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Impact of Hydrogen Peroxide Treatment on Environmental *Escherichia coli* Strains

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Abstract The impact of three hydrogen peroxide (H_2O_2) concentrations (250, 300 and 350 mg.L^{-1}) at 30, 60, 90 and 120 min time intervals was determined on environmental and ATCC reference *E. coli* strains (n=11). Variation between strains was evident and treatment resulted in significantly different log reductions after the 120 min contact time. The environmental strains were generally more resistant than the reference strains. A H_2O_2 resistant environmental strain (M53) and a potential pathogenic strain (W1371) were used to determine bactericidal effect at higher (and a lower) H_2O_2 concentrations of 50, 350, 700 and 1 000 mg.L^{-1} on the microbial inactivation. Bacterial inactivation increased as concentration increased, with 50 mg.L^{-1} resulting in low microbial inactivation and 1 000 mg.L^{-1} resulting in an effective (>4 log) reduction. A significant difference in microbial reduction was not observed at H_2O_2 concentrations between 350 and 700 mg.L^{-1}. The potential influence of the COD (chemical oxygen demand) of river water on the H_2O_2 treatment was also determined. It was observed that the water COD, at the levels investigated, might influence H_2O_2 efficacy treatment over shorter treatment times (30 min), but not over longer periods (90-120 min). Different levels of catalase activity were also measured for the test strains. A trend was observed between H_2O_2 resistance and an increased amount of HPII catalase activity. However, it was also observed that *E. coli* can also employ other protection mechanisms, as two of the most resistant environmental *E. coli* strains only indicated average catalase activity.

Keywords: *H_2O_2, biocide, irrigation, water treatment, E. coli, resistance, catalase*

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1. Introduction

Water scarcity is a global problem, and a reality in South Africa [1,2]. In many developing countries, water scarcity can be linked to poverty due to the decrease in food production [3]. It is therefore of the utmost importance that the South African agricultural industry keep up with growing food demands in spite of a limited water supply.

Due to a variety of reasons the quality of irrigation water sources is deteriorating [4]. This a major cause for concern since foodborne disease outbreaks have been linked to irrigating fresh produce with faecally contaminated water [5]. On-farm treatment options of water are therefore needed in order to decrease the high microbial loads prior to irrigation.

The disinfectant properties of H_2O_2 have been known for many years. It acts as a disinfectant due to the formation of highly reactive hydroxyl and superoxide radicals with strong oxidising properties [6]. The main advantage associated with the use of H_2O_2 is that it degrades into non-toxic by-products (oxygen and water) [6]. This is seen as a significant advantage for fresh produce farmers when considering water treatment options.

Factors such as concentration, contact time and organic matter content can all influence the efficacy of H_2O_2 as a treatment option [7,8]. Presence of catalases might furthermore contribute to microbial tolerance at lower concentrations [9]. Generally there is limited literature available on H_2O_2 concentrations used for irrigation water treatment. Studies on wastewater have, however, shown that a 250 mg.L^{-1} H_2O_2 treatment resulted in a 2.2 log reduction after 120 min [10], and that the organic matter content in water can also decrease the efficiency of H_2O_2 treatment [8].

H_2O_2 has been used for microbial inactivation of a variety of viruses and other pathogenic microorganisms, as well as for both gram positive and gram negative vegetative bacteria [11]. According to the WHO (World Health Organization) [12] faecal coliforms must not exceed 1000 faecal coliforms per 100 mL water intended for irrigation of fresh produce to ensure food safety. Therefore, in this study the effect that H_2O_2 can have on *E. coli* strains was studied.

It is furthermore evident that variation between bacterial strains exists with regards to survivability after exposure to different treatment options. A study done by

Cherchi and Gu [13] on chlorine disinfection indicated that *E. coli* O157:H7 was more sensitive to chlorine disinfection compared to *E. coli* K12. Wojcicka *et al*. [14] has also reported that *E. coli* O157:H7 were less sensitive than reference strains (after a free chlorine and monochloramine treatment). However, other heterotrophic environmental strains were also included in the study, and the results indicated that they were easily inactivated compared to reference strains [14]. Mazzola *et al*. [15], on the other hand, reported that environmental strains isolated from a water purification system showed higher resistance against chemical disinfectants compared to standard reference strains. Environmental strains might not always have the same inactivation kinetics as standard reference strains. It is, however, important to investigate strain-strain variation and determine the most resistant strains. These strains should then be used during optimization studies of water treatment options in order to determine the most effective treatment parameters. Therefore, in this study the effect that H_2O_2 can have on different environmental and reference *E. coli* strains was studied.

2. Materials and Methods

2.1. *Escherichia coli* Cultures

In this study, 12 *E. coli* strains (three ATCC strains and nine environmental strains) were used (Table 1). The environmental *E. coli* strains had been isolated previously from river water and produce (Department of Food Science, Stellenbosch University) and stored in 40% ($v.v^{-1}$) glycerol (Fluka Analytical, Germany) at -80°C. Strains were resuscitated in Nutrient Broth (NB) (Biolab, Merck), after which single colonies was obtained by streaking on Levine Eosin Methylene-Blue Lactose Sucrose Agar (L-EMB) (Oxoid, South Africa. The *Escherichia coli* colonies had a metallic green sheen on L-EMB agar [16].

Each strain's identification was confirmed using API 20E (BioMèrieux, South Africa) as described previously [17].

Table 1. *E.coli* strains' characteristics (environmental and reference strains) in terms of their isolation sources and antibiotic resistances

Strain	Source	Resistance
M29	River	C, TM
MJ58	Parsley	None
MJ56	Parsley	None
M53	River	T,TM,AMP,S
E12.1	River	None
E22.1	River	None
E11.1	River	None
F11.2	River	T
ATCC 11772	ATCC	None
ATCC 25922	ATCC	None
ATCC 35218	ATCC	AMP,C,S
W1371 (EPEC)	River	TM, AMP, S

C: chloramphenicol, T: tetracycline, TM: trimethoprim,
AMP: ampicillin, S: streptomycin,
ATCC: American Type Culture Collection.

2.2. *E. coli* Inoculum Preparation

Physiological Saline Solution (PSS) (0.85% $m.v^{-1}$ NaCl) was used as the test medium for all the laboratory studies discussed in this report. Before each treatment an inoculum was prepared for each strain by diluting an overnight culture (incubated in NB at 35°C) in SSS to a achieve a turbidity equal to 0.5 McFarland standard (BioMèrieux, South Africa).

2.3. *E. coli* Enumeration

A dilution series was prepared both before (control), and after all specific treatments and time intervals. Enumeration of *E. coli* were done on Violet Red Bile Agar (VRBA) (Biolab, Merck, South Africa). Plates were poured in duplicate, inverted and incubated at 35°C for 24h.

2.4. H_2O_2 Treatments

H_2O_2 30% ($v.v^{-1}$) (Merck, South Africa) was used to prepare the H_2O_2 concentrations (50, 250, 300, 350, 700 and 1 000 $mg.L^{-1}$) that were used in this study. The H_2O_2 concentrations were confirmed by a Spectroquant® Hydrogen Peroxide Cell Test (2.0 – 20.0 $mg.L^{-1}$) (Merck, South Africa).

2.5. Study Design

2.5.1. Study 1: *E. coli* Resistance to H_2O_2 Treatment/ Effect of H_2O_2 Concentration and Contact Time on Selected *E. coli* Strains

The impact of three H_2O_2 concentrations (250, 300 and 350 $mg.L^{-1}$) were determined on all the *E. coli* strains at five intervals (0 (initial counts), 30, 60, 90 &120 min) in triplicate in PSS. One of the most resistant environmental strains was then selected and exposed to a wider range of H_2O_2 concentrations (50, 350, 700 & 1000 $mg.L^{-1}$) at five time intervals (0 (initial counts), 30, 60, 90 & 120 min) in PSS. An environmental *E. coli* strain identified as carrying enteropathogenic (EPEC) gene sequences, W1371, was also exposed to the wider range of H_2O_2 concentrations (50, 350, 700 & 1000 $mg.L^{-1}$).

2.5.2. Study 2: Influence of COD (Chemical Oxygen Demand)

The impact that COD content (taken as an indicator of organic matter content) could have on H_2O_2 treatment was also determined. The most resistant environmental strain from Study 1 was inoculated into PSS, as well as into autoclaved river water from two different sources (River 1 and River 2), and exposed to 350 $mg.L^{-1}$ H_2O_2 at five time intervals (0 (initial counts), 30, 60, 90 & 120 min).

2.5.3. Study 3: Catalase Activity of *E. coli* Strains

The catalase activity of all 12 *E. coli* strains was measured to determine if higher catalase activity corresponded with increased H_2O_2 resistance. Two different methods were used in this study. The first method (method 1) [18], only gave an indication of total catalase activity and was based on interpreting the visual reduction of 30 % ($v.v^{-1}$) H_2O_2 by each culture on a glass slide. Results were recorded based on the robustness of the bubbling: '- (no bubble formation)'; '+ (little bubble formation); '++ (average bubble formation)' and; '+++ (immediate, strong bubble formation)'.

The second method (method 2) [19], was used to quantify HPI (hydroperoxidase I), HPII (hydroperoxidase II) as well as total catalase activity. The method involved the transfer of cell suspension (0.01 g of wet weight and 100 uL of PSS) to a test tube, after which 100 µL Triton X-100 was added, followed by the addition of 100 µL H_2O_2 solution (30% $v.v^{-1}$). The height of the foam in the test tube was measured after the foam height had remained constant for 15 min. This value was then used as an indication of total catalase activity. In order to distinguish between the two types of catalase, HPI and HPII, a second aliquot in PSS was also prepared. This aliquot underwent a heat treatment of 55°C for 15 min. The heat treatment was done before adding the cell suspension to the test tube with Triton X-100 and H_2O_2. The heat-stable catalase (HPII activity) was determined by measuring the height of foam that had been stable for 15 min of the heat treated aliquots. The heat–labile (HPI activity) catalase activity was then determined by subtracting the heat-stable catalase activity from the total catalase activity.

2.6 Statistical Analysis

Main effects ANOVA using Statistica 12.5 (Statsoft Inc.) was used, with *E. coli* strain and time as the two effects. The main purpose of doing the ANOVA was to determine significant differences between means. A mixed model repeated measures ANOVA was used to analyze the *E. coli* counts and a two-way ANOVA was used to analyze the log reductions achieved in study 1. In study 2, a mixed model repeated measures ANOVA was used to analyze the *E. coli* counts in the PSS, River 1 and River 2 samples.

Post hoc tests were conducted using the Fisher least significant difference (LSD) testing. A 5% significance level ($p < 0.05$) was used as guideline for determining significant results. If no *E. coli* growth was observed it was recorded as 30 $cfu.mL^{-1}$ (1.48 log $cfu.mL^{-1}$) (as the lower limit) for all the statistical analyses done in studies 1 and 2.

3. Results

3.1. Study 1: *E. coli* Resistance to H_2O_2 Treatment/ Effect of H_2O_2 Concentration and Contact Time on Selected *E. coli* Strains

In order to determine the bactericidal effect that the three H_2O_2 concentrations (250, 300 and 350 $mg.L^{-1}$) had at the individual time intervals, the colony counts of all the *E. coli* strains, were pooled and are presented in Figure 1. The highest H_2O_2 concentration (350 $mg.L^{-1}$) was the most effective and resulted in a higher log reduction over 120 min (Figure 1). When comparing the 250 and 300 $mg.L^{-1}$ treatments, 300 $mg.L^{-1}$ resulted in a slightly higher log reduction (Figure 1). This indicated a trend that the higher H_2O_2 concentration would result in a greater log reduction. However, although a trend was observed when comparing results at the different H_2O_2 concentrations, a significant difference could not be proven ($p < 0.05$) between the 250, 300 and 350 $mg.L^{-1}$ H_2O_2 treatments. The average colony counts obtained for each strain at all three H_2O_2 concentrations, were, therefore, pooled before log reductions of the individual strains were compared.

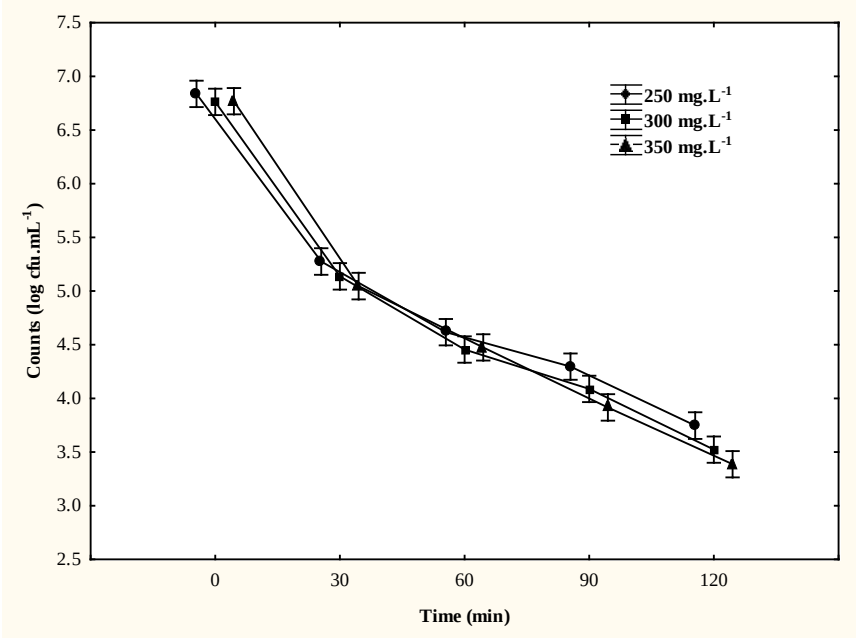

Figure 1. Effect of H_2O_2 concentration and contact time (0, 30, 60, 90 and 120) on the 11 *E. coli* strains used in study B, the *E. coli* strains were pooled in order to observe only the effect of H_2O_2 concentration and contact times on the *E. coli* strains. Error bars represent error at a 95% confidence interval

The log reductions achieved for the individual *E. coli* strains exposed to H_2O_2 for the 120 min contact time varied from 2.13 – 5.48 (Figure 2). It was observed that ATCC 11775 was the most sensitive to the H_2O_2 treatment (5.48 log reduction). ATCC 25922 showed a 5.14 log reduction after 120 min. A significant difference was not seen between the log reductions achieved for ATCC 11775 and ATCC 25922 at 120 min ($p = 0.19$). These strains were considered the most sensitive of all the strains tested. ATCC 35218 was the most resistant ATCC strain to H_2O_2 treatment and only a 2.14 log reduction was achieved after 120 min (Figure 2).

The environmental *Escherichia coli* strains MJ58 and M53 showed the same log reduction (2.17 log reduction

after 120 min), while M29 resulted in a 2.27 log reduction after 120 min (Figure 2). A significant difference was not seen between M53, MJ58 and M29 ($p > 0.05$) after 120 min, and these strains were considered the most resistant of the environmental *E. coli* strains evaluated in this study. Environmental strains F11.2, E22.1 and E11.1 indicated 2.82, 3.08 and 2.9 log reductions, respectively, after 120 min.

A significant difference was not observed between F11.2, E22.1 and E11.1 ($p > 0.05$), although these strains did however, differ significantly from, M53, MJ58 and M29 ($p < 0.05$) (Figure 2). A 3.52 log reduction was achieved after the 120 min contact time for E12.1. Consequently, *E. coli* E12.1 was considered one of the more sensitive environmental strains to the H_2O_2 treatment. E12.1 differed significantly from M29, M53, MJ58, F11.2 and E11.1, however a significant difference was not seen between E12.1 and E22.1 ($p = 0.08$) (Figure 2). MJ56 was the most sensitive environmental strain to the H_2O_2 treatment, resulting in a 3.92 log reduction after 120 min. MJ56 differed significantly ($p < 0.05$) from all the other environmental *E. coli* strains used in the study, except for *E. coli* E12.1 ($p = 0.11$). Growth was observed for all the environmental *E. coli* strains after 120 min, however for ATCC 11775 no growth was present after 120 min and therefore a statistical lower limit of 30 cfu.mL^{-1} (1.48 log cfu.mL^{-1}) was used for the data analysis.

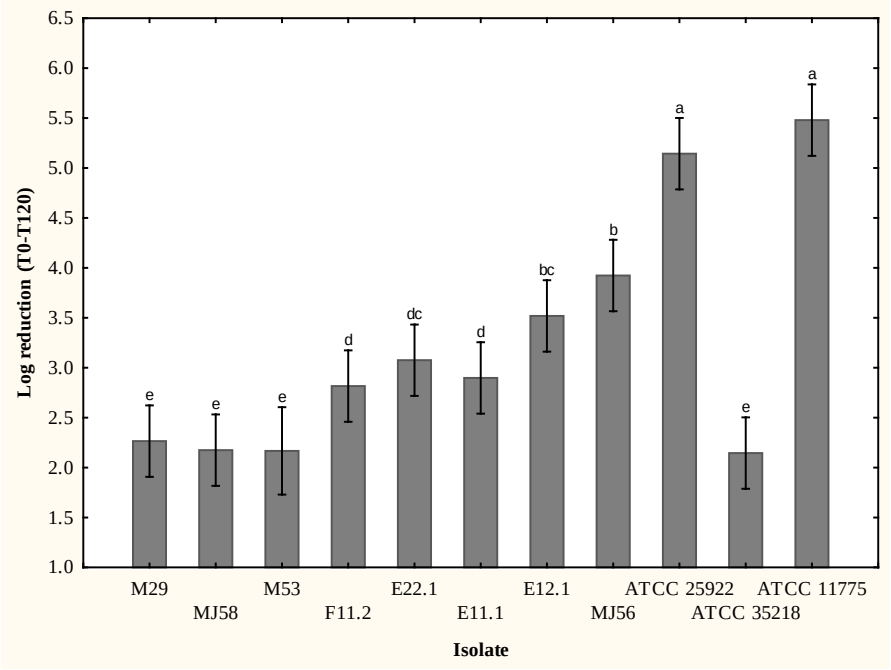

Figure 2. Log reductions achieved for the 11 *E. coli* strains after the 120 min contact time (T0 – T 120) (Pooled H_2O_2 concentrations). Error bars represent error at a 95% confidence interval

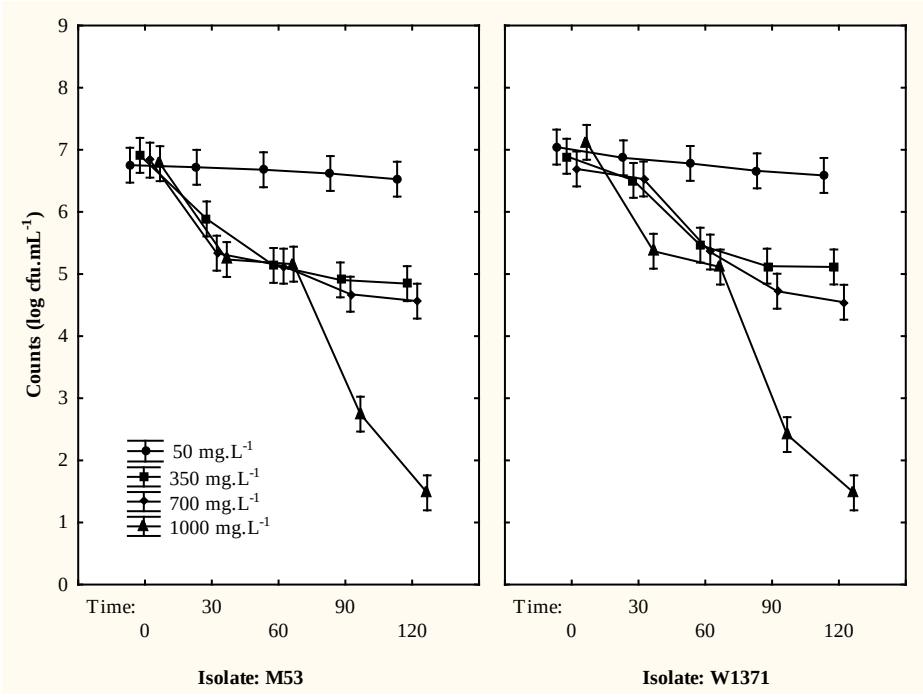

Figure 3. Effect of four H_2O_2 (50, 350, 700 and 1000 mg.L^{-1}) concentrations on M53 and W1371. No growth was recorded as 30 cfu.mL^{-1} (1.48 log cfu.mL^{-1}). Error bars represent error at a 95% confidence interval

M53 was selected as one of the most resistant environmental *E. coli* strains to H_2O_2 treatment and exposed to four H_2O_2 concentrations (50, 350, 700 and 1 000 mg.L^{-1}), to determine the effect that both low and high H_2O_2 concentrations would have. When 50 mg.L^{-1} H_2O_2 was used, a significant difference was not seen between the counts obtained at 0, 30, 60, 90 and 120 min (Figure 3) ($p > 0.05$), with an overall log reduction of 0.22. At 350 mg.L^{-1}, the overall log reduction increased to 2.06 (Figure 4). When the H_2O_2 concentration was doubled to 700 mg.L^{-1} the log reduction increased slightly from 2.06 to 2.27, and no significant difference was observed between the log reductions achieved with the 350 and 700 mg.L^{-1} H_2O_2 treatments ($p = 0.24$) (Figure 4). At 1 000 mg.L^{-1} growth was observed at 90 min (Figure 3), where a 4.3 log reduction was achieved. However, no growth was present after the 120 min contact time. For W1371, as for M53, no significant difference was seen between the log reductions at 350 and 700 mg.L^{-1} ($p = 0.05$) (Figure 4). At 1 000 mg.L^{-1} growth was observed at 90 min, where a 4.7 log reduction was achieved. However, no growth was present after the 120 min contact time (Figure 3). To facilitate statistical analyses no growth was recorded as 30 cfu.mL^{-1} (1.48 log cfu.mL^{-1}).

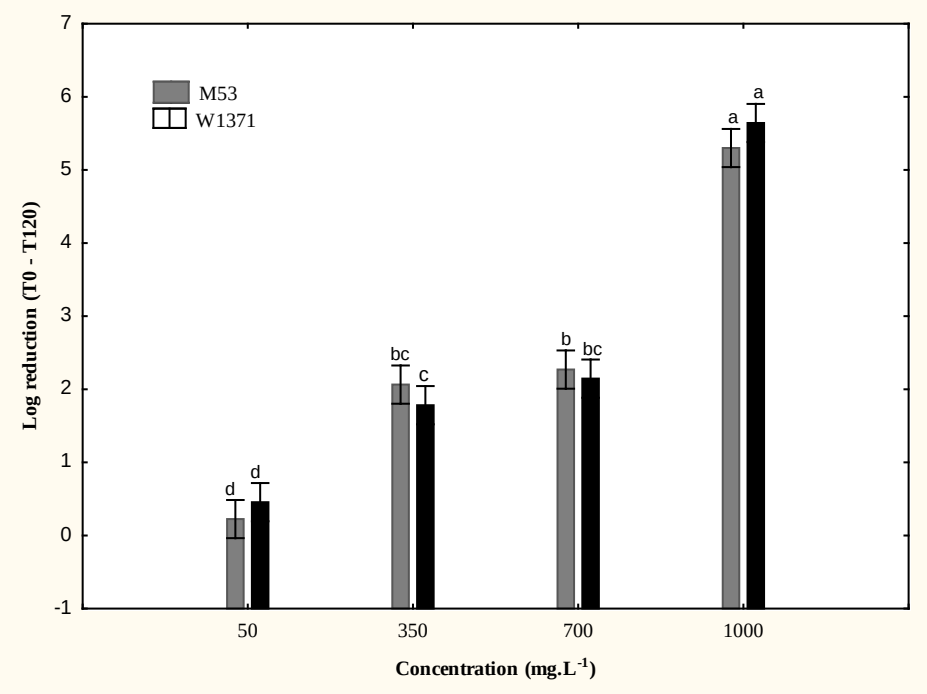

Figure 4. Log reductions achieved after 120 min for M53 and W1371 at 50, 350, 700 and 1 000 mg.L^{-1}. Error bars represent error at a 95% confidence interval

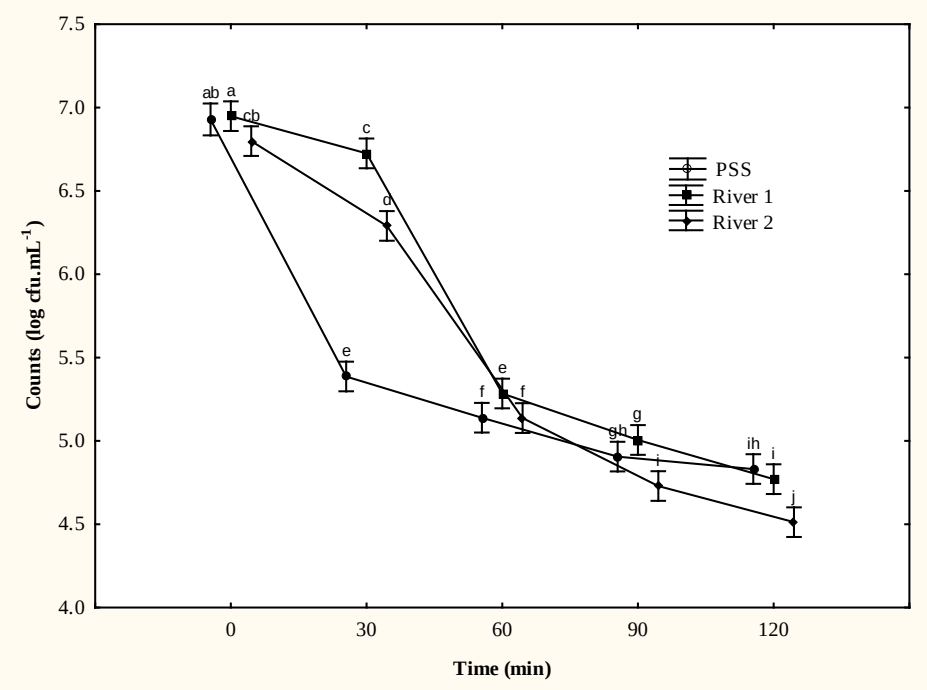

Figure 5. Effect of 350 mg.L^{-1} H_2O_2 on M53 in PSS, River 1 and River 2 water samples. Error bars represent error at a 95% confidence interval

3.2. Study 2: Effect of Organic Matter (COD) on the Efficacy of the H_2O_2 Treatment

River water was sampled from two rivers, and autoclaved before the samples were inoculated with *E. coli* M53. The COD values for River 1 and River 2 were 18 and 55 mg.L^{-1}, respectively. A comparison was made between the log reductions achieved after the H_2O_2 treatment (350 mg.L^{-1}) in PSS (which served as a control), River 1 and River 2 at time intervals at 0, 30, 60, 90 and 120 min (Figure 5).

After 30 min, a significant difference was seen between the log reductions achieved for strain M53 in the PSS and River 1 ($p = 0.01$), as well as the PSS and River 2 ($p = 0.02$), with numbers decreasing quicker in the PSS sample. After 120 min, 2.12, 2.18 and 2.28 log reductions were achieved in the PSS, River 1 and River 2 samples, respectively. These did not differ significantly (PSS and River 1, $p = 0.90$; PSS and River 2, $p = 0.70$; River 1 and River 2, $p = 0.80$).

3.3. Study 3: Catalase Activity of *E. coli* Strains

The total catalase activity was determined for all 12 *E. coli* strains using method 1 [19]. MJ58, M53, M29, as well as E22.1, was observed to have the highest catalase activity (+++) according to the amount of bubbles formed. F11.2, E12.1, E11.1, MJ56, W1371 and ATCC 35218 showed a slightly lower catalase activity (++). The ATCC strains 25922 and 11775 had the lowest catalase activity (+) in comparison to all the other *E. coli* strains tested.

In order to quantify the catalase activity, all the strains were evaluated using method 2 [20]. The amount of total catalase activity varied between 120.2 - 557 units (U per 1 mg wet weight) for all strains tested (Figure 6).

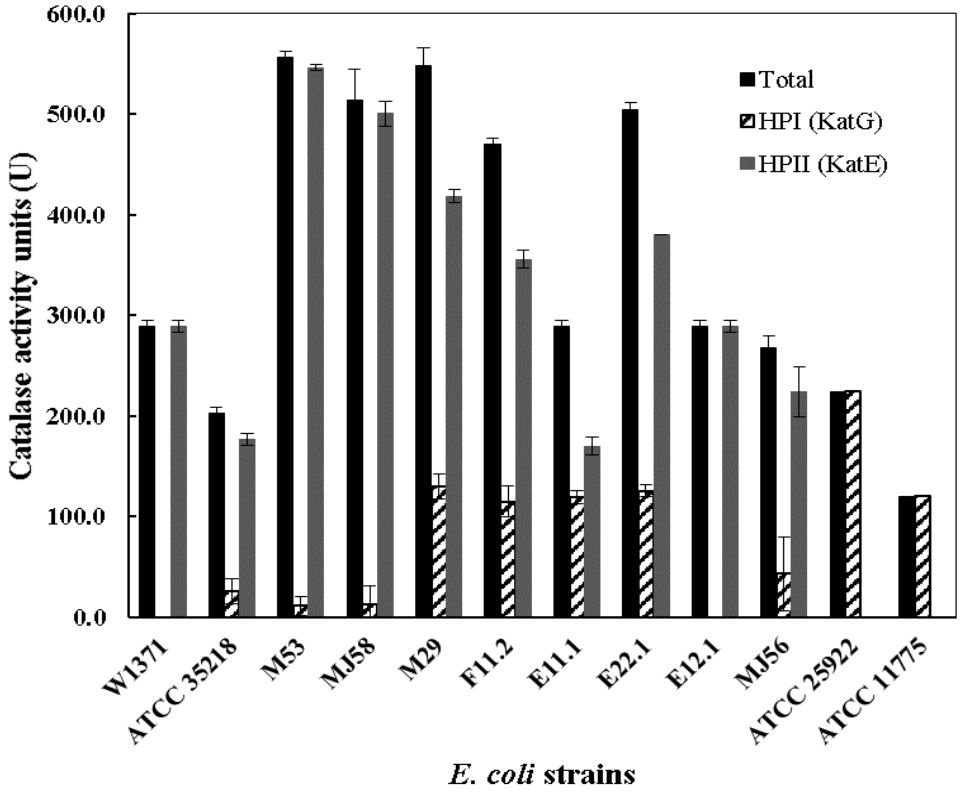

Figure 6. Catalase activity of all 12 *E. coli* strains in the study. Error bars represent error at a 95% confidence interval

M53, M29, MJ58 and E22.1 had 557, 548.3, 513.7 and 505.1 U total catalase, respectively, and had the highest amount of total catalase activity. These results corresponded with the observations of method 1. These four strains also had the highest amount of HPII activity (Figure 6). The HPI activity varied between 0 – 224 U catalase for all strains tested (Figure 6). It was observed that H_2O_2 resistant strain (W1371) indicated no HPI activity and ATCC 25922, a sensitive strain to the H_2O_2 treatment, had 224 U HPI catalase activity

4. Discussion

Although a trend was observed in Study 1, no significant differences could be proven between the bactericidal activity of the 250, 300 and 350 mg.L^{-1} H_2O_2 treatments. Similar results were seen in a study done by Vargas *et al.* [8] where little statistical significant variation was found between the following H_2O_2 concentrations: 100, 150, 200, 250 and 300 mg.L^{-1}. ATCC 35218 was the most resistant ATCC strain to H_2O_2 treatment. ATCC 35218 is also resistant to three antibiotics including ampicillin, chloramphenicol and streptomycin (Table 1). Log reductions obtained for ATCC 35218 differed significantly from both those obtained for ATCC 11775 and ATCC 25922. Thus, significant variations exist between the H_2O_2 resistance of the three ATCC *E. coli* strains tested.

Variation in strain resistance to the H_2O_2 treatment was also clearly evident for the eight environmental strains tested at the 250, 300 and 350 mg.L^{-1} H_2O_2 treatments. The log reductions of the three most resistant

environmental strains (M53, M29 and MJ58) did not differ significantly from the reduction observed for ATCC 35218. Interestingly, M53 and M29 also exhibited multiple antibiotic resistances (Table 1), as was observed for ATCC 35218. The other five environmental strains all showed varying resistance to H_2O_2 treatments, but were all significantly more resistant than the two ATCC strains (25922 and 11775) (Figure 2). This highlights the fact that careful consideration should be given to the choice of strain when testing the efficacy of water treatment strategies. Choosing a sensitive strain, like ATCC 25922, might result in the development of strategies which is not properly optimized for the reduction of environmentally adapted strains.

Studies by Britz *et al.* [20,21] indicated high faecal contamination of certain South African rivers and recommended a 3 – 4 log reduction to achieve irrigation water of a safe quality. The log reductions obtained for the four most resistant strains (Figure 2) were all in the range between 2.14-2.27. Orta de Velásquez *et al.* [10] reported similar reductions in a study done using H_2O_2 to treat wastewater. They found that faecal coliforms decreased by 2.2 log reduction after 120 min, in the presence of 250 $mg.L^{-1}$ H_2O_2. However it must be noted that the treatment was done in wastewater, and therefore high levels organic matter present in the water could have further reduced the efficiency of the H_2O_2 treatment [7,10]. The impact of higher concentrations of H_2O_2 on the log reduction of resistant (M53) and pathogenic strains (W1371) were, therefore, also evaluated.

A study done by Linley *et al.* [6] hypothesized that at lower H_2O_2 concentrations, bacterial inactivation is a result of DNA damage, and is closely related to the Fe^{2+} ions associated with the DNA molecule. They argued that DNA-associated iron may be the limiting factor when H_2O_2 reacts with DNA, thus indicating that increasing the H_2O_2 concentration may not result in an increased rate of DNA damage. It was hypothesized that the biocidal effect of H_2O_2 at higher concentrations is a result of oxidation of proteins and lipids [6]. A low and high H_2O_2 concentration was thus included to determine if it would result in at least a three log reduction.

Results indicated that survival decreased as the concentration increased (Figure 3 & Figure 4). The fact that no significant differences were observed between the log reductions of both strains at 350 and 700 $mg.L^{-1}$ H_2O_2 treatments suggests that there might be a certain resistance threshold in resistant *E. coli* strains which enables the strains to withstand higher H_2O_2 concentrations up to a certain level. The same "threshold" can be observed for the 1000 $mg.L^{-1}$ H_2O_2 treatment up to 60 min (Figure 3). After 60 min a rapid decrease in numbers were observed for both strains. This is an important factor to consider, as the concentration selected to treat irrigation water has cost implications. It would not be worth doubling the concentration of H_2O_2, and in turn the chemical cost, if similar log reductions could be achieved at both concentrations. Less contaminated water might only need a 2 log reduction in *E. coli* levels to reach the recommended irrigation water limit. For more contaminated sources, implementing a 1 000 $mg.L^{-1}$ treatment for at least 90 min could result in a sufficient log reduction, unless water quality parameters interfere with H_2O_2 efficacy.

High organic matter may limit the efficiency of H_2O_2 [7]. Study 1 was done in PSS, but since H_2O_2 will oxidize all the organic matter present, log reductions achieved in river water might be negatively impacted if the water has a high COD. It was therefore expected in Study 2 that the H_2O_2 treatment of samples from River 1 and River 2 would be less effective that the control sample in PSS. This was true for the first 30 min, where the levels of M53 decreased quicker in PSS than in the river water samples (Figure 5). After the 120 min treatment it was however clear that the PSS control was not more efficient that the River water samples. It was therefore concluded that, a low COD value (18 – 55 $mg.L^{-1}$) did not appear to influence the efficiency of the H_2O_2 treatment over the 120 min exposure time.

H_2O_2 can be catalyzed into water and oxygen by the catalase enzymes hydroperoxidase I (HPI) and hydroperoxidase II (HPII), therefore reducing the toxicity of H_2O_2 [22]. *Kat*G genes are responsible for encoding HPI and *Kat*E genes are responsible for encoding HPII [19,22,23,24]. These two catalase enzymes have different functions. HPI is induced by low H_2O_2 concentrations, whereas HPII is induced during the stationary phase or other stresses [25,26]. Thus, catalase is an enzyme that can protect the cell against sub-lethal concentrations of H_2O_2. Catalase levels may be increased due to phenotypic adaptation to protect the cell against oxidative stress. Consequently, active resistance against H_2O_2 may develop [27]. The aim of Study 3 was to determine if the *E. coli* strains which showed resistance to the H_2O_2 treatments in Study 1, also had high catalase activities. This would in turn indicate the importance of catalase as a mechanism by which *E. coli* strains can protect themselves against H_2O_2 treatment.

The results from Study 3 indicated that no correlation existed between H_2O_2 resistance and HPI catalase activity. In fact the two strains with the highest HP1 catalase activity (ATCC strains 25922 and 11775), were the most sensitive strains to the H_2O_2 treatment (Figure 6). In contrast, it was observed that HPII could be an important enzyme to protect strains against H_2O_2 treatment. The H_2O_2 resistant strains M53, M29, MJ58 also had the highest HPII catalase activity, while the most sensitive strains (ATCC strains 25922 and 11775) showed no HPII catalase activity.

It was however concluded that HPII catalase activity could not be the only mechanism that determines resistance against H_2O_2 treatment. Two of the most resistant strains to H_2O_2 treatment (ATCC 35218 and W1371) only indicated average HPII catalase activity. These strains may have other properties that enable them to withstand high H_2O_2 concentrations. Both these strains, for instance, display multiple antibiotic resistances (Table 1). The mechanisms that enable them to resist certain antibiotics might also aid in protecting the strains against high H_2O_2 concentrations. Loui *et al.* [28] determined the resistance of *E. coli* to H_2O_2 in the presence of chloramphenicol, which inhibits protein synthesis by inhibiting peptide bond formation. Their results indicated that chloramphenicol enhanced the effect of H_2O_2, as greater bacterial inactivation was seen with the use of H_2O_2 and chloramphenicol than either one of the substances alone. It was concluded that the synergistic effect of using H_2O_2 and chloramphenicol indicated that

protein synthesis is important for *E. coli* resistance to H_2O_2. In this study both M29 and ATCC 35218, showed antibiotic resistance against Chloramphenicol, and both these strains were considered highly resistant to the H_2O_2 treatment. It could be that, as a result of their unique genetic make-up, the same physiological properties that protect protein synthesis in these strains from Chloramphenicol, will also protect these strains from H_2O_2 treatment. Thus, the resistance of the *E. coli* strains may not be due to catalase activity alone, especially in the strains indicating H_2O_2 resistance, yet displaying only average catalase activity. Non-specific efflux pumps is another strategy that bacteria can employ to protect them against non-specific biocides (such as H_2O_2) as well as against target-specific antibiotics [29]. High efflux pump activity might explain why certain multi-drug resistant strains in this study with only average catalase activity, (W1371 and ATCC 35218), were highly resistant to H_2O_2 treatment. This will, however, need to be investigated further.

5. Conclusion

Overall, a trend was seen between the H_2O_2 concentrations tested, with the highest H_2O_2 concentration resulting in the highest log reduction. Strain-to-strain variation was observed for all the strains tested indicating that environmental strains are usually better adapted to withstand the H_2O_2 treatment that ATCC strains. It was concluded that resistant environmental *E. coli* strains are capable of surviving high H_2O_2 concentrations (1 000 $mg.L^{-1}$), for long time periods (up to 90 min) provided high microbial loads are present. Careful consideration should therefore be given to the choice of a test strain for treatment method optimization studies. Exposure time during H_2O_2 treatment appear to be a critical parameter for two reasons: Firstly, when considering the "threshold" tolerance that certain H_2O_2 resistant strains exhibited in this study at concentrations of 1000 $mg.L^{-1}$ H_2O_2 and lower; and secondly, considering the influence of water COD levels (18 – 55 $mg.L^{-1}$) on H_2O_2 efficacy at shorter treatment times. Finally, although catalase activity (more specifically HPII activity) appears to be an important microbial defense mechanism against H_2O_2 treatment, it is evident that *E. coli* can also employ other protection strategies.

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References

[1] Department of Water Affairs & Forestry, South Africa. *National Water Resource Strategy – first edition*. The Government Printer, Pretoria, 2004.

[2] Norton-Brandão, D., Scherrenberg, S.M. and Van Lier, J.B, "Reclamation of used urban waters for irrigation purposes – A review of treatment technologies," *Journal of Environmental Management*, 122. 85-98. 2013.

[3] FAO (Food and Agriculture Organization). *Coping with water scarcity challenge of the twenty-first-century- UN Water*. 2007. [Internet document]. Available: http://www.fao.org/nr/water/docs/escarcity.pdf. [Accessed Aug. 31, 2013].

[4] Ijabadenyini, O.A. and Buys, E.M, "Irrigation water and microbiological safety of fresh produce; South Africa as a case study: A review," *African Journal of Agricultural Research*, 7 (35). 4848-4857. 2012.

[5] Heaton, J.C. and Jones, K, "Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review," *Journal of Applied Microbiology*, 104. 613-626. 2008.

[6] Linley, E., Denyer, S.P., McDonnell, G., Simons, C. and Maillard, J-Y, "Use of hydrogen peroxide as a biocide: new consideration of its mechanism of biocidal action," *Journal of Antimicrobial Chemotherapy*, 67 (7). 1589-1596. 2012.

[7] Newman, S.E, "Disinfecting Irrigation Water for Disease Management." in *20th Annual Conference on Pest Management on Ornamentals. San Jose, California*, Colorado State University. 2004.

[8] Vargas, G.D.L.P., Moreira, R.F.P.M. and José, D.S.H.J, "Treated domestic sewage: Kinetics of *Escherichia coli* and total coliform inactivation by oxidation with hydrogen peroxide," *Quim Nova*, 36 (2). 252-256. 2013.

[9] McDonnell, G. and Russell, A.D, "Antiseptics and disinfectants: activity, action and resistance," *Clinical Microbiology Reviews*, 12. 148-179. 1999.

[10] Orta de Velásquez, M.T., Yáñez-noguez, I., Jiménez-cisneros, B. and Luna Pabello, V.M, "Adding silver and copper to hydrogen peroxide and peracetic acid in the disinfection of an advanced primary treatment effluent," *Environmental Technology*, 29. 1209-1217. 2008.

[11] Labas, M.D., Zalazar, C.S., Brandi, R.J. and Cassano, A.E, "Reaction kinetics of bacteria disinfection employing hydrogen peroxide", *Biochemical Engineering Journal*, 38. 78-87. 2008.

[12] WHO (World Health Organization), "*Health Guidelines for the Use of Wastewater in Agriculture and Aquaculture*," WHO, Technical Report Series no. 778. Geneva, Switzerland. 1989.

[13] Cherchi, C. and Gu, A.Z, "Effect of bacterial growth stage on resistance to chlorine disinfection," *Water Science & Technology*, 64 (1). 7-13. 2011.

[14] Wojcicka, L., Hofmann, R., Baxter, C., Andrews, R.C., Lière, J., Miller, T., Chauret, C. and Baribeau, H, "Inactivation of environmental and reference strains of heterotrophic bacteria and *Escherichia coli* O157:H7 by free chlorine and monochloramine," *Journal of Water Supply: Research and Technology-AQUA*, 56 (2). 137-149. 2007.

[15] Mazzola, P.G. Martins, A.M.S. and Penna, T. C.V, "Chemical resistance of gram-negative bacteria to different sanitizers in a water purification system," *BMC Infectious Disease*, 6. 131-142. 2006.

[16] Merck, *Microbiology Manual, 12th edition*. Merck, Darmstadt, Germany. 2005.

[17] Lamprecht, C., Romanis, M., Huisamen, N., Carinus, A., Schoeman, N., Sigge, G.O. and Britz, T.J, "*Escherichia coli* with virulence factors and multidrug resistance in the Plankenburg River," *South African Journal of Science*, 110(9/10), Art.#2013-0347. October 2014. [Online] Available: http://www.scielo.org.za/scielo.php?pid=S0038-23532014000500013&script=sci_arttext. [Accessed May 30, 2015].

[18] Chen, C-H., Hofmann, C.S., Cottrell, B.J., Strobaugh, T.P., Paoli, C.G., Nguyen, L-H., Yan, X. and Uhlich, G.A, "Phenotypic and genotypic characterization of biofilm forming capabilities in non-O157 Shiga toxin producing *Escherichia coli* strain," PLoS ONE, 8(12), e84863, December 2013. [Online]. Available:

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0084863. [Accessed May 30, 2015].

[19] Iwase, T., Tajima, A., Sugimoto, S., Okuda, K., Hironaka, I., Kamata, Y., Takada, K. and Mizunoe, Y, "A simple assay for measuring catalase activity: A visual approach," *Scientific Reports*, 3. 3081; October 2013. [Online]. Available: http://www.nature.com/srep/2013/131030/srep03081/full/srep03081.html?message-global=remove. [Accessed May 30, 2015].

[20] Britz, T.J., Sigge, G.O., Buys, E.M., Schmidt, S., Potgieter, N. and Taylor, M.B, "Baseline study on extent (types and quantities) of contamination found in irrigation water at selected sites (K5/1773)" In: *Quantitative investigation into the link between irrigation water quality and food safety* (Volume 2). WRC Report No. K5/1773/4, Pretoria, 2012.

[21] Britz T.J., Sigge, G.O., Huisamen, N., Kikine, T., Ackermann, A., Lötter, M., Lamprecht, C. and Kidd, M, "Fluctuations of indicator and index microbes as indication of pollution over three years in the Plankenburg and Eerste Rivers, Western Cape, South Africa," *Water SA*, 39 (4). 457-465. 2013.

[22] Loewen, P.C., Switala, J. and Triggs-Raine, B.L. "Catalase HPI and HPII in *Escherichia coli* are induced independently," *Archives of Biochemistry and Biophysics*, 243 (1). 144-149. (1985).

[23] Heimberger, A. and Eisenstark, A, "Compartmentalization of catalases in *Escherichia coli*,"*Biochemical and Biophysical Research Communications*, 154 (1). 392-397. 1988.

[24] Storz, G. and Imlay, J, "Oxidative stress," *Current Opinion Microbiology*, 2. 188-194. 1999.

[25] Mulvey, M.R., Switala, J., Borys, A. and Loewen, P.C, "Regulations of transcription of *Kat*E and *Kat*F in *Escherichia coli*," *Journal of Bacteriology*, 172 (12), 6713-6720. 1990.

[26] Visick, J.E. and Clarke, S, "RpoS- and OxyR-Independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of *rpo*S mutations in common laboratory strains," *Journal of Bacteriology*, 179 (13). 4158-4163. 1997.

[27] Morató, J., Mir, J., Codony, F., Mas, J. and Ribas, F, "Microbial response to disinfectants" in *The Handbook of Water and Wastewater Microbiology* (edited by D. Mara & N. Horan), Academic Press, London, 2003, 657-670

[28] Loui, C., Chang, A.C. and Lu, S, "Role of the ArcAB two-component system in the resistance of *Escherichia coli* to reactive oxygen stress," *BMC Microbiology*, 9, 183-196.

[29] Ortega Morente, E., Fernández-Fuentes, M.A., Burgos, M.J.G., Abriouel, H., Publido, R.P. and Gálvez, A, "Biocide resistance in bacteria," *International Journal of Food Microbiology*, 162 (1). 13-25. 2013.