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Influence of peracetic acid and hydrogen peroxide on *Escherichia coli* inactivation in wastewater

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DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING

TECHNICAL UNIVERSITY OF DENMARK

DEPARMENT OF ENVIRONMENTAL ENGINEERING

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Abstract

Commercial peracetic acid (PAA) is a quaternary mixture at equilibrium among PAA, acetic acid and hydrogen peroxide (HP). After PAA disinfection, acetic acid and HP remain in water as residues. HP displays a very slow degradation, compared to PAA, and a relevant toxicity effect. For this reason, the study was oriented on the evaluation of the disinfection efficiency of PAA against *E. coli* when HP is eliminated from commercial PAA, in view of preparing more sustainable commercial solutions of PAA. After HP removal from commercial PAA solution, PAA decay and HP re-appearance were monitored in order to detect the range of time when the concentration of PAA remains high and the concentration of HP is negligible. Disinfection experiments were carried out using commercial PAA, PAA without HP and commercial HP, with constant *E. coli* initial concentration in water, constant contact time and different initial concentration values of disinfectant. Results of disinfection tests gave the chance to estimate dose-inhibition curves, determining median effect concentration (EC_{50}) and comparing the efficiency of the disinfectants used: high disinfection efficiency was observed for commercial PAA and PAA without HP and a very low disinfection efficiency was observed for commercial HP. For both commercial PAA and PAA without HP, other disinfection tests were performed studying different combinations of initial PAA concentration and time. Experimental results seem to indicate that higher concentration values lead to higher inhibition, even at shorter contact times. The effect of HP on *E. coli* was also observed after the inactivation of catalase enzyme contained in *E. coli* and using commercial HP as disinfectant: results obtained were compared with the previous ones, to better understand the contribution of HP to disinfection and the consequence of its removal. Moreover, the possibility to develop a colorimetric method to detect *E. coli* concentration in water was studied.

Keywords: wastewater, disinfection, peracetic acid, hydrogen peroxide, *Escherichia coli*, decay kinetics, inhibition curve.

Sommario

La soluzione commerciale di acido peracetico (PAA), è costituita da PAA, acido acetico ed acqua ossigenata (HP) in equilibrio tra loro. In seguito a disinfezione con PAA, acido acetico ed HP restano in acqua come sottoprodotti. L'HP è caratterizzato da una degradazione molto lenta, rispetto al PAA, ed elevata tossicità. Per questo motivo, obiettivo dello studio è stato valutare l'efficienza disinfettante rispetto ad *E. coli* di una soluzione di PAA priva di HP, in vista dell'utilizzo di soluzioni commerciali di PAA più sostenibili. In seguito alla rimozione di HP dal PAA commerciale, il decadimento di PAA e la ricomparsa di HP sono stati monitorati individuando l'intervallo di tempo in cui la concentrazione di PAA resta elevata e la ricomparsa di HP risulta trascurabile. Diversi esperimenti di disinfezione sono stati svolti utilizzando PAA commerciale, PAA senza HP ed HP commerciale, con concentrazione iniziale di *E. coli* in acqua e tempo di contatto costanti e diversi valori di concentrazione iniziale di disinfettante. Dai risultati dei test di disinfezione sono state ottenute curve di inibizione-dose ed è stato stimato il valore di concentrazione che genera un'inibizione del 50%, comparando i diversi disinfettanti: il PAA commerciale e il PAA senza HP sono risultati avere alto potere disinfettante; più debole, invece, quello dell'HP. Il PAA commerciale e il PAA senza HP sono stati utilizzati in test di disinfezione aggiuntivi per valutare l'effetto di diverse combinazioni di concentrazione iniziale di PAA e tempo di contatto. I risultati hanno mostrato come alte concentrazioni portino ad alti valori di inibizione, anche in brevi tempi di contatto. L'effetto dell'HP sull'*E. coli* è stato valutato inattivando l'enzima catalasi contenuto nel batterio e utilizzando HP commerciale come disinfettante: i risultati sono stati confrontati con quelli ottenuti in precedenza per meglio comprendere il contributo dell'HP nel processo di disinfezione e le conseguenze della sua rimozione. Infine, è stata valutata la possibilità di utilizzare un metodo colorimetrico per la quantificazione di *E. coli* in acqua.

Parole chiave: acque reflue, disinfezione, acido peracetico, perossido di idrogeno, *Escherichia coli*, cinetiche di decadimento, curva di inibizione.

1. Introduction

Peracetic acid (PAA) is one of the most used disinfectants in wastewater treatment. Commercial solution of PAA is a quaternary mixture at equilibrium among PAA, acetic acid and hydrogen peroxide (HP). When this commercial solution is added to water, acetic acid and HP persist during time. As PAA, also HP has a germicidal power, but it displays a very slow decay, a relevant toxicity effect and in Denmark there is a very strict limit on its residual concentration in surface water (a discharge must not result in more than a 10 µg/L increase in the HP concentration in the receiving water).

The current study was developed to investigate the possibility to use as disinfectant a solution of PAA without HP, removing it from the commercial solution. This aim was chased through four experimental phases: the new disinfectant preparation, the study of its decay and its disinfection efficiency, the analysis of the influence of disinfection parameters and the study of the influence of HP in *E. coli* disinfection.

The first step was to optimize the preparation of the disinfectant, selectively removing HP from the commercial solution of PAA and studying the stability of the solution obtained.

Then, the disinfectant was used in a set of disinfection experiments, monitoring its decay in water and selecting *Escherichia coli* (*E. coli*) as indicator microorganism. To better analyse the germicidal power of the new disinfectant produced, the experiments were run using commercial PAA, PAA without HP and commercial HP and the results obtained were compared.

Commercial PAA and PAA without HP were better analysed considering different time-concentration combinations and observing the disinfection efficiency. The specific aim here was to identify which parameter (concentration or time) gave the main contribution to *E. coli* inactivation.

The last experimental phase was dedicated to study the interference between catalase enzyme contained in *E. coli* and HP, in order to better understand the influence of HP in *E. coli* disinfection and the consequences of its removal.

In all the experiments carried out, *E. coli* was enumerated by traditional spread plate count, that is a very reliable but also time consuming method. For this reason, the possibility to measure *E. coli* by a colorimetric assay was also preliminary assessed.

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2.State of the art

In wastewater treatment, disinfection plays the main role to ensure water quality conform to different destinations: bathing water, drinking water, agricultural reuse water. According to Thcobanoglous et al. (2003), an ideal disinfectant should guarantee maximum efficiency in pathogenic microorganism removal, without generating toxic and undesirable disinfection by-products (DBPs).

Various well known disinfectants are currently used, such as chlorine dioxide and hypochlorite (White G.C., 1992). Their use as disinfectants has been recently restricted due to the risk related to DBPs occurrence in water, chlorite and chlorate for chlorine dioxide and trihalomethanes for hypochlorite. WHO set 700 µg/L as guideline value for chlorite and chlorite in water, while European countries regulations on wastewater reuse set a very strictly limits for total trihalomethanes (30 µg/L according to Italian regulation D. Lgs. 152/06 and 10 µg/L according to Danish regulation LBK 879, 26/06/2010), which practically exclude chlorination as main disinfection in wastewater treatment plants (Antonelli et al., 2013).

An alternative solution is represented by organic peracids. The main drivers of their diffusion have been the increased awareness of DBPs, resulting from traditionally used chlorine based compounds, and recalcitrant micropollutants occurring in wastewater (Luukkonen et al., 2015). Organic peracids are typically available as an equilibrium solution containing: peracid, hydrogen peroxide, corresponding carboxylic acid and water. With regards to industrial applications, the most relevant peracids are peracetic acid and performic acid (Luukkonen et al., 2015).

2.1.   Characteristics of peracetic acid

PAA is a strong disinfectant with a wide spectrum of antimicrobial activity and without DBPs generation at low dosages ($\leq 5 - 10$ mg/L) (Monarca et al., 2002; Crebelli et al., 2005; Nurizzo et al., 2005). It was introduced to wastewater treatment around 30 years ago (Kitis, 2004; Falsanisi et al., 2006; Luukkonen et al., 2015), with an increasing diffusion in the recent years. This is due to its efficacy even in presence of organic matter, the negligible toxicity of its DBPs and its low dependence to water pH value (Kitis, 2004). With regards to the presence of total suspended solid (TSS) in water, this affects peracetic acid efficiency that increases lowering TSS concentration;

anyway, at low concentration values, between 10 and 40 mg/L, PAA seems to be not affected by TSS (Stampi et al., 2001) and it displays a good efficiency until 100 mg/L, where inactivation is 1 log reduced (Lefevre et al., 1992). An important advantage from PAA usage is represented by the chance to use it in the same disinfection tanks designed and used for chlorine based compounds, avoiding any expensive and invasive structural adjustment (Antonelli et al., 2013): for this reason, PAA can be chosen as the natural substitute of chlorine based compounds.

2.1.1. Chemistry of peracetic acid, main reactions and decay

PAA is an unstable chemical: commercially PAA used as disinfectant is made stable as a quaternary equilibrium mixture with HP, acetic acid and water as shown by the following equation (Block, 1991; Alasri et al., 1992; Gehr et al., 2002):

$$CH_3COOH + H_2O_2 \underset{\leftarrow}{\overset{\rightarrow}{}} CH_3CO_3H + H_2O \tag{2.1}$$
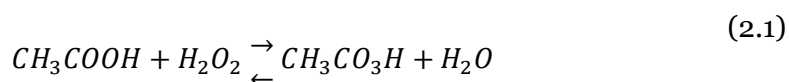
HP also contributes to disinfection, even if it is very weak compared to PAA that displays a good efficiency already at low concentration (Baldry, 1983; Fraser and Godfree, 1984; Baldry and French, 1989). On the other side, HP is a more stable compound: considering a 1% concentrated solution of PAA, this losses half of his disinfection efficiency in 6 days. For this reason, commercial PAA solutions are usually prepared at higher concentration (10% - 15%); over 15%, PAA – that is thermodynamically instable – displays risk of explosion (Block, 1991).

When PAA is added to water, two main consumption phenomena occur (Antonelli et al., 2013): an initial instantaneous oxidative consumption, due to the presence of organic and inorganic oxidable compounds, and a slower consumption, due to PAA decay in water. Overall, PAA displays a decay over time, that approximately corresponds to a 25-30% decrease in 1 hour, even in tap water. This means that PAA decays in any case, independently by the presence of oxidable compounds (Rossi et al., 2007). According to Yuan et al. (1997), the slow decay during time, under pH conditions between 5.5 and 9, is due to three kinds of reaction:

1) Spontaneous decomposition to acetic acid and oxygen:

$$2CH_3COOOH \rightarrow 2CH_3COOH + 2O_2 \tag{2.2}$$

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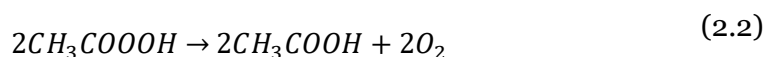
that causes a loss of oxidative power.

2) Hydrolysis reaction to acetic acid and HP:

$$CH_3COOOH + H_2O \rightarrow CH_3COOH + H_2O_2$$ (2.3)
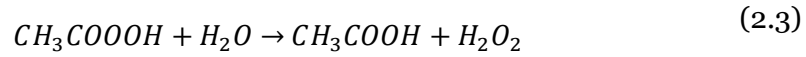
that becomes more relevant increasing solution alkalinity.

3) Interaction with reduced metals (as Fe^{2+} and Mn^{2+}), which can be oxidized by PAA:

$$CH_3COOOH + M \rightarrow O_2 + other\ decemposition\ products$$ (2.4)
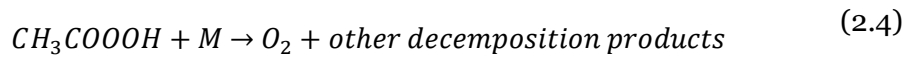
According to reactions 2.2 and 2.3, HP and acetic acid remain in water as residues after PAA disinfection treatment (Lefevre et al., 1992; Sanchez-Ruiz et al., 1995; Colgan and Gehr, 2001). Acetic acid is then degraded to carbon dioxide, while HP is degraded to oxygen and water: no one of these final compounds is toxic to aquatic life (Liberti and Notarnicola, 1999). Anyway, HP degrades very slowly compared to PAA (Wagner et al., 2002) and, while it stays in water, it displays proved toxicity effect on aquatic organisms, with an effect concentration value as low as 2.4 mg/l (ECETOC, 1993).

The concentration value of the disinfectant in water can be predicted through a mathematical function, called decay model, that corresponds to disinfectant concentration profile during time. This way, when PAA is chosen as disinfectant for wastewater treatment, both decay and initial oxidative consumption will be considered to properly design the disinfection treatment unit.

With regard to PAA, one of the simplest equations used is a first order kinetic model, that describes disinfectant concentration profile as an exponential decay during time (Feben and Taras, 1951):

$$C_t = C_0 \cdot e^{-k \cdot t_c}$$ (2.5)

where:

- C_t (mg/L) is the residual concentration of disinfectant at time *t*;
- C_0 (mg/L) is the initial concentration of disinfectant added to water;
- k (min^{-1}) is the first order decay constant;
- t_c (min) is the instant that corresponds to disinfectant-water contact time.

In many cases, this model is not suitable to well describe the decay, since it doesn't keep into account the initial oxidative consumption that diverge from the first order decay trend (Hua et al., 1999; Warton et al., 2006). Different other models were so developed: the most used is described by Haas & Finch kinetic (Haas and Finch, 2001). In this case, the first order decay was corrected by an oxidative demand coefficient, to include the initial instantaneous oxidative consumption:

$$C_t = (C_0 - OD) \cdot e^{-k' \cdot t_c} \qquad (2.6)$$

where:

- OD (mg/L) is the initial oxidative demand of disinfectant;
- k' (min^{-1}) is the Haas & Finch decay constant.

In literature, both models were used to describe the same kind of water and comparing results, as in Falsanisi et al. (2006). In Rossi et al. (2007) case of study, a first order model was used to describe disinfectant decay in tap water, while decay in wastewater was described using both first order model and Haas & Finch model, to keep in account the presence of oxidable compounds and the consequent consumption. The values of the parameters are reported and compared in Table 2.1.

Table 2.1 – First order model parameter k *and Haas & Finch model parameters k' and OD values for tap water and wastewater.*

Authors	Types of water	Initial concentration of PAA (mg/L)	First order model	Haas & Finch model	
			k (min^{-1})	k' (min^{-1})	OD (mg/L)
Falsanisi et al., 2006	Wastewater	$1.5 - 8.5$	0.00514	0.0028	0.44
Rossi et al., 2007	Tap water	$1 - 15$	0.007		
	Wastewater	$1 - 15$	$0.008 - 0.013$	$0.007 - 0.009$	$0.415 - 0.785$
Santoro et al., 2015	Wastewater	$0.5 - 10$		$0.0008 - 0.0012$	$0.2 - 1.6$

OD parameter values observed are relevant only for the lower concentration values (1 to 5 mg/L).

2.1.2. Microbial inactivation by peracetic acid

PAA disinfectant activity is based on the release of active oxygen (Liberti and Notarnicola, 1999). It is suggested that PAA disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane and moves through dislocation or rupture of cell walls (Leaper, 1984; Baldry and Fraser, 1988). Then PAA oxidizes sensitive sulfhydryl and sulfur bonds in proteins, enzymes and other metabolites, acting on the bases of the DNA molecule (Tutumi et al., 1973).

Once this capacity of PAA to destroy bacterial cells was proved, PAA disinfection efficiency was study by different authors on different kinds of water and with a particular attention to coliform bacteria. Rossi et al. (2007) analysed PAA effect on a secondary effluent: PAA gave $1-5$ log inactivation on *E. coli* and fecal coliforms, with dependence to initial concentration of bacteria, dose used, residual PAA concentration and contact time. For instance, using $5-10$ mg/L PAA in $35-50$ minutes, 4 log inactivation on *E. coli* was obtained: this result respects Italian legislation limits about surface waters protection (5000 CFU/100 mL for *E. coli.,* D. Lgs. 152/06) and wastewater reuse (10 CFU/100 mL for *E. coli,* D. M. 185/03). In the Italian legislation, *E. coli* is chosen as indicator microorganism to set limits about surface waters protection and wastewater reuse since 1999: in fact, to achieve the same removal efficiency obtained for *E. coli* on total and faecal coliforms, lower dosages and contact times are required (Azzellino et al., 2011).

PAA effect was also studied with regards to tertiary effluents – where microbial load is lower – by Luukkonen et al. (2015), using both *E. coli* and total coliform as indicators, $1.5-2$ mg/L PAA concentration and $10-15$ minutes contact time. These conditions result sufficient to reach 2 log abatement and to respect current limits set by European bathing water directive for excellent water quality (250 CFU/100 mL for *E. coli*).

Other cases of PAA effect on *E. coli* were studied and reported in different papers. Some of these results are reported in Table 2.2, together with disinfectant concentration value and contact time used.

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Table 2.2 – PAA concentration and contact time and consequent E. coli inactivation.

Authors	Concentration (mg/L)	Contact time (min)	Log reduction on *E. coli*
Antonelli et al., 2006	5 - 15	12	2 to 5
Baldry and French, 1989	25	5	5
Dell'Erba et al., 2004	4 - 8	≤ 10	~ 3.5
Flores et al., 2014	≥ 5	≤ 2.1	~ 4
Gehr et al., 2003	1.5 - 3	60	1
Kitis, 2004	5	20	4 to 5

Gehr et al. (2003) used as disinfection parameters lower concentration values and higher contact time, compared to the other studies reported: the lower inactivation obtained is coherent with the conclusions of Azzellino et al. (2011) study, namely that low concentration values are not able to give high inhibition even after a long contact time and, for this reason, PAA disinfection effect results more influenced by concentration value than contact time.

Disinfection experiments are fundamental to define appropriate models that describe the correlation between the disinfectant dose used and the inhibition obtained and to identify the right dose needed when a certain abatement is required. The inactivation of *E. coli* and fecal coliform bacteria by PAA can be satisfactorily modelled by the S-model (Azzellino et al., 2011; Antonelli et al., 2013; Luukkonen et al., 2015), proposed for PAA disinfection by Profaizer (1998). A peculiar behaviour can be outlined for lower doses and contact times, especially for *E. coli*, for which an initial lag in the PAA disinfection was observed. For higher doses, inactivation increases rapidly in the early stages of the process and then follows an asymptotic trend, while, for lower doses, the inactivation trend is more time dependent. This behaviour was probably due to an initial resistance to PAA diffusion through the cell membrane, appreciable in the early process stages at low disinfectant concentrations but negligible at higher PAA doses (Antonelli et al., 2013).

2.2.   Characteristics of hydrogen peroxide

The possibility to use HP as disinfectant was investigated by Poffe et al. (1978): doses of HP as high as 5500 mg/L and a contact time of 2 hours were needed to achieve adequate disinfection of the municipal wastewater that they tested. Concentration values like this suggest that HP is not suitable to be used as disinfectant. The only instance of HP use at reasonable doses in wastewater application seems to be in controlling sludge bulking in the activated sludge process (Jenkins et al., 1986). The range of dosages used is quite wide (20 – 200 mg/L) and the dosing point could be anywhere between the activated sludge biological reactor and the final clarifier. Contact time is not listed as a variable; HP is simply added at the point found to be most suitable for bulking control. Another recent application in wastewater treatment is in Advanced Oxidation Processes (AOPs) in combination with ozone or UV radiation to treat refractory pollutants resistant to other treatment (Boczkaj and Fernandes, 2017). HP can be used in aquaculture systems where preventive or curative water treatments occasionally are required (Arvin and Pedersen, 2015).

2.2.1. Chemistry of hydrogen peroxide, main reactions and decay

Although almost pure (98%) HP exists, it is commercially available as a water solution in a wide range of concentrations (Snell and Ettre, 1971). Main reactions where HP is involved are: decomposition into oxygen gas and water reduction-oxidation reactions, peroxide group transfer and addition-compound formation (Schumb et al., 1955). HP decomposition is substantially accelerated in the presence of some metallic impurities, particularly iron, copper, manganese, nickel, and chromium (Snell and Ettre, 1971) and it is particularly favoured at alkaline pH. For this reason, stabilizers such as aluminium are commonly added to commercial solutions (Schumb et al., 1955). In contrast, however, the addition of salts such as iron, copper or chromium, has been shown to increase the disinfection efficiency of HP (Schumb et al., 1955).

Since HP finds application in different fields, its decay in water can be described by different models: first order decay (Arvin and Pedersen, 2015), second order decay (Wagner et al., 2002) or, most commonly, specific polynomial models to better describe the decay in the different water conditions analysed (Tao et al., 2009; Flores et al., 2012). When the presence of metals before reported and oxidable compounds

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is not relevant, HP decay is well described by a first order decay (equation 2.5). Otherwise, when HP displays a more rapid decay, a second order decay can be used to describe its concentration profile:

$$C_t = \frac{1}{\frac{1}{C_0} + k'' \cdot t_c} \qquad (2.7)$$

where:

- k'' ([mg·min/L]⁻¹) is the second order decay constant.

Typical values of the second order decay constant in wastewater are reported in Wagner et al. (2002) study, where different decay constant values were observed for different initial concentration values, as reported in Table 2.3.

Table 2.3 – Second order decay constant k" *in wastewater (Wagner et al., 2002).*

Initial concentration of HP (C_o, mg/L)	Second order kinetic constant (k'', [mg·min/L]⁻¹)
466	$3 \cdot 10^{-4}$
233	$8.5 \cdot 10^{-4}$
116	$2.8 \cdot 10^{-3}$
58	$7 \cdot 10^{-3}$

It is possible to notice that for higher initial concentration values (necessary to obtain an adequate disinfection effect), HP displays a slower decay in water and, consequently, more residues remain in water after treatment.

2.2.2. Microbial inactivation by hydrogen peroxide

With regards to HP microbial inactivation properties, it can be classified as a nonspecific bactericide that is more effective against Gram-positive than Gram-negative bacteria such as *E. coli* (Wagner et al., 2002). HP is permeable to *E. coli* membrane and it reacts with the bioavailable metals in the cell membrane. The cytotoxicity of HP primary occurs through its ability to generate a hydroxyl radical through metal-catalysed reactions, such as Fenton reactions (Flores et al., 2016).

Alasri et al. (1992) studied the inactivation of typical bacteria found in water and wastewater with HP alone and in combination with PAA. High doses of HP were required to achieve 5 log reduction of *E. coli* (more than 700 mg/L with contact time of 2 hours). However, when high concentrations of HP were used with PAA the effect was synergistic: approximately 100 mg/L of HP was needed in the presence of 0.75 mg/L of PAA. When PAA was used alone, 3 mg/L was required for the same effect. Alasri et al. (1992) also established that the action of HP was rather slow, with bactericidal effects found even after 4 hours. On the other hand, the action of PAA was almost instantaneous and, in many cases, no further change was observed after 30 minutes.

The disinfection efficiency of HP alone was also studied in Flores et al. (2012): a 30 – 500 mg/L concentration range was analysed and the inhibition effect on *E. coli*, represented in Figure 2.1, was expressed as the ratio between the bacterial concentration in water after the treatment and the initial bacterial concentration.
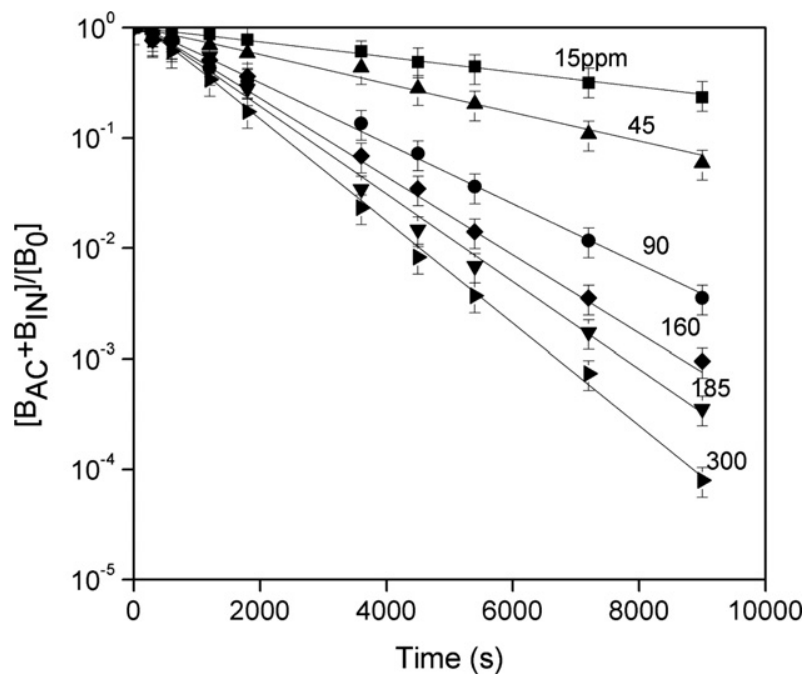
Figure 2.1 – E. coli inactivation by hydrogen peroxide (Flores et al., 2012): B_{AC} is the concentration of active bacteria, B_{IN} is the concentration of injured bacteria and B_0 is the initial bacterial concentration.

It is possible to note that, even after 2 hours, the disinfection effect is still pronounced, as represented by the relevant decreasing in the inactivation kinetic curves, in particular for high concentration values.

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An important aspect to consider about *E. coli* disinfection by HP is that HP loses his effectiveness when it reacts with catalase enzyme, releasing water and oxygen (Flores et al., 2016), and – since this enzyme is naturally contained in *E. coli* – this affects HP disinfection efficiency on *E. coli* and represents a possible explanation to HP weak disinfection effect on these kind of bacteria.

3. Materials and methods

All the reagents and the instruments used are reported in chapter 3.1 Commercial PAA, PAA without HP and commercial HP were used in disinfection experiments described in chapter 3.2. The specific experimental plan followed to analyze the influence of catalase enzyme on HP disinfection efficiency is reported in paragraph 3.3. The procedure followed to prepare PAA without HP solution is reported in chapter 3.4. Disinfectant concentration measurement and *E. coli* concentration measurement methods adopted are reported respectively in chapters 3.5 and 3.6. Software and methods used for data analysis are reported in chapter 3.7.

3.1.   Reagents and instruments

Commercial PAA solution (36% – 40%) and commercial HP solution (30%) were purchased by Sigma-Aldrich (Brøndby, Denmark), such as all the other reagents used.

For PAA and HP concentration measurement, the following reagents were used: ABTS (2,2"-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt), acetic acid (≥ 99.8%), potassium titanium oxide oxalate dihydrate (≥ 98%), sulfuric acid (≥99.99%). A Varian Cary® 50 UV-Vis spectrophotometer and BRAND® semi-micro 1 cm polypropylene cuvettes were used.

For PAA without HP preparation, potassium permanganate (≥99%), sodium hydroxide (99.99%) were used. Phosphate buffer solution (pH approx. 6.5, 1.7 M) was prepared with 16.1 g monopotassium phosphate, 8.4 g sodium phosphate dibasic and 0.28 g EDTA (ethylenediaminetetraacetic acid) in 100 mL of water. Crison's Medidor pH Basic 20 pH-meter, burette, 10 mL syringe and Sartorius Minisart® 45 μm syringe filter were used for the disinfectant preparation.

For the disinfection experiment, *E. coli* strain ATCC 25922 was used. It was grown in LB (Luria Bertani) agar. Laboratory tap water was used as medium. Sodium thiosulphate (≥99.99%) and catalase from bovine liver (2000 – 5000 units/mg protein) were used for residual disinfectant quenching.

For *E. coli* colorimetric assay, Red-Gal® (6-Chloro-3-indolyl-β-D-galactopyranoside), X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside), SDS (Sodium lauryl sulfate) and DMF (N,N-Dimethylformamide, 99.8%) were used.

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B-PER™ (Bacterial Protein Extraction Reagent) was purchased by Fisher Scientific (Roskilde, Denmark). LTB (Lauryl Tryptose Broth) was prepared with 20 g Tryptose, 5 g Lactose, 2.75 g Dipotassium Phosphate, 2.75 g Monopotassium Phosphate, 5 g Sodium Chloride, 0.1 g Sodium Lauryl Sulfate in 1 L of demineralized water. Nunc® MicroWell™ 96 well plates and a BioTeck® 96 well plate reader spectrophotometer and incubator were used.

All the operations conducted on *E. coli* (as growth or dilution) were made with sterilized equipment and sterilized water.

Reagents preparation and dilutions were made using Milli-Q water.

3.2.   Disinfection experiment

Disinfection experiments were prepared partially filling 50 mL volumetric flasks with laboratory tap water, adding 0.5 mL of *E. coli* grown in LB broth, adding the disinfectant and then filling to the mark with tap water again. One more sample was prepared just with tap water and *E. coli* and used as blank. *E. coli* pure culture had a concentration of 10^9 CFU/mL and, consequently, *E. coli* concentration in the samples prepared resulted 10^7 CFU/mL. *E. coli* initial concentration in water and the contact time (60 minutes) were maintained constant for each experiment.

For each sample, another volumetric flask was prepared with Milli-Q water and the same amount of disinfectant was added. This sample had the function to measure disinfectant concentration in a stable environment right after the start of the experiment to check the actual value of disinfectant initial concentration and eventually individuate any error related to pipette.

The disinfectants used, the initial concentration values used and the number of experiments carried out were reported in Table 3.1.

Table 3.1 – Experimental plan of disinfection experiments with constant contact time (60 min) and E. coli *concentration (10^7 CFU/mL).*

Number of experiments		Initial concentration of disinfectant (mg/L)											Tot.
		0.5	0.75	1	1.5	2	3	6	10	50	100	200	
Disinfectant	Commercial PAA	1	1	2	2	2	1						9
	PAA without HP	2	1	2	2	2	1	1					11
	Commercial HP								1	1	1	1	4

During the experiments, disinfectant residual concentration was measured at 1, 10, 20, 30, 40, 50 and 60 minutes. Absorbance values should be corrected subtracting back colour absorbance of the blank with the colorimetric reagents, since blank had a light yellow colour due to the presence of LB broth.

After 60 minutes, the experiment was stopped quenching the residual amount of disinfectant. According to Wagner et al. (2002), PAA was quenched by sodium thiosulphate (1 g/L), adding 1 mL to 10 mL of sample, that corresponds to 100 mg/L concentration of sodium thiosulphate in water. HP was quenched by catalase (1 g/L), adding 0.5 mL to 10 mL of sample, that corresponds to 50 mg/L concentration of catalase in water. Sodium thiosulphate and catalase were dosed in the same concentrations for each sample studied. After that, PAA and HP concentrations were measured again in order to verify the complete removal from water. Absorbance values given by PAA and HP must be corrected considering that catalase added naturally has yellow colour.

After disinfectant quenching phase, residual amount of *E. coli* was measured in the blank and in each disinfected sample.

Additional disinfection tests, using commercial PAA and PAA without HP, were carried out changing contact time as reported in Table 3.2. Concentration values were

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measured every 10 minutes until the end of the experiments, with the exception of the 90 minutes contact time experiment, where the measurements were run every 10 minutes for the first 60 minutes and then one more measurement at 90 minutes.

Table 3.2 – Experimental plan of disinfection experiments changing contact time value.

Contact time (t_c, min)	Commercial PAA initial concentration (C_o, mg/L)	PAA without HP initial concentration (C_o, mg/L)
30	1.21	1.69
60	0.60	0.84
90	0.40	0.56

3.3.   Catalase enzyme inactivation

This experiment consisted in two consequent phases, reported in Table 3.3.

Table 3.3 – Experimental plan of catalase enzyme inactivation experiment.

	Disinfectant	Contact time (t_c, min)	Initial concentration (C_o, mg/L)
First treatment	Commercial PAA	60	To inactivate half of *E. coli* population
	PAA without HP	60	To inactivate half of *E. coli* population
Second treatment	Commercial HP	60	1
		60	5
		60	10
		60	50
		60	100

The first treatment was run using commercial PAA and PAA without HP, as comparison, and inactivating half of *E. coli* population. Disinfectant decay was

monitored during time, residual disinfectant was quenched and *E. coli* concentration was measured at the end of the treatment. On the *E. coli* survived to commercial PAA and to PAA without HP, a disinfection experiment was run with commercial HP, as done in the disinfection experiments described in chapter 3.2, monitoring again disinfectant decay, quenching residual disinfectant and measuring residual *E. coli*.

In this specific experiment, an important modification was introduced: when PAA was quenched at the end of the first treatment, it was important to identify the right amount of sodium thiosulphate that completely quenched PAA without interfering with HP used in the second treatment. For this reason, a preliminary study was necessary, analyzing different concentrations of sodium thiosulphate in water (0.5, 1, 2 mg/L).

3.4.   Hydrogen peroxide consumption from peracetic acid commercial solution

A 1 g/L PAA solution was prepared in a 10 mL volumetric flask. PAA concentration was checked and HP concentration was measured too. HP was removed by titration using potassium permanganate (KMnO$_4$), as in Flores et al. (2016). Permanganate reacts with HP according to the following reaction:

$$2MnO_4^- + 5H_2O_2 + 6H^+ \rightarrow 2Mn^{2+} + 5O_2 + 8H_2O \qquad (3.1)$$
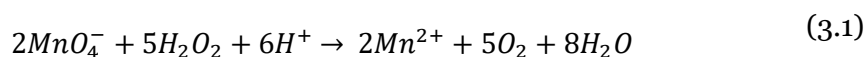
Potassium permanganate was prepared in a concentration of 0.02 M. A volume of 5 mL of the PAA solution prepared was separated and put in a beaker. At first, HP consumption was obtained by titration with a burette: potassium permanganate has pink colour and when it is added in a solution that contains HP, potassium permanganate is consumed in reaction 3.1 and manganite remains in the solution; since manganite doesn't give any colour, a transparent solution is obtained. When potassium permanganate remained in water giving pink colour to the solution, titration was completed and HP was removed. To remove manganite from the solution, a pH adjustment was necessary, in order to obtain a solid manganite that could be removed by filtration. The pH value of the peracetic acid solution right after the titration was 3.24. pH was raised until at least 8.5 adding sodium hydroxide (NaOH) (1 M). Sodium hydroxide was pipetted 10 µL at a time in the beaker containing the peracetic acid solution, always monitoring pH value. It was then

possible to filter the solution and remove solid manganite. After the filtration, pH was adjusted again, close to 6.5, using a phosphate buffer solution. In this solution, PAA and HP concentration values were measured again.

Titration procedure was repeated using different amounts of potassium permanganate and studying the effect of these on PAA and HP concentration values after titration. Fifteen samples of 10 mL were prepared (four amounts of potassium permanganate to study with three replicates per each and three replicates of control sample) and, after titration, PAA and HP concentration values were monitored during 48 hours, at 1, 2, 4, 6, 24, 48 h, meanwhile stocking samples in the dark at 5°C.

3.5.   Peracetic acid and hydrogen peroxide concentration measurement

To monitor PAA and HP concentration values in water, a colorimetric method was adopted. The main concept of colorimetric methods is to add a reagent that is able to react with the chemical species to measure, giving some colour. A spectrophotometer is used to measure the absorbance value and, through an absorbance-concentration curve, a concentration value is obtained.

According to Chhetri et al. (2014), the concentration of PAA in water has been quantified by selective oxidation reaction for organic peroxide with ABTS, to form a green product, which is quantified by measuring absorption at 405 nm. The method is a variation of the method proposed by Pinkernell et al. (1997), where potassium iodide was used to quantify peroxides along with ABTS, acetic acid buffer and sample. With Pinkernell et al. (1997) method, higher signal from HP was observed. For this reason, potassium iodide is omitted to minimize the cross reaction of HP and potassium iodide.

All the reagents and dilutions must be prepared using Milli-Q water. 1.0 g/L ABTS and 1.0 M acetic acid must be prepared, adjusting acetic acid pH to 3.50 with NaOH (1 M). A cuvette must be filled with Milli-Q and read as zero. Sample analysis consists in filling a 1.0 cm polypropylene cuvette with 350 μL acetic acid, with 350 μL ABTS and then adding 350 μL of sample, mixing using the pipette tip and running the measurement after 10 minutes.

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The recorded absorbance value has been transformed to concentration using the following equation:

$$C_{PAA} = \frac{Abs - 0.002341}{0.1675}$$

(3.2)

where:

- C_{PAA} (mg/l) is the concentration of PAA;
- *Abs* is the absorbance value measured at 405 nm.

The absorbance value used in the equation 3.2 has been corrected subtructing the absobance value measured on a cuvette filled with 350 µL of ABTS, 350 µL of acetic acid and 350 µL of Milli-Q water, to exclude any back colour interference from reagents.

This equation comes from a calibration curve, represented in Figure 3.1, obtained measuring the absorbance of different concentrations of peracetic acid (0.5, 1.0, 1.5, 2.0 mg/L).
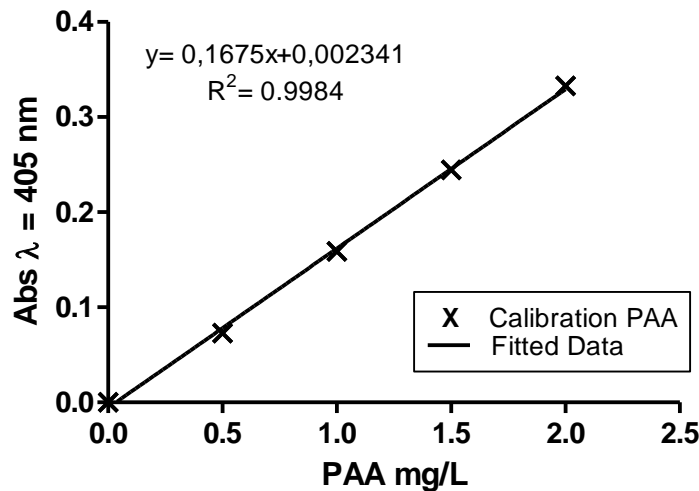
Figure 3.1 – Absorbance-concentration calibration curve for peracetic acid (Chhetri et al., 2014).

HP is measured as described by Antoniou and Andersen (2015). HP reacts with potassium titanium oxalate in acid solution to form a yellow pertitanic acid complex. The colored complex absorbance is measured by a spectrophotometer set at 400 nm. The method is reliable for the determination of HP in the range of 0,054 − 88 mg/L.

50 g/L potassium titanium oxalate solution has been prepared and 100 g/L sulfuric acid was also necessary to lowering pH and catalase the colour reaction. A cuvette has been filled with 1 mL of sample, 0.1 mL of sulfuric acid and 0.1 mL of potassium titanium oxalate. The colour reaction was instantaneous and measurement has been run as the cuvette was filled.

The absorbance value obtained has been transformed to concentration using the following equation:

$$C_{HP} = \frac{Abs - 0.0005}{0.0282} \qquad (3.3)$$

where:

- C_{HP} (mg/L) is the concentration of HP;
- *Abs* is the absorbance value measured, corrected subtracting reagents back colour absorbance.

Calibration curve is reported in Figure 3.2.
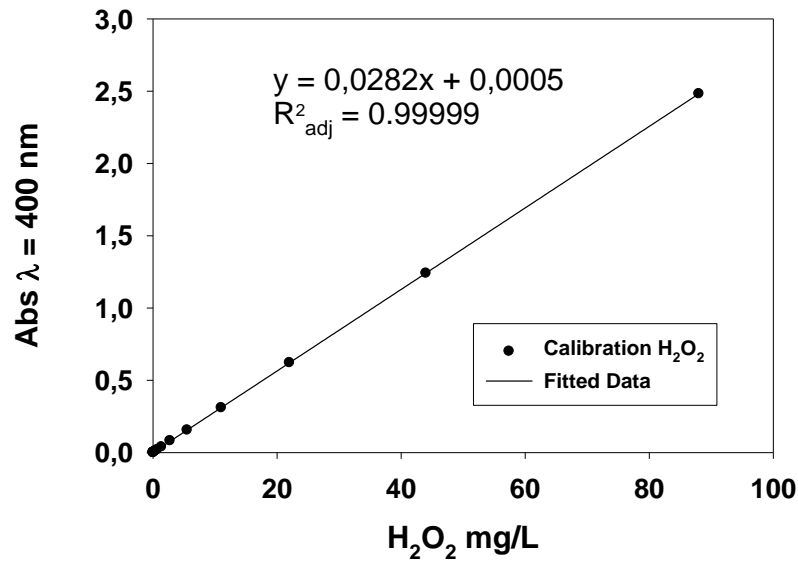
Figure 3.2 – Absorbance-concentration calibration curve for hydrogen peroxide (Antoniou and Andersen, 2015).

With regard to both methods, every time PAA or HP concentration must be detected in a sample that is expected to be more concentrated than the higher calibration range

value, the sample must be diluted with Milli-Q water in a volumetric flask, obtaining a sample within the range of concentration where the method was optimized and considered reliable. Starting from the absorbance values, it was possible to calculate the concentration of PAA and HP in the diluted sample and then, considering the dilution factor, the concentration of PAA and HP in the original sample.

3.6. *Escherichia coli* concentration measurement

E. coli concentration at the end of disinfection tests was measured by spread plate method, described in paragraph 3.6.1. From the disinfection tests reported in Table 3.1, one replicate for each test was selected and *E. coli* concentration was also evaluated by the colorimetric assay described in paragraph 3.6.2.

3.6.1. Spread plate method

In the present study, spread plate method was adopted following indication reported in Standard Methods for Examination of Water and Wastewater (Rice et al., 2012). Agar medium suitable to *E. coli* growth has been prepared, sterilized and maintained at the right temperature to remain in liquid phase. Then, it is poured in Petri's dishes (around 20 mL per each dish). Plates has been dried for 20-25 minutes and stored in the dark at 5°C until usage. Starting from the sample to analyze, a dilution series has been prepared (Figure 3.3), shaking every single microtube by a vortex (set at 1000 rpm) during the series preparation: this is important to avoid *E. coli* deposit on the bottom of the tube and to have a homogeneous *E. coli* concentration when the inoculum is taken and pipetted in the following tube. From each diluted sample, 100 µL has been pipetted on the plate and carefully and uniformly spread using a metal stick. All these operations have been conducted under a metallic fume in a sterilized environment. When the inoculum was absorbed, plates have been labeled and incubated at 37 °C for 22-24 hours.

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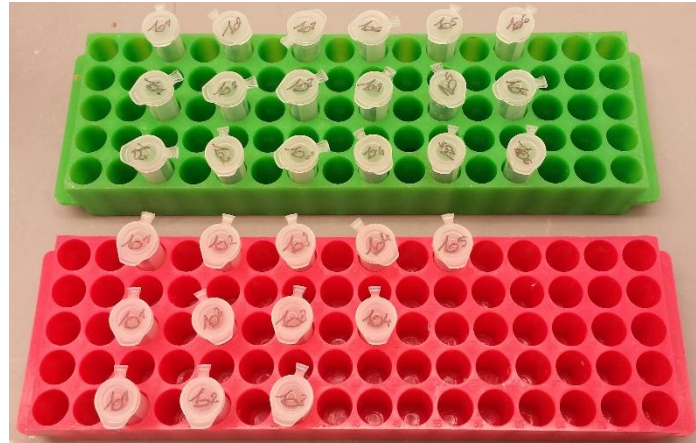
Figure 3.3 - Dilution series prepared in microtubes for spread plate method.

After the incubation phase, meanwhile *E. coli* could grow forming colonies, from each incubated plate a number of colonies (CFU) was observed (Figure 3.4). If the dilution factor used is too low, *E. coli* concentration on the plate will be too high to clearly see every single colony (Figure 3.4): these results are reported as "too numerous to count" (TNTC). These plates and all the plates with more than 300 colonies will be excluded from the calculation.
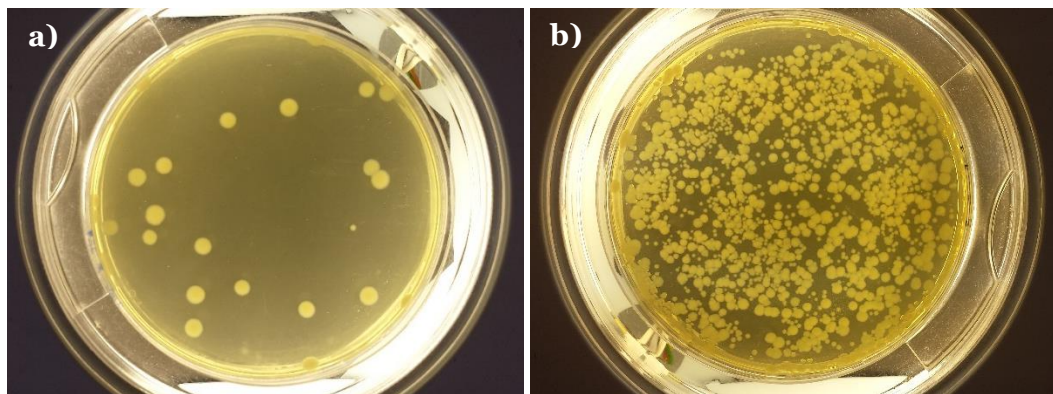
Figure 3.4 – E. coli *colonies on LB agar: a) countable* E. coli *colonies; b) uncountable* E. coli *colonies.*

E. coli concentration has been calculated using the following formula:

$$CFU/mL = \frac{number\ of\ colonies\ counted\ on\ plate}{actual\ volume\ of\ sample\ spread\ on\ plate\ (mL)} \qquad (3.4)$$

The actual volume of sample spread has been calculated considering the dilution factor (10^1, 10^2, 10^3 and so on) and the amount of the inoculum spread:

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$$actual\ volume\ of\ sample\ spread\ on\ plate\ (mL)$$
$$= \frac{volume\ spread\ on\ plate\ (mL)}{dilution\ factor} \qquad (3.5)$$

From each plate, a CFU/mL value was obtained and the mean value can be assumed as the *E. coli* concentration in the original sample analyzed.

3.6.2. Colorimetric method

The colorimetric method was optimized starting from the experimental work reported in Gunda et al. (2016) and Mitra et al. (2014). A 96 well microplate (Figure 3.5) was used, adding in each well the sample to test and the reagents. A single well has a capacity of 300 µL.
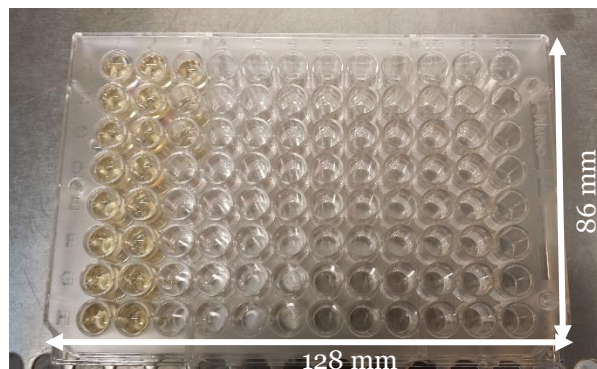
Figure 3.5 – 96 well microplate preparation.

Different reagents were necessary: they are reported in Table 3.4, classified according to their function.

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Table 3.4 – List of reagents necessary for the colorimetric assay.

Name	Function	Chemicals	Preparation	Volume [μL]
Bacteria		Water to analyse		100
Reagent X	Growth media (provides nutrients for the growth of *E. coli*)	LTB	Dissolve a mixture of LTB in demineralized water	100
Reagent Y	Cell lysing agent	SDS (2 %)	Dissolve 0.02 g of SDS in 10 mL of Milli-Q water	25
		B-PER™		
Reagent Z	Chromogenic enzymatic substrate	Red-Gal (red colour)	Dissolve 30 mg of Red-Gal in 1 mL of 1:1 DMF and Milli-Q water solution	50
		X-Gal (blue colour)	Dissolve 30 mg of X-Gal in 1 mL of 1:1 DMF and Milli-Q water solution	

The plate must be incubated in the microplate reader at 37° C, temperature value compatible with *E. coli* growth, and maintained under 60 rpm shaking conditions, to have a homogeneous concentration in the well avoiding *E. coli* deposit.

The first step of the method development was to identify a wavelength for the absorbance measurement, observing the whole spectrum and looking for an absorbance peak. X-Gal was the first chromogenic enzymatic substrate studied: it is able to react with β-D-galactosidase (GAL) enzyme giving blue colour. A well was filled with LTB, SDS, and X-Gal. Starting from the *E. coli* pure culture, a dilution series was prepared, obtaining an *E. coli* concentration of 10^6 CFU/mL. This diluted sample was added in the well prepared. Red-Gal absorbance peak was derived from literature (530 nm).

The second step was the choice between SDS and B-PER™ and between Red-Gal and X-Gal. The 96-well microplate were prepared as reported in Table 3.5 scheme.

Table 3.5 – Microplate layout and reagents.

	1	2	3	4	5	6	7
A	*E. coli* 10^7 LTB X-Gal	*E. coli* 10^7 B-Per LTB X-Gal	*E. coli* 10^7 SDS LTB X-Gal	LTB	*E. coli* 10^7 LTB Red-Gal	*E. coli* 10^7 B-Per LTB Red-Gal	*E. coli* 10^7 SDS LTB Red-Gal
B	*E. coli* 10^6 LTB X-Gal	*E. coli* 10^6 B-Per LTB X-Gal	*E. coli* 10^6 SDS LTB X-Gal	Milli-Q water		*E. coli* 10^6 B-Per LTB Red-Gal	*E. coli* 10^6 SDS LTB Red-Gal
C	*E. coli* 10^5 LTB X-Gal	*E. coli* 10^5 B-Per LTB X-Gal	*E. coli* 10^5 SDS LTB X-Gal			*E. coli* 10^5 B-Per LTB Red-Gal	

The microplate reader was set to run a measurement every one hour, for 24 hours. Three different absorbance values, around the peak previously identified, were selected to make a comparison. An absorption kinetic during time was registered for each sample at the three wavelengths considered.

3.7.   Data analysis

All the calculations were made using Excel®.

Graphs were built and analysed using the software Prism5®. In particular, Prism5® was used to fit concentration decay data obtaining decay parameters. Inhibition-dose response curves were estimated by Prism5® using a nonlinear regression and assuming a lognormal distribution of the dose; by inverse estimation, EC_{50} values were determined. The software also gives the chance to obtain a 95% interval of confidence of the curve and the EC_{50} parameter.

4.Results and discussion

The results obtained from the experimental work were collected, processed and here presented. With regards to HP removal from commercial PAA, a procedure was defined and the new disinfectant characteristics were reported (chapter 4.1). From the disinfection experiments, decay kinetics (chapter 4.2) and inhibition-dose curves (chapter 4.3) were obtained for each disinfectant studied. Results obtained from disinfection experiments using the same dose obtained by different parameters were compared (chapter 4.4). Decay kinetics and inhibition results were also obtained in the disinfection experiment with HP and inactive catalase enzyme (chapter 4.5). Colorimetric assay conditions were defined, *E. coli* concentration values were calculated and compared with plate count results (chapter 4.6).

4.1.   Peracetic acid without hydrogen peroxide: preparation procedure and characteristics

Titration procedure of HP with potassium permanganate gave as results that 0.3 mL were necessary to completely removed HP. In Table 4.1 are reported the characteristic of the commercial solution and the characteristics of the solution obtained at the end of the procedure. Starting from PAA and HP concentration data, PAA mass and HP mass after titration were calculated and compared to initial PAA mass and HP mass in the original sample, obtaining consumption data expressed in term of percentage. Results are reported in Table 4.1.

Table 4.1 – Solution's characteristics before and after titration procedure.

	PAA	HP
Initial concentration (mg/L)	1140	140
Concentration after titration procedure (mg/L)	290	0.23
Mass reduction	-74.6 %	-99.8 %

As expected, a relevant reduction of HP was obtained. At the same time, also a reduction of PAA mass was observed.

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With the aim to define a HP removal procedure that entails a lower PAA reduction, the effect of lower amounts of potassium permanganate was studied, repeating the titration procedure with four different permanganate concentration values. In Table 4.2 are reported the characteristics of the commercial PAA solution prepared. Titration procedure data are also reported in Table 4.2.

Table 4.2 – Titration procedure results with different amounts of potassium permanganate (mean and standard deviation).

Commercial PAA				
	PAA		HP	
Concentration (g/L)	1.16±0.01		0.15±0.01	
Titration procedure				
Sample	*A*	*B*	*C*	*D*
Volume of sample (mL)	10	10	10	10
Volume of KMnO$_4$ (0.02 M) (mL)	0.05	0.1	0.2	0.3
KMnO$_4$ / sample volume ratio	0.005	0.01	0.02	0.03
Concentration of KMnO$_4$ (mg/L)	16	31	62	92
pH value after KMnO$_4$ addition	3.19±0.01	3.30±0.02	3.38±0.03	3.53±0.03
Volume of NaOH (μL)	340	340	340	340
pH value after NaOH addition	8.63±0.04	8.62±0.01	8.63±0.02	8.62±0.02
Volume of phosphate buffer solution (μL)	700	700	600	600
pH value after phosphate buffer addition	6.59±0.01	6.61±0.01	6.64±0.01	6.65±0.02
Final sample colour	clear	clear	pink	pink
PAA concentration after titration (g/L)	1.05±0.02	1.01	0.93±0.03	0.83±0.04
HP concentration after titration (mg/L)	5.13±0.11	1.46±0.11	0.66±0.09	0.59±0.03

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Titration procedure is also represented in the pictures in Figure 4.1, where it is possible to observe the colour evolution of the solution, that was initially transparent (Figure 4.1), then it became pink as potassium permanganate was added (Figure 4.1); when potassium permanganate and HP reaction occurred the solution became clearer (Figure 4.1) and then transparent again (Figure 4.1); manganite precipitate gave orange colour to the solution (Figure 4.1), until it was removed by filtration (Figure 4.1), obtaining again a transparent solution (Figure 4.1).
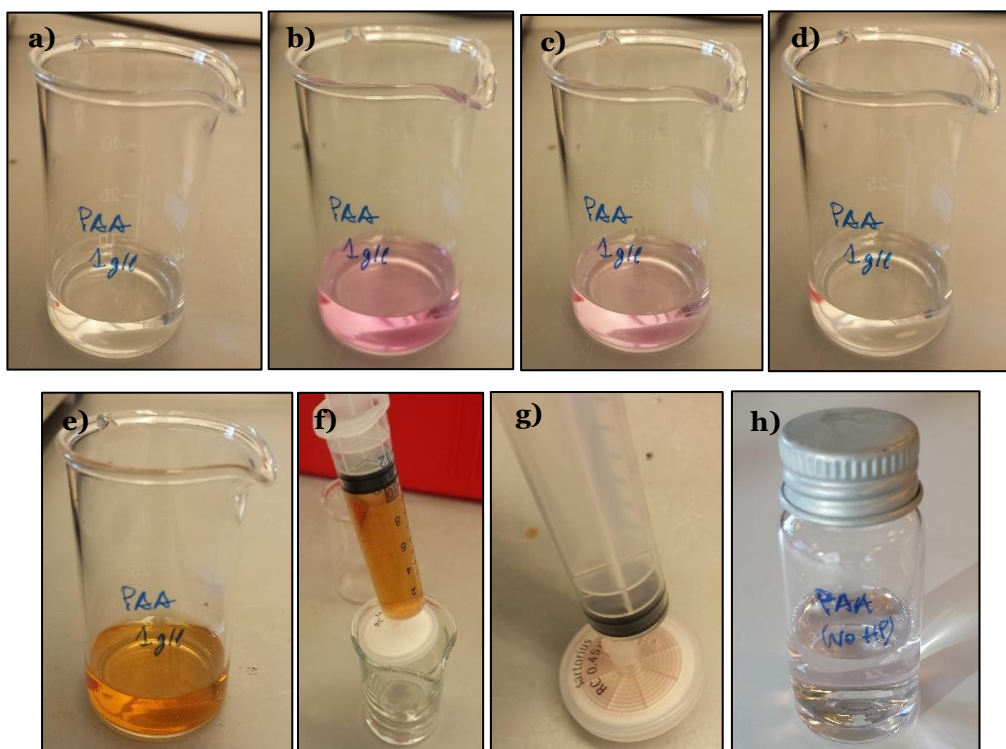
Figure 4.1 - Titration procedure of sample B: a) 1 g/L PAA solution was prepared; b) potassium permanganate was added to peracetic acid solution; c) KMnO$_4$ and HP reaction occurred; d) potassium permanganate was completely consumed with HP; e) manganite precipitated; f) manganite was filtered; g) manganite remained in the filter; h) PAA without HP solution was obtained.

PAA and HP concentrations measured in the samples at the end of the titration procedure (Table 4.2) were compared to potassium permanganate concentration values used for HP removal in Figure 4.2.
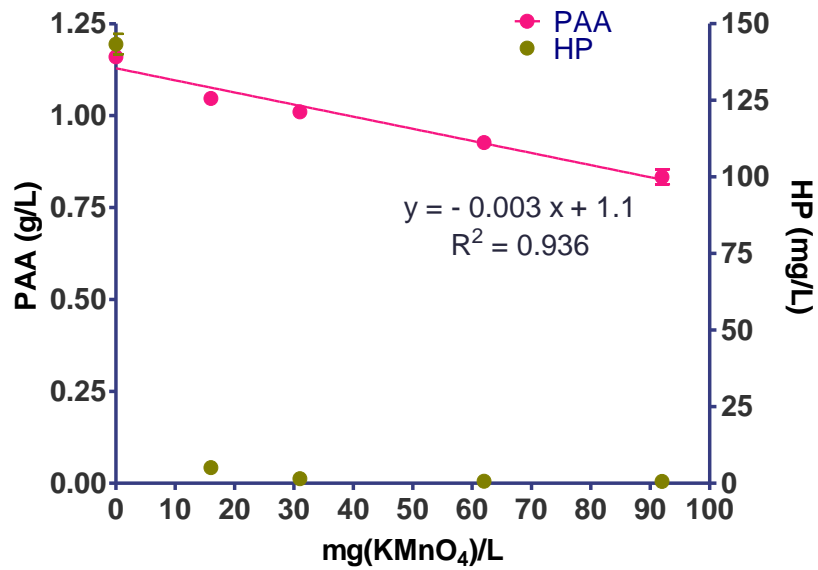
Figure 4.2 – PAA and HP concentrations measured at the end of titration procedure with different concentrations of potassium permanganate.

With regards to PAA, increasing potassium permanganate concentration, a light linear decreasing was observed. Looking at HP concentration values, even with the lowest concentration of potassium permanganate tested, a relevant HP mass reduction was obtained. The side reaction between potassium permanganate and PAA is definitely weaker than the main wanted reaction with HP.

PAA and HP concentrations monitoring during 48 hours gave the results reported in Figure 4.3.

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a)

b)
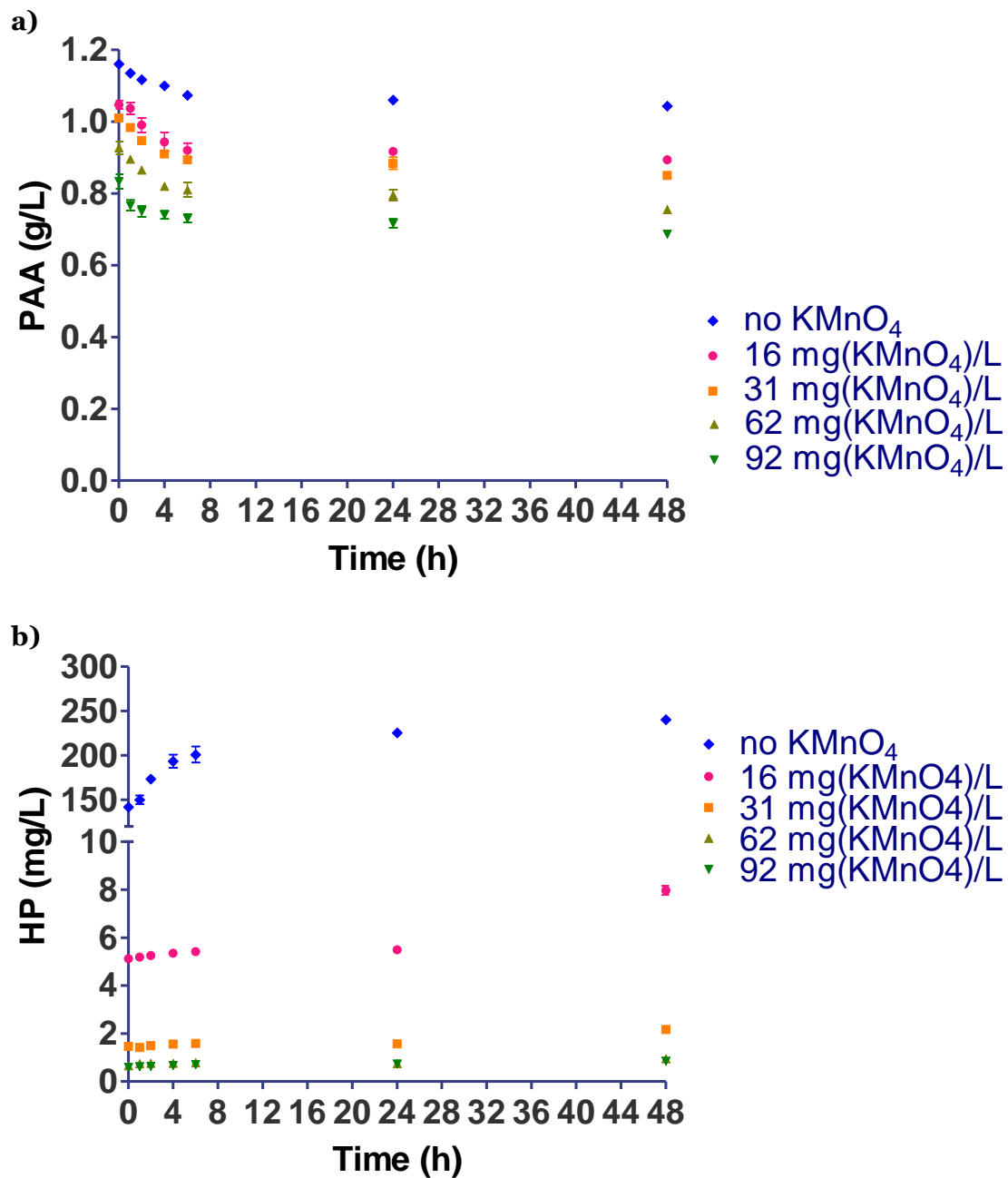
Figure 4.3 – PAA (a) and HP (b) decay and reappearance during time after titration.

It is possible to note that PAA decayed during time: an initial relevant consumption was observed within the first 6 hours; after that, concentration tended to be more stable. This happened independently to the potassium permanganate amount added in the solution; in fact, the same behaviour was observed also in the 1 g/L PAA solution initially prepared where no potassium permanganate was added (blue dots in Figure 4.3). On the other side, HP concentration values suggest the reappearance of HP during time, with an increasing trend that continued after 6 hours (while PAA seemed

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to be stable). Even in this case, the reappearance was presented in every sample studied, included the original 1 g/L PAA sample.

According to the obtained results, it seems that potassium permanganate acts reducing PAA and HP concentration after titration but it doesn't influence PAA decay and HP reappearance trend during time, since it is the same decay trend observed in the sample without potassium permanganate. PAA decay observed could be due to natural PAA hydrolysis reaction (reaction 2.3), that is independently to the potassium permanganate presence in water; moreover, one of the products of this reaction is HP and this can explain its reappearance in water here observed.

PAA and HP mass consumptions were calculated in term of percentage, with reference to PAA and HP mass mean values in the original 1 g/L PAA sample (named *O*). Results are reported in Table 4.3 and Table 4.4.

Table 4.3 –PAA mass consumption (mean and standard deviation).

	Sample				
	O	*A*	*B*	*C*	*D*
0 h	0±1%	0±2%	-4%	-13±3%	-22±3%
1 h	-2±1%	-1±2%	-7%	-16±1%	-28±3%
2 h	-4±1%	-6±3%	-10±1%	-18±1%	-29±2%
4 h	-5±1%	-10±4%	-13±1%	-22±2%	-30±2%
6 h	-7±1%	-12±4%	-15±1%	-24±2%	-31±2%
24 h	-9±1%	-13±1%	-16±3%	-25±2%	-32±2%
48 h	-10±1%	-15±1%	-19±2%	-29±1%	-35±1%

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Table 4.4 –HP mass consumption (mean and standard deviation).

	Sample				
	O	A	B	C	D
0 h	-2±2%	-96%	-99%	-99%	-100%
1 h	3±5%	-96%	-99%	-99%	-99%
2 h	19±2%	-96%	-99%	-99%	-99%
4 h	33±8%	-96%	-99%	-99%	-99%
6 h	38±9%	-96%	-99%	-99%	-99%
24 h	55±1%	-96%	-99%	-99%	-99%
48 h	65±2%	-94%	-98%	-99%	-99%

Looking at the results, the most interesting sample could be *B* (obtained with a potassium permanganate concentration equal to 31 mg/L). In fact, after the titration procedure, the solution is used as disinfectant in no more than 1 or 2 hours: in this small range of time, the loss of PAA after titration was around 10% and the HP reappearance was negligible (under 2 mg/L) compared to PAA concentration in the samples (higher than 0.9 g/L).

An additional analysis was carried out, calculating loss mole of PAA and gained mole of HP after titration, expressed in term of mmole/L. Results obtained for each sample studied are reported in Figure 4.4.

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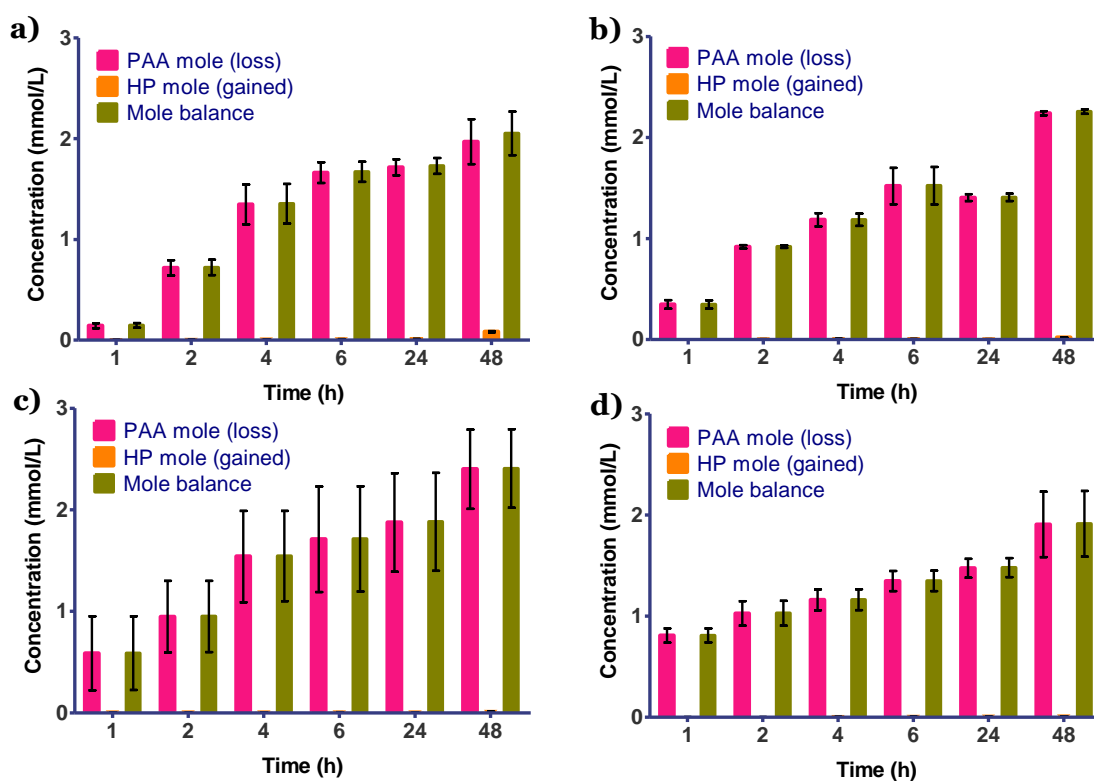
Figure 4.4 – PAA and HP mole balance during time: a) sample A; b) sample B; c) sample C; d) sample D.

Only a small part of peracetic acid (pink column) becomes hydrogen peroxide (orange column): around 4% for sample A and less than 1% for the other samples. Seen the relevant difference in PAA and HP mole balance (green column), it is possible to affirm that PAA degrades to HP peroxide and, above all, to other compounds probably mainly to acetic acid, according to the reactions 2.2 and 2.3.

4.2. Peracetic acid and hydrogen peroxide decay kinetics

Disinfection experiments were carried out studying different initial concentration values, excluding or including some values from one disinfection session to another, re-defining the initial concentration range to study in order to identify, with the highest precision possible, the EC_{50} value (dose value that corresponds to a 50% microbial inactivation) of each disinfectant and use this parameter as comparison. All the results obtained are here reported and discussed.

From the measurement of disinfectant concentration values in water during the experiments, concentration profiles were obtained. For each disinfectant, an initial rapid decay was observed: this could be due to the presence in water of *E. coli* growing broth, rich of oxidable compounds. Haas & Finch model (equation 2.6) was chosen to describe these concentration profiles, fitting data from 10 to 60 minutes, considering the initial oxidative consumption concluded within 10 minutes. From each curve obtained, it was possible to estimate decay parameters, *OD* (mg/L) and *k'* (min^{-1}). Once decay parameters were known, it was possible to calculate the actual dose of disinfectant in water. It was calculated as the area under the kinetic curve, that can be written as:

$$D = \int_o^{t_c} C(t)\, dt = \int_0^{t_c} (C_0 - OD) \cdot e^{-k' \cdot t_c}\, dt = \frac{C_0 - OD}{k'} \cdot (1 - e^{-k' \cdot t_c}) \qquad (4.1)$$

where:

– *D* is the dose of disinfectant (mg·min/L).

Commercial PAA, PAA without HP and commercial HP concentration decays during time are reported in Figure 4.5. HP concentration in commercial PAA was also measured during the experiments, but the values measured resulted lower than the range where the colorimetric method was calibrated, with exception of the first five measurements in test at 2 mg/L PAA, the first three measurement at 1.5 mg/L PAA and the first measurement at 1 mg/L PAA and 0.75 mg/L PAA: these data were not sufficient to define a kinetic and calculate a dose value in 60 minutes and, for this reason, HP dose in commercial PAA was excluded from the study.

a)

b)

c)
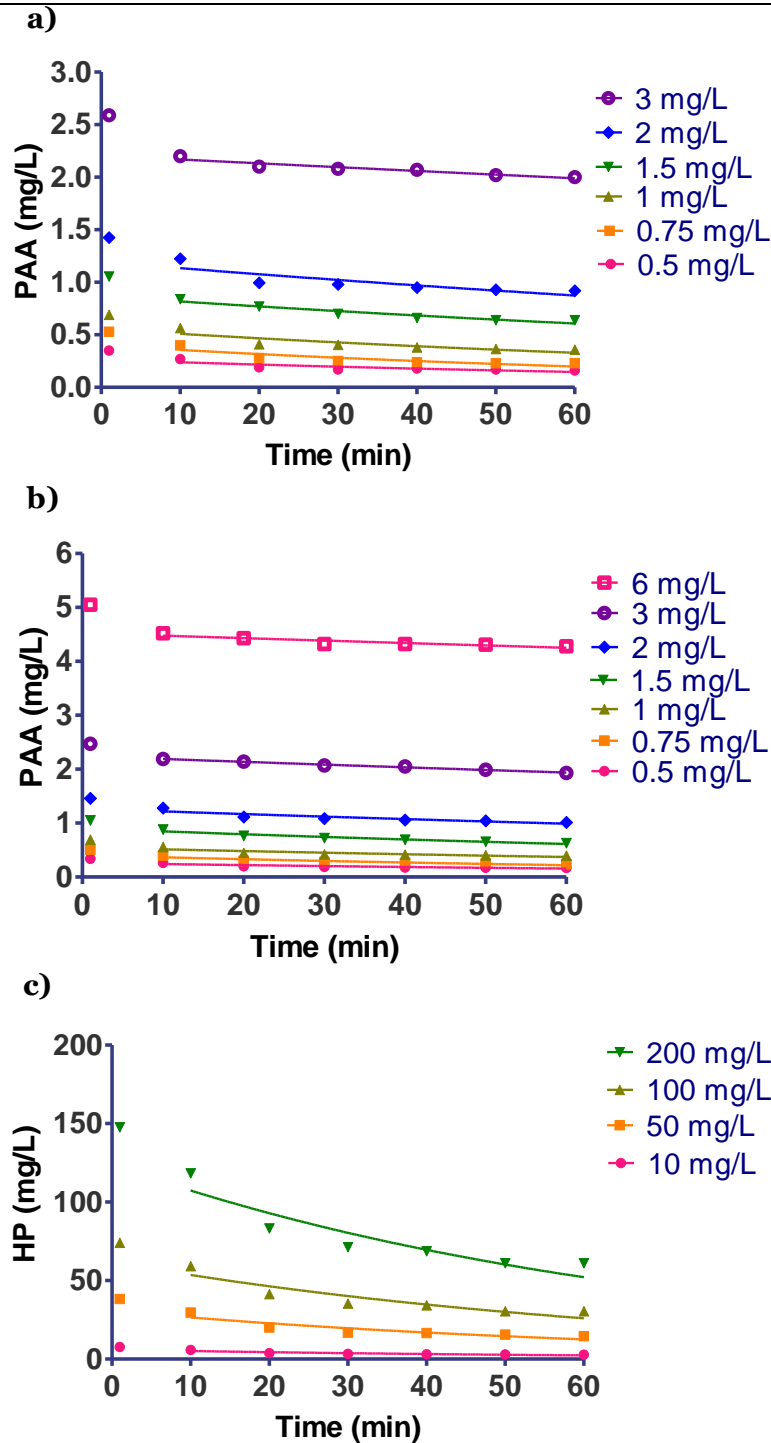
Figure 4.5 – Decay of commercial PAA (a), PAA without HP (b) and commercial HP (c): dots represent measurement results, lines represent the Haas & Finch model decay.

Seen the relevant difference between the concentration at 1 minutes and at 10 minutes, for each disinfectant, it could be interesting to deeper look what happens to PAA and HP in the first 10 minutes, running more measurements in that interval (e.

g. one measurement every 1 minute), in order to build an accurate concentration profile even in that range of time.

In Table 4.5 the initial concentration values, contact time, decay parameters and the disinfectant dose calculated by equation 4.1 are reported, without considering HP dose in commercial PAA.

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Table 4.5 – Experimental conditions, decay parameters and disinfectant dose.

	Initial concentration (C_0, mg/L)	Contact time (t_c, min)	Oxidative demand (OD, mg/L)	Haas & Finch decay constant (k', min^{-1})	R^2	PAA dose (D, mg·min/L)	HP dose (D, mg·min/L)
Commercial PAA	0.5	60	0.24	0.0098	0.68	12	
	0.75	60	0.35	0.0117	0.75	17	
	1	60	0.44	0.0087	0.72	26	
	1.5	60	0.63	0.0059	0.84	44	
	2	60	0.81	0.0052	0.68	62	
	3	60	0.79	0.0017	0.9	126	
PAA without HP	0.5	60	0.24	0.0087	0.73	12	
	0.75	60	0.34	0.0103	0.83	18	
	1	60	0.45	0.0065	0.55	27	
	1.5	60	0.60	0.0065	0.91	45	
	2	60	0.73	0.0042	0.65	67	
	3	60	0.76	0.0025	0.99	125	
	6	60	1.48	0.0010	0.82	263	
Commercial HP	10	60	3.98	0.0156	0.79		151
	50	60	19.20	0.0150	0.81		893
	100	60	38.09	0.0145	0.83		1800
	200	60	75.90	0.0145	0.84		3609

As previously supposed looking at the Figure 4.5, *OD* values confirm that a relevant PAA and HP initial consumption occurred. In Figure 4.6, *OD* values obtained are compared with the corresponding initial concentration values.
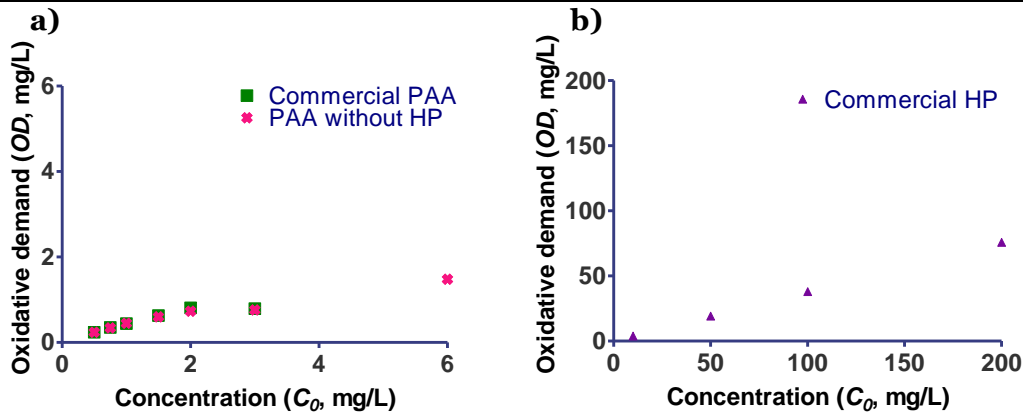
Figure 4.6 – Initial concentration value and corresponding oxidative demand: a) commercial PAA and PAA without HP; b) commercial HP.

For all the disinfectants analyzed, it is possible to observe that oxidative demand value was higher for the higher concentration values. In the lower range of concentration (commercial PAA 0.5 – 2 mg/L and PAA without HP 0.5 – 2 mg/L), the oxidative consumption was responsible of the 40 – 50 % consumption of the initial concentration. In the upper range of initial concentration (commercial PAA 3 mg/L, PAA without HP 3 – 6 mg/L and commercial HP 10 – 200 mg/L), it was responsible of the 25 – 40 % consumption of the initial concentration. These results show that, even if the oxidative consumption increases with the initial concentration value, in the higher concentration range the consumption is less relevant. In fact, the oxidative consumption is probably mainly due to bacteria growing broth that was present in water always with the same concentration; consequently, the oxidative consumption is limited by the amount of broth in water and it can't affect the disinfectant concentration always with the same magnitude. In any case, this value can't definitely be considered negligible and the Haas & Finch model, that keeps into account this kind of consumption, results suitable to describe what was observed.

With regards to the decay constant, different values were obtained for the different initial concentrations studied. In Figure 4.7, initial concentration and corresponding constant decay observed are presented.

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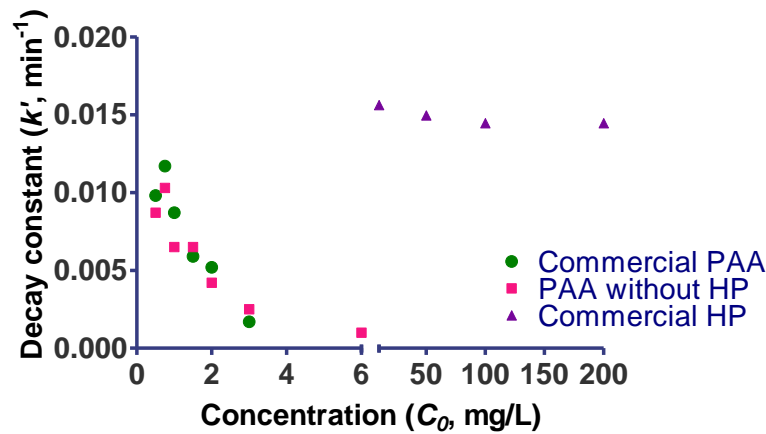
Figure 4.7 – Initial concentration value and corresponding decay constant of PAA in commercial PAA and PAA without HP and of HP in commercial HP.

Looking at commercial PAA and PAA without HP, it is possible to observe a negative trend: this suggests that more concentrated samples are less affected by decay during time. For commercial HP, the same negative trend is observed, even if it is less pronounced. Anyway, possible interferences due to bacteria growing broth must be keep into account: other measurements using centrifugated *E. coli* solution (excluding growing broth) would be useful to better evaluate the disinfectant decay.

4.3. Peracetic acid and hydrogen peroxide inhibition on *Escherichia coli*

After *E. coli* residual concentration was estimated by plate count method, the corresponding percentage inhibition was calculated, comparing plate count results of disinfected samples to control sample.

Percentage inhibition values obtained using different kind of disinfectant and different doses (not considering again HP dose in commercial PAA) were reported in Table 4.6.

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Table 4.6 – Disinfectant dose and corresponding percentage inhibition.

	PAA dose (*D*, mg·min/L)	HP dose (*D*, mg·min/L)	Inhibition (mean value)
Commercial PAA	11		4%
	16		32%
	22		46%
	30		100%
	41		100%
	68		100%
PAA without HP	12		18%
	17		25%
	24		47%
	32		89%
	46		100%
	116		100%
	243		100%
Commercial HP		217	16%
		1003	51%
		1642	74%
		3338	93%

Dose and inhibition data were fitted by the Prism5® equation "log(agonist) vs. response -- Variable slope", suggested by the software for this kind of elaborations, when the aim is to evaluate the response of an agent that cause an inhibition effect, obtaining an inhibition–dose curve that approaches the S-model curve and with the

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same main characteristics: initial resistance to inactivation, exponential inactivation and asymptotic inactivation, from low to high dose values.

Three curves were obtained, one for each disinfectant: they are shown in Figure 4.8.
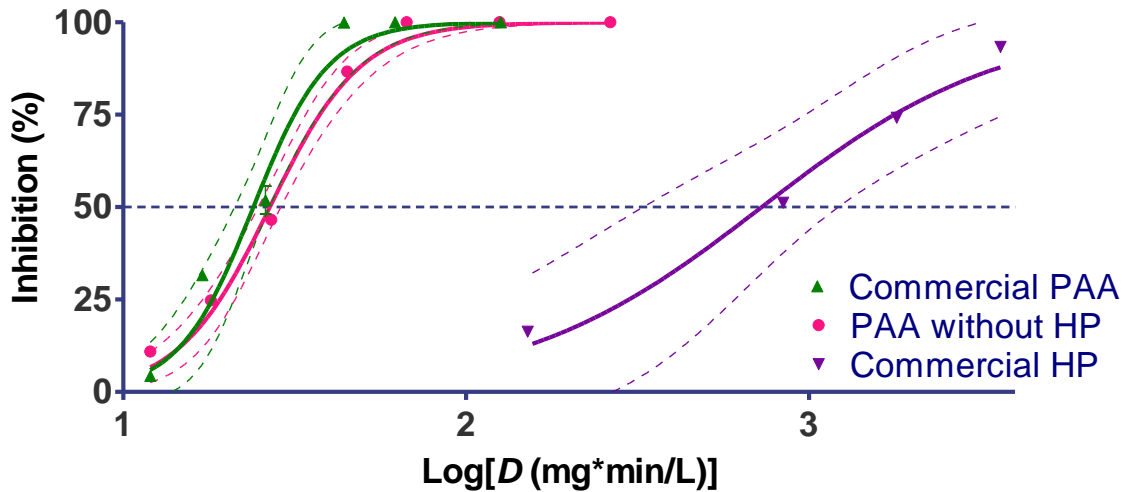
Figure 4.8 – Inhibition-dose curves (thick lines) represented with their 95% confidence intervals (dashed lines). Observations fittings are characterized by the following coefficients of determination: commercial PAA, $R^2 = 0.99$; PAA without HP, $R^2 = 0.97$; commercial HP, $R^2 = 0.98$.

Looking at the curves obtained, different observations can be made. First of all, it is possible to notice that commercial PAA and PAA without HP effects are described by curves characterized by a higher slope, compared to commercial HP one. This could be due to the different microbial response of *E. coli* to PAA and HP. For PAA, once that the initial diffusion resistances are passed, PAA is able to kill *E. coli*, going from a null to a complete inactivation in a very small range of dose. For HP, instead, other resistance phenomena affect the disinfection treatment, as probably the catalase enzyme interference with HP action. Another important difference is the confidence intervals largeness: the wide confidence interval for commercial HP curve could be due to the lack of replicates (in fact the curve was built using only four disinfection test results). The possibility to run each experiment at least with three replicates would give a more robust result, even for commercial PAA and PAA without HP.

From these curves, the EC_{50} value for each disinfectant was observed and used to made a disinfection efficiency comparison. Values are reported in Table 4.7 with the corresponding 95% confidence interval.

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Table 4.7 – Disinfection efficiency of each disinfectant studied.

	EC_{50} [mg·min/L]	95% confidence interval
Commercial PAA	24	(22; 27)
PAA without HP	27	(25; 29)
Commercial HP	727	(407; 1300)

Commercial PAA and PAA without HP seem to display very similar disinfection efficiency: EC_{50} value estimated are very close to each other and confidence intervals are partially overlapped, as it is possible to observe in Figure 4.9, where curve's parameters (EC_{50} and Hillslope) are compared.
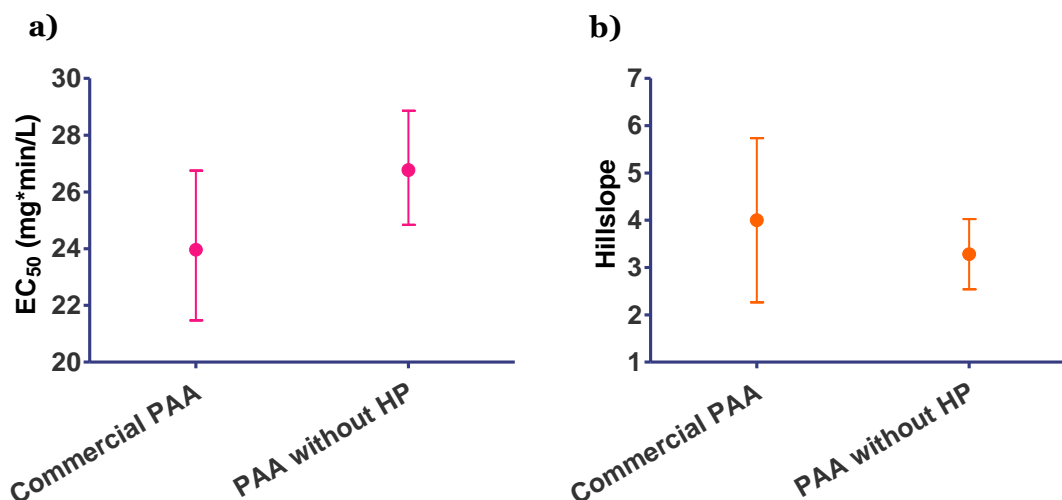
Figure 4.9 – a) EC_{50} values (points) with confidence intervals (lines); b) Hillslope values (points) with confidence intervals (lines).

Since there are relevant overlaps of confidence intervals, it is not possible to conclude which disinfectant actually displays the highest efficiency.

In particular, looking at the lower section of the curves for commercial PAA and PAA without HP (Figure 4.8), the overlapping is more pronounced: this means that, for lower doses, the difference between these two disinfectants is almost negligible. To better understand this, HP disinfection efficiency must be considered: HP curve shows how its disinfection efficiency is really weak (high EC_{50} value). For this reason, for low doses of commercial PAA – and, consequently, low concentration of HP – there is no relevant difference between commercial PAA and PAA without HP

behaviour; while, for high doses of commercial PAA, HP plays a major role and a disinfection efficiency difference is observed.

4.4. Inhibition-dose curve validation

Disinfection tests carried out with a different contact time value gave the results reported in Table 4.8.

Table 4.8 – Commercial PAA decay parameters and inhibition on E. coli.

Sample	Initial concentration (C_0, mg/L)	Contact time (t_c, min)	Oxidative demand (OD, mg/L)	Haas & Finch decay constant (k', min^{-1})	R^2	PAA dose (D, mg·min/L)	Inhibition
A	1.21	30	0.60	0.0101	0.99	16	61%
B	0.60	60	0.39	0.0055	0.91	11	43%
C	0.40	90	0.30	0.0062	0.89	7	21%

Inhibition-dose curve previously obtained for commercial PAA and these inhibition-dose pairs are compared and reported on the same graph in Figure 4.10, in order to verify if the curve well describes also the disinfection effect of doses obtained with a different contact time.

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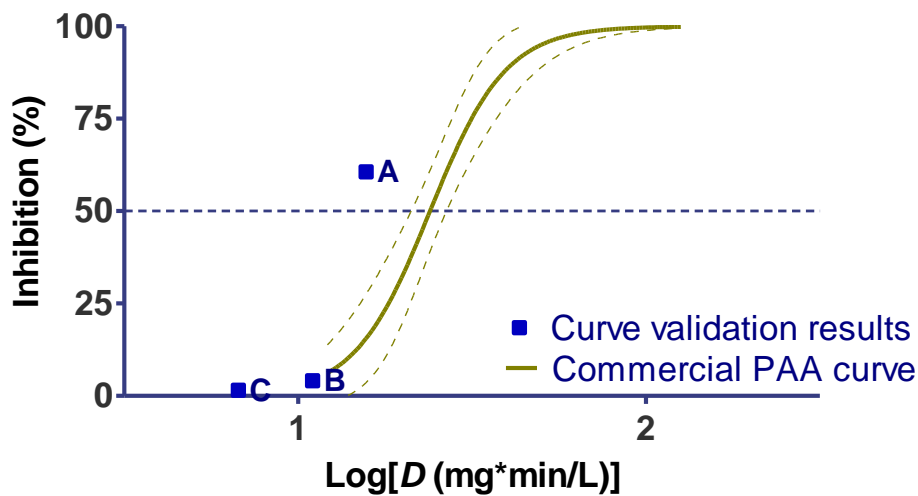
Figure 4.10 – Comparison between commercial PAA inhibition-dose curve and results
obtained with a different contact time.

Looking at the graph, points B and C result in a zone of the graph at very low inhibition values where the curve is not completely defined, but they seem to be included in the 95% confidence interval. Looking at point A, it is possible to observe that this dose value gave a higher inhibition compared to the curve prediction. Dose used to disinfect sample A was obtained with a contact time lower than that one used in the previous experiments (60 minutes). It is possible to suppose that, if the same dose value is obtained with a lower contact time and with a higher initial concentration value, the dose gives a higher inhibition effect. For this reason, concentration results to be the parameter that mostly affects the disinfection treatment efficiency (compared to contact time), as also reported in Azzellino et al. (2011).

With regards to PAA without HP, initial concentration – time pairs studied are reported in Table 4.9.

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Table 4.9 – PAA without HP decay parameters and inhibition on E. coli.

Sample	Initial concentration (C_0, mg/L)	Contact time (t_c, min)	Oxidative demand (OD, mg/L)	Haas & Finch decay constant (k', min^{-1})	R^2	PAA dose (D, mg·min/L)	Inhibition
D	1.69	30	0.89	0.013	0.97	20	68%
E	0.84	60	0.52	0.009	0.97	15	8%
F	0.56	90	0.36	0.015	0.95	10	4%

As done for commercial PAA, in Figure 4.11 the results obtained with a different contact time were compared to the curve previously obtained.
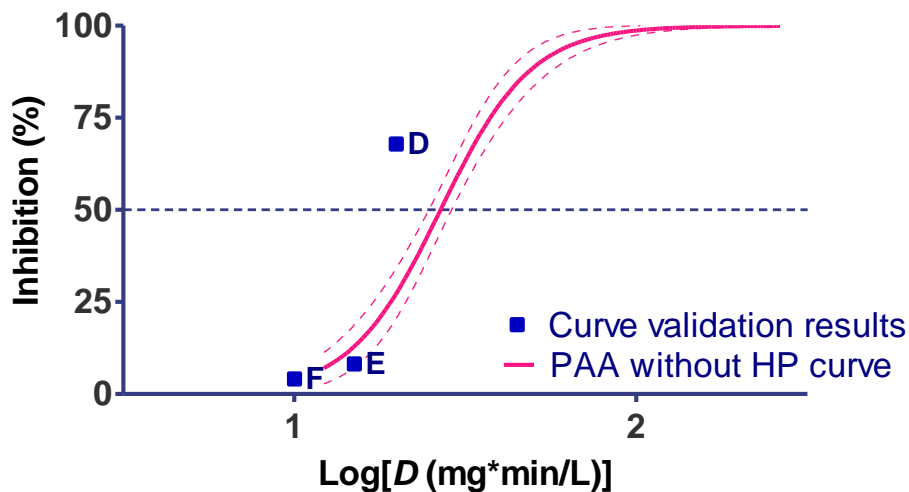
Figure 4.11 – Comparison between PAA without HP inhibition-dose curve previously obtained and new results.

The graph displays a situation similar to the previous one. Points E and F seem to be included in the 95% confidence interval. Looking at the sample D, same considerations done before can be made for PAA without HP.

A more accurate evaluation could be done repeating the experiments with two more replicates for each sample: this would give the chance to estimate a confidence interval for each point, comparing points' interval to the curve's interval.

4.5.   Catalase enzyme inactivation by peracetic acid

Half of *E. coli* population was inactivated in 60 minutes contact time using the following initial concentration values: 0.95 mg/L for commercial PAA and 1 mg/L for PAA without HP. To quench residual PAA (0.37 mg/L for commercial PAA and 0.34 mg/L for PAA without HP) a concentration of sodium thiosulphate of 2 mg/L was identified as the right amount of sodium thiosulphate to completely remove residual PAA with no interference on HP action in the second treatment.

Actually, for commercial PAA, at the end of the treatment, a residual amount of HP was present in water. Anyway, quenching it with catalase was not a possibility in this case: if this would be done, an inhibition of HP in the second treatment would occur. HP residual concentration after the first treatment was measured and it resulted around 0.01 mg/L. In the previous disinfection experiment, an initial concentration value of 43 mg/L was necessary to reach a 50% inhibition in 60 minutes: for this reason, the residual HP concentration measured was considered negligible and not sufficient to kill more *E. coli* and it was not quenched.

PAA decay is reported in Figure 4.12 for commercial PAA and PAA without HP, while decay parameters and inhibition obtained are reported in Table 4.10.
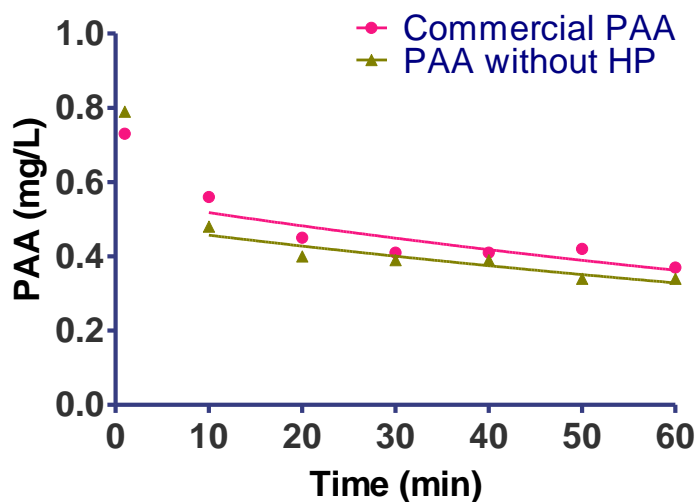
Figure 4.12 – Decay of commercial PAA and PAA without HP.

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Table 4.10 – Disinfectant decay parameters and inhibition on E. coli.

	Initial concentration (C_0, mg/L)	Contact time (t_c, min)	Oxidative demand (OD, mg/L)	Haas & Finch decay constant (k', min^{-1})	R^2	PAA dose (D, mg·min/L)	Inhibition
Commercial PAA	0.95	60	0.39	0.0071	0.75	27	54%
PAA without HP	1	60	0.51	0.0066	0.86	24	52%

On the samples obtained (that one disinfected by commercial PAA and that one disinfected by PAA without HP), the disinfection experiment by commercial HP was run testing five different concentration values. Decays are represented in Figure 4.13 and all decay parameters are reported in the Table 4.11 together with the results obtained in the previous disinfection experiments.

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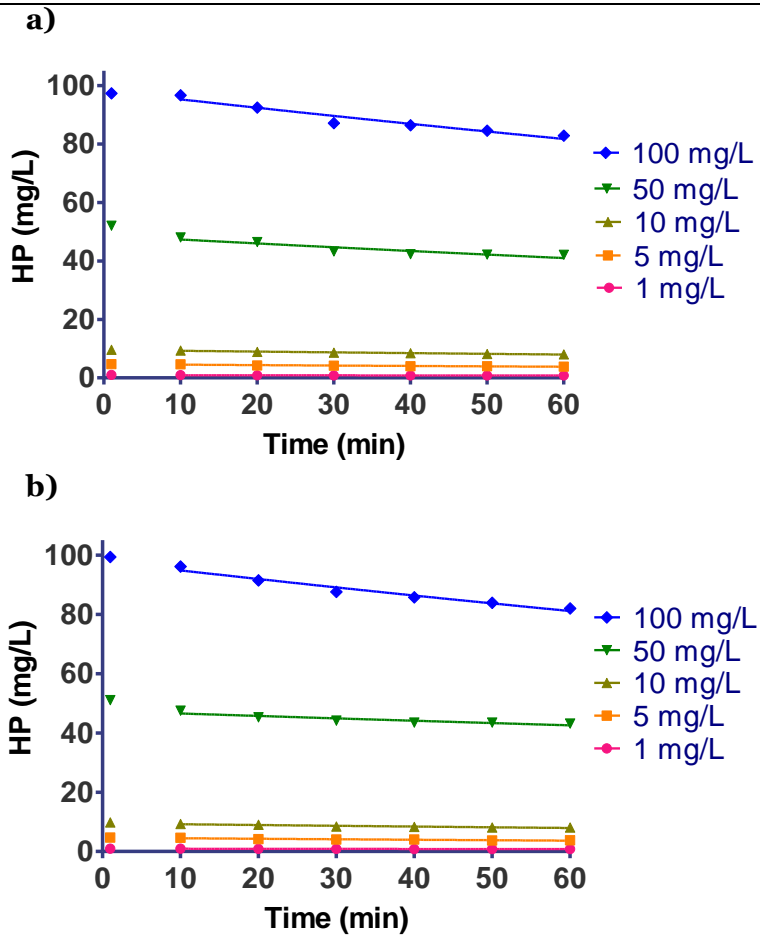
Figure 4.13 – HP decay in the samples previously disinfected by commercial PAA (a) and PAA without HP (b).

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Table 4.11 – Commercial HP disinfectant decay parameters.

	Initial concentration (C_0, mg/L)	Contact time (t_c, min)	Oxidative demand (*OD*, mg/L)	Haas & Finch decay constant (*k'*, min^{-1})	R^2	HP dose (*D*, mg·min/L)	Inhibition
Commercial HP, after commercial PAA	1	60	0.07	0.0027	0.89	52	14%
	5	60	0.33	0.0034	0.91	253	26%
	10	60	0.45	0.0030	0.98	524	37%
	50	60	1.31	0.0029	0.84	2683	82%
	100	60	1.77	0.0031	0.93	5383	94%
Commercial HP, after PAA without HP	1	60	0.05	0.0028	0.88	53	12%
	5	60	0.34	0.0040	0.88	248	27%
	10	60	0.53	0.0030	0.91	521	36%
	50	60	2.61	0.0018	0.82	2696	83%
	100	60	2.15	0.0031	0.96	5353	95%
Commercial HP (previous experiment)	10	60	3.98	0.0156	0.79	151	16%
	50	60	19.20	0.0150	0.81	893	51%
	100	60	38.09	0.0145	0.83	1800	74%
	200	60	75.90	0.0145	0.84	3609	93%

Looking at decay constant values, in this experiment HP displayed a lower decay. Wagner et al. (2002) reported that PAA is able to inactivate catalase enzyme; consequently, *E. coli* that survived the first treatment contained inactive catalase enzyme. Beyond catalase inactivation, an important difference between this experiment and the previous one must be considered: *E. coli* broth in the sample, this time, was probably mostly consumed during the first treatment with peracetic acid: after this, even if some by-products are probably still contained in water, HP should face less interference with *E. coli* broth. This can also be deduced looking at oxidative demand that results lower than before. For this reason, it is hard to say how much

catalase inactivation exactly contributes to this lower decay. This could be better observed repeating the previous disinfection experiment with commercial HP using centrifugated *E. coli* – without the growing broth – in the sample preparation, to exclude broth's oxidative consumption phenomena.

Results obtained were used to build other inhibition-dose curves to compare with the previous one obtained: they are represented in Figure 4.14.
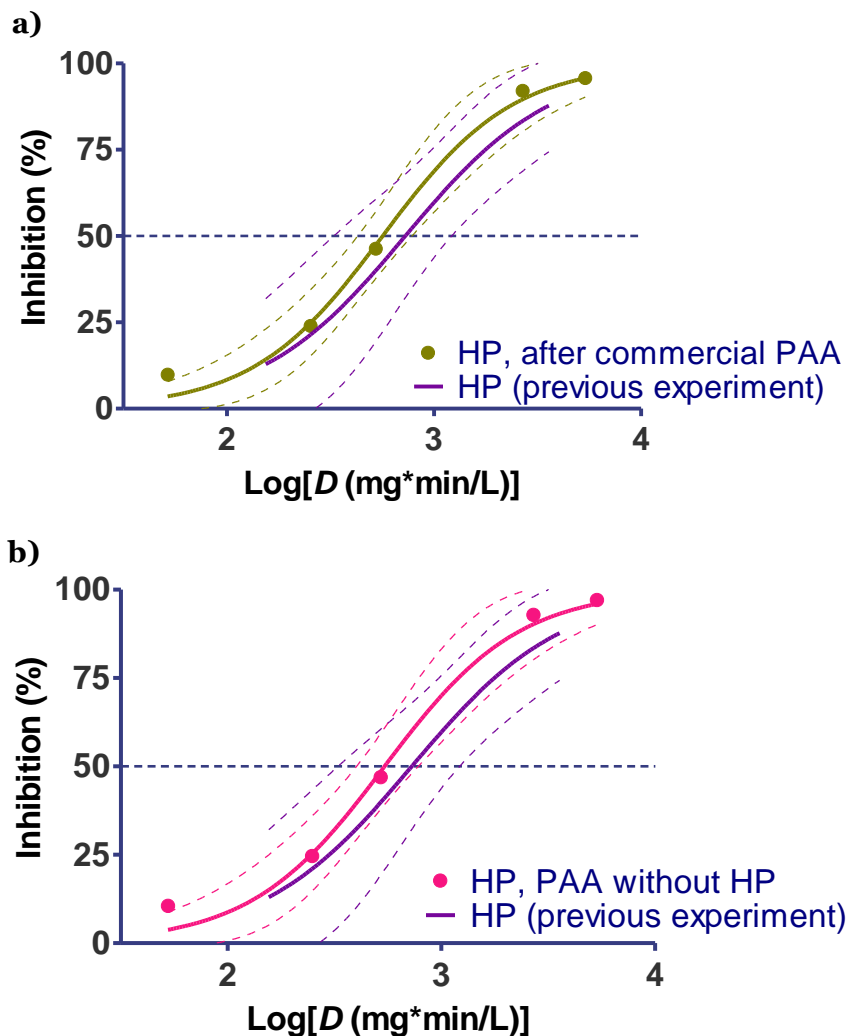
Figure 4.14 – Disinfection efficiency of commercial HP after commercial PAA (a) and after PAA without HP (b) compared to commercial HP used alone.

It is possible to note that the two curves obtained after preliminary treatment with PAA are characterized by a slightly higher slope, compared to the curve obtained from the commercial HP experiment previously carried out. This could be due to the inactivation of catalase enzyme and a consequently variation of the *E. coli* microbial

response that show less resistance to HP action. Anyway, even in this case, the overlap of confidence intervals doesn't allow to come to a conclusion about what was observed: a more numerous set of disinfection tests would give a more robust result and a more robust explanation to what was observed.

4.6. Colorimetric assay: definition of measurement conditions and results

The enzymatic substrate X-Gal presented the absorbance spectrum reported in Figure 4.15.
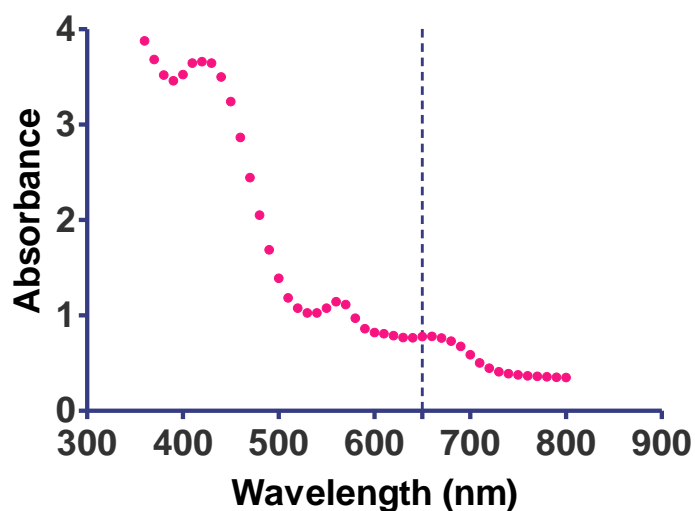
Figure 4.15 – Absorbance spectrum of 10⁶ CFU/mL E. coli with LTB broth, SDS and X-Gal.

An absorbance peak can be notice around at 650 nm. In fact, X-Gal displays (namely, reflects) blue colour and blue light has a wavelength of about 480 nm: according to colour theory, the complementary colour absorbed has a wavelength of 600-650 nm.

The analysis of the 96-well microplate represented in Table 3.5 was run measuring absorbance at 600, 650 and 660 nm, for the wells containing X-Gal, and at 520, 530 and 540 nm, for the wells containing Red-Gal. Absorption kinetics obtained are represented in Figure 4.16 for X-Gal and Figure 4.17 for Red-Gal.

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Figure 4.16 – Absorption kinetic obtained using X-Gal: a) E. coli 10^7 CFU/mL, absorbance at 600 nm; b) E. coli 10^7 CFU/mL, absorbance at 650 nm; c) E. coli 10^7 CFU/mL, absorbance at 660 nm; d) E. coli 10^6 CFU/mL, absorbance at 600 nm; e) E. coli 10^6 CFU/mL, absorbance at 650 nm; f) E. coli 10^6 CFU/mL, absorbance at 660 nm; g) E. coli 10^5 CFU/mL, absorbance at 600 nm; h) E. coli 10^5 CFU/mL, absorbance at 650 nm; j) E. coli 10^5 CFU/mL, absorbance at 660 nm.
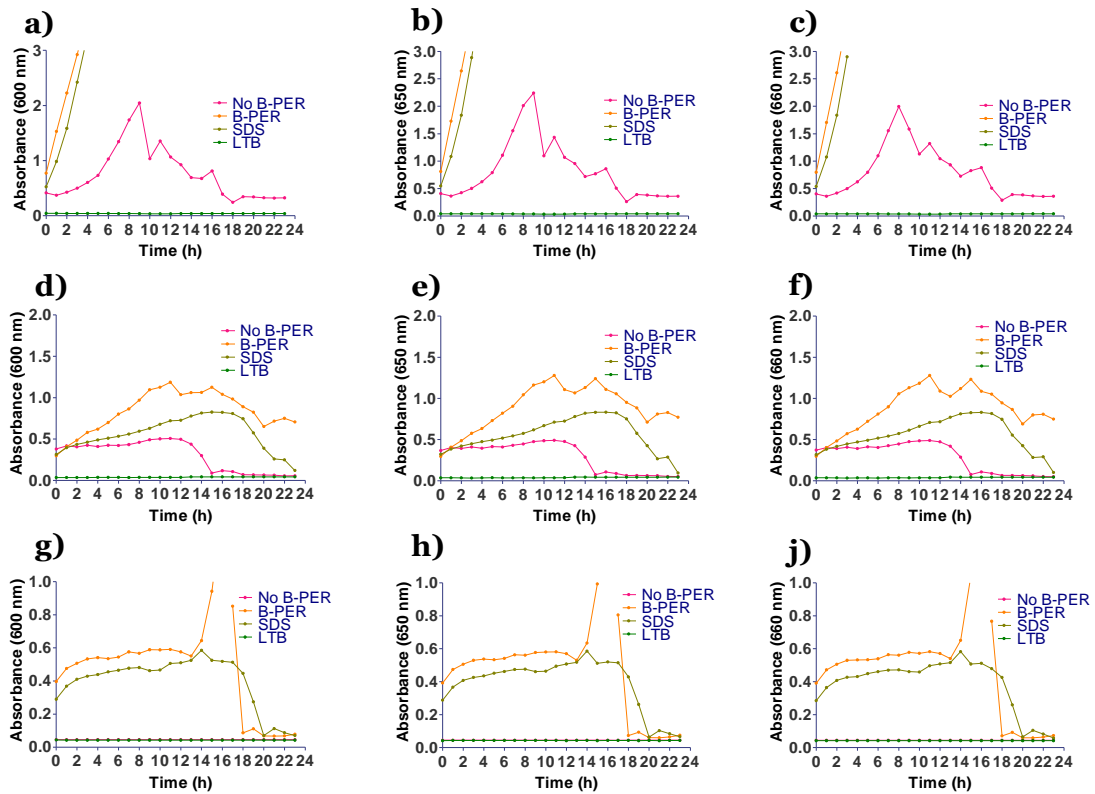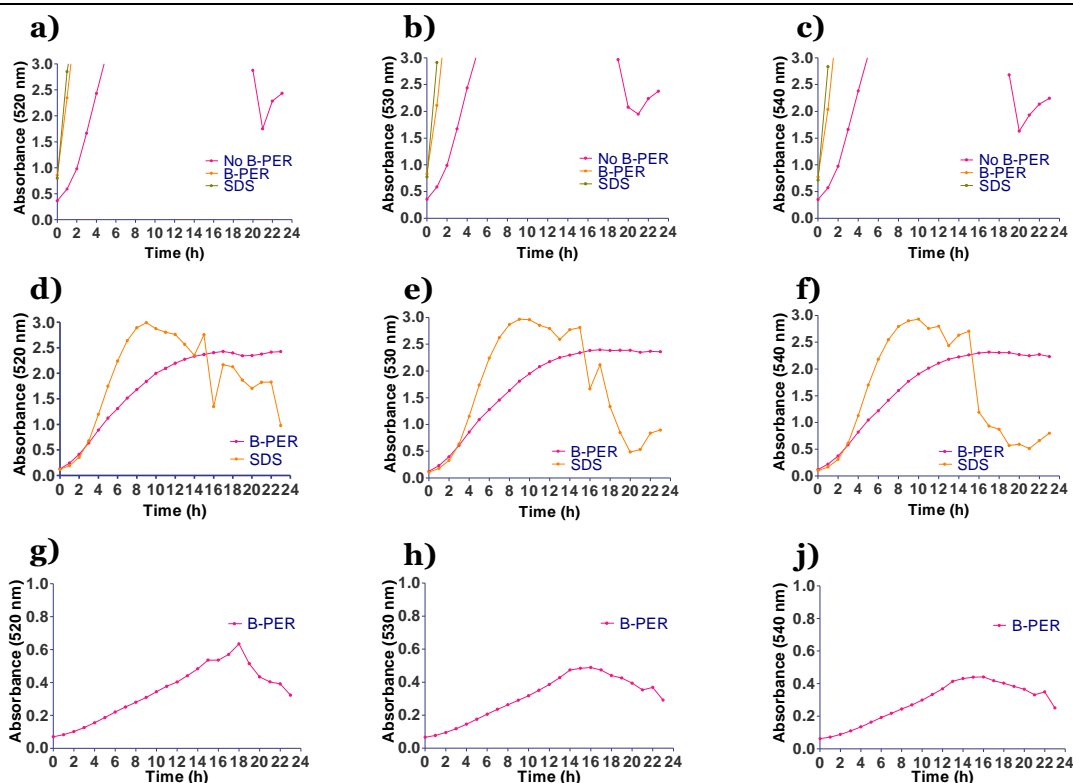
Figure 4.17 – Absorption kinetic obtained using Red-Gal: a) E. coli 10^7 CFU/mL, absorbance at 520 nm; b) E. coli 10^7 CFU/mL, absorbance at 530 nm; c) E. coli 10^7 CFU/mL, absorbance at 540 nm; d) E. coli 10^6 CFU/mL, absorbance at 520 nm; e) E. coli 10^6 CFU/mL, absorbance at 530 nm; f) E. coli 10^6 CFU/mL, absorbance at 540 nm; g) E. coli 10^5 CFU/mL, absorbance at 520 nm; h) E. coli 10^5 CFU/mL, absorbance at 530 nm; j) E. coli 10^5 CFU/mL, absorbance at 540 nm .

Red-Gal displayed higher absorbance value than X-Gal (looking at Figure 4.17 compared to Figure 4.16) and, for this reason, Red-Gal was preferred to X-Gal. Looking at the graphs related to Red-Gal (Figure 4.17), cell lysing agent SDS displayed higher absorbance value compared to B-PER™ so SDS was preferred to B-PER™. With regards to the wavelength, a peak was observed at 530 nm. Looking at Figure 4.17, when *E. coli* concentration is high (10^7 CFU/mL), absorbance measurement must be done within 2 hours. For lower concentration value (as 10^6 CFU/mL, Figure 4.17), absorbance reaches high values after 8 hours.

Resuming:

- Red-Gal will be used as chromogenic enzymatic substrate;
- SDS will be used as cell lysing agent;
- the absorbance will be measured at 530 nm;
- the measurement will be made at 2 h (for high *E. coli* concentration) and at 8 h (for medium – low *E. coli* concentration).

These results gave the chance to build a calibration curve, to obtain an *E. coli* concentration value (CFU/mL) starting from the absorbance value measured with the reagents and under the conditions defined.

To build the calibration curve, a solution of *E. coli* in LB broth was prepared and, from this, a dilution series was made. *E. coli* concentration was estimated by plate count method and reported in Table 4.12.

At the same time, 100 μL were taken from 10^1 - 10^3 diluted sample range and analysed by the colorimetric assay. This high concentration range was studied since the idea is to use the colorimetric method on the original not diluted sample, as a very fast concentration detection. A well with reagents and no *E. coli* was also analysed: absorbance value measured was 0.067 after 2 hours and 0.07 after 8 hours. These values must always be registered and used to correct the absorbance values obtained from *E. coli* (also reported in Table 4.12)

Table 4.12 – Absorbance values measured after 2 hours and 8 hours.

Dilution factor	*E. coli* concentration (CFU/mL)	Absorbance value corrected with reagents back colour (2 h)	Absorbance value corrected with reagents back colour (8 h)
10^1	$3{,}36 \cdot 10^8$	2,7895	
10^2	$3{,}36E \cdot 10^7$	0,119	1,683
$3 \cdot 10^2$	$2{,}35 \cdot 10^7$	0,0785	1,0115
$6 \cdot 10^2$	$1{,}34 \cdot 10^7$	0,043	0,411
10^3	$3{,}36 \cdot 10^6$	0,008	0,063

Two calibration curves were obtained and represented in Figure 4.18.

a)

b)
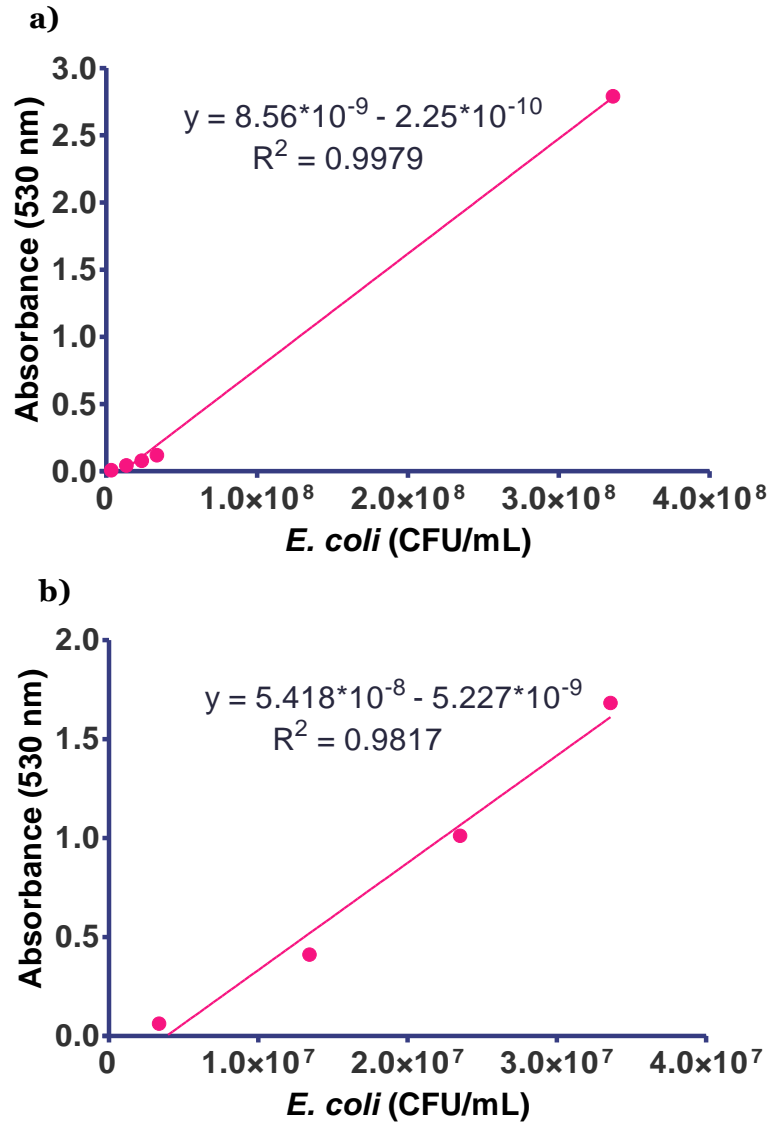
Figure 4.18 – Absorbance-concentration calibration curves obtained after 2 hours (a) and after 8 hours (b) incubation.

Looking at the first graph in Figure 4.18, some more measurements between $1 \cdot 10^8$ and $3 \cdot 10^8$ CFU/mL should be included in the analysis to better evaluate if there actually is a linear trend.

At the end of three experiments carried out, one with commercial PAA, one with PAA without HP and one with commercial HP, absorbance was measured, as described before, in the blank and in the disinfected samples. Absorbance observed are represented in Figure 4.19.

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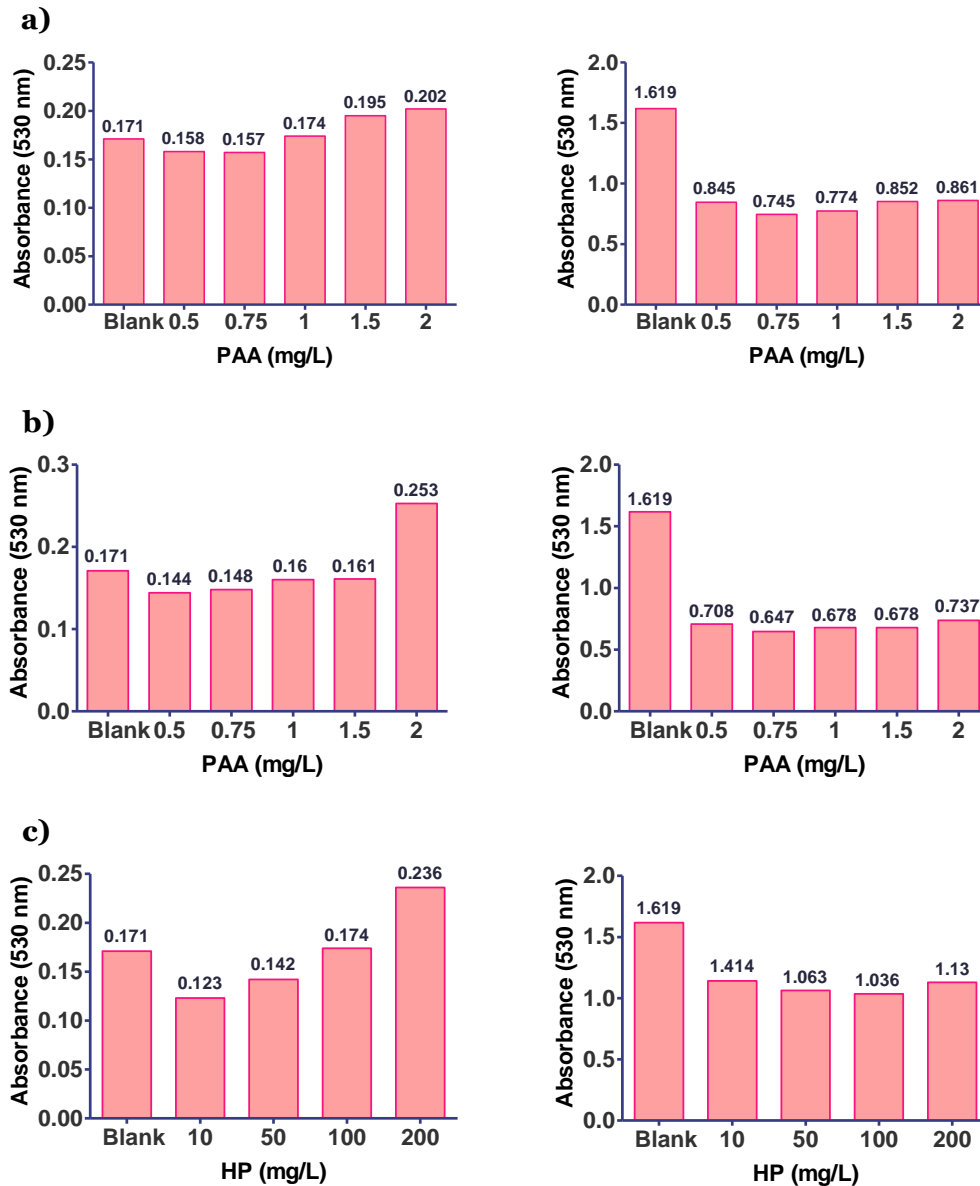
Figure 4.19 - Absorbance values of the samples disinfected by commercial PAA (a), PAA without HP (b) and commercial HP (c), measured after 2 hours (graphs on the left) and after 8 hours (graphs on the right).

A negative trend of absorbance value from the blank to the sample with the higher disinfectant concentration was expected. At 2 hours, this was not observed. At 8 hours, the blank displayed a higher absorbance value than the other samples, but even here, the disinfected samples didn't display a negative absorbance trend.

Residual *E. coli* concentration measured by plate count method was compared to the corresponding *E. coli* concentration value obtained by the colorimetric assay, using both the calibration curves built. This comparison was made only for the samples where *E. coli* concentration – calculated by plate count – was included in the

concentration range used to calibrate the curves. Results are reported in Table 4.13, Table 4.14 and Table 4.15 and compared using a scatter plot (Figure 4.20).

Table 4.13 –Concentration estimated by plate count and by colorimetric assay for the samples disinfected by commercial PAA.

Commercial PAA initial concentration (mg/L)	*E. coli* residual concentration from plate count (CFU/mL)	*E. coli* residual concentration from colorimetric assay (2 h) (CFU/mL)	*E. coli* residual concentration from colorimetric assay (8 h) (CFU/mL)
Blank (0 mg/L)	$2.19 \cdot 10^7$	$1.20 \cdot 10^6$	$9.13 \cdot 10^6$
0.5	$1.94 \cdot 10^7$	$1.11 \cdot 10^6$	$4.77 \cdot 10^6$
0.75	$1.50 \cdot 10^7$	$1.10 \cdot 10^6$	$4.20 \cdot 10^6$
1	$9.73 \cdot 10^6$	$1.22 \cdot 10^6$	$4.37 \cdot 10^6$

Table 4.14 - Comparison between concentration estimated by plate count and concentration estimated by colorimetric assay for the samples disinfected by PAA without HP.

PAA without HP initial concentration (mg/L)	*E. coli* residual concentration from plate count (CFU/mL)	*E. coli* residual concentration from colorimetric assay (2 h) (CFU/mL)	*E. coli* residual concentration from colorimetric assay (8 h) (CFU/mL)
Blank (0 mg/L)	$2.22 \cdot 10^7$	$1.20 \cdot 10^6$	$9.13 \cdot 10^6$
0.5	$2.03 \cdot 10^7$	$1.01 \cdot 10^6$	$3.99 \cdot 10^6$
0.75	$1.67 \cdot 10^7$	$1.04 \cdot 10^6$	$3.65 \cdot 10^6$
1	$1.07 \cdot 10^7$	$1.13 \cdot 10^6$	$3.82 \cdot 10^6$

Table 4.15 - Comparison between concentration estimated by plate count and concentration estimated by colorimetric assay for the samples disinfected by commercial HP.

Commercial HP initial concentration (mg/L)	*E. coli* residual concentration from plate count (CFU/mL)	*E. coli* residual concentration from colorimetric assay (2 h) (CFU/mL)	*E. coli* residual concentration from colorimetric assay (8 h) (CFU/mL)
Blank (0 mg/L)	$2.19 \cdot 10^7$	$1.20 \cdot 10^6$	$9.13 \cdot 10^6$
10	$1.84 \cdot 10^7$	$8.66 \cdot 10^5$	$6.44 \cdot 10^6$
50	$1.07 \cdot 10^7$	$9.99 \cdot 10^5$	$6.00 \cdot 10^6$
100	$5.65 \cdot 10^6$	$1.22 \cdot 10^6$	$5.84 \cdot 10^6$

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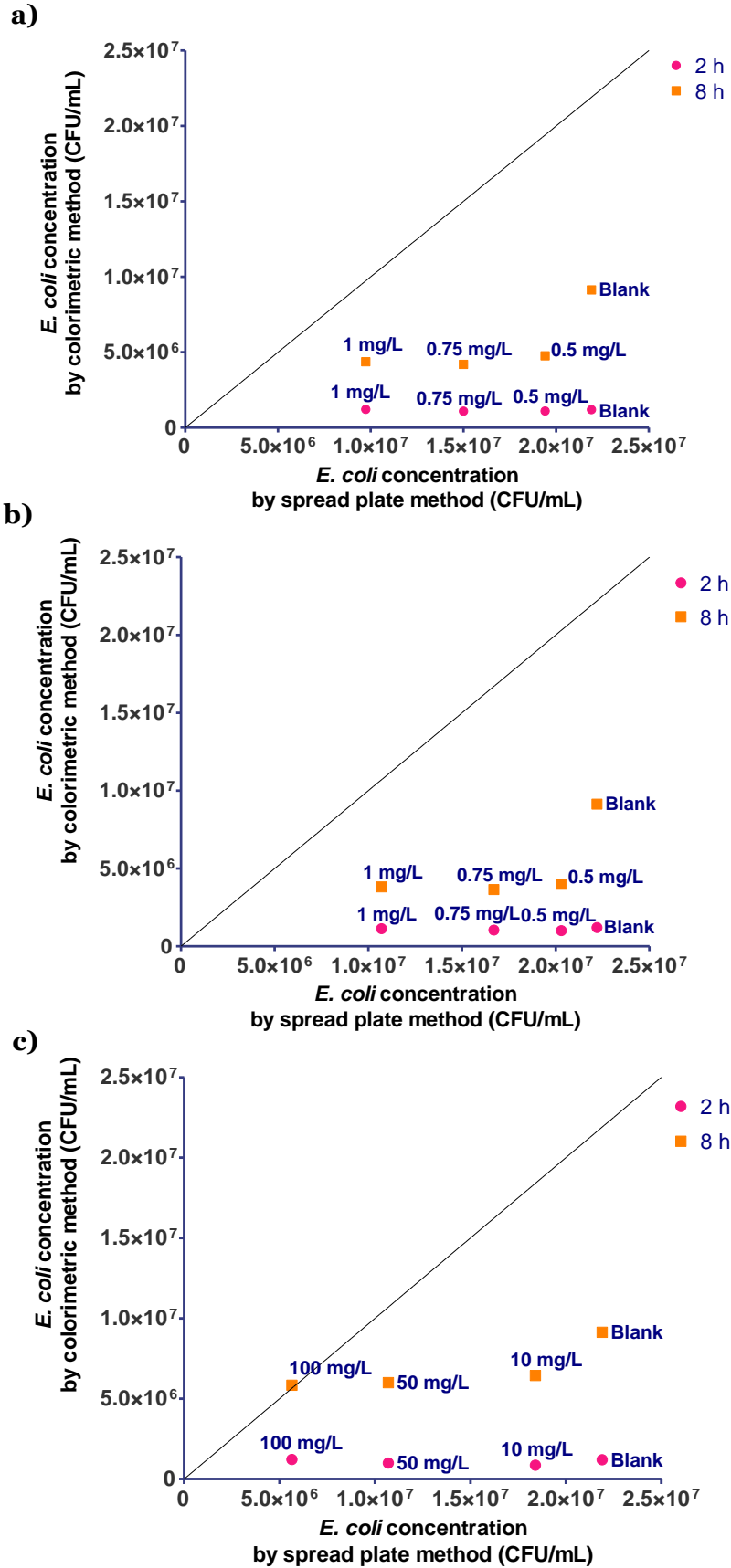*inactivation in wastewater*

Figure 4.20 - Scatter plot for disinfection experiment with: a) commercial PAA, b) PAA without HP; c) commercial HP.

Looking at the *E. coli* concentration values calculated by the colorimetric assay, they didn't approximate the results obtained by the plate count method. This is clearly observed also looking at the scatter plots.

Since the traditional plate count method can be considered very reliable, results suggest that this colorimetric assay still needs more studies and optimization before being adopted.

5. Conclusions and future developments

A new solution of PAA without HP was obtained by HP selectively consumption with potassium permanganate. Solution stability was studied and the results suggest to use it within 2 hours after preparation, to have a solution rich of PAA and with a negligible HP amount.

Disinfectant decay during the disinfection experiments was observed and modelled by Haas & Finch kinetic model. The initial disinfectant consumption could be reduced using a centrifugated *E. coli* solution for the preparation of the samples, without growing broth rich of oxidable compounds. Choose a centrifugated *E. coli* solution would also be relevant to better evaluate the catalase inactivation effect on HP, with less interferences.

Disinfection efficiency was assessed, observing *E. coli* inhibition under different doses action and obtaining an inhibition-dose curve for each disinfectant considered. Results show a disinfection efficiency close to the commercial PAA one. The new disinfectant would give the advantage to avoiding all the problems related to the commercial solution usage, as residues in water and high toxicity effect due to the presence of HP, and obtaining the same disinfection efficiency. Anyway, more disinfection tests would be useful to obtain a more statistically robust inhibition-dose curve.

The good disinfection efficiency of the new disinfectant could not be sufficient to guarantee a possible applicability of this disinfectant in a real water treatment plant: it must be considered that the disinfectant preparation procedure here presented entails the need of many tanks (for commercial PAA, potassium permanganate and pH adjustment reagents, in addition to a filtration device): all these practical aspects, as the related economical aspects, seems not to permit an applicability of this disinfectant, at the current conditions.

As for the colorimetric assay presented for *E. coli* concentration measurement, by now the method didn't display the results expected and it's not ready to substitute the spread plate method. Possible additional studies to correct the method could be focused on the actual ability of the method to distinguish between dead and alive *E. coli* and any possible interferences between reagent used for the colorimetric assay and all the reagents used in the disinfection process.

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