Free-Living Bacteria or Attached Bacteria: Which Contributes More to Bioremediation?

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ABSTRACT

Researchers have implied that natural bioremediation in karst or fractured rock is unlikely to occur because of the lack of bacteria biofilm in karst aquifers. Hydrologic and geologic characteristics of fractured rock aquifers have been described as not being suited for natural bioremediation because of small microbial populations. If bioremediation in bedrock aquifers is dependent upon contact between surface-attached bacteria and contaminants, then bioremediation would be limited by the low surface area to volume ratio (SA/V) of karst aquifers. A quantitative basis, however, for accepting or rejecting the assumption that attached bacteria dominate the biodegradation process in karst conduits has not been shown. The objective of this research was to determine if free-living karst bacteria contributed as much to toluene biodegradation as attached bacteria. Two flow-through reactor systems were established to test the different biodegradation rates. Each reactor system consisted of four 1.24-liter cylinders connected together with glass tubing for a total open volume of approximately 5 liters. The second reactor system was similar to the open system except the cylinders were filled with acid-washed, circular glass spheres that increased surface area to volume ratio approximately fivefold compared to the open system. Rhodamine dye was used to calculate the different residence-time distributions in each system. A sterile control study established that less than 3 percent of the toluene was lost to abiotic processes. Next, raw water from a karst aquifer containing live, indigenous bacteria was pumped through each system for 5 days to establish a biofilm on the glass surfaces. Colonization of the surface was confirmed by microscope visualization before toluene was added to the systems. The resulting first-order rate constants were computed to be 0.014 per hour for the open system and 0.0155 per hour for the packed reactor system. If surface-attached bacteria were the main contributors to the biodegradation process and the SA/V ratio was increased fivefold, a significantly higher biodegradation rate should have occurred in the packed reactor. The results of this study indicate that the free-living bacteria indigenous to a karst aquifer contribute as much to the toluene biodegradation process as attached bacteria.

INTRODUCTION

The lack of studies examining biodegradation in karst aquifers may be due to the widespread perception that contaminants are rapidly flushed out of karst aquifers. In highly developed and well-connected conduit systems, the rate of contaminant migration is expected to be much faster than the rate of biodegradation. Field (1993) states that remediation techniques such as ground-water extraction or bioremediation are impractical in karst aquifers dominated by conduit flow; however, he also states that the belief that contaminants are rapidly flushed out of karst aquifers is a popular misconception. Large volumes of water may be trapped in fractures along bedding planes and other features isolated from active ground-water flow paths in karst aquifers (Wolfe and others, 1997). In areas isolated from the major conduit flow paths, contaminant migration may be slow enough that biodegradation could reduce contaminant mass if favorable microorganisms, food sources, and geochemical conditions are present.

Researchers have implied that natural bioremediation in karst or fractured rock is unlikely to occur because of the microbiological characteristics of karst aquifers; small microbial populations and low surface area to volume (SA/V) ratio (Vogel, 1994). Typical microbial numbers for material from

unconsolidated aquifers have been reported to range from 1 x 10^4 to 1 x 10^7 cells per milliliter (cells/mL) (Ghiorse and Wilson, 1988). Studies have shown that water from bedrock (granite and karst) aquifers also may contain microbial populations within this range. For example, total microbial populations of 9.7 x 10^5 to 8.5 x 10^6 cells/mL and heterotrophic bacteria populations of 3.5 x 10^3 to 5.0 x 10^5 cells/mL were detected in ground-water samples collected from a gasoline-contaminated karst aquifer in Missouri (O'Connor and Brazos, 1991). The fact that greater than 70 percent of bacteria in consolidated aquifers are attached to solid surfaces (Harvey and others, 1984; Harvey and Barber, 1992) may have led to the assumption that natural bioremediation in karst conduits is negligible because contact between attached bacteria and contaminants would be limited by the SA/V ratio.

Research currently underway at Tennessee State University in cooperation with the U.S. Geological Survey focuses on modeling biodegradation of contaminants in karst systems. The research presented in this paper compares the biodegradation of toluene by attached and free-living bacteria in two laboratory karst systems. Conservative tracer studies, sterile controls and quantified toluene biodegradation were used to mathematically determine biodegradation rates for two laboratory karst systems representing a different SA/V ratio. The toluene-biodegradation results from the laboratory karst systems were analyzed in terms of chemical reaction kinetics and mass transfer principles. The math used to calculate whether the degradation was predominantly a function of volume through free-living microbes or a function of surface area through attached bacteria is described in the Methods and Materials section of this paper.

METHODS AND MATERIALS

Flow-through microcosms were constructed using a 20-liter glass reservoir, a multi-channel peristaltic pump, 10-milliliters (mL) stirred injection cells, four 1-liter volumetric flasks (actual volume when full = 1,240 mL), and 3-millimeter (mm) inner-diameter glass tubing connecting the pieces (fig. 1). One system was packed with a sufficient number of flat, glass spheres to increase the surface to volume area fivefold in the packed system as compared to the unpacked system. Water was pumped into both systems by using a high-performance peristaltic pump. A stirred injection cell (10 mL volume) was placed at the entrance of each replicate system for the injection of dye or toluene. The water traveled from the stirred injection cell

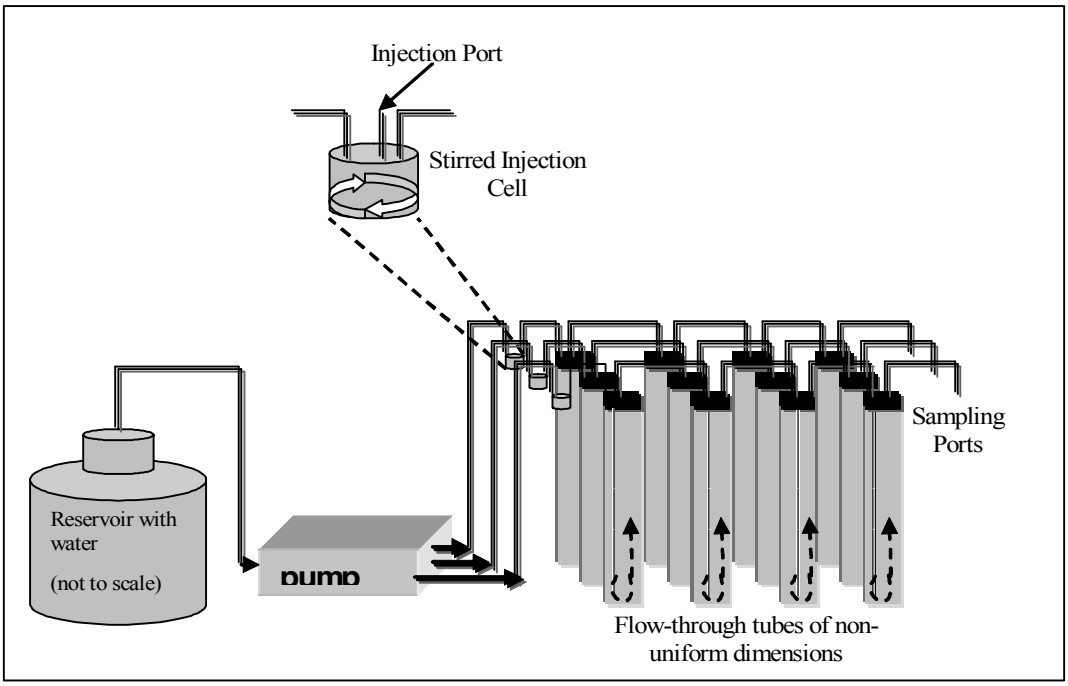


Figure 1. A schematic of the experimental karst systems.

through a thin glass tube to the bottom of graduated cylinders arranged in series. The water and injected constituents traveled through a series of nonuniform-size cylindrical glass tubes ranging from 3- to 56-mm in diameter. The non-uniform dimensions in the systems contributed to non-ideal flow conditions such as eddies and currents.

During the conservative dye tracer study, a constant flow rate of approximately 3 milliliters per minute (mL/min) was established for both systems. The pump was stopped at the beginning of the tracer study, 476 micrograms (μg) of Rhodamine dye was injected into each stirred injection cell, and the pump was restarted. The Rhodamine concentration at the discharge port was monitored through time by collecting samples at 1- to 2-hour time intervals over a 4-day period. A Turner 700® fluorometer was used to quantify the Rhodamine in the water samples. The lower detection limit on the fluorometer was established at 100 parts per trillion.

Before the toluene biodegradation study was initiated, the experimental systems were sterilized with bleach. The bleach was neutralized with sterile sodium thiosulfate. Filter-sterilized toluene (87 μg) dissolved in 100 microliters (μL) of methanol was delivered into the injection cell and pushed through the system with sterile water that had a pH of 10. Previous work indicated that elevating the pH to 10 to maintain an abiotic system.

Toluene concentration was monitored at the discharge port over the next 5 days. Water samples were collected in clean 40-mL volatile organic compound (VOC) vials every 1 to 4 hours. The water samples were immediately analyzed on a Syntex® gas chromatograph (GC) equipped with a purge-and-trap system, 30 meter (m) X 0.32 mm, 1.8-micrometer (μm) silica-film capillary column, argon-carrying gas, and micro-argon ionization detector. The lower detection limit for toluene on the GC was 0.5 microgram per liter (μg/L). Every fourth sample was either a duplicate sample or a standard of known toluene quantity, and a complete calibration curve was run every 6-12 hours.

The biodegradation experiments used water containing live bacteria collected from a 120-foot-deep well completed in a karst aquifer in south-central Kentucky. An 87-μg aliquot of toluene was dissolved in 100 μL of methanol and placed in the stirred injection chamber at time zero. The flow rate used in all experiments was kept constant at approximately 3.0 mL/min. Data results from the tracer tests and the biodegradation studies were entered into a computer spreadsheet and all calculations regarding residence-time distribution (RTD) and biodegradation rates were documented in the spreadsheets.

Experimental runs consisted of running the packed system and an unpacked system in parallel under similar conditions. In order to document the presence of attached bacteria, glass slides were suspended in both the packed and unpacked systems. The suspended slides were removed prior to and at the end of the experiments and viewed using an epifluorescent microscope and the direct-count method (Eaton and others, 1995).

Toluene was selected as the experimental contaminant because it is a component in most fuels and because previous work indicated Pseudomonad bacteria, which are heterotrophic aerobic bacteria (HAB), from the Kentucky site could grow using toluene as a food source (Byl and others, 2001; Byl and others, 2002). The concentration of HAB in the water was determined by using the most probable number (MPN) method (Eaton and others, 1995). The MPN bacteria concentrations in the abiotic systems were less than one colony-forming unit per 100 millimeter. The bacteria concentration in ground water from the karst aquifer ranged from 600,000 to 700,000 HAB/mL at the beginning and end of the experiment.

DESCRIPTION OF THE MATHEMATICS USED TO CALCULATE BIODEGRADATION RATES

The fate of biodegradable contaminants in a karst aquifer system is dependant upon the rate of their biodegradation and the amount of time they spend in the system with the bacteria (referred to as residence-time distribution or RTD). As a result, the difference in amount of toluene that is biodegraded in each of the systems is not sufficient to numerically predict the fate of contaminants in a karst

aquifer. The biodegradation-rate equation must be coupled to the RTD formula since the removal of the contaminant is a function of RTD and biodegradation reaction rate. Once the equation is established, it can be re-arranged to solve for the biodegradation rate using the experimental data. Following is a description of how the biodegradation rate and RTD equation were coupled.

For a sparingly soluble contaminant (A) reacting with a rate-limiting constituent (B), which may be the microbes themselves or different electron acceptors, the reaction may be second order (symbols are defined in Appendix):

$$\frac{dC_A}{dt} = kC_AC_B$$

In a situation where the microbes are acclimated and at steady state and electron acceptors are not limiting the concentration, the reaction, C_B may not change appreciably while C_A changes. Treating C_B like a constant, the equation can be rewritten as a first-order equation:

$$\frac{dC_A}{dt} = kC_AC_B \Rightarrow k'C_A$$

where $k' = kC_B$ is the pseudo first-order rate constant. Oxygen and microbes were assumed to be not limiting in this laboratory system because the initial water was saturated, allowing use of the pseudo first-order equation.

In this context, the relative contributions of surface and volumetric biodegradation to the observed biodegradation rate can be determined by experiments with varying SA/V. If free-living bacteria dominate, the biodegradation reaction is volumetric and the observed pseudo first-order rate constants for the packed and unpacked reactors will be of similar magnitude. If, on the other hand, attached bacteria dominate, then the reaction is surface controlled and the observed pseudo first-order rate constants will not be of similar magnitude. If attached bacteria dominate the biodegradation reaction, the rate constant will be directly proportional to SA/V ratio.

The non-ideal hydraulic and mass transfer characteristics for systems with different SA/V ratios would lead to different lengths of residence time in each system. The dissimilar residence times in the packed and unpacked systems used in this study can be offset by using the RTD formula (Bischoff, and Levenspiel, 1962). The RTD of solutes in each system was determined using a conservative dye study to compensate for the shorter residence time in the system with less volume (that is, packed with glass spheres). The residence time (τ) is related to the mean residence time obtained from the RTD as:

$$t_m = \left(1 + \frac{2}{P_e}\right)\tau .$$

The Peclet number *(Pe)* is obtained from the variance of the RTD according to:

$$\frac{\sigma^2}{P_e^2} = \frac{2}{P_e} + \frac{8}{P_e^2} .$$

The Peclet numbers and the amount of toluene biodegraded (X_m) obtained from the biotic experiments in the packed and unpacked systems were used to obtain the rate of biodegradation by rearranging the following:

$$X_m = 1 - \frac{4qe^{(P_e/2)}}{(1+q)^2 e^{(P_e q/2)} - (1-q)^2 e^{(-P_e q/2)}}$$

where:

$$q = \sqrt{1 + 4D_A/P_e}$$

The Damkohler number (D_A) incorporates the biodegradation reaction rate (k') and time. The equation can be rearranged and solved for k'.

RESULTS AND DISCUSSION

The RTD for each system was calculated from the conservative dye study. The data were numerically integrated to determine the mean residence time (t_m) and the variance (σ^2) for the packed and unpacked laboratory karst systems. These parameters where then used to calculate the Peclet numbers, which are an indicator of the dispersion as the solute moves through the system. The results of the conservative dye study are shown in figure 2. After the dye study, the reactor systems were sterilized and toluene was injected to measure the amount of removal by abiotic processes. A mass balance was done on the toluene injected and recovered from the sterile

systems. Approximately 3 percent of the injected toluene was lost to abiotic processes in each of the experimental systems during the sterile run.

In the third phase of the study, water containing live bacteria was pumped through the laboratory systems for 4 days to establish a biofilm on the glass surfaces. Bacteria counts using MPN and microscopic methods were used to confirm that bacteria covered the glass surfaces and were suspended in the water at the beginning and end of the experiments (photos 1 and 2). A solution containing 87.0 µg of toluene was injected into each system. Numerical integration of the resulting effluent toluene concentration and time (fig. 3) indicated recovery of 61 µg toluene from the unpacked reactor and 69 µg toluene from the packed reactor. The resulting observed toluene biodegradation value (X_m) for the packed and unpacked systems was 0.21 and 0.31, respectively.

These X_m values where used in the equation listed above to calculate the observed reaction rate constants ($k'_{observed}$). The values of $k'_{observed}$ were 0.014 per hour and 0.0155 per hour for the packed and unpacked systems, respectively. The above results for conversion and rate constants for the packed and unpacked systems seem counter-intuitive at first glance; that is, the packed system has lower conversion and higher reaction rate of the two systems. This occurs because of the complex relation between hydraulic and chemical reaction kinetics in a non-ideal flow system. When the residence time is taken into consideration in each system, however, the rate of biodegradation for the free-living bacteria alone, or volumetric rate, $k'_{volumetric}$ = 0.0135 per hour for both systems.

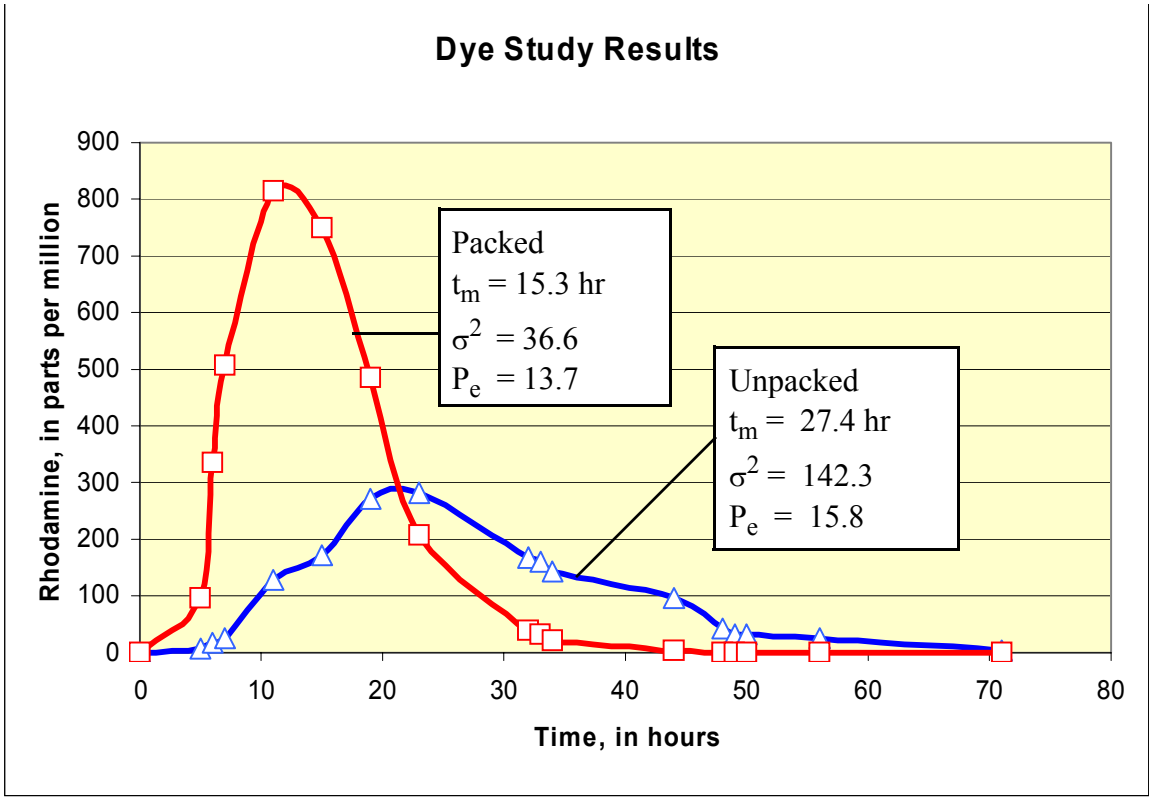
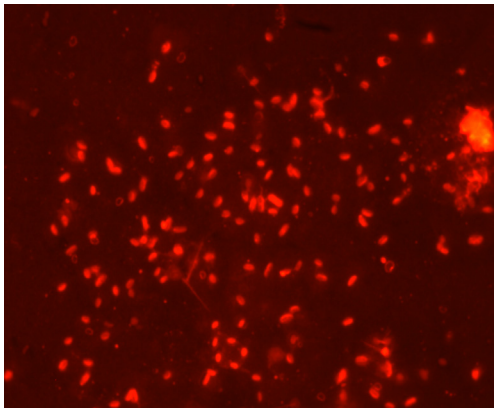
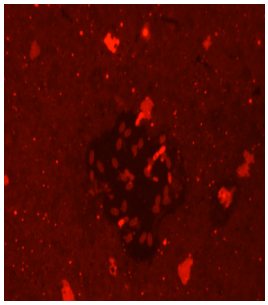


Figure 2. Rhodamine concentration at the end sampling port as a function of time for the high-surface area (packed) system and low-surface area (unpacked) system and the mean residence time (t_m), variability (σ^2) and Peclet value (P_e) for each system.

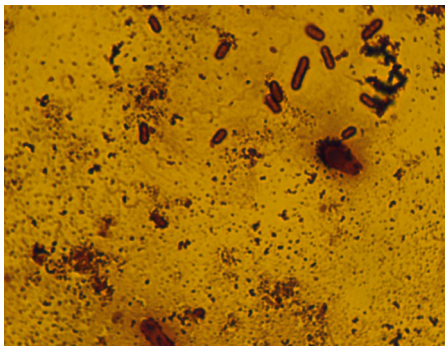
(A)



(B)



Photograph 1. (A) Bacteria (white objects) attached to the surface of the glass after 3 days of pumping water through the system (400x magnification, epifluorescent), and (B) close up of a bacteria cluster on the surface of the glass (800x magnification, epifluorescent).



Photograph 2. Free-living bacteria (dark objects) collected from the water column after 3 days. Flagella can be observed attached to the rod-shaped bacteria (1,000x magnification, bright field).

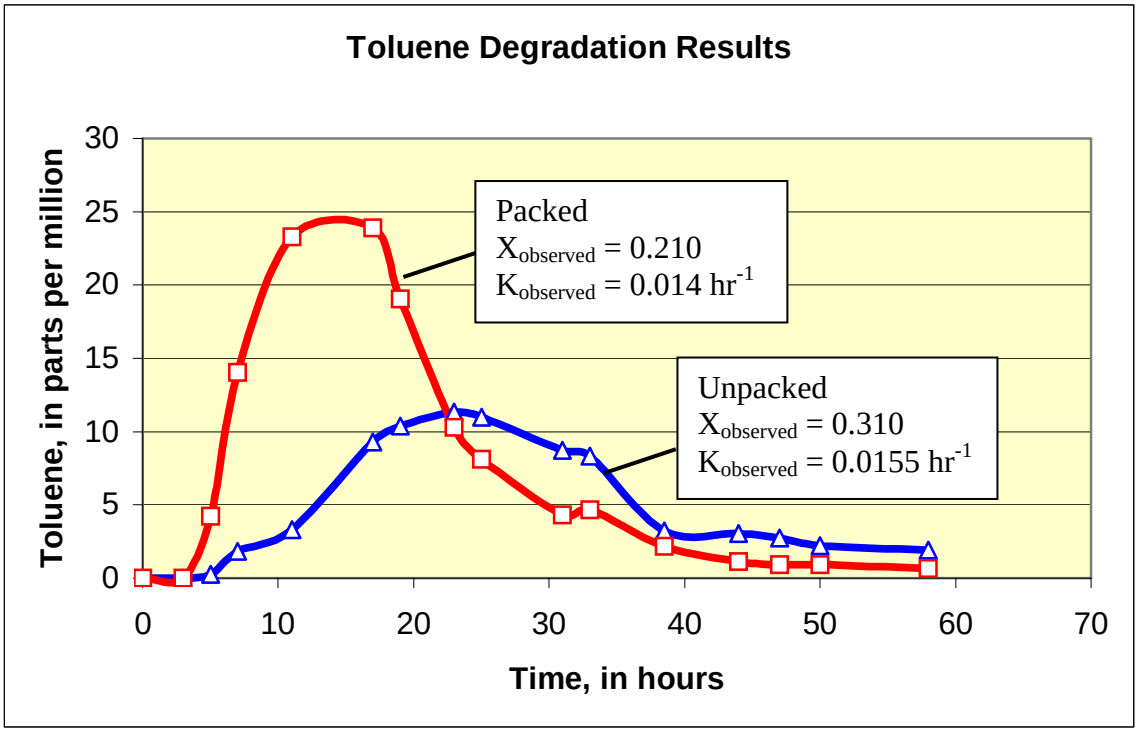


Figure 3. The concentration of toluene as a function of time in the packed system and unpacked systems. The $X_{observed}$ refers to the amount biodegraded, and the $K_{observed}$ refers to the first-order exponential rate of biodegradation calculated from the experimental data.

SUMMARY AND CONCLUSION

Biodegradation of toluene in flow-through laboratory karst systems of varying SA/V indicated that the observed biodegradation of toluene was a function of free-living and attached bacteria. This was evidenced by the fact that the system with fivefold greater surface area had only a 10-percent increase

in biodegradation. If attached bacteria were primarily responsible for biodegradation, a proportional increase in biodegradation with an increase in surface area would be expected; however, the free-living bacteria appear to contribute as much to biodegradation processes as attached bacteria. The volumetric reaction rate constant ($k'_{volumetric}$) of 0.135 per hour corresponds to a half-life for toluene of approximately 51 hours without consideration of surface bacteria. Thus, dissolved toluene that resided for several days in a karst conduit with characteristics similar to those in this study could experience substantial biodegradation regardless of interaction with the surface area.

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Appendix. Symbol notation and unit of measure

C	solute concentration, moles per liter [M/L]
C_A	initial contaminant concentration [M/L]
C_B	bacteria-electron-acceptor concentration [M/L]
$C(t)$	concentration of tracer as a function of time [M/L]
D_A	Damkohler Number
k'	pseudo first-order rate constant [T^{-1}]
P_e	Peclet Number [VL/D]
τ	space time [T]
t	time [T]
t_m	mean residence time [T]
$X(m)$	observed or calculated value of chemical biodegraded [M/L]
σ^2	variance [T^2]