

# The effect of various abiotic factors on the survival growth of *Escherichia coli* and *Pseudomonas aeruginosa* in bathroom greywater

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## ABSTRACT

This study was conducted to examine the effect of various abiotic (non-living chemical and physical) factors, which enhance or limit the growth of *Escherichia coli* and *Pseudomonas aeruginosa* in bathroom greywater (GW). These factors included: temperature ( $6 \pm 2$ ,  $23 \pm 2$  and  $42 \pm 2$  °C); aeration (aerobic and anaerobic); salinity (1.75 and 3.5%); GW with deionised water (DW) (50% GW:50% DW); and nutrient-rich and -poor medium. The survival period and decay constant ( $K$ ) were less at  $42 \pm 2$  °C than at  $23 \pm 2$  °C and  $6 \pm 2$  °C. The optimum survival of *E. coli* and *P. aeruginosa* was measured under aerobic and anaerobic condition, respectively. *P. aeruginosa* showed better survival in low salinity concentrations than *E. coli*. Cell size and zeta potential were also measured for both species under the test conditions.

**Key words** | abiotic factors, decay constant, greywater, pathogenic microorganism

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## INTRODUCTION

Greywater (GW) is usually defined as all the wastewater produced in a household except toilet wastes (black water). Typically, GW includes water from bathroom sinks, baths and showers, and may also include water from laundry facilities and dishwashers. Some definitions include wastewater from kitchen sinks although there is no consensus on this (Queensland Government 2003). GW represents the largest potential source of water savings in domestic residences, accounting for as much as 50–80% of the total water uses (Christova-Boal *et al.* 1996; Kargi & Dincer 1996; Eriksson *et al.* 2002; Jenssen & Vrale 2003; Flowers 2004). This study focuses on bathroom GW, which comes from hand washing and bathing and generates 50–60% of total GW, and is considered to be the least contaminated type of GW. Common chemical contaminants include soap, shampoo, hair dye, toothpaste and cleaning products. It also has some faecal contamination (and the associated bacteria and viruses) through body washing. It may further contain microbial agents that present a public health hazard, according to previous

studies (Rose *et al.* 1991; Dixon *et al.* 1999; Casanova *et al.* 2001; Friedler *et al.* 2006; Gilboa & Friedler 2008). In addition to enteric pathogens, there are several human-associated opportunistic pathogens present in GW. Because of the large volume produced and low pollutant concentration, many people propose that GW could be used as irrigation water in dry regions. However, there has been little consideration of the survival of potential pathogenic microorganisms and use of GW without treatment. Few studies have investigated the effect of abiotic factors on pathogenic bacteria in real GW. In this study we chose two bacterial species *Escherichia coli* and *Pseudomonas aeruginosa*.

*E. coli* is commonly found in the lower intestine of warm-blooded organisms; it is one of the most diverse bacterial species with several pathogenic strains with different symptoms and only 20% of the genome is common to all strains (Lukjancenko *et al.* 2010). *P. aeruginosa* is widely distributed in aquatic and terrestrial habitats. Concentrations of *P. aeruginosa* in sewage may

exceed  $10^5$  CFU/100 mL (Howard *et al.* 2004). While usually not a significant risk to healthy individuals, *P. aeruginosa* has been associated with cases of folliculitis, dermatitis, and ear and urinary infections.

Many factors could affect the growth rate or survival of bacteria. This paper focuses on four factors: temperature, aeration, salt concentration and nutrient concentration. Cell size and zeta potential for *E. coli* and *P. aeruginosa* were also determined in relation to the medium composition.

## MATERIALS AND METHODS

### Bacterial growth conditions and parameters

The experimental work concerning pathogenic microorganisms in GW was carried out with *E. coli* and *P. aeruginosa*. Both species have been isolated from bathroom GW from Nantes, France. *E. coli* and *P. aeruginosa* were generally grown in different media: nutrient broth (NB: composition g/L, beef extract 3.0 g, peptone 5.0 g); minimum mineral medium with glucose (MMG: composition g/L,  $\text{Na}_2\text{HPO}_4$  4.8 g,  $\text{KH}_2\text{PO}_4$  4.4 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g,  $\text{NH}_4\text{Cl}$  1.0 g, glucose 1 g (Mian *et al.* 1978)); and GW. The survival or growth measurement was done by plate counting using a selective medium (TTC Tergitol 7Biokar diagnostics) at 37 or 44 °C.

To characterise the rate of bacteria decay and the time required for 90% reduction in bacterial cell, we used decay rate  $K$  (h) and  $T_{90}$ :

- (i) The decay rate  $K$  (h) were calculated according to Chick's law (Alkan *et al.* 1995) which is represented by:

$$-Kt = (\log N_t/N_0) \quad (1)$$

where:  $N_0$  and  $N_t$  are respectively the bacterial numbers initially and at time  $t$  measured in hours.

- (ii) The time required for 90% reduction in cell viability,  $T_{90}$ .

$$T_{90} = -t/\log(C/C_0) \quad (2)$$

where:  $C_0$  and  $C$  are respectively the bacterial concentrations initially and at time  $t$  measured in hours.

### Greywater samples

GW samples were collected from a domestic bathroom shower. The samples were immediately stored at 4 °C in a dark polyethylene bottle. The typical chemical composition of GW which was used in this study is presented in Table 1 (Chaillou *et al.* 2011). For each experiment, the strain was first cultured in NB, harvested by centrifugation and washed with deionised water (DW) and added to GW at the appropriate initial concentration; all experimental survival curves was done in triplicate.

### Temperature

To investigate the effect of temperature, three Erlenmeyer flasks were filled with 250 mL of GW seeded with *E. coli* or *P. aeruginosa* suspension at pH 7 and incubated in the dark at different temperatures ( $6 \pm 2$ ,  $23 \pm 2$  and  $42 \pm 2$  °C) under shaking at 300 rpm. The three temperatures were chosen depending on the mean temperatures in France and Egypt, and on the known survival characteristics of *E. coli* and *P. aeruginosa*.

### Aeration

Two bottles were used for *E. coli* and *P. aeruginosa*. The bottles were each filled with 250 mL of GW, seeded with *E. coli* or *P. aeruginosa* suspension and incubated at  $23 \pm 2$  °C. To produce anaerobic and aerobic conditions, one bottle was closed air-tight and the other closed with a cotton-wool plug to allowed air to enter with shaking at 300 rpm.

### Salinity

Three salinity concentrations were investigated: 1.75 and 3.5% and GW dilution at 50% GW:50% DW seeded with

**Table 1** | Chemical analysis of greywater samples used in this study (Chaillou *et al.* 2011)

Parameters	Average (range)
COD (mg/l)	170 (116–233)
Turbidity (FNU)	28 (9–68)
pH	7.8 (7.6–7.9)
Conductivity ( $\mu\text{S}/\text{cm}$ )	399 (360–465)

COD: chemical oxygen demand.

FNU: formazin nephelometric units.

**Table 2** | Zeta potential and pH for *E. coli* and *P. aeruginosa* in NB and GW media at 25 °C

Samples	Temperature (°C)	pH	Zeta potential (mV)
EC GW	25	7.47	-14.5
EC NB	25	6.72	-33.3
PA GW	25	7.13	-23.2
PA NB	25	6.82	-6.61

EC: *Escherichia coli*.

PA: *Pseudomonas aeruginosa*.

GW: Greywater.

NB: Nutrient broth.

*E. coli* or *P. aeruginosa* suspensions. The flasks are protected from light and incubated at  $23 \pm 2$  °C with shaking (300 rpm). The two levels of salinity were chosen according to the mean levels of salt in seawater: 3.5‰ salinity is the mean seawater salinity; half of this concentration (1.75‰ salinity) represents the effect of dilution by GW.

### Zeta potential and cell size measurement

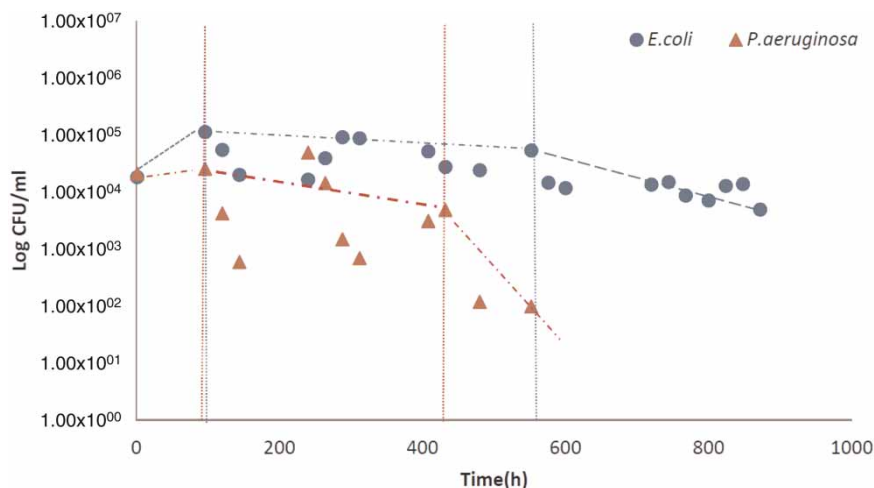
The zeta potential and the cell size of the study microorganisms were measured by dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). For this experiment, the microorganisms were grown in NB as a nutrient-rich medium or in MMG and GW as a nutrient-poor medium at 25 °C with shaking at 300 rpm (Table 2). The bacteria were harvested by centrifugation for 10 min at  $8,545 \times g$  rpm and washed twice with NaCl solution

( $9 \text{ g L}^{-1}$ ). The measures were recorded after 6 days for GW and after the end of exponential growth phase for other media.

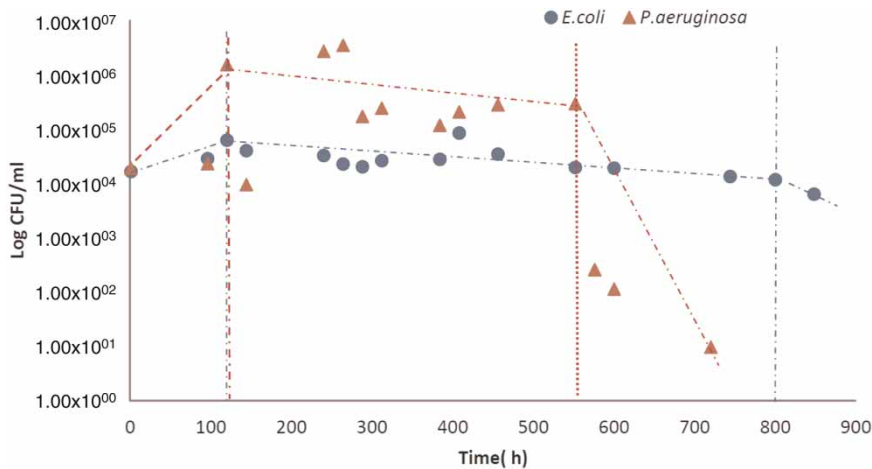
## RESULTS AND DISCUSSION

### Effects of temperature on the survival of *E. coli* and *P. aeruginosa*

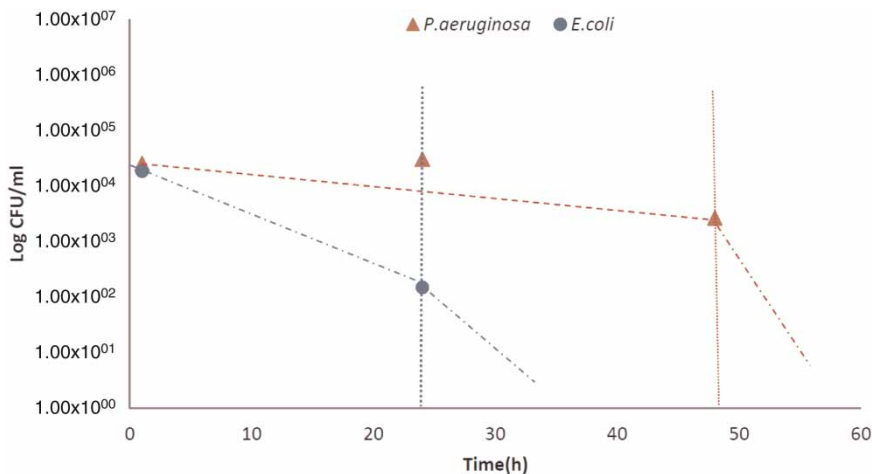
To study the effects of temperature on abundances of *E. coli* and *P. aeruginosa*, we incubated these species in GW at different temperature ( $6 \pm 2$ ,  $23 \pm 2$  and  $42 \pm 2$  °C). As shown in Figures 1, 2 and 3, the survival of bacteria decreased with increasing temperature, in accordance with other studies (Ostrolenk *et al.* 1947; Kristiansen 1981; Stevik *et al.* 2004). *E. coli* survived better at  $6 \pm 2$  and  $23 \pm 2$  °C. The survival period was close to 860 h for both temperatures. *P. aeruginosa* also survived better at  $6 \pm 2$  and  $23 \pm 2$  °C, with survival periods of 552 and 720 h, respectively. At low temperature, the bacteria limit energy expenditure by reducing metabolic activities, allowing much longer survival than at high temperatures. Many authors have shown that low temperatures prolong the survival of pathogenic bacteria, in freshwater (Barcina *et al.* 1986; Wang & Doyle 1998; Mizunoe *et al.* 1999), in estuarine water (Faust *et al.* 1975) and in marine water (Pike & Gameson 1970). The results also show that  $42 \pm 2$  °C has a greater effect on



**Figure 1** | Effects of temperature on growth curve of *P. aeruginosa* and *E. coli* at  $6 \pm 2$  °C. (The vertical lines indicate change in survival stage.)



**Figure 2** | Effects of temperature on growth curve of *P. aeruginosa* and *E. coli* at  $23 \pm 2$  °C. (The vertical lines indicate change in survival stage.)



**Figure 3** | Effects of temperature on growth curve of *P. aeruginosa* and *E. coli* at  $42 \pm 2$  °C. (The vertical lines indicate change in survival stage.)

the survival of *E. coli* and *P. aeruginosa* than other study temperatures. Extremes of temperature seem to be most disruptive to bacterial survival (McFeters *et al.* 1974; Flint 1987). Three distinct stages were observed during the incubation; a short survival growth stage, a stationary survival stage and decrease or death stage (Figures 1, 2, and 3). During the initial stage the bacteria grow rapidly, so the cell numbers increase. Then cell numbers reach the stationary phase, then slowly decrease ( $K_1$ ), followed by a rapid decrease ( $K_2$ ). So the overall decrease in numbers could be determined by two rate factors, the two decay rates constant, a slow one  $K_1$  ( $\text{h}^{-1}$ ) and a rapid one  $K_2$  ( $\text{h}^{-1}$ ). There are limited data in the literature for

these constants. Tables 3 and 4 present the constant values for *E. coli* and *P. aeruginosa* determined for the three experimental temperatures. The main observations, is that for both species the optimal survival condition is  $23 \pm 2$  °C. In this case the time required for loss of 90% of bacterial cells ( $T_{90}$ ) is relatively similar at 730 h for *E. coli* and 590 h for *P. aeruginosa*. At  $6 \pm 2$  °C the two species also show good survival, better one for *E. coli* ( $T_{90} = 590$  h) than *P. aeruginosa* ( $T_{90} = 460$  h). In contrast, *P. aeruginosa* is more resistant than *E. coli* at  $42 \pm 2$  °C. The time required for 90% reduction in cell viability is lower for *E. coli* than *P. aeruginosa*. Incubation at  $42 \pm 2$  °C has the strongest effect on the bacterial mortality.

**Table 3** | The absolute magnitude values  $T_{90}$  and  $K$  for *Escherichia coli* incubation in laboratory conditions

Factors		Parameters		
		$K1(h^{-1})$	$K2(h^{-1})$	$T_{90}(h)$
Temperature	$6 \pm 2^{\circ}C$	1.6	8.0	590
	$23 \pm 2^{\circ}C$	2.0	13.3	730
	$42 \pm 2^{\circ}C$	0.01	–	11
Salinity	50% DW:50% GW	0.4	5.6	680
	1.75% NaCl	0.4	5.6	150
	3.5% NaCl	0.4	–	43
Aeration	Aerobic	2.0	12.4	490
	Anaerobic	2.0	13.7	380

$K$ : Decay rate (h).

$T_{90}$ : Time required for 90% reduction in cell viability.

**Table 4** | The absolute magnitude values  $T_{90}$  and  $K$  of *Pseudomonas aeruginosa* incubation in laboratory conditions

Factors		Parameters		
		$K1(h^{-1})$	$K2(h^{-1})$	$T_{90}(h)$
Temperature	$6 \pm 2^{\circ}C$	7.2	1.6	460
	$23 \pm 2^{\circ}C$	2.0	9.2	590
	$42 \pm 2^{\circ}C$	0.01	–	48
Salinity	50% DW:50% GW	0.4	8.5	300
	1.75% NaCl	0.4	6.47	247
	3.5% NaCl	0.4	–	115
Aeration	Aerobic	1.6	9.2	520
	Anaerobic	0.01	4.4	100

$K$ : Decay rate (h).

$T_{90}$ : Time required for 90% reduction in cell viability.

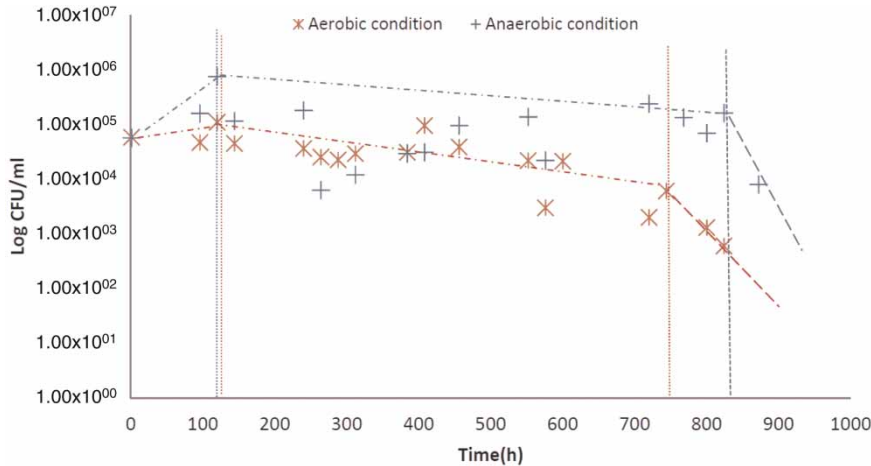
These results are in agreement with [Abdulkarim et al. \(2009\)](#) who investigated the effect of  $44^{\circ}C$  on *E. coli*.

### Effect of oxygen on survival of *E. coli* and *P. aeruginosa*

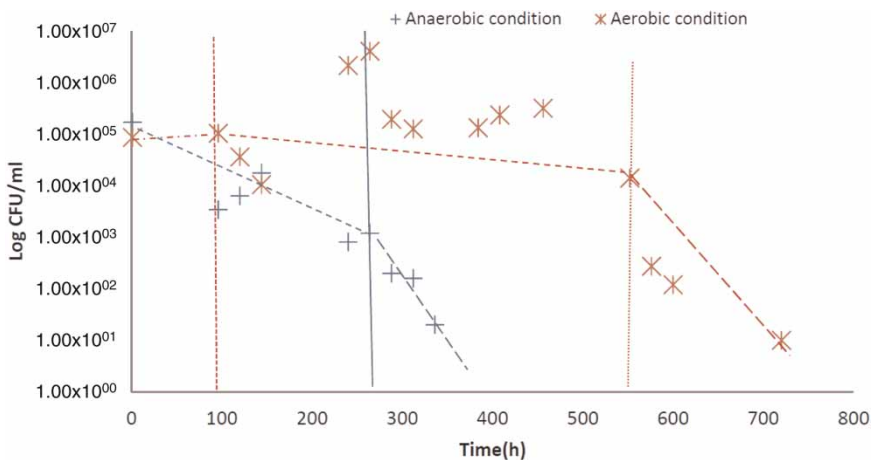
Figures 4 and 5 show the effects of oxygen (aerobic and anaerobic conditions) on the survival of *E. coli* and *P. aeruginosa* in GW. Three distinct stages could be noted. The related constants are presented in Tables 3 and 4. Under anaerobic conditions in GW, *P. aeruginosa* did not show growth, while *E. coli* did grow.

The aerobic condition causes a marked decrease in the survival of *E. coli*. In contrast, the anaerobic conditions cause a marked decrease in the survival of *P. aeruginosa*. This is consistent with other studies which had shown that aeration conditions affect the

abundance of pathogenic bacteria ([Attrassi et al. 1996](#); [Yoon et al. 2002](#); [Mahendran et al. 2005](#)). The number of viable cells remains relatively constant in the stationary stage and the decay rate was slow. This was followed by a rapid death stage (greatest  $K2$ ). In general the rapid die-off could be attributed to the fact that bacterial structures get weaker in the stationary period, so the bacteria were not strong enough to show more resistance to environmental changes. The time required for a 90% decrease in CFU ( $T_{90}$ ) for *P. aeruginosa* was estimated to be 520 and 100 h under aerobic and anaerobic conditions, respectively (Tables 3 and 4). Thus, *P. aeruginosa* maintained cultivability five times longer when incubated under aerobic compared with anaerobic conditions. For *E. coli*,  $T_{90}$  was estimated to be 490 h under anaerobic conditions and 380 h under aerobic conditions. Thus, *E. coli* survival



**Figure 4** | Effects of oxygen availability on survival curve of *E. coli*. (The vertical lines indicate change in survival stage.)



**Figure 5** | Effects of oxygen availability on survival curve of *P. aeruginosa*. (The vertical lines indicate change in survival stage.)

only decreased 1.2-fold when incubated under anaerobic compared with aerobic conditions.

#### Effect of salinity on survival of *E. coli* and *P. aeruginosa*

Salinity is also an important stressor against microorganisms in GW (Gauthier *et al.* 1987; Rose *et al.* 1991; Winward *et al.* 2008; Hrenovic & Ivankovic 2009), where the bacteria must restore the osmotic balance between the external environment and the cytoplasm. This restoration involves complex mechanisms, including increasing the concentration of certain solutes (osmoregulators) in the bacteria. The change in abundance of *E. coli* and

*P. aeruginosa* in GW adjusted to different salinities (50% GW:50% DW, 1.75 and 3.5% salinity) is illustrated in Figures 6, 7 and 8. In laboratory conditions of incubation of the bacteria (in dark at 25 °C), survival curves of *E. coli* and *P. aeruginosa* show the same shape for the different conditions.

Bacterial growth goes through three distinct stages: survival growth, stationary survival and death. At the highest salinity level (3.5%) the bacterial abundance decreases very quickly. However at lower salinity (50% GW:50% DW and 1.75%) the cell numbers decrease very slowly. In Tables 3 and 4 the results show the adverse effect of increasing salinity on the survival of both species. So for 3.5%

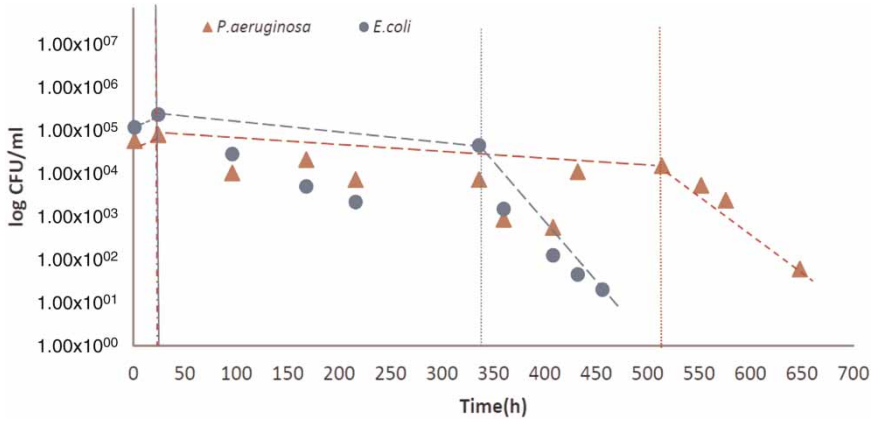


Figure 6 | Survival curve of *E. coli* and *P. aeruginosa* in 50% GW to 50% DW. (The vertical lines indicate change in survival stage.)

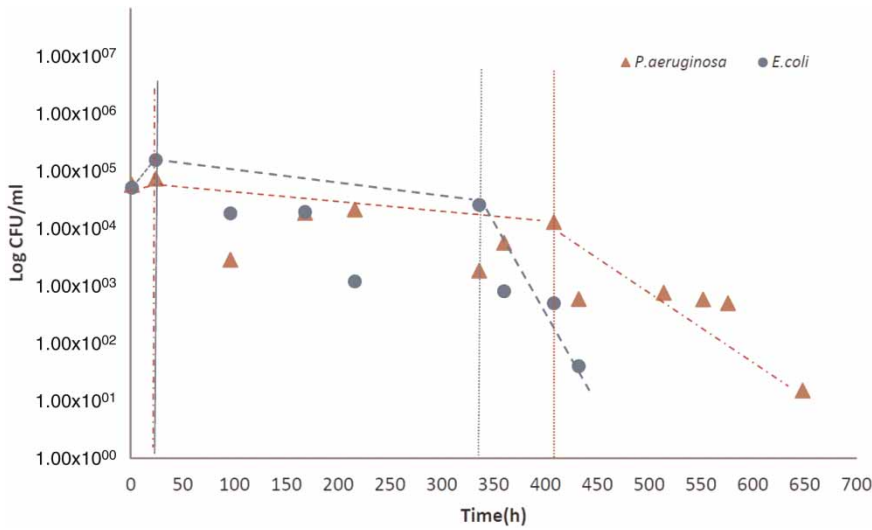


Figure 7 | Survival curve of *E. coli* and *P. aeruginosa* in 1.7% salinity. (The vertical lines indicate change in survival stage.)

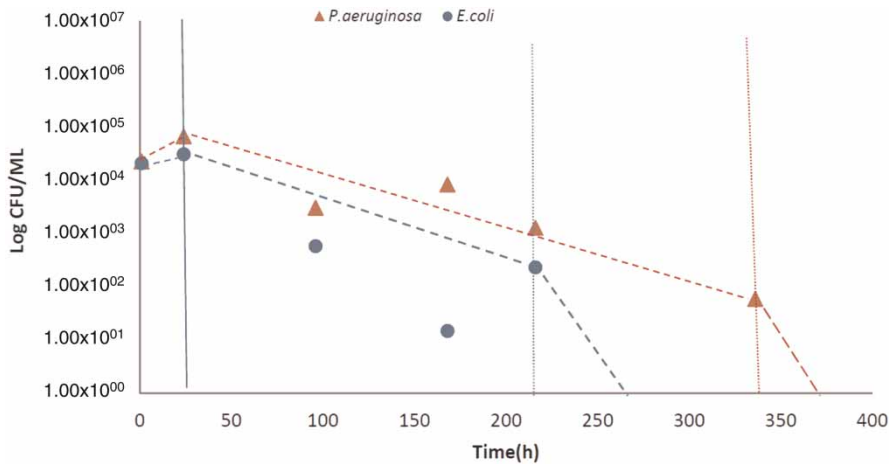


Figure 8 | Survival curve of *E. coli* and *P. aeruginosa* in 3.5% salinity. (The vertical lines indicate change in survival stage.)

salinity, the  $T_{90}$  for *E. coli* is only 43 h. This is consistent with other studies which illustrated that at high salinities the survival of faecal coliforms in water is lower (Bordalo *et al.* 2002). When the salinity decreases by 50% the survival time has shown a two-fold (*P. aeruginosa*) or three-fold (*E. coli*) increase. Table 3 shows the time required for a 90% decrease in CFU ( $T_{90}$ ) for *E. coli* at low salinity was estimated to 150 and 43 h for 1.75 and 3.5% salinity respectively. For *P. aeruginosa* the  $T_{90}$  (Table 4) was estimated to 247 and 115 h at low and high salinity respectively; *P. aeruginosa* is more resistant than *E. coli*.

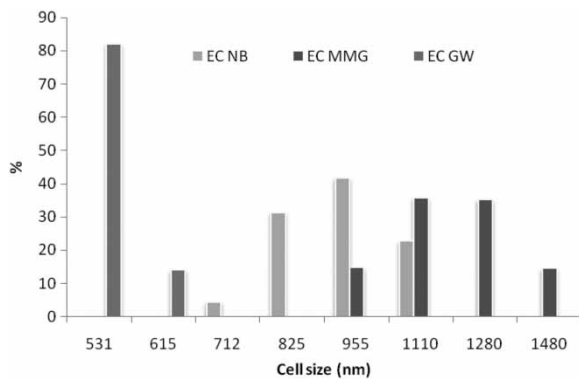


Figure 9 | Effects of different media on particle size of *E. coli*.

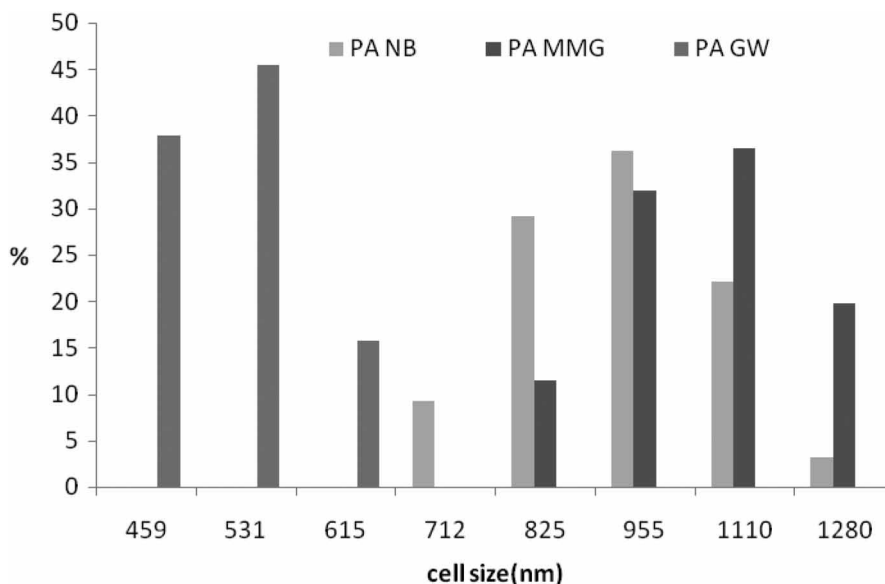


Figure 10 | Effects of different media on particle size of *P. aeruginosa*.

Incubation under low nutrient concentrations (50% GW:50% DW) led to a reduction in both the decay rate ( $K$ ) and  $T_{90}$  (see Tables 3 and 4).

#### Effect of different media on zeta potential and cell size of *E. coli* and *P. aeruginosa*

The zeta potential of *E. coli* and *P. aeruginosa* exhibit a global negative charge of the cells at pH close to neutrality (Table 2). The electric charge on the surface of a bacterial cell is attributable to a large extent to the kind of ionisable groups present on the cell surface and to their spatial distribution. In gram-negative bacteria the major contribution to their surface charge is made by ionisable amino ( $\text{NH}_2$ ) and carboxyl ( $\text{COOH}$ ) groups of proteins exposed at the cell surface (Gittens & James 1963; Sherbet & Lakshmi 1973). Such bacteria have been shown to have an overall net negative surface charge. Acid lipopolysaccharides present in the outer membrane of gram-negative bacteria also contribute to the negative charge level (Sutherland 1977). In general, there was no clear influence of the medium on the zeta potential of *E. coli* and *P. aeruginosa*.

The cell size distribution of *E. coli* and *P. aeruginosa* cultivated in nutrient-rich (NB, MMG) and poor (GW) media are shown in Figures 9 and 10. For *E. coli* grown in



rich media bacterial cells have mean particle size around 1,050 nm (range 825–1,480 nm). For the same micro-organism starved 1 week in GW the size was smaller and close to 550 nm. For *P. aeruginosa* bacterial cells grown in rich media have mean particle size around 1,050 nm (range 700–1,280 nm). For the *P. aeruginosa* cells maintained 1 week in GW the size decrease to a value between 450 and 530 nm. These results highlight that if the medium used to develop the bacteria is rich like NB or MMG, the cells size will be bigger than in poor medium like bathroom GW.

## CONCLUSION

After this study, we can conclude that the treatment of GW with various abiotic factors (temperature, aeration, salt concentrations and nutrient starvation) inhibited the growth of some pathogenic bacteria (*E. coli* and *P. aeruginosa*). We conclude: (1) temperature and salinity have the greatest impact on survival of these bacteria; (2) if the medium has a low concentration of nutrients survival will be affected; (3) substrate and nutrients concentration in the medium also affect cell size and zeta potential. These parameters are of importance in the retention of microorganisms by filtration processes and will condition the interaction between particles and filtration material such as membrane or sand. Our study highlights the fact that for safe reuse of GW it is necessary to remove biological pollution. High temperature is an abiotic factor which could be used as a simple and economic treatment in arid and semi-arid areas in order to make safe use of GW for irrigation. This research could be more widely used and applied in sunny countries such as arid area countries.

## ACKNOWLEDGEMENTS

The study was supported by doctoral scholarships from the Egyptian Government; South Valley University and Ecole des mines de Nantes. We would like to express special thanks to Lionel Limousy, Lorient University.

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First received 21 December 2011; accepted in revised form 23 March 2012