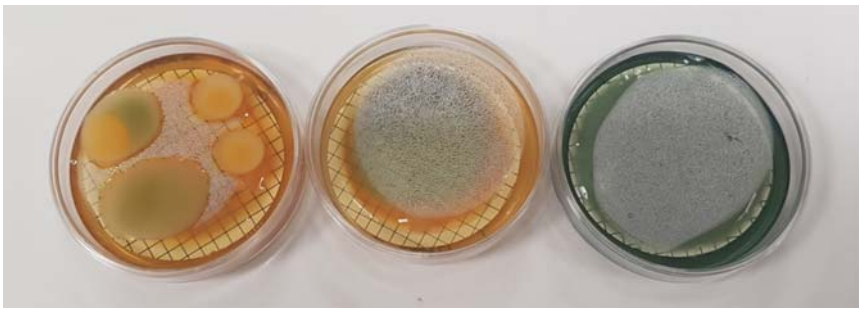
Chapter 2

Fecal bacteria regrowth and viability after disinfection with PFA



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2.1 INTRODUCTION

The bactericidal efficacy of PFA against fecal contamination control germs is now well documented and has been demonstrated in this study (see Chapter 1). However, when using a tertiary disinfection treatment, it is important to evaluate the regrowth potential of the germs contained in the treated water. This is especially critical when wastewater reuse applications are being considered. Bacterial inactivation can in fact lead to the production of viable non-cultivable bacteria as an initial response, and various repair processes can then renew the ability to reproduce after a certain length of time (usually several hours). This phenomenon has been studied with well-known disinfection processes and used at the industrial scale for several years (UV-C, chlorination, ozone) or with advanced oxidation processes (e.g., photocatalysis, UV/H_2O_2).

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The phenomenon of cellular repair has been demonstrated in several studies during the UV-C disinfection of wastewater (Bohrerova & Linden, 2007; Bohrerova Zuzana *et al.*, 2015). Such a disinfection process seems to depend on the UV dose applied and moreover appears to be reached 24 hours after irradiation (Oguma *et al.*, 2002). Several repair mechanisms have been identified, some in the dark while others photoinitiated (Shang *et al.*, 2009).

Similarly, the simple ozonolysis of a secondary effluent leads to the regrowth of *E. coli* and coliforms (Malvestiti & Dantas, 2018). The presence of hydroxyl radicals (generated from coupling O_3/H_2O_2 or UV-C/H_2O_2) seems to mitigate this phenomenon. On the other hand, the presence of OH inhibitors (nitrates, carbonates) would seem to favor it. Some studies mention a synergistic effect when ozone is introduced in combination with photocatalysis (Mecha *et al.*, 2017): while ozone and photocatalysis used separately lead to bacterial regrowth, their combined use seems to prevent it. Tertiary treatment by photocatalysis alone (TiO_2/UV-A) does not prevent regrowth on various heterotrophic microorganisms, including *E. coli* and enterococci (Biancullo *et al.*, 2019). Several studies suggest that bacterial regrowth is slower during H_2O_2/sun disinfection than with chlorination (Fiorentino *et al.*, 2015; Giannakis *et al.*, 2015; Li *et al.*, 2013).

The chemical disinfection of secondary effluents is another widely employed process. In the case of chlorination, bacterial regrowth can occur, especially when low doses are applied (Li *et al.*, 2013), and is heavily dependent on the microorganisms under study. In the case of disinfection with peracids, recent results with peracetic acid (PAA) have been published; Zhang *et al.* (2019b) determined the minimum dose of PAA to prevent the regrowth of planktonic cells. This dose is dependent on the amount of dissolved organic matter (DOM) present in the wastewater. A comparison with chlorination shows that for this microorganism, chlorination requires lower doses yet is much more sensitive to the presence of DOM. Zhang *et al.* (2019a) also showed that while both PAA and chlorine prevented bacterial regrowth, they were inactive against a plasmid incorporated into the DNA of bacteria. To the best of our knowledge, no data have been published with PFA concerning these investigations; consequently, the objectives of this chapter are twofold: (1) to study the potential for regrowth of fecal contamination control germs in a performic acid-treated environment; and (2) to provide information on the effect of performic acid on these germs in order to explain the effect being observed.

2.2 EXPERIMENTAL DESCRIPTION

2.2.1 Fecal bacteria regrowth after PFA disinfection

The experimental procedure used to study bacterial regrowth is summarized in Figure 9 (Steps 1 and 2.1); it has been adapted from previous studies (Fiorentino *et al.*, 2015; Zhou *et al.*, 2017). In Step 1, two bacterial inocula (*E. coli* and intestinal enterococci) were prepared from SEV WWTP discharge water using

restrictive agar media. Once these strains had been isolated, they were stored at −20°C in a mixture of 30% glycerol for 70% culture broth.

The bacteria were first cultured from agar and then from a liquid medium once the strain had developed sufficiently, resulting in a solution in a decreasing exponential phase whose bacterial concentration could be estimated by an absorbance measurement at 600 nm (0.1 absorbance unit = 10^7 bacteria/mL). The bacterial suspension was then diluted to a realistic bacterial concentration compared to the usual concentrations of the SEV discharge (Rocher & Azimi, 2016): *E. coli* between 10^5 and 10^6 MPN/100 mL and intestinal enterococci between 10^4 and 10^5 MPN/100 mL.

This step of the experimental procedure allows working at controlled concentration of bacteria composed of pure strains but coming from real SEV discharge samples.

The kinetic monitoring of bacterial regrowth was performed by either microplate or agar plate enumeration. Agar analyses were preferred on samples treated with PFA since this method has a lower quantification level (LOQ <3 NPP/100 mL). Microplates were used for untreated control samples with bacterial concentrations greater than 10^3 NPP/100 mL. The initial point was established by both methods in order to verify that they yielded equivalent results.

In Step 2.1, a bacterial inoculum containing *E. coli* and intestinal enterococci was introduced into autoclaved discharge water in order to achieve realistic concentrations. Some of this bacterial suspension was treated with PFA at a treatment rate of 0.8 ppm for 10 min (C × t of 8 ppm.min) and another part was used as a control. The sample treated with PFA was then mixed with autoclaved Seine River water (90/10 volume mixture) to best simulate actual mixing of the SEV discharge and Seine River water at the WWTP discharge point (unfavorable case of the Seine at a low water flow rate). A cultivable bacteria count according to standard methods was carried out at different times after disinfection (up to 24 hours) on both samples.

Two experimental conditions were applied during the tests as follows:

- The first considered a regrowth at room temperature under laboratory light at constant temperature (26°C). This experiment was carried out in triplicate.
- The second condition considered a regrowth under irradiation simulating the solar spectrum (visible lamps + UV-A lamps) at room temperature (26°C), with the experiment being performed in duplicate.

These experiments were carried out in a temperature-controlled chamber equipped with four fluorescent tubes (either three tubes emitting in the visible light and one tube emitting in UV-A, or two tubes emitting in the visible spectrum and two emitting in UV-A). The distance of the tubes from the sample was adjusted such that the light power was comparable to that of sunlight (i.e., between 4 and

6 mW/cm^{-2} in the UV-A range). Measurements were controlled by a spectroradiometer (CAS 120, Instrument Systems). In the two experiments, the UV-A light power was 4 and 8 mW/cm^2.

2.2.2 Viability tests

Two types of analyses were conducted for this part of the study: (1) bacterial counting of samples before disinfection by the methods described above; and (2) determination of bacterial viability before and after disinfection by cytometry (cytometric counting).

For the tests carried out on untreated samples, both methods produced comparable results (at uncertainties close to those of the method, i.e., 20%). Only untreated samples were monitored by both methods.

The count performed by flow cytometry was performed on a BD Accuri C6 device (BD Biosciences – Becton Dickinson, France SAS, Grenoble, France); this apparatus is equipped with a scatter detector and three fluorescence detectors (WL1, WL2 and WL3).

The viability analyses were carried out using the BD Cell viability kit provided by BD Biosciences. This kit contains various solutions, namely:

- a 'rainbow balls' solution to ensure a response from the device's various detectors;
- a calibration bead solution for a quantitative determination of the analyzed objects;
- an orange thiazole (OT) solution (500 µL at a concentration of 42 µmol/L in DMSO); this compound responds to both WL1 and WL2 detectors and colors all cells;
- a solution of propidium iodide (PI) (500 µL at a concentration of 4.3 mmol/L in water); this compound responds to WL3 and colors the lysed cells.

A typical experiment necessitates several control cytometric analyses:

(1) 500 µL of physiological water + rainbow beads (instrument validation);
(2) 500 µL of physiological water (matrix validation);
(3) 500 µL of physiological water + calibration beads (validation quantification);
(4) 500 µL of bacterial suspension (concentration around 10^5 bacteria/mL) without dyes (sample control);
(5) 500 µL of bacterial suspension (around 10^5 bacteria/mL) with fluorescent PI and TO dyes (marking control, qualitative analysis);
(6) 500 µL of bacterial suspension (around 10^5 bacteria/mL) with PI+TO fluorescent dyes + calibration beads (sample measurement, qualitative and quantitative analyses);
(7) 500 µL of bacterial suspension + NaN_3 + PI+TO fluorescent dye (lysed bacterial control).

After these controls, the sample analysis by cytometry could be achieved by adding 5 µL of each dye and 50 µL of calibration beads to 500 µL of each sample (bacterial suspension treated with 0.8 ppm performic acid and an untreated suspension). The BD Accuri™ C6 Plus software interface provided quick access to the collection, analysis and statistics functions. The raw data could also be analyzed using the R program 'rattle package' (Williams, 2011).

The interpretation of results is obtained by plotting WL1 and WL3 fluorescence intensities on a 2D graph. Given the emission properties of the dyes, the best distinction between populations is derived by monitoring the fluorescence intensities on WL1 and WL3:

- a high intensity on WL1 and a low intensity on WL3 corresponds to viable bacteria;
- an increase in the WL3 intensity corresponds to bacteria that have already been lysed.

2.3 RESULTS

2.3.1 Regrowth kinetics

The regrowth results, under dark conditions of samples reconstituted from real autoclaved matrices doped with isolated *E. coli* (high) and intestinal enterococci (low) germs and then disinfected with 0.8 ppm PFA, are shown in Figure 10. This experiment was carried out in triplicate with different initial matrices and identical pure strains. At the initial time ($t = 0$), the three histograms correspond to the initial concentrations of fecal bacteria in the non-disinfected control samples. At the other times ($t = 1$, 3, 6, 8 and 24 h), the three histograms on the left part of the figure correspond to the non-disinfected control samples, while the three histograms on the right part of the figure correspond to these same samples yet disinfected with PFA (8 ppm.min). Each color thus corresponds to the same experiment.

For *E. coli* and intestinal enterococci, the concentrations of the three untreated controls (July 17, 2018, July 10, 2018 and Sept 25, 2018) increase over 24 hours, mostly after 3 hours. This result indicates that the conditions applied during the experiments are indeed favorable to bacterial growth in the controls. This phenomenon is less obvious for intestinal enterococci.

Adding 0.8 ppm PFA induces results systematically lower than LOQ: no cultivable bacteria are quantified even after 24 hours, which proves the absence of regrowth. It should be noted that the treatment efficacy on *E. coli* tends to be quite good, as well observed in the present case. Studies conducted at other sites have yielded comparable results (Ragazzo *et al.*, 2013).

For intestinal enterococci, the treatment effectiveness of 0.8 ppm PFA is lower: for both the July 10, 2018 and Sept 25, 2018 experiments, 70 and 80 CFU/100 mL

were quantified after disinfection, respectively; however, no increase over 24 hours was observed experimentally. The concentration does tend to decrease slightly for the July 10, 2018 experiment (from 70 to 35 between 1 and 24 h) and remain stable for the Sept 25, 2018 experiment (80 CFU/100 mL). The July 17, 2018 experiment results are similar to *E. coli*, i.e. below the LOQ until 24 hours. The conclusion is thus the same as for *E. coli*: no regrowth of enterococci after PFA treatment even though the disinfection efficacy is lower, as previously demonstrated in Chapter 1 and by other studies (Ragazzo *et al.*, 2013). To the best of our knowledge, these are the first results describing regrowth experiments involving the PFA disinfection of wastewater. Some of the recent results published for PAA disinfection have shown inhibited regrowth of planktonic and biofilm bacteria after disinfection (Zhang *et al.*, 2019a).

These same experiments were repeated with simulated solar irradiation. Sunlight was simulated by a set of lamps emitting in both the visible and the UV-A wavelength. Several studies have revealed that light can stimulate cell repair processes after disinfection treatment and moreover that bacterial regrowth can then be observed (Giannakis *et al.*, 2016).

The UV-irradiated bacterial regrowth results of samples reconstituted from real autoclaved matrices doped with isolated *E. coli* (high) and intestinal enterococci (low) germs, and then disinfected with 0.8 ppm PFA, are shown in Figure 11.

This experiment was carried out in duplicate with different initial matrices and identical pure strains. At the initial time (i.e., $t = 0$), the two histograms correspond to the initial concentrations of fecal bacteria in the non-disinfected control samples. At the other times ($t = 2$, 4 and 6 h), the two histograms on the left part of the figure still correspond to the non-disinfected controls, while the two histograms on the right part correspond to the same samples but disinfected with PFA (8 ppm.min). Each color thus corresponds to the same experiment. Note that for one experiment (Feb 10, 2018, gray), the UV-A light power is 8 $mW \cdot cm^{-2}$ while for the other the UV-A light power is 4 $mW \cdot cm^{-2}$ (Oct 16, 2018, black).

UV-A exerts a major short-term effect (<2 h) on *E. coli* (total disinfection). The simulated solar radiation had a sublethal effect on *E. coli* populations over a brief time exposure by causing a loss of culturability and the formation of viable yet nonculturable cells (Muela *et al.*, 2000). The presence of colored DOM in the water matrix may also favor the formation under irradiation of Reactive Oxygen Species (e.g., singlet oxygen, superoxide anion), whose bactericidal properties are well known (Häder *et al.*, 2015; Maraccini *et al.*, 2016).

The effects of light on enterococci are less apparent, but they display greater resistance to UV-A irradiation. A latency period of 4 hours seems to be required

for the UV-A power of 8 $mW \cdot cm^{-2}$, while a continuous decrease is observed for irradiation at 4 $mW \cdot cm^{-2}$.

After adding PFA, the bacterial concentration remains below the limit of quantification after 4 hours of irradiation. The increase in concentration after 2 hours in one experiment remains unexplained to this day and could be due to an external contamination. In any case, regrowth was never observed in this study either in the dark or under irradiation.

2.3.2 Fecal bacteria viability after disinfection with PFA

The objective of these experiments has been to provide additional information regarding the actual effect of PFA on fecal bacteria and, in particular, to better understand their viability after disinfection. The testing method employed, that is, flow cytometry, is applicable to monitoring the effect of a biocidal treatment or comparing various biocidal treatments (Whitton *et al.*, 2018).

Figure 12 presents the results obtained on a suspension of *E. coli* germs treated with 0.8 ppm PFA for 10 min; the initial concentration of *E. coli* was approximately 10^5 NPP/100 mL. Figure 12 shows the flow cytometry maps derived in the single test performed, both before and after disinfection, while Figure 13 provides these same results as percentages of detected bacteria, which were classified as dead, alive or damaged by the methodology used.

In Figure 12, the illustrations before disinfection (left) show the bacterial population of *E. coli* before treatment. As anticipated for a population composed mainly of living bacteria, high fluorescence intensity in WL1 and low fluorescence intensity in WL3 are observed. After treatment with 0.8 ppm PFA (right part of the figure), a low fluorescence intensity in WL1 and a high intensity in WL3 can be noted, which means that the *E. coli* are mostly dead (99.3% dead, see Figure 13). Nearly identical results have been obtained from a suspension of enterococci (99.6% dead bacteria after PFA treatment).

Figures 12 and 13 reveal that PFA causes irreversible damage to fecal contamination indicator bacteria cells and moreover that such cells are not in a 'viable non-cultivable' (VNC) state after disinfection. It does appear that the entire population of *E. coli* and intestinal enterococci are lysed after disinfection at 8 ppm.min PFA, whereas they were for the most part alive or damaged before disinfection. These results tend to agree with the regrowth experiments previously described in this chapter and those obtained with PAA, where no regrowth phenomena were observed with these bacteria (Antonelli *et al.*, 2013). In the case of PAA, several observations recorded by electron microscopy indicate that the cells are damaged and holes appear in the cell centers (Zhang *et al.*, 2019b); these results are attributed to reactions between PAA and cellular components. As for PFA, its ability to oxidize disulfide links (S-S) in cysteic acid RSO_3^- and the sulfide function of methionine to methionine sulfone in protein residues may help explain its irreversible effect on bacteria (Voet & Voet, 1995).

Key points

- PFA allows for stable disinfection of wastewater, with no regrowth after 24 hours without irradiation, even though the *E. coli* concentration in the control samples increased.
- UV irradiation, which had been identified in the literature as a parameter that could mitigate the effects of disinfectants by stimulating cell repair, did not lead to regrowth after 6 hours; moreover, it even enhanced the disinfection effects.
- The flow cytometry analysis of a bacterial suspension containing either *E. coli* or enterococci shows that the cells appear to be irreversibly inactivated.
- These results, combined with those from studies conducted with PAA, suggest that PFA reacts irreversibly on cell membrane components, most likely by means of chemical oxidation on chemical functions present in proteins (disulfides, sulfides) and unsaturated fatty acids (C = C double bond).