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Inactivation of *Enterococcus faecium* in Water and Hospital Laundry Wastewater by Disinfection Processes Utilizing Peroxyacetic Acid or Ultraviolet Radiation

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In this study two disinfection processes were used to determine the disinfection effect of water and hospital laundry wastewater artificially contaminated with *Enterococcus faecium*. Different concentrations of peroxyacetic acid and different exposure times with ultraviolet radiation were tested on inoculated water and wastewater. The number of cfu after incubation on agar base was determined for each experiment. 70 mg/LPAA was sufficient to reach a 5-log^{10} reduction within 35 min treatment for hospital laundry waste water, whilst 5 min treatment time was reached by 110 mg/L PAA. 80 mg/L ensured a 5-log^{10} reduction after 15 min treatment time. For water inoculated with *Enterococcus faecium* 80 mg/LPAA was sufficient to reach a 5-log^{10} reduction within 5 min. However minimal recovery was noted and subsided after 40 min, thus proving that the hospital laundry wastewater already contained inhibitory substances shortening the necessary treatment time. Ultraviolet radiation for 1½ hours also proved to be efficient for hospital laundry wastewater with inhibitory substances preventing any dark repair after 18 hours. Thus indicating that such method could be used for laundry wastewater or similar water that is stored overnight and then reused, if scale-up and cost-effective studies prove to coincide with these results.

Key words: *Enterococcus faecium,* Laundry wastewater, Peroxyacetic acid, UV radiation.

The availability of freshwater to meet different water needs has raised serious concerns in the last decades all around the world. Water scarcity, deterioration of quality and increasing demand has led to the development and use of alternative sources of water. Reclamation and recycling are now considered as key components of water and wastewater management policies around the world[1].

Disinfection is considered to be the essential process for the inactivation and destruction of waterborne pathogens, in order to protect human health and also the environment[2]. Chemical disinfectants such as chloramines, chlorine dioxide etc. and especially chlorine are commonly used for drinking water disinfection as well as disinfection of various wastewaters such as from procedures for laundering hospital textiles because of their low cost, ease of handling, and their ability to provide disinfectant residual[3-4]. However disinfection procedures using chlorine substances are not environmentally friendly due to the formation of carcinogenic or mutagenic by-products such as trihalomethanes and haloacetic acids[6], especially with waters containing organic matter such as laundry wastewater[6]; therefore alternative disinfection procedures such as UV radiation, ozonation, chemical decontaminants such as oxidizing agents etc. need to be investigated.

Peroxyacetic acid has shown to be a very efficient biodegradable decontaminant that produces very little disinfection by-products and

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is useful for drinking water pipelines, surface drinking water, wastewater treatment plants, sterilization of surfaces and equipment in pharmaceutical and food industries etc[7-8].

Ultraviolet radiation has been noted as an important alternative for disinfection of drinking water or wastewater due to its excellent biocidal properties with very limited formation of disinfection by-products, extremely short contact times, cost-effectiveness and environmentally sustainable[9-11].

Although several strains of enterococci including *Enterococcus faecium* that normally colonize the intestinal tract of humans and animals, have been used as human probiotics and in a variety of fermented foods; they have also been known to be opportunistic pathogens with strains highly resistant to vancomycin[12-13]. It must also not be overlooked that the presence of enterococci in water is considered as an indication of faecal contamination and the possible presence of enteric pathogens[12]. Therefore assessing efficient methods for inactivating enterococci in waters is important to public health in order to protect public from outbreaks of waterborne diseases[4]. On the other hand it is also important that microorganisms in wastewaters do not contribute to bio-burden whether they are sent to a municipal wastewater treatment plant or reused in industrial processes such as laundering of hospital textiles where there is a risk of cross-contamination hospital textiles due to laundering procedures, serving as a subsequent vehicle for the transmission of hospital-acquired infections[14-15].

In the present study the inactivation of *Enterococcus faecium* in drinking water and in simulated hospital laundry wastewater by disinfection processes utilizing peroxyacetic acid or ultraviolet radiation was investigated.

MATERIALS AND METHODS

Preparation of microbial culture and water sample

A 48 hour culture of *Enterococcus faecium* (ATCC 6057) was prepared by inoculating 1 mL of the frozen stock culture into 35 mL of tryptic soy broth and incubating at 37±1°C in order to prepare a log phase suspension. The cells were harvested by centrifugation at 10000 rpm for 10 min followed by washing with 0.9% NaCl and resuspension in 50 mL sterile distilled water.

Preparation of simulated hospital laundry wastewater

Hospital laundry wastewater was simulated according to the research study by Altenbaher *et al.*,[16]. Briefly, 1.2 g/L detergent; 3.65 g/L of disinfecting agent and 1 mL/L acetic acid was added to 50 mL samples of sterile distilled water with an inoculated suspension of *Enterococcus faecium*. The detergent consisted of sodium hydroxide: 15-30%, potassium hydroxide: 5-15%, non-ionic surfactants: 5-15%, hydroxyacetic acid: 1-5% andphosphonates<5%. The disinfecting agent contained hydrogenperoxide: 15-30%, peroxyaceticacid: 5-15%andaceticacid: 5-15%.

Set-up for experiments with peroxyacetic acid

50 mL of water or simulated hospital laundry wastewater containing an inoculation of Gram positive bacteria *Enterococcus faecium* (initial bacterial count between $1.74x10^8$ and $1.48x10^9$ cfu/mL) was prepared and various concentrations of peroxyacetic acid was added. The 50 mL centrifuge tubes that were added onto a centrifuge rack atop a mixing device (Heidolphvibramax 100) at 450 rpm where the samples were mixed for 10 min followed by adding a drop of sodium thiosulphate ($Na_2S_2O_3$) to quench the residual peroxyacetic acid and a drop of catalase to neutralize the residual hydrogen peroxide[17]. After this serial dilutions were made and inoculated onto the chosen selective agar.

Set-up for experiments with ultraviolet radiation

The calibrated 30 W germicide ultraviolet light (wavelength of the UV lamp was 253.7 nm thus being in the UV-C region) from the biological cabinet Telstar bio II advance was used. All experiments with 50 mL samples were conducted in glass Petri dishes (diameter 9 cm) thus forming an 11 mm deep suspension. The distance between the germicide ultraviolet light and the Petri dishes was 10 cm. After determined contact times (max 12 hours) aliquots of the suspension were taken and serial dilutions were made and inoculated onto the chosen selective agar.

Bacterial enumeration

Enterococcus faecium inactivation was determined using the spread plate method with serial 10-fold dilutions in sterile 0.9% NaCl solution plated in duplicate on kanamycin esculinazide agar

for identification and enumeration of enterococci (Sigma - Aldrich 17151) and incubated for 48 hours at 37±1°C. After incubation the plates were counted and the average was calculated as cfu/mL.

RESULTS AND DISCUSSION

Enterococci are facultative anaerobic organisms, *i.e.*, they are capable of cellular respiration in both oxygen-rich and oxygen-poor environments. Though they are not capable of forming spores, enterococci are tolerant of a wide range of environmental conditions: extreme temperature (10-45°C), pH (4.5–10.0)[18] as well as high salt, (6.5% NaCl) and high bile salt- (40 %) concentrations[19] etc. Several enterococci can also survive thermal treatment for 1 min at 80°C and 150 ppm active chlorine for 5 min [20]; 30 min heating at 60°C[21], laundering for 3 min at 71°C[22]; laundering at 60°C with 3.36 g peroxyacetic acid/kg textiles[23] etc. Enterococci are among the most common nosocomial pathogens and they have been implicated as an important cause of endocarditis, bacteremia, and infections of the urinary tract, central nervous system, intra-abdominal and pelvic infections, as well as of multiple antibiotic resistances[21]. Although the most important and widely recognized indicator of faecal contamination of drinking water is *Escherichia coli;* enterococci are also an important faecal indicator especially in combination with *E. coli*; where *Escherichia coli* is an indicator of fresh faecal pollution, whilst the presence of only enterococci in drinking water indicates 'old' faecal pollution due to the ability of enterococci to persist in the environmental for long periods[24].

Limit value of successful disinfection of water or wastewater

There are several different limit values with regard to reusable water after disinfection. The value is always dependent on the end use of the disinfected water. Many countries have regulations that specifically address different end-uses of disinfected water such as recreational and environmental uses of reclaimed water. The most commonly used limit values for drinking water is 0 cfu *Escherichia coli*/100 mL and 0 cfu enterococci/ 100 mL; these values are also part of the guidelines for drinking water valid in the Republic of Slovenia[25]. A common limit value for wastewater reuse is according to the 'California title 22' [26] where the limit value is 2 cfu total coliforms/100 mL. According to the EPA-USA Guidelines[27], California's recommended treatment for each type of recreational water reuse is linked to the degree of body contact in that use. Disinfection to 2.2 total coliforms/100 ml averages is required for recreational water bodies where fishing, boating, and other non-body contact activities are permitted[27]. For water reused for recirculating cooling towers the limit value is < 200 faecal coliforms/100 mL (or 2 FC/mL). This limit value is also recommended for industrial reuse. Swift *et al.,*[28] stated that the goal of UV disinfection in water reuse applications typically has to inactivate 99.999 % or more of the target pathogens. Where 99,999 % means at least 5 $\log^{10}$ reduction for water reuse, this limit value was also our goal in this research for wastewater.

Efficiency of water and wastewater disinfection with peroxyacetic acid against *Enterococcus faecium*

According to literature conflicting evidence exists on PAA performance and effectiveness[17]. Sanchez-Ruiz *et al.*,[29] reported a 2-logs microbial reduction of total coliforms when disinfecting mechanically pre-treated raw sewage with 80 mg PAA/L dosage at a contact time of 20 min. A 4-logs reduction of total and faecal coliforms was achieved when disinfecting a primary settled effluent (PSE) with 50 mg PAA/L at a 30-min contact time by Morris *et al.*[30]. Up to 4-logs total coliforms inactivation was also reported when disinfecting secondary settled effluent (SSE) in a plug-flow contactor with 11 and 15 mg PAA/ L at 30-min and 60-min contact times by Lefevre *et al.*,[31]. Liberti *et al.*,[32] noted that a 30 min treatment time of 10 mg/ Lperacetic acid was enough to reach the WHO faecal coliform guideline (1000 cfu/100 mL), whilst much higher dosages (> 400 mg/L) at the same treatment time was needed to achieve the California limit of 2 cfu/100 mL. Liberti and co-workers also stressed that the necessity of constant mixing that can be slow (90 rev/min) was stressed as important. In pilot designs it was found that quick mixing with a recirculation pump (60 m^3/h) only minimally influences the results after 30 min and the results after 60 min are analogue to the slow mixing results with the four-bladed stirrer. Another important finding in the research of Libertiand co-workers

was that the most important factor for the overall disinfection effectiveness was the peracetic acid dosage, whilst no systematic correlation was found with total coliform content in wastewater ranging from $7x10^4$ to 9×10^5cfu/100 mL. Another study by Ditommaso *et al.*,[33] proved that peroxyacetic acid was not effective for the disinfection of a hospital water system contaminated with *Legionella* species and resembled the decontamination pattern observed in water distribution systems treated with conventional intermittent disinfection methods such as superheating and hyperchlorination. From these results it is obvious that for achieving proper disinfection yielding higher disinfection levels for safe laundry wastewater reuse the limit concentration should be in the range of at least over 100 mg/L PAA or higher. Of course, in all these studies different types of wastewaters were used thus resulting in different optimum results and proving that optimum dosages must be determined for each case experimentally.

In our research several initial experiments showed very puzzling results and after research it was found that peroxyacetic acid is much less stable in diluted form[7] and we realized that our prepared solutions that were not prepared exactly on the day of the experiments but several days before, had actual concentrations much lower than anticipated. This led to an important conclusion namely, that the solutions for the treatment with peroxyacetic acid must be freshly prepared daily from concentrated form in order to achieve the exact concentration of peroxyacetic acid. We also found in some experiments that the log reduction directly after addition of peroxyacetic acid was below 5-log[10] steps but after a certain time a small number of microbial recoveries was observed followed by the repeated log reduction after certain contact time with peroxyacetic acid. This could also be related to the fact that the sample was not yet

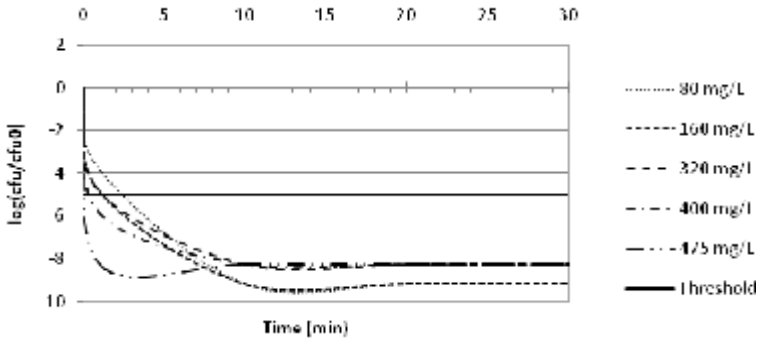

Fig. 1. Log_{10} reduction of *Enterococus faecium* inoculated in water after treatment with peroxyacetic acid at various concentrations and initial bacterial concentration $1.74x10^8$cfu/mL (for 80 to 320 mg/L PAA) and $1.48x10^9$ cfu/mL(for 400 and 475 mg/L PAA)

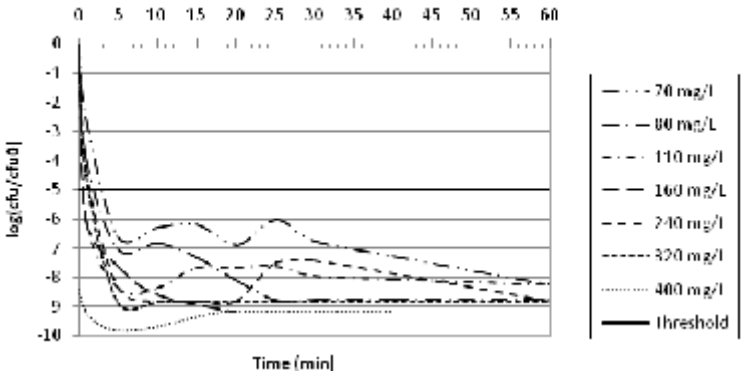

Fig. 2. Log_{10} reduction of *Enterococus faecium* inoculated in hospital laundry wastwaterafter treatment with peroxyacetic acid at various concentrations and contact times and initial bacterial concentration between $1.74x10^8$cfu/mL (for 80 to 320 mg/L PAA) and $1.48x10^9$cfu/mL (for 70 and 400 mg/L PAA)

uniformly homogeneous at the time of taking samples. In several experiments another important observation included that the water to prepare the diluted concentration of peroxyacetic acid must not have a temperature higher than room temperature as again very puzzling results were found and after extensive duplicate testing it was found that this was due to the too high temperature of sterilized water used to prepare the diluted peroxyacetic acid resulting in shifting the equilibrium to the left and thus resulting in a lower concentration of peroxyacetic acid. This also complies with the findings[34] that the peroxyacetic acid concentration is influenced by temperature and the actual concentrations used in these experiments were much lower than anticipated.

From the results noted in Fig.1 we can see that a dosage of 80 mg/L peroxyacetic acid is sufficient to reach a 5-log[10] reduction within 5 min for wastewater inoculated with the faecal indicator *Enterococcus faecium*. However minimal recovery was noted for this sample.

From the results noted in Fig.2 we can see that there are several possibilities to ensure disinfection of hospital laundry wastewater. A minimum dosage of 70 mg/L peroxyacetic acid is sufficient to reach a 5-log[10] reduction within 35 min treatment. The minimum treatment time of 5 min ensuring a 5-log[10] reduction is reached by adding 110 mg/L peroxyacetic acid. A dosage of 80 mg/L ensures a 5-log[10] reduction after a treatment time of at least 15 min. These optimized Figs take into account that shorter contact time is correspondent with higher peroxyacetic dosages and vice versa. Depending on which factor is more important for a particular kind of wastewater, either higher dosages with shorter contact times or lower dosages with longer contact times could be chosen. A scale-up and cost effectiveness experiment should however be conducted beforehand.

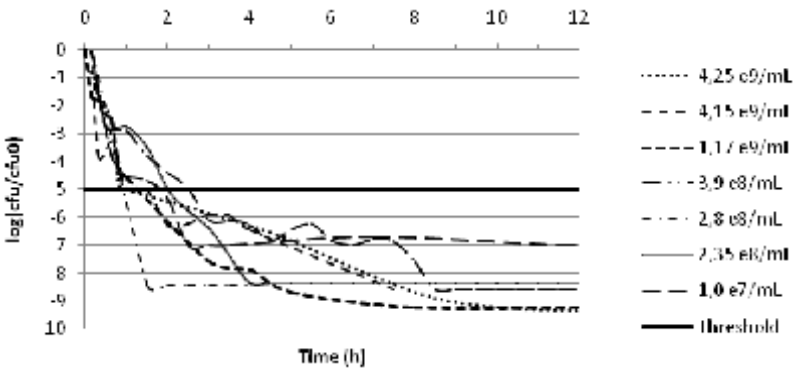

Fig. 3. Log_{10} reduction of *Enterococus faecium* inoculated in water after treatment with germicial UV radiation

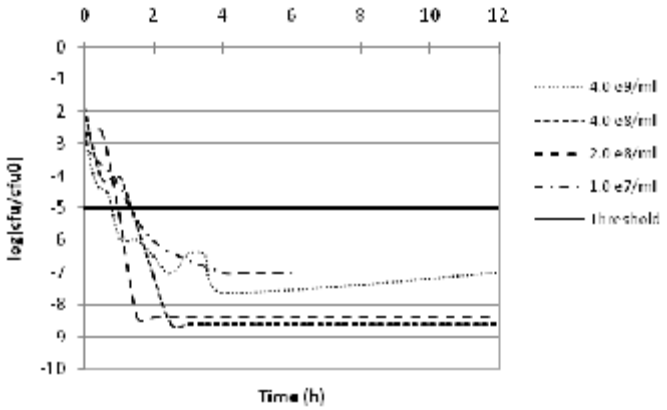

Fig. 4. Log_{10} reduction of *Enterococus faecium* inoculated in simulated hopsital laundry wastewater after treatment with UV radiation

Efficiency of water and wastewater disinfection with ultraviolet radiation against *Enterococcus faecium*

In Fig. 3, which presents the data for experiments with only water inoculated with *Enterococcus faecium*, we can observe that the time of ultra violet radiation required to achieve a 5-log[10] reduction is not uniform and in average is around 3 hours. This is perhaps due to the fact that some samples had higher bacterial concentrations, thus increasing the turbidity and the actual UV dosage was also influenced by the exact distance and angle of samples from the UV germicidal light, which perhaps was not exactly uniform in all experiments. The turbidity of samples can significantly influence the germicidal effects of UV as the UV treatment dose decreases due to turbidity[35]. This is perhaps why some of the samples needed larger UV doses than others as the concentrations were different and thus the turbidity was also different. In any case no bacteria were recovered indicating that the UV dosages were sufficient after 3 hours. This also implies that in pilot-scale the microorganisms nearer to the UV lamp will be inactivated much quicker than microorganisms that are further away from the lamp, therefore it is necessary to add agitation. This is an important factor to consider then planning the UV disinfection design full-scale.

In the experiments using simulated hospital laundry wastewater inoculated with faecal indicator *Enterococcus faecium* (Fig. 4) the threshold of 5-log[10] steps for the simulated hospital laundry wastewater with added detergent and disinfectant was reached within 1 ½ hours. Therefore, we can conclude that the addition of detergents and disinfectants in the laundry wastewater do not adversely influence the disinfection effect of UV. On the contrary, the disinfection effect is amplified. We even found no live bacteria after 24 hours in sample 4 (initial bacterial count 1.0×10^7cfu/mL) that was exposed for 6 hours and then left in the dark for 18 hours after exposure followed by a second colony counting. Thus if any cells remained intact, the concentration was not sufficient to increase the population over an 18 h period. Bacterial population otherwise commonly exhibit dark repair after 18 hours[10] regardless of delivered dose of UV treatment, therefore the added detergent and disinfectant in the laundry wastewater obviously created a hostile environment minimising the bacterial recovery in our research.

From all the above noted experiments it is obvious that the germicidal wavelength of the UV lamp (253.7 nm) does penetrate through water and microorganisms are inactivated by UV light as a result of damage to nucleic acids (4). The amount of cell damage depended on the dose of UV energy absorbed by the microorganisms. Most bacteria and viruses require relatively low UV doses for inactivation; however photochemical damage caused by UV may be repaired by some organisms via repair pathways such as photoreactivation and dark repair[10]. Studies show that the amount of cell damage and subsequent repair is directly related to the UV dose. The amount of repair will also depend on the dose (intensity) of photo-reactivating light. For low UV doses the resulting minimal damage can be more readily repaired than for high doses where the number of damaged sites is greater[36]. Although UV disinfection is not affected by temperature or pH, however turbidity caused by i.e. accumulation of inorganic and organic solids on the quartz sleeve decreases the intensity of UV light that enters the surrounding water[3]. In the study by Patoczka *et al.*,[37] it was found that a major impediment to UV performance is the presence of UV-adsorbing organics that adsorb UV light in the critical wavelengths and thus render the system ineffective. This was not the case in our experiments as the bacterial inactivation is much more consistent and effective than for the sample with only water thus proving that UV radiation could be typically used for hospital laundry wastewater that is stored overnight in reservoirs[35] and awaits for the following day for reuse before any repair mechanisms take place. A scale-up and cost effectiveness experiment should however be conducted beforehand.

CONCLUSIONS

The findings of the research of the disinfection of water and hospital laundry wastewater inoculated with *Enterococcus faecium* using peroxyacetic acid or ultraviolet radiation can be summarized as follows:

1. Both investigated methods are applicable

for hospital laundry wastewater as a log reduction of 5-log^{10} steps was reached for both methods.

2. For the use of peroxyacetic acid there are several possibilities to ensure disinfection of hospital laundry wastewater. These optimized Figs take into account the time to reach the reduction after the observed slight recovery:

a A minimum dosage of 70 mg/Lperoxyacetic acid is sufficient to reach a 5-log^{10} reduction within 35 min of contact time.

b The minimum contact time of 5 min ensuring a 5-log^{10} reduction is reached by adding 110 mg/L peroxyacetic acid.

c A dosage of 80 mg/L ensures a 5-log^{10} reduction after a short treatment time of 15 min.

3. Less than 10 min treatment of water with 80 mg peroxyacetic acid/L results in a 7 to 8 $\log_{10}$ reduction thus proving that it could be used for various industrial applications such as cooling towers etc. When cooling tower water is tapped from a river or lake, and must be discharged into the same water body after it has been used, it must meet certain discharge demands. Peroxyacetic acid disinfection could be an efficient and quick disinfection method for this kind of water.

4. In the experiments using UV radiation it was found for the model hospital laundry wastewater that the threshold of 5-log^{10} steps was reached within 1 ½ hours with no observed bacterial recovery. This method could be used for hospital laundry wastewater or similar water that is stored overnight and then reused the following day.

Further research on the long-term disinfection effect of these methods for water or wastewater should be taken into account as well as scale-up experiments and cost-effectiveness calculations.

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