Contribution of microbial activity to virus reduction in saturated soil

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Abstract

Application of wastewater to soil may result in the contamination of groundwater and soil with pathogenic microorganisms and other biological and chemical agents. This study was performed to determine the antiviral microbial activity of soil saturated with secondary effluent. Low concentrations (0.05 mg/ml) of protease pronase resulted in the inactivation of more than 90% of seeded Cox-A9 virus, whereas Poliovirus type 1, Hepatitis A virus (HAV) and MS2 bacteriophages were found to be insensitive to the enzyme activity. Exposure of Cox A9 virus to \textit{P. aeruginosa} extracellular enzymes resulted in 99% inactivation of the seeded virus. Hepatitis A virus was found to be as sensitive as the Cox A9 virus, whereas Poliovirus 1 and MS2 were found to be insensitive to \textit{P. aeruginosa} extracellular enzymatic activity. Furthermore, the time required for 99% reduction (T99) of Cox A9 and MS-2 Bacteriophage, at 15°C, in soil saturated with secondary effluent was found to be 7 and 21 days, respectively. Faster inactivation was observed for MS2 and Cox A9 in soil saturated with secondary effluent incubated at 30°C, T99 of 2 and 0.3 days, respectively. Although the concentration of the total bacterial count in the soil samples increased from $10^3$ cfu/g to $10^5$ cfu/g after 20 days of incubation at 30°C, the proteolytic activity was below the detection level. The results of this study indicate that the virucidal effect of microbial activity is virus type dependent. Furthermore microbial activity in the soil material can be enhanced by the application of secondary effluent at higher temperature. The results also showed that MS2 bacteriophage can be used to predict viral contamination of soil and groundwater.

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1. Introduction

Viruses in groundwater can originate from numerous sources. The most frequently reported sources include sewage from septic tanks, land application of sewage and sewage sludges, domestic landfills, oxidation ponds and deep well sewage injection [1].

The survival and the potential of viruses to migrate through the soil matrix control the viral contamination of groundwater. Virus persistence in the subsurface is influenced mainly by temperature [2], virus type and microbial activity [3–6]. Clay minerals in soil and aquatic environment may protect viruses from biological biodegradation of the viral genome by nucleases and viral capsid by protease’s [1].

Very few studies have addressed the effect of soil microbial activity on virus survival [7–9]. For example, the effects of antiviral activity in soil have been attributed to aerobic microorganisms, which were found to result in two to threefold increases in virus
inactivation [8]. Furthermore, Lipson and Stotzky [9] demonstrated that bacteria could utilize viruses as growth substrates.

The present study was performed to determine the effect of microbial activity on virus reduction in soil material. The correlation between temperature, soil saturation, water quality with microbial activity was also evaluated.

2. Materials and methods

2.1. Soil

Samples of loess soil used in this study were obtained from Gai Levy, Volcani Center, Israel. The loess soil was composed of 65% sand, 10% silt, and 25% clay. Organic material in the loess soil was found to be 2.3%. Un-sterile loess soil was used throughout the study, which contained 10^5 cfu/g heterotrophic bacteria (Total Plate Count, TPC) (APHA, 1998).

2.2. Water samples

Secondary effluent (activated sludge) samples used for the die-off experiments were obtained from the Tel-Aviv central wastewater treatment plant. The composition of the secondary effluent was as follows: pH—7.7, dissolved O_2—5.1 mg/l, conductivity—1788 μs/cm, turbidity—5 nephelometric turbidity units (NTU) and the TPC was 5 x 10^8 cfu/ml (APHA, 1998).

2.3. Virus enumeration

Buffalo Green Monkey Kidney (BGMK) cells were used for cultivation and enumeration of Coxsackie A9 virus and Poliovirus 1 according to procedures described previously by Guttman-Bass and Nasser [10]. Poliovirus 1, Lsc vaccine strain (ATCC VR-192) was received from the American Type Culture Collection. Coxsackievirus type A (Bozek) (ATCC 7797), was kindly supplied by R. Hindsher, Central Virology Laboratory, Ministry of Health, Israel. Enteroviruses were enumerated by dilution in 0.01M phosphate buffered saline, pH=7.2 (PBS) and duplicate plating in BGMK cultures.

A cytopathic strain of Hepatitis A virus (HAV), strain HM-175, was cultivated in Fetal Rhesus Kidney cells (FRhK-4) as described by Nasser and Metcalf [11]. HAV was enumerated by the plaque-assay technique as previously described by Cromeans et al. [12].

2.4. Bacteriophage enumeration

MS2 bacteriophage was cultivated and enumerated on Salmonella typhimurium WG-49 Havelaar and Hoge-

boom [13]. Bacteriophages were enumerated by the double-layer technique [14].

2.5. Bacteria and enzymatic activity

P. aeruginosa (PAO1) was used in this study as a model for the production of extracellular bacterial enzymes. P. aeruginosa was obtained from N. Garber, Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel. This strain originates from strain 15692 of the American Type Culture Center (ATCC).

P. aeruginosa were seeded into Brain Heart Infusion (BHI) medium (Difco USA) and incubated for 24 h at 37°C, for extracellular enzymes production, the startup culture was incubated for 72 h in a rotary shaker water bath (37°C) at 150 rpm.

P. aeruginosa extracellular enzymatic activity was determined on the pure supernatant. The supernatant was obtained by centrifugation for 90 min. at 10,000 rpm. Proteolytic activity was measured by color development following casein digestion and calculated as units per hour Laskowsky [15]. Enzymatic activity over 200 units/hr (equivalent to 0.05 mg/ml Proteinase–pronase) was considered as suitable for use in the study.

Proteinase–pronase activity served as a reference for the bacterial extracellular enzymatic activity. Protease type XIV-bacterial from Streptomyces griseus was purchased from Sigma (USA).

2.6. Experimental design—virus survival in saturated soil

Virus inactivation in loess soil saturated with secondary effluent and with distilled water was determined at 15°C and 30°C. Soil saturation was achieved by the addition of 5 ml of either distilled water or secondary effluent to 1 g of loess soil. MS2 bacteriophage and Coxsackie A9 virus were seeded at a concentration of 10^5 pfu/ml. Samples were removed at 0, 0.3, 1, 2, 5, 10, 15 and 21 days and stored at −20°C for later analysis of viruses, while bacterial total plate count (TPC) was determined on the same sampling date.

3. Results and discussion

3.1. Effect of pure proteinase–pronase on virus inactivation

To determine the antiviral effect of proteolytic enzymes, MS2 bacteriophage, CoxA9, Polioviruses 1 and HAV were incubated for 30 min in various concentrations (0–1.0 mg/ml; 1 mg = 4.2 units) of protease pronase at 37°C, in a shaking water bath (Fig. 1). The survival rate was calculated as log C_t/C_0, where C_0 is the virus concentration with no enzyme activity and C_t is the virus concentration after treatment at the indicated enzyme
The results indicate that the effect of the protease on virus survival is virus type dependent. MS2 bacteriophage was not affected by the protease activity, whereas more than 90% of the seeded Cox-A9 virus was inactivated at a low enzyme concentration (0.05 mg/ml). No increase in the inactivation of Cox A9 was observed when the concentration of protease was increased from 0.05 to 1.0 mg/ml, this can be due to the fact that only a fraction of Cox A9 population is sensitive to the enzyme activity. The effect of the pure enzyme on the survival of Hepatitis A and Polio 1 viruses was negligible (Fig. 1). Cox A9 virus was also the only virus type affected by elastase (a microbial enzyme), a concentration of 1.2 mg/ml resulted in 99% inactivation of Cox A9. Polio 1 and MS2 were found to be insensitive to elastase (data not presented). Since all the tested viruses are SSRNA viruses with a protein capsid, it seems that the protease and elastase cleave a specific site in the capsid of Cox A9, which is not found in the other tested viruses. Ward et al. [16] reported similar results for echovirus particles in fresh water, where cleavage of virus proteins by proteolytic bacterial enzymes exposed virus RNA to nuclease digestion.

3.2. Effect of extracellular activity of P. aeruginosa on virus inactivation

*P. aeruginosa*, a gram-negative bacterium is capable of producing many different extracellular enzymes, such as phospholipase [17], alkaline protease [18] and chitinase/lysozymes [19]. Callan et al. [20] demonstrated that *P. aeruginosa* secreted a protease and elastase that cleaved influenza virus hemagglutinin. *P. aeruginosa*, POA1, was included in this study to simulate the antiviral effect of microbial activity in saturated soil. Exposure of the Cox A9 virus to *P. aeruginosa* extracellular enzymatic activity resulted in 99% inactivation of the seeded virus (Fig 2). Hepatitis A virus was found to be as sensitive as the Cox A9 virus to extracellular enzymes, whereas Poliovirus 1 and MS2 bacteriophage were found to be insensitive to this extracellular activity. The survival rate in this experiment was calculated as $\log C/C_p$, where $C$ is the virus concentration in the *P. aeruginosa* extracellular suspension at the indicated incubation times and $C_p$ is the virus concentration in 0.1 M phosphate buffered saline (PBS), pH = 7.2. The enzymatic activity of the extracellular suspension was always between 200 and 240 Units/h (equivalent to 0.05 mg/ml of pure protease pronase). These results indicate a wide range of extracellular enzyme activity released from the bacterial cells, which causes inactivation of a wide range of viruses, including HAV. Although, the viruses studied
have the same size and basic composition, they possess different isoelectric points indicating that their surface composition varies accordingly [21]. The different surface composition of viruses may affect their sensitivity to extracellular bacterial activity.

Deng and Cliver [22,23] have demonstrated that in addition to the microbial enzymatic activity, a fraction of bacterial suspension prepared by ultrafiltration caused the inactivation of HAV, suggesting that their mode of action is not enzymatic. Furthermore, a unique feature of \textit{P. aeruginosa} is the production of high level of rhamnolipid biosurfactant, especially during the stationary phase of growth [24]. The rhamnolipid may also have a role in virus inactivation. The results of HAV inactivation by the extracellular activity of \textit{P. aeruginosa} are in agreement with the previously reported data on the antiviral activity of bacteria isolated from manure [22,23].

3.3. Virus adsorption to soil saturated with secondary effluent or with distilled water

MS2 bacteriophage was included for further study of virus persistence in saturated soil to simulate a worst case scenario in which a virus is insensitive to a wide range of proteolytic microbial activity. However, Cox A9 represents viruses which are sensitive to proteolytic activity of pure enzymes as well as to extracellular activity of \textit{P. aeruginosa}. MS2 bacteriophage and Cox A9 virus served as indexes for the evaluation of virus adsorption and inactivation in loess soil saturated with secondary effluent. Less than 10% of the seeded MS2 bacteriophages adsorbed to loess soil saturated either with distilled water or with secondary effluent (data not shown), whereas more than 80% of the seeded Cox A9 virus adsorbed onto loess soil (data not shown). The adsorption results indicate that virus type is the most important factor in virus adsorption to soil.

3.4. Virus inactivation in loess soils saturated with secondary effluent

The poor adsorption of MS2 bacteriophage to loess soil combined with its insensitivity to extracellular \textit{P. aeruginosa} activity strengthens the worst case scenario in which MS2 represents virus survival in saturated soil. The time required for 99% inactivation (T99) of Cox A9 virus and MS2 bacteriophage in secondary effluent and in distilled water and in loess soil saturated with either distilled water or with secondary effluents are presented in Table 1. In loess soil saturated with secondary effluent, at 15°C, the T99 of Cox A9 virus was reached within 7 days, whereas 21 days were required for 99% inactivation of MS2 bacteriophage. In loess soil saturated with secondary effluent, at 30°C, the inactivation of both viruses was faster than that observed at 15°C, and the T99 was reached within 0.3 and 2.0 days for Cox A9 virus and MS2 bacteriophage, respectively (Fig. 3 and Table 1). Virus inactivation in soil saturated with distilled water, at 15°C were found to be slower than that observed in soil saturated with secondary effluent. In loess soil saturated with distilled water the T99 of both viruses was not reached after 21 days incubation at 15°C, indicating that, at least for Cox A9 virus, secondary effluent saturated soil enhances virus inactivation.

In loess soil saturated with distilled water at 30°C, the T99 of Cox A9 was reached within 1 days, whereas that of MS2 was reached after 6 days of incubation (Table 1). It is important to indicate that microbial proteolytic activity was below the detection level in the saturated soil at the various incubation times. Furthermore, no appreciable differences were observed in the numbers of the heterotrophic bacterial counts (TPC) in soil samples saturated with either distilled water or with secondary effluent. The concentration of heterotrophic bacteria in soil suspended in distilled water and secondary effluent

<table>
<thead>
<tr>
<th>Time (days) required for the reduction of 99% (T99) of the seeded viruses</th>
<th>Experiment conditions</th>
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<tbody>
<tr>
<td>Coxsackievirus A9</td>
<td>Bacteriophage MS2</td>
</tr>
<tr>
<td>6.0</td>
<td>21.0</td>
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<tr>
<td>1.0</td>
<td>10.0</td>
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was \(1 \times 10^3\) cfu/ml and \(2 \times 10^3\) cfu/ml, respectively. The greatest heterotrophic bacterial count (\(10^5\) cfu/ml) was reached after 5 days incubation at 30°C in loess soil saturated with secondary effluent and distilled water. The enhanced inactivation of both MS2 and Cox A9 when incubated in soil saturated with secondary effluent, at 30°C, can be due to a synergistic effect, which is caused by the interaction of components of microbial activity effluent and loess soil. Although the proteolytic activity was below the detection level other microbiobically excreted enzymes could enhance virus inactivation; furthermore, none-enzymatic microbial activity has been shown to enhance virus inactivation [22,23]. This antiviral activity seems to affect a broader range of viruses, since MS2 was insensitive to either protease pronase or to \(P.\ aeuruginosa\) extracellular activity. The enhanced inactivation of Cox A9 and MS2 can be only partially explained by irreversible attachment, because MS2 has been shown as a poor adsorbent to loess soils (10%) regardless of water quality (data not presented). For MS2 and Cox A9 the presence of loess soil in distilled water resulted in reduced inactivation. Grant et al. [25] observed similar data, when compared virus inactivation in suspension with and without soil. The rapid inactivation observed for Cox A9 in distilled water (99% inactivation within 8 h) is in agreement with data presented by Mattess et al. [26] for the inactivation of Cox A9 in deionized water as compared with untreated groundwater.

The results of the effect of temperature on the reduction of Cox A9 virus are presented in Fig. 4. The die-off of Cox A9 virus was greater at 30°C than at 15°C. Furthermore, the die-off of the Cox A9 was greater in soil saturated with secondary effluent than in secondary effluent alone. The T99 for Cox A9 was reached within 8 hr in soil saturated with secondary effluent at 30°C, whereas 7 days were needed to reach the same T99 at 15°C (Table 1). This enhanced inactivation of Cox A9 could be the result of greater microbial activity in saturated soil. The results of this study are in agreement with previously reported data concerning the effect of temperature on virus inactivation [3–6].

The effect of bacterial enzymatic activity on viruses was found to be virus type dependant. For example, HAV did not exhibit appreciable sensitivity to pure proteinase–pronase, while, it was found to be sensitive to \(P.\ aeuruginosa\) extracellular bacterial activity. On the contrary, Poliovirus 1, MS2 bacteriophage as well as PRD-1 bacteriophage were found to be insensitive to the same microbial activity. Therefore, enteroviruses could be classified according to their sensitivity to microbial activity. The results of this study have shown the Cox
A9 and HAV to represent the sensitive group and MS2, PRD bacteriophages and Poliovirus 1 the insensitive group of viruses to microbial activity. The effect of microbial activity on virus inactivation depends on other environmental factors such as temperature, soil composition and water quality. Furthermore, since adsorption of viruses to soil is virus type dependent and also the effect of microbial activity is virus type dependent, it is important to emphasize the importance of virus type as a major factor in the retardation efficiency of virus when wastewater effluents are applied to soil. Previous studies have shown that the association of viruses to soil may reduce inactivation by protecting the viruses from proteolytic enzymes or other virus inactivating substances [27]. This study is in agreement with previous reports on the enhancement of virus inactivation by microbial activity Hurst [8], Sobsey et al. [28]. Both of these studies reported faster inactivation of viruses under nonsterile conditions than under sterile conditions.

4. Conclusions

1. The effect of proteolytic activity on viruses is virus type dependent. Structural differences apparently govern the sensitivity of enteric viruses to proteolytic enzymes.
2. Extracellular enzymatic activity of *P. aeruginosa* affected wider range of viruses than the protease and it was also found to be virus type dependent.
3. Soil microbial activity may be the result of the enhanced virus inactivation in soil saturated with secondary effluent incubated at higher temperatures.
4. The results of this study indicate that MS2 bacteriophage might be used as an indicator for viral contamination of soil and groundwater.

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