FUNGAL BIOFILMS IN DRINKING WATER STORAGE CONTAINERS: FLIPPING THOUGH THEIR HEALTH RISKS. A CASE-STUDY OF HOUSEHOLDS AT DIOBU, PORT HARCOURT, NIGERIA.

ABSTRACT

The formation of fungal biofilm in drinking water storage containers has not been giving considerable attention over the years despite the challenges posed by bacterial biofilms. Hence, the study aimed to determine the fungal occurrence and the associated biofilm formation capacity from domestic drinking water storage containers in Diobu homes of Port Harcourt. Fifty water samples were obtained from domestic storage containers in different house units, and the sample was subjected to the standard microbiological procedure. The spread and streak techniques were applied on antibiotics treated Saboraud Dextrose and Congo red growth media. The result shows that the mean fungi count of 6.2 x 10^2 and 6.1 x 10^2 CFU/ml in household sizes of 4 and 5 persons respectively were less than that of the household population of 2, 6, and 8 persons with the fungal count of 8.7 x 10^2, 8.3 x10^2 and 8.0 x10^2 CFU/ml respectively. Positive, negative and zero relationships were established between the household sizes and the fungal recovered. The analysis further revealed a total of 25 fungal isolates of which three genera were distinguished morphologically, namely, *Aspergillus flavus*, *Candida albicans* and *Penicillium notatum*. All isolates were able to form a heavy mass of biofilm; except *Candida albicans* which had a delayed formation after 24hours with less biofilm development potential, and thus signifies its inability to persist, despite having the highest count or load. The success of fungi biofilm is based on the constant growth rate of Aspergillus *flavus* and *Penicillium notatum* biofilms for which, the household size has reasonably be attributed. The study recommends that storage facilities in the homes be regularly cleaned and always disinfected to inhibit the successes of fungal biofilm.

Keywords: Fungal Biofilm, Drinking Water, Storage Containers, Household Size

Introduction

Fungal contaminated drinking water has become essential to researchers and health practitioners alike due to the severe implications of fungal infection on immune-suppressed patients (Tekere *et al.*,2019). Fungi infection has also been linked to allergic diseases, including worsening asthmatic symptoms and conditions, hypersensitivity, and skin irritation (Tekere *et al.*,2019). The role of fungal contaminated water in the transmission of diseases was not given the required attention (Babic *et al.*,2017). Fungi and other microbes such as bacteria, viruses and protozoa were reported as biological pollutants in drinking water (Hamid *et al.*, 2014). Several years earlier, Arvanitidou *et al.* (2000) analysed the presence of fungi in drinking water samples obtained from different sources such as spring water, shower water, tap water, groundwater, storage tank as well as water consumed in hospitals and schools from which the following fungi genera: *Fusarium, Aspergillus, Cladosporium, Epicoccum* and *Trichoderma* were isolated and

identified. According to Grabinska-Lonesiska (2007), water can be contaminated by fungi in distribution pipes, thus adversely affecting the water's odour and taste. Pipe-borne water, when consumed, as reported in studies carried out by Hamid *et al.* (2014), is known to be strongly allergenic or successful in immune-suppressed persons of cancer, asthma and AIDS patients. Like Fusarium spp and Aspergillus spp, Fungi isolates were reported by Anaissie *et al.* (2003) to occur in water storage containers of some households where the fungi build up biofilm, which encourages its persistence.

Perhaps, a deep look into the definition of biofilms will help elucidate the subject matter of discuss. According to the American Environmental Protection Agency (2002), " biofilm is a complex mixture of microbes, organic and inorganic material accumulated amid a microbially produced organic polymer matrix attached to the inner surface of the water distribution system or storage containers" (EPA 2002). This definition of biofilm, points to the fact they protect microbes from disinfection and allow microbes injured by environmental stress and disinfectants to recover and grow afterwards; causing deterioration of water quality, generation of bad tastes and odors, and proliferation of macroinvertebrates. Such contamination and the materials in the biofilm may subsequently be slowly released into the flowing water under various circumstances for prolonged constant contamination of the slowing water systems or storage water. This underpined the importance of biofilms and their significance to public health.

Meanwhile, over three decades ago, fungal biofilm was reported significant in distribution pipes. The water-borne in the underground piping line is contaminated (LeChavallier & Babcock, 1987), affecting the water storage containers. The risk of fungal infection resulting from water consumption calls for the investigation of the fungal biofilm success in the containers of stored drinking water. Fungi are unevenly distributed in water because they are held in biofilms, capable of establishing and multiplying, and have a long residence time in the water. The significance of this study is that the occurrence of fungal biofilm in water storage containers accounts for some related fungal infections in humans. Moreover, it is reported that fungal biofilm occurrence increases the risk of fungal transmission in food handling facilities and the risk of human illnesses (Eduar *et al*., 2011). Fungal biofilm has also been noted by Bravo *et al.* (2010) to have increased resistance to disinfectants, antibiotics and environmental stressors such as salt, pH, which thus makes it challenging to address problems that arise from their formation. Therefore, the study focuses on the determination of fungal density in stored drinking water with respect to household size of homes, which is a critical factor in the quality of drinking water stored in homes.

Materials and Methods

Study Area

The study area for this study is Diobu, Port Harcourt, Nigeria. Diobu was chosen due to its several squatter settlement homes with rooms that are averagely overcrowded. Broadly, the houses have a structure that gives it an average calibrated indoor temperature of 37 degrees

celsius, and such home condition encourages fungal growth with implications for fungal biofilm formation and associated health risk

Sample Collection

Sources of water to these settlements are private borehole service providers.Sample collectors were trained on hygienic collection method and aseptic sample handling, storage and transport. Based on the number of inhabitants or household sizes, 50 water samples were aseptically collected. Ten samples were collected from various housing units/settlements and grouped into five (5) household sizes(2,4,5,6 and 8).

Fungal Bioload Evaluation

Total heterotrophic fungi biomass were determined on abroad dextrose medium as prepared and described by Wemedo *et al*. (2016) using the spread plate technique, where a 0.2ml volume potion of the water samples was inoculated into a freshly prepared medium using a1 ml pipette and a glass spreader to spread the sample over the Saboraud dextrose medium evenly. It was then followed by incubation at 25^{o}C for five days. Biomass/fungal density results were obtained and reported as fungal forming unit per ml.

Pearson's Correlation Coefficient

The population size or household size of the homes were correlated against the density of mould recovered in the study as carried out by Mbah & Oselebe (2004).

Morphological Identification

The taxonomical identity of the fungal was determined based on Ellis and Ellis, 1988. The identified isolates were then sub-cultured to get pure forms for further study.

Biofilm Test

Biofilm analysis was achieved by preparing the biofilm agar and inoculating the test fungal organisms into it by streaking, as Amadi-Ikpa et al. (2020) described.

Result

Enumeration of Fungi Biomass in the Home

Table 1 shows the mean fungi count of 6.2 x 10^2 and 6.1 x 10^2 CFU/ml in the household sizes of 4 and 5 persons respectively were less than that of the household population of 2, 6, and 8 with the fungal count of 8.7 x 10^2, 8.3 x10^2 and 8.0 x10^2 CFU/ml respectively. Although statistically, the mean count of fungi in household sizes of 4 and 5 persons was not significant to 2,6 and 8 persons at a P >0.05. Household sizes of 2, 5, 6, and 8 persons had higher yeast counts than mould counts at a significant difference of P<0.05.

Table 1: Mean Enumeration of Fungi Biomass in the Home

S/No	Household Size	Yeast (CFU/ml)	Mould (CFU/ml)	Total Count	Mean

				(CFU/ml)
I	2	4.4 x10^2	4.3 x10^2	8.7 x10^2
II	4	1.4 x10^2	4.8 x10^2	6.2 x10^2
III	5	5.9 x10^2	2.0 x10^1	6.1 x10^2
IV	6	8.1 x10^2	2.0 x10^1	8.3 x10^2
V	8	7.9 x10^2	1.0 x10^2	8.0 x10^2

Pearson's Correlation Coefficient Between Household Sizes and Fungi Isolates from the Water Sample

Figure 1A below shows a positive relationship between the household population size and the density of yeast recovered from the water sample. The correlation coefficient shows a positive R^2 value of 0.4819. whereas, Figure 1B shows a negative relationship between the household population size and the density of mould recovered from the water sample. The correlation coefficient shows an R2 value of 0.6379. Figure 1C shows no relationship between the household population size and the density of fungi recovered from the water sample. The correlation coefficient shows an R^2 value of zero (0).

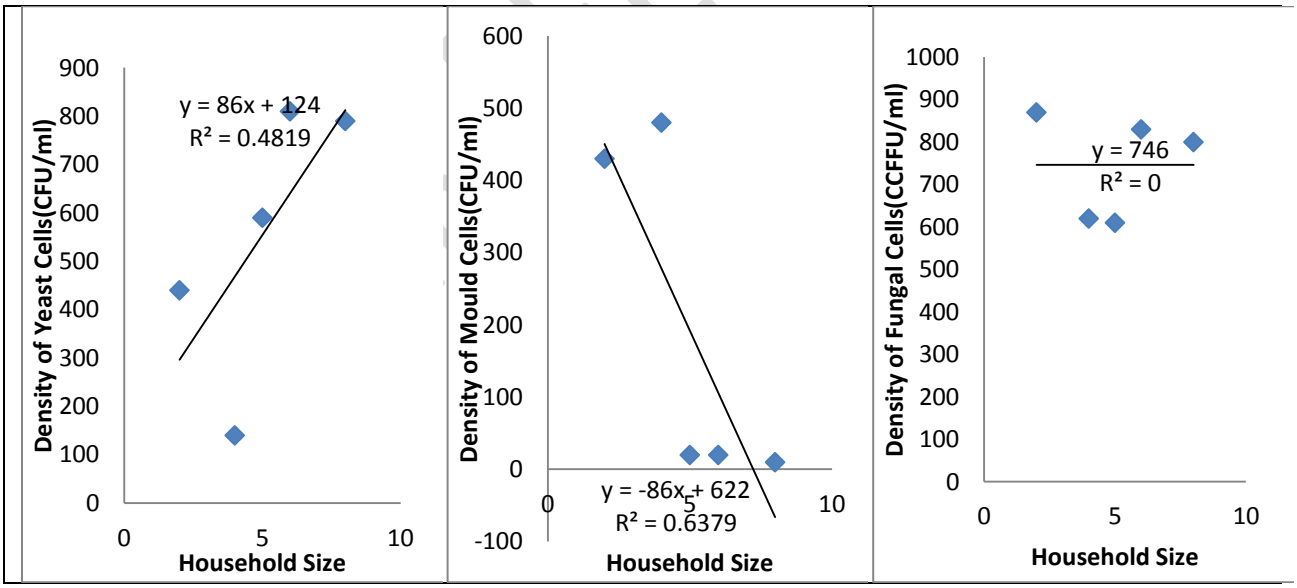


Figure 1A Figure 1B Figure 1C

Figure 1 Pearson's Correlation Coefficient Between Household Sizes and Fungal Isolates from the Water Samples.

Morphological Identification of the Fungal Isolates

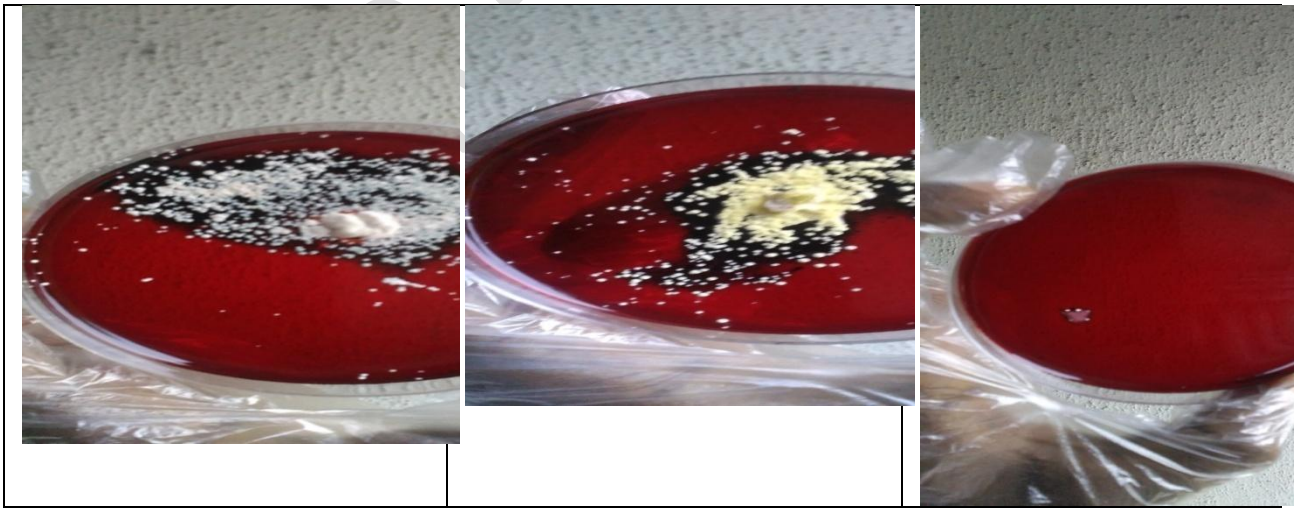
In Table 2 below, a total of 25 fungal isolates were obtained in the study, from which two fungal classifications (mould and yeast) were derived, making up three genera, namely; *Aspergillus flavus, Candida albicans* and *Penicillium notatum* with the yellow, milky and blue cob web appearance on culture media respectively.

Table 2 Morphological Identification and Occurrence of Fungal Population

Morphological Features	Fungal Occurrence	Percentage Fungal Occurrence (%)	Fungal Isolates
Yellow pigmentation with white background surface circular in shape with an elevated centre	7	28	*Aspergillus flavus*
Milky colonies with mainly surface growth growing singly	15	60	*Candida* spp
Blue cobweb or hair-like appearance growing to cover plate	3	12	*Penicillium notatum*

Biofilm Forming Ability

Plate 1: bellow shows a biofilm test of the fungal isolates obtained in the investigation. *Aspergillus flavus* and *Penicillium notatum* isolates were able to form a biofilm, while *Candida albicans* did not constitute a biofilm



Penicillum notatum *Aspergillus flavus* *Candida albican*

Plate 1; Fungal Biofilm Formation

Discussion

Biofilms are the predominant mode of microbial growth within the drinking water distribution systems, constituting a key public health challenge as consumption of contaminated water with pathogenic biofilms has been linked to human infections and waterborne outbreak in recent past (Angles et al., 2007; Prest et al., 2016; Douterelo et al., 2016). And the major biofilm producing bacteria in drinking water are *P. aeruginosa, Campylobacter jejuni, Legionella pneumophila, Mycobacteria, Aeromonas hydrophila*, and *Klebsiella pneuminiae* (Chan et al., 2019). However, from this study, household sizes of 2, 5, 6 and 8 persons had higher counts of yeast than mould counts obtained. Also, it suggests the impact of seasonal variation, specifically, the summer season, as reported by Urooj *et al*.(2018) and observed in the present circumstance. The mean counts of Fungi in the household sizes of 4 and 5 persons were less than that of the household population of 2, 6, and 8 persons. Statistically, the mean count of fungi in household units of 4 and 5 persons was not significant at a $P>0.05$. Thus, a high microbial count in a densely polluted city, as Amin (2008) reported, indicates an unhygienic condition of a food processing area. Following this, Household sizes of 2, 5, 6 and 8 persons had higher yeast counts than mould counts. Thus, counts of yeast were far more significant than mould. A positive relationship exists between the household population size and the density of yeast recovered from the water sample, which signifies that the yeast count increases as the household population size increases. Perhaps, this may have resulted from favourable growth factors such as optimum temperature, nutrient and hydrogen ion concentration (pH) (Mbah & Oselebe, 2004). The negative relationship between the household population size and the density of mould recovered from the water sample signifies that the household population size increases. Hence, the mould counts decrease, thus collaborating with studies carried out by Mbah & Oselebe (2004), where they listed temperature, pH, nutrients and light as factors that could inhibit mould growth if not harnessed. However, the household population sizes and the density of fungi recovered from the water sample showed no relationship exist; that is to say, the size of a household does not affect fungi growth (Hall & Noverr, 2017). A total of 43 fungal isolates were obtained in the study, thus disagrees with investigations carried out by Mhlongo *et al.* (2019), who got more than 43 isolates. Similarly, Goussou *et al*. (2009) recovered eight (8) indigenous fungal genera, in which this study obtained only three genera, namely, *Aspergillus* spp, *Candida* spp and *Penicillium* spp. The high prevalence of *Candida albicans* in this study suggests why it is the most common fungi isolated from water (Goussou *et al*., 2009). *Aspergillus flavus* has been isolated from drinking water in many studies. Thus, this study agrees with Annaise *et al.* (2003), who submitted that water is increasingly recognised as an environmental source of Aspergillus spp with augmenting that suggests water is considered an important route of transmission pathogenic *Aspergillus* spp. The presence of *Penicillium notatum* in the drinking water sample signifies it as a source of exposure. However, the source of Penicillium infection is not yet known (Rodrignes & Nosanchuk, 2020)**.** *Aspergillus flavus* and *Penicillium notatum* isolate formed a heavy biofilm mass, while *Candida albicans* developed a low biofilm density. This study is inconsonant with the one carried out by Siqueira (2013), who evaluated the capacity of some fungi isolates to form biofilms and reported in their findings that *Aspergillus flavus* and *Penicillium notatum* showed

better ability in terms of higher biomass to form biofilm when compared to other fungi isolates, in which case *Candida albicans.* Similarly, the inability of *Candida albicans* to form a biofilm of heavy biomass has also been reported by Pathak *et al.* (2012). This initial activation of efflux pumps, which happens in the initial phases of biofilm production, is a major factor in biofilm resistance to antifungal drugs. This quick regulatory responsiveness could have developed in reaction to suppressive substances produced by other microbiota contending for about the same biological niche inside the host, according to one theory. Nonetheless, antifungals and traditional polyene formulations, for all, are ineffective against *C. albicans* biofilms. Resistance to antifungal medicines in *C. albicans* biofilms is multidimensional and molecular mechanisms complicated, but it is largely owing to three basic elements: activation of efflux transporters, the extrinsic framework, and the occurrence of obstinate, physiologically dormant cells known as "persister" cells (FOX and Nobile, 2013). An earlier activation of efflux pumps, which arises inside the beginning stages of biofilm production, is a major factor in biofilm resistance to antifungal drugs. This quick transcriptional response could have developed in reaction to inhibitory various chemicals by other microbial species competing for the same environmental niche within the host, according to one theory. Even though the molecular processes by which *C. albicans* proliferation in the biofilm state influences relations with the innate immunity are understood at this time, there really are hints that biofilm development potentially valuable guidance and support versus innate and adaptive immunity (Xie et al., 2012). *C. albicans* is a safe commensal that coexists with other parts of the microbiota in most people with a sound immune response. Interruptions in this sensitive equilibrium, such as changes in the local environment (pH shifts or dietary changes), antibiotic use, or immune system abnormalities (induced by an infection or immunosuppressant medication), might allow *C. albicans* to quickly multiply and lead to infection (Gulati and Nobile, 2016). Once a *C. albicans* biofilm has formed on medical equipment, it serves as a repository for infectious cells, is resistant to antibiotics and immune regulation, and has the ability to seed circulated bloodstream infections (candidemia), which could also ultimately led to invasive severe infections of organs and tissues. Approximately five million venous blood catheters are implanted in the United States annually, and biofilm infection occurs in more than half of these catheters, despite recent improvements in treatment modalities (Fox and Nobile, 2013).

Despite the fact that fungi have been detected in drinking water distribution systems and biofilms, they have yet to be definitively linked to waterborne disease. The fungal pathogens found in the distribution system, on the other hand, are opportunistic and seldomly cause any sickness, regardless of the source of infection. *A. flavus* and several other *Aspergillus* species found in drinking water, on the other hand, create powerful toxins (mycotoxin). Aflatoxins, cyclopiazonic acid, ochratoxin A, and sterigmatocystin are examples of mycotoxins produced by *Aspergillus* species. Citrinine, patulin, and penicillic acid, which are likewise produced by *Penicillium* species, and several *Aspergillus* species (CDC, 2020). Aflatoxin, a species-specific toxin generated by *Aspergillus flavus*, a specie implicated in this research, is the most important *Aspergillus* mycotoxin. According to Bennett and Kich (2003) and Gülhan (2020), acute

aflatoxicosis outbreaks have been documented in a number of locations around the world, despite the fact that acute poisoning in humans is uncommon. Acute aflatoxicosis in humans causes vomiting, stomach pain, jaundice, pulmonary edema, unconsciousness, convulsions, and death. Aflatoxins are carcinogens that cause DNA damage. Aflatoxin exposure over time has been linked to liver disorders such as cancer, cirrhosis, hepatitis, and jaundice. The *Penicillium* mycotoxins that affect liver or kidney function, by acute or chronic exposure, are usually asymptomatic in humans or animals (Gülhan, 2020; Bennett and Kich, 2003). Consequently, the American Environmental Protection Agency (2002) recommended consistent monitoring of standards. However, in the Nigerian context, standards monitoring of drinking water supplies in households or neighbourhoods is a herculian task, if not non-existent.

Candida albicans infection (candidiasis), a harmful yeast, implicated in this research, can also colonize the GI tract. (Schaechter et al., 1998) and thus could potentially be spread by the fecal-oral route. It has been found in seawater and in sand at marine beaches. *C. albicans* and other important fungal pathogens are also associated with soil. Thus, the potential for waterborne disease caused by fungi in the biofilm exists, but the significance is unknown as wasb the opinion of Azuonwu et al., (2019) which underpinned that water storage containers and underground water distribiution systems could potentially be a source of harmful pathogens to humans. Thus, suggesting the need for consistent monitoring, surveillance and proactive water quality evaluation; in their words, "keeping a careful watch at all times from the public health point of view, over safety and acceptability of drinking water supply" standards (Azuonwu et al., 2019). Perhaps with proper monitoring and evaluation of drinking water quality, prevention of the effects of biofilm forming microbes that could lead to life-threatening infections and diseases in humans such as cystic fibrosis (CF), otitis media, periodontitis, infective endocarditis (IE), chronic wounds, and osteomyelitis would be achieved (Southey-Pillig et al., 2005; Akyildiz et al., 2013; Masters et al., 2019). Similarly, U.S. Food and Drug Administration (FDA 2020) and the European Food Safety Authority standards guidelines could be used as the spring board for strategic monitoring of these standards.

Conclusion

The isolates, *Aspergillus flavus* and *Penicillium notatum* created a high mass of biofilm for their survival. *Candida albicans* made a low mass of biofilm, despite *Candida albicans* high recovery density or biomass count. Thus, the success of fungal in this study is based on the persistence rate of biofilm formed by *Aspergillus flavus* and *Penicillium notatum* which are notable toxin producers and potential public health risks. The household size has somewhat been implicated. The study recommends that storage facilities in the homes be cleaned and disinfected always, and regular turn-over of water in storage cans should be adhered to consistently. Therefore, standards monitoring, evaluation and enforcement, should be strengthened more especially in low income settings like this as those who are immunocompromised might be at serious risk of contracting varying degree of fungal infections. Nonetheless, it is firmly advocated that pipe-

borne water in storage containers from these affected areas, irrespective of household size, should be treated, boiled, and filtered before use for drinking and washing fresh fruits to avert the possibility of an outbreak of water-borne epidemic in the area.

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