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DEGRADATION OF CHLOROPHENOLS IN SWINE WASTE

A Thesis
Presented to
The Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Srilatha Gangula

May 2010

DEGRADATION OF CHLOROPHENOLS IN SWINE WASTE

Date Recommended April 15, 2010

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Finally, I dedicate my thesis to **My Parents** and **My Husband.**

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LIST OF ABBREVIATIONS AND SYMBOLS

General

Cm	Centimeter
CO_2	Carbondioxide
CH_4	Methane
$^{\circ}C$	Celsius
DI	Deionised
EPA	environmental protection agency
FID	flame ionization detector
GC	gas chromatograph
HPLC	high performance liquid chromatograph
HS-SPME	head space solid-phase microextraction
LLE	liquid liquid extraction
Ml	Milliliter
µm	Micrometer
µl	Microliter
µg/L	microgram per liter
mg/L	milligram per liter
N_2	Nitrogen
PCP	Pentachlorophenol
PCDD	polychlorinated dibenzodioxins
PCDF	polychlorinated dibenzofurans
PCB	polychlorinated biphenyls

PDMS	polydimethyl siloxane
PDVB	polydivinyl benzene
PA	Polyacrylate
Ppm	parts per million
Ppmv	parts per million by volume
Psig	pounds per Square Inch Gauge
Psi	pound per square inch
Rpm	revolutions per minute
$socl_2$	sulfuryl chloride
SPME	solid phase microextraction
SPE	solid phase extraction
TR	template poly resin
TCDD	tetrachloro dibenzodioxin

DEGRADATION OF CHLOROPHENOLS IN SWINE WASTE

Srilatha Gangula May 2010 51 Pages

Directed by: Dr. Eric D Conte

Department of Chemistry Western Kentucky University

Naturally occurring plant derived phenols can be degraded through bacteria in swine waste. Chlorinated phenols, which are not naturally present in the environment, are toxic and generated from industrial activities as such petrochemical, pharmaceutical, plastic, rubber, pesticide, iron, steel, paper production, coal conversion, wood preserving, and cellulose bleaching. Large scale coal gasification and carbonization plants are another source of chlorinated phenols. Although not normally present in the environment, chlorinated phenols are structurally similar to many plant derived phenolics.

It is our hypothesis that bacteria located in swine wastes may also have the ability to degrade chlorinated phenols. Identifying situations (and organisms) in which degradation of pollutants occurs is important field of research.

Experimental work was focused on measuring the degradation of seven chlorinated phenols in swine waste using solid-phase micro-extraction (SPME) and gas chromatography(GC). Microbes in the waste perform respiration or fermentation to obtain the energy they need to carry out their life processes. Fermentation is a process in which electrons are transferred from one organic substrate to another and which results in incomplete degradation of organic compounds. Anaerobic respiration is a process in which organic substrates are degraded completely to CO_2, but using substances other than oxygen as the terminal electron acceptor (such as Fe(III), NO_3^- or SO_4^{2-}). Anaerobic

respiration using these alternative electron acceptors provides an easier pathway for degradation of aromatics than fermentation alone. Usually the abundance of these electron acceptors in waste is low since microbes consume them readily and thus they must be added to the mixture. Our work focused on development of methods for the quantification of chlorinated phenols in swine wastes and results of bioremediation research.

In this study, chlorophenols were extracted by SPME and analysed by GC. This research project mainly focused on the anaerobic degradation of chlorophenols in swine waste. It was observed that the decreased concentration of the chlorophenols was likely due to partitioning of the chlorophenols to solids, sticking to glass bottles and by bacteria present in the swine waste.

In summary, it was observed that by ANOVA and gas production analysis 2,6-dichlorophenol and 2,4,5-trichlorophenol were likely to be degraded by bacteria present in swine waste.

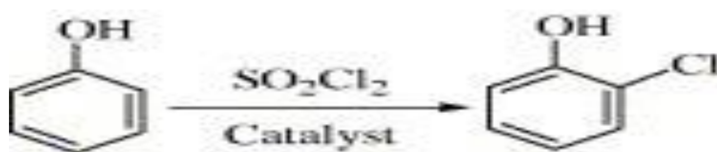
I. INTRODUCTION

Phenols simple, which are non toxic, can result from the degradation of plants into tannin-like compounds. These tannin compounds are responsible for the flavor and color of potable water and also have natural occurrence in the environment. Alkyl phenols are used in the manufacture of nonionic surfactants used in detergents.[1] In contrast, the unusual phenolic compounds are chlorinated phenols, being very toxic and caustic, for that reason they are considered priority pollutants.[2] These pollutants are present in the waste waters which are generated from industrial activities as such petrochemical, coal conversion, pharmaceutical, wood preserving, plastic, rubber proofing, pesticide use, iron, steel, paper and cellulose bleaching. Large scale coal gasification and carbonization plants are also the waste water generators containing large quantities of highly toxic phenolic compounds. The United States Environmental Protection Agency regulates the content of phenols in waste water from less than 1mg/L to the several thousand mgs/L.[2] Chlorophenols are formed as the byproducts of many industrial activities such as chlorination of drinking water, production of antioxidants, dyes and drugs.[3]

Chlorophenols are organic chemicals formed from phenol (1-hydroxybenzene) by substitution in the phenol ring with one or more chlorine atoms.[4]

Phenol reacts with sulfuryl chloride in the presence of a catalytic amount of t-butylaminomethyl polystyrene which is a heterogeneous amine catalyst, in a nonpolar solvent and proceeds with high conversion (~98%) and with high selectivity (~89%) to

ortho-chlorophenol. The catalyst used in this process is stable under these chlorination conditions, and it can be easily regenerated by filtration and reused.[4]



Chlorophenols are a group of 19 compounds ranging from monochlorophenols to the fully chlorinated pentachlorophenol (PCP). Some chlorophenols, particularly trichlorophenols, tetrachlorophenols and PCP are also available as sodium or potassium salts increasing acidity. These different chlorinated phenols differ in degree and position of chlorination.[5] These compounds consist of mono-, di-, tri-, and tetrachloro isomers and PCP.

The chlorinated phenols are effective disinfectants, antiseptics, fungicides, slimicides, bactericides, wood preservatives, herbicides, insecticides and molluscicides. In industry and agriculture, the widespread use of these chlorophenols resulted in contamination of food producing animals and the environment. Byproducts of water chlorination resulted in the inadvertent formation of 2-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol.[5] 2-chlorophenols is a commercially produced chemical which is used as an intermediate in the production of higher chlorinated phenols.[5] PCP and its salts have been widely used in industry and agriculture since 1936. Approximately 200 million pounds of PCP were produced worldwide in 1977.[5]

Though its use has been restricted after 1980's, it is widely distributed in the environment and considered as a chemical pollutant of concern. Main pathways for human exposure to this compound are air and food chains, and it is possible to determine

significant concentrations of PCP in the plasma of various groups including in newborns. Industrial exposure and consumption of contaminated water and food are the principal sources of exposure for humans.[5] Acute toxicity of PCP cause damage to various organs and chronic exposure results in severe health disorders. It was reported that in blood, concentrations of PCP of exposed individuals can vary widely, and the values ranging from 1.5μg/L to 90μg/L. Also, PCP is distributed widely in the tissues and has a relatively long elimination half-life. Though PCP is not directly classified as a human carcinogen, in animal studies it has shown to be involved in the initiation and/or promotion of carcinogenesis.[6]

One of the most well-known hazardous chlorinated compounds is 4-chlorophenol, during anaerobic degradation 4-chlorophenol is released into the environment. As 4-chlorophenol cannot be easily degraded under anaerobic conditions, it accumulates in the environment.[7]

Once the chlorophenols are released into the environment, they are subjected to a series of physical, chemical, and biological transformations. Processes that govern the fate and transport of chlorophenols include sorption, volatilization, degradation and leaching. A major factor affecting the fate and transport of chlorophenols is pH in water, soil and sediment, since the degree of ionization of the compounds increases with increasing pH. As the number of chlorine atoms increase, chlorophenols vapor pressure decreases, boiling point increases and water solubility also decreases.[8] Therefore, the tendency of chlorophenols to partition into sediments and lipids and to bioconcentrate increases with increasing chlorination. Chlorophenols are subject to abiotic and biotic processes, including photodegradation, volatilization, plant and animal uptake. A major

factor that controls the above processes is the distribution of the pollutant between the solid phase and dissolved phase which is governed by the physical and chemical properties of the solute, the sorbent, and the solvent.[8]

Treatment of Chlorophenols:

There are different physicochemical and biological methods which are proposed to treat chlorophenols, including 4-chlorophenols, such as chemical oxidation, aerobic and anaerobic biologic degradation, and activated carbon adsorption. Physicochemical techniques are very expensive and also do not yield complete purification and thus a post treatment process is required to degrade the pollutant and undesirable byproducts from the contaminated environment.[7]As the chlorophenols show inhibitory effect on microbial metabolism, biodegradation of these compounds by conventional activated sludge systems is usually slow and also fails to achieve greater efficiency in removing them from waste water. Another method for practical remediation of these pollutants is biodegradation by way of direct application of adapted microorganisms which are capable of degrading chlorophenols. As the bacteria need organic nutrients for growth and for degrading pollutants, addition of nutrients to the polluted area is required.[7] This makes practical remediation of pollutants at low concentrations difficult by the bacterial method. As physicochemical and biologic treatments of contaminants are expensive and result in incomplete purification and hazardous byproduct formation, new remediation techniques have been developed in which advanced oxidation processes are reported as one of the techniques for the degradation of chlorophenols in water and soils.

Advanced oxidation technique also involves great cost and large amounts of reactants are required for the degradation of chlorophenols. Recently, enzymes such as

peroxidases have been used in many remediation processes to target specific pollutants for treatment. In comparison to biologic or physicochemical methods, this technique has more advantages. Here, handling and storage of isolated enzymes are easier than microorganism manipulation and concentration of enzyme is not simply related to bacterial growth. Moreover, conventional methods are not that selective, where as specificity of isolated enzymes is greater when compared to other catalysts.[7] In addition, formation of insoluble polymers during enzymatic removal gets precipitated and is separated by simple filtration or flocculation. However, this treatment has not been applied for large scale industries because of the adsorption of enzyme molecules on end product polymers that cause losses in enzymatic activity and high cost of enzymatic treatment. Finally it was found that plant materials are useful for the decontamination of the phenolic compounds which are present in water, during which the detoxification effect was caused by enzyme peroxidases present in plant tissues.[7]

Roper et al. explained that by using horseradish roots as plant material has shown good substrate specificity to treat waters contaminated with phenols and anilines and was found to be useful in wide array of potential waste-treatment applications.[7] Though the plant materials from agricultural wastes can be used as enzyme source, which is inexpensive, a toxicologic test was not conducted to ascertain whether or not plant materials release potentially hazardous compounds into the water. Also, significant amounts of plant material are needed to treat the contamination and also handling of plant materials might be another environmental problem.[7]

Chlorophenols can be modified by plants and actinomyces, often making them more soluble and to degrade easily by other microorganisms. Thus, phyto remediation,

which has proven to be an effective and economic way of treating recalcitrant contaminants, might also be used to treat chlorophenols, especially at low concentrations, when degradation by bacteria is not feasible.[7]

Chlorinated phenols are used in forest industries and also they are used as disinfectants. These chlorinated phenols are the chemicals which were widely produced and are wide spread in our environment and have a long half-life. Physicochemical properties of these chemicals depend on the electron donor-acceptor behavior of the hydroxyl group in solution. The pKa values of chlorinated phenols are such that they dissociate partially in aqueous solution. The value of pKa depends on the number and position of the chlorine atoms on the aromatic ring.[9]

Another source of ground water and surface water contamination is the leaching of these chlorinated phenols from point sources, such as landfills. As the chlorinated phenols are toxic to humans and most aquatic organisms and because they bioaccumulate in the food chain, their presence in the environment is a particular concern.[10] Toxicity of chlorinated phenols depends on the total number of chlorine atoms present in the molecule with PCP being the most toxic. Even in low concentrations, phenols can impart an unpleasant taste and odor to drinking water and food products. Chlorinated phenols are classified by EPA as priority pollutants due to their toxicity and carcinogenicity.[10]

Chlorinated organic byproducts are commonly formed during the combustion of chlorine-containing materials. Industrial process like waste incineration, nonferrous metal smelting, cement kiln combustion and distributed combustion processes like open burning and brush fires are identified as sources of hazardous organochlorides, such as polychlorinated dibenzodioxins (PCDD), polychlorinated dibenzofurans (PCDF), and

polychlorinated biphenyls (PCB).[10] Chlorinated organic pollutants are environmentally persistent compounds and they tend to accumulate in living organisms because of their high lipid solubility.[11] Acute exposure to PCDD, PCDF, and PCB in humans causes skin lesions and abnormalities of liver and nervous system functions. Long-term effects of acute exposure include an increased frequency of certain kinds of cancers.[11]

Laboratory experiments have shown that low temperatures are more favorable for the formation of some of the more complex and hazardous organo chlorine pollutants PCDD and PCDF from organic chlorinated precursors (chlorobenzenes and chlorophenols) via homogeneous and catalytic pathways. Field measurements of the exhaust from combustors fed with different organic fuels have shown that PCDD and PCDF levels are frequently associated with high concentrations of chlorophenols and chlorobenzenes.[11] Chlorination of organic species is thermodynamically favored only at intermediate and low temperatures (700°C) and under conditions of excess oxygen. Born et al. studies have shown that gas-phase reactions of benzene, oxygen, and hydrochloric acid can form trace amounts of chlorobenzenes and chlorophenols at temperatures between 450^{0}C and 900^{0}C but only in the presence of large reactant concentrations and at long residence times–conditions which are not representative of practical combustors.[11]

In recent years, many phenolic compounds have been discharged into water, soil and sediments from a variety of industrial activities. Most commonly discharged products and are toxic to many organisms and the environment are phenols, alkylphenols, chlorophenols and nitrophenols.[12] Burttschell et al. also determined the maximum dilution for the chlorophenols at which odor can be detected by an individual with an average olfactory sensitivity. This is known as threshold odor concentration.

Additionally, Burttschell noticed that 2-chlorophenol, 2,4-dichlorophenol and 2,6-dichlorophenol are the compounds found to have the strongest organoleptic properties and also found that these were detectable at concentrations of 2 to 3 μg/L. In contrast, phenol, 4-chlorophenol, 2,4,6-trichlorophenol were detectable only at much higher concentrations.

Consequently, from Burttschell's studies it appears that 2-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol are the compounds primarily responsible for the chlorophenolic tastes and odors in water supplies arising from the chlorination of phenol. In their important study, chlorophenols formed from the chlorination of phenol in dilute aqueous solutions were for the time isolated and identified.[13] From the knowledge of how chlorophenols are formed, they proposed a reaction scheme to account for the production and subsequent elimination of chlorophenolic tastes and odors in water supplies that arise from the phenol chlorination. According to Burttschell et al, chlorination of phenol proceeds by a step wise substitution of 2, 4 and 6 positions of the aromatic ring. In the first step, phenol is chlorinated to form either 2-chlorophenol or 4-chlorophenol. Then 2-chlorophenol is chlorinated to either 2,4-dichlorophenol or 2,6-dichlorophenol, while 4-chlorophenol is chlorinated further to form 2,4-dichlorophenol. In the third step, both 2,4-dichlorophenol and 2,6-dichlorophenol are chlorinated to form 2,4,6-trichlorophenol. In the final step 2,4,6-trichlorophenol reacts with aqueous chlorine to form a mixture of non-phenolic oxidation products.[13]

From Burtschell's experiments it was found that the rate of reaction of aqueous chlorine and phenol or the chlorophenols formed obey a second-order rate expression in which the rate of reaction is proportional to the product of the formal concentrations of

aqueous chlorine and phenolic compound. The rates of these reactions is highly pH dependent with the maximum rate occurring, dependent on the compound being chlorinated, in a neutral or slightly alkaline pH range. From the results of his experiments, it was found that chlorination of phenol-bearing waters should be conducted with the maximum possible free chlorine of pH ranging from 7-8.[13]

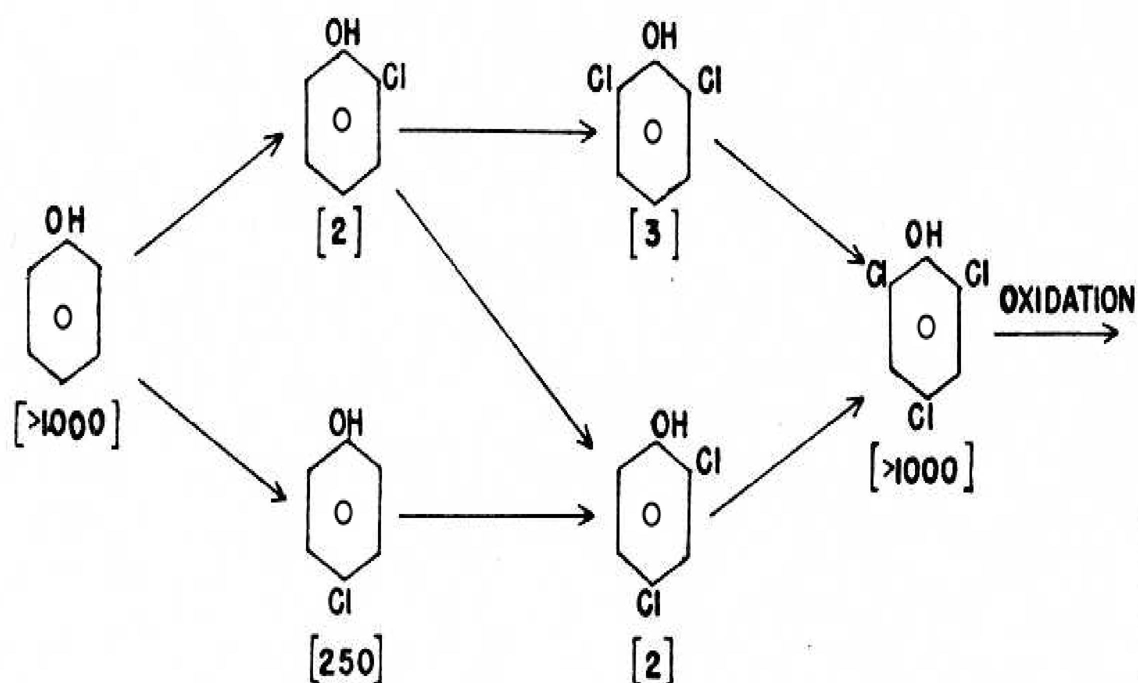


Figure 1.1. Reaction scheme for chlorination of phenol.

Chlorophenols are typically biorefractory or toxic in aqueous environment. Related studies noticed that partial oxidation of chlorophenols by ozone or other oxidants will promote biodegradability for subsequent biological treatment and to reduce the effluent toxicity. Byproducts formed in these oxidation processes are presumed to be more biodegradable or less toxic in comparison to their parent compounds.[14]

Degradation of Chlorophenols:

Removal of toxic organic pollutants from ground water and waste water is one of the most important and critical topics in environmental research.[15] The microorganisms responsible for the degradation of phenols in an anaerobic environment are explained to found in three interacting physiological groups of bacteria. Those include: phenol metabolizers, hydrogen utilizing methanogens and acetotrophic methanogens which are responsible for the complete degradation of phenol to methane and carbon dioxide.[16]

In water, chlorophenols will get sorb onto the particulate material and if not degraded, eventually they form sediments. In anoxic sediments, sulfate, carbonate and nitrate may serve as the electron acceptors and degrade the organic material. Anaerobic degradation of the chlorophenols has been studied under methanogenic conditions. These studies shown that degradation of chlorophenols was by reductive dechlorination leading to the formation of less toxic and more biodegradable compounds with complete mineralization to CO_2 and CH_4.[17] Reductive dechlorination of 2,4-dichlorophenol was followed by carboxylation, ring fission. Then, it undergoes acetogenesis and methanogenesis which led to complete mineralization of 2,4-dichlorophenol[18]. Among chlorophenols, 4-chlorophenol was chosen as a model compound as its anaerobic degradation was found to be most difficult. Conventional and advanced oxidation processes are the treatment methods which were employed for the degradation of 4-chlorophenol. Advanced oxidation processes are known for efficient removal of 4-chlorophenol by using hydroxyl radical, a highly reactive species capable of degrading a wide range of other organic pollutants.[19] Krumme and Boyd in their studies shown monochlorophenols could be dehalogenated and partially mineralized by an anaerobic

upflow bioreactor. But this method was found to be limited in its ability to degrade more-chlorinated phenols.[20] Microbial degradation of chlorophenols was found to be more advantageous in comparison to physicochemical treatment methods as the former results in complete removal of the toxic compounds without any undesirable byproducts formation.[21]

Extraction of Chlorophenols:

Many different techniques have been developed for measuring the volatile compounds in water. These techniques include liquid liquid extraction (LLE), supercritical fluid extraction and many solid-phase extraction (SPE) techniques such as partitioning onto a porous polymer.[22]

Solid-phase micro extraction (SPME), was developed by Pawliszyn and coworkers and it has been marketed by Supelco in order to redress the limitations inherent in solid phase extraction and liquid liquid extraction.[23] SPME is well known for rapid sampling and sample preparation.[24] Since that time, SPME has become an alternative method for extracting organic compounds from aqueous and gaseous media.[25] SPME method can integrate sampling, extraction, concentration and sample introduction into a single step and provides a simple, solvent-free alternative to traditional methods of sample preparation. SPME is known for its simplicity, low cost, rapidity, selectivity and sensitivity.[23,26] Headspace-SPME (HS-SPME) is a modified SPME in which fused-silica fibers coated with a thin polymer is used to trap and concentrate the analytes from the head space.[26] The choice of choosing appropriate coating to SPME fiber is most important for SPME method. Sensitivity of each fiber differs depending on the polarity and molecular mass of the analytes that are to be extracted.[23] Temperature, appropriate

time period for the extraction; stirring rate and ionic strength are the important parameters to be considered which influence the HS-SPME process.[23]

II. EXPERIMENTAL

A. Chemicals and Materials:

All chemicals used were ACS reagent grade. All standard solutions were prepared by using methanol which was B&J ACS certified solvent and was purchased from Honeywell Burdick & Jackson Chemicals (Jackson, MI, USA) and swine waste was obtained from USDA labs in Bowling Green.

Different chlorophenols were used in my experiment:

A.1. 4- chloro-m-cresol was purchased from Tokyo Kasel Kogyo Co., Ltd., Tokyo, Japan

A.2. 2,4- dichlorophenol, 2,6-dicholrophenol, 2-chlorophenol, 4- chlorophenol and 2,4,5-trichlorophenol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

A.3. Solid Phase Micro-Extraction Holder (Manual) 57330-U and Solid Phase Microextraction Fiber Assemblies were purchased from Supelco., Bellefonte, PA, USA.

A.4. Stirrer, Barnsted/Thermolyne, Model No. S46415 was used in the experiment.

B. Instrumentation:

B.1. Weighing balance: Denver Instrument M- 220D.

B.2. Gas Chromatograph: Hewlett Packard 5890 series II

B.3. Integrator: Agilent 3396 series III

B.4. Forma Anaerobic System: Model 1025/1029

B.5. Autoclave: Market Forge sterilmatic, STM-E-Model

B.6. Nitrogen Gas Manifold prepared by Marty Haley, USDA labs, Bowling Green, KY.

C.1. Solid-Phase Micro Extraction (SPME):

The SPME fiber itself is a thin fused-silica optical fiber, which is coated with a thin polymer film which serves as a coating material in chromatography.

C.2. Choosing a Fiber:

Supelco has provided users with several coatings which included three poly (dimethyl siloxane) (PDMS) films of different thickness (7, 30 and 100µm), 85µm poly (acrylate) (PA) and three 65µm mixed phases, poly (dimethyl siloxane)/poly(divinyl benzene) (PDMS/PDVB), poly(ethylene glycol)/poly(divinyl benzene) (carbowax/DVB) and poly(ethylene glycol)/template poly (divinylbenzene) resin (carbowax/TR). The more polar phases are polyacrylate and carboxen.

Fiber Coating	Film Thickness	Polarity	Coating Method	Technique	Compounds to be analyzed
PDMS	100 µm	Non-polar	Non-bonded	GC/HPLC	Volatiles
PDMS	30 µm	Non-polar	Non-bonded	GC/HPLC	Non-polar semi volatiles
PDMS	7 µm	Non-polar	Bonded	GC/HPLC	Medium to nonpolar semi volatiles
PDMS-DVB	65 µm	Bipolar	Cross-linked	GC	Polar volatiles
PDMS-DVB	60 µm	Bipolar	Cross-linked	HPLC	General purpose
PDMS- DVB	65 µm	Bipolar	Cross-linked	GC	Polar volatiles
PA	85 µm	Polar	Cross-linked	GC/HPLC	Polarsemivolatiles

Carboxen-PDMS	75 µm	Bipolar	Cross-linked	GC	Gases and volatiles
Carboxen-PDMS	85 µm	Bipolar	Cross-linked	GC	Gases and volatiles
Carbowax-DVB	65 µm	Polar	Cross-linked	GC	Polar analytes
Carbowax- DVB	70 µm	Polar	Cross-linked	GC	Polar analytes
TPR	50 µm	Polar	Cross-linked	HPLC	Surfactants
DVB-PDMSCarboxen	50/30 µm	Bipolar	Cross-linked	GC	Odors and flavors

Figure 2.1. Summary of commercially available SPME fibers.

The most important feature in determining the analytical performance of SPME is the type and thickness of the coating material. Figure 2.1 lists the most common commercially available polymer coatings. Stationary phases are immobilized by non-bonding, partial cross-linking or high cross-linking. Non-bonded phases are stable with some water-miscible organic solvents. Bonded phases are compatible with majority of organic solvents except some non-polar solvents. Partially cross-linked are stable in most water-miscible solvents. Highly cross-linked phased are equivalent to partially cross-linked phases.

The fibers selected in my experiment were 1cm long and were coated with a 85µm polyacrylate phase. Polyacrylate phase is suitable for polar analytes. Polyacrylate polymer is a solid at room temperature and is a rigid material. As the diffusion coefficients are lower compared to PDMS, it allows the analytes to diffuse into the coating with longer extraction times.

D. Conditioning Instructions:

A new fiber generally has very poor adsorptive properties. Its optimum adsorptive properties development is generally attained by a conditioning step, consists in repeated heating under a convenient gas flow until the fiber results in intended chromatogram without any extraneous peaks. The desorption temperature was limited by considering the stability of the fiber with which polymer it was coated. Heating temperatures for this purpose was maintained up to 300°C. Gas chromatograph used for this purpose was a 3800 GC Varian. Injection port temperature was set to 280°C as mentioned in the Figure 2.3 below. Helium gas was used as a carrier gas.

SPME needle was then inserted into the GC injection port at 280^0C and the fiber was exposed for 2 hours as mentioned in the Figure 2.3. After conditioning was completed, the fiber was retracted and the needle was removed from the injection port.

Stationary phase	Film Thickness	Maximum Temperature	Recommended OperatingTemp.	Conditioning Temperature	Time (Hrs)
PDMS	100µm	280^0C	200-280^0C	250^0C	0.5
PDMS/DVB	65 µm	270^0C	200-270^0C	250^0C	0.5

POLYACRYLATE	85 µm	320^{0}C	220-310^{0}C	300^{0}C	2
CAR/PDMS	75 µm	320^{0}C	250-310^{0}C	300^{0}C	1-2
CW/DVB	65 µm	260^{0}C	200-250^{0}C	220^{0}C	0.5
DVB/CAR/PDMS	50/30 µm	270^{0}C	230-270^{0}C	220^{0}C	1

Figure 2.2. Temperature Conditioning Recommendations for GC Use.

E. Blank Analysis:

Once the conditioning of the SPME fiber was done, a blank analysis was performed in order to make sure that there were no unidentified signals which might interfere with analytical results. All analysis was carried out using a Hewlett Packard 5890 Series II gas chromatograph coupled to flame ionization detector (FID). The injection port temperature of the GC was set to 250^{0}C and the GC oven was cooled to 50^{0}C. Split injection port valve was closed for 2 minutes and the SPME needle was inserted into the injection port and was allowed to desorb for 2 minutes. After 2 minutes

the fiber was retracted and removed from the injection port. Extraneous peaks were observed which may be either from the previous extraction or from the polymer Polyacrylate which was used to fix the SPME fiber on its metallic fiber holder. Then the fiber was redesorbed a couple of times until a decrease in the intensity of the peaks was seen on subsequent chromatograms.

F. Extraction Procedure:

SPME needle shown in Figure 2.3 was used for the extraction of different chlorophenols from swine waste.

Figure 2.3. Supelco SPME needle with PA 85µm fiber.

Solid Phase Micro Extraction can be used in two principle modes. They are

- Head space Extraction
- Direct-Extraction.

In the Direct extraction method, the fiber is inserted directly into the sample and the analytes are extracted directly from the sample matrix to the extraction phase. In this extraction method, the fiber may be damaged or the properties could be changed through adsorption as the samples of swine waste are dirty. In the head-space mode, the vapor present above the sample matrix is sampled. Volatile analytes are transported from the bulk matrix to the head space then the fiber coating. Thus, head-space mode was used for the extraction of chlorophenols in swine waste. More volatile components in the head-space are extracted faster than less volatile components.

F.a. Sample Preparation:

One hundred milliliters of swine waste was spiked at added concentration levels of 0.5ppm, 1.25ppm, 2.5ppm, 3.75ppm, 5ppm, 6.25ppm from the mixture of all six chlorophenols i.e., 2- chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, 4-chloro-m-cresol and 2,4,5-trichlorophenol.

Briefly, 0.5g of 2,4-dichlorophenol, 0.5g of 2,6-dichlorophenol, 0.5g of 4-chloro-m-Cresol, 0.5g of 2,4,5-trichlorophenol, 380µl of 2-chlorophenol and 42 0µl of 4-chlorophenol were mixed in a 150 ml Erlenmeyer flask with 10 ml of methanol. This was allowed to stir for a couple of minutes so that all the chlorophenols were dissolved in methanol. From this solution different concentrations (as mentioned above) were added to 100 ml of swine waste collected in 125 ml round neck glass bottles which were closed with a rubber septum.

F.b. SPME Determination:

A typical SPME determination was carried out within three steps as follows:

- Fiber cleaning
- Adsorption
- Desorption and Chromatography

F.b.1. Fiber Cleaning:

Fiber cleaning was done before each analysis in order to remove any contaminants that were present in the fiber from the previous extraction. For cleaning, the injection port temperature of the gas chromatograph was set to 280˚C and then the SPME fiber was introduced into the gas chromatograph, followed by heating up for 2 hours at 280˚C as shown in Figure 2.4 below. In this process any adsorbed species were desorbed and fed to the inlet of the capillary column. After 2 hours the fiber was retracted into the needle and the needle was removed slowly from the hot injection port.

Normally, the first blank run was carried out with the cleaned SPME fiber and some unidentified signals were noticed in the chromatogram. The same procedure was repeated for a couple of times until the blank signal became reproducible.

Figure 2.4. Fiber cleaning by using gas chromatograph.

F.b.2. Adsorption:

Swine waste sample bottles, each with different concentrations of chlorophenols, were subjected to constant stirring by using Barnsted/Thermolyne, model no. S46415 stirrer and a small magnetic stir bar for one hour.

Then the cleaned fiber was immersed in the head space (HS) of the stirred swine waste sample bottles, where the analytes were concentrated as shown in Figure 2.5. During this step, adsorption occurred. After 20 minutes the fiber was retracted into the needle and the needle is removed from the bottle slowly.

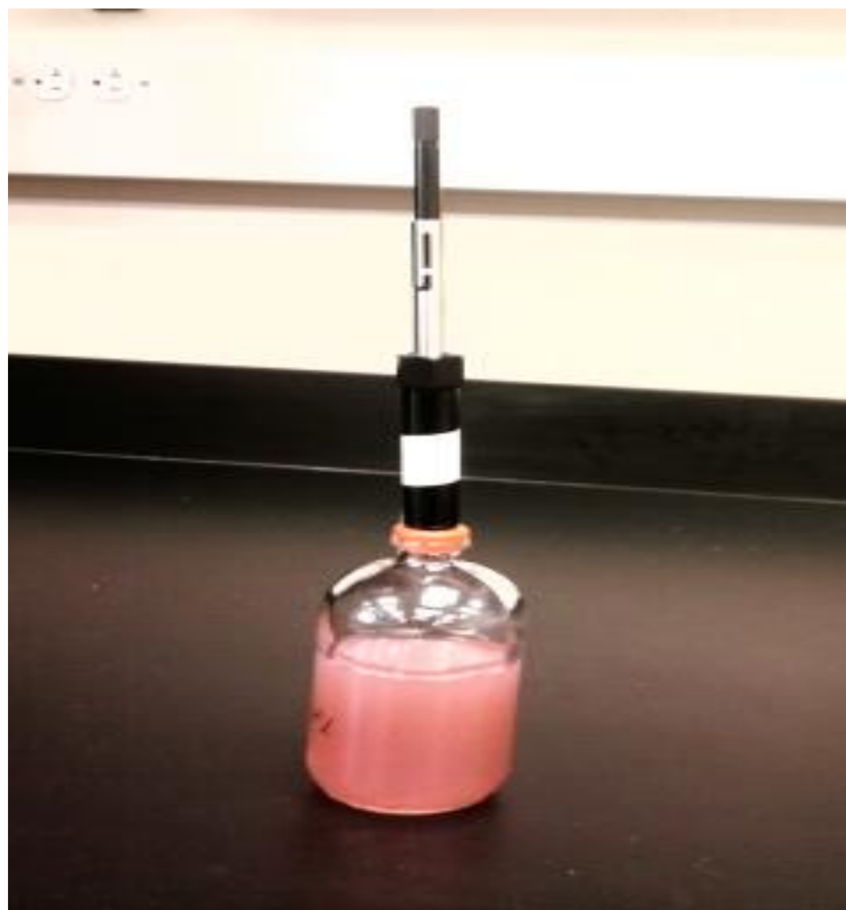


Figure 2.5. Adsorption of the chlorophenols from swine waste samples by SPME fiber.

F.b.3. Desorption:

Once the fiber was retracted and the SPME needle was removed from the swine waste sample bottle, it was transferred to the hot injection port of the gas chromatograph Hewlett Packard 5890 series II equipped with a flame ionization detector as shown in Figure 2.6, where the chlorophenols were desorbed thermally and transferred directly to the separation column for analysis. Sample injection was done in splitless mode for two minutes so that a larger portion of the analytes went to the analytical column directly without any portion of analytes exhausting through the split vent.

Figure 2.6. Gas Chromatograph-FID detector.

F.d. Analysis:

Hewlett Packard 3396 series III is an integrator connected to the GC which processes an analog signal from the GC to which it is connected. It plots the signal sent by the FID detector into a chromatogram. It also quantified the sample by determining the area under a peak by comparing it to the reference or standard peaks.

Figure 2.7. Analysis of chlorophenols by GC connected to HP 3396 series III integrator.

By sampling different concentrations of chlorophenols three times and calculating the average area of each chlorophenol the following calibration curves were plotted.

F.d.1. Calibration of Chlorophenols:

Dependencies between compound concentration and the peak area as a result of chromatographic analysis of swine waste samples with added 2-chlorophenol, 2,4-dichlorophenol, 4-chlorophenol, 2,6-dichlorophenol, 4-chloro-m-cresol and 2,4,5-trichlorophenol respectively. Standard solutions were used with the concentration level of the compounds ranging from 0.5 to 6.25ppm. The final peak area was taken as an average of three experiments in turn.

The calibration plot was explained with the general equation: y=mx+c

Where y- Gives the peak area

x- The amount of determined compound in ppm,

c- Y intercept

PPM	2-chloro phenol	2,4-Dichloro phenol	4-chloro phenol	2,6-dichloro phenol	4-chloro-m-Cresol	2,4,5-trichloro phenol
0.5	1044	1530	-	-	1104	1183
1.25	1700	2760	896	644	1788	2126
2.5	4031	5052	1138	1620	2770	4180
3.75	4852	6307	1725	2523	3822	5477
5	6338	7912	2027	3443	5151	7638
6.25	6976	9039	2691	4305	5465	8519

Table 2.1. calibration of chlorophenols at different concentrations.

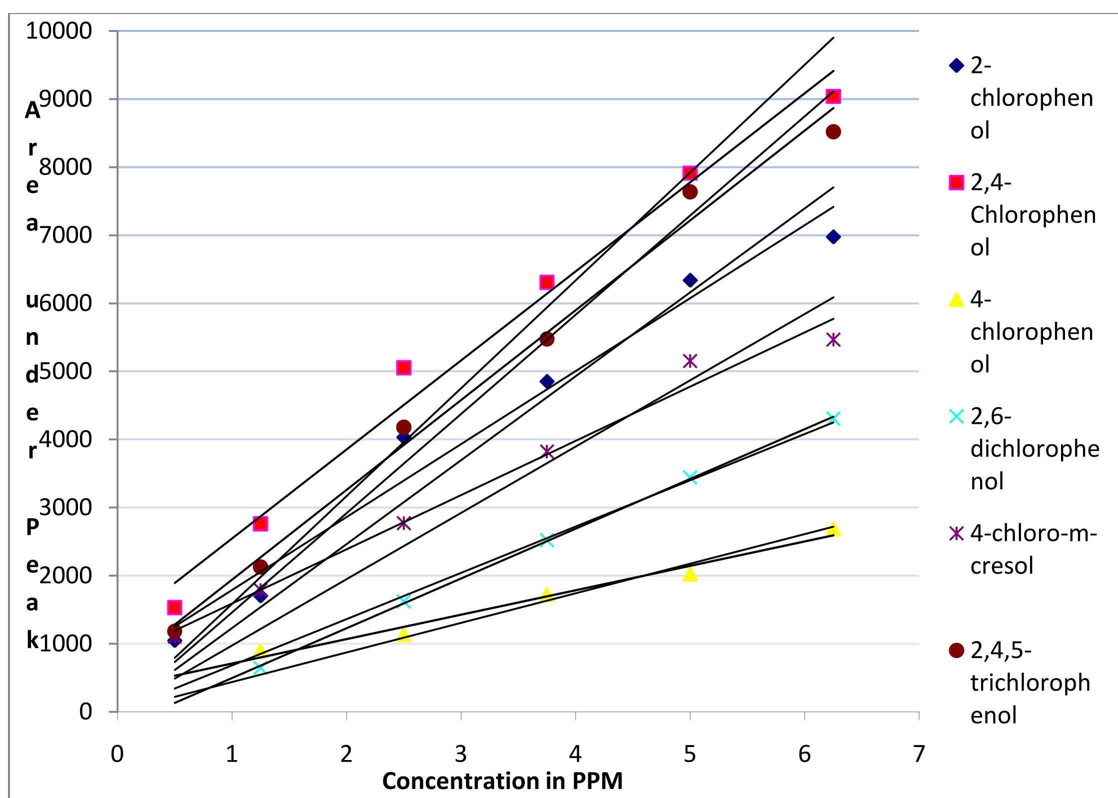


Figure 2.8. Calibration curve of chlorophenols.

Compound	Best fit line	R^2 value
2-Chlorophenol	Y=10721x+717.26	0.9707
2,4-Dichlorophenol	Y=1308.3x+1236	0.9853
4-Chlorophenol	Y=358.3x+351.86	0.9777
2,6-Dichlorophenol	Y=731.63x+236.67	0.9996
4-Chloro-m-cresol	Y=797.05x+236.67	0.9845
2,4,5-Trichloropphenol	Y=1319.7x+619.93	0.9906

Table 2.2. Best fit line and R^2 value calculated from the calibration curves.

G. Anaerobic Degradation of Chlorophenols:

To study the degradation of chlorophenols under the anaerobic conditions, experimental work was carried out under the anaerobic hood i.e., Forma Anaerobic System: Model 1025/1029 (Thermoscientific). For this purpose, nine sampling bottles were used (three for each treatment) among which three were of non-sterile swine waste, three were of sterile swine waste and three were of DI water. The step wise procedure of this process was as follows:

Firstly, 500ml of swine waste was placed in 1000 ml Erleyenmeyer flask and was autoclaved by using Market Forge sterilmatic, STM-E-Model autoclave for 20 minutes rendering sterile and thus free of bacteria.Empty sampling bottles were also autoclaved for 20 minutes to make them sterile.In another 1000ml Erleyenmeyer flask , 500ml of non-sterile swine was collected. These were all placed under the anaerobic hood.

G.a. Making the Samples Anaerobic:

The purpose of this procedure was to remove all the atmospheric gas, particularly oxygen, from the work chamber and replace it with a special anaerobic gas mixture consisting of nitrogen, hydrogen and carbondioxide.

Procedure:

1. The nitrogen gas supply was connected to the anaerobic gas connection.
2. The nitrogen gas supply was turned on at the source and the low stage pressure was adjusted to 10-15psig.
3. The outer door was closed and secured and the inner, incubator doors were opened.

4. Then vacuum and Nitrogen was turned on and the switches were equalized to the auto position and then the gas System was switched on.

5. The Manual Fill Knob was turned fully to the counter clock-wise position.

6. Then the start button was pushed, which caused the vacuum system to evacuate atmospheric gas. After that, it automatically shuts off and the work chamber was filled with nitrogen.

7. Once the procedure was completed, the Manual Fill Knob and nitrogen at the source were turned off.

8. The nitrogen was then removed from the special gas compression fitting and was connected to the nitrogen fitting and the special gas was connected to the appropriate fitting on the back of the unit.

9. With the gas System switch on and the vacuum, nitrogen, and equalize switches in the auto position, the above steps from 4 to 8 were repeated five more times.

10. Once the procedure was completed, Manual Fill Knob was turned off and the Catalyst Fan Switch was turned on.

11. After one hour, chamber was verified to be anaerobic by checking if the cabinet was below 1% oxygen.

12. The cabinet was not disturbed overnight which permitted the catalyst wafer to remove any traces of oxygen that might be present.

13. Then the two Erleyenmeyer flasks containing either sterile or non sterile swine waste samples and 500 ml DI water in a beaker were placed in the interchange. This transferred them to and from the work chamber without contaminating the work chamber with the atmospheric gas.

Figure 2.9. Forma Anaerobic system used for setting anaerobic conditions.

14. The vacuum pump was programmed to operate up to 20 inHg vacuum. Nitrogen was injected until the interchange reached atmospheric pressure and the interchange equalized with anaerobic gas mixture in the work chamber.

15. The transfer cycle was factory set for three evacuations, two nitrogen purges and one equalizing purge of anaerobic gas mixture.

16. Both the inner and the outer door were securely closed during the transfer cycle. Doors were not opened until the cycle was completed.

17. With the inner door securely closed and clamped, the outer door was opened and the material was then placed in the interchange.

18. The outer door was secured and for an automatic transfer cycle, the Cycle Start button was pressed.

19. When the cycle was completed, the equalize switch, both door lights became off, and the Cycle Complete light were on.

20. Now it was safe to open the inner door and remove the material.

To remove the material from the chamber:

With the inner and outer doors secure, press “start cycle”.

When the “sequence complete” light comes on, then inner door was opened and the material was transferred to the interchange.

Then the inner door was closed and secured and the outer door was opened to remove any material.

Then outer door was closed and secured.

Now within the anaerobic hood sterile, non-sterile swine waste and DI water of 100ml each were transferred to empty 125ml bottles three of each kind.

From the stock solution of chlorophenols 1250 µl volume was added to all nine sample bottles in the anaerobic hood to give initial concentrations of 6.25ppm. The three bottles containing sterile swine waste served as control in order to account for possible sorption of chlorophenols to organic material. All the nine bottles were sealed tightly under the anaerobic hood, placed in the interchange chamber and then taken out through the outer door.

G.b. Measurement of the Degradation of Chlorophenols:

Possible degradation of the chlorophenols was monitored by sampling the treatments using SPME at different time intervals. Relative areas of each chlorophenol with 6.25ppm of stock solution in anaerobic samples were compared with that of the standard chlorophenol areas at same volume as shown in Table 2.1. The SPME determination was carried out as same as for extracting the standard chlorophenols for plotting the calibration curves.

H. Measurement of the Production of CO_2 and CH_4 Gas:

CO_2 and CH_4 gas production from all the nine sample bottles were measured from which the activity of the bacteria was measured. Gas production was measured by using Varian CP-3800 Gas Chromatograph.

Procedure:

Head space samples were collected in 25 ml vials sealed with 20 mm grey butyl septa (Supelco) and aluminium crimp-top rings (Fisher Scientific). Prior to gas sampling, 25 ml vials were filled with nitrogen gas up to 20psi using a bench top gas manifold. Nitrogen gas was flushed through each vial for five minutes to remove any atmospheric gases. Two syringes used and were fixed as shown in Figure 2.10.

Figure 2.10. Nitrogen gas manifold.

Gas is inserted into the vial through one syringe and a second syringe is inserted into the septa to restrict the gas flow coming out. This is carried out for 5minutes.

Composition of dry atmosphere by volume:

Average atmosphere has:

Methane (CH_4) : 1.745ppmv (0.0001745%)

Carbon dioxide (CO_2): 383ppmv (0.0383%)

After five minutes the valve was shut off prior to removing the output syringe to minimize over pressure. Now gas production can be measured by using this sealed vial of 'N_2'. If any pressure above that of atmospheric was present in the 'N_2' filled gas vials, it was removed by using a tem milliliter disposable syringe. For measuring gas production, 200 µl of gas was withdrawn by using one milliliter syringe from the nitrogen filled gas in order to make space for replacing the head space gas from each of the sample bottle. Then, from the sample bottles, 200 µl of head space gas was removed by using the same 1ml. syringe and it was put back into the 'N_2' filled vial. Now from the 'N_2' gas filled vials CH_4 and CO_2 gas production measurements were made by gas chromatograph

for different time periods at which the sampling was done and corresponding values were shown in Table 3.5-3.7.

III. RESULTS AND DISCUSSION

A.Objectives:

Experimental work focused on measuring the anaerobic degradation of six different chlorinated phenols namely, 2-chlorophenol, 2,4-dichlorophenol, 4-chlorophenol, 2,6-dichlorophenol, 4-chloro-m-cresol and 2,4,5-trichlorophenol in swine waste using solid-phase micro extraction and gas chromatography.

E. Analysis:

A Hewlett Packard 3396 series III integrator, connected to GC, which plotted the signal sent by the FID detector into a chromatogram. Samples were quantified by determining the area under a peak and compared them to the areas of standard peaks for further analysis.

Calibration curves were plotted, as shown in Figure 2.9., by sampling different concentrations of chlorophenols each three times and calculating the average area of each chlorophenol.

Possible degradation of the chlorophenols was monitored in swine waste sample bottles while being continuously sparged with an O_2-free gas mixture of 70% N_2-30% CO_2 stirred. 100 ml of nonsterile swine waste was transferred using a graduated cylinder into each of the three 125 ml glass bottles, 100 ml DI water into another three which were also being sparged. Three bottles, each with 100ml. of swine waste, were autoclaved for 12 hours to serve as sterile controls. After transfer of the swine waste into the amended bottles, they were added with 6.25ppm of above referred stock solution of chlorophenols. After this, all the sample bottles Chlorophenol concentrations were monitored by sampling the sample bottles at different time periods for degradation. All

the sample bottles were sampled on the same day by using the SPME device. Then, all the bottles were incubated with shaking at 100rpm at 37°C for 4days and sampled on the fourth day by using SPME. Again the samples were incubated for another eight days and sampled on the twelfth day by using the SPME device. Analysis was made by Hewlett Packard Gas Chromatograph consisting of a model 5800, with to FID detection, connected to a Hewlett Packard 3396 series III integrator.

The reduction in concentrations and peak areas of the corresponding chlorophenols in non-sterile swine waste samples was shown in Table 3.1.

Sample	Conc. /Day 1 ppm	Conc./ Day 12 ppm
2-Chlorophenol	2..9±0.4	1.1±0.2
2,4-Dichlorophenol	3.4±0.2	1.1±0.1
4-Chlorophenol	0.9±0.7	0.4±0.2
2,6-Dichlorophenol	1.8±0.3	0.7±0.2
4-Chloro-m-cresol	2.6±0.7	0.5±0.3
2,4,5-Trichlorophenol	3.2±0.7	0.8±0.7

Table 3.1. Concentrations and peak areas of non-sterile swine waste samples.

The percent reduction in the areas of the corresponding chlorophenols from day one to day twelve was plotted as shown in the Figure 3.1.

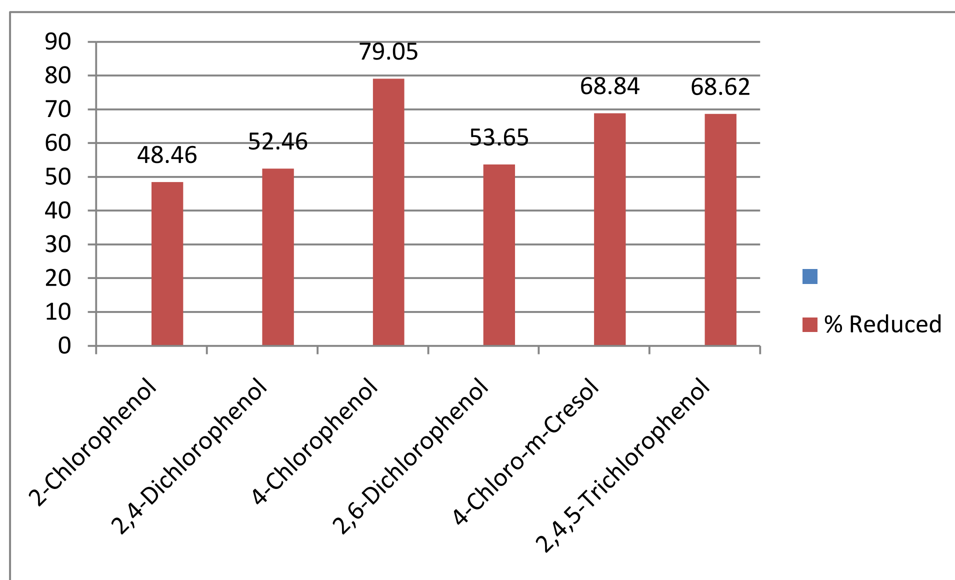


Figure 3.1. Graph showing percent reduction in peak areas from day 1 to day 12.

The reduction in concentrations and peak areas of the corresponding chlorophenols sterile swine waste samples was shown in Table 3.1.

Sample	Conc. /Day 1 ppm	Conc./ Day 12 Ppm
2-Chlorophenol	2.9±0.2	0.6±0.1
2,4-Dichlorophenol	4.5±0.2	1.1±0.2
4-Chlorophenol	0.6±0.2	0.5±0.2
2,6-Dichlorophenol	3.6±0.6	0.9±0.7
4-Chloro-m-cresol	2.6±0.3	0.4±0.3
2,4,5-Trichlorophenol	4.4±0.4	1.6±0.4

Table 3.2. Concentrations and peak areas of sterile swine waste samples.

The percent reduction in the areas of the corresponding chlorophenols from day one to day twelve was plotted as shown in the Figure 3.2.

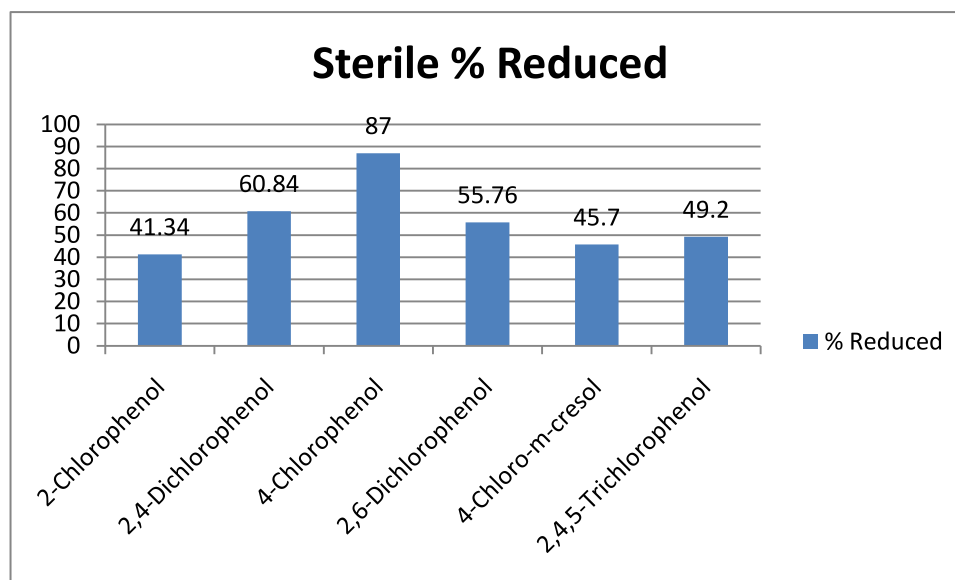


Figure 3.2. Graph showing percent reduction in peak areas from day 1 to day 12.

The reduction in concentrations and peak areas of the corresponding chlorophenols DI water samples was shown in Table 3.1.

Sample	Conc. /Day 1 (ppm)	Conc./ Day 12 (ppm)
2-Chlorophenol	2.7±0.2	0.7±0.1
2,4-Dichlorophenol	4.9±0.4	1.4±0.2
4-Chlorophenol	1.5±0.4	0.3±0.1
2,6-Dichlorophenol	4.9±0.5	2.7±0.2
4-Chloro-m-cresol	3.2±0.4	0.9±0.1
2,4,5-Trichlorophenol	6.5±1.1	5.1±0.4

Table 3.3. Concentrations and peak areas of DI water samples from day 1 to day 12 .

The percent reduction in the areas of the corresponding chlorophenols in DI water samples from day one to day twelve was plotted as shown in the Figure 3.3.

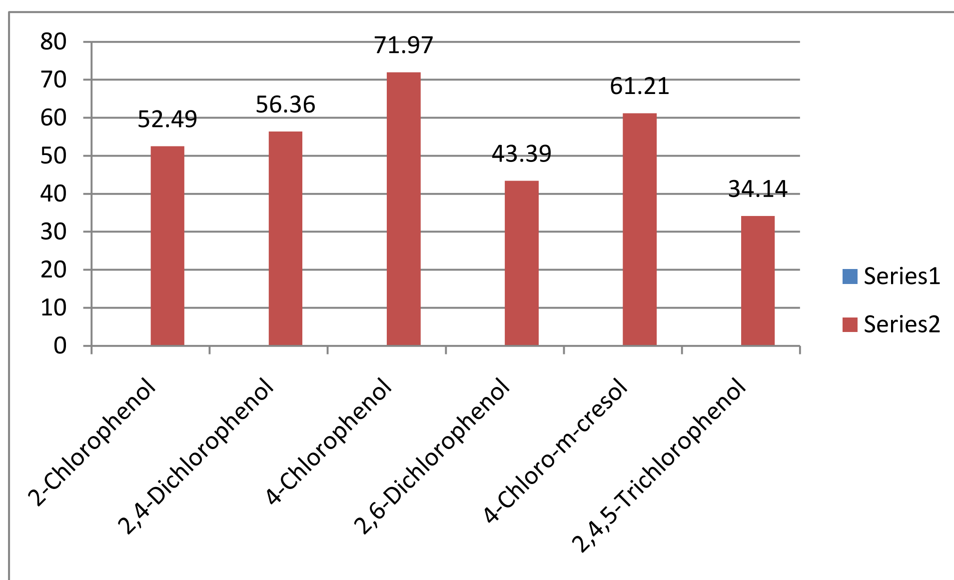


Figure 3.3. Graph showing percent reduction in peak areas from day 1 to day 12.

It was observed from the figure3.1-3.3, that there was loss of all the six chlorophenols in non-sterile, sterile and DI samples.

ANOVA test was done to compare the means of the decrease in concentration levels on day sixteen for all the three different samples. The values were shown in Table 3.4.

Compound	Water ppm	Sterile ppm	Non-Sterile ppm	Pr>F*
2-Chlorophenol	0.747 b	0.617 b	1.08 a	**0.0207**
2,4-Dichlorophenol	**1.42** a	1.05 b	1.20 ab	0.1092
4-Chlorophenol	**0.25** a	0.15 a	0.133	0.7578
2,6- Dichlorophenol	**2.72** a	0.967 b	0.650 b	**0.0027**
4-Chloro-*m*-cresol	**0.817** a	0.373 b	0.543 ab	**0.0692**
2,4,5-Trichlorophenol	**5.05** a	1.55 b	0.757 b	**0.0004**

Table 3.4. Duncan's Multiple Comparison to compare means of three samples. a= highest level, b= lower level, means labeled with a different letter are significantly different at P=0.05 by analysis of variance and Duncan's multiple range test.

From the above data, it was observed that only 2,6-dichlorophenol and 2,4,5-trichlorophenol are highly significant. Also, it was noticed that for 2-chlorophenol, 2,4-dichlorophenol and 4-chloro-m-cresol mean concentration values are less in the sterile swine waste samples than the non-sterile swine waste samples.

H. Measurement of the Production of CO_2 and CH_4 Gas:

Production of CO_2 and CH_4 gas was measured from all the nine sampling bottles, refer to the procedure already mentioned in Chapter 2, from which the activity of the bacteria was measured.

From all the nine sample bottles production of CO2 and CH_4 was measured on first day after sampling by SPME.

Sample	Quantity/CO_2 (ppm)	Quantity/CH_4 (ppm)
Sterile	179±46	1,102±1818
Non sterile	322±41	15,254±287
DI water	414±13778	5,852±9999

Table 3.5. Measurement of CO_2 and CH_4 gas on day one.

Then all the nine sample bottles were incubated for four days, production of CO_2 and CH_4 gas was measured on the fourth day after sampling.

Sample	Quantity/CO_2 (ppm)	Quantity/CH_4 (ppm)
Sterile	164±12	55±3
Non sterile	283±17	12,702±4772
DI water	228±26865	83±19

Table 3.6. Measurement of CO_2 and CH_4 gas on day four.

Again all the nine sample bottles were incubated for eight days and production of CO_2 and CH_4 gas was measured on the twelfth day incubation.

Sample	Quantity/CO_2 (ppm)	Quantity/CH_4 (ppm)
Sterile	211±40	67±11
Non sterile	431±67	20,530±7487
DI water	355±7156	3212±5350

Table 3.7. Measurement of CO_2 and CH_4 gas on day twelve.

Methane concentration from day one to day twelve was plotted as shown in Figure 3.4.

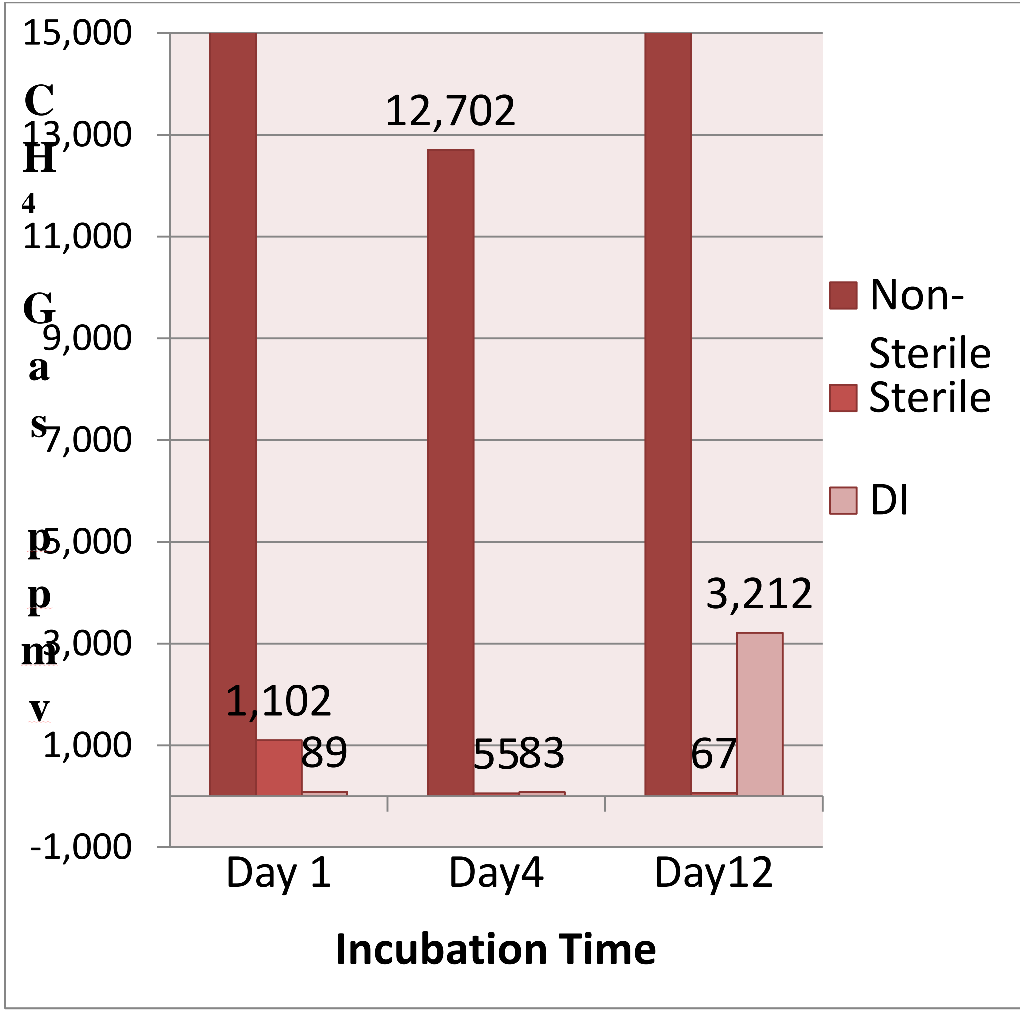


Figure 3.4. Quality of methane gas from day 1 to day 12.

It was observed that the concentration of chlorophenols in the sterile swine waste samples were changed which were kept as controls and thus those were not likely be completely sterile. From Figure, 3.1-3.3, it was observed that all the six chlorophenols were showing decreased response at different rates in all the samples. So, therefore decreased response of the chlorophenols observed was likely to be by partitioning of the compounds to the solids (refer to $logk_{ow}$ values in chapter one) present in the swine waste samples, sticking of the compounds to that of the glass bottles used for sampling and by the bacteria present in the swine waste. From Table 3.4., it was noticed that the mean concentration values for 2,6-dichlorophenol, 2,4,5-trichlorophenol and 4-chlorophenol non-sterile swine waste samples were less than that of the sterile swine waste samples. The Probability factor for 2,6-dichlorophenol and 2,4,5-trichlorophenol was found to be significant where 4-chlorophenol probability factor was not significant. From this analysis, results indicate that 2,6-dichlorophenol and 2,4,5-trichlorophenol are likely to be degraded by the bacteria.

Further analysis was made by comparing the CO_2 and CH_4 gas concentration values for all samples. From Figure 3.4., it was observed that CH_4 gas production was high in the non sterile swine waste samples compared to sterile swine waste and DI water samples. In non-sterile swine waste samples, the CH_4 gas production was likely to be by the methanogenic bacteria which produce CO_2 and CH_4 gas during metabolism as its byproducts. Sterile swine waste and DI water samples also found to produce CO_2 and CH_4 gas whose values were relatively much less in comparison to non-sterile swine waste samples. By considering all the above obtained data and from the concentration mean

values shown in Table 3.4., 2,6-dichlorophenol and 2,4,5-trichlorophenol were found likely to be degraded by the bacteria present in the swine waste samples.

IV. CONCLUSIONS

As mentioned in Results and Discussion, the main objectives of this study was to measure the anaerobic degradation of chlorophenols, namely 2-chlorophenol, 2,4-dichlorophenol, 4-chlorophenol, 2,6-dichlorophenol, 4-chloro-m-cresol and 2,4,4-trichlorophenol in swine waste. For this purpose, Solid-phase microextraction method in head space extraction mode was used for the extraction of chlorophenols from the swine waste. Analysis was made by gas chromatograph with an FID detection.

To measure the degradation of chlorophenols, nine sampling bottles (three of each experiment) among which one were non-sterile swine waste, sterile swine waste and DI water were used. From the stock solution of chlorophenols 6.25ppm concentration was added to each of the sampling bottles under anaerobic conditions. All the sample bottles were monitored for degradation on the first day without incubation, after four and twelve days of incubation. It was observed that the decreased response of the chlorophenols in comparison to standard calibration curves was likely to be by partitioning of the chlorophenols to the solids, sticking to the glass bottles and by the bacteria present in the swine waste.

Further analysis was made by comparing the CO_2 and CH_4 gas production values for all the samples. In non-sterile swine waste samples, gas production was likely to be by the methanogenic bacteria which produced CO_2 and CH_4 gas as its byproducts. In sterile swine waste samples gas production values measured were much less in comparison to non-sterile swine waste samples. By ANOVA and gas production analysis, it was found that 2,6-dichlorophenol and 2,4,5-trichlorophenol were likely to be degraded by the bacteria present in the swine waste.

V. FUTURE WORK

1. Identifying situations (and organisms) in which degradation of pollutants occurs is important field of research.
2. Measure the degradation of chlorophenols by changing the substitution patterns of the chlorophenols.

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