Disinfection of *Burkholderia pseudomallei* in potable water

Kay Howard, Timothy J. J. Inglis*

Division of Microbiology and Infectious Diseases. Western Australian Centre fro Pathology and medical Research, Locked Bag 2009, Nedlands W A 6909, Australia

*Corresponding author
Telephone: +618-9346-3461; fax: +618-9381-7139.
Email address: Tim.Inglis@health.wa.gov.au

Disinfection of *Burkholderia pseudomallei* in potable water

Kay Howard, Timothy J. J. Inglis

Division of Microbiology and Infectious Diseases. Western Australian Centre fro Pathology and medical Research, Locked Bag 2009, Nedlands W A 6909, Australia

ABSTRACT

The effect of chlorine, monochloramine and UV disinfection on the water-borne pathogen *Burkholderia pseudomallei* was assessed. Persistence of *B. pseudomallei* was verified by MPN involving a one-step recovery procedure. Chlorine proved the most effective disinfectant with a 99.99% reduction of a 10^6 CFU/mL pure bacterial culture followed by 99.9% reduction by monochloramine and 99% reduction by UV. Co-culture of *B. pseudomallei* with *Acanthamoeba astronyxis* was found to greatly enhance survival of *B. pseudomallei* in the presence of all disinfecting agents tested. For example, when amoebae were present 100 times more monochloramine was required to maintain the disinfectant efficacy. Given the results obtained from these co-culture experiments, more research is needed to investigate the role of amoeba and biofilms in survival of *B. pseudomallei* in potable water.

Keywords: Burkholderia pseudomallei, Acanthamoeba astronyxis, Disinfection, Melioidosis, Drinking water

1.0 Introduction

Burkholderia pseudomallei causes the potentially fatal infection known as melioidosis. A recent small outbreak of melioidosis in a northern Australian community occurred during failure of the community's potable water supply chlorine treatment plant (Inglis *et al*., 1999; Inglis *et al*., 2000). There has also been evidence that the water supply may have been the principal means of infection in other melioidosis case clusters (Ketterer *et al*., 1986; Currie and Fisher, 2000).

The minimum residual chlorine concentration and contact time of 1 mg/L chlorine for 30 min, as prescribed by potable water providers in Australia, was insufficient to reduce a *B. pseudomallei* population by more than 2 $\log_{10}$ (Howard and Inglis, 2003a). Using flow cytometry to estimate viable bacteria and a recovery procedure to determine survival of chlorine-treated *B. pseudomallei* suspensions, the recovery of viable bacteria from water containing up to 1000 mg/L free chlorine indicated that chlorine had a bacteriostatic effect on *B. pseudomallei* (Howard and Inglis, 2003a). This study highlighted the need for assessment of alternative water disinfection processes for *B. pseudomallei*.

Intra-protozoal growth of bacterial pathogens has been associated with increased environmental survival, enhanced virulence, and resistance to biocides and antibiotics (Kilvington and Price, 1990). Protozoa may serve as reservoirs for bacteria with human pathogenic potential (Marciano-Cabral and Cabral, 2003). These protozoa can survive and grow after exposure to levels of free chlorine residuals that kill free-living bacteria. For example, *Salmonella* and *Legionella* were greater than 50 times more resistant to free chlorine when ingested by *Tetrahymena pyriformis* (King *et al*., 1988). Both trophozoites and cysts of *Acanthamoeba* can retain viable bacteria. Endamebic bacterial survival of *B. pseudomallei* has recently been observed in *Acanthamoeba* (Inglis *et al*., 2000).

Monochloramine, another oxidant, is a weaker disinfectant than free chlorine (Kouame and Haas, 1991) but has been shown to be superior on some indicator

organisms (Pretorius and Pretorius, 1999) and has greater penetrating power on biofilms compared to free chlorine. UV light disinfects by damaging bacterial DNA to prevent replication (Gadgil *et al*., 1997). The UV dose for a 90% bactericidal effect ranges from 380 to 5500 $\mu Ws.cm^{-2}$, while *Giardia, Cryptosporidium* and cysts required 60 000 to 80 000 $\mu Ws.cm^{-2}$ minimum (Wolfe, 1990).

In this paper we compare the effectiveness of chlorine, monochloramine and UV light for the disinfection of *B. pseudomallei* from drinking water in the presence and absence of *Acanthamoeba astronyxis* trophozoites.

2.0 Methods

2.1 Bacteria

B. pseudomallei strains for this study were selected from a group of 45 isolates previously tested for tolerance to chlorine (Howard and Inglis, 2003a). Three of the most tolerant strains (NCTC 13177, NCTC 10276 and BCC11) were selected for most of the experiments. In addition, BCC16 and BCC30 (less tolerant to chlorine), BCC11 (persistently mucoid) and BCC69 (mucoid) were also included for comparison.

Bacterial strains were stored in 15% glycerol in brain heart infusion broth at -70°C and incubated on blood agar for a maximum of 3 days, then transferred to 10 mL trypticase soy broth (TSB) for 18 h incubation at 37ºC in the dark. Prior to use in the experiments, these overnight cultures were washed twice in cold sterile water after centrifugation at 300 *g* for 15 min at room temperature. They were incubated in water at room temperature for 2 h to bring the cultures to mid-lag phase, before exposure to disinfectant or amoeba.

2.2 Amoebae

A. astronyxis (WACC111) was grown axenically at 30ºC in 25mL PYG (peptone, yeast extract, glucose) broth containing 100 mg/L penicillin and 60 mg/L streptomycin (Excel Laboratory Products, Belmont, Western Australia). After 14 days incubation as monolayers in tissue culture flasks, the amoeba were harvested by centrifugation (300 *g*, 5 min, room temperature) washed once and resuspended to give a density of 10^7 trophozoites/mL. The concentration of amoebae was determined by counting viable trophozoites in a Kova® cell (Hycor Biomedical Inc., Garden Grove, California) with 0.04% trypan blue.

2.3 Co-cultures

A. astronyxis suspensions were exposed to mid lag phase cultures (Inglis *et al*., 2000) of *B. pseudomallei* (strain NCTC 13177) to yield a multiplicity of infection of

approximately 10 amoebae (10^6) per bacteria (10^5). The tubes were gently rolled to mix the two suspensions and left for 10 min. The co-cultures were washed once and resuspended in water, or phosphate buffer (pH 8) for the monochloramine experiments, to facilitate ingestion of the bacteria, and incubated at room temperature in the dark for 1 h. In this study the term co-culture is used for a 10:1 ratio of *A. astronyxis* (WACC111) / *B. pseudomallei* (NCTC 13177).

2.4 Disinfection methods

2.4.1 Chlorine

Chlorine was added to cell suspensions as a solution of sodium hypochlorite. Concentrations of free chlorine (Cl_2, HOCl, OCl^-) were measured ($\pm$ 0.02 mg/L) using a pocket colorimeter analysis system (HACH Test Kit, HACH Company, Colorado, USA). All water used for experiments was microfiltered at 18Ω.

The three tolerant strains of bacteria (each at 10^6 CFU/mL) were exposed to 0, 0.25, 0.5, 1, 2 and 4 mg/L chlorine for 30 min. Four concentrations (10^3, 10^4, 10^5 and 10^6 CFU/mL) of these three strains of bacteria were also exposed to 1 mg/L chlorine for 30 min to determine the effect of cell density on efficacy of chlorine disinfection. All six strains of bacteria were exposed to 1 mg/L chlorine for 30 min.

Survival of amoebae 10^2, 10^3, 10^4 and 10^5 trophozoites/mL was determined after 30 min exposure to 0, 5, 10, 20, 50 or 100 mg/L chlorine.

Co-cultures and 10^5 CFU/mL bacteria (controls) were exposed to 0, 10, 20, 50 and 100 mg/L chlorine for 30 min. The treatment was ended by dilution and the cultures were washed twice by centrifugation, resuspended in water, incubated at 4ºC for 30 min, then sonicated (15 min, Branson B-12 ultrasonic bath) to disrupt amoeba membranes. Viable counts of bacteria were done using the most probable number (MPN) method (Howard and Inglis, 2003a).

2.4.2 Monochloramine

NaOCl and NH_4Cl were combined in a 4:1 ratio and the solutions were tested with a pocket colorimeter analysis system to ensure that there was no free chlorine present. All bacterial, amoebae and co-culture suspensions were made in phosphate buffer (pH 8) for monochloramine treatments.

The three chlorine tolerant strains of bacteria were exposed to 0, 0.5, 1, 2 and 4 mg/L monochloramine. Aliquots to determine survival were taken at 1, 2 and 7 days. An untreated control for each strain was used to determine the reductive power of the monochloramine. Three concentrations (10^4, 10^5 and 10^6 CFU/mL) of these three strains of bacteria were also exposed to 1 mg/L monochloramine for 24 h to determine the effect of cell density on efficacy of chlorine disinfection. All six strains of bacteria were exposed to 1 mg/L monochloramine for 24, 48 and 72h.

Survival of 10^2, 10^3, 10^4 and 10^5 amoebal trophozoites/mL was determined after 24 h exposure to 0, 10, 20, 50 or 100 mg/L monochloramine in phosphate buffer (pH 8). Co-cultures were exposed to 0, 1, 5, 10, 50 and 100 mg/L monochloramine and aliquots were taken at 3 h, 24 h and 7 days. The aliquots were washed twice, incubated at 4ºC for 30 min, and then viable counts done by MPN.

2.4.3 UV light

The UV treatment used a Spectroline model EA-160/FC (Spectronics Corp., New York, USA) 365 nm wavelength, 230v 50hz 0.17 amp UV lamp at 5 L/min producing approximately 90 000 $\mu Ws.cm^{-2}$.

The three chlorine tolerant strains of bacteria at three concentrations (10^4, 10^5 and 10^6 CFU/mL), then all six strains (10^6 CFU/mL) of *B. pseudomallei*, were exposed to UV. Survival of amoebae (10^2, 10^3, 10^4 and 10^5 trophozoites/mL) and bacteria from a co-culture was determined after exposing to UV. All cultures were centrifuged after exposure before viable counts.

2.5 Recovery and Counting Methods

2.5.1 Bacteria

The concentration of bacteria (CFU/mL) prior to treatment was determined by plating in triplicate on plate count agar (PCA) using a spiral plater (Don Whitely Scientific Ltd, Shipley, UK) and by MPN. There were 10 replicate tubes for each MPN dilution. Each tube with growth was plated onto PCA for confirmation of *B. pseudomallei* recovery.

2.5.2 Amoebae

The cultures were washed twice and 5 x 10 µL aliquots of the washed pellet were dropped onto non-nutrient agar (NNA) plates spread with a concentrated suspension of live *Escherichia coli* (WACC9) to ascertain if there were surviving amoebae after each treatment. The plates were examined at 40x magnification daily for evidence of migration from the inoculation zone.

Results

3.1 Bacteria

Viable bacteria were recovered from all chlorine, monochloramine and UV concentrations tested. For the shortest exposure time tested, chlorine was the most effective disinfectant agent, for three strains of bacteria (Figure 1) with a 10-fold greater reduction than either monochloramine or UV. There was a more marked difference in the sensitivity of the mucoid strain which was least affected by UV.

As the concentration of monochloramine increased there a continual decline in survival, in contrast, there was no extra reduction in survival after 1 mg/L chlorine (Figure 2). Monochloramine at 4 mg/L had a greater disinfectant power than chlorine at 4 mg/L when bacteria were exposed for 48 h. Increasing the bacterial concentration increased the survival rate of *B. pseudomallei* in chlorine, monochloramine and UV (Figure 3). In each treatment there was no difference ($p=0.08$) in survival when starting with concentrations of 10^4 or 10^5 CFU/mL.

There was no difference ($p=0.1$) in the effectiveness of monochloramine or UV on the chlorine "tolerant" and "sensitive" strains (Figure 4). However, the mucoid strains were more sensitive to monochloramine ($p=0.04$) and more resistant to UV ($p=0.01$) than the non-mucoid strains. The mucoid strains (BCC11 and BCC69) had a higher resistance ($p=0.02$) to chlorine than the chlorine "sensitive" strains.

3.2 Amoebae

The amoeba survived all three disinfectants at all concentrations tested. Migration across the NNA plates was observed at all trophozoite concentrations and all disinfectant concentrations. While at 50 and 100 mg/L chlorine, and 50 and 100 mg/L monochloramine, there was considerable reduction in migration rates, more than 2 migration zones were seen from each aliquot of approximately 200 live amoebae/mL.

3.3 Co-cultures

When co-cultured with amoebae, there was no reduction ($p=0.06$) in the bacterial concentration by any of the disinfecting methods (Figure 5).

Bacterial recovery from co-cultures was significantly higher ($p = 0.006$) than the pure culture at all chlorine concentrations tested (Figure 6a). In the presence of amoebae, *B. pseudomallei* were 1000- and 10,000-fold more resistant to 1 mg/L and 10 mg/L chlorine, respectively. At 100 mg/L chlorine, the presence of amoebae conferred a 100-fold increase in survival of bacteria.

Over time, the number of surviving bacteria increased in the presence of amoebae consistent with bacterial replication within amoebae. At 1 and 10 mg monochloramine/L, there was a 1000-10,000-fold reduction on pure cultures at 24 and 48 h, and 7 days (Figures 6b, c and d), while there was no effect ($p=0.08$) when amoebae were present.

4.0 Discussion

This study highlights the capacity of *B. pseudomallei* to survive in treated potable water. Chlorine proved the most effective disinfectant of 10^6 CFU/mL pure bacterial cultures with a 99.99% reduction, followed by 99.9% reduction by monochloramine and 99% reduction by UV. This is in agreement with the findings of Kim *et al*. (2002) who showed that, in general, oxidising disinfectants (chlorine and monochloramine in this study) were more effective than non-oxidising ones against *Legionella* in water systems. The reduction by chlorine calculated by MPN in this study is greater than those estimates produced by flow cytometry (Howard and Inglis, 2003a), however flow cytometry was not used due to the lower detection limit of 10^4 CFU/mL. Underestimation of viable bacteria by MPN can also occur due to aggregates formed by bacteria, but the large number of MPN tubes used in this study has provided a more sensitive quantification of the viable cells than flow cytometry.

In pure bacterial cultures, disinfection is density dependant, with UV treatment least effective at the highest bacterial concentration. There is a significant reduction in the disinfectant power of monochloramine and UV when the bacterial population is increased from 10^5 to 10^6 CFU/mL. However, monochloramine can be utilised as a successful disinfectant if there is sufficient retention time as it can reduce the viability of *B. pseudomallei* by a greater degree than chlorine. At 7 days the reduction in viable cells can reach 99.9999% of a population of 10^6 CFU/mL in pure culture at as low as 0.5 mg/L monochloramine.

Survival of groups of chlorine “tolerant”, chlorine “sensitive” and mucoid strains from the earlier study (Howard and Inglis, 2003a) showed a difference between the three disinfecting agents used in this study. When exposed to chlorine the mucoid strains were as resistant to chlorine as the tolerant group. This is in contrast to Morris *et al*. (1996) who showed rugose variants of *Vibrio cholerae* displayed

resistance to killing by chlorine due to expression of an amorphous exopolysaccharide that promotes cell aggregation. However, monochloramine is more effective at reducing survival of mucoid strains than non-mucoid strains, while UV was least effective at killing mucoid strains. The reasons for these differences in survival have not been explored further. The mucoid strain may be least affected by UV due to protection against UV light by the mucoid polysaccharide coating. This is an important finding as mucoid strains may have evaded detection in diagnostic laboratories due to limitations of isolation techniques. These identification problems may be more prevalent than previously thought. The recent development of a selective agar designed to improve recovery of the more easily inhibited strains of *B. pseudomallei* (Howard and Inglis, 2003b) is expected to increase our ability to isolate the mucoid strains from environmental and clinical specimens.

Pure cultures of amoebae were able to survive all disinfecting agents. At most there was a 99.9% reduction in amoeba. Migration of trophozoites showed a rapid recovery of *Acanthamoeba* and contradicts the earlier findings of Cursons *et al*. (1980) that 1.25 mg/L of total available chlorine was amoebicidal after 30 min exposure for 10^4 amoebic cells/mL of two *Acanthamoeba* species. Amoebic trophozoites probably encyst in response to the disinfecting agents resulting in greater protection for the bacteria. In an equivalent study, Kilvington and Price (1990) reported cysts produced from co-cultures of *Legionella* with *A. polyphaga* protected the bacteria from 50 mg/L free chlorine.

Co-culture of *B. pseudomallei* with *A. astronyxis* greatly enhanced survival of *B. pseudomallei* in the presence of the disinfecting agents tested. As amoebae occur commonly in drinking water supplies, this may be an important mechanism for persistence of *B. pseudomallei.* Aquatic amoebae play a central role in *Legionella* ecology by supporting intracellular multiplication and providing protection against a

hostile environment. *Acanthamoebae* also protect *Legionella* against disinfecting agents (Cirillo *et al*., 1999). In view of the results of this study, this effect is likely to hold true for *B. pseudomallei*.

When amoebae are present, 100 times more monochloramine is required to maintain the disinfectant efficacy. There are at least two possible explanations. Bacteria are capable of low level intracellular multiplication within protozoa, for example, a 7-fold increase in *B. cepacia* per *Acanthamoeba* trophozoite occurs after co-culturing *in vitro* (Marolda *et al*., 1999). A 10-fold increase was seen in the 7 day co-cultures of *B. pseudomallei* and *A. astronyxis* in the current study. A second reason for enhanced bacterial survival in the presence of amoebae may be the greater disinfectant demand created by the amoebae.

Intracellular survival within amoebae may induce a stress-resistant phenotype (James *et al*., 1999) which may manifest as an increased resistance to disinfectant agents. James *et al*. (1999) proposed that as well as providing protection and supporting proliferation in hostile environments, amoebae may contribute to bacterial persistence by inducing a polyhydroxybutyrate rich phenotype that is more physiologically prepared for extracellular survival in low nutrient environments.

While the recommended dosage of the disinfectant agents will never totally eradicate *B. pseudomallei*, they can significantly reduce the concentration of the pathogen in the absence of amoebae. We believe that there are several lines of research to be undertaken to ensure that water supplies in endemic areas can be monitored for potential contamination by *B. pseudomallei.* Firstly, it is important to monitor potable water supplies in endemic regions for the presence of amoeba as well as *B. pseudomallei*, and it will be essential to assess the ability of *B. pseudomallei* to survive and multiple within a range of commonly found aquatic

amoebae. Formation of biofilms containing amoebae and/or *B. pseudomallei* is likely to enhance the survival of this bacterium and therefore the effect of disinfecting agents on *B. pseudomallei* biofilms warrants investigation. Further studies into the effect of sequential disinfecting methods should be considered, as they could be employed in areas where disease is present. Finally, it has not been determined if *B. pseudomallei* damaged by disinfectants, or after survival within amoebic hosts, are more virulent than an untreated bacterial population.

Acknowledgements

This work was sponsored by the CRC for Water Quality and Treatment which is supported by the Australian Government's Cooperative Research Centres Program. In particular, we thank Dr Martha Sinclair of Monash University, Darryl Day of NT PowerWater and Dr Chris Saint of the Australian Water Quality Centre. We gratefully acknowledge the gift of *Burkholderia* isolates used in this study by Professor BJ Currie and Dr G Lum, Northern Territory and Dr R Norton, Queensland. We thank our colleagues at PathCentre for their support. We appreciate the information and equipment supplied by the water service providers and extend special thanks to Water Corporation of WA members. We also thank Professor Tom Riley of The University of Western Australia for reviewing this manuscript.

References

Cirillo, J., Cirillo, S., Yan, L., Bermudez, L., Falkow, S. and Tompkins, L. (1999) Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infection & Immunity*, **67,** 4427-4434.

Currie, B. J. and Fisher, D.A. (2000) The epidemiology of melioidosis in Australia and Papua New Guinea. *Acta Tropica* **74,** 121-127.

Cursons, R. T. M., Brown, T. J. and Keys, E. A. (1980) Effect of disinfectants on pathogenic free-living amoebae in axenic conditions. *Applied and Enivronmental Microbiology*, **40,** 62-66.

Gadgil, A., Drescher, A., Greene, D., Miller, P., Motau, C. and Stevens, F. (1997) Field-testing UV disinfection of drinking water. *Water and Sanitation for All. 23rd WEDC Conference*, Durban, South Africa, Water Engineering Development Centre, University of Loughborough, UK.

Howard, K. and Inglis, T. J. (2003a) The effect of free chlorine on *Burkholderia pseudomallei* in potable water. *Water Research*, **37,** 4425-4432.

Howard, K. and Inglis, T. J. (2003b) Novel selective medium for the isolation of *Burkholderia pseudomallei*. *Journal of Clinical Microbiology*, **41,** 3312-3316.

Inglis, T. J., Garrow, S. C., Adams, C., Henderson, M., Mayo, M. and Currie, B. J. (1999) Acute melioidosis outbreak in Western Australia. *Epidemiology & Infection* **123,** 437-43.

Inglis, T. J., Rigby P., Robertson, T. A., Dutton, N. S., Henderson, M., and Chang, B. J. (2000) Interaction between *Burkholderia pseudomallei* and *Acanthamoeba* species results in coiling phagocytosis, endamebic bacterial survival, and escape. *Infection & Immunity* **68,** 1681-1686.

James, B., Mauchline, W., Dennis, P., Keevil, W. and Wait, R. (1999) Poly-3-hydroxybutyrate in *Legionella pneumophila*, and energy source for survival in low-nutrient environments. *Applied and Environmental Microbiology*, **65,** 822-827.

Ketterer, P. J., Webster, W. R., Shield, J., Arthur, R. J., Blackall, P. J. and Thomas, A. D. (1986) Melioidosis in intensive piggeries in south eastern Queensland. *Australian Veterinarian Journal* **63,** 146-149.

Kilvington, S. and Price, J. (1990) Survival of *Legionella pneumophilia* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *Journal of Applied Bacteriology* **68,** 519-525.

Kim, B. R., Anderson, J. E., Mueller, S. A., Gaines, W. A. and Kendall, A. M. (2002) Literature review - efficacy of various disinfectants against *Legionella* in water systems. *Water Research*, **36,** 4433-4444.

King, C. H., Shotts, E. B., Wooley, R. E. and Porter, K. G. (1988) Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Appl Environ Microbiol* **54,** 3023-3033.

Kouame, Y. and Haas, C. N. (1991) Inactivation of *Escherichia coli* by combined action of free chlorine and monochloramine. *Water Research*,**25,** 1027-1032.

Marciano-Cabral, F. and Cabral, G. (2003) *Acanthamoeba* spp. as agents of disease in humans. *Clinical Microbiological Reviews* **16,** 273-307.

Marolda, C., Hauroder, B., Michel, M. J. R and Valvano, M. (1999): Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae. *Microbiology* **145,** 1509-1517.

Morris, J. G. Jr., Sztein, M. B., Rice, E. W., Nataro, J. P., Losonsky, G. A., Panigrahi, P., Tacket, C. O. and Johnson, J. A. (1996) *Vibrio cholerae* O1 can assume a chlorine-resistant rugose survival form that is virulent for humans. *J Infect Dis*, **174,** 1364-1368.

Pretorius, P. C. and Pretorius, W. A. (1999) Disinfection of purified sewage effluent with monochloramine. *Water SA* **25,** 463-467.

Wolfe, R. L. (1990) Ultraviolet disinfection of potable water. *Environmental Science and Technology* **24,** 768-773.

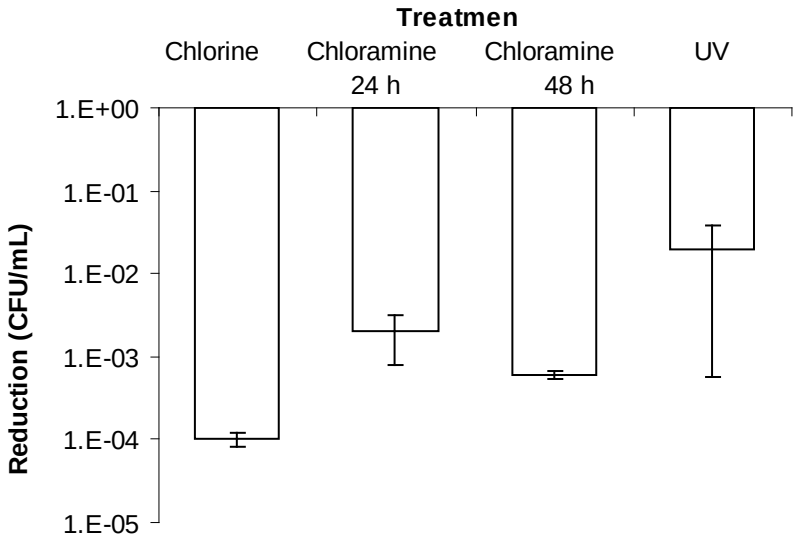

Figure 1 Mean reduction in viable cell counts of 10^6 CFU/mL of 3 strains of *Burkholderia pseudomallei* (NCTC 13177, NCTC 10276 and BCC11) subjected to chlorine (1 mg/L for 30 min), monochloramine (1 mg/L for 24 and 48 h) or UV (90 000 $\mu Ws.cm^{-2}$).

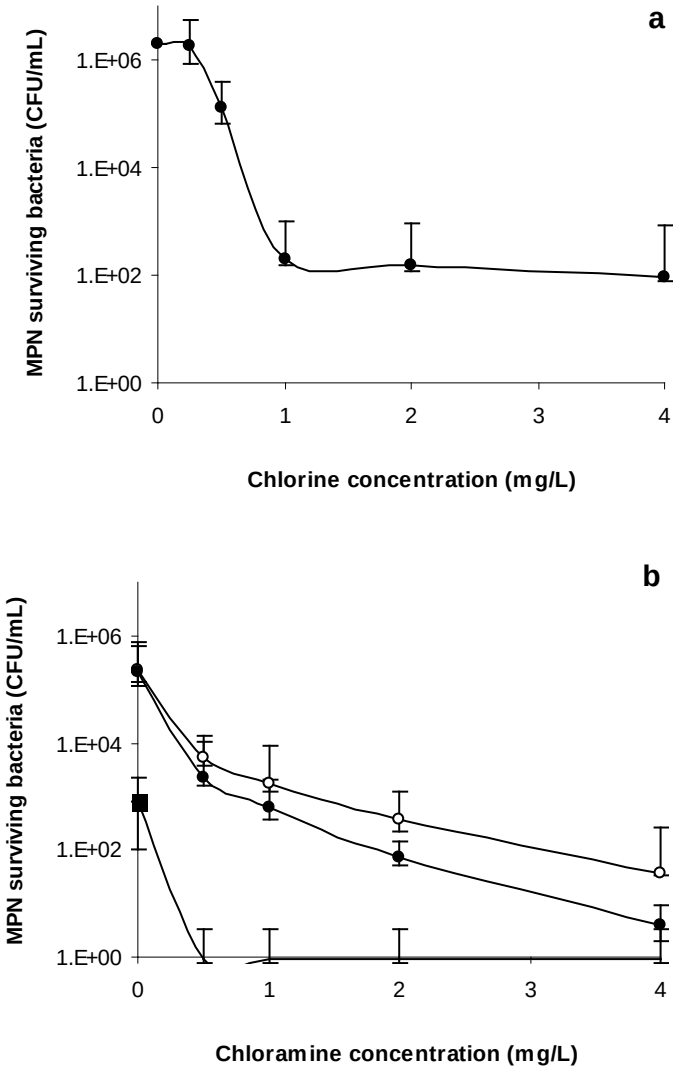

Figure 2 The effect of a) chlorine (30 min) and b) monochloramine (O 24 h, ● 48 h, and ■ 7 days) on survival of 3 strains of 10^6 CFU/mL *Burkholderia pseudomallei*. Viable bacteria presented as most probable number of colony forming units/mL.

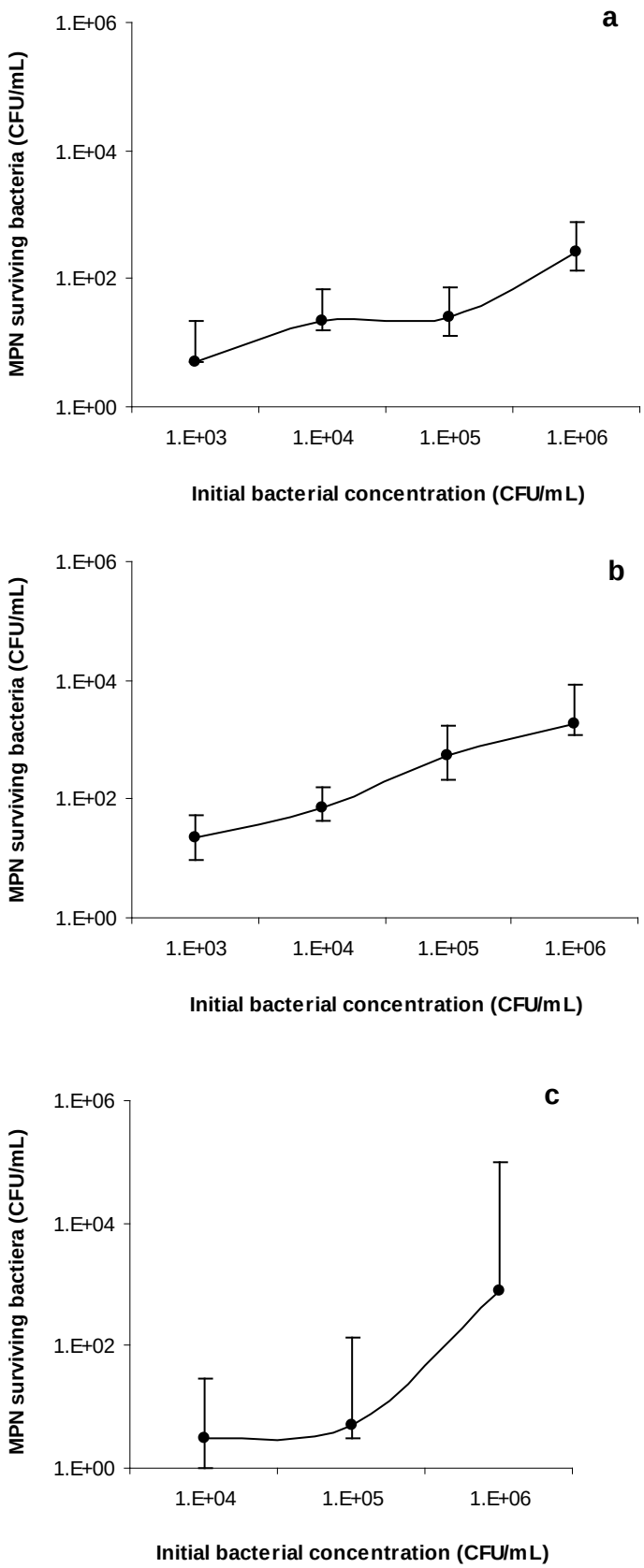

Figure 3 The effect of a) 1 mg/L chlorine, b) 1 mg/L monochloramine and c) UV (90 000 μWs.cm^{-2}) on the survival of different starting concentrations of 3 strains of *Burkholderia pseudomallei* (NCTC 13177, NCTC 10276 and BCC11). Surviving bacteria presented as most probable number of colony forming units/mL.

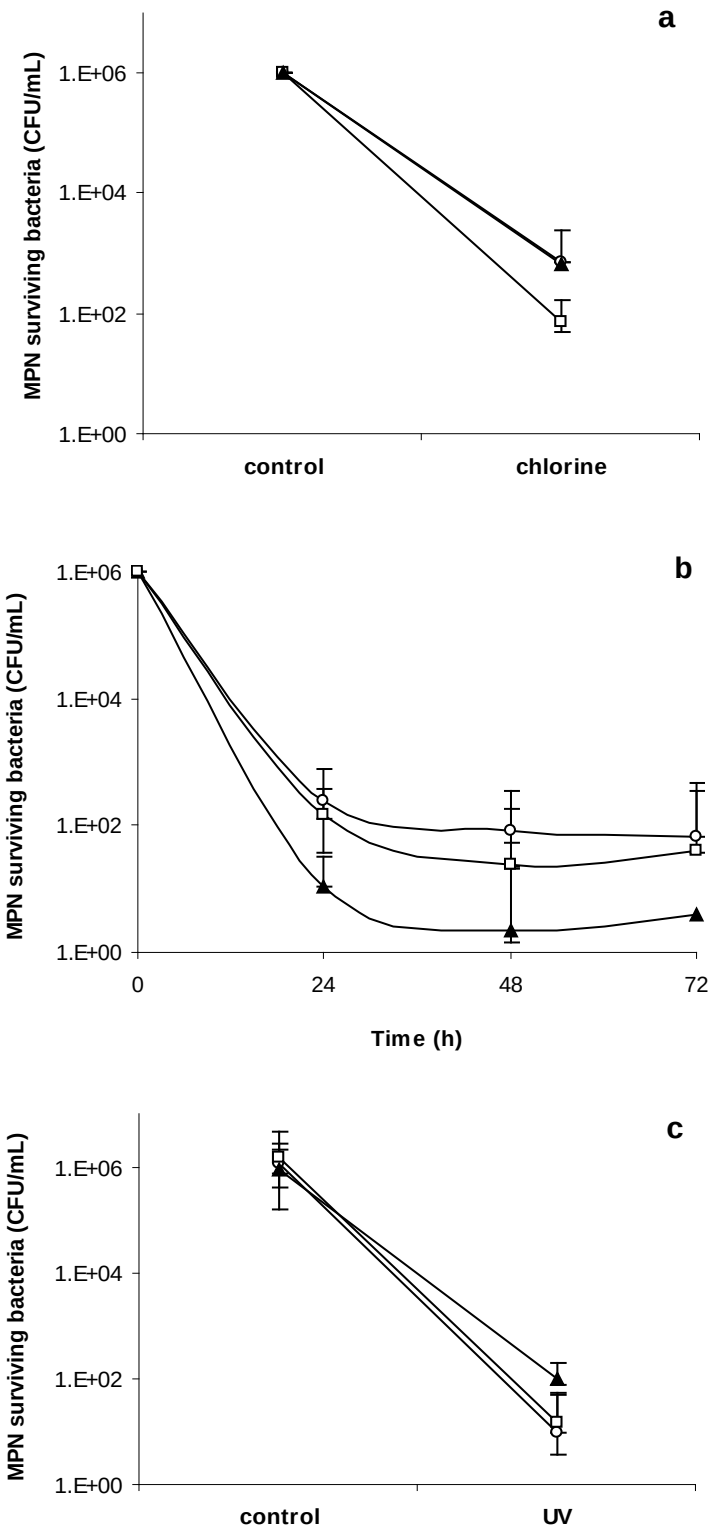

Figure 4 Effect of a) chlorine, b) monochloramine and c) UV on survival of chlorine sensitive strains (□), chlorine tolerant strains (○) and mucoid strains (▲) of *Burkholderia pseudomallei*.

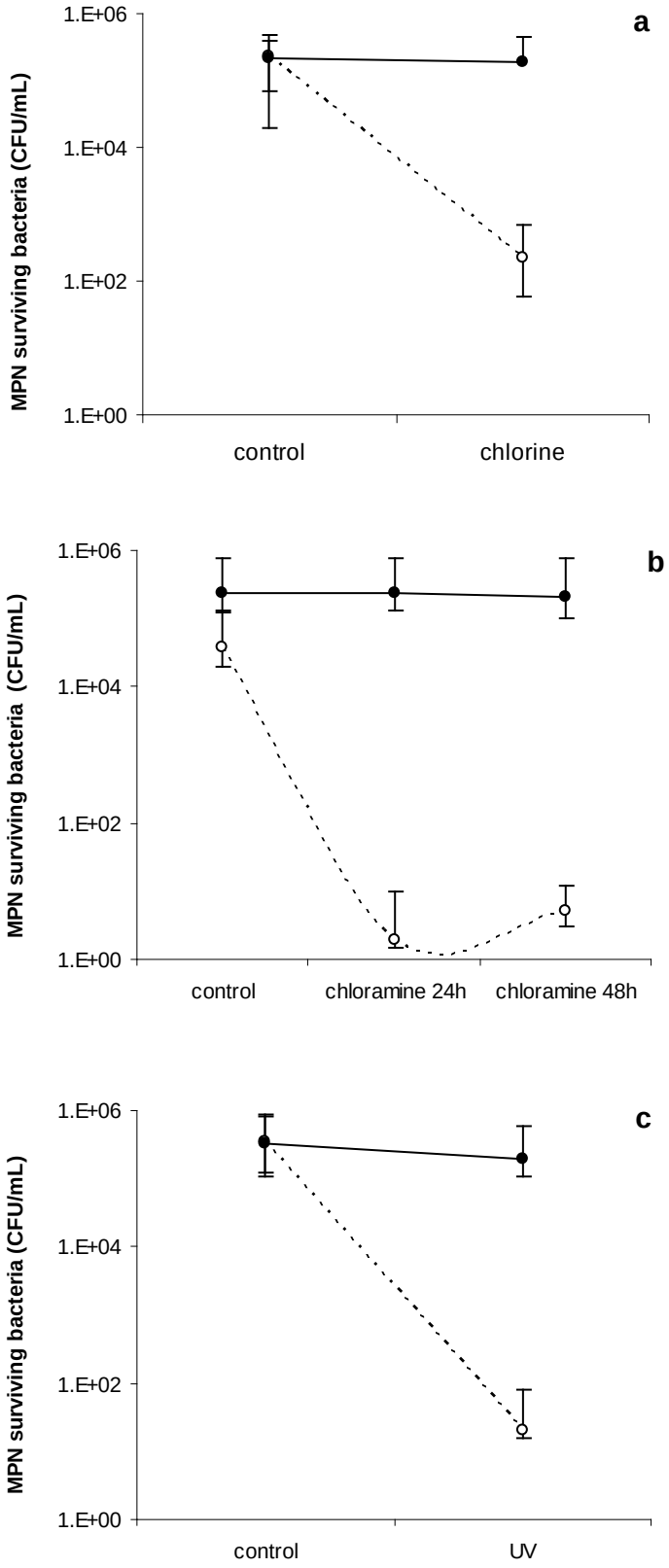

Figure 5 Survival of pure bacterial cultures (dashed line) of *Burkholderia pseudomallei* (NCTC 13177 at 10^5 CFU/mL) compared to a co-culture with *Acanthamoeba astronyxis* (10^6 trophozoites/mL) after a) 30 min exposure to 1 mg/L chlorine, b) 1 mg/L monochloramine for 24 h, c) and 48 h and d) UV (90 000 µWs.cm^{-2}). Viable counts determined by most probable number. Error bars are 95% confidence interval.

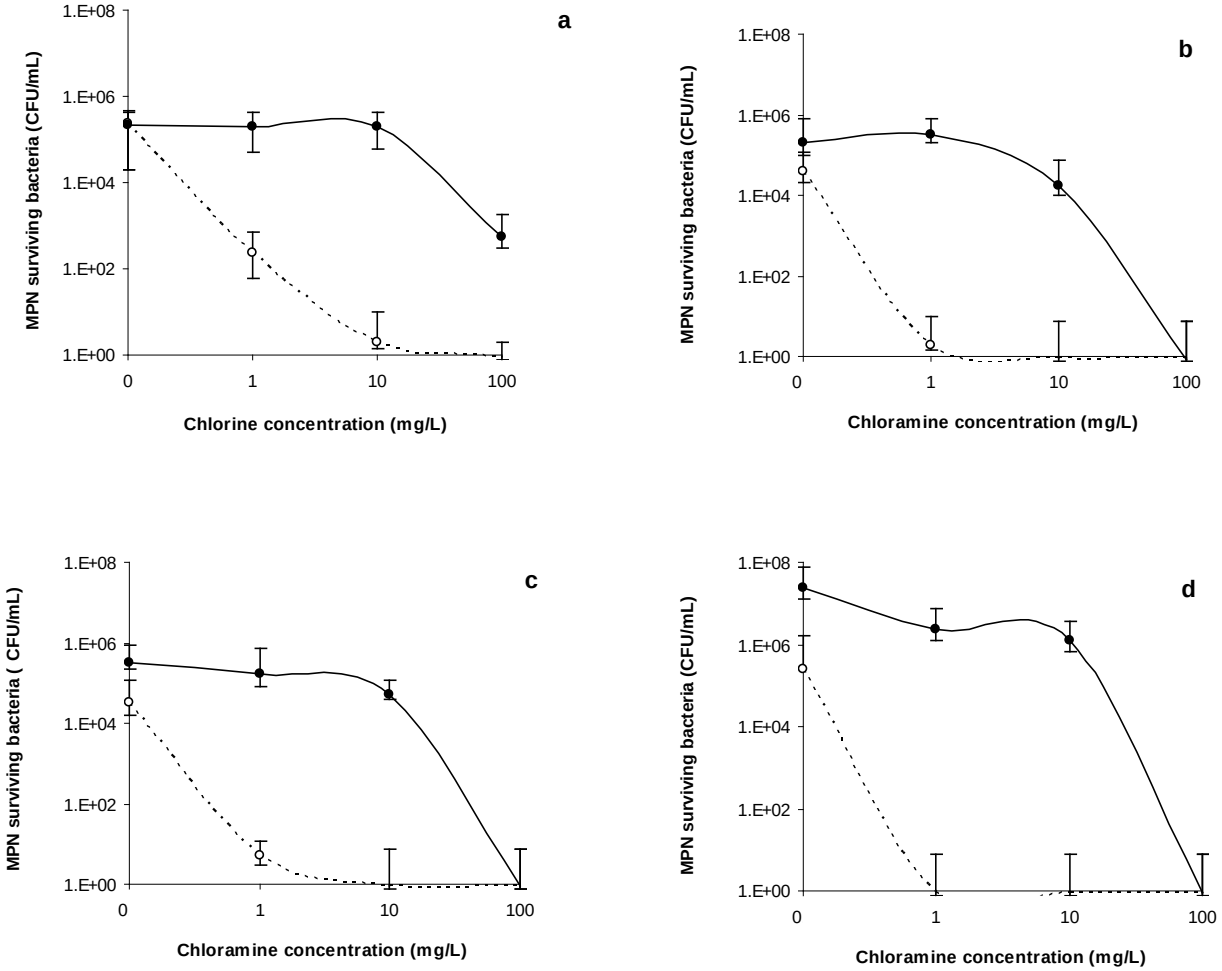

Figure 6 Survival of pure bacterial cultures (dashed line) of *Burkholderia pseudomallei* (NCTC 13177 at 10^5 CFU/mL) compared to a co-culture with *Acanthamoeba astronyxis* (10^6 trophozoites/mL) after a) 30 min exposure to 1 mg/L chlorine, b) 24 h, c) 48 h and d) 7 day exposure to 1 mg/L monochloramine.