Ministry of Environment of Denmark
Environmental Protection Agency

REBAHS-Disinfection of antibiotic-resistant bacteria in hospital wastewater

MUDP Report

September 2022

Publisher: The Danish Environmental Protection Agency

Editors:
Ravi Kumar Chhetri,
Diego Francisco Sanchez,
Sabine Lindholst,
Thomas Eilkær,
Birgitte Krogh Løppenthien,
Caroline Kragelund Rickers,
Henrik Rasmus Andersen

Photos:
Ravi Kumar Chhetri, DTU
Sabine Linholst, DTI
Alexander Valentin Hansen, Alumichem

ISBN: 978-87-7038-442-1

Miljøteknologisk Udviklings- og Demonstrationsprogram

Projektet, som er beskrevet i denne rapport, er støttet af Miljøteknologisk Udviklings- og Demonstrationsprogram, MUDP, som er et program under Miljøministeriet, der støtter udvikling, test og demonstration af miljøteknologi.

MUDP investerer i udvikling af fremtidens miljøteknologi til gavn for klima og miljø i Danmark og globalt, samtidig med at dansk vækst og beskæftigelse styrkes. Programmet understøtter dels den bredere miljødagsorden, herunder rent vand, ren luft og sikker kemi, men understøtter også regeringens målsætninger inden for klima, biodiversitet og cirkulær økonomi.

Det er MUDP's bestyrelse, som beslutter, hvilke projekter der skal modtage tilskud. Bestyrelsen betjenes af MUDP-sekretariatet i Miljøstyrelsen.

MUDP-sekretariatet i Miljøstyrelsen
Tolderlundsvej 5, 5000 Odense| Tlf. +45 72 54 40 00

Mail: ecoinnovation@mst.dk
Web: www.ecoinnovation.dk

Denne slutrapport er godkendt af MUDP, men det er alene rapportens forfatter/projektlederen, som er ansvarlige for indholdet. Rapporten må citeres med kildeangivelse.

Contents

Preface		**5**
Introduction		**6**
Summary		**8**
Resume		**10**
1.	**Chapter I-Overview of antibiotic resistant bacteria levels in wastewater with and without hospital contribution**	**12**
1.1	Determination of Ciprofloxacin-resistant bacteria in raw wastewater in WWTPs without hospital contribution	12
1.2	Occurrence of multi-resistant bacteria	17
1.3	Summary	19
2.	**Chapter II-Determination of disinfectant dose and contact time for pilot experiment**	**21**
2.1	Determination of disinfection dose and reaction time in different types of wastewater	21
2.2	Residual determination and degradation products	24
2.3	Effect on Pharmaceuticals	25
2.4	Summary of chapter	26
3.	**Chapter III- Development and testing of continuous disinfection technology for low-dose**	**27**
3.1	Pilot container	27
3.2	Experiments with long reaction time	27
3.3	Testing of available sensors for improved control	30
3.3.1	Results for turbidity-, conductivity- and flowsensors	31
3.3.2	Sensors for PAA control by living bacteria	33
3.4	Disinfection technology rebuilt and adjusted for operation at SK-Forsyning with short contact time	37
3.5	Demonstration of the disinfection concept for continuous disinfection	40
3.6	Summary of chapter	41
4.	**Chapter IV- Dimensioning for full-scale disinfection solutions for Hillerød forsyning and SK-forsyning**	**43**
4.1	Overall design	43
4.2	Daily operation	43
4.3	Safety	43
4.4	Design parameters and financials	44
4.5	Summary of chapter	44
5.	**Conclusion**	**45**
6.	**References**	**46**
Appendix		

Preface

The purpose of the project was to develop a treatment technology to reduce the amount of antibiotic resistant bacteria (ARB) from raw hospital wastewater using the unbranched wastewater pipe from super hospitals to the wastewater treatment plants using peracetic acid (PAA).
The advantage of this treatment is that there is no need of additional construction and that the wastewater pipe from the hospital to the wastewater treatment plant can be used as a reaction tank giving a long reaction time. A similar approach with a shorter reaction time was used to reduce the ARB from hospital wastewater before mixing it with municipal wastewater. Peracetic acid was selected to treat raw wastewater as it does not form toxic by-products.

The project presented the amount of ARB that need to be reduced from the hospital wastewater before mixing it with municipal wastewater. This gives the municipalities the opportunity to specify requirements for the level of disinfection for hospital wastewater. During the project period, pilot experiments were conducted at two different locations in Denmark. In total 8 pilot experiments were conducted using wastewater from two hospitals namely Hillerød hospital and Slagelse hospital and two wastewater treatment plants namely Hillerød and Slagelse (SK Forsyning). A continuous disinfection experiment on raw wastewater using PAA was performed in pilot scale for 6 weeks to demonstrate the disinfection concept.

The project was funded by the Danish EPA as project: MST 117-00661: "REBAHS" Desinfektion af Resistente Bakterier i HospitalsSpildevand. The partners participating in the project were as follows:

- Technical University of Denmark: Ravi Kumar Chhetri, Diego Francisco Sanchez & Henrik Rasmus Andersen (Project leader)
- Danish Technological Institute: Sabine Lindholst, & Caroline Kragelund Rickers
- Alumichem: Thomas Eilkær, Birgitte Krogh Løppenthien & Alexander Valentin Hansen
- Hillerød Forsyning: Jørgen Skaarup & Henning Gade
- SK Forsyning: Jan M. Jørgensen & Natascha Kock Pedersen
- Hjørring Vandselskab: Jacob Andersen & Didde Lindholm Jürgensen

Introduction

Hospital wastewater is a point source for antibiotic-resistant bacteria (ARB) and the most important point source for multi-resistant, pathogenic bacteria, which are highly undesirable in the environment and at the same time pose a work environment risk at wastewater treatment plants and sewer workers.
In Denmark, national hospital service is currently being centralized into super hospitals servicing entire regions by replacing many local hospitals. This centralisation is leading to higher ARB concentrations in hospital wastewater. The higher concentration of ARB might pose increased risks to workers servicing these wastewater treatment plants. Moreover, during heavy precipitation in combined sewers system, concentrations of ARB can be expected to be increased in effluent and any overflow that occur servicing these super hospitals. To mitigate this wastewater issue, several new super hospitals have constructed separate wastewater pipes linking them with wastewater treatment plants, in order to limit the spread of pathogens and resistant bacteria to combined sewers. However, the risk from antibiotic-resistant bacteria compromising the working environment for sewer workers is still need consideration. In the professional debate, both central and decentralized treatment solutions to treat both antibiotic-resistant bacteria and pharmaceuticals residual from hospital wastewater have been discussed. In the discussion of hospital wastewater management, neither technology nor the location of the same was defined; although there was consensus that the treatment technology should correspond to BAT (Nielsen et al., 2011). Environmental friendly and economical treatment of hospital wastewater is planned in centralized super hospitals such as: at DNU, DNV Gødstrup and Nyt Hospital Nordsjælland. The project aim to develop treatment system for continuous dosing of disinfectants directly to the raw wastewater from hospitals and thereby reduce the number of antibiotic resistant bacteria significantly (> 90%). The raw wastewater from super hospitals will thus be able to be handled without increased risk to the sewer workers from the wastewater treatment plant.

There are several disinfection solutions available to reduce the number of microorganisms from the wastewater. Chlorine based disinfectants is not suitable for disinfection of raw wastewater as it reacts with organic matter forming high concentration of organohalogens and with nitrogen compounds forming toxic by-products (Watson et al., 2012). Ozonation is not attractive for disinfection of raw wastewater, as the ozone dose is directly proportional to the amount of organic matter. Peracetic acid (PAA) was used as disinfectant in this project. PAA is a potent antimicrobial agent that is stable over time and inexpensive (Du et al., 2018). PAA reacts effectively against a broad spectrum of microorganisms and is able to attack microbes in different ways (Block, 1991). The disinfection mechanism for PAA is still being investigated, but one suggestion is that PAA release the active oxygen or active hydroxyl radicals, which interfere with sulfhydryl (-SH) and sulfur (SS) bonds in proteins and enzymes. In addition, PAA play a role in disrupting the chemiosmotic function of lipoprotein cytoplasmic membrane transport through dislocation or rupture of the cell wall (Block, 1991; Santoro et al., 2007). Performic acid (PFA) is also strong disinfectant and does not form the toxic by-products (Gehr et al., 2009). Both PAA and PFA were used to disinfect the combined sewer overflows to reduce the number of E. coli and Enterococcus sp in the project funded by Danish EPA (Chhetri et al., 2015, 2014, 2016; FRODO, 2014). Permanent disinfection system using PAA is used at Viby wastewater treatment plant in Aarhus and PFA have been used to disinfect wastewater effluent from Hjørring Vandselskab at Nørre Lyngby. However, PAA and PFA were not used to treat raw hospital wastewater till date.

In this project, the focus was to test the disinfection solution in pilot scale, so that hospital wastewater can be treated continuously. The developed technology was targeted for disinfection of ARB from hospital wastewater, which requires that the technology must be able to handle dosing and mixing in relation to short and long disinfection times. At new super hospitals, Nyt Hospital Nordsjælland and DNV-Gødstrup, a direct and independent sewer line will be established from hospital to the wastewater treatment plant, which can potentially be used as a "reaction tank" for the disinfection treatment. At the New University Hospital (DNU), Aarhus Municipality and Aarhus vand have entered into an agreement that the disinfection must take place before the wastewater leaves the hospital premises. The similar sewer configuration is found at SK-Forsyning and Slagelse Hospital where disinfection was tested. This project investigated the disinfection of raw hospital wastewater to reduce the number of ARB utilizing the unbranched wastewater pipe from hospital to the wastewater treatment plant and in the wastewater pipe at the hospital before mixing to the municipal sewer network . Moreover, the effect of disinfectant on the selected pharmaceuticals was studied. During disinfection, short and long contact time and high and low PAA dose was used for disinfection of hospital wastewater. Residual PAA concentration and degradation products post PAA disinfection was determined.

Summary

The spread of antibiotic-resistant bacteria (ARB) in the environment is increasing due to the excessive use of antibiotics in humans, animals and plants. Among many environmental conduits known to spread ARB, wastewater has been reported as an important vehicle. Multi-resistant bacteria are abundant in the hospital wastewater compared to the municipal wastewater, as the hospital is the point source for multi-resistant ARB. The Danish national hospital service is currently being centralised into super hospitals servicing entire regions and replacing many local hospitals. The wastewater from these large hospitals will be connected directly to the wastewater treatment plant using a direct pipe. This centralization means antibiotic resistant bacteria in hospital wastewater will pose an increased risk to the workers in the few receiving wastewater treatment plant. ARB can be reduced by disinfecting hospital wastewater at the source that could reduce the risk for infection from ARB to the workers in the WWTP as well as reduce the spread of ARB from the WWTP effluent to the environment.

In this project, raw wastewater from 7 different WWTPs without hospital wastewater contribution was analyzed to survey the average concentration of ciprofloxacin-resistant bacteria in municipal wastewater. The analysis was done to specify disinfection requirements to reduce the ARB from hospital wastewater to the level of ARB in municipal wastewater. A pilot scale disinfection using PAA (peracetic acid) was conducted in a container using raw wastewater from Hillerød and Slagelse hospital and using raw wastewater from Hillerød WWTP and SK Forsyning. The removal of the antibiotic resistant bacteria was studied using different PAA doses and different contact times. Degradation of PAA and hydrogen peroxide in PAA stock solution were studied in the raw wastewater.

In total 8 pilot experiments were conducted using hospital and municipal wastewater in two different locations in Denmark. Different PAA doses were applied with long contact time in the pilot scale that resembled the unbranched wastewater pipe from super hospital to the wastewater treatment plant at Hillerød. In SK Forsyning at Slagelse, pilot experiments were conducted with high PAA doses and short contact time, as the number of ARBs in hospital wastewater had to be reduced in a short period of time before mixing to the municipal sewer network. In the pilot experiment, fast degradation of PAA was observed due to the high organic content in the raw wastewater. Therefore, there was no residual effect of PAA to the process of wastewater treatment plant. Removal of ciprofloxacin-resistant bacteria from pilot experiment was 99.9% when 50 mg/L PAA was used with 20 min contact time. Removal of ciprofloxacin-resistant bacteria increased by increasing contact time. Using 75 mg/L PAA and 2 min contact time, ciprofloxacin- resistant bacteria were reduced with 99.9%, and the numbers of ciprofloxacin-resistant bacteria from hospital wastewater were reduced to less than the level found in municipal wastewater ($1.5 \cdot 10^4$ cfu/mL).

In addition to the disinfection of antibiotics resistant bacteria, degradation of pharmaceuticals from wastewater was studied using both PAA and Performic acid (PFA). PAA showed potency to be used to remove ARB and partial removal of pharmaceuticals from raw hospital wastewater. Finally, continuous disinfection experiment in the pilot scale was done for 6 weeks using raw wastewater and PAA to demonstrate the disinfection concept. Operation cost has been estimated for DKK 0.63 per m^3 treated water when 50 mg/L PAA with 20 min contact time and DKK 0.7 per m^3 treated water when 75 mg/L PAA with 2 min contact time is used to remove 99.9% of selected ARB.

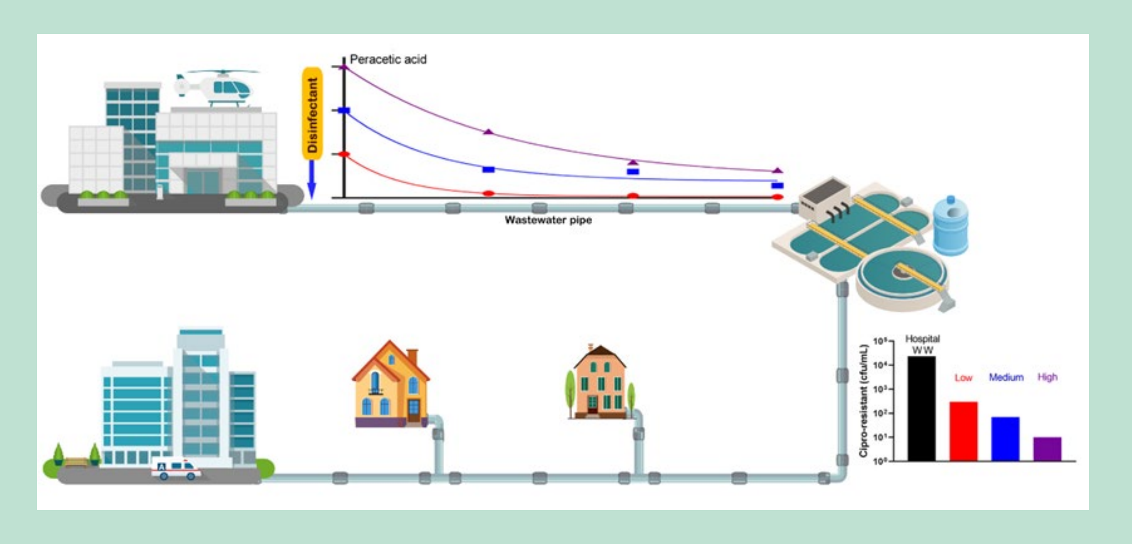


FIGURE 1. : Schematic diagram of proposed disinfection system for disinfection of raw hospital wastewater with separate pipe from hospital to WWTP (top of diagram) and mixing of hospital wastewater with municipal wastewater in a combined sewer (bottom of diagram).

The findings of this study demonstrated that the PAA disinfection of wastewater constitutes a promising treatment technology for the elimination of selected hospital-derived ARB. The method appears an ideal technology to minimize the risk of antibiotic resistant bacteria to the sewage workers when new centralized super hospitals are constructed in Denmark utilizing the unbranched direct connection of wastewater from hospital to the WWTP. Similarly, this method is also appropriate to reduce the ARB from hospital wastewater with short contact time (1-3 minutes) before wastewater is mixed in the existing sewer network.

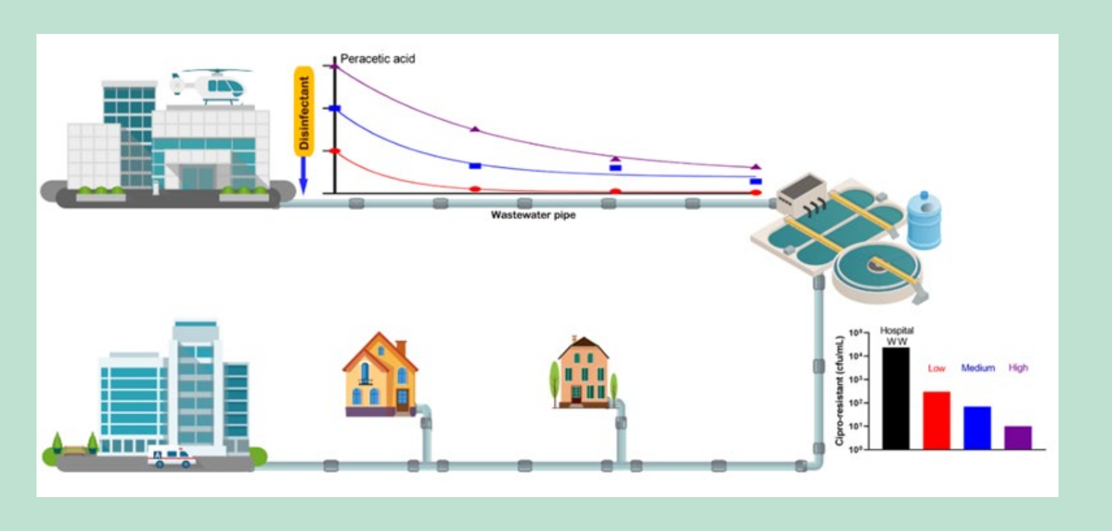
Resume

Spredningen af antibiotika-resistente bakterier (ARB) i miljøet er stigende på grund af overdreven brug af antibiotika hos mennesker, dyr og planter. Blandt mange veje, der vides at sprede ARB i miljøet, er spildevand blevet rapporteret som en vigtig faktor. Multiresistente bakterier forekommer i signifikant højere koncentrationer i hospitalsspildevand sammenlignet med det kommunale spildevand, da hospitalet er punktkilden for multiresistent ARB. Det danske hospitalsvæsen er i øjeblikket ved at blive centraliseret til nye supersygehuse, der servicerer hele regioner og erstatter mange lokalsygehuse. Spildevandet fra disse store hospitaler tilsluttes direkte til renseanlægget ved hjælp af et direkte rør. Denne centralisering betyder, at antibiotikaresistente bakterier i hospitalsspildevand vil udgøre en øget risiko for medarbejderne i de få modtagende spildevandsrensningsanlæg. ARB kan reduceres ved at desinficere hospitalsspildevand ved kilden, hvilket kan reducere risikoen for infektion fra ARB til medarbejderne i renseanlægget samt reducere spredningen af ARB fra spildevandsvandet til miljøet.

I dette projekt blev råspildevand fra 7 forskellige renseanlæg uden hospitalsspildevandsbidrag analyseret for at kortlægge den gennemsnitlige koncentration af ciprofloxacin-resistente bakterier i kommunalt spildevand. Analysen blev udført for at specificere desinfektionskrav til at reducere ARB fra hospitalsspildevand til niveauet af ARB i husholdningsspildevand. Desinfektion i pilotskala med PAA (pereddikesyre) blev udført i en behandlingssløjfe, installeret i en container, som blev tilført henholdsvis råspildevand fra Hillerød og Slagelse sygehus og råspildevand fra Hillerød renseanlæg og SK Forsyning. Fjernelsen af de antibiotikaresistente bakterier blev undersøgt ved forskellige PAA-doser og kontakttider. Nedbrydning af PAA og hydrogenperoxid i PAA-stamopløsningen blev undersøgt i råspildevandet.

I alt blev der udført 8 pilotforsøger med sygehus- og kommunalt spildevand to forskellige lokationer i Danmark. Forskellige PAA koncentrationer blev påført med lang kontakttid i pilotskalaen, der efterlignede det uforgrenede spildevandsrør fra supersygehuset til spildevandsanlægget i Hillerød. Hos SK Forsyning ved Slagelse blev der gennemført pilotforsøg med høje PAA- koncentrationer med kort kontakttid, hvor antallet af ARB i sygehusspildevand skal reducere på kort tid inden opblanding med andet vand i det kommunale kloaknet. I pilotforsøget blev der observeret hurtig nedbrydning af PAA på grund af det høje organiske indhold i råspildevandet. Derfor var der ingen resteffekt af PAA på renseanlægsprocessen. Fjernelse af ciprofloxacin-resistente bakterier fra pilotforsøg var 99,9 %, når 50 mg/L PAA blev anvendt med 20 minutters kontakttid. Fjernelse af ciprofloxacin-resistente bakterier blev øget med øget kontakttid. Ved at bruge 75 mg/L PAA og 2 minutters kontakttid blev de ciprofloxacin-resistente bakterier reduceret med 99,9 %, og antallet af ciprofloxacin-resistente bakterier fra hospitalsspildevand blev reduceret til mindre end det niveau, der findes i kommunalt spildevand (1,5 10^4 cfu/mL).

Udover desinfektion af antibiotikaresistente bakterier blev nedbrydning af lægemidler fra spildevand undersøgt ved brug af både PAA og Permyresyre (PFA). PAA viste potentiale til at blive brugt til at fjerne ARB og delvis fjerne lægemidler fra rå-hospitalsspildevand. Endelig blev der udført et kontinuerligt desinfektionsforsøg i pilotskalaen i 6 uger med råt spildevand og PAA for at demonstrere desinfektionskonceptet. Driftsomkostninger er estimeret til 0,63 kr. pr. m^3 behandlet vand ved 50 mg/L PAA og 20 min kontakttid og 0,7 kr. pr. m^3 behandlet vand, ved 75 mg/L PAA og 2 min kontakttid til at fjerne 99,9 % af udvalgte antibiotikaresistente bakterier.



FIGUR 1. Skematisk diagram af foreslået desinfektionssystem til desinfektion af rå hospitals-spildevand ved separat ledning fra hospitalet til renseanlægget (øverst) og sammenblanding af hospitalsspildevand med husholdningsspildevand i fælles kloakeringsledning.

Resultaterne af denne undersøgelse viste, at PAA-desinfektion af spildevand udgør en lovende behandlingsteknologi til eliminering af udvalgte hospitals-afledte ARB. Metoden fremstår som en ideel teknologi til at minimere risikoen for antibiotikaresistente bakterier for kloakarbejderne, når nye centraliserede supersygehuse opføres i Danmark, der udnytter den ikke forgrenede, direkte kloakledning til spildevand fra hospital til renseanlæg. Tilsvarende er denne metode også egnet til at reducere ARB fra hospitalsspildevand med kort kontakttid (1-3 minutter), før spildevand blandes i det eksisterende kloaknet.

1. Chapter I-Overview of antibiotic resistant bacteria levels in wastewater with and without hospital contribution

Currently, there are no requirements for the number of resistant bacteria that may be discharged to the water treatment plant or the recipients. However, it is expected that a regulation hereof will become relevant, as the large super-hospitals currently only have a temporary connection permit. In order to give the municipalities, which are the decision-making authority in this area, guidelines for potential disinfection requirements, the average level of resistant bacteria was mapped in several treatment plants in Denmark with and without hospital contribution.

A relatively high concentration of antibiotic-resistant bacteria in the samples collected are necessary to be able to document the effectiveness of the disinfection technology through a combination of disinfectant dose and reaction time. Ciprofloxacin-resistant bacteria meet these requirements. Ciprofloxacin is a broad-spectrum antibiotic that is used in the primary sector and in hospitals (number DDD in 2020: primary sector: 570,000, hospitals: 183,000 according to The Danish Health Data Authority (Sundhedsstyrelsen), 2021 at medstat.dk). However, the proportion of several types of multi-resistant bacteria was also quantified, as these bacteria are extremely problematic for the handling at WWTPs and discharge to recipients.

1.1 Determination of Ciprofloxacin-resistant bacteria in raw wastewater in WWTPs without hospital contribution

Raw wastewater from 7 different WWTPs without hospital wastewater contribution was analyzed to survey the average concentration of ciprofloxacin-resistant bacteria in municipal wastewater, as this value could be used as a guideline to municipalities for setting disinfection requirements for hospital wastewater. Total cell counts were derived after cultivation of the wastewater samples on standard culture media after the addition of ciprofloxacin and/or other antibiotics, as described in the paper I (Appendix 1)(Nielsen et al., 2011). The WWTPs contributing with samples are located across the entire Denmark, which means that regional differences, if present, could be addressed

FIGURE 2. Locations for sampling of municipal raw wastewater without connected with hospital wastewater.

The locations without contribution of hospital wastewater were Hjørring (sewer Fuglsigvej), Vildbjerg WWTP near Herning, 3 WWTPs in Aarhus (Åby, Viby, Marselisborg), Korsør WWTP, and Skævinge WWTP near Hillerød.

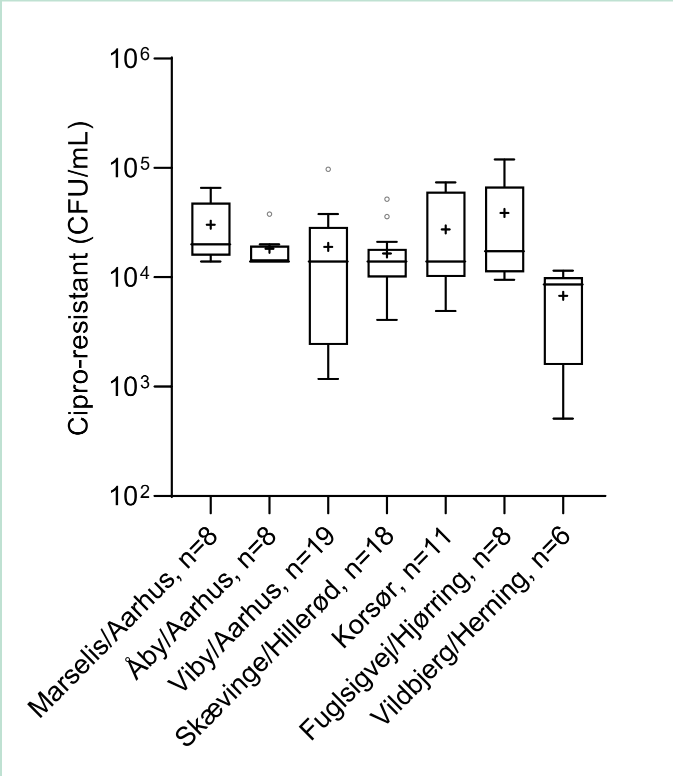


FIGURE 3. Concentration of ciprofloxacin-resistant bacteria in sewer without hospital wastewater. The horizontal line in the box represents the median value, while the '+' shows the mean value. The number of samples analyzed is indicated by n. Data in the graph was plotted using box plot using Tukey method and open circle symbol represent the outliers.

No significant regional variances were found in the concentration of resistant bacteria in wastewater without hospital contribution. The number of analyses for Vildbjerg WWTP was too low to draw a conclusion that the average value is lower compared to the other WWTPs. Generally, the concentration varied a lot due to dilution by rain at some places and some location rainwater was separated from the wastewater.

The calculation of the mean value of resistant bacteria in the domestic wastewater without hospital contribution was based on 78 measurements on samples collected by 24-hour sampling from the 7 different WWTPs. The number of samples from each plant varies, which is why it was not possible to determine the mean value accurately.

Method 1

The mean value was calculated for each plant, after which a weighted mean value of the individual plant mean values was calculated.

The mean value of each plant was based on measurement points excluding outliers. Outliers have been defined as measurement points that do not lie between Q1 – 1.5·IQR and Q3+1.5 IQR corresponding to 99.3 % of data in a normal distribution. Q1 and Q3 are the 25% and 75% percentiles, respectively, that is, corresponding to 50% of the data in a normal distribution. IQR is the difference in the value of data at Q1 and Q3 (Figure 4).

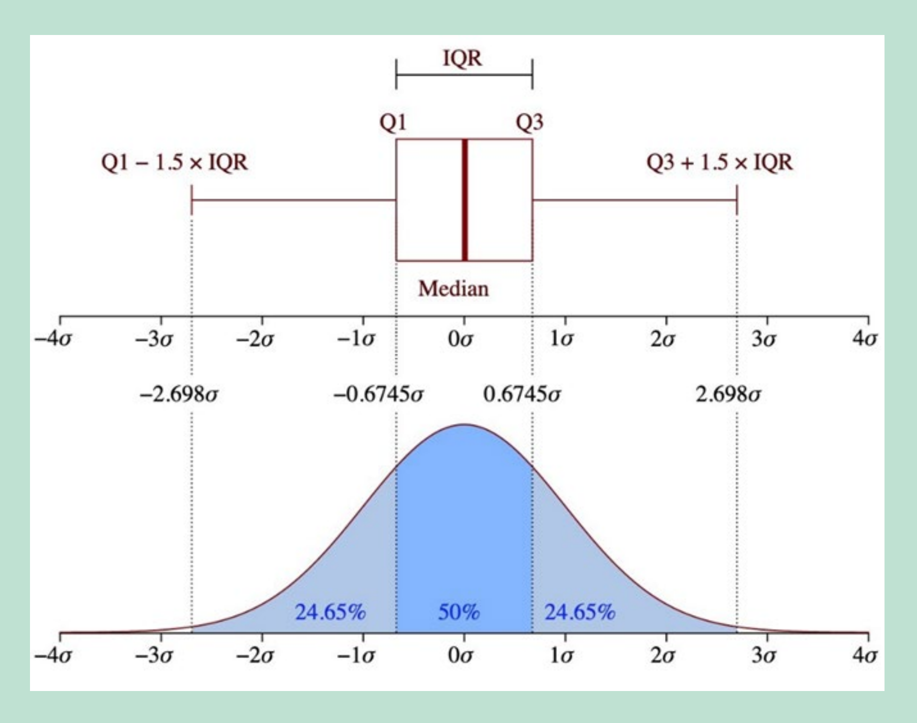


FIGURE 4. Visualization of mean value calculation.

The total mean value was calculated as a weighted average of the plants' individual mean values. The weight w_i for the i'th plant is given as:

$$w_i = \frac{n_i}{IQR_i'} \qquad \text{Equation 1}$$

where n_i is the number of data points (excluding outliers) that were available from each plant. Thus, a plant with many data points has a greater weight than plants with only a few data points. In the same way, plants with a small spread are weighted higher than plants with a large spread. The total weighted average was calculated as follows:

$$avg = \frac{\Sigma_i w_i \cdot avg_i}{\Sigma_i w_i} \qquad \text{Equation 2}$$

where avg_i is the mean value for plant *i*.
In method 1, the total mean value was $1.5 \cdot 10^4$ CFU/mL.

Method 2
All measurements are pooled together, and a weighted mean value was calculated based on the number of measurements for each plant as weight:

$$w_i = n_i \qquad \text{Equation 3}$$

where n_i is the number of measurement points for the i'th plant excluding outliers. Outliers are defined in method 1 and have not been included in the calculation of mean value:

$$avg = \frac{\Sigma_i w_i \cdot m_i}{\Sigma_i w_i} \qquad \text{Equation 4}$$

where m_i is the value of the i'th measurement point.
In method 2, the total mean value is $2.1 \cdot 10^4$ CFU/mL (not weighted) and the weighted mean value according to method 2 is $2.0 \cdot 10^4$ CFU/mL.

To be able to specify a level for disinfection requirements, the most conservative approach for indicating the mean value would be the lowest value: $1.5 \cdot 10^4$ CFU/mL.

The average contribution of Ciprofloxacin-resistant bacteria in the analyzed samples was 3.3 % of the total bacteria with a standard deviation of 3.1 %.

The analyses results on concentrations of Ciprofloxacin-resistant bacteria in hospital wastewater vs. municipal wastewater without hospital contribution are shown in Figure 5.

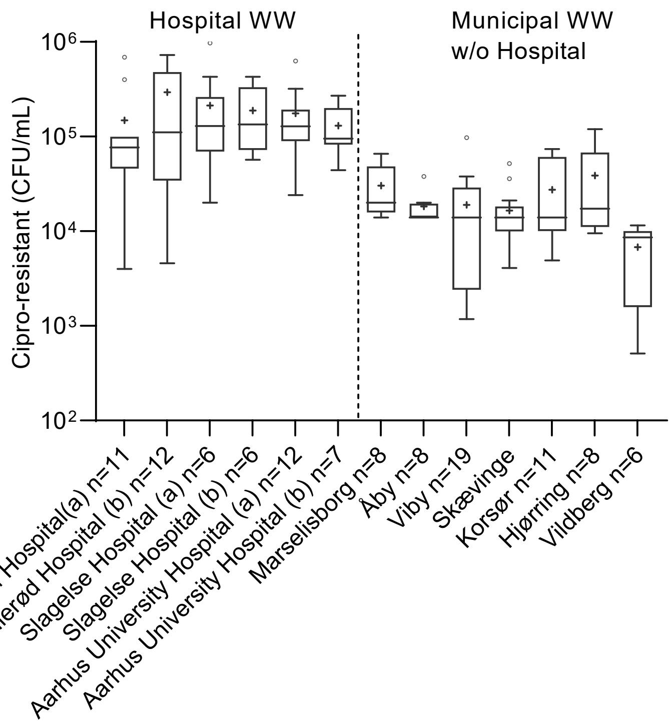


FIGURE 5. Concentration of Ciprofloxacin-resistant bacteria in hospital wastewater vs. municipal wastewater without hospital contribution. Hillerød (a) = Brønd 1, Nordsjællands Hospital Hillerød, Hillerød (b) = Brønd 15, Nordsjællands Hospital Hillerød, Slagelse (a) = Parkvej, Slagelse Hospital, Slagelse (b) = Fælledvej, Slagelse Hospital, Aarhus University Hospital (a) = West, Aarhus University Hospital (b) = East. Grab sampling in hospital sewers from Nordsjællands Hospital Hillerød and Slagelse Hospital, 24-hour sampling at Aarhus University Hospital and the WWTPs. The horizontal line in the box represents the median value, while the '+' shows the mean value. The number of samples analyzed is indicated by n. Data in the graph was plotted using box plot using Tukey method and open circle symbol represent the outliers.

Large variances were found in the analyses of hospital wastewater, especially in the data from Hillerød and Slagelse hospitals. The reasons could be related to the fact that it was only possible to take grab samples in these sewers, and it is expected that the content of bacteria in the wastewater will vary throughout the day. Sewers 15A at Hillerød and Fælledvej at Slagelse hospital are connected to the medical departments with patients suffering from serious infections caused by multi-resistant bacteria. This could easily be seen in the higher concentrations of resistant bacteria in the wastewater collected from these sewers. At AUH 24-hour flow proportional samples were analyzed, where the two sewers East and West receive all watewater, so that a minor contribution from one department will not contribute substantially.

The level of the concentrations of Ciprofloxacin-resistant bacteria in hospital wastewater was approximately 10 times as high as the concentration in municipal wastewater.

Analyses from WWTP with hospital contribution (65 samples collected by 24-hour sampling from 5 WWTPs) and without hospital contribution (78 samples collected by 24-hour sampling from 7 WWTPs) were compared. The average percentage of Ciprofloxacin-resistant bacteria was 5.2 % (standard deviation 6.5 %) in the influent samples from WWTPs with hospital contribution, while it was 3.3 % (standard deviation 3.1 %) of total cell counts in the influent samples of WWTPs without hospital contribution. As the standard deviations are high, the values are fraught with great uncertainty.

1.2 Occurrence of multi-resistant bacteria

The occurrence of multi-resistant bacteria in hospital wastewater and in the influent of WWTPs without hospital contribution was examined with the following broad-spectrum antibiotics: Ciprofloxacin (Ci), Cefuroxime (Ce), Gentamicin (Ge) and Sulfamethoxazole (Su) and sales in Denmark from the period 2014 - 2019 are shown in table 1.

TABLE 1. Sales of broad-spectrum antibiotics in Denmark during the period 2015 - 2019 as defined daily dose (DDD) in 1,000 units (The Danish Health Care Authority (Sundhedsdatastyrelsen), 2021: medstat.dk)

	Primary sector	Secondary sector (hospitals)	Ratio primary sector/ secondary sector	Total sales
Cefuroxime	212	1971	1 : 9	2183
Sulfamethoxazole and trimethoprim	2	1128	1 : 564	1130
Gentamicin	4	372	1 : 93	376
Ciprofloxacin	4237	1216	3.5 : 1	5453

During this period, Ciprofloxacin was by far the most widely applied antibiotic and most evenly distributed between primary and secondary sector, thus being the most suitable model antibiotic for this project. The multi-resistance was investigated by combining Ciprofloxacin with one of the other three antibiotics and as a combination of all four antibiotics.

Figure 6 illustrates the average amount of resistant bacteria from 9 influent samples collected during 24-hour from Skævinge WWTP (near Hillerød without hospital contribution) collected during the period of 8/9/2020 until 17/11-2020.

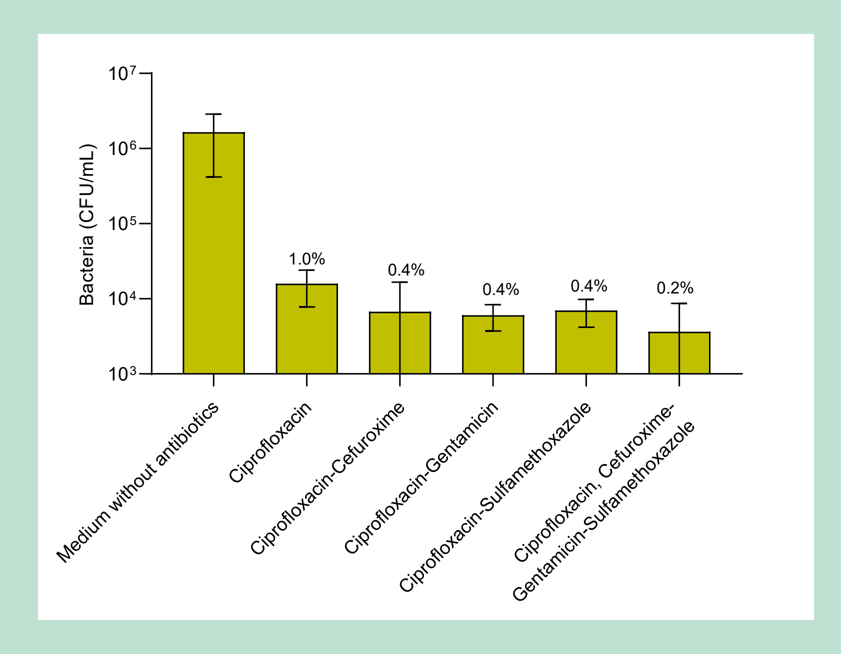


FIGURE 6. Average number of heterotrophic bacteria enumerated in the medium without antibiotics and medium with Ciprofloxacin, combination of Ciprofloxacin, Cefuroxime, Gentamicin and Sulfamethoxazole and combination of all four antibiotics (Ciprofloxacin and Cefuroxime, Gentamicin and Sulfamethoxazole) from 9 samples from Skævinge WWTP without hospital contribution. The percentage of resistant bacteria from total heterotrophic bacteria is shown above the columns while the error bars represent standard deviations.

Multi-resistant bacteria were present in municipal wastewater only to a lower degree (0.2 to 0.4 %) compared to hospital wastewater. The percentage of multi-resistant bacteria in hospital wastewater in the case of Hillerød Hospital was 1% to 26 %, depending on samples analyzed, which is shown in Figure 7 illustrating the two sewers from Hillerød Hospital.

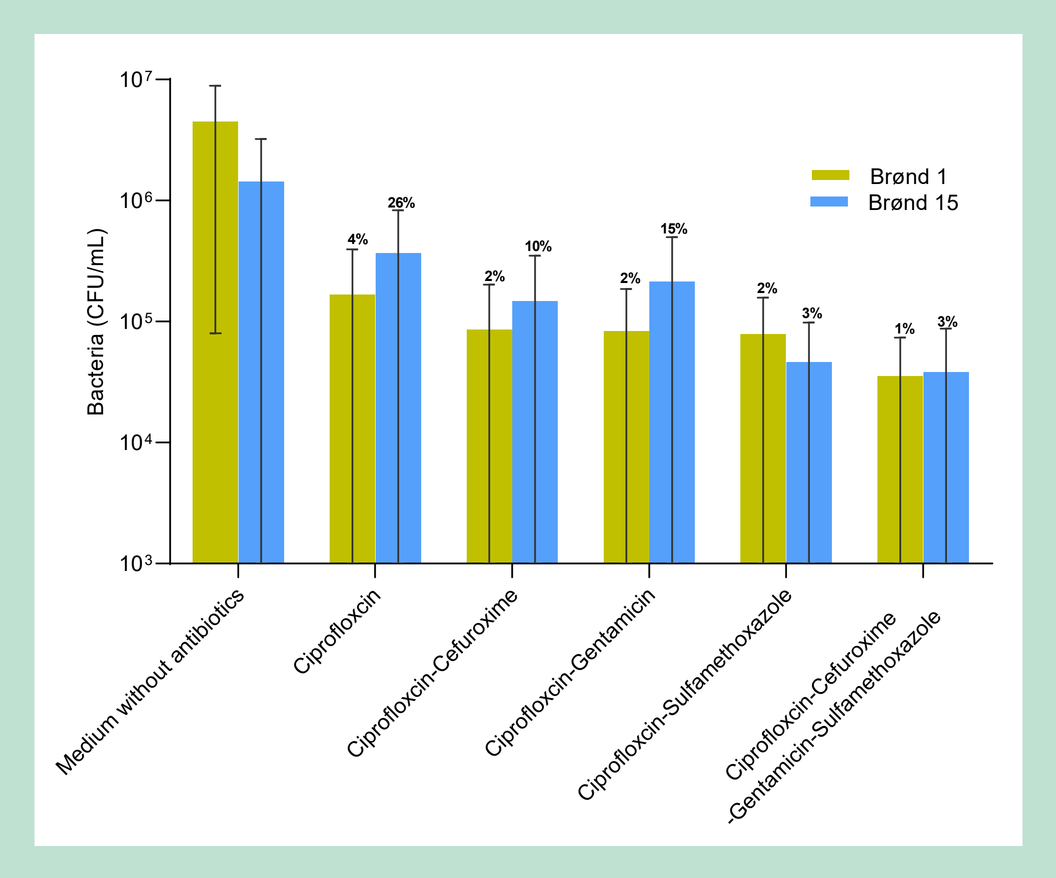


FIGURE 7. Average values (n = 9) of grab samples in a 2-months period from two sewers, brønd 1 and brønd 15, collected from Hillerød Hospital. The heterotrophic bacteria were enumerated without adding antibiotics in the growth medium while resistant-bacteria were enumerated in the growth medium added with different combinations of antibiotics. The percentage of antibiotic-resistant bacteria from total heterotrophic bacteria is shown above the columns while the error bars represent standard deviations.

It was not possible to collect 24-hour samples from the hospitals due to the location of the sewers and the ongoing Covid-19 situation. The great deviation of the samples can be explained by the grab sampling, as large variances in the wastewater composition throughout the day can be expected. Nevertheless, the occurrence of resistant and multi-resistant bacteria in hospital wastewater was significantly higher than in municipal wastewater. The data undoubtedly indicates the sewer that was connected to the hospital unit for treating infectious diseases (blue bars).

1.3 Summary

To give the municipalities an idea for the specification of disinfection level requirements for hospital wastewater, the average level of Ciprofloxacin-resistant bacteria in wastewater without hospital contribution was analyzed in 78 samples collected by 24-hour sampling from 7 different WWTPs across Denmark (different number of samples from each WWTP).

- The most conservative approach for specifying a mean value in the dataset is the lowest achieved value of $1.5 \cdot 10^4$ CFU/mL.
- On average, the concentration of Ciprofloxacin-resistant bacteria in hospital wastewater was approximately 10 times as high as the concentration in municipal wastewater.
- The wastewater in WWTPs with hospital contribution showed a slightly higher concentration of Ciprofloxacin-resistant bacteria compared to the ones without hospital contribution, but the variances in the numbers are too high for final conclusion.

- Multi-resistance bacteria for up to 4 broad-spectrum antibiotics was detected in both wastewater without hospital contribution and in hospital wastewater. The level in hospital wastewater was significantly higher in all sewers and much higher in the sewer connected to the infectious disease unit of the hospital.

2. Chapter II-Determination of disinfectant dose and contact time for pilot experiment

The purpose of this chapter was to identify concentrations and reaction times for disinfectant, PAA, to disinfect hospital wastewater in the pilot scale. Commercial PAA is available in the market consisting of diverse PAA:hydrogen peroxide ratios. In this study, commercial PAA was purchased from Novadan (Kolding, Denmark) consists of 15% w/w PAA, 5% (w/w) acetic acid and 15% w/w hydrogen peroxide. To determine the optimal disinfection dose of PAA, two scenarios were examined. The infrastructure between WWTP and the new super-hospitals varies; either an independent sewer line will be established from hospital to WWTP without the possibility of overflow; alternatively, the disinfection of the raw wastewater take place before the wastewater is mixed in the existing sewer network. Hospital wastewater contains antibiotic-resistant bacteria and pharmaceuticals residual, which have adverse effect in recipients. In addition to the disinfection of antibiotics resistant bacteria, degradation of pharmaceuticals residual from wastewater was studied using both PAA and PFA. Concentration of disinfectant residuals as well as degradation products during disinfection was studied, which is necessary to design and obtain regulatory approval of the treatment systems.

2.1 Determination of disinfection dose and reaction time in different types of wastewater

A design experiment was conducted in laboratory to determine the PAA dose that is required to disinfect at long and short contact time to achieve reasonable disinfection level.

PAA degradation was studied in the raw wastewater from two municipal WWTPs (Hillerød and SK forsyning) and two hospitals (Hillerød and Slagelse hospital). Concentration profiles and removal of ciprofloxacin-resistant bacteria using different PAA dose and contact time was studied.

To study concentration profiles and removal efficiency of antibiotic resistant bacteria, different concentrations of PAA (25, 50, 75, 100 and 150 mg/L) were spiked in raw wastewater from different location mentioned above in a separate bottles. PAA concentration in wastewater samples was determined using the colorimetric method described in Chhetri et al., (2020) at different contact time.

Ciprofloxacin resistant bacteria were enumerated using spread plate method where serial dilutions of each sample were prepared in a solution with 4 mg/L ciprofloxacin mixed with sterile PBS, and 1mL of diluted samples were added onto the CompactDry plate in duplicate and incubated for 24 h at 37 ± 1 °C. Bacteria counts were expressed as colony forming units per mL (CFU/mL), with the limit of quantification (LOQ) of ciprofloxacin resistant bacteria equal to 10 CFU/ mL. The detail method description of enumeration of Ciprofloxacin resistant bacteria is presented in the Paper I (Appendix 1). Experiments were repeated on two different days one week apart, to test wastewater quality variability. Residues of PAA in the samples were neutralized by adding 100 mg/L sodium thiosulphate to destroy PAA, followed by 50 mg/L catalase to destroy hydrogen peroxide.

In an experiment with wastewater from Hillerød WWTP, concentration of PAA was quantified after 3, 10, 30, 60 and 120 min and in parallel samples were taken for quantification of antibiotics-resistant bacteria. A half of the PAA concentration was degraded between 1 and 5 minutes (Figure 8A). Complete PAA degradation was observed after 60 minutes when 50 and 100 mg/L PAA was used and after 120 minutes contact time when 150 mg/L PAA was used. The differences on degradation of PAA concentration can be explained by different physico-chemical factors affecting the decay of PAA. These factors tend to be related to the presence of suspended solids (SS), pH, temperature, COD, salinity and water hardness (Luukkonen and Pehkonen, 2017; Sarathy et al., 2016).

TABLE 2. Characteristics of wastewater samples collected from Hillerød WWTP, Hillerød hospital, SK forsyning and Slagelse hospital.

Parameters	Hillerød WWTP	Hillerød hospital	SK forsyning	Slagelse hospital
pH	7.0	7.4	7.1	7.4
COD (mg/L)	NA	830	1067	1782

N/A: Not analyzed

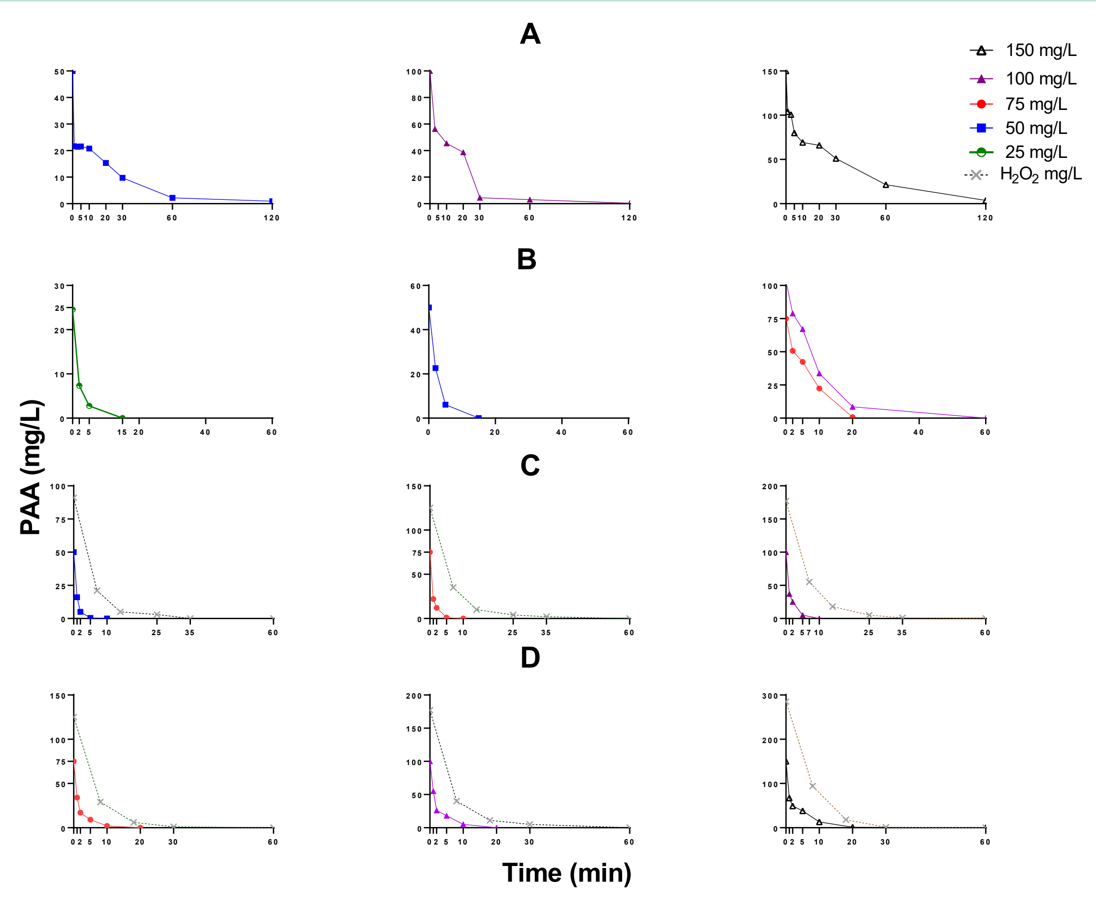


FIGURE 8. Concentration profiles of PAA in wastewater from Hillerød WWTP (A), Concentration profiles of PAA in wastewater from Hillerød hospital (B), Concentration profiles of PAA in wastewater from Slagelse WWTP (C), Concentration profiles of PAA in wastewater from Slagelse hospital (D). Dotted line in the graph represent the concentration profiles of hydrogen peroxide quantified from PAA solution.

Raw hospital wastewater was collected from two outlets from Hillerød hospital namely: Brønd 1 and 15. Three samples from Brønd 15 and Brønd 1 were collected on three different days and mixed equally prior measuring COD, pH and concentration profiles of PAA. Average COD of mixed wastewater from Brønd 15 and Brønd 1 was 830 mg/L COD and pH 7.4. Three PAA concentrations (25, 50 and 75 mg/L) were added in a separate bottles and PAA was quantified after 2, 5, 10, 20 and 60 min. Moreover, samples for quantification of Ciprofloxacin-resistant bacteria were collected at the same time. An apparent high initial consumption of PAA was observed in all samples with initial consumption of PAA increasing with increased nominal PAA dose. Rapid degradation of PAA was observed where almost all PAA was degraded within 10 min contact time (Figure 8B). Similar degradation of PAA was observed in hospital wastewater consisting of an equal mixture of water collected from Brønd 1 and Brønd 15 except for the highest dose of 100 mg/L PAA degraded at 60 min contact time.

To determine the optimal concentration of PAA with short contact time for disinfection, degradation of PAA and removal efficiency of ciprofloxacin-resistant bacteria was studied in the raw wastewater collected from SK forsyning and Slagelse hospital. Three PAA concentrations (50, 75 and 100 mg/L) was added in separate bottles containing wastewater from Slagelse wastewater treatment plant and 75, 100 and 150 mg/L PAA was added in a separate bottles containing wastewater from Slagelse hospital. PAA was quantified after 1, 2, 5, 10 and 20 min. In parallel, samples were collected for quantification of Ciprofloxacin-resistant bacteria. A rapid degradation of PAA was observed in both samples and complete degradation of PAA was observed after 10 min contact time except in an experiment with 100 mg/L and 150 mg/L PAA in the hospital wastewater from Slagelse where PAA was degraded after 20 min contact time (Figure 8 C &D).

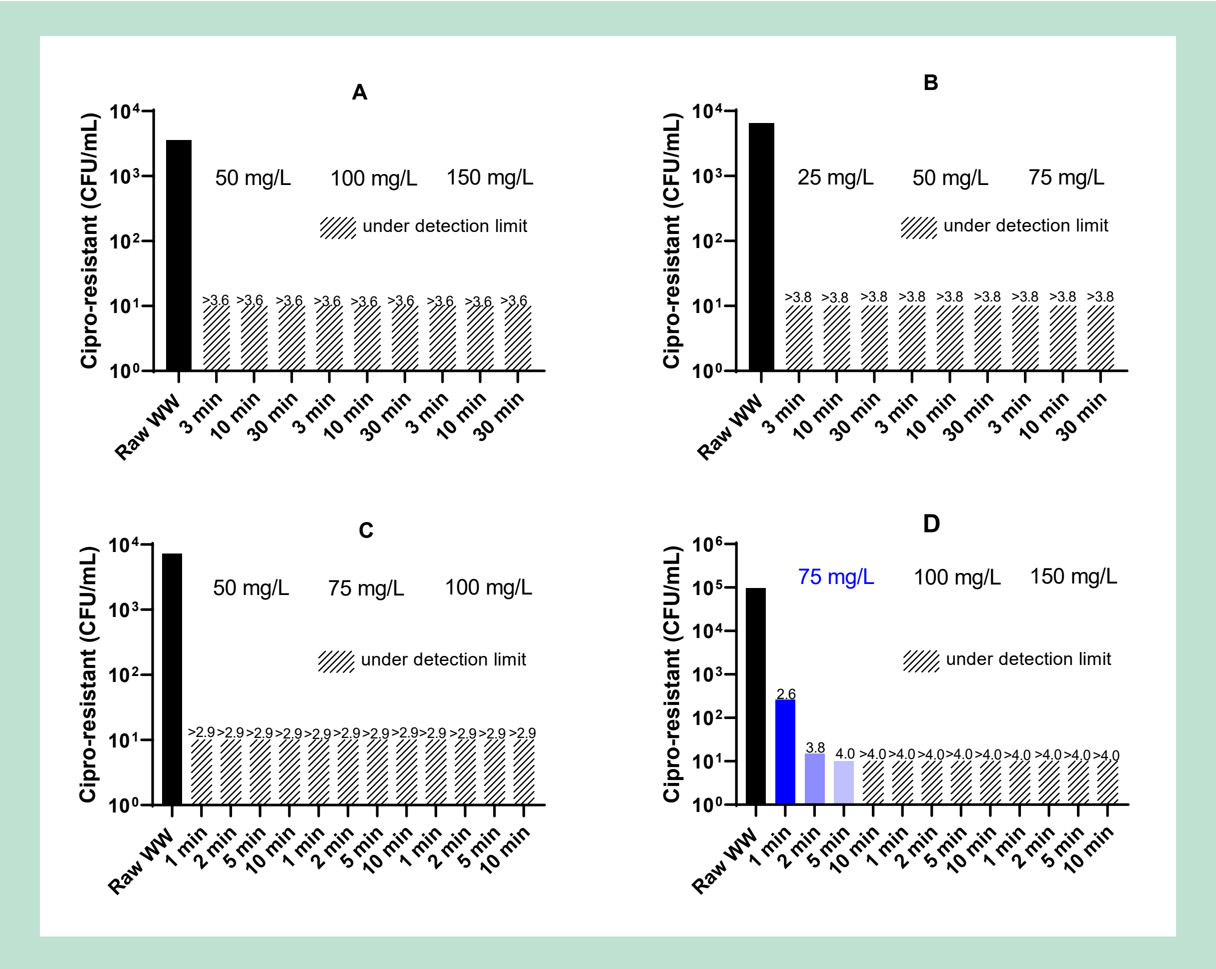


FIGURE 9. Microbiology results from batch experiment using wastewater from Hillerød WWTP (A), Microbiology results from batch experiment using wastewater from Hillerød Hospital (B), Microbiology results from batch experiment using wastewater from SK forsyning (C), Microbiology results from batch experiment using wastewater from Slagelse hospital (D). The logarithm removal of ciprofloxacin-resistant bacteria is indicated above each bar in the graph.

A complete removal of Ciprofloxacin-resistant bacteria was observed when PAA concentrations ranging from 25 mg/L to 150 mg/L were used with 1-30 min contact time (Figure 9). The reason for complete removal of Ciprofloxacin- resistant bacteria could be prolonged storage of wastewater. Wastewater samples were collected a day before experiment and viable bacteria are more vulnerable and sensitive with prolonged storage time.

2.2 Residual determination and degradation products

The determination of residual concentration of chemical disinfectant and their degradation products is utmost important when chemical disinfection is used. The determination of the residual concentration allows to identify the adverse effect on an aquatic life in a recipient so that appropriate dilution level could be applied to minimize the toxicity from residual concentration of disinfectant. Similarly, study on the concentration and fate of degradation products in a recipient is important while using chemical disinfectant. Commercial PAA is available as an acidic quaternary equilibrium mixture of PAA, hydrogen peroxide, acetic acid, and water:

$$CH_3COOH + H_2O_2 \rightleftharpoons CH_3CO_3H + H_2O \qquad \text{Equation 5}$$

The residues after PAA use are acetic acid, hydrogen peroxide, and water. Acetic acid is further biodegraded to carbon dioxide and water and not considered toxic to aquatic life (Liberti and Notarnicola, 1999), while hydrogen peroxide degrades to oxygen and water. Degradation of hydrogen peroxide in the wastewater was slower than PAA therefore, degradation of hydrogen peroxide that was present in the PAA solution were studied in the design experiments using wastewater from SK forsyning and pilot experiment using wastewater from Hillerød hospital, SK forsyning and Slagelse hospital. Concentration of PAA and hydrogen peroxide were quantified simultaneously using the method described by Chhetri et al., (2020). Slow degradation of hydrogen peroxide was observed compared to PAA. This could be due to the destruction of catalase enzyme present in the gram-negative bacteria by PAA as catalase enzyme degrades hydrogen peroxide to oxygen and water.

2.3 Effect on Pharmaceuticals

Pharmaceuticals degradation using PAA and PFA was studied in the influent and effluent wastewater collected from Hjørning Vand at Nørre Lyngby and tap water.
At laboratory, the pharmaceutical spiking solution dissolved in methanol was added into a serum bottle. Prior to adding 100 mL wastewater into the bottle, it was left inside the fume hood to evaporate methanol. Afterwards, different concentration of PAA or PFA were added into the serum bottle and the reaction solution was kept stirring. The samples were taken when PAA or PFA were degraded completely. Afterwards samples were filtered with the syringe filters (PTFE, 0.22 µm, Agilent, USA) and 0.9 mL of the filtered samples were transferred to the HPLC vial with 0.1 mL of internal standards. 10 µL of sample from the HPLC vial was injected and analysed by HPLC-MS/MS (Agilent, USA). The detailed descriptions for the sample preparation and the instrumental settings is presented in (Tang et al., 2021).
The concentrations of PAA/PFA and removal of pharmaceuticals is presented in the Figure 10. When influent wastewater was treated with 50, 75 and 100 mg/L PAA complete degradation was observed after 40 minutes and when 25 and 50 mg/L PFA was used, complete degradation was observed after 36 hours. However, when treating tap water, PAA (1, 4 and 8 mg/L) and PFA (2, 4 and 12 mg/L) complete degradation was observed after 9 hours and 19 hours, respectively.

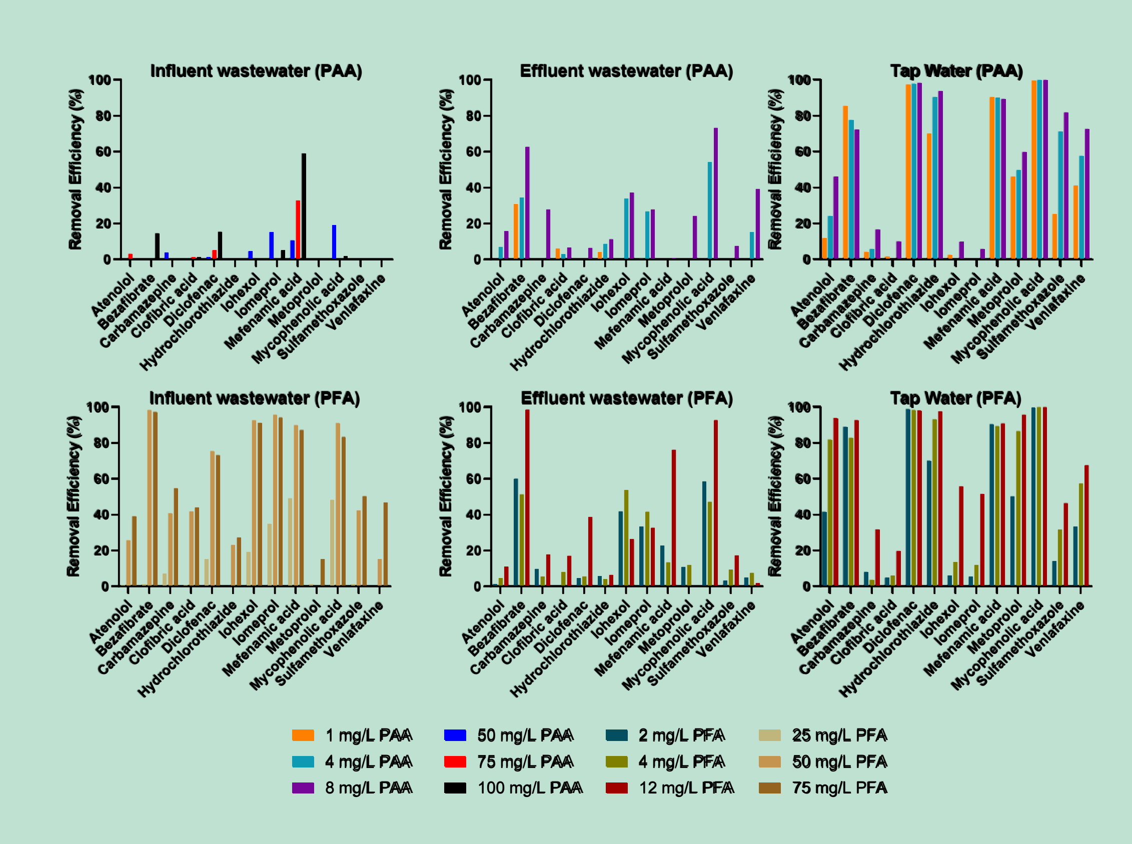


FIGURE 10. Removal of pharmaceuticals using different concentration of PAA and PFA in the wastewater influent and effluent from Hjørring Vand and tap water.

When 75 mg/L PFA was added in the influent wastewater, 75% diclonefac, 95% bezafibrate, 90% mefenamic acid and 95% Iomeprol was removed whilst by applying 100 mg/L PAA, 18% diclonefac, 15% bezafibrate, 60% mefenamic acid and 5% Iomeprol was removed. In an experiment where effluent wastewater was treated with 4 mg/L PFA, 5% diclonefac, 55% bezafibrate, 15% mefenamic acid and 42% Iomeprol was removed whilst by applying 4 mg/L PAA, 35% bezafibrate, and 25% Iomeprol was removed and no removal of diclonefac and mefenamic acid was observed.

2.4 Summary of chapter

To determine the concentration of PAA for pilot-scale experiments with long and short contact time experiments were conducted in the wastewater from two different sites consisting of municipal and hospital wastewater. To disinfect the municipal WWTP with long contact time 25, 50, 75 mg/L PAA concentration was selected with 20,40,60 min contact time. For disinfection of hospital wastewater with long contact time 50, 75, 100 mg/L PAA was selected. Similarly, for disinfection in pilot container with short contact time, 25, 50 & 75 mg/L PAA was selected for municipal wastewater and 50, 75 and 150 mg/L PAA for hospital wastewater. Residual PAA was quantified while degradation of PAA was studied in different types of wastewater from different locations and rapid degradation of PAA was observed. Hydrogen peroxide, degradation product of PAA during disinfection, was also quantified. Degradation of hydrogen peroxide was slow compare to PAA.
PAA showed potency to be used to remove ARB and partial removal of pharmaceuticals from raw hospital wastewater.

3. Chapter III- Development and testing of continuous disinfection technology for low-dose

3.1 Pilot container

Alumichem designed and constructed a container for disinfection of wastewater in pilot scale. The container was 20-foot in length. A plug flow reactor was constructed by folding a long pipe in the container to reflect the retention time of wastewater from a hospital to a wastewater treatment plant. Several sampling points were constructed in the container to take samples of the wastewater after different reaction times. The system was equipped with a submersible centrifugal feed pump to feed the wastewater and a peristaltic dosing pump to ensurea continuous addition of disinfectant. A static mixer downstream of the dosing point ensured that the disinfectant was thoroughly mixed with the wastewater. PAA concentrations and contact times were adjusted by adjusting the flows of the dosing pump and the feed pump.

3.2 Experiments with long reaction time

Pilot experiments with untreated municipal and hospital wastewater were carried out on two different days one month apart. The first pilot experiment was conducted by using 25, 50 and 75 mg/L PAA doses with three contact times using the wastewater from Hillerød forsyning. To verify the degradation of PAA and disinfection efficiency, similar experiment was conducted in a batch scale in parallel to the pilot experiment same day. Same PAA concentrations and contact time as the pilot experiments were applied in the batch experiments.
A fraction of sample was collected to quantify the PAA concentration and to enumerate ciprofloxacin-resistant bacteria after different contact times. PAA concentrations were quantified until 60 min in the remaining sample for pilot experiments whilst in batch experiments, PAA quantification was done until complete degradation was observed. Furthermore, raw wastewater was collected at the inlet of the pilot container prior to the beginning and the end of the experiment, to enumerate the initial number of ciprofloxacin-resistant bacteria and to measure pH and COD.

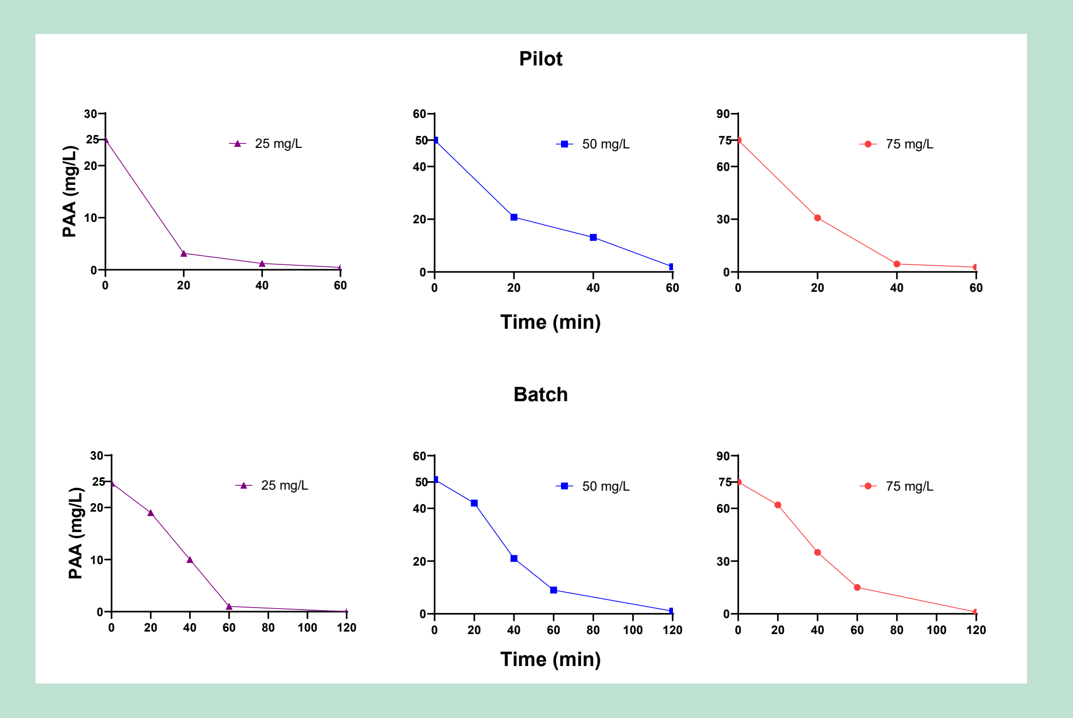


FIGURE 11. Concentration profiles of PAA from the pilot and batch experiment using wastewater from Hillerød WWTP.

In the pilot experiment using municipal wastewater from Hillerød forsyning, 25, 50 and 75 mg/L PAA were used in the pilot and batch experiments. PAA and hydrogen peroxide concentrations were measured after 20, 40 and 60 min. Low COD (349 mg/L) was measured in the WW, resulting in the slow degradation of PAA in the pilot experiment, whilst complete degradation was observed after 120 min in the batch experiment (Figure11). Low PAA dose was used for disinfection that that was used for hospital wastewater based on low COD and the fact that municipal wastewater contains less ciprofloxacin-resistant bacteria compared to hospital wastewater.
The pilot experiment using wastewater from Hillerød hospital was conducted using 50, 75 and 100 mg/L of PAA. Wastewater from the Hillerød hospital was collected from Brønd 1 and Brønd 15 in the same day when pilot experiment was conducted. Wastewater from Brønd 1 and Brønd 15 were mixed equally in a IBC next to the pilot container and used for pilot experiment. Concentrations of PAA and hydrogen peroxide were measured after 1, 5, 10 and 20 min. Fast PAA degradation was observed and PAA concentration was less than 0.5 mg/L after 10 min. However, around 40% of the initial hydrogen peroxide remained when measured after 40 min contact time (Figure 13). A similar degradation of PAA and hydrogen peroxide was observed in the batch experiment (Figure 13). The faster decay of PAA in the raw hospital wastewater was observed, which might be due to higher COD and organic matter compared to municipal wastewater (Table 3).

TABLE 3. : Characteristics of wastewater samples.

Parameters	Hillerød WWTP	Hillerød WWTP	Hillerød hospital	Hillerød hospital
pH	7.3	7.3	7.7	7.8
COD (mg/L)	349	473	743	445

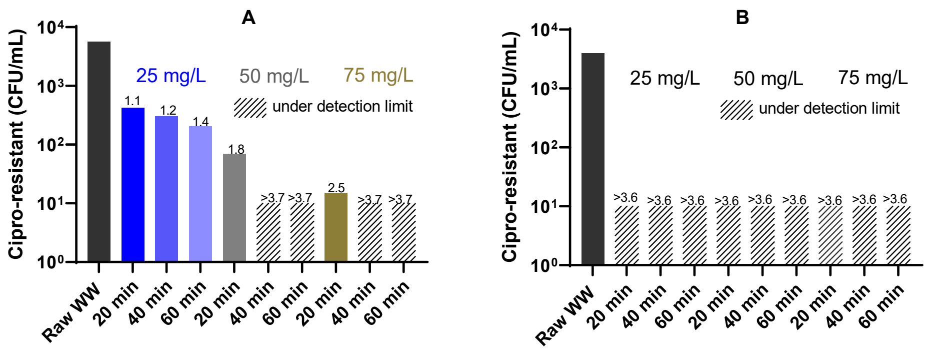


FIGURE 12. Microbiology results for the removal of ciprofloxacin- resistant bacteria from the Pilot (A) and Batch (B) experiments using wastewater from Hillerød WWTP. Logarithm to the removal of ciprofloxacin-resistant bacteria is indicated above each bar in the graph.

Results from the pilot experiment using municipal wastewater showed the progressive removal of ciprofloxacin-resistant bacteria. With a PAA dose of 25 mg/L with 60 min contact time, the removal of ciprofloxacin-resistant bacteria was 1.4 log, while at dosages of 50 and 75 mg/L PAA, the removal of ciprofloxacin-resistant bacteria was under the detection limit after a contact time of 60 min (Figure 12A). However, in the batch experiment with 20 min exposure to 25 mg/L PAA, ciprofloxacin-resistant bacteria were under the detection limit (Figure 12 B).

In a pilot experiments using hospital wastewater, the removal of ciprofloxacin-resistant bacteria increased in line with an increase in contact time. The removal rate was 0.8 log when 50 mg/L of PAA was used and with 1 min of contact time. This increased to 2.4 log when 50 mg/L of PAA was used along with a 10 min contact time. Results from second pilot experiments using wastewater from Hillerød WWTP and Hillerød hospital is presented in Appendix 3. Moreover, selected results on concentration profiles and disinfection efficiency of PAA against antibiotics resistant bacteria from this section was published in the peer-reviewed journal in Journal of Water process engineering (Appendix 1).

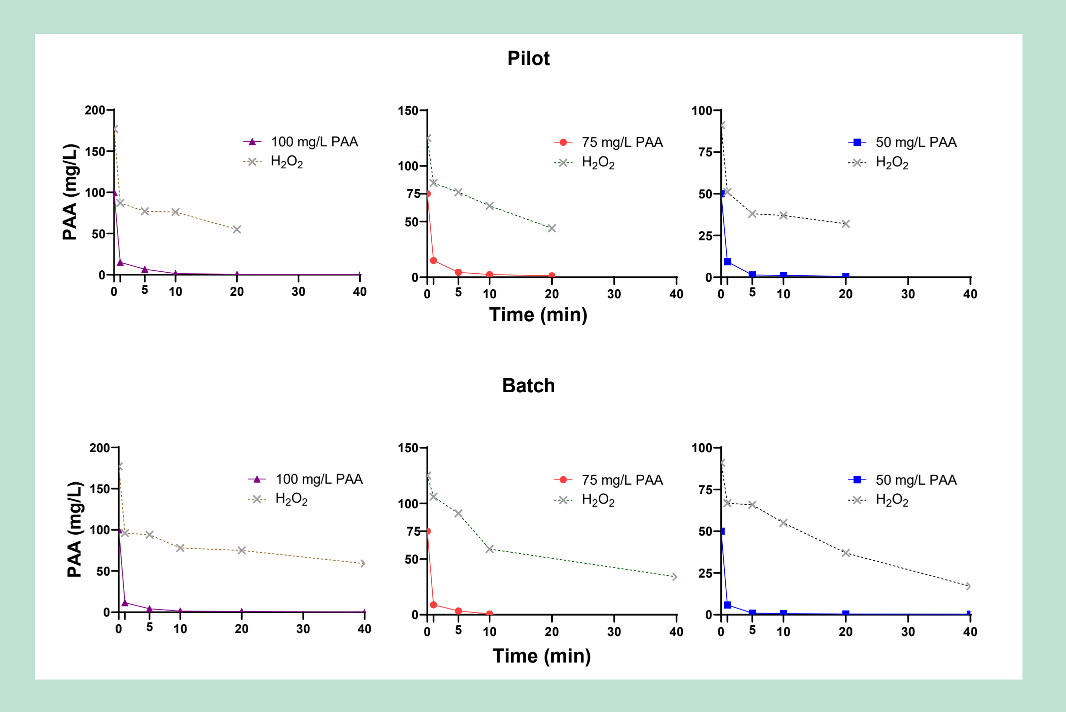


FIGURE 13. Concentration profiles of PAA and H_2O_2 in wastewater from Hillerød hospital in pilot and batch experiments

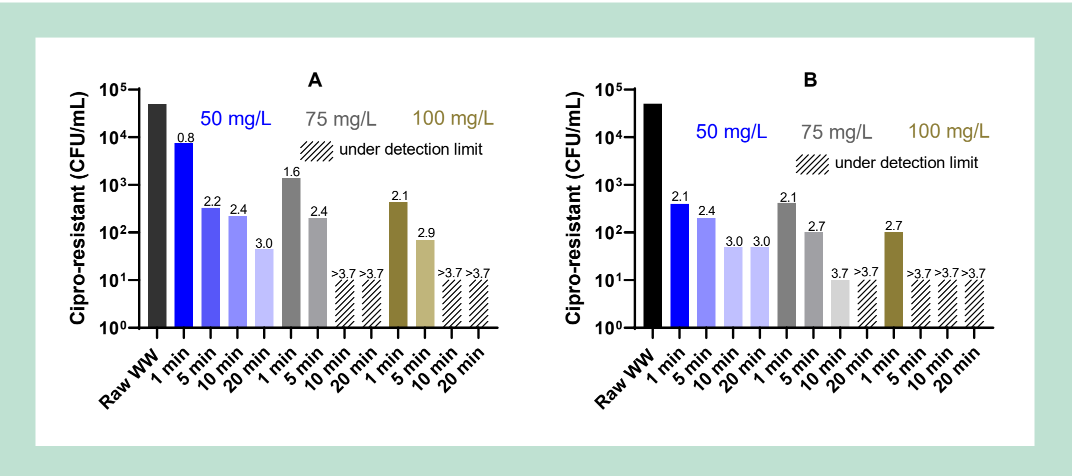


FIGURE 14. Microbiology results for the removal of ciprofloxacin- resistant bacteria from the Pilot (A) and Batch (B) experiments from Hillerød hospital. The logarithm to the removal of ciprofloxacin-resistant bacteria is presented above each bar in the graph.

3.3 Testing of available sensors for improved control

Different sensors were tested to check if efffective control of disinfection dosage could be done and measure the effect of peracetic acid online. This creates the possibility of a feed-back solution that can increase or decrease a disinfection dosage regardless of the flow of wastewater.

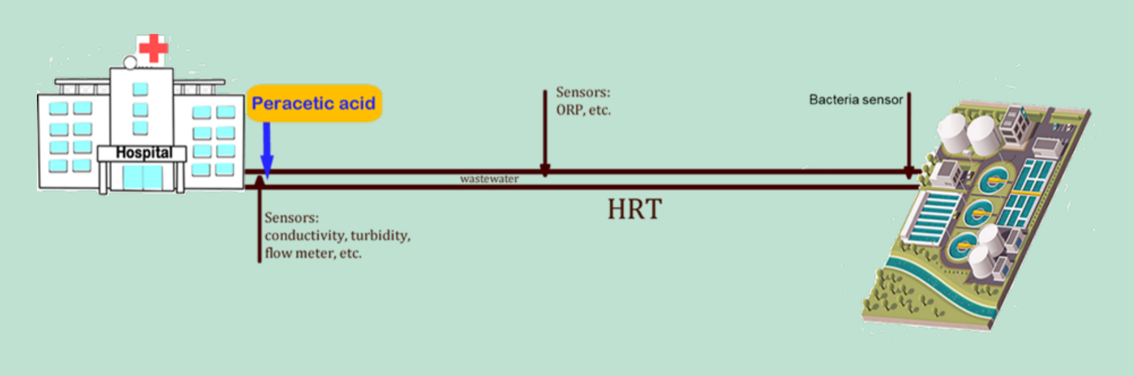


FIGURE 15. Schematic diagram of disinfection system of hospital wastewater using different sensors.

3.3.1 Results for turbidity-, conductivity- and flowsensors

Different sensors were investigated including turbidity, conductivity, and dissolved oxygen. In cooperation between Alumichem, DTU, TI it was decided to move forward with turbidity and conductivity sensors. A multichannel measuring device (JUMO AQUIS touch P) was installed in the pilot unit to measure turbidity and conductivity (Figure 16 and 17. The multichannel device has an integrated recorder that log the data from both sensors every 5 second. The function of the turbidity sensor is based on an infrared light measurement according to the 90 ° scattered light method with a measuring range of 0 - 4000 NTU. The conductivity sensor detects the electrolytic conductivity of the hospital sewage with a measuring range of 0-100 mS/cm.

FIGURE 16. Multichannel measuring device to record the measurement from turbidity and conductivity sensors.

FIGURE 17. Conductivity and turbidity sensors installed in the pilot container.

Two sensors were used to measure the turbidity and conductivity of raw wastewater during pilot experiments using wastewater from SK forsyning and Slagelse hospital. During pilot experiment using wastewater from SK forsyning, measurement from conductivity and turbidity were compared with COD analyzed in the laboratory. However, no correlation was observed between conductivity , turbidity and COD. In a pilot experiment using wastewater from Slagelse hospital, wastewater was collected in a IBC and pumped in a container. No variance on turbidity, conductivity and COD was observed due to wastewater was pumped from the same IBC and quality of wastewater was unchanged (Table 4).

TABLE 4. Turbidity, conductivity and COD measured from hospital wastewater. Turbidity and conductivity was measured using sensors.

Conductivity (mS/cm)	Turbidity (NTU)	TSS (mg/L)	COD (mg/L)	Wastewater pumped
1.11	207.8	263	463	IBC 4
1.09	203.8	200		
1.09	198.4	238		
1.10	186.9	138	470	IBC 3
1.11	191.8	138		
1.10	196.6	113		
1.11	201.1	138		
1.09	235.6	150	450	IBC 2
1.07	237	138		
1.06	256.9	150		
1.05	275.1	138		
0.98	392.6	138	479	IBC 1

3.3.2 Sensors for PAA control by living bacteria

Two different methods based on living/dead bacteria were tested for their potential to control the varying need for PAA addition and both methods are described in the following sub-section. Both methods do not measure antibiotic-resistant bacteria selectively but measure total bacterial cells. A decrease of total bacteria due to PAA treatment is assumed to reflect also what happens to the antibiotic resistant bacteria and is therefore applied as a potential indirect measure of PAA dose. The analytical time is much lower (minutes) compared to conventional growth-based enumeration of antibiotic resistant bacteria.

3.3.2.1 LIVE/DEAD staining

The Live/dead staining was performed with the LIVE/DEAD BacLightTM Viability Kit (ThermoFisher Scientific, USA), staining intact cells with a fluorescent stain of a certain wavelength (SYTO®9), while cells with damaged cell membrane are stained with another fluorescent stain (propidium Iodide). The emissions of the different wavelengths were measured and compared with a standard curve based on known percentages of live/dead cells. The following figure 19 shows the results of cell reduction in a wastewater sample from Hillerød hospital before and after treatment with different concentrations of PAA and different reaction times measured with the Live/dead staining method.

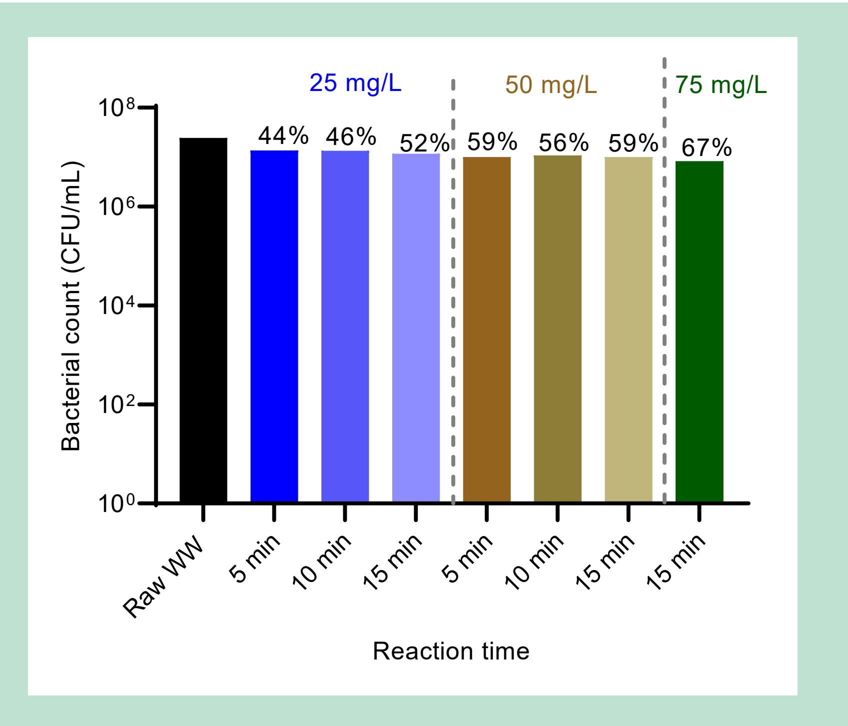


FIGURE 18. Live/dead staining results on a raw wastewater sample from Hillerød Hospital (first bar) and reduction of cells with intact cell membrane after treatment with PAA in different concentrations and reaction times. The reduction in % is shown above each bar.

No difference was observed between the samples with the same PAA concentration added with 5, 10 and 15 min of contact time. However, there might be a minor difference between wastewater sample treated with 25, 50 or 75 mg/L PAA with 15 min contact time, although the data must be validated by further measurements.

For comparison, figure 19 shows the same sample analyzed by culturing on Compact Dry TC plates (TC plates) (Nissui Pharmaceutical CO., LTD., Japan) achieving the total viable bacterial count on nutrient standard agar. Both the total bacteria and the Ciprofloxacin-resistant bacteria in this sample were cultured.

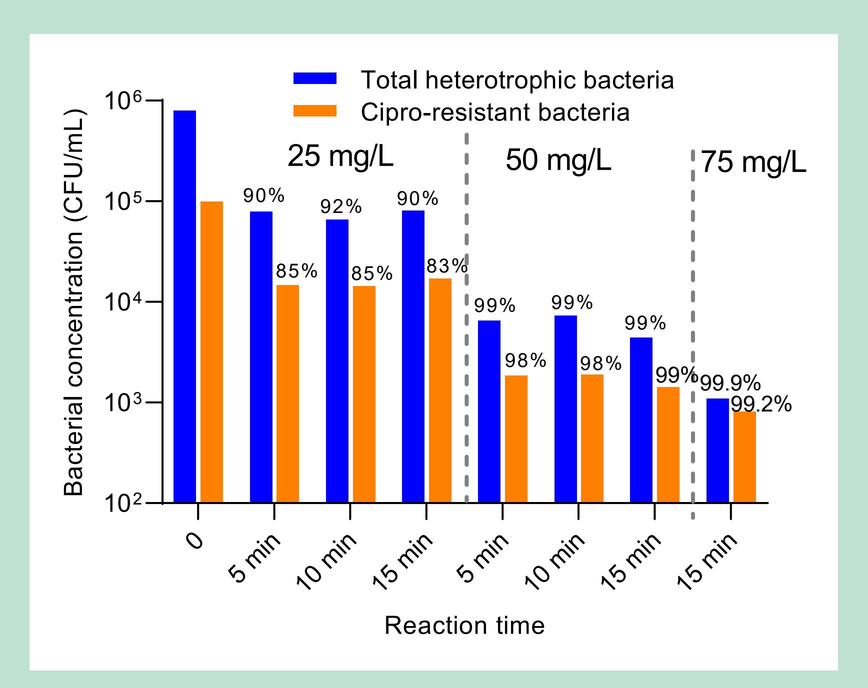


FIGURE 19. Culturing of total bacteria (blue) and Ciprofloxacin-resistant bacteria (orange) in a raw wastewater sample from Hillerød Hospital before (bar 1) and after treatment with PAA in different concentrations and reaction times. The reduction in % is shown above each bar.

As shown in figure 19, the reduction of ciprofloxacin-resistant bacteria was consistent with the reduction of total bacteria in all cases. Therefore, it is possible to use the total living bacterial count as a measure for disinfection effectivity.

It was observed that a factor 50 less bacteria was detected with the culturing method in the raw wastewater sample than with the LIVE/DEAD staining method (Figure 18 & 19). A reason for this may be the fact, that < 2% of all bacteria can be cultivated (Wade, 2002). Plating involved a percentage reduction of more than 82 % for both total and Ciprofloxacin-resistant bacteria, whereby the addition of 25 mg/L peracetic acid left 105 CFU/mL of bacteria, while the LIVE/DEAD method could detect a reduction of no more than 68% with bacteria still present at 107 CFU/mL after addition of 75 mg/L peracetic acid.

A comparison of the two methods (culturing and live/dead staining) with a pure *E.coli* culture showed that there was no significant difference between the methods (t-test: $p = 0.66$), but the complexity in the wastewater matrix can cause insufficient staining. Furthermore, extracellular material can interfere with the propidium iodide stain resulting in high background fluorescence (Biggerstaff et al., 2006; ThermoFisherScientific, 2004). Some gram-negative bacteria membranes are less permeable for the stains (Boulos et al., 1999; Stiefel et al., 2015). Several experiments must be conducted to determine whether the staining method could be a reliable tool for the control of PAA addition.

Since the results can only be achieved within an hour resulting in the fact that the method would give a relatively long delay for the control of PAA dosage, this was not pursued any further.

3.3.2.2 Impedance flow cytometry

Another potential online sensor for measuring the bacteria before and after PAA treatment was tested in the laboratory. Impedance measurements of the PAA treated wastewater were performed with the BactoBox® instrument (SBT Instruments) to investigate whether intact cells could be differentiated from damaged cells.

FIGURE 20. Impedance flow cytometry instrument BactoBox® (SBT Instruments) and measuring principle.

Impedance changes are measured when intact single cells pass a capillary (Impedance flow cytometry (sbtinstruments.com)) (Figure 20). The method can differentiate intact cells from cells with broken cell walls/other particles in the sample. Pre-condition for the successful measurement is the bacterial cell concentration in the range of 10.0000 to 2.000.000 IC/mL (intact cells/mL), which makes dilution of the wastewater samples necessary.

In all experiments with inlet wastewater from WWTP Åby (impedance flow cytometry and culturing on Compact Dry TC plates), a 20-fold dilution of the raw wastewater from inlet water of Åby WWTP was used, as this dilution corresponded to the concentration requirements of the BactoBox® instrument. The results of two experiments with PAA treatment of inlet wastewater from Åby WWTP (20-fold dilution) and 80 mg/L PAA in the diluted samples is presented in Figure 21. The high PAA concentration was selected in the first experiments to ensure complete disinfection.

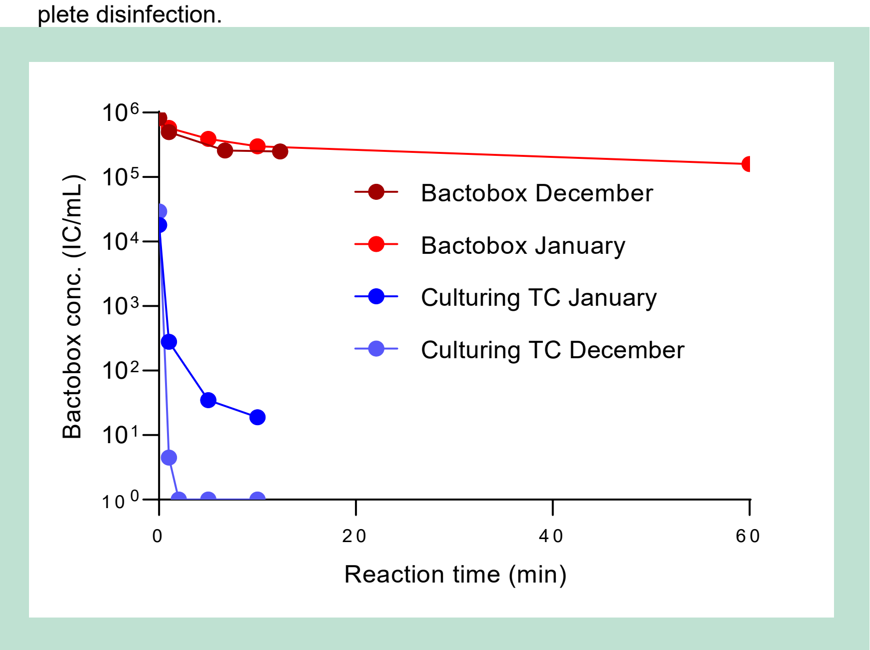


FIGURE 21. Disinfection experiments with inlet wastewater from Åby WWTP in a 20-fold dilution and 80 mg/L PAA. A comparison of impedance flow cytometry (Bactobox - IC/mL = intact cells/mL) and cultured bacteria (TC - CFU/mL) as detection methods.

As showed in figure 21 the LIVE/DEAD staining method had a much higher start concentration (45 times) with the impedance flow cytometry than with the culturing method, which again can be explained by the fact that only a very small part of the bacteria is culturable (Wade, 2002). While the reduction of culturable bacteria was 99.9 % after 2 minutes reaction time with PAA, the reduction in impedance flow cytometry was 63 % after 10 minutes and 80 % after 60 min in the experiment conducted on January (comparable results in the experiment conducted on December). The ongoing reduction after 10 minutes shows that the PAA was still active, and the high slope in the first 5 minutes corresponds very well to the experiments with PAA consumption in the chapter 2.1.

The impedance flow cytometry was also used in an experiment with wastewater from Hillerød Hospital presented in the figure 22. Here a 30-fold dilution was necessary and a PAA concentration of 80 mg/L in the undiluted wastewater was applied in this experiment, as this is a realistic concentration for disinfection with PAA.

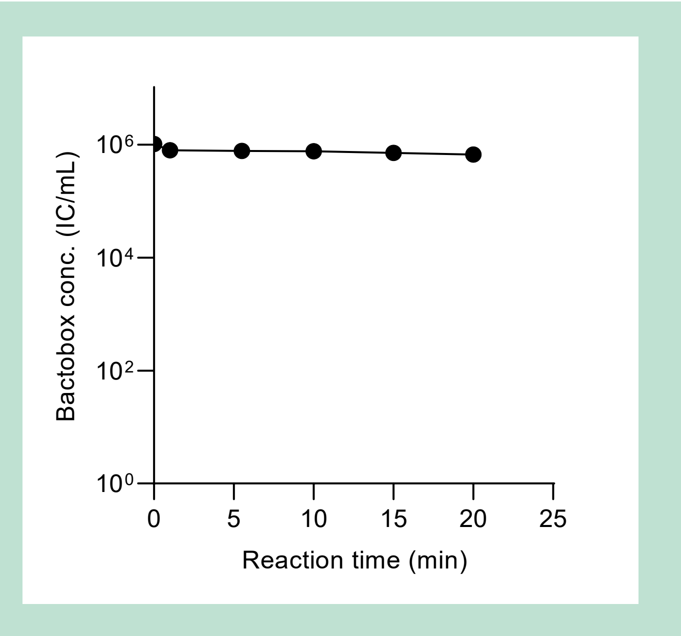


FIGURE 22. PAA disinfection (80 mg/L) of raw wastewater from Hillerød hospital measured in a 30-fold dilution with impedance flow cytometry.

The fast reduction of intact cells in the first few minutes was observed in this experiment, and the reduction of intact cells was lower (35 % after 20 minutes) than in the experiments with wastewater from Hillerød WWTP, most likely due to the different dilutions and thus lower PAA concentration.

The impedance flow cytometry showed very promising results. Further experiments with different types of wastewaters are necessary to gain experience with:

- the dilution requirements in different samples
- the intact cell level of the impedance measurement after disinfection required to ensure that the concentration of antibiotic-resistant bacteria is below the required level.

Further development of the instrument is necessary to make the stand alone instrument an on-line version with automated dilution of the wastewater and self-cleaning. A very strong advantage of this method is the availability of the measurement results within 2 to 3 minutes, which would allow on-time control of the PAA dosage.

3.4 Disinfection technology rebuilt and adjusted for operation at SK-Forsyning with short contact time

In this section, study was focused on the disinfection of the raw wastewater before the wastewater is mixed in the existing sewer network. The retention time of wastewater will be short as the length of wastewater pipe from hospital to the sewer network is short allowing only few minutes of contact time. The container for pilot experiments with short contact time were adjusted by increasing the flow of wastewater and disinfectant in the pipe. Moreover, different sensors were tested to control the disinfection (section 3.3). The study on pre-filtration of raw wastewater prior disinfection was also studied by applying pre-filtration unit using filter with pore size of 600 µm. Experiments with short contact time and high PAA dose were used that was determined during the pre-experiment (section 2). The pilot container was placed next to the primary tank in the WWTP at Slagelse. In the pilot experiment, municipal wastewater was collected after grit and grease chamber. Two pilot experiments were conducted in different days with two weeks in a part to see the variation in the wastewater quality. Batch experiments were conducted in the container in parallel to pilot experiments to verify the PAA dose delivered and removal of ciprofloxacin resistant bacteria. Three PAA concentration, 25, 50 and 75 mg/L, was used in the pilot experiments and batch experiments. PAA and hydrogen peroxide present in the commercial PAA solution was quantified after 1, 2, 5 and 10 min and samples were taken for enumeration of ciprofloxacin resistant bacteria from the pilot and batch experiments. pH and COD of the raw wastewater is presented in the table 5.

TABLE 5. Characteristics of wastewater samples collected during pilot experiments.

Parameters	Slagelse WWTP	Slagelse WWTP	Slagelse hospital	Slagelse hospital
pH	7.5	7.5	7.5	8.0
COD (mg/L)	2010	1164	598	473

COD was 2010 and 1164 mg/L and pH was 7.5 in the wastewater sampled on 8th March and 15th March 2021, respectively. A rapid degradation of hydrogen peroxide was observed in both pilot and batch experiments compared to PAA. Complete degradation of PAA was observed within 10 min contact time for 25 and 50 mg/L PAA and 91% PAA was degraded within 10 min contact time for 75 mg/L PAA in the pilot experiment. In the batch experiment, complete degradation of 25, 50 and 75 mg/L PAA was observed at 20 min contact time (Figure 23). The removal of ciprofloxacin resistant bacteria increased with increasing PAA concentration and increasing contact time. The removal increased from 0.2 log to 1.1 log when 25 mg/L PAA was used and contact time increased from 1 min to 10 min (Figure 24). Similarly, by using 75 mg/L PAA with 1 min contact time, removal of ciprofloxacin resistant bacteria was 1.6 log and the removal increased to more than 3.6 log with 10 min contact time then the bacteria concentration was under the detection level. The removal of ciprofloxacin resistant bacteria was similar in both pilot and batch experiment when the same PAA concentration and contact time were used.

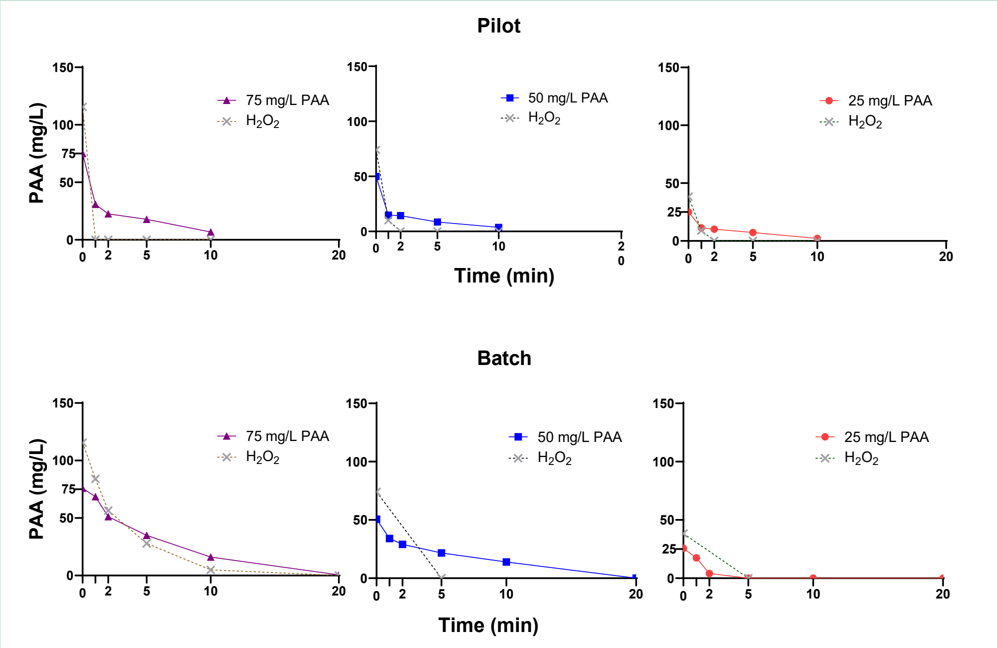


FIGURE 23. Concentration profiles of PAA in municipal wastewater from SK forsyning from pilot and batch experiment.

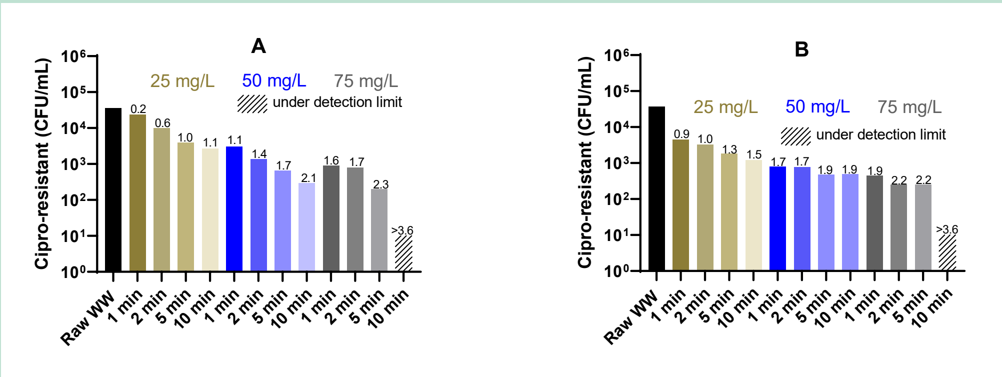


FIGURE 24. Microbiology results for the removal of ciprofloxacin- resistant bacteria from the Pilot (A) and Batch (B) experiments from Slagelse municipal wastewater SK forsyning. The logarithm to the removal of ciprofloxacin-resistant bacteria is presented above each bar in the graph.

Two pilot experiments were conducted using wastewater from Slagelse hospital. Five IBCs were filled with the wastewater collected from the outlet (Brønd Parkvej) of the Slagelse hospital and used for disinfection in a pilot container. In the first pilot experiment, pre-filtration unit was installed in between the IBC containing wastewater and container. Samples were collected before and after filtration to measure COD and antibiotics resistant bacteria. However, no significant different in COD and number of bacteria was observed. During experiment, filter was clogged several time. Moreover, sludge collected from filtration could be difficult to manage in the purposed disinfection system where ARB and pharmaceuticals are abundant among other pollutants that require special treatment. Therefore, second pilot experiment was conducted without pre-filtration unit. In the pilot experiment with hospital wastewater (COD 600 mg /L), 50, 75 and 150 mg/L PAA was used for disinfection with 1, 2, 5 and 10 min contact time. In parallel, batch experiment were conducted using the same PAA concentration however, concentration of PAA was measured until complete degradation was observed. Samples were collected for microbiological analysis when samples were collected for PAA quantification. Slow degradation of PAA and hydrogen peroxide was observed in a hospital wastewater compare to PAA and hydrogen peroxide degradation in the municipal wastewater. A slow degradation of PAA in wastewater could be due to the dilution of wastewater with rainwater. In the pilot experiment, PAA concentration was quantified until 10 min however, in the batch experiment PAA was quantified until complete degradation was observed (Figure 25).

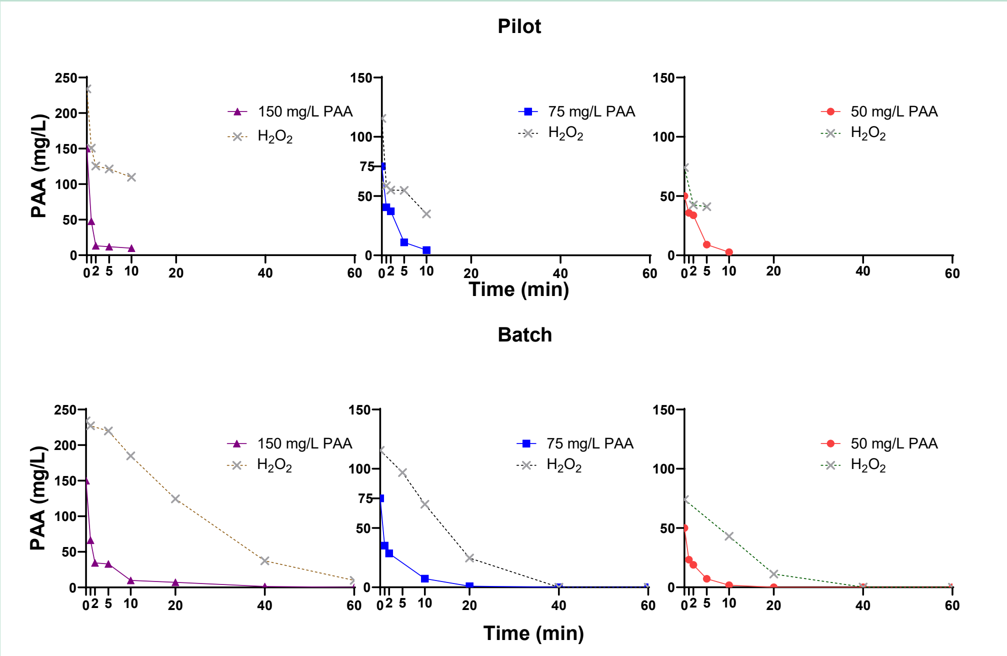


FIGURE 25. Concentration profiles of PAA in wastewater from Slagelse hospital and H_2O_2 from the PAA solution.

The number of ciprofloxacin resistant bacteria was higher in hospital wastewater than the municipal wastewater. The removal of ciprofloxacin resistant bacteria was 0.6 log when 50 mg/L PAA was used in pilot experiment with 1 min contact time and removal increased to 2.7 log with 10 min contact time using same PAA concentration (Figure 26A). Complete removal of ciprofloxacin resistant bacteria (under detection level) was observed with 75 mg/L with 5 and 10 min contact time and 150 mg/L PAA with 1 min contact time. Similar removal of ciprofloxacin resistant bacteria was observed in batch experiment (Figure 26B) that verify the disinfection of wastewater in pilot experiment. The removal of antibiotics resistant bacteria from hospital wastewater using PAA showed a potential on using it for continuous disinfection for prolonged period.

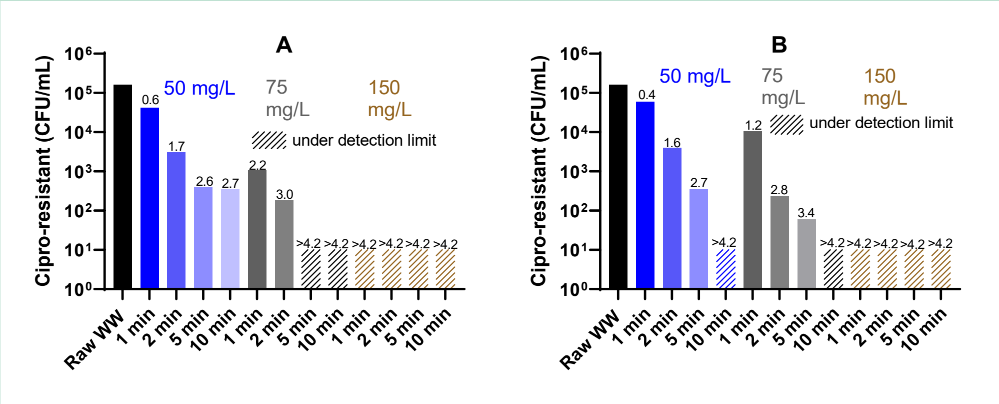


FIGURE 26. Microbiology results for the removal of ciprofloxacin- resistant bacteria from the Pilot (A) and Batch (B) experiments using wastewater from Slagelse hospital. The logarithm to the removal of ciprofloxacin-resistant bacteria is presented above each bar in the graph. Selected concentration profiles and results for disinfection efficiency of PAA against antibiotics resistant bacteria from this section was compiled in the manuscript and submitted to the ISI journal for peer-review (Appendix 2). Results from second pilot experiments using wastewater from SK Forsyning and Slagelse Hospital is presented in Appendix 3.

3.5 Demonstration of the disinfection concept for continuous disinfection

To document a robust disinfection technology that effectively reduces antibiotics resistant bacteria in hospital wastewater, the continuous disinfection operation was demonstrated for 6 weeks. Municipal wastewater was used for disinfection instead of wastewater from hospital. It was technically difficult to place the pilot container next to the sampling well (brønd Parkvej) to pump the wastewater for disinfection since brønd Parkvej was located in the middle of parking lot at Slagelse hospital. Alternative solution was collecting the wastewater from hospital, unloading it to the IBC and pumped for disinfection in the pilot container. However, variation in the chemical and microbiological quality of wastewater will not observed as it will be collected from same IBC. Therefore, municipal wastewater was used for continuous disinfection to observed the variation on wastewater quality. Demonstration of continuous disinfection was done for 6 weeks by applying different PAA dose and contact time. Each week, three samples were taken before and after disinfection to see the variation on wastewater quality by applying same PAA dose and contact time. A detail on PAA dose and contact time used for continuous disinfection is presented in the table 6:

TABLE 6. Overview of PAA dose and contact time for continuous disinfection for 6 weeks in the pilot container.

Week no.	PAA dose (mg/l)	Contact time (min)
Week 1	25	1
Week 2	25	2
Week 3	50	1
Week 4	50	2
Week 5	75	2
Week 6	75	2

COD and pH were measured from the wastewater to see the variability in the quality of wastewater disinfected in the container. The overview of sampling date, time and chemical analysis of wastewater is presented in table 7.

TABLE 7. The overview of sampling date, time and chemical analysis of wastewater during continuous disinfection experiment.

Date	Time	pH	COD (mg/L)
28-07-2021	07:47	6.7	1230
	11:26	6.9	1806
	14:15	6.7	1382
02-08-2021	07:32	6.9	1242
	12:26	N/A*	1336
	14:15	6.9	1466
10-08-2021	07:55	6.8	1800
	12:35	7.4	1266
	14:10	7.3	1198
18-08-2021	N/A*	6.7	861
	11:38	6.9	943
	14:00	6.5	784
07-10-2021	08:21	7.4	1385
	12:52	7.6	1964
18-10-2021	08:45	6.6	2162
	11:30	6.6	1506
	14:00	7.0	884

*N/A: Not available

In the first week of the pilot disinfection using 25 mg/L PAA with 1 min contact time, three samples were collected before and after disinfection with COD 1230, 1806 and 1382 mg/L. Samples arrived after 2 days of collection that resulted a huge difference in the removal. The removal of ciprofloxacin resistant bacteria ranged from 35% to 93%. In the second week onwards, removal of ciprofloxacin resistant bacteria remained about 40% on average and the removals did not increased with increasing PAA concentration and contact time. In hindsight, accumulation of sludge was observed in the pipe used as the reaction tank in the pilot container during the pilot experiments - which was not observed in previous pilot experiments with shorter trials where the pipe was flushed between experiments. The accumulated sludge likely consumed PAA fast through reaction with bacteria and organic matter, which decreased the removal of bacteria by the added PAA. Additionally bacteria can be shielded in the particles (Domínguez Henao et al., 2018).

3.6 Summary of chapter

Disinfection results from pilot experiment with long contact time showed that PAA can remove ciprofloxacin resistant bacteria before it reaches to the inlet of wastewater treatment plant. 50 mg/L PAA with 20 min contact time removed the ciprofloxacin resistant bacteria to the 1.5x104 cfu/mL, the level defined in the section 1.
Two sensors, turbidity and conductivity, were used to test for online control of PAA disinfection. No correlation of COD, turbidity and conductivity were observed when turbidity and conductivity sensors were used in the pilot experiment.

Another approach to the development of sensors for the PAA control is the detection of living bacterial cells. Two methods were tested: the LIVE/DEAD staining method (BacLightTM Viability Kit, ThermoFisher) and the impedance flow cytometry (BactoBox®, SBT Instruments). Both methods detected far more bacterial cells (factor 45 to 50) than the standard culturing method (cell cultures on Compact Dry Plates), supporting the knowledge that the culturing method has some limitations. Both methods showed the potential to measure the living intact bacterial cells in wastewater, but the impedance flow cytometry is clearly superior to the staining method due to very short analysis times (2 minutes), which could enable on-time control of the PAA dosage. Both methods require further development to be able to measure wastewater as well as there is a need for automation before they can be used on-line for the control of PAA dosage.

Disinfection result from pilot experiments with short contact time showed that 75 mg/L PAA with 1 min contact time reduce the ciprofloxacin resistant bacteria to the level $1.5 \cdot 10^4$ cfu/mL (defined in section 1).
Results from continuous disinfection experiment showed around 40% removal of ciprofloxacin resistant bacteria. The uncontrolled experiments during continuous disinfection experiment resulted a poor removal of resistant bacteria compare to the pilot experiments conducted earlier.

4. Chapter IV- Dimensioning for full-scale disinfection solutions for Hillerød forsyning and SK-forsyning

4.1 Overall design

The disinfection system is designed for automatic operation, in the sense that it will vary the dosing according to the wastewater flow. The automation also involves web access, monitoring of chemical consumption and alarms.

The system design is based on two scenarios: 1) disinfection of wastewater from large hospitals (super hospitals) connected directly to the wastewater treatment plant using a separate pipe. 2) disinfection of the raw wastewater before the wastewater is mixed in the existing sewer network. , where parameters found in the experimental work are used. The main operational parameters are dosing volume and contact time, where a larger dose can compensate for a shorter contact time and vice versa.

The chemical dosing system consists of monitoring equipment, such as flow, temperature and COD, and the control system. The contact time is a closed volume where the chemical can react. The volume can be either an existing pipeline or a tank installed onsite. Please see a diagram above of the system setup

4.2 Daily operation

The system is designed to be completely automated, in the sense that there is no need for it to be supervised constantly. The chemical use is supervised, and appropriate warning will be sent out when the level in the chemical tank runs low and needs to be refilled.

4.3 Safety

The system will be designed for dosing of peracetic acid from either IBCs or from a bulk tank. The decision between the two are made considering chemical price (bulk is usually cheaper than IBC) and space availability onsite. A system fed by PAA from a bulk tank ensures that there is no manual labor involved in the operation. When level is running low in the tank, it is simply refilled by means of a tank truck. The filling itself is made safe by an alarm system, informing the truck driver by light and sound when the tank is nearly full, in order to stop the filling in due time.

A system fed from IBCs involves manual handling of the tanks. When an IBC is used for disinfection, it is placed on a tray which contains any spill. The risk is highest when the empty IBC is removed and the full one is placed.

Safety precautions when handling IBCs with PAA involves placement in a well-ventilated area, and places where the temperature does not exceed 30°C. Safety measures according to the material safety data sheet must be followed at all times.

4.4 Design parameters and financials

The system design can be adapted to local conditions in the sense that control cabinets and dosing pumps can be placed somewhat freely, still taking safety into consideration. The following financial calculations assumes that the system can be placed in an area where:

- Storage of PAA can be done according to safety measures
- Retention time of the treated water is sufficient before entering into the main sewage system
- The system pricing is for equipment alone, excluding civil works and installation
-

System 1: Utilizing existing sewer pipe for long retention time

The scope of system 1 is:

- Bulk storage of PAA
- Sufficient retention time in the existing sewage pipeline
- A wastewater flow of maximum 30 m^3/h, with a daily volume of 240 m^3

System 2:Treatment in a tank with short retention time

The scope of system 2 is:

- IBC storage of PAA
- Retention time obtained in a tank
- A wastewater flow of maximum 20 m^3/h, with a daily volume of 160 m^3

TABLE 8. Overview of financial costs for removal of 99.9% of selected ARB from hospital wastewater.

	System 1 (Utilizing existing sewer pipe)	**System 2 (Treatment in a tank)**
Dosing	50 mg/L	75 mg/L
Retention time	20 min	2 min
Volume for retention[1]	10 m^3	0,7 m^3
Budget price	DKK 500.000,-	DKK 625.000,-
Operational costs (excluding manpower, including chemical cost, maintenance, electricity)	DKK 0,63 per m^3 treated water	DKK 0,95 per m^3 treated water

The decision process of the correct setup must entail an analysis of the existing facilities, including:

- Does the wastewater have a common discharge point?
- Available space above ground
- Potential for placement below ground
- Variation in wastewater flow

4.5 Summary of chapter

Operation cost has been estimated for DKK 0.63 per m^3 treated water when 50 mg/L PAA is used with 20 min contact time to remove 99.9% of selected ARB in an existing sewer pipe. And operation was estimated for DKK 0.7 per m^3 treated water using 75 mg/L PAA with 2 min contact time to remove 99.9% selected ARB when treatment was done in a tank (Table 8). This estimate was done excluding manpower and including chemical cost, maintenance, electricity cost.

[1] Retention time is defined as the time between chemical dosing and discharge into another sewage system with additional wastewater streams

5. Conclusion

The removal of antibiotic-resistant bacteria (ARB) from raw hospital wastewater using peracetic acid was evaluated. Disinfection treatment in direct unbranched sewers as it is used in the superhospitals was done by using different PAA dose and long contact time ranging from 20 to 60 min. Additionally, pilot experiments were conducted using high PAA dose and short contact time ranging from 1-5 min when ARB from hospital wastewater needs to be reduced before mixing to the municipal sewer network. The degradation of PAA was fast, resulting in no residual effect on the process in wastewater treatment plant. Removal of ciprofloxacin-resistant bacteria increased by increasing the contact time and the PAA concentration. Removal of ciprofloxacin-resistant bacteria from pilot experiment was 99.9% when 50 mg/L PAA was used with 20 min contact time. Removal of ciprofloxacin-resistant bacteria increased by increasing contact time. Using 75 mg/L PAA and 2 min contact time, ciprofloxacin- resistant bacteria were reduced with 99.9 %. PAA showed potency to be used to remove ARB and partial removal of pharmaceuticals from raw hospital wastewater.

The typical levels of ARB in wastewater with and without hospitals connections were determined to find the necessary level of disinfection to reduce hospital wastewater’s ARB content to the background level for municipal sewage. Different sensors were tested for online control of PAA dosing for continuous disinfection. The disinfection method used in this project showed potency to minimize the risk of antibiotic resistant bacteria to the sewage workers when new centralized super hospital are constructed in Denmark utilizing the unbranched direct connection of wastewater from hospital to the WWTP or reducing the number of antibiotic resistant bacteria in wastewater pipe from hospital before mixing it to the existing sewer network.

6. References

Biggerstaff, J.P., Le Puil, M., Weidow, B.L., Prater, J., Glass, K., Radosevich, M., White, D.C., 2006. New methodology for viability testing in environmental samples. Mol. Cell. Probes 20, 141–146. https://doi.org/10.1016/j.mcp.2005.11.006

Block, S.S., 1991. Disinfection, Sterilization, and Preservation, 4th ed. ed. Philadelphia (Pa.): Lea and Febiger.

Boulos, L., Prévost, M., Barbeau, B., Coallier, J., Desjardins, R., 1999. LIVE/DEAD(®) BacLight(TM): Application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J. Microbiol. Methods 37, 77–86. https://doi.org/10.1016/S0167-7012(99)00048-2

Chhetri, R.K., Flagstad, R., Munch, E.S., Hørning, C., Berner, J., Kolte-Olsen, A., Thornberg, D., Andersen, H.R., 2015. Full scale evaluation of combined sewer overflows disinfection using performic acid in a sea-outfall pipe. Chem. Eng. J. 270, 133–139. https://doi.org/10.1016/j.cej.2015.01.136

Chhetri, R.K., Kaarsholm, K.M.S., Andersen, H.R., 2020. Colorimetric Quantification Methods for Peracetic Acid together with Hydrogen Peroxide for Water Disinfection Process Control. Int. J. Environ. Res. Public Health 17, 4656. https://doi.org/10.3390/ijerph17134656

Chhetri, R.K., Thornberg, D., Berner, J., Gramstad, R., Ojstedt, U., Sharma, A.K., Andersen, H.R., 2014. Chemical disinfection of combined sewer overflow waters using performic acid or peracetic acids. Sci. Total Environ. 490, 1065–1072. https://doi.org/10.1016/j.scitotenv.2014.05.079

Chhetri, R.K.R.K., Bonnerup, A., Andersen, H.R.H.R., 2016. Combined Sewer Overflow pretreatment with chemical coagulation and a particle settler for improved peracetic acid disinfection. J. Ind. Eng. Chem. 37, 372–379. https://doi.org/10.1016/j.jiec.2016.03.049

Domínguez Henao, L., Cascio, M., Turolla, A., Antonelli, M., 2018. Effect of suspended solids on peracetic acid decay and bacterial inactivation kinetics: Experimental assessment and definition of predictive models. Sci. Total Environ. 643, 936–945. https://doi.org/10.1016/j.scitotenv.2018.06.219

Du, P., Liu, W., Cao, H., Zhao, H., Huang, C.H., 2018. Oxidation of amino acids by peracetic acid: Reaction kinetics, pathways and theoretical calculations. Water Res. X 1, 100002. https://doi.org/10.1016/j.wroa.2018.09.002

FRODO, 2014. Forbedret rensning og desinfektion af overløbsvand [WWW Document]. URL http://www.udviklingssamarbejdet.dk/en/projekter/frodo (accessed 1.2.14).

Gehr, R., Chen, D., Moreau, M., 2009. Performic acid (PFA): Tests on an advanced primary effluent show promising disinfection performance. Water Sci. Technol. 59, 89–96. https://doi.org/10.2166/wst.2009.761

Liberti, L., Notarnicola, M., 1999. Advanced treatment and disinfection for municipal wastewater reuse in agriculture. Water Sci. Technol. 40, 235–245. https://doi.org/10.1016/S0273-1223(99)00505-3

Luukkonen, T., Pehkonen, S.O., 2017. Peracids in water treatment: A critical review. Crit. Rev. Environ. Sci. Technol. 47, 1–39. https://doi.org/10.1080/10643389.2016.1272343

Nielsen, U., Klausen, M.M., Pedersen, B.M., Lentz, J.W., la Cour Jansen, J., 2011. Hospitalsspildevand - BAT og udvikling af renseteknologier.

Santoro, D., Gehr, R., Bartrand, T.A., Liberti, L., Notarnicola, M., Dell'Erba, A., Falsanisi, D., Haas, C.N., 2007. Wastewater Disinfection by Peracetic Acid: Assessment of Models for Tracking Residual Measurements and Inactivation. Water Environ. Res. 79, 775–787. https://doi.org/10.2175/106143007X156817

Sarathy, S., Murray, A., Neofotistos, P., Walton, J., Lawryshyn, Y., Santoro, D., 2016. Wastewater disinfection using peracetic acid: Innovative process design, optimization, and control. WEFTEC 2016 - 89th Water Environ. Fed. Annu. Tech. Exhib. Conf. 4, 4722–4731. https://doi.org/10.2175/193864716819707472

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K., Ren, Q., 2015. Critical aspects of using

bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. BMC Microbiol. 15, 1–9. https://doi.org/10.1186/s12866-015-0376-x
Tang, K., Rosborg, P., Rasmussen, E.S., Hambly, A., Madsen, M., Jensen, N.M., Hansen, A.A., Sund, C., Andersen, H.G., Torresi, E., Kragelund, C., Andersen, H.R., 2021. Impact of intermittent feeding on polishing of micropollutants by moving bed biofilm reactors (MBBR). J. Hazard. Mater. 403, 123536. https://doi.org/10.1016/j.jhazmat.2020.123536
ThermoFisherScientific, 2004. LIVE/DEAD™ BacLight™ Bacterial Viability Kit, for microscopy.
Wade, W., 2002. Unculturable bacteria - The uncharacterized organisms that cause oral infections. J. R. Soc. Med. 95, 81–83. https://doi.org/10.1258/jrsm.95.2.81
Watson, K., Shaw, G., Leusch, F.D.L., Knight, N.L., 2012. Chlorine disinfection by-products in wastewater effluent: Bioassay-based assessment of toxicological impact. Water Res. 46, 6069–6083. https://doi.org/10.1016/j.watres.2012.08.026

Appendix

Appendix 1-Scientific article I

Disinfection of hospital-derived antibiotic-resistant bacteria at source using peracetic acid.
Published in the *Journal of Water Process Engineering*. DOI:
https://doi.org/10.1016/j.jwpe.2021.102507

Journal of Water Process Engineering 45 (2022) 102507

Contents lists available at ScienceDirect

Journal of Water Process Engineering

journal homepage: www.elsevier.com/locate/jwpe

Disinfection of hospital-derived antibiotic-resistant bacteria at source using peracetic acid

Ravi Kumar Chhetri [a,*], Diego Francisco Sanchez [a], Sabine Lindholst [b], Alexander Valentin Hansen [c], Jesper Sanderbo [c], Birgitte Krogh Løppenthien [c], Thomas Bilkær [c], Henning Gade [d], Jørgen Skaarup [d], Caroline Kragelund [b], Henrik Rasmus Andersen [a]

[a] *Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet, Building 115, 2800 Kgs. Lyngby, Denmark*
[b] *Department of Chemistry and Biotechnology, Danish Technological Institute, Kongsvang Alle 29, DK-8000 Aarhus C, Denmark*
[c] *Alumichem (Norlex Systems A/S), Bistrupvej 172, 3460 Birkerød, Denmark*
[d] *Hillerød Forsyning, Solrødgårds Alle 6, 3400 Hillerød, Denmark*

ARTICLE INFO

Keywords:
Antibiotic-resistant bacteria
Disinfection
Hospital wastewater
Peracetic acid
Wastewater treatment plant

ABSTRACT

This work evaluated the removal of hospital-derived antibiotic-resistant bacteria (ARB) from source, using peracetic acid (PAA). Four pilot experiments, two using raw hospital wastewater and two using municipal wastewater, were conducted using three PAA concentrations at different contact times. These contact times were selected in order to mimic the retention time of wastewater in a pipe running from a hospital to a wastewater treatment plant (WWTP) with high and low flows. In order to confirm the PAA dose delivered in the pilot experiments, comparable PAA treatments were made in parallel in batch experiments on the untreated wastewater. PAA degradation was swift in the pilot and batch experiments, and no adverse effects were envisioned for the WWTP from the residual PAA. The numbers of multi-resistant and ciprofloxacin-resistant bacteria were higher in the hospital wastewater compared to the municipal WW, as the hospital was the point source of ARB. The estimated cost of 0.06 € is needed to remove 99.9% ciprofloxacin-resistant bacteria using 50 mg/L PAA and 20 min contact time. Similarity on the removal of ciprofloxacin-resistant bacteria from the pilot experiment and batch experiments performed in the laboratory was observed, and it increased based on increasing contact time and PAA concentration. The method appears to be an ideal technology to minimise the risk ARB poses to sewage workers when new centralised super hospitals are being constructed in Denmark and utilise an unbranched direct wastewater pipe from the hospital to the WWTP.

1. Introduction

The spread of antibiotic-resistant bacteria (ARB) in the environment is increasing, due to the excessive use of antibiotics in humans, animals and plants [1,2], which in turn has caused a considerable reduction in the effectiveness of antibiotic-based therapeutic treatment [3]. Among many environmental conduits known to spread ARB, wastewater has been reported as an important vehicle [4]. One study reported that approximately 50% to90% of antibiotics administered to humans or animals are discharged through urine and excrement [5,6].

Multi-resistant bacteria are non-susceptible to at least one antibiotic in three or more families, and they can be Gram-positive as well as Gram-negative bacteria [7]. Resistance to multiple types of antibiotics is now commonplace, and it is increasing for some of the major bacterial pathogens, thus limiting medical treatment alternatives [8,9]. Multi-resistant bacteria are known for their high capacity to spread epidemics, and hospitals are seen as major point sources.

Denmark's national hospital service is currently being centralised into super hospitals servicing entire regions and replacing many local hospitals. This centralisation is leading to higher ARB concentrations in hospital wastewater and fewer sewers and treatment plants, which might pose increased risks to workers servicing these wastewater treatment plants. Furthermore, increased concentrations of ARB can be expected in effluent and any overflow that occur during heavy precipitation in combined sewers servicing these super hospitals. To mitigate this wastewater issue, several new super hospitals have constructed separate wastewater pipes linking them with wastewater treatment plants, in order to limit the spread of pathogens and resistant bacteria to

* Corresponding author.
E-mail address: rakc@env.dtu.dk (R.K. Chhetri).

https://doi.org/10.1016/j.jwpe.2021.102507
Received 6 August 2021; Received in revised form 6 December 2021; Accepted 6 December 2021
Available online 29 December 2021

Appendix 2-*Draft* for Scientific article II

Disinfection of antibiotic-resistant bacteria in municipal and hospital raw wastewater using peracetic acid

Ravi Kumar Chhetri, *[a] Diego Francisco Sanchez[a], Lina Kristine[a], Sabine Lindholst[b], Alexander Valentin Hansen[c], Jesper Sanderbo[c], Birgitte Krogh Løppenthien[c], Thomas Eilkær[c], Natascha Kock Pedersen [d], Jan Jørgensen [d], Caroline Kragelund[b], Henrik Rasmus Andersen[a]

Abstract This work evaluated the removal of multi-resistant and ciprofloxacin-resistant bacteria from municipal and hospital raw wastewater, using peracetic acid (PAA). A laboratory design experiments were conducted to determine the PAA dose for pilot-scale experiments. Using PAA concentrations (25-150 mg/L PAA) at different contact times two pilot scale experiments with raw municipal wastewater and two experiments with raw hospital wastewater were conducted. These contact times were selected in order to mimic the retention time of wastewater in a pipe running from a hospital to a municipal wastewater pipe with high and low flows. For confirmation of PAA dose in the pilot experiments, batch experiments were conducted in parrallel under the same experimental and wastewater conditions. PAA degradation was swift in the pilot and batch experiments, and no adverse effects were envisioned for the WWTP from the residual PAA. The numbers of multi-resistant and ciprofloxacin-resistant bacteria were higher in the raw hospital wastewater compared to the raw municipal wastewater, around per one CFU unit. Similarity on the removal of ciprofloxacin-resistant bacteria from the pilot experiment and batch experiments performed in the laboratory was observed. The removal of resistant bacteria increased by increasing contact time and PAA concentration. Peracetic acid is shown to be an effective desinfectant able to minimize risk of multi-resistant and ciprofloxacin-resistant bacteria in both municipal and hospital raw wastewater.

1. INTRODUCTION

Antibacterial resistance is a huge threat to global health recognised by the World Health Organization. Excessive use, or use without prescription foster antibiotic-resistant bacteria (ARB) to develop in humans, animals, plants and the environment [1]. Furthermore, treatment of common infections and injuries with the first-line antibiotics becomes ineffective while novel treatment alternatives are under development [2].

It is reported that 10-90% of active compound of ingested antibiotic is excreted and urinated with a variation per specific compound[2,3].
Studies show that pharmaceuticals, ARB and antibiotic-resistant genes (ARG) are able to pass through conventional municipal WWTP and reach surface water bodies [4,5]. Antibiotic residues, together with ARB and ARG eventually are exposed to favourable conditions to strengthen antimicrobial resistance due to replication or horizontal genes transfer in wastewater treatment plants (WWTP) [6,7].

Hospitals play an important role in a concentrated wastewater stream filled with pharmaceuticals, ARB and ARG.
The numbers of β-lactamase (ESBL)-producing *E. coli* and multi-resistant *P. aeruginosa* were more in hospital wastewater compared to municipal WWTP [8]. In Denmark due to organizational optimisation of health service, small hospitals are being centralised into big hospitals, therefore contribution from such big hospitals may play a bigger role to the local WWTP. Since municipal WWTP provides the final treatment step and direct discharge to the environment, it is vital to prevent the spreading of ARB and implement an appropriate treatment step in WWTP[6].

Multi-resistant bacteria, non-susceptible to at least one antibiotic, are known for their high capacity to spread epidemics, and hospitals are seen as major point sources[9]. Resistance to multiple types of antibiotics is now commonplace, and it is increasing for some of the major bacterial pathogens, thus limiting medical treatment alternatives [6,10].

Disinfection is the most common technology to reduce the number of bacteria and viruses from wastewater. Chlorine based disinfectant have been used for tertiary treatment of wastewater due to the economic cost and bactericidal capacity. However, chlorine is not suitable for disinfection of raw wastewater as it reacts with organic matters forming acutely toxic, mutagenic and carcinogenic undesired disinfection by-products [11–15]. Chlorination of secondary effluent in full-scale municipal WWTP in China showed that increase in the abundance both ARB and ARG[16]. It was also found that chlorination was not efficient to remove 80 % of tetracycline resistance genes[15] and showed an abundance of different ARGs after chlorination of wastewater effluent from three different hospitals[17]. UV disinfection is widespread technology however, it is not suitable for disinfection due to the higher suspended solids and turbidity in the raw wastewater.

Peracetic acid (PAA) is strong, efficient disinfectant with a wide spectrum of antimicrobial activity and it is widely used to treat wastewater, and combined sewer overflows [18–20]. PAA oxidises sulphur and sulfhydryl bonds into enzymes and proteins [21]. The disruption of the chemiosmotic conditions of the microbial cell by breaking the cell's walls and altering its transport systems is considered the primary ARB inactivation mechanism. A secondary inactivation mechanism can be attributed to the formation of hydroxyl radicals [22]. Residues remaining after PAA treatment are acetic acid, hydrogen peroxide and water. Acetic acid is further biodegraded into carbon dioxide, whilst hydrogen peroxide degrades to oxygen and water – neither is considered toxic to aquatic life. PAA was used to disinfect antibiotic resistant *E. coli* from secondary effluent [23]. In our previous study, we presented the effect of long contact on PAA to disinfect the ARB from raw hospital wastewater. However, to the best of authors' knowledge, the present study is one of the first to report on the disinfection effect of PAA on ARB and antibiotics in the raw wastewater from hospital and WWTP with short contact time.

The aim of this study were to: (i) reduce the number of selected ARB before hospital wastewater is mixed in the municipal sewer network in the pilot scale experiment, (ii) compare the disinfection effect of PAA on ARB in the hospital and municipal wastewater.
Bacteria resistant to ciprofloxacin were used as indicator bacteria to study the disinfection process, since they are abundant in wastewater both connected and not connected with hospital wastewater, thus leading to measurable concentrations that help monitor the effects of disinfection. Moreover, multi-drug resistant bacteria, resistant to four antibiotics (ciprofloxacin, gentamicin, cefuroxime and sulfamethoxazole) were enumerated from raw wastewater to get the information of ARB fraction in the hospital and municipal wastewater.

2. Materials and Methods

2.1 Chemicals

Sulphuric acid (95% (w/v), ciprofloxacin, cefuroxime sodium salt, gentamicin sulphate salt, sulfamethoxazole, ABTS (2,2-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt), sodium thiosulphate, potassium titanium oxide oxalate and catalase from bovine liver (2000—5000 units/mg protein) were all reagent grade and purchased from Sigma-Aldrich (Brøndby, Denmark).
PAA solution containing 15% w/w of commercial-grade disinfectant was purchased from Novadan (Kolding, Denmark). Using this solution, a working solution of 10 g/L of PAA was prepared.

2.2 Peracetic acid and hydrogen peroxide quantification

PAA and hydrogen peroxide (H_2O_2) concentration in the samples were quantified using the colorimetric method adopted from Chhetri et al., [24] . Actual PAA and hydrogen peroxide concentrations in the stock solution were quantified by two-step iodometric titration based on the procedures described by [25].

2.3 Microbiological analysis

Raw and PAA-disinfected wastewater samples were collected from the municipal wastewater treatment plant (SK forsyning- wastewater utility) and Slagelse Hospital, located in the southern part of Copenhagen, Denmark. Samples were processed within 2 h after collection. PAA residues were neutralised by adding 100 mg/L sodium thiosulfate, followed by adding 50 mg /L catalase to destroy any hydrogen peroxide.

Total heterotrophic bacteria, ciprofloxacin-resistant bacteria and multi-resistant bacteria were enumerated on CompactDry plates (Nissui Pharmaceutical, Tokyo, Japan), using the spread plate technique. Compact Dry TC is a medium for total viable bacterial count, which contains nutrient agar (0.5% peptone, 0.25% yeast extract, 0.1% glucose, 1.5% agar). For enumeration of total heterotrophic bacteria, serial dilutions of each sample were prepared in sterilized sterile phosphate buffer saline (PBS), and 1mL of diluted samples were added onto the CompactDry plate in duplicate and incubated for 24 h at 37 ± 1 °C. For enumeration of ciprofloxacin resistant bacteria, serial dilutions of each sample were prepared in a solution with 4 mg/L ciprofloxacin mixed with sterile PBS, and 1mL of diluted samples were added onto the CompactDry plate in duplicate and incubated for 24 h at 37 ± 1 °C. Bacteria counts were expressed as colony-forming units per mL (CFU/mL), with the limit of quantification (LOQ) of ciprofloxacin-resistant bacteria equal to 10 CFU/ mL.

Multidrug-resistant bacteria (multi-resistant bacteria) were enumerated using the spread plate technique. Serial dilutions of each sample were prepared in a solution mixed with 4 mg/L ciprofloxacin, 15 mg/L gentamicin, 20 mg/L cefuroxime and 0.6 mg/L sulfamethoxazole with sterile PBS. Diluted samples were added onto the CompactDry plate in duplicate and incubated for 24 h at 37 ± 1 °C. Antibiotic concentrations were chosen based on a study conducted by Nielsen et al. [26] for Danish Environmental Protection Agency where authors already conducted validation and quality control of antimicrobial susceptibility of antibiotics therefore, it was not repeated and presented in this study.

2.4 Experimental design

A laboratory design experiment were conducted in 100 mL beaker to determine the PAA dose and contact time for pilot scale experiment using raw municipal and hospital wastewater. To study concentration profiles and removal efficiency to determine the dose of PAA, experiment were carried out by spiking 50, 75 and 100 mg/L PAA in the raw municipal wastewater in separate bottles using 1, 2, 5 and 10 min contact time. A laboratory design experiments were repeated on two different days one week apart, to test wastewater quality variability. Similar experiments were repeated using raw hospital wastewater using 75, 100 and 150 mg/L PAA that resulted in a reasonable disinfection level using 1, 2, 5 and 10 min contact time.

2.4.1 Pilot and batch experiments

Pilot experiments were conducted in a 20-foot container containing a long pipe folded into the container, thereby reflecting the retention time of wastewater running from a hospital to a wastewater treatment plant. The pipe served as a reaction tank facilitating plug flow and provided different sampling points. The system was equipped with a submersible centrifugal feed pump and a peristaltic dosing pump, to aid the continuous addition of disinfectant. A static mixer downstream of the dosing point ensured that the disinfectant was thoroughly mixed with the wastewater. PAA concentrations and contact times were adjusted by adjusting the flows of the dosing pump and the feed pump. Two pilot experiments using municipal wastewater were conducted by using 25, 50 and 75 mg/L PAA with 4 contact times. Two pilot experiments using

hospital wastewater were conducted by using 50, 75 and 150 mg/L PAA with 4 contact times. Pilot experiments with untreated municipal and hospital wastewater were carried out on two different days one month apart.
While conducting the pilot experiments, in-situ disinfection, on municipal and hospital raw wastewater, the batch experiment was performed in parallel, in order to confirm the PAA dose delivered in the pilot experiment. Moreover, to ensure the same conditions for both tests and to avoid changes in wastewater, such as hydrolysis over a prolonged storage time. Samples were collected for PAA and H_2O_2 quantification and microbiological analysis at different contact times. Contact times were selected based on PAA degradation on a laboratory design experiments. For batch experiments contact times range from 1-20 minutes whereas for pilot experiments between 1-10 minutes.

After different contact times were applied for the pilot and batch experiments, a fraction of each sample was collected to enumerate ciprofloxacin-resistant bacteria. In parallel, PAA concentrations were quantified until 10 min in the remaining sample for pilot experiments whilst in batch experiments, PAA quantification was done until complete degradation was observed. Furthermore, raw wastewater was collected at the inlet of the pilot container prior to the beginning and the end of the experiment, to enumerate the initial number of ciprofloxacin-resistant bacteria, multi-resistant bacteria, heterotrophic bacteria, pH and COD.

3. RESULTS AND DISCUSSION

3.1 A laboratory design experiment to optimize PAA dose

In a laboratory-design experiment, PAA degradation was studied in the raw wastewater from SK Forsyning and Slagelse hospital. Moreover, removal of ciprofloxacin-resistant bacteria was studied in different contact time using different PAA dosages (Figure 1).

In both laboratory-design experiments, half of the PAA concentration was degraded between 1 and 2 minutes (Figure 1A & C). In the experiment with municipal wastewater, complete PAA degradation was observed after 10 minutes when 50 75 and 100 mg/L PAA was used. In the experiment with raw hospital wastewater, complete PAA degradation was observed after 10 minutes when 75 mg/L PAA was used and after 20 minutes contact times when 100 and 150 mg/L PAA was used. The differences for both PAA concentration profiles in the laboratory design experiments can be explained by different physico-chemical factors affecting the decay of PAA. These factors tend to be related to the presence of suspended solids (SS), pH, temperature, COD, salinity and water hardness[27,28]. Furthermore, a huge initial consumption of PAA was observed in all experiments using wastewater from different sources.
Ciprofloxacin-resistant bacteria were removed below the detection limit (<10 CFU/mL) after 1 minutes of exposure to 50, 75 and 100 mg/L PAA (Figure 1B & D). The laboratory design experiments carried out on different days achieved similar removal results when inactivating the ciprofloxacin-resistant bacteria. Ciprofloxacin-resistant bacteria were more abundant in hospital wastewater than municipal wastewater as the hospital is the point source for ARB.

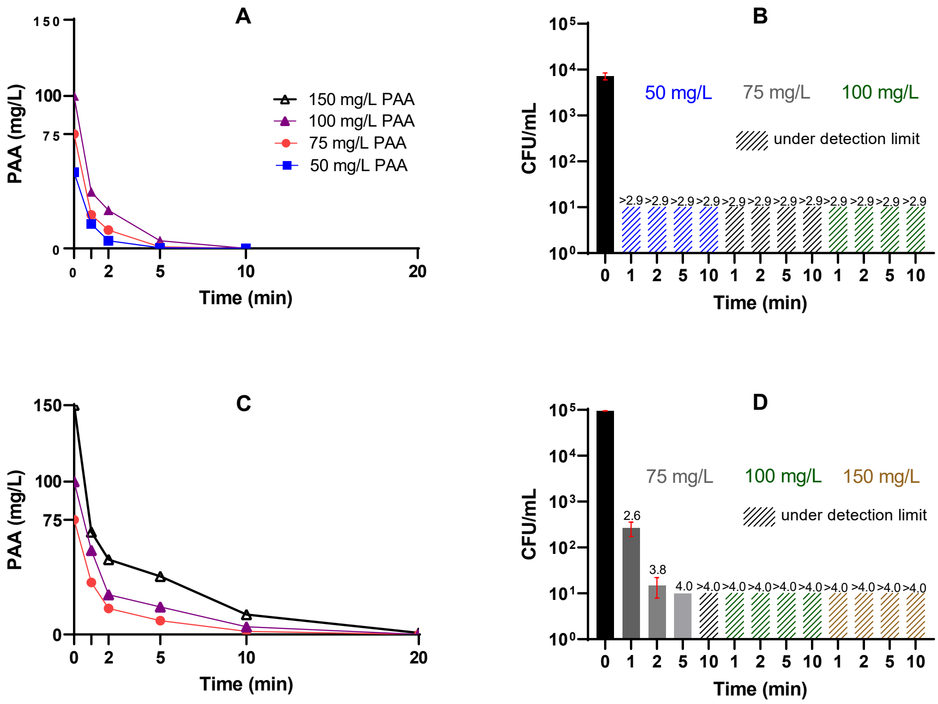


Figure 1: Concentration profiles of PAA in raw municipal wastewater from SK forsyning in a laboratory design experiment (A), the removal of ciprofloxacin-resistant bacteria from the raw municipal wastewater from SK forsyning (B), concentration profiles of PAA in raw wastewater from Slagelse hospital in a laboratory design experiment (C), the removal of ciprofloxacin-resistant bacteria in raw wastewater from Slagelse hospital (D).

3.2 Pilot scale disinfection

COD of raw municipal wastewater from SK Forsyning used for two pilot experiments were 2010 and 1164 mg/L and pH was 7.5 in both samples. COD of raw wastewater from Slagelse hospital used for two pilot experiments were 598 and 473 mg/L and pH was 7.5 and 8.0, respectively.
In the pilot experiment using municipal wastewater, three PAA dosages (25, 50 and 75 mg/L PAA) were used in the pilot and batch experiments, based on COD measurements and the fact that municipal wastewater contains less ciprofloxacin-resistant bacteria compared to hospital wastewater thus a lower PAA dose would be needed for ciprofloxacin-resistant bacteria inactivation. PAA and hydrogen peroxide concentrations were measured until 10 min. Rapid degradation of PAA was observed as COD in the wastewater was 2010 mg/L (Figure 2A). Complete degradation of PAA was observed after 20 min in the batch experiment (Figure 2B and S1).

Results from the pilot experiment using municipal wastewater demonstrated the progressive removal of ciprofloxacin-resistant bacteria. With a PAA dose of 25 mg/L with 1 min contact time, the removal of ciprofloxacin-resistant bacteria was 0.2 log, and the removal was increased to 1.1 log with 10 min contact time. Using 50 mg/L PAA, the removal of ciprofloxacin-resistant bacteria was 1.1 and the removal was increased to 2.1 long with 10 min contact time. The removal of ciprofloxacin-resistant bacteria was 1.6 log with 75 mg/L PAA with 1 min contact time and with 10 min contact time ciprofloxacin-resistant bacteria was reduced to under the detection (Figure 2C).

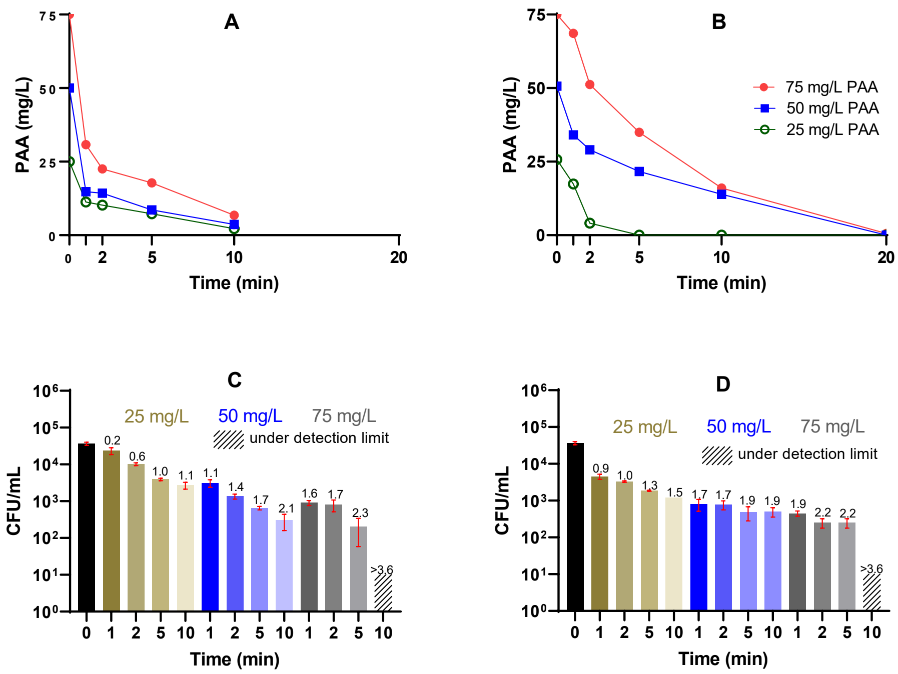


Figure 2: Concentration profiles of PAA in raw municipal wastewater from SK forsyning in the pilot-scale experiment (A), and from the batch experiment (B). the removal of ciprofloxacin-resistant bacteria in the raw municipal wastewater from SK forsyning after disinfection using different PAA concentrations in the pilot-scale experiment (C) and from the batch experiment (D). The logarithm removal of ciprofloxacin-resistant bacteria is indicated above each bar in the graph. The range of the duplicate analysis is indicated by T-bars.

Similarity on the removal of ciprofloxacin-resistant bacteria were observed in the batch experiment, in that after 10 min exposure to 75 mg/L PAA, ciprofloxacin-resistant bacteria were under the detection limit (Figure 2D).

The pilot experiment using wastewater from Slagelse Hospital was performed using 50, 75 and 150 mg/L of PAA. Concentrations of PAA and hydrogen peroxide were measured after 1, 2, 5 and 10 min. Fast PAA degradation was observed at a concentration less than 0.5 mg/L after 10 min. However, around 40% of the initial hydrogen peroxide remained when measured after 20 min contact time (Figure 3A, S2 &S3). A similar degradation of PAA and hydrogen peroxide was observed in the batch experiment (Figure 3B, S2& S3). The faster decay of PAA in the raw hospital wastewater was observed, which might be due to higher organic content than found in the municipal wastewater.

Results from the pilot experiment using wastewater from Slagelse hospital showed the progressive removal of ciprofloxacin-resistant bacteria. The removal of ciprofloxacin-resistant bacteria increased in line with an increase in contact time. The removal rate was 0.2 log when 50 mg/L of PAA was used and with 1 min of contact time. This increased to 2.7 log when 50 mg/L of PAA was used along with a 10 min contact time (Figure 3C). The number of ciprofloxacin-resistant bacteria was under detection limit, and the log removal was more than 4.2 log when 75 mg/L PAA was used for disinfection of hospital wastewater with a 5 min contact time (Figure 3C). Similar and better removal of ciprofloxacin-resistant bacteria was observed in the batch experiment, likely due to the better mixing of wastewater and PAA compared to only initial mixing in the pilot experiment. Even though most of the PAA concentration was consumed after 5 minutes, the disinfection process continued, due to the remaining concentration of H_2O_2, which is known as a supplementary source for hydroxyl radicals and a weak disinfectant if dosed alone, resulting in ineffective disinfection, since the catalase enzyme will protect the microorganisms from the H_2O_2 [32]. In this case, the catalase first has to be inactivated by peracetic acid, in order to accomplish combined disinfection with H_2O_2

The removal of ciprofloxacin-resistant bacteria were similar in the batch experiment where same concentration of PAA and contact time were used. In the pilot and batch experiment, ciprofloxacin-resistant bacteria were reduced under the detection limit in all contact time used with150 mg/L PAA (Figure 3 C & D).

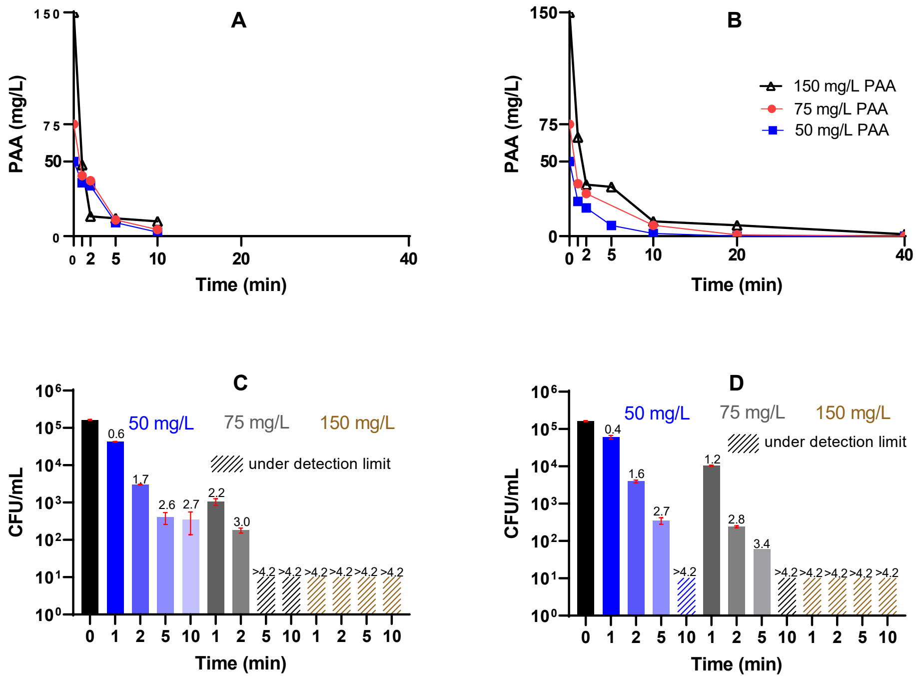


Figure 3: Concentration profiles of PAA in raw wastewater from Slagelse hospital in the pilot-scale experiment (A), and from the batch experiment (B). The removal of ciprofloxacin-resistant bacteria in the raw wastewater from Slagelse hospital after disinfection using different PAA concentrations in the pilot-scale experiment (C) and from the batch experiment (D). The logarithm removal of ciprofloxacin-resistant bacteria is indicated above each bar in the graph. The range of the duplicate analysis is indicated by T-bars.

Dominguez Henao et al.[29] revealed a correlation between soluble matter and PAA consumption and demonstrated that higher the soluble COD faster the PAA degradation with an additive effect to the influence of total suspended solids in PAA consumption. The study conducted by Koivunen & Heinonen-Tanski [30] showed the need for higher PAA doses in primary effluent than in secondary and tertiary effluent, whereas this study used higher PAA doses, as the quality of raw wastewater was different. Raw wastewater consists of high COD, TSS and turbidity, factors known to affect PAA degradation and thus disinfection.

Profaizer [34] described a survival curve consisting of three stages: an initial resistance phase, the exponential inactivation of bacteria and, finally, asymptotic inactivation. The initial resistance phase is attributed by resistance of PAA diffusion through the cellular membrane, which results in an inactivation lag [35]. This survival curve is present in PAA concentrations lower than 5 mg/L; at higher PAA concentrations, the resistance of PAA to the cell membrane is reported as being insignificant [35]. The initial resistance phase was not observed in these experiments since the lowest concentration used was 25 mg/L PAA.
Koivunen and Heinonen-Tanski [30] demonstrated that the inactivation of bacteria occurred in two phases in the disinfection process. In the first phase, a steep decline in the total number of ciprofloxacin-resistant bacteria was observed. This is a known outcome at the beginning of the disinfection process, when mainly free-swimming microbes are inactivated. The first phase was observed when 50 mg/L PAA with 1-5 min contact time removed around 90% of ciprofloxacin resistant bacteria and removal was increased to more than 99% at 10 min contact time (Figure 2C). This steep decline of ciprofloxacin-resistant bacteria was also observed in the batch experiment with municipal wastewater at 1 min reaction time with different PAA concentrations (Figure 2C & D). In the second phase, which is the most stable phase, the inactivation rate decreases (tailing of the inactivation curve) is seen in the Figures 2C at from 2 to 5 min contact time in all PAA concentrations used for disinfection. Tailing of the inactivation curve was observed in the Pilot experiment with municipal wastewater (Figure 3B) where the log removal increases only by 0.1 log between 5 and 10 minutes when 50 mg/L PAA was used for disinfection. These two bacterial inactivation phases were observed in the pilot experiments using wastewater from Slagelse Hospital and municipal wastewater (Figures 2C & 3C), as well as in the batch experiment with municipal wastewater (Figure 2D). Studies by Domínguez Henao et al., [31] & Koivunen and Heinonen-Tanski [30] suggested the possibility that bacteria might aggregate to survive PAA disinfection, or that the presence of suspended solids might protect bacteria against disinfection; however, this can be overcome with a higher PAA dose or a longer contact time. This bacteria protection by aggregating itself was not observed in the batch experiment with municipal wastewater (Figure 3D), likely due to the better and continuous mixing of the wastewater and PAA.

Kitis [19] showed that PAA can cause different levels of specific damage to biomolecules. PAA oxidises sulfhydryl, disulphide, enzymes and double bonds in proteins. PAA has the ability to cause protein denaturation and make DNA bases react adversely. Furthermore, PAA can inactivate the peroxidase enzyme. The damage PAA causes to bacteria makes them incapable of recovery, and no re-growth occurs [33]. In this study, re-growth of ciprofloxacin resistant bacteria was not studied. Furthermore, disinfected wastewater ends up in the municipal sewer network and to the WWTP where wastewater is treated further. Therefore, recovery of injured bacteria will be negligible.

4. CONCLUSION

The findings of this study demonstrate that the PAA disinfection of wastewater constitutes a promising treatment technology for the elimination of selected hospital-derived ARB. PAA can be used to treat hospital wastewater at source, thereby enabling a retention time through structures that transport the wastewater from the hospital to the municipal sewer network. Four pilot experiments, using wastewater from two different sources, were performed with different PAA concentrations and contact times. In parallel to the pilot experiments, in order to confirm the PAA dose, comparable PAA treatments were applied in batch experiments on the untreated wastewater. The degradation of PAA was fast in the pilot and batch experiments, thus ensuring no residual PAA effect on wastewater treatment plant processes. Similar results for the removal of ciprofloxacin-resistant bacteria from the pilot and the batch experiments were observed when different PAA concentrations were used.

To remove 99.9% ciprofloxacin resistant bacteria from municipal and hospital wastewater, it is estimated 50 mg/L PAA and 5 min contact time would be needed and 75 mg/L PAA would be needed if only 2 min contact time was available. In summary, disinfection of hospital wastewater at hospital using PAA seems a feasible method for reducing the numbers of ARB and thus minimising the risk spreading ARB in the municipal sewer network thereby reducing the risk of infection from ARB for plant workers in the WWTP.

5. Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. ACKNOWLEDGEMENTS

The authors wish to acknowledge the Danish Environmental Protection Agency for funding the MUDP (Miljøteknologisk udviklings- og demonstrationsprogram/ Research, development, demonstration, test and adaptation) project, grant number MST-117-00661, for the REBAHS project (Desinfektion af REsitstente BAkterier i HospitalsSpildevand/Disinfection of resistant bacteria from hospital wastewater) and supporting partners.

7. REFERENCES

1 K. Kümmerer, *Chemosphere*, 2009, **75**, 417–434.

2 WHO, *WHO 2014 AMR Rep.*, 2014, 1–8.

3 J. Oteo, G. Bou, F. Chaves and A. Oliver, *Enfermedades Infecc. y Microbiol. Clin. (English ed.)*, 2017, **35**, 667–675.

4 C. M. Manaia, A. Novo, B. Coelho and O. C. Nunes, *Water. Air. Soil Pollut.*, 2010, **208**, 335–343.

5 Y. Yoon, H. J. Chung, D. Y. Wen Di, M. C. Dodd, H. G. Hur and Y. Lee, *Water Res.*, 2017, **123**, 783–793.

6 J. Oteo, A. Ortega, R. Bartolomé, G. Bou, C. Conejo, M. Fernández-Martínez, J. J. González-López, L. Martínez-García, L. Martínez-Martínez, M. Merino, E. Miró, M. Mora, F. Navarro, A. Oliver, Á. Pascual, J. Rodríguez-Baño, G. Ruiz-Carrascoso, P. Ruiz-Garbajosa, L. Zamorano, V. Bautista, M. Pérez-Vázquez and J. Campos, *Antimicrob. Agents Chemother.*, 2015, **59**, 3406–3412.

7 T. U. Berendonk, C. M. Manaia, C. Merlin, D. Fatta-Kassinos, E. Cytryn, F. Walsh, H. Bürgmann, H. Sørum, M. Norström, M. N. Pons, N. Kreuzinger, P. Huovinen, S. Stefani, T. Schwartz, V. Kisand, F. Baquero and J. L. Martinez, *Nat. Rev. Microbiol.*, 2015, **13**, 310–317.

8 E. Marti and J. L. Balcázar, *Appl. Environ. Microbiol.*, 2013, **79**, 1743–1745.

9 L. Domínguez-Henao, A. Turolla, D. Monticelli and M. Antonelli, , DOI:10.1016/j.talanta.2018.02.078.

10 G. Cabot, A. A. Ocampo-Sosa, M. A. Domínguez, J. F. Gago, C. Juan, F. Tubau, C. Rodríguez, B. Moyà, C. Peña, L. Martínez-Martínez and A. Oliver, *Antimicrob. Agents Chemother.*, 2012, **56**, 6349–6357.

11 J. P. Pasternak, D. R. J. Moore and R. S. C. Teed, *Hum. Ecol. Risk Assess.*, 2003, **9**, 453–482.

12 G. C. White, *Handbook of chlorination and alternative disinfectants*, John Wiley & Sons, Inc., New York, 5th edn., 2010.

13 K. Watson, G. Shaw, F. D. L. Leusch and N. L. Knight, *Water Res.*, 2012, **46**, 6069–6083.

14 C. Nurizzo, M. Antonelli, M. Profaizer and L. Romele, *Desalination*, 2005, **176**, 241–253.

15 Q. Bin Yuan, M. T. Guo and J. Yang, *PLoS One*, , DOI:10.1371/journal.pone.0119403.

16 S. S. Liu, H. M. Qu, D. Yang, H. Hu, W. L. Liu, Z. G. Qiu, A. M. Hou, J. Guo, J. W. Li, Z. Q. Shen and M. Jin, *Water Res.*, 2018, **136**, 131–136.

17 S. Yao, J. Ye, Q. Yang, Y. Hu, T. Zhang, L. Jiang, S. Munezero, K. Lin and C. Cui, *Environ. Sci. Pollut. Res.*, 2021, **28**, 57321–57333.

18 M. G. C. Baldry, M. S. French and D. Slater, *Water Sci. Technol.*, 1991, **24**, 353–357.

19 M. Kitis, *Environ. Int.*, 2004, **30**, 47–55.

20 R. K. Chhetri, D. Thornberg, J. Berner, R. Gramstad, U. Ojstedt, A. K. Sharma and H. R. Andersen, *Sci. Total Environ.*, 2014, **490**, 1065–1072.

21 B. K. Biswal, R. Khairallah, K. Bibi, A. Mazza, R. Gehr, L. Masson and D. Frigon, *Appl. Environ. Microbiol.*, 2014, **80**, 3656–66.

22 C. Lubello, C. Caretti and R. Gori, *Comparison between PAA/UV and H2O2/UV disinfection for wastewater reuse*, Affiliation: Dipartimento di Ingegneria Civile, Università di Firenze, Via S. Marta 3, 50139 Firenze, Italy; Correspondence Address: Lubello, C.; Dipartimento di Ingegneria Civile, Università di Firenze, Via S. Marta 3, 50139 Firenze, Italy, 2002, vol. 2.

23 N. Campo, C. De Flora, R. Maffettone, K. Manoli, S. Sarathy, D. Santoro, R. Gonzalez-Olmos and M. Auset, *Water Res.*, , DOI:10.1016/j.watres.2019.115227.

24 R. K. Chhetri, K. M. S. Kaarsholm and H. R. Andersen, *Int. J. Environ. Res. Public Health*, 2020, **17**, 4656.

25 R. Gehr, D. Chen and M. Moreau, *Water Sci. Technol.*, 2009, **59**, 89–96.

26 U. Nielsen, M. M. Klausen, B. M. Pedersen, J. W. Lentz and J. la Cour Jansen, *Hospitalsspildevand - BAT og udvikling af renseteknologier*, 2011.

27 T. Luukkonen and S. O. Pehkonen, *Crit. Rev. Environ. Sci. Technol.*, , DOI:10.1080/10643389.2016.1272343.

28 S. Sarathy, A. Murray, P. Neofotistos, J. Walton, Y. Lawryshyn and D. Santoro, *WEFTEC 2016 - 89th Water Environ. Fed. Annu. Tech. Exhib. Conf.*, 2016, **4**, 4722–4731.

29 L. Domínguez Henao, M. Cascio, A. Turolla and M. Antonelli, *Sci. Total Environ.*, 2018, **643**, 936–945.

30 J. Koivunen and H. Heinonen-Tanski, *Water Res.*, 2005, **39**, 4445–4453.

31 L. Domínguez Henao, A. Turolla and M. Antonelli, *Chemosphere*, 2018, **213**, 25–40.

32 T. Luukkonen and S. O. Pehkonen, *Crit. Rev. Environ. Sci. Technol.*, 2017, **47**, 1–39.

33 M. Antonelli, S. Rossi, V. Mezzanotte and C. Nurizzo, *Environ. Sci. Technol.*, 2006, **40**, 4771–4775.

34 M. Profaizer, Politecnico di Milano, 1998.

35 S. Rossi, M. Antonelli, V. Mezzanotte and C. Nurizzo, *Water Environ. Res.*, 2007, **79**, 341–350.

Appendix 3-Concentration profiles and microbiology results

Concentration profiles of PAA from the pilot and batch experiment using wastewater from different source are presented below. Microbiology results for the experiment performed on wastewater from different source showing the quantification of total heterotrophic, ciprofloxacin- resistant and multi-resistant bacteria prior disinfection and microbiology results for the removal of ciprofloxacin- resistant bacteria from the pilot and batch experiments using wastewater are presented below.

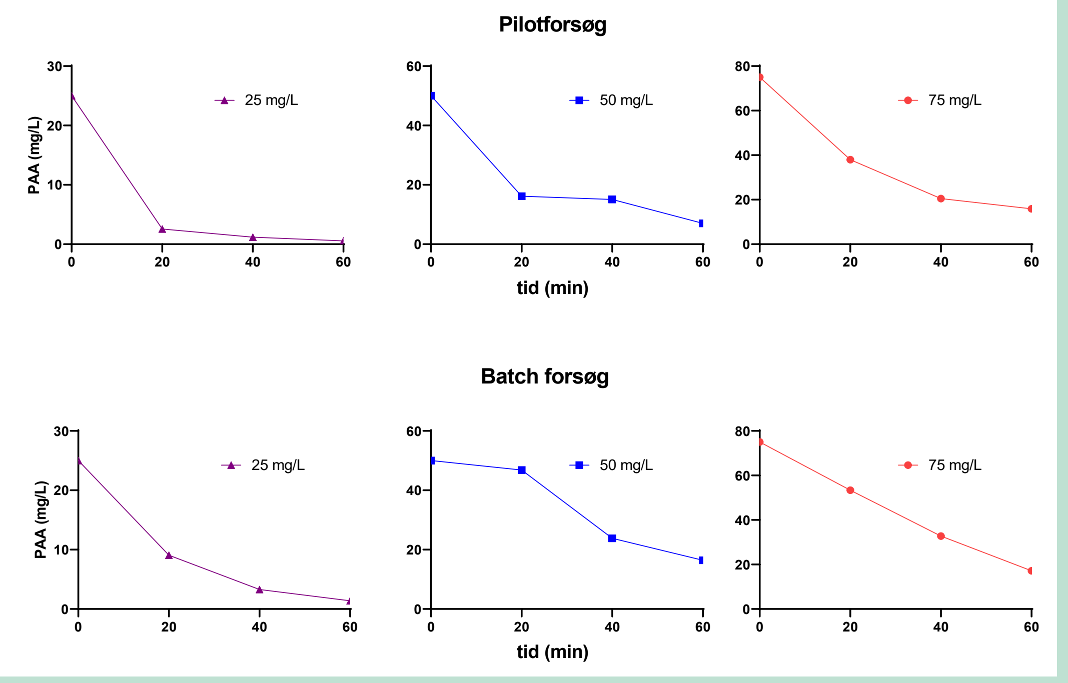


FIGURE A1. Concentration profiles of PAA from the pilot and batch experiment using wastewater from Hillerød WWTP.

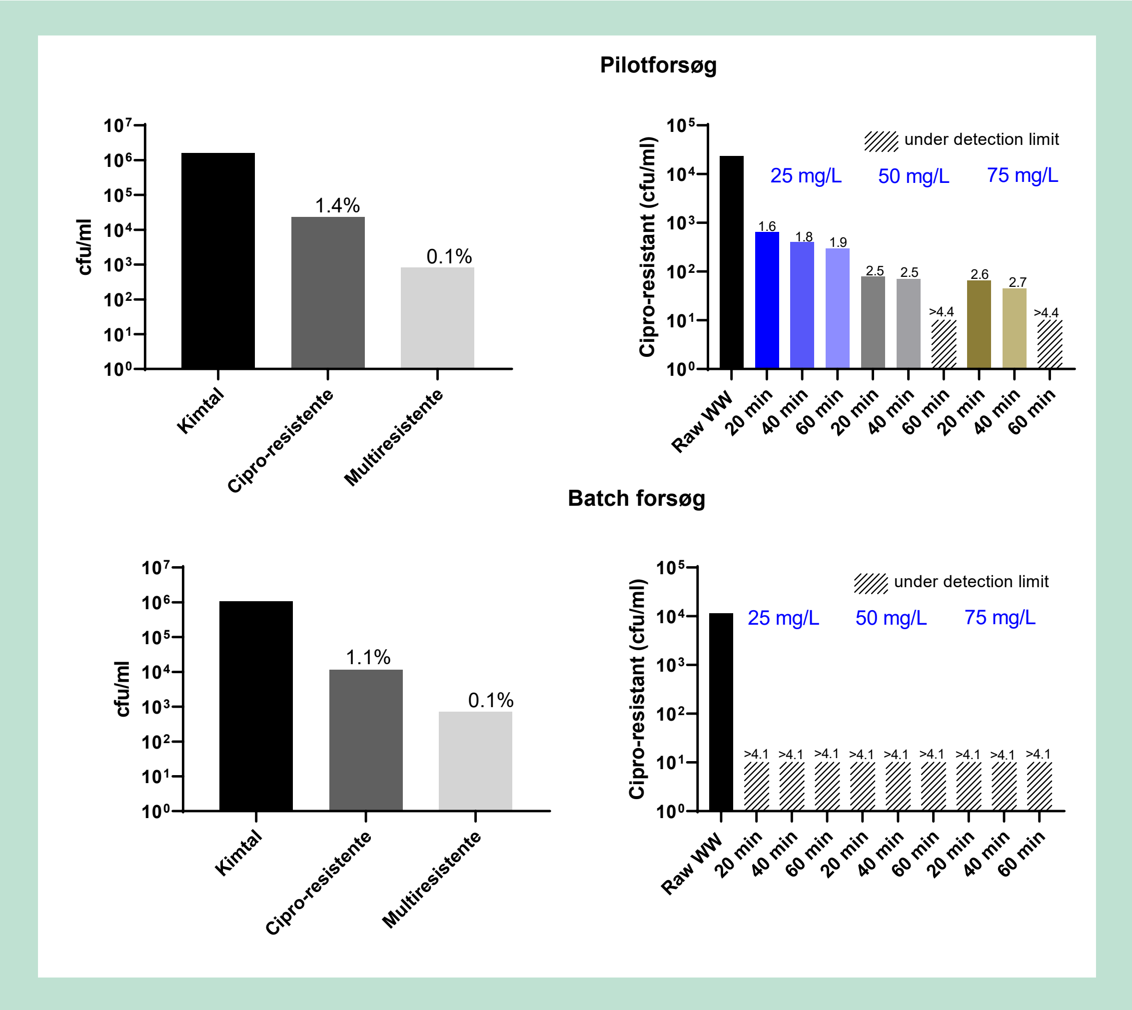


FIGURE A2. Microbiology results for the pre-experiment performed on municipal water (Hillerød WWTP) showing the quantification of total heterotrophic, ciprofloxacin-resistant and multi-resistant bacteria prior disinfection (left). Microbiology results for the removal of ciproflox-acin- resistant bacteria from the pilot and batch experiments using wastewater from Hillerød WWTP.

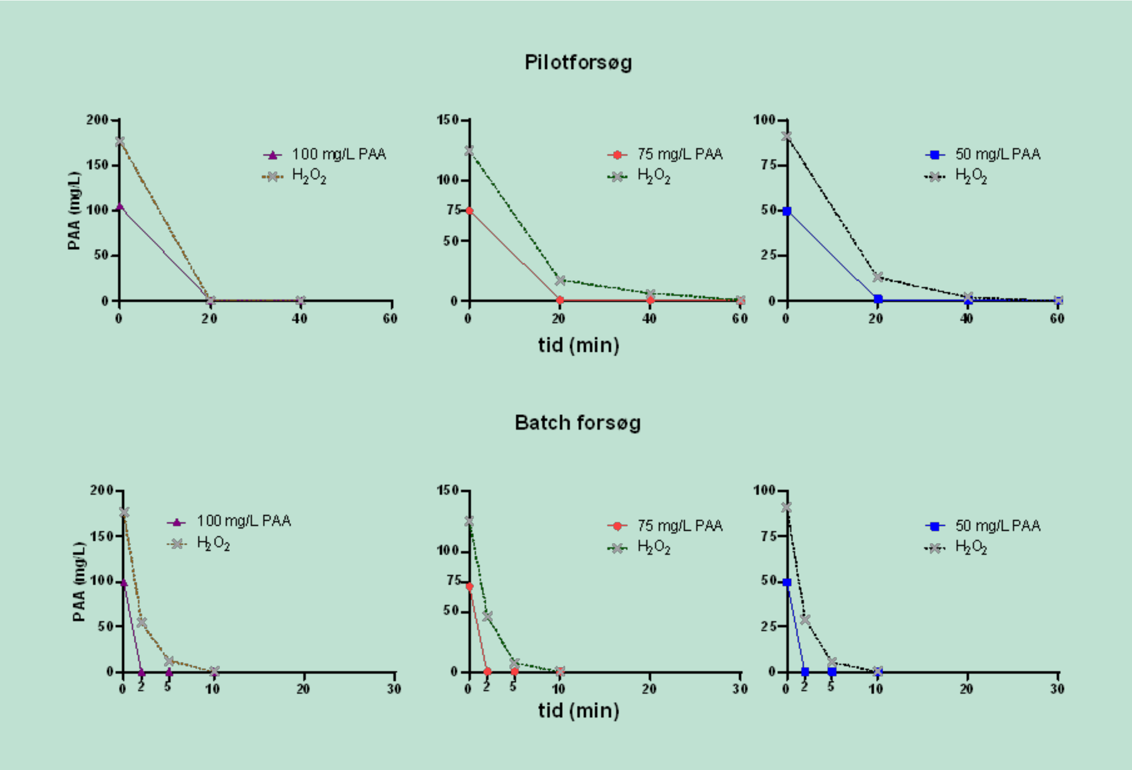


FIGURE A3. : Concentration profiles of PAA from the pilot and batch experiment using wastewater from Hillerød hospital.

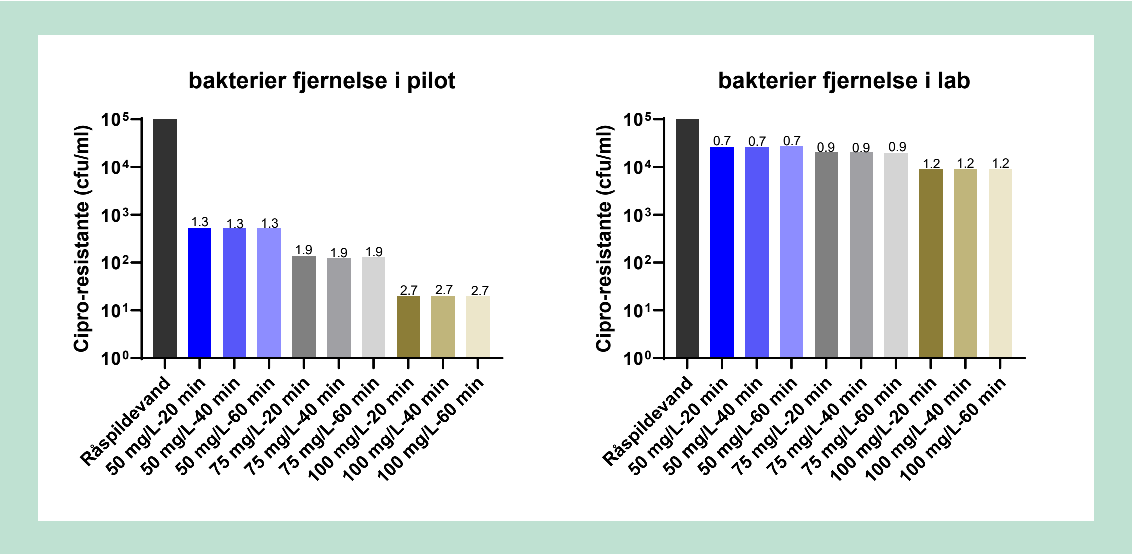


FIGURE A4. Microbiology results for the removal of ciprofloxacin-resistant bacteria from the pilot and batch experiments using wastewater from Hillerød hospital.

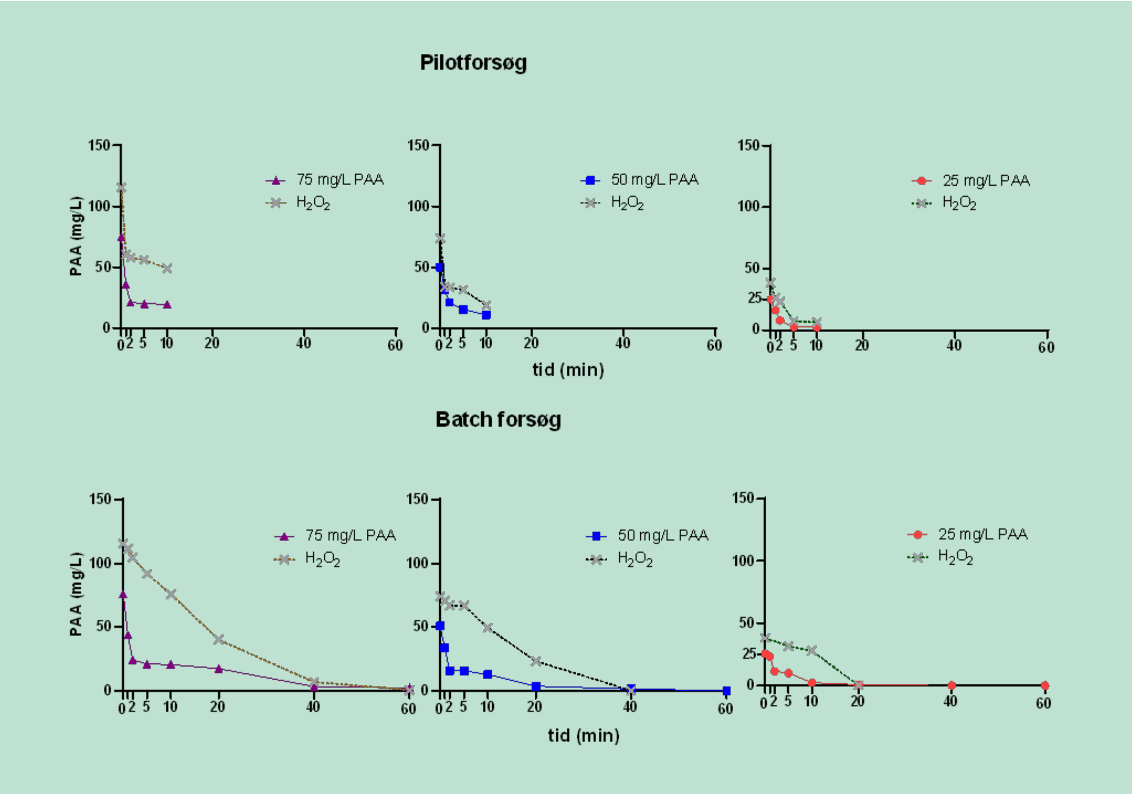


FIGURE A5. Concentration profiles of PAA from the pilot and batch experiment using wastewater from SK forsyning.

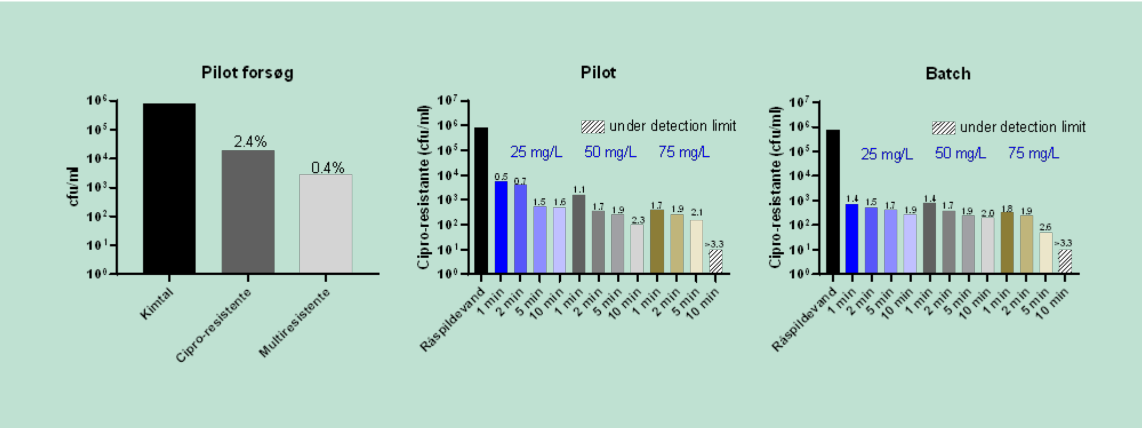


FIGURE A6. Microbiology results for the experiment performed on wastewater from SK forsyning showing the quantification of total heterotrophic, ciprofloxacin-resistant and multi-resistant bacteria prior disinfection (left). Microbiology results for the removal of ciprofloxacin- resistant bacteria from the pilot and batch experiments using wastewater from SK forsyning.

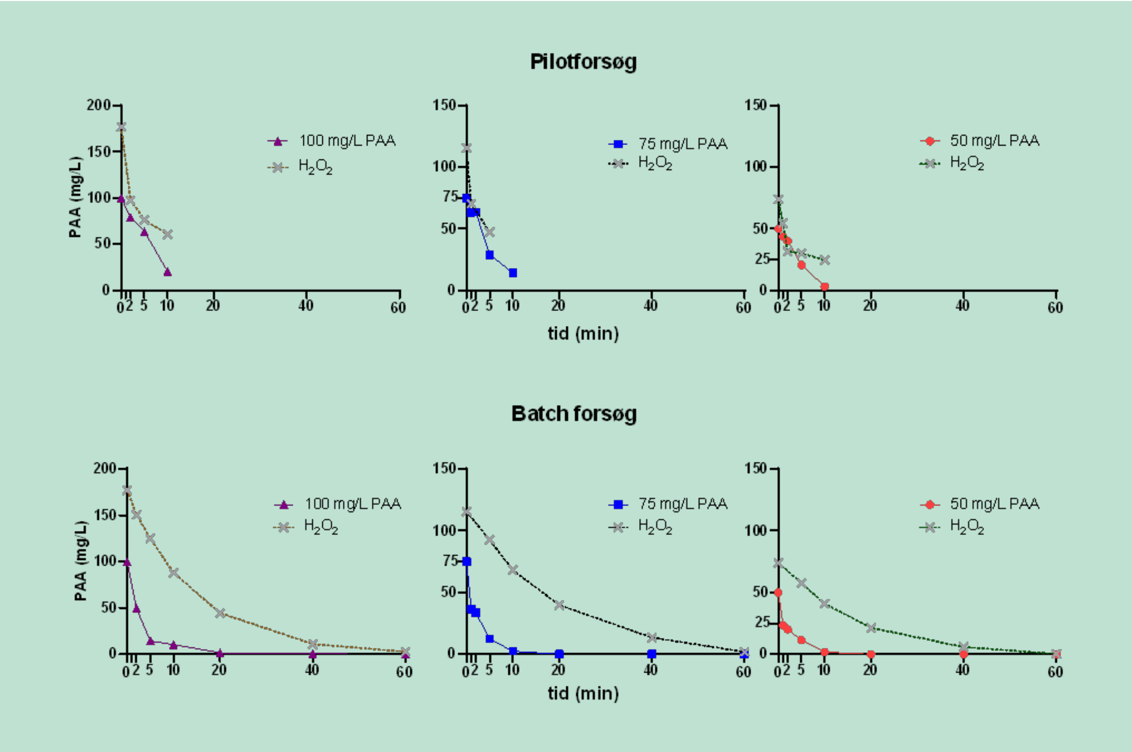


FIGURE A7. Concentration profiles of PAA from the pilot and batch experiment using wastewater from Slagelse hospital.

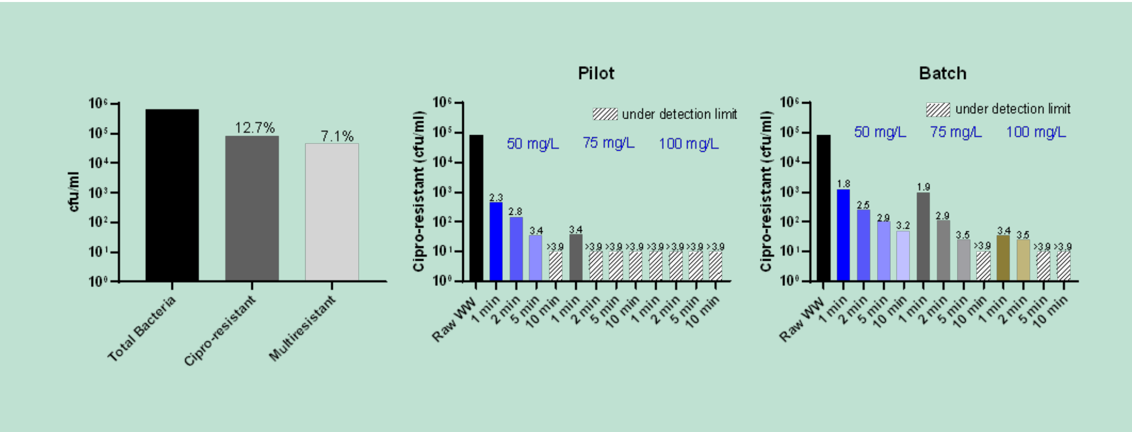


FIGURE A8. Microbiology results for the experiment performed on wastewater from Slagelse hospita showing the quantification of total heterotrophic, ciprofloxacin-resistant and multi-resistant bacteria prior disinfection (left). Microbiology results for the removal of ciprofloxacin-resistant bacteria from the pilot and batch experiments using wastewater from Slagelse hospital.

REBAHS-Disinfection of antibiotic-resistant bacteria in hospital wastewater

The spread of antibiotic resistant bacteria (ARB) in the environment is increasing due to the excessive use of antibiotics in humans, animals and plants. Hospital wastewater is the most important point source for multi-resistant, pathogenic bacteria, which are highly undesirable in the environment and at the same time pose a work environment risk at wastewater treatment plants for sewer workers. This study demonstrated that peracetic acid (PAA) disinfection of wastewater constitutes a promising treatment technology for the elimination of selected hospital-derived ARB. The method appears to be an ideal technology to minimize the risk of ARB exposure of the sewage workers when new centralized super hospitals are constructed in Denmark utilizing unbranched direct connections of wastewater from hospitals to the WWTPs. Similarly, this method is appropriate to reduce ARB from hospital wastewater with short contact time before wastewater is mixed in the existing sewer network.

Spredningen af antibiotikaresistente bakterier (ARB) i miljøet er stigende på grund af overdreven brug af antibiotika hos mennesker, dyr og planter. Hospitalsspildevand er den vigtigste punktkilde til multiresistente, patogene bakterier, som er meget uønskede i miljøet, og som samtidig udgør en arbejdsmiljørisiko for spildevandsarbejdere. Dette projekt har demonstreret, at desinfektion af spildevand med pereddikesyre er en lovende teknologi til elimination af udvalgte, hospitalsafledte ARB. Metoden virker som en ideel teknologi til nye centraliserede supersygehuse, der opføres i Danmark, og som tilsluttes ved direkte rørføring til renseanlæg. Samtidig er metoden anvendelig til at reducere ARB i hospitalsspildevand, der har kort kontakttid, før spildevandet blandes op i det eksisterende spildevandsnet.

The Danish Environmental
Protection Agency
Tolderlundsvej 5
DK - 5000 Odense C

www.mst.dk