientific journals in the English language. We considered the search terms present in the title, abstract, and keywords without restriction of place and time; (2) the search response variable was information describing the different methods of disinfection of fungi in natural and artificial or aquatic environments; (3) systematic reviews, editorials, and policy statements were excluded.

Search for Articles

For the selection of articles, initially, the keywords were delimited, and the potential articles were identified with the search algorithm (Figure 1). Then, the bibliographic information regarding the main author, date of publication, journal, title, and abstract was tabulated in a spreadsheet. The papers that were relevant and provided useful information for our work (focused on fungi disinfection, antifungal resistance, and water treatment) were selected and subsequently reviewed in their entirety. The articles selected from the previous step were re-read under the inclusion and exclusion criteria. Additionally, a secondary search was conducted in congress abstracts, Colombian and foreign consensus on the subject, and the references of the articles selected for full-text reading ("snowball" strategy).

Data Extraction

All articles included were reviewed by two independent reviewers using standardized data extraction tools prepared in a Microsoft Excel spreadsheet. Disagreements were resolved by reviewing the entire article. When the two people did not reach a consensus on a specific article, a third person was introduced. The PRISMA flow chart shows the number of articles at each step of the article selection process (**Figure 1**).

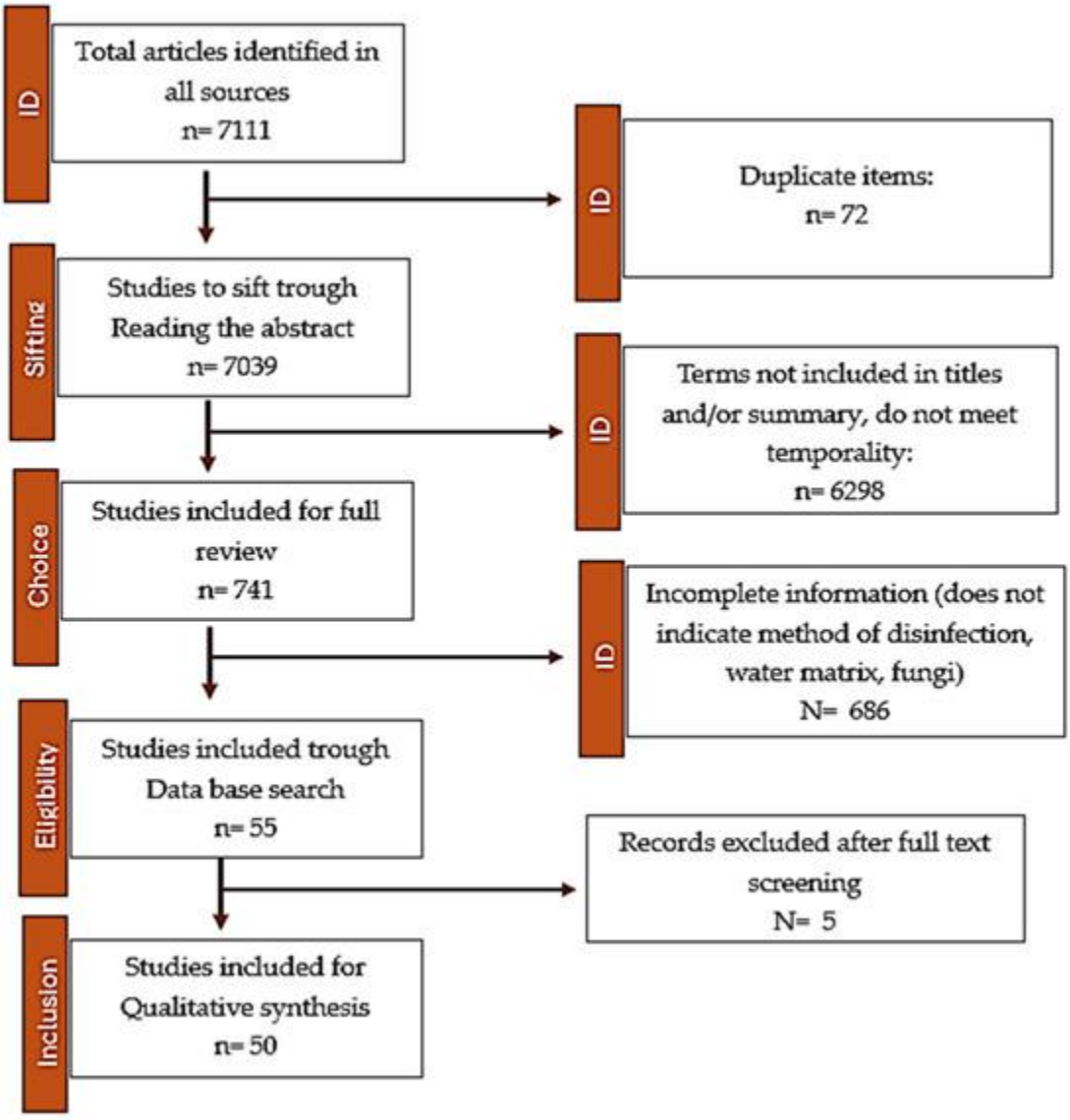

Figure 2S1. PRISMA flowchart of the search strategy and selection of articles reporting disinfection methods in aquatic environments.

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ANNEX 2.

Chapter 3 (Systematic Review)

Table 1S1. Removal of fungi by chlorination

Tab

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
1983	DW	*A. fumigatus* *A. niger* *Cladosporium* sp. *C. laurentii* *P. oxalicum* *R.glutinis* *R. rubra*	Cl_2	$[Cl_2]_{Free}$: 7 mg L^{-1}. [conidia] 1.0×10^5 to 5.0×10^6 [Yeast]: 10^5 to 10^6 pH: 5, 7, or 8. t: 10, 30, 60 min, Inactivate by $Na_2S_2O_3$ 0.25%	pH 5 > 7 > 8. Inactivation level: Conidia's: 1.0 log yeast: 1.0 log. Cl_2 demand (mg per cell or conidium) after 60 min: *A. fumigatus* 1.2×10^{-8} *A. niger* 3.2×10^{-8} *Cladosporium* sp. 3.6×10^{-9} *C. laurentii* 8.0×10^{-9} *P. oxalicum* 5.9×10^{-9} *R. glutinis*, 2.6×10^{-9} *R. rubra* 2.4×10^{-9}	[122]

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2017	DW	*P. purpurogenum A. fumigatus*, *A. versicolor*	Cl_2 NH_2Cl.	$[Cl_2]$ 1 mg L^{-1} of 5% NaClO $[NH_2Cl]$ 4–10 mg L^{-1} pH 7.0 for Cl_2 pH 8.0 for NH_2Cl $[Spore]_0$: 4.3 log spore's mL^{-1}. t: 0, 5, 15, 30, 60 min. Stirred: 300 rpm, T: 22.5 °C.	Ct for $Cl_{2\ free}$ 60 mg min/L. *A. fumigatus*: 2.9 log. *A. fumigatus* 4.6 log *A. versicolor*: 1.9 log *P. purpurogenum*: 0.9 log The disinfection kinetics: Chick–Watson model incorporating an initial lag phase and Markov Chain Monte Carlo model.	[124]
2017	GW	*C. cladosporiodes* *T. harzianum* *P. polonicum*	ClO_2	$5 \pm 0.5 \times 10^5$ CFU mL^{-1}, $[ClO_2]$ 0.5–3 mg L^{-1}. 10 or 27 °C, 130 rpm. 10, 20, 30, 50, and 60 s $Na_2S_2O_3$: 0.1 mol L^{-1} pH 6.0 and 7.0. [Humic acid]: 0, 0.5, 2.0, or 4.0 mg/L, as total organic carbon (TOC). The inactivation kinetics Chick, Chick–Watson, and Hom models.	*Penicillium* sp., *Trichoderma* sp., and *Cladosporium* sp. 100%, 99.6%, and 70% for t: 60 s. $k_{inactivation}$ to ClO_2: *Penicillium* sp., *Trichoderma* sp., *Cladosporium* sp., and *E. coli* were 1.182, 0.615, and 0.398, $k_{inactivation}$ to Cl_2: *Penicillium* sp., *Trichoderma* sp., and *Cladosporium* sp. were 0.045, 0.079, and 0.037.	[14]
2021	GW	*A. niger* *P. polonicum* *T. harzianum*	Cl_2 ClO_2 NH_2Cl	$[Spores]_0$ (4.5–5.5) $\times 10^5$ CFU mL^{-1}, pH: 7.2–7.4, T: 28 ± 1 °C. $[Cl_2]$, $[ClO_2]$, $[NH_2Cl]$: 0.1, 0.3, 0.5, 0.7 and 1.0 mg L^{-1}.	$[Cl_2]_0$ 1.0 mg L^{-1}: k_{max} *A. niger*, *P. polonicum*, and *T. harzianum* 25.0%,43.6%, and 34.6%, respectively. $[ClO_2]_0$ 1.0 mg L^{-1}: k_{max} *A. niger*, *P. polonicum*, and *T. harzianum* decreased by 26.7, 44.4%, and 37.8%, respectively. $[NH_2Cl]_0$ 1.0 mg L^{-1}, k_{max} *A. niger*, *P. polonicum*, and *T. harzianum* decreased by 23.7%, 32.4%, and 31.8%, respectively.	[125]
2021	PFW	*A. flavus* *A. fumigatus*	Cl_2 ClO_2 NH_2Cl	$[Spores]_0$: 5–8 $\times 10^5$ CFU mL^{-1} $[Cl_2]$: 2.0, 3.0 mg L^{-1} pH: 7.0 ± 0.2; T: 25 ± 2 °C	The inactivation rate constants of *Aspergillus fumigatus* at 30% and 63% aggregation degree were 1.5- and 4-fold lower than that of monodisperse spores, respectively.	[126]

Notes: * drinking water (DW), laboratory-grade water (LGW), settled water (SW), hot water system (HWS), groundwater (GW), and pathogenic fungi in water (PFW).

Table 1S2. Removal of fungi by ozonation

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
1994	WW	*T. verrucosum*	O_3	$[O_3]$ up to 25 mg L^{-1} Flow rate: 4 L h^{-1} 6×10^3 CFU mL^{-1}	Total inactivation (100%). Ozone consumption 200–210 mg O_3 $min\ L^{-1}$	[130]
2020	GW	*T. harzianum* *P. polinicum* *A. niger*	O_3	$1–2 \times 10^5$ CFU mL^{-1} $[O_3]_0$: 2.0 mg L^{-1} T: 20 °C, pH = 7.0 40 mmol L^{-1} with 20 mmol L^{-1} t-BuOH	O_3 to inactivation 2 log (99%) (mg $min\ L^{-1}$): *A. niger* 5.65 *T. harzianum* 2.36 *P. polonicum* 0.82	[15]
2021	GW	*A. niger* *T. harzianum*	O_3 Cl_2	$[O_3]_0$ = 1.0, 2.0 mg L^{-1} $[Cl_2]_0$ = 1.0, 2.0, and 3.0 mg L^{-1} in 40 mM PBS pH = 7.0, T = 20 °C; $[Spores]_0$: $1–2 \times 10^5$ CFU mL^{-1}.	The log reduction in survival of *A. niger* and *T. harzianum* spores at 10 min: 0.28- and 0.13 log. Ct O_3 and Cl_2 inactivation 2log *A. niger*: 2.56 mg O_3 $min\ L^{-1}$ and 9.68 mg chlorine $min\ L^{-1}$ *T. harzianum* 2.09 mg O_3 $min\ L^{-1}$ and 6.59 mg chlorine $min\ L^{-1}$	[131]

Notes: * wastewater (WW), groundwater (GW), and phosphate buffer solution (PBS).

Table 1S3. Removal of fungi by UV radiation

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2012	DW	*A. fumigatus* *A. flavus* *A. niger*	UV	UV fluence: 4.15–25 mJ cm^{-2} $10^2–10^3$ CFU mL^{-1} t: 5–30 s Turbidity 1–5 NTU $[Fe^{2+}]$: 0.1–0.5 mg L^{-1}	4 log inactivation achieved at UV fluence (mJ cm^{-2}), respectively: *A. fumigatus* 12.45 *A. flavus* 16.6 *A. niger* 20.75	[149]
2013	UDW GW SW	*Candida* sp. *C. carnescens* *M. viticola* *C. kofuensis* *R. babjevae* *R. minuta* *R. mucilaginosa*	LPUP 254 nm.	UV fluences 0, 5, 10, 20, 30, 40, 70, and 100 mJ cm^{-2} T: 21 ± 2 °C. 2 and 6×10^6 cells mL^{-1}. t: up to 13 min.	2 log inactivation for all yeasts using UV fluences lower than 111 mJ cm^{-2}. UV fluences lower than 32 mJ cm^{-2} to achieve 99% inactivation levels for the tested *Cryptococcus*, *Candida*, and *Metschnikowia* species	[150]
2019	GW	*P. polonicum*, *A. niger* *T. harzianum*	UV 254 nm	UV fluence: 0.112 mW cm^{-2}. $[Spore]_0$: 10^6 CFU mL^{-1}. Room temperature. After 2 log10 UV inactivation: photoreactivation and dark repair	Fungal spores were more resistant compared with *E. coli*. The photoreactivation (k) rate constant of *T. harzianum*, *A. niger*, and *P. polonicum*: 0.0066 min^{-1}, 0.0054 min^{-1}, 0.0107 min^{-1}. respectively.	[93]
2020	GW	*A. niger* *P. polonicum*, *T. harzianum*	UV/LEDs LPUV	Irradiance 0.215 mW/cm^2 for the 265 nm LEDs, 0.214 mW cm^{-2} for the 280 nm LEDs, 0.185 mW cm^{-2} for the 265/280 nm combination UV-LEDs and 0.120 mW/cm^2 for the 254 nm (LP) Initial spore 10^6 CFU mL^{-1}.	UV inactivation efficiency (UV-LEDs and LP UV) was not influenced by the incubation time of spores. UV-LEDs emitting at 265, 280, and 265/280 nm were more effective compared with the 254 nm (LP).	[151]

Table 1S3. Cont.

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2020	GW	*A. niger* *A. terreus* *A. fumigatus*	LED (255 nm, 265 nm)	10^8 spores mL^{-1}, pH 7, Temperature 20 °C. t: 0, 0.5, 1, 5, 10, 15, 30, 45, and 60 min. UV fluence of 2.33 mJ cm^{-2} Monitoring disinfection by plate counting, flow cytometry with viability staining, and electron microscopy.	*A. fumigatus*: 2 log reduction, with 10 min. *A. terreus*: 3 log reduction, with 1.64 mJ cm^{-2} (5 min) for the 265 nm. *A. niger*: reduction < 1 log was obtained even for the highest UV fluence (60 min) using both LEDs.	[152]
2021	GW	*A. niger* *A. terreus* *A. fumigatus*	LEDs (255 nm, 265 nm)	Irradiance 54 μW cm^{-2} (255 nm) and 250 μW cm^{-2} (265 nm). 10^8 spores/mL Exposure: 0, 30 and 60 min Natural light and dark as controls.	LEDs (255 nm) are less efficient in the inactivation of *A. fumigatus* and *A. terreus*, having no inactivation effect on *A. niger*. LEDs that emit at 265 nm showed 3 log, 2 log, and 4 log reduction for *A. fumigatus*, *A. niger*, and *A. terreus*, respectively	[153]
2021	GW	*A. niger* *A. terreus* *A. fumigatus*	UV Mercury lamp and UVH-Lamp Type Z.	10^8 spores/mL, pH 7, T: 20 °C. t: 0, 0.5, 1, 5, 10, 15, 30, 45, and 60 min Efficacy monitoring by plate counting, scanning electron microscopy, flow cytometry analysis, DNA damage, proteome analysis, photoreactivation, and dark repair experiments.	*A. fumigatus*, *A. niger*, and *A. terreus* were 3.05 log, 0.23 log, and 3.50 log after 1 min of inactivation and 5.58 log, 1.90 log, and 5.63 log after 45 min of inactivation. Resistance: *A. niger* > *A. fumigatus* > *A. terreus* to UV MP radiation.	[154]

Notes: * Drinking water (DW), untreated drinking water (UDW), groundwater-surface (GW), spring water (SW). Low-pressure ultraviolet photolysis (LPUP), and light-emitting diodes (LED).

Table 1S4. Removal of fungi by SODIS.

Date	Matrix	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2005	Drinking water	*C. albicans* *F. solani*	SODIS	$[C. albicans]_0 = 2.5 \times 10^5$ CFUmL^{-1} $[F. solani]_0 = 3.4 \times 10^5$ CFU mL^{-1} Global irradiances of 870 W m^{-2} 300 nm–10 μm range and 200 W m^{-2} in the 300–400 nm UV range	*C. albicans* 4.1 log inactivation after 2 h; total inactivation 5.4 log at 6 h. Conidia of *F. solani*, 1.75 log inactivation occurred at 2 h and a total (5.5 log) inactivation at 8 h	[161]
2022	Groundwater	*A. niger* *P. polonicum*	SODIS	$[Spores]_0$: 1–2 $\times 10^5$ CFU mL^{-1} $\lambda \geq 300$ nm. pH = 7.4 ± 0.2. T = 20 ± 2 °C. Irradiance simulated sunlight: 900 W m^{-2} 300–800 nm. Agitation at 200 rpm. The pH 5–9; T: 30–40 °C for *A. niger*, and 20–30 °C for *P. polonicum*.	SL: *A. niger* (64.30 min) > *P. polonicum* (32.27 min) ($p < 0.05$), k_{max}: *A. niger* (0.033 min^{-1}) < *P. polonicum* (0.062 min^{-1}) ($p < 0.05$). 110 min *P. polonicum* to achieve 2 log inactivation, while for *A. niger*, 220 min. pH: 5.0–9.0 and [humic acid]: 1.0, 3.0 mg L^{-1} did not affect the solar inactivation of spores.	[162]

Table 1S5. Removal of fungi by Fenton-based processes.

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2012	SMWWE	*F. solani*	LDFO Solar radiation H_2O_2 oxidation in the dark	$[Spore]_0$: 10^3 CFU mL^{-1} LDFO: $[Fe^{2+}]$: 5 mg L^{-1}, $[H_2O_2]$: 10 mg L^{-1} pH 3, Solar radiation 21.1 kJ L^{-1}, pH 3–8 H_2O_2 oxidation alone up to 20 mg L^{-1} in the dark	Solar irradiation + 10 mg L^{-1} peroxide = ≤2 CFU mL^{-1}, at 11.9 kJ L^{-1}, pH 3 and 16.9 kJ L^{-1} at pH 4–8, but no mineralization occurred. Complete inactivation required 17.1 kJ L^{-1} accompanied by 36% mineralization.	[173]
2013	DW	*P. capsici*	Photo-Fenton Fe^{2+}/H_2O_2	H_2O_2/solar radiation: 2.5, 5, and 10 mg L^{-1} of H_2O_2 Photo-Fenton 1/2.5 mg L^{-1}, 2.5/5 mg L^{-1}, 5/10 mg L^{-1} of Fe^{2+}/H_2O_2 and Solar photo-inactivation. $[Spores]_0$ 315 (±85) CFU mL^{-1}.	Best inactivation results were achieved with 10 mg L^{-1} of H_2O_2 which required only 1 h of solar exposure (4 kJ L^{-1} of QUV) to attain the detection limit (2 CFU mL^{-1}). 1 log spore reduction was attained with 5 mg L^{-1} of Fe^{3+}	[175]
2014	DW, SMWWE MWWE	*F. solani*	Photo-Fenton (Fe^{2+}, Fe^{3+})	Sunlight and 10 mg L^{-1} H_2O_2 in dW. Photo-Fenton with $FeSO_4$ in SMWWE at several concentrations.	The best *F. solani* inactivation rate was with photo-Fenton treatment (10/20 mg L^{-1} of Fe^{2+}/H_2O_2) at pH 3, followed by H_2O_2/Solar (10 mg L^{-1}) and finally TiO_2/Solar was the slowest. Complete inactivation with 27 kJ L^{-1}.	[174]
2017	WW, DW	*Curvularia* sp.	Solar/H_2O_2 Solar photo-Fenton	Several oxidant concentrations, 10, 20, 30, 40, and 60 mg L^{-1} in DW under natural solar irradiation. Acid and near-neutral pH	Complete inactivation with 30, 40, and 60 mg L^{-1} of H_2O_2 with solar UV dose between 14.7 and 15.2 kJ L^{-1}	[176]

Notes: * Simulated municipal wastewater treatment plant effluent (SMWWE), distilled water (DW), real effluents (MWWE), and wastewater (WW). Like dark-Fenton oxidation (LDFO).

Table 1S6 Removal of fungi by photocatalysis with semiconductors

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2005	dW	*C. albicans* *F. solani*	SPC-DIS	$[C. albicans]_0$: 2.5×10^5 CFU mL^{-1} $[F. solani]_0$: 3.4×10^5 CFU mL^{-1}	*C. albicans* 4.1 log inactivation after 2 h with a total inactivation of 5.4 log at 4 h. Conidia of *F. solani*, 1.75 log inactivation occurred at 2 h and a total (5.5 log) inactivation at 4 h	[161]
2009	DW, WW	*F. equiseti* *F. solani*	PC	5 or 6 h exposure Natural sunlight. $[TiO_2]$: 0, 50, and 100 mg L^{-1}, 30 L min^{-1} of flow rate	The highest *Fusarium* spore inactivation with 100 mg L^{-1} TiO_2. Resistant: *Chlamydospores* > macroconidia > microconidia	[179]
2014	DW, SMWWE, MWWE	*F. solani*	PC Solar photoassis-ted H_2O_2	Solar photocatalysis $[TiO_2]$:100 mg L^{-1} SMWWE: Fe^{2+}/H_2O_2 DW: Fe^{3+}/H_2O_2 Solar photo-Fenton ($FeSO_4$, pH: 3), TiO_2, and H_2O_2 in MWWE. PC, $Fe(NO_3)_3$ in SMWWE at pH 3 and several concentrations.	Complete spore inactivation, 31.8 kJ/L of *QUV* were required, and DOC was reduced 56% at the end of the experimental time with 55.42 kJ/L of *QUV*. The highest temperature was 44.1 °C and the pH was 7.8.	[174]
2015	TP	*C. albicans*	UV/TiO_2/Fe^{3+}	$[Spores]_0$:1×10^6 CFU cm^{-3}. λ = 289–365 nm pH 3, 7 and 9. $[FeCl_3]$:1×10^{-2}, 1×10^{-3}, 1×10^{-4} mol L^{-1}.	Log removal: pH 9: 5 log, pH 3: 3.5 log pH 7: 4 log $[TiO_2]$ 0.5; 0.25 and 0.1 g L^{-3} constitutes, respectively, 0.24, 0.37, and 0.5 log	[180]
2017	WW	*A. fumigatus* *Penicillium* spp.	PC (UVA/TiO_2)	$[TiO_2]_{P25}$: 0.125 mg L^{-1}	Inactivation after 60 min of irradiation. Inhibition of 98.5% and 99.7% were by *A. fumigatus* and *Penicillium* sp. respectively, after 180 min under UVA irradiation	[181]
2017	WW, DW	*Curvularia* sp.	SPC-DIS with H_2O_2	TiO_2/solar and TiO_2/H_2O_2/solar in vessel reactors. $[TiO_2]$: 35, 50, and 100 mg L^{-1} Natural solar radiation in dW, $[H_2O_2]$: 10, 20, 30, 40 and 60 mg L^{-1}	(~1 log). light inactivation under natural sunlight. The water temperature varied from 26.1 to 38.2 ± 0.1 °C. 3 h.	[176]

Notes: * Drinking water (dW), distilled water (DW), simulated municipal wastewater effluent (SMWWE), real municipal wastewater effluent (MWWE), wastewater (WW), Solar photocatalytic (TiO_2) disinfection (SPC-DIS), TiO_2 photocatalysis (PC).

Table 1S7. Removal of fungi by UV/chlorination

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2013	dW	*A. flavus*	UV Cl_2 UV-Cl_2	UV fluence: 73.33 to 2239.5 mJ cm^2. Contact time 1–60 s. [NaClO]: 0.5 to 3 mg L^{-1}. Contact time: 1, 5, 10, 15, 30, 60, 120, 240, 480, and 1440 min. 10 °C. Ascorbic acid (25 mg L^{-1}) was used to quench Cl_2, pH: 7.	UV of 1119 mJ cm^2: 2 log after the 30 s Chlorination: 0.5 mg L^{-1}: 3 log at 120 min 1 mg L^{-1}: 3 log at 60 min 2 mg L^{-1}: 3 log: 10 min, 4 log: 2 h. 100% elimination: 24 h. 3 mg L^{-1}: 2 log after 1 min, 4 log after 2 h, 100% inactivation after 24 h. UV for 5 s followed by Cl_2 (0.5–3 mg L^{-1}) showed better results than treatments used alone.	[185]
2017	dW RSW	*A. fumigatus* *A. flavus* *A. niger* *A. terreus* *A. alutaceus* *A. sulfurous* *P. chrysogenum* *P. gloprum* *T. viride*	UV Cl_2 UV-Cl_2	UV: t:15, 30, 60, 90, 120, 150 and 180 s [NaClO]: 0.5–4 mg L^{-1}. t: 5, 15, 30, 60 and 120 min. [Ascorbic acid]: 25 mg L^{-1} to quench Cl_2. UV- Cl_2: UV exposure 15–120 s and [NaClO]: 1–0.125 mg L^{-1}	[NaClO] 0.125 mg L^{-1} and UV exposure 15 s were required to eliminate the fungal contamination from a water sample.	[186]
2020	GW	*P. polonicum* *A. niger* *T. harzianum*	UV-LEDs/Cl_2 LPUV/Cl_2 UV	UV, Cl_2, UV-LEDs/Cl_2 and LPUV/Cl_2. $[Spores]_0$: 2–4 × 10^6 CFU mL^{-1}, $[Cl_2]$: 2 mg L^{-1}. UVA intensity: 0.25 mW cm^{-2}.	UV-LEDs/Cl_2 exhibited better inactivation compared to UV alone and Cl_2 alone. The inactivation rate constants (k) by Cl_2 alone for *P. polonicum*, *A. niger*, and *T. harzianum* were only 0.022, 0.011, and 0.008 min^{-1}, respectively.	[184]
2022	GW	*Penicillium polonicum* *Aspergillus niger* *Trichoderma harzianum*	UV-Cl_2, Cl_2-UV, UV/Cl_2-UV, UV-UV/Cl_2,	UV fluence: 40 mJ cm^{-2} $[Cl_2]$: 2 mg L^{-1} and 30 min at each stage.	UV-Cl_2 (UV_{265}-Cl_2, UV_{280}-Cl_2), treatments by UV_{265} and UV_{280} with the fluence of 40 mJ cm^{-2} caused the LCR of 1.75 log and 2.23 log, 2.20 log and 2.10 log, 0.76 log and 0.87 log for *P. polonicum*, *A. niger*, and *T. harzianum* respectively.	[9]

Notes: * drinking water (dW), river surface water (RSW), and groundwater (GW).

Table 1S8. Removal of fungi by UV/PS or UV/PMS systems

Date	Matrix *	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2022	GW	*T. harzianum* *P. polinicum* *A. niger*	UV-LEDs/PS UV-LEDs/PMS	$[Spores]_0$: 2–4 × 10^6 CFU mL^{-1} T: 25 ± 2 °C. UV irradiance 254, 265, 280 and 265/280 nm was 0.215, 0.214, 0.185 and 0.120 mW cm^{-1} respectively [PS or PMS]: 0.1 mmol L^{-1}	2 log *P. polonicum* y *T. harzianum*: fluence 20–40 mJ cm^{-2} 2 log *A. niger*: fluence 60 mJ cm^{-2}	[16]
2019	GW	*A. niger* *T. harzianum* *P. polinicum*	UV/PMS	The final concentration of fungal spores: 10^6 CFU mL^{-1}. UV fluence: 0.109 mW cm^{-2}. UVA_{365nm} irradiance: 0.10–0.25 mW cm^{-2}. 1 mL $Na_2S_2O_3$ (1 mol L^{-1}) to terminate the process. 1 mL reagent (PMS) was added to 98 mL of PBS solution without a chloride	UV inactivation rate constants (k) of *T. harzianum*, *P. polonicum*, and *A. niger*: 0.0638, 0.0859, and 0.0368 mJ cm^{-2} respectively.	[189]
2017	PBS GW	*Trichoderma* sp. *Acremonium* sp. *Penicillium* sp. *Cladosporium* sp.	UV/PMS	UV_{254} in PBS solution pH = 7.0 T = 20 ± 2 °C Fluence rate: 0.109 mW cm^{-2}. $[Spores]_0$: 2–7 × 10^5 CFU mL^{-1} UV-AOPs (UV/PMS, UV/PS): [PMS] = [PS] = $[H_2O_2]$ = 0.1 mmol L^{-1} UV dose 40 mJ cm^{-2}	UV dose (mJ cm^{-2}) for 99% inactivation in PBS: *Trichoderma* sp. 45; *Acremonium* sp. 50; *Penicillium* sp. 65; *Cladosporium* sp. 130. UV + PMS dose (mJ cm^{-2}) for 99% inactivation in PBS: *Trichoderma* sp. 35; *Acremonium* sp. 35; *Penicillium* sp. 45; *Cladosporium* sp. 85.	[17]

Notes: * groundwater (GW), peroxydisulfate (PS), and peroxymonosulfate (PMS).

Table 1S9. Removal of fungi by UV/H_2O_2, UV/PAA, and UV/O3.

Date	Matrix	Fungi	Method	Experimental Conditions	Efficiency	Ref.
2011	DW SWWE	*F. equiseti*	H_2O_2/UV-Vis	10 mg L^{-1} of H_2O_2 in 60 L CPC photoreactor 325 (±70) CFU mL^{-1} Exposure: 2–5 h	Removal: 2 log dW: 27.1 kJ L^{-1} SWWE: 31.8 kJ L^{-1} DW: 44.5 kJ L^{-1}	[192]
2009	DW	*F. solani*	H_2O_2/ UV solar	$[H_2O_2]$: 5–500 mg L^{-1} Triplicates Temperature: >25 °C Sunlight + H_2O_2	Log removal: 0.6 log; 1.5 log 30.4 kJ L^{-1}; 38.5 kJ L^{-1}	[191]
2017	SWWE	*A. niger* *R. rubra*	UV/O_3	UV 254 nm Contact time: 30 min Density: 10^3 CFU/100 mL	Log inactivation 2.3 ± 0.7 for *Rhodotorula rubra* and *Aspergillus niger*, corresponding to 99.98 ± 0.03% and 98.25 ± 2.20%, respectively. 30 min was sufficient time to achieve log reductions of 3.3 ± 0.2 for fungi	[193]
2019	GW	*A. niger* *T. harzianum* *P. polinicum*	UV/H_2O_2	The final concentration of fungal spores: 10^6 CFU/mL. UV fluence rate: 0.109 mW cm^{-1}. UVA_{365nm} irradiance intensity: 0.10–0.25 mW cm^{-1}. An aliquot of 1 mL reagent (H_2O_2) was added to 98 mL PBS without a chloride ion.	The UV inactivation rate constants (k) of *T. harzianum*, *P. polonicum*, *and A. niger* are 0.0638, 0.0859, and 0.0368 cm^{-2} mJ, respectively.	[189]
2022	GW	*T. harzianum* *P. polinicum* *A. niger*	UV-LEDs/H_2O_2	Initial concentration: 2–4 × 10^6 CFU/mL Temperatura (25 ± 2 °C). The irradiance for the 265, 280, and 265/280 nm combination was 0.215, 0.214, 0.185, and 0.120 mW cm^{-2}, respectively $[H_2O_2]$: 0.1 mM	UV fluence to achieve 2 log reduction in *P. polonicum* y *T. harzianum*: 20–40 mJ cm^{-2} for each wavelength. UV fluence to achieve 2 log reduction in *A. niger*: 60 mJ/cm^2	[16]
2022	PBS SW	*A. niger* *A. flavus*	UV/PAA	[PAA]0 = 7.0 mg L−1, UV irradiance = 0.120 mW cm^{-2}; pH = 7.2 ± 0.2; T = 25 ± 2 °C; initial concentration of fungal spores: 2.5 × 10^5 CFU mL^{-1}. Concentrations of PAA (5.0, 7.0, and 10.0 mg L^{-1}). pH value (5.0, 7.0 and 9.0).	The k of *A. niger* and *A. flavus* was similar at pH 5.0 and 7.0, while it decreased 60.00% and 39.13% at pH 9.0 compared with that at pH 7.0. The inactivation of *A. niger* in the UV/PAA system: 48.23%. The inactivation of *A. flavus*: 64.91%. k of *A. niger* by UV/PAA: 0.48 min^{-1}, and k of *A. flavus* by the UV/PAA: 0.91 min^{-1}.	[146]

Notes: distilled water (dW), drinking water (DW), simulated wastewater effluent (SWWE), groundwater (GW), phosphate buffered solution (PBS), and surface water (SW).

ANNEX 3.

Chapter 4 (Article)

Table 3S.1. Yeast strains were analyzed in this study.

Strain	**Species**	Identification m**ethod**	**GenBank Accession**	Reference
CS11	*Rhodotorula paludigena*	ITS	MT161369	[26]
CS12A	*Rh. mucilaginosa*	ITS	-	[26]
CS13	*Rh. paludigena*	ITS	MT161370	[26]
CS14	ND	-	-	
CS15	*Vanrija humicola*	API 20C	-	
CS16	*Candida parapsilosis*	API 20C	-	
CS17	*Diutina catenulata*	ITS	OP696682	
CS17A	*Rh.mucilaginosa*	LSU	OP658772	[26]
CS18	*D. catenulata*	ITS	OP696683	
CS19	*C. tropicalis*	LSU	OP658773	
CS1A	*C. glabrata*	API 20C	-	
CS1B	*Rh. mucilaginosa*	ITS	-	[26]
CS2	*Candida* sp. 1	ITS	OP696681	

CS20A	*C. tropicalis*	LSU	OP658775	
CS20B	*Rhodotorula paludigena*	ITS	MT161371	[26]
CS21	*Pichia fermentans*	ITS	OP696684	
CS22	ND	-	-	
CS23	*Cryptococcus neoformans*	API 20C	-	
CS24	*Cr. neoformans*	API 20C	-	
CS4	*Trichosporon mucoides*	API 20C	-	
CS45	*P. kluyveri*	ITS	OP696685	
CS51A	*D. catenulata*	ITS	-	
CS51B	*C. tropicalis*	API 20C	-	
CS55	*C. albicans*	ITS	OP696686	
CS7	*C. tropicalis*	ITS/LSU	OP658776	
CS7B	*Geotrichum candidum*	API 20C	-	
CS7C	ND	-	-	
CS9	*Candida* sp. 1	ITS	MK256275	
CS9A	*Rh. mucilaginosa*	ITS	MT161372	[26]
CS9B	*Rh. mucilaginosa*	ITS	OP696687	[26]
M10	*Rh. paludigena*	ITS	-	
M12	ND	-	-	
M14	*P. kudriavzevii*	ITS/LSU	OP658777	
M2	ND	-	-	
M23A	*P. kudriavzevii*	LSU	OP658778	
M23B	*Rh. paludigena*	ITS	MT161375	[26]

Table 2S1 Cont.

P1	***C. albicans***	**ITS**	**OP696688**	
P10A	*Rh. mucilaginosa*	ITS	MT161376	[26]
P10B	*Rh. mucilaginosa*	ITS	-	[26]
P12	*P. kudriavzevii*	ITS/LSU	OP658782	
P13A	*Candida* sp. 1	ITS	MK256275	
P13B	*C. albicans*	API 20C	-	
P14	*C. aaseri*	ITS/LSU	OP658783	[81]
P15	*P. fermentans*	ITS	-	
P16A	*C. tropicalis*	API 20C	-	
P16B	*Papiliotrema laurentii*	ITS	-	
P20A	*Debaryomyces hansenii*	API 20C	-	
P22	*P. kudriavzevii*	API 20C	-	

P24A	*D. hansenii*	ITS	-
P24B	*Rh. mucilaginosa*	ITS	OP696689
P3	*Saccharomyces cerevisiae*	API 20C	-
P3A	*Hanseniaspora pseudoguilliermondii*	LSU	OP658784
P4	*C. tropicalis*	API 20C	-
P46	*Candida* sp. 2[b]	ITS	MK256277
P5	*Meyerozyma guilliermondii*	ITS	-
P7	*Rh. mucilaginosa*	LSU	OP658843
P8	*Rh. mucilaginosa*	ITS	-
P9A	*P. fermentans*	ITS	OP696690
PM14	*G. candidum*	API 20C	-
PM15	*Pichia* sp.	ITS	OP696691
PM18	*T. coreemiforme*	ITS	OP696692
PM19	*P. fermentans*	ITS	OP696693
PM20	*Naganishia* sp.	ITS	OP696697
PM22	*C. albicans*	API 20C	-
PM24	*Candida* sp. 1	ITS	-
PM4A	*T. mucoides*	API 20C	-
PM4B	*P. laurentii*	API 20C	-
PM54	*Candida* sp. 1	ITS	MK256279
PM54A	*C. parapsilosis*	API 20C	-
PM59	*Candida* sp. 1	ITS	MK256278
PM64	*Candida* sp. 1	ITS	MK256276
PM79	*P. fermentans*	ITS	OP696694
PM7B	*Pichia* sp.	ITS	OP696695

[a]Closely related to *Candida intermedia*. [b]Closely related to *Candida pseudolambica*

We used two methods of identification: Biochemical tests (API 20C) and sequence analysis. Yeast strains labeled with "CS" were isolated from the South Channel, Navarro; yeast strains labeled with "P" were isolated from WWTP “PTAR”; yeast strains labeled with "PM" were isolated from DWTP “Puerto Mallarino”, Cauca River; and yeast strains labeled with "M" were isolated from Melendez River. ND: not determined. ITS Internal Transcribed Spacers; LSU D1/D2 domain of the Large Subunit of the Ribosome.

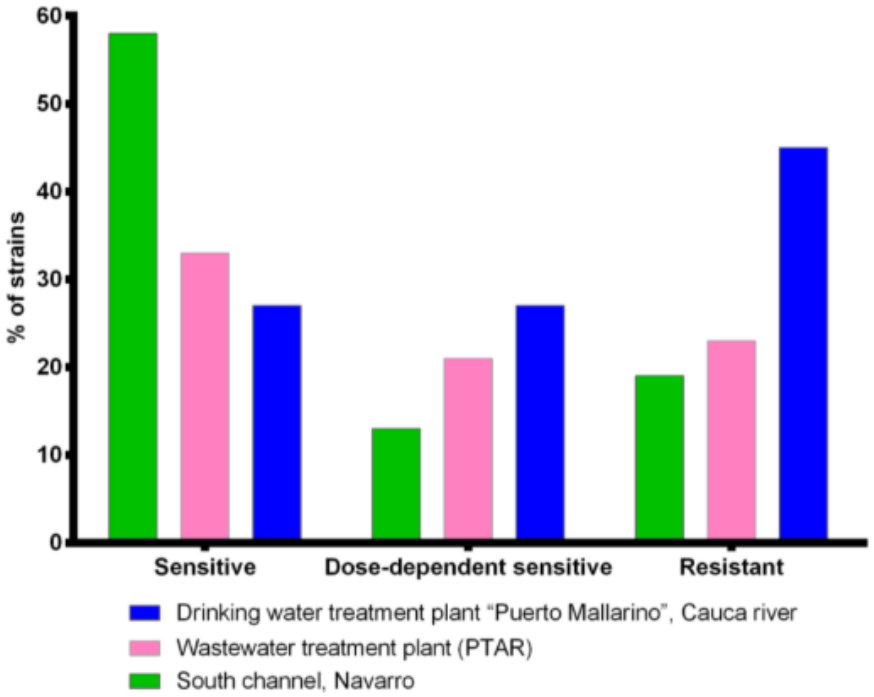

Figure 3S.1. Percentage of sensitive, dose-dependent sensitive, or resistant strains for each source isolation, depending on the ranges of MIC established for FCZ.

ANNEX 4 (Methodology Chapter 5)

Article: Evaluation of ultraviolet light-based oxidative systems for the inactivation and susceptibility of a fluconazole-resistant *Candida albicans* strain

Reagents

The culture media used were: YPD agar (Sigma-Aldrich) and Sabouraud agar (Merck). The inorganic peroxides were: Peroxydisulfate (PDS, 99.0% purity) and peroxymonosulfate (PMS, as Oxone®, in analytical grade) were purchased from Sigma-Aldrich, and hydrogen peroxide (H2O2, 35% v/v), sodium chloride (99%, purity), and ethanol (96%, purity) were provided by Merck. All reagents were prepared with deionized water obtained from a Merck Milli-Q water system. All dilutions of the fungus for the different assays were performed in physiological solution (NaCl, 0.85% w/v). The peroxide solutions were prepared on the same day as they were assayed. The concentrations of the oxidants were 50 and 500 µmol L-1, which were obtained by dilution of concentrated PMS 123 (0.1537 g in 10 mL), PDS (0.1190 g in 10 mL), and H2O2 (57 µL in 10 mL) solutions.

Fungal strain and inoculum

A fluconazole-resistant *Candida albicans* strain supplied by the Secretaría de Salud Municipal de la ciudad de Cali (Colombia) was used for the assays. The strain was preserved in YPD agar and then transferred to YPD broth, where it was incubated for 12 hours with constant agitation at 28°C. The concentration of the inoculum under study was measured using a spectrophotometer at a wavelength of 530 nm to obtain an optical density to 0.5 (OD530 = 0.5), which corresponds to $1x10^8$ CFU mL^{-1}. The OD measurements for the fungi were performed in a Spectronic spectrophotometer.

Reaction system

Assays were performed in a homemade aluminum reflector box equipped with a UVC lamp (OSRAM HNS®) of 8 W and maximum emission at 254 nm, which has an actual intensity of 2.3 µW cm^{-1} (measured by hydrogen peroxide actinometry based on Kuhn et al. [34]). For the disinfection tests, 5 mL of the yeast inoculum with 45 mL of sterile water (pH 7.2 ± 0.2) and constant agitation (200 rpm) at 25 ± 2 °C were mixed, according to the protocol of Serna-Galvis et al.[1] [2]. This mixture was left for 30 min in darkness, and three serial dilutions were made in physiological saline (10^{-1} to 10^{-4}) to check the initial concentration of the inoculum. Then, it was exposed to UVC light, and sampling was performed at 15, 30, 45, 60, and 80s of treatment, and the corresponding serial dilutions (10^{-1} to 10^{-4}) were made duplicate. For testing the photooxidation, the same procedure was repeated, but adding PMS, PDS, and H_2O_2 (at 500 µmol L^{-1}, this concentration was selected based on previous works, [1][2] individually before turning on the UVC

lamp. Also, the chemical oxidation control (i.e., the peroxides without the UVC light) was set up following the same procedure. Each disinfection experiment was carried out at least in triplicate.

Yeast counting

During the assays, 1.0 mL of the reactor content was transferred to screw-capped 149 tubes containing 10 mL of sterile physiological solution (0.85% NaCl) at a 10^{-1} dilution. This was followed by subsequent serial dilutions from 10^{-2} to 10^{-4}. Finally, 100µL of the dilution tubes were seeded on a YPD plate and incubated (24-48 h at 25 °C) for yeast enumeration. During the different assays, aliquots were taken for yeast observation with an Olympus CX21 microscope.

Dark reactivation of yeasts

The reactivation study was conducted using the UVC, UVC/PMS, UVC/PDS, and UVC/H^2O^2 treatments, performed at exposure times of 30, 45, 60, 80, and 120 s. For such tests, 100 µL of each sample was taken and inoculated in 900 mL of Sabouraud broth, left in the dark for 5 days, and observed for the presence of turbidity. Then, 100 µL of the incubated broth was inoculated into Petri dishes containing Sabouraud agar, and the plates were incubated in the dark. Fungal growth was observed at 48 h. Tubes with turbidity were considered positive, and yeast growth was checked in the agar cultures. A possible fungistatic action was observed in the tubes without turbidity. The tests were performed at least in triplicate.

Susceptibility of C. albicans to fluconazole using the macrodilution technique

The methods standardized by CLSI for studying antifungal sensitivity (Documents M27-A3, M38-A, and M44-A)[3] were followed. Sterile tubes of 11 mm × 70 mm and a final volume of 1 mL were used in each tube. The culture medium used was synthetic RPMI 1640 medium with glutamine and without sodium bicarbonate (Gibco, ICN, Oxoid, Sigma), 10.40 g buffered with 0.164 µmol L^{-1} morpholino propane sulfonic acid (MOPDS) (Sigma), 34.53 g adjusted to pH 7.0 ± 0.1 and containing 0.2% glucose. Preparation of the culture medium and antifungal stock solution was consistent with the microdilution method [3]. The indicated amounts were dissolved in 900 mL of distilled water, with stirring until complete dissolution. The pH was adjusted to 6.9 - 7.1, using 1N NaOH, and distilled water was added up to 1 liter. A 0.22 µm Millipore filter was used, and the sample was filtered under sterile conditions. The solution was kept refrigerated (4-8 °C).

The fluconazole standard was weighed to obtain a concentration at least 10 times the maximum concentration of the antifungal to be tested (1.28 mg/mL in sterile distilled water). It was divided into 1.1-mL aliquots and frozen at -40 °C. Dilutions were made starting from the concentration of 640 μg mL^{-1} up to dilution 1.25 μg mL^{-1}. On the day of testing, the tubes were thawed and diluted 1:10 by adding 0.9 mL of inoculated RPMI to each tube, thus obtaining a dilution of 640 µg mL^{-1}.

Before sensitivity testing, two passages were performed on 20% Sabouraud dextrose agar (Merck 103873). An inoculum for *Candida albicans* was prepared by transferring 5 colonies ≥1 mm in diameter and 24 h of growth on an SDA plate with a culture loop and then suspending it in a tube

of physiological solution (0.85% NaCl). The initial inoculum was shaken and adjusted to an optical density of 0.5 McFarland at a wavelength of 530 nm, corresponding to an approximate concentration of $1x10^6$ - $5x10^6$ CFU mL^{-1}. Subsequently, a 1:2000 dilution was performed with RPMI medium. The final concentration of yeast on the plates was $0.5x10^3$ - $2.5x10^3$.

A control strain was included in each assay to detect any abnormalities or inactivation of the antifungal. The strain used was *C. parapsilosis* ATCC 22019. This test was performed in triplicate. The reading was performed visually at 48 h of incubation by comparing the turbidity of the tubes with that of the diluted control 1/20 (0.2 mL of the control tube plus 0.8 mL of RPMI). The MIC of the azoles was the lowest antifungal concentration that produced 80% growth inactivation. The MIC range of the antifungals for quality control strains of *C. parapsilosis* 22019, determined by the M27-A3 macrodilution method, was 2-8 g mL^{-1} [3,4].

References

1. Serna-Galvis, E.A.; Salazar-Ospina, L.; Jiménez, J.N.; Pino, N.J.; Torres-Palma, R.A. Elimination of Carbapenem Resistant Klebsiella Pneumoniae in Water by UV-C, UV-C/Persulfate and UV-C/H2O2. Evaluation of Response to Antibiotic, Residual Effect of the Processes and Removal of Resistance Gene. *J. Environ. Chem*. Eng. 2020, 8, 102196, doi:10.1016/j.jece.2018.02.004.
2. Verbel-Olarte, M.I.; Serna-Galvis, E.A.; Salazar-Ospina, L.; Jiménez, J.N.; Porras, J.; Pulgarin, C.; Torres-Palma, R.A. Irreversible Inactivation of Carbapenem-Resistant Klebsiella Pneumoniae and Its Genes in Water by Photo-Electro-Oxidation and Photo-Electro-Fenton - Processes Action Modes. *Sci. Total Environ*. 2021, 792, 148360, doi:10.1016/j.scitotenv.2021.148360.
3. Cantón Emilia, Martín Mazuelos Estrella, Espinel-ingroff, A. Métodos Estandarizados Por El CLSI Para El Estudio de La Sensibilidad a Los Antifúngicos (Documentos M27-A3, M38-A y M44-A); 2007; ISBN 978-84-611-8776-8.
4. Espinel-ingroff, A. Commercial Methods for Antifungal Susceptibility Testing of Yeasts : Strengths and Limitations as Predictors of Resistance. *J. fungi* 2022, 8, 309, doi:https://doi.org/10.3390/ jof8030309.

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ANNEX 4

Chapter 5 (Article) (Inactivation and reactivation study)

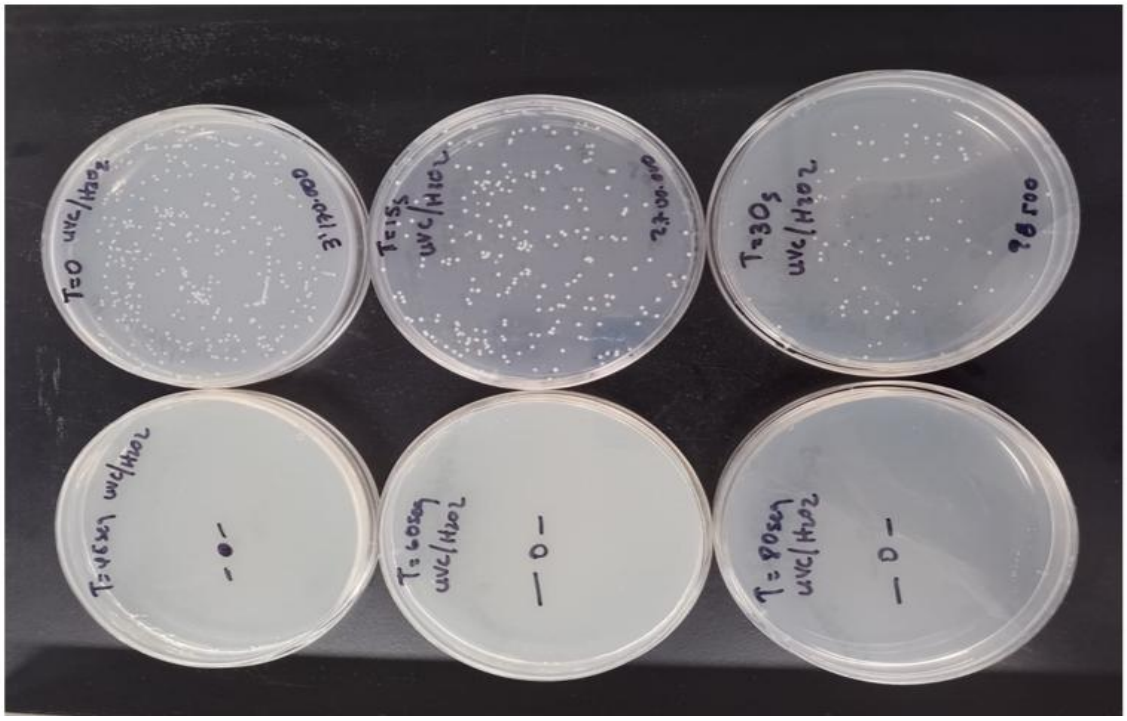

Figure 4S.1. Inactivation of fluconazole-resistant *Candida albicans* /UVC/H_2O_2. Tiempo 0, 15,30, 45, 60 y 80 s

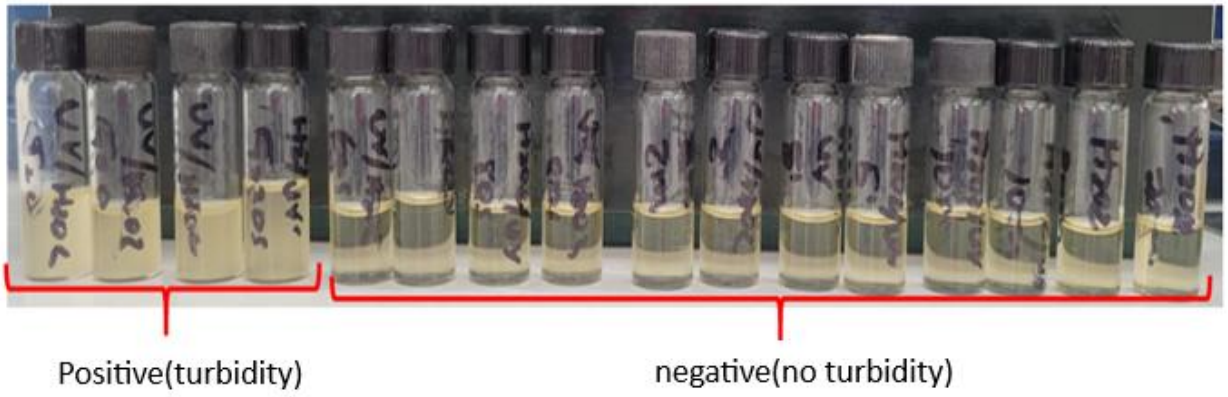

Figure 4S.2. Reactivation in the dark. *Candida albicans* resistant/ UVC/H_2O_2. Exposure time 0, 15, 30, 45, 45, 80, 120, 120, 300, 600, and 1200 sec. Reactivation only occurred when exposure was up to 30s. After 45 seconds, there was inactivation and no reactivation in the dark.

Chapter 5. Susceptibility study

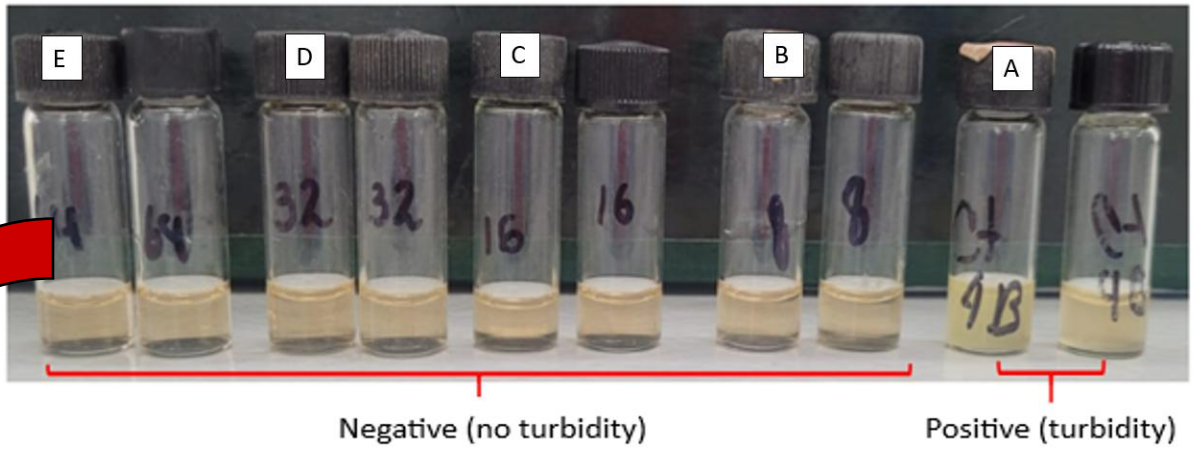

Figure 5S1. Fluconazole-resistant *Candida Albicans* UVC/H_2O_2. 8 μg/mL to 64 μg/mL
A: Positive control without antifungal, B: 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL

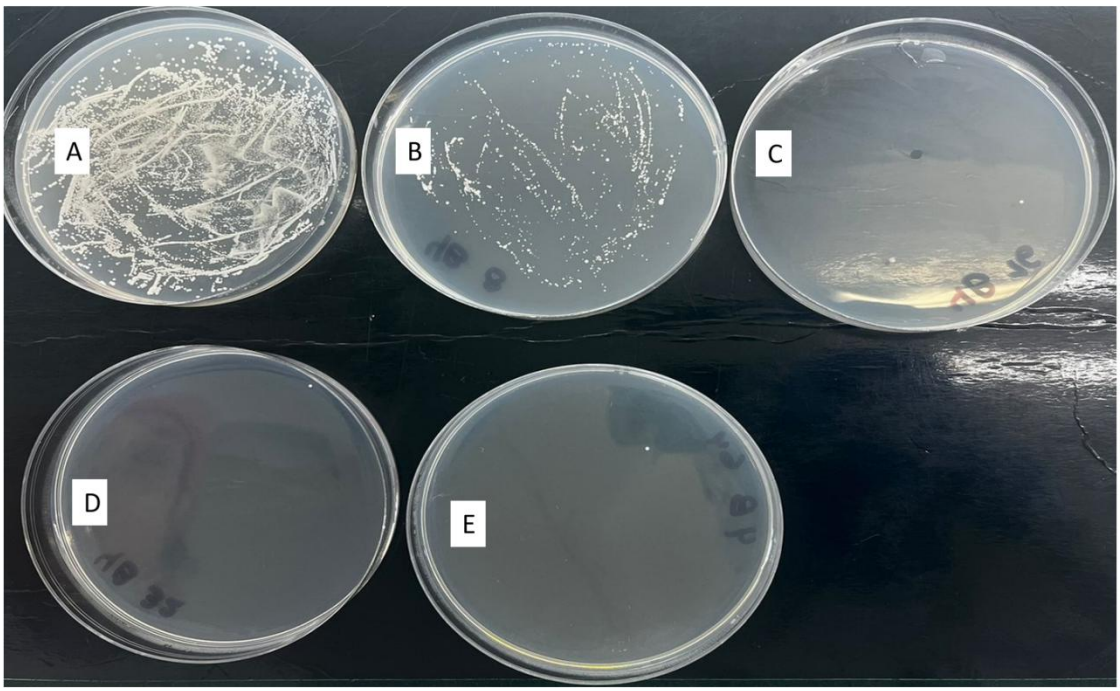

Figure 5S2. Fluconazole-resistant *Candida Albicans* UVC/H_2O_2. Dilution 10^{-1}
8 μg/mL to 64 μg/mL:. Positive control without antifungal, B: 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL

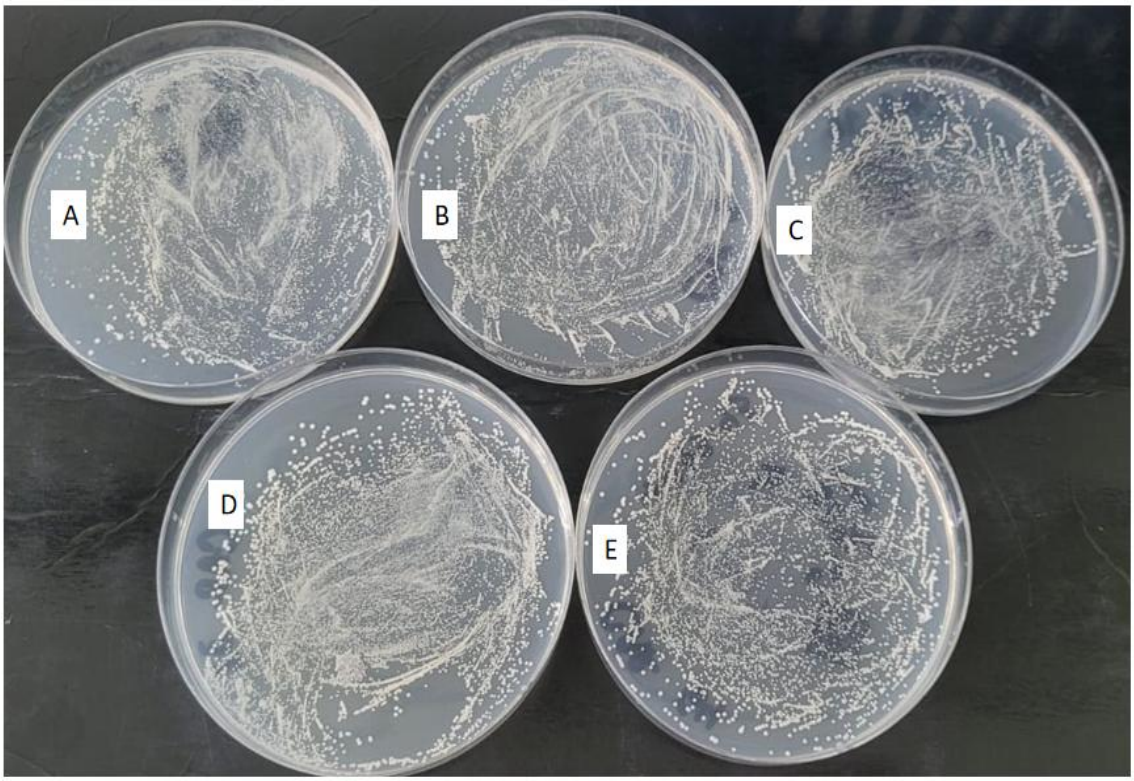

Figure 5S3. Fluconazole-Resistant *Candida Albicans*. Dilution 10^{-1} No treatment.
8 μg/mL to 64 μg/mL: Positive control without antifungal, B: 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL