International Journal of Environmental Bioremediation & Biodegradation, 2016, Vol. 4, No. 3, 68-79
Available online at http://pubs.sciepub.com/ijebb/4/3/1

DOI:10.12691/ijebb-4-3-1

Effectiveness of Various Carbon Amendments in the Bioremediation of Perchlorate Contaminated Soils

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Abstract A laboratory bioremediation study was conducted on perchlorate contaminated soils from the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas, USA. In this study, the effects on perchlorate bioremediation of five carbon substrates, fertilization and prior exposure to the contaminant by the native soil microorganisms were explored in microcosms over a five week period. Biostimulation of native soil microorganisms to degrade pollutants is widely practiced as it increases the rate of natural attenuation, which is otherwise slow. The five carbon substrates studied were: chicken litter, biosolids, yeast extract, sodium acetate, and cornstarch. Among the five carbon sources, unfertilized chicken litter treatment in unsterilized soil was quickest in reducing perchlorate. In these flasks, perchlorate concentrations fell below detection limit from an initial mean concentration of 220 mg/L by the end of first week. There was a significant negative effect ($p < 0.05$) of fertilization in chicken litter treatments on perchlorate degradation. In unsterilized condition, unfertilized chicken litter treated flasks reduced perchlorate quicker than the fertilized treatments. In fertilized treatments there was a significant effect ($p < 0.05$) of soil sterilization on perchlorate degradation. Chicken litter treatments with unsterilized soil degraded perchlorate to below detection limit quicker (< 2 weeks) than chicken litter with sterilized soil (4 weeks). The results suggest that application of inexpensive carbon substrates such as chicken litter without the need for addition of chemical fertilizers may be a feasible remediation strategy in perchlorate contaminated soils with large active, native microbial populations.

Keywords: *bioremediation, biostimulation, perchlorate, soil contamination, carbon amendments, native soil microorganisms*

Cite This Article: Sarat Kannepalli, and Kenneth W. Farrish, "Effectiveness of Various Carbon Amendments in the Bioremediation of Perchlorate Contaminated Soils." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 4, no. 3 (2016): 68-79. doi: 10.12691/ijebb-4-3-1.

1. Introduction

Perchlorate (ClO_4^-), the soluble anion associated with the solid salts of ammonium, potassium, and sodium perchlorate is a contaminant of increased concern. Ammonium perchlorate has long been used in the defense industry as an oxidant in solid propellants and explosives. The primary sources of soil and groundwater contamination with perchlorate are related to the production of the compound for aerospace and military applications. Because of limited shelf life, perchlorate salts require periodic removal and replacement from applications [7]. Improper disposal of this material has resulted in perchlorate contamination in 34 states, the most severely contaminated being California, Nevada, New Mexico, Utah, and Texas [22]. United States Food and Drug Administration has detected perchlorate in food crops and milk [13].

Currently, there is no federal clean-up standard for perchlorate in groundwater or soil such as a Maximum Contaminant Level (MCL). Different states have set different enforceable standards for perchlorate in drinking water ranging from 2 µg/L in Massachusetts to 6 µg/L in California [5,12]. The United States Environmental Protection Agency (USEPA) has established an Interim Lifetime Drinking Water Health Advisory of 15 µg/L, which is the concentration of perchlorate in drinking water that is not expected to cause any adverse non-carcinogenic effect for a lifetime exposure [19].

Once released to the environment, perchlorate is highly soluble in water, and relatively stable and mobile in surface and subsurface aqueous systems [18]. The primary pathways for human exposure to perchlorate are ingestion of contaminated food and drinking water [1]. Different physical characteristics of perchlorate such as low reactivity, low volatility, and high solubility make many treatment technologies such as ultra filtration, air stripping, carbon adsorption, and advanced oxidation either ineffective or uneconomical [7]. *In situ* bioremediation of perchlorate is a promising technology in which naturally occurring microbes can be used to biodegrade perchlorate. *In situ* bioremediation can be accomplished either by bioaugmentation and/or biostimulation. In the process of biostimulation, various forms of rate limiting organic or inorganic compounds are introduced into a contaminated system. These can be carbon substrates, electron donors or electron acceptors which would increase the population of indigenous microorganisms there by speeding up the

degradation process. In biostimulation of perchlorate degradation an electron donor (i.e., carbon substrate) is added to the perchlorate-contaminated soil. In a microbial mediated energy yielding redox reaction, perchlorate accepts these electrons and is reduced to oxygen and chloride [4]. Microbial respiration couples the oxidation of an organic substrate to the reduction of a final electron acceptor. In perchlorate bioremediation, perchlorate acts as final electron acceptor by accepting electrons from a carbon substrate.

Most perchlorate-reducing microorganisms are capable of functioning under variable environmental conditions and can use oxygen, nitrate, and chlorate, but not sulfate, as electron acceptors [4]. In the presence of oxygen, microbes prefer oxygen as a terminal electron acceptor to perchlorate and so microbial induced perchlorate reduction can only take place under anaerobic conditions [10].

Though perchlorate-reducing bacteria are widely distributed in the environment, their populations are found to be enriched at previously contaminated sites. It has been shown that prior exposure to a chemical contaminant or its natural analog may stimulate development of a group of microbes, which can degrade a contaminant more effectively [11]. Prior presence of perchlorate degrading bacteria is an important factor, since in some materials such as dredge tailings; perchlorate-degrading microbes took a long time to develop a population large enough to reduce perchlorate, even when a carbon substrate was available [17]. A 1998 Remedial Investigation/Feasibility Study has indicated that surface water, groundwater and soils at LHAAP, from which the soil sample was collected for this study, had a long history of perchlorate contamination [14].

In the current study, five different carbon substrates were used: chicken litter, sodium acetate, cornstarch, biosolids, and yeast extract. Chicken litter, apart from being inexpensive and abundantly available in the east Texas region, was found to be the most effective amendment among five others in a bench scale experiment conducted on perchlorate contaminated LHAAP soils [14].

Tipton et al., [17] found that sodium acetate used in a bioremediation experiment degraded perchlorate more effectively than glucose, with a reduction of perchlorate from 50 mg/L to below the detection limit within 15 days. It was also reported that perchlorate-degrading microbes have the ability to grow by combining oxidation of acetate, reduction of perchlorate and dismutation (enzymatic degradation) of chlorite into chloride and oxygen.

Biosolids was another carbon source included in the study because *Perc 1 ace*, a known perchlorate degrading bacteria has been isolated from biosolids [9]. Further, biosolids are widely available and inexpensive. Biosolids are the solid material left as a result of wastewater treatment. Biosolids are of two types based on the method of stabilization. The first type is that stabilized with lime and the second type is stabilized with physical, chemical and biological processes. Biological processes stabilized the biosolids used in this study. In a study on yeast extract as a carbon amendment it was found that high protein organic nutrients were found to support perchlorate reduction better than non-protein carbon sources [8]. Finally, though it has been reported that fermentable substrates such as starch, glucose, and lactose do not directly support perchlorate reduction [10], starch was included in this study as a carbon substrate as it is a simple and inexpensive carbon substrate capable of being easily metabolized.

In a study on bioreactor processes for the remediation of perchlorate-contaminated wastewater, it was determined that perchlorate reduction occurred at a temperature range from 25 to 42°C [10]. The same work showed that the enzyme chlorite dismutase, which is essential for conversion of toxic chlorite to non-toxic chloride and oxygen, works best at 30°C. In another study, *Perc 1 ace* was found to work best between 25 and 30ºC [8].

In a field project at an aquifer in Pasadena, California, the biodegradation of perchlorate was most rapid at a pH of 8.0 and levels of perchlorate declined from 250 µg /L to less than 4 µg/L in 28 days. Perchlorate was also completely degraded in samples at a pH of 7.0 during the 28-day incubation. However, at pH values of 4.0, 5.0, and 6.0, little or no perchlorate degradation was observed in the aquifer microcosms [7]. In another experiment conducted on perchlorate degradation on Yolo loam soil, perchlorate degradation occurred between pH 6.95 to 7.55 [17].

Usually, environments polluted with organics have low redox potentials as microbes utilize the available oxygen for decomposition process [2]. A perchlorate bioremediation system works best at low redox potentials, as facultative anaerobic bacteria use alternate electron acceptors like perchlorate at low redox potentials instead of oxygen. In a bioreactor process for the remediation of perchlorate contaminated water, it was found that a redox potential of less than –110 mV is required for perchlorate reduction [10].

The specific objectives of this study were to

1) Test effectiveness of five inexpensive carbon substrates on perchlorate reduction

2) Ascertain the effect of native soil microorganisms from a perchlorate contaminated site in perchlorate reduction

3) Determine the effectiveness of nitrogen and phosphorus nutrient amendments in promoting perchlorate reduction.

2. Materialas and Methods

2.1. Soil materials and Experimental Set up

Surface soil (approximately 0-10 cm deep) was collected from Site 04 located in the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. The soil sample collected was homogenized, and sent to Ana Lab, Kilgore, Texas for initial measurement of perchlorate concentration. The soil had an initial water extractable perchlorate concentration of 19.6 mg/kg on dry weight basis. The initial soil pH was determined to be 4.27 using an Accumet® Basic AB15 pH meter. In the experimental setup, moist soil (25 grams dry) was placed in 80, 125 mL Erlenmeyer flasks. Forty of these flasks were sterilized in an autoclave at 118°C for 30 minutes. The remaining 40 flasks were left unsterilized. In 30 of the sterilized flasks and 30 of the unsterilized flasks, 60 mL unsterilized nutrient solution containing 93 mg/L of NH_4PO_4 (MC&B),

151 mg/L of $MgSO_4.7H_2O$ (EM Science), 200 mg/L of KCl (Fisher Scientific) and 15 mg/L of $CaCl_2 \cdot 2H_2O$ (EMD^{TM}) was added (Vogel, 1994). In the remaining ten sterilized flasks and ten unsterilized flasks, 60 mL of nanopure water (18 mΩ) was added. A solution of 200 mg/L HPLC grade sodium perchlorate (Fisher Scientific, New Jersey) (F.W.140.46) was added to all flasks for a final concentration of 220 mg/L in each flask. Initially ammonium perchlorate was considered for spiking the soil samples with perchlorate. But it was not used due to concern that ammonium toxicity might hamper bacterial activity. All flasks were titrated with 0.1 M stock solution of KOH (MCB reagents, Ohio) to adjust the soil pH to 7.0. Initially, NaOH was considered for adjusting the pH. This idea was abandoned as sodium present in the NaOH deflocculated the soil, perhaps making the environment less hospitable for microbes.

The five different carbon sources were added at 5% dry weight by weight of the soil (1.25 g to 25 g soil). The chicken litter used was collected from a chicken (broiler) farm near Nacogdoches, Texas. Chicken litter usually contains a mixture of pine sawdust or fine shavings, chicken manure, feathers and a remainder of 10-15% of the composted litter from the previous litter cycle. So, it was expected that nitrogen, phosphorus, other nutrients and a variety of microorganisms were present in the chicken litter. Analysis of 383 litter samples in the Stephen F. Austin State University Soil, Plant and Water Analysis Laboratory was found to have nitrogen, phosphorus ratio of 3.4: 1.7 (Personal communication from Prof. Leon J. Young of Stephen F. Austin State University). After moisture correction, 1.64 g of moist chicken litter (1.25 grams dry) was added to 10 of the sterilized and 10 of the unsterilized flasks. Anhydrous sodium acetate (1.73 g) (EMD^{TM}, Gibbstown, NJ) was added to have 1.25 grams of acetate. Cornstarch (1.25 g) containing 2% total carbohydrates was used. Moist biosolids (1.92 g) was used. The biosolids used was from the Neches River Authority Compost facility, Jacksonville, Texas. The biosolids used had an earthy smell, 40-60% moisture content, and approximately 2-2-2 NPK nutrient value. The biosolids had been composted for 15 d at temperatures as high as 55ºC at the compost facility. Granulated yeast extract (1.25 g) containing 10.5% nitrogen and 5% chloride was added to the yeast extract treated flasks.

Each carbon source had five replicates, except for chicken litter, which had ten replicates. Five of the chicken litter replicates had an added nutrient solution (containing N, P, K, Ca, Mg, and S in the above mentioned chemical forms and concentrations) and the other five had only nanopure water. There were ten sterilized controls without any carbon source. Five of these had the added nutrient solution (containing N, P, K, Ca, Mg, and S) and the other five had only nanopure water. The same procedure was repeated with the 40 unsterilized flasks. A summary of the treatments is shown in Table 1.

Table 1. Number of flasks in each treatment cell of the perchlorate degradation experiment.

		Control	Chicken Litter	Acetate	Starch	Biosolids	Yeast Exract
Sterilized soil	*Fertilized*	5	5	5	5	5	5
	Unfertilized	5	5	–	–	–	–
Unsterilized soil	*Fertilized*	5	5	5	5	5	5
	Unfertilized	5	5	–	–	–	–

Immediately after setting up the flasks, redox potential and pH were measured using Orion® redox and pH electrodes and a 5 mL sample was also collected at time zero. All the flasks were incubated at 30ºC for 6 weeks in a VWR® scientific incubator (Model # 2020). Every seven days, redox potential and pH were measured and a 5 mL sample was collected from each flask. The samples were kept frozen until analyzed.

2.2. Sample Preparation and Analysis

Before analyzing the samples on the Ion Chromatograph, each sample was thawed and diluted 10 fold. All the samples were passed through conditioned Silica (Fisher Scientific, Fair Lawn, NJ) and C-18 cartridges (Fisher Scientific, Fair Lawn, NJ) to eliminate matrix interferences. Silica cartridges (500 mg/6 mL packing) were conditioned with 4 mL of nanopure water. C-18 cartridges (1 g/6 mL packing) were conditioned with 2 mL of Hexane, 2 mL of acetone and 2 mL of nanopure water. Silica cartridges were used to adsorb non-polar solvents like hydrocarbons, chloro or fluoro substituted hydrocarbons, esters and ethers. C-18 cartridges were used to remove neutral organic compounds. Finally, the samples were filtered through a 0.45 µm nylon filters (Gelman Laboratory, Buffalo, NY) to remove any particulates, which might block the connecting tubing, column end frits or other hardware components of Ion Chromatograph.

To measure the amount of perchlorate present, all the pretreated samples were analyzed by ion chromatography using a Dionex DX-500 Ion Chromatography system equipped with a CD20 Conductivity Detector, an AS 40 Automated Sampler, and a GP 50 Gradient Pump (Dionex Corp., 2000). Ion separation was made with a Dionex IonPac AS 16 (4 x 250 mm) analytical column. EPA method 314.0 specific to perchlorate was used. This method had a detection limit of 0.53 µg/L and an average recovery range between 85 to 113%. [20]. In this method 100 mN NaOH was used as eluent. A four point standard curve was constructed from constant volume injections of calibration standards of 2 µg/L, 50 µg/L, 500 µg/L, 50 mg/L and 500 mg/L. The calibration standards were made from perchlorate stock standard solution purchased from the manufacturer. Dilution factors were included to the computer generated concentration values to determine the final perchlorate concentration. Each 10 fold diluted sample was analyzed five times to have confidence in the results as the lower range of recovery for EPA method 314.0 was ~85%. Each time a 1 mL sample was taken from the 10 fold diluted sample and was run through the system. In effect, the replicates are the repeated analysis of the same 5 mL sample taken from the treatment flasks.

2.3. Statistical Analysis

The statistical analysis was carried out by dividing the experiment into fertilized treatments and unfertilized treatments. Three balanced one-way analysis of variances (ANOVAs) for completely randomized designs were used to test for changes in perchlorate concentrations, pH values and redox potential values by the fifth week compared to the initial time. A balanced two-way ANOVA was carried out to test the change in perchlorate concentration by the fifth week and the change in perchlorate concentration in sterilized vs. unsterilized soils by the fifth week. A balanced three-way ANOVA was used to test change in perchlorate concentration by the fifth week for control and chicken litter treatments, change in perchlorate concentration by the fifth week for sterilized and unsterilized soil in control and chicken litter treatments, and change in perchlorate concentration for fertilized versus and unfertilized control and chicken litter treatments by the fifth week.

For all ANOVAs, a post hoc Tukey's test was performed to separate means that were statistically different from each other in hypotheses tests with more than two levels. All appropriate interactions between the 12 treatments, two soil conditions, and two fertilizer regimes were also tested to insure appropriate interpretations of the tests on hypotheses of main effects. SAS software version 6.12 [16] was used to run all ANOVAs.

3. Results

3.1. Change in Perchlorate Concentration Over Time

A one-way ANOVA comparing all the fertilized treatments showed a significant difference ($p<0.0001$) among carbon amendments in the change in perchlorate concentration from time 0 to the fifth week. A three-way ANOVA performed on control and chicken litter treatments, which had both fertilized and unfertilized treatments also showed significant difference ($p<0.0001$) among the treatments in the change in perchlorate concentration from time 0 to the fifth week. Table 2 shows mean perchlorate concentrations (mg/L) and standard deviation during the five week experimental period.

3.1.1. In the Fertilized Treatments

In the fertilized treatments, Tukeys' mean separation test showed that there was no significant difference in change in perchlorate concentration by the fifth week among sterilized and unsterilized chicken litter, sterilized starch, sterilized and unsterilized yeast extract (Table 3). However, chicken litter and yeast extract treated flasks with unsterilized soil, reduced perchlorate quicker than the other carbon substrates in the fertilized condition (Table 2). Perchlorate concentrations in the fertilized chicken litter and yeast extract treated flasks with unsterilized soil reached below the detection limit by the second week from an initial (time 0) concentration of 150.3 and 192.4 mg/L respectively (Figure 1A). The next fastest reduction in perchlorate occurred with the chicken litter treatments with sterilized soil. In these flasks, perchlorate concentrations reached below the detection limit by the fourth week (Figure 1B). Another carbon substrate, which degraded perchlorate to below the detection limit was fertilized yeast extract with sterilized soil. In these flasks, perchlorate concentration reached below the detection limit by the fifth week (Figure 1B). In starch treatments with sterilized soil, perchlorate concentrations fell to 34.7 ppm by the fifth week (Figure 1B). The remaining carbon substrates in the fertilized condition (i.e., biosolids, and acetate) did not show significant reduction in perchlorate concentration by the fifth week.

Table 2. Mean perchlorate concentrations (mg/L) by time of incubation under various treatments.

Time (in weeks)	0	1	2	3	4	5
Fertilized Treatments						
Sterilized Control	147.2 ± 0.6	197.0 ± 2.8	207.8 ± 13.6	232.7 ± 41.6	214.2 ± 18.9	177.7 ± 37.1
Unsterilized Control	170.3 ± 10.9	225.6 ± 24.5	216.2 ± 21.7	184.6 ± 15.8	190.8 ± 5.9	186.7 ± 6.5
Sterilized Chicken litter	170.6 ± 9	130.6 ± 43	17.3 ± 51.6	14.8 ± 21.4	BDL	BDL
Unsterilized Chicken litter	192.4 ± 15.5	46.5 ± 102.1	BDL	BDL	BDL	BDL
Sterilized Acetate	144.7 ± 85.7	234.6 ± 86.1	231.6 ± 85.5	187.1 ± 96	202.0 ± 93.4	191.7 ± 76.3
Unsterilized Acetate	158.3 ± 12.2	182.3 ± 100	180.6 ± 33.5	132.3 ± 63.7	191.3 ± 38.6	225.5 ± 17.6
Sterilized Starch	187.6 ± 6.5	214.6 ± 24.6	210.6 ± 42.4	193.7 ± 25	60.8 ± 83.8	34.7 ± 46.2
Unsterilized Starch	160.4 ± 13.8	201.0 ± 32.1	200.0 ± 19.8	170.4 ± 37.9	150.4 ± 33.3	180.1 ± 36.7
Sterilized Biosolids	168.7 ± 31.1	212.5 ± 50.4	199.4 ± 58.9	153.2 ± 52.4	172.9 ± 37.9	149.6 ± 9.8
Unsterilized Biosolids	157.0 ± 16.1	141.8 ± 38.7	156.8 ± 55.8	115.7 ± 61.7	169.4 ± 81.9	145.1 ± 90
Sterilized Yeast extract	181.3 ± 39.3	132.0 ± 58.8	32.3 ± 47	21.5 ± 47.7	14.1 ± 25.3	BDL
Unsterilized Yeast extract	150.3 ± 50.2	17.8 ± 7.9	BDL	BDL	BDL	BDL
Unfertilized treatments						
Sterilized Control	134.6 ± 26.5	231.7 ± 78.3	226.4 ± 43.3	185.8 ± 46.6	196.0 ± 39.4	213.6 ± 8.4
Unsterilized Control	198.1 ± 12.9	221.5 ± 42	220.4 ± 52.1	153.5 ± 36.6	218.7 ± 12.6	262.4 ± 56.3
Sterilized Chicken litter	150.2 ± 36.8	163.8 ± 10.2	91.7 ± 26.3	13.9 ± 22.8	BDL	BDL
Unsterilized Chicken litter	162.0 ± 5.8	BDL	BDL	BDL	BDL	BDL

Values are mean ± standard deviation of five replicates.
BDL = below detection limit of 2 µg /L (the lowest standard used in the standard curve).

Table 3. Tukey's difference in mean change in perchlorate concentrations during experimental period.

	n	Control	Chicken litter	Acetate	Starch	Biosolids	Yeast extract
		Fertilized treatments					
Sterilized	5	31.7 (a,b)	-171.3 (c)	46.9 (a,b)	-146.6 (c)	-19.1 (b)	-181.3 (c)
Unsterilized	5	14.9 (a,b)	-190.1 (c)	67.1 (a)	9.5 (a,b)	-11.4 (a,b)	-164.3 (c)
		Unfertilized treatments					
Sterilized	5	45.3 (a)	-158.1 (b)				
Unsterilized	5	47.4 (a)	-160.4 (b)				

Means in a condition (fertilized or unfertilized) followed by the same letter are not significantly different at the 0.05 level.

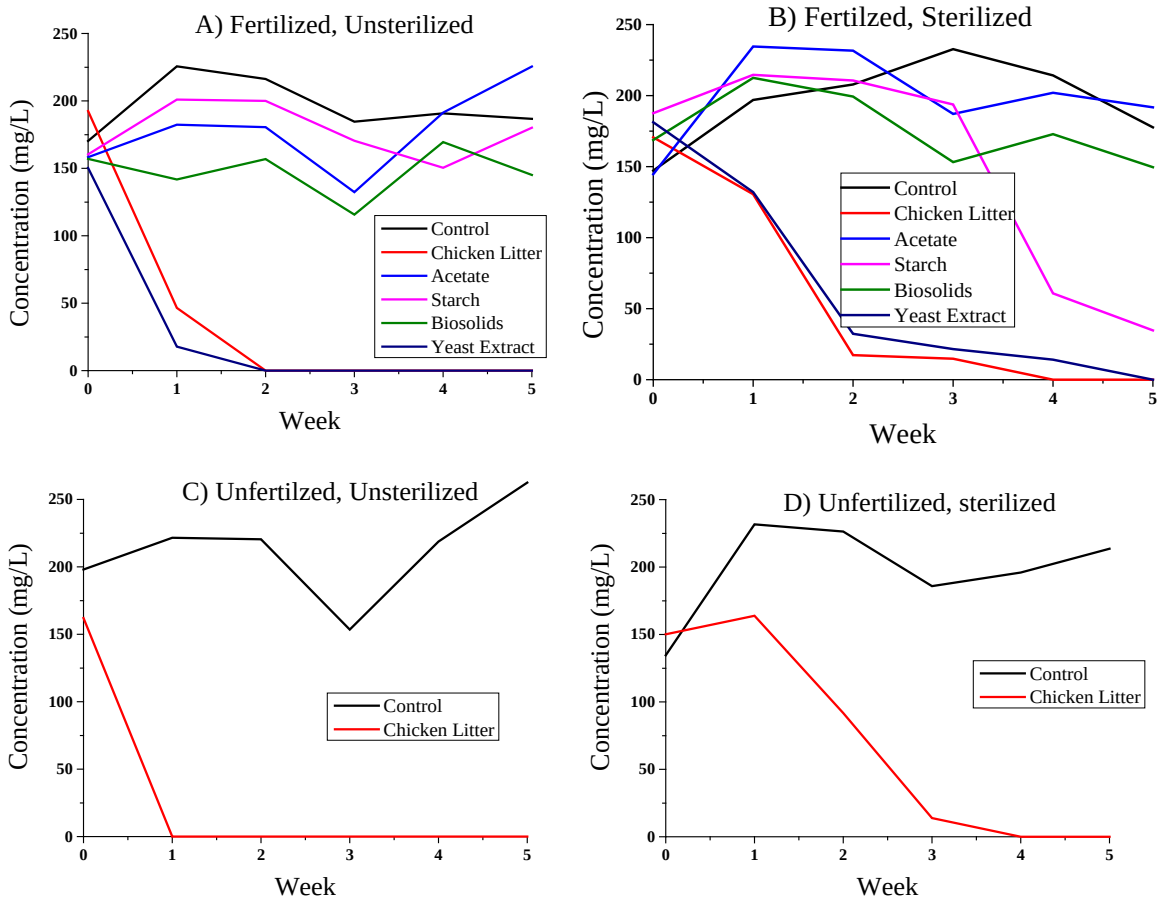

Figure 1. Change in perchlorate concentration during experimental period (Each data point is mean value of five replicates)

3.1.2. In the Unfertilized Treatments

Tukey's mean separation test on the unfertilized treatments showed that there was no significant difference in change in perchlorate concentration by the fifth week between chicken litter treatments with sterilized and unsterilized soil (Table 3). However, chicken litter treated flasks with unsterilized soil were generally faster at degrading perchlorate than chicken litter treatments with sterilized soil (Table 2). In fact, chicken litter treatments with unsterilized soil in the unfertilized condition were the fastest among all the treatments in both fertilized and unfertilized condition in degrading perchlorate. In these flasks, perchlorate concentrations fell below the detection limit by the first week (Figure 1C). However, in the sterilized soil, perchlorate concentrations reached below the detection limit by the fourth week (Figure 1D). On the whole, sterilized and unsterilized soil with chicken litter treatment in both fertilized and unfertilized conditions had the greatest change in perchlorate concentration among all the treatments by the fifth week.

3.2. Evaluation of the Effect of Fertilization on the Bioremediation of Perchlorate

A three-way ANOVA showed that there was significant effect (p=0.03) of fertilization on the change in perchlorate concentration observed by the fifth week from the initial time. The results showed that chicken litter with unsterilized soil in the unfertilized condition appeared to degraded perchlorate somewhat more quickly than chicken litter with unsterilized soil in the fertilized condition (Figure 2). Perchlorate concentration in chicken litter treatments with unsterilized soil in the unfertilized condition decreased below the detection limit within the first week from an initial concentration of 162 ppm. In the fertilized condition, unsterilized chicken litter treated flasks took two weeks to degrade perchlorate to below the detection limit from an initial concentration of 192.4 ppm. In the sterilized soil, perchlorate concentrations in both the fertilized and unfertilized chicken litter treated flasks took four weeks to decrease below the detection limit.

Significant differences in perchlorate reduction were not observed in controls in both fertilized and unfertilized condition in both sterilized and unsterilized soil.

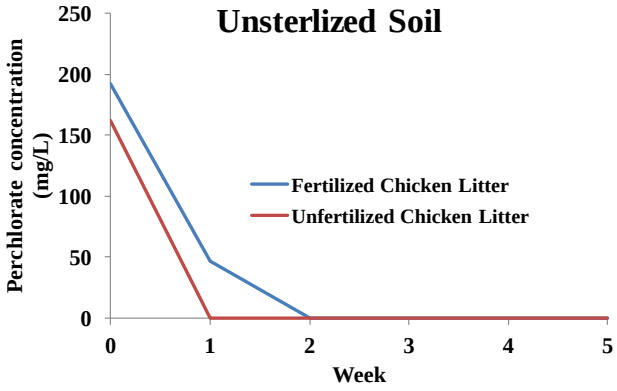

Figure 2. Effect of fertilization on perchlorate degradation

3) Importance of Resident Soil Microorganisms in Perchlorate Reduction

A two-way ANOVA performed on all the fertilized treatments showed that there was a significant effect ($p=0.009$) of soil sterilization on the change in perchlorate concentration observed by the fifth week from the initial time. A three-way ANOVA performed on the control and chicken litter treatments, however, showed that there was no significant effect of sterilization ($p=0.38$) on the change in perchlorate concentration by the fifth week from the initial concentration.

3.2.1. In the Fertilized Treatments

Chicken litter with unsterilized soil degraded perchlorate to below the detection limit by the second week. However, chicken litter with sterilized soil took four weeks to degrade perchlorate to below the detection limit. Perchlorate concentration in the yeast extract treated flasks with sterilized soil reached below the detection limit by the fifth week. Where as in the unsterilized soil perchlorate concentration reached below the detection limit by the second week (Figure 3). Starch treated flasks with sterilized soil degraded perchlorate quicker than starch treatments with unsterilized soil. There was no significant degradation of perchlorate observed in other treatments in both sterilized and unsterilized soil.

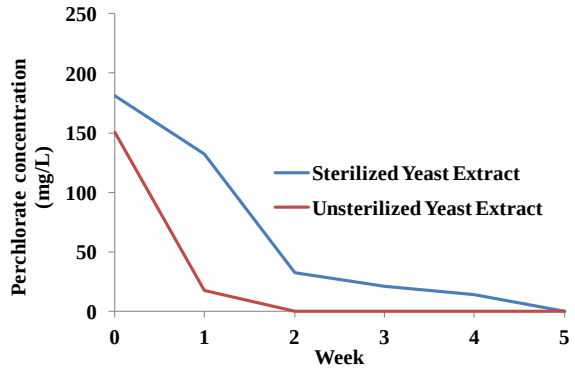

Figure 3. Importance of native soil microorganisms on perchlorate degradation

3.2.2. In the Unfertilized Treatments

Although the three-way ANOVA performed on control and chicken litter treatments showed that there was no significant effect of sterilization on perchlorate degradation, chicken litter with unsterilized soil degraded perchlorate more rapidly than chicken litter in the sterilized soil. Perchlorate in the chicken litter treatments with unsterilized soil reached below the detection limit by the second week, while chicken litter treatments with sterilized soil took four weeks to fall below the detection limit.

3.3. Change in pH and H^+ ion Concentration Values over the Five-week Period

A one-way ANOVA performed on all the fertilized treatments showed that there was a significant effect ($p<0.0001$) of carbon substrate on the change in H^+ ion concentration by the fifth week from the initial time. A one-way ANOVA performed on the unfertilized treatments also showed that there was significant effect ($p<0.0001$) of carbon substrate on the change in H^+ ion concentration, by the fifth week from the initial time. Table 4 shows mean pH values and standard deviations during the experimental period.

3.3.1. In the Fertilized Treatments

Tukey's mean separation test on the fertilized treatments showed that only starch treatments with unsterilized soil differed significantly with other treatments in the change in H^+ ion concentration by the fifth week from the initial time (Table 5). In fact, pH decrease observed in these flasks was the greatest among all the treatments (Figure 4). In these flasks, pH fell to 4.43 by the fifth week from an initial pH of 7 (Table 4). Even though the Tukey's mean separation test showed no significant H^+ ion concentration change by the fifth week in yeast extract treatments, a small increase in pH although not statistically significant was observed in these flasks. In yeast extract treated flasks with unsterilized soil, pH increased to 8.66 by the fifth week from an initial concentration of 6.79 (Figure 4A). This increase in pH was also observed in the sterilized yeast extract treated flasks with sterilized soil. In these flasks, pH increased to 8.1 by the fifth week from an initial pH value of 6.63 (Figure 4B). No significant change in pH was observed in other treatments in the fertilized condition.

3.3.2. In the unfertilized Treatments

Tukey's mean separation test performed on the unfertilized treatments showed that change in H^+ ion concentration by the fifth week in controls with sterilized soil to be significantly different from other treatments in the unfertilized condition (Table 5). In these flasks, pH fell to 6.13 by the fifth week from an initial value of 6.67 (Figure 4C). Tukeys' mean separation test also showed that there was no significant change in H^+ ion concentration by the fifth week in the chicken litter treatments with sterilized and unsterilized soil. The pH in the chicken litter treatments with unsterilized soil decreased slightly to 7.43 from an initial value of 7.89 (Figure 4C). In chicken litter treatments with sterilized soil, the pH also decreased slightly to 7.3 from an initial value of 7.5 (Figure 4D). In the unfertilized condition, there was an initial slight decrease in pH values in all the treatments.

Table 4. Mean pH values by time of incubation under various treatments.

Time (in weeks)	0	1	2	3	4	5
Fertilized treatments						
Sterilized Control	6.61 ± 0.30	5.92 ± 0.6	6.12 ± 0.48	5.31 ± 0.89	5.13 ± 0.51	5.90 ± 0.34
Unsterilized Control	6.95 ± 0.33	6.67 ± 0.18	6.21 ± 0.11	6.60 ± 0.08	6.61 ± 0.03	6.58 ± 0.35
Sterilized Chicken litter	7.32 ± 0.008	6.47 ± 0.1	6.77 ± 0.18	6.96 ± 0.13	7.19 ± 0.08	7.22 ± 0.22
Unsterilized Chicken litter	7.64 ± 0.07	6.56 ± 0.05	6.76 ± 0.1	6.82 ± 0.1	7.30 ± 0.05	7.51 ± 0.06
Sterilized Acetate	6.86 ± 0.06	6.29 ± 0.04	6.70 ± 0.11	6.92 ± 0.09	7.47 ± 0.05	7.70 ± 0.08
Unsterilized Acetate	7.11 ± 0.06	6.61 ± 0.07	7.10 ± 0.09	7.22 ± 0.05	7.48 ± 0.02	7.64 ± 0.04
Sterilized Starch	6.22 ± 0.204	5.24 ± 0.2	5.54 ± 0.17	5.61 ± 0.3	5.81 ± 0.47	5.74 ± 0.5
Unsterilized Starch	7.00 ± 0.337	4.50 ± 0.09	4.55 ± 0.1	4.42 ± 0.08	4.43 ± 0.05	4.43 ± 0.05
Sterilized Biosolids	6.19 ± 0.16	5.67 ± 0.12	5.15 ± 0.12	5.09 ± 0.12	5.17 ± 0.11	5.10 ± 0.1
Unsterilized Biosolids	6.72 ± 0.16	5.96 ± 0.33	5.81 ± 0.27	5.67 ± 0.26	5.69 ± 0.12	5.76 ± 0.22
Sterilized Yeast extract	6.63 ± 0.05	6.94 ± 0.2	7.14 ± 0.41	7.27 ± 0.5	7.68 ± 0.56	8.10 ± 0.57
Unsterilized Yeast extract	6.79 ± 0.04	6.31 ± 0.25	6.99 ± 0.46	7.66 ± 0.18	8.12 ± 0.1	8.66 ± 0.22
Unfertilized treatments						
Sterilized Control	6.67 ± 0.1	5.67 ± 0.13	5.34 ± 0.18	5.27 ± 0.13	5.40 ± 0.04	6.13 ± 0.21
Unsterilized Control	6.93 ± 0.21	6.69 ± 0.11	6.50 ± 0.15	6.54 ± 0.13	6.74 ± 0.07	7.14 ± 0.37
Sterilized Chicken litter	7.55 ± 0.16	6.47 ± 0.19	6.70 ± 0.19	6.85 ± 0.13	7.02 ± 0.21	7.30 ± 0.13
Unsterilized Chicken litter	7.89 ± 0.17	6.46 ± 0.17	6.99 ± 0.08	6.93 ± 0.08	7.26 ± 0.07	7.43 ± 0.20

Values are mean ± standard deviation of five replicates. .

Table 5. Tukey's differences in change in H^+ ion concentration during experimental period.

	n	Control	Chicken litter	Acetate	Starch	Biosolids	Yeast extract
		Fertilized treatments					
Sterilized	5	1377.0 (b)	19.0 (b)	-119.0 (b)	2908.0 (b)	7412.0 (b)	-232.0 (b)
Unsterilized	5	205.0 (b)	7.0 (b)	-55.0 (b)	29898.0 (a)	1686.0 (b)	-159.0 (b)
		Unfertilized treatments					
Sterilized	5	590.2 (a)	21.8 (b)				
Unsterilized	5	-34.6 (b)	26.7 (b)				

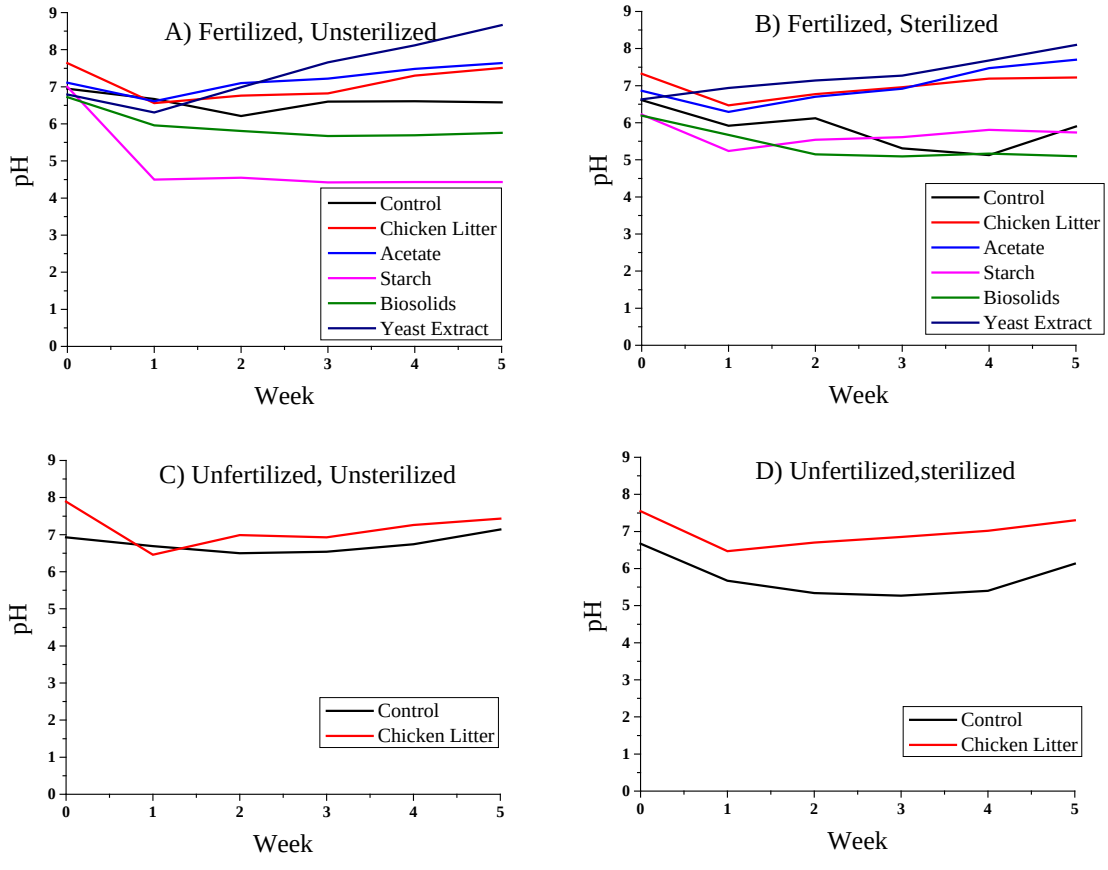

Figure 4. Change in pH values during experimental period

3.4. Change in Redox Potential Values over the Five-week Experimental Period

A one-way ANOVA performed on all the fertilized treatments showed that there was a significant effect ($p<0.0001$) of carbon substrate used on the change in redox potential values observed by the fifth week from the initial time. A one-way ANOVA performed on the unfertilized treatments also showed that there was a significant effect ($p<0.0001$) of carbon substrate used on the change in redox potential values observed by the fifth week from the initial time. Table 6 shows mean redox potential values (mV) and standard deviation during the experimental period.

3.4.1. In the Fertilized Treatments

A Tukey's mean separation test performed on the fertilized treatments showed that there was no significant difference in redox potential values by the fifth week from the initial time among yeast extract treatments with sterilized and unsterilized soil, chicken litter treatments with sterilized soil (Table 7). Among all the treatments, the greatest decrease in redox potential value was observed in the yeast extract treated flasks with unsterilized soil in the fertilized condition (Table 6). In these flasks, the mean redox potential values reached –432.6mV by the fourth week from an initial value of 169.5 mV. But a slight increase in redox potential was observed from the fourth to fifth week (Figure 5A). This decrease in redox potential value was also observed in the yeast extract treated flasks with sterilized soil. The next largest decrease in redox potential values was observed in the chicken litter treated flasks with the sterilized soil (Figure 5B). In these flasks, redox potential values reached –363.4 mV by the fifth week from an initial value of 139.3 mV. This decrease in redox potential was also observed in the chicken litter treated flasks with unsterilized soil. In these flasks, redox potential values reached –344.1 mV by the fourth week. But an increase in redox potential was observed between the fourth and fifth week (Figure 5A). Among other fertilized treatments, notable decrease in redox potential was observed in the starch treatments in the unsterilized soil and in the acetate treated flasks in both sterilized and unsterilized soil. Significant decrease in redox potential values was not observed in the control and biosolids treated flasks in the fertilized condition.

3.4.2. In the Unfertilized Treatments

Tukey's mean separation test performed on the unfertilized treatments showed that change in redox potential by the fifth week from the initial time was not significantly different between chicken litter treatments with sterilized and unsterilized soil (Table 7). In the unsterilized soil, redox potential values in the chicken litter treated flasks reached –381.5 mV by the fifth week (Figure 5C). In the sterilized soil, redox potential values in the chicken litter treatments reached –387.3 mV by the fifth week (Figure 5D). The Tukey's mean separation test also showed that there was significant difference in change in redox potential by the fifth week from time 0 between control with sterilized soil and control with unsterilized soil (Table 7). In the unsterilized soil, redox potential values in the chicken litter treated flasks reached –381.5 mV by the fifth week from an initial value of 158.7 mV (Figure 5C). In the sterilized soil, redox potential values in the chicken litter treated flasks reached –387.3 mV by the fifth week from an initial value of 116.8 mV (Figure 5D).

Table 6. Mean redox potential values (mV) by time of incubation under various treatments.

Time (in weeks)	0	1	2	3	4	5
Fertilized treatments						
Sterilized Control	192.6 ± 6.3	221.4 ± 41.6	220.8 ± 23.7	181.6 ± 25.2	246.3 ± 36.2	243.0 ± 16.4
Unsterilized Control	195.7 ± 9.3	111.5 ± 24.8	110.4 ± 16.3	119.1 ± 16.1	164.2 ± 12.2	143.3 ± 17.9
Sterilized Chicken litter	139.3 ± 3.2	-211.5 ± 39.6	-234.2 ± 50.0	-280.2 ± 33.5	-296.7 ± 68.6	-363.4 ± 58.5
Unsterilized Chicken litter	172.4 ± 1.6	-234.0 ± 6.5	-306.0 ± 10.9	-332.1 ± 18.5	-344.1 ± 13.2	-178.4 ± 109.4
Sterilized Acetate	199.8 ± 2.5	152.0 ± 28.9	-118.8 ± 41.6	-35.8 ± 26.3	-70.2 ± 33.7	-57.7 ± 27.0
Unsterilized Acetate	217.4 ± 1.7	38.0 ± 6.7	-92.0 ± 22.3	-30.2 ± 54.0	-173.1 ± 108.8	-84.3 ± 94.9
Sterilized Starch	219.7 ± 6.2	43.4 ± 124.9	47.0 ± 11.0	129.5 ± 52.5	109.4 ± 65.0	12.5 ± 136.9
Unsterilized Starch	215.3 ± 9.1	-49.7 ± 140	83.5 ± 91.6	-98.5 ± 242.0	23.1 ± 156.0	-66.2 ± 127.2
Sterilized Biosolids	186.4 ± 4.8	164.3 ± 34.5	120.2 ± 15.8	206.0 ± 27.1	187.1 ± 30.6	172.8 ± 14.4
Unsterilized Biosolids	232.5 ± 3.8	116.5 ± 20.4	113.4 ± 45.1	111.6 ± 11.4	124.2 ± 27.7	132.2 ± 15.4
Sterilized Yeast extract	132.2 ± 4.3	-204.6 ± 66.5	-359.9 ± 41.8	-387.3 ± 59.3	-388.2 ± 71.9	-417.8 ± 92.1
Unsterilized Yeast extract	169.5 ± 4.3	-181.0 ± 42.1	-293.9 ± 67.5	-238.0 ± 76.1	-432.6 ± 9.3	-404.8 ± 97.0
Unfertilized treatments						
Sterilized Control	186.3 ± 6.3	267.7 ± 17.1	225.3 ± 9.0	167.4 ± 6.8	261.3 ± 5.6	248.0 ± 2.8
Unsterilized Control	199.0 ± 7.6	142.7 ± 10.3	53.7 ± 15.9	118.6 ± 8.2	120.5 ± 10.3	120.2 ± 55.6
Sterilized Chicken litter	116.8 ± 16.8	-179.8 ± 69.3	-268.2 ± 48.3	-227.6 ± 96.9	-302.4 ± 53.1	-387.3 ± 28.2
Unsterilized Chicken litter	158.7 ± 5.4	-183.4 ± 4.0	-243.7 ± 73.2	-295.3 ± 29.4	-258.4 ± 56.0	-381.5 ± 15.9

Values are mean ± standard deviation of five replicates.

Table 7. Tukey's differences in mean change in redox potential values during experimental period.

	n	Control	Chicken litter	Acetate	Starch	Biosolids	Yeast extract
		Fertilized treatments					
Sterilized	5	50.3 (a)	-502.7 (e,f)	-257.5 (c,d)	-207.2 (b,c,d)	-13.6 (a)	-550.2 (f)
Unsterilized	5	-52.4 (a,b)	-350.8 (e,d)	-301.7 (d)	-281.6 (d)	-100.2 (a,b,c)	-574.3 (f)
		Unfertilized treatments					
Sterilized	5	61.7 (a)	-504.2 (c)				
Unsterilized	5	-78.8 (b)	-540.3 (c)				

Means in a condition (fertilized or unfertilized) followed by the same letter are not significantly different at the 0.05 level.

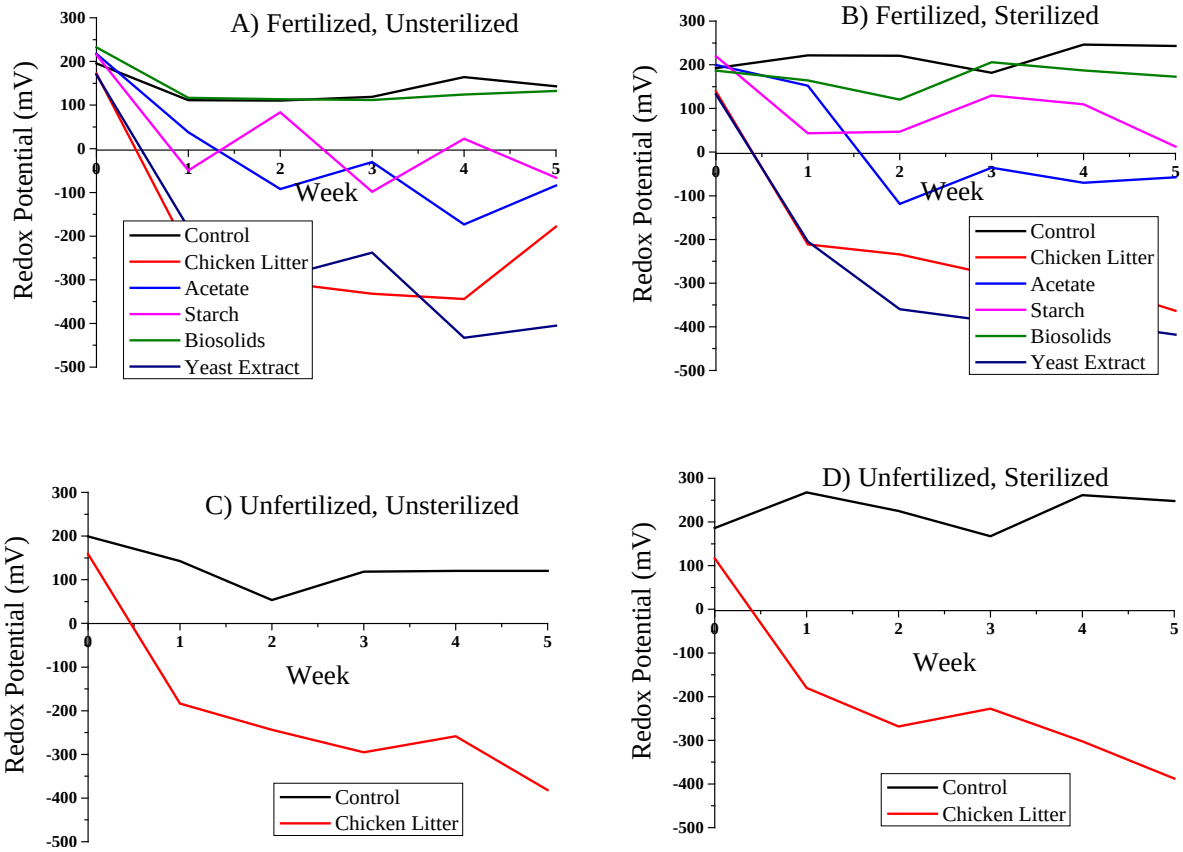

Figure 5. Change in redox potential (mV) values during experimental period

4. Discussion

4.1. Change in Perchlorate Concentration over Time

The high fluctuation in measurements, especially at time 0 could be due to poor filtration of samples that could have resulted in interference by total dissolved solids (TDS), which is a drawback that has been observed when using USEPA Method 314.0. Although, USEPA Method 314.0 is the most widely used and least expensive commercially available perchlorate detection method, it involves many sources of uncertainty. Some of these include non-specificity for perchlorate, possible interferences and absence of systematic validation in matrices other than potable water [3]. Newer methods such as USEPA Methods 314.1, 314.2, 331.0 and 332.0 do not have these limitations and could be used in studies that involve matrices other than drinking water. The results of the one-way ANOVA on all the fertilized treatments and three-way ANOVA on control and chicken litter support the finding that some of the carbon amendments acting as electron donors promoted perchlorate reduction. The results showed that unfertilized chicken litter treatments with unsterilized soil were the best combination for degrading perchlorate. Chicken litter is a very good carbon substrate that supports rapid microbial growth. The efficiency of chicken litter in degrading perchlorate is likely due to the availability of large amounts of easily metabolized organic material, nutrients (both macro and micronutrients) and microbes. The presence of pine shavings in the chicken litter also serves as a carbon source and might reduce toxicity caused by the accumulation of ammonia. Nitrates in the chicken litter might have also stimulated development of denitrifying bacteria, which are capable of degrading perchlorate. In a previous study by Envirogen Inc., [7] it was found that the degradation of perchlorate was more rapid in samples that were initially amended with nitrates compared to those that did not receive the anion. The next most effective carbon sources were chicken litter and yeast extract treatments with unsterilized soil in the fertilized condition. The efficiency of yeast extract as carbon amendment could be due to the availability of large amounts of

nutrients to the microorganisms in the form of carbon, nitrogen, amino acids, and vitamin factors. Yeast extract has high amounts of nitrogen, different amino acids like aspartic acid, glutamic acid, isoleucine, lysine, valine etc., and a variety of vitamin factors like pyridoxine, nicotinic acid, riboflavin which might have helped in the growth of microorganisms, especially bacteria. High protein organic substrates like yeast extract are better at supporting perchlorate reduction than non-protein carbon sources [10].

The next most effective carbon sources were fertilized chicken litter and unfertilized chicken litter in the sterilized soil. The result that chicken litter treatments in the unsterilized soil were more efficient than chicken litter treatments in the sterilized soil shows that native microbes present in the soil contributed early to the degradation of perchlorate. However, microbes present in the chicken litter or those that were introduced inadvertently later eventually reduced the perchlorate given a little more time. Another effective carbon source was found to be fertilized yeast extract with sterilized soil. Perchlorate degradation in these flasks was probably was carried out by microbes introduced with the addition of the carbon amendments. Once in the flasks, the microbes had access to the high protein and nutrient rich carbon amendment, which allowed them to multiply rapidly and reduce the perchlorate. The next most effective carbon source was found to be fertilized starch treatments in the sterilized soil. The better efficiency of starch in the sterilized soil to starch in the unsterilized soil was unexpected. Starch fermentation has a tendency to decrease pH and this was evident in the study. Biosolids and acetate treated flasks did not show notable reduction in perchlorate by the fifth week. Non-degradation of perchlorate in the biosolids treated flasks could be because of the composted nature of the biosolids used in the experiment. During the composting process, bacteria and other microbes breakdown and feed on the organic material present in the biosolids. These processes cause the temperature of the compost to rise to 55°C at which many of the microbes present might have died. Also the microbes probably used up the easily metabolized organic substrates, leaving more resistant organic molecules behind. Absence of sufficient number of microbes and nutrients could be the reason for non-performance of biosolids in perchlorate reduction. Though, fresh sewage sludge would probably be more successful in promoting perchlorate reduction, it would be more risky to use because of the presence of human pathogens. Though Tipton et al., [17] found that sodium acetate degraded perchlorate more effectively than glucose, with a reduction of perchlorate from 50 mg/L to below the detection limit within 15 days, sodium acetate did not degrade significant quantities of perchlorate by the fifth week in this study. Non-performance of sodium acetate may have been because of sodium toxicity to the bacteria.

4.2. Evaluation of the Effect of Fertilization on the Bioremediation of Perchlorate

The reason for testing the fertilization effect only on chicken litter amendment and not on other carbon amendments is due to the presence of significant amount of macro and micronutrients inherently in the chicken litter. This test was done to see if addition of extra nutrients added with chicken litter was necessary for better reduction of perchlorate. The results of 3-way ANOVA showed that there was actually a significant negative effect of fertilization on the biodegradation of perchlorate in the control and chicken litter treatments. Unfertilized chicken litter in the unsterilized soil degraded perchlorate more efficiently than fertilized chicken litter in the unsterilized soil. This can be explained as possibly due to the adverse effect of excess amount of nitrogen added as fertilizer in addition to that already present in the chicken litter. The nitrogen present might have been converted into ammonia by the microbes resulting in ammonia toxicity to the microbes. However, in the unfertilized condition this extra nitrogen was not available for conversion to ammonia.

4.3. Importance of Resident soil Microorganisms in Perchlorate Reduction

The results of the two-way ANOVA performed on all the fertilized treatments showed that soil microorganisms are necessary for rapid perchlorate reduction to take place. The better efficiency and faster reduction of perchlorate in the fertilized chicken litter treatments in the unsterilized soil was due to the reduction of perchlorate by the microbes present in the soil which were well adapted to the degradation of perchlorate. However, perchlorate reduction in the fertilized chicken litter treatments in the sterilized soil was due to the microbes present inherently in the chicken litter or perhaps inadvertently introduced later. Though these microbes were able to degrade perchlorate to below the detection limit by the fourth week, it was much slower and less efficient than with the chicken litter treatments in the unsterilized soil due to the absence of microbes adapted to perchlorate reduction. The same explanation can be given for quicker reduction of perchlorate in yeast extract treatments with unsterilized soil to yeast extract treatments with sterilized soil. The reason for perchlorate degradation observed in the yeast extract treatments in the sterilized soil was probably due to degradation by microbes, which were introduced into the flasks along with yeast extract. The reason for the results of the three-way ANOVA showing that there was no effect of soil sterilization on perchlorate degradation is due to the degradation observed even in the chicken litter treatments with sterilized soil. This degradation may be due to the microbes inherently present in the chicken litter or those that have entered inadvertently later. This is because perchlorate-reducing bacteria are ubiquitous in nature and are present in diverse ecosystems. Sterilization had no effect in the controls due to absence of carbon amendments.

4.4. Change in pH Over the Five-Week Experimental Period

The results of one-way ANOVA in both fertilized and unfertilized conditions showed that the carbon amendments had an effect on pH by the fifth week from the initial time. The observation of greatest decrease in pH in starch treated flasks was unexpected. It could be due to the preferential and rapid fermentation of starch by lactate producing bacteria causing a decline in pH. This explanation is further supported by the result that this decrease was observed only in the unsterilized soil where

lactate producing bacteria were probably present. The greatest increase in pH was observed in the yeast extract treated flasks in the unsterilized soil. The probable reason for this increase could be ammonia production by microbes growing on protein rich carbon sources like yeast extract. Ammonia production by microbes growing on protein rich carbon substrates could be due to dissimilation of amino nitrogen produced in excess of that required for growth [15]. The significant difference in pH by the fifth week in the unfertilized controls with sterilized soil (Table 4) is not understood and needs to be further explored. It was observed in this study that acidic conditions reduce perchlorate degradation. Although starch is a simple carbon substrate, which can be easily metabolized, perchlorate degradation was not observed in the starch treatments with unsterilized soil due to development of acidic conditions in these flasks. This finding was consistent with previous findings where perchlorate degradation was not carried out in acidic conditions. It was previously found that perchlorate-degrading bacteria are able to survive at low pH, but do not degrade perchlorate at this pH. So, it was suggested that there might be a pH below which perchlorate biodegradation is physiologically inhibited [17].

4.5. Change in Redox Potential Values over the Five-week Experimental Period

The results of one-way ANOVA performed on all the fertilized and unfertilized treatments showed that the carbon substrates used had a significant effect on change in redox potential values. The proportion of the oxidized to reduced compounds constitutes the redox potential (Eh) value of a solution. As the reduced compounds in a solution increases, the redox potential value decreases. In this work, as the redox potential value decreased, perchlorate ion was reduced. As the reduction of perchlorate ion is dependent on the carbon substrate potential for rapid decomposition, the redox potential value is also dependent on the decomposition potential of the carbon source used. So, more easily degraded carbon sources tend to decrease redox potential to negative values more quickly than more complex carbon sources and therefore cause reduction of perchlorate more quickly. Accordingly, easily degradable and more organic carbon sources like chicken litter and yeast extract had lower redox potential values than less readily metabolized substrates like composted biosolids and sodium acetate. Once the easily metabolized components of the organic substrate are used up, the redox potential values begin to increase again. Large amounts of carbon and nitrogen present in chicken litter and yeast extract treated flasks might have also helped in decreasing the redox potential values in these flasks.

5. Conclusions

Perchlorate contamination of the environment is a major concern. Methods aimed at reducing this compound efficiently and economically are needed. There is a need for testing different carbon amendments including liquid amendments in perchlorate bioremediation. However, the selection of carbon amendments has to be made taking into consideration economic aspects and environmental compatibility. The results of this bench scale research project showed that chicken litter was a good carbon amendment leading to the reduction of perchlorate. It is recommended that the results of this research project be tested in the field, as the use of chicken litter in reducing perchlorate is economically advantageous particularly in regions where poultry farms are prevalent. The results of this study suggest that carbon substrates with high protein content, which are easily metabolized and promote rapid cell growth and rapid decrease in redox potential are ideal for use in bioremediation of perchlorate. The other important finding of this research was the better efficiency of unfertilized chicken litter than the fertilized chicken litter in the unsterilized soil. This finding saves cost of fertilizers and also avoids unnecessary application of chemical fertilizers. In future research, a microbiological study to identify and characterize how the differences in carbon amendments might affect the microorganism communities can be explored. Another important area that can be studied is the ability of different soil types in binding perchlorate and making it unavailable for measurement.

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