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Use of the aqueous extract of *Eucalyptus microcorys* for the treatment in microcosm, of water containing *Enterococcus faecalis*: hierarchisation of cells' inhibition factors

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Abstract

An experimental study in aquatic microcosm was carried out to determine the major factors involved in the inhibition of *Enterococcus faecalis* in the presence of aqueous extract of *Eucalyptus microcorys*. The planktonic bacterial cells remained in various concentrations of the aqueous solution at light intensities which fluctuated between 0 and 3,000 lx and incubation periods which ranged from 3 to 24 hours. A hierarchisation of studied factors revealed that the aqueous extract concentration, followed by experimental temperature, light intensity and incubation duration influence the inhibition of *E. faecalis* cells, respectively, with a rate of 86.82%, 7.03%, 5.25% and 0.90%. The cell abundances dropped significantly at 1.5% ($\lambda = 0.491$ and $F = 5.518$) and 2% ($\lambda = 0.568$ and $F = 4.055$) concentrations coupled with 1,000, 2,000 and 3,000 lx. The highest light intensities and extract concentration produce the highest log removal values. The disinfectant properties of *E. microcorys* were evaluated by the Chick–Watson model. This Chick–Watson model so obtained varied between $\log (N/No) = -0.09$ Ct and $\log (N/No) = -0.17$ Ct for extract concentrations of 1, 1.5 and 2%. Aqueous extract of *E. microcorys* could be used for water disinfection.

Key words: aquatic microcosm, aqueous extract, *Enterococcus faecalis* inhibition, *Eucalyptus microcorys*, log removal values

INTRODUCTION

Water is now known as the world's largest food commodity (Noah Ewoti *et al.* 2011). Water used for human consumption is not always of good quality (Nola *et al.* 1998). These waters are mostly at risk of physicochemical and microbiological contamination (Eheth *et al.* 2016). Microbiological

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contaminants, especially bacteria, when present, have abundances that vary in time and space depending on meteorological and hydrological factors (Eheth *et al.* 2016). Alternatively, people resort to underground waters for their apparent clarity, but ignore their microbiological quality (Njiné *et al.* 2001). Groundwater intakes are often obtained by traditional techniques, most often without compliance of hygiene and cleansing rules. This situation raises the problem of vulnerability of these water resources to anthropogenic pollution (Allahdin *et al.* 2008). The increase of the pollution of groundwater has led populations to adopt various methods of water treatment before consumption, such as decanting/filtration, boiling, solar disinfection, and most recently, plant treatment (Lontsi Djimeli *et al.* 2013; Tamsa Arfao *et al.* 2016).

The use of extract plants in the treatment of drinking water has undergone major advances in recent years. Several studies have been focused on this subject and shown that the aqueous extracts of *Lantana camara*, *Cymbogon citritus* and *Hibiscus rosa-sinensis* have bactericidal effects in the aquatic environment (Sunda *et al.* 2008). Moreover, in the presence of light, *Artemisia annua* extract inhibits the growth of *Enterococcus faecalis* in aquatic milieu (Mobili *et al.* 2015). Similarly, it has been shown that the aqueous extract of *Eucalyptus microcorys* decreases the concentration of free planktonic bacteria cells and those adhere on substrates in aquatic environments (Tamsa Arfao *et al.* 2013, 2016). Other authors reported the antibacterial activity of *Aujan* extract in water treatment (Sunil & Nitin 2012). Despite this knowledge, few data are available on the impact of some abiotic factors, such as temperature, light intensities and incubation duration on the anti-bacterial properties of these plants. The present study aimed to determine the impact of the above factors on the inhibition/anti-bacterial properties of *Eucalyptus microcorys* acqueous extract against *Enterococcus*.

MATERIALS AND METHODS

Collection of plant materials and preparation of the aqueous extracts

Fresh leaves of *E. microcorys* were harvested in Yaounde, Centre region (Cameroon) and dried at room temperature (23 ± 2 °C) in the laboratory for 30 days. The leaves were thereafter ground and sifted to obtain powder which was used to prepare a decoction. The latter was dried in an oven at 45–50 °C (Tamsa Arfao *et al.* 2016). The crystals obtained were used to prepare the crude extract. Six ranges of extract concentration termed $C_1 = 0.05\%$, $C_2 = 0.1\%$, $C_3 = 0.5\%$, $C_4 = 1\%$, $C_5 = 1.5\%$ and $C_6 = 2\%$ were prepared using sterile physiological water. For each concentration, the solution was filtered through a nitrate cellulose membrane of 0.45 μm porosity. A qualitative phytochemical screening was done according to the protocols developed by Odebiyi & Sofowora (1978) and Trease & Evans (1983).

Isolation and identification of bacterial species

The bacterial species used in this study is *Enterococcus faecalis*. This bacterium was chosen because of its importance in hygiene and public health (Le Minor & Véron 1989). *E. faecalis* cells were isolated from urban streams in the equatorial region of Cameroon. Isolation of *E. faecalis* was performed using Bile Esculin Azide (BEA) agar medium, incubated at 37 °C for 24 hours. The identification and the preparation of bacterial stocks were made according to standard methods (Holt *et al.* 2000).

Experimental design

The experiment tests consisted of introducing a specific bacterial density of 27×10^8 CFU/mL in six Erlenmeyer flasks each containing 200 mL of extract solution of *Eucalyptus microcorys* at different

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concentrations ($C_1 = 0.05\%$, $C_2 = 0.1\%$, $C_3 = 0.5\%$, $C_4 = 1\%$, $C_5 = 1.5\%$ and $C_6 = 2\%$). For each replicate, a control was prepared using only 200 mL of physiological water (NaCl: 0.85%). The incubation times were 3, 6, 9, 12 and 24 hours. The light intensity was of the order of 0, 1,000, 2,000 and 3,000 lx. We followed the protocol described by some authors who recommend the use of 100 W Tungsten filament lamps (TESLA HOLESOVICE) to illuminate the solution (Nola *et al.* 2010a). A lamp was placed at 25 cm above each microcosm. Bacteriological analysis was performed for each incubation time in triplicate.

Statistical analysis

To explain the variation of cell abundances and investigate the influences of extract concentration of *Eucalyptus microcorys* and light intensity on the survival of the bacterial cells, multiple factor analysis was conducted. This discriminant analysis is a one-dimensional test of equality of mean classes that can highlight the different observed abundances and rank the involved factors in a hierarchical decreasing order of influence levels. The Wilks' lambda test (λ) and the asymptotic approximation of Fisher (F) were used for this ranking. The Wilks' lambda varies between 0 and 1. If the value is low, this reflects a slight variation of cell abundances obtained in the presence of each concentration of *Eucalyptus microcorys* and therefore a large variation between concentrations of extract. This test was performed using XLSTAT 2007 software.

To quantify the contribution of the effect of temperature comparatively to the contribution of photoinactivation (light intensity), the MANOVA tests were used to determine the sum of squares of the percentage of factors implicated on inactivation. This analysis was performed using 'R' software.

A log removal value (LRV) was calculated to measure the ability of a treatment process to remove bacteria. LRVs are determined by taking the logarithm of the ratio of cell concentration in the influent and effluent water of a treatment process as shown in Equation (1) – calculation of LRV from pathogen concentration data (Water Research Australia 2014):

$$LRV = log\left(\frac{\text{Influent } E.\ faecalis \text{ Concentration}}{\text{Effluent } E.\ faecalis \text{ Concentration}}\right) \quad (1)$$

where influent *E. faecalis* concentration = number of bacteria in control (NaCl: 0.85%), effluent *E. faecalis* concentration = remaining bacteria after the action of *Eucalyptus microcorys* extract.

To compare the results obtained from different experimental data such as those mainly involving disinfectant concentration and reaction time (Ct), a disinfection kinetic model described by the Chick–Watson model was used. In general, disinfection systems are designed by the Ct values derived from Chick–Watson kinetics based on the data obtained from laboratory inactivation studies (Sunil & Nitin 2012). This Chick–Watson function was done using Excel program to apply the following formula: $\log (N/No) = -K\, C^n t$ (Chick 1908; Watson 1908).

In many cases, the n value for Chick–Watson's law is close to 1.0 and hence a fixed value of the product of concentration and time (Ct product) results in a fixed degree of inactivation (AWWA 1999). In this formula, N is the number of microorganisms at contact time t exposure in the extract concentration; N_0 is the number of bacteria in control (NaCl: 0.85%); t is contact time; k is the disinfection rate constant; C represents the extract concentration and log is logarithm to the base 10.

RESULTS AND DISCUSSION

In the absence of *Eucalyptus microcorys* extract (Control), the abundance of *Enterococcus faecalis* varied between 19.59 and 20.27 (Ln (CFU/100 mL)); 19.18 and 19.87 (Ln (CFU/100 mL)); 18.99 and 19.16 (Ln (CFU/100 mL)) and between 18.90 and 19.79 (Ln (CFU/100 mL)) at 0 lx, 1,000 lx,

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2,000 lx and 3,000 lx, respectively (Figure 1). A relative reduction of planktonic cells was noted in most cases in the presence of *Eucalyptus microcorys* extract. In the absence of light (0 lx), the abundance of *Enterococcus faecalis* ranged from 17.32 to 19.32 (Ln (CFU/100 mL)), 14.91 to 16.86 (Ln (CFU/100 mL)), 13.75 to 16.05 (Ln (CFU/100 mL)), 11.37 to 15.70 (Ln (CFU/100 mL)), 11.11 to 13.59 (Ln (CFU/100 mL)) and from 9.90 to 11.78 (Ln (CFU/100 mL)) at the extract concentration $C_1 = 0.05\%$, $C_2 = 0.1\%$, $C_3 = 0.5\%$, $C_4 = 1\%$, $C_5 = 1.5\%$ and $C_6 = 2\%$, respectively. The lowest cell densities were observed at C_5 and C_6 under 2,000 lx and 3,000 lx. A variation of cell abundance from 8.52

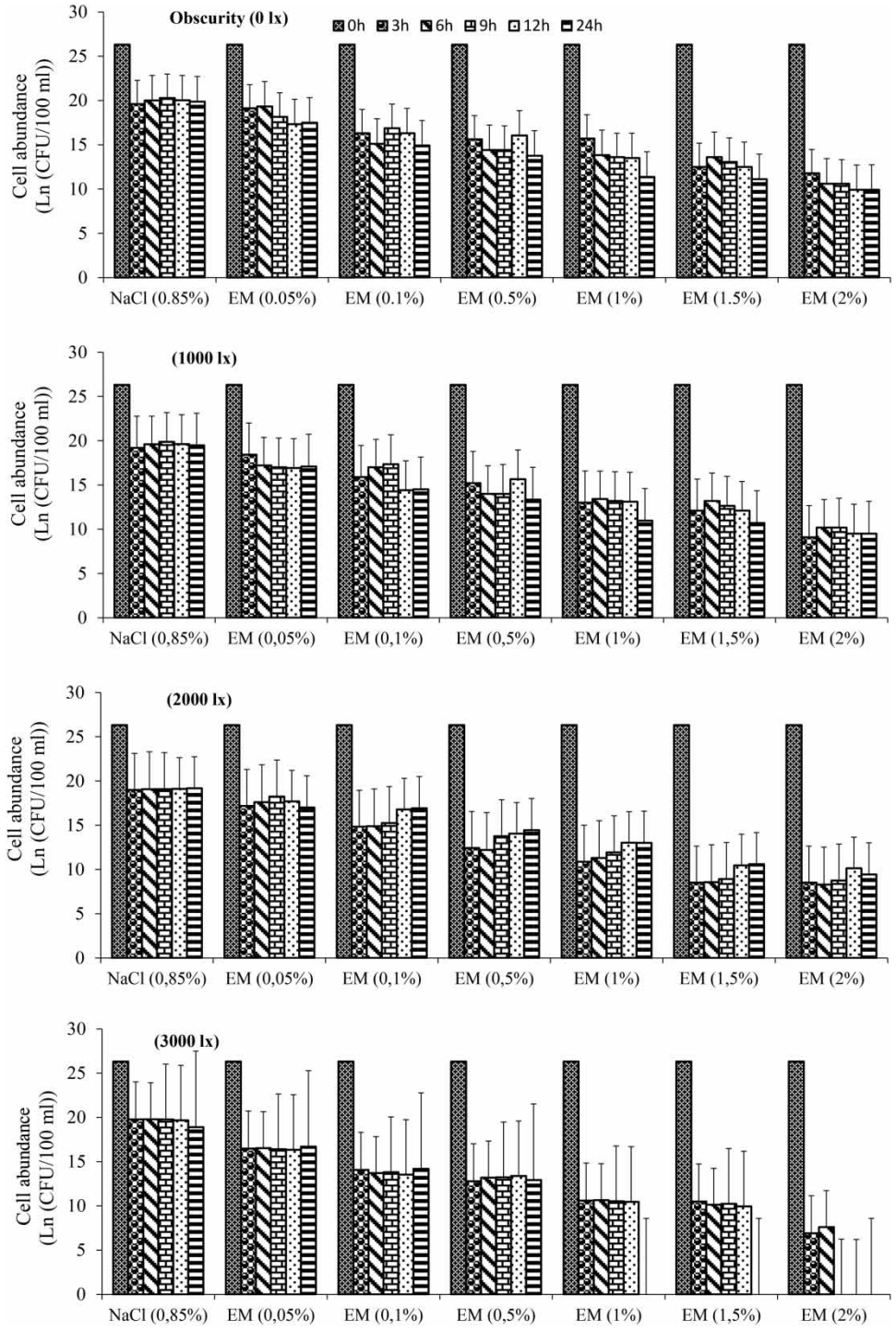

Figure 1 | Temporal variation (with standard deviations) of the abundance of planktonic *Enteroccocus faecalis* in the presence of *Eucalyptus microcorys* extract under each light condition.

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to 10.60 (Ln (CFU/100 mL)) and from 8.29 to 10.13 (Ln (CFU/100 mL)) under 2,000 lx were recorded at C_5 and from 0 to 10.49 (Ln (CFU/100 mL)) and from 0 to 7.60 (Ln (CFU/100 mL)) under 3,000 lx at C_6 (Figure 1). This reduction mostly depends on concentration of aqueous extract and the intensity light.

The percentages of inhibited cells were calculated in each experimental condition. They are presented in Table 1. In the presence of the plant extract, the inhibition level of *E. faecalis* varied from one concentration to another. Indeed, it appears that the percentages of cells' inhibition generally vary at each luminous intensity according to the incubation period and to the extract concentration to which *E. faecalis* was exposed (Table 1). In the absence of light (0 lx), the inhibition percentage of *E. faecalis* oscillated between 38% (observed after 3 hours of incubation when the concentration of the extract was C_1) and 100% observed at almost all incubation periods when the concentration ranges were C_4, C_5 and C_6.

This result could be explained by the bactericidal effect of the aqueous extract. In general, it has been observed that the inhibition of bacteria growth in aquatic environments is linked to the presence of the bactericidal and bacteriostatic compounds from plants. The most commonly known for *E. microcorys* are quinones and anthraquinones. Other compounds, such as furocoumarins, can inhibit DNA replication and stop the growth of microorganisms in the aquatic environment (Serrano *et al.* 2008). It has also been shown *in vitro* that the essential oils of *E. microcorys* have a strong antibacterial activity against *E. faecalis* and *Salmonella typhimurium* (Younes *et al.* 2012), but the degrees of inhibition of these oils vary according to the bacteria species.

There is thus a gradual increase in cell inhibition rate in tested cells in the presence of extract after light exposure. The hourly inhibition rate is very low in dark conditions and increases as light intensity augments. At 1,000, 2,000 and 3,000 lx intensities, after 3 hours incubation, the inhibition percentages

Table 1 | Variation of the percentage of inhibition (PI) of *Enterococcus faecalis* under each light condition and under extract concentration

Light intensity and extract concentration of *Eucalyptus microcorys*		PI values (%) after each incubation period				
		3 h	6 h	9 h	12 h	24 h
Obscurity	0.05%	38	49	88	93	91
	0.1%	96	99	97	98	99
	0.5%	98	100	100	98	100
	1%	98	100	100	100	100
	1.5%	100	100	100	100	100
	2%	100	100	100	100	100
1,000 lx	0.05%	53	91	94	93	91
	0.1%	96	93	92	99	99
	0.5%	98	100	100	98	100
	1%	100	100	100	100	100
	1.5%	100	100	100	100	100
	2%	100	100	100	100	100
2,000 lx	0.05%	84	77	57	76	88
	0.1%	98	98	98	90	90
	0.5%	100	100	100	99	99
	1%	100	100	100	100	100
	1.5%	100	100	100	100	100
	2%	100	100	100	100	100
3,000 lx	0.05%	96	96	97	96	89
	0.1%	100	100	100	100	99
	0.5%	100	100	100	100	100
	1%	100	100	100	100	100
	1.5%	100	100	100	100	100
	2%	100	100	100	100	100

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of 53%, 84% and 96%, respectively, were observed when the concentration of the aqueous extract was C_1 (Table 1). This result could be explained by the bactericidal synergistic effect of the luminous intensity and of the aqueous extract of the plant used. Some research has revealed that solar radiation has a strong influence on the survival of certain bacteria such as *Escherichia coli*, *E. faecalis* or *S. typhi* in wastewater when comparing rates obtained in the presence and absence of light (Maiga 2010). Moreover, the growth of faecal coliforms and enterococci is more inactivated during the day than the night in rivers (Sinton *et al.* 2002). An evolution of *E. faecalis* to a non-cultivable viable state when exposed to visible light can be observed in seawater and freshwater (Barcina *et al.* 1990). Visible light would act on the bacteria via the photosensitizers present in the medium (Cooper & Yogi 2002).

In general, the bacteria have photosensitive sites P which, in the presence of light, are activated into reactive form P*. These activated forms convert dissolved oxygen (O_2) into singlet oxygen ($_1O^{2-}$), which is a powerful oxidant capable of destroying bacterial cells (Stanier *et al.* 1990). The toxicity is linked to superoxide radicals, hydrogen peroxides and hydroxyls which are produced during oxidation reactions (Stanier *et al.* 1990). Indeed, it has been shown that the irradiation of *E. faecalis* only has no significant effect and its exposure to classical conventional photosensitizers does not significantly reduce its viability (Pileggi 2013). The observed inhibition rate would therefore be due to the presence of the ranges of aqueous extracts of *E. microcorys*. This result could be explained by the effect of compounds of the extract which could induce photosensitization. Certain plants used in traditional pharmacopoeia to treat microbial infections contain photosensitive molecules which trigger photosensitization (Mobili *et al.* 2015). The established photodynamic activity is attributable to the presence of psoralens or furocoumarins and/or quinones and anthraquinones from some plants (Serrano *et al.* 2008). These latter compounds are often described as singlet oxygen generators (Fufezan *et al.* 2007). Thus, the luminous intensity acts as a facilitator of *E. microcorys* extract activities.

The discriminant factorial analysis was performed in order to determine the gradual influence of *E. microcorysis* aqueous extract and light intensity on the survival of *Enterococcus faecalis*. The illumination globally discriminates cellular abundances as compared to darkness with values of $\lambda = 0.454$ and $F = 5.783$ for 1,000 lx, $\lambda = 0.232$ and $F = 15.865$ for 2,000 lx and $\lambda = 0.020$ and $F = 235.425$ for 3,000 lx (Table 2). When the dependent variable is the light intensity, the correlation circle shows that the main axis that contains 84.80% correlates with the cell densities recorded at concentrations C_5 ($r = 0.813$) and C_6 ($r = 0.868$). The cell concentrations recorded at 0 lx light intensity are distributed in positive coordinates compared to the cell densities, recorded at the light intensities of 1,000 lx, 2,000 lx and 3,000 lx, distributed in negative coordinates. The extract concentrations C_5 ($\lambda = 0.491$ and $F = 5.518$) and C_6 ($\lambda = 0.568$ and $F = 4.055$) are the ones that best discriminate the cell abundances obtained under different illumination conditions (Table 2).

Table 2 | Equal statistical test of mean cell abundances of *Enterococcus faecalis* under different lighting conditions and in the presence of extract solution at different concentrations

		Value of λ de Wilks, asymptotic approximation of Fisher F and *p*-value		
Experimental conditions		**λ de Wilks**	**F**	***p*-value**
Illumination condition	Obscurity	0.408	6.971	0.000
	1,000 lx	0.454	5.783	0.001
	2,000 lx	0.232	15.865	<0.0001
	3,000 lx	0.020	235.425	<0.0001
Concentration of the extract of *Eucalyptus microcorys*	C_1 (0.05%)	0.598	3.584	0.037
	C_2 (0.1%)	0.757	1.708	0.206
	C_3 (0.5%)	0.663	2.714	0.079
	C_4 (1%)	0.742	1.854	0.178
	C_5 (1.5%)	0.491	5.518	0.009
	C_6 (2%)	0.568	4.055	0.025

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The percentages of the sum of squares of the factors considered during experiments with planktonic cells show that these factors influence, at varying degrees, the effect of *Eucalyptus microcorys* extracts on the bacteria used. The first parameter that stands out is the concentration of the aqueous extract that may have a considerable impact on the cultivability of bacterial cells. A negative and significant correlation ($p < 0.01$) was observed between cell abundances and concentration of *Eucalyptus microcorys* extract. The Wilks' lambda test (λ) at 5% threshold reinforces these results for extract concentrations of 1.5 and 2%. For some diseases caused by bacteria (sinusitis, sore throat, angina, cough, bronchitis, urinary tract infection), *Eucalyptus* leaves are recommended in the form of a decoction of 10 to 20 g of leaves per litre of water to drink in daytime (Nicolas 2012). Under illumination conditions, the cultivability of *Enterococcus faecalis* was influenced by the aqueous extract concentration, followed by experimental temperature, light intensity and incubation duration, respectively with a rate of 86.82%, 7.03%, 5.25% and 0.90%. The incubation temperature may increase the efficiency of *Eucalyptus microcorys* extract; cellular inhibition is considerable at mesophilic temperatures and lower at psychrophilic temperatures. The Wilks' lambda test (λ) at 5% threshold reinforces these results for incubation temperatures of 37 °C and 44 °C.

In microcosm condition, an increase in temperature is usually correlated with an increase in the rate of metabolic and biochemical reactions, with the accumulation of certain toxic compounds that may occur, thereby inhibiting bacterial growth (Nola *et al.* 2010b). Concerning incubation duration, some authors have shown that the effectiveness of a low-level disinfectant on microorganisms is high if the duration of exposure increases significantly (Rutala *et al.* 2000).

As for light intensity, Meierhofer & Wegelin (2002) showed that UV-A radiation and high temperature lead to the elimination of 99.99% of viruses and bacteria that cause diarrhoeal diseases. During the study, there was a change in temperature registered during the experiment. In dark condition, the temperature fluctuated between 22 and 24 °C. Under 1,000 lx, it ranged from 38 to 42 °C. Under 2,000 lx and 3,000 lx, respectively, it changed from 39 to 50 °C and 40 to 54 °C (Figure 2). A variation of the temperature of the solution between 40 and 50 °C, under an intensity of 2,000 lx and 3,000 lx, could be responsible for inactivation of the cells. A study using waters highly contaminated with *E. coli* have shown that thermal inactivation occurs only from 45 °C, where it is possible to note a synergistic action between optical and thermal inactivation (McGuigan *et al.* 1998). A water temperature threshold of about 50 °C favours the inactivation of bacterial cells (Wegelin *et al.* 1994).

The effectiveness of wastewater treatment processes is measured using a concept called log removal values (LRVs). Our study showed that the highest light intensities and extract concentration produce the highest log reduction values. For example, from 1,000 lx and from C_4, a LRV ranging between 3 and 6 was observed. The value of LRV = 6 registered under 3,000 lx (Table 3). According to Water Research Australia (2014), an LRV of 1 is equivalent to 90% removal of a target pathogen, an LRV of 2 is equivalent to 99% removal and an LRV of 3 is equivalent to 99.9% removal and so on.

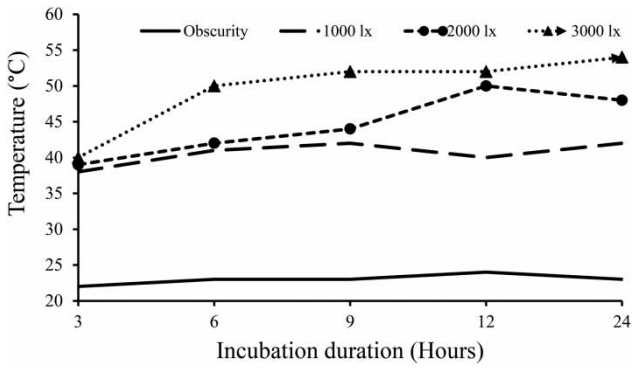

Figure 2 | Fluctuation of temperature during study.

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Table 3 | Log_{10} removal value (LRV) after each incubation time at each extract concentration under different light intensity

Light intensity and each extract concentration		Log10 removal value after each incubation time				
		3 h	6 h	9 h	12 h	24 h
0 lx	0.05%	0	0	1	1	1
	0.1%	1	2	1	2	2
	0.5%	2	2	3	2	3
	1%	2	3	3	3	4
	1.5%	3	3	3	3	4
	2%	3	4	4	4	4
1,000 lx	0.05%	0	1	1	1	1
	0.1%	1	1	1	2	2
	0.5%	2	3	3	2	3
	1%	3	3	3	3	4
	1.5%	3	3	3	3	4
	2%	5	4	4	5	5
2,000 lx	0.05%	1	1	0	1	1
	0.1%	2	2	2	1	1
	0.5%	3	3	3	3	2
	1%	4	4	4	3	3
	1.5%	5	5	5	4	4
	2%	5	5	5	4	5
3,000 lx	0.05%	1	1	1	1	1
	0.1%	2	3	3	3	2
	0.5%	3	3	3	3	3
	1%	4	4	4	4	6
	1.5%	4	4	4	4	6
	2%	6	5	6	6	6

As the disinfection kinetic models are the basis for assessing the disinfectants' performance, the experimental results were used to derive a suitable kinetic model. Table 4 shows the decimal logarithm values of the concentration ratios of *Eucalyptus microcorys* in a given solution after exposure in a concentration range of the aqueous extract of *E. microcorys* at a given luminous intensity. It was found that at the concentration C_1, C_2, C_3 and C_4 plant extract ranges, the low log (N/No) −0.08 Ct, −0.09 Ct, −0.14 Ct and −0.5 Ct, respectively, were obtained at the highest intensity exposure of 3,000 lx. The highest values log (N/No) −0.02 Ct, −0.04 Ct, −0.07 Ct and −0.09 Ct, respectively, were obtained at the intensity exposure of 2,000 lx. At C_5 and C_6 aqueous extract of *E. microcorys*, the highest log (N/No) values were −0.14 Ct and −0.17 Ct, respectively. These values were obtained at a light intensity of 3,000 lx and represent, generally, the lowest values.

Table 4 | Chick–Watson model for different *E. microcorys* extract concentration

Experimental condition (light intensity)	Value of log (N/No) with respect to each *E. microcorys* extract concentration					
	0.05%	0.1%	0.5%	1%	1.5%	2%
0 lx	−0.03 Ct	−0.06 Ct	−0.08 Ct	−0.10 Ct	−0.11 Ct	−0.14 Ct
1,000 lx	−0.03 Ct	−0.06 Ct	−0.08 Ct	−0.10 Ct	−0.11 Ct	−0.17 Ct
2,000 lx	−0.02 Ct	−0.04 Ct	−0.07 Ct	−0.09 Ct	−0.13 Ct	−0.14 Ct
3,000 lx	−0.04 Ct	−0.08 Ct	−0.09 Ct	−0.14 Ct	−0.5 Ct	−0.17 Ct

CONCLUSION

The use of medicinal plants as water disinfection offers many research opportunities in a world where access to drinking water remains a permanent concern for the public authorities. In general, the

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results of this study show that the presence of *Eucalyptus microcorys* extract significantly reduces the cultivability of planktonic bacteria in water. A hierarchical ranking of the various factors which could influence the effect of the aqueous extract of *E. microcorys* on the growth of planktonic cells showed that the extract's concentration is the most influencing parameter, followed by experimental temperature, light intensity and incubation duration. Data obtained from this exploratory research make it possible to consider the use of *E. microcorys* aqueous extract as an alternative method in water disinfection.

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