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ESTIMATION OF SAFE SETBACK DISTANCE BETWEEN WELL AND CONTAMINATION SOURCE USING BACTERIOPHAGE – A CASE STUDY

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Abstract – To determine the safe setback distances between groundwater abstraction wells and contamination sources, quantitative information is needed about the removal of micro-organisms during its passage through soil. Column experiments were conducted using soil samples collected from areas in and around an artificial canal in India, which is receiving sewage generated in an urban area through various outlets. Lambda phage isolated from the canal was used as a biological tracer and its movement and sorption mechanism was studied to determine the safe set back distance. Two-way analysis of variance was conducted to identify the significant factors affecting the phage survival in soil. Phage adsorption to soil was assessed by Freudlich isotherm. Our study suggests that the survival of lambda phage was highest during low temperature, high moisture content and at a pH range of 7-9. The soil types identified from the study area also had a significant impact on phage survival (p= >*0.05*). The highest and lowest sorption capacity was obtained in sandy clay and sandy soil respectively. There was a strong negative correlation (r = 0.914) with a significant ($p < 0.01$) value between adsorption coefficient and the calculated safe distance which means that high sorption capacity score go with low safe distance and vice versa.

INTRODUCTION

Water and sanitation infrastructure in Kerala is extensive and valuable. In the rural sector the percentage of households with toilets in the State is currently 94.9%. An estimated 4.5 million wells have been constructed in Kerala and about 80% of the population is reported to be relying on the groundwater from these wells for drinking purposes. In spite of this high percentage of sanitation coverage, about 85% of the drinking water wells are reported to be fecally contaminated (CWRDM, 2007). This can be attributed to pollution by domestic sewage and inefficient on site sanitation measures adopted in the State.

A study conducted by Megha *et al.,* (2015) reported a higher incidence of bacterial count present in the wells of Koditathur village in Kozikode district were the latrine distance was >10 m, and also the houses with higher incidence of water borne infections with a latrine to well distance between4 - 6 m. The main sources of dug well contamination were found to be on site sanitation systems (Soil Absorption Systems) like septic tanks and associated leach pits and also some unscientific pit disposal systems (Biju *et al.,* 2011). If the distance between the soil absorption system (SAS) and the well is not allowed to fall below a certain limiting value (which is characteristics of a given soil type) the contamination can be greatly reduced, thereby saving on treatment costs and diversifying water use.

To protect drinking-water wells against microbial contamination, government authorities have often used arbitrarily determined safe distances, from septic tank and other contamination systems. According to the earlier legislation which prevailed in Kerala State, India, the minimum distance between dug wells and septic system was 15m, which underlines the importance of a sufficient soil-path length in contaminant removal. Currently, these arbitrary setback distances in Kerala have been made to be 7.5 meters (KMBR 1999). Setback distances are site-specific and the guidelines

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published by many countries and agencies have suggested distances ranging from 15 m to as high as 60 m between well and septic systems to avoid microbial contamination of wells used as source of drinking water (USEPA, 2002; CMHC, 2008; MSDH, 2008). As the distance suggested by Kerala Municipality Building Rules (KMBR) 1999 is much less than these values, a critical study of the issue becomes relevant.

The ability of microorganisms to migrate through soil increases the probability of water contamination. Both field and laboratory observations have shown that microorganisms can migrate significant distances through soil in both vertical and horizontal directions (Viraraghavan, 1978; Stewat and Reneau, 1981; Keswick *et al.,* 1982; Chen, 1998). The chances of contamination will increase if the microorganisms have the ability to survive in these soils for longer periods of time. Viral survival in the subsurface generally involves simple function of time and temperature (Yates *et al.,* 1987). Although temperature was found to be most important, a number of other factors like moisture content, pH, soil properties, virus type etc can also affect the inactivation of viral pathogens in soil (Sobsey 1983, Gerba 1984).

Sorbtion of virus in transport model is generally assumed to be reversible and is often described mathematically by Langmuir or Freundlich isotherms. Absorption behaviour of the virus in the presence of soil varies greatly with virus and soil types (Burge *et al.,* 1978; Vilker *et al.,* 1980). On the other hand, Hurst *et al.,* 1980 has reported that virus survival was likely to be greater in the soil that was most effective in preventing groundwater contamination.The purpose of this study was (i) to compare and statistically evaluate the effects of environmental variables on bacteriophage survival, (ii) to calculate the sorption capacity of bacteriophage on different textural classes of soil and (iii) also to conduct a case study, to estimate the safe distance between well and contamination source using bacetriophage.

MATERIALS AND METHODS

Collection of Soil Samples

Eleven set of soil samples were collected from the surrounding areas of Canoli Canal, which is in the heart of Kozhikode City, Kozhikode District, Kerala, India. From our earlier studies (Megha *et al.,* 2015), it was evident that both the canal and the open wells in and around the canal were contaminated with pathogenic bacteria. The soil samples were also collected from the same sites and stored in air tight, solvent washed glass jars, sealed with tefflon foil liner fitted with new screw caps. After collection, the soil samples were spread in trays to dry in air. Drying was carefully carried out to avoid secondary reaction. The samples were dried as rapidly as possible. Visible plant debris and fauna were removed from the collected soil samples by hand. Large lumps of soil were crushed and the soils were gently sieved (< 2 mm fraction), sterilized and stored in sealed polythene bottles for further analyses.

Soil Quality Characterization

The soil samples were air dried crushed and passed through a 2-mm sieve and then mixed thoroughly to obtain a homogeneous mixture. Soil texture analysis was performed using the hydrometer method (Bouyoucous, 1962). Bulk density was determined by Clod method (Black 1965). The soil pH and electrical conductivity of the soil-water suspension was determined using Systronics Water Analyzer 371.

Bacteriophage Isolation and Characterization

Water samples from Canoli canal, were processed for isolation of bacteriophages by soft agar overlay technique. The bacterial pathogen *E. coli* isolated from the canal water was used as host for the recovery of phages. Ten mL of water samples in sterile polypropylene tubes were centrifuged at 10400 rpm at 4°C, and the supernatant was filtered through 0.22 mm filter (Millipore, Bedford, USA). One ml of this filtrate was used as phage sample and mixed with 0.1 mL of log phase cultures (OD600=0.3) of *E.coli* hosts grown in Tryptone soya broth (TSB, Hi-media, Mumbai, India) and incubated at 30°C for 30 min. After incubation, 5 mL of molten soft agar (TSB with 1.5% NaCl, 0.3% glycerol and 0.7% agar) held at 46°C in a water bath was added, mixed and overlaid on TSA supplemented with 1.5% NaCl and 0.3% glycerol. The plates were incubated at 37°C for 24 h and observed for the presence of plaques.

Purification of the phages was done by plate lysis and elution method. Five mL of sterile SM buffer was added to the surface of each plate and the phages were aseptically eluted using sterile glass beads (Carlson, 2005). The phage and cell

suspensions were harvested and centrifuged (5 min, 10,400 ×g, 4°C) and the supernatant was then filtered through a 0.22 µm pore size membrane filter to remove any remaining bacterial cells. Phage stocks were stored in SM buffer at 4°C until further use. Bacteriophage morphology studies were performed using Transmission Electron Microscope (TEM) as described by Oliveira *et al.,* (2009). Further, the quality of phage DNA was evaluated on 0.8% Agarose gel to obtain a single band of high molecular weight.

Phage survival studies in soil

Experiments were conducted to determine the survival of phages in the collected soil samples. The effect of pH, temperature, soil moisture content and the time of exposure on the survival rate of lambda phage were checked in laboratory conditions. The soils were air dried at room temperature and homogenized to remove large aggregates. A 10 mL phage suspension having (150 PFU/mL) was subjected to various pH conditions ranging from 4-9, temperature conditions ranging from 20^0C- 45^0C and moisture content ranging from 5%-30% individually. After 12 hours, five gram of soil sample from each set was assayed using the double agar layer method. The phages were also subjected to direct contact with the soil samples for a period of three months, and the soil samples were collected every ten days to detect the PFU count as per the protocol mentioned above.

Data analysis

All experiments were performed at least in duplicate. In order to determine the significant parameters that affect phage survival, a two-way ANOVA which aims to compare the effects of several levels of two factors in a factorial experiment with a two-way layout was conducted. The parameters were evaluated using a 5% (p=0.05) significance level. The survival rate of phage was reported as percentage, which was calculated by equation:

Survival percentage = $(Nt/N_0) \times 100$

where, N_0= is the initial plaque count at day 0, and Nt = is the final plaque count every day of the experiment (Garcia Estrada *et al.,* 2014).

Batch sorption study

Batch sorption experiments were performed as per the method formulated by Shwan *et al.,* 1997, in 15-ml glass screw cap tubes. Glass tubes were washed with detergents, soaked in 6 N HCl, rinsed thoroughly in deionised water, and oven dried at 105^0C overnight. A minimum of five different soil types were used to establish the isotherm curve. Physiological conditions were used in an effort to promote virus stability and thereby eliminating potential confounding factors which could contribute to inactivation. Experimental tubes received 10 mL of virus stock solution and 10 g of soil; control tubes received only virus solution (10 mL). A ratio of 1:1 ratio of soil to virus solution was used in each batch experiment. Soil virus suspension was mixed and kept 37^0C for 3 h, further the suspension was centrifuged for 10 min at approximately 12000 rpm. Control tubes were treated the same manner as the experimental tubes. All the experiments were conducted in triplicate. Virus sorption was determined using equation (1)

$$C_S = [(C_I - C_L)/M] \quad \text{.....} \quad (1)$$

Where, C_I, C_L, and C_S are, respectively, the concentrations of phage in the control liquid phase (PFU per milliliter), in the experimental liquid phase (PFU per milliliter), and adsorbed to the soil (PFU per gram) and M is the total mass of soil per unit volume of virus suspension (grams per milliliter) used in each batch experiment.

Experiments using packed soil column

The kinetic studies of the adsorption of bacteriophages on the soil media was carried out using a column study. A set of five acrylic columns (diameter - 5 cm, length- 40 cm) was filled to a depth of 30 cm with oven dried soil collected from areas in and around the canal and flushed with sterile distilled water for 3- 4 days prior to the introduction of the phage. Steady state flow conditions were established and the head difference between the standing water in soil column and the reservoir were adjusted until the desired flow rate was achieved. Before adding phage to the column, a background sample was collected in order to determine the presence of any phage already in the column. Twenty ml of purified phage suspension was allowed to pass through the packed columns at a flow rate of 0.59 mL/min. The outflow samples were collected by holding a sterile glass beaker under the outflow port and the recovery time was also noted. The collected aliquot was further assayed for the quantification of phages.

Using the travel time of phages in the soil columns and considering various soil properties,

safe distance between contamination source and drinking water wells in different soil types identified around the canal area were calculated using equation (Freeze and Cherry, 1979) (2):

$$D = (tKi)/\theta \quad \ldots (2)$$

where, D is the safe distance (m), t is the travel time of the active phages in the column; K is the hydraulic conductivity of the soil (m/day); i is the hydraulic gradient (m/m); and θ is the effective porosity of the soil. Porosity (θ) was obtained from bulk density (P_b) using the relation described by Hillel (1980) (3):

$$\theta = 1\text{-}P_b/P_s \quad \ldots (3)$$

where, the particle density (P_s) is taken as 2.75 g/cm^3(Muthuvel and Udayasoorian, 1999). This porosity value was used for characterizing the packing in the soil columns and for the velocity calculations. The movement of water through a saturated soil is governed in part by the saturated hydraulic conductivity (K) of the soil. The saturated hydraulic conductivity is a function of the pores and fractures in the soil as well as the kinematic viscosity and density of the water flowing through it. The hydraulic conductivity values for each sample were calculated using constant head method. The hydraulic gradient for this study was taken to be 0.01 (Molin *et al.*, 2010).

RESULTS AND DISCUSSION

Soil characteristics

The quality of 11 soil series was analyzed and the results are tabulated in Table 1. As per the results five textural classifications were observed based on the sand, silt and clay percentage. Majority of the samples were found to be loamy sand type, three samples came under the sandy clay loam category and the next three samples were categorized as sandy clay, sandy and sandy loam respectively. Pathogen movement in the soil may be facilitated by the physical conditions and the composition of the soil. The higher percentages of sand in the soils enhance permeability, hence promoting the movement of pathogens deep into soil (Sinton, 1986; Abu Ashour *et al.*, 1994). Spatial distribution of soil texture classes in various areas near Canoli canal is shown in Fig. 1. The pH of the soil samples ranged from 4.5-8.3. The soil sample collected from CC1 showed acidic nature and the samples from CC5 and CC6 showed slightly alkaline nature. Experiments indicated that there is increased sorption rate of pathogens at slightly acidic or neutral pH and adsorption was found to be reduced substantially at pH values above 8 (Toze, 1997).

Phage isolation and characterization studies

The results of soft agar overlay method showed the presence of plaques which were clear and circular in nature with an average plaque diameter in the range of 5-7 mm. The presence of the zones of clearing indicated that, amplifying bacteriophage from a raw sewage sample and inoculating the phage into *E.coli* host was an effective way of isolating (and visualizing) phage. The TEM results revealed the morphology of the phage to be assigned to the family Siphoviridae and order Caudovirales because of the presence of a non enveloped head and long contractile tail. Heads were measured between opposite apices with an approximate of 49.7 ± 5.37 nm in size and the length of tails was 143 ± 3.25 nm in size. The DNA of the phage was

Table 1. Major characteristics of soil samples collected from the study area

Soil code	Sand (%)	Clay (%)	Silt (%)	pH	Conductivity (micro siemens/cm)	Bulk density
CC1	68.04	28.02	3.94	4.5	111	1.09
CC2	64.82	4.78	30.4	6.2	872	1.15
CC3	73.5	12.5	14	6.5	82	1.11
CC4	78.5	8.5	13	7.7	83	1.12
CC5	68.88	4.44	26.68	8.1	192	1.15
CC6	82.5	1.25	16.25	8.3	150	1.11
CC7	87.5	7.75	4.75	7.5	117	1.12
CC8	84.75	4.75	10.5	6.9	57	1.11
CC9	64.1	5.8	30.1	6.7	101	1.16
CC10	83.75	1.25	15	6.8	40	1.9
CC11	93.75	2.75	3.5	7.9	89	1.17

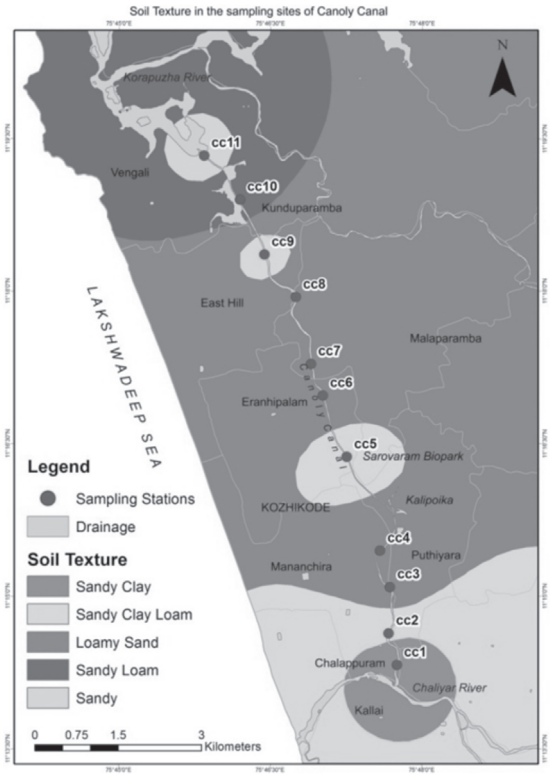

Fig. 1. Spatial distribution of soil texture in the study area

isolated and electrophoresed on 0.8% agarose gel with a λ Hind/III marker (Banglore Genei, Banglore, India). Molecular weight of the phage DNA was found to be 31 kb. The characterization results revealed that the isolated phages were sensitive to Hind*f* I and BsuRI, and exhibited different banding patterns confirming that the phage harboured double stranded DNA as genetic material and was confirmed to be a lambda phage.

Phage survival studies in soil samples

The effect of soil pH, temperature, moisture content and soil type on phage survival was studied and the results showed that the isolated bacteriophage was highly dependent on these factors. The survival results for various experimental conditions are shown in Figure 2 on a time scale of 24 hours. Lambda phage showed maximum stability between pH 7.0 and 9.0. The rapid transition towards instability over a lesser pH range is characteristic of the acid denaturation of protein. Similar results have been obtained with various animal, plant and bacterial viruses (Best and Samuel, 1936, Finkelstein *et al.*, 1940; Miller 1944; Putnam *et al.*, 1949; Riverin *et al.*, 1970).

Influence of temperature upon the biological

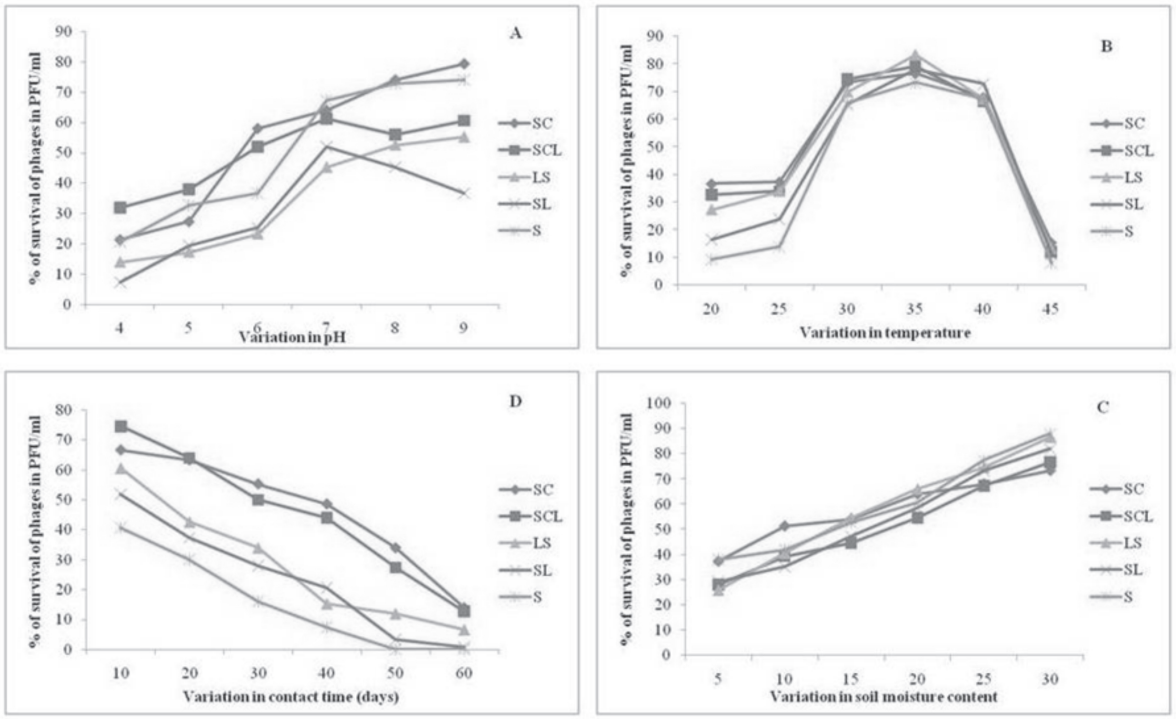

Fig. 2. Effect of pH (A), temperature (B), soil moisture (C) and time on the survival of phage in different soil types. SC-sandy clay, SCL-sandy clay loam, LS- loamy sand, SL-sandy loam, S-sandy

system is very vivid and it has been observed that evolution of phenotypic traits, species distributions, and extinction in many cases can be traced to changes in temperature regimes (Vale *et al.,* 2008). Present study results are in confirmation with the above findings as during the experimental studies it was observed that persistence of phage was highly temperature dependent. Optimum temperature for virus survival in all soil types were found to be at a range of 30 - 40^{0}C. These findings are in line with Groman (1962), who observed a decline in phage at an incubation temperature below 37^{0}C. The temperature of 45^{0}C showed a negative impact on the survival of phages. Study results regarding the inactivation are in confirmation with those observed by Basdew and Laing (2014) who reported that increase in temperature decreases virus survival and lytic activity. In the same way, findings by Pope *et al.,* (2004) indicated an increase in phages at 30^{0}C and 39^{0}C corroborates the present study results revealing that 35^{0}C was ideal temperature for bacteriophage activity in soil.

Soil moisture content was also found to have an impact on the phage survival; higher moisture content was found to be favourable for the persistence of phages in different soil types. Greatest survival of phage occurred at 25 and 30% soil moisture; survival was intermediate at 15 and 20% moisture and least at 5% soil moisture, indicating an optimum survival zone in 25-30% of moisture range. Phage survival decreased significantly over time and reached a uniform low on days 50 and 60. Phage survival was greatest in sandy clay and sandy clay loam; it is because the soil nature has a major role in determining phage survival and retention. Sandy clay exhibited the highest moisture retention and supported the greatest phage survival. Survival time was found to be lowest in sandy and sandy loam because of its lower water holding capacity. Similar results were reported by Yates *et al.,* 1987; they reported that viruses were found to show best survival rate in moist soil under low temperature and the soil type also influenced survival with relation to the degree of adsorption.

Statistical studies using ANOVA revealed a greater 'F' calculated value than the 'F' critical value, indicating a significant impact of the experimental factors on phage survival. F table value for the degree of freedom (5, 20) is 2.71. All the ANOVA values obtained for the factors were found to be more than the critical F value indicating a rejection of null hypothesis, which also confirms that the factors selected for the survival studies had a significant impact on the persistence of phages (Table 2). Also for every combination of soil types with pH, temperature, moisture content and contact time significant impact was observed in terms of phage survival and all the F calculated value was found to be greater than the critical F value. All the values fall in the rejection region with a degree of freedom (4, 20) which is 2.87. As the calculated F values were greater than the critical F value, the *p* value was found to be *>0.05.*

Table 2. Two-way ANOVA analysis results for phage survival during different environmental conditions

Factors	Calculated F value	Critical F value	*p* value
pH	29.72	2.71	*>0.05*
Temperature	113.2	2.71	*>0.05*
Moisture content	86.25	2.71	*>0.05*
Contact time	70.24	2.71	*>0.05*

Batch sorption studies

There was no substantial loss of lambda phage in the control blanks, permitting the quantification of sorbed-phase phage. Adsorption of phage to different types of soil was quantified by the Frundlich isotherm $Cs = K_F C_L^{1/n}$, where C_S is the quantity of virus sorbed to the soil (PFU per gram), C_L is the concentration of virus remaining in the liquid phase (PFU per milliliter), and K_F (Freundilich constant) and $1/n$ are constants. The parameters K_F and $1/n$ were estimated by linear regression of the $\log_{10}$ transformed data (i.e., $\log_{10}$ Cs versus $\log_{10} C_L$). Figure 3 presents the isotherm and corresponding Freudlich constants for phage adsorption to different soil types from batch experiments.

The adsorption coefficient values obtained from the isotherm plots, it was evident that a higher sorption was found in sandy clay soil type with a K_F value of 82.794 than other soil types. Increasingly large K_F value indicates greater adsorption capacity. As $1/n$ is a function of the strength of the used absorbent material, a higher $1/n$ value showed a weaker adsorption bond. If $1/n > 1$, the K_F value increases with increase in concentration leading to an increase in hydrophobic surface characteristics of the monolayer. When the value of $1/n < 1$, there is a decrease in the K_F value with decrease in concentration (Hamidi *et al.,* 1992). Here all the $1/n$ values obtained were less than 1 indicating a lesser adsorption of the lambda phage to soils with

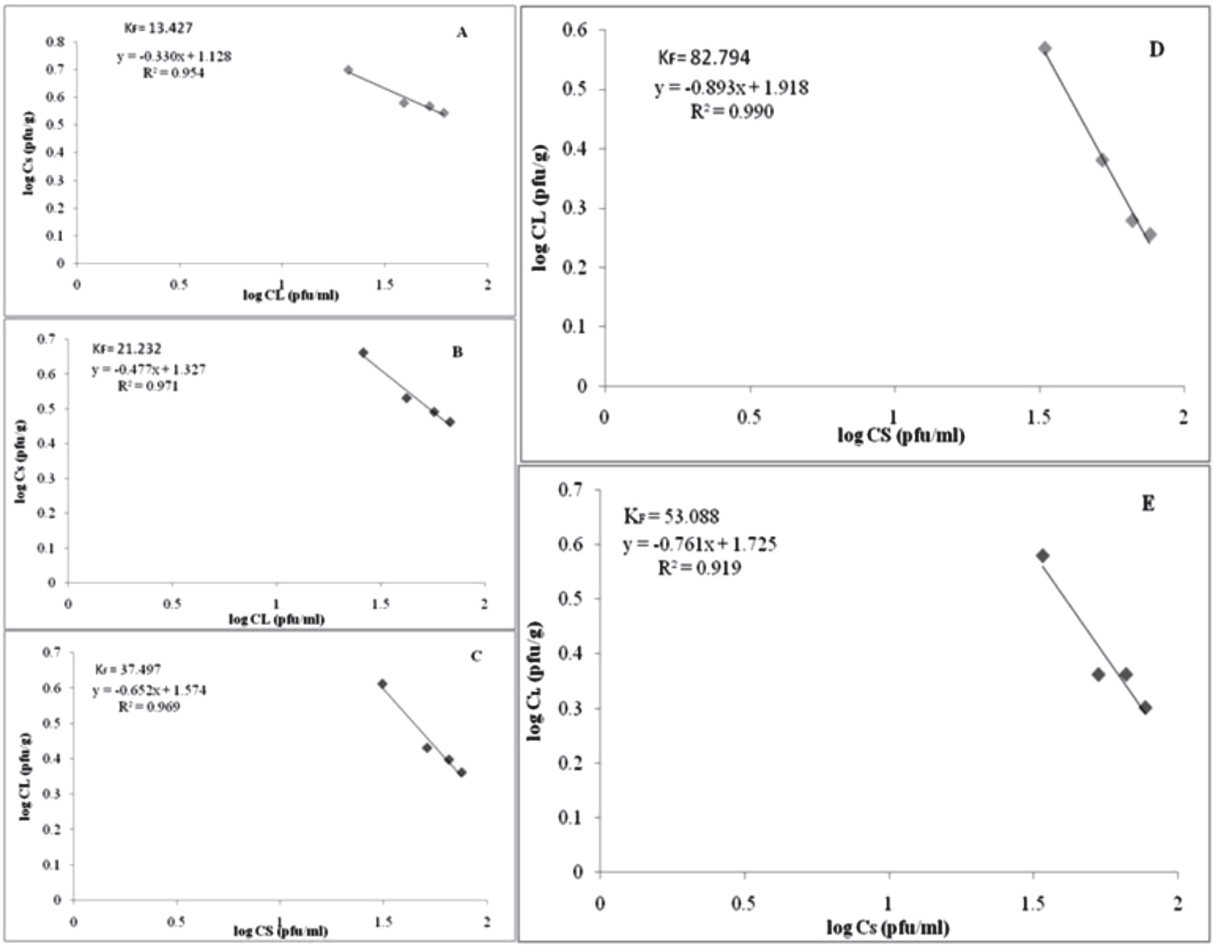

Fig. 3. Freundlich isotherm plots for sandy soil (A), sandy loam (B), loamy sand (C), sandy clay (D) and sand clay loam (E). All values of K_F are in millilitres per gram

concentration. Thus it is evident from the study that adsorption has an important role in removal of bacteria and virus in soil.

Experiments using packed soil column

Based on the phage migration rate in different soil types, the safe distance between contamination source and well were calculated as shown in Table 3. The results showed a safe distance range of 6.5-22 meters in the five different soil profiles identified from the study area. The sorption of phage was found to be very less in sandy soil (K_F=13.427) owing to the easy transportation of phages through this soil type (Table 4). Sandy clay soil type exhibited poor movement of phages owing to the high K_F value (82.794). Viral adsorption to soils can be explained in terms of surface interaction between amino acids on the capsid and biological and non-

Table 3. Safe horizontal distances for different soil types

Soil type	Distance moved per day (m)	Effective porosity	Soil permeability in saturated condition (m/day)	Horizontal safe distance (m)
Sandy	3.32	0.43	5.17	22.0
Sandy loam	2.46	0.45	1.98	16.0
Loamy sand	1.96	0.56	1.79	12.0
Sandy clay	1.0	0.59	0.61	6.5
Sandy clay loam	1.10	0.55	1.44	8.0

Table 4. Adsorption Isotherm Parameters with different soil types

Soil type	R^2	K_F	$1/n$	Isotherm Freundlich's
Sandy	0.954	13.427	0.330	$x/m=0.05807C_L^{0.330}$
Sandy loam	0.971	21.232	0.477	$x/m=0.88471\ C_L^{0.477}$
Loamy sand	0.969	37.497	0.652	$x/m=0.73194\ C_L^{0.652}$
Sandy clay	0.990	82.794	0.893	$x/m=0.65316\ C_L^{0.893}$
Sandy clay loam	0.919	53.088	0.761	$x/m=0.65170\ C_L^{0.761}$

biological surfaces; this could also include both electrostatic and hydrophobic interactions (Bitton 1975 and Tanford, 1978).

The efficiency of a soil absorption system likes leach/soakage pits depend on the ability of the soil to remove contaminants, which in turn depend on the soil type. We did correlation study to examine the impact of those systems on the water quality. There was a strong negative correlation (r = 0.914) with a significant ($p < 0.01$) value between adsorption coefficient and the calculated safe distance which means that high sorption capacity score go well with low safe distance and vice versa. The sorption results were correlated with the calculated safe distance in sandy clay soil type, indicating a higher adsorption of phage to the soil whereby a lesser safe distance. The scatter plot for correlation study also gave a R^2 value of 0.836 (Fig. 4).

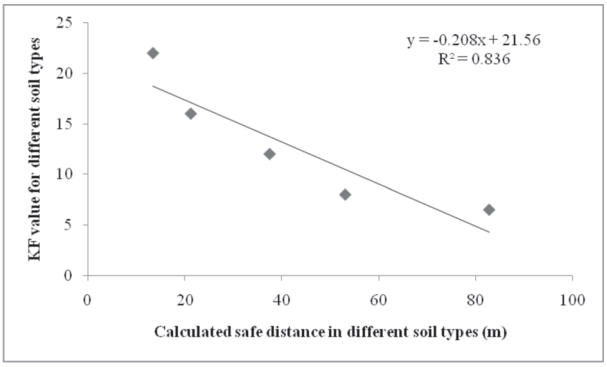

Fig. 4. Correlation scatter plot for KF value versus safe distance in different soil type

The safe distances were calculated in and around the canal. Majority of the study area came under the loamy sand soil profile. The results indicated that in loamy sand profile (CC3, CC4, CC6, CC7, CC 8) a minimum distance of 12 meters, in sandy clay loam (CC2, CC5 and CC9) 8 meters, in clay loam (CC1) 6.5 meters, in sandy loam (CC10) 16 meters and in sandy soil (CC11) 22 meters is the safe distance to be maintained in order to avoid bacteriological contamination from the canal to the drinking water wells.

Non-hazardous microbial tracers (bacteriophage) that mimic the movement of pathogenic microorganisms through soil were successfully used in this study. The results obtained from evaluating effects of environmental variables on phage survival indicated that all the factors selected for study had a significant impact on phage survival in soil. Phage adsorption to soil and the calculated setback distances correlated well; as the adsorption increases the safe distance got decreased. There is a strong negative correlation (r = 0.914) with a significant ($p < 0.01$) value between adsorption coefficient and the calculated safe distance. So study conducted on these soil types can be applied to regions with similar soil profile. Thus the distances calculated as per this study can be suggested as the minimum safe path length to avoid microbial contamination for the selected soil zones.

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