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Occurrence, Isolation, and Morphological Characterization of Free-Living Amoebae in Different Drinking Water Sources: Implications for Public Health

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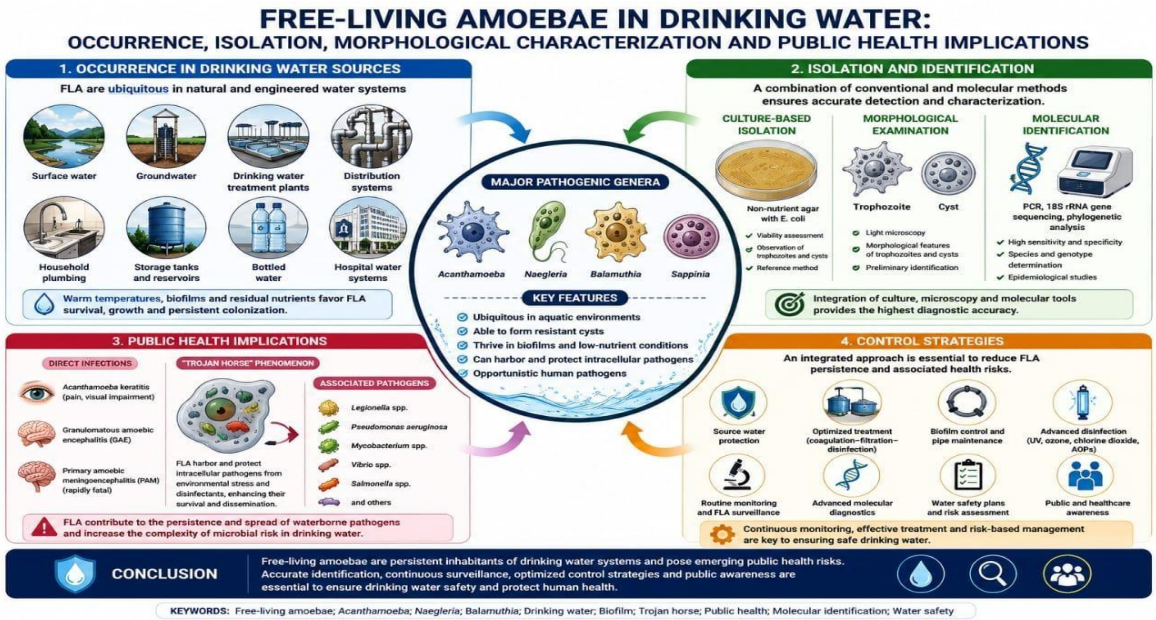
ABSTRACT

Free-living amoebae (FLA) are ubiquitous protozoan microorganisms widely distributed in natural and engineered aquatic environments, including surface water, groundwater, drinking water treatment plants, distribution systems, household plumbing, and storage reservoirs. Although many FLA are non-pathogenic, several genera, particularly Acanthamoeba, Naegleria, Balamuthia, and Sappinia, are recognized as opportunistic pathogens capable of causing severe human diseases, including Acanthamoeba keratitis, granulomatous amoebic encephalitis, and primary amoebic meningoencephalitis. Moreover, FLA act as environmental reservoirs and intracellular hosts for numerous amoeba-resistant microorganisms, enhancing the persistence, survival, and dissemination of clinically important bacterial pathogens in drinking water systems. This review comprehensively examines the occurrence, ecology, isolation, and morphological characterization of FLA in different drinking water sources, emphasizing their public health significance and current control strategies. Conventional culture-based techniques and microscopic examination remain valuable for preliminary detection; however, their limited taxonomic resolution necessitates integration with molecular approaches, including polymerase chain reaction (PCR), 18S rRNA gene sequencing, and phylogenetic analysis, to achieve accurate species identification and genotype characterization. The review also highlights the ecological role of FLA in biofilm formation and the "Trojan horse" phenomenon, whereby intracellular pathogens are protected from environmental stress and conventional disinfection.Current evidence indicates that FLA represent an emerging challenge for drinking water safety because their resistant cysts and biofilm-associated populations frequently survive conventional treatment processes. Therefore, effective risk management requires integrated surveillance, optimized treatment technologies, biofilm control, advanced molecular diagnostics, and public health awareness. Continued multidisciplinary research and internationally standardized monitoring protocols are essential to improve drinking water quality and reduce the global health risks associated with free-living amoebae.

KEYWORDS: Free-living amoebae; Acanthamoeba; Drinking water; Morphological characterization; Waterborne pathogens; Public health.



Graphic abstract: Free-Living Amoebae in Drinking Water: Occurrence, Isolation, Morphological Characterization and Public Health Implications.

List of Abbreviations

- AK: Acanthamoeba Keratitis
- ARMs: Amoeba-Resistant Microorganisms
- BBB: Blood-Brain Barrier
- CDC: Centers for Disease Control and Prevention
- CNS: Central Nervous System
- eDNA: Environmental DNA
- FLA: Free-Living Amoebae
- GAE: Granulomatous Amoebic Encephalitis
- ICP: Intracranial Pressure
- NGS: Next-Generation Sequencing
- NNA: Non-Nutrient Agar
- PAM: Primary Amoebic Meningoencephalitis
- PBS: Phosphate-Buffered Saline
- PCR: Polymerase Chain Reaction
- qPCR: Quantitative Polymerase Chain Reaction
- QMRA: Quantitative Microbial Risk Assessment
- RNA: Ribonucleic Acid
- SEM: Scanning Electron Microscopy
- UV: Ultraviolet
- WHO: World Health Organization
- WSPs: Water Safety Plans

1. INTRODUCTION

Access to microbiologically safe drinking water is a fundamental prerequisite for human health and remains one of the primary objectives of global public health programs. Considerable progress has been achieved in drinking water treatment through the implementation of coagulation, filtration, and chemical disinfection technologies. Nevertheless, the persistence of certain environmental microorganisms within water treatment facilities and distribution systems continues to represent a significant challenge for water utilities worldwide.

Among these microorganisms, free-living amoebae (FLA) have attracted increasing scientific attention because of their ubiquitous distribution, remarkable environmental adaptability, and growing clinical importance. Unlike obligate parasitic protozoa, FLA naturally inhabit aquatic and terrestrial ecosystems and complete their entire life cycle independently of a host organism. Their widespread occurrence in both natural and engineered water environments has raised important concerns regarding drinking water safety and environmental health (Thomas et al., 2021).

Free-living amoebae comprise a heterogeneous group of unicellular eukaryotic microorganisms belonging to several taxonomic lineages. More than twenty genera have been described, although only a limited number are considered medically important. Among these, *Acanthamoeba*, *Naegleria*, *Balamuthia*, and *Sappinia* have received particular attention because of their ability to cause severe human infections under specific environmental and host conditions. These organisms have been isolated from a wide variety of environmental sources, including rivers, lakes, groundwater, hot springs, recreational waters, wastewater, cooling towers, swimming pools, soil, dust, air-conditioning systems, and drinking water distribution networks, demonstrating their exceptional ecological versatility (Shi et al., 2022).

The ecological success of FLA is primarily attributed to their biphasic life cycle, consisting of an actively feeding trophozoite stage and a metabolically inactive cyst stage. Under favorable environmental conditions, trophozoites actively migrate, feed on bacteria, algae, fungi, and organic debris, and reproduce through mitotic binary fission. However, when exposed to adverse conditions such as nutrient depletion, desiccation, osmotic stress, unfavorable temperatures, ultraviolet radiation, or chemical disinfectants, trophozoites transform into double-walled cysts that exhibit remarkable resistance to environmental stress. Encystment enables these protozoa to survive for prolonged periods until favorable conditions return, when excystment allows trophozoites to resume active growth. This adaptive mechanism is largely responsible for the persistence of FLA in drinking water systems despite conventional treatment processes (Abdelaziz et al., 2025).

Figure 1. Schematic illustration of the ecology, life cycle, environmental resilience, and public health significance of free-living amoebae (FLA) in drinking water systems.

In recent years, FLA have become increasingly recognized not only as opportunistic pathogens but also as important environmental indicators of microbial water quality. Their presence in treated drinking water frequently reflects deficiencies in treatment efficiency, inadequate maintenance of distribution systems, or extensive biofilm development within water pipelines and storage reservoirs. Numerous investigations have demonstrated that FLA are capable of colonizing biofilms that develop on the internal surfaces of drinking water distribution networks. Biofilms provide physical protection, stable nutrient availability, and favorable ecological conditions that promote amoebal survival, multiplication, and dissemination throughout water systems. Consequently, FLA may remain detectable even in chlorinated water supplies that satisfy conventional microbiological quality standards (El-Wakil et al., 2022).

The public health importance of free-living amoebae extends beyond their environmental occurrence. Although most species are non-pathogenic, several members of the genera *Acanthamoeba*, *Naegleria*, and *Balamuthia* are recognized as emerging opportunistic pathogens capable of causing severe and frequently fatal infections in humans. *Acanthamoeba* spp. are responsible for amoebic keratitis, particularly among contact lens users, as well as granulomatous amoebic encephalitis (GAE), which predominantly affects immunocompromised individuals. *Naegleria fowleri*, commonly known as the "brain-eating amoeba," causes primary amoebic meningoencephalitis (PAM), an acute, rapidly progressive disease associated with an exceptionally high fatality rate. Although infections caused by *Balamuthia mandrillaris* and *Sappinia pedata* are relatively uncommon, they are associated with severe neurological complications and continue to represent important emerging infectious diseases worldwide (Siddiqui et al., 2016).

The growing interest in FLA is also closely linked to their ecological interactions with other microorganisms. As efficient microbial predators, amoebae regulate bacterial populations in aquatic ecosystems through phagocytosis. However, numerous bacterial species have evolved sophisticated mechanisms that enable them to survive intracellular digestion and exploit amoebae as protective hosts. This evolutionary relationship has considerable implications for environmental microbiology because intracellular survival within amoebae enhances bacterial persistence under environmental stress and may increase resistance to conventional water disinfection procedures. Consequently, FLA are increasingly regarded as key components of the microbial ecology of drinking water systems and as valuable indicators of potential microbiological hazards.

The occurrence of FLA has been widely documented in drinking water systems worldwide, including raw and treated water, distribution networks, storage tanks, and household plumbing. Their prevalence varies according to

environmental conditions, water treatment efficiency, and detection methods; however, their repeated isolation from treated water indicates that conventional treatment processes are often insufficient for complete removal. The persistence of FLA is strongly influenced by environmental factors such as temperature, pH, nutrient availability, disinfectant residuals, and biofilm formation. Beyond their direct pathogenicity, FLA serve as intracellular hosts for numerous amoeba-resistant microorganisms (ARMs), including *Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa*. This "Trojan horse" relationship protects bacterial pathogens from adverse environmental conditions and conventional disinfection, enhancing their persistence and potentially increasing their virulence and transmission through drinking water systems (Price et al., 2024). These characteristics highlight the limitations of conventional microbiological monitoring based solely on bacterial indicator organisms. Therefore, incorporating FLA surveillance into drinking water quality assessment and microbial risk management programs is increasingly recognized as an essential strategy for improving public health protection and ensuring the microbiological safety of drinking water supplies.

Accurate detection and identification of FLA are essential for understanding their environmental distribution, evaluating potential health risks, and improving drinking water surveillance programs. Culture-based methods remain the most commonly used techniques because they are cost-effective, relatively simple, and allow the recovery of viable amoebae for subsequent morphological and molecular analyses. Standardization of sampling, concentration, and cultivation procedures is critical to ensure reliable and reproducible results across different investigations (Thomas & Ashbolt, 2011). Environmental water samples are typically concentrated by membrane filtration (0.22–0.45 μm) and cultured on non-nutrient agar (NNA) plates seeded with heat-killed *Escherichia coli*. This method provides a suitable nutritional source for trophozoites while limiting bacterial overgrowth, thereby facilitating amoebal growth and microscopic observation. Incubation is generally performed at 25–37°C, although higher temperatures may be used to recover thermotolerant species such as *Naegleria fowleri*.

Morphological examination remains the primary approach for preliminary identification of FLA. Trophozoites and cysts can be differentiated based on their morphology, motility, and developmental characteristics. For example, *Acanthamoeba* trophozoites possess distinctive acanthopodia, whereas *Naegleria* trophozoites exhibit lobose pseudopodia and can transform into a temporary biflagellate stage. Cyst morphology also provides valuable taxonomic information, with *Acanthamoeba* producing characteristic double-walled cysts and *Naegleria* forming smooth, single-walled spherical cysts (Muchesa, 2017). Despite its diagnostic value, morphological identification alone has limitations because amoebal morphology may vary with culture conditions, and closely related species often exhibit similar structural features. Therefore, microscopy alone may not provide reliable species-level identification.

Molecular techniques, particularly PCR amplification of the 18S rRNA gene followed by DNA sequencing, have substantially improved the accuracy of FLA identification and classification. These methods enable species identification, genotype determination, and phylogenetic analysis, while advanced approaches such as quantitative PCR and next-generation sequencing facilitate the detection of mixed amoebal populations directly from environmental samples. Currently, combining culture-based isolation, microscopic examination, and molecular methods is considered the most reliable strategy for investigating FLA in drinking water systems, providing accurate identification while supporting environmental surveillance and public health risk assessment (Krol-Turminska & Olender, 2017). Collectively, these characteristics highlight the ecological, medical, and environmental significance of free-living amoebae and explain the growing international interest in monitoring their occurrence in drinking water systems (Thomas & Ashbolt, 2011).

Figure 2. Integrated workflow for the isolation, detection, and identification of free-living amoebae (FLA) from drinking water using culture-based, microscopic, and molecular approaches.

2. Global Occurrence and Distribution of Free-Living Amoebae in Drinking Water Sources

Free-living amoebae (FLA) have been reported from drinking water systems on every inhabited continent, demonstrating their remarkable ecological adaptability and widespread environmental distribution. Over the past three decades, increasing numbers of investigations have confirmed the presence of FLA in both untreated and treated drinking water, highlighting their persistence throughout the water supply chain. Their occurrence has been documented in municipal water treatment plants, distribution networks, storage reservoirs, domestic plumbing systems, bottled water, hospital water supplies, and rural drinking water sources. Reported prevalence varies considerably among countries and geographic regions, reflecting differences in climatic conditions (such as higher isolation rates reported in tropical and subtropical zones compared to temperate regions), water source characteristics, treatment efficiency, sampling protocols, and laboratory identification methods. Consequently, geographical variation in prevalence should be interpreted cautiously because methodological differences may significantly influence recovery rates (Thomas & Ashbolt, 2011).

Natural water bodies constitute the primary environmental reservoirs of FLA. Rivers, lakes, ponds, reservoirs, wetlands, and streams continuously receive organic matter, microorganisms, and nutrients that support the growth of bacterial communities, which represent the principal food source for amoebae. Consequently, surface water generally harbors greater concentrations and diversity of FLA than other freshwater sources. Seasonal rainfall, agricultural runoff, wastewater discharge, and sediment resuspension further contribute to the introduction and dissemination of amoebae within surface water ecosystems, creating favorable habitats that facilitate the proliferation of both trophozoites and cysts before water enters treatment facilities (Choi et al., 2024).

Although groundwater is traditionally regarded as microbiologically safer than surface water because of natural filtration through geological formations, numerous studies have demonstrated that wells, springs, and aquifers are not exempt from FLA contamination. Amoebae may enter groundwater systems through fractured bedrock, permeable soils, surface infiltration following heavy rainfall, inadequate wellhead protection, defective borehole construction, or contamination during groundwater abstraction and storage. Moreover, poorly maintained groundwater systems may permit biofilm formation and microbial colonization, allowing FLA to persist despite the relatively stable physicochemical conditions characteristic of subsurface environments.

Water treatment plants are designed to reduce microbial contamination through sequential processes that include coagulation, flocculation, sedimentation, filtration, and final disinfection. Although these treatment steps substantially reduce microbial loads, complete removal of FLA is not consistently achieved. Trophozoites may occasionally survive filtration, whereas cysts exhibit significantly greater resistance because of their double-layered cell wall and reduced metabolic activity. Consequently, viable amoebae have been isolated from treated drinking water in numerous countries, suggesting that conventional treatment processes alone may not be sufficient to eliminate these protozoa under all operational conditions (Thomas & Ashbolt, 2011).

Following treatment, drinking water enters extensive distribution systems that provide favorable ecological niches for the long-term survival of FLA. Pipelines, storage tanks, service reservoirs, and household plumbing

frequently support the development of multispecies biofilms composed of bacteria, fungi, algae, and extracellular polymeric substances. These biofilms provide physical protection against hydraulic disturbances and disinfectants while simultaneously supplying abundant bacterial prey for amoebal growth. Within these complex microbial communities, trophozoites actively graze on bacteria, reproduce by binary fission, and undergo encystment when environmental conditions become unfavorable. Consequently, biofilms act as persistent reservoirs from which amoebae may be continuously released into the flowing water, allowing recolonization of distribution systems even after routine maintenance and disinfection procedures (Choi et al., 2024).

Table 1 provides a comprehensive comparative synthesis of the global distribution patterns, prevalent genera, and primary environmental drivers of Free-Living Amoebae (FLA) across major geographical regions. The data highlights that the occurrence and isolation frequency of FLA are heavily dictated by an interplay between regional climatic parameters and municipal water infrastructure efficiency.

- **Climatic Influence on Thermophilic Strains:** Regions characterized by arid, tropical, and subtropical climates (such as Africa, the Middle East, and parts of Asia) experience elevated ambient and water temperatures. These thermal conditions create an optimal environment for the proliferation of thermophilic and pathogenic species, notably *Naegleria fowleri* and *Acanthamoeba* spp.
- **Infrastructural Dynamics in Developed Sectors:** Conversely, regions like Europe and North America generally report lower isolation frequencies in treated municipal supplies due to rigorous regulatory monitoring and advanced water treatment technologies. However, persistent colonization remains a challenge in domestic plumbing and stagnant hot-water loops, where amoebae thrive within protective biofilms.

Ultimately, the matrix underscores that effective management and risk assessment of drinking water systems cannot rely solely on standard disinfection protocols; they require region-specific control strategies that account for local climatic conditions and biofilm-associated microbial communities.

Table 1: ***Comparative distribution, dominant genera, and environmental drivers of Free-Living Amoebae (FLA) across major global regions.***

Geographical Region / Continent	Estimated Isolation Frequency	Predominant Genus / Genera	Key Environmental Drivers
Africa & Middle East	Moderately High (Particularly in arid/semi-arid zones)	*Acanthamoeba* spp., *Naegleria fowleri*	Elevated ambient and water temperatures; seasonal evaporation; aging municipal distribution networks.
Asia	Moderate to High (Varying by infrastructure levels)	*Acanthamoeba* spp., *Vahlkampfia*	Climatic diversity (tropical/subtropical belts); reliance on surface water reservoirs in developing sectors.
Europe	Moderate (Concentrated in domestic hot water systems)	*Acanthamoeba* spp.	High standards of advanced water treatment; persistent biofilmed colonization in domestic plumbing and heating loops.
North America	Low to Moderate in treated supplies; higher in surface waters	*Naegleria fowleri* (southern tiers), *Acanthamoeba*	Rigorous regulatory monitoring of municipal supplies; thermal impacts on southern recreational water bodies.

The occurrence and abundance of FLA in drinking water are strongly influenced by environmental and operational factors. Water temperature is considered one of the most important determinants because many species, particularly thermotolerant *Acanthamoeba* spp. and *Naegleria fowleri*, exhibit accelerated growth under warm conditions. Increased water temperatures during summer months or in warmer climates have frequently been associated with higher isolation rates and increased amoebal densities in both natural waters and engineered water systems. Climatic warming may therefore contribute to the expansion of suitable habitats for thermophilic amoebae and increase the likelihood of human exposure in regions previously considered at low

risk (Pelley, 2015). In addition to temperature, several physicochemical characteristics influence the survival and proliferation of FLA, including pH, turbidity, conductivity, dissolved oxygen, nutrient availability, dissolved organic carbon, residual chlorine concentration, hydraulic residence time, and the composition of resident microbial communities.

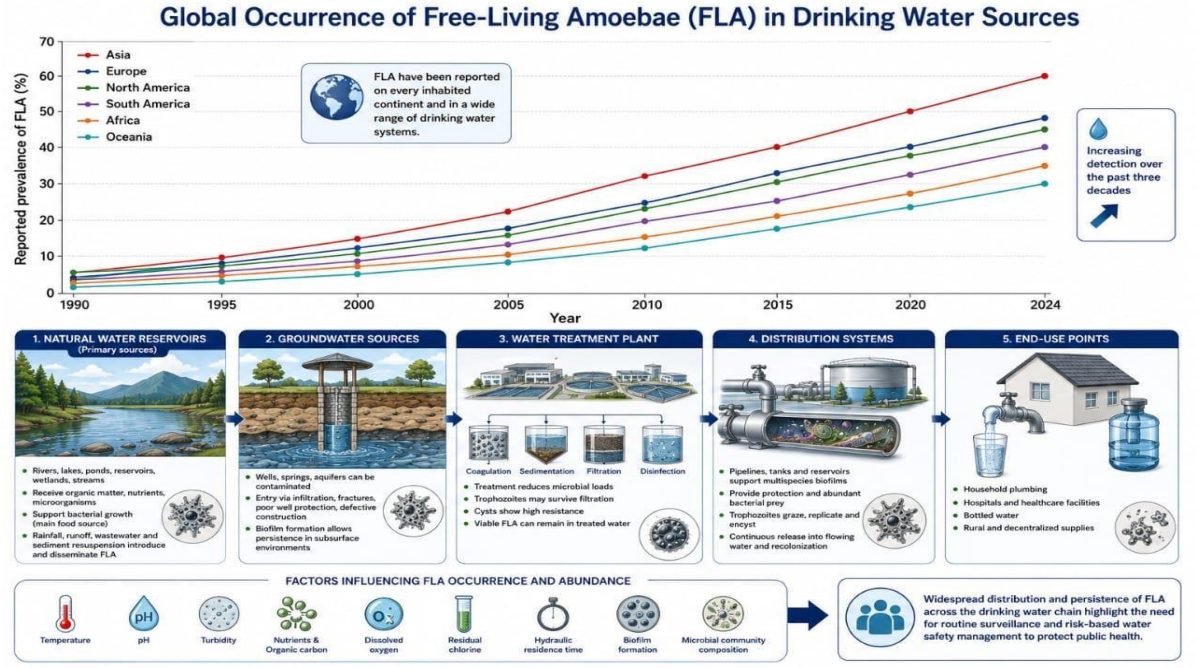


Figure 3. Global distribution and occurrence trends of free-living amoebae (FLA) in drinking water systems and the major environmental factors influencing their persistence.

3. Isolation Methodologies of Free-Living Amoebae from Drinking Water Sources

The successful recovery of free-living amoebae (FLA) from drinking water samples depends largely on the efficiency of sample collection, concentration, cultivation, and purification procedures. Because amoebae are often present in low numbers and may exist either as metabolically active trophozoites or highly resistant cysts, sensitive isolation techniques are required to maximize recovery while minimizing contamination by competing microorganisms. Although molecular approaches have gained increasing importance in recent years, culture-based isolation remains the gold standard for obtaining viable amoebae suitable for morphological, physiological, and molecular characterization (Pawelec et al., 2026).

3.1 Sample Collection and Concentration

Representative sampling is the first and one of the most critical steps in the isolation of FLA from drinking water systems. Sampling protocols vary according to the objectives of the investigation and the characteristics of the water source; however, relatively large sample volumes are generally recommended because amoebae are frequently present at low concentrations. In most environmental surveys, between 10 and 50 L of drinking water are collected aseptically in sterile polyethylene or polypropylene containers to improve the probability of recovering both trophozoites and cysts. When sampling distribution systems, water is usually allowed to flow for several minutes before collection to minimize the influence of stagnant water and to obtain representative samples from the pipeline network.

Following collection, water samples are concentrated to increase the likelihood of detecting amoebae. Membrane filtration is the most widely used concentration technique due to its simplicity, reproducibility, and cost-effectiveness. In this method, water is passed through sterile cellulose nitrate or mixed cellulose ester membrane filters with pore sizes ranging from 0.22 to 0.45 µm, which effectively retain both trophozoites and cysts. Depending on the quality and turbidity of the water sample, multiple filters may be required to prevent clogging caused by suspended particulate matter. For highly turbid samples, such as raw surface water or drinking water sources affected by wastewater contamination, continuous-flow centrifugation or low-speed centrifugation may be used either as an alternative concentration method or as a complementary step following filtration. These techniques allow the processing of larger volumes of water while minimizing cellular damage and enhancing organism recovery efficiency (Mathison & Pritt, 2023).

3.2 Culture-Based Isolation

Following concentration, the retained material is transferred to culture media that selectively support amoebal growth while limiting the proliferation of other microorganisms. The most widely accepted isolation method involves placing the membrane filter directly onto non-nutrient agar (NNA) previously seeded with a dense lawn of bacteria serving as a food source. Traditionally, live or heat-killed *Escherichia coli* has been used because it provides an abundant and readily available nutrient source for trophozoites without excessively overgrowing the

agar surface. Some laboratories also employ *Enterobacter aerogenes* or other non-pathogenic bacterial species that support comparable amoebal growth.

The absence of nutrients within the agar itself suppresses the growth of many competing microorganisms while encouraging amoebae to migrate toward the bacterial lawn in search of food. Culture plates are incubated under controlled laboratory conditions, typically between 28°C and 30°C for environmental isolates of *Acanthamoeba* and many other FLA. When thermotolerant species such as *Naegleria fowleri* are suspected, additional incubation at 37–42°C is frequently performed because elevated temperatures favor the growth of pathogenic thermophilic strains while inhibiting many non-pathogenic amoebae. Culture plates are examined daily for up to two weeks using an inverted or stereomicroscope, as the time required for amoebal emergence varies according to species, initial cell density, and environmental conditions (Visvesvara et al., 2007).

3.3 Detection of Amoebal Growth and Purification

The appearance of characteristic feeding zones on the bacterial lawn represents one of the earliest indicators of successful amoebal isolation. As trophozoites migrate across the agar surface, they actively consume bacteria, producing distinctive transparent tracks or plaques that can be readily observed under low magnification. These migration patterns are considered a hallmark of viable amoebal growth and guide the selection of colonies for further purification. Individual agar blocks containing actively migrating trophozoites are carefully excised and transferred onto freshly prepared NNA plates seeded with bacteria.

Repeated subculturing is generally required to eliminate bacterial contaminants, fungi, and mixed amoebal populations, thereby establishing clonal or near-clonal cultures suitable for downstream analyses. For detailed physiological studies or molecular investigations, purified isolates may subsequently be adapted to axenic culture, in which amoebae are maintained in specialized liquid media without bacterial food sources. Axenic cultivation facilitates investigations of amoebal metabolism, pathogenicity, antimicrobial susceptibility, host–pathogen interactions, and genomic characterization while reducing interference from bacterial DNA during molecular analyses. However, not all environmental isolates adapt readily to axenic conditions, and prolonged laboratory cultivation may alter morphological features or reduce virulence in some species. Consequently, many investigators recommend combining short-term culture with molecular identification to preserve the original biological characteristics of environmental isolates (Santos, 2025).

3.4 Advantages and Limitations of Culture-Based Isolation

Despite the rapid development of molecular diagnostic methods, culture-based isolation continues to play a fundamental role in FLA research because it enables the recovery of viable organisms for morphological examination, pathogenicity assessment, antimicrobial susceptibility testing, and genetic characterization. Nevertheless, conventional isolation techniques are relatively time-consuming, labor-intensive, and highly dependent on sample quality, incubation conditions, and operator expertise. Slow-growing species may be overlooked because of bacterial or fungal overgrowth, while some viable amoebae may enter a dormant state and fail to excyst under laboratory conditions. Therefore, many recent studies advocate integrating culture-based isolation with molecular techniques, including polymerase chain reaction (PCR), quantitative PCR (qPCR), and DNA sequencing, to improve detection sensitivity, species identification, and epidemiological surveillance of free-living amoebae in drinking water systems (Perez et al., 2025).

3.5 Alternative Solutions and Methodologies for Managing Bacterial and Fungal Overgrowth in Environmental Samples During Prolonged Incubation

During the prolonged incubation of environmental samples (such as drinking water or surface water isolates) which can extend up to two weeks for the isolation of Free-Living Amoebae (FLA) microbial contamination by fast-growing bacteria and fungi frequently poses a significant challenge. To effectively suppress contaminants without harming target amoebae, the following advanced alternative strategies and protocols are utilized in academic and research laboratories:

3.5.1. Targeted Antimicrobial and Antifungal Supplementation

Because FLA (such as *Acanthamoeba* spp.) are eukaryotic organisms possessing high resistance to many antibacterial agents, specific antimicrobial compounds can be incorporated directly into non-nutrient agar (NNA) media to control severe bacterial and fungal proliferation:

- **Antibacterial Agents:** A carefully optimized combination of **penicillin and streptomycin**, or alternatively **ceftazidime**, is frequently added to suppress accompanying environmental bacteria without impeding amoebic motility.
- **Antifungal Agents:** The inclusion of **nystatin** or **amphotericin B** serves as an effective prophylactic measure to inhibit the rapid growth of molds and yeasts that typically compromise culture plates during extended incubation periods.

3.5.2. Serial Washing and Cyst Plating Techniques

When environmental samples experience severe fungal or bacterial overgrowth:

- Small agar plugs containing visible advancing fronts of amoebic trophozoites or cysts are excised and transferred to fresh, nutrient-depleted agar plates.
- **Cyst Washing Protocols:** Because encysted amoebae exhibit significantly higher resistance to mild antimicrobial washes compared to vegetative trophozoites or contaminating microbes, washing isolated cysts through sterile phosphate-buffered saline (PBS) solutions effectively separates them from vegetative biological contaminants.

3.5.3. Controlled Bacterial Seeding Protocols

Standard cultivation relies on supplying live bacteria (such as *Escherichia coli*) as a food source for FLA. However, unchecked bacterial multiplication can rapidly overwhelm the culture plate:

- **Thermally Killed Bacteria:** Utilizing heat-killed or UV-irradiated bacterial suspensions provides the necessary nutrients for phagocytosis while completely eliminating the risk of bacterial overgrowth and subsequent media degradation.
- **Low-Density Inoculation:** Restricting the initial bacterial food density and replenishing it incrementally prevents excessive organic enrichment during early incubation stages.

3.5.4. Utilization of Selective Media formulations

In instances involving highly complex environmental matrices, specialized selective media formulations can be introduced. These formulations employ specific inhibitory agents to suppress both Gram-positive and Gram-negative bacterial competition simultaneously, thereby granting slow-growing FLA a distinct competitive advantage.

3.5.5. Exploiting the "Amoebic Migration" Phenomenon

Leveraging the natural biological behavior and chemotactic motility of FLA away from contamination sites offers a reliable physical separation method:

- Centrifuged or filtered sample concentrates are inoculated at the exact center of an agar plate.
- As fungal mycelia or dense bacterial colonies dominate the primary inoculation zone, viable amoebae actively migrate outward toward the periphery of the plate in search of clean nutrients.
- Clean subcultures are subsequently established by excising peripheral agar blocks well away from the contaminated focal point.

4. Morphological Characterization of Free-Living Amoebae

Morphological characterization remains one of the most widely applied approaches for the preliminary identification of free-living amoebae (FLA) recovered from environmental and clinical samples. Despite the continuous growth of molecular techniques, microscopic examination remains an essential initial diagnostic step because it is relatively rapid, cost-effective, and well-suited for routine laboratory workflows. Identification at this stage relies primarily on observing trophozoites and cysts after cultivation on non-nutrient agar plates seeded with live or heat-killed Escherichia coli. Diagnostic features are typically evaluated using bright-field, phase-contrast, and differential interference contrast microscopy, alongside scanning electron microscopy (SEM) when available to provide high-resolution views of surface ultrastructures (Nageeb et al., 2022).

4.1 Morphology of the Trophozoite Stage

The trophozoite represents the metabolically active, feeding, motile, and reproductive stage of the amoebal life cycle. During this phase, amoebae actively migrate across culture surfaces, feeding on bacteria, yeasts, algae, and other microorganisms primarily through phagocytosis. Under favorable conditions, trophozoites multiply via mitotic binary fission and display characteristic morphological traits that facilitate preliminary genus-level classification. Among medically relevant FLA, *Acanthamoeba* trophozoites generally measure roughly 15–45 μm in diameter and feature slender, spine-like cytoplasmic extensions known as acanthopodia, which drive locomotion, adherence, and prey capture. The cytoplasm is typically segregated into a clear ectoplasm and a granular endoplasm loaded with food vacuoles, mitochondria, and a distinct osmoregulatory contractile vacuole. A single vesicular nucleus housing a central, densely stained nucleolus serves as a key diagnostic landmark. The irregular cell outline created by numerous acanthopodia readily differentiates *Acanthamoeba* trophozoites from most other free-living amoebae (Siddiqui & Khan, 2012).

Conversely, *Naegleria* trophozoites are typically smaller, spanning 10–30 μm, and travel via rapid, eruptive movements driven by broad lobose pseudopodia (lobopodia). Unlike *Acanthamoeba*, *Naegleria* lacks acanthopodia and maintains a smooth cellular profile during active displacement. A defining biological feature of *Naegleria* is its capacity to transition reversibly into a temporary biflagellate form when moved into distilled water or low-ionic-strength solutions. Although non-feeding and non-dividing, this flagellate stage acts as a crucial diagnostic marker separating *Naegleria* from other free-living amoebae genera. Meanwhile, *Balamuthia mandrillaris* trophozoites are characteristically pleomorphic with broad, variable pseudopodia, whereas *Sappinia* species feature distinct binucleate trophozoites—an uncommon trait among pathogenic amoebae that provides high diagnostic value (Visvesvara et al., 2007).

4.2 Morphology of the Cyst Stage

The cyst represents the dormant and highly resistant stage of free-living amoebae (FLA), enabling survival under adverse environmental conditions such as nutrient deprivation, desiccation, osmotic stress, extreme temperatures, and exposure to chemical disinfectants. Because cyst morphology is considerably more stable than that of the trophozoite stage, it has long served as the basis for the classical taxonomy of FLA. Acanthamoeba cysts typically measure 10–25 μm in diameter and possess a characteristic double-layered wall composed of an outer ectocyst and an inner endocyst. The ectocyst may appear smooth, wrinkled, or slightly undulating, whereas the endocyst exhibits considerable morphological diversity, ranging from polygonal, stellate, and triangular to oval or spherical, depending on the species and genotype. The shape of the endocyst and the arrangement of cyst pores have historically formed the basis of major taxonomic classifications and continue to serve as important microscopic diagnostic features (Marciano & Cabral, 2007).

In contrast, Naegleria cysts are generally spherical, measure approximately 7–15 µm in diameter, and possess a smooth, single-layered wall perforated by one or more pores (ostioles) that facilitate excystment. Unlike Acanthamoeba, Naegleria cysts lack a double-layered wall and the distinctive stellate or polygonal endocyst, making them relatively easy to distinguish by experienced microscopists. Balamuthia mandrillaris cysts are typically spherical and characterized by a thick, triple-layered wall. In contrast, Sappinia cysts are rarely observed in environmental samples because cultivation more frequently yields trophozoites than cysts (Lorenzo et al., 2015).

4.3 Diagnostic Value and Limitations of Morphological Identification

Microscopic evaluation remains an indispensable pillar of laboratory diagnostics, enabling rapid screening of environmental samples without demanding complex equipment. It allows direct tracking of amoebal viability, motility, encystment, excystment, and interactions with bacterial food sources. Furthermore, skilled analysts can frequently separate major pathogenic genera strictly through structural analysis, making microscopy a frontline tool for routine drinking water surveillance.

Table 2 presents a structured comparative diagnostic matrix designed to mitigate the inherent challenges of conventional microscopic screening, such as human subjectivity and morphological plasticity. Because environmental stressors, osmotic fluctuations, and nutrient variations can induce significant structural alterations in the vegetative and encysted stages of Free-Living Amoebae (FLA), relying solely on general visual inspection often leads to misidentification.

This table directly addresses these limitations by emphasizing specific, invariant structural markers for each major genus:

- **Structural Differentiation:** It contrasts the distinctive spine-like acanthopodia and stellate double-walled cysts of *Acanthamoeba* with the smooth, pore-bearing cysts of *Naegleria*, the complex triple-layered walls of *Balamuthia*, and the unassuming smooth forms of *Hartmanella*.
- **Minimizing Diagnostic Errors:** By highlighting specific differential criteria and common morphological pitfalls—such as size overlaps with fungal spores or yeast cells—the matrix serves as a reliable practical tool for rapid laboratory screening.

Ultimately, Table 2 equips researchers with clear comparative guidelines to navigate phenotypic overlaps, reduce operator-dependent errors, and improve the precision of initial microscopic surveillance before confirmatory molecular assays are applied.

Table 2: ***Comparative morphological diagnostic matrix for rapid microscopic screening of major Free-Living Amoebae (FLA) genera.***

Genus	Trophozoite Morphology & Locomotion	Cyst Morphology & Key Features	Diagnostic Challenges (Morphological Plasticity & Pitfalls)
Acanthamoeba	Characterized by spine-like projections called *acanthopodia*; sluggish movement via broad hyaline pseudopodia.	Typically double-walled; outer exocyst is usually wrinkled/polyhedral, and inner endocyst is star-, polygonal-, or spherical-shaped.	Cysts can mimic fungal spores or small yeast cells if wall structures are not clearly resolved under high magnification.
Naegleria	Exhibits rapid, directional movement using broad lobopodia; possesses a transient flagellate phase when transferred to distilled water.	Spherical with a smooth, single-layered wall containing distinct pore-like germinal openings (ostrioles).	Vegetative forms can be misidentified with other small flagellates or amoeboflagellates without testing the transformation phase.
Balamuthia	Large multinucleate trophozoites with broad, irregular pseudopodia; frequently exhibits branching or multiple pseudopodial extensions.	Characterized by a distinctive triple-layered wall (thin outer envelope, thick wavy inner wall, and inner spherical endocyst).	Often confused with *Acanthamoeba* cysts due to size overlap; requires careful observation of the three-layered cyst wall.
Hartmanella	Small, slow-moving trophozoites with clear, hemispherical pseudopodia; lacks distinctive spine-like acanthopodia.	Spherical to oval with a smooth, thin, single or double wall lacking complex polygonal or stellate configurations of *Acanthamoeba*.	Easily overlooked or misclassified as non-pathogenic environmental amoebae due to unassuming, generic round cyst structures.

Nonetheless, morphological assessment carries clear limitations. Structural parameters fluctuate in response to culture conditions, nutrient levels, incubation temperatures, and culture age, creating notable phenotypic plasticity. Closely related species also frequently display overlapping traits, and non-pathogenic isolates often look identical to pathogenic ones. Consequently, morphology alone cannot definitively establish species identity, genotype, or virulence. Modern diagnostic pipelines therefore merge conventional microscopy with

molecular assays such as polymerase chain reaction (PCR), 18S rRNA gene sequencing, mitochondrial gene tracking, and phylogenetic profiling to secure precise taxonomic identification and elevate environmental surveillance standards in drinking water supplies (Qvarnstrom et al., 2006).

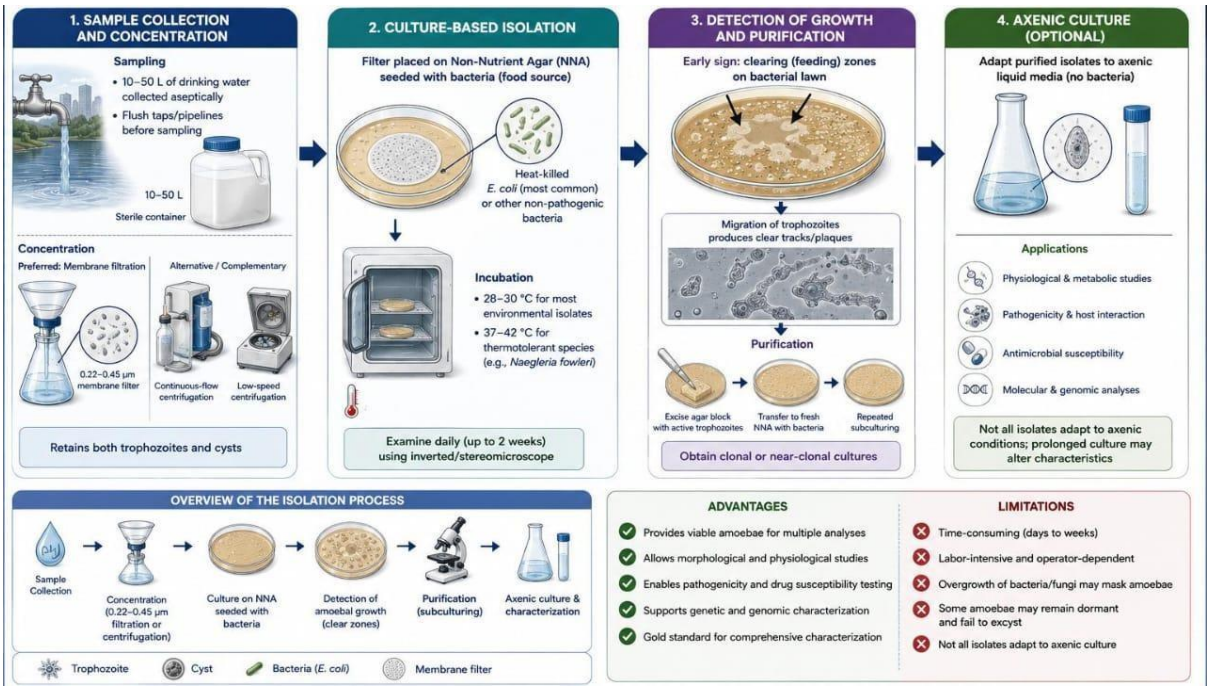


Figure 4. Schematic overview of culture-based isolation, purification, and characterization of free-living amoebae (FLA) from drinking water sources, highlighting major methodological steps, advantages, limitations, and integration with molecular identification.

5. Public Health Implications

The occurrence of free-living amoebae (FLA) in drinking water systems represents a critical public health concern because these protozoa function both as opportunistic human pathogens and as environmental reservoirs for numerous clinically significant microorganisms. Their widespread distribution across treated drinking water supplies, hospital networks, domestic plumbing systems, and recreational water environments heightens the risk of human exposure, particularly among vulnerable populations. Although clinical infections caused by FLA are relatively uncommon, they are frequently characterized by severe health outcomes, delayed diagnosis, limited therapeutic options, and exceptionally high mortality rates. Consequently, the World Health Organization (WHO) recognizes several FLA genera as emerging public health threats that require structured environmental surveillance and quantitative risk assessment (Siddiqui & Khan, 2012).

5.1 Human Diseases Caused by Free-Living Amoebae

Among pathogenic FLA, species within the genus *Acanthamoeba* account for the vast majority of documented human infections. *Acanthamoeba* keratitis (AK) is a painful, progressive corneal infection primarily affecting contact lens wearers, especially those with poor hygiene practices or habits of rinsing lenses with tap water. Clinical symptoms include severe ocular pain, photophobia, blurred vision, excessive tearing, and corneal ulceration. Delayed diagnosis often leads to permanent corneal scarring, visual impairment, or blindness, frequently necessitating corneal transplantation in advanced cases. The global incidence of AK has risen steadily over the past three decades, driven by increased contact lens adoption and heightened awareness of waterborne environmental exposure (Perspectives et al., 2024).

In addition to ocular disease, *Acanthamoeba* species can cause granulomatous amoebic encephalitis (GAE), a rare but devastating central nervous system infection predominantly affecting immunocompromised individuals, such as patients on immunosuppressive regimens, organ transplant recipients, and those with advanced HIV infection. GAE typically has an insidious onset, manifesting with persistent headaches, low-grade fever, focal neurological deficits, seizures, altered mental status, and progressive cerebral deterioration. Despite aggressive antimicrobial treatment, mortality remains extremely high because clinical recognition is often delayed until advanced disease stages.

Meanwhile, *Naegleria fowleri* is the causative agent of primary amoebic meningoencephalitis (PAM), an acute, rapidly progressive central nervous system infection marked by extensive destruction of brain tissue. Infection occurs when water containing trophozoites enters the nasal cavity during swimming, diving, ritual ablution, recreational water sports, or nasal irrigation using untreated water. Following attachment to the olfactory mucosa, trophozoites migrate along the olfactory nerves into the central nervous system, triggering severe neuroinflammation and tissue necrosis. Although relatively rare, PAM carries a case-fatality rate exceeding 95%, positioning it among the most lethal waterborne infectious diseases known to humanity (Priya et al., 2025).

Although infections caused by *Balamuthia mandrillaris* and *Sappinia pedata* are diagnosed much less frequently, both are linked to severe, highly fatal encephalitis. The rising number of reported cases globally suggests these pathogens remain widely underdiagnosed due to their non-specific clinical presentations and the limited availability of specialized diagnostic assays (Siddiqui & Khan, 2012).

5.2 Free-Living Amoebae as "Trojan Horses" for Waterborne Pathogens

Beyond their direct pathogenicity, FLA profoundly influence the ecology and transmission dynamics of waterborne microorganisms by acting as intracellular sanctuaries. During phagocytosis, trophozoites engulf various environmental bacteria; however, several species have evolved specialized survival mechanisms that block intraphagolysosomal digestion, allowing them to replicate within amoebal vacuoles. This symbiotic interaction—widely known as the "Trojan horse" phenomenon shields intracellular microbes from environmental stressors, predatory protozoa, and conventional chemical disinfection processes.

A broad spectrum of amoeba-resistant microorganisms (ARMs) has been identified, including *Legionella pneumophila*, *Mycobacterium avium* complex, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Burkholderia* species, *Vibrio cholerae*, *Campylobacter jejuni*, and members of the family Chlamydiaceae. Intracellular residence within FLA bolsters bacterial tolerance against chlorination, ultraviolet irradiation, oxidative stress, and nutrient deprivation encountered throughout drinking water treatment and distribution networks. Furthermore, bacteria released from host amoebae frequently display heightened virulence and enhanced infectivity compared to free-living bacterial counterparts, indicating that FLA serve as evolutionary incubators that sustain and amplify bacterial pathogenicity (Fan et al., 2024).

5.3 Public Health Challenges and Future Perspectives

The persistence of FLA in drinking water distribution systems poses a fundamental challenge for routine water quality monitoring, which relies primarily on tracking conventional bacterial indicators rather than protozoan hosts and their endosymbionts. Consequently, treated water supplies that successfully comply with standard bacteriological criteria may still harbor viable FLA capable of supporting pathogens within biofilms and pipeline networks.

This limitation underscores the critical need to integrate FLA surveillance into comprehensive Water Safety Plans (WSPs) and microbial risk management frameworks. Routine environmental monitoring, optimization of physical and chemical treatment barriers, proactive management of biofilm development, and the combination of conventional microscopy with molecular diagnostic assays represent essential strategies for mitigating human exposure risks. Enhancing clinical awareness among healthcare professionals and water utility operators will likewise facilitate earlier diagnosis of FLA-related infections and strengthen overarching public health interventions (Thomas & Ashbolt, 2011).

5.4. Comprehensive Therapeutic Protocols for Fatal Neurological Infections Caused by Free-Living Amoebae

While early diagnosis remains the single most critical determinant of survival in primary amoebic meningoencephalitis (PAM) and granulomatous amoebic encephalitis (GAE), modern clinical management relies heavily on aggressive, combinatorial antimicrobial and antiparasitic regimens. Due to the rapid progression and high mortality rates associated with these central nervous system (CNS) infections, standard monotherapy is typically ineffective.

5.4. 1. Primary Regimens for *Naegleria fowleri* (PAM)

Management of PAM requires synergistic drug combinations capable of crossing the blood-brain barrier (BBB) effectively:

- **Amphotericin B Deoxycholate:** Administered both intravenously and intrathecally, it serves as the cornerstone of anti-amoebic therapy, directly binding to sterols in the amoebic cell membrane.
- **Azoles (Fluconazole or Voriconazole):** These agents are co-administered to inhibit ergosterol synthesis, acting synergistically with Amphotericin B.
- **Miltefosine:** An alkylphospholipid originally developed as an anti-leishmanial drug, miltefosine has demonstrated significant amoebicidal activity and is now a critical component of recommended combination protocols.
- **Adjunctive Agents:** Rifampin and azithromycin are frequently integrated into the regimen for their broad-spectrum synergistic properties. Furthermore, aggressive management of intracranial pressure (ICP) using osmotic diuretics (like mannitol) and therapeutic hypothermia are vital clinical interventions to prevent fatal cerebral edema.

5.4.2. Therapeutic Strategies for *Balamuthia mandrillaris* and *Acanthamoeba* Encephalitis (GAE)

Because GAE typically manifests with a subacute or chronic presentation in immunocompromised hosts, treatment protocols require prolonged, multi-drug regimens sustained over several months or even years:

- **First-Line Combinations:** A multi-drug cocktail typically comprising **miltefosine, albendazole, pentamidine isethionate, and fluconazole or voriconazole** is utilized.
- **Surgical Intervention:** In cases involving well-defined cerebral granulomas or mass lesions, surgical excision is occasionally considered alongside medical therapy to reduce pathogen load and relieve severe mass effect.

5.4.3. Clinical Challenges and Future Directions

Despite these intensive protocols, survival rates for PAM remain exceptionally low (under 10%), primarily due

to irreversible tissue necrosis occurring before treatment initiation. Current clinical research emphasizes the urgent need for novel blood-brain barrier-penetrant compounds, rapid molecular point-of-care diagnostics, and immunomodulatory therapies to mitigate destructive host inflammatory responses during infection.

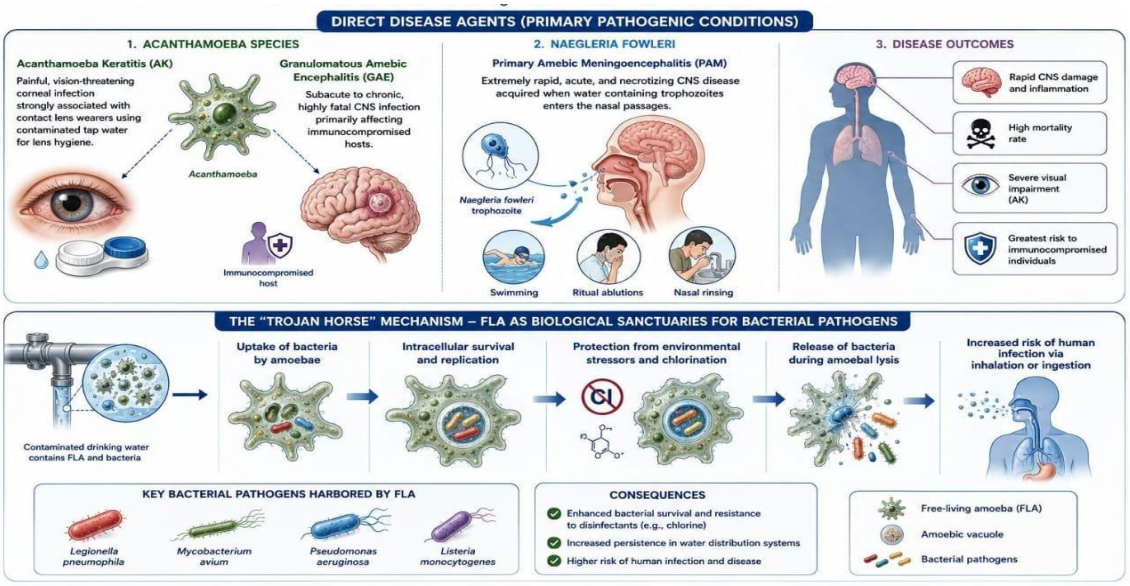


Figure 5: Overview of direct disease agents (pathogenic free-living amoebae such as *Acanthamoeba* and *Naegleria fowleri*) causing clinical conditions, alongside the "Trojan Horse" mechanism illustrating free-living amoebae acting as biological sanctuaries and vectors for bacterial pathogens.

6. Control and Prevention Strategies

The effective control of free-living amoebae (FLA) in drinking water systems requires a comprehensive, multi-barrier strategy that integrates source water protection, optimized treatment protocols, routine monitoring, distribution network management, and public health education. Because FLA can survive harsh environmental conditions and frequently colonize biofilms within water infrastructure, relying on a single control measure is insufficient to eradicate these organisms or prevent their recolonization. Consequently, international water safety frameworks recommend deploying multiple preventive safeguards across the entire drinking water supply chain, from source abstraction to point-of-use consumption (Nisar et al., 2025).

6.1 Optimization of Water Treatment Processes

The primary defense against FLA relies on optimizing conventional water treatment trains. Effective coagulation and flocculation enhance the aggregation of suspended particulate matter and microorganisms, facilitating their subsequent removal via sedimentation and rapid sand filtration. High-performance dual-media filters and advanced membrane technologies—such as microfiltration and ultrafiltration demonstrate superior efficiency in stripping amoebal trophozoites, cysts, and associated debris prior to final disinfection. Sustaining optimal operating parameters, including precise coagulant dosages, stable filtration rates, and routine filter maintenance, is essential to minimize the breakthrough of FLA into treated drinking water supplies (WHO, 2022).

6.2 Advanced Disinfection Technologies

The remarkable resistance of FLA cysts to conventional chemical disinfectants poses one of the most stubborn challenges in drinking water treatment. The resilient double walls of *Acanthamoeba* cysts and the hardy structures of other FLA can endure chlorine concentrations typically applied in municipal water treatment facilities. Therefore, implementing supplementary or alternative disinfection methods is increasingly vital to enhance inactivation rates. Ultraviolet (UV) irradiation, ozonation, chlorine dioxide, and advanced oxidation processes achieve significantly higher efficacy in destroying viable amoebae and neutralizing intracellular pathogens. Combining multiple disinfection modalities often yields synergistic effects, maximizing microbial reduction while lowering the risk of downstream recolonization. Continuous tracking of disinfectant residuals throughout the distribution grid remains equally crucial for preserving microbiological water safety (Siddiqui & Khan, 2012).

6.3 Biofilm Control and Distribution System Management

Because biofilms provide an ideal ecological refuge for the persistence and proliferation of FLA, rigorous biofilm management forms a cornerstone of water safety programs. Routine cleaning of storage reservoirs, periodic flushing of water mains, mitigation of hydraulic stagnation, and maintenance of stable disinfectant residuals can substantially suppress biofilm accumulation and microbial colonization. Incorporating corrosion-resistant pipeline materials and designing well-engineered distribution layouts further restrict surface conditions that favor biofilm development. Periodic environmental surveillance using both culture-based and molecular assays enables the early detection of FLA contamination, allowing operators to execute corrective interventions before widespread colonization occurs (Ngoc & Khanh, 2024).

6.4 Public Health Education and Point-of-Use Prevention

Public awareness serves as an indispensable pillar of FLA prevention strategies. Communities must be educated on the health risks tied to using untreated water, particularly in activities involving direct exposure to the eyes or nasal passages. Contact lenses should never be rinsed or stored in tap water, and stringent compliance with lens hygiene guidelines must be encouraged. Similarly, only sterile, distilled, boiled-and-cooled, or properly filtered water (using filters with a pore size of 0.2 m or smaller) should be utilized in nasal irrigation devices, including neti pots and squeeze bottles.

Comparable precautions apply when operating household humidifiers, respiratory therapy apparatuses, and other aerosol-generating medical equipment. Public health outreach aimed at healthcare practitioners, water utility managers, and everyday consumers can significantly cut down preventable infections by promoting better hygiene practices and early symptom recognition (Centers for Disease Control and Prevention [CDC], 2025).

6.5 Future Perspectives

Despite considerable advancements in water treatment engineering, the complete eradication of free-living amoebae remains a formidable challenge due to their environmental resilience and intimate ties to biofilms and intracellular microorganisms. Future control paradigms should prioritize coupling conventional microbiological testing with molecular diagnostic tools, quantitative microbial risk assessment (QMRA), and real-time monitoring of distribution networks.

Additional research is likewise needed to innovate disinfection technologies capable of neutralizing both amoebal cysts and their intracellular endosymbionts without compromising overall water quality. Bolstering global surveillance networks and embedding FLA metrics into standard drinking water evaluations will ultimately drive down waterborne disease transmission and strengthen public health protection worldwide (Vijayan et al., 2026).

7. DISCUSSION

The increasing detection of free-living amoebae (FLA) in drinking water systems worldwide highlights their growing significance as environmental microorganisms with important implications for water quality and public health. Although FLA have long been regarded as naturally occurring protozoa with limited clinical relevance, accumulating evidence demonstrates that their persistence in both natural and engineered aquatic environments represents a complex microbiological challenge. Their remarkable ability to withstand adverse environmental conditions, colonize water distribution systems, and interact with diverse microbial communities has shifted scientific attention from their traditional role as environmental organisms to their broader significance as opportunistic pathogens and reservoirs of other infectious microorganisms (Thomas & Ashbolt, 2011).

One of the most consistent observations reported in the literature is the widespread occurrence of FLA in virtually all types of drinking water sources, including untreated surface water, groundwater, treated municipal water, household storage tanks, hospital water systems, bottled water, and premise plumbing. This broad ecological distribution reflects the extraordinary adaptability of these protozoa rather than deficiencies in individual water treatment facilities alone. The ability of FLA to survive under oligotrophic conditions, tolerate fluctuating environmental parameters, and encyst in response to stress enables them to persist even after conventional water treatment processes have substantially reduced other microbial populations. Consequently, their detection in treated drinking water should not necessarily be interpreted as treatment failure but rather as evidence of the exceptional biological resilience of these microorganisms. This finding also explains why FLA have been repeatedly isolated from drinking water systems that satisfy routine bacteriological standards based on indicator organisms such as *Escherichia coli* and total coliforms (Visvesvara & Stehr-Green, 1990).

Environmental Factors and Ecological Distribution

The considerable variation in reported prevalence among published studies deserves careful interpretation. Surveys conducted in tropical and subtropical regions generally report higher detection rates than those performed in temperate climates. Elevated water temperatures promote trophozoite growth, accelerate bacterial proliferation, and stimulate biofilm development, thereby creating favorable ecological conditions for amoebal multiplication. In contrast, lower temperatures may reduce trophozoite activity while increasing the proportion of dormant cysts, potentially influencing recovery rates depending on the isolation technique employed.

These observations suggest that geographical differences in FLA occurrence are not solely determined by climatic conditions but also by methodological variations, including sample volume, filtration procedures, culture media, incubation temperature, incubation period, and the diagnostic methods used for identification. Therefore, direct comparison of prevalence data among different investigations should be interpreted cautiously because methodological heterogeneity may substantially influence reported isolation rates (Arberas et al., 2024).

Another important factor contributing to the persistence of FLA is their close association with biofilms. Biofilms represent highly structured microbial communities attached to pipe surfaces, storage reservoirs, and household plumbing systems. Within these communities, amoebae benefit from abundant bacterial prey, stable nutrient availability, and protection from hydraulic disturbances and disinfectant exposure. Biofilm-associated amoebae are therefore considerably more difficult to eliminate than planktonic cells suspended in the water column. This observation has important practical implications because routine water sampling often focuses on bulk water while neglecting attached biofilms that may serve as long-term reservoirs for recolonization of drinking water distribution systems. Consequently, the actual prevalence of FLA may be underestimated when surveillance

programs rely exclusively on water samples without evaluating biofilm-associated microorganisms (Potgieter et al., 2021).

Resilience, Risk Assessment, and Indicator Potential

The resistance of FLA cysts to environmental stress further complicates efforts to control these organisms. Encystment represents a highly efficient adaptive response that allows amoebae to survive nutrient depletion, osmotic stress, desiccation, ultraviolet radiation, and exposure to disinfectants commonly applied in drinking water treatment. Several investigations have demonstrated that conventional chlorination alone is insufficient to completely eliminate cysts of *Acanthamoeba*, particularly when cysts are embedded within biofilms or particulate matter. This biological resilience partly explains why viable amoebae continue to be recovered from chlorinated water supplies and why recolonization frequently occurs after routine maintenance or disinfection procedures. These findings support the current recommendation that FLA management should rely on integrated preventive strategies rather than dependence on a single disinfection method. Recent WHO guidance similarly emphasizes preventive, risk-based water safety planning and continuous surveillance throughout the distribution system rather than reliance on end-point microbiological testing alone (WHO, 2022).

An equally important aspect emerging from recent investigations is the growing recognition of FLA as potential indicators of microbial ecosystem instability rather than merely independent pathogens. Because amoebae depend on bacterial populations for nutrition, their presence frequently reflects favorable ecological conditions for microbial growth within drinking water systems. Several authors have therefore proposed that routine monitoring of FLA could complement traditional bacterial indicators by providing additional information regarding biofilm development, treatment efficiency, and the potential presence of amoeba-resistant microorganisms. Although this concept remains under active investigation, recent studies suggest that integrating FLA surveillance into drinking water monitoring programs may improve microbial risk assessment and strengthen preventive public health strategies. Nevertheless, standardized protocols for environmental sampling, culture conditions, and species identification remain necessary before FLA can be adopted as routine biological indicators of drinking water quality.

Critical Evaluation of Isolation, Microscopy, and Molecular Methods

Accurate detection and identification of free-living amoebae are fundamental prerequisites for understanding their environmental distribution, assessing their public health significance, and developing effective surveillance programs. Although numerous studies have investigated the occurrence of FLA in drinking water systems, considerable variation in reported prevalence remains evident. This inconsistency is largely attributable to differences in sampling strategies, concentration methods, culture conditions, incubation periods, and identification techniques rather than genuine geographical variation alone. Consequently, direct comparison of prevalence rates among studies should be undertaken with caution, particularly when different laboratory protocols have been employed (Coulon et al., 2010).

Among the available detection methods, culture-based isolation on non-nutrient agar (NNA) seeded with *Escherichia coli* remains the most widely accepted conventional technique for environmental investigations. The method is inexpensive, technically straightforward, and enables direct observation of trophozoite migration, bacterial feeding behavior, encystment, and excystment. In addition, successful cultivation confirms organism viability, an important advantage over molecular techniques that may amplify DNA from non-viable cells. Consequently, NNA culture continues to serve as the reference method for routine isolation of FLA from environmental water samples, particularly in laboratories with limited access to advanced molecular facilities (Choi et al., 2024).

Despite these advantages, culture-based isolation has several limitations that may influence recovery rates. Amoebal growth is affected by incubation temperature, nutrient availability, bacterial food source, and the physiological condition of environmental isolates. Some strains grow slowly or enter prolonged dormant states, while others fail to proliferate under standard laboratory conditions despite remaining viable in the environment. Furthermore, competitive interactions among microorganisms present in environmental samples may suppress amoebal growth or obscure microscopic observation. As a result, culture-based methods may underestimate the true abundance of FLA, particularly in chlorinated drinking water where amoebae are often present in low numbers or predominantly in the cyst stage.

Morphological characterization remains an indispensable component of laboratory diagnosis because it provides rapid preliminary identification based on the structural characteristics of trophozoites and cysts. Diagnostic features such as the acanthopodia of *Acanthamoeba*, the eruptive locomotion and transient flagellate stage of *Naegleria*, and the distinctive architecture of cyst walls continue to represent valuable taxonomic criteria. Experienced investigators can often distinguish the principal pathogenic genera using conventional light microscopy alone, making morphological examination particularly valuable for routine environmental surveillance and preliminary screening of large numbers of samples (Trabelsi et al., 2015).

However, morphological identification alone is insufficient for reliable species-level classification. Considerable phenotypic plasticity exists among environmental isolates, and morphological characteristics may change according to nutrient availability, culture age, osmotic conditions, temperature, and other environmental variables. Furthermore, closely related species frequently exhibit overlapping cyst morphology, making accurate differentiation difficult even for experienced microscopists. Similar limitations have been reported for distinguishing pathogenic and non-pathogenic isolates because virulence cannot be inferred solely from

trophozoite or cyst morphology. These observations explain why earlier epidemiological studies based exclusively on microscopy may have underestimated species diversity or misclassified environmental isolates (Choi et al., 2024).

During the past two decades, molecular diagnostic techniques have substantially improved the accuracy of FLA identification. Polymerase chain reaction (PCR), sequencing of the 18S rRNA gene, and phylogenetic analyses have enabled precise discrimination among genera, species, and genotypes that cannot be reliably differentiated using morphology alone. Molecular approaches have been particularly valuable for identifying pathogenic *Acanthamoeba* genotypes, especially the T4 genotype, which is responsible for the majority of clinical cases of *Acanthamoeba* keratitis worldwide. Likewise, molecular tools have facilitated epidemiological investigations of *Naegleria fowleri*, *Balamuthia mandrillaris*, and other clinically significant FLA by providing greater taxonomic resolution and allowing comparison of environmental and clinical isolates (Fallah et al., 2017).

Nevertheless, molecular methods are not without limitations. PCR-based assays require specialized laboratory infrastructure, trained personnel, and rigorous contamination control. Environmental inhibitors present in water samples may reduce amplification efficiency, while DNA detection does not necessarily indicate organism viability or infectivity. Consequently, exclusive reliance on molecular detection may overestimate the public health significance of environmental contamination because non-viable organisms may still yield positive amplification results. For this reason, an integrated diagnostic strategy combining culture, microscopic examination, and molecular confirmation is increasingly recommended as the most reliable approach for environmental surveillance of FLA (WHO, 2022).

Another important consideration emerging from recent investigations is the lack of standardized methodologies for FLA surveillance. Significant differences remain regarding filtration pore size, sample volume, incubation temperature, duration of cultivation, DNA extraction protocols, and PCR primer selection. These methodological inconsistencies complicate inter-study comparisons and limit the establishment of internationally accepted prevalence estimates. The development of harmonized laboratory protocols would therefore improve data comparability, facilitate global surveillance, and strengthen quantitative microbial risk assessments. Such standardization has become increasingly important as climate change, aging water infrastructure, and expanding urban populations continue to influence the ecology of drinking water microorganisms (Zheng & Shu, 2025).

Economic and Practical Feasibility Constraints of Advanced Water Treatment Technologies in Developing Nations

While advanced water treatment technologies—such as ozonation, ultraviolet (UV) irradiation, and ultrafiltration demonstrate exceptional efficacy in inactivating and eliminating Free-Living Amoebae (FLA) and associated waterborne pathogens, their large-scale implementation in developing countries remains severely limited by economic and operational barriers.

1. Capital and Operational Cost Disparities

The widespread adoption of advanced disinfection systems is largely hindered by prohibitive financial expenditures:

- **High Capital Outlay (CapEx):** Procurement, importation, and specialized infrastructural setup for ozone generators, UV reactors, and membrane filtration systems require substantial capital investments that often exceed the municipal budgets of developing economies.
- **Ongoing Operational and Maintenance Costs (OpEx):** Unlike conventional chlorination, advanced treatments demand continuous energy supplies, frequent replacement of high-grade UV lamps, specialized membrane cleaning, and highly skilled technical personnel. In regions facing energy grid instability and limited financial resources, these continuous expenses render long-term operation unsustainable.

2. Infrastructural and Technical Limitations

Beyond economic constraints, practical deployment is obstructed by structural inadequacies:

- **Grid Reliability:** Advanced disinfection technologies require uninterrupted, stable electrical power. Frequent power outages and voltage fluctuations prevalent in many developing regions disrupt treatment continuity and compromise effluent safety.
- **Maintenance Expertise:** The shortage of locally trained instrumentation and maintenance technicians complicates rapid troubleshooting and repairs, frequently leading to prolonged system downtime.

3. Strategic Policy Implications

Consequently, drinking water treatment facilities in developing nations often rely heavily on conventional, cost-effective disinfection methods (such as standard chlorination). However, because FLA cysts exhibit high resistance to standard chlorine residuals, bridging this gap requires the development of localized, cost-optimized multi-barrier frameworks and international technological partnerships that account for the economic and infrastructural realities of the region.

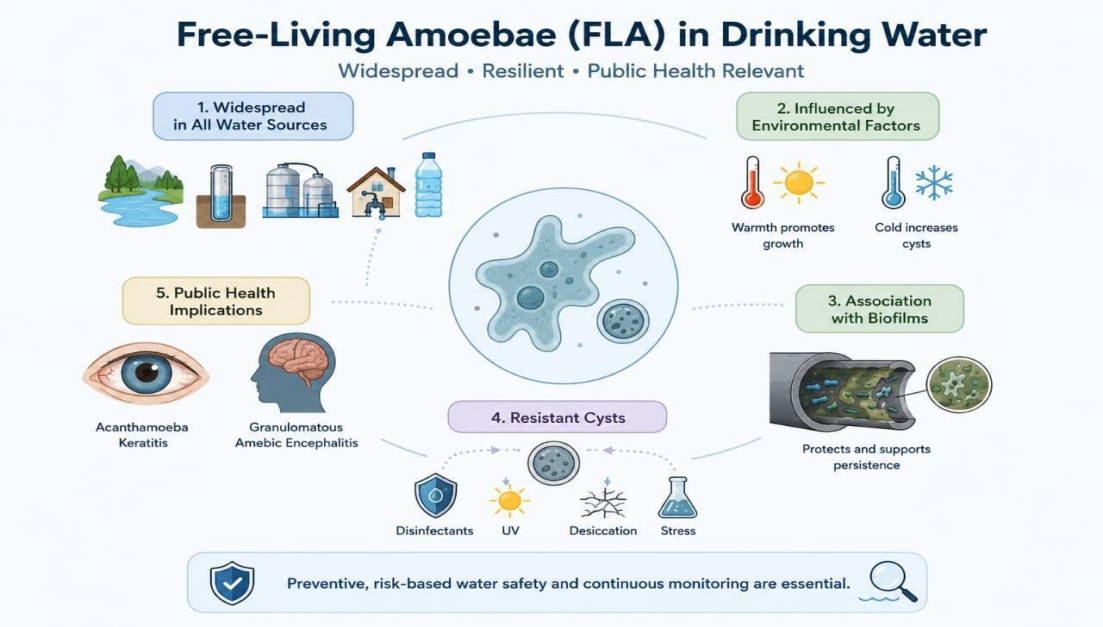


Figure 6. Graphical Summary of the Distribution, Persistence, and Public Health Significance of Free-Living Amoebae (FLA) in Drinking Water.

Overall, the available evidence indicates that no single diagnostic technique is sufficient for comprehensive assessment of free-living amoebae in drinking water systems. Conventional culture remains indispensable for demonstrating organism viability, morphological examination provides rapid preliminary identification, and molecular methods ensure accurate taxonomic classification and epidemiological characterization. Integrating these complementary approaches offers the highest diagnostic accuracy and provides a more reliable foundation for environmental monitoring, public health surveillance, and future research aimed at understanding the ecology and pathogenic potential of free-living amoebae.

8. CONCLUSION

The comprehensive evaluation of free-living amoebae (FLA) in drinking water systems underscores their growing recognition as critical environmental and public health concerns. Due to their exceptional biological resilience, robust encystment mechanisms, and intimate association with pipeline biofilms, these protozoa frequently evade conventional water treatment barriers. Consequently, their persistence in treated water supplies poses a dual threat: they act directly as opportunistic agents of severe, often fatal infections—such as *Acanthamoeba* keratitis, granulomatous amoebic encephalitis, and primary amoebic meningoencephalitis—and indirectly as protective intracellular reservoirs ("Trojan horses") for numerous waterborne pathogens including *Legionella pneumophila*, *Mycobacterium* species, and *Pseudomonas aeruginosa*.

Addressing these microbiological challenges requires shifting away from sole reliance on end-point bacteriological testing toward integrated, risk-based management strategies. Effective control demands a multi-barrier framework combining source water protection, advanced treatment technologies, rigorous biofilm management, and continuous distribution system surveillance. Furthermore, while conventional culture-based isolation and morphological assessment remain essential frontline screening tools, their limitations necessitate integration with advanced molecular diagnostics, such as 18S rRNA gene sequencing and quantitative PCR, to achieve precise species and genotype identification. Ultimately, standardizing global laboratory protocols, fostering interdisciplinary collaboration, and raising public health awareness are vital steps toward safeguarding drinking water safety and minimizing the health risks associated with free-living amoebae worldwide.

9. Recommendations for the Control and Management of Free-Living Amoebae in Drinking Water

1. **Expand Surveillance & Integration:** Integrate routine screening for medically important free-living amoebae (*Acanthamoeba* spp. and *Naegleria spp.*) into drinking water monitoring programs alongside traditional bacterial indicators, and incorporate them directly into Water Safety Plans (WSPs).
2. **Standardize Laboratory & Diagnostic Protocols:** Adopt internationally harmonized protocols for sampling, cultivation, and microscopy, while combining conventional methods with advanced molecular tools (such as PCR and 18S rRNA gene sequencing) to improve species and genotype identification accuracy.
3. **Optimize Treatment & Infrastructure Management:** Upgrade water treatment plants with advanced technologies—including ultrafiltration, UV irradiation, ozonation, chlorine dioxide, and advanced oxidation processes—while strictly managing biofilms through regular pipeline flushing, storage maintenance, and optimized disinfectant residuals.
4. **Public Health & Clinical Awareness:** Increase public education regarding the risks of using untreated tap

water for contact lens care, nasal irrigation, and aerosol-generating devices, while simultaneously enhancing clinical awareness to achieve earlier diagnosis of FLA-associated keratitis and encephalitis.

5. **Advanced Research & Interdisciplinary Collaboration:** Foster close cooperation among scientists, engineers, and public health authorities to investigate environmental prevalence, explore the mechanisms behind the "Trojan horse" phenomenon, and leverage emerging technologies like metagenomics, whole-genome sequencing, eDNA, and artificial intelligence for early detection.

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