Sensitivity of *Vermamoeba* (*Hartmannella*) *vermiformis* cysts to conventional disinfectants and protease

Emilie Fouque, Yann Héchard, Philippe Hartemann, Philippe Humeau and Marie-Cécile Trouilhé

ABSTRACT

Vermamoeba vermiformis is a free-living amoeba (FLA) widely distributed in the environment, known to colonize hot water networks and to be the reservoir of pathogenic bacteria such as *Legionella pneumophila*. FLA are partly resistant to biocides, especially in their cyst form. The control of *V. vermiformis* in hot water networks represents an important health issue, but there are very few data on their resistance to disinfection treatments. The sensitivity of cysts of two strains of *V. vermiformis* to three disinfectants frequently used in hot water networks (chlorine, heat shock, peracetic acid (PAA) mixed with hydrogen peroxide (H_2O_2)) was investigated. *In vitro*, several concentrations of biocides, temperatures and exposure times according to the French regulation were tested. Cysts were fully inactivated by the following conditions: 15 mg/L of chlorine for 10 min; 60 °C for 30 min; and 0.5 g/L equivalent H_2O_2 of PAA mixed with H_2O_2 for 30 min. For the first time, the strong efficacy of subtilisin (0.625 U/mL for 24 h), a protease, to inactivate the *V. vermiformis* cysts has been demonstrated. It suggests that novel approaches may be efficient for disinfection processes. Finally, *V. vermifomis* cysts were sensitive to all the tested treatments and appeared to be more sensitive than *Acanthamoeba* cysts.

Key words | disinfectant, free-living amoeba, hot water networks, subtilisin, *Vermamoeba* (*Hartmannella*) *vermiformis*

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INTRODUCTION

Free-living amoebae (FLA) are protozoa widely distributed in the environment and isolated from water, soil, air, compost and sediments (Rodriguez-Zaragoza 1994). FLA also colonize the aquatic environment related to human activities, such as water treatment plants (Thomas *et al.* 2008; Garcia *et al.* 2013), water distribution networks (Thomas *et al.* 2006b), cooling towers (Srikanth & Berk 1993; Berk *et al.* 2006), cooling systems of nuclear power plants (Behets *et al.* 2007) and hot water networks (Rohr *et al.* 1998; Lasheras *et al.* 2006). Even if FLA are not considered to constitute a major threat to human health, those belonging to *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri* can potentially be responsible for severe brain pathologies (Marciano-Cabral & Cabral 2003; Schuster & Visvesvara 2004). The genus *Acanthamoeba* is also responsible for many cases of keratitis (Schuster & Visvesvara 2004).

FLA have two stages of development: a vegetative form named trophozoite and a dormant form named cyst; this differentiation is called encystment (Fouque *et al.* 2012). Encystment occurs when environmental conditions become unfavourable such as nutrient starvation or osmotic stress. Encystment induces extensive morphological changes: FLA become spherical and a cyst wall is formed, leading to increased resistance of cysts to treatments and harsh conditions.

FLA are mainly encountered in biofilms and feed by grazing on microorganisms. Importantly, several pathogenic bacterial species like *Legionella pneumophila*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Mycobacterium* spp.

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resist amoebic phagocytosis (Greub & Raoult 2004). Thus, it is recognized that FLA act as reservoirs of pathogenic bacteria, resulting in protection from hostile conditions and promoting bacterial dissemination in the environment (Horn *et al.* 2000; Greub & Raoult 2004; Thomas *et al.* 2010; Santic *et al.* 2011). For example, the relationship between FLA and *L. pneumophila* is particularly well documented and it has been demonstrated that the majority of legionellosis outbreaks were linked to hot water networks contaminated by *L. pneumophila* associated with FLA (Fields *et al.* 2002; Lau & Ashbolt 2009). The management of FLA in hot water networks is therefore a first issue in the control of public health risks.

Despite the awareness of the importance of FLA control in management of health risks related to devices linked to human activities, few data regarding FLA resistance to various biocides and disinfection procedures are available. Most studies dealing with FLA resistance to treatment focus on the genus *Acanthamoeba* (De Jonckheere & van de Voorde 1976; Coulon *et al.* 2010; Dupuy *et al.* 2013). Also, *Vermamoeba* and *Hartmannella* have been poorly studied, probably because they are not pathogenic by themselves, although these genera seem to be predominant in hot water networks (Rohr *et al.* 1998; Buse *et al.* 2013; Ovrutsky *et al.* 2013). Only two studies have investigated the resistance of *V. vermiformis* to biocides used in hot water networks and cooling towers. A study conducted on the impact of chlorine and heat, alone and in combination, on both *V. vermiformis* trophozoites and cysts showed that trophozoites were more sensitive than cysts (Kuchta *et al.* 1993). Critchley & Bentham (2009) have evaluated the efficacy of three biocides (chlorine, bromine and isothiazolinone) used in cooling towers on trophozoites and cysts of three tested strains: *Acanthamoeba* sp., *Vahlkampfia* sp. and *V. vermiformis*. They have shown that cysts were more resistant than trophozoites for the three strains. Also, the *Acanthamoeba* strain was more resistant than the *V. vermiformis* strain which was more resistant than the *Vahlkampfia* strain (Critchley & Bentham 2009). These studies were conducted on one strain of *V. vermiformis,* which does not allow us to assess the differences within the same species.

The lack of knowledge about the sensitivity of *V. vermiformis* cysts to disinfection treatments led us to investigate the cyst sensitivity of two strains (environmental and reference) to biocides frequently used in hot water networks. Three treatments (chlorine, heat shock and peracetic acid (PAA) mixed with hydrogen peroxide) were selected according to the French circular in relation to *Legionella* risk prevention in health facilities (DGS/SD7A/DHOS/EA No. 2002/243 2002-04-22).

Finally, several studies have shown that classical biocides are not sufficient to eliminate FLA in water networks; thus, it is necessary to develop new treatments (Rohr *et al.* 1998; Thomas *et al.* 2004; Loret *et al.* 2005; Loret & Greub 2010). In this context, the efficacy of an innovative enzymatic treatment has been tested. A protease was selected because the major components of the cyst wall of *H. glebae*, which is close to *V. vermiformis,* are proteins (Upadhyay *et al.* 1984). Thus, subtilisin was chosen because it has recently been identified for its antifouling activity on marine biofilms (Leroy *et al.* 2008).

METHODS

Amoebal strains

Two strains of *Vermamoeba vermiformis* were used in this study: *V. vermiformis* Page ATCC 50237, a reference strain isolated from a hospital cooling tower in South Dakota (USA), and *V. vermiformis* 172A, an environmental strain isolated from a hospital water network (Lausanne, Switzerland) (Thomas *et al.* 2006a), where it is referenced as *H. vermiformis* strain 2. Indeed, since 2011 the species *H. vermiformis* has been reclassified in the genus *Vermamoeba* which contains only the species *vermiformis* (Smirnov *et al.* 2011).

Cultivation

V. vermiformis were grown axenically in 15 mL of modified PYNFH medium (pH = 6.5, 1% bacto-peptone, 1% yeast extract, 0.1% RNA of torula yeast type VI, 33 μmol/L folic acid, 1.5 μmol/L hemin, 3.6 mmol/L Na_2HPO_4, 26 mmol/L KH_2PO_4, 10% fetal bovine serum), in a 75 cm^3 tissue culture flask at 28 °C. After 3–5 days of culture, the strains were subcultured into modified PYNFH medium.

Encystment

Cysts were prepared from trophozoites using Neff's encystment medium (pH 8.8, 0.1 mol/L KCl, 8 mmol/L $MgSO_4$, 0.4 mmol/L $CaCl_2$, 20 mmol/L Tris (2-amino-2-hydroxymethyl-1,3-propanediol), $NaHCO_3$ 1 mmol/L). Trophozoites (3 days old) were harvested by centrifugation ($500 \times g$ for 7 min), washed twice in encystment medium and suspended in 15 mL of encystment medium at approximately 1×10^6 trophozoites/mL. The cell concentration was estimated by enumeration in a counting chamber (Fast-Read 102®). Then trophozoites were incubated in a 75 cm^3 tissue culture flask at 28 °C for 7 days to obtain mature cysts. Mature cysts were harvested by centrifugation ($1{,}000 \times g$ for 7 min), washed twice in phosphate buffer (pH 7, 50 mmol/L) and suspended in the same phosphate buffer at approximately 1×10^6 cells/mL and stored at 4 °C for up to 2 weeks.

Inactivation tests

For all experiments, cysts concentration was adjusted to 5×10^5 cysts/mL in phosphate buffer (pH 7, 50 mmol/L) before treatment. The cell concentration was estimated by enumeration in a counting chamber (Fast-Read 102®). Biocide concentrations (chlorine and PAA mixed with hydrogen peroxide), heat shock temperatures and exposure times were chosen according to the French circular in relation to *Legionella* risk prevention in health facilities (DGS/SD7A/DHOS/EA No. 2002/243, 2002-04-22). Table 1 summarizes the disinfection procedures recommended by the French circular. For each condition, cyst viability was evaluated by the most probable number (MPN) method and all experiments were performed in triplicate.

Chlorine

Tests were performed with a commercial sodium hypochlorite solution containing 13% active chlorine (Acros Organics). Before each experiment the concentration in free chlorine was assessed by titrimetric method with *N,N*-diethyl-1,4-phenylenediamine (NF EN ISO 7393-1) and a chlorine solution at 1 g/L was prepared. The cysts suspension was distributed in sterile 15 mL tubes and chlorine treatments were applied with the previous solution.

Table 1 | Disinfection procedures recommended by French regulation (DGS/SD7A/DHOS/EA No. 2002/243 2002-04-22) in hot water networks

Disinfection	Continuous treatment	Discontinuous treatment	Curative treatment shock
Chlorine (free)	1 mg/L	10 mg/L for 8 h	100 mg/L for 1 h or 50 mg/L for 12 h or 15 mg/L for 24 h
Heat shock	60/50 °C in the network and 50 °C in bathrooms	70 °C for 30 min	/
PAA mixed with hydrogen peroxide	/	/	1 g/L equivalent H_2O_2 for 2 h

Different concentrations of free chlorine and exposure times were tested: 2.5, 5, 10 and 15 mg/L during 10 to 60 min at 25 °C. At the end of the treatment, free chlorine residual was neutralized by the addition in excess (5 μL/mL) of a solution of sterile sodium thiosulfate (0.1 mol/L).

Heat shock

Heat shocks were performed by incubation of cysts in a dry heat oven at different temperatures and exposure times. The cysts suspension was distributed in sterile 25 cm^3 tissue culture flasks and the flasks were incubated at 50, 55, 60 and 70 °C for 30 and 60 min. The control sample was incubated at 25 °C for 30 and 60 min.

PAA mixed with hydrogen peroxide

Experiments were performed with a commercial solution of PAA mixed with hydrogen peroxide (H_2O_2) (Ferrocid 8591, BKG Water Solutions). A solution at 100 g/L expressed in H_2O_2 equivalent was prepared. The cyst suspension was distributed in sterile 15 mL tubes and PAA-H_2O_2 treatments were applied with the previous solution. Different concentrations of this biocide and contact times were tested: 0.1, 0.5 and 1 g/L for 30 and 60 min at 25 °C. Seven minutes before the end of contact time, cysts were recovered by centrifugation ($1{,}000 \times g$ for 7 min), resuspended for 5 min in Dey-Engley neutralizing broth (D3435, Fluka Analytical) with 0.02% bovine catalase (C1345, Sigma) to neutralize

PAA and H_2O_2. Then cysts were centrifuged (1,000 × *g* for 7 min) and suspended in phosphate buffer (pH 7, 50 mmol/L).

Protease

Protease tests were performed using a commercial solution of subtilisin (protease isolated from *Bacillus licheniformis*, Novozymes). The cysts suspension was distributed in sterile 25 cm^3 tissue culture flasks and subtilisin treatments were applied. Flasks were incubated at 37 °C (optimal enzymatic activity) for 24 h with different concentrations of subtilisin: 0.625, 1.25 and 2.5 U/mL. The control sample was also incubated at 37 °C for 24 h.

Cyst viability

Cyst viability was evaluated by using the MPN method. For each sample a serial dilution ranging from 10^0 to 10^7 was performed in phosphate buffer (pH 7, 50 mmol/L). Each dilution was inoculated in triplicate on non-nutritive agar (15 g/L) and recovered by living *Escherichia coli* XL1-Blue. During 2 weeks of incubation at ambient temperature, the Petri plates were examined daily with an inverted microscope (CKX41, Olympus) to observe the presence or absence of trophozoites. According to these results, the number of viable cysts by mL for each sample was calculated using an MPN calculation programme (http://www.wiwiss.fu-berlin.de/institute/iso/mitarbeiter/wilrich/MPN_ver2.xls). Log_{10} reductions were calculated by comparison to the non-treated control. Limit of detection (LOD) of this analysis was set at log_{10} reduction = 6.

RESULTS

Chlorine treatment

The amoebicidal effect of chlorine on *V. vermiformis* cysts was assessed at different concentrations and treatment times (Figure 1). At 2.5 mg/L, chlorine had a low (less than 1 log) amoebicidal effect. At 5 mg/L, the 50237 strain and 172A strain had log_{10} reductions between 0.45 and 1 unit and between 0.95 and 1.7 units, respectively. Considering a 10 mg/L free chlorine concentration, the strain 50237 presented a log_{10} reduction between 2 and 3.4 units while the strain 172A was totally inactivated after a 10 min exposure. For the highest free chlorine concentration tested (15 mg/L), a 10 min exposure was enough to completely inactivate both strains. The results illustrate that the strain 172A might be slightly more sensitive than strain 50237. In addition, for a given concentration, there was hardly any difference between contact times.

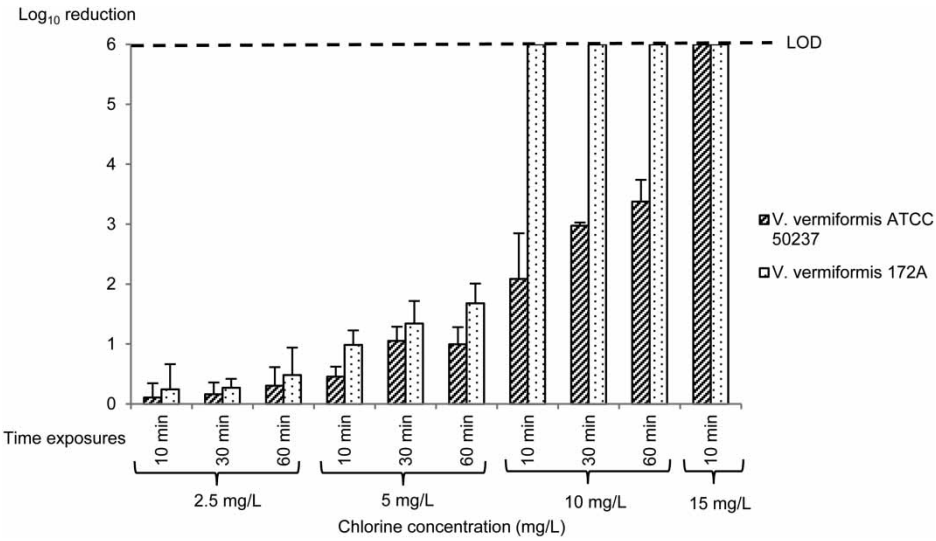

Figure 1 | Amoebicidal effect of chlorine at different concentrations and exposure times on *V. vermiformis* cysts (LOD).

Heat shock treatment

The cystidal effect of heat shock on *V. vermiformis* cyst activity was assessed at various temperatures and exposure times (Figure 2). At 50 °C, inactivation was low (up to 1 log). At 55 °C for 30 min, the $\log_{10}$ reductions were 2.7 units for the strain 50237 and 4.6 for the strain 172A. At the same temperature for a 60 min exposure, $\log_{10}$ reductions were approximately equal to 5.6 units for the two strains. At 60 and 70 °C, cysts of the two strains were completely inactivated after 30 min. The two strains showed a similar sensitivity to heat treatment. For a given temperature, the extension of exposure led to greater inactivation.

PAA mixed with hydrogen peroxide treatment

The amoebicidal effect of PAA mixed with H_2O_2 on *V. vermiformis* cysts was assessed at different concentrations and treatment times (Figure 3). For the strain 50237, at 0.1 g/L equivalent H_2O_2, $\log_{10}$ reductions were 0.68 and 1.25 units for 30 and 60 min exposures, respectively. For the strain 172A, at 0.1 g/L, $\log_{10}$ reductions were 1.28 and 2.37 units for 30 and 60 min, respectively. Cysts of the two strains were completely inactivated when 0.5 and 1 g/L equivalent H_2O_2 were applied for both 30 and 60 min.

Protease treatment

We evaluated the cystidal effect of subtilisin (protease isolated from *Bacillus licheniformis*) at various concentrations on *V. vermiformis* cysts (Figure 4). Subtilisin had a strong cystidal effect, as complete inactivation was obtained from 1.25 U/mL. For the strain 172A, at 0.625 U/mL, $\log_{10}$ reduction was approximately 5 units. Cysts of the 50237 strain were completely inactivated by the three tested concentrations.

DISCUSSION

V. vermiformis is a FLA frequently found in hot water networks (Rohr *et al.* 1998; Buse *et al.* 2013; Ovruskty *et al.* 2013) and very often associated with *Legionella pneumophila* and other pathogenic bacteria (Fields *et al.* 1990; Thomas *et al.* 2006a, 2006b). There are very few data in the literature on the resistance of *V. vermiformis* cysts to the disinfection treatments used in hot water networks. Therefore, the present study investigated the cyst sensitivity of *V. vermiformis* to chlorine, heat, and PAA mixed with H_2O_2, which are treatments frequently used in hot water networks. Biocide concentrations (chlorine and PAA mixed with H_2O_2) and heat shock temperatures were chosen according to the

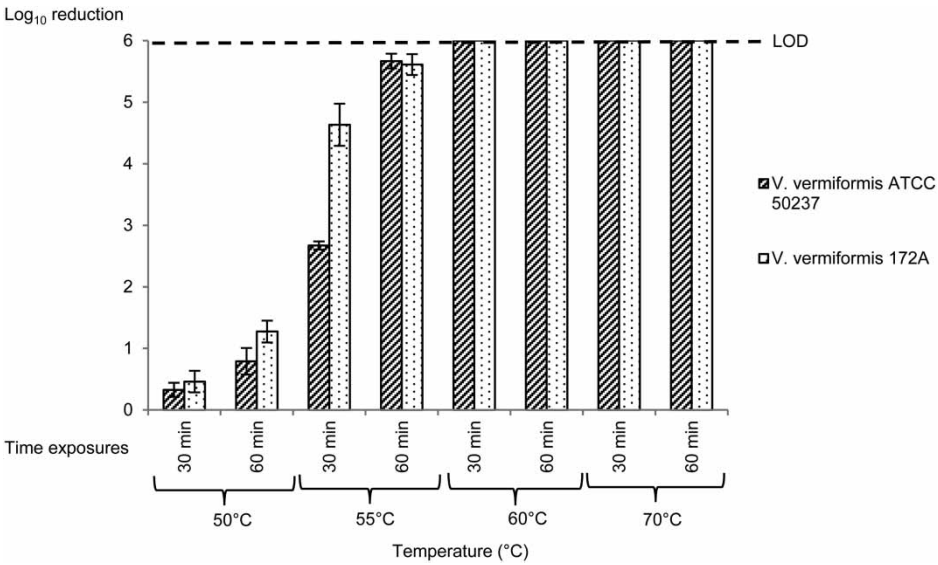

Figure 2 | Amoebicidal effect of temperature at different temperatures and exposure times on *V. vermiformis* cysts (LOD).

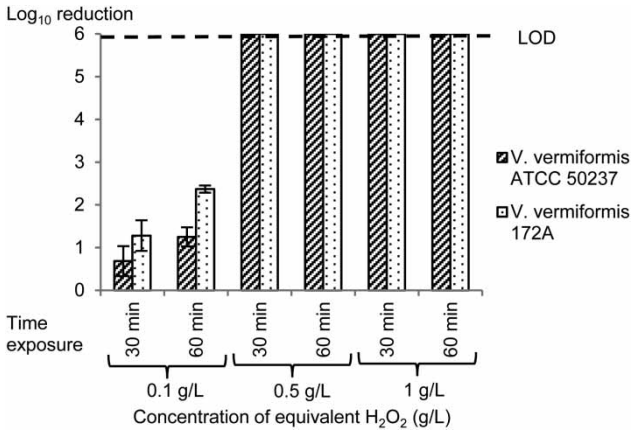

Figure 3 | Amoebicidal effect of PAA mixed with hydrogen peroxide at different concentrations and exposure times on *V. vermiformis* cysts (LOD).

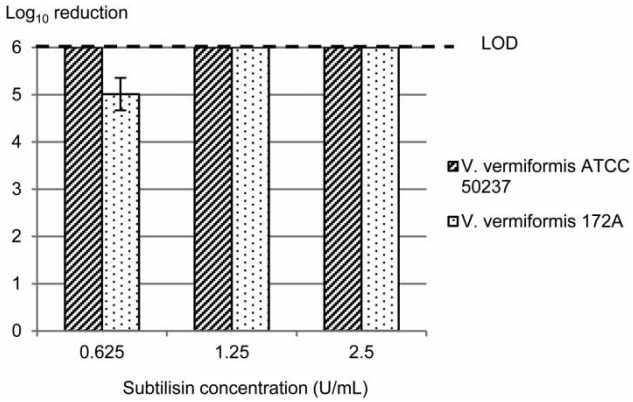

Figure 4 | Amoebicidal effect of subtilisin (protease) at different concentrations on *V. vermiformis* cysts (LOD).

French circular (DGS/SD7A/DHOS/EA No. 2002/243, 2002-04-22) in relation to *Legionella* risk prevention in health facilities. Also, sensitivity of *V. vermiformis* cysts to an innovative treatment, protease, was evaluated.

In hot water networks, chlorine curative shock treatments may vary from 15 mg/L to 100 mg/L for 1 h to 24 h exposures (Table 1). First, the sensitivity of *V. vermiformis* cysts to a 10 min exposure at 15 mg/L free chlorine was tested; the cysts were totally inactivated by this treatment. *In vitro*, free chlorine concentrations recommended by the French regulation (Table 1) for curative shock treatment in hot water systems seem to inactivate *V. vermiformis* cysts very efficiently. Then, free chlorine concentrations were decreased in order to define the sensitivity range of *V. vermiformis* cysts and also to compare the results with those already published. At 10 mg/L for 10, 30 and 60 min exposures, the sensitivity of *V. vermiformis* cysts varied depending on the strain. Indeed, $\log_{10}$ reductions for the strain 50237 were between 2 and 3.4 units, whereas 172A strain cysts were completely inactivated. These results are consistent with a previous study in which the authors tested the sensitivity of cysts of one strain of *V. vermiformis* to a 30 min exposure at 10 mg/L free chlorine and found that only 0.01% of cysts had resisted this treatment (Kuchta *et al.* 1993). At 5 mg/L, chlorine seems to have a limited action on *V. vermiformis* cysts (Kuchta *et al.* 1993). Nevertheless, another work (Critchley & Bentham 2009) found that 5 mg/L free chlorine was efficient when *V. vermiformis* cysts were exposed for 8 h at 25 °C. Finally, 2.5 mg/L were inefficient for the inactivation of *V. vermiformis* cysts, in agreement with Kuchta *et al.* (1993), who tested the sensitivity of *V. vermiformis* cysts to a 30 min exposure at 2 mg/L free chlorine and who noted that 49% of cysts resisted this treatment. For a given concentration, the duration of exposure had a limited effect suggesting that chlorine was rapidly consumed by cysts.

Water temperature in hot water networks is well regulated in France. At any point of a hot water network, the temperature must be above 50 °C (Table 1). Heat shocks can be used as a discontinuous treatment at 70 °C for at least 30 min (Table 1). Thus, the sensitivity of *V. vermiformis* cysts between 50 and 70 °C was studied. At 60 and 70 °C, cysts of both strains were completely inactivated. This shows that, in these conditions, the heat shock procedure was efficient to inactivate *V. vermiformis* cysts. These results were in accordance with an older study (Kuchta *et al.* 1993) which showed that cysts of a *V. vermiformis* strain were totally inactivated by a 30 min exposure at 60 °C. Also at 50 and 55 °C, the results were similar to this referenced work (Kuchta *et al.* 1993) which found that, for the same treatment, 0.05% and 16% of *V. vermiformis* cysts survived at 55 °C and 50 °C, respectively. Thus, *in vitro*, a temperature of 50 °C seemed to be poorly effective on the viability of *V. vermiformis* cysts.

PAA mixed with H_2O_2 can be used only as a curative shock treatment in hot water networks according to the following procedure: 2 h exposures at 1 g/L equivalent H_2O_2 (Table 1). Thus, the sensitivity of *V. vermiformis* cysts to an exposure at 1 g/L equivalent H_2O_2 for 30 and 60 min was tested. Cysts of the two strains tested were completely inactivated by this treatment and the same results were observed

when *V. vermiformis* cysts were exposed to 0.5 g/L equivalent H_2O_2. *In vitro*, concentrations of PAA mixed with H_2O_2 recommended by French regulation (Table 1) for curative treatments seemed to be very efficient at eliminating *V. vermiformis* cysts. These results are original since there is no publication referring to inactivation of *V. vermiformis* cysts, or of other FLA cysts, with PAA mixed with H_2O_2.

In vitro, all these procedures were very efficient at inactivating *V. vermiformis* cysts. Nevertheless, this study was limited to *in vitro* tests using axenical *V. vermiformis* cultures. In real hot water networks, there is a large microbial diversity and chemical biocides are consumed by all these microorganisms. The materials of which the network is made can consume part of the chemical disinfectants too. Furthermore, in hot water networks, the majority of FLA are not suspended in the aqueous phase but associated with biofilm. Several publications have underlined that biofilm can protect FLA from disinfection treatments (Srikanth & Berk 1993; Goudot *et al.* 2014). Therefore, to evaluate the real efficacy of these disinfection treatments in hot water networks, it will be necessary to test them in real networks or in pilot networks, as performed in applied research (Farhat *et al.* 2010, 2011).

The compilation of these results and literature clearly suggest that cysts of *V. vermiformis* are more sensitive to chlorine and heat than cysts of *Acanthamoeba* spp. and *Balamuthia mandrillaris*. For example, *A. culbertsoni* cysts resisted a concentration of 40 mg/L free chlorine for 3 h (De Jonckheere & van de Voorde 1976) and those of *A. polyphaga* resisted for 18 h at 50 mg/L (Kilvington & Price 1990). Cysts of *B. mandrillaris* have survived to a concentration of 25 mg/L free chlorine for 1 h (Siddiqui *et al.* 2008). Also, cysts of thermotolerant strains of *Acanthamoeba* have resisted moist heat at 80 °C for a 10 min exposure (Storey *et al.* 2004). In addition, cysts of *B. mandrillaris* were recognized to withstand a 60 min exposure at 60 °C and 70 °C (Siddiqui *et al.* 2008). Also, there is no clear-cut difference with the sensitivity of *Naegleria* cysts (De Jonckheere & van de Voorde 1976; Chang 1978).

Several studies have shown that conventional treatments are not efficient at eliminating FLA in water networks, so it is essential to develop innovative treatments (Rohr *et al.* 1998; Thomas *et al.* 2004; Loret *et al.* 2005; Loret & Greub 2010). Thus the efficacy of an original treatment with protease was tested because the cyst wall of *H. glebae*, a FLA close to *V. vermiformis*, mainly contains proteins (Upadhyay *et al.* 1984). Subtilisin was selected because it has recently been identified for its antifouling activity on marine biofilms (Leroy *et al.* 2008). First, a concentration of 2.5 U/mL was tested, and the cysts of the two strains of *V. vermiformis* were totally inactivated. Then, the concentration was decreased to 1.25 and 0.625 U/mL, and even the lowest concentration tested inactivated the cysts very efficiently. However, the subtilisin probably cannot be used as such in hot water networks. Indeed, its cost is important and its impact on the environment must be addressed. However, this type of approach can allow identification of new targets and ways to develop the next generation of disinfection treatments.

CONCLUSION

In conclusion, the efficacy of disinfection treatments frequently used in hot water networks on the cysts of two strains of *V. vermiformis* was tested. This study shows that cysts of *V. vermiformis* were fully inactivated under the following conditions: 15 mg/L of chlorine for 10 min, 60 °C for 30 min and 0.5 g/L equivalent H_2O_2 of PAA mixed with H_2O_2 for 30 min. It also underlines the strong efficacy of subtilisin in inactivating the cysts of *V. vermiformis*. Thus, the concentrations of biocides (chlorine and PAA mixed with H_2O_2), the heat shocks, and the exposure times recommended by French regulation (DGS/SD7A/DHOS/EA No. 2002/243 2002-04-22) were efficient *in vitro* in the inactivation of *V. vermiformis* cysts. Although further research needs to be conducted to work out the mechanisms involved in the response of cysts of *V. vermiformis* to disinfection treatments, this work is innovative in the management of health risks linked to FLA within hot water systems. The next step will be to evaluate the efficacy of these treatments in a pilot or a real hot water network.

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