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Color image analysis of contaminants and bacteria transport in porous media

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ABSTRACT

Transport of contaminants and bacteria in aqueous heterogeneous saturated porous systems have been studied experimentally using a novel fluorescent microscopic imaging (FMI) technique. The approach involves color visualization and quantification of bacterium and contaminant distributions within a transparent porous column. By introducing stained bacteria and an organic dye as a contaminant into the column and illuminating the porous regions with a planar sheet of laser beam, contaminant and bacterial transport processes through the porous medium can be observed and measured microscopically. A computer controlled color CCD camera is used to record the fluorescent images as a function of time. These images are recorded by a frame accurate high resolution VCR and are then analyzed using a color image analysis code written in our laboratories. The color images are digitized this way and simultaneous concentration and velocity distributions of both contaminant and bacterium are evaluated as a function of time and pore characteristics. The approach provides a unique dynamic probe to observe these transport processes microscopically. These results are extremely valuable in in-situ bioremediation problems since microscopic particle-contaminant-bacterium interactions are the key to understanding and optimization of these processes.

Keywords: color imaging, transport, flow, contaminants, bacteria, porous media, fluorescent techniques

1. INTRODUCTION

The immediate need for the remediation of contaminated ground water and soils is apparent from the latest report of the National Research Council¹. The report discloses that well over 400,000 sites across the nation have contaminated ground water and will require remediation.

Biological degradation has been studied and applied over the past few decades to clean up contaminated ground water and soil^{2,3}. In-situ soil and ground water remedial procedure, or simply in-situ bioremediation, is one of the progressive technologies for the remediation of contaminated ground waters and subsurface soils by stimulating the growth of in-situ microflora with the addition of carbon and energy sources and trace mineral elements in place or, alternatively, by introduction of bacteria into the subsurface^{4,5}. Therefore, the contaminated system is not physically moved from its original geographical location. Total treatment occurs in-situ, on site. While other in-situ remediation processes have been tested and examined for contaminated soils such as in-situ vitrification^{6,7} or in-situ dynamic stripping of NAPL's⁸, in-situ bioremediation continues to be an economical and effective method of remediation. In fact, passive (i.e., nonengineered) microbial processes can also be exploited to achieve cleanup in many instances⁹. The total treatment is a commercial venture, the cost of which is determined by the volume and type of contaminant at the site, the location of the contaminant in the subsurface, and the concentration of the contaminant. In-situ bioremediation is a way to manage the subsurface bioreactor to carry out biological degradations of contaminants. A successful implementation of bioremediation should include a complete aquifer characterization, removal of contaminant source and free product, plume containment, laboratory feasibility studies, installation and operation of biostimulation controls, and continuous monitoring¹⁰.

Microbial transport can have an extreme influence on the rate of contaminant degradation. There are different bioremediation scenarios. In one, the abundance of natural, in-situ bacteria is stimulated through the addition of nutrients or electron donors that promote bacteria growth and additional degradation. This is because the bacterial population may not be sufficiently large to degrade the contamination that is there. The assumption here is that the bacteria are attached and their movement through the system is of secondary relevance. The major concerns of this is that when nutrients are added (by injection in a fluid from the surface) they necessarily displace contaminants that are around the bacteria away from the simulated bacteria. Hence, the bacteria and contaminants are in different locations, preventing degradation. If the bacteria move themselves, they may not mix with the nutrients, as they are

displaced at the same rate. Another concern is that too much growth will clog the pores and prevent flow and contaminant migration, defeating the purpose of bioremediation. So, this approach can be laced with implementation problems in general, and its successful use must be carefully engineered (appropriate nutrient levels, understanding of contaminant and microbial distributions, understanding of flow patterns, etc.). Another scenario deals with natural in-situ bacteria that do the job, passively, without any aid from the mankind⁹. These types of approaches fall under a broad category called "natural attenuation", which means letting (aka assuming that) nature will take care of the problem. There is a knowledge gap, of course, that defines the difference between "assuming" and "letting", to which microscopic experimental studies can help close. In a third, bacteria grown at the surface are injected into the ground, allowed to attach (becoming immobile), therefore allowing dissolved contaminants to move past and undergo degradation^{4,5,11}. This approach requires a good knowledge of bacterial transport and attachment/detachment processes. In addition, bacterial transport in ground water is becoming increasingly important due to dense rural septic systems or ground water recharges that are derived from percolating treated waste waters into recharge basins or large irrigated landscape areas. These will have an increasing impact on ground water quality that is only now becoming recognized. In all of the above cases, the significant issues are understanding how bacteria move, attach, detach, interact with grain particles and contaminants, and distribute as a function of various hydrological, geological, and chemical conditions.

A large number of studies¹²⁻¹⁸ have therefore focused attention on bacteria transport in relation to contaminants in porous media. In general, these studies have concentrated on *macroscopic* transport of bacteria in porous media due to the difficulty of obtaining *microscopic* measurements within the interstices of the solid porous matrix. One of the major problems in bioremediation is the lack of ability to quantitatively evaluate the technology. Its benefits can not be realized unless it is clearly evaluated and shown that these processes do actually work. Therefore, there is an essential need to observe microscopic interactions, measure the pore-scale quantities, and analyze the overall processes systematically (see Rashidi et al.^{19,20}).

Our research at University of California's Lawrence Livermore National Laboratory is an extensive effort to study microscopic transport processes within porous media through direct measurements and observations. It concentrates on the problem of microbial and contaminant flow and transport in the subsurface. This is being accomplished using two high-resolution imaging techniques: Particle Tracking Velocimetry (PTV) to measure velocity and Fluorescence Microscopic Imaging (FMI) to measure bacterial concentration. In particular, it addresses important issues such as: factors controlling the transport of bacteria and contaminants in heterogeneous subsurface systems, bacterial attachment/detachment processes, pore-scale bacterium-grain particle-contaminant interactions, optimum conditions for acceleration of contaminant consumption in the subsurface environment, and successful applications of laboratory experiments and findings to the scaled up field processes for enhancement of in-situ bioremediation.

The derived information will be extremely valuable in understanding of in-situ microbial/contaminant transport and interactions, and as a result, provide the basis for improved modeling and biological treatment of contaminants in subsurface.

2. BIOREMEDIATION PROCESSES

The word, bioremediation, originates from two other words, namely biology and remedy. Bioremediation, therefore, is a remedy for cleaning up contaminants by employing a biological process. The major sources of contaminants that pollute the soil and subsurface ground water are illustrated in Figure 1. Tests have proven that many of these contaminants are known or suspected carcinogens.

Ground water is the only source for drinking water for many people around the world, especially in rural areas. In the United States ground water supplies 42.4% of the population served by public water utilities. More than half of the population of the United States relies upon a ground water source for drinking water²¹. Enzymes have shown to break up these contaminants in hazardous sites into nonhazardous compounds. Enzymes are complex proteins that are produced by living cells and are responsible for catalyzing specific biochemical reactions such as the degradation of organic contaminants. Microorganisms, mainly bacteria, use enzymes to degrade organic contaminants simply by digesting them.

In general, bioremediation processes degrade organic contaminants to nontoxic and less harmful products. The degrading reaction, called oxidation-reduction, is initiated by microorganisms such as bacteria or fungi. The degradation process has the usual oxidation reaction mechanism. Typical oxidation reactions are the combustion of alkanes, such as octane (C_8H_{18}). As shown below in equation (1) for the typical hydrocarbon, octane, a two mole sample of octane (228 grams), requires exactly twenty five moles of molecular oxygen (800 grams) for its

quantitative oxidation to give sixteen moles of carbon dioxide (704 grams) and eighteen moles of water (324 grams).

Similarly, Phenol ($\text{C}_6\text{H}_6\text{O}$), another common contaminant, also undergoes oxidation to give the same products as shown by equation (2).

Degradation processes of hydrocarbons that require molecular oxygen (*aerobic oxidation-reduction reactions*) yield products of gaseous or dissolved carbon dioxide (CO_2), bicarbonate ion (HCO_3^-), water, and new cell matter (regenerated microbes). Microorganisms that consume contaminants without the need of oxygen (*anaerobic oxidation-reduction reactions*) give products that may include methane gas (CH_4), nitrogen gas (N_2), hydrogen sulfide (H_2S), and reduced forms of metals such as iron (Fe^{2+}), and manganese (Mn^{2+}). For example, the anaerobic degradation of benzene (C_6H_6) by denitrification, as shown in equation (3), gives the following products.

Microorganisms responsible for biological degradation of contaminants have rates of reaction that are extremely sensitive to a number of different conditions, such as temperature, pH, oxygen, and nutrients²²⁻²⁵. The most favorable conditions for bacteria feeding and digestion are pH values between 6.0 and 10.0, temperatures ranges of 15°C to 45°C and a ratio of organic carbon to nitrogen and phosphorus of 300:15:1.

3. EXPERIMENTAL FACILITIES AND MEASUREMENTS TECHNIQUES

Figure 2 shows the detail of the experimental apparatus. Experiments were performed at various flow conditions (see Table I) in a clear Plexiglas rectangular packed column 3.0 cm in width and about 22.0 cm in length. The column was filled with natural mineral particles (crystals of fluoride salt) of nonuniform sizes with average diameters of about 0.15 cm. The experimental setup has been designed such that at the test section, flow is free from any wall or entrance effects.

The difficulty in performing experiments in aqueous systems has been to be able to find optically clear materials that have refractive indices close to water's refractive index of about 1.33 at 20.0°C and 514.5 nm. This task has been accomplished after many searches and tests. Some natural minerals have been discovered that are found in nature or made as crystals in the laboratory with refractive indices close to that of water. These minerals are the crystals of Lithium fluoride salts (LiF) that have been made in the laboratory to the range of size specification for these experiments. Therefore, the column was filled with these natural mineral particles of nonuniform sizes with average diameters of about 0.15 cm. An aqueous solution has been chosen as the fluid which matched the particles' refractive index at 20.0°C and a wavelength of 514.5 nm. The refractive index of the aqueous solution has been matched to the refractive index of the particles by adjusting the temperature and small addition of dissolved sugar (sucrose) to water. The refractive index matching is critical up to third digit after decimal point for the refractive indices of the packing particles and the aqueous solution. The column was maintained at this temperature throughout all runs by being immersed in a circulating constant temperature bath. A syringe pump was used to provide a constant volumetric flow rate of the above fluid.

The experiments were done with the refractive matched fluid seeded with fluorescent latex microspheres of about 5 μm in diameter (for velocity measurements), an organic dye (for contaminant concentration measurements), or stained bacteria (for bacterial concentration and velocity measurements). The column was illuminated by an Argon-ion laser (coherent) operated at 475 nm for velocity measurements and 488 nm for concentration measurements. A CCD camera was used to record the experimental runs. Since, the dye emission peaked around 514.5 nm, a band pass filter was used on the video camera to pass a narrow range of 514.5 nm $\pm$ 5 nm wavelength associated with the dye excitation. As seen in Figure 2, the video camera was placed perpendicular to the laser propagation beam on a remotely operated platform such that it moved with the beam in order to scan various cross-section of the column. The refractive index-matching yielded a transparent porous medium, free from any scattering and refraction at the solid-liquid interfaces, thus allowing direct optical probing at any point within our porous system.

Figure 1. Sources of contamination in soil and ground water.

Figure 2. Experimental setup and measurement techniques.

In our experiments, first the velocity measurements was taken by sequentially scanning the velocity fields in vertical cross-sections. Velocity measurements were obtained by tracking of the seeded microspheres in the fluid. This provided flow velocity measurements at every point within the cylindrical test section. Then, a neutrally buoyant dye was steadily introduced into the column and its concentration was imaged within the same segment where the velocity fields were determined. The video camera recorded fluorescence images at every vertical plane location while sweeping back and forth (with the illumination plane) across the column at every minute. This allowed us to obtain interstitial chemical concentrations as a function of time in the same planes where velocity measurements were taken. For bacterial transport studies, fluorescently marked bacteria (stains) were used and experimental data were taken similar to velocity and concentration measurements.

The experimental runs were recorded through the video camera by a computer controlled VCR (Sony, EVO-9650) in Hi-8 mm format. The VCR is a frame accurate model that produces high quality still images of specified frames. It is computer controlled through an RS-232 interface for automated velocity and concentration analysis. In order to capture the recorded images for data analysis, two image processing systems were used. One system was utilized for gray-scale (B & W) analysis. For this system, a frame grabber board (ITI PC Vision Plus) with 640 by 480 pixel resolution was used in conjunction with an IBM compatible 486-133 MHz computer. The other system was used for color analysis and had a PCI frame grabber board at 640 x 480 pixel resolution with an IBM compatible PentiumPro 200 MHz computer. The image analysis was done using the OPTIMAS (BioScan) and the AIDA (our laboratory's developing) softwares. Several detailed programs have been developed in our laboratories as interface softwares for automated experimental run and analysis softwares for velocity and concentration measurements.

A test run was performed to evaluate measurement uncertainties. The uncertainty in the values of average axial velocity is about 2% at 95% confidence level for 200 frames analyzed (see Rashidi and Banerjee²⁶, for details on uncertainty analysis of particle tracking techniques). Similarly, the concentration and porosity measurements reveals an uncertainty estimate of about 2% at 95% confidence level. Rashidi²⁷ provides more details on the image analysis techniques. In particular, the three velocimetry techniques of Particle Imaging Velocimetry (PIV), Particle Streak Velocimetry (PSV), and Particle Tracking Velocimetry (PTV) have been compared in details. It is shown that because of the desire for rapid, automated, and dynamic data collection, the present technique (PTV) is more advantageous in studying the flow and transport in porous media. The present technique also provides an improvement in measurement uncertainties and resolution over our previous experiments.

Table I. Experimental Conditions.

Run	T (°C)	Fluid Kinematic Viscosity ν (cm ² /s)	Average Column Porosity ϕ	Volumetric Flow Rate Q (cm ³ /min)	Average Pore Velocity $\bar{v}$ (cm/s)	Average Particle Diameter d_p (cm)	Reynolds Number Re ($\bar{v} d_p / \nu$)	Peclet Number Pe ($\bar{v} d_p / D_m$)
1	20.0	0.0381	0.4	0.23	0.0011	0.15	0.0040	4.8
2	20.0	0.0381	0.4	0.57	0.0027	0.15	0.010	12
3	20.0	0.0381	0.4	1.2	0.0053	0.15	0.020	24
4	20.0	0.0381	0.4	2.3	0.011	0.15	0.040	48
5	20.0	0.0381	0.4	5.7	0.027	0.15	0.10	121
6	20.0	0.0381	0.4	12	0.055	0.15	0.20	251
7	20.0	0.0381	0.4	23	0.11	0.15	0.40	482

4. RESULTS

Experiments were performed using the described facilities. Details of the experimental conditions are summarized in Table I. For Run 5 (volumetric flow rate of 5.7 cm³/min), the aqueous fluid was injected into the column with both a contaminant dye and stained bacteria. The experiment was continued till the column was saturated with the contaminant. As the experiment was initiated, a color CCD camera with series of close up lenses was used to record the color images at the foremost slice (vertical cross section) of the porous column. These color images were recorded using the computer-controlled frame-accurate VCR. The recorded images were later transferred to the computer for processing using a frame grabber and written algorithms through a RS-232 interface. Figure 3 illustrates the actual images of contaminant and bacteria distributions for the same slice at eight different times. Figure 3a shows an image slice where concentration of contaminant is zero while figure 3h illustrates the saturated

Figure 3a. Imaged slice where dye concentration is zero.

Figure 3b. Dye concentration and bacterial distributions after 2 minutes that the dye and bacteria have been introduced into the column.

Figure 3c. Dye concentration and bacterial distributions after 4 minutes that the dye and bacteria have been introduced into the column.

Figure 3d. Dye concentration and bacterial distributions after 6 minutes that the dye and bacteria have been introduced into the column.

Figure 3e. Dye concentration and bacterial distributions after 8 minutes that the dye and bacteria have been introduced into the column.

Figure 3f. Dye concentration and bacterial distributions after 10 minutes that the dye and bacteria have been introduced into the column.

Figure 3g. Dye concentration and bacterial distributions after 14 minutes that the dye and bacteria have been introduced into the column.

Figure 3h. Dye concentration and bacterial distributions after 18 minutes that the dye and bacteria have been introduced into the column.

slice at minute 18. From the onset of the introduction of contaminant till saturation of the column by contaminant, bacteria were transporting through the porous system. In all these images, the bacteria are appeared as white-greenish color particles while the contaminant is shown in yellowish orange color. In addition, the scale of the images in Figure 3 is about 5.5 mm in width and 4.0 mm in height.

A color image processing code was written in order to evaluate the changes in bacteria and contaminant velocity and concentration distributions with time. The program distinguished between the two color ranges and identified the bacteria and contaminant. It evaluated in each image, the overall mean concentration of bacteria & contaminant, the number of bacteria, the concentration of bacteria/contaminant in each pixel/cell, and the velocity of bacteria in the pores as a function of time. Since the flow was at steady state, the fluid velocity could also be evaluated using microspheres as tracers²⁷.

For velocity measurements, some bacteria were not clearly distinguished, some preprocessings were done to enhance the images being studied. A sharpening filter was applied to the images to detect the sharp edges of the microspheres. The convolution mask used in the sharpening filter had a positive coefficient in its center and mostly negative coefficients around the outer edge. It used a high pass filter mask, which removed the low frequency component and showed only high details. This convolution was a high pass 5x5 filter that specifically sharpened edges and enhanced details. Then, the image was binarized using a certain threshold to separate the foreground from the background. Binarizing distinguished which pixels were microspheres. Then, bacteria were more readily trackable and velocities could be determined with much higher accuracy.

Two components of the velocity were observed in the plane of the light. Denoting the light sheet as the x-z plane, one could determine x- and z- velocities over the range of the x- and z- positions in the field-of-view. Here, z axis is the axial coordinate in the flow direction along the column length and x axis is in the transverse direction. The Particle Tracking Velocimetry (PTV) technique was used to analyze and calculate velocities. In this particle tracking algorithm all of the bacteria in the first frame were located and their positions were recorded. Then the tape was advanced one or several frames (depending on the flow rate), and each microsphere was relocated by using the previous bacteria positions and the new positions were recorded. The bacterium's velocity was calculated by fitting the time history of its path, $x(t)$ and $z(t)$, using linear regression. The algorithm also evaluated the number of bacteria in each image analyzed as a function of time.

For bacteria and contaminant concentration analysis, separate color filtering was performed on individual images. For contaminant concentration analysis, the color contribution of bacteria was removed from all images by removing the green color and then performing a gray-scale analysis on the images. Similarly for bacteria concentration analysis, the color contribution of contaminant was removed by filtering out the orange color associated with the contaminant and as before doing a gray-scale analysis on the images. Then, some preprocessings were done to enhance the gray-scale images under study. Due to a nonuniform illumination problem of the laser plane, the intensities of pixels values of recorded images were higher on the left side of the image. Figure 4a shows a typical image sample at a couple of minutes after dye has been introduced to the column and it shows that the intensities of pixel values are higher on the left side of the image. This nonuniform problem can be seen in the histogram of Figure 4a, as shown in Figure 5a. The vertical bars of the histogram are tightly clustered together and a low range of all the possible pixel values is used. A Contrast Stretching technique works best on these images since most of the pixels are concentrated in one part of the histogram. It can be applied to the image to stretch the histogram to fill the full dynamic range of the image intensity.

Contrast Stretching is a useful technique to enhance images that have low contrast, such as the image shown in Figure 4a. The resulting image exhibits a wide range of pixel values and no single shade dominates the image. The following equation is the basis for the Contrast Stretching technique:

$$NewPixelValue = \frac{(CurrentPixelValue - LowPixelValue)}{(HighPixelValue - LowPixelValue)} \times 255. \quad (4)$$

Here, the Low and High Pixel Values are the images with no dye concentration (Figure 4b- black image) and with the highest dye concentration (Figure 4c- saturated image), respectively. These images have varying dye concentration intensity values, but they have the same exact solid phase topology. In equation (4), when the Low Pixel Value (Figure 4b) is subtracted from Current Pixel Value (Figure 4a), the histogram in Figure 5a slides to the left, as shown in Figure 5b. The lowest pixel value is now zero. When the nominator is divided by the denominator in equation (4), each pixel value is scaled so that the image fills the entire dynamic range. The result is an image that spans the pixel values from the Low Pixel Value (black image) to High Pixel Value (saturated image), as shown in Figure 4d. Figure 5c shows the histogram of Figure 4d. Figure 4d shows that noise was introduced in the solid phase. In these areas there are false and meaningless pixel values, which are converted to zero by multiplying the image in Figure 4d by the Bead image (Figure 4g).

4a. Typical image slice sample at a couple of minutes after dye and bacteria have been introduced to the column.

4b. Image slice with dye concentration equal to zero.

4c. Image slice after saturation of dye.

4d. Resulting image from equation (4).

4e. Sharpening filter is applied to image resulting from subtraction of Figure 4b from Figure 4c.

4f. Median filter is applied to the image in Figure 4e.

4h. Final image after all preprocessing has been completed.

5a. Histogram of a typical image slice sample at a couple of minutes after dye has been introduced to the column.

5b. Histogram of Figure 4b subtracted from Figure 4a and the lowest pixel value is now zero.

5c. Histogram of Figure 4d. Image spans the entire dynamic range of pixel values.

The Bead image is a binarized image that has the same topology of the image being analyzed. The Bead image is formed by first subtracting the Low Pixel Value image (Figure 4b) from High Pixel Value image (Figure 4c) and then applying the sharpening filter to increase the image's contrast and emphasis details, such as solid phase's sharp edges. Figure 4e shows the result of the applied sharpening filter. Since the high pass filter mask, in the sharpening filter, enhances fine details in an image, it also tends to amplify the noise in an image. An effective method for removing noise is the application of a median filter which also does a good job of preserving sharp edges of the image. This is important in our Bead image, since it is crucial in preserving the exact topology of the solid phase. Figure 4f is the result of the use of a median filter on Figure 4e. This image shows the median filtering forces points with very distinct intensities to be more like their neighbors, thus eliminating intensity spikes. Then the Bead image is binarized using a certain threshold, as shown in Figure 4g. Finally, Figure 4h shows the result of multiplying the normalized image, Equation (4) (Figure 4d), by the Bead image (Figure 4g). Figure 4h shows that pixel values in the solid phase have been successfully converted to 0 values.

Using the color imaging technique described above, the overall mean concentration of bacteria & contaminant, the number of bacteria, the concentration of bacteria/contaminant in each pixel/cell, and the velocity of bacteria in the pores as a function of time was evaluated. Figure 6 shows the volumetrically averaged contaminant concentration as a function of time evaluated as described for Run 5 (volumetric flow rate of $5.7 \text{ cm}^3/\text{min}$). These results show the successful application of our novel automated and color imaging system to obtain bacteria and contaminant flow and transport information in porous media. The new color imaging system can be employed to get detailed insights not only in porous medium problems but also in various industrial processes where color imaging is important for analysis of the systems.

6. CONCLUSIONS

Development of a color image processing system has been described with applications to transport of bacteria and contaminant in porous media. The approach involves color visualization, discrimination, and quantification of bacterium and contaminant distributions within a transparent porous column. By introducing stained bacteria (green color) and a contaminant (orange color) into a transparent porous column and illuminating the porous regions with a planar sheet of laser beam, contaminant and bacterial transport processes can be observed and measured microscopically. Using the color imaging technique, the overall mean concentration of bacteria & contaminant, the number of bacteria, the concentration of bacteria/contaminant in each pixel/cell, and the velocity of bacteria in the pores as a function of time was evaluated. The approach shows the successful application of our novel automated and color imaging system to obtain bacteria and contaminant flow and transport information in porous media. The new color imaging system can be employed to get detailed insights not only in bioremediation problems but also in various environmental and industrial processes where color imaging is important for analysis of the systems.

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