

Assessment of Pathogenic Free-Living Amoebae in a Wastewater Treatment Plant in Beheira Governorate, Egypt

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ABSTRACT

Among the genera of free-living amoebae (FLAs) existing in nature, some *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea* have an association with human disease. Among these, *Acanthamoeba* spp. and *Balamuthia mandrillaris* are recognized as opportunistic pathogens that may lead to severe infections affecting the CNS, lungs, sinuses, and skin, primarily in immunocompromised individuals. However, their occurrence in sewage-related environments still needs to be systematically documented. The present study aimed to isolate and morphologically identify different FLA species from the Kom El Tarfaya Wastewater Treatment Plant in Beheira Governorate, Egypt. Wastewater samples were collected from this plant between January and December 2024. Samples were processed for the detection of free-living amoebae using non-nutrient agar (NNA). Based on the morphological criteria, FLA isolates were identified to the genus level. By morphological identification, FLA species of three genera: *Naegleria*, *Vahlkampfia*, and *Vermamoeba* were encountered. Members of the *Naegleria* were recorded in inlet wastewater samples (16.7%), while genus *Vahlkampfia* spp. had the same occurrence of 4.2% in both inlet and outlet wastewater samples. Genus *Vermamoeba* spp. had the same occurrence of 8.33% in both wastewater samples. Overall, the findings indicate a high risk associated with sewage-related environments, as the prevalence of FLAs, including pathogenic strains, is even found in treated sewage water. These results may be valuable for both risk remediation actions against amoebic infections and future research endeavors.

INTRODUCTION

Around the world, water contamination is a major problem (Fawell & Nieuwenhuijsen, 2003). According to Sharma and Bhattacharya (2016), microbial pollutants include diseases such as parasites, bacteria, viruses, protozoa, and microscopic worms, which can be unintentionally or intentionally transferred by human and animal

wastes. Furthermore, FLAs are found in soil, a range of aquatic ecosystems, and even the air, indicating their widespread prevalence (Al-Herrawy *et al.*, 2014; Al-Herrawy *et al.*, 2015).

FLAs are water and soil-dwelling protozoa, that have been identified as possible repositories of harmful bacteria, and some are pathogenic (Morsy *et al.*, 2016). Trophozoites and cysts are the two morphological stages in which they exist. The vegetative form, also known as the trophozoite, is the amoeba's metabolic activity phase during which it divides, feeds, and moves (Trabelsi *et al.*, 2012). When environmental conditions are unfavorable, such as when temperature, osmotic pressure, and pH are at extremes, or when nutrient levels are low, the cyst stage develops. FLA can stay in the cyst stage for extended periods of time before developing into active trophozoites when environmental conditions improve (Marciano-Cabral & Cabral, 2003; Visvesvara *et al.*, 2007).

A few of the FLAs are harmful to people (Martinez & Visvesvara, 1997). The majority of *Acanthamoeba* species are linked to human conditions such as granulomatous amoebic encephalitis (GAE), kidney and lung infections of the nasopharynx lesions, and cutaneous lesions, particularly in people with impaired immune systems. Immunocompetent individuals can also get amoebic keratitis due to the *Acanthamoeba* species. Mostly in healthy youngsters, *Balamuthia mandrillaris*, another amoeba, and in addition to skin and lung infections, a close related of *Acanthamoeba* causes fatal GAE infections. The non-opportunistic disease known as primary amoebic meningoencephalitis (PAM) is caused by *Naegleria fowleri* in healthy children and young adults. It has also been reported that brain infection in a healthy man is caused by *Sappinia pedate* (Gelman *et al.*, 2001).

Additionally, FLAs may serve as harbors for harmful bacteria, including environmental *mycobacterium* species, *Legionella* species, especially *Legionella pneumophila*, *Vibrio cholerae*, and methicillin-resistant *Staphylococcus aureus* (Goni *et al.*, 2014). These "amoebae-resistant bacteria" (ARB) can replicate, live, and colonize aquatic water systems in addition to infecting and thwarting FLA's digestive processes (Greub & Raoult, 2004; Abd & Saeed, 2007; Lone & Syed, 2009). To date, 102 species of verified amoeba-resistant bacteria have been identified, and the number continues to grow (Thomas *et al.*, 2010). Amoebae-resistant bacteria depend on their amoebal hosts for protection and nourishment in unfavorable environmental conditions, such as when biocides like chlorine are applied in water treatment (when amoebae form cysts). It is thought that certain microorganisms, especially *L. pneumophila* and those in the *M. avium* complex, become more virulent as they travel through their amoebal hosts (Schuster 2002; Loret & Greub, 2010). Thus, in water systems, FLAs may serve as distributors and multipliers of harmful bacteria.

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This study aimed to monitor the presence and distribution of FLAs in wastewater samples, assessing their prevalence in a wastewater treatment facility in Beheira Governorate, Egypt, to determine whether any potentially harmful FLAs were present and how well the treatment processes removed these FLAs from the wastewater.

MATERIALS AND METHODS

Sample Site

The Kom El Tarfaya Wastewater Treatment Plant is in a rural area within Kafr El Dawar, Beheira Governorate, Egypt. The plant primarily treats domestic sewage from surrounding villages and serves a moderate population size. It works by conventional treatment processes, including primary sedimentation, biological treatment, and secondary clarification. The facility is situated near agricultural lands, and the treated effluent is discharged into nearby water channels used for irrigation. The sampling points include the inlet (raw sewage entry point) and the outlet (post-treatment discharge point), allowing for comparative analysis of microbial content before and after treatment.

Samples Collection

Throughout the period from January to December 2024, a total of 48 wastewater samples (24 from the inlet and 24 from the outlet) were collected every two weeks from the treatment plant. Each sample, comprising one liter, was collected in a sterile, autoclavable polypropylene container. Then, samples were transported under ambient conditions to the Environmental Parasitology Lab., Water Pollution Research Dept., (National Research Center), Giza, where they were immediately processed upon arrival on the same day of collection.

Isolation, cultivation, and morphological identification of FLAs

Each wastewater sample (1 liter) was concentrated by the filtration method. Filtration was carried out through a 0.45µm pore size, 47mm diameter nitrocellulose membrane using a stainless-steel filter holder connected to a vacuum pump. Then, each membrane was aseptically inverted onto the surface of non-nutrient (NN) agar plates pre-seeded with live *Escherichia coli* and incubated at 30°C for up to two weeks. Daily microscopic examination was performed using an inverted microscope, in accordance with the **Health Protection Agency guidelines (2004)**. Plates exhibiting growth of FLAs were sub-cultured and cloned on fresh NN agar-*E. coli* plates for further morphological assessment. Isolated FLAs from the wastewater were identified based on the morphological characteristics of both trophozoite and cyst of each amoeba, following the taxonomic criteria described by **Pussard and Pons (1977)**, **Page (1988)** and **Al-Herrawy (1992)**.

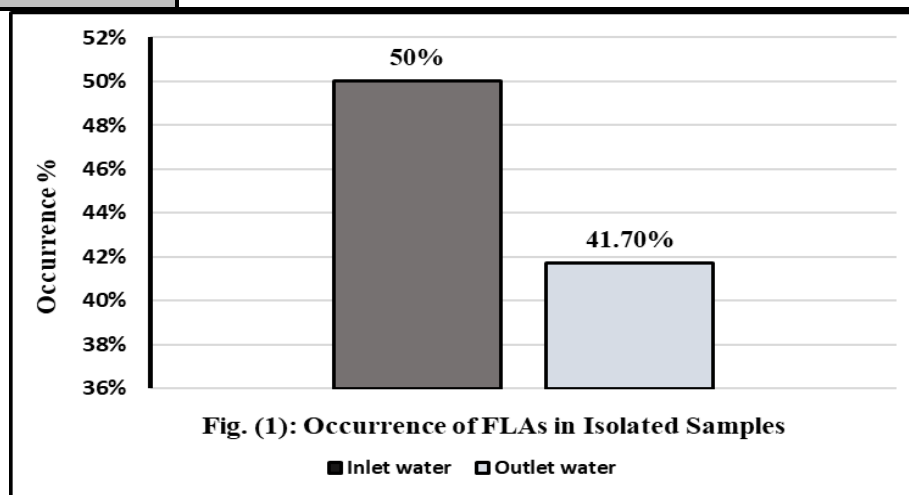
RESULTS

Isolation of FLAs

As shown in Table (1), examination of 48 wastewater samples revealed that 22 (45.8%) had characteristic amoebic trophozoites and cysts with live bacteria. Wastewater samples collected from the inlet water were significantly higher in percentage positive for FLAs, with 50%, than process outlet water samples, with 41.7% (Fig. 1).

Table 1. Occurrence of FLAs in isolated samples

Samples Results	Inlet Water	Outlet Water	Total
Examined	24	24	48
+ ve FLAs (%)	12 (50%)	10 (41.7%)	22 (45.8%)
Removal %	16.7		



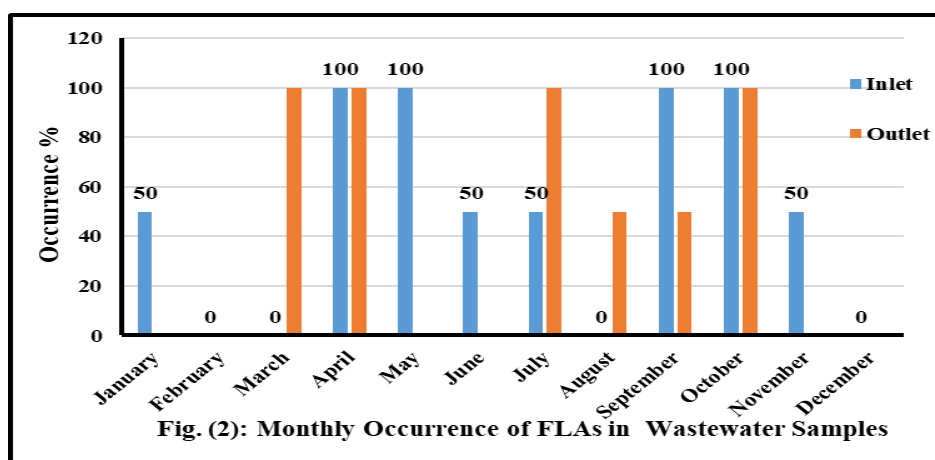
Monthly occurrence of FLAs in wastewater samples

Concerning the monthly occurrence of FLAs, on one hand, it was observed that the highest occurrence percentage in inlet wastewater samples was 100% for four months: April, May, September, and October, then it decreased to 50% in four months: January, June, July, and November. It completely disappeared during the four months of February, March, August, and December. On the other hand, the highest occurrence percentage of FLAs in outlet wastewater samples was 100% in March, April, July, and October. It decreased to 50% in both August and September, while the percentage disappeared completely in the rest of the year (Table 2 & Fig. 2).

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Table 2. Monthly occurrence of FLAs in wastewater samples

Month	Waste Samples					
	Inlet			Outlet		
	Examined	Positive	%	Examined	Positive	%
January	2	1	50	2	0	00
February	2	0	00	2	0	00
March	2	0	00	2	2	100
April	2	2	100	2	2	100
May	2	2	100	2	0	00
June	2	1	50	2	0	00
July	2	1	50	2	2	100
August	2	0	00	2	1	50
September	2	2	100	2	1	50
October	2	2	100	2	2	100
November	2	1	50	2	0	00
December	2	0	00	2	0	00



Concerning the seasonal occurrence of FLAs in inlet wastewater samples, it showed that the highest seasonal occurrence of FLAs (83.3%) in inlet wastewater samples was in spring, then it decreased to 50% in both summer and autumn seasons. While the lowest occurrence, 16.7%, was observed in winter. Whereas, in outlet

wastewater samples, the highest occurrence, 66.7%, was observed in summer, followed by a decrease to 33.3 % in the rest of the year (Table 3 & Fig. 3).

Table 3. Seasonal occurrence of FLAs in wastewater samples

Season	Number of Examined Samples	Positive Samples No. (%)	
		Inlet	Outlet
Winter	6	1 (16.7)	2 (33.3)
Spring	6	5 (83.3)	2 (33.3)
Summer	6	3 (50)	4 (66.7)
Autumn	6	3 (50)	2 (33.3)

Occurrence of detected FLA genera

Morphological examination of FLAs from the positive wastewater samples revealed the occurrence of several amoebae species belonging to three genera: *Naegleria*, *Vahlkampfia*, and *Vermamoeba*. The occurrences of these amoebae all over the year indicated that the highest prevalence of FLAs of the *Naegleria* spp. was recorded in inlet wastewater samples (16.7%), while genus *Vahlkampfia* spp. had the same occurrence (4.2%) in both inlet and outlet wastewater samples. In addition, genus *Vermamoeba* spp. had the same occurrence (8.33%) in both wastewater samples (Table 4; Figs. 4, 5).

Table 4. Occurrence of detected FLA genera

Genera of FLAs	Inlet		Outlet	
	Positive /total examined	%	Positive /total examined	%
<i>Naegleria</i> spp.	10/24	41.7	4/24	16.7
<i>Vahlkampfia</i> spp.	1/24	4.2	1/24	4.2
<i>Vermamoeba</i> spp.	2 /24	8.33	2/24	8.33

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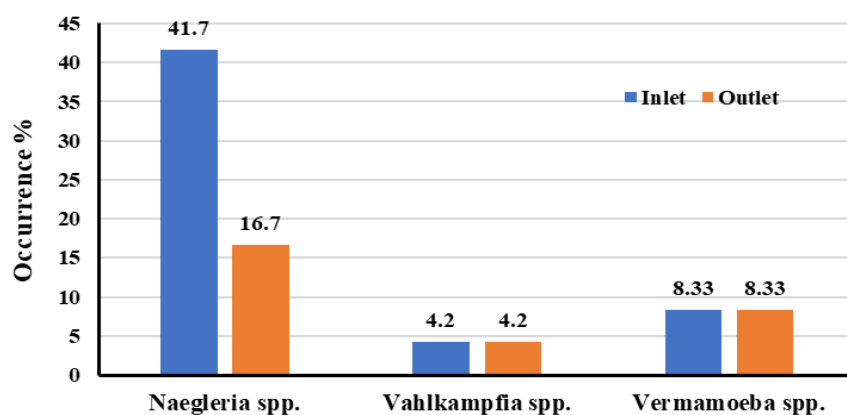


Fig. (4): Occurrence of Detected FLA Genera

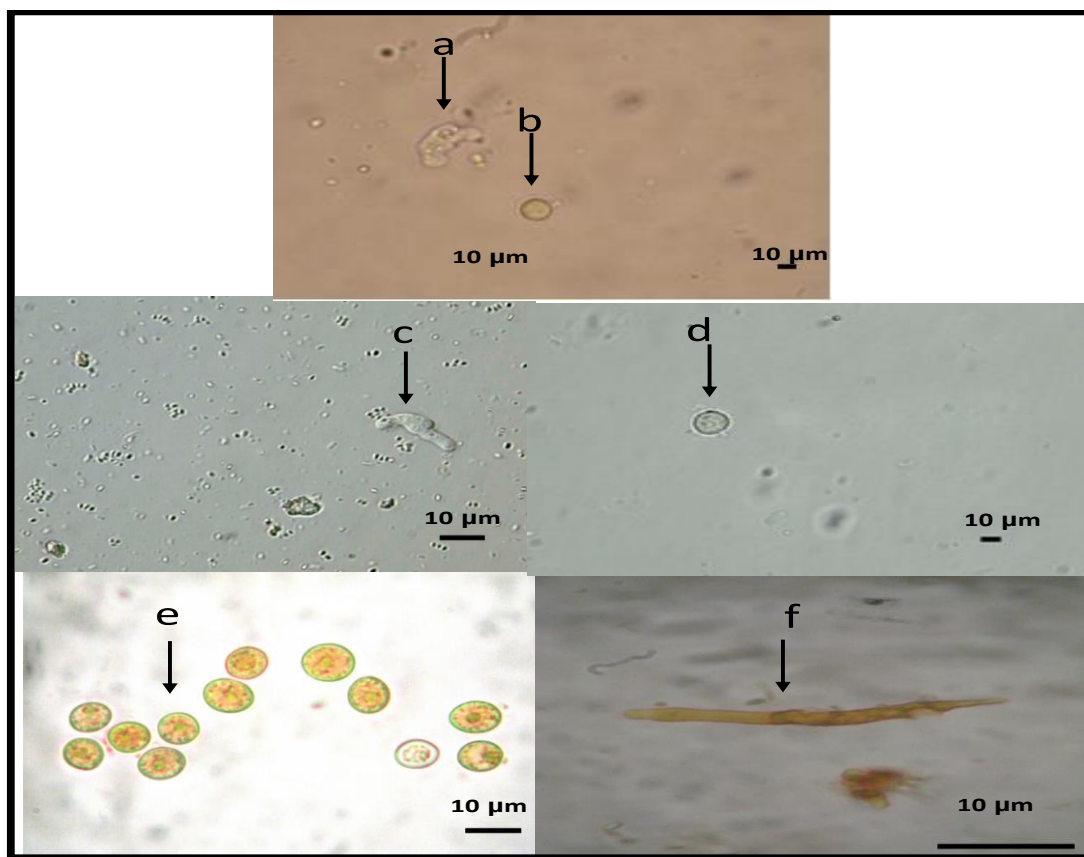


Fig. 5. Photomicrographs of the isolated FLAs: (a & b) *Naegleria* spp. trophozoite and cyst, respectively, (c & d) *Vahlkampfia* spp. trophozoite and cyst, respectively, (e & f) *Vermamoeba* spp. trophozoite and cyst, respectively

DISCUSSION

Wastewater is usually susceptible to contamination in many ways, and bacteria and viruses are often found to be major causes of many waterborne disease outbreaks. Furthermore, it has been established that both free-living and parasitic protozoa are important sources of infections in humans. It's interesting to note that protozoa often survive at higher concentrations and are more resistant to disinfectants than bacteria and viruses (**Briancesco & Bonadonna, 2013**).

During the present work, the use of the centrifugation concentration method did not result in the identification of FLAs in any of the wastewater samples gathered throughout the year. In contrast, **Muchesa *et al.* (2014)** in Gauteng Province, South Africa, demonstrated that FLAs were easily isolated using filtration-based propagation techniques rather than centrifugation, and that FLAs were found throughout the wastewater treatment process. Similarly, the Health Protection Agency (**HPA, 2004**) mentioned that filtration outperformed centrifugation in terms of amoebae recovery from water samples. **Winiecka-Krusnell and Linder (1998)** also mentioned that although the sample amount can affect centrifugation effectiveness, amoebae are more easily retrieved utilizing filtration techniques. While filtration techniques are usually used for greater amounts of 500 to 1000mL, studies that use centrifugation frequently treat relatively modest sample volumes (around 50mL) (**Pagnier *et al.*, 2008**).

In the present investigation, FLAs were found in 22 of the 48 samples (45.8%) collected from the Kom El Tarfaya Wastewater Treatment Plant's input and output, located in Kafr El Dawar, Beheira, Egypt, over one year. Similarly, **Al-Herrawy *et al.* (2020)** found FLAs in 11 of the 24 samples (45.8%) that were taken from Helwan University's Wastewater Treatment Plant in Cairo, Egypt. Whereas, in Bulgaria, **Tsvetkova *et al.* (2004)** reported the existence of FLAs in 42 out of 45 samples (93.3%) collected from nine different wastewater treatment plants. In Porto Alegre, located in Southern Brazil, **Borella da Silva *et al.* (2024)** reported the isolation of FLAs from all 34 (100%) of the examined sewage treatment plants. **Al-Herrawy *et al.* (2020)** observed that geographic variations, the standard of raw water sources, and extra technology for treatment employed in every region are the main causes of the diversity in FLAs detection rates across various places and nations.

The present study's findings indicated a removal rate of only 16.7% for FLAs in the examined wastewater treatment plant. **Mosteo *et al.* (2013)** reported that the low efficiency of treatment may be attributed to FLA's cysts strong resistance to both treatment processes and environmental conditions. They added that although FLAs were found in the wastewater samples, *Cryptosporidium* and *Giardia* were not (**Mosteo *et al.*, 2013**). The cellulose in their cyst walls, which creates a physical barrier against chlorine, is what gives bare FLAs this resistance (**Thomas *et al.*, 2010**). **Storey *et al.* (2004)**

showed that certain amoebic cysts could withstand ten minutes of exposure to up to 100 mg/L of chlorine.

The current investigation found that the maximum prevalence of FLAs, 83.3% and 66.7%, respectively, was observed in spring and summer months in both inlet and outlet wastewater samples. Whereas the wintertime samples showed the lowest prevalence (16.7% and 33.3%) in both inlet and outlet samples, respectively. These results are in line with those of **Muchesa *et al.* (2014)** in Gauteng Province, South Africa reporting a notably high prevalence of FLAs in both untreated and treated wastewater samples throughout the year, with detection rates reaching up to 100%. **Griffin (1972)** reported that elevated water temperatures support the proliferation of thermophilic FLA strains. Similarly, **Dimmick *et al.* (1979)** and **Lundholm (1982)** highlighted that amoebae's capacity to create cysts allows them to endure prolonged exposure to extremely high or low temperatures. According to **Hoffmann and Michel (2001)** and **Marciano-Cabral *et al.* (2010)**, seasonal temperature fluctuations affect the quantity and diversity of bare FLAs at the point of application, in line with **Carlesso *et al.* (2010)**, stating that the occurrence of FLAs rose with warmer months. The potential for some FLAs to result in skin infections, eyes, and the neurological system in general makes their resistance to biocidal treatments like chlorine a serious concern for the spread of disease. In addition to being directly harmful, FLAs serve as natural hosts and carry a variety of intracellular pathogenic microorganisms (**Greub & Raoult, 2004**).

During the current study, 22 (45.8%) of the ambient samples had characteristic amoebic trophozoites and cysts with live bacteria, suggesting the possible inside the cell presence of bacteria resistant to amoeba. The removal rate of 16.7% still indicates a remarkably high occurrence of potentially pathogenic intracellular bacteria, possibly because other microorganisms interfered with detection. Given that pathogenic bacteria may be released from the wastewater treatment facility, this observation suggests a decline in the receiving water body's (in this case, sewage) microbiological quality. As a result, nearby populations who depend on the river water for drinking, farming, and enjoyment are at serious risk for health problems (**Toze, 2004**).

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