A COMPARISON OF INACTIVATION OF BACTERIOPHAGE AND ENTEROVIRUS DURING ACTIVATED SLUDGE TREATMENT

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ABSTRACT

Inactivation of indigenous bacteriophages was followed during extended-aeration activated sludge treatment of domestic sewage. Also, a bacteriophage was isolated from the sewage and used to follow the inactivation of phages contained inside a dialysis membrane chamber immersed in an activated sludge aeration reactor. Finally, inactivation of Poliovirus T-1 and Coxsackievirus B-1 contained inside immersed membrane chambers were followed.

Statistical comparisons showed no detectable difference between phage inactivation rate constants measured in the aeration reactors and in immersed membrane chambers. No significant differences were detected between inactivation rate constants for Poliovirus T-1 and Coxsackievirus B-1. Finally, no significant differences were detected between inactivation rate constants for phage and enteroviruses inside immersed membrane chambers.

The results suggest that immersed dialysis membrane chambers may provide an accurate, reliable method for measuring virus inactivation during activated sludge treatment, and that bacteriophages may provide a convenient indicator system for virus inactivation during this process.

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INTRODUCTION

Since the 1930's there has been increasing concern among environmentalists about the possibility of water acting as a transmission vehicle for virus diseases. Recent trends toward the use of renovated wastewater to supplement domestic water supplies make accurate characterization of viral water pollution factors imperative (12, 16).

Viruses of major concern in water pollution are primarily of the human enterovirus group. Members of this group infect and multiply in the wall of the intestine and are excreted in the feces of infected individuals. Sabin (29) estimated that human populations excrete enteroviruses in concentrations up to 10^5 $TCID_{50}$ (50% tissue culture infective dose) per gram of feces. Consequently, entry of these viruses into domestic wastewater systems is inevitable. Specific viruses of concern are polioviruses, which can cause paralytic poliomyelitis; coxsackieviruses, which can cause pleurodynia, infantile myocarditis, and aseptic meningitis; the agent of infectious hepatitis, which is assumed to be viral; echoviruses, adenoviruses and reoviruses, all of which can cause a variety of rashes and respiratory diseases; and the agents of viral gastroenteritis. The agents of infectious hepatitis and gastroenteritis are considered most important due to the relatively high incidence of the diseases and the lack of reliable vaccines against them (11). In fact, several cases of epidemics caused by water transmission of viruses

have appeared in the literature, including hepatitis (3, 26), adenovirus (5) and coxsackievirus (13) epidemics.

Research is needed in two general areas to facilitate the eventual control of viral water pollution. First, virus removal and inactivation during exposure to natural water environments and during common wastewater treatment processes must be quantified (9, 11). Studies in this area will enable present and future sewage treatment plants to optimize processes for maximum reduction of virus numbers. Second, an indicator of viral pollution and virus inactivation is needed so that the prohibitive expense and difficulty of direct enterovirus assay can be circumvented. The Coliform Test for bacteriological water quality has been shown to be inadequate as an indicator of viral pollution (12). Ideally, a virus indicator system should be easily, quickly and inexpensively assayed, and results obtained must reliably indicate the presence and fate of human enteroviruses.

Recent research has examined inactivation or removal of human enteroviruses by wastewater treatment processes (2, 17). Nearly every common treatment method has been examined, and the activated sludge process has frequently been found to effectively reduce virus numbers in sewage (6, 8, 18, 20, 21, 22, 27). Activated sludge is an aerobic biological treatment method in which sewage (mixed liquor), cleared of large debris, is held in a basin (reactor) and heavily aerated (28). In normal applications, aeration periods vary from six to eight hours. Bacteria indigenous to sewage aerobically convert organic materials to

carbon dioxide and bacterial mass. Indigenous protozoans consume bacteria and convert the cell mass to carbon dioxide and protozoan cell mass. Excess protozoa and bacteria settle to the bottom of the basin to form a sludge, part of which can be pumped back to the head of the reactor to supplement the microflora of incoming sewage. The treated effluent is removed, usually by overflow, from the upper regions of the liquid in the reactor. A modification of the activated sludge process is extended aeration activated sludge, in which the aeration period lasts from 24 to 36 hours. The concentration of organic materials in the sewage is greatly reduced and autodigestion by indigenous microorganisms subsequently occurs, yielding a much cleaner effluent (7).

Most previous quantitative studies of enterovirus removal by activated sludge have been done in small volume bench-scale reactors. Viruses commonly employed in such experiments are poliovirus (virulent and vaccine strains) and coxsackieviruses. All workers have found significant removal rates by the process, but the variation among rates seen by different authors is considerable. Clarke, et al. (8) reported a 99% removal of coxsackievirus after 45 minutes of aeration; Kelly, et al. (18) indicated that poliovirus and coxsackievirus were 99% removed after four hours of aeration; Malina, et al. (21) noted a 98% removal of poliovirus after 24 hours of aeration and, in another study (22), a 90% removal after six hours of aeration. Reductions in virus numbers during activated sludge treatment are usually ascribed to adsorption of virus particles to

suspended solids and subsequent settling into the bottom sludge, rather than to destructive actions of the process on the particles. It is interesting to note that all of the above studies were done using sewage containing comparable concentrations of mixed liquor suspended solids (approximately 1000-2000 mg/l). In the only quantitative study of virus removal done in a full-scale municipal activated sludge unit (presumably with an aeration period of six to eight hours), Mack, et al. (20) found that indigenous poliovirus and coxsackievirus concentrations were reduced 16% by the process. The discrepancies among virus removal rates found by different authors suggest that caution must be exercised in extrapolating from bench-scale reactors to full-scale units.

The objectives of the work presented here are to evaluate a new method of monitoring virus inactivation rates during activated sludge treatment and to compare bacteriophage inactivation rates with virus inactivation rates.

The technique for monitoring virus inactivation should be applicable in full-scale operating activated sludge units so that possible variables introduced by the use of small-volume reactors can be eliminated. It should be easy to use, pending possible applications in optimization of present and future activated sludge units for maximum reduction of virus numbers. Finally, the method must give consistently accurate results over many trials. The technique employed here made use of dialysis membrane chambers, as described by McFeters and Stuart (23), containing viruses and

immersed in the aeration reactor of an extended aeration pilot plant. Inactivation of poliovirus T-1 and coxsackievirus B-1 during exposure to aerated mixed liquor were followed.

Some bacteriophages are structurally similar to human enteroviruses and may be vulnerable to the same inactivating mechanisms. Furthermore, phages are much more easily and inexpensively assayed than enteroviruses. These considerations make bacteriophages an attractive possibility as an indicator system for enterovirus inactivation. In fact, phages have been proposed as indicators of viral pollution (19). To be a reliable indicator system, the phage utilized should be found commonly in sewage and should be inactivated at the same or a slower rate than enteroviruses. Calabro, et al. (4) and Dhillon and Dhillon (14) have shown that bacteriophages can be isolated from domestic sewage.

A bacteriophage was isolated from the sewage used in these experiments. The isolate was then used to compare inactivation rate constants observed inside immersed membrane chambers with inactivation rate constants observed in aeration reactors. Also, inactivation rate constants for the phage isolate were compared with virus inactivation rate constants to evaluate its feasibility as an indicator.

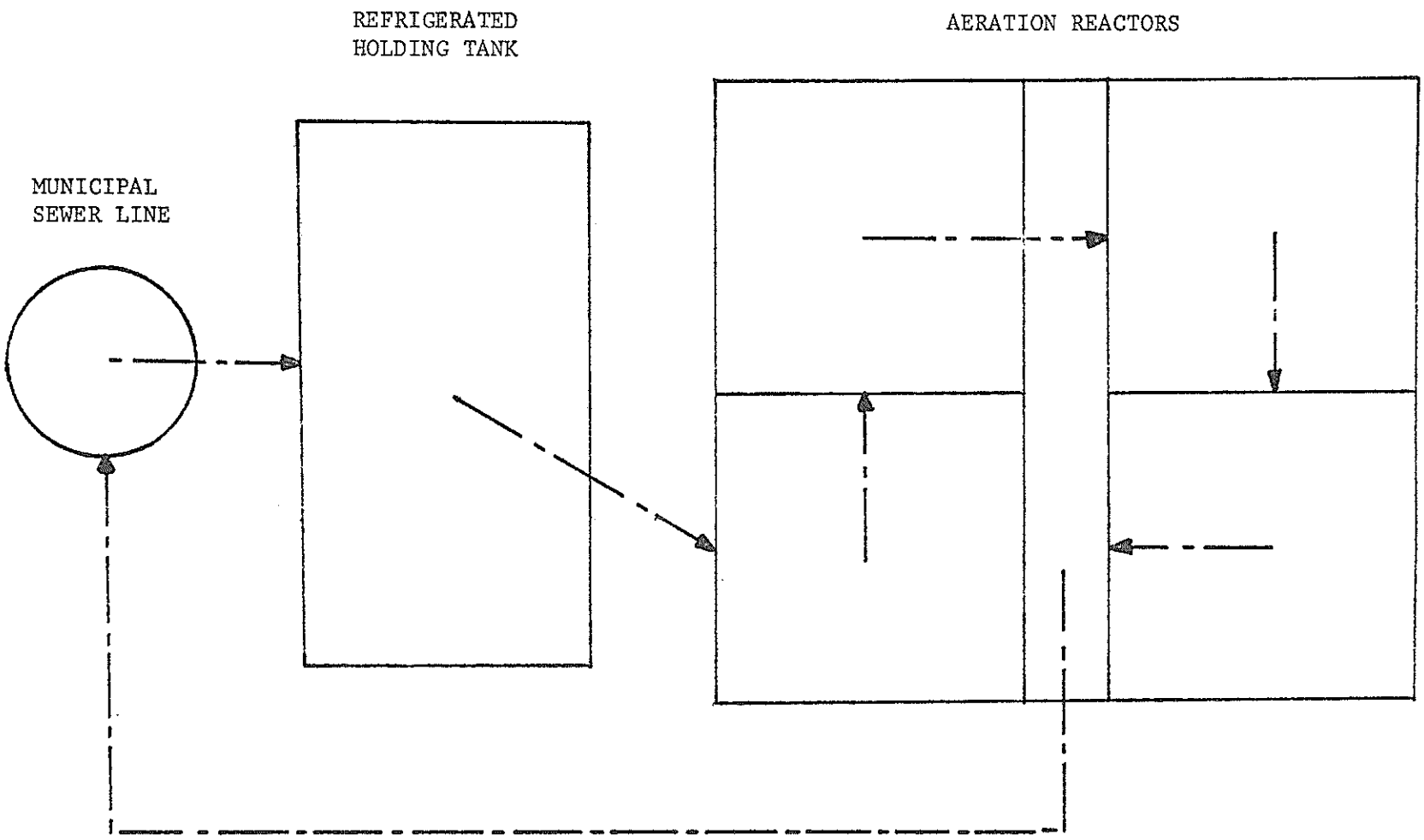
MATERIALS AND METHODS

Activated Sludge Unit

The activated sludge unit used in these experiments was an extended aeration pilot plant obtained from Sanilogical, Inc. The unit was owned and operated by the Civil Engineering Department at New Mexico State University. It consisted of four sequential aeration reactors of 200 gallon capacity each (Fig. 1), and was operated on a continuous-flow basis. Sewage was obtained from a municipal sewer line, and was pumped once-daily into a 1000-gallon capacity refrigerated (4 C) holding basin where it was mixed continuously to ensure homogeneity. Mixed liquor was pumped from the holding basin into the first reactor with variable-speed peristaltic pumps. The flow rate of mixed liquor through the system was accurately controlled with the pumps, and flow from reactor to reactor was by overflow. Aeration and mixing were provided by aerators located at the bottom of each reactor. Aerators were supplied by an air pump located outside the unit. Oxygen tension in the mixed liquor was maintained at approximately 8 mg/l. Mixing was sufficient to prevent settling of suspended solids. Unit effluent was collected by overflow from the fourth reactor and was returned by gravity-flow to the municipal sewer line. No sludge return was employed.

Sewage mixed liquor suspended solids remained constant at approximately 900 mg/l during experimentation. Mixed liquor temperature was maintained at 25 $\pm$ 2 C with electric heating coils

Figure 1. Schematic of four-reactor sequential extended aeration activated sludge pilot plant. Arrows indicate flow of mixed liquor through system.


REFRIGERATED
HOLDING TANK
AERATION REACTORS
MUNICIPAL
SEWER LINE

immersed in the first reactor. Mixed liquor pH was 7.0-7.5. Total-unit (i.e. four-reactor) detention times examined were 36, 72 and 144 h.

Media

A. Bacteriophage studies (24)

1) R-Medium

Bacto tryptone	10 g
Bacto yeast extract	1 g
NaCl	8 g
CaCl	0.2 g
Glucose	1 g
Distilled water	1000 ml

2) Dilution Broth

Bacto tryptone	10 g
NaCl	5 g
Distilled water	1000 ml

3) Hard Agar: R-medium with 1.5% Bacto agar

4) Soft Agar: R-medium with 0.5_ Bacto agar

B. Cell Culture

1) MEM Medium

Eagle's Basal Diploid (Gibco; Berkley, CA; Cat. No. G-13)

Penicillin	100 µg/ml
Streptomycin sulfate	100 µg/ml
Fungizone	25 µg/ml
Newborn calf serum	10%

(Inactivated at 56 C for 30 minutes before use)

$NaHCO_3$ solution	2.5% (v/v)
Bacto tryptose phosphate broth	10% (v/v)

2) GKN Medium (10x)

Glucose	10 g
NaCl	80 g
KCl	4 g
Phenol red (1% in 0.3N NaOH)	1 ml
Distilled water	1000 ml

3) $NaHCO_3$ solution (7.5%)

$NaHCO_3$	75 g
NaCl	9 g
Distilled water	1000 ml

4) EDTA Solution

Disodium ethylenediaminetetraacetic acid	1.86 g
Tetrasodium ethylenediaminetetraacetic acid	1.90 g
Distilled water	1000 ml

5) Trypsin stock (1%)

Bacto trypsin (1:250)	10 g
$NaHCO_3$	2 g
GKN medium (10x)	100 ml
Distilled water	900 ml

6) Working Trypsin (0.05%)

Stock trypsin (1%0	50 ml
$NaHCO_3$ solution	1.5 ml

EDTA solution	50 ml
Distilled water	900 ml

C. Enterovirus Assays

1) Phosphate-buffered saline (PBS)

KCL	2 g
NaCl	80 g
Na_2HPO_4	11.5 g
K_2HPO_4	4 g
Distilled water	1000 ml

2) Noble Agar Stock

Noble Agar	2.5 g
GKN medium (10x)	100 ml

3) Overlay Agar

MEM medium	73.5 ml
$MgCl_2$ (0.3M in GKN medium)	10 ml
Noble agar stock	16.5 ml

4) Alcoholic Crystal Violet

Crystal Violet	5 g
Ethanol (95%)	40 ml
Distilled water	960 ml

Indigenous Bacteriophage

Bacteriophages were isolated directly from mixed liquor by the soft-agar overlay technique (1). Escherichia coli A-19, an Hfr strain, was used as a host due to the large numbers of plaques obtained with this organism in preliminary determinations of indigenous phage

concentrations in mixed liquor. Mixed liquor samples were chloroformed 30 min at 37 C to reduce the viable numbers of indigenous bacteria. Samples were then diluted in dilution broth and 0.1 ml of appropriate dilutions combined with 0.2 ml of an exponential phase culture of E. coli A-19, grown in R-medium. Mixtures were incubated in 13 mm x 100 mm glass test tubes at 37 C with slow rotary shaking (120 rpm) for 30 min to allow attachment of phage particles to host bacteria. Mixtures were then combined with 2.0 ml of molten soft-agar, mixed gently and poured over a base of hard agar in 60 mm plastic petri plates. Plates were incubated 24 h at 37 C and observed for plaques in the bacterial lawn. Several plaque morphologies were observed, and all morphologies were counted for determinations of bacteriophage concentration in mixed liquor.

A lysate of indigenous phage was prepared in liquid culture. Two plaques of the predominant morphological type were punched out of the soft agar on a phage isolation plate (above) with a sterile glass tube, and both were inoculated into a single 50 ml exponential phase culture of E. coli A-19 in R-medium. The culture was incubated at 37 C overnight with slow rotary shaking (120 rpm). After incubation, the culture was centrifuged for 10 min at 7700 x g to remove cell debris and chloroformed 30 min at 37 C to eliminate remaining viable bacteria. The lysate was decanted into a sterile bottle and stored in the R-medium at 4 C. Soft-agar overlay assay of lysate titer, using E. coli A-19 as the host, showed a concentration of 1.1×10^{10}

plaque-forming units/ml (PFU/ml), which remained constant throughout the experimentation period.

Enterovirus Studies

All enterovirus experiments were done using poliovirus T-1 and coxsackievirus B-1. Viruses were stored as suspensions in PBS at 4 C. Titers were in the range of 1×10^8 PFU/ml.

Virus concentrations were determined with the Dulbecco plaque assay technique (15) on HeLa cells. Cells were grown 48 h at 37 C under MEM medium in a sealed rotating cylindrical bottle of 2 l capacity. Monolayers were removed with 20 ml working trypsin and resuspended in fresh MEM medium to a concentration of $1\text{-}2 \times 10^5$ cells/ml. The cell suspension was then distributed in 5 ml aliquots to 2 oz square prescription bottles ("plaque bottles"). Bottles were incubated horizontally for 48 h at 37 C in a 5% CO_2 atmosphere to allow monolayers to reach confluency.

Virus samples were diluted in PBS and 0.2 ml of appropriate dilutions inoculated onto monolayers drained completed of MEM medium. After 1 h, the monolayers were overlaid with 5 ml of over-lay agar and incubated 50 h at 37 C in a 5% CO_2 atmosphere. Agar overlays were then poured off into a solution of commercial bleach (Clorox). Monolayers were stained with alcoholic crystal violet and the plaques counted.

Dialysis Membrane Chambers

Plexiglas chambers were made by the New Mexico State University Physical Plant shops as described previously (23). The chambers consisted of three plates, each 10 cm x 8.7 cm x 0.7 cm with a central opening 6 cm in diameter. Dialysis membrane was obtained in sheets from Bolab, Inc. The membrane pore size was 48 Å, and the molecular weight exclusion was 12,000 d. Sheets of membrane were placed on both sides of the middle plexiglas plate and held in place by the outer plates, secured with stainless steel bolts. The central chamber, bound on either side by dialysis membrane, had a capacity of 20 ml. Sampling access to the central chamber was provided by two 18-gauge needles mounted in the middle plexiglas plate and extending into the chamber interior. While immersed, the access needles were sealed with plastic syringe tips to prevent leakage.

Reactor Phage Inactivation

Mixed liquor samples were taken simultaneously from the unit influent line and from each aeration reactor. Samples were assayed immediately for infective phage concentration as described earlier using *E. coli* A-19 as the host.

The order of the phage inactivation reaction in the aeration reactors was determined by applying a modification of the complete mixing mass-balance relationship around each reactor (7), as follows:

$$V\frac{dC}{dt} = QC_0 - QC_1 - KC_1^{n}V \qquad [1]$$

where V is the volume of the reactor; Q is the volumetric flow rate

of mixed liquor through the reactor; C_0 and C_1 are the infective phage concentrations in the reactor influent and effluent, respectively; n is the order of the inactivation reaction; and K is the reaction rate constant. Influent total bacterial counts were constant at approximately 10^6 colony-forming units/ml and influent coliphage counts were constant at approximately 10^3 PFU/ml. Previous experience in this laboratory indicated that these concentrations were too low to allow significant phage replication, and it was assumed that no phage replication occurred in the aeration reactors. At steady state during continuous-flow conditions, the concentration of infective phage particles in a reactor does not change with time, so that:

$$\frac{dC}{dt} = 0 \text{ (at steady state)} \qquad [2]$$

The activated sludge unit employed here was maintained at steady state during experimentation. Substituting from Equation 2 into Equation 1,

$$0 = \frac{Q}{V}(C_0 - C_1) - KC_1^n \qquad [3]$$

Rearranging and taking the logarithm of both sides,

$$\log \left[\frac{Q}{V}(C_0 - C_1)\right] - \log K + n \log C_1 \qquad [4]$$

The order of the inactivation reaction was found by plotting log $[\frac{Q}{V}(C_o - C_1)]$ vs log C_1 for each detention time, and determining the best-fit line by linear regression (30). The slope of the plot was the order of the reaction. Slopes, shown in Table 1, were close to unity and, since removal of many substances from sewage by activated sludge is known to follow first-order kinetics (W. A. Barkely, pers.

Table 1. Slopes of best-fit lines of plots of log $[\frac{Q}{V}(C_0 - C_1)]$ vs. log C_1 for reactor phage inactivation.[a]

Detention Time (h)[b]	Number Replicates	Slope[c]
36 h	4	0.414
72 h	3	0.930
144 h	2	0.716
all times	9	0.820

[a]See text for definition of terms.

[b]Total-unit detention time.

[c]Slope = order of phage inactivation reaction.

comm., Department of Civil Engineering, New Mexico State University, 1976), the reaction order (n) was assumed to be 1 (i.e. phage inactivation was assumed to follow first-order kinetics).

A value of 1 was substituted for n in Equation 3, and the equation solved for K.

$$K = \frac{Q}{V} \left(\frac{C_0}{C_1} - 1\right) \qquad [5]$$

Using Equation 5, a value for K (phage inactivation rate constant) was calculated for each aeration reactor at each detention time. Each experiment was replicated two to four times, and phage inactivation rate constants for the reactors were compared by a one-way analysis of variance (30).

Chamber Phage and Enterovirus Inactivation

Dialysis membrane chambers were filled with 20 ml of mixed liquor containing enteroviruses or bacteriophages in concentrations of approximately 10^6 PFU/ml. Chambers were then suspended by steel chains in the contents of the first or second aeration reactor during unit operation. At 2 h intervals, 0.5 ml samples of chamber contents were removed. Chamber contents were thoroughly mixed before sampling. Sampling was continued for the duration of a single-reactor detention time. Phage concentrations were assayed as described earlier immediately after sampling. Samples containing enteroviruses were diluted with an equal volume of MEM medium, incubated 2 h at 25 C to eliminate indigenous bacteria through antibiotic action, and stored at -70 C until assayed.

Least-squares analyses (30) were done on PFU/ml vs. exposure time data for each chamber experiment to determine the type of inactivation kinetics exhibited. Analyses were done with an APL computer program obtained from the New Mexico State University Civil Engineering Department. Basic equation forms evaluted were $C_t = at + b$ (zero-order kinetics), $C_t = 10^{(at + b)}$ (first-order kinetics) and $C_t = b \cdot a^t$ (second-order kinetics). The program generated an equation (of the basic form evaluated) for the best-fit line and a correlation coefficient for spread of the data points around the line.

Highest correlation coefficients were consistently found with the first-order model [$C_t = 10^{(at + b)}$], indicating that phage and virus inactivation inside immersed membrane chambers followed first-order kinetics. Inactivation rate constants were calculated from the basic equation form for first-order kinetics as follows:

$$C_t = 10^{(at + b)} \qquad [6]$$

Differentiating and reducing,

$$\frac{dC_t}{dt} = \ln (10) \times 10^{(at + b)} \cdot \frac{d(at + b)}{dt} \qquad [7]$$

$$\frac{dC_t}{dt} = 2.303a \times 10^{(at + b)} \qquad [8]$$

However, by rearranging Equation 6,

$$t = \frac{\log C_t - b}{a} \qquad [9]$$

Substituting into Equation 8,

$$\frac{dC_t}{dt} = 2.303a \times 10^{\left[\frac{a(\log C_t - b)}{a} + b\right]} \qquad [10]$$

Simplifying,

$$\frac{dC_t}{dt} = 2.303a \cdot C_t \qquad [11]$$

Equation 11 is of the form $\frac{dc}{dt} = KC$ (i.e. first-order kinetics), and

$$K = 2.303a \qquad [12]$$

for phage and enterovirus inactivation inside immersed dialysis membrane chambers. Using Equation 12 and values of 'a' obtained from least-squares analysis of each chamber experiment, values of K (i.e. inactivation rate constants) for phage and enterovirus inactivation were calculated. Each experiment was replicated two to four times, and inactivation rate constants were compared by Student t-test (30).

Comparison of Reactor and Chamber Phage Inactivation Rate Constants

Since reactor and chamber phage inactivation rate constants were calculated by different methods with data taken from two presumably independent populations, a nonparametric statistical method was necessary to make comparisons between them. Comparison was done by the Wilcoxon Test (30).

RESULTS

Membrane Diffusion Studies

In order for viruses suspended inside dialysis membrane chambers to be exposed to the actual mixed liquor environment, the chamber and reactor environments must remain at equilibrium through diffusion of solutes across the chamber membranes. To determine if chamber interiors maintained equilibrium with surrounding mixed liquor, experiments were conducted to examine the diffusion of various solutes across the chamber membranes.

Oxygen tension is a critical factor in the activated sludge process. Sufficient oxygen must be maintained in the mixed liquor to allow maximum aerobic metabolism by microorganisms. To study the diffusion of oxygen across the dialysis membrane, four chambers were filled with aerated mixed liquor and suspended in the contents of the second aeration reactor. At 3 h intervals, one of the chambers was removed from the reactor and its contents withdrawn. Simultaneously, 20 ml of mixed liquor were withdrawn from the aeration reactor. Samples were diluted with equal volumes of distilled water and slowly poured into separate 40 ml air-tight bottles. Dissolved oxygen in each sample was determined with a Delta, Inc. Oxygen Probe. Oxygen tension in the distilled water was low (on the order of 1 mg/l) so that measurement of dissolved oxygen in diluted mixed liquor samples was probably not accurate. However, since all samples were diluted identically, relative concentrations could be compared. Results,

presented in Fig. 2, showed that chamber interiors maintained the same oxygen tension as reactor contents during 24 h of immersion, indicating that oxygen diffused instantaneously across the membrane.

Diffusion of water and bicarbonate and phosphate ions were measured with $^{3}H_2O$, $Na_2{}^{14}CO_3$ and $H_3{}^{32}PO_4$, respectively. Beakers were filled with 700 ml of mixed liquor supplemented with all three isotopes at concentrations shown in Fig. 3. Mixed liquor in the beakers was stirred and aerated vigorously. Two membrane chambers were filled with mixed liquor containing no isotopes and one chamber was suspended in each beaker. At 10 min intervals over a 3 h period, 0.5 ml samples were withdrawn from each chamber. The samples were added to 5 ml of Omniflour scintillation cocktail (New England Nuclear) and counted on a Packard scintillation counter set for triple-label counting. Diffusion rates, measured as cpm/ml/min, were converted to mole/min values as described in Table 2. For comparisons of diffusion rates, differences in original external concentrations of the compounds were normalized by calculating specific diffusion rates. Specific diffusion rates were calculated by dividing the moles of each labelled compound which diffused per unit time by the initial number of moles of that compound in the external liquid. Diffusion rates for the three compounds are presented in Table 2.

Diffusion of water can be more accurately estimated if the diffusion rate of $^{3}H_2O$ is assumed to be representative of the diffusion rate of H_2O. The original concentration of $^{3}H_2O$ in the external liquid was 24 nC_i/ml or 51,500 cmp/ml. Since 1 ml is 0.0556 mole H_2O,

Figure 2. Oxygen tension in immersed dialysis membrane chambers (Δ ——— Δ) and in aerated mixed liquor in extended aeration reactors (× - - - ×). Each line is an average of two replicate experiments.

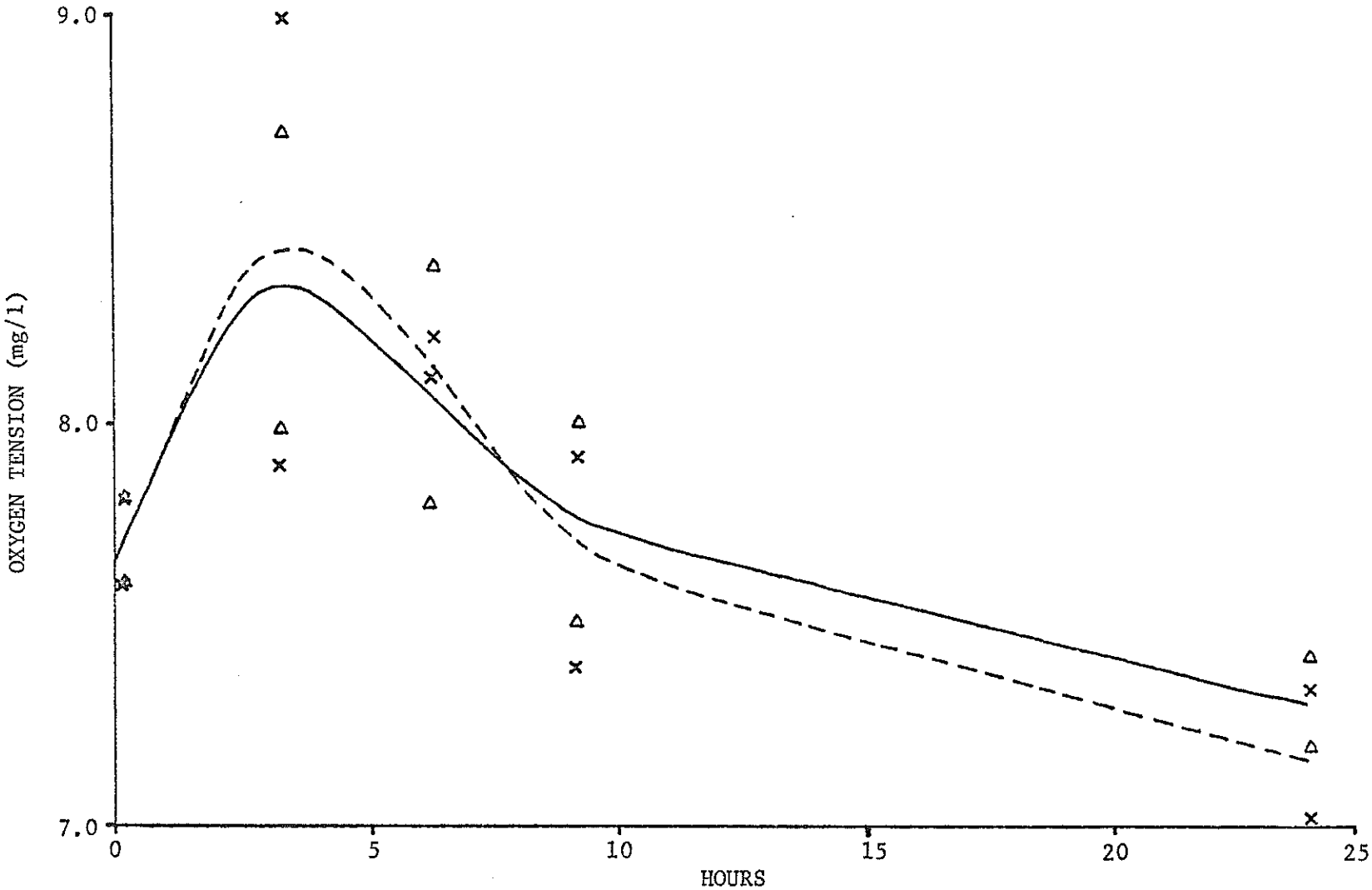

9.0
8.0
7.0
OXYGEN TENSION (mg/l)
0
5
10
15
20
25
HOURS

Figure 3. Diffusion of $^{3}H_2O$ (× ——— ×), $H^{14}CO_3^-$ (Δ - - - Δ) and $H^{32}PO_4^=$ (◇ —·— ◇) into dialysis membrane chambers immersed in aerated mixed liquor. Initial external concentrations of the isotopes were : $^{3}H_2O$, 24 nCi/ml; $Na_2{}^{14}CO_3$, 1 nCi/ml; $H_3{}^{32}PO_4$, 2 nCi/ml.

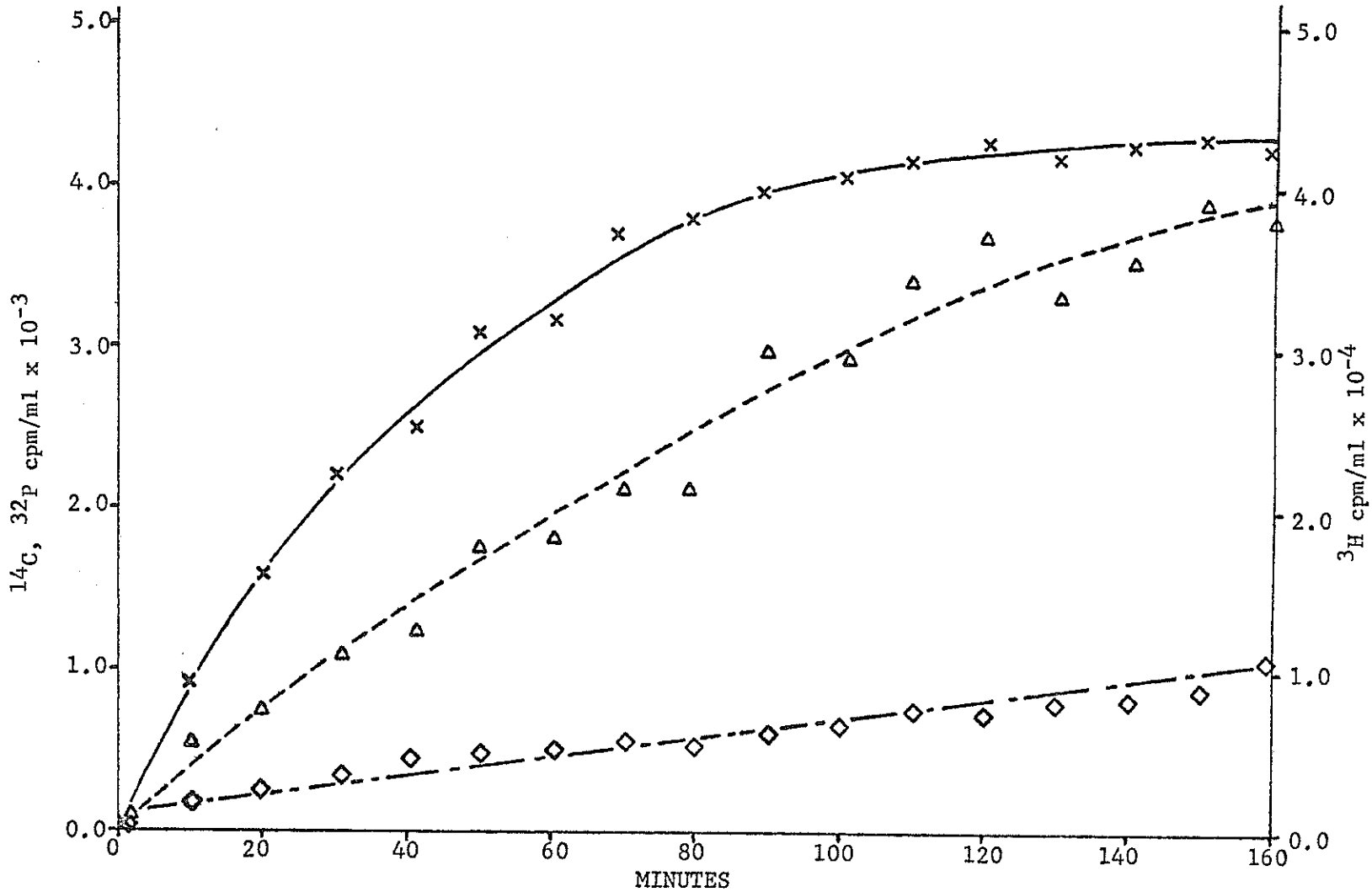

5.0
4.0
3.0
2.0
1.0
0.0
^{14}C, ^{32}P cpm/ml x 10^{-3}
^{3}H cpm/ml x 10^{-4}
0
20
40
60
80
100
120
140
160
MINUTES

Table 2. Diffusion rates of $^{3}H_2O$, $H^{32}PO_4^{=}$ and $H^{14}CO_3^{-}$ in immersed dialysis membrane chambers.

Compound[a]	Diffusion Rate		Specific Diffusion Rate (min^{-1})[d]
	cpm/ml/min[b]	Mole/min[c]	
$^{3}H_2O$	90.91	1.61×10^{-14}	5.5×10^{-5}
$H^{14}CO_3^{-}$	31.75	1.18×10^{-11}	1.03×10^{-3}
$H^{32}PO_4^{=}$	6.25	1.22×10^{-17}	7.97×10^{-5}

[a] Compounds are presented as species existing at pH 7-7.3.

[b] Measured from plot (Fig. 4).

[c] $$\text{mole/min} = \frac{[(\text{cpm/ml/min}) \times (\text{chamber volume}) \times (\frac{\text{mole}}{\text{Ci}} \text{ for compound})]}{[(\text{counting efficiency}) \times (2.22 \times 10^{12} \text{ dpm/Ci})]}$$

[d] Specific diffusion rate = (mole/min)/(original number of moles of compound in external liquid).

the original concentration of isotope can be expressed as 9.27×10^5 cpm/mole H_2O. The raw diffusion rate of ^3H_2O into the membrane chamber (Fig.4;p.29) was 90.91 cpm/ml/min, or 1818.2 cpm/min for the 20 ml of chamber contents. Thus, the molar diffusion rate was:

$$(1818.2 \frac{\text{cpm}}{\text{min}}) / (9.26 \times 10^5 \text{ cpm/mole } H_2O) = 1.96 \times 10^{-3} \frac{\text{mole } H_2O}{\text{min}}$$

Similar calculations for diffusion of HCO_3^- and $HPO_4^=$ were not possible since the concentrations of unlabelled HCO_3^- and $HPO_4^=$ in the external mixed liquor were not known. However, it is apparent from the results that these solutes diffused across the dialysis membrane, although at slower rates than oxygen.

Bacteriophage Inactivation

Reactor Rate Constants. Rate constants for bacteriophage inactivation in the aeration reactors, as calculated with Equation 5, are shown in Table 3. Constants are positive because the inactivation term (i.e., KC_1V) is subtracted in Equation 1. Negative rate constants at 36 h detention time indicate an increase in infective phage concentration. These anomalies can be explained by fluctuations in the influent phage concentration, in combination with the small decreases in phage numbers which occurs during short detention times (see Appendix Table 11). Errors arising from the variability of phage assay were also a contributing factor. Because of the variability of results at the 36 h detention time, rate constants associated with this detention time were not used in rate comparisons.

Table 3. Rate constants for bacteriophage inactivation in aeration reactors.[a]

Detention Time (h)[b]	Reactor Number	Replicate[c] 1	2	3	4
36	1	-.0845	-.1063	-.1077	N.D.[d]
	2	.2708	.0101	.0018	-.0043
	3	.0413	.0960	.0324	-.0469
	4	.2424	.0528	-.0141	.0675
72	1	.2653	.0760	N.D.	N.D.
	2	.1520	.2963	.0688	N.D.
	3	.0404	.0556	.1063	N.D.
	4	.5556	.0278	.0196	N.D.
144	1	.3426	.1037	N.D.	N.D.
	2	.1389	.0139	N.D.	N.D.
	3	.0139	.2500	N.D.	N.D.
	4	.0278	.2247	N.D.	N.D.

[a] Rate constants (K) have units of h^{-1}; constants were calculated with Equation 5 from phage concentrations found by simultaneous sampling of unit influent and each aeration reactor.

[b] Total-unit detention time.

[c] Rate constants were calculated for each reactor in each replicate experiment; negative values indicate an increase in infective phage concentration.

[d] N.D. = no data.

From Equation 4 and regression theory, it can be seen that the logarithms of inactivation rate constants are distributed normally. Since an analysis of variance requires normally-distributed observations, logarithms of K-values were used to compare between reactor phage inactivation constants. The results of the comparisons are shown in Table 4. No significant differences between rate constants in different reactors were detected in these experiments. Furthermore, no significant differences between rate constants at different detention times were detected.

Chamber Rate Constants. Inactivation rate constants calculated with Equation 12 and values of 'a' from regression lines of PFU/ml vs. exposure time are shown in Table 5 (Fig.4,p.29 for regression lines). Constants are negative because values of 'a' for inactivation plots are negative. Comparisons between rate constants for different detention times were done by a Student t-test (30). Constants found from 36 h detention time data were not used in the comparisons, for reasons mentioned above (see Appendix Table 12). Results of the comparisons are shown in Table 8. No significant difference between phage inactivation rate constants at 72 h and 144 h detention times was detected in these experiments.

Comparison Between Chamber and Reactor Phage Inactivation Rate Constants. Chamber and reactor phage inactivation rate constants were compared by the Wilcoxon test (30). Absolute values of K were used to normalize rate constants for comparison purposes. The percentile of the confidence level for differences between rate

Table 4. Comparison of rate constants for bacteriophage inactivation in aeration reactors.[a]

Detention Time (h)[b]	Mean (log K) in Reactor 1	2	3	4	F-ratio[c]	p[d]
72 h	.8447	.8363	1.2075	1.1731	0.4556	>50%
144 h	.7225	1.3572	1.2296	1.1022	0.3278	>50%
72 and 144 h	(composite of above)				0.3571	>50%

[a] Rate constants were compared by an analysis of variance using logarithms of K-values.

[b] Total-unit detention time.

[c] F = treatment mean square/error mean square.

[d] Percentile of confidence level for differences between rate constants.

Table 5. Rate constants for bacteriophage inactivation inside immersed dialysis membrane chambers.[a]

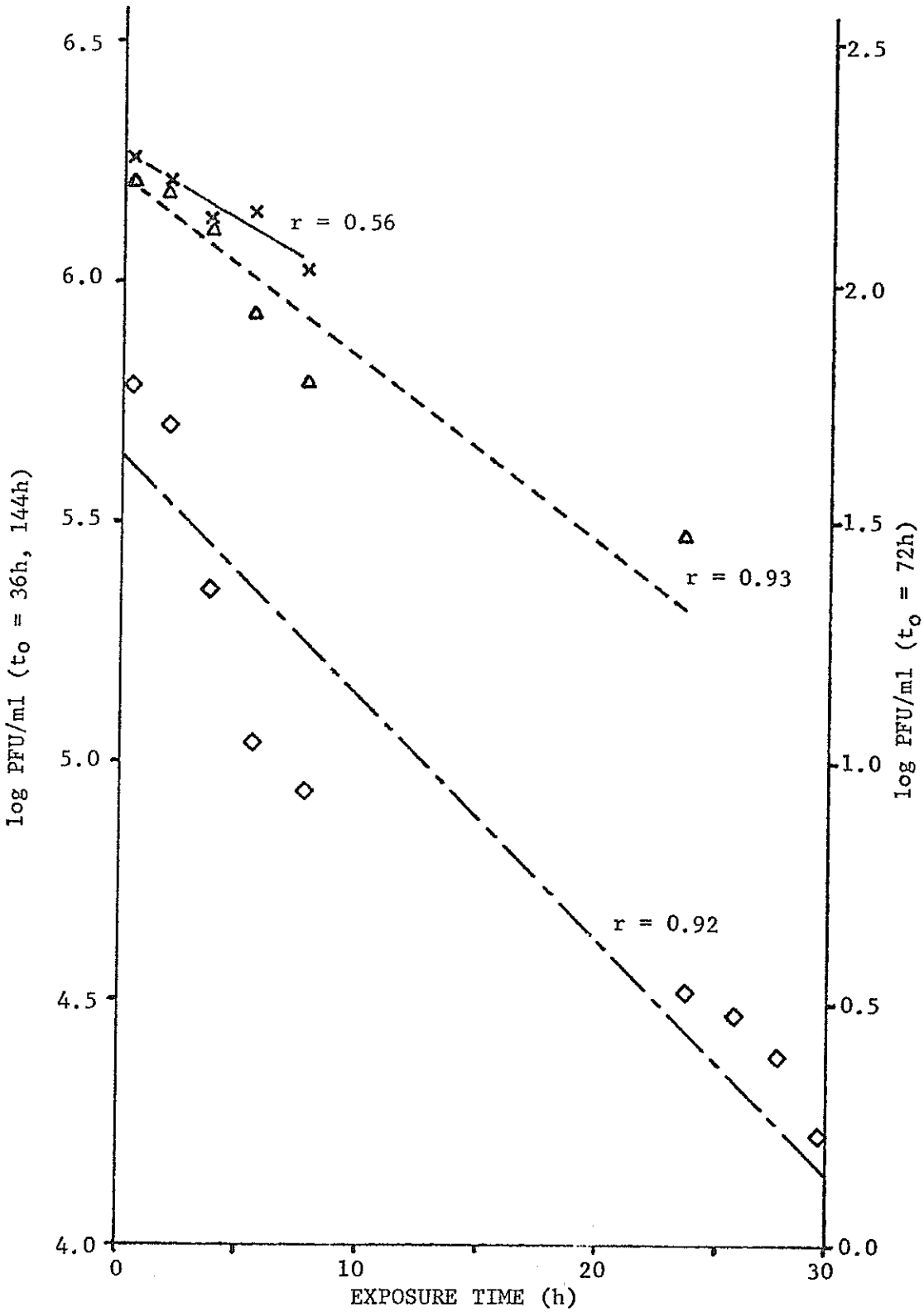
Detention Time (h)[b]	Replicate[c] 1	2	3	4	5
36	-.0695	-.0924	-.0238	-.0331	-.0294
72	-.0858	-.0744	--	--	--
144	-1.323	-.0955	-.1163	-.0892	--

[a]Rate constants were calculated with Equation 12 with values of 'a' from regression lines of PFU/ml vs. exposure time data. Constants have units of h^{-1}.

[b]Total-unit detention time.

[c]Rate constants were determined for each replicate chamber experiment.

Figure 4. Inactivation of indigenous bacteriophage in dialysis membrane chambers immersed in extended aeration reactors at detention times of 36 h (× ——— ×), 72 h (Δ - - - Δ) and 144 h (◇ —· —◇). Correlation coefficients for the regression lines are denoted by "r". Each line is an average of two to five replicate experiments. t_0 = total-unit detention time.


6.5
6.0
5.5
5.0
4.5
4.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
EXPOSURE TIME (h)
log PFU/ml (t_o = 36h, 144h)
log PFU/ml (t_o = 72h)
r = 0.56
r = 0.93
r = 0.92

constants was greater than 10%, indicating that no significant differences between rate constants were detected in these experiments.

Enterovirus Inactivation

Inactivation rate constants for enteroviruses inside immersed membrane chambers, calculated with Equation 12 with values of 'a' from regression lines of enterovirus PFU/ml vs. exposure time data, are presented in Table 6 (see Figs. 5 and 6 for regression lines). Values of K are negative because values of 'a' from inactivation plots are negative. Comparisons between enterovirus inactivation rate constants were done by a Student t-test (30). Constants calculated from 36 h detention time data were not used in the comparisons because of the variability of results, as cited earlier (see Appendix Tables 13 and 14). The results of the comparisons are shown in Table 7. No significant differences between rate constants for either virus at either detention time were detected in these experiments.

Comparison Between Phage and Enterovirus Inactivation Rate Constants

Inactivation rate constants for phage and enteroviruses inside immersed membrane chambers were compared by a Student t-test (30). Constants calculated from 36 h detention time data were not used in the comparisons for reasons cited earlier (see Appendix Tables 12-14). The results of the comparisons are shown in Table 8. No significant differences were detected between phage and enterovirus inactivation rate constants, with the exception of coxsackievirus B-1 at 72 h detention time.

Table 6. Rate constants for enterovirus inactivation inside immersed dialysis membrane chambers.[a]

Dentention Time (h)[b]	Replicate[c]					
	poliovirus T-1			coxsackievirus B-1		
	1	2	3	1	2	3
36	-.0238	-.0331	-.0294		N.D.[d]	
72	-.1237	-.1992	-.0963	-.1926	-.1879	-.1871
144	-.0913	-.1074	N.D.	-.1195	-.1668	N.D.

[a]Rate constants were calculated with Equation 12 from values of 'a' from regression lines of PFU/ml vs. exposure time data. Constants have units of h^{-1}.

[b]Total-unit detention time.

[c]Rate constants were calculated for each replicate chamber experiment.

[d]N.D. - not determined.

Figure 5. Inactivation of poliovirus T-1 in dialysis membrane chambers immersed in extended aeration reactors at detention times of 36 h (× ——— ×), 72 h (Δ - - - Δ) and 144 h (◊ — · — ◊). Correlation coefficients for regression lines are denoted by "r". Each line is an average of two to three replicate experiments.

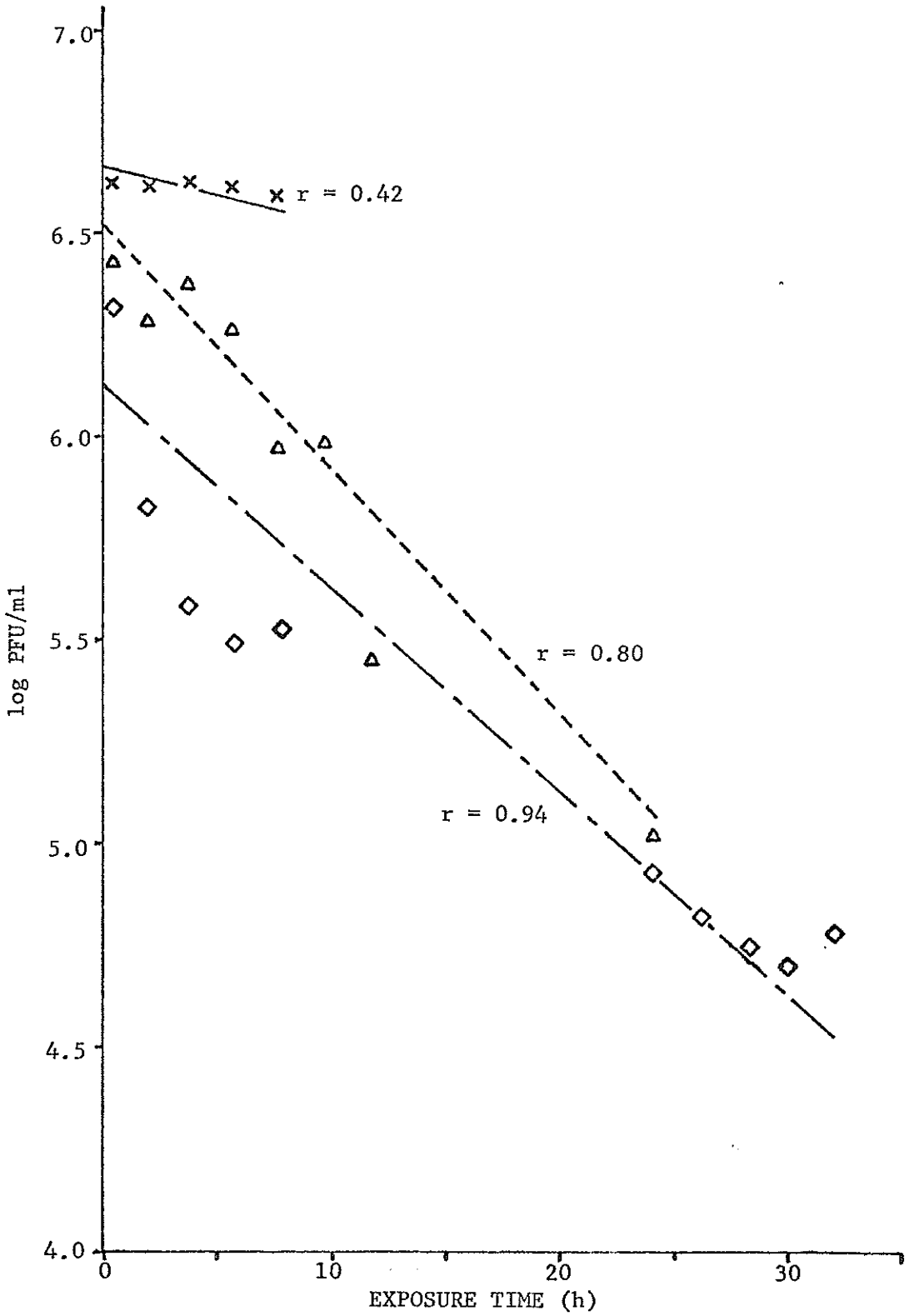

7.0
6.5
6.0
5.5
5.0
4.5
4.0
log PFU/ml
r = 0.42
r = 0.80
r = 0.94
0
10
20
30
EXPOSURE TIME (h)

Figure 6. Inactivation of coxsackievirus B-1 in dialysis membrane chambers immersed in extended aeration reactors at detention times of 72 h (Δ - - - Δ) and 144 h (◇ — · — ◇). Correlation coefficients for regression lines are denoted by "r". Each line is an average of two to three replicate experiments.

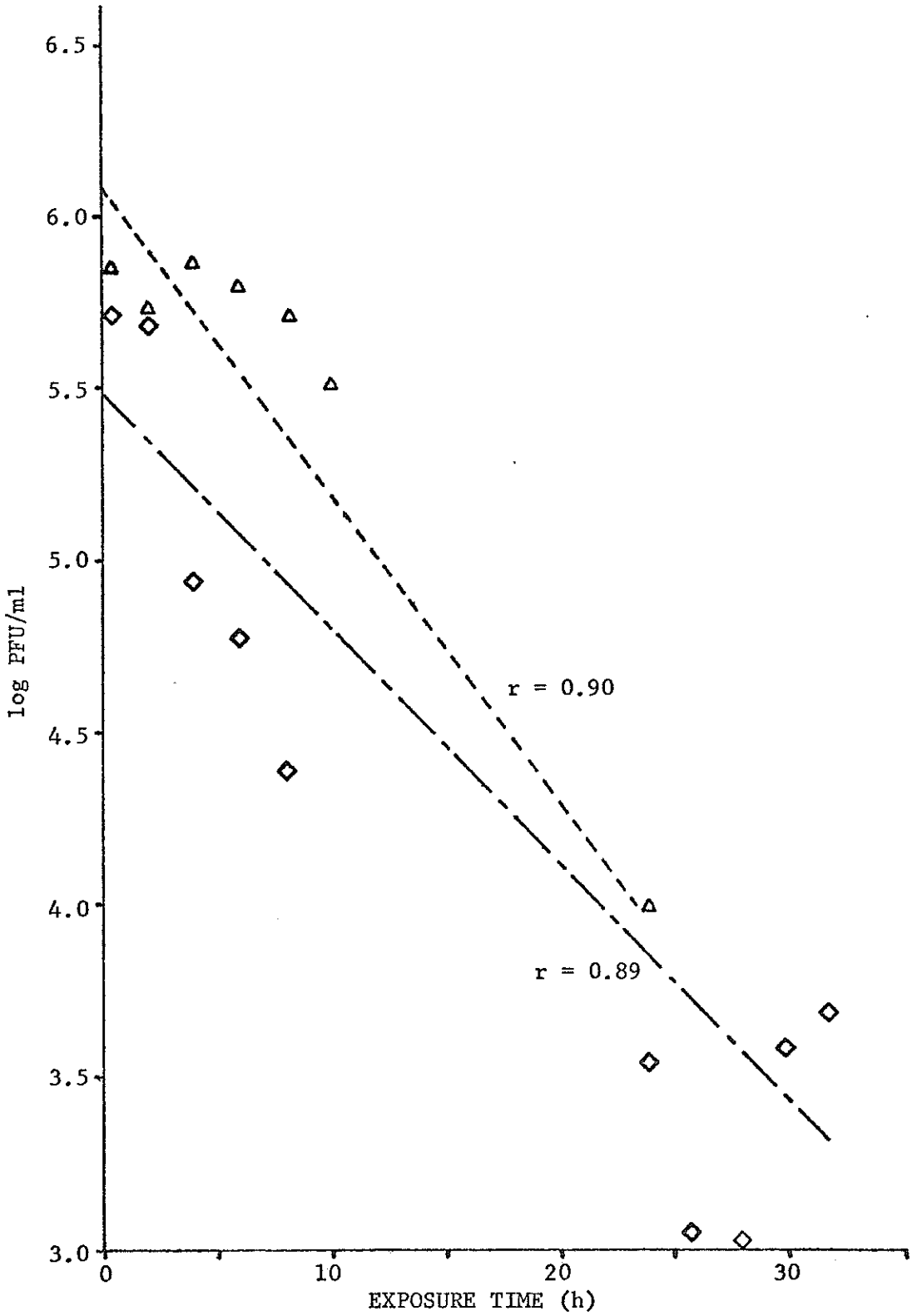

6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
log PFU/ml
r = 0.90
r = 0.89
0
10
20
30
EXPOSURE TIME (h)

Table 7. Comparison of rate constants for enterovirus inactivation inside immersed dialysis membrane chambers.[a]

Virus	Detention Time(h)[b]	Mean[c] K		Virus	Detention Time (h)	Mean K	P[d]
Poliovirus T-1	72	-.140	vs.	Poliovirus T-1	144	-.099	50%
Coxsackievirus B-1	72	-.186	vs.	Coxsackievirus B-1	144	-.143	>50%
Poliovirus T-1	72	-.140	vs.	Coxsackievirus B-1	72	-.186	>50%
Poliovirus T-1	144	-.099	vs.	Coxsackievirus B-1	144	-.143	>50%

[a] Rate constants were compared by Students t-test.

[b] Total-unit detention time.

[c] Mean K calculated from K-values from all replicates.

[d] Percentile of confidence level for differences between rate constants.

Table 8. Comparison of rate constants for phage and enterovirus inactivation inside immersed dialysis membrane chambers.[a]

	Detention Time (h)[b]	Mean[c] K		Virus	Detention Time (h)	Mean K	P[d]
Phage	72	-.080	vs.	Phage	144	-.108	50%
Phage	72	-.080	vs.	poliovirus T-1	72	-1.40	>50%
Phage	72	-.080	vs.	coxsackievirus B-1	72	-.186	0.5%
Phage	144	-.180	vs.	poliovirus T-1	144	-.099	>50%
Phage	144	-.180	vs.	coxsackievirus B-1	144	-.143	>50%

[a]Rate constants were compared by Student t-test.

[b]Total-unit detention time.

[c]Mean K calculated from K-values of all replicates of each experiment.

[d]Percentile of confidence level for differences between rate constants.

Log Reduction Times

Perhaps a more direct way to consider phage or enterovirus inactivation is by examining the T_{90}, or the time required to inactivate 90% of the original number of particles. An expression for T_{90} can be easily derived if phage and enterovirus inactivation is assumed to follow first-order kinetics. A first-order reaction can be described as follows (7):

$$\frac{dc}{dt} = KC \qquad [13]$$

where $\frac{dc}{dt}$ is the instantaneous change in concentration of substrate (i.e. phage or enterovirus infective particles); K is the inactivation rate constant; and C is the concentration of substrate. Rearranging and integrating,

$$\frac{1}{C}\, dc = Kdt \qquad [14]$$

$$\ln C \Big|_{C_0}^{C_1} = Kt \Big|_{t_2}^{t_1} \qquad [15]$$

$$\ln (C_1/C_0) = Kt, \text{ (when } t_0 = 0) \qquad [16]$$

where C_0 and C_1 are defined previously. However, at T_{90}, $C_0 = 10\ C_1$ and

$$C_1/C_0 = 0.1 \qquad [17]$$

Substituting into Equation 16 and simplifying,

$$\ln (0.1) = K \cdot T_{90} \qquad [18]$$

$$T_{90} = -2.303/K \qquad [19]$$

Using Equation 19, and mean values for K, a T_{90} value was calculated

for each set of replicate experiments. Results are shown in Table 9. Data from 36 h detention time is not shown because of high probability of error, as mentioned previously. The mean T_{90} value for all experiments was 25.33 h, with a standard deviation of 12.00 h. The mean K value for all experiments was 0.1130 h^{-1}, with a standard deviation of 0.0476 h^{-1}.

Table 9. Log-reduction times (T_{90}) for phage and enterovirus inactivation.

	Location	Detention Time(h)[a]	Mean K(h^{-1})[b]	$\lvert T_{90}(h) \rvert$[c]
Phage	Reactor 1	72	.1420	16.22
Phage	Reactor 2	72	.1458	15.80
Phage	Reactor 3	72	.0620	37.15
Phage	Reactor 4	72	.0671	34.32
Phage	Reactor 1	144	.1895	12.15
Phage	Reactor 2	144	.0439	52.46
Phage	Reactor 3	144	.0589	39.10
Phage	Reactor 4	144	.0790	29.15
Phage	Chamber	72	-.0800	28.79
Phage	Chamber	144	-.1080	21.32
Poliovirus T-1	Chamber	72	-.1400	16.45
Poliovirus T-1	Chamber	144	-.0990	23.26
Coxsackievirus B-1	Chamber	72	-.1860	12.36
Coxsackievirus B-1	Chamber	144	-.1430	16.10

[a] Total-unit detention time.

[b] Mean K was calculated from K-values for all replicates of each experiment. Signs are different due to different methods of calculation.

[c] $T_{90} = -2.303/K$. Absolute values shown due to differences in signs of of rate constants calculated by different methods.

DISCUSSION

Since dialysis membrane chambers were filled with mixed liquor before immersion in aeration reactors, chamber and reactor environments were identical at the beginning of each experiment. Diffusion studies with oxygen and water showed that these substances were exchanged rapidly between chamber interiors and reactor contents. Furthermore, phosphate and carbonate ions also diffused across the membranes, although at slower rates than oxygen. The slower diffusion rates were possibly due to size and charge of the phosphate and carbonate ions. However, the diffusion of these ions suggests that other solutes were also exchanged between chamber and reactor mixed liquor. Therefore, the accumulation of metabolic end products inside the chambers was probably minimal. These considerations suggest that the dialysis membrane chamber interior environment remained in equilibrium with the surrounding mixed liquor during chamber immersion. Further support for this conclusion can be found in the comparison of bacteriophage inactivation rate constants observed inside chambers and in aeration reactors. No significant differences were detected between chamber and reactor phage inactivation rate constants. This result suggests that the inactivation mechanisms which operated inside the membrane chambers were probably similar to inactivation mechanisms in the reactors. Furthermore, the variability in values of enterovirus T_{90} with respect to the mean T_{90} value measured using membrane chambers was less significant than the

variability in log-reduction times measured by various authors in small-volume model activated sludge reactors (see Table 10).

Thus, dialysis membrane chambers may provide a method for measuring enterovirus inactivation rates during activated sludge treatment which is more accurate and reliable than methods which employ small-volume model aeration reactors. However, more extensive studies will be necessary to verify this proposal.

Comparison of phage and enterovirus inactivation rate constants measured inside immersed dialysis membrane chambers at different detention times showed, for the most part, no detectable differences. This suggests that the rates of inactivation for phages and enteroviruses in these experiments were similar. If mechanisms and rates of inactivation for phages and enteroviruses are similar during activated sludge treatment, bacteriophages may provide a reliable indicator system for enterovirus inactivation during this process. The use of phages as such an indicator would be highly desirable. For example, no addition of potentially pathogenic virus particles to sewage would be necessary. Furthermore, assay procedures for bacteriophages are simpler and much less expensive than those for enteroviruses. Finally, results could be obtained as quickly as eight hours after sampling for phage, in comparison with a minimum waiting period of fifty hours with enteroviruses

Although other authors (8, 18, 20, 21, 22) have suggested that virus removal during activated sludge treatment is primarily due to adsorption of virus particles to suspended solids and subsequent

Table 10. Log-reduction time variability for enterovirus inactivation measured by two different methods.

Method	Mean T_{90}(h)	Variance(h)	Standard Deviation(h)
Small-volume[a] model reactor	5.09	20.09	4.48
Immersed dialysis[b] membrane chamber	19.71	29.22	5.41

[a] T_{90} values for model reactors estimated from results published by previous authors (Carlson, et al., 6; Clarke, et al., 8; Malina, et al., 21; Malina, et al., 22; Nupen, et al., 27.)

[b] T_{90} values measured for phage and enteroviruses at detention times of 72 and 144 h employed in calculations.

equally vulnerable to inactivation in aerated mixed liquor. Furthermore, it has not been shown that virus inactivation follows the same kinetics in commercial activated sludge units as in the extended aeration unit employed here. However, continued investigation in this area should lead to accurate quantification of phage inactivation in large-scale commercial units.

Following adequate quantification of phage inactivation during the activated sludge process, it should be possible to establish a standard protocol, using a bacteriophage, to estimate the efficiency of activated sludge units for enterovirus inactivation. Although the reduction of total phage populations can be measured by comparing influent and effluent titers (20), a technique which employs membrane chambers would be preferable due to the accuracy of exposure time, the easily-sampled volume of chambers, and the possibility of using a single, well-characterized bacteriophage as an indicator. With such a protocol available, optimization of activated sludge unit operation (with respect to detention time, aeration rate, mixed liquor temperature, BOD and COD loading, etc.) for maximum reduction of enterovirus numbers would be possible. Clearly, such optimization is imperative if the results presented here can be generalized to commercial activated sludge processes. Present operation of activated sludge units would seem to be inadequate to inactivate a significant fraction of enteroviruses in sewage. Detention times of six to eight hours, which are normally employed (7), are much shorter than the T_{90} observed for enteroviruses in this study.

Another factor which must be considered in activated sludge optimization is mixed liquor temperature. Preliminary experiments in this laboratory indicated that a reduction of 10 C in aerated mixed liquor temperature increases the T_{90} for bacteriophages five-fold. The effect of temperature changes on enterovirus inactivation by activated sludge, as well as all other factors which can be varied to optimize the process, must be thoroughly investigated if elimination of the virus hazard in water is to be achieved.

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APPENDIX: RAW DATA

Table 11. Reactor phage inactivation.

Detention Time (h)[a]	Reactor	Phage concentration[c] in replicate experiments 1	2	3	4
36	Influent	7.9×10^{2}	5.2×10^{2}	1.9×10^{3}	N.D.[b]
	1	3.3×10^{3}	1.2×10^{4}	6.1×10^{3}	2.5×10^{3}
	2	9.6×10^{2}	1.1×10^{4}	6.2×10^{3}	2.6×10^{3}
	3	7.0×10^{2}	5.9×10^{3}	4.8×10^{3}	4.5×10^{3}
	4	2.2×10^{2}	4.0×10^{3}	5.5×10^{3}	2.8×10^{3}
72	Influent	4.1×10^{3}	9.0×10^{2}	N.D.	
	1	7.1×10^{2}	3.8×10^{2}	1.5×10^{3}	
	2	1.9×10^{2}	6.0×10^{1}	6.7×10^{2}	
	3	1.1×10^{2}	3.0×10^{1}	2.3×10^{2}	
	4	1.0×10^{1}	2.0×10^{1}	1.7×10^{2}	
144	Influent	2.4×10^{3}	7.1×10^{2}		
	1	1.8×10^{2}	1.5×10^{2}		
	2	3.0×10^{1}	1.0×10^{2}		
	3	2.0×10^{1}	0		
	4	1.0×10^{1}	0		

[a] Total-unit detention time.

[b] N.D.=no data

[c] Measurement in PFU/ml.