ABSTRACT

BAEZA, ANA CAROLINA. Using Fluorescent Microspheres as a Non-Biological Surrogate Indicator for Sequential Disinfection Performance. (Under the direction of Dr. Joel Ducoste)

Fluorescent YG-microspheres (Polysciences Inc.) were evaluated to simulate Cryptosporidium inactivation in treatment systems that utilize multiple disinfectants. Experiments were performed in batch reactors including an ozone primary stage at pH 7 and a secondary free chlorine treatment at pH 6. The impact of exposure to the chemical disinfectants was accomplished by tracking the changes in fluorescence distribution using a flow cytometer. Microsphere survival ratios (N/No) were calibrated to replicate the inactivation of different Cryptosporidium strains by selecting an appropriate threshold in a histogram analysis. The threshold value corresponds to a boundary between the beads representing the viable and non-viable Cryptosporidium cysts. The results suggest that YG-fluorescent microspheres are adequate non-biological surrogate indicators for the evaluation of sequential disinfection performance. In addition, it was found that microspheres had collateral reactions with sodium sulfite, affecting the physical integrity of the particle, a phenomenon that does not occur with the organism cyst. Analysis of the data showed that dot/density plot, which display the bead morphology characteristics, should be performed along with the histogram analysis to ensure the correct microsphere survival ratio outcome. Lastly, microsphere structural tests showed that the sequential disinfection mechanism consists of a polystyrene surface damage caused by ozone. This polystyrene damage enhances the diffusion of the secondary disinfectant into the microsphere, where it degrades the dye available in the opened polymer layer.

USING FLUORESCENT MICROSPHERES AS A NON- BIOLOGICAL SURROGATE INDICATOR FOR SEQUENTIAL DISINFECTION PERFORMANCE

by
ANA CAROLINA BAEZA

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APPROVED BY:

_____
Dr. Francis de los Reyes

_____
Dr. Detlef R.U. Knappe

_____
Dr. Joel Ducoste
Chair of Advisory Committee

BIOGRAPHY

Ana Carolina Baeza was born on November 21, 1974 in Southfield MI. She is the daughter of Jaime Baeza and Juanita Freer, and has one brother, Jaime Baeza. Although she is an American citizen, she had lived most of her life in Concepcion-Chile. She graduated from Saint John's English School in 1992. She received her Degree of Licentiate of Engineering Sciences in 1996 and graduated in July 1999 with a B.S. in Chemical Engineering from University of Concepcion-Chile. After graduating, Carolina's interest in environmental engineering made her pursue a Postgraduate Diploma in Environmental Engineering (January 2000) and work in environmental issues as a research assistant in the University of Concepcion for 2 years.

In August 2001, Carolina came to the United States and began her research on her Master of Science in Civil, Construction and Environmental Engineering at North Carolina State University.

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1. Introduction

Water treatment plants are faced with increasing challenges to ensure appropriate levels of disinfection to safeguard against waterborne disease outbreaks. This assurance of adequate disinfection is a daunting task when there are resistant organisms such as *Cryptosporidium*. *Cryptosporidium* is a protozoan parasite that causes a disease called cryptosporidiosis. In 1984 *Cryptosporidium* was first reported and since then has been responsible for several outbreaks. EPA, in response to community health concerns, has promulgated the 1998 Interim Enhanced Surface Water Treatment rule (IESWTR), which requires a 2-log removal for a public water system with conventional treatment serving over 10,000 people [1-2]. The IEWSTR was followed by two Long Term Enhanced Surface Water Treatment rules: LT1ESWTR and the second stage LT2ESWTR [3]. Both regulations are more stringent, they require a strengthen turbidity performance, and enhance the monitoring of the disinfectant concentration through the entire distribution system, disinfection by-products and *Cryptosporidium* inactivation.

Due to stricter regulations, some researchers have devoted their time to study the removal of *Cryptosporidium*. Different studies showed that for free and combined chlorine at typical drinking water facility doses, no *Cryptosporidium* inactivation was observed [4-6]. On the other hand, stronger oxidants, such as ozone and chlorine dioxide, are more effective for *Cryptosporidium* inactivation, however with a high chemical consumption [7].

Instead of applying individual disinfectants, newer strategies have investigated *Cryptosporidium* inactivation by combining two of them [4-5, 7-13]. Currently, drinking

water facilities apply ozone or chlorine dioxide as a primary disinfectant and free chlorine or monochloramine as a secondary disinfectant. Li *et al.* [13] found synergistic effects using either ozone/free chlorine or ozone/monochloramine disinfectant combinations. Driedger *et al.* [5] showed that when ozone/free chlorine were used sequentially to inactivate *Cryptosporidium*, their results demonstrated that the inactivation rate constant was higher for free chlorine than when chlorine was used alone. As a consequence, Driedger *et al.* [5] showed a 2-log inactivation of *Cryptosporidium* using O_3/free chlorine combination with a Ct value 4.5 times lower than what would be required without synergy.

The synergy mechanism has been explained by an improved chlorine permeation through the *Cryptosporidium* cell wall, damaged by ozone during the first stage. Conversely, slower kinetics will be found when *Cryptosporidium* has thicker or denser cell walls due to a minor reaction with ozone. As a result, the organisms will be more resistant to the access of the secondary disinfectant. This aspect of cell wall density variation also explains the discrepancies between inactivation kinetics for *Cryptosporidium* lots of different origin and age [5].

Disinfection kinetic constants are obtained by fitting inactivation data to different models. Chick-Watson and Delayed Chick-Watson (equation 1) are the most commonly used for describing *Cryptosporidium* decay. The kinetic constants for single and sequential disinfection reported by Driedger *et al.* [5] and Rennecker *et al.* [10] are summarized in Table 1.

The results in Table 1 clearly show the impact of sequential disinfection on the inactivation rate constant.

$$\frac{N}{No} = \begin{cases} \left(\frac{N}{No}\right)_C \text{ if } Ct \le Ct_{lag} = \frac{1}{k}\ln\left\{\left(\frac{N1}{No}\right)\left(\frac{No}{N}\right)_C\right\} \\ \frac{N1}{No}\exp(-k\,Ct) = \exp\left(-k\left\{Ct - Ct_{lag}\right\}\right) \text{ if } Ct > Ct_{lag} = \frac{1}{k}\ln\left\{\left(\frac{N1}{No}\right)\left(\frac{No}{N}\right)_C\right\} \end{cases} \quad (1)\ [10]$$

- With : $(N/No)_C$ = viability *Cryptosporidium* control (empirical), k= post-shoulder inactivation rate constant, N1/No = intercept with the Y-axis resulting from extrapolation of the pseudo-first order, C= disinfectant concentration, t= contact time in min, Ctlag= lag phase Ct.
- For Chick Watson model there is no lag phase, therefore $(N/No)_C$ = N1/No =1.0.

The majority of *Cryptosporidium* disinfection studies have been performed at the bench scale. The main problem of designing full-scale reactor is that Ct values from batch reactors do not take into account the hydraulics and mixing of the system. If an under-designed system is built, there is a high risk of *Cryptosporidium* breakthrough. Contrary, if the plant is over-designed, the system could be using an excessive amount of disinfectant, implying higher costs [14] and higher formation of disinfection by-products.

To better understand/evaluate the disinfection system, direct quantification of disinfection performance is required. Researchers have used techniques to continuously monitor the disinfectant distribution and hydraulics characterization of the treatment system. These techniques include the use of chemical tracer tests for hydraulic characterization, disinfectant demand-decay models and inactivation models simulating the organism disinfection behavior [15-18]. Other researchers have investigated better analytical procedures for quantifying *Cryptosporidium* inactivation [19].

Newer approaches include the use of surrogate indicators: biological [20-22] and non-biological [14,23-24]. Non-biological methods are of particular interests because (1) there are no specialized biological facilities necessary, (2) it is a direct measurement, therefore the collection of just one sample is necessary instead of several tracer test samples, and (3) it already takes into account the system hydraulics. These non-biological surrogate methods involve the use of fluorescent dye polystyrene microspheres to assess the ozone disinfection efficiency in a drinking water facility. Chiou *et al.* [23] published the first effort on using YG Fluoresbrite™ fluorescent microspheres to mimic the inactivation of *Giardia* with ozone treatment. Mariñas *et al.* [14] used the same beads to imitate *Cryptosporidium* inactivation with ozone in batch reactors and in full-scale drinking water treatment. Both studies showed reasonable results in using microspheres to evaluate microbial inactivation in bench and full-scale water treatment systems. Yet, researchers have not investigated whether the microspheres could be used to analyze disinfectant combinations.

The purpose of this research is to investigate the use of fluorescent YG microspheres for evaluating the efficiency of *Cryptosporidium* sequential disinfection using ozone as the primary disinfectant and free chlorine as the secondary disinfectant.

2. Materials and Methods

2.1. Microspheres

Experiments were done using YG Fluoresbrite™ fluorescent microspheres of 0.94 μm diameter (2% coefficient of variation) and a density of 1.045 g/cm^3 (Figure 1). The commercial microspheres come stored in an aqueous solution of 2.6% solid content. As part of this study, microspheres were pretreated to lower the original fluorescence intensity to better mimic the kinetics of *Cryptosporidium* inactivation. The pretreatment procedure will be explained in section 2.2.1. [14].

The dye is a proprietary chemical formula of Polysciences Inc. The fluorescence of the YG fluoresbrite matches the fluorescence characteristics of fluorescein isothiocyanate [14]. Fluorescein isothiocyanate reactions with ozone and free chlorine were observed in this study to understand the behavior of the fluorescence dye decay similar to the one incorporated in the microspheres. Fluorescence intensity was monitored in a Shimadzu RF-5301PC fluorometer. The fluorescence decay spectra were developed in an emission range of 500 – 650 nm (excitation wavelength 497 nm). The reactions were carried out at the same experimental conditions given in 2.2.2. for ozone treatment and in section 2.3. for free chlorine treatment, at a dye concentration of 0.1 mg/L.

In this study, additional tests were performed at pH between 5 and 7 to assess the effect of pH on the microspheres fluorescence intensity (FITC). Results of these tests showed no significant impact on the FITC value in the pH range studied.

2.2. Ozone experimental system

A system composed of a 0.25-liter gas equalizer reservoir and a subsequent 1-liter borosilicate semi-batch reactor was used for all ozone experiments. All experiments were performed at room temperature (20 ± 2 °C). Ozone was generated with dried industrial air by a Model G-11, Ozone-Pacific corona discharge generator. The ozone flow was fed at a flow of 10 SCFH (standard cubic feet per hour) through a porous diffuser in the center of the reactor. Mixing was enhanced by a magnetic stirrer. The ozone concentration was controlled with a variable output knob that operates from 0 to 100%.

2.2.1. Microsphere pretreatment*:* One liter of 0.01 M phosphate buffer at pH 7 was set up in the semi-batch reactor. Ozone was bubbled through the reactor for at least 30 minutes to ensure a steady state concentration of 0.30 ± 0.03 mg/L. The ozone concentration was measured (50 mL of sample) by an Indigo Pocket Colorimetric Method, Hach Co. (Loveland, Co). After a steady state ozone concentration was reached, approximately 1.2×10^{10} microspheres suspended in 50 mL of pH 7 buffer were injected in to the system. The reaction was carried out for a period of 90-100 minutes, then the knob was turned to 0% and air was bubbled for 20 minutes to remove the residual ozone in the microspheres batch. The 1-L of pretreated microspheres was stored at 4°C.

2.2.2. Primary disinfection with ozone: These experiments were performed in the same way described for the pretreatment step. Experiments were performed at ozone concentrations of 0.30 ± 0.03 mg/L and 0.60 ± 0.03 mg/L. After steady

state was achieved in the reactor, the reaction was initialized by injecting 50 mL of pretreated microspheres, obtaining a microspheres concentration of 270 μg/L. Samples of 3.5 mL were taken from the reactor to quantify the progression of the reaction. The sampling was more frequent in the beginning of the reaction, achieving a total of 9 -13 data points per experiment. The target Ct was 4 mg-min/L. The ozone in the samples was quenched by adding 0.4 mL of 0.1N sodium thiosulfate (Fisher Scientific), and mixed with a vortex mixer. The ozone concentration was measured before the last sample was extracted, so that an average ozone concentration in the reactor can be calculated.

2.3. Sequential disinfection:

In this study sequential disinfection consisted of an ozone primary stage and a free chlorine secondary stage. The methodology used for the ozone primary stage was presented in section 2.2.2, with the exception of the extent of the reaction. In order to simulate an overall inactivation of 1-log removal, a Ct of 1.4 mg-min/L was selected [5,10]. Two methods for stopping the primary ozone stage were evaluated. The first method was to quench the reaction with 15 mL of 0.1N sodium thiosulfate solution. Subsequently, the quenched suspension was filtered through 0.22-μm GV membrane filters (Millipore) and the residue was re-suspended by sonication in a pH 6 phosphate buffer solution, previously made oxidant free [25]. The second method was to discontinue the ozone supply to the reactor. At 10 – 20 seconds before a Ct of 1.4 mg-min/L was reached, the ozone knob was turned to 0% and air was bubbled for 20 minutes to eliminate any residual ozone.

At the conclusion of the primary stage of ozone treatment, 0.5 L of the micropheres solution was placed on 1-L Erlenmeyer flask reactor. The flask was made oxidant free by the procedure of Finch *et al.*[25]. The free chlorine secondary disinfection stage was performed at room temperature (20 ± 2 °C) and pH 6. Where necessary, the pH of the microspheres solution was adjusted to 6 with phosphoric acid (Fisher Scientific). A 1 g/L free chlorine stock solution was prepared with 5% sodium hypochlorite (ACROS Organics) in pH 6 phosphate buffer, and adjusted to 6 with phosphoric acid. The stock solution was added to the reactor to produce free chlorine concentrations of 3 and 8.5 ± 0.25 mg/L as Cl_2. Free chlorine was measured by the DPD Pocket Colorimetric Method, Hach Co. (Loveland, Co). Samples of 3.5 mL were taken more frequently in the beginning of the reaction, achieving 10 - 13 data points per experiment. The free chlorine reaction was stopped with 0.4 mL of quenching solution. Three quenching solutions were evaluated: a low sodium sulfite concentration (0.15 M), a high sodium sulfite concentration (0.5 M) (ACROS Organics) and a 0.1 N sodium thiosulfate. These experiments were performed until a target Ct of 400 mg-min/L was reached.

Additional tests of free chlorine were made with non-pretreated microspheres at pH 6 and an initial free chlorine concentration of 1 g/L. The purpose of these experiments was to observe the effect of free chlorine on the fluorescence decay of microspheres without ozone pretreatment.

Sample storage: All samples were stored in 12 × 75 mm Falcon (B.D. Lab Ware) tubes at 4°C before analysis. The samples were analyzed within 24 hours from conducting the experiments.

2.4. Flow cytometer analysis

Cytometry is a method to differentiate and count cells and microparticles. The flow cytometer model used for measuring the microspheres fluorescence was a Becton Dickinson FACSCalibur[+]. Microspheres showed the highest signal on the FL1 (FITC fluorescence) detector. The flow cytometer is restricted by two FITC resolutions: 256 or 1024 channels. Settings were adjusted to see the microspheres at a resolution of 256. Gating was performed in a dot/density plot using the characteristic forward (FSC) versus orthogonal light scatter (SSC) for eliminating any extraneous element. The light scattering properties, FSC and SSC are used to assess differences in the particles' morphology. FSC is related to the particle size, while the SSC is related to the internal granularity and complexity [26]. At least 10,000 event-microspheres were acquired per sample. Flow cytometer settings used in this research are displayed in Table 2.

2.4.1. Histogram Analysis:

The fluorescence intensity results were obtained as histograms. Data were analyzed by a compatible shareware software called WinMDI Version 2.8 - (Windows Multiple Document Interface for Flow Cytometry) developed by Joe Trotter, Scripps Institute [27].

The microspheres survival ratio (N/No) was calculated [14] by selecting a threshold or marker where the N/No is equal to the number of events with fluorescence intensity greater than the threshold divided by the total number of events in the whole histogram. Survival ratios are normalized by the survival ratio at time

[+] Flow cytometer: B.D. Model FACSCalibur, Microbiology and Immunology. College of Veterinary Medicine. NCSU Raleigh NC 27606.

zero of each experiment. Figure 2 shows the histograms obtained with the software. Using Figure 2A symbols, the survival ratio was described by the following equation:

$$\frac{N}{No} = \left(\frac{M0 - M1}{M0}\right) \Big/ \left(\frac{N}{No}\right)_{Ct=0\,min\text{-}mg/L} \quad (2)$$

- With: (Mo-M1) = number of microspheres-events with fluorescence intensity greater than the threshold, Mo= number of microspheres-events in the whole histogram.

Figure 2B shows an example of fluorescence intensity histograms after an ozone treatment of 0.28 mg/L. The movement to the left of the histograms produces the decay of the survival ratio (N/No) that is used to develop the inactivation curves: N/No versus Ct (mg-min/L) disinfection values.

2.5. Microsphere Morphology

Additional tests were performed to study the surface changes of the microsphere after ozone treatment. These tests include Scanning Electron Microscopy (SEM)[*] and Atomic Force Microscopy (AFM)[++]. For both analyses, microsphere samples were prepared by sonication for 10 minutes followed by filtering through 0.1-μm polycarbonate – 13 mm diameter filters. The filters were rinsed with 0.5 mL of distilled water and were allowed to air dry in a vacuum desiccator overnight.

For SEM tests, the filters were placed onto the SEM stubs using double-sided tape and sputter-coated with 25 nm gold/palladium. For AFM tests, the filters were placed on glass slides and transported in glass capsules.

[*]SEM: JEOL. Model 5900LV. CALS - NCSU, Raleigh NC 27695. [++] AFM: Digital Instruments, Inc. Model Dimension 3000. Analytical Instrument Facility (AIF). NCSU, Raleigh NC 27695.

3. Results and Discussion.

3.1. Microspheres pretreatment:

The fluorescence average intensity decay with an ozone concentration of 0.26 and 0.66 mg/L for ozone pretreated microspheres at pH 7 and 20 ± 2ºC is illustrated in Figure 3A. The convergence between the data depicted in Figure 3A confirms that the Ct concept is applicable to microspheres exposed to ozone. Since the pretreatment stage was easier to control at the lower ozone concentration, the 0.30 ± 0.03 mg/L was selected for all subsequent pretreatments. The ozone pretreatment time was determined by trial and error. A pretreatment time between 90 to 100 minutes appeared to work best for representing *Cryptosporidium* inactivation. However, the selected pretreatment time was lower than what was reported in the literature [14]. In this study, the results suggest that the microsphere inactivation has the same decay rate independent of the initial fluorescence intensity for pretreatment times greater than 90 minutes. However, a different flow cytometer and software was used in this study than what was reported in the literature. Different flow cytometers have different relative fluorescence scales/FITC resolution available. Fluorescence intensity values are directly related to the instrument settings, and for that reason, threshold values are not absolute and differ depending on the flow cytometer settings.

The pretreatment with free chlorine at pH 6 and 20 ± 2ºC is displayed in Figure 3B. Each data point represents the average fluorescence intensity decay when microspheres are treated with an initial free chlorine concentration of 1 g/L. Contrasting from the ozone pretreatment, free chlorine presents significantly lower fluorescence

intensity decay. It is thought that free chlorine does not react with the polystyrene of the microspheres. Therefore, the chlorine diffusion is reduced and the chance of contact with the dye is negligible. As a result, free chlorine pretreatment was not employed in this study. Reactions between the disinfectant and the polystyrene matrix will be discussed in a later section

3.2. Primary disinfection with ozone:

Results for the microsphere inactivation with ozone are shown in Figures 4 – 9. The conditions of each experiment are tabulated in Tables 3-5. The histogram analysis (section 2.4.1.) was used to develop these figures by selecting the appropriate threshold level that matches the reference inactivation rate. Figure 4 displays experiment P1-1 (Table 3) inactivation curves with threshold values from 40 to 90. The reference curve in the figure represents the *Cryptosporidium* inactivation data observed by Driedger *et al*. [5] (Table 1A). As in Mariñas *et al*. [14], an incorrect threshold selection produced erroneous results when using microspheres to replicate *Cryptosporidium* inactivation.

Figures 5, 6 and 7, show the microsphere inactivation rates for three different pretreatment batches (Pretreatment 1, 2 and 3). As in Figure 4, the thresholds of all microsphere inactivation curves were chosen to simulate the Chick-Watson kinetic model for *Cryptosporidium* inactivation observed by Driedger *et al.* [5]. For the 100-minute pretreatment (batch 1), a threshold of 65 was found to best fit the *Cryptosporidium* data. For the 90-minute pretreatments (batches 2 and 3), thresholds of 82 and 56, respectively, were found to best replicate *Cryptosporidium* inactivation. The inequality in thresholds obtained for the same time of pretreatment could be due to ozone concentration

variability during pretreatment. This slight variability could be attributed to the instability of the ozone output from the generator.

The results in Figure 6 also show the ozone inactivation experiments performed at two different concentrations: 0.30 ± 0.03 mg/L and 0.60 ± 0.01 mg/L. These results continue to support the validity of the Ct concept for microspheres exposed to ozone under the experimental conditions investigated.

In Figures 5-7, the microsphere inactivation rates showed excellent agreement with the reference data during the first log removal. Beyond one log removal, the data showed a tailing region. This tailing region experienced with the microspheres could be due to either a slower fluorescence decay of the microspheres compared to the ozone *Cryptosporidium* inactivation in Driedger *et al.*[5] or due to experimental error when the histogram analysis calculations are made with a small microspheres population on the high fluorescence tail of the histogram (see Figure 1A).

Rennecker *et al.* [10] reported *Cryptosporidium* lots that were more resistant to ozone disinfection than those used by Driedger *et al.* [5]. These lots differ in origin and age, and were characterized by a short lag phase and slow inactivation rates. In this study, experiments P1-1 (Table 3) and P3-1 (Table 5) were fitted to lot A, B and C *Cryptosporidium* inactivation reference (Table 1B) to demonstrate the versatility of the histogram analysis. Figures 8 and 9 displayed the fitting of Rennecker *et al.* [10] inactivation curves. As shown in Figures 8 and 9, microspheres post-shoulder inactivation slopes were obtained by finding the most suitable threshold through the histogram analysis. Unlike the *Cryptosporidium* lag phase, microspheres have a successive fluorescence decay from the beginning of the reaction. Therefore,

microspheres simulation for the Delayed Chick-Watson model was done by including the terms $(N/No)_C$ and N1/No in the histogram analysis, as shown in Equation 3, and the inactivation N/No results are limited by the Ct region beyond the Ct_{lag}.

$$\frac{N}{No} = \begin{cases} \left(N/No\right)_C & \text{if } Ct \le Ctlag \\ \left(\frac{N1}{No}\right)\left(\frac{M0 - M1}{M0}\right) \Big/ \left(\frac{N}{No}\right)_{Ct=0\,min\text{-}mg/L} & \text{if } Ct > Ctlag \end{cases} \tag{3}$$

- With: $(N/No)_C$ = viability *Cryptosporidium* control, N1/No = intercept with the Y-axis resulting from extrapolation of the pseudo-first order and Ctlag= lag phase Ct. These three parameters are obtained from the *Cryptosporidium* inactivation reference.
- $(N/No)_{Ct=0\ min\text{-}mg/L}$= microsphere survival ratio obtained with the histogram analysis at time zero.

As in Mariñas *et al.* [14], the data from the primary treatment of the microspheres suggest that microspheres can represent the extent of ozone treatment as seen by *Cryptosporidium* and could be used as a non-biological indicator for disinfection performance. The major findings of these primary ozone tests include: the threshold variability for the same pretreatment time and the flexibility of the histogram analysis for simulating different *Cryptosporidium* resistance.

3.3. Analysis of sequential disinfection using fluorescent microspheres.

3.3.1. Impact of sodium sulfite concentration.

Sodium sulfite is widely used for quenching free chlorine in *Cryptosporidium* disinfection studies. Sodium thiosulfate could also be used to quench residual chlorine for laboratory use. However, for large-scale treatment, thiosulfate is typically not recommended because it is a stepwise reaction and the reaction stochiometry is function of pH [28]. In this study, tests showed sodium sulfite may have a collateral effect with microspheres by changing the physical characteristics of the microsphere population. Measurements of the physical characteristics were done by analyzing the changes in light scattered from the microspheres. Light scattered is quantified using the forward light scatter (FSC) and the 90º light scatter change (SSC) parameters using the flow cytometer. FSC is related to the particle size while the SSC is related to the internal granularity or complexity of the particle. It is thought that sodium sulfite could damage the beads by reacting with the polystyrene matrix or the dye by an oxidation-reduction reaction. Figures 10-12 display dot/density plots of light scatter for microsphere samples quenched with either sodium thiosulfate or sodium sulfite. When sodium thiosulfate is used, the dot/density plots (Figures 10 A-D) show that the microsphere population is unmodified through all the sequential inactivation treatment. Figures 10 E-H show the typical change in FITC that is caused by the reaction of the dye with the disinfectant. The figures are plotted in relation to the FSC and SSC parameters. However, with the low sodium sulfite concentration (0.15 M) the dot/density plot (Figure 11) changed from the test P1-4 (Figure 11B) to the test P1-4S (Figure 11C). The change is easier to visualize using gates. Gate R2 represent the microsphere population when the samples are quenched with

sodium thiosulfate during the ozone primary stage treatment. Gate R1 represent the population of the same microspheres when the samples are quenched with the low sodium sulfite concentration during the secondary free chlorine treatment. The microsphere cluster moves to the right in the dot/density plot, changing their FSC value. Further tests were done at higher concentrations of sodium sulfite (0.5 M). The augmented change resulting from this higher sulfite dose is observed as a smaller region in Figures 12C and 12D. Figure 12G shows that sodium sulfite not only affects the FSC but also the SSC value, suggesting a complex interaction between this chemical and the polystyrene matrix.

As a consequence, sodium sulfite influenced the overall fluorescence intensity distribution of the microsphere samples. In Table 3, the population of microspheres at time zero on the secondary free chlorine disinfection had a higher fluorescence intensity average than the last data point obtained in the primary ozone disinfection stage. As will be discussed in the next section, the low sulfite also increases the variability between successive data points within the same experiment. The results of these sodium sulfite/thiosulfate tests, suggests that quenching the residual free chlorine should be performed with sodium thiosulfate and not sodium sulfite.

3.3.2. Analysis of free chlorine secondary disinfection

The results of secondary free chlorine disinfection experiments are displayed in Figures 13-14. The conditions of these experiments are tabulated in Tables 3 and 5. All free chlorine experiments were carried out at pH 6 that followed a primary ozone treatment at pH 7 and an ozone exposure of Ct=1.4 mg-min/L. N/No was obtained from

the histogram analysis technique (Section 2.4.1.). The threshold value used in the free chlorine stage was the same value selected in the primary ozone treatment stage. The results were normalized by N/No ratio at time zero. The normalization was done due to the variability of the microspheres initial survival ratio, which range from 0.203 to 0.52 for pretreatment 1 (Table 3) and ranged from 0.0756 to 0.153 for pretreatment 3 (Table 5). The variability depends on the moment when the ozone primary stage was stopped. For pretreatment 3, it was found that for 10-20 seconds before Ct of 1.4 mg-min/L was achieved, the N/No value was approximately to 0.1.

Figure 13 displays the results of the secondary disinfection when the samples were quenched with a low sodium sulfite concentration. In addition, Figure 13 illustrates the impact of the methods used to remove the residual ozone from the primary ozone stage (see methods 2.3). For sodium thiosulfate/filtering (experiments P1-2S and P1-4S) or the air bubbling step (experiments P1-5S, P1-6S and P1-7S), the same inactivation trend was obtained regardless of the method used to remove the residual ozone. In addition there was no significant effect of sodium thiosulfate/filtering or air bubbling step on the secondary treatment outcome. Figure 13 also shows a higher irregularity between successive data points. Successive data points irregularity could be explained by random changes in the microsphere samples due to the sodium sulfite quenching, as explained in the previous section.

Figure 14 displays the results of the secondary disinfection treatment when the samples were quenched with sodium thiosulfate. The most significant result in Figures 13 and 14 is that microspheres show an inactivation rate comparable with the reference kinetic data of *Cryptosporidium* inactivation. These results suggest that microspheres

could be used to analyze sequential disinfection performance in disinfection processes. However, it is not clear what allows microspheres to exhibit a sequential synergistic behavior like *Cryptosporidium* in the presence of ozone followed by chlorine. Tests were performed to help elucidate the mechanism of sequential disinfection with fluorescent microspheres and are presented in the following section.

3.4. Understanding microspheres fluorescence intensity decay under sequential disinfection.

To understand the influence of ozone and chlorine on fluorescent microspheres, tests were performed to examine structural changes as well as the decay rates on the dye used inside the microspheres. As discussed in the methods section, Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) were done to examine microsphere surface changes with ozone treatment. A fluorometer was used to qualitatively examine the reaction kinetics of fluorescein isothiocyanate, which is considered analogous to the YG-dye of the microspheres [14].

SEM results are shown in Figures 15 and 16. Figure 15 is an image of the original non-pretreated microspheres. Figure 16 corresponds to an ozone-pretreated microsphere after 46 minutes at a concentration of 0.66 mg/L. The results in Figure 16 show no visible crack or holes in the polystyrene matrix of the pretreated microspheres. However, the non-pretreated microspheres show a smoother surface than the pretreated ones. AFM analyses were done to quantify the surface damage of the bead and are shown in Figures 17-19. In Figures 17-19, the root mean square deviation of the surface roughness (RMS) was 0.828 for the non-pretreated microspheres, 3.409 for 90 minutes pretreatment at an

ozone concentration of 0.30 ± 0.03 mg/L, and 5.69 for 120 minutes pretreatment at an ozone concentration of 0.30 ± 0.03 mg/L. These RMS results indicate that the polymer surface was affected physically by ozone and at higher ozone exposure times increasing damage will occur to the polystyrene surface.

Fluorescein isothiocyanate degradation spectra are shown in Figures 20A and 20B for ozone and free chlorine treatment, respectively. The result in Figure 20A suggest that the ozone reaction with the dye alone occurs very quickly. If the YG-dye behavior is analogous to fluorescein isothiocyanate, the degradation of the dye is much faster than the fluorescence decay intensity of the microspheres in the pretreatment experiments (Figure 3A). This result is consistent with the findings of Chiou *et al.* [23], where they found that the polystyrene reaction was the slowest reaction step compared to the dye reaction in the overall microsphere fluorescence intensity decay. Additional information could be obtained from the ozone pretreatment results. During the pretreatment stage, the fluorescence decay was extremely fast in the first hour of ozone exposure, suggesting that the high concentration of the dye is in the outer polystyrene layer of the bead. While it is difficult to quantify the fraction of the dye in different regions of the polystyrene matrix, work done by Chiou *et al*. [23] seems to support the fast reaction of ozone with the dye on the microsphere surface. Ozone is then faced with a higher mass transfer resistance through the polystyrene matrix as it progresses towards the inside of the microsphere.

In case of free chlorine pretreatment, the slow fluorescence decay suggests that the polystyrene reaction with free chlorine is very slow compared to the reaction with ozone (Figure 3B). However, free chlorine was able to degrade fluorescein isothiocyanate dye much faster (Figure 20B) than when it was part of the microsphere. For that reason, it

is believed that for non-pretreated microspheres, the chlorine reaction with the dye is limited by the diffusion of chlorine molecules through the polystyrene matrix.

Researchers have proposed a model for understanding how ozone reacts with each microsphere [23]. This model involves the following steps: (1) negligible contribution of aqueous diffusion through interfacial laminar film formed around the particle due to ozone mass transfer, (2) reaction of the disinfectant with polystyrene matrix, (3) diffusion of the disinfectant inside the microspheres and (4) the reaction with YG-dye. The results of this study suggest that the microsphere sequential disinfection mechanism is analogous to the existing model represented by an overall parallel reaction process defined by each disinfectant stage. The microsphere sequential disinfection reaction steps include: (1) a primary ozone reaction with the dye and the polystyrene located on the surface of the microsphere, (2) ozone begins to damage the microspheres matrix surface producing a higher surface roughness and increase access to the inner microsphere layer, where ozone continues to react with the YG-dye, (3) the damaged polystyrene allows chlorine to readily diffuse through the outer portion of the microsphere matrix, reacting with the dye in the secondary disinfectant stage, (4) reactions between the disinfectant and the dye occurs faster than in non-pretreated microspheres and results in an overall fluorescence decay. In summary, it is believed that the polymer matrix is eroded and that subsequent free chlorine can readily react with the dye that is available on the layers opened by the ozone exposure. The fluorescence intensity decay mechanism, elucidated in this section, allows the beads to mimic inactivation rate of *Cryptosporidium* at pH 7 and 20°C for ozone primary treatment and pH 6 and 20°C for the secondary free chlorine treatment.

4. Summary

Experimental research was conducted to demonstrate that the YG Fluoresbrite™ microspheres could imitate *Cryptosporidium* disinfection under sequential disinfectant application. The impact of ozone on the particle surface was also investigated. The microsphere inactivation survival ratios (N/No) were developed by choosing the best threshold using a histogram analysis technique so that better agreement can be obtained between the *Cryptosporidium* inactivation data and the microsphere fluorescence intensity decay data. For simulated Delayed Chick-Watson models, the term N1/No and $(N/No)_C$ were included in the histogram analysis. The experimental analysis of the microsphere inactivation yielded the following findings:

- YG microsphere is an appropriate non-biological surrogate indicator to simulate *Cryptosporidium* sequential disinfection.
- Because of the pretreatment variability, a histogram analysis technique must be done for each new pretreatment batch to determine the threshold value that best characterizes *Cryptosporidium* inactivation data.
- In analyzing treatment systems that utilize two disinfectants the same threshold value must be used. The histogram analysis technique is capable of replicating different *Cryptosporidium* strains, with different resistance levels.
- Sodium sulfite affects the microsphere's physical properties. The microsphere physical characteristics were described by analyzing forward scatter and side scatter dot/density plots that relate changes in bead size and granularity. Therefore, microsphere inactivation ratios could be affected by tertiary reactions

with compounds that could damage the bead but not influence the *Cryptosporidium* inactivation. For that reason it is recommended that dot/density plots must be observed along with the histogram analysis to reduce errors in the survival ratio calculations.

- Microsphere sequential disinfection mechanism is thought to be a process that involves damaging the polymer matrix in the primary ozone treatment to enhance any subsequent free chlorine diffusion in the secondary free chlorine disinfection. Damaging the polystyrene allows a faster reaction between the free chlorine and the YG-dye.

Currently the main disadvantage of applying this technique in a full-scale drinking water treatment plant is the high cost of microspheres. However, the methodology presented in this study is strongly recommended for use at pilot scale and will likely become cost effective in the future when the price of the microspheres decreases. Moreover, data from microsphere analysis of a sequential disinfection system study could be used to validate models that can characterize and optimize disinfection processes.

5. Future Work

In this study, fluorescent microspheres have been calibrated in a batch mode in order to simulate *Cryptosporidium* sequential disinfection. In future studies, Ct, pH, and temperature conditions used during this calibration stage will also be used in a flow-through reactor system. Preliminary tests will be conducted to characterize the mixing behavior of the reactors. The sampling methodology of the dye-microspheres is similar to the tracer test methodology, although utilizing microspheres only requires the collection of just one sample instead of several samples for the tracer test [23]. The purpose of this future research is to use microspheres to evaluate the efficiency of sequential disinfection using ozone as the primary disinfectant and chlorine as secondary disinfectant, taking into account the hydraulics and mixing of the flow-through system. The data obtained in the flow-through system will eventually used to validate CFD models that can characterize and optimize these processes in full-scale drinking water treatment plants.

6. Acknowledgements

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- Water Resources and Environmental Engineering. Department of Civil, Construction and Environmental Engineering, NCSU.
- Microbiology and Immunology. College of Veterinary Medicine where the flow cytometer analyses were done.

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Table 1:

(a) Kinetic constants (k) for *Cryptosporidium* single disinfectant and sequential disinfection. Reported by Driedger *et al.* [5]. (Oocyst viability determined by the modified *in-vitro* excystation assay developed by Rennecker *et al.* [29])

For single disinfectant			
Using ozone: Shape: characterized by a very small lag phase and follows a Chick-Watson model.			
k (20ºC) [L/mg-min]	1.502	**N1/No**	-
Using free chlorine: Shape: characterized by an initial lag phase and follows a Delayed Chick-Watson model.			
k (20ºC) [L/mg-min]	0.00134	**N1/No**	1.49

Sequential disinfections: secondary free chlorine treatment

- Level of ozone pretreatment: a Ct of 1.4 mg-min/L
- Shape: double slope, characterized by an initial faster decline and a secondary slower inactivation decay.

	First slope	**Second slope**
Factor	6	2
k (20ºC) [L/mg-min]	0.00804	0.00268

(b) Kinetic constant (k) for *Cryptosporidium* single disinfectant and sequential disinfection. Reported by Rennecker *et al.* [10] (Oocyst viability determined by the modified *in-vitro* excystation assay developed by Rennecker *et al.* [29])

For single disinfectant

Using ozone:
Shape: characterized by an initial lag phase and follows a Delayed Chick-Watson model.

	k (20ºC) [L/mg-min]	**N1/No**	**$(N/No)_C$**
Lot A	0.942	1.4	0.9
Lot B	1.04	2.9	0.815
Lot C	0.845	0.81	0.525

Using free chlorine:
Shape: characterized by an initial lag phase and follows a Delayed Chick-Watson model.
According to Arrhenius equation:

$k = A \exp\left(-Ea/RT\right)$

A= frequency factor = 1.29×10^{10} L/mg-min
Ea = apparent activation energy = 71,610 J/mole
R = ideal gas constant = 8.314 J/(mole K)
T = absolute temperature in K

k (20ºC) [L/mg-min]	0.002207

Sequential disinfections: secondary free chlorine treatment

Level of ozone pretreatment: a Ct of 1.4 mg-min/L
Shape: single slope, no lag phase present.

	Factor	**k (20ºC) [L/mg-min]**
Lot A and C	1.6	0.00353

Table 2: Flow cytometer settings.

Detector/Amps				
Parameter	**Detector**	**Voltage**	**Amp.Gain**	**Mode**
P1	FSC	EO2	4.16	Log
P2	SSC	564	1.00	Linear
P3	FL1	999	9.99	Linear
Threshold:				
Primary parameter: FSC - Value: 192 Secondary parameter: SSC - Value: 279				

Detector notation: FSC (forward light scatter, related to cell size), SSC (90° light scatter, related to the internal granularity or complexity of the particle), FL1 (Green-FITC fluorescence)

Table 3: Ozone primary and free chlorine secondary disinfection tests and experimental conditions for pretreatment batch 1.

Pretreatment 1: 100 min at 0.30 ± 0.03 mg O_3/L, pH 7.0 and 20± 2°C				
Ozone primary stage:				
Test	Ozone (mg/L)	Time (min)/ Nº of samples	Threshold fitted	Final N/No
P1-1	0.29	13.21 / 12	65	0.00771
P1-2	0.29	4.5 / 9	65	0.16
P1-3	0.29	13.25 / 12	65	0.00548
P1-4	0.29	4.5 / 9	65	0.169
Free chlorine secondary treatment: 0.15 M sodium sulfite for free chlorine quenching				
Test	Free Chlorine (mg/L)	Time (min)/ Nº of samples	Threshold fitted	Initial N/No
P1-2S	8.54	45.1 / 12	65	0.241 [f]
P1-4S	3.02	117.2 / 13	65	0.203 [f]
P1-5S	3.27	117 / 13	65	0.41 [*nf]
P1-6S	3.12	119.2 / 13	65	0.52 [*nf]
P1-7S	8.85	45.4 / 12	65	0.51 [*nf]

S: secondary sequential free chlorine stage.
f: sodium thiosulfate/ filtered methodology for quenching the primary ozone treatment
nf :Discontinued ozone/air bubbling methodology for quenching the primary ozone treatment
* : the primary ozone stage of these sequential disinfections were not monitored.

Table 4: Ozone primary disinfection tests and experimental conditions for pretreatment batch 2.

Pretreatment 2: 90 min at 0.30 ± 0.03 mg O_3/L, pH 7.0 and 20± 2°C			
Test	Ozone (mg/L)	Time (min)/ Nº of samples	Threshold fitted
P2-1	0.30	13.17 / 12	82
P2-2	0.33	13.4 / 12	82
P2-3	0.31	13.17 / 12	82
P2-4	0.60	7.25 / 11	82
P2-5	0.59	7.05 / 11	82

Table 5: Ozone primary and free chlorine secondary disinfection tests and experimental conditions for pretreatment batch 3.

Pretreatment 3: 90 min at 0.30 ± 0.03 mg O_3/L, pH 7.0 and 20± 2°C				
Ozone primary stage:				
Test	Ozone (mg/L)	Time (min)/ Nº of samples	Threshold fitted	Final N/No
P3-1	0.28	4.27 / 7	56	0.0756
P3-2	0.27	4.28/ 6	56	0.114
P3-3	0.27	5.33 / 8	56	0.153
P3-4	0.28	13.08 / 12	56	0.0139
Free chlorine secondary treatment: 0.1 N sodium thiosulfate for free chlorine quenching				
Test	Free Chlorine (mg/L)	Time (min)/ Nº of samples	Threshold fitted	Initial N/No
P3-1S	8.67	45.9 / 10	56	0.0756^{nf}
P3-2S	8.72	45.9 /10	56	0.114^{nf}
P3-3S	8.58	45 / 10	56	0.153^{nf}

S: secondary sequential free chlorine stage.
nf :Discontinued ozone/air bubbling methodology for quenching the primary ozone treatment

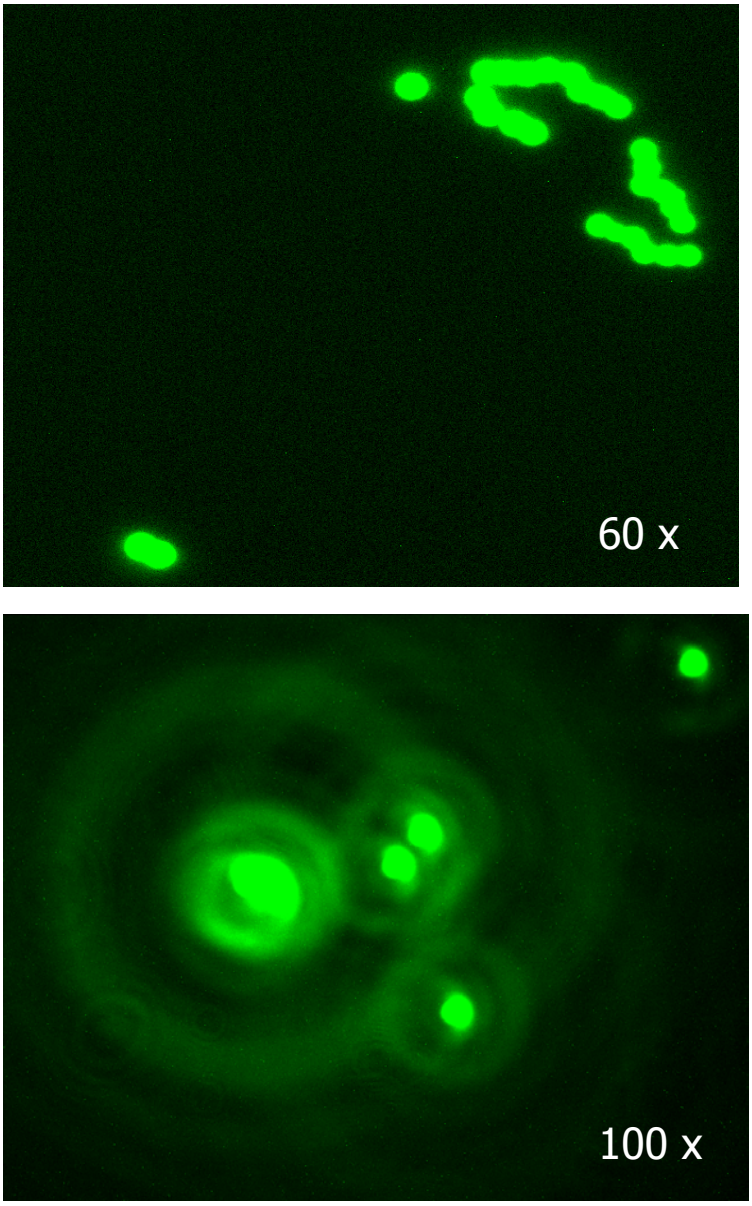
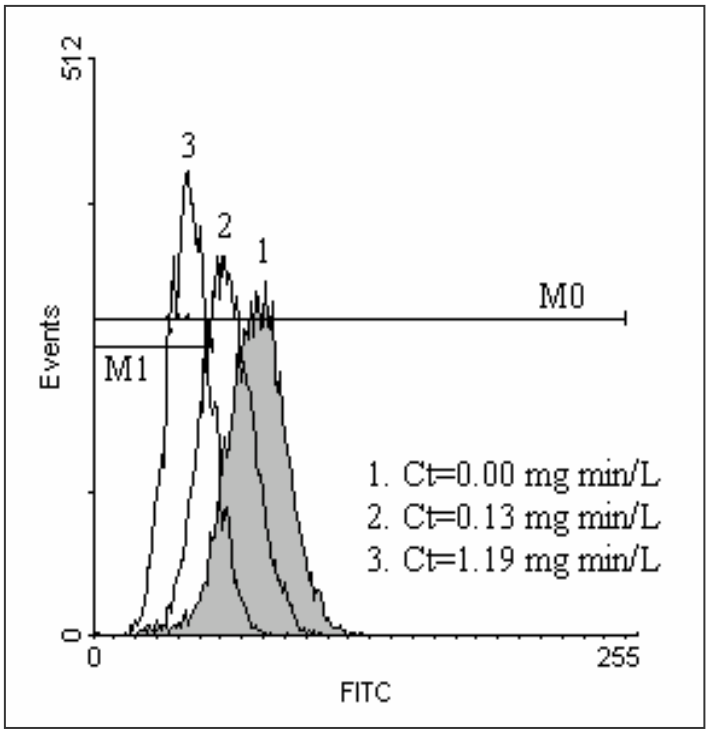


Figure 1: YG Fluoresbrite™ fluorescent microspheres of 0.94 μm diameter seen in an epifluorescence microscope.

A)



B)

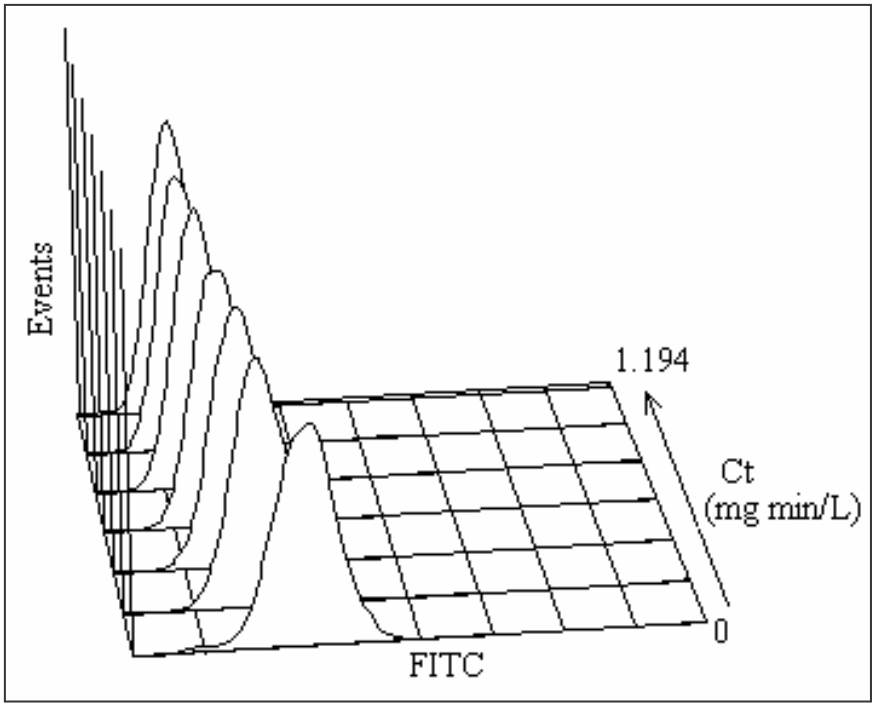


Figure 2: Fluorescence intensity histogram analysis. A) Figure descriptive of the N/No survival ratio calculation. B) Microsphere histograms for ozone treatment at pH 7 and 20 ± 2 ºC. Microsphere results correspond to pretreatment 3- experiment 1 (P3-1, Table 3).

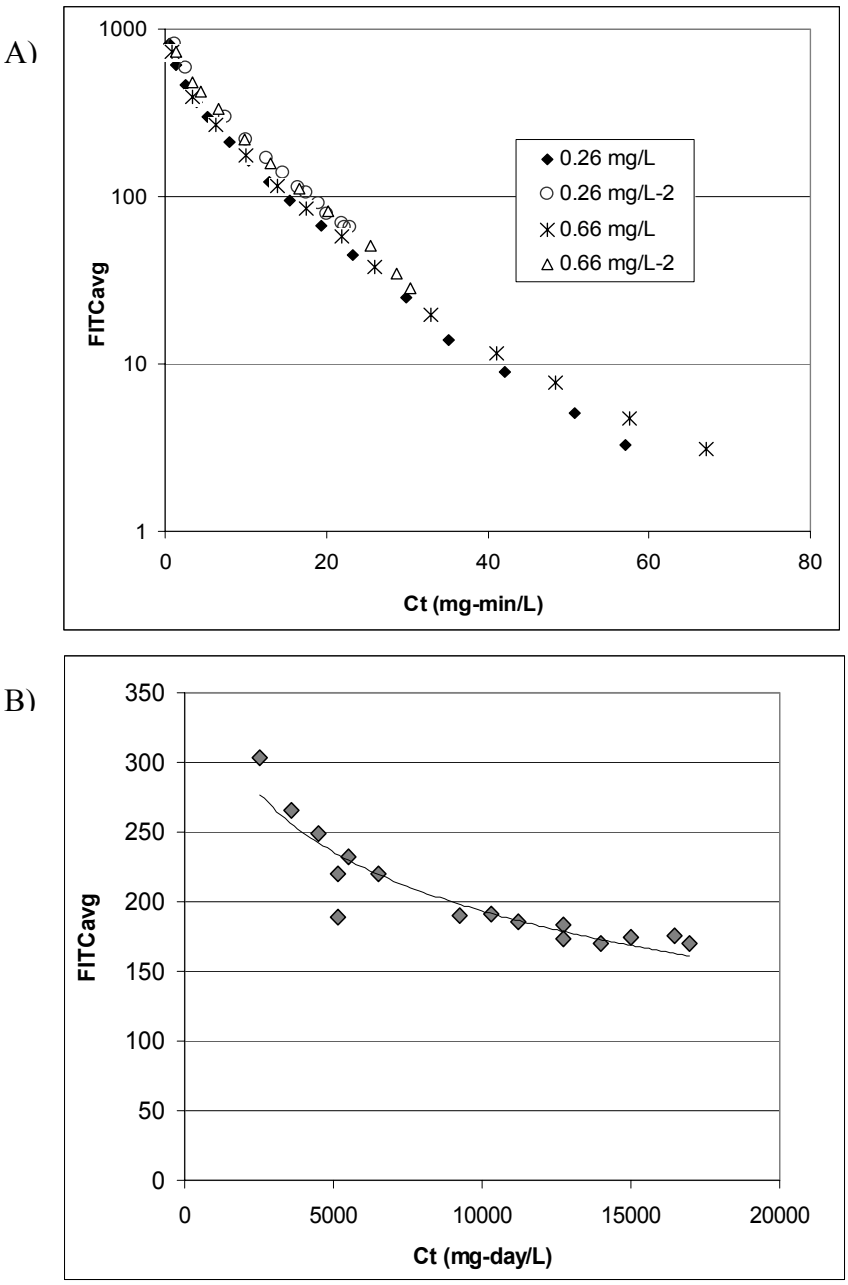


Figure 3: A) Ozone pre-treatment of microspheres at pH 7 and 20 ± 2ºC. B) Free chlorine pretreatment of microspheres at pH 6 and 20 ± 2ºC, with an initial free chlorine concentration of 1 g/L.

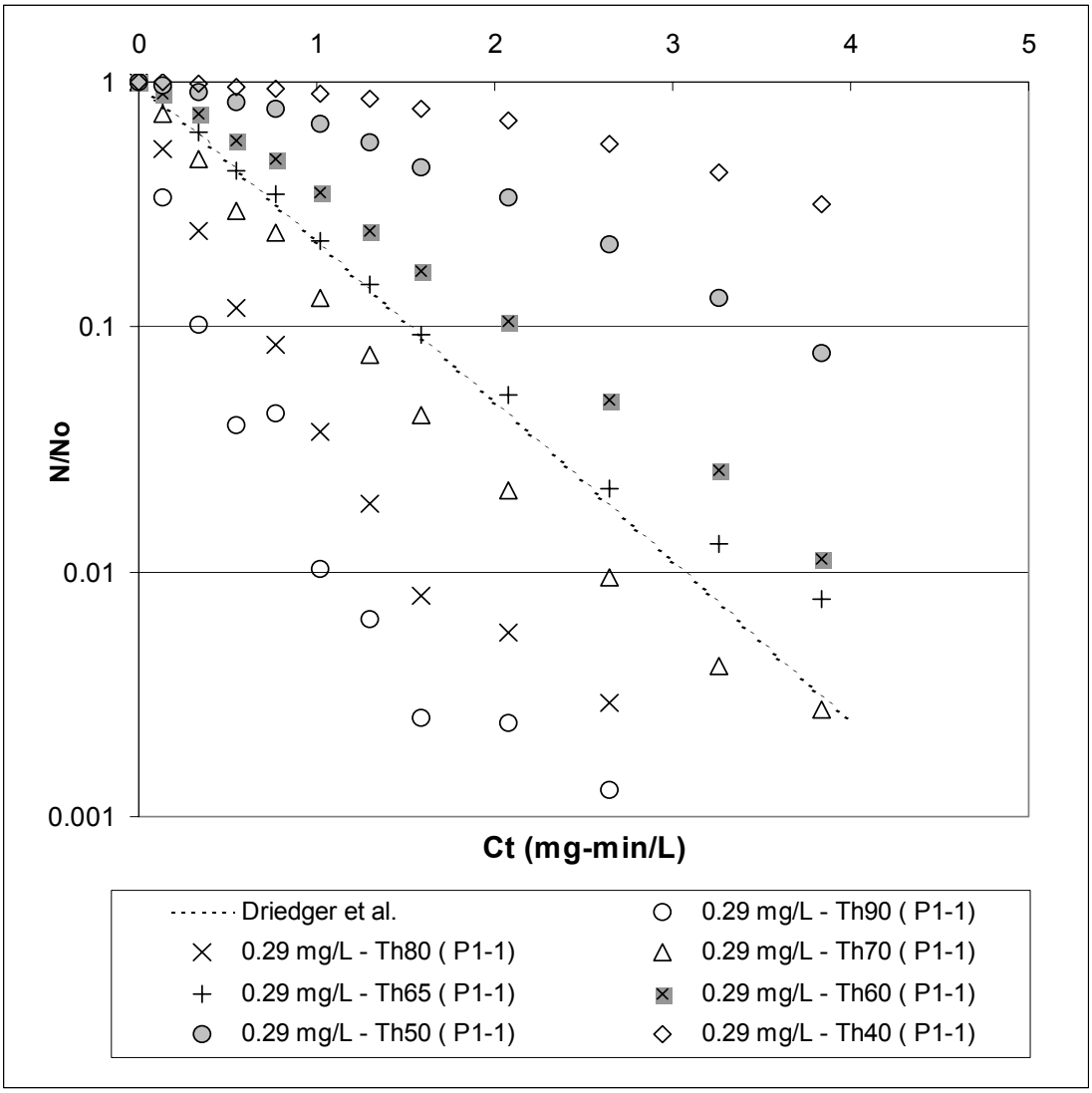


Figure 4: Microspheres inactivation with ozone at pH 7 and 20 ± 2 Cº. Microsphere pretreated batch corresponds to pretreatment 1- Table 3. Inactivation ratio curve variability depending on the fluorescence intensity threshold selected. Reference inactivation curve based on Driedger *et al.* [5] (Table 1A)

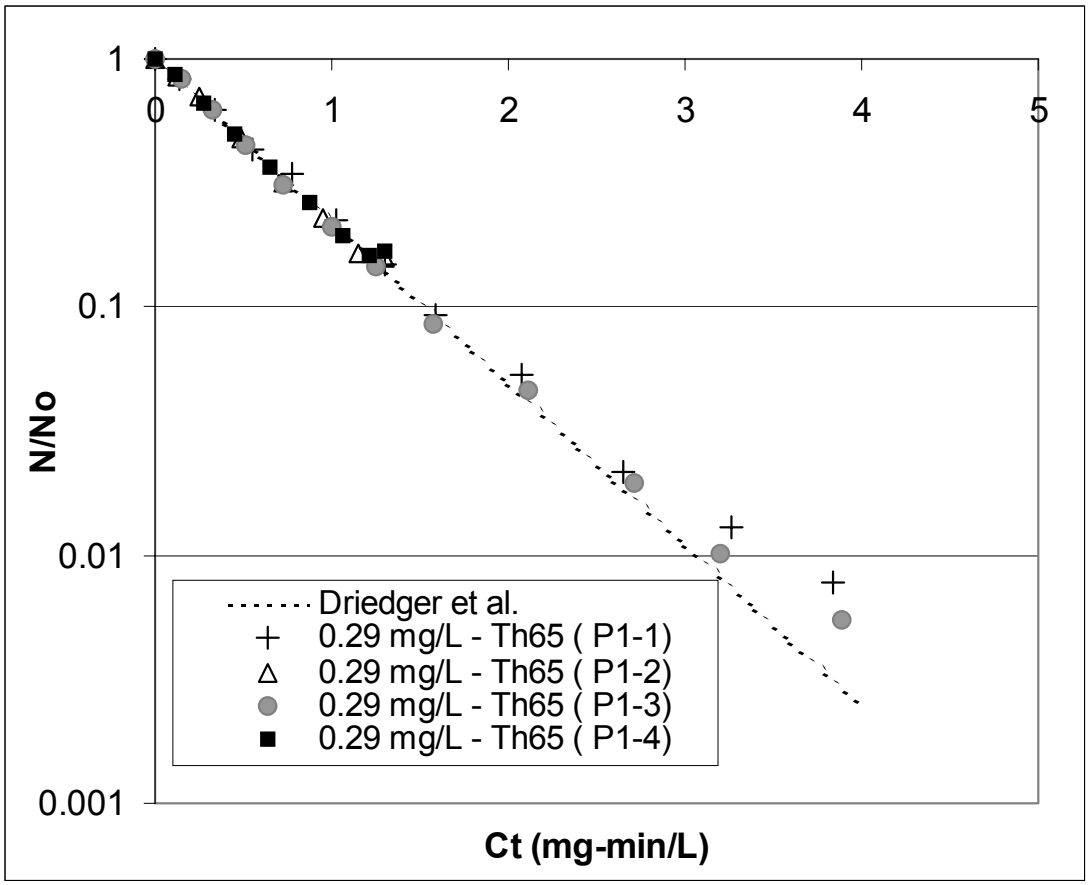


Figure 5: Microspheres primary ozone inactivation at pH 7 and 20 ± 2 Cº. Microspheres pretreated batch corresponds to pretreatment 1- Table 3. Fluorescence intensity threshold fitted at 65 (Th65) and the N1/No intercept of 1.0. Reference inactivation curve based on Driedger *et al.* [5] (Table 1A).

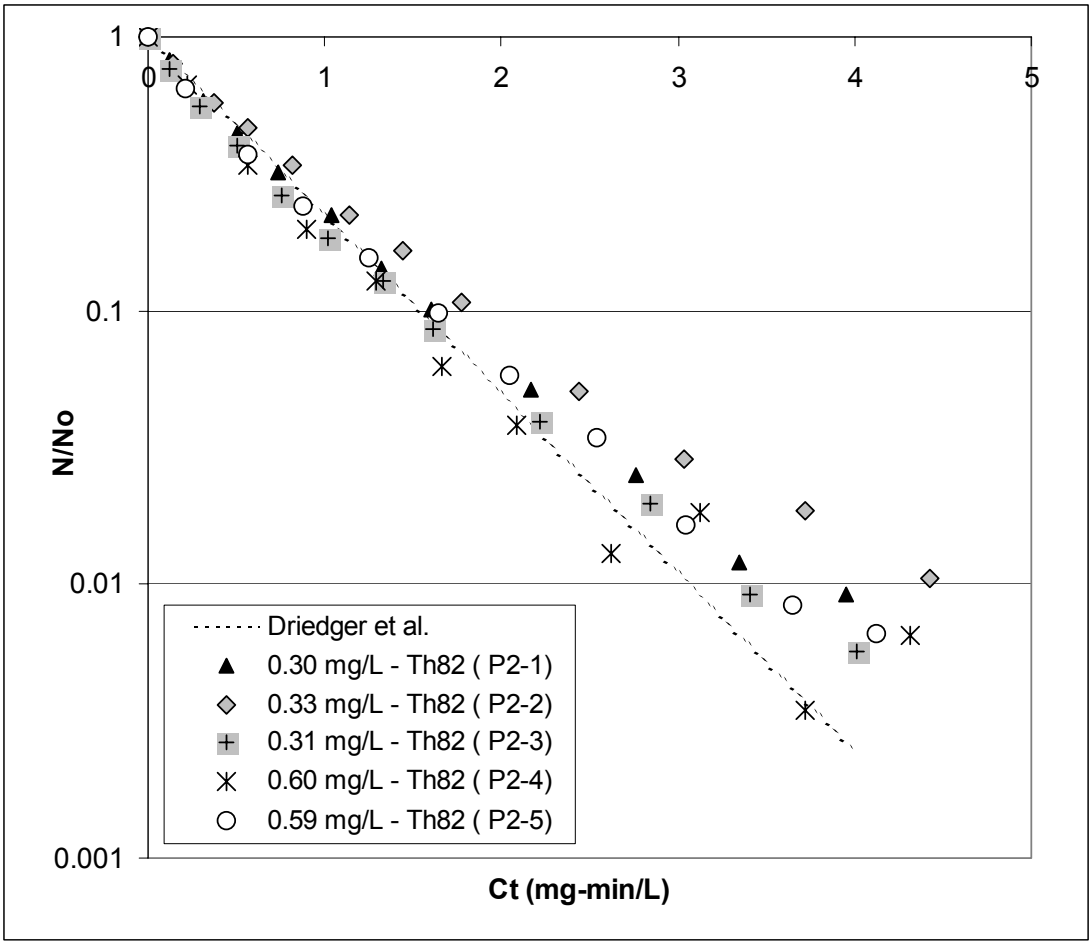


Figure 6: Microspheres primary ozone inactivation at pH 7 and 20 ± 2 Cº. Microspheres pretreated batch corresponds to pretreatment 2- Table 4. Fluorescence intensity threshold fitted at 82 (Th82) and the N1/No intercept of 1.0. Reference inactivation curve based on Driedger *et al*. [5] (Table 1A).

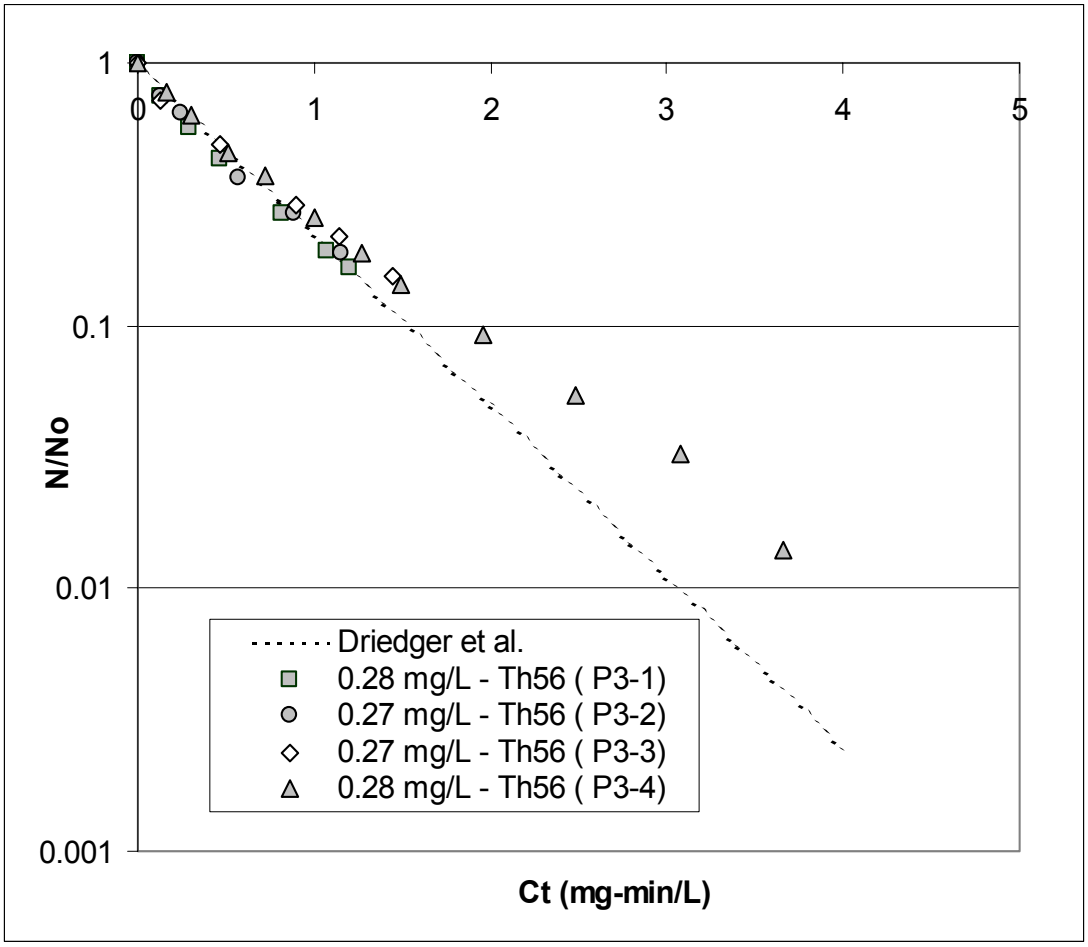


Figure 7: Microspheres primary ozone inactivation at pH 7 and 20 ± 2 Cº. Microspheres pretreated batch corresponds to pretreatment 3- Table 5. Fluorescence intensity threshold fitted at 56 (Th56) and the N1/No intercept of 1.0. Reference inactivation curve based on Driedger *et al*. [5] (Table 1A).

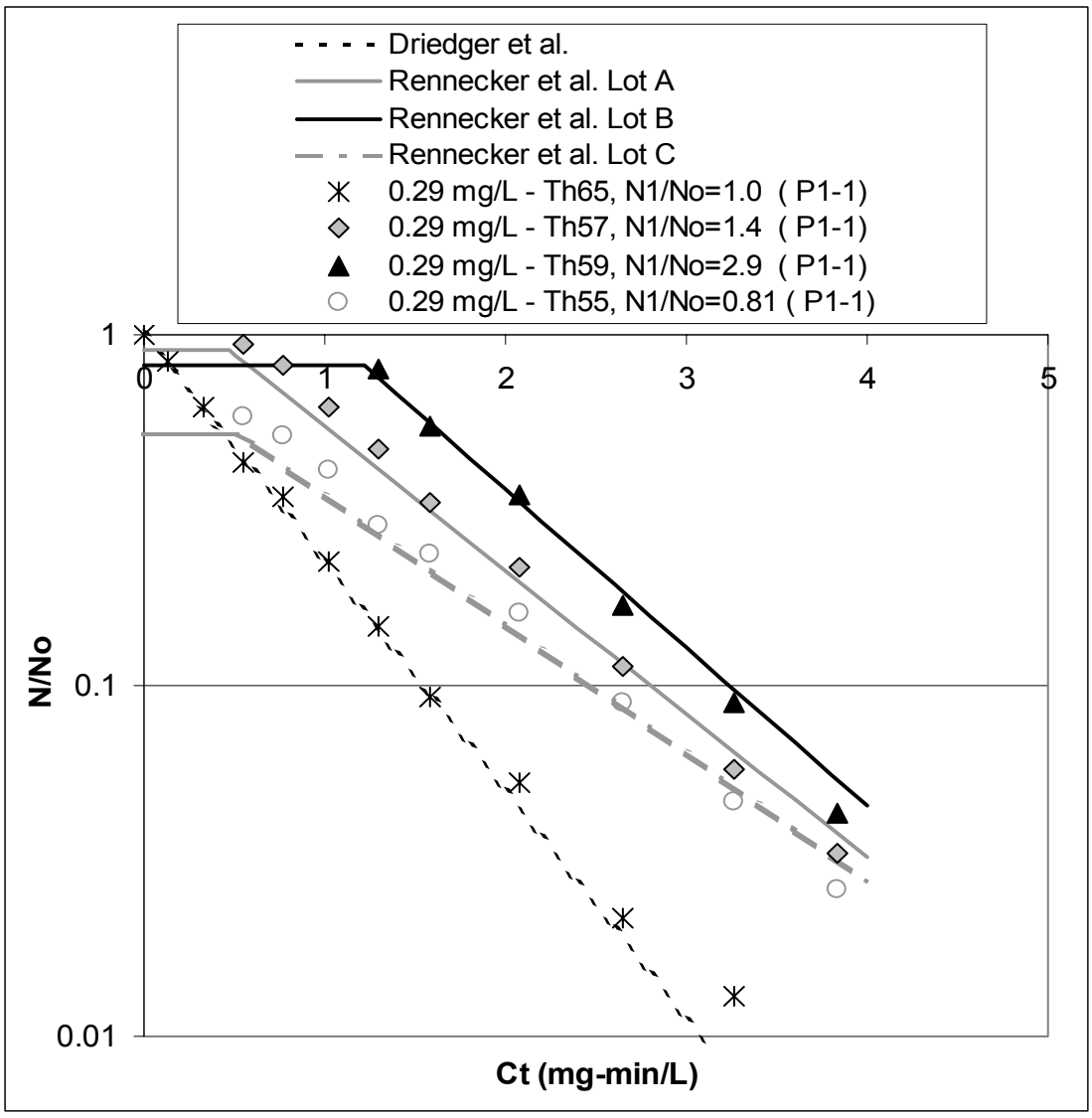


Figure 8: Microspheres primary ozone inactivation at pH 7 and 20 ± 2 Cº. Microspheres corresponds to pretreatment 1- experiment 1 (P1-1, Table 3). Each fluorescence intensity threshold was determined following the kinetics, $(N/No)_c$ and the N1/No intercept of each reference inactivation curve (Table 1A-B).

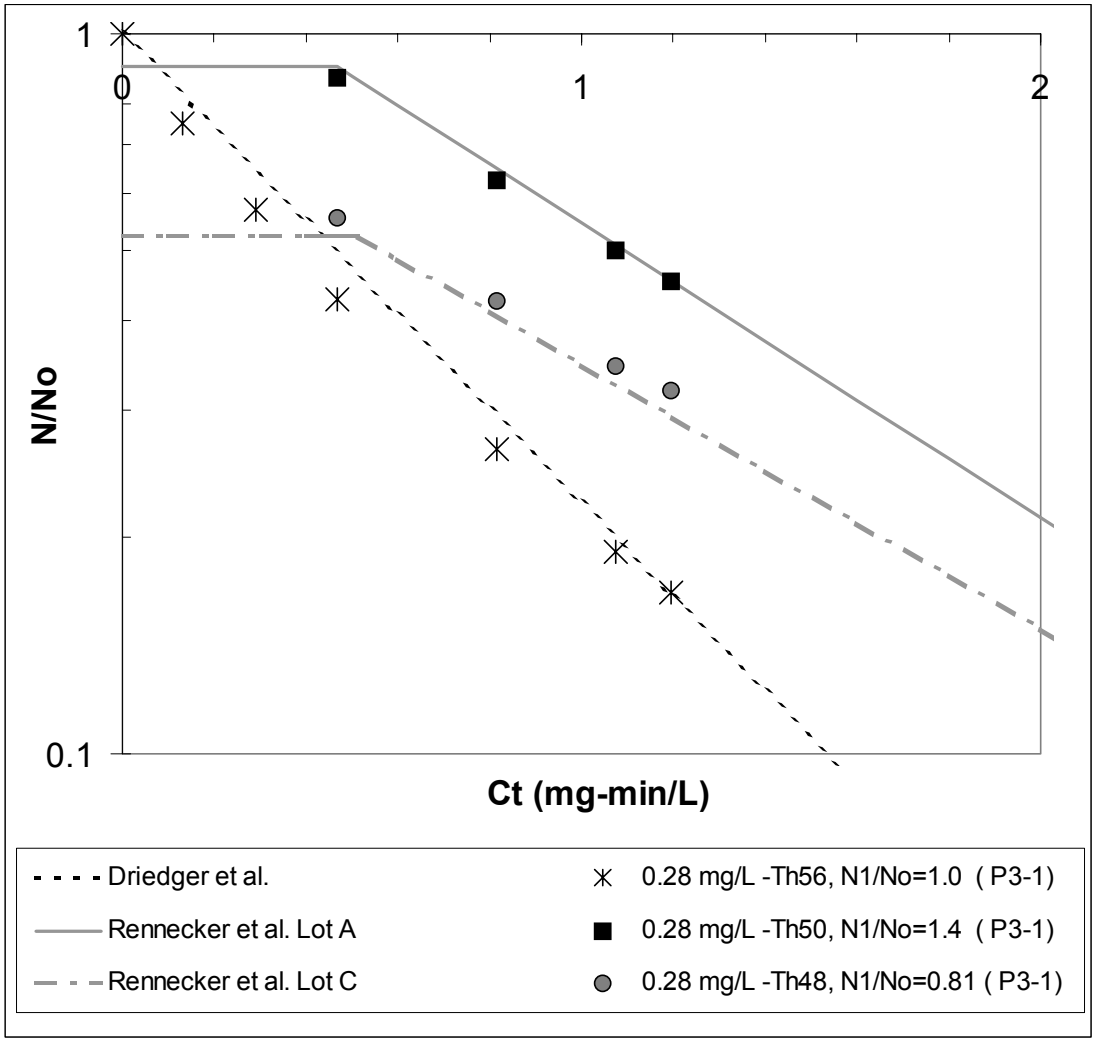


Figure 9: Microspheres primary ozone inactivation at pH 7 and 20 ± 2 Cº. Microspheres corresponds to pretreatment 3- experiment 1 (P3-1, Table 5). Each fluorescence intensity threshold was determined following the kinetics, $(N/No)_c$ and the N1/No intercept of each reference inactivation curve (Table 1A-B).

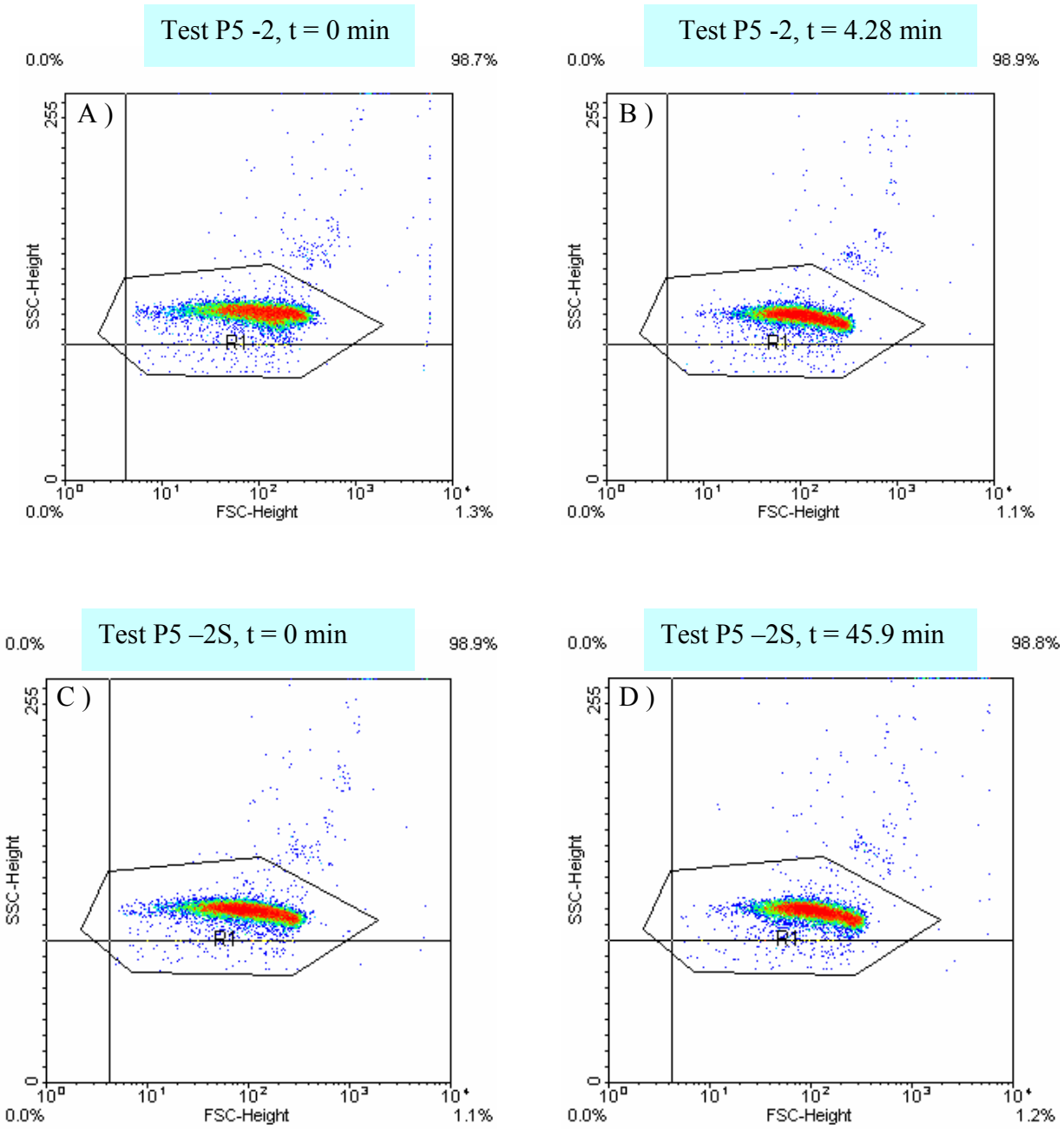


Figure 10: Dot/density plot showing forward ligth scatter (FSC) on the X-axis and 90º light scatter (SSC) on the Y-axis aqcuired for experiment P5-2 (A and B) and P5-2S (C and D). Sodium thiosulfate was used to quench the secondary disinfectant samples.

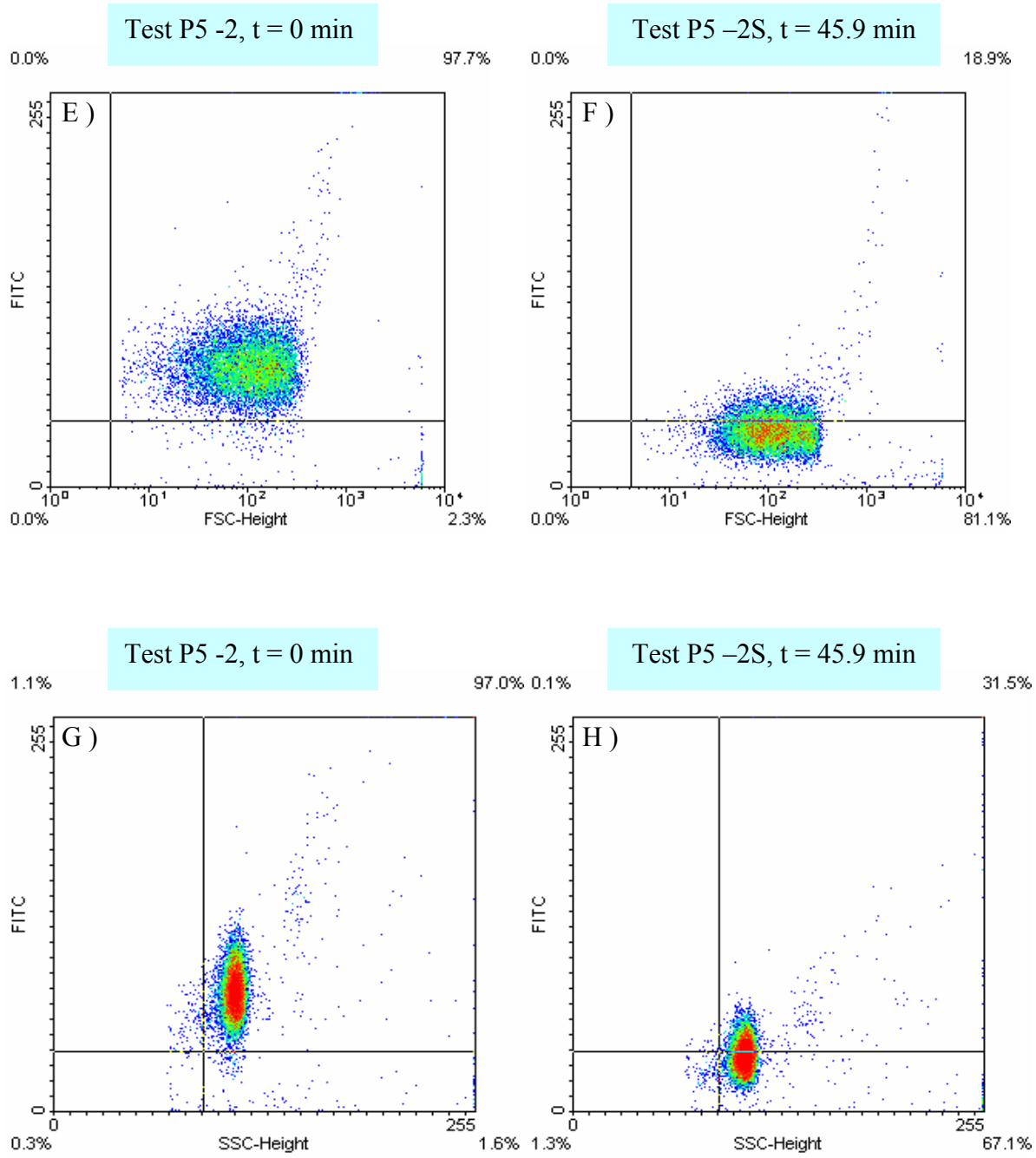


Figure 10: Dot/density plot showing: E and F : forward ligth scatter (FSC) on the X-axis and FITC on the Y-axis. G and H: 90º light scatter (SSC) on the X-axis and FITC on the Y-axis. For experiment P5-2 and P5-2S. Sodium thiosulfate was used to quench the secondary disinfectant samples.

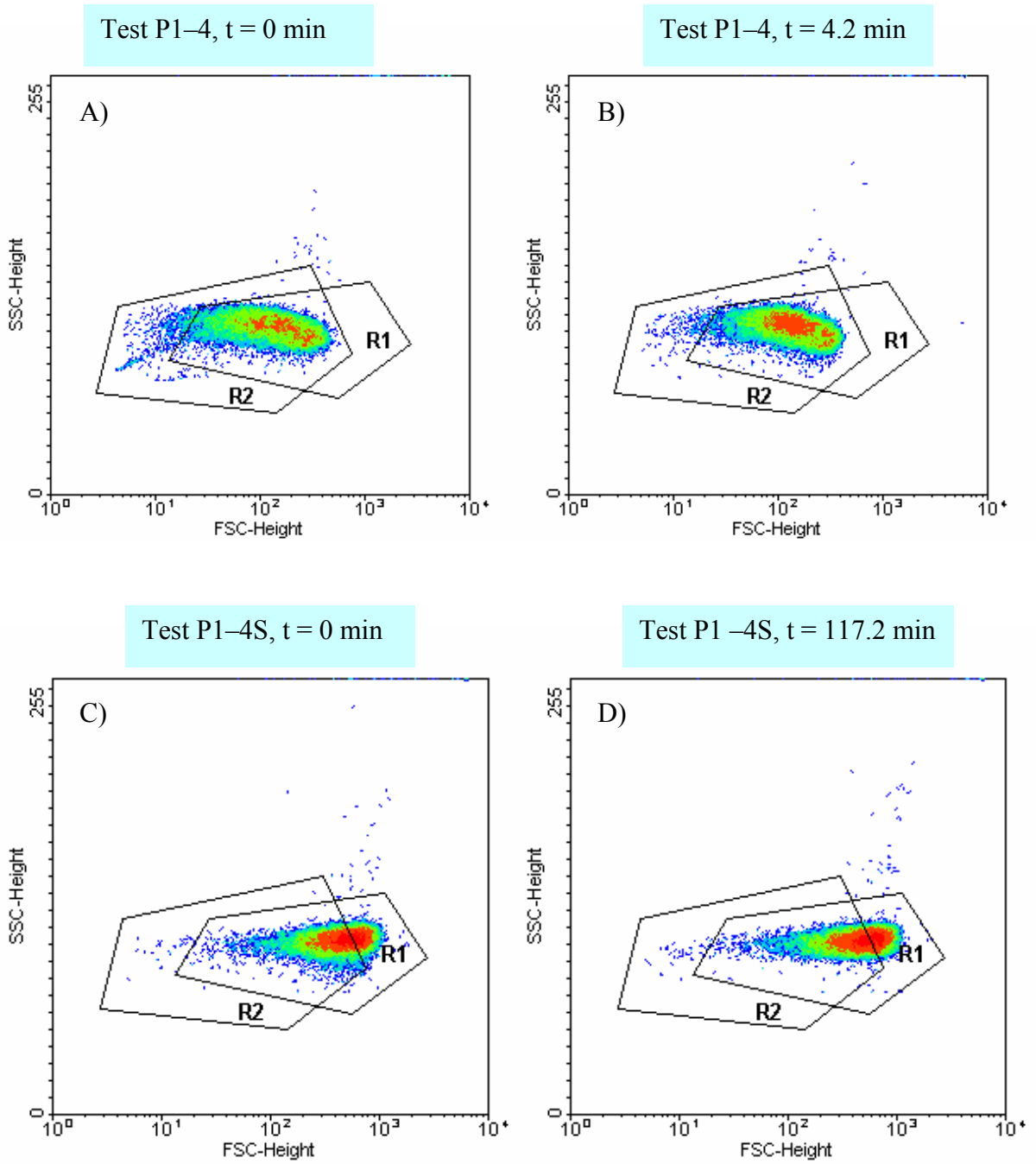


Figure 11: Dot/density plot showing forward ligth scatter (FSC) on the X-axis and 90º light scatter (SSC) on the Y-axis aqcuired for experiment P1-4 (A and B) and P1-4S (C and D). Low sodium sulfite concentration was used to quench the secondary disinfectant samples.

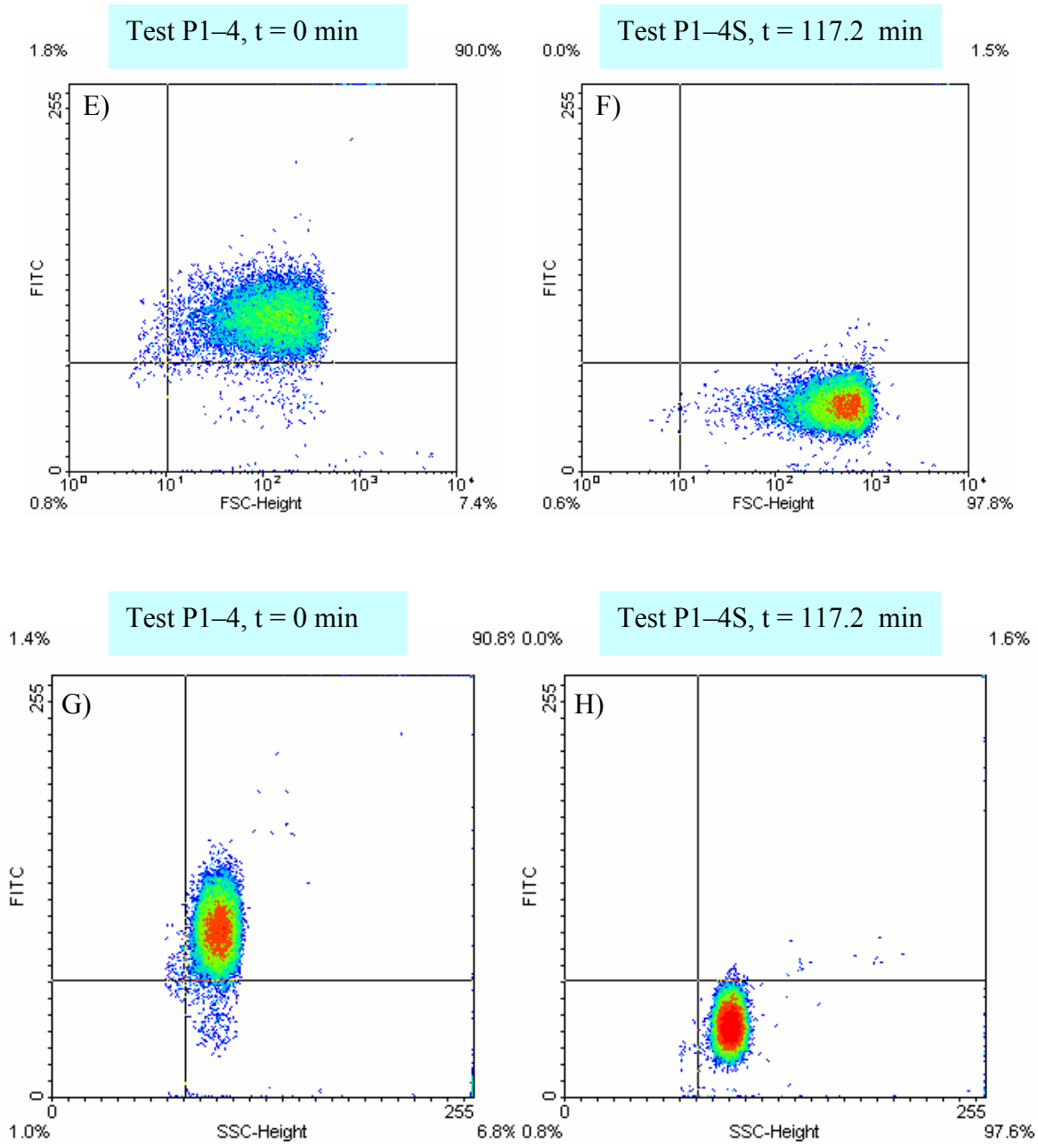


Figure 11: Dot/density plot showing: E and F : forward ligth scatter (FSC) on the X-axis and FITC on the Y-axis. G and H: 90º light scatter (SSC) on the X-axis and FITC on the Y-axis. For experiment P1-4 and P1-4S. Low sodium sulfite concentration was used to quench the secondary disinfectant samples.

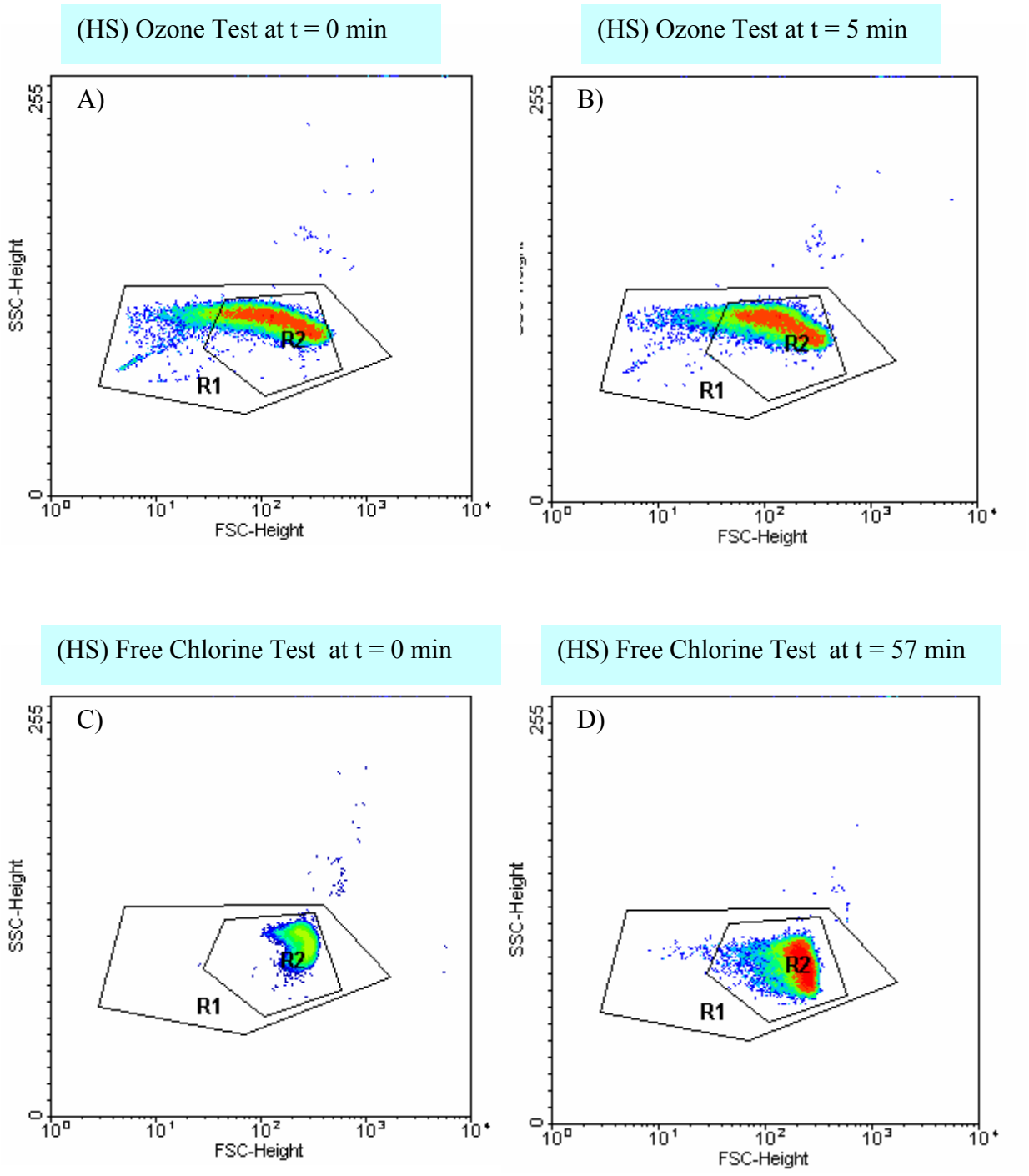


Figure 12: Dot/density plot showing forward ligth scatter (FSC) on the X-axis and 90º light scatter (SSC) on the Y-axis. High sodium sulfite concentration was used to quench the secondary disinfectant samples.

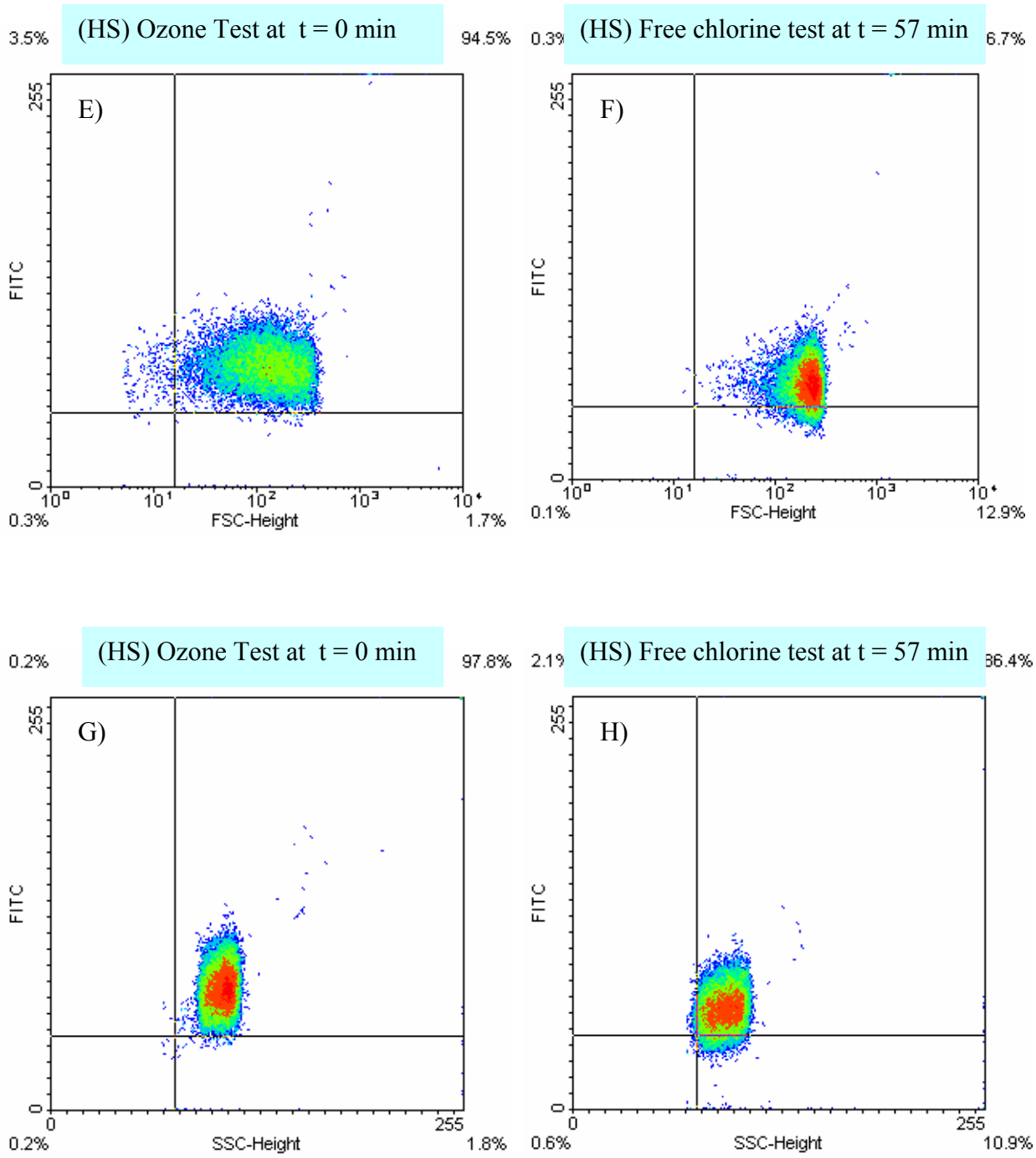


Figure 12: Dot/density plot showing: E and F : forward ligth scatter (FSC) on the X-axis and FITC on the Y-axis. G and H: 90º light scatter (SSC) on the X-axis and FITC on the Y-axis. High sodium sulfite concentration was used to quench the secondary disinfectant samples.

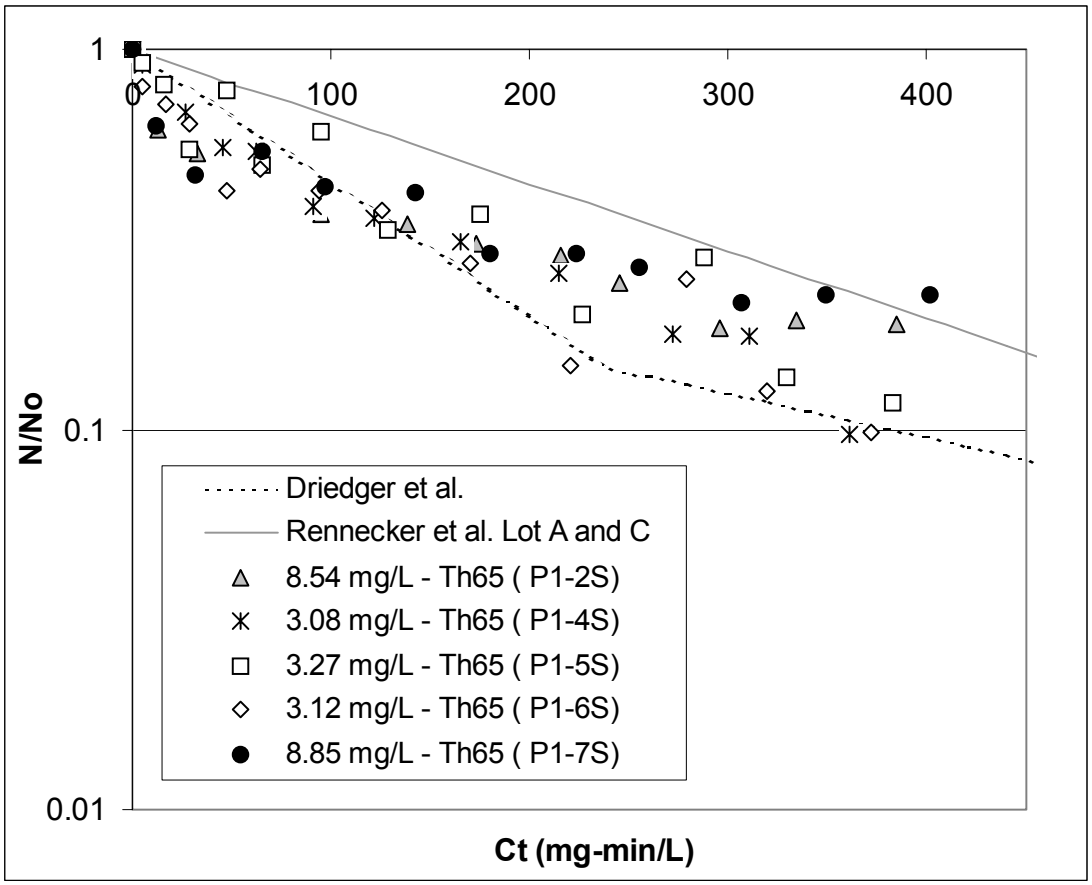


Figure 13: Normalized microspheres secondary free chlorine inactivation at pH 6 and 20 ± 2 Cº. Microspheres pretreated batch corresponds to pretreatment 1- Table 3. Fluorescence intensity threshold fitted at 65 (Th65). Ozone pretreatment primary stage- Ct = 1.4 mg-min/L. Reference inactivation curve based on Driedger *et al*. [5] and Rennecker *et al.* [10] (Table 1A-B).

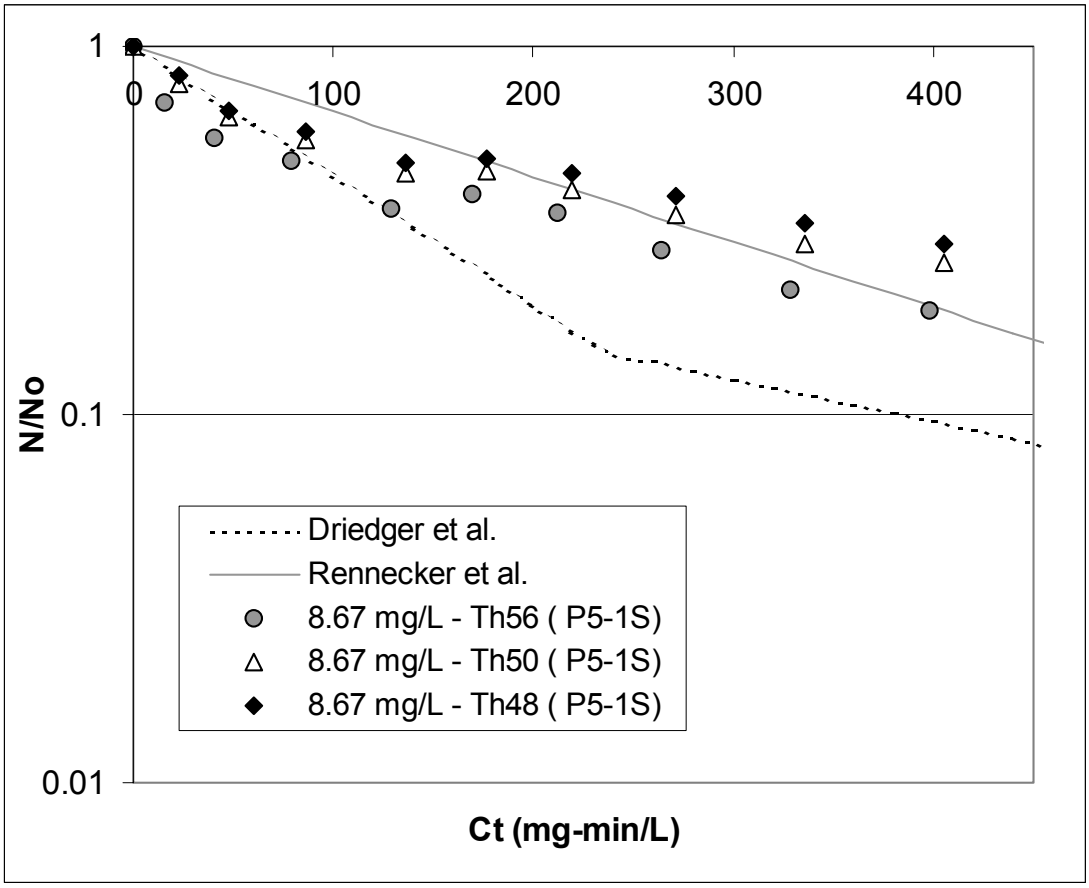


Figure 14: Normalized microspheres secondary free chlorine inactivation at pH 6 and 20 ± 2 Cº. Microspheres pretreated batch corresponds to pretreatment 3- Table 5. Fluorescence intensity threshold fitted at 56 (Th56). Ozone pretreatment primary stage- Ct = 1.4 mg-min/L. Reference inactivation curve based on Driedger *et al*. [5] and Rennecker *et al.* [10] (Table 1A-B).

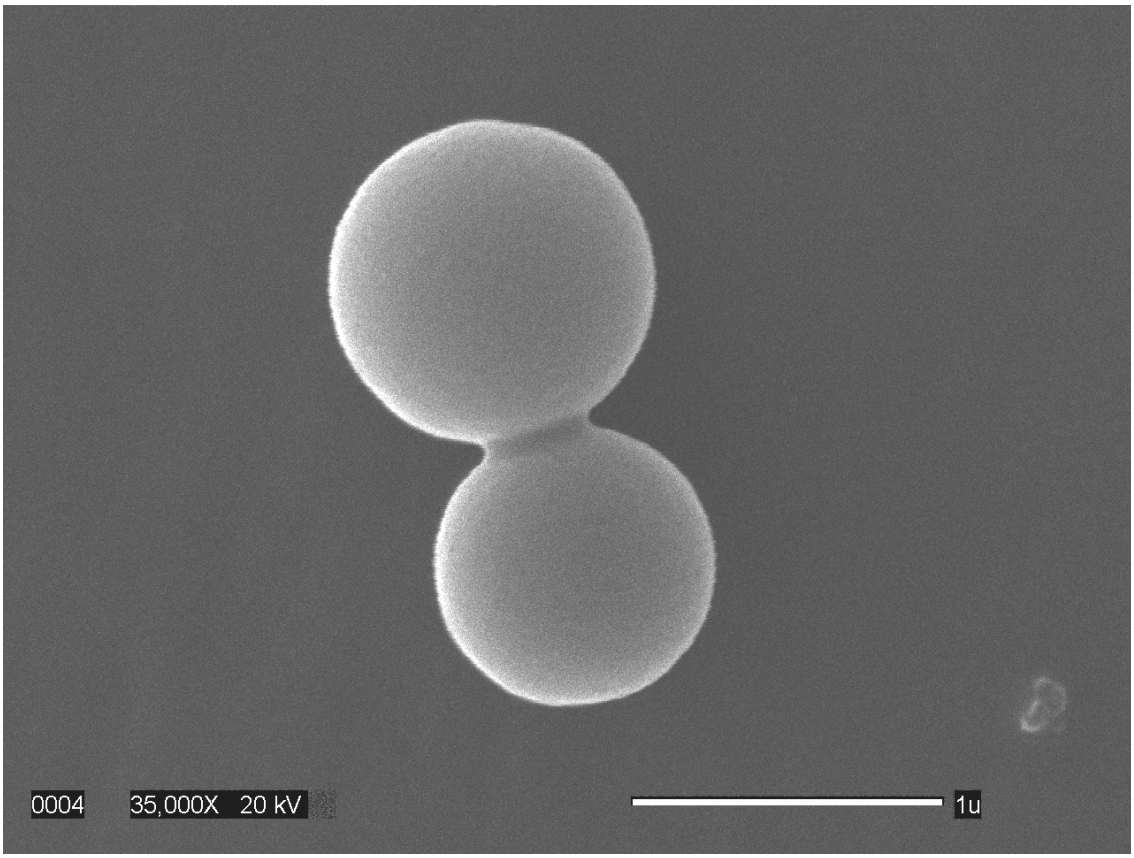


Figure 15: Scanning electron micrograph of the original non-pretreated microspheres (35,000 magnification).

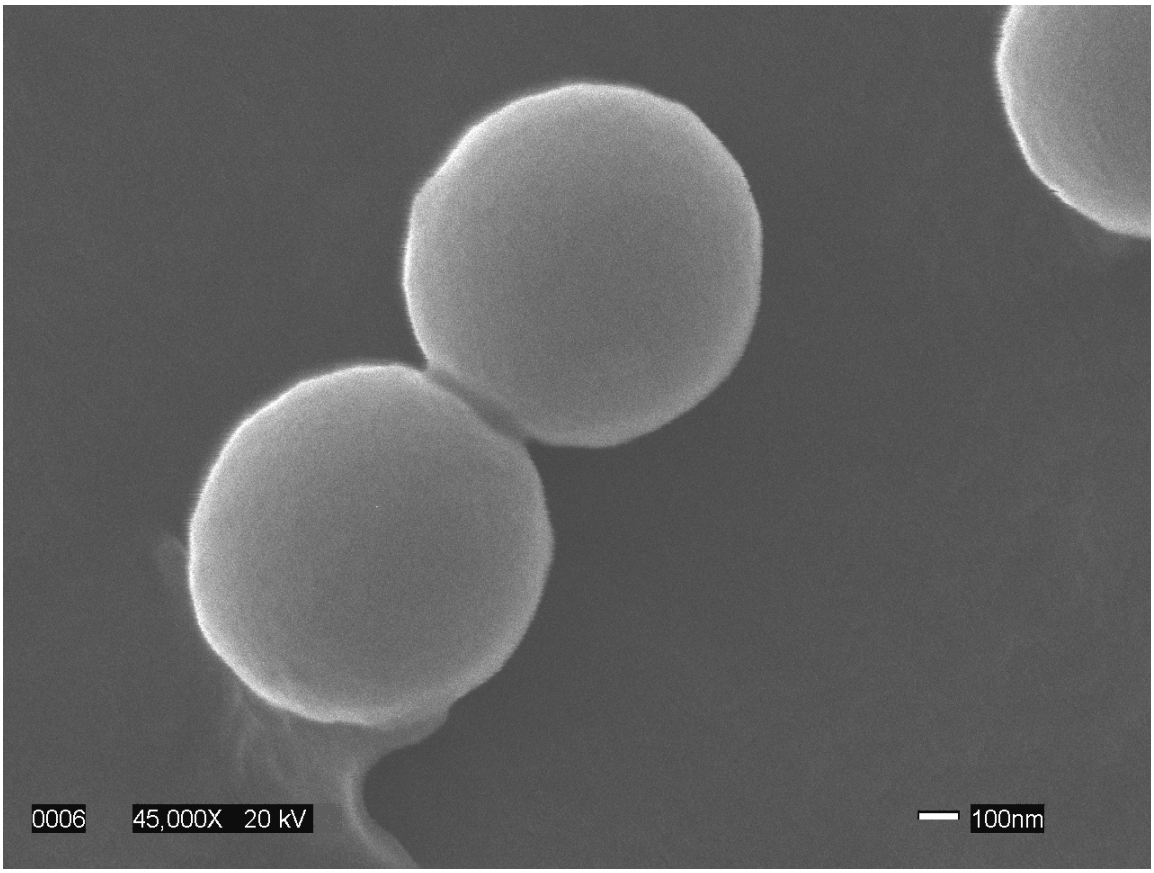


Figure 16: Scanning electron micrographs of ozone pretreated microspheres after 46 minutes at an ozone concentration of 0.66 mg/L (45,000 magnification).

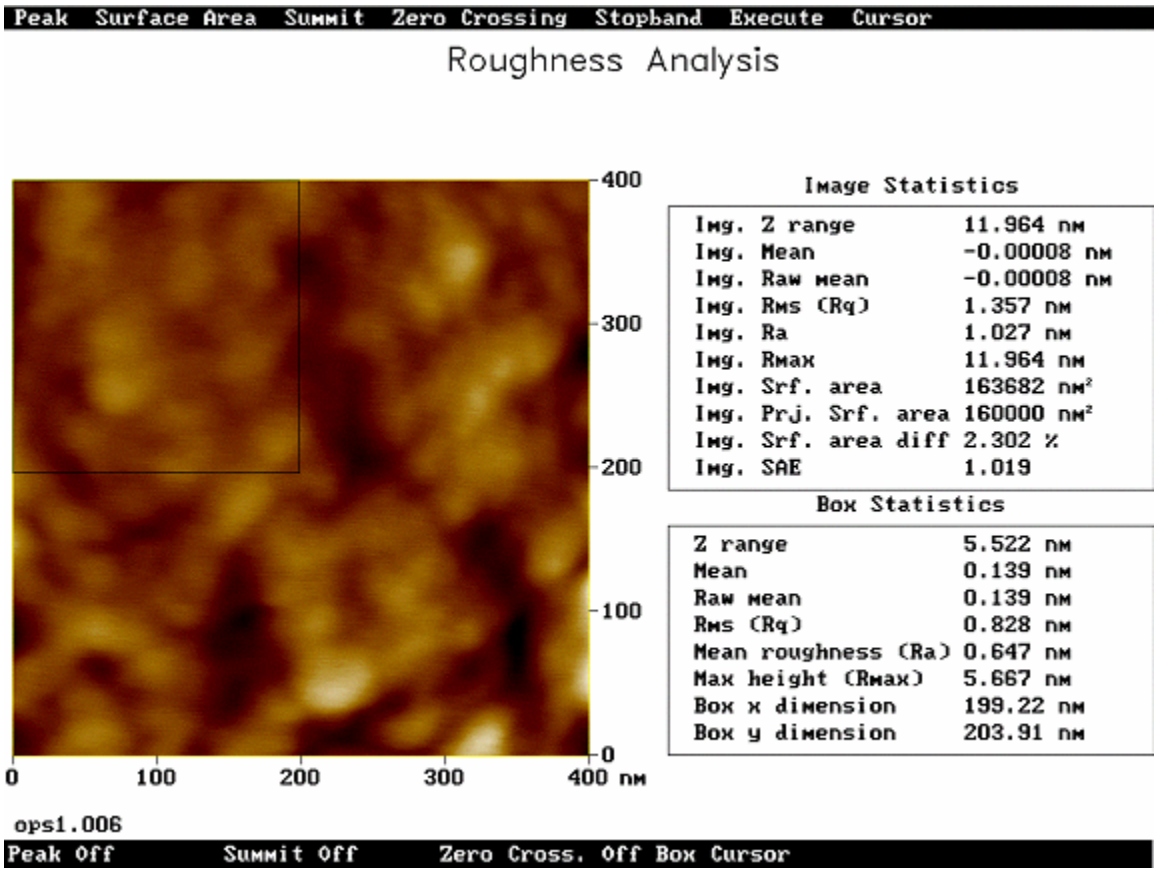


Figure 17: Atomic force microscopy (AFM) roughness analysis for original non-pretreated microspheres.

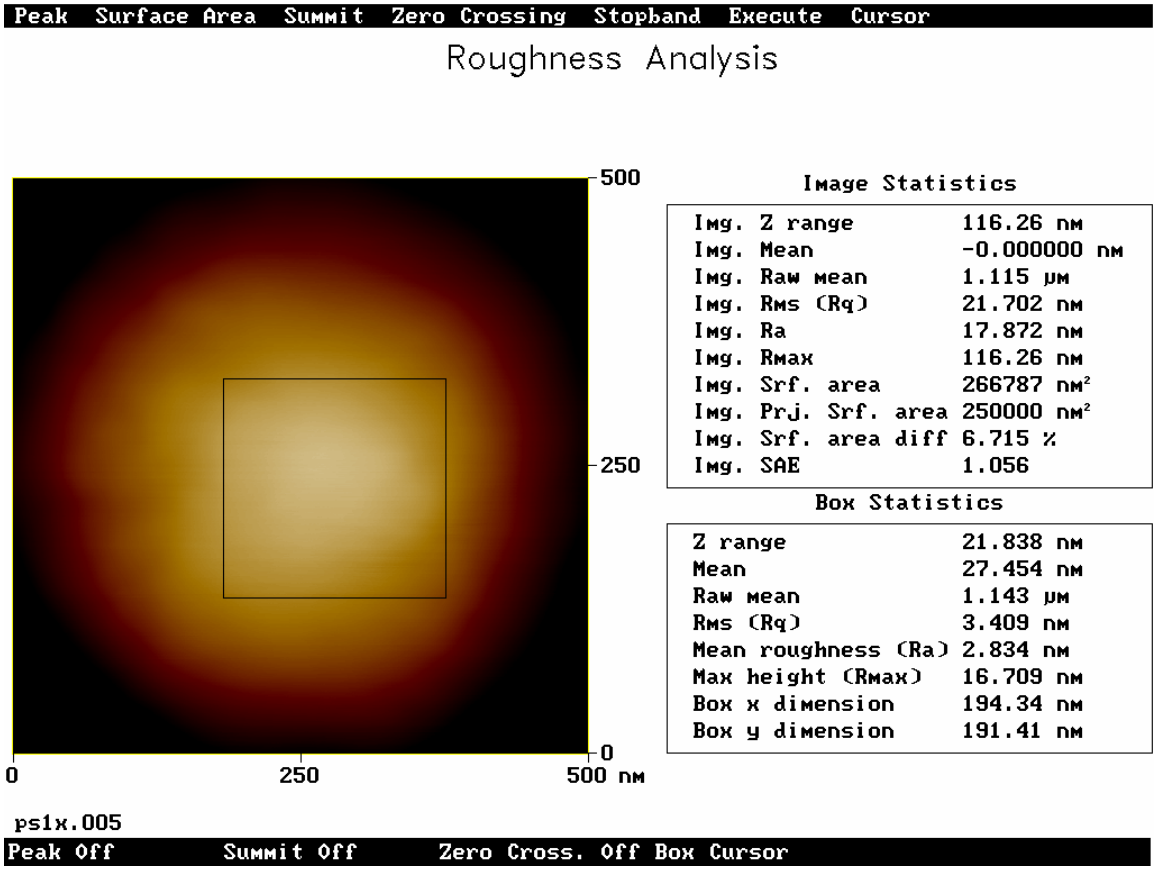


Figure 18: Atomic force microscopy (AFM) roughness analysis for microspheres pretreated 90 minutes at an ozone concentration of 0.3 ± 0.03 mg/L.

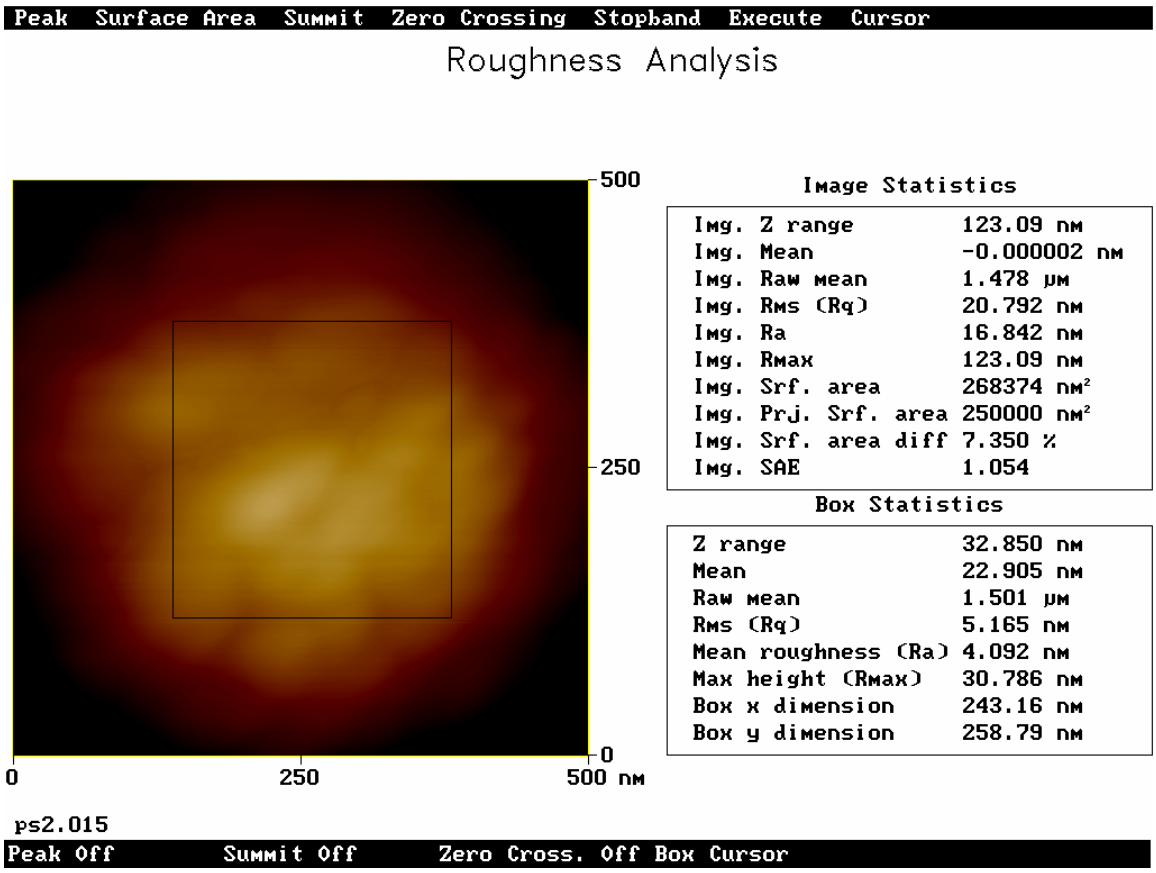
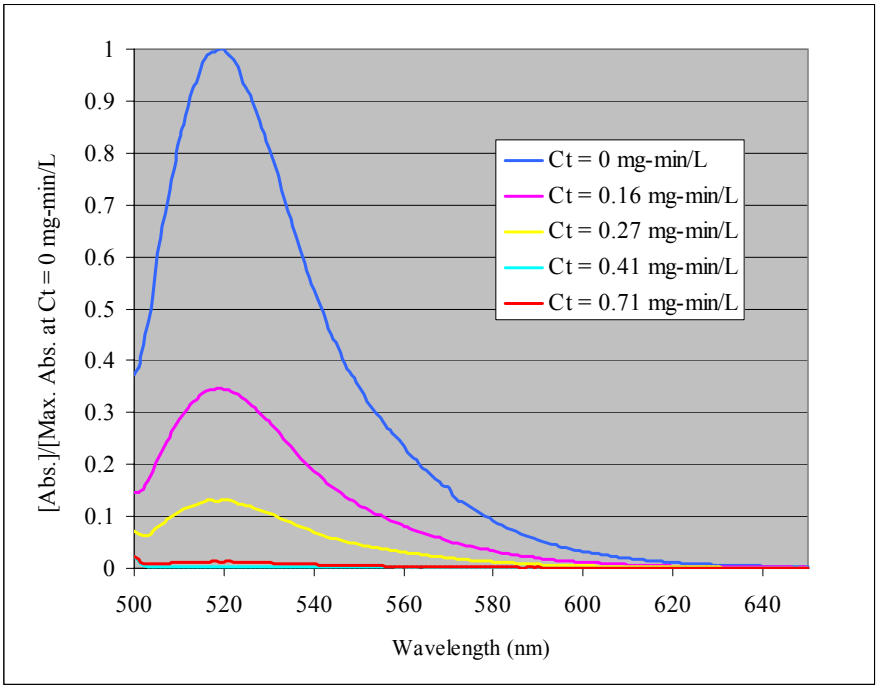


Figure 19: Atomic force microscopy (AFM) roughness analysis for microspheres pretreated 120 minutes at an ozone concentration of 0.3 ± 0.03 mg/L.

A)



B)

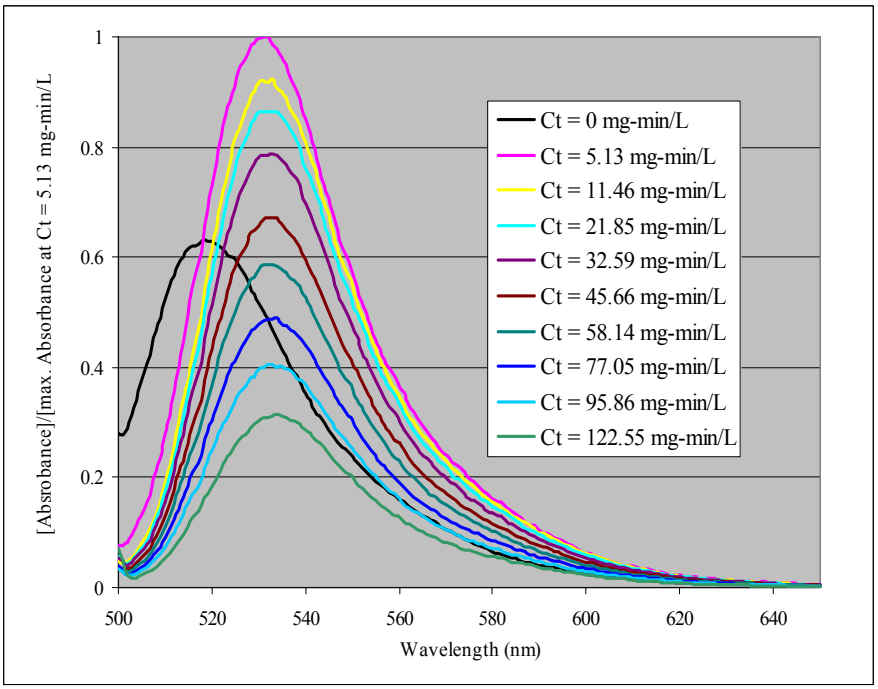


Figure 20: Normalized fluorescein isothiocyanate (initial concentration of 0.1 mg/L) degradation spectra for: (A) ozone treatment at pH 7 and 0.27 mg/L ozone concentration (B) free chlorine treatment at pH 6 and 5.7 mg/L free chlorine concentration.