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Tracer studies with fluorescent-dyed microorganisms—A new method for determination of residence time in chlorination reactors

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ABSTRACT

The *Ct* value, a product of free chlorine concentration and contact time, is the most significant factor in the disinfection of pathogenic microorganisms. Pulse introduction of a tracer at the reactor inlet and a collection of probes at the outlet are often used to determine contact time *t* from the residence time distribution (RTD) curve. Introduction of inert soluble tracers such as chloride, fluoride, and fluorescent dyes has long been considered sufficient to determine the flow-through path in chlorination reactors. It was generally agreed that the microorganisms are following the path of the soluble tracers.

The current study compares RTD curves of soluble tracers with curves of viruses and bacteria under a hypothesis that residence time can be influenced by the size of the solute. Tracer studies were performed in a bench-scale chlorination contact tank with fluorescent dyes rhodamine B (RB) and acridine orange (AO), viruses MS2 and T4, and bacterium *Escherichia coli*. The viruses and bacterium were stained with RB and AO, respectively.

An inverse relation between residence time and size of solute has been observed. Mean residence times of 10.8, 7.8, 7.1, and 6.1 min were found for rhodamine B, MS2, T4, and *E. coli*, respectively. The RTD curves of labeled and native microorganisms closely resemble each other, and were significantly different from the RTD curves of soluble dyes. Fluorescence intensity of the dyes was found to be sensitive to free chlorine concentration *C*. A linear correlation over a free chlorine concentration between 0 and 3 mg/L was found.

Determination of contact time in chlorination reactors by fluorescent labeling of microorganisms is a novel, simple, fast, and convenient procedure. The staining can provide precise microorganism-specific residence time in a chlorination reactor; in that sense the new tool has the potential to replace the inert soluble tracers used so far in RTD experiments.

1. Introduction

Chlorination of drinking and waste water is the most widely implemented disinfection technology. The main advantages of the chlorination, in comparison with other disinfection techniques, are its low cost and the unprecedented efficiency [1]. Chlorination is based on *the inactivation* of pathogenic microorganisms and not on their physical retention. Efficiency of chlorination is expressed by the log *inactivation* value (log_{in}). The log_{in} is defined as the base 10 logarithm of the ratio of microorganism concentrations in the feed N_0 and in disinfected water N, $log_{10}(N_0/N)$ [2]. The main factors that influence chlorination efficiency are concentration of free chlorine C, time t of contact between the disinfectant and pathogen, temperature, and pH of the solution. Reaching a lab-determined Ct value for each pathogen [3] is the single most important parameter in the design of chlorination reactors [1,2]. The U.S. Environmental Protection Agency (US EPA) guidelines use a more strict Ct_{10} value, where C is the residual concentration of chlorine at the reactor outlet and t_{10} is the contact time during which the first 10% of the water passes through the disinfection reactor [4].

Accurate determination of both C and t is therefore of vital importance in design and daily operation of chlorination reactors. The conservative approach requires that C will be measured as a residual concentration of free chlorine after the disinfection reactor and before first water consumer [4,5]. Determination of contact time t is more complicated. According to US EPA regulations and American Water Works Association (AWWA) manuals [4,6], in mixing basins and storage reservoirs the utilities are required to apply tracer studies or other methods approved by a state for accurate evaluation of t. Determining theoretical detention time (TDT), defined as the ratio of reactor volume V to flow rate Q, is not allowable. The reason is tht the actual flow through the reactor is far from an ideal plug and cannot be characterized by a single flow rate. The tracer studies are based on introduction of a passive

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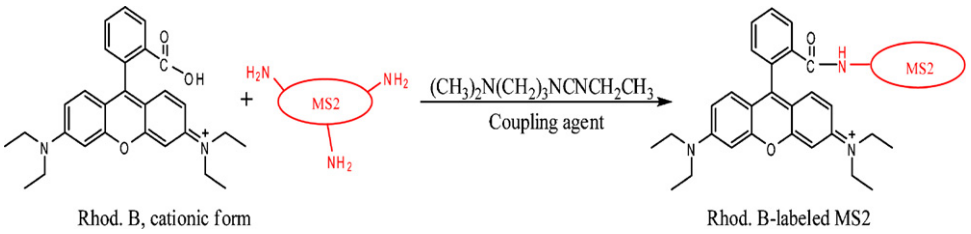


Fig. 1. Labeling reaction scheme of MS2 phage with rhodamine B.

tracer at the reactor inlet and tracking its concentration at the reactor outlet. The range of outlet tracer concentrations is plotted as a function of their residence time inside the reactor, to provide the residence time distribution (RTD) curve. Several characteristics of the reactor are derived from the RTD curve [7]. The contact time t_{10} and the mean residence time inside the reactor θ are among the most important [1]. The tracer is usually introduced by pulse or step injection. In a former mode, a large quantity of tracer is injected into a reactor in a relatively short period of time. In step input the tracer is introduced at a constant quantity until its concentration at the outlet reaches a steady-state level [8]. The step input requires significantly larger tracer doses to generate concentration signals that are not subject to random fluctuations; therefore the contact time is often determined by a spike (pulse).

The need for *on-site* monitoring of the reactor outlet for RTD profiling governs the tracer selection. Anions such as chloride and fluoride [9,10], and fluorescent dyes such as rhodamine B [4] are the most common chemical tracers implemented. The anions are considered nontoxic and approved for potable water use without limitations. Maximum concentrations of 10 mg/L and 0.1 μg/L in reactor inlet and outlet, respectively, are maximum allowable values when rhodamine B is injected. The tracers are important in studies of water flow inside the reactor, but do not necessarily reflect the flow-through path of microorganisms. Accurate evaluation of *Ct* value can best be obtained with deliberate introduction of pathogenic microorganisms into the reactor [3,4]. As this is prohibited for health reasons, use of surrogate microorganisms is a reasonable alternative.

The current study describes implementation of surrogate tracers made of a microorganism of concern and fluorescent dye. Conjugation of the dye results in inactivation of the microorganism and therefore use of the fluorescent-dyed microorganism can only be limited by concentrations, similar to those applied for fluorescent dyes. Significant dilution of the tracer inside the reactor will bring its concentration at the outlet closer to that required. Application of *microorganisms of interest* instead of soluble dyes might help in more accurate determination of the residence time.

Two types of bacteriophages (viruses that infect bacteria), MS2 and T4 [11], and *Escherichia coli* (*E. coli*) bacteria were labeled with fluorescent dyes, and used for tracer studies in a specially designed chlorine contact tank (CCT). The free dyes were used as reference soluble tracers. A screening of fluorescent dyes was performed initially. We searched for the dye with two features—conjugation with a broad variety of microorganisms, and bleaching in the presence of free chlorine. A linear correlation between fluorescence intensity and concentration of free chlorine is needed to find contact time t and chlorine dose C from a single RTD curve.

2. Materials and methods

2.1. Cultivation and enumeration of MS2 and T4 bacteriophages

E. coli K12, ATCC 23631 and *E. coli* B/r H266 (DSMZ, Germany) were used as host bacteria for cultivation and enumeration of MS2 and T4 bacteriophages, respectively. The cells were incubated overnight at 37 °C in Lauria–Bertani (LB) medium. One milliliter of the overnight culture was added to 100 mL LB medium for additional incubation. Incubation was continued until the cells reached exponential growth phase, determined by suspension turbidity of 0.2–0.3 OD at 600 nm. Propagation of the bacteriophages was performed by incubating each phage with the corresponding cells at the stage of exponential growth. Cultivation of the phages continued until the suspension became clear. Extraction of the phages was performed by lysis of bacterial cells with 1 mL chloroform added per 100 mL suspension. The obtained solution was then centrifuged at 3800 × *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 37,000 × *g* to retrieve purified phages. The pellet was re-suspended in phosphate buffered saline (PBS) at pH = 7.2. Bacteriophages were enumerated by a plaque forming unit (PFU) assay using a double-layer technique [12]. Concentrations of up to 10^{10} PFU/mL were obtained.

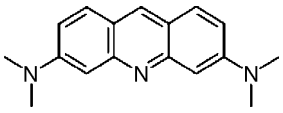
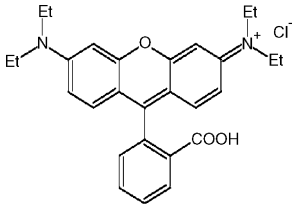
2.2. Microorganism staining

MS2 and T4 bacteriophages were labeled with rhodamine B (RB), (9-(o-carboxyphenyl)-6-(diethylamino)-3H-xanthen-3-ylidene) diethylammonium chloride (Aldrich), according to the procedure described earlier [7,13]. The labeling reaction (Fig. 1) was performed in the presence of coupling agent DEC (1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride), which was needed to attain permanent attachment. The solution was placed into a dialysis membrane (MWCO = 6000–8000 Da) and free dye molecules were washed out. The membrane dialysis was carried out in distilled water under stirring at 4 °C, until no fluorescence was observed in the rinsing water (approximately 1 week). The purified labeled bacteriophages were stored at 4 °C in a dark room.

E. coli were stained with acridine orange (AO), 3,6-bis [dimethylamino] acridinium chloride (Aldrich). *E. coli* cells were incubated in LB medium for 16 h at 37 °C. The suspension was then diluted (100-fold) and re-incubated until an OD of 0.6 at 600 nm was observed. The solution was then centrifuged for 10 min at 6000 × *g*; the supernatant was discarded and the pellet was resuspended in PBS (pH = 4). *E. coli* concentration (as colony forming units, CFU) was determined by overnight incubation at 37 °C on LB agar. For dyeing, AO stock solution in 0.5 M sodium acetate was added to the bacterial suspension for 3 min. The solution was purified by several cycles of centrifugation and resuspension until all free dye molecules were removed. Pertinent properties of RB and AO are summarized in Table 1.

Fluorescence measurements were conducted using TECAN Infinite M200 plate reader equipped with a monochromator. The intensity was reported in the instrument's relative fluorescence units (RFU). The excitation at 530 nm (9 nm bandwidth) and emission at 580 nm (20 nm bandwidth) for RB and RB-labeled phages were used. For AO and AO-labeled cells we used the literary values of 492 and 525 nm, respectively [14,15].

Table 1
Properties of acridine orange and rhodamine B fluorescent dyes.

	Acridine orange	Rhodamine B
Structure		
Molecular formula	$C_{17}H_{19}N_3$	$C_{28}H_{31}N_2O_3Cl$
Molecular weight (g/M)	265.35	479.02
$\lambda_{excit}/\lambda_{emiss}$ (nm)	492/525[a]	530/580[b]
Solubility in water	High	Very high

[a] Back and Kroll [14].
[b] Measured values.

2.3. Effect of chlorination on fluorescence intensity

Kinetics of fluorescence bleaching in the presence of hypochlorite in a concentration range of 1 and 100 mg/L was recorded at time intervals of 10 s for at least 1 h. Concentration of hypochlorite was determined with a colorimetric method [16] using N,N-diethyl-p-phenylenediamine (DPD) and a DR/890 colorimeter (Hach Company, Loveland, CO, USA).

Changes in fluorescence intensity at the reactor outlet were linked to contact time *t* or to chlorine concentration *C*. The observed decay in fluorescence intensity was used as an indication of log inactivation values for labeled microorganisms.

2.4. Experimental setup

A bench-scale disinfection system, designed and built for the study, is illustrated in Fig. 2. The system consists of two feed vessels, two peristaltic pumps, a static mixer, a chlorine contact tank (CCT), and a sample collector (FC 203B, Gilson Inc., Middleton, WI, USA) connected to the CCT outlet. Design of the system was based on common knowledge in disinfection systems design including mixing before the CCT and baffling inside the reactor to prevent short-circuiting [4]. The CCT is 162 mm long, 40 mm wide, and 40 mm deep, its holdup volume is 200 mL. The CCT was designed for a theoretical detention time (TDT) of 10 min. The internal volume was divided by 10 baffles to 11 compartments. The exact CCT design is detailed in Fig. 3. Inlet and outlet pipes of 4 mm inside diameter were connected to points 1 and 2. The outlet pipe was connected to a fraction collector, which was programmed to collect samples at fixed times (every 0.5 min for 0.2 min) for further analysis.

The system operated with a constant 20 mL/min flow rate. Chlorination experiments were performed with 2–3 mg/L NaOCl. The residual chlorine was neutralized at the CCT outlet with sodium thiosulphate, $Na_2S_2O_3$ (final concentration 0.084%), prior to collection in the fraction tubes [17]. The experiments were carried out at room temperature (20 °C) and pH 5.5. Tracers were injected

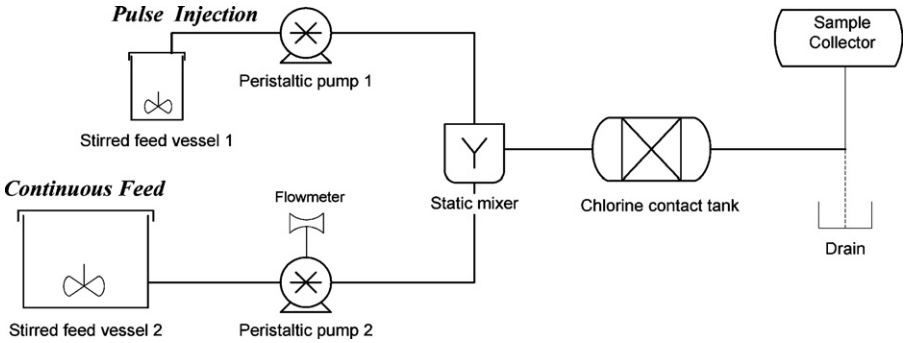


Fig. 2. Schematic diagram of the laboratory disinfection system.

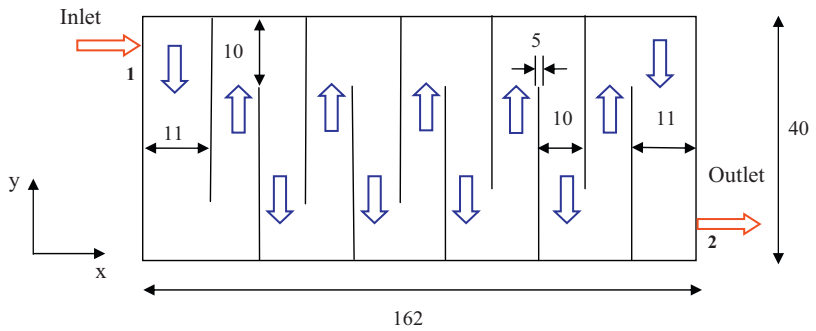


Fig. 3. Details of chlorine contact tank (dimensions in mm). Main stream is indicated by arrows.

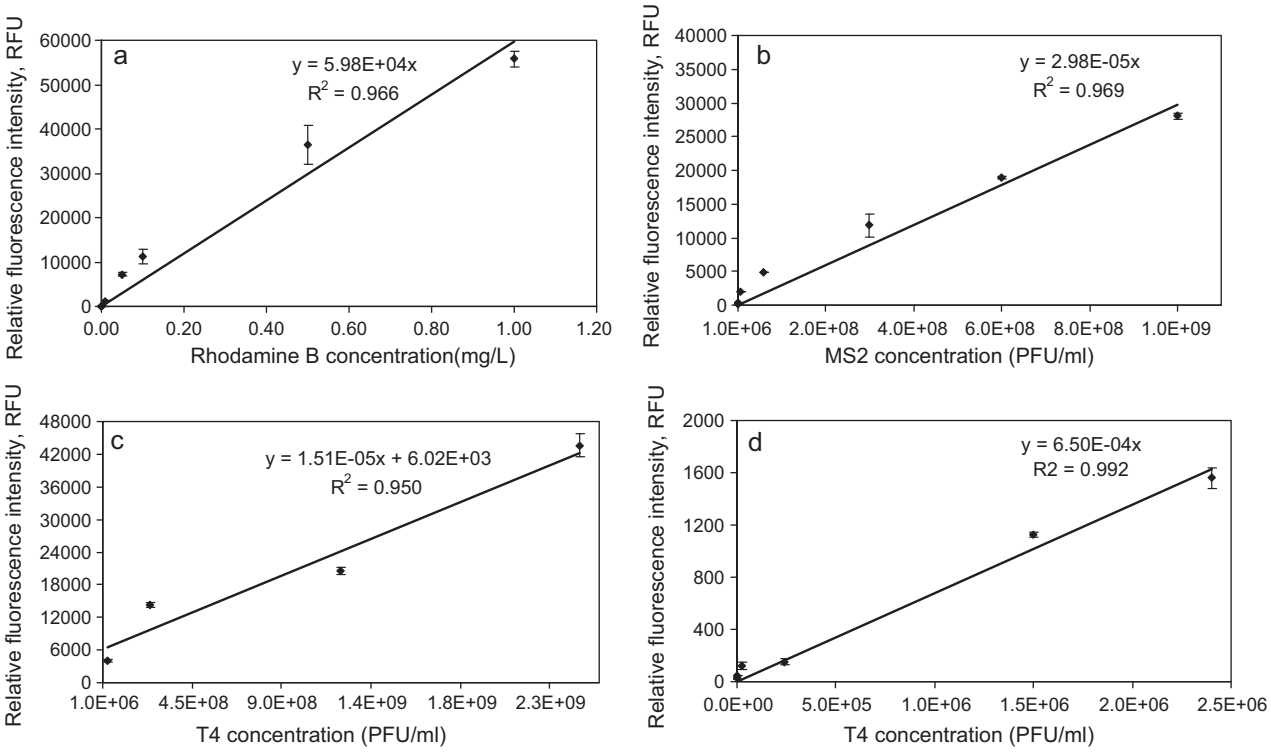


Fig. 4. Calibration curves of (a) free RB (optimal gain = 70; $\lambda_{exit}/\lambda_{emiss}$ = 530/580 nm); (b) RB-MS2 (optimal gain = 70; 530/580 nm); (c) RB-T4 (high concentrations, optimal gain = 79, 530/580 nm); (d) RB-T4 (low concentrations, optimal gain = 79, 530/580 nm).

in 10 mL spikes over ~20 s injection time. Separate experiments with free fluorescent dyes RB and AO, with RB-labeled phages MS2 and T4, and with AO-labeled *E. coli*, were performed. The collected fractions were transferred to 96-well plates for fluorescence measurements. The CCT was operated in the dark due to the sensitivity of fluorescent dyes to light exposure.

2.5. Modeling of the RTD curves

The residence time distribution (RTD) curves were characterized by several values including arrival times t_f and t_{10}, mean residence time θ, maximal residual ratio C/C_0, and distribution variance σ^2 [1]. The arrival times t_f and t_{10} in minutes are the times between injection and appearance of 1% and 10% of the total tracer mass, respectively, at the CCT outlet. Values lower than 0.01 C/C_0 were referred to as background noise. The values were determined from the area under RTD curves (taken as the time at 1% or 10% of total area), using the OriginLab® programming.

The mean residence time θ was calculated by summing the areas of discrete time intervals, as exemplified in Eq. (1):

$$\theta = \frac{\int_0^\infty t \cdot C(t)dt}{\int_0^\infty C(t)dt} \approx \frac{\sum_i t_i \cdot C_i \, \Delta t_i}{\sum_i C_i \, \Delta t_i} \quad (1)$$

where C_i is concentration of the tracer, t_i is elapsed time since the injection, and Δt_i is the collection time period of individual samples. In experiments with fluorescent dyes the concentration was replaced with fluorescence intensity in relative fluorescence units (RFU). Calculation of θ in experiments performed with fluorescent tracers in the presence of hypochlorite demanded complex calculations that included changes in fluorescence intensity due to the reaction between the dye and hypochlorite. Eq. (2) includes an exponential term with a constant decay rate, k_{dec} (min^{-1}), in a pseudo first-order reaction with constant hypochlorite concentration.

$$\theta = \frac{\sum_i C_i \cdot \exp^{-k_{dec}t} \cdot t_i \cdot \Delta t_i}{\sum_i C_i \cdot \exp^{-k_{dec}t} \cdot \Delta t_i} \quad (2)$$

Residence time distribution variance σ^2 (min^2) was defined as in Eq. (3):

$$\sigma^2 = \frac{\sum_i C_i \cdot \exp^{-k_{dec}t} t_i^2 \, \Delta t_i}{\sum_i C_i \cdot \exp^{-k_{dec}t} \, \Delta t_i} - \theta^2 \quad (3)$$

3. Results and discussion

3.1. Calibration of the probes

Fluorescence intensities of free RB and RB-conjugated MS2 and T4 were calibrated against the concentration of the probe. Calibration of viruses was performed as fluorescence intensity *vs.* concentration of native viruses before labeling. The results are depicted in Fig. 4. The calibrations were carried out under various conditions in order to achieve as much *accuracy* and *sensitivity* as possible for a wide fluorescence range. Since the applied gain affects the sensitivity and the detection range of the fluorescence measurements (high gain values lead to lower minimum detection limit, and *vice versa*), the calibrations were conducted at several gain values. This allows a wide concentration detection range for each fluorescent species, as needed for analyzing the series of samples collected during RTD experiments. The free dye and the labeled MS2 exhibited a linear curve throughout the entire concentration range. Calibration of dyed T4 was performed in two concentration ranges, presented in Fig. 4c and d. Still, the resulting slopes are of the same order of magnitude for both ranges.

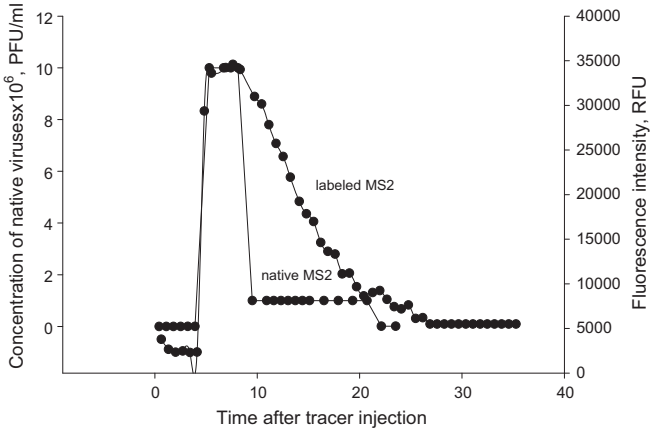


Fig. 5. RTD curves of native and RB-labeled MS2 bacteriophages in CCT reactor.

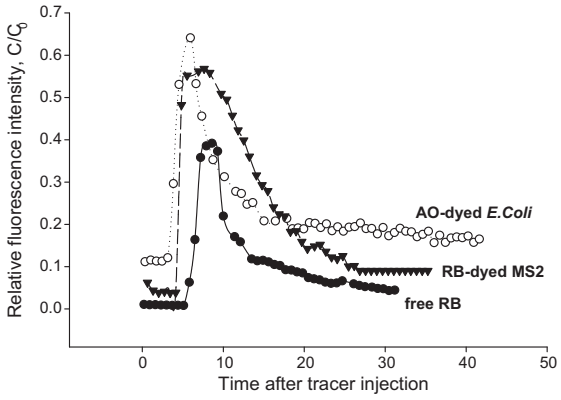


Fig. 6. Normalized RTD curves of free RB dye, RB-MS2, and AO-labeled *E. coli*.

3.2. Determination of residence time

RTD curves of native and RB-conjugated MS2 bacteriophages are plotted in Fig. 5. Ten milliliter mixtures of native (10^{10} PFU) and RB-labeled MS2 (6×10^4 RFU) were injected into a static mixer immediately before the flow enters the CCT. The spike was performed 30 min after the experiment started. The curves have skewed Gaussian forms. The pertinent properties of two curves are detailed in Table 2. A good match was observed. Differences in hydraulic indices including t_f, t_{10}, and θ do not exceed 10%, which is well within the accuracy margins of PFU enumeration. It was assumed that the observed step-like shape of the native virus plot is due to lower resolution of the plaque assay method. Behavior of native and labeled viruses in processes of coagulation, flocculation, sedimentation, and filtration was compared previously [7,13,18]. Similar retention levels and hydraulic indices were found. Based on present and previous studies, it was concluded that behavior of native viruses in CCT can be adequately imitated by labeled bacteriophages.

RTD curves of separate experiments with free RB, RB-labeled MS2, and AO-labeled *E. coli* are plotted in Fig. 6 for analysis. All three curves exhibited a skewed Gaussian form with a right tail indicating dispersion of the probe inside the reactor. Along with that, a clear shift in the three curves was observed. The peak of *E. coli* appeared before the peak of MS2, and both peaks appeared significantly earlier than the peak of free dye. Breakthrough of microorganisms occurred much earlier than that of solutes. The hydraulic indices of the probes, including t_f, t_{10}, θ and σ^2, are detailed in Table 3. The 5 probes, namely free RB, free AO, RB-labeled MS2, RB-labeled T4, and AO-labeled *E. coli*, were subdivided into three groups—two free dyes, two viruses, and one bacterium. Comparison of the relative characteristics reveals that each group displays significantly different values. At the same time little variation is observed within each group. For example, the t_{10} time of both dyes is significantly larger – 7.2 and 7.8 min for RB and AO, respectively, than the t_{10} time of viruses – 5.6 and 5.4 min for MS2 and T4, respectively. The smallest t_{10} value of 4.8 min was found for *E. coli*. The mean residence times θ of free dyes are 10.8 and 12.3 min for RB and AO, respectively. Values of 7.8 and 7.1 min for MS2 and T4, respectively, and 6.1 min for *E. coli* were found. Other calculated indices followed the same path.

Observations of curves in Fig. 6 and their hydraulic parameters point to a noticeable tendency. Hydraulic parameters within a group are much closer than between the groups. The variation within a group is usually less than a minute; the gap to another group is 2–3 min. There is a clear opposite relation between size of the tracer and its residence parameters, *i.e.*, the bigger the tracer, the less time it spends inside the reactor. Typical hydraulic diameter of free dyes is ~1 nm [19], of MS2 ~28 nm [18], and of *E. coli* ~1 μm [20,21]. Clearly, soluble tracers are much smaller than viruses, and viruses are much smaller than bacteria. But even comparing the tracers within each group reveals a similar trend. For example, MS2 is a smaller phage than T4 and therefore spends more time in the reactor.

The Stokes–Einstein equation suggests a relation between size of a particle and its diffusive motion:

$$D = \frac{K_B T}{6\pi r \mu} \tag{4}$$

here D is the diffusion coefficient (m^2/s), K_B is the Boltzmann constant (J/K), T is absolute temperature (K), r is the radius of a spherical particle (m), and μ is water viscosity (kg/m s) [22]. The diffusion coefficients for the tracers were calculated based on provided estimated values of the tracers' radii and assuming their sphericity. The calculations were based on single particles, assuming no aggregation. Concentration of free dye molecules in stock solution was intentionally taken below the initial aggregation concentration of 10^{-4} M [15,23]. Results of the calculations are presented in Table 4. Diffusion coefficients of 4.3×10^{-10} m^2/s, 1.4×10^{-11} m^2/s, 7.2×10^{-12} m^2/s, and 2.1×10^{-13} m^2/s were found for free RB, MS2, T4, and *E. coli*, respectively. There is an order of magnitude difference between diffusion coefficients of free dye, viruses, and the bacterium. The three groups are, once again, clearly observable.

Transport of solutes through CCT can be governed by convection and diffusion. The evident difference in the diffusion coefficient of investigated solutes suggests that for some of them the diffusion

Table 2
Calculated residence time-related parameters for native and fluorescent dyed MS2 (as of Fig. 5).

Hydraulic indices [min]	Native MS2	RB-labeled MS2
t_f	3.9	4.1
t_{10}	5.0	5.6
θ	8.7	7.8

Table 3
Characteristics of the RTD curves of free dyes, RB-MS2, RB-T4, and AO-*E. coli* during RTD test in the laboratory CCT (as of Fig. 6).

Hydraulic indices	Free dyes		Viruses		Cell
	Free RB	Free AO	RB-labeled MS2	RB-labeled T4	AO-labeled *E. coli*
t_f (min)	5.1	5.6	4.1	3.8	3.1
t_{10} (min)	7.2	7.8	5.6	5.4	4.8
θ (min)	10.8	12.3	7.8	7.1	6.1
σ^2 (min^2)	26.0	31.3	13.7	12.3	21.2

Table 4
Diffusion coefficients of the tracers in water.

	Free RB	RB-labeled MS2	RB-labeled T4	AO-labeled *E. coli*
Diameter (nm)	~1[a]	~28	Head—90 nm Tail—20 nm	~1000
D (m^2/s)	$4.3e^{-10}$	$1.4e^{-11}$	$7.2e^{-12}$ [c]	$2.1e^{-13}$
Reference value	($4.4e^{-10}$)[b]	(N.A.)	(N.A.)	(N.A.)

[a] Guodong and Minquan [19].
[b] Gendron et al. [24].
[c] Calculated for spherical particle with estimated diameter of 70 nm at pH = 5.5 [20].

might be less important. A difference between 10^{-10} m^2/s for free dyes and 10^{-13} m^2/s for bacteria suggests significant diffusion for the dyes and dominant convection for microorganisms. Low diffusion may result in flow through the reactor following a mean path without axial movement to "dead" compartments near the baffles. The actual residence time θ might be much shorter than the theoretical detention time (TDT) of 10 min. Greater diffusivity of free dyes can result in elevated θ values due to the dye's ability to spread out into the entire volume of CCT including the "dead" areas where the large solutes will not travel.

3.3. Influence of free chlorine on determination of residence time

The influence of free chlorine on values of hydraulic indices was evaluated through RTD curves of experiments performed with and without NaOCl addition. Results of experiments with RB-labeled MS2 and T4 are depicted in Figs. 7 and 8, respectively. In both figures relative fluorescence intensity was plotted as a function of time elapsed since the pulse. The curves exhibited a skewed Gaussian form with a right tail indicating dispersion of the probe inside CCT. A greater decay in fluorescence intensity was observed in the presence of hypochlorite, due to oxidation of the dye by NaOCl. Both curves, however, resembled a similar general shape. Differences in hydraulic indices of each pair, detailed in Table 5, did not exceed 15%. It was believed that more precise determination of a decay rate constant k_{dec} would decrease the variance between indices.

Kinetics of fluorescence decay in the presence of hypochlorite was investigated in batch conditions. RB-labeled MS2 and T4 were exposed to 0, 1, 1.5, 2, and 3 mg/L NaOCl for time periods of 2, 3, 4, 6, and 10 min. The decay was calculated as a log of the ratio of initial to oxidized dye fluorescence intensities, and plotted against hypochlorite concentration. The plots for MS2 and T4 are depicted in Figs. 9 and 10, respectively. In the absence of the disinfectant, no inactivation was observed and C_0/C was equal to 1 (*i.e.*, $\log C_0/C = 0$). An increment in NaOCl concentration yielded more rapid decrease in fluorescence intensity. A linear correlation between fluorescence intensity and free chlorine dose was observed for hypochlorite concentrations below 3 mg/L. Exponential decay was observed at NaOCl concentrations greater than 3 mg/L, and no traceable fluorescence was observed at concentrations greater than 10 mg/L. Higher slopes with longer contact time but similar NaOCl concentration indicated an increase in fluorescence decay with exposure time. Similar behavior was observed for the additional fluorescent species. Greater decay in fluorescence intensity was observed with either increased chlorine dose C or with prolonged contact time t. The observed linear dependence can be useful in prediction of the actual disinfectant dosage Ct by measuring the tracer fluores-

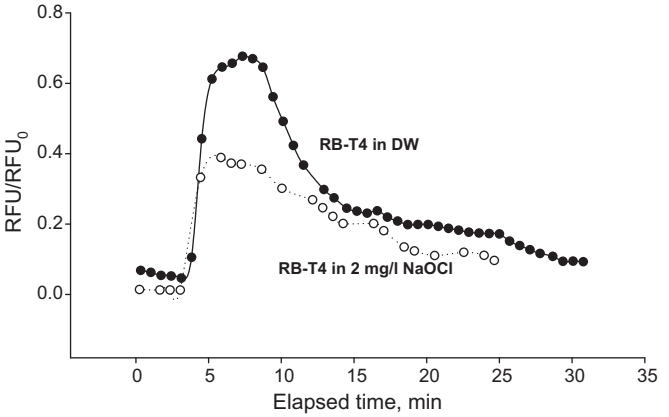


Fig. 8. Normalized RTD curves of RB-T4 phage in water with and without 2 mg/L NaOCl.

Table 5
Hydraulic indices of RTD curves of RB-labeled MS2 and RB-labeled T4 in test with and without NaOCl addition.

Hydraulic indices	With NaOCl addition		Without NaOCl addition	
	RB-labeled MS2	RB-labeled T4	RB-labeled MS2	RB-labeled T4
t_f (min)	4.3	3.7	4.1	3.8
t_{10} (min)	5.7	5.5	5.6	5.4
θ (min)	6.8	5.9	7.8	7.1
σ^2 (min^2)	9.6	9.0	13.7	12.3

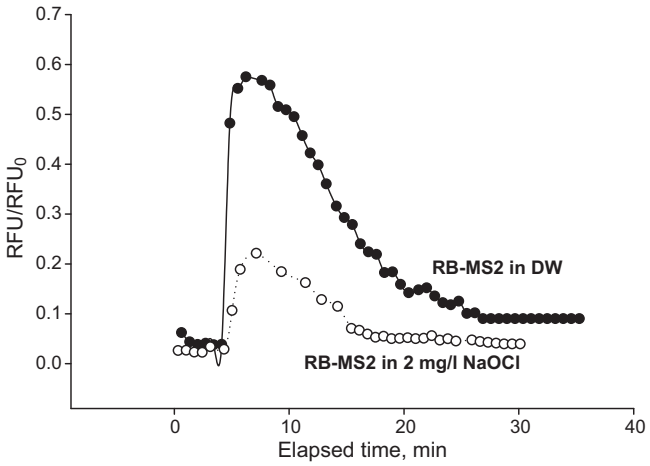


Fig. 7. Normalized RTD curves of RB-MS2 phage in water with and without 2 mg/L NaOCl.

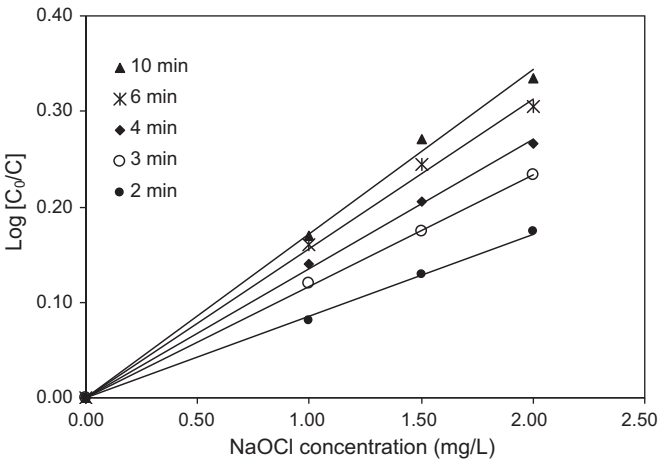


Fig. 9. Linear range of dye-labeled MS2 log fluorescent decay *vs.* NaOCl concentration at different exposure times.

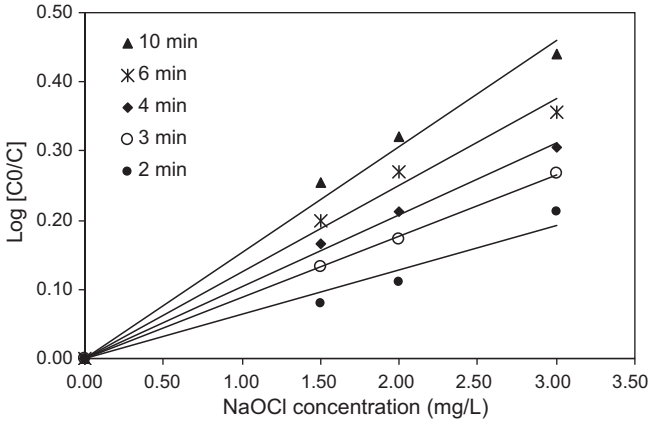


Fig. 10. Linear range of dye-labeled T4 log fluorescent decay *vs*. NaOCl concentration at different exposure times.

cence intensity solely at the system downstream, avoiding the use of native pathogenic organisms.

4. Closure

Studies of residence time in chlorination reactors are often performed with a pulse injection of a tracer. The RTD curves at the reactor outlet are used to calculate hydraulic indices such as θ, t_f, and t_{10}. Until now, calculation of *Ct* values was based on the indices obtained with soluble tracers despite the need to disinfect the microorganisms.

Significant and consistent difference between the hydraulic indices of soluble and particle tracers was observed. The difference was attributed to size of the solutes and was related to diffusion. Diffusion coefficients of free dyes were two orders of magnitude higher than those of viruses and three orders of magnitude higher than that of bacteria. It was argued that the solutes with a higher diffusion coefficient are spread more evenly in the chlorination reactor and are able to give a more accurate determination of residence time of water. However, the residence time of microorganisms was significantly lower than expected based on TDT calculations and soluble tracer studies. The difference of up to 4 min in the 10-min TDT reactor suggests that viruses and bacteria with lower diffusion coefficients are following the main stream path of water in the middle of reactor without lateral movement to the "dead" areas near baffles.

A specific fluorescent method based on dye-labeling of a vast variety of microorganisms (viruses and bacteria) was applied for careful examination of CCT. Separate pulse tracer experiments performed in a lab-scale chlorination reactor showed distinct similarity in RTD curves of dyed and native microorganisms. Based on a comparison of native and dyed microorganisms it was suggested that pulse injection of dye-labeled microorganisms can be used for determination of precise residence time in chlorination reactors. The suggested method provides two major advantages over soluble tracers. It helps to determine the exact residence time and disinfectant dose for each microorganism from a single RTD curve. Labeling results in inactivation of microorganisms and therefore the method is not limited to surrogate entities, but can express behavior of the dyed pathogens. The method can be diversified for parallel determination of *Ct* values of various pathogens by using fluorescent dyes with different excitation/emission spectra.

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