Egypt. J. Chem. **Vol. 66**, No. 9 pp. 363 - 374 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Alkaline pH Caused Complete Viral Inactivation And Reduced Chemical Pollutants In Wastewater

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Abstract

Although different wastewater treatment methods were developed but none of them could guarantee viral removal and inactivation. That made reuse of treated wastewater a cause of viral transmission. Here we evaluated the effect of increasing pH values of wastewater on adenovirus, Herpes simplex virus type 1, rotavirus, coxsackievirus B and Influenza A virus infectivity. On inoculating each virus at a time in distilled water with pH range 8-13, results showed that viral inactivation was directly proportional to increasing both pH and incubation time and that complete inhibition of all tested viruses was achieved upon co-incubation for 1 hour with water at pH12. Same conditions also caused complete inactivation of the same viruses inoculated in sterile-autoclaved wastewater. Viral inactivation due to increasing pH value to 12 was also achieved in field samples. Physico-chemical properties of wastewater samples were characterized before and after increasing pH to 12. Results showed that increasing pH caused decrease in heavy metals as well as sulphate, phosphate, bicarbonate concentrations which also suggest pH increase as mean to reduce chemical pollutants of wastewater.

Keywords: Alkaline pH; loss of infectivity; safe reuse; wastewater; waterborne viruses.

1. Introduction

Different wastewater treatment methods as chlorination, filtration, activated sludge processes, lime coagulation and others could only eliminate 50-90% of viruses presented in wastewater [1] causing presence of un-neglected viral amount in effluent [2] and that made the reuse of treated wastewater a cause for viral transmission. WHO and FAO set guidelines for the safe use of wastewater based on specific aspects of each country [3, 4].Types and amount of pathogens in wastewater varied among regions according to both sanitary and socioeconomic conditions of each community. Mostly, the efficacy of a wastewater disinfection process was regulated and monitored based on measurements of the

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Received date 22 November 2022; revised date 29 December 2022; accepted date 15 January 2023
DOI: 10.21608/EJCHEM.2023.176414.7220

responses of indicator bacteria. However, inactivation of indicator bacteria did not guarantee an acceptable degree of inactivation of other pathogens including viruses [5].

Commonly found viruses in wastewater arehepatitis A, hepatitis E, rotaviruses, noroviruses, astroviruses, adenoviruses and enteric viruses [6], this in addition to SARS-CoV-2 [7]. Viruses detected both in the inlet and outlet of wastewater treatment plants suggested that treatment processes was not sufficient to remove all viruses [2].

One of the most important uses of treated wastewater was its usage in irrigation as it consumed high percentage of available water and so usage of treated wastewater might be an ideal way to solve water shortage problem. Reports showed presence of waterborne viruses in irrigation water and on number of crops as well [8]. For example, hepatitis A virus (HAV) and human norovirus were detected in strawberry, coriander and watercress [9]. Also, human adenovirus and rotavirus were detected in green onion and lettuce [10]. Those reports attributed presence of viruses on crops to the usage of treated wastewater in irrigation.

Waterborne viruses were found to be stable at pH ranges between 5-9. On the other hand, many enteric viruses were more stable at pH 3-5 than at pH 9-12. Adenoviruses and rotaviruses were delicate to pH 10 or greater which lead to their inactivation [11].

Treated wastewater could be used in irrigation of crops, industrial purposes and others so it must be of quality that would not harm crops or soil and also be of no harm to workers [12].

According to Egyptian code (ECP 501/2015) [13] that states wastewater criteria accepted for irrigation, there was no specifications for pH value however in Australia and Italy [14, 15] pH values between 9.5 - 10 are accepted for water reuse.

Here we tested the inhibitory effects of different alkaline pH values on structurally different viruses, enveloped and non-enveloped viruses, to identify both the optimum pH and duration of its application to achieve complete viral inhibition. We also evaluated the influence of increasing pH value on the physico-chemical properties of wastewater.

2. Experimental:

2.1.Tested Viruses and cell lines:

Virus stocks of structurally different viruses, enveloped and non-enveloped, were prepared and exact viral count was determined.
Non-enveloped viruses: Coxsackie B virus (Cox B) virus : viral count was $0.9x10^5$ viral particle /ml. Rotavirus, ATCC VR-2018: viral count was 4.4 $x10^5$ PFU/ml and Adenovirus type 1, ATCC VR-1: viral count was $1.6x10^4$ PFU/ml.
Enveloped viruses: Influenza A virus: viral count was $5.2x10^6$ PFU/ml and Herpes simplex virus type 1 (HSV-1): $9.2x10^6$ PFU/ml.

Cells: MDCK cell line was used for Influenza A virus propagation. MA104 cells were used for rotavirus and vero cells were used for adenovirus 1, HSV-1 and cox B virus propagation. MDCK cell line was propagated in RPMI medium (Lonza,USA) and DMEM medium (Lonza,USA) was used for both MA104 and vero cells. Media was supplemented with 10% fetal bovine serum (Gibco,USA), 1% antibiotic–antimycotic mixture (Lonza,USA).

2.2,Identifying optimum alkaline pH and duration for viral inactivation in distilled water:

Different tubes containing 10 ml distilled water were prepared and pH of each group of tubes was raised to single alkaline pH value ranging from 8-13 with 5% calcium oxide (CaO) solution, water was sterilized then seeded with tested viruses each at a time, one group of tubes was left without changing its pH (7.2) and inoculated with same viral count of each virus as a positive control. 100 µl of different water treatments were inoculated over susceptible cells for each virus after different time points: 1, 2, 3, 4 and 5 hours.

2.3.Test to loss of viral infectivity:

2.3.1.For influenza A virus and adenovirus type 1:100 µ1 of water of different alkaline pHs as well as positive control of tested viruses were inoculated over cells and incubated for 2 h at 37°C with shaking every 15 min. Cells were washed twice with PBS (Phosphate buffer saline) to remove any unadsorbed viral particles. Cells were observed for 48 hours till appearance of cytopathic effect (CPE) then plate was

freezed and thawed twice and cell lysates of different treatments were used in ELISA test. 96 well plates were coated with 50µg/ml in 3 replicates of each lysed cell treatment for 2.5 hours (carbonate buffer, PH=9.6). Wells were blocked with 150 µl/ well blocking buffer (PBS-0.05%-Tween20 (PBST), 5%-FBS) for 2 hours then incubated with50 µl/ well of either 1:2000 dilution of Influenza antibody or 1:200 of adenovirus antibody for 1 hour. This was followed by the application of 1:1000 dilution of horseradish-peroxidase (HRP)-conjugated anti-mouse IgG antibody (Bio-Rad Laboratories, Germany) for 1 hour. Wells were then incubated with HRP specific substrate (0.04% *O*-phenylenediamine, Sigma-Aldrich; Germany; in Citrate buffer + 30% H_2O_2). Color development was stopped by adding 50µl/well 2M H_2SO_4. The optical density values (OD; absorbance) were recorded using a multi-well plate reader (Tecan, Switzerland) at wavelength λ 492 nm and a 620 nm filter as a reference wavelength. All assay incubations were carried out at 37ºC, wells were washed 4 times prior to each step with 300 µl per well with PBST.

Calculations: Readings of all wells (cell control, positive control (cells inoculated with virus seeded in water without changing its pH) and wells of viruses subjected to different alkaline pHs at different time points) were obtained as OD readings, first we calculated the mean of the three readings of every treatment then subtract the mean of the reading of cell control wells from all other treatments including the positive control then the following equation was applied:

(Positive control reading - Treated virus reading)/ Positive control reading x100

2.3.2.For HSV-1, cox B and Rotavirus: Plaque count assay was used to detect viral count before and after treatment [16]. In a 12 well plate cells (10^5cell/ml) were cultivated for 1 day at 37ºC. 100 µ1 of water of different alkaline pHs as well as positive control seeded with tested viruses were inoculated over cells and incubated for 2 h at 37ºC with shaking every 15 min.. Cells were washed twice with PBS (Phosphate buffer saline) to remove any unadsorbed viral particles. 1 mL of Dulbecco's Modified Eagles Media (DMEM) supplemented with 2% agarose was added onto the cell monolayer, plates were left to solidify and incubated at 37ºC till formation of viral plaques. Formalin (10%) was added for 2 h then plates were stained with crystal violet. Finally plaques were counted and percentage reduction in plaques formation in comparison to control wells was recorded.

Calculations: (Number of PFU of virus control - Number of PFU of treated virus)/ Number of PFU of virus control x100

2.4.Test effect of optimum alkaline pH and duration on inactivating viruses inoculated in wastewater.

2 bottles of 500 ml wastewater were prepared, pH of wastewater was raised to 12 (based on results of previous experiment) by using 5% CaO for one bottle and the second was left without changing its pH as control. Wastewater of both bottles was autoclaved then aliquoted in 10 ml tubes where each virus was inoculated at a time once in wastewater of pH 12 and once in control waste water and incubated for 1 hour. 100 µl of both wastewater of pH 12 and control wastewater were applied over cells according to viral type for 2 hours with shaking every 15 min then inoculum was removed and cells were washed twice with PBS. Steps were continued to detect % reduction for each virus as previously described.

Test effect of optimum alkaline pH and duration on inactivating viruses in wastewater field samples.

2.5.Wastewater samples: collection , treatment and concentration

Wastewater samples, 2 liters each, were collected weekly through the period between 6 February 2022 and 13 March 2022 (6 samples in total) from Zenin waste water treatment plant (WWTP): it is one of the largest WWTPs in Giza government, Egypt. It receives 275,000 to 550,000 m^3/day with an average of 385,000 m^3/day of waste water flow [17].

For each sample, pH of 1 L was raised to 12 using 5% CaO and incubated at room temperature for 1hour those samples were named (S1-pH12, S2-pH12, S3-pH12, S4-pH12, S5-pH12 and S6-pH12) and the other liter was left without any change as control and were named as (S1, S2, S3, S4, S5 and S6).

Wastewater concentration: A volume of 50 ml of 1M aluminium chloride was added to each liter to give a final concentration 0.05M. pH was adjusted to be 3.5 by 1N HCl. Waste water samples were filtered through 0.45 µm nitrocellulose membrane followed by passing 200 mL 0.5 mM H_2SO_4, pH 3.0 to ensure viral particle retention and eliminate all biosolids. Membrane was removed from holder and soaked in a Petri dishin11 mL of 1 mMNaOH; pH 10.5 for 10 minutes. Later, the upper surface of the membrane was scraped to elute the viruses. The elute was neutralized by adding 50 µL 50 mM H_2SO_4 and 50 µL 100X 1nM Tris–EDTA pH 8.0 and stored at -20ºC till use [18].

2.6.Detection of viruses present in each samples.

Concentrated samples were subjected to viral RNA/DNA extraction (Qiagen, Germany), reverse transcription (super script III Reverse transcriptase, Invitrogen, USA) then PCR (QIAGEN, Germany), Primers of targeted viruses and the size of their products were summarized in table (1). Reaction mixture was subjected to the following temperature conditions, 50ºC for 30 sec., 94ºC for 15 sec., denaturation at 94ºCfor 30 sec., annealing at 57ºC for 1 min. for HAV, cox A, coxB and rotavirus and at 55ºC for 1 min. for adenovirus, extention at 68ºC for 2 min., for 40 cycles followed by extension at 68ºC for 7 min. . For adenovirus, PCR was made directly after DNA extraction as it is DNA virus and doesn't need reverse transcription.

Table 1: Sets of in house primers used for viral detection.

Virus	Primer sequence	Product seize/bp
Adenovirus	F: CCCACGGTGGCGCCTAC	
	R: AACCGCAGCGTCAAACGCT	80
HAV	F:TGATTAGCATGGAGCTGTAGG	
	R: CAAAGCATCTCTTCATAGAAGTA	190
Rotavirus	F: TCAGTCTATTTTAAAGAGTACTCA	
	R: TTTGATTCTCCCGATTGTTGATA	202
Cox B	F: TACTGACATGGTGCGAAGAGT	
	R: ACTGGATTGTGATTGCCTGCT	100
Cox A	F: ACGGTGGTCCAGGCTGCG	
	R: AAGGAAACACGGACACCCAAA	219

2.7.Test inactivation of viral infectivity due to effect of alkaline pH (12):

This test was made to test inactivating effect of pH 12 on tested viruses, all 12 samples, 6 control wastewater samples (S1 to S6) and 6 wastewater samples incubated at pH 12 for 1 hour before sample preparation for concentration (S1-pH12 to S6-pH12). In a 12 well plate seeded with vero cells and incubated at 37ºC overnight, 150 µl of each concentrated sample was inoculated and incubated for 2 hours to ensure viral adsorption then inoculum was removed and fresh media was added and wells were incubated at 37ºC for 5 days then examined for appearance of cytopathic effect as a result of viral replication. Plates were freezed and thawed then subjected to RNA/DNA extraction followed by reverse transcription and PCR as previously described.

2.8.Physico-chemical criteria of waste water samples before and after increasing pH to 12.

Parameters were measured in three wastewater samples according to APHA 2017 (110). Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) Model Agilent 5100 Synchronous Vertical Dual View (SVDV) was used for the determination of the trace levels of environmentally sensitive element including metals. Samples were digested with the analytical method [19]. A blank and five standards from Merck (Germany) were used to create an intensity calibration curve for each series of data. External reference standards from Merck, and a quality control samples from the National institute of standards and technology (NIST) were used to confirm the accuracy and precision of metal measurements

3. Results and Discussion:

Different wastewater treatment techniques were not optimized to eliminate and disinfect suspended viral particles in wastewater completely making wastewater reuse a source for viral spreading. Viruses as Adenovirus, Cox A and B, rotavirus and others were detected in wastewater [6]. Since the beginning of COVID-19 pandemic and the usage of wastewater to detect SARS-Co-V2 and predict number of infected persons in a given area based on viral count detected in wastewater samples [20] it became clear that we must not focus only on gastrointestinal or waterborne viruses in wastewater research. Here we focused on structurally different group of viruses, enveloped and nonenveloped ones. HSV-1, cox B, Rotavirus, Influenza A and adenovirus type 1 were inoculated in distilled water of alkaline pH ranging from 8-13 and their infectivity was tested at different time points (1-5 hours), viruses inoculated in distilled water at its normal pH (7.2) was used as positive control.

CaO was used to increase pH of water of this experiment and of wastewater in the coming experiment as well. CaO can make 90-95% turbidity removal due to ability of divalent cations as Ca^{+2} to improve coagulation process [21], sludge resulting from its usage is easier to dewater than ferric or aluminium sludges, and also it allows higher surface overflow rates on sedimentation tanks than does ferric and aluminium salts [22]. This beside its cheap cost [23].

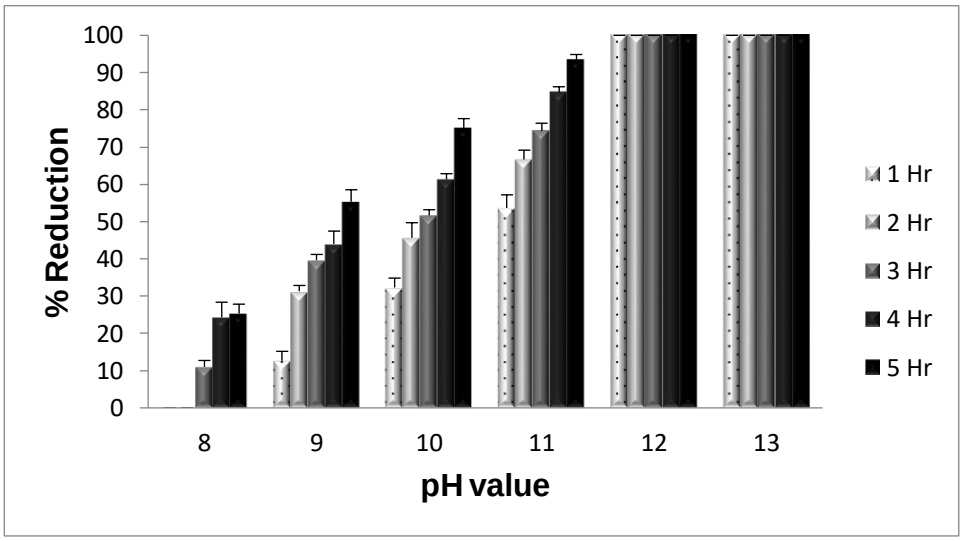
Results showed that for HSV-1(Figure 1A), cox B (Figure 1B) and Influenza A virus (Figure 1C) gradual increase in % reduction appeared that started

from pH 8 and increased by increasing time to be 100 % after 1 hour incubation in pH10 for Influenza A virus, 4 hours incubation at pH 11 for cox B and 1hour incubation at pH 12 for HSV-1. Rotavirus was more stable as pH 8 showed no effect on its infectivity and reduction in viral count started from pH 9 with gradual increase with time till being maximum at 3 hours incubation at pH 11 (Figure 1D), that agreed with Meng et al., 1987 [24] who incubated rotavirus at different ranges of acidic and alkaline pH (2-12) for 1 hour and showed that the virus was not affected by pHs 3-10 at 1 hour incubation and that reduction starts from pH 11 and was also maximum at first hour incubation at pH 12. Adenovirus in Figure 1E showed the highest stability as pHs 8 and 9 had completely no effect on its infectivity and reduction started from pH 10 with gradual increase by time till being 100 % at 3 hours incubation at pH 11. From this experiment we could conclude that 1 hour incubation at pH 12 was the common among enveloped, non enveloped, RNA and DNA viruses.

This can be discussed as follows: Knowing that the building unit of the outer shell of non-enveloped virus (capsid) is proteins [25] and envelop of enveloped viruses composed of Lipids or glycoproteins [26].

High pH enhances protein unfolding and aggregation and causes changes in its tertiary and quaternary structure and composition [27], It can cause lipids damage as well [28]. That changes in structural outer components of the virus unable it to bind successfully to its antigenic binding site for causing infection.

A



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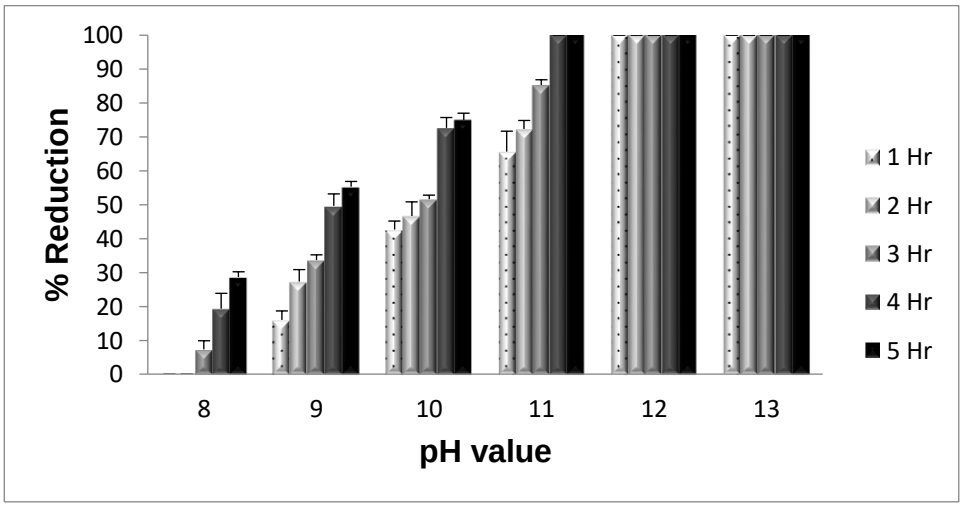

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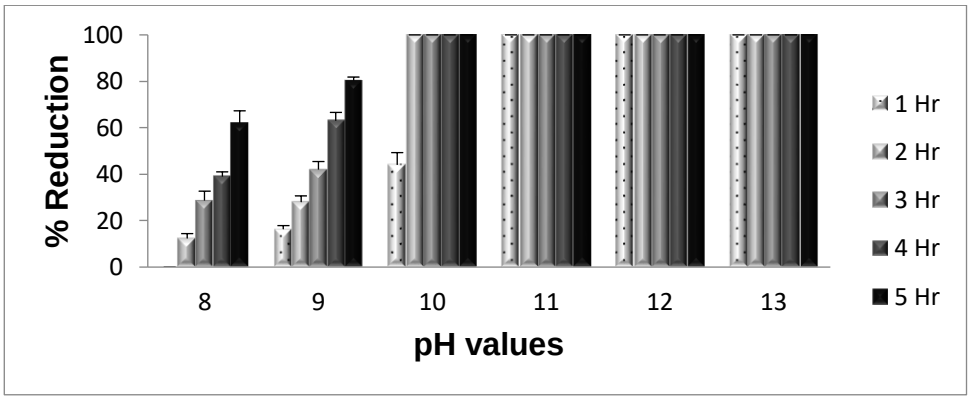

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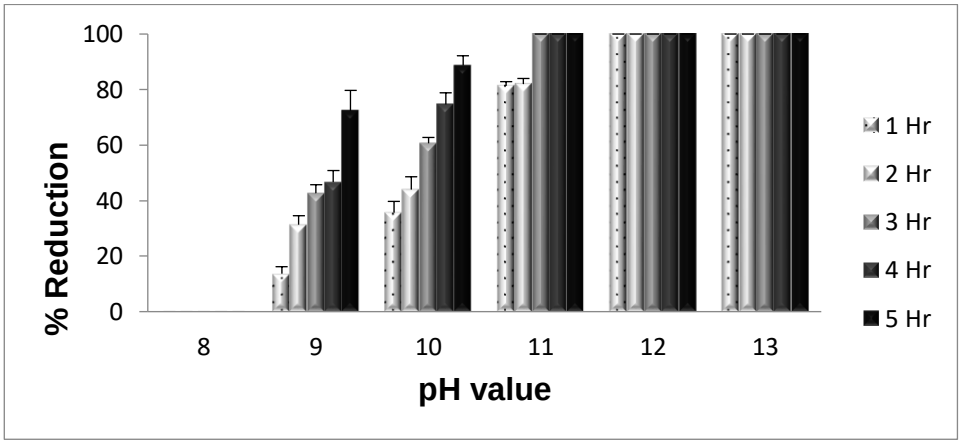

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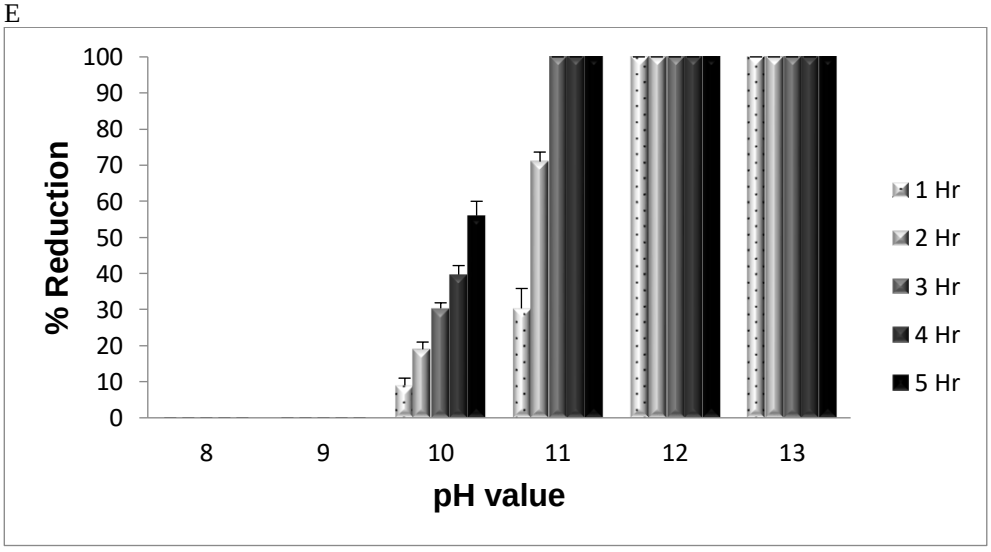

Figure 1: Effect of different alkaline pH values on infectivity of viruses inoculated in distilled water and tested at different time intervals where A: HSV-1, B: cox B, C: Influenza A, D: rotavirus and E: adenovirus type 1.

From the previous experiment the optimum pH that caused complete inactivation to all tested viruses was pH 12 and the optimum time duration was 1 hour. pH of wastewater samples obtained from Zenin wastewater treatment plant was raised to 12 then samples were autoclaved to inactivate any infectious viral particles of any kind that might be found in it naturally and also to get rid of bacteria or fungus as those samples would be inoculated over cells in sterile conditions. Another wastewater samples were kept at its normal pH but was sterilized as well. Both wastewater samples were inoculated with tested viruses and incubated for 1 hour before being concentrated, sterilized and applied over cells, Figure 2 showed that alkaline pH 12 treatment was able to make complete inactivation to the 5 tested viruses after 1 hour incubation, the small % reduction in viral count shown by wastewater control of each virus might be due to adsorption of viral particles on protein particles suspended in wastewater.

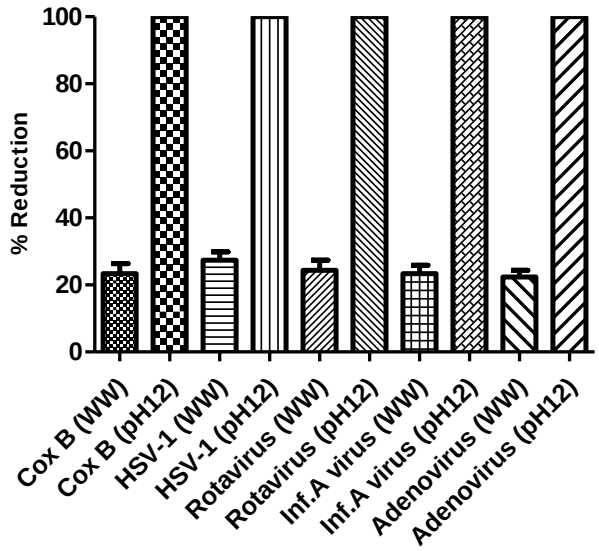

Figure 2:% Reduction in viral count inoculated in waste water at its normal pH and at pH 12, where WW: wastewater at normal pH, pH12: wastewater after raising its pH to 12.

To test effect of optimum alkaline pH and duration on inactivating viruses in waste water field samples, Concentrated wastewater samples were subjected to PCR test to detect presence or absence of viruses before inoculating them over cells to confirm 1- presence of viruses and identify their kind, 2- confirm that both halves of each sample (1 liter left as control and the 1 L of the same sample subjected to pH 12 for 1 hour) were PCR positive for the same viruses to be able to compare them. Results in table 2 showed that number of PCR positive samples were 4/6 and 1/6 for adenovirus and cox A virus respectively. Also table 2 showed that the 2 liters from each sample were homogenous when divided into two as both halves were positive and/or negative for the same viruses. On the other hand samples gave negative PCR results for cox B, HAV and rotavirus.

Table 2: PCR results of concentrated waste water samples

Sample ID	Adenovirus	HAV	Rotavirus	Cox B	Cox A
S1	+	-	-	-	-
S2	-	-	-	-	-
S3	+	-	-	-	-
S4	-	-	-	-	-
S5	+	-	-	-	+
S6	+	-	-	-	-
S1-pH12	+	-	-	-	-
S2-pH12	-	-	-	-	-
S3-pH12	+	-	-	-	-
S4-pH12	-	-	-	-	-
S5-pH12	+	-	-	-	+
S6-pH12	+	-	-	-	-

(-): Sample was PCR negative

(+): Sample was PCR positive

To confirm viral loss of infectivity, concentrated wastewater samples were inoculated on vero cells as it was susceptible to both adenovirus and cox A virus, after 5 days incubation samples S3,S5 and S6 showed cytopathic effect indicating viral infection and replication and at the same time samples S3-pH12, S5-pH12 and S6-pH12 which were the same wastewater samples but subjected to alkaline pH treatment showed no cytopathic effect (Figure 3) and that was a great sign of the ability of alkaline pH to cause complete loss to infection ability of viral particles presented in the sample.

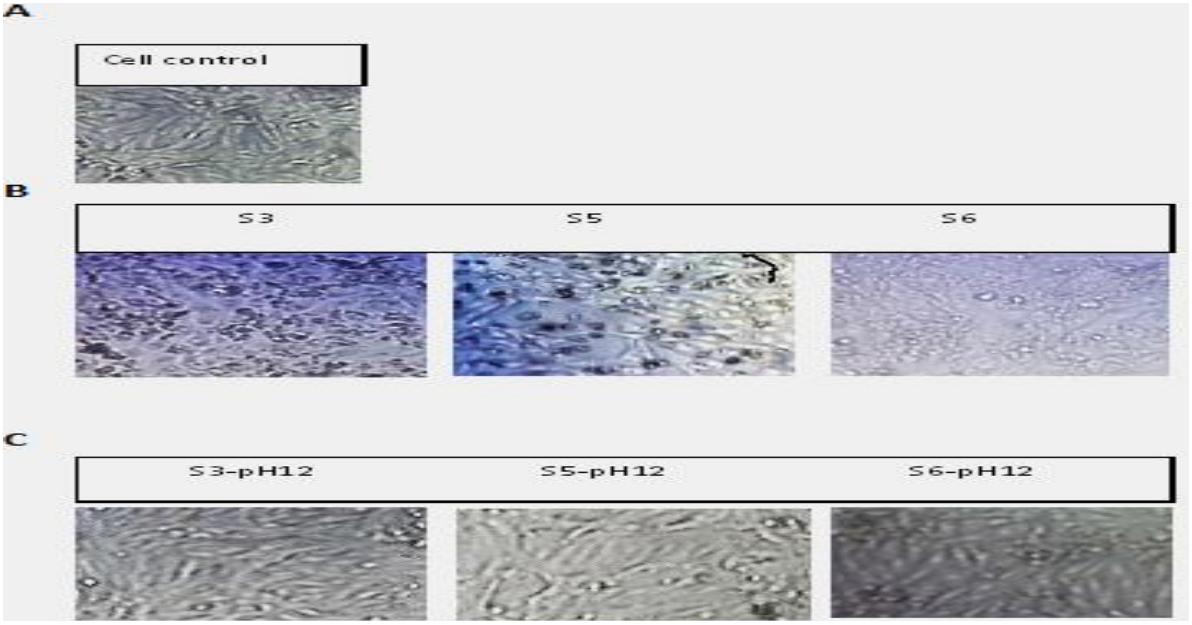

Figure 3:vero cells inoulated with concentrated wastewater samples, where A was the cell control, B: cells inoculated with samples S-3, S-5 and S-6 showing rounding of cells due to viral replication and C: cells inoculated with samples S-3-pH12, S-5-pH12 and S-6-pH12 showing decrease in or no rounding of cells.

On subjecting cell culture harvest to PCR, Results showed that for Adenovirus, only S3, S5 and S6 gave specific bands which was not the case with cell culture harvest of sample S1 which previously showed specific PCR band before being inoculated on cells, that might be due to effect of any other component in the waste water that cause its inactivation. On the other hand samples S3-pH12, S5-pH12 and S6-pH12 showed no specific bands indicating success of pH treatment to cause its complete inactivation (Table 3, Figure 4A).

Cox A virus was PCR positive in only sample (S5) and its pH treatment (S5-pH12) and after their inoculation on vero cells specific band appeared with sample S5 and disappeared completely with sample S5-pH12 showing success of that treatment to inactivate virus (Table 3, Figure 4B).

Appearance of faint bands in PCR experiment results from our usage of small wastewater inoculum (containing low viral titre) over the cells to avoid cell toxicity resulting from wastewater composition.

Table 3: PCR results of cell culture harvest inoculated with concentrated waste water samples before and after treatment to pH 12

Virus	Sample ID	PCR result	Sample ID	PCR results
Adenovirus	S1	Negative	S1-pH12	--
	S3	Positive	S3-pH12	Negative
	S5	Positive	S5-pH12	Negative
	S6	Positive	S6-pH12	Negative
Cox A	S5	Positive	S5-pH12	Negative

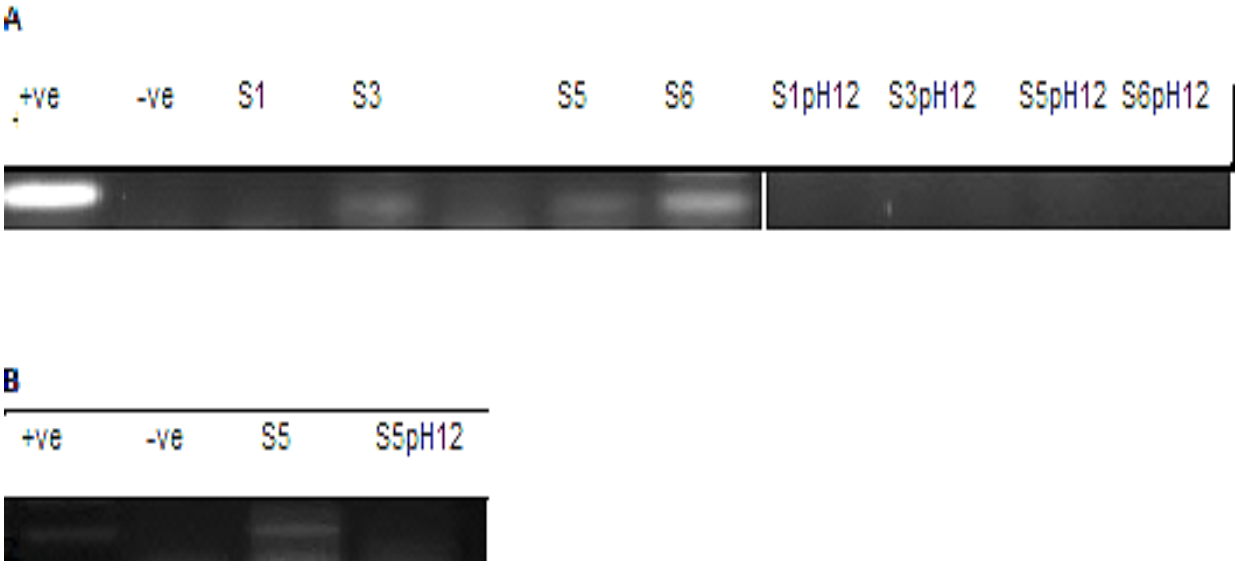

Figure 4: Electrophoresis agarose gel showing PCR products of cell culture harvest of vero cells inoculated with concentrated wastewater samples and incubated for 5 days till development of cytopathic effect, A: Detection of adenovirus type 1 where +ve: positive control, -Ve: negative control, S1,S3,S5 and S6 are cell culture harvest inoculated with concentrated wastewater samples at their normal pH and S1-pH12, S3-pH12, S5-pH12 and S6-pH12 are cell culture harvest inoculated with concentrated wastewater samples after rising their pH to 12 for 1 hour. B: Detection of cox A virus where where +ve: positive control, -Ve: negative control, S5 is cell culture harvestinoculated with concentrated wastewater samples at its normal pH and S5-pH12 is cell culture harvest inoculated with concentrated wastewater samples after rising their pH to 12.

Treated wastewater could be used in irrigation of crops, industrial purposes and others so it must be of quality that would not harm crops or soil and also be of no harm to workers [12].

Although pH 12 was very high and might cause harm to plants and soils if used in irrigation but the fact that there was no treatment protocol that could result in complete viral elimination and inactivation made it a tool that deserve thinking.

We might also recommend that after viral inactivation wastewater could return again to it normal or accepted pH as our results showed that viral inactivation is irreversible upon returning to neutral pH as during (loss of infectivity assay steps) we inoculated small inoculum of treated water over culture media covering the cells so its surrounding pH returned to neutral (pH 7.2) and that did not affect loss of infectivity caused by 1 hour incubation at pH 12.

To be sure that the increase in pH would not cause undesired changes to wastewater, physic-chemical criteria of three wastewater samples were studied once at their normal pH and once after increasing their pH to 12. Results in Table 3 showed that increasing pH of wastewater improved its quality and made it nearer to the permissive limits of waste water that could be used for irrigation according to Egyptian code (ECP 501/2015) [13]. Due to increase in pH all heavy metals concentration decreased except for calcium as CaO is used to increase pH value but also still below the accepted concentration [29], also sodium adsorption ratio (SAR) levels decreased which was of great benefit as increasing sodium amount in irrigation water caused soil crusting and decrease in its permeability also if it was absorbed by plant it accumulated in leaves causing plant injuries. Results showed that total suspended solids (TSS), Chemical oxygen demand (COD) as well as biological oxygen demand (BOD) decreased by increasing pH to 12.

Although bicarbonate is not harmful to plants but its presence with high concentration might affect availability of Fe and Zn in soil [30], it might also increase soil pH affecting its permeability, so increase wastewater pH cause significant decrease in % of bicarbonate.

Precipitation of sulfate ions as ($CaSO_4$ $2H_2O$) by addition of Cao was the most extensively used technique in sulfate removal [31] as it caused high % in sulphate ions removal. However any decrease in the concentration of essential elements to plants as the micronutrients (N, K, P) were found not to be affected by increasing pH except for P ions which could be compensated by fertilizers containing all those metals and compounds.

Table 3: Physico-chemical criteria of wastewater samples before and after treatment by raising pH according to Egyptian code (ECP 501/2015)

Parameter	Unit	Sample 1-Initial	Sample 1-pH 12	Sample 2-Initial	Sample 2-pH 12	Sample 3-Initial	Sample 3-pH 12	Permissible limits Long term-short term
pH	--	7.29	12	7.6	12	7.3	12	
Total suspended Solids (TSS)	mg/l	280	2.2	130	10	184	4	50--300
Chemical Oxygen Demand (COD)	mg/l	750	234	240	101	179	72	
Biological Oxygen demand (BOD)	mg/l	422	127	135	54	89	39	80--350
Total Dissolved Solids (TDS)	mg/l	1022	3476	396	1052	481.6	1034	2000-3000
Phosphate (PO_4-P)	mg/l	6.2	0.05	1.17	0.01	6.1	1.12	30
Sulfate (SO_4^-)	mg/l	35	6.14	65.4	7.8	62.5	8.16	500
Bicarbonate (HCO_3)	mg/l	40	139	180	64	74	48	400
Sodium adsorption ratio (SAR)	---	4.34	3.14	3.03	2.09	2.4	1.95	6-9
Sodium	mg/l	158	130	86	79	70	65	230
Magnesium	mg/l	21.5	4.1	14	1.4	15	3.2	100
Calcium	mg/l	65	123	38.2	106	40	82	230
Aluminum	mg/l	2.9	ND	0.18	0.02	0.04	ND	5.0-20
Arsenic	mg/l	ND	ND	ND	ND	ND	ND	0.1-2.0
Beryllium	mg/l	ND	ND	ND	ND	ND	ND	0.1-0.5
Copper	mg/l	0.02	ND	ND	ND	ND	ND	0.2-5.0
Iron	mg/l	2.2	ND	0.16	ND	0.06	ND	5.0-20.0
Lithium	mg/l	ND	ND	ND	ND	ND	ND	2.5
Manganese	mg/l	0.06	ND	0.07	ND	0.01	ND	0.2-10
Nickel	mg/l	0.02	ND	ND	ND	ND	ND	0.2-2.0
Lead	mg/l	0.05	ND	0.02	ND	0.03	ND	5.0-10.0
Selenium	mg/l	ND	ND	ND	ND	ND	ND	0.2
Cadmium	mg/l	ND	ND	ND	ND	ND	ND	0.01-0.05
Zinc	mg/l	0.09	ND	0.02	ND	0.05	ND	5.0-10.0
Chromium	mg/l	ND	ND	ND	ND	ND	ND	0.1-1.0
Mercury	mg/l	ND	ND	ND	ND	ND	ND	0.002
Vanadium	mg/l	ND	ND	ND	ND	ND	ND	0.1-1.0
Cobalt	mg/l	ND	ND	ND	ND	ND	ND	0.05-5.0
Boron	mg/l	0.03	ND	0.01	ND	0.01	ND	1.0-2.0
Molybdenum	mg/l	ND	ND	ND	ND	ND	ND	0.01-0.05
Total Nitrogen	mg/l	5.6	14	19.6	11.2	19.6	11.2	--
Phosphorus	mg/l	19	0.15	286	150	272	53.8	--
Potassium	mg/l	23	22	12	11.5	14	14	--

The results of the study were summarized in Figure 5 that showed that complete viral inactivation in wastewater seeded with 5 tested viruses occurred on applying pH 12 for 1 hour compared to control wastewater sample and also showed the removal of suspended particles and decrease in heavy metals and salt concentration on applying same conditions.

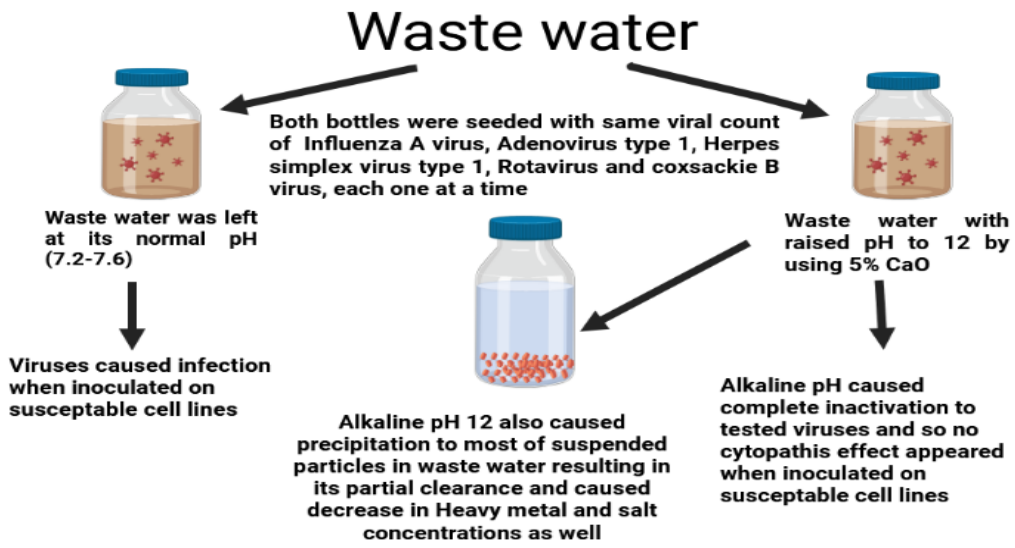

Figure 5: Graphical representation summarizing the results of the study.

4. Conclusion.

On increasing pH of wastewater to 12 for 1 hour, complete loss of infectivity to viruses present in wastewater occurred. Noteworthy increasing wastewater pH up to 12 with CaO cause improvement in wastewater quality by getting rid of heavy metals and salts found in it and made them reach accepted levels for wastewater reuse in irrigation. More research is needed to confirm acceptable usage of wastewater in alkaline pH without affecting plants or we should return water to its normal pH after viral inactivation. Although appeared to be more steps and more money but on comparing it to Human health and money that should be spent on treating diseased people and children it might be saving money.

5. Conflict of interest

All authors declare no conflict of interest.

6. Funding:

This work was funded by National research Center (Grant number: E-120806).

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