Chlorine disinfection of \textit{Pseudomonas aeruginosa}, total coliforms, \textit{Escherichia coli} and \textit{Enterococcus faecalis}: revisiting reclaimed water regulations

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\textbf{ABSTRACT}

Pathogenic organisms can be transmitted orally through drinking water or through skin and mucosae by both direct and indirect contact, and their presence in water thus has a negative impact on public health. In wastewater treatment plants (WWTP), water is disinfected to inactivate pathogens. The quantification of several microbial indicators in aquatic systems is required to estimate the biological quality of such systems. So far, coliform bacteria have been used as traditional indicators worldwide. This study has assessed the resistance of total coliforms, \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa} and \textit{Enterococcus faecalis} to three dosages of sodium hypochlorite (NaClO) at two exposure times. The bacteria were isolated from secondary effluents of a WWTP located in Hidalgo, Mexico. The results show that the number of colony-forming units of all studied bacterial types decreased when both the NaClO concentration and exposure times increased. However, they were not eliminated. The inclusion of the species \textit{Pseudomonas aeruginosa} in regulations for treated wastewater quality as a new indicator is highly recommended due to its importance as an opportunistic pathogen. The detection of this species along with the traditional organisms could be particularly significant for reclaimed water to be used with direct human contact.

\textbf{Key words} | inactivation, indicators, NaClO, pathogens, reclaimed water, resistance

\textbf{INTRODUCTION}

Water reuse is a cost-effective answer to the increasing pressure exerted on fresh water resources. Reclaimed water is now considered as a resource, which can even be sold as a product (Lazarova \textit{et al.} 2001). Around the world, reclaimed water has been used mainly for nonpotable applications such as irrigation, industrial process water and environmental enhancement (Mujeriego & Asano 1999). In Mexico, agricultural use is the most important, not only of reclaimed water but also of wastewater discharged with any or limited treatment. The main public concern lies in the microbiological risk, as water polluted with human faeces is likely to contain human-specific pathogens such as bacteria, protozoan parasites or enteric viruses.

Due to the constraints associated with pathogen monitoring, indicator organisms are employed as surrogates. Ideally, indicators must be nonpathogenic, rapidly detected, easily enumerated and strongly associated with the presence of pathogens (Scott \textit{et al.} 2002). Total coliforms (TC) and faecal coliforms (FC), as well as \textit{Escherichia coli}, have been used extensively as indicators of faecal pollution in reclaimed water and as a measure of efficiency of disinfection processes. Following the guidelines of the World
Health Organization (WHO 2006), Mexican regulations for reclaimed water are based on the monitoring of faecal coliforms and helminth eggs (DOF 1997, 1998).

However, it has been shown that coliform bacteria do not reflect properly the occurrence of pathogens in disinfected wastewater due to their higher susceptibility to chemical disinfection (Harwood et al. 2005). Besides, recent studies show that the ability of E. coli to grow in contaminated soils limits its reliability as an indicator in tropical and subtropical regions (Scott et al. 2002). Therefore, Mazari-Hiriart et al. (2008) have suggested the use of alternative indicators as Enterococcus spp. on the basis of their prevalence in reclaimed water used around Mexico City.

Due to their sanitary importance, genera such as Pseudomonas, Flavobacterium, Acinetobacter, Klebsiella, Proteus, Aeromonas and Mycobacterium could be also used for this purpose (Coronel-Olivares 2007).

The need to obtain a realistic overview of reclaimed water microbial quality in temperate countries has been previously recognized (Mazari-Hiriart et al. 2008). This need is aggravated by the widespread use of wastewater or reclaimed water not adequately treated. Thus, the main objective of this work was to assess the quality of secondary effluents in terms of conventional indicators such as TC and E. coli but also of Enterococcus faecalis and Pseudomonas aeruginosa, which could constitute surrogates better suited to temperate conditions. The adequacy of these organisms as indicators was evaluated by their resistance to sodium hypochlorite (NaClO), which is the disinfectant most commonly used in Mexico.

Resistance to disinfection was studied on strains isolated from secondary effluents of a municipal wastewater plant (WWTP) and on certified strains. Three concentrations of disinfectant and two exposure times were tested to simulate typical conditions of disinfection treatments (Metcalf & Eddy 2003).

**METHODS**

The study was undertaken in samples from a small plant treating municipal wastewater in Pachuca, Hidalgo (Mexico). Wastewater is treated by a conventional activated sludge process of the extended aeration type. The tertiary treatment condition is disinfection with NaClO (11%). Twenty samples were taken weekly at the following points of the WWTP: raw influent (I), treated effluent (E) and disinfected water (D). Water samples were collected in sterilized polyethylene containers with a capacity of 1000 mL. Temperature and pH were measured in situ according to protocols described in the Standard Methods for the Examination of Water and Wastewater (APHA/AWWA/WEF 1998).

For the quantification of mesophilic aerobic bacteria, serial decimal dilutions were prepared from $1 \times 10^{-1}$ to $1 \times 10^{-5}$, for water samples coming from the three points (I, E and D), using isotonic saline solution (ISS) 0.85%. Of each dilution, 100 μL were plated in standard count agar media (Dioxon) and incubated for 48 h at 37 °C. Subsequently, the average number of colony-forming units was quantified and reported as log (CFU/100 mL).

For the disinfection tests, a volume of 200 mL of E water was placed in each of three sterilized flasks and varying concentrations of NaClO (8, 20 and 30 mg/L) were added. These concentrations correspond to 7.56, 18.90 and 28.35 mg/L of available chlorine according to the conversion table of Metcalf & Eddy (2003). Flasks were continuously stirred using a magnetic stirring apparatus. Two typical exposure times were studied (20 and 30 min). For each disinfectant concentration studied, samples of 100 μL obtained before and after the exposure times were cultured by the spread-plate method in triplicate. The media were composed of a dual layer; on the surface, one of the following selective media, i.e. MacConkey Agar (Dioxon), Pseudomonas Agar (Difco), Enterococcus Agar (Difco), Violet Red Bile Agar (Dioxon), and for all plates, Tripticase Soy Agar (Dibico) as a base. Petri dishes were incubated at 37 °C for 48 h. Then, quantification of colonies was carried out, as well as confirmatory biochemical tests and reported as log (CFU/100 mL). The pyocyanin-positive test, involving irradiation with UV light and catalase-positive activity by adding H$_2$O$_2$ 50%, confirmed the isolation of P. aeruginosa. The catalase-negative test confirmed the isolation of E. faecalis.

The experimental disinfection procedure was validated by comparison against independent tests on reference strains of the American Type Culture Collection: E. coli ATCC 35218, P. aeruginosa ATCC 19433 and E. faecalis ATCC 10145, inoculated in treated effluent previously filtered through a sterilized nitrocellulose membrane, with a pore size of 0.45 μm.

**DATA ANALYSIS**

The removal percentage of microorganisms in the disinfection experiments is given by:

\[
R_\% = \frac{(T_0 - T_1)}{T_0} \times 100
\]
where the removal percentage, $R\%$, the number of CFU quantified before the disinfectant addition, $T_0$, and the number of CFU quantified at the retention times of 20 and 30 min, $T_1$. From raw data the media ($X$), maximum (MAX), minimum (MIN) and standard deviation (SD) values were calculated.

For the purpose of statistical analysis, data were transformed to natural logarithms. To determine bacterial resistance among the various exposure times and treatments, two-way ANOVA tests were performed for each type of microorganism (i.e. total coliforms and the other three species) as well as for each treatment (concentration and exposure times). Variance analyses were carried out with the software Power Analysis and Sample Size (PASS) from NCSS (USA).

### RESULTS AND DISCUSSION

#### Physicochemical parameters

The success of microorganisms in aquatic environments is determined by their growth rate, propagation and activity. While the determination of physicochemical parameters is of great importance to understand water quality, it is also important to acknowledge the natural growth conditions of microorganisms at the time of sampling. The values of the parameters (pH and temperature) were satisfactory for potential growth of microorganisms in all sampling points of the WWTP.

The average values of pH were 7.32, 7.49 and 7.44 in the three sampling points (I, E and D, respectively), which were similar to what has been determined by Martínez-Hernández (2006) and Coronel-Olivares (2007) for the same WWTP. Salgot et al. (2006) made a compendium of the thresholds reported in the regulations of different countries and proposed a pH range of 6 to 9.5 for reclaimed water with residential, urban and irrigation uses. Countries and proposed a pH range of 6 to 9.5 for reclaimed water supplies for sanitary services or car washing, the amount of these bacteria should be between $1.0 \times 10^5$ to $1.0 \times 10^3$. For instance, for garden irrigation, water supplies for sanitary services or car washing, the amount of these bacteria should be between $1.0 \times 10^5$ and $1.0 \times 10^4$; for street cleaning and ornamental water fountains, the values should be around $1.0 \times 10^4$, and for water ponds and lakes – with public contact but excluding bathing – they should range from $1.0 \times 10^4$ to $1.0 \times 10^5$.

#### Resistance to NaClO

Before disinfection tests the total average values (log UFC/100 mL) of each type of microorganism were $4.46 \pm 0.55$ for TC; $4.41 \pm 0.55$ for E. coli; $3.47 \pm 0.42$ for P. aeruginosa and $3.76 \pm 0.75$ for E. faecalis.

Table 2 shows the removal percentages for each type of microorganism at different dosages and after two exposure times with the disinfectant. It should be noted that the water quality changed continually. For some water samples, over 300 CFU could grow in the plates while in others the plates displayed no growth at all. Therefore, the standard deviation was high for some samplings.

Total coliform bacteria and E. coli were selected for this study, as they are considered traditional indicators of water quality. However, they were not removed entirely as expected. The results show that there is no guarantee of disinfection in treated water using the NaClO concentrations of these experiments. With the higher exposure time of bacteria to the disinfectant and at the greater disinfectant dosage, only 85.95% of total coliforms was removed, while

### Table 1: Content of mesophilic aerobic bacteria in log (CFU/mL) isolated from the points raw influent (I), treated effluent (E) and disinfected water (D) of a WWTP

<table>
<thead>
<tr>
<th></th>
<th>(I)</th>
<th>(E)</th>
<th>(D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>8.16</td>
<td>7.20</td>
<td>6.46</td>
</tr>
<tr>
<td>Maximum</td>
<td>8.99</td>
<td>8.29</td>
<td>8.03</td>
</tr>
<tr>
<td>Minimum</td>
<td>6.39</td>
<td>5.30</td>
<td>4.70</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.67</td>
<td>0.84</td>
<td>1.15</td>
</tr>
</tbody>
</table>

By guest

Downloaded from https://iwaponline.com/wst/article-pdf/64/11/2151/444113/2151.pdf
for *E. coli* the removal was of 87.40% under the same conditions. Lazarova *et al.* (1999) determined that at chlorine dosages of 8 mg/L with an exposure time of 30 min, a total coliform quantification <10⁴ CFU/100 mL was assured, but complete elimination was not possible.

*Pseudomonas aeruginosa* had the lowest removal percentage, confirming its high resistance to the chemical action of the disinfectant at all the tested concentrations and for both exposure times. At the greatest concentration of NaClO and at the maximum exposure time, removal of these bacteria hardly reached 53.57%; hence the experimental conditions employed were not enough to assure the inactivation of the strains studied. Stewart *et al.* (2001) found that with 1,000 mg/L of the same disinfectant and after an exposure time of 11.6 min, the cell density of *P. aeruginosa* decreased by 0.85 log in relation to the number of viable cells. Reports by Shrivastava *et al.* (2004) demonstrated a decrease from 400 to 90 ± 5 CFU in strains of *P. aeruginosa* when exposure to a dosage of chlorine of 500 µg/L during 30 min was tested. When concentration was lower, even greater colony counts were found. Jjemba *et al.* (2010) confirmed an important regrowth of *Pseudomonas* spp. in reclaimed water systems of the USA after chlorine disinfection in three different WWTPs. Therefore, the need to include this bacterial species as a new indicator of reclaimed water quality is evident, especially if we consider its importance as an opportunist human pathogen.

*Pseudomonas aeruginosa* is known for producing opportunistic infections in immunocompromised individuals (de Victorica & Galván 2001). The primary route of infection is through skin contact or inhalation rather than ingestion. These microorganisms can cause cutaneous infections, as well as infections of the mucosa of the eyes, ears, nose and throat (cf. Shrivastava *et al.* 2004). The obvious resistance of this species to the action of NaClO, as reflected in its abundance in treated water, reveals the urgency of adding this bacterium to the list of indicators of reclaimed water quality regulations in all countries. Rutala & Weber (1997) demonstrated that there was no decrease in logarithmic units of *P. aeruginosa* after an exposure time of 100 min with a residual chlorine concentration of 100 mg/L at 20 °C and a pH value comprised between 8.2 to 9.2.

All the previous reports support the conclusion that *P. aeruginosa* is not totally removed by chemical treatment with chlorine. According to our results, the species that was removed in a greater proportion was **E. faecalis** (98.54%), although it was not eliminated. This last species denotes faecal contamination in treated water, either of human or animal origin. However, it would not be advisable to use this species as an indicator to assess the quality of reclaimed water, especially as it is more sensitive to disinfection than the other bacteria tested in this study.

### Statistical analysis

ANOVA tests demonstrated that the CFU/100 mL quantified for the different types of microorganisms studied varied

**Table 2** | Mean (X), maximum (MAX), minimum (MIN) and standard deviation (SD) values of removal percentages (R%) of CFU for total coliforms (TC), *E. coli*, *P. aeruginosa* and *E. faecalis* exposed to different concentrations of NaClO (8, 20 and 30 mg/L), with exposure times of 20 and 30 min

<table>
<thead>
<tr>
<th>Microorganism type</th>
<th>Values</th>
<th>8 mg/L</th>
<th></th>
<th>20 mg/L</th>
<th></th>
<th>30 mg/L</th>
<th></th>
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<tr>
<td>Total coliforms (TC)</td>
<td></td>
<td>56.96</td>
<td>78.32</td>
<td>67.12</td>
<td>83.06</td>
<td>79.14</td>
<td>85.95</td>
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<tr>
<td></td>
<td>MAX</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
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<tr>
<td></td>
<td>MIN</td>
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<td>28.90</td>
<td>12.65</td>
<td>54.11</td>
<td>12.50</td>
<td>25.00</td>
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<tr>
<td></td>
<td>SD</td>
<td>34.29</td>
<td>23.77</td>
<td>33.97</td>
<td>18.16</td>
<td>29.56</td>
<td>20.46</td>
</tr>
<tr>
<td>E. coli</td>
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<td>54.82</td>
<td>75.56</td>
<td>55.76</td>
<td>77.51</td>
<td>75.08</td>
<td>87.40</td>
</tr>
<tr>
<td></td>
<td>MAX</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>MIN</td>
<td>0.24</td>
<td>16.20</td>
<td>3.46</td>
<td>40.06</td>
<td>16.70</td>
<td>36.70</td>
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<tr>
<td></td>
<td>SD</td>
<td>36.80</td>
<td>28.17</td>
<td>37.29</td>
<td>23.23</td>
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<td>20.35</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td>39.08</td>
<td>50.39</td>
<td>39.33</td>
<td>50.35</td>
<td>50.31</td>
<td>53.57</td>
</tr>
<tr>
<td></td>
<td>MAX</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>MIN</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>34.58</td>
<td>30.45</td>
<td>32.68</td>
<td>32.36</td>
<td>35.70</td>
<td>32.69</td>
</tr>
<tr>
<td>E. faecalis</td>
<td></td>
<td>72.78</td>
<td>94.75</td>
<td>91.47</td>
<td>94.88</td>
<td>93.16</td>
<td>98.54</td>
</tr>
<tr>
<td></td>
<td>MAX</td>
<td>100.00</td>
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<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>MIN</td>
<td>8.55</td>
<td>74.72</td>
<td>55.11</td>
<td>50.06</td>
<td>16.70</td>
<td>36.70</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>24.25</td>
<td>7.39</td>
<td>13.72</td>
<td>12.11</td>
<td>20.55</td>
<td>6.34</td>
</tr>
</tbody>
</table>
significantly with the concentration of disinfectant and with the exposure time (Table 3).

The interactions between the ANOVA models showed no significant values, which means that the concentration of NaClO and the exposure times were independent for each microbial type studied. Figure 1 shows a significant decrease in the abundance of the different types of microorganisms in relation to the disinfectant concentration. Each type of microorganism displayed a unique trend with regard to the CFU decrease under the experimental conditions.

The most resistant species was *P. aeruginosa* while *E. faecalis* was removed to the greatest extent. Total coliform and *E. coli* showed a similar removal trend. It can be seen that standard errors do not overlap for any treatment. This demonstrates that there were significant differences between the types of microorganisms subjected to the exposure of varying concentrations of disinfectant and the exposure times described.

Figure 2 shows the decreasing trend in the mean abundance of the different types of microorganisms studied (expressed as log CFU/100 mL), with regard to the concentration of the disinfectant. Removal trends differed significantly in relation to NaClO concentrations. The difference in ANOVA test values between the lowest and the highest concentration of disinfectant were 2.41 log for *E. coli*, 2.23 log for total coliforms, 1.55 log for *E. faecalis* and 0.96 log for *P. aeruginosa*. The order in which the trends appear in the figure do not represent the real magnitude in which the various types of microorganisms were removed. In fact, percentages followed the decreasing sequence: *E. faecalis* > *E. coli* > total coliforms > *P. aeruginosa*.

The removal of all types of microorganisms according to the exposure times to the disinfectant is presented in Figure 3. There were significant differences in the removal of microorganisms as a function of time. The tendency of the mean abundance of each type of microorganism decreased.

The difference between the results from the ANOVA tests as opposed to the initial time and 30 min of exposure to the disinfectant was 1.54 log for *E. coli*, 1.21 log for total coliforms, 1.38 log for *E. faecalis* and 0.55 log for *P. aeruginosa*. Not even with the longer exposure to the disinfectant could the elimination of the different types of microorganisms be guaranteed. The order of decrease in abundance according to the exposure times to the disinfectant followed the same sequence as in the concentration tests.

### Resistance of certified bacterial strains

The experimental procedures and results were validated with a second set of tests on certified bacterial strains. With the dosages and exposure times studied, it was confirmed that only *E. faecalis* was eliminated. The removal percentage for certified strains increased when the dosage of the disinfectant and the exposure times were raised. At a 20 mg/L dosage after a contact of 20 min the removal was 57.14, 50 and 20% for *E. coli*, *E. faecalis* and *P. aeruginosa*, respectively. With the same dosage but after 30 min of exposure, the results were 100, 71.42 and 55% for *E. faecalis*, *E. coli* and *P. aeruginosa*. When the dosage was augmented to 30 mg/L with 20 min of exposure, the corresponding results were 100, 69.23 and 50%, and for an exposure time of 30 min, the results were 100, 84.61 and 60%.

These results were very similar to the percentages of removal of microorganisms isolated from the plant (98.54, 87.40 and 53.57%). The results on the certified strain of *P. aeruginosa* also confirmed the resistance of this species to disinfection processes based on moderate concentrations of NaClO. The greatest removal was 60%, very similar to the experimental result for the same species when isolated from the WWTP at the largest dosage and longest exposure time. This highlights once again the bacterial resistance to disinfection with NaClO, and renders evident the urgency to include *P. aeruginosa* in the regulations of reclaimed water quality as a new biological indicator.

### Table 3 | Obtained values from two-way ANOVA tests for each type of microorganism studied

<table>
<thead>
<tr>
<th>Type of microorganism</th>
<th>TC</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>E. faecalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>14.77</td>
<td>2</td>
<td>0.001</td>
<td>18.06</td>
</tr>
<tr>
<td>Interval</td>
<td>25.31</td>
<td>2</td>
<td>0.005</td>
<td>33.28</td>
</tr>
<tr>
<td>Interaction a and b</td>
<td>0.17</td>
<td>4</td>
<td>0.951</td>
<td>0.21</td>
</tr>
</tbody>
</table>

*F* = value of the ANOVA test; *d.f.* = degrees of freedom; *P* = significance of the analyses; TC = total coliforms.
CONCLUSIONS

The resistance to chlorination of total coliforms, *E. coli* and *P. aeruginosa* at different dosages of NaClO and contact times was demonstrated: when varying the concentrations of the NaClO disinfectant (8, 20 and 30 mg/L) at two exposure times (20 and 30 min) the studied bacteria were not eliminated. Larger dosages and longer exposure times might be explored. But it must be reminded that higher dosages lead to organo-chlorinated compound formation. *E. faecalis* reached higher removal percentages than total coliforms at all tested concentrations, and at both exposure times. This species does not pose a potential health risk for humans as its elimination is assured even at low concentrations of NaClO.

According to ANOVA tests there were significant differences in the percentage of removal of the studied microorganisms in relation to the concentration of the disinfectant. These results were independent for each type of bacteria. In all cases, the percentage of removal varied according to the concentration. With regard to the exposure times of the disinfectant, the results were also independent for each type of microorganism.
It is thus strongly recommended that the regulations of reclaimed water should include indicators other than TC bacteria, particularly *P. aeruginosa*, because of its opportunistic pathogenic capability. The inclusion of microorganisms other than those currently specified in reclaimed water regulations will allow an improvement of water quality and will ensure a safer reutilization.

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