Fluorescent dye labeled bacteriophages—a new tracer for the investigation of viral transport in porous media: 1. Introduction and characterization

Vitaly Gitis\textsuperscript{a,1}, Avner Adina\textsuperscript{a}, Abed Nasser\textsuperscript{b}, Jenny Gun\textsuperscript{a}, Ovadia Lev\textsuperscript{a,∗}

\textsuperscript{a} Division of Environmental Sciences, Fredy and Nadine Herrmann School of Applied Science, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
\textsuperscript{b} Water Quality Research Laboratory, Ministry of Health, Tel Aviv, Israel

Received 29 June 2001; received in revised form 1 January 2002; accepted 19 March 2002

Abstract

A new method for the study of pathogen transport in porous media is presented. The method is based on conjugation of fluorescent dyes to target bacteriophages and application of the modified bacteriophages for tracer studies. We demonstrate that the relevant transport determining properties of Rhodamine and several fluorescein-labeled phages are practically identical to those of the native bacteriophages.

The advantages of the proposed method relative to direct enumeration of bacteriophages by plaque forming unit method, turbidity, fluorescent microspheres, and other alternative tracers are discussed. Notable advantages include simple quantitation by optical methods, unbiased signals even when virus aggregates are formed, and the ability to decouple inactivation kinetics from transport phenomena. Additionally, the signal reflects the removal and transport of the studied microorganism and not a surrogate.

© 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Tracer studies; Deep-bed filtration; Porous media; MS2 bacteriophage

1. Introduction

Considerable research efforts were devoted during the recent decade for the understanding and modeling of waterborne virus transport and fate in over-ground and subsurface porous media [1–3]. Pollutant transport in saturated and unsaturated subsurface zones, river bank filtration, soil aquifer treatment and ground water recharge are examples of environmental processes for which the elucidation of a virus transport mechanism is of vital importance. Deep-bed filtration and slow sand filtration of drinking water and wastewater effluents are typical environmental unit operations involving viral pathogen removal by porous media. Despite some differences between the hydrodynamics of virus transport in natural and engineered porous media, the probing tools and mathematical models used to investigate these over-ground and underground processes are rather similar, and the need for better probing tools of virus transport and inactivation is common for both fields.

Recent trends in environmental engineering include a demand for information pertaining to virus transport and fate in natural and manmade porous media. The intensified use of wastewater effluents for irrigation and groundwater recharge increases the risks associated with the gradual contamination of aquifers. The demand for a better mechanistic understanding is further increased by stricter water treatment regulations. One of the remaining bottlenecks for reliable implementation of
viral decontamination models is the limited information of virus decontamination during their transport through complex environments [1,4]. In order to accumulate more information on virus removal, the scientific community requires simple and rapid methods for the investigation of virus transport. Additionally, it is generally accepted that virus removal in porous media is performed by a dual mechanism involving attachment/detachment and inactivation steps. However, all current methodologies for the investigation of virus transport measure (at best) the result of the coupled inactivation/physical transport process and thus cannot distinguish between the two.

Current techniques for mechanistic studies of virus transport and fate in porous media rely on the following probes. (1) Turbidity. This is the cheapest, simplest, and most portable probe technique. However, viruses differ from light scattering particles in most of the relevant transport determining properties including surface charge, hydrophobicity, specific density, inactivation vulnerability, and size. (2) Particle size analysis. This technique overcomes the size limitation of the turbidity measure but not the inherent dissimilar nature of natural inorganic particles and viruses. Also, determining virus-size particle concentrations by particle analyzers is yet impossible. (3) Conservative tracers. Various chemical substances including anions (e.g., bromide and boron [5–7], surfactants [8] and soluble organic fluorescent dyes [9] are important probes for understanding and modeling of the hydrodynamics of environmental unit operations [10–12] and natural porous media [1] but their transport properties bear little resemblance to viral pathogens. (4) Fluorescent latex microspheres. Latex microspheres of various dimensions can imitate the transport of viral pathogens in porous media [13]. The drawbacks of this technology stem from the difference between the surface properties and size of viruses and those of microspheres, their high cost, and the time consuming enumeration process of microspheres by fluorescence microscopy. (5) Attenuated virus and bacteriophage indicators. Bacteriophages, a class of viruses that infect bacteria, often replace viral pathogens as microbial tracers [13,14]. Since laboratory and field experiments with native pathogens are associated with considerable health risks, bacteriophage tracers are useful surrogates. Bacteriophages evolved to be the most important and useful probes for viral pathogen transport and survival since they are closest in size, shape, and surface properties to viral pathogens. However, bacteriophage inactivation and transport phenomena are always coupled and it is a formidable task to distinguish the physicochemical retention from the biological inactivation. Other notable disadvantages of bacteriophage transport studies include complicated and time consuming plaque forming unit (PFU) enumeration; plaque techniques enumerate a virus cluster as a single unit rather than reflecting the actual number of viruses; bacteriophage studies require careful sample handling and preservation to prevent post-sampling inactivation; and the test results are received only after 12–48 h [15]. Our research and the proposed method were largely influenced by the success of the last two probing tools, and in fact the proposed tracer can be viewed as a combination of bacteriophage indicators and fluorescence microspheres.

In this study, we introduce a new tool for the investigation of viral transport in porous media. Since we believe that this tool is equally useful for the investigation of virus retention in soils and underground media the following introduction does not distinguish between the two fields. Three levels of investigations using labeled bacteriophages were conducted. First, we determined the aggregation state and surface charge characteristics of the labeled bacteriophages as compared to the native ones. A second level of investigation was aimed at assessing the applicability of optical monitoring of labeled bacteriophages as surrogates for PFU enumeration of the native bacteriophages. For that we compared the removal efficiency and retention time distribution (RTD) characteristics of native and labeled bacteriophages in a Jar test and a deep-bed filtration test. A third level of investigation which is reported in a subsequent article [16] was then carried out with the labeled MS2 bacteriophages in order to probe the state of the porous media during different filtration stages and thus demonstrate the usefulness of the new approach.

2. Experimental section

2.1. Cultivation and enumeration of MS2 bacteriophages

Cultivation and enumeration of MS2 bacteriophages were performed using Escherichia coli K-12, (ATCC 23631) as the host bacterium. Bacteriophage MS2 was enumerated by the plaque-assay method using the double-layer technique [17]. Generation of phage stock suspension for seeding studies was obtained by the inoculation of 1:1 ratio of phage and host cell, to the overlayer. After 24-h incubation at 37°C, the overlayer containing the infected bacterial cells was scraped into 50-mL tubes. Purification of the bacteriophage cultures was accomplished by chloroform extraction. Titer of up to 10¹² plaque forming units (PFU/ml) was obtained by this procedure.

2.2. Preparation of fluorescence-labeled bacteriophages

MS2 bacteriophage is comprised of a maturation protein (MW = 44,000) and a single-stranded RNA (3569 bases) surrounded by a protein capsid. The virus does not have a lipid outer coating, and its capsid is...
constructed of 180 copies of a protein ($MW = 13,600$ for each) having positively and negatively charged amino acid residues in the polypeptide chain [18]. Protein capsids can be easily derivatized due to the high reactivity of the amine group on the L-lysine amino acid.

We labeled MS2 bacteriophages with fluorescein-5-isothiocyanate, FITC (1), fluorescein (2), 5-(4,6-dichlorotriazinyl)aminofluorescein, 5-DTAF (3), and rhodamine B (4). FITC and DTAF were conjugated according to a detailed procedure provided by Banks and Paquette [19] for the conjugation of amine reactive probes to the L-lysine group of myoglobin. In our current study, we used the same procedure to modify the protein capsid of the MS2 phages instead of the current study, we used the same procedure to modify the protein capsid of the MS2 phages instead of the myoglobin. Briefly, 1.2 mL of $10^{11}$ PFU/mL MS2 protein capsid of the MS2 phages instead of the current study, we used the same procedure to modify the protein capsid of the MS2 phages instead of the myoglobin. Briefly, 1.2 mL of $10^{11}$ PFU/mL MS2 phages in 0.1 M borate buffer, pH 9.2, were mixed with 0.021 g FITC or 0.0102 g 5-DTAF and 5 mL N,N-Dimethylformamide (DMF). The solutions were stirred overnight at 4°C and then purified by dialysis.

Rhodamine B and fluorescein labeling of MS2 phages were performed by mixing 0.2 g of DEC, 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (Aldrich) coupling agent and 0.02 g of the dye (Rhodamine B (Aldrich) or sodium fluorescein (BDH)) in 25 mL of bacteriophage stock solution in phosphate buffer (pH 5.6). This procedure resulted in permanent attachment without covalent conjugation of the dye, probably due to DEC assisted caging of the fluorescent labels in the hydrophobic environment of the dye. Without addition of DEC all the dye molecules leached out through the dialysis membrane within 2 h.

In all cases, the labeled bacteriophage mixture was purified by membrane dialysis (The Scientific Instrument Center Ltd., London, UK) under stirring to remove low molecular weight organic compounds and free dye molecules. Approximately 3 days of dialysis were needed to obtain leak free fluorescein and Rhodamine fluorescent-labeled bacteriophages, and several hours (ca. 10) dialysis were sufficient to achieve leak-free FITC and DTAF-labeled phages. However, for our tests, we used in all cases at least 6 days dialysis to assure leak-free phage suspensions. The modified bacteriophages were stored at 4°C in the dark. We did not detect changes in fluorescence of the labeled bacteriophages during 2 months storage in a refrigerator. We did not attempt to optimize the viability of the bacteriophages; instead we used a mixture of labeled and native bacteriophages when both viability evaluation and fluorescence detection were compared.

2.3. Bacteriophage characterization

Optical spectra were recorded using a Cary E1 Varian double beam UV–visible spectrophotometer. Fluorometric measurements were conducted using a PerkinElmer LS-50B FL fluorescence spectrometer (PerkinElmer, Norwalk, USA) equipped with a 1-cm optical path length cuvette. Intensity is reported in the instrument’s fluorimetric intensity units (FIU).

Zeta potential measurements of the fluorescent bacteriophages were conducted, using a ZetaMaster S 1.26 (Malvern Instr., Malvern, England) particle size analyzer. The experiments were conducted with a 633 nm laser. Conventional 488 nm laser irradiation was inappropriate for such studies due to optical interferences of the dye labels.

Electrophoretic mobility in agarose gel was determined with an Ortec 4100 - EG&G (Princeton, USA) gel electrophoretic unit. The pH of the agarose gel was maintained constant in the range 2.5–9.3 by appropriate citrate or phosphate buffer solutions. The ionic strength was kept constant at 0.06 M for all buffer solutions.

The isoelectric point of the labeled and native MS2 bacteriophages were independently determined by isoelectric focusing using an Immobiline DryStrip kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Isoelectric focusing was conducted according to the supplier’s recommended procedure. A 20-μL solution of phages was applied onto the hydrated polyacrylamide gel strip (Immobiline DryStrip pH 3–10 L, 11 cm). Electrophoretic focusing was conducted at 500 V for 5 h and then at 3500 V for an additional 14.5 h. Protein spot visualization was performed with Coomassie R 350 stain solution.

The particle size distribution was determined using a Malvern Autosizer IIc (Malvern Instr., Malvern, England) in order to determine the extent of cluster formation of the labeled bacteriophages. The tests were conducted with a 633 nm laser, as for the zeta potential tests. We had to use a rather concentrated suspension of the MS2 bacteriophages ($10^{10}$ PFU/ml) in these studies in order to achieve a stable and significant signal. It is not possible to elucidate the particle size distribution of the native bacteriophages by a similar method due to their low light scattering.

2.4. Jar test coagulation tests

A conventional multiple stirrer jar apparatus (Phipps and Bird 7790-402, Richmond, USA) was used. The following program was used in all tests: Rapid mixing at 100 rpm for 1 min, flocculation at 30 rpm for 20 min, and quiescent settling for 30 min. Tests were performed at room temperature (~20°C). Samples were taken from a point located 4-cm below water level. Alum $Al_2(SO_4)_3 \cdot 18H_2O$ was used for particle destabilization. The stock solution was prepared by mixing 1 g/L Alum with tap water using a T-310 Elma Ultrasonic Rapid Mixer (Elma-Hans Schmidbauer GmbH & Co KG, Singen, Germany). The solution was stored in a 2-L container under continuous stirring to prevent concentration changes by sedimentation.
2.5. Deep-bed filtration

Details of the filtration setup are provided in a subsequent paper [16] and will be described here only briefly. A 7.6 cm diameter, 80-cm high, transparent Plexiglas filter was used. Filtration media was 20–60 cm height Haifa bay quartz sand of 1.32-mm effective size with 1.55 uniformity coefficient and 0.45 porosity. The sand was acid washed, washed with deionized water and screened before use. Flow rate measurements and control were performed with a Dwyer flowmeter installed at the column outlet. Influent and effluent suspension turbidities were monitored with a Hach Ratio/ RX turbidimeter (Loveland, USA). The filtration tests were performed at a constant flowrate of 8.55 L/h in a contact downward mode. The influent was prepared from Jerusalem tap water and Kaolin clay 0.778 ± 0.315 μm diameter. Concentrated clay slurry was processed for 2 min in an Ultra-Turrax T-50 homogenizer (Staufen, Germany) at a speed of 10,000 rpm to ensure reproducible colloidal suspension. The mixture was poured into the feed tank and mixed for a minimum of 2 h before the filtration test. The filtration tests were performed with 10 mg/L Kaolin, 12.8 NTU, 10 mg/L Alum, pH = 7.5 at ambient temperature. Since chlorine residue is detrimental to native bacteriophages, the influent was treated with 0.2 mM Na₂S₂O₃.

Native and labeled bacteriophage and dye removal tests were performed using impulse introduction mode. One milliliter of 10¹¹ PFU/mL tagged MS2 bacteriophages was injected 30 cm above the sand bed. Samples were collected manually from the outlet at 1-min time intervals starting from the injection time.

3. Results

3.1. Characterization of fluorescent-labeled bacteriophages

Fig. 1a–d presents the optical absorption and fluorescence spectra of FITC, fluorescein, 5-DTAF, and Rhodamine B labeled bacteriophages as compared to the spectra of the free dyes. Substantial 20–40 nm bathochromic shifts were observed for fluorescein and Rhodamine B absorbance and fluorescence peaks due to the chemical association to the hydrophobic phage environment. The conjugation of FITC and DTAF did not influence the absorption and fluorescence spectra. At this stage, we cannot provide a good estimate for the number of attached dye molecules per MS2 virus because the extinction coefficient of the conjugated dye is unknown. The specific fluorescence intensity (per dye molecule) decreases when more labels are attached to each protein [19]. In addition, the PFU is a measure of the viable bacteriophages only and does not predict the absolute virus count accurately.

Fig. 2 presents zeta potential measurements of fluorescein-conjugated bacteriophages in 0.1 M potassium phthalate buffer solutions. As expected, the bacteriophages are more negatively charged at higher pH. Note that the true surface charge of the bacteriophage is substantially larger (in absolute units) because these tests were conducted in rather concentrated solutions. The point of zero charge is approximately pH 3.7, in accordance with earlier reported data for unlabeled bacteriophages where the pH is 3.8 [18]. Fig. 3 depicts the zeta potential–pH dependence for the three different fluorescein-labeled phages (1,2,3) and shows that they exhibit very similar pH–zeta potential dependencies. The point of zero charge ranged between 3.6 for FITC and 3.9 for DTAF labeled MS2.

The isoelectric point (IEP or point of zero charge (p.z.c)) determines to a large extent the interaction between viruses and the sand grains. Therefore, we reaffirmed the fact that fluorescein and Rhodamine B labeling had no significant effect on the IEP of the bacteriophage by gel electrophoresis studies. Fig. 4 presents the electrophoretic mobility of fluorescein-labeled bacteriophages in a 1% (w) agarose gel. These measurements show that the IEP of the fluorescein-labeled phages is around pH 3.6 in accordance with the zeta potential measurements and earlier reports for the native MS2 phages [18].

Finally, since each of these modes of measurement is based on the potential at the shear plane we performed straightforward gel isoelectric focusing tests on commercial polyacrylamide gel strips (Immobiline DryStrip, Amersham Pharmacia Biotech). This technique provides unambiguous reading of the IEP of proteins. The IEP of the native phage and FITC and DTAF-labeled phages were all 3.5 ± 0.4, in excellent agreement with Figs. 2–4.

The secondary structure of MS2 bacteriophages was resolved by Valegard and coworkers [20]. A capsid of a spherically shaped phage (app. 27 nm in diameter) is comprised of 180 copies of a single polypeptide arranged on the viral surface in isocahedral symmetry. The number of amine groups of the side chains of the amino acids arginine and lysine were calculated to be around 2520 per virus. Therefore, the addition of several charges to each bacteriophage had apparently only a limited effect on the potential and isoelectric point of the virus.

Some of the discrepancy between reports on bacteriophage removal efficiency in environmental unit operations and underground transport is caused by different states of aggregation of the bacteriophages in the different studies. The absolute size of the bacteriophage was found to be 41 nm instead of the reported 27 nm. This difference could be attributed to experimental inaccuracy of the Malvern instrument at the low end of the instrument analysis range or to the uncertainty in
determining the viscosity and refraction index of the phage solution, which again contributed some degree of aggregation. At this stage we cannot preclude the possibility that a certain degree of dimer and trimer aggregation is also possible, though thorough sonication of the concentrated phage suspension did not sharpen the particle size distribution peak.

In the current set up the minimum detection limit (MDL) of fluorescein-labeled MS2 phages was $10^6$ PFU/mL in distilled water (corresponding to signal to noise $= 3$). This is a rather high MDL compared to reported MDLs of fluorescein labels, which goes well below subattomole levels (i.e., $< 600$ dye molecules) [21]. However, since the sensitivity of the labeled fluorescein was at least an order of magnitude higher than the level required for the tracers’ studies of deep-bed filtration and several orders of magnitude higher than the level required for the Jar test studies we did not make any deliberate effort to reduce the detection limit or to optimize the fluorescence set-up.

3.2. Comparison of removal efficiencies of free dyes, labeled, and native bacteriophages

This set of experiments was aimed at assessing the hypothesis that the transport and physical removal of labeled phages reflects that of the native viruses.

---

**Fig. 1.** Optical absorbance (curves 1,3) and fluorescence (curves 2,4) spectra of the free (curves 1,2) and protein conjugated dyes (3,4). (a) Rhodamine B, (b) Fluorescein, (c) 5-DTAF, (d) FITC.
Jar test studies were performed in order to compare the removal efficiency of Kaolin turbidity, native MS2 bacteriophage, labeled MS2 bacteriophage, and the free dye by Alum coagulation. The removal efficiency is defined as the ratio between the precipitated and the initial number of viruses (or PFU) in the sample. The Jar test studies were performed with 10-mg/L Kaolin giving 12.8 NTU initial turbidity in 1-L beakers. 1-mg Rhodamine B, 10^{11} PFU of labeled MS2 (as measured before the conjugation step) or 10^{11} PFU of native MS2 bacteriophages were inserted into each test beaker. Fig. 5 shows the removal curves of Rhodamine B and the bacteriophages as a function of Alum dosage. Most of the free Rhodamine B was removed by adsorption to the negatively charged Kaolin, in striking contrast to the bacteriophage labeled with Rhodamine B. Turbidity removal was significantly higher compared to the sedimentation of the native and labeled MS2 bacteriophages. Thus, both turbidity and dye molecules are poor indicators for virus removal in flocculation sedimentation units, in accordance with common know-how [22].

Removal efficiency of Rhodamine-modified bacteriophages was somewhat superior as compared to the removal of the native bacteriophages. This small difference is consistent, repeatable and is induced by the different methods for quantification of the labeled and native bacteriophages. Both the labeled and native bacteriophages form clusters, at least to some extent. The flocculation/sedimentation processes remove the larger clusters more efficiently. Since native bacteriophage quantification was performed by enumeration of PFUs and since a stable cluster forms only a single plaque, then native bacteriophage enumeration is always negatively biased compared to the actual number of

3.3. Jar test studies

Jar test studies were performed in order to compare the removal efficiency of Kaolin turbidity, native MS2 bacteriophage, labeled MS2 bacteriophage, and the free dye by Alum coagulation. The removal efficiency is defined as the ratio between the precipitated and the initial number of viruses (or PFU) in the sample. The Jar test studies were performed with 10-mg/L Kaolin giving 12.8 NTU initial turbidity in 1-L beakers. 1-mg Rhodamine B, 10^{11} PFU of labeled MS2 (as measured before the conjugation step) or 10^{11} PFU of native MS2 bacteriophages were inserted into each test beaker. Fig. 5 shows the removal curves of Rhodamine B and the bacteriophages as a function of Alum dosage. Most of the free Rhodamine B was removed by adsorption to the negatively charged Kaolin, in striking contrast to the bacteriophage labeled with Rhodamine B. Turbidity removal was significantly higher compared to the sedimentation of the native and labeled MS2 bacteriophages. Thus, both turbidity and dye molecules are poor indicators for virus removal in flocculation sedimentation units, in accordance with common know-how [22].

Removal efficiency of Rhodamine-modified bacteriophages was somewhat superior as compared to the removal of the native bacteriophages. This small difference is consistent, repeatable and is induced by the different methods for quantification of the labeled and native bacteriophages. Both the labeled and native bacteriophages form clusters, at least to some extent. The flocculation/sedimentation processes remove the larger clusters more efficiently. Since native bacteriophage quantification was performed by enumeration of PFUs and since a stable cluster forms only a single plaque, then native bacteriophage enumeration is always negatively biased compared to the actual number of

3.3. Jar test studies

Jar test studies were performed in order to compare the removal efficiency of Kaolin turbidity, native MS2 bacteriophage, labeled MS2 bacteriophage, and the free dye by Alum coagulation. The removal efficiency is defined as the ratio between the precipitated and the initial number of viruses (or PFU) in the sample. The Jar test studies were performed with 10-mg/L Kaolin giving 12.8 NTU initial turbidity in 1-L beakers. 1-mg Rhodamine B, 10^{11} PFU of labeled MS2 (as measured before the conjugation step) or 10^{11} PFU of native MS2 bacteriophages were inserted into each test beaker. Fig. 5 shows the removal curves of Rhodamine B and the bacteriophages as a function of Alum dosage. Most of the free Rhodamine B was removed by adsorption to the negatively charged Kaolin, in striking contrast to the bacteriophage labeled with Rhodamine B. Turbidity removal was significantly higher compared to the sedimentation of the native and labeled MS2 bacteriophages. Thus, both turbidity and dye molecules are poor indicators for virus removal in flocculation sedimentation units, in accordance with common know-how [22].

Removal efficiency of Rhodamine-modified bacteriophages was somewhat superior as compared to the removal of the native bacteriophages. This small difference is consistent, repeatable and is induced by the different methods for quantification of the labeled and native bacteriophages. Both the labeled and native bacteriophages form clusters, at least to some extent. The flocculation/sedimentation processes remove the larger clusters more efficiently. Since native bacteriophage quantification was performed by enumeration of PFUs and since a stable cluster forms only a single plaque, then native bacteriophage enumeration is always negatively biased compared to the actual number of

3.3. Jar test studies

Jar test studies were performed in order to compare the removal efficiency of Kaolin turbidity, native MS2 bacteriophage, labeled MS2 bacteriophage, and the free dye by Alum coagulation. The removal efficiency is defined as the ratio between the precipitated and the initial number of viruses (or PFU) in the sample. The Jar test studies were performed with 10-mg/L Kaolin giving 12.8 NTU initial turbidity in 1-L beakers. 1-mg Rhodamine B, 10^{11} PFU of labeled MS2 (as measured before the conjugation step) or 10^{11} PFU of native MS2 bacteriophages were inserted into each test beaker. Fig. 5 shows the removal curves of Rhodamine B and the bacteriophages as a function of Alum dosage. Most of the free Rhodamine B was removed by adsorption to the negatively charged Kaolin, in striking contrast to the bacteriophage labeled with Rhodamine B. Turbidity removal was significantly higher compared to the sedimentation of the native and labeled MS2 bacteriophages. Thus, both turbidity and dye molecules are poor indicators for virus removal in flocculation sedimentation units, in accordance with common know-how [22].

Removal efficiency of Rhodamine-modified bacteriophages was somewhat superior as compared to the removal of the native bacteriophages. This small difference is consistent, repeatable and is induced by the different methods for quantification of the labeled and native bacteriophages. Both the labeled and native bacteriophages form clusters, at least to some extent. The flocculation/sedimentation processes remove the larger clusters more efficiently. Since native bacteriophage quantification was performed by enumeration of PFUs and since a stable cluster forms only a single plaque, then native bacteriophage enumeration is always negatively biased compared to the actual number of

3.3. Jar test studies

Jar test studies were performed in order to compare the removal efficiency of Kaolin turbidity, native MS2 bacteriophage, labeled MS2 bacteriophage, and the free dye by Alum coagulation. The removal efficiency is defined as the ratio between the precipitated and the initial number of viruses (or PFU) in the sample. The Jar test studies were performed with 10-mg/L Kaolin giving 12.8 NTU initial turbidity in 1-L beakers. 1-mg Rhodamine B, 10^{11} PFU of labeled MS2 (as measured before the conjugation step) or 10^{11} PFU of native MS2 bacteriophages were inserted into each test beaker. Fig. 5 shows the removal curves of Rhodamine B and the bacteriophages as a function of Alum dosage. Most of the free Rhodamine B was removed by adsorption to the negatively charged Kaolin, in striking contrast to the bacteriophage labeled with Rhodamine B. Turbidity removal was significantly higher compared to the sedimentation of the native and labeled MS2 bacteriophages. Thus, both turbidity and dye molecules are poor indicators for virus removal in flocculation sedimentation units, in accordance with common know-how [22].

Removal efficiency of Rhodamine-modified bacteriophages was somewhat superior as compared to the removal of the native bacteriophages. This small difference is consistent, repeatable and is induced by the different methods for quantification of the labeled and native bacteriophages. Both the labeled and native bacteriophages form clusters, at least to some extent. The flocculation/sedimentation processes remove the larger clusters more efficiently. Since native bacteriophage quantification was performed by enumeration of PFUs and since a stable cluster forms only a single plaque, then native bacteriophage enumeration is always negatively biased compared to the actual number of
bacteriophages. This negative bias is larger before the flocculation/sedimentation test than for the residual phage suspension simply because sedimentation processes remove clusters more efficiently than individual viruses. Thus, actual removal of viruses surpasses the measured PFU removal. Since the fluorescence measurement is insensitive to cluster formation and represents more accurately the virus concentration, the reduction of fluorescence due to the removal of labeled MS2 surpassed the PFU removal. This test clearly shows a significant advantage for the labeled over the native bacteriophages since PFU counts of native bacteriophages underestimate the virus removal efficiencies while fluorescence is a less biased measure.

3.4. Deep-bed filtration

Fig. 6 compares tracer studies conducted with the free fluorescein dye and the native and labeled MS2 bacteriophage. The test was carried out 2 h after the beginning of a filter run with an approach velocity of 10 m/h and bed height, \( L = 40 \) cm. Other parameters were reported in the experimental section. 1-mL mixture of fluorescein (4.5 \( \times \) \( 10^{-6} \) M), fluorescein-labeled MS2 (2 \( \times \) \( 10^{5} \) fluorometric intensity units, FIU, at 1 cm pathlength, which corresponded to \( 10^{11} \) PFU before the derivatization step) and native bacteriophage (\( 10^{11} \) PFU) were injected 30 cm above the filter bed. PFU and fluorescence of the native and labeled bacteriophages and fluorescence intensity of the free fluorescein were measured as a function of the number of bed volumes.

The residence time distribution (RTD) curves of Fig. 6 have skewed Gaussian forms, as previously reported [23]. There was a clear difference between the RTD curves of the free fluorescein and the MS2 bacteriophages. In contrast, a very good fit was observed between the RTD curves of the native and labeled bacteriophages and the removal efficiencies were practically identical, ca. \( 54 \% \pm 4 \% \) (Table 1). Linear regression coefficient of the RTD points of the native and labeled bacteriophages was \( R^2 = 0.94 \) (number of degrees of freedom, \( n = 9 \), zero points in both curves were excluded to prevent over estimate of the regression coefficient). The calculated average retention times and the variances of the RTD curves of the labeled and native bacteriophages differed by only \( 8 \% \) (Table 1), which is well within the accuracy margins of PFU enumeration.

4. Concluding remarks

A new dye tracer based on dye-conjugated bacteriophages was introduced and applied for the investigation of deep-bed filtration. The advantages of the new tracer compared to existing tracers include the ability to (1) decouple inactivation processes from transport phenomena; (2) enumerate true virus concentrations instead of PFU; (3) work in a non-pathogenic environment; (4) keep all the pertinent charge transport properties of true viruses; (5) avoid costly sample preservation processes. Additionally, the new tracer offers rapid detection by fluorescence and convenient sample handling. Currently, the major drawback of the method is its higher MDL compared to PFU count, which limits its application to pilot scale or small system studies. This obstacle will be overcome by enzyme labeling which will provide signal amplification.

Acknowledgements

The support of the Infrastructure Program of the Ministry of Science and Culture, Israel and Mekorot Ltd are gratefully acknowledged. We thank Prof. I. Runbinstein and Dr. S. Srebnik for valuable discussions.
References


