Study of disinfection efficiency of peracetic acid (PAA) on *Escherichia coli* by rapid colorimetric assay based on enzymatic substrates after eliminating hydrogen peroxide from the commercial PAA mixture

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Abstract Disinfection of combined sewer overflows (CSO) is a quick approach to reduce the indicator bacteria (*E. coli* and *Enterococcus spp*) to maintain the bathing water quality on the receiving waters when CSO are discharged. Peracetic acid (PAA) has been used to disinfect combined sewer overflows.

This study was conducted to investigate the disinfection efficiency of PAA against *E. coli* when hydrogen peroxide was removed from the commercial PAA mixture. Furthermore, disinfection efficiency of PAA, commercial PAA and hydrogen peroxide against *E. coli* was compared. Disinfection efficiency of PAA against *E. coli* was studied by using rapid colorimetric assay using enzymatic substrates 6-Chloro-3-indolyl-β-D-galactopyranoside (Red-Gal) which develops dark red when it reacts to the β-galactosidase enzyme of *E. coli*. The resulting color intensity from the enzymatic substrate interaction was correlated to the bacterial concentration by using plate count method. Median inhibition concentration (IC_{50}) of PAA without hydrogen peroxide, commercial PAA and hydrogen peroxide on *E. coli* was determined by using the area under curve (Ct). IC_{50} of PAA alone on *E. coli* was 32 mg·min/L whilst IC_{50} of commercial PAA was 23 mg·min/L.

Keywords: Disinfection, Peracetic acid, *Escherichia coli*, Red-Gal, Combined sewer overflow

1. Introduction

Disinfection is essential for elimination or inactivation of the number of microorganisms to ensure the public health and safety and environmental protection. However, when untreated combined sewer overflows (CSO), a variable mixture of wastewater and rain water, are discharged the quality of receiving surface waters gets deteriorated. European Union defined the bathing water standards for recreational purposes in the directive 2006/7/EC and to qualify for a good quality of bathing water, the number of indicator bacteria should not exceed 500 MPN *E. coli* and 200 MPN *Enterococcus* per 100 mL water intended for recreational purposes (Directive 2006/7/EC, 2006).

Disinfection of the inflowing CSO to the receiving waters will reduce the number of indicator bacteria to maintain the good bathing water quality. There are various well known disinfectants used in the water industries and recently, peracetic acid (PAA) and performic acid (PFA) have been used to disinfect CSO (Chhetri *et al*., 2016, 2015, 2014). PAA is a well-known disinfectant which was introduced to wastewater treatment approximately 30 years ago (Antonelli *et al*., 2013, 2006; Baldry, 1983; Falsanisi *et al*., 2006; Kitis, 2004; Luukkonen *et al*., 2015). Commercial PAA is a quaternary equilibrium mixture of PAA, acetic acid and hydrogen peroxide:

$$CH_3COOH + H_2O_2 \rightleftharpoons CH_3COOOH + H_2O \quad \text{Eq. 1}$$

$$CH_3CO - OOH + 2e^- \rightarrow CH_3CO - O^- + HO^- \quad \text{Eq. 2}$$

The residues after PAA use are acetic acid, hydrogen peroxide and water. The degradation of hydrogen peroxide is slower than PAA (Chhetri *et al*., 2014; Wagner *et al*., 2002) and it has a stringent discharge limit to the surface water.

The aim of this study was to investigate the disinfection efficiency of PAA when hydrogen peroxide was eliminated from the commercial mixture of PAA. Furthermore, to study the disinfection efficiency, a rapid colorimetric assay based on enzymatic substrate 6-Chloro-3-indolyl-β-D-galactopyranoside (Red-Gal) for *E. coli* enumeration was used which later was compared to direct plate count method for *E. coli*.

2. Materials and methods

2.1. Chemicals and chemical analysis

ABTS (2,2”-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt), sodium thiosulphate, potassium permanganate, Red-GaL and technical grade PAA solution

[30–40% (w/w) PAA, 5% (w/w) H_2O_2] were purchased from Sigma–Aldrich (Brøndby, Denmark).

PAA concentration was analyzed using the colorimetric method described by Chhetri *et al.* (2014) based on selective oxidation of ABTS by PAA without interference from hydrogen peroxide. Hydrogen peroxide was analyzed using the titanium oxide-oxalate colorimetric assay (Antoniou and Andersen, 2015). Hydrogen peroxide from commercial PAA solution was removed by titration with potassium permanganate ($KMnO_4$). Endpoint of titration was determined by slight appearance of pink color of manganate where hydrogen peroxide was eliminated from the commercial PAA.

2.2. PAA disinfection

From the stock solution, a working solution of 1 g/L PAA was prepared which was further titrated with 0.02 N potassium permanganate until the end point of light pink appearance of manganate. The excess manganate was removed from the PAA by raising pH of the solution to pH 8.5 where manganate crystals were formed which was filtered with 0.45µm filter. To make PAA solution stable pH of PAA solution was maintained at 6.5 using phosphate buffer. For disinfection experiment, laboratory water was spiked with known concentration of *E. coli* (ATCC 25922) and five dose of PAA ranging from 0.5 mg/L to 3 mg/L were used. Concentration profiles of PAA were observed for 60 min and residual PAA was neutralized by adding sodium thiosulphate and samples were processed for *E. coli* enumeration.

2.3. E. coli enumeration

To enumerate the *E. coli*, chromogenic substrate Red-Gal was used which reacts with the β-galactosidase enzyme present in *E. coli*. The 96-well plates detection method was applied for *E. coli* detection as described by Gunda *et al.*, (2016). In short, 100µl of *E. coli* mixture was mixed with 100 µl Lauryl Tryptose Broth, 25 µl 0.2% (w/v) sodium dodecyl sulfate and 50 µl Red-Gal (30 mg in 1 mL of a 1:1 mixture of N,N-Dimethylformamide and DI water) in a 96-well plate. The plate was incubated for 7 h at 37°C and the appearance of red color was quantified by measuring absorption spectra at 530 nm. Furthermore, the number of *E. coli* was quantified by direct plate count method in LB agar to calculate the disinfection efficiency. Inhibition and concentration response curves were estimated by use of a nonlinear regression program assuming lognormal distribution. By use of logistic curve fitting and inverse estimation inhibition concentration (IC) were determined with corresponding 95% confidence limits.

3. Results and discussion

3.1. Concentration profiles

Concentration profiles were obtained by measuring concentrations of PAA, commercial PAA and hydrogen peroxide over time in the tap water (Figure 1). A first order degradation kinetics model was used for curve fitting in Figure 2:

$$C_t = C_0 \cdot e^{-kt} \quad \text{Eq. 3}$$

In Equation (3) C_t is the residual disinfectant concentration at time t, C_0 is the applied disinfectant dose, k is the rate constant, and t is time. Area under the curve of each disinfectant was calculated by measuring the concentration of residual disinfectant at time t ($C \cdot t$). Ct was used to calculate the median inhibition concentration of disinfectants to the *E. coli*. A slow degradation of PAA, commercial PAA and hydrogen peroxide was observed in the tap water whilst higher concentration degraded faster compared to the low concentration of disinfectants used. There was a 33% degradation of 6 mg/L PAA without hydrogen peroxide when it was measured after 60 min of contact time whilst 67% degradation of commercial PAA was observed when 3 mg/L was measured after 60 min. Similarly, 77% of hydrogen peroxide was degraded when 500 mg/L was measured after 60 min. Slow degradation of commercial PAA was observed when it was used to disinfect combined sewer overflows (Chhetri *et al.*, 2016, 2015) and wastewater effluents (Hey *et al.*, 2012). In this study, PAA degraded faster compared to our previous studies. Tap water does not contains organic mattes which could have reacted with PAA for rapid degradation. Degradation of PAA might be due to the reaction of PAA with existing catalase enzyme in *E. coli*. Faster degradation of hydrogen peroxide was due to the reaction of catalase enzyme of *E. coli* which converts hydrogen peroxide to water and oxygen.

3.2. Disinfection efficiency

Disinfection efficiency of PAA, commercial PAA and hydrogen peroxide was calculated by estimating the growth inhibition of *E. coli* exposed to disinfectants compared to the one without disinfectants. Median inhibition concentration (IC_{50}) of PAA was 1 mg/L whilst IC_{50} of commercial PAA was 0.97 mg/L and IC_{50} of hydrogen peroxide was 43 mg/L. Inhibition concentration of PAA was higher than commercial PAA that means commercial PAA was effective against *E. coli* than PAA without hydrogen peroxide. Commercial PAA mixture has hydrogen peroxide in equilibrium and hydrogen peroxide is a weak disinfectant. IC_{50} of hydrogen peroxide was 43 times higher than PAA and commercial PAA. Hydrogen peroxide was not effective against the bacteria containing catalase enzyme which destroy hydrogen peroxide to water and oxygen. However, when commercial PAA was used to disinfect *E. coli*, it inactivated the catalase enzyme, which inhibits hydroxyl radical oxidation (Kitis, 2004). The difference on IC_{50} of PAA and commercial PAA was due to the synergic effect of hydrogen peroxide present on commercial PAA for disinfection. The two stages attacking scheme of commercial PAA to the bacteria was also explained by Flores *et al.*, (2014) where synergic action of PAA and hydrogen peroxide was explained.

Disinfection efficiency of PAA and commercial PAA was calculated using colorimetric assay using Red-GAL enzymatic substrate besides traditional plate count method described in previous paragraph. In Red-GAL colorimetric assay, β-galactosidase enzyme present in *E. coli* was induced when it was exposed to lactose in the medium. Simultaneously, sodium dodecyl sulfate lyses the *E. coli*

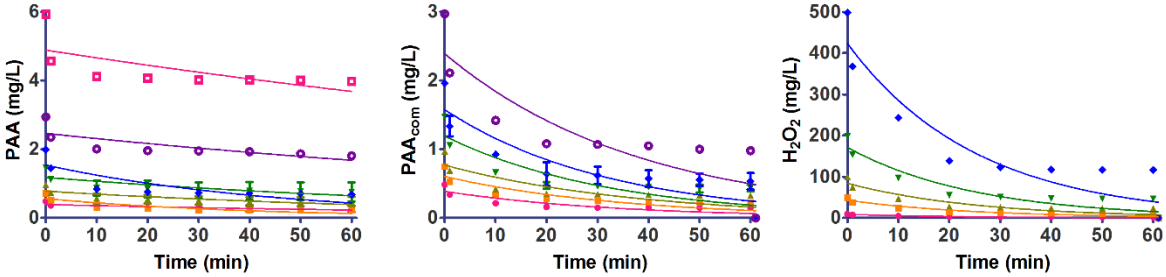

Figure 1. Concentration profiles of PAA without hydrogen peroxide, commercial PAA and hydrogen peroxide in tap water. Curves were fitted with first order degradation kinetics.

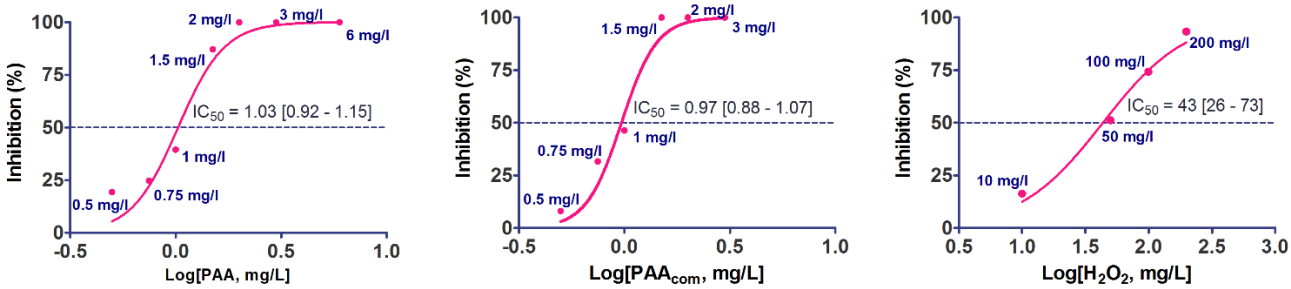

Figure 2. Concentration dose response curve of PAA without hydrogen peroxide, PAA commercial PAA and hydrogen peroxide on *E. coli*.

cell to release the β-galactosidase enzyme. Red-GAL reacts with β-galactosidase enzyme giving red color that was quantified spectrophotometrically at 530 nm. The disinfected samples showed lower absorbance compared to the non-disinfected (control) samples (Figure 3). Absorbance of samples disinfected with commercial PAA showed lower absorbance with increasing concentration. However, no trend on effect of increasing concentration of PAA and hydrogen peroxide was observed. The results obtained from both plate count method and colorimetric assay showed that commercial PAA results in better *E. coli* inhibition in comparison to PAA alone and hydrogen peroxide. Moreover, disinfection efficiency of PAA alone is 43 times higher than hydrogen peroxide. Area under the curve (Ct) of PAA, commercial PAA and hydrogen peroxide was calculated to obtain the inhibition concentration of *E. coli*. Median inhibition concentration (IC_{50}) of PAA, commercial PAA and hydrogen peroxide was 32 mg·min/L, 23 mg·min/L and 866 mg·min/L, respectively when area under the curve (Ct) was used. Ct of commercial PAA was 2.75 times less than PAA alone to obtain the 3 orders of magnitude removal of *E. coli* from the results published by Flores *et al*., (2014). In this study, the difference on Ct from commercial PAA and PAA alone was 1.3 times to obtain the median inhibition concentration of *E. coli*. This might be due to the use of two different methods to destroy the hydrogen peroxide from the commercial PAA mixture.

Overall, it was evident that PAA without hydrogen peroxide was as effective as commercial PAA when it was used to disinfect *E. coli*. Furthermore, PAA without hydrogen peroxide showed the potential as an alternative disinfectant to commercial PAA where strict regulation on discharge of hydrogen peroxide can be avoided.

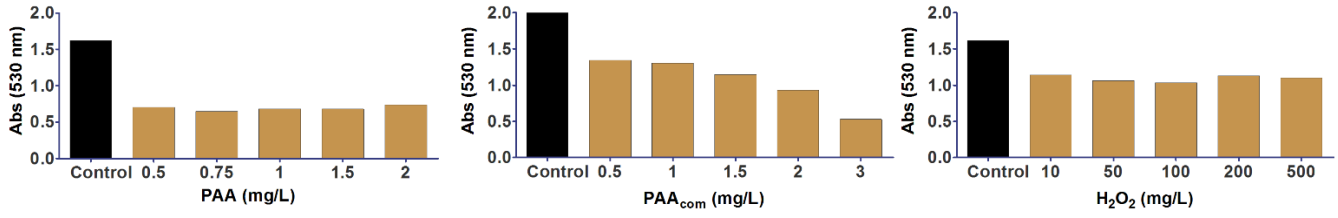

Figure 3. Absorbance of reaction products (Red-GAL and β-galactosidase enzyme from *E. coli*) of samples with and without disinfection.

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