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# Growth dynamic of *Naegleria fowleri* in a microbial freshwater biofilm

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ABSTRACT

The presence of pathogenic free-living amoebae (FLA) such as *Naegleria fowleri* in freshwater environments is a potential public health risk. Although its occurrence in various water sources has been well reported, its presence and associated factors in biofilm remain unknown. In this study, the density of *N. fowleri* in biofilms spontaneously growing on glass slides fed by raw freshwater were followed at 32 °C and 42 °C for 45 days. The biofilms were collected with their substrata and characterized for their structure, numbered for their bacterial density, thermophilic free-living amoebae, and pathogenic *N. fowleri*. The cell density of *N. fowleri* within the biofilms was significantly affected both by the temperature and the nutrient level (bacteria/amoeba ratio). At 32 °C, the density remained constantly low (1–10 *N. fowleri*/cm$^2$) indicating that the amoebae were in a survival state, whereas at 42 °C the density reached 30–900 *N. fowleri*/cm$^2$ indicating an active growth phase. The nutrient level, as well, strongly affected the apparent specific growth rate ($\mu$) of *N. fowleri* in the range of 0.03–0.23 h$^{-1}$. At 42 °C a hyperbolic relationship was found between $\mu$ and the bacteria/amoeba ratio. A ratio of $10^6$ to $10^7$ bacteria/amoeba was needed to approach the apparent $\mu_{max}$ value (0.23 h$^{-1}$). Data analysis also showed that a threshold for the nutrient level of close to $10^4$ bacteria/amoeba is needed to detect the growth of *N. fowleri* in freshwater biofilm. This study emphasizes the important role of the temperature and bacteria as prey to promote not only the growth of *N. fowleri*, but also its survival.



## 1. Introduction

Free-living amoebae (FLA) are highly diverse and ubiquitous organisms that have been isolated from various natural environments (rivers, lakes, springs) and man-made water systems (drinking water networks, poorly chlorinated swimming pools) (Jamerson et al., 2009; Sibille et al., 1998; Thomas et al., 2008). *Naegleria fowleri* is a free-living thermotolerant amoeboflagellate of health interest, because it is the causative agent of a primary amoebic meningoencephalitis (PAM) (Marciano-Cabral, 1988), a fatal central nervous system disease. This infection is rare but the acute illness severe (Pond, 2005). To date, less than 300 cases of PAM have been reported worldwide from 1965 to 2008 and attributed to *N.*

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*fowleri* (Caruzo and Cardozo, 2008), 15 new cases were known in India, Pakistan and Guadeloupe (France) (Angrup et al., 2010; Shakoor et al., 2011; De Jonckheere, 2011).

Studies postulate that both natural and man-made factors such as temperature rises disturb the environment and contribute to the spread of *N. fowleri*. Thus, this amoeba was shown to be the most frequently encountered thermotolerant amoebae in the thermally enriched cooling water of a Belgian power plant (Behets et al., 2007). In addition, the survey of a newly created cooling lake in southern California showed that during periods of higher temperatures, the concentration of *N. fowleri* increased by as much as 2 orders of magnitude (Tyndall et al., 1989). In a similar way, the survey of the cooling water in Lake Anna (Virginia, US) showed that of 16 sites sampled during the summer of 2007, nine were found to be positive for *N. fowleri* (Jamerson et al., 2009).

Due to their surface-associated lifestyle, FLA graze effectively on attached preys (Parry, 2004). The soil was assumed to be the ideal habitat for FLA and that the rainfall runoff introduced amoebae into water (Kyle and Noblet, 1985). However, from recent studies, the biofilm from various environments has been considered to be the major reservoir for FLA including pathogenic species (Huws et al., 2005; Parry, 2004; Pickup et al., 2007; Puzon et al., 2009), where they found nutrients (bacterial cells and dissolved organics) (Barbeau and Buhler, 2001) and protection from disinfectants (Thomas et al., 2004). Several authors suggested that high temperatures (30–40 °C) are involved in the environmental occurrence of the pathogen and appear to facilitate its growth (Huizinga and McLaughlin, 1990; Marciano-Cabral, 1988; Sheehan et al., 2003; Wellings et al., 1977). However, to our knowledge, the influence of the temperature on *N. fowleri* dynamic in a complex biofilm (where other species of amoebae coexist with bacteria) has never been documented.

The main goal of the present work was therefore to determine the growth dynamic of *N. fowleri* during the formation of a freshwater biofilm at 32 and 42 °C. We assumed 32 °C as the threshold temperature for the presence of the pathogen in water (Huizinga and McLaughlin, 1990) and 42 °C as the optimal growth temperature (according to Griffin, 1983; from laboratory experiments). After being artificially spiked in an open channel reactor, *N. fowleri* density was followed within the biofilms as well as the structure of the biofilms, the bacterial density and the thermophilic FLA density for several runs at 42 and/or 32 °C conducted during 45 days each.

## 2. Materials and methods

### 2.1. *Naegleria fowleri* strain and growth conditions

The strain of *N. fowleri* AMI005 (EDF collection, LNHE, Chatou, France) had been previously isolated from cooling water of power station (unpublished data). The strain was grown (3–5 days) at 43 °C on non-nutrient agar (NNA, Indicia Biotechnology, Oullins, France) previously overlaid with an *Escherichia coli* suspension and identified by the enzyme-linked immunosorbent assay (Indicia Biotechnology) using monoclonal antibody 5D12 (Pougnard et al., 2002).

### 2.2. *Biofilm reactor*

The biofilm reactor setup consisted of a flat-plate open channel made of polyvinylchloride and had external dimensions of 41 cm wide, 12 cm deep and 136 cm long with a working volume of 2.7 L including the tubing volume (Fig. 1). All tubing was made of silicone platinum and heat-sterilized. A set of two identical reactors was used. Each reactor was previously acid-cleaned (HCl 1 M, 4 h), disinfected (100 mg $Cl_2$/L, 4 h) and finally rinsed by deionized water (5 times the working volume). The reactors were operated in continuous flow mode ensured by peristaltic pumps. The inlet flow and the recycle flow rate were respectively maintained at 1.9 and 810 mL/min. The hydraulic retention time was 24 h. According to Lewandowski and Beyenal (2007), a high recycle ratio (recycle flow/inlet flow), maintained at 427, provides uniform substrate concentration along the reactor. The flow presents a laminar velocity profile in the length direction characterized with a Reynolds number of 241. The shear rate exerted by the flow on the biofilm was 17 $s^{-1}$.

The biofilms grew on the glass slides (8 × 2.5 × 0.1 cm, VWR, France) at the bottom of the reactor. For each run, 78 acid- and chlorine-cleaned glass slides were positioned at the bottom of the reactor at the distance of 30 cm from the inlet (Fig. 1). This distance was calculated to give uniform hydrodynamic conditions (Lewandowski and Beyenal, 2007) in order to produce a homogeneous development of the biofilms on all of the glass slides. Analyzing biofilms randomly collected at three locations of the reactor served as an experimental control for this point. No significant difference has been obtained (data not shown).

### 2.3. *Experimental set-up*

Ten runs were accomplished for a period of 45 days each. Four runs were conducted with two reactors at the same time at two temperatures and using the same inlet water. That is to say, within the two serial runs named R1 and R2, there were two reactors (R1-32 and R1-42 or R2-32 and R2-42) fed with the same water each but carried out at 32 °C or 42 °C, respectively. Six other runs named R3-42 °C to R8-42 °C were independently (a new freshwater inlet for each run) carried out at 42 °C.

For each run, 11 samples from three coupons were randomly and regularly collected at 2–7 day intervals over a 45-day period for analysis. The coupons were gently washed with 10 mL of bacteria-free phosphate buffer saline solution pH 7.4 (PBS, previously 0.2 μm filtered and heat sterilized), in order to remove cells and deposits not strongly attached to the substrata (i.e. not considered a part of the biofilm).

#### 2.3.1. *Characteristics of freshwater at the inlet and outlet*

For each run, the reactors were fed with inlet freshwater (Loire River, Dampierre-en-Burly, France) stored in an agitated and refrigerated (4 °C) tank for the duration of a run (~6 weeks). The freshwater was collected between November 2009 and February 2011. Microbial and physico-chemical characteristics of the inlet water are presented in Table 1. Except for thermophilic FLA, no drastic variation in water quality was noted between the inlet and outlet. The high values of the