REVIEW ARTICLE

Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality

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Received 5 May 2009; revised 4 August 2009; accepted 5 August 2009.
Final version published online 10 September 2009.

DOI:10.1111/j.1574-6976.2009.00190.x

Editor: Colin Berry

Keywords
amoebae; *Acanthamoeba*; intracellular; survival; biocides; cysts.

Abstract

An increasing number of microorganisms, including bacteria but also viruses and eukaryotes, have been described as benefiting from interaction with free-living amoebae (FLA). Beneficial interaction can be due to resistance to predation conferring ecological advantage, intracellular survival and/or intracellular proliferation. This review highlights the potential risk associated with amoebae by listing all known pathogenic microbial species for which growth and/or survival promotion by FLA (mainly *Acanthamoeba* spp.) has been demonstrated. It focuses on the susceptibility of amoebal and intra-amoebal bacteria to various categories of biocides, the known mechanisms of action of these biocides against trophozoites and cysts and the various methods used to demonstrate efficacy of treatments against FLA. Brief descriptions of FLA ecology and prevalence in domestic/institutional water systems and their intrinsic pathogenicity are also presented. The intention is to provide an informed opinion on the environmental risks associated with the presence of FLA and on the survival of cysts following biocidal treatments, while also highlighting the need to conduct research on the roles of amoebae in aquatic ecosystems.

Introduction

Free-living amoebae (FLA) are widespread in nature and are normal inhabitants of freshwater microbial ecosystems (Rodriguez-Zaragoza, 1994; Khan, 2006). They are thought to have a major impact on the dynamics of multimicrobial biofilms by feeding on various microorganisms and contributing to nutrient recycling (Pedersen, 1982). Several FLA species are potentially pathogenic to humans and animals but infections are not commonly reported. FLA *per se* are not considered to constitute a major threat to public health, although some have been involved in specific diseases. However, it has been recently recognized that FLA can interact with a variety of microorganisms (Greub & Raoult, 2004; Khan, 2006), in a way that benefits those microorganisms (particularly studied with bacteria but also observed with fungi and viruses). Bacteria can benefit from interactions with FLA due to (1) their ability to escape predation and grow in the presence of a protozoan that would normally phagocytose and digest nonresistant bacterial species; (2) their ability to resist intracellular digestion (intracellular survival, with the possible subsequent survival within a protozoan cyst); and (3) their ability to resist digestion but also to grow within the protozoan vegetative form (trophozoite; intracellular multiplication). Furthermore, several studies have demonstrated that the virulence of known pathogenic bacteria toward their protozoan hosts can reflect virulence toward humans and/or animals (Fields *et al.*, 1986; Fenner *et al.*, 2006; Goy *et al.*, 2007; Steinberg & Levin, 2007). It has been postulated that the newly discovered amoebae-resisting bacteria (ARB) are likely to be pathogenic for humans and/or animals (Greub & Raoult, 2004; Molmeret *et al.*, 2005). Consequently, FLA have been used as a tool to isolate new potentially pathogenic ARB species from various sources (Collingro *et al.*, 2005; Thomas *et al.*, 2006b).

From a public health perspective, amoebae, and notably amoebal cysts, can be highly resistant to various physical and chemical stresses and can thus protect any intracellular microorganism from deleterious environmental conditions that would normally kill them (King *et al.*, 1988; Kilvington & Price, 1990; Barker *et al.*, 1992). This protective effect is of

increasing concern because it is speculated that partially efficient biocide treatments might select for FLA together with their intracellular microorganisms. They may even provide favorable conditions directly [the biocide treatment itself stimulating FLA growth (Srikanth & Berk, 1993)] or indirectly [biocidal treatment killing extracellular bacteria that are then used as a food source by *Legionella pneumophila* as demonstrated by Temmerman *et al.* (2006)].

A good understanding of ways to control FLA in water or other liquids is therefore most important. There is general paucity of information on the efficacy and mechanisms of action of biocides against amoebae. Most of the information available concerns other water-transmitted protozoal species such as *Cryptosporidium* and *Giardia* spp. that have been involved in gastrointestinal disease outbreaks. For FLA, mainly *Acanthamoeba* spp. have been evaluated against biocides used in contact lens solutions because of their association with keratitis and contamination of these solutions. Several studies were also published concerning the resistance of *Acanthamoeba* spp., *Hartmannella* spp. and *Naegleria* spp. to drinking water treatments but there is a general lack of information and clear discrepancies between studies on the efficacy of biocides against amoebal trophozoites and particularly their resistant forms (cysts). The reported variability of results between studies can be attributed to the lack of an international consensus in standard efficacy protocols to grow amoebal cysts and measure the cysticidal activity of biocides (Mercer, 2008).

The prevalence of amoebae in water networks and their association with biofilms

FLA and other protozoa are normal inhabitants of freshwater sources and soils (Rodriguez-Zaragoza, 1994). In these environments, the exact FLA population composition is dependent on the actual physicochemical parameters present, such as annual temperature fluctuation and pH changes (Kyle & Noblet, 1986). FLA can also colonize domestic and institutional water systems. It has been demonstrated that although clarification steps used in drinking water production plants dramatically reduce their numbers, some cells can spread from the water source to the distribution network despite disinfection of water with ozone and chlorine (Hoffmann & Michel, 2001; Corsaro *et al.*, 2008; Thomas *et al.*, 2008). Once in the distribution network, low disinfectant levels have only limited activity on FLA (Thomas *et al.*, 2004). They can thus colonize virtually any kind of water system and have been isolated from a number of diverse environments, some containing harsh physical and/or chemical conditions such as elevated temperature (hot tubes and cooling towers) or a high concentration of a biocide or combination of biocides such as chlorine or chlorine-releasing agents (CRAs), biguanides and other agents. By way of example, they have been recovered from domestic tap water (Jeong & Yu, 2005), hospital water networks (Rohr *et al.*, 1998; Thomas *et al.*, 2006b), swimming pools (Vesaluoma *et al.*, 1995), hydrotherapy baths (Scaglia *et al.*, 1983), dental unit waterlines (Singh & Coogan, 2005), eyewash stations (Paszko-Kolva *et al.*, 1991) and cooling towers (Barbaree *et al.*, 1986; Berk *et al.*, 2006). In large studies including many sampling points, amoebae were found in 20–30% of domestic tap water samples (Shoff *et al.*, 2008). An even higher prevalence has been reported in hospitals, with as much as 68.9% of samples collected from hot water faucets, showers, hot water tanks and cooling towers being colonized (Lasheras *et al.*, 2006). In a study of FLA colonization of domestic water networks in homes of patients suffering keratitis, Kilvington *et al.* (2004) found 89% of homes colonized, with FLA recovered from 76% of bathroom sink cold taps sampled. They concluded that water storage tanks promote colonization of domestic water with FLA (Kilvington *et al.*, 2004). Amoebae cultivated from domestic water systems mostly belong to the genera *Acanthamoeba*, *Hartmannella* and *Naegleria*, but species belonging to the *Echinamoeba*, *Vannella*, *Vahlkampfia* and *Saccamoeba* genera have also been isolated (Rohr *et al.*, 1998; Barbeau & Buhler, 2001; Hoffmann & Michel, 2001; Thomas *et al.*, 2006b, 2008) (Fig. 1). Most species of these genera can form cysts that present various degrees of resistance to harsh environmental conditions.

It has been demonstrated that suspended bacteria do not provide particularly favorable conditions for FLA growth (Pickup *et al.*, 2007a) and that FLA proliferation in water systems is mainly due to grazing on bacterial biofilms (Barbeau & Buhler, 2001) with which FLA are integrally associated (Huws *et al.*, 2005). Protozoal grazing on bacterial biofilms happens to be a key factor regulating biofilm composition and dynamics (Pedersen, 1982; Kalmbach, 1998). Not all bacteria seem to be equally suitable food sources for amoebae; this will depend on the specific amoebae and bacterial strains, but antipredatory mechanisms may arise, including microcolony formation, toxin production and the presence of an intact capsule that can prevent feeding by FLA on bacteria (Weekers *et al.*, 1993; Pickup *et al.*, 2007b). Some of these mechanisms are thought to be predation-driven and regulated by *N*-acylhomoserine lactone (AHL)-dependent quorum-sensing systems, as demonstrated with the violacein toxin production by *Chromobacterium violaceum* (Matz *et al.*, 2004). Interestingly, AHLs might also directly interact with eukaryotic cells (Joint *et al.*, 2002). Other mechanisms that are not under direct dependency of quorum sensing have been described, such as those demonstrated for *Pseudomonas aeruginosa* type III secretion system that is used to kill biofilm-

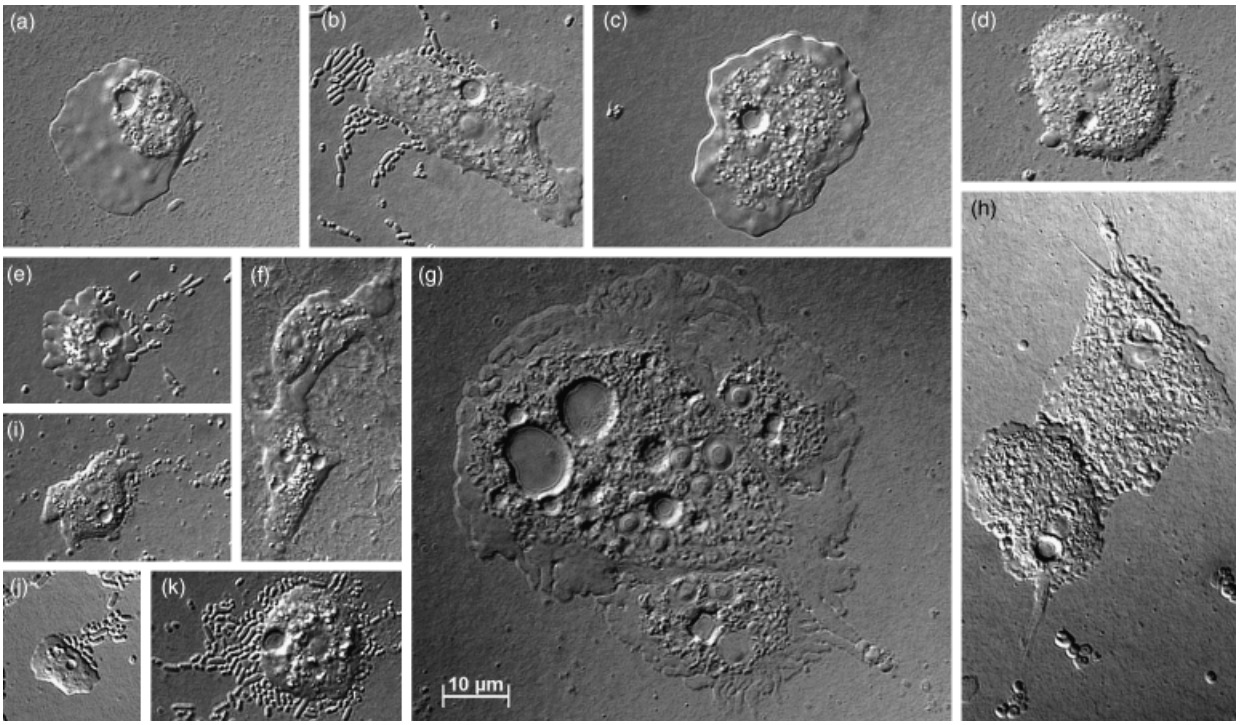

Fig. 1. Example of various FLA isolates recovered from a drinking water treatment plant (reproduced from Thomas *et al*., 2008). (a) Isolate closely related to *Playtamoeba* genus; (b) isolate closely related to *Acanthamoeba* genus; (c) isolate closely related to *Vanella* genus; (d) isolate closely related to *Acanthamoeba* genus; (e) isolate closely related to *Glaeseria* genus; (f) isolate closely related to *Hartmannella* genus; (g) isolate closely related to *Naegleria* genus; (h) isolate closely related to *Naegleria* genus; (i) isolate closely related to *Hartmannella* genus; (j) isolate closely related to *Echinamoeba* genus; (k) isolate closely related to *Acanthamoeba* genus. Scale bar for all pictures = 10 µm.

associated amoebae (Matz *et al*., 2008). Of note, whereas it is well known that microorganisms embedded within a biofilm are less susceptible to the effects of antimicrobial treatments including disinfection and antibiotic chemotherapy, the fate of trophozoites and cysts within microbial biofilms following biocidal treatment is under-reported and requires further investigation.

The intrinsic pathogenicity of FLA and the speculative role of intracellular bacteria

FLA have been associated with a number of diseases in humans (for reviews, see Visvesvara *et al*., 2007 and Marciano-Cabral & Cabral, 2003). Encephalitis and meningoencephalitis are life-threatening infections affecting immunocompetent children and immunocompromised adults. They mainly involve *Acanthamoeba* spp. and *Balamuthia mandrillaris* for encephalitis and *Naegleria fowleri* for meningoencephalitis. *Sappinia diploidea* has also been isolated from one human case of encephalitis (Visvesvara *et al*., 2007). Treatment is problematic due to difficulty in diagnosis, speed of the infection in the case of meningoencephalitis, resistance to some therapeutic compounds and the dormancy of cysts that can lead to subsequent patient relapse (Schuster & Visvesvara, 2004). Other types of infections have been reported: local or disseminated infections in immunocompromised adults but also in immunocompetent children; these infections mainly involve *Acanthamoeba* spp. (Marciano-Cabral & Cabral, 2003). *Acanthamoeba* spp. are also the main cause of corneal infection leading to keratitis associated with contact lenses (Ahearn & Gabriel, 1997; Illingworth & Cook, 1998), although several cases involving *Vahlkampfia* sp., *Vannella* sp. and *Hartmannella* sp. have also been described (Aitken *et al*., 1996; Michel *et al*., 2000b). Interestingly, some of these keratitis-associated FLA were found to be infected by various bacterial species (Fritsche *et al*., 1993; Michel *et al*., 2000b; Xuan *et al*., 2007; Yu *et al*., 2007; Scheid *et al*., 2008), thus raising concerns about the possible role of these intracellular bacteria in amoebal resistance to biocides and/or pathogenicity (Murdoch *et al*., 1998; Cengiz *et al*., 2000). Marciano-Cabral *et al*. (2003) also recovered an infected acanthamoebal isolate from an immunosuppressed patient who was diagnosed postmortem with disseminated acanthamoebiasis. They observed gram-negative rods in the trophozoites but did not indicate to which bacterial species these rods belonged. They speculated that amoebal pathogenicity may be a result of both tissue damage from amoeba-secreted products

and the induction of high levels of proinflammatory cytokines (tumor necrosis factor-α) caused by intracellular bacteria. It should also be mentioned that *Mycobacterium avium* and *L. pneumophila*-infected FLA have been proved to be infectious particles in murine models of infection, and in these cases infected FLA were more pathogenic than an equivalent number of bacteria or a coinoculum of bacteria and uninfected amoebae (Brieland *et al.*, 1997; Cirillo *et al.*, 1997).

FLA interactions with pathogenic microbial species

Symbiotic and pathogenic interactions with bacteria

The association of various bacterial species with amoebae is thought to be common. Microscopic observation of cytoplasmic endosymbionts in *Acanthamoeba castellanii* were reported in 1967 (Jeon & Lorch, 1967) and 1975 (Proca-Ciobanu *et al.*, 1975). Over a 40-year period, Jeon and colleagues published several studies describing an endosymbiotic bacteria (X-bacteria) that infected the amoebae initially as intracellular parasites, transferred from one amoebal isolate to another and thus established a new endosymbiotic relationship with the new host, which ultimately became necessary for amoebal survival (Jeon & Jeon, 1976). They later demonstrated that X-bacteria could switch expression of host genes coding for *S*-adenosylmethionine synthetase (sams) from one sams-encoding gene to another (Jeon & Jeon, 2004). Interestingly, X-bacteria were further demonstrated to belong to the *Legionella* order, being proposed as '*Legionella jeonii*' (Park *et al.*, 2004). A major interest in the interactions between FLA and pathogenic bacteria was triggered by the finding that human pathogenic species such as *L. pneumophila* proliferate in various amoebal hosts (Rowbotham, 1980). Fritsche *et al.* (1993) later reported that 25% of clinical and environmental *Acanthamoeba* spp. isolates harbored a variety of endosymbiotic bacteria. Another study reported that amoebae were more frequently infected in cooling towers (55%) than in natural environments (7.5%), suggesting that artificial conditions might favor association of bacteria with amoebae (Berk *et al.*, 2006). In several cases, a single amoebal host was even reported to be infected with two different bacterial species (Michel *et al.*, 2006; Heinz *et al.*, 2007).

Bacterial species pathogenic to humans are also likely to resist digestion by FLA

It has been proposed that through their capacity to resist digestion by FLA, potential intracellular bacterial species (many yet to be discovered) are also likely to resist digestion by macrophages and thus may represent new human pathogenic species (Greub & Raoult, 2004). In our review, we studied demonstrated pathogenic species and particularly screened the Environmental Protection Agency (EPA) list of microorganisms that are known to cause disease in humans ('CCL 3 Universe' list, available at http://www.epa.gov/safewater/ccl/pdfs/report_ccl3_microbes_universe.pdf). This was compared with the current list of bacterial species that have been demonstrated elsewhere in the literature to 'benefit' interaction with protozoa. The CCL 3 Universe list is based on the comprehensive review by Taylor *et al.* (2001) that identified 538 bacterial species pathogenic to humans and/or animals. The EPA list has since been extended by two species but both from the genus '*Legionella*,' recognizing that all species that belong to this genus are potentially pathogenic. Overall, there are now 539 bacterial species in the February 2008 EPA list. Of these 539 species, a comprehensive review of the literature allowed us to identify 102 (18.9%) that have been described as surviving and/or flourishing when in contact with various amoebal species (Table 1). Among these 102 species, 40 (39.2%) were isolated by amoebal coculture without complete demonstration of intracellular survival, 30 (29.4%) have been demonstrated to survive in one or several amoebal species and 32 (31.4%) have been shown to survive and grow in one or several amoebal species. These are likely to be underestimates of the true level of FLA–bacterial associations, when the following limitations are considered. First, most of the published studies used *Acanthamoeba polyphaga* strain Linc-Ap1 (available at the Culture Collection of Algae and Protozoa as CCAP 1501/18) or *A. castellanii* ATCC 30234 as the cell background, thus considerably limiting the possible range of bacteria–amoeba interactions that are often host specific. For example, it has been demonstrated that a *L. pneumophila* strain virulent to humans can grow within *Acanthamoeba lenticulata* strain PD2 whereas another virulent strain cannot (Molmeret *et al.*, 2001). This might be the reason why, to our knowledge, there is no published report demonstrating intraprotozoal survival for several *Legionella* species: *Legionella birmighamensis*, *Legionella cherrii*, *Legionella cincinnatiensis*, *Legionella jordanis*, *Legionella lansigensis*, *Legionella sainthelensi*, *Legionella wadsworthii* and *Tatlockia maceachernii* (now *Legionella maceachernii* but still listed under its previous name in the CCL3 list). It is probable that these listed pathogenic species can also grow in specific amoebal hosts that have not been identified so far. Second, various bacterial strains reported in the CCL3 list have obviously never been tested for their interactions with protozoa. For example, the 12 *Rickettsia* strains listed should be considered as being strongly suspect of being resistant to protozoa because one species belonging to this genus, *Rickettsia bellii* (not in the CCL3 list), has been demonstrated to be able to survive for at least 6 weeks in *A. polyphaga* Linc-Ap1 (Ogata *et al.*, 2006). Furthermore, *Ehrlichia*-like organisms have also been reported in *Saccamoeba* species (Michel *et al.*, 1995b). In this case, the lack of published evidence for

Table 1. Described interactions of pathogenic bacterial species with FLA

Bacterial species	Described interaction with protozoa	Threat list					References
		Emerging infectious diseases	CDC notifiable	NIAID bioterror agents	Food and water pathogens	HHS select agents	
Achromobacter xylosoxidans	Coculture and cell lysis (Ap Linc AP-1)						Greub *et al*. (2004); Pagnier *et al*. (2008)
Acinetobacter baumannii	Coculture without cell lysis (Ap Linc AP-1, Ac ATCC 30010)	x					Pagnier *et al*. (2008); Thomas *et al*. (2008)
Acinetobacter calcoaceticus	Coculture without cell lysis (Ap Linc AP-1)	x					Pagnier *et al*. (2008)
Acinetobacter haemolyticus	Coculture (Ac ATCC 30010)						Thomas *et al*. (2008)
Acinetobacter johnsonii	Co-culture (Ac ATCC 30010)						Thomas *et al*. (2008)
Acinetobacter junii	Coculture without cell lysis (Ap Linc AP-1, Ac ATCC 30010)						Pagnier *et al*. (2008); Thomas *et al*. (2008)
Acinetobacter lwoffii	Coculture (Ac ATCC 30010)						Thomas *et al*. (2008)
Acinetobacter radioresistens	Coculture (Ac ATCC 30010)	x					Thomas *et al*. (2008)
Aeromonas caviae	IC survival (Ac ATCC 30234)						Rahman *et al*. (2008)
Aeromonas hydrophila	IC survival (Ac ATCC 30234)						Rahman *et al*. (2008)
Aeromonas veronii	IC survival (Ac ATCC 30234)						Rahman *et al*. (2008)
Bacillus cereus	IC multiplication (Ap Linc Ap-1)	x			x		Pagnier *et al*. (2008); Evstigneeva *et al*. (2009)
Bacillus licheniformis	Coculture (*Naegleria fowleri* HB-1, Ac ATCC 30010)						Lebbadi *et al*. (1995); Thomas *et al*. (2008)
Bacillus pumilus	Coculture (Ac ATCC 30010)						Thomas *et al*. (2008)
Bacillus subtilis	Coculture without cell lysis (Ap Linc Ap-1)						Pagnier *et al*. (2008)
Brevundimonas diminuta	IC multiplication (Ap Linc Ap-1)						Evstigneeva *et al*. (2009)
Brevundimonas vesicularis	Coculture without cell lysis (Ap Linc Ap-1)						Pagnier *et al*. (2008)
Burkholderia cepacia	IC multiplication (Ap Linc Ap-1), IC survival (Ap ATCC 50372)	x					Landers *et al*. (2000); Lamothe *et al*. (2004)
Burkholderia pseudomallei	IC survival (*Acanthamoeba astronyxis* CCAP 1534/1)	x		x		x	Inglis *et al*. (2000)
Campylobacter coli	IC multiplication (Ap Linc Ap-1)						Axelsson-Olsson *et al*. (2007)
Campylobacter hyointestinalis	IC multiplication (Ap Linc Ap-1)						Axelsson-Olsson *et al*. (2007)
Campylobacter jejuni	IC multiplication (Ap Linc Ap-1)	x		x	x		Axelsson-Olsson *et al*. (2007)
Campylobacter lari	IC multiplication (Ap Linc Ap-1)						Axelsson-Olsson *et al*. (2007)
Chlamydophila pneumoniae	IC survival (Ac ATCC 30234)	x					Essig *et al*. (1997)
Chromobacterium violaceum	Coculture with complete cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Chryseobacterium meningosepticum	Coculture with partial cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Citrobacter freundii	IC survival (Ac ATCC 30234), coculture (Ac ATCC 30010)						King *et al*. (1988); Thomas *et al*. (2008)
Coxiella burnetii	IC survival (Ac ATCC 30234)		x	x		x	La Scola & Raoult (2001)
Delftia acidovorans	Coculture with partial cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Edwardsiella tarda	IC survival (Tp)						King *et al*. (1988)
Enterobacter aerogenes	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Enterobacter amnigenus	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)

Table 1. Continued.

Bacterial species	Described interaction with protozoa	Threat list					References
		Emerging infectious diseases	CDC notifiable	NIAID bioterror agents	Food and water pathogens	HHS select agents	
Enterobacter cancerogenus	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Enterobacter cloacae	IC multiplication (Ap Linc Ap-1), IC survival (Ac ATCC 30234), coculture (Ac ATCC 30010)						King *et al*. (1988); Thomas *et al*. (2008); Evstigneeva *et al*. (2009)
Escherichia coli (includ. 0157H7)	IC multiplication (Ac, Ap), IC survival (Tp)	x	x	x	x		Barker *et al*. (1999); Alsam *et al*. (2006); Steinberg & Levin (2007)
Francisella tularensis	IC multiplication and IK survival (Ac ATCC 30234)	x	x	x		x	Abd *et al*. (2003)
Hafnia alvei	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Helicobacter pylori	IC survival (Ac)	x					Winiecka-Krusnell *et al*. (2002)
Klebsiella oxytoca	IC survival (Ac ATCC 30234), coculture without cell lysis (Ap Linc-Ap1)						King *et al*. (1988); Pagnier *et al*. (2008)
Klebsiella pneumoniae	IC survival (Ac ATCC 30234), coculture without cell lysis (Ap Linc-Ap1)	x					King *et al*. (1988); Pagnier *et al*. (2008)
Kluyvera cryocrescens	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Legionella anisa	IC multiplication (Tp, Ac ATCC 30010, Ap Linc-Ap1)						Fields *et al*. (1990); La Scola *et al*. (2001); Thomas *et al*. (2006a, b)
Legionella feeleii	IC multiplication (Tp), coculture (*Acanthamoeba culbertsoni*)						Fields *et al*. (1986); Kuroki *et al*. (2007a)
Legionella hackeliae	IC multiplication (Tp)						Fields *et al*. (1986)
Legionella longbeachae	IC multiplication (*Acanthamoeba* sp., Tp)						Steele & McLennan (1996); Doyle *et al*. (1998)
Legionella oakridgensis	IC multiplication (Tp)						Fields *et al*. (1986)
Legionella pneumophila	IC multiplication and IK survival (> 20 FLA species*)	x	x				For a review, see Kuiper (2006)
Legionella rubrilucens	Coculture (Ap)						La Scola *et al*. (2003b)
Listeria ivanovii	IC survival (Ac ATCC 30234)						Zhou *et al*. (2007)
Listeria monocytogenes	IC multiplication (Tp), IC survival (Ac ATCC 30234)		x	x	x		Ly & Muller (1989); Ly & Muller (1990); Zhou *et al*. (2007)
Listeria seeligeri	IC survival (Ac ATCC 30234)						Zhou *et al*. (2007)
Listeria welshimeri	IC survival (Ac ATCC 30234)						Zhou *et al*. (2007)
*Methylobacterium mesophilicum**	Coculture and cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Morganella morganii	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al*. (2008)
Mycobacterium abscessus	IC and IK survival (Ap Linc-Ap1)	x					Adekambi *et al*. (2006)
Mycobacterium avium	IC multiplication (Ac ATCC 30234 and CCAP 1501/1B, Ap Linc-Ap1 and CCAP 1501/3B, Dd AX2, Tp ATCC 30202), IK survival (Ap ATCC 30872 and Linc-Ap1)	x					Cirillo *et al*. (1997); Steinert *et al*. (1998); Adekambi *et al*. (2006) Strahl *et al*. (2001); Skriwan *et al*. (2002); Mura *et al*. (2006); Whan *et al*. (2006)
Mycobacterium bovis	IC and IK survival (Ac CCAP 1501/1A)	x					Taylor *et al*. (2003)
Mycobacterium chelonae	IC and IK survival (Ap Linc-Ap1)						Adekambi *et al*. (2006)

Mycobacterium fortuitum	IC multiplication (Ac ATCC 30234), IC and IK survival (Ap Linc-Ap1)	x				Cirillo *et al*. (1997); Adekambi *et al*. (2006)
Mycobacterium gordonae	IC and IK survival (Ap Linc-Ap1), coculture (Ac ATCC 30010)					Adekambi *et al*. (2006); Thomas *et al*. (2006a, b); Thomas *et al*. (2008)
Mycobacterium kansasii	IC multiplication (Ac ATCC 30010), IC and IK survival (Ap Linc-Ap1)	x				Adekambi *et al*. (2006); Goy *et al*. (2007)
Mycobacterium leprae	IC survival (*Acanthamoeba culbertsoni* ATCC 30171)		x			Jadin (1975); Lahiri & Krahenbuhl (2008)
Mycobacterium malmoense	IC and IK survival (Ap Linc-Ap1)					Adekambi *et al*. (2006)
Mycobacterium marinum	IC multiplication (Ac ATCC 30234, Dd AX2), IC and IK survival (Ap Linc-Ap1)	x				Cirillo *et al*. (1997); Solomon *et al*. (2003); Adekambi *et al*. (2006)
Mycobacterium mucogenicum	IC and IK survival (Ap Linc-Ap1)					Adekambi *et al*. (2006)
Mycobacterium peregrinum	IC and IK survival (Ap Linc-Ap1)					Adekambi *et al*. (2006)
Mycobacterium porcinum	IC and IK survival (Ap Linc-Ap1)					Adekambi *et al*. (2006)
Mycobacterium scrofulaceum	IC multiplication and IK survival (Tp ATCC 30202)					Strahl *et al*. (2001)
Mycobacterium simiae	IC and IK survival (Ap Linc-Ap1), IC survival (Ac)					Krishna Prasad & Gupta (1978); Adekambi *et al*. (2006)
Mycobacterium smegmatis	IC and IK survival (Ap Linc-Ap1), IC survival (Ac)					Krishna Prasad & Gupta (1978); Adekambi *et al*. (2006)
Mycobacterium szulgai	IC and IK survival (Ap Linc-Ap1)					Adekambi *et al*. (2006)
Mycobacterium ulcerans	IC survival (Ac, Ap)	x				Krishna Prasad & Gupta (1978); Eddyani *et al*. (2008)
Mycobacterium xenopi	IC multiplication and IK survival (Ap Linc-Ap1), coculture (Ac ATCC 30010)	x				Drancourt *et al*. (2006); Thomas *et al*. (2006a, b)
Ochrobactrum anthropi	Coculture without cell lysis (Ap Linc-Ap1)					Pagnier *et al*. (2008)
Pantoea agglomerans	Coculture without cell lysis (Ap Linc-Ap1)					Pagnier *et al*. (2008)
Pasteurella multocida	IC multiplication (Ap ATCC 50372)					Hundt & Ruffolo (2005)
Porphyromonas gingivalis	IC multiplication (Ac ATCC 30234)					Wagner *et al*. (2006)
Prevotella intermedia	IC multiplication (Ac ATCC 30234)					Wagner *et al*. (2006)
Providencia alcalifaciens	Coculture with partial cell lysis (Ap Linc-Ap1)					Pagnier *et al*. (2008)
Providencia rettgeri	Coculture without cell lysis (Ap Linc-Ap1)					Pagnier *et al*. (2008)
Pseudomonas aeruginosa	IC multiplication (Ap ATCC 30461, *Echinamoeba* sp.)	x				Michel *et al*. (1995a); Hwang *et al*. (2006)
Pseudomonas alcaligenes	IC multiplication (Ap Linc-Ap1)					Evstigneeva *et al*. (2009)
Pseudomonas fluorescens	Coculture with cell lysis (Ap Linc-Ap1, Ac ATCC 30010)					Pagnier *et al*. (2008); Thomas *et al*. (2008)
Pseudomonas putida	Coculture with partial cell lysis (Ap Linc-Ap1, Ac ATCC 30010)					Pagnier *et al*. (2008); Thomas *et al*. (2008)
Rahnella aquatilis	Coculture and cell lysis (Ap Linc-Ap1)					Pagnier *et al*. (2008)
Ralstonia pickettii	IC multiplication (Ac, *Naegleria lovaniensis* ATCC 30808)	x				Michel & Hauroder (1997)
Rhodococcus equi	Coculture (Ac ATCC 30010)	x				Thomas *et al*. (2008)
Rhodococcus erythropolis	Coculture (Ac ATCC 30010)					Thomas *et al*. (2008)
Rothia dentocariosa	IC multiplication (Ap Linc Ap-1)					Evstigneeva *et al*. (2009)
Salmonella typhimurium	IC multiplication (Ap Linc-Ap1)	x	x	x	x	Gaze *et al*. (2003)
Serratia ficaria	Coculture without cell lysis (Ap Linc-Ap1)					Pagnier *et al*. (2008)

Table 1. Continued.

Bacterial species	Described interaction with protozoa	Threat list					References
		Emerging infectious diseases	CDC notifiable	NIAID bioterror agents	Food and water pathogens	HHS select agents	
Serratia marcescens	Coculture with complete cell lysis (Ap Linc-Ap1)						Pagnier *et al.* (2008)
Serratia plymuthica	IC multiplication (Ap Linc Ap-1)						Evstigneeva *et al.* (2009)
Serratia proteamaculans	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al.* (2008)
Shigella boydii	Coculture without cell lysis (Ap Linc-Ap1)		x	x	x		Pagnier *et al.* (2008)
Shigella dysenteriae	IC multiplication (Ac ATCC 30234)		x	x	x		Saeed *et al.* (2009)
Shigella sonnei	IC multiplication (Ac ATCC 30234 and 30010)		x	x	x		Jeong *et al.* (2007a); Saeed *et al.* (2009)
Staphylococcus aureus (MRSA)	IC multiplication (Ap), IK survival (?)		x		x		Marciano-Cabral (2004); Huws *et al.* (2006)
Stenotrophomonas maltophilia	Coculture with complete cell lysis (Ap Linc-Ap1)						Pagnier *et al.* (2008)
Streptococcus pneumoniae	IC multiplication (*A. polyphaga* Linc-Ap1)		x				Evstigneeva *et al.* (2009)
Tatlockia micdadei[†]	IC multiplication and IK survival (Ap Linc Ap-l, Ac, Hv, Tp, *A. culbertsoni*, *H. cantabrigiensis*)	x					Fields *et al*. (1986); Fallon & Rowbotham (1990)
Vibrio cholerae	IC multiplication and IK survival (Ac ATCC 30234, *Naegleria gruberi* 1518/1e)		x	x			Thom *et al.* (1992); Abd *et al*. (2005)
Vibrio parahaemolyticus	Coculture (Ac ATCC 30234)	x		x	x		Laskowski-Arce & Orth (2008)
Yersinia enterocolitica	IC survival (Ac ATCC 30234)			x	x		King *et al.* (1988)
Yersinia pestis	IC survival (*Hartmannella rhysodes*)		x	x			Nikul'shin *et al.* (1992)
Yersinia pseudotuberculosis	Coculture without cell lysis (Ap Linc-Ap1)						Pagnier *et al.* (2008)

Bacterial species classified as high priority infectious microorganisms in several official lists are indicated (Ecke *et al*., 2005). Interactions with the soil amoeba *Dictyostelium discoideum* and the ciliate *Tetrahymena pyriformis* are also reported but were not taken into account for bacteria–FLA interactions calculation.

Ac, *Acanthamoeba castellanii*; Ap, *Acanthamoeba polyphaga*; Dd, *Dictyostelium discoideum*; Hv, *Hartmannella vermiformis*; Tp, *Tetrahymena pyriformis*; IC, intracellular; IK, intracyst.

*Amoebal species that were demonstrated to support growth of *Legionella pneumophila*: *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*, *Acanthamoeba griffini*, *Acanthamoeba lenticulata*, *Acanthamoeba palestinensis*, *Acanthamoeba royreba*, *Acanthamoeba polyphaga*, *Balamuthia mandrillaris*, *Dictyostelium discoideum*, *Echinamoeba exundans*, *Hartmannella cantabrigiensis*, *Hartmannella vermiformis*, *Naegleria australiensis*, *Naegleria fowleri*, *Naegleria jadini*, *Naegleria lovaniensis*, *Naegleria gruberi*, *Platyamoeba placida*, *Saccamoeba* spp., *Vahlkampfia jugosa*, *Vexillifera* spp. and *Willaertia* spp.

[†]Now '*Legionella micdadei*'. x, Bacterial species listed in threat list (Ecke *et al*., 2005). ?, not clearly demonstrated.

other rickettsial species survival within protozoa might be due to the difficulty in cultivating these obligate intracellular microorganisms (the majority classified as biosafety level 3). Other BSL3 species such as *Mycobacterium leprae* and *Yersinia pestis* were initially cited in the literature as being resistant to amoebae (Jadin, 1975; Nikul'shin *et al.*, 1992) but these studies are very limited and lack strong scientific evidence. A more recent study tends to demonstrate that the leprae bacillus can survive for at least 72 h in *A. castellanii* ATCC 30010 (Lahiri & Krahenbuhl, 2008). Furthermore, symbiotic interactions between *Acanthamoeba lugdunensis* and a mycobacterial species related to *M. avium* and *Mycobacterium intracellulare* has been described recently; mycobacteria were propagated in protozoa for >6 years without any obvious detrimental effect for both microorganisms (Yu *et al.*, 2007), demonstrating that amoebae can readily act as a stable environmental reservoir for mycobacteria.

Other reasons why various bacterial species listed in the CCL3 list have not been tested for their interaction with amoebae includes a limited number of laboratories investigating these interactions and the theoretical assumption that many of these interactions would not make sense. It is almost counterintuitive that obligate anaerobic bacteria such as *Clostridium* spp. (16 species in the CCL3 list), *Prevotella* spp. (20 species) and *Porphyromonas* spp. (seven species) might survive or even grow within amoebae. However, intra-amoebal persistence and/or multiplication have been demonstrated for several obligate anaerobic species that belong to these genera: *Clostridium frigidicarnis* (Pagnier *et al.*, 2008), *Porphyromonas gingivalis* and *Prevotella intermedia* (Wagner *et al.*, 2006). Persistence and multiplication in *Acanthamoeba* spp. has also been demonstrated for *Mobiluncus curtisii*, an obligate anaerobe that causes vaginosis (Tomov *et al.*, 1999). Interestingly, other species that require microaerobic conditions for cultivation in artificial media, such as *Campylobacter* spp. and *Helicobacter pylori*, are also able to persist and grow within amoebae (Winiecka-Krusnell *et al.*, 2002; Axelsson-Olsson *et al.*, 2007). Thus, it seems that protozoa provide an ecological niche that can respond to the needs of various, and very different, bacterial species.

It should also be emphasized that various ARB bacterial species that are not in the CCL3 list have been demonstrated or are strongly suspected of being significant pathogens for humans and/or animals (Table 2). They consist mainly in several mycobacterial species (listed in Herdman & Steele, 2004), *Alpha-* and *Gammaproteobacteria* and new *Chlamydiales* species (Horn, 2008; Greub, 2009).

Interactions with fungi and parasitic protozoa

In addition to bacteria, other microorganisms pathogenic to humans have also been reported to interact with amoebae in a way that can promote their survival and their transmission to susceptible hosts (Table 3). *Cryptococcus neoformans* is able to grow inside (in the case of *C. neoformans* var. *neoformans*) or in the presence of (with *C. neoformans* var. *gattii*) *A. castellanii* ATCC 30324 (Steenbergen *et al.*, 2001; Malliaris *et al.*, 2004), whereas nonvirulent acapsular strains and phospholipase mutants cannot. Importantly, it has also been demonstrated that the murine virulence of *C. neoformans* was enhanced by passage through live *Dictyostelium discoideum* culture (Steenbergen *et al.*, 2003). Similar studies with other fungi also demonstrated their capacity to use amoebae after ingestion (*Sporothrix schenckii* and *Histoplasma capsulatum*) or to exert extracellular cytotoxic effect (*Blastomyces dermatitidis*), leading in both cases to the release of nutrients from killed amoebae that might then be used for fungal growth (Steenbergen *et al.*, 2004). It has also been demonstrated that *H. capsulatum* virulence to mice was enhanced after growth in the presence of *A. castellanii* ATCC 30324. Other studies suggested that *Acanthamoeba* spp. could assist in disseminating *Cryptosporidium parvum* oocysts; however, it was not clearly demonstrated that ingested forms were still alive (Stott *et al.*, 2003; Gomez-Couso *et al.*, 2006). The parasite *Toxoplasma gondii* was recently demonstrated to survive for up to 2 weeks in *A. castellanii* without reducing the infectivity and the pathogenicity of oocysts (Winiecka-Krusnell *et al.*, 2009).

Interactions with viruses

Significantly, it has been demonstrated that several human pathogenic enteroviruses interact with protozoa. Enhanced survival of echoviruses bound to amoebae has been reported (Danes & Cerva, 1981). The study by Mattana *et al.* (2006) reported that coxsackie virus b3 was able to survive within *A. castellanii* trophozoites, and detected significant infectious virus after a 6 months cycle of encystment and excystment; to our knowledge, there is no other report of a well-known pathogenic virus being able to survive inside FLA. It has only been recently suggested that *Acanthamoeba* spp. could act as reservoirs for adenoviruses, with viral DNA (mostly adenovirus type 2) being detected from 34 of 236 (14.4%) environmental amoebal isolates (Lorenzo-Morales *et al.*, 2007). A newly described giant virus '*Acanthamoeba polyphaga* Mimivirus' has also been isolated from an environmental *Acanthamoeba* strain (La Scola *et al.*, 2003a) and might be involved in pneumonia (La Scola, 2005; Raoult *et al.*, 2006).

Potential impact on public health

From a public health perspective, it is important to note that a large number of bacterial species interacting successfully with protozoa are included in various lists of pathogenic microorganisms created by several medical and governmental organizations (see Ecke *et al.*, 2005 for a complete review of these lists) (Table 1). Furthermore, 20 bacterial species in

Table 2. Described interactions of highly suspected pathogenic bacterial species with FLA

Bacterial species	Described interaction with protozoa	References
Chlamydiales		
Parachlamydia acanthamoebae	IC multiplication (various *Acanthamoeba* spp.)	Amann *et al*. (1997); Marrie *et al*. (2001); Borel *et al*. (2007); Baud *et al*. (2008)
Protochlamydia amoebophila	IC multiplication (*Acanthamoeba* UWC1, Dd AX2)	Fritsche *et al*. (2000); Skriwan *et al*. (2002); Haider *et al*. (2008)
Protochlamydia naegleriophila	IC multiplication (various FLA species)	Michel *et al*. (2000a); Casson *et al*. (2008)
Simkania negevensis	IC multiplication (*N. clarki*, *B. mandrillaris*, Ac, Hv), IC multiplication and IK survival (Ac Linc-Ap1)	Kahane *et al*. (1998); Kahane *et al*. (2001); Lieberman *et al*. (2002); Michel *et al*. (2005)
Waddlia chondrophila	IC multiplication (various FLA species)	Dilbeck *et al*. (1990); Henning *et al*. (2002); Dilbeck-Robertson *et al*. (2003); Michel *et al*. (2004); Baud *et al*. (2007); Haider *et al*. (2008)
Alphaproteobacteria		
Agrobacterium tumefaciens	IC multiplication (Ap Linc-Ap1)	Evstigneeva *et al*. (2009)
Bosea sp.	Coculture (Ap Linc-Ap1, Ac ATCC30010)	La Scola *et al*. (2003b); Berger *et al*. (2006); Thomas *et al*. (2007)
Bradyrhizobium sp.	Coculture (Ap Linc-Ap1, Ac ATCC30010)	La Scola *et al*. (2003b); Berger *et al*. (2006), Thomas *et al*. (2006a, b)
Rasbo bacterium	Coculture (Ap Linc-Ap1, Ac ATCC30010)	La Scola *et al*. (2003b); Berger *et al*. (2006); Thomas *et al*. (2006a, b)
Roseomonas gilardii	Coculture (Ac ATCC30010)	Thomas *et al*. (2006a, b)
Gammaproteobacteria		
Aeromonas eucrenophila	Partial cell lysis (Ap Linc-Ap1)	Evstigneeva *et al*. (2009)
Aeromonas salmonicida	IC multiplication (Ap Linc-Ap1)	Evstigneeva *et al*. (2009)
Legionella-like amoebal pathogens	IC multiplication (various FLA species depending on LLAP isolate considered)	Birtles *et al*. (1996); Birtles *et al*. (1997); McNally *et al*. (2000)
Pseudomonas mendocina	IC multiplication (Ap Linc-Ap1)	Evstigneeva *et al*. (2009)
Klebsiella variicola	Complete cell lysis (Ap Linc-Ap1)	Evstigneeva *et al*. (2009)
CFB group bacteria		
Chryseobacterium indologenes	Coculture and cell lysis (Ap Linc AP-1)	Pagnier *et al*. (2008)
Sphingobacterium multivorum	IC multiplication (Ap Linc-Ap1)	Evstigneeva *et al*. (2009)
High GC% Gram+		
Kocuria kristinae	Coculture (Ac ATCC 30010)	Thomas *et al*. (2008)
Mobiluncus curtisii	IC multiplication (*A. culbertsoni* A1 and others *Acanthamoeba* spp.)	Tomov *et al*. (1999)
Mycobacterium bohemicum	IC and IK survival (Ap Linc-AP1)	Adekambi *et al*. (2006)
Mycobacterium goodii	IC and IK survival (Ap Linc-AP1)	Adekambi *et al*. (2006)
Mycobacterium immunogenum	IC and IK survival (Ap Linc-AP1)	Adekambi *et al*. (2006)
Mycobacterium lentiflavum	IC and IK survival (Ap Linc-AP1)	Adekambi *et al*. (2006)
Mycobacterium mageritense	IC and IK survival (Ap Linc-AP1)	Adekambi *et al*. (2006)
Mycobacterium septicum	IC and IK survival (Ap Linc-AP1)	Adekambi *et al*. (2006)
Mycobacterium tusciae	IC and IK survival (Ap Linc-AP1)	Adekambi *et al*. (2006)
Bacilli		
Staphylococcus pasteuri	IC multiplication (Ap Linc-Ap1)	Evstigneeva *et al*. (2009)

Ac, *Acanthamoeba castellanii*; Ap, *Acanthamoeba polyphaga*; Dd, *Dictyostelium discoideum*; Hv, *Hartmannella vermiformis*; IC, intracellular; IK, intracyst.

the CCL3 list have been shown to survive in amoebal cysts; these are mainly mycobacteria (16 species) interacting with *Acanthamoeba* Linc-Ap1 (Adekambi *et al*., 2006; Thomas & McDonnell, 2007). Other species include *Francisella tularensis* (Abd *et al*., 2003), *L. pneumophila* (Kilvington & Price, 1990), *Legionella micdadei* (Fallon & Rowbotham, 1990) and *Vibrio cholerae* (Thom *et al*., 1992). This may be of importance because when bacteria internalized within trophozoites have already increased resistance to biocides (King *et al*., 1988; Howard & Inglis, 2005), this level of resistance increases significantly for bacteria internalized in amoebal cysts (Kilvington & Price, 1990). Some species impair

Table 3. Described interactions of microorganisms other than bacteria with FLA

Microorganism	Described interaction with protozoa	Reference
Viruses		
Acanthamoeba polyphaga Mimivirus	IC multiplication (various *Acanthamoeba* spp. including Ap Linc-Ap1)	La Scola *et al*. (2003a); Suzan-Monti *et al*. (2006)
Coxsackie virus b3	IC and IK survival (Ac), IC survival (Tp)	Teras *et al*. (1988); Mattana *et al*. (2006)
Adenovirus	IC survival (various *Acanthamoeba* isolates)*	Lorenzo-Morales *et al*. (2007)
Echoviruses	Enhanced survival (Ac strain Neff)	Danes & Cerva (1981)
Fungi		
Cryptococcus neoformans	IC multiplication (Ac ATCC 30324, Dd AX-4)	Steenbergen *et al*. (2001); Steenbergen *et al*. (2003)
Blastomyces dermatitidis	Coculture with cell lysis (Ac ATCC 30324)	Steenbergen *et al*. (2004)
Sporothrix schenckii	Coculture with cell lysis (Ac ATCC 30324)	Steenbergen *et al*. (2004)
Histoplasma capsulatum	Coculture with cell lysis (Ac ATCC 30324)	Steenbergen *et al*. (2004)
Others		
Toxoplasma gondii	IC survival (Ac)	Winiecka-Krusnell *et al*. (2009)

*Indirect proof because only viral DNA was detected from axenic *Acanthamoeba* isolates.
Ac, *Acanthamoeba castellanii*; Ap, *Acanthamoeba polyphaga*; Dd, *Dictyostelium discoideum*; Tp, *Tetrahymena pyriformis*; IC, intracellular; IK, intracyst.

encystation once inside their amoebal host (Garcia *et al*., 2007). An impressive picture of amoebal cysts heavily infected with *Staphylococcus aureus* has been published by Marciano-Cabral (2004) (Fig. 2), although there is no study on the role of cysts in survival and protection of *S. aureus* in this paper. Additional studies testing intracyst survival of waterborne pathogens such as *Pseudomonas* spp. and *Burkholderia* spp. are also warranted. Resuscitation of bacteria existing in the 'viable-but-nonculturable' (VBNC) state due to starvation or biocide treatment has been demonstrated for *L. pneumophila* (Steinert *et al*., 1997) and *Campylobacter jejuni* (Axelsson-Olsson *et al*., 2005) after coculture with FLA, and might thus be investigated for other bacterial species.

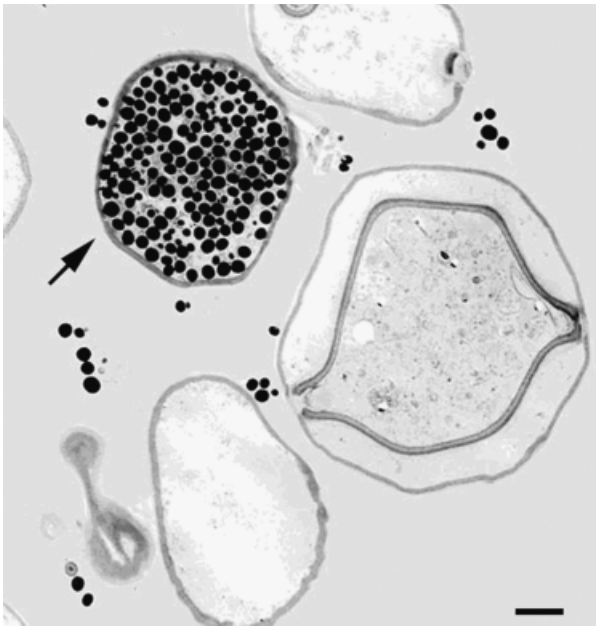

Fig. 2. An *Acanthamoeba* cyst containing *Staphylococcus aureus* (arrow). Scale bar = 1 μm. Reproduced from Marciano-Cabral (2004) with kind authorization of author and editors.

Amoebal coculture as a tool to isolate new microorganisms

Because of the similarities between mechanisms allowing microorganisms to escape phagocytosis and/or digestion by FLA and the mechanisms allowing these same microorganisms to escape phagocytosis and/or digestion by macrophages, FLA have been proposed as a tool to recover potentially new pathogenic species from various environments (Greub & Raoult, 2004). The method is relatively straightforward and has been extensively described in previous publications (La Scola *et al*., 2000; Greub & Raoult, 2004; Thomas & McDonnell, 2007). Briefly, it mainly consists in seeding samples onto axenic amoebal cells and observing whether microorganisms develop in amoebae using appropriate staining methods such as Gimenez staining. If microorganisms are observed, and depending on the results obtained with specific colorations such as Ziehl–Neelsen staining for mycobacteria, samples can be subcultured on specific media or on new axenic amoebae in case of obligate intracellular organisms (Fig. 3). PCR and sequencing with universal primers targeting 16S rRNA- or RpoB-encoding genes can be used to identify isolated bacteria. When using classical staining and microscopic methods to observe potentially infected amoebae, one should remember that FLA have also been found to be infected by small eukaryotic cells (microsporidies) (Hoffmann *et al*., 1998; Michel *et al*., 2000b) that can resemble bacteria, and that giant viruses infecting amoebae can resemble gram-positive cocci (La Scola *et al*., 2003a).

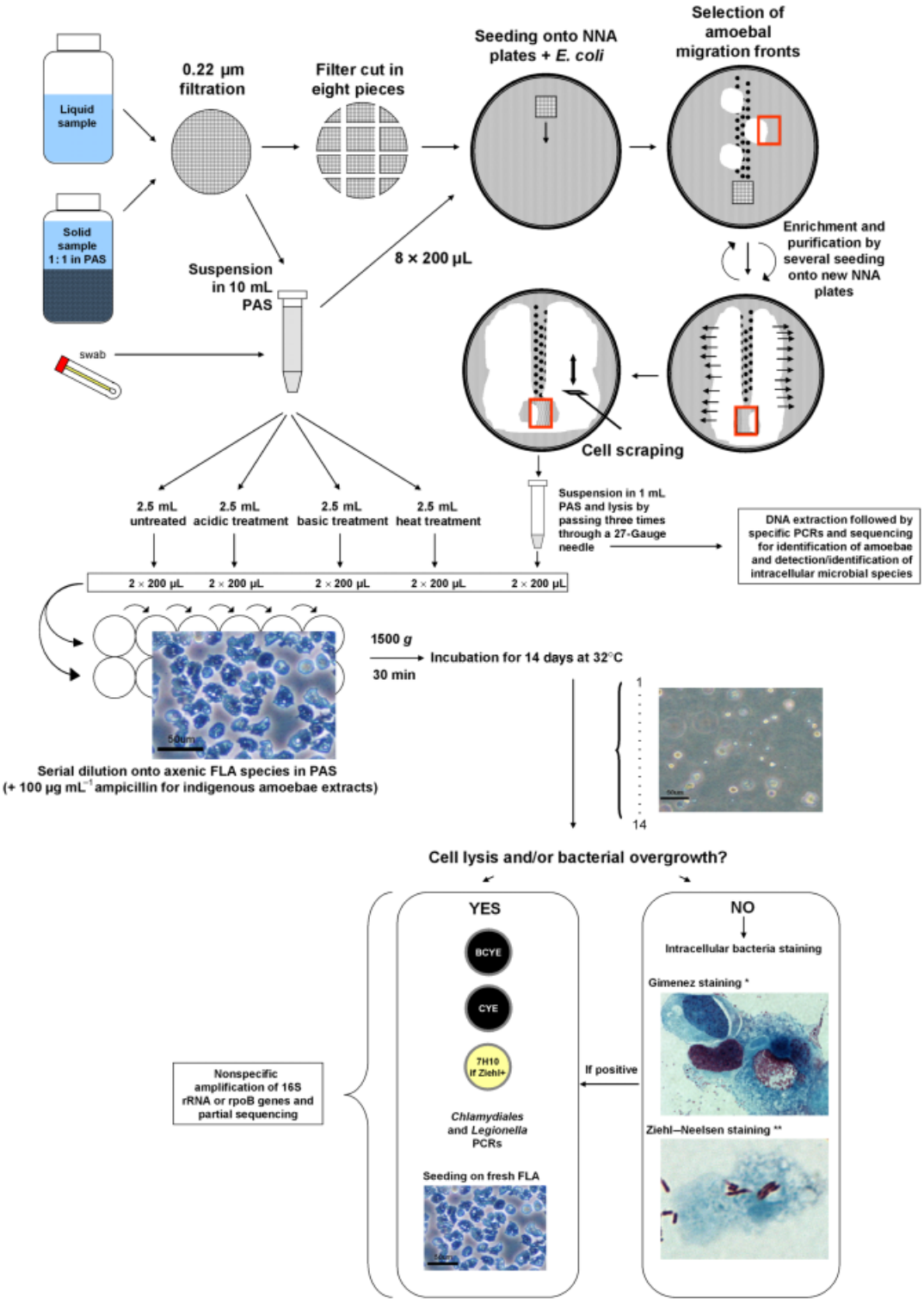

Fig. 3. Proposed method used to recover FLA and intracellular microorganisms from various samples. Reproduced from Thomas *et al*. (2008) and Thomas & McDonnell (2007). *Gimenez-staining of *Candidatus* 'Criblamydia sequanensis' in *Acanthamoeba castellanii* ATCC 30010 (unpublished data); **Zielh–Neelsen staining of *Mycobacterium fluoranthenivorans* in *A. castellanii* ATCC 30010 (Loret *et al*., 2008a).

Using this technique with complex environmental samples generally leads to retrieval of a wide variety of microorganisms, including *Alpha*-, *Beta*- and *Gammaproteobacteria* species (La Scola *et al*., 2000; Thomas *et al*., 2006b; Pagnier *et al*., 2008), members of the *Cytophaga–Flavobacterium–Bacteroides* group (Horn *et al*., 2001; Pagnier *et al*., 2008; Evstigneeva *et al*., 2009), *Actinomycetales* species (Wang *et al*., 2006; Pagnier *et al*., 2008, 2009), bacilli and clostridii (Pagnier *et al*., 2008; Evstigneeva *et al*., 2009) and new *Chlamydiales* (Amann *et al*., 1997; Horn *et al*., 2000; Thomas *et al*., 2006a; Corsaro *et al*., 2009). Attempts to use amoebae to isolate microorganisms directly from clinical samples are less common. *Legionella pneumophila* was isolated from feces and sputa of patients with community-acquired legionnaires' disease (Rowbotham, 1998), *Legionella anisa* from sputum of an immunocompromised man with pneumonia for whom conventional diagnostic tests were negative (La Scola *et al*., 2001). A new mycobacterial species, *Mycobacterium massiliense*, was isolated from the sputum and bronchoalveolar fluid of a patient with hemoptoic pneumonia by plating on axenic media and amoebal coculture with *A. polyphaga* (Adekambi *et al*., 2004). Mura *et al*. (2006) used cocultivation with *A. polyphaga* to examine gut samples from patients with various intestinal disorders; after cocultivation, they were able to detect *M. avium* ssp. *paratuberculosis* from 13 of 39 coculture samples using PCR and *in situ* hybridization (Mura *et al*., 2006). Initial surgical samples were all Ziehl–Neelsen negative whereas auramine–rhodamine staining detected mycobacteria in six of the 13 coculture samples demonstrated positive by molecular methods, suggesting that in these cases acid-fast phenotype changed from negative to positive during incubation with amoebae. Of note, albeit *Chlamydia*-like bacteria are thought to be responsible for pneumonia and miscarriages, to our knowledge, they have never been isolated from clinical samples using the amoebal coculture method; indirect evidence of their role in these infections was provided using only molecular and serological methods. However, the first described *Parachlamydia* isolate was recovered from an *Acanthamoeba* sp. cultivated from the nasal mucosa of a female volunteer (Michel *et al*., 1994; Amann *et al*., 1997), suggesting that amoebae and their intracellular host may colonize humans in close proximity of potential infection sites. A new *Alphaproteobacteria* species proposed as '*Rhodobacter massiliensis*' was isolated from the nose of a patient with aspiration pneumonia using amoebal coculture with *A. polyphaga* (Greub & Raoult, 2003b). Authors used the same method in another study and isolated seven gram-negative bacteria (*Alpha*- and *Betaproteobacteria*, bacteria belonging to the *Bacteroides–Cytophaga–Flexibacter* group) from the nose of patients presenting severe concomitant viral and bacterial infections for most of them (Greub *et al*., 2004).

Thus, amoebal coculture can be considered as a valuable method to isolate fastidious species from complex environmental samples as well as from clinical samples. However, as described above, the main limitation of this technique might be the restricted host range that was described for several intracellular species (Michel *et al*., 2004, 2005), including clearly demonstrated pathogenic species (Dey *et al*., 2009). Further optimization of the method is thus needed and could be achieved by proposing the concomitant or sequential use of a range of selected amoebal species/strains, in parallel with a shell-vial culture assay using mammalian cell lines for clinical samples (Gouriet *et al*., 2005).

Amoebal survival strategies to inimical factors

Upon exposure to detrimental conditions, both physical (temperature changes, UV light and radiation) or chemical (e.g. pH changes, exposure to biocide and starvation) trophozoites can undergo encystation (Greub & Raoult, 2004). Cysts can survive for many years in the environment; Mazur *et al*. (1995) showed that 14 of 17 encysted acanthamoebal isolates stored at $+4\,^{\circ}C$ in water survived for 24 years without apparent loss of virulence in the mouse model. Acanthamoebal cysts have also been reactivated after storage for > 20 years in a completely dry environment (Sriram *et al*., 2008). Encystation is a relatively rapid process that can be divided into a number of development stages (Table 4) (Chavez-Munguia *et al*., 2005, 2009). During encystation, the forming cysts become resistant to a number of agents and these events can be used as markers of encystations progress (Turner *et al*., 2000a, b; Lloyd *et al*., 2001). Pre-emergent *A. castelanii* cysts (committed to excystation) have been found to be less resistant than mature cysts when challenged with chlorhexidine (CHA) or polyhexamethylene biguanide (PHMB), although trophozoites were still more susceptible (Khunkitti *et al*., 1998b). Several proteins have been demonstrated to be secreted mainly during encystation: this includes but is not limited to the proteins necessary for cellulose synthesis, several cyst cell wall proteins and polyphenol oxidase (Chen *et al*., 2004). Preliminary studies also demonstrated that gene expression is modulated during encystment, with upregulation of genes coding for cyst-specific protein 21, protein kinase C, proteasome, heat shock protein, various proteinases, cullin 4, autophage protein 8 and ubiquitin-conjugating enzymes (Moon *et al*., 2008). Cysts are a dehydrated structure with a double wall composed of cellulose and relatively small numbers of proteins (Turner *et al*., 2000b; Lloyd *et al*., 2001). The outer ectocyst wall is composed mainly of protein and lipid-containing materials (Bauer, 1967; Neff & Neff, 1969; Rubin *et al*., 1976) and its appearance is fibrillar

Table 4. Encystment and excystment stages

Stages	Appearance	Events	References
Encystment			
Induction	Ameboid	Degradation of cellular components	Neff *et al*. (1964); Neff & Neff (1969)
Immature cyst	Spherical	Cell wall synthesis – first cell wall layer observed	Weisman (1976)
Mature cyst	Spherical	Synthesis of second layer wall	Weisman (1976)
Excystment			
Initiation stage	Cytoplasm of the cyst loses its granular appearance and larger globules are observed		Mattar & Byers (1971)
Pre-emergent stage	Detachment of the amoeba from the endocyst wall and reappearance of a contractile vacuole		Weisman (1976)
Emergence stage	Digestion of an operculum	Amoeba passes out of the cyst walls	Weisman (1976)

in nature, associated with ill-defined amorphous substances (Bowers & Korn, 1969). In contrast, the inner endocyst wall contains cellulose and its structure appears to be composed of fine fibrils embedded in a granular matrix (Bowers & Korn, 1969). Both walls meet at ostioles, covered by opercula (Fig. 4). The exact composition and morphological aspect of the cyst wall, and notably the spatial separation between endocyst and ectocyst (Smirnov & Michel, 1999), may vary between species and strains but also depends upon the composition of the media used during encystation (Griffiths & Hughes, 1969; Stratford & Griffiths, 1971; Chagla & Griffiths, 1974; De Jonckheere & Brown, 2005). Such a media effect accounts in part for discrepancies in inactivation data between studies (Kilvington & Anger, 2001; Hughes *et al*., 2003). Interestingly, it has also been demonstrated recently that *Acanthamoeba* strains lose their ability to encyst synchronously after prolonged axenic culture, suggesting that in the 'perfect' environments FLA down-regulate genes that are no longer required for survival (Kohsler *et al*., 2008).

Because cysts are the major contributor to protozoal resistance to biocides, encystation is a crucial step in producing resistance and the factors inducing encystation need to be understood. A number of chemical biocides have been shown to induce the formation of cysts in *Acanthamoeba* spp., including diamidines such as diminazene aceturate and pentamidine isethionate and CHA at a low concentration (Byers *et al*., 1991; Chomicz *et al*., 2005). A study investigating the development of resistance during encystation highlighted that exponentially growing trophozoites of *A. castellanii* were more susceptible to CHA and PHMB than pre-encystation trophozoites (i.e. trophozoites committed to encystation) or mature (7 days old) cysts (Khunkitti *et al*., 1998b). Indeed, other investigations showed a correlation between encystation, notably the synthesis of the cellulose endocyst wall, and the development of resistance to different cationic biocides and oxidizing agents (Turner *et al*., 2000a, b; Lloyd *et al*., 2001). The development of the cyst wall has been shown to correspond to a decrease in CHA and PHMB adsorption during encystment in *A. castellanii* (Turner *et al*., 2004).

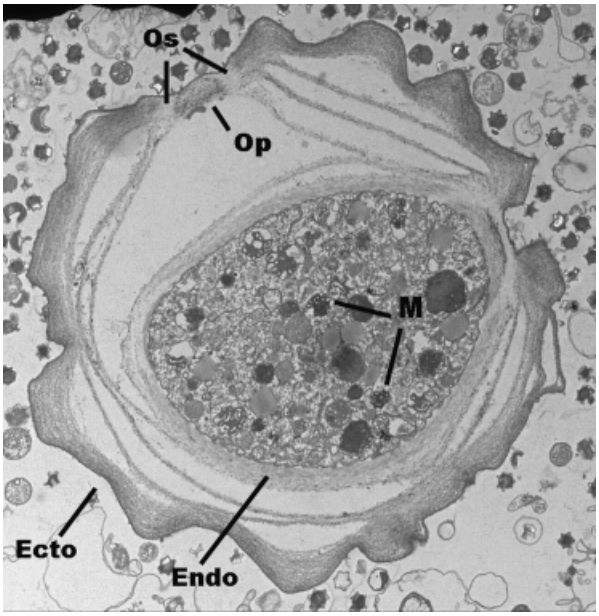

Fig. 4. Structure of an *Acanthamoeba castellanii* ATCC 30010 cyst obtained after 5–7 days incubation of trophozoites at 33 °C in Page's modified Neff's amoeba saline (magnification 4500 ×). Endocyst (Endo), ectocyst (Ecto), ostioles (Os), operculum (Op) and mitochondria (M) are indicated. In this experiment, amoebal trophozoites were used to grow the new *Chlamydiales* species: *Criblamydia sequanensis* (Thomas *et al*., 2006a) that can be observed as star-shaped extracellular bacteria in this picture.

Overall, little is known about the mechanisms of resistance of *Acanthamoeba* spp. to biocides. The double cyst

wall is presumed to represent a permeability barrier and an intrinsic resistance mechanism for CHA and PHMB (McDonnell & Russell, 1999) as well as various diamidines (Perrine *et al.*, 1995). Uptake isotherms of cysts challenged with CHA and PHMB (Khunkitti *et al.*, 1997b), as well as the kinetics of biocide action (Perrine *et al.*, 1995; Khunkitti *et al.*, 1996), provide possible evidence for penetration barrier type resistance mechanisms. Additionally, the metabolically dormant nature of the cyst may negate some of the effects of biocides such as the aromatic diamidines, which inhibit polyamine synthesis and mitochondrial function (Byers *et al.*, 1991). Superior homologues of these biocides such as hexamidine and octamidine were found to be more efficient against trophozoites and cysts. These compounds were found to diffuse better through the cytoplasmic membrane and cell wall although their lethal activity was linked to the protonated amide groups rather than their longer side chain (Perrine *et al.*, 1995).

The resistance of *Acanthamoeba* spp. to chlorine has been described for 30 years (De Jonckheere & van de Voorde, 1976). Comparatively, there have been few reports of protozoal resistance to PHMB (Murdoch *et al.*, 1998; Wysenbeek *et al.*, 2000). Chlorine and CRAs are heavily used in the water industry, while PHMB is used in both the water industry and the medical contact lens industries.

Although the relative biocide sensitivity for most commonly isolated FLA genera is more or less predictable (*Acanthamoeba* spp. cysts > *Hartmannella* spp. cysts > *Naegleria* spp. cysts), it is more difficult to classify species according to this criterion. Concerning *Acanthamoeba* genus, species that were historically defined based on morphological criteria were later divided into 15 genotypes based on rRNA gene sequences (Visvesvara *et al.*, 2007). Genotype T4, which includes *A. castellanii* and *A. polyphaga*, is the most frequently encountered in human infection (Booton *et al.*, 2002, 2005), but the authors have demonstrated that isolates belonging to suspected nonpathogenic genotypes can be more resistant to disinfectants (Shoff *et al.*, 2007). Furthermore, and similar to bacteria, different strains of FLA but of the same taxonomic species may present rather different susceptibilities to biocides (Jeong *et al.*, 2007b). This was observed by Srikanth & Berk (1993) with the *Acanthamoeba hatchetti* ATCC isolate being more susceptible to cooling tower biocides than an environmental isolate that belonged to the same species, the latter even being speculated to grow using biocides as a carbon source. The same authors observed in a further study that isolates can adapt to biocides and reported emerging cross-resistance in amoebae following treatment with sub-inhibitory levels of biocides. Such a phenomenon is of particular interest considering the common but controversial practice of rotating biocides for water treatment (Srikanth & Berk, 1994).

Efficacy of chemical biocides and physical agents against amoebae

Although the lethal effects of biocides against trophozoites and cysts have been described in a number of amoebae (Illingworth & Cook, 1998; Lindquist, 1998; Turner *et al.*, 1999, 2004), our understanding of the interaction between cysts or trophozoites and biocides is limited. The mechanisms of biocidal action against bacteria and other organisms are often presumed to be similar to those upon comparable structures present in amoebae. Some studies aiming to elucidate the effect of cationic agents against *Acanthamoeba* spp. have been published. A transmission electron microscope study has demonstrated both structural and cytoplasmic membrane damage to *A. castellanii* trophozoites treated with biguanides (CHA and PHMB) and this is comparable to the effects observed during similar studies using bacteria (Khunkitti *et al.*, 1998a, 1999). Both CHA and PHMB have been shown to induce pentose leakage in trophozoites at low concentrations, which is also thought to be an indication of cytoplasmic membrane damage (Khunkitti *et al.*, 1997b; Turner *et al.*, 2004). Biocides generally lack selective toxicity and many target sites are likely involved in their lethal effects (Khunkitti *et al.*, 1998a). More specific interactions have been sometimes described. For instance, diamidines have been shown to inhibit *S*-adenosylmethionine decarboxylase and consequently polyamine synthesis in trophozoites of *Acanthamoeba* (Byers *et al.*, 1991); it has also been demonstrated that they can affect mitochondria (Akins & Byers, 1980; Sands *et al.*, 1985) and nucleic acids (Lindquist, 1998).

The efficacy of a limited number of biocides, principally biguanides, quaternary ammonium compounds (QACs), chlorine and CRAs, and oxidizing agents, have been investigated in studies focusing on medical application (contact lenses) and water disinfection. Some discrepancies in results can be observed in trophocidal and cysticidal activity, and these will be discussed later.

Activity of cationic biocides

Trophozoites of *Acanthamoeba* spp., *A. castellanii*, *A. polyphaga* and *Acanthamoeba culbertsoni* have been found to be susceptible to low concentrations (0.005–0.006%) of CHA (Penley *et al.*, 1989; Silvany *et al.*, 1990; Connor *et al.*, 1991; Hugo *et al.*, 1991). Brandt *et al.* (1989) reported CHA (0.005%) in formulation with thimerosal 0.001% (an organomercury compound) and/or EDTA 0.1% to be effective against acanthamoebal cysts within 24 h.

PHMB was described as trophocidal and cysticidal against *A. castellanii* (Silvany *et al.*, 1991; Burger *et al.*, 1994; Khunkitti *et al.*, 1996), *A. polyphaga* (Silvany *et al.*, 1991; Burger *et al.*, 1994) and clinical isolates of *Acanthamoeba* spp. associated with keratitis (Elder *et al.*, 1994; Hay *et al.*, 1994; Seal *et al.*, 1996; Kim & Hahn, 1999). However,

very low concentrations of PHMB (0.00005%), polyaminopropyl biguanide (0.00005%) and the QAC polyquaternium-1 (0.055–0.001%) were reported to be inactive against *A. castellanii* (Penley *et al.*, 1989; Davies *et al.*, 1990; Silvany *et al.*, 1990; Zanetti *et al.*, 1995; Cengiz *et al.*, 2000), *A. polyphaga* (Penley *et al.*, 1989; Davies *et al.*, 1990; Silvany *et al.*, 1990) and *A. culbertsoni* (Connor *et al.*, 1991). Several investigations showed that the concentration of polymeric biguanides in multipurpose solutions commercially available was not cysticidal within the manufacturer's recommended contact time (Buck *et al.*, 1998; Kilvington, 1998; Niszl & Markus, 1998). Indeed, a prolonged contact time of 24 h was necessary for significant cysticidal activity of PHMB (0.02%) against *A. castellanii* (Aksozek *et al.*, 2002). The QAC benzalkonium chloride, at a concentration of 0.04%, was shown to be efficacious against trophozoites and cysts of *A. castellanii* (Silvany *et al.*, 1991; Zanetti *et al.*, 1995; Khunkitti *et al.*, 1996) and *A. polyphaga* (Silvany *et al.*, 1991). Much lower concentrations (0.003–0.004%) were found to be noncysticidal (Penley *et al.*, 1989; Connor *et al.*, 1991; Hugo *et al.*, 1991), although Turner *et al.* (2000b) showed that the minimum cysticidal concentration for *A. castellanii* was 0.004%.

Oxidizing agents

Chlorine at 10 mg L^{-1} (free and combined) was reported to be effective against *Hartmannella vermiformis* cysts after 30 min exposure (Kuchta *et al.*, 1993), but it is clearly ineffective against acanthamoebal cysts because they can resist exposure to 100 mg L^{-1} chlorine for 10 min (Storey *et al.*, 2004) or 50 mg L^{-1} for 18 h (Kilvington & Price, 1990). Limited activity of chlorine was also reported against *B. mandrillaris* cysts and trophozoites (Siddiqui *et al.*, 2008), whereas Chang (1978) reported that exposure to 1–7 mg L^{-1} free chlorine (for 5–30 min) was cysticidal against *Naegleria* spp. and that cyst inactivation conformed to a first-order kinetic. Differences in cysticidal activity to chlorine, and also to chlorine dioxide and ozone, have been reported with *Acanthamoeba* spp. being less susceptible than *Naeglaria* spp. (Cursons *et al.*, 1980). Monochloramine at 3.9 mg L^{-1} killed *Naegleria lovanensis* cysts within 1 h (Ercken *et al.*, 2003). However, this biocide has also been demonstrated to induce VBNC state in *L. pneumophila* that could then be resuscitated by coculture with *A. castellanii* (Alleron *et al.*, 2008).

Hydrogen peroxide formulations are commonly used for the disinfection of contact lenses and the trophocidal and cysticidal efficacy of liquid hydrogen peroxide against *Acanthamoeba* spp. has been well studied (Brandt *et al.*, 1989; Davies *et al.*, 1990; Silvany *et al.*, 1990, 1991; Niszl & Markus, 1998; Hughes & Kilvington, 2001; Aksozek *et al.*, 2002). Efficacy has been reported to depend upon the type of formulation and contact time (Hughes & Kilvington, 2001), with two studies reporting commercial products containing 3% hydrogen peroxide to be ineffective against *Acanthamoeba* spp. within the manufacturer's recommended contact time of 30 min (Ludwig *et al.*, 1986; Zanetti *et al.*, 1995), but they were cysticidal after 4 h (Aksozek *et al.*, 2002). Hydrogen peroxide as a gas has been demonstrated to be effective against *A. polyphaga* and *A. castellanii* cysts (Thomas & McDonnell, 2008).

When tested against *Acanthamoeba* and *Naegleria* spp. trophozoites, chlorine dioxide in water was efficacious after treatment for 30 min at concentrations of approximately 2 mg L^{-1} (*Naegleria* spp.) or 3 mg L^{-1} (*Acanthamoeba* spp.) (Cursons *et al.*, 1980). However, the same active had only limited effect on *A. polyphaga* cysts exposed to 5 mg L^{-1} for 60 min (Loret *et al.*, 2008b), and continuous injection in water pipes at 0.5 mg L^{-1} could not completely inactivate FLA (Thomas *et al.*, 2004).

Ozone, the most potent oxidizing agent, has been shown to considerably reduce the FLA population in a drinking water plant (Thomas *et al.*, 2008) and to be an efficient cysticidal against *A. polyphaga* (Loret *et al.*, 2008b). However, its use is limited to the treatment of a limited volume of circulating water and it has no residual activity against FLA (Thomas *et al.*, 2004).

Unformulated peracetic acid (PAA) was demonstrated to be effective against *Acanthamoeba* and *Naegleria* trophozoites after exposure to 15 mg L^{-1} for 2 h, but activity against *Acanthamoeba* cysts required longer incubation (18 h) and higher concentration (150 mg L^{-1}). One commercial PAA-based product, when tested at room temperature, was shown to kill all trophozoites of *A. polyphaga* within 30 min, but failed to completely inactivate cysts within 24 h (Greub & Raoult, 2003a). Other FLA species seem more susceptible to inactivation by PAA, and cysticidal activity against *N. lovanensis* was observed after exposure to 5.33 mg L^{-1} for 1 h (Ercken *et al.*, 2003).

Other biocides

The trophocidal and cysticidal activities of some other biocides have also been reported, but to a lesser extent. Iodine and bromine were found to have some cysticidal activity against *N. fowleri* (De Jonckheere & van de Voorde, 1976). These actives have generally poor efficacy against a number of protozoa (De Jonckheere & van de Voorde, 1976), and they have been proven to be inefficient against *Acanthamoeba* spp. cysts (Lim *et al.*, 2000) under the conditions tested. Only a few studies reporting testing of aldehydes against FLA have been published. In the work by Aksozek *et al.* (2002), 10% formalin was reported to be cysticidal within 30 min against *A. castellanii*. Glutaraldehyde might be less active because a study focusing on

A. polyphaga reported that a large proportion of trophozoites were still viable after exposure to a commercial formulation containing 2% glutaraldehyde for 30 min to 3 h (Greub & Raoult, 2003a). Isothiazolones consist of a range of biocides extensively used in the water industry, and as a consequence, their cysticidal activity, as well as their activity against intracellular bacterial pathogens, has been investigated. Cysts of environmental isolates of *A. hatchetti* and *Cochliopodium bilimbosum* were shown to be resistant to an isothiazolin derivative (5-chloro-2-methyl-4-isothiazolin-3-one) used according to the manufacturer's recommendations for the disinfection of cooling towers (Srikanth & Berk, 1993; Sutherland & Berk, 1996) and were shown to reproduce faster at a low concentration of the biocide (Srikanth & Berk, 1993). Diamidines have been shown to be trophocidal but they are not cysticidal (Osato *et al.*, 1991; Perrine *et al.*, 1995; Gray *et al.*, 1996; Kim & Hahn, 1999; Wysenbeek *et al.*, 2000). Although propamidine is poorly cysticidal, other homologous biocides such as hexamidine and octamidine appeared to have greater activity (Perrine *et al.*, 1995). Interestingly, products containing 20% isopropyl alcohol were demonstrated to present good activity against *A. polyphaga*, *A. castellanii* and *A. culbertsoni* cysts (Penley *et al.*, 1989; Connor *et al.*, 1991; Aksozek *et al.*, 2002). There are very few publications available on the efficacy of metallic salts against amoebae. Rohr *et al.* (2000) showed that the concentrations of copper ($100\,\mu g\,L^{-1}$) and silver ($10\,\mu g\,L^{-1}$) used within the limit of drinking water regulations were not active against *H. vermiformis* and the ciliate *Tetrahymena pyriformis*.

Combination of treatments

Owing to the low cysticidal activity of biocides when used over short contact times, combinations of biocides have been tested for their efficacy. A combination of hydrogen peroxide (3%) with catalase and potassium iodide (50 μM) significantly enhanced the cysticidal activity against *A. polyphaga* (Hughes *et al.*, 2003); this may be due the increased oxidation potential of these mixtures. Sokmen *et al.* (2008) showed that the combination of silver-titanium dioxide activated by UV light, while inducing irreversible damage to the cell wall and intracellular structure of *Giardia intestinalis* cysts, was ineffective against cysts of *A. castellanii*. Heat pretreatment of *H. vermiformis* rendered them more susceptible to chlorine in the study by Kuchta *et al.* (1993). Likewise, although a number of publications have described the lack of efficacy of a range of QACs against environmental amoebal isolates, a combination of QACs with tributyltin neodecanoate (TBT/QAC) produced better activity (Srikanth & Berk, 1993; Sutherland & Berk, 1996). CHA (0.005%) was also found to be effective against cysts within 24 h (Brandt *et al.*, 1989) or in combination with thiomersal (0.005%) within 6–9 h (Zanetti *et al.*, 1995) or 6–24 h (Brandt *et al.*, 1989).

Efficacy of physical agents, heat and irradiation against protozoa

Heat

Moist heat is generally considered to be trophocidal and cysticidal, although higher temperatures are needed to inactivate cysts. This difference in inactivation may arise as a consequence of dehydration of the cyst cytoplasm during cyst cell wall synthesis (Turner *et al.*, 2000b; Lloyd *et al.*, 2001).

Turner *et al.* (2000b) observed that trophozoites of *A. castellanii* were inactivated following a 30-min exposure at 46 °C, while a temperature of 56 °C was necessary to inactivate the same number of cysts. Aksozek *et al.* (2002) reported that a temperature of 65 °C for > 5 min was cysticidal for *A. castellanii*, while Ludwig *et al.* (1986) reported that cysts of *A. castellanii* and *A. polyphaga* were inactivated only after exposure to moist heat at 80 °C for 10 min. However, the same exposure conditions did not completely inactivate thermotolerant *Acanthamoeba* spp. cysts in other studies (Storey *et al.*, 2004). *Balamuthia mandrillaris* trophozoites may be more resistant to heat, because a temperature of 60 °C was not trophocidal within 60 min; however, a temperature of 80 °C maintained for at least 60 min did inactivate cysts (Siddiqui *et al.*, 2008). Thermotolerant *Hartmannella* strains have been cultivated at 53 °C (Rohr *et al.*, 1998), but cysts of *H. vermiformis* (environmental strain) were inactivated after exposure at 60 °C for 30 min (Kuchta *et al.*, 1993). One *Echinamoeba* sp. strain has been shown to grow at 57 °C, but the cyst temperature resistance was not studied (Baumgartner *et al.*, 2003). *Naegleria* spp. are generally considered more sensitive to heat (Chang, 1978; Rohr *et al.*, 1998).

UV disinfection

While UV radiation is widely used for water disinfection, its efficacy against FLA has not been widely reported when compared with other protozoa such as *C. parvum*. Hijnen *et al.* (2006) provide a comprehensive overview of the ability of UV radiation to inactivate cysts and reported that *Acanthamoeba* spp. are highly UV resistant (Hijnen *et al.*, 2006). UV exposure has good activity against trophozoites (Maya *et al.*, 2003), but cysts are much more resistant. For example, *Acanthamoeba* spp. cysts were demonstrated to be resistant to exposure to UV-C at 253.7 nm, $1.1\,mJ\,s^{-1}\,cm^{-2}$ (Hwang *et al.*, 2004); *A. castellanii* cysts were shown to be resistant to UV-B irradiation ($800\,mJ\,cm^{-2}$) and *B. mandrillaris* cysts to exposure at $200\,mJ\,cm^{-2}$ UV irradiation (Siddiqui *et al.*, 2008). Others studies reported a $4\log_{10}$

reduction of *A. polyphaga* cysts after exposure to 40 mJ cm^{-2} (Loret *et al.*, 2008a), suggesting that there might be some differences between strain and species sensitivities.

Exposure of *A. polyphaga* cysts to simulated global solar irradiance in water (85 mJ s^{-1} cm^{-2}) did not achieve cyst inactivation when the temperature was kept $< 40\,^{\circ}C$ but gave better results at 45 °C (1.2 $\log_{10}$ reduction after 6 h), 50 °C ($> 3.6 \log_{10}$ reduction after 6 h) and 55 °C ($> 3.3 \log_{10}$ reduction after 4 h) (Heaselgrave *et al.*, 2006). Sixty one percent of *Naegleria gruberi* cysts were still alive after UV irradiation with 21.6 mJ cm^{-2}, and treating amoebae with inhibitors of DNA repair mechanisms improved UV irradiation efficacy (Hillebrandt & Muller, 1991). Photosensitized inactivation of *Acanthamoeba palestinensis* in the cystic stage has also been demonstrated by incubating cysts with tetracationic Zn(II)-phthalocyanine before exposure to 600–700-nm wavelength light sources (Ferro *et al.*, 2006).

Filtration

Physical removal by clarification and filtration processes has been proposed as an effective way to remove protozoa from drinking water (Loret *et al.*, 2008a). Several studies demonstrated that a clarification step by sand filtration removed approximately 2–3 $\log_{10}$ FLA from water, with successive filtration on granular activated carbon (GAC) removing an additional 1–2 $\log_{10}$ (Hoffmann & Michel, 2001; Jeong & Yu, 2005; Thomas *et al.*, 2008; Loret *et al.*, 2008a). Of note, some of the FLA isolated in these studies harbored endosymbiotic bacterial species. Interestingly, most of the species that were recovered after the sand and GAC filters in these studies belonged to *Hartmannella* and *Echinamoeba* genera, suggesting that these small amoebae (cyst diameter generally $< 10\,\mu m$) are more likely to spread through drinking water treatment processes than *Acanthamoeba* and *Naegleria* spp. (cyst diameter generally $> 10\,\mu m$). Nanofiltration of water as a final stage of drinking water production is also used to remove microbial contaminants; however, membrane fouling is a major constraint in this process (Her *et al.*, 2007), and it does not prevent recolonization downstream in a drinking water network. Terminal point-of-use filters are also effective (Exner *et al.*, 2005). In our view, however, additional studies are needed to investigate the potential growth of biofilms and amoebae on these supports, as well as intracellular growth and release of bacteria that can pass through 0.2-μm filters (Hahn, 2004; Silbaq, 2009) that might then grow in the presence of FLA, such as *Microbacterium* spp. (Thomas *et al.*, 2008).

Other physical treatments

Pulsed electric fields have been used against *Naegleria lovaniensis* trophozoites (Vernhes *et al.*, 2002); however, this is not a particularly challenging organism. *Acanthamoeba castellanii* cysts were resistant to 2500 Gy of γ irradiation (Aksozek *et al.*, 2002), and *N. gruberi* cysts have been demonstrated to resist X-ray irradiation with a dose of 1.74×10^4 Gy (Hillebrandt & Muller, 1991), suggesting that some, if not all, FLA have very efficient DNA repair mechanisms. Cryotherapy (repeated quick freezing to around $-100\,^{\circ}C$) has been used to treat *Acanthamoeba* spp. cysts *in vitro* and in clinical settings but was not fully efficient (Meisler *et al.*, 1986; Matoba *et al.*, 1989). Repeated freeze–thawing cycles were also demonstrated to have limited cysticidal activity against *B. mandrillaris* (five cycles from -80 to 37 °C) (Siddiqui *et al.*, 2008) and against *A. castellanii* (five cycles from -160 to 45 °C) (Aksozek *et al.*, 2002).

Biocide efficacy against intracellular pathogens

Bacteria associated with FLA generally display higher resistance to biocides than their planktonic counterparts. Two kinds of mechanisms can potentially explain this observation: the physical barrier to the action of biocides constituted by the trophozoite and the cyst and the resistant bacterial phenotype triggered by intracellular growth. These two phenomena are intimately associated and might be difficult to differentiate. They should, however, be taken into account in future studies aiming at comparing resistance to biocides of bacteria grown under different conditions. For example, comparing the resistance of intracellular vs. planktonic *Legionella* spp. to biocides should consist in treating both infected FLA and intracellular bacteria just released from their host; comparing infected FLA with bacteria grown on agar plates might lead to different results. When growing in FLA, *L. pneumophila* differentiate into mature intracellular forms (MIFs) (Garduno *et al.*, 2002). These MIFs are short, stubby rod-like structures with an electron-dense, laminar outer membrane layer. They contain inclusions of poly-β-hydroxybutyrate and laminations of internal membranes originating from the cytoplasmic membrane (Garduno *et al.*, 2002). Stationary phase bacteria obtained after growth on artificial media are morphologically distinct. They appear as dull rods and present a typical gram-negative cell wall ultrastructure (Garduno *et al.*, 2002). Interestingly, MIFs have a very low respiration rate, increased resistance to detergent-mediated lysis and tolerate higher pHs compared with stationary-phase bacteria (Garduno *et al.*, 2002). In addition, it has also been observed that genes that are necessary for intracellular infection and virulence increase *L. pneumophila*'s resistance to the bactericidal effects of cationic antimicrobial peptides (Robey *et al.*, 2001). It should also be recognized that intracellular pathogens use a variety of different mechanisms to survive

oxidative stress encountered in phagocytic cells. These same mechanisms can also enhance resistance to disinfection treatments. For example, this was demonstrated for the expression of the DNA-binding protein gene (*dps*X) from starved cells in '*Candidatus* Legionella jeonii'; the expression of this gene was enhanced by phagocytic activities, conferring upon bacteria higher resistance to liquid hydrogen peroxide (Park *et al.*, 2006). Barker *et al.* (1992) reported the lack of efficacy of isothiazolinones against *L. pneumophila* grown within *A. polyphaga*. The decreased susceptibility was linked to an iron-depletion phenotype. Differences in phenotypes of a bacterial strain grown in different amoebal hosts are also thought to influence biocide susceptibility of released bacteria; this was recently evidenced with *L. pneumophila* replicated from *H. vermiformis* showing greater chlorine resistance than the cells replicated from *A. castellanii* (Chang *et al.*, 2009).

Other studies have focused on the fate of bacterial pathogens within amoebae following biocide exposure from a more 'mechanical' perspective, without investigation of phenotypic changes induced by intracellular growth. King *et al.* (1988) investigated the survival and 'resistance' of a range of intracellular bacterial pathogens to free chlorine and concluded that *A. castellanii* trophozoites and, to some extent, *T. pyriformis* had a predominant role in the survival of these pathogens. Similar studies were reported with *Burkholderia pseudomallei* being more resistant to monochloramine, chlorine and UV once protected in *Acanthamoeba astronyxis* trophozoites (Howard & Inglis, 2005). Hwang *et al.* (2006) reported the decreased efficacy of silver ($0.1\ mg\ L^{-1}$) and copper ($1\ mg\ L^{-1}$) against *L. pneumophila* and *P. aeruginosa* within *A. polyphaga* trophozoites. *Campylobacter* survival to chemical disinfection with an iodine-based product has been reported to increase when associated with *A. castellanii* and *T. pyriformis* trophozoites (Snelling *et al.*, 2005). Encystment of FLA is preceded by the expulsion of food vacuoles and vesicles (Schuster, 1979). These vesicles can also contain intracellular bacteria that have been observed to stay viable for up to 6 months (Bouyer *et al.*, 2007). They can also directly protect intracellular bacteria from the effect of biocides (Berk *et al.*, 1998). Encystation itself might be either beneficial or detrimental to the intracellular bacteria. Mycobacteria (Adekambi *et al.*, 2006), *F. tularensis* (Abd *et al.*, 2003) and *V. cholerae* (Thom *et al.*, 1992; Abd *et al.*, 2005) survive in cysts and might therefore benefit from protection toward biocide treatments. However, in some instances, the bacterial pathogen loses its viability within the cyst. This was reported with encystation of *L. monocytogenes* in *A. castellanii* (Ly & Muller, 1990). An additional phenomenon has been recently described with *L. pneumophila* infection of *A. polyphaga* trophozoites. Although both microorganisms seemed to exhibit an increase in resistance to sodium hypochlorite (available chlorine concentration was not measured), the amoebal host lost its ability to encyst. It thus provided an intracellular niche immediately available for resuscitation of extracellular *L. pneumophila* that enter a VBNC state upon treatment with chlorine (Garcia *et al.*, 2007).

Methods used for inactivation studies

Antimicrobial testing against amoebae is compounded by the absence of a standard test method (Mercer, 2008). A variety of test protocols have been used (e.g. flow cytometric analysis, plaque assay and colorimetric assay) and ultimately it is difficult to compare the results between these studies (Khunkitti *et al.*, 1997a; McBride *et al.*, 2005). Buck *et al.* (2000) reviewed several studies that evaluated the efficacy of contact lens preservative/disinfectant against *Acanthamoeba* spp. cysts; she reported great variability in test organisms, growth conditions, inoculum preparation, neutralization, recovery and quantitation methods for survivors. The critical parameters have been demonstrated to be the age of the test culture, the type of medium used for induction of cysts and the maturity of cysts (Kilvington & Anger, 2001; Hughes *et al.*, 2003). Cysts produced from isolates that have not been subcultivated for many generations in axenic media are more resistant than isolates cultivated in artificial broth, as are cysts obtained from monoculture with bacteria (Hughes *et al.*, 2003). Various neutralization methods have been used to halt the activity of test biocides after specified contact times, and the Dey–Engley neutralizing broth, which is recommended in European Standards for bactericidal efficacy testing, has been proven to be nontoxic to amoebal trophozoites (Buck & Rosenthal, 1996). Despite this, neutralization should always be confirmed as an internal control and can be product/biocide specific. It has been reported that centrifugation might be more readily adapted than filtration for the recovery of trophozoites; both methods giving the same recovery rates with cysts (Pernin *et al.*, 1998). Another critical step is the method used to evaluate and enumerate survivors after treatment. Excystation is more efficient with cysts grown on a live bacteria lawn than with cysts grown on artificial media, and the most probable number technique for the enumeration of amoebae grown on *Escherichia coli* lawns has been proposed as the basis of a national standard for amoebicidal efficacy testing (Beattie *et al.*, 2003). The choice of bacteria used may be a further variable in the efficiency of recovery. Alternative enumeration methods based on AlamarBlue staining (McBride *et al.*, 2005) and viability staining with fluorescein diacetate and propidium iodide have been proposed (Khunkitti *et al.*, 1997a; Borazjani *et al.*, 2000), but they may prove difficult to use with cysts due to low metabolic activity and their low permeability to chemicals.

Concluding remarks

There can be little doubt that amoebae play a major role in the composition of microbial flora of water systems. Currently, however, this role is largely overlooked. The microbiological quality of water is still based on the presence of coliforms and the presence of amoebae, and more generally of protozoa and ciliates is not considered unless they are pathogenic (i.e. *Cryptosporidium* spp. and *Giardia* spp.) and involved in infection. In some environments such as in hospitals and dental practices, amoebae are seldom, if at all, considered. When they are investigated in these environments, amoebae are found together with a range of intracellular microorganisms, some being demonstrated pathogenic species and others for which a pathogenic role remains to be elucidated. FLA offer a protective intracellular environment in which to harbor microbial pathogens, thereby significantly reducing the efficacy of biocide treatment. In some cases, they might also favor persistence and extracellular proliferation of pathogenic bacterial species, thus leading to selection of these predation-resistant microorganisms in water systems. A number of investigations are needed to increase our understanding of these microorganisms together with the means for their control. The ecology of microorganisms in water systems and the impact of biocides have to be approached from a global perspective, taking into account interactions of microorganisms with biofilms and amoebae and the potential selection of pathogenic species during disinfection. These are suggested to include studies investigating (1) the interaction of physical and chemical biocides with amoebal trophozoites and cysts and the mechanisms of resistance to disinfection, (2) the survival of amoebae in complex microbial biofilms, (3) the fate of intracellular pathogens following biocidal treatment and (4) trophozoite and cyst detection. Finally, the standardization of cyst production and the efficacy testing for disinfection and sterilization investigations are required to produce meaningful and reproducible data. FLA have also been demonstrated to constitute a preferential place for intra- and interspecies genetic exchanges between intracellular microorganisms and their hosts (Ogata *et al.*, 2006). They could thus act as true reservoirs and cross-kingdom vehicles of genetic information, including bacterial virulence or resistance factors. The availability of *A. castellanii* Neff genome (see http://www.hgsc.bcm.tmc.edu) should bring valuable information in this field of investigation and will trigger other FLA genome sequencing.

Increasing knowledge of the resistance of trophozoites and cysts to disinfection, their impact on selecting potential pathogenic species from complex microbial flora and their capacity to release intracellular pathogens will undoubtedly raise important questions. It will be our responsibility to use this information to develop new mechanisms for control.

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