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AQUEOUS REMEDIATION OF 4,4′-DICHLOROBIPHENYL
BY FENTON'S REAGENT: A STUDY OF OXIDATIVE
DEGRADATION, BYPRODUCT PRODUCTION, AND
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ABSTRACT

AQUEOUS REMEDIATION OF 4,4′-DICHLOROBIPHENYL BY FENTON'S REAGENT: A STUDY OF OXIDATIVE DEGRADATION, BYPRODUCT PRODUCTION, AND TOXICOLOGICAL EFFECT

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2-biphenylol (2BP), 3-biphenylol (3BP), 2,2′-biphenyldiol (2,2′BP), 3,3′-biphenyldiol (3,3′BP), 3-chloro-2-biphenylol (3C2BP), and 4,4′-dichloro-3-biphenylol (4,4′DC3BP), considered to be representative of potential byproducts of Fenton's remediation of polychlorinated biphenyls (PCBs), were studied for the correlation between their chemical/structural properties and observed epigenetic toxicity and cytotoxicity. The scrape-loading/dye transfer (SL/DT) technique was performed to determine the in vitro modulation of gap junctional intercellular communication (GJIC) in a normal rat liver epithelial cell line as a measure of the epigenetic toxicity. Cytotoxicity was determined using the neutral red uptake assay. Only 3,3′BP and 4,4′DC3BP induced cytotoxicity within a dose range of 0 to 300 μM. 4,4′DC3BP was most inhibitory to GJIC at the lowest dose. 3C2BP was least inhibitory to GJIC. Although cells were capable of complete recovery of GJIC after removal of each of the chemicals, only with 2,2′BP and 4,4′DC3BP did the cells demonstrate partial recovery without removal of the chemical. In view of the cytotoxicity and GJIC inhibitory effects observed for 4,4′DC3BP, the PCB congener 4,4′-dichlorobiphenyl (4,4′DCBP) was selected for Fenton's reagent remediation studies. For toxicology studies performed for 4,4′DCBP, over a dose range of 0 to 260.87 μM, very slight to no inhibition of GJIC was observed for incubation times of 30 minutes, 2 hours, 6 hours, and 24 hours.

The methylene blue (MB) dye test was developed to qualitatively indicate the presence of hydroxyl radicals through an immediate, distinct bleaching of the MB dye on a paper test strip. When applied to Fenton's remediation, MB dye tests were capable of indicating the formation of hydroxyl radicals during the Fenton's reaction and indicating the completion of quenching. The presence of hydroxyl radicals was verified by benzoic acid chemical probe hydroxyl radical detection methods using thin layer chromatography (TLC) and spectrophotometric wavelength scans.

Fenton's remediation of 4,4′DCBP in 50/50 Milli-Q water/acetonitrile was performed, followed by an examination of the toxicity of the final Fenton's remediation solution and disappearance of 4,4′DCBP as a result of Fenton's remediation. The Fenton's reaction conditions were pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM. Partial inhibition of GJIC occurred for test volumes greater than 40 μL (30 minute incubation), and a maximum level of inhibition was attained at 60 μL (FOC = 0.75 ± 0.03). For the time-response GJIC bioassay (60 μL test volume), a maximum level of inhibition was attained at 30 minutes (FOC = 0.78 ± 0.02), followed by complete recovery of GJIC without removal of the chemical by 240 minutes. Although the final Fenton's remediation solution was determined to be more toxic than the parent PCB, and the inhibitory effects were similar to those observed for 4,4′DC3BP, the byproducts responsible for the toxicity observed could not be ascertained by the detection methods employed. Only 27.22 ± 1.32% of the 4,4′DCBP remained by 15 minutes of remediation and no further decrease occurred through 60 minutes.

I dedicate this dissertation to my family, whose unending support has encouraged me on this long journey, and to my Grandpa, Dr. John S. Evans, who taught me to believe in my dreams.

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ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of Variance
BA	Benzoic Acid
2BP	2-Biphenylol
2,2′BP	2,2′-Biphenyldiol
3BP	3-Biphenylol
3,3′BP	3,3′-Biphenyldiol
BSA	Bovine Serum Albumin
3C2BP	3-Chloro-2-biphenylol
CI	Confidence Interval
4,4′DCBP	4,4′-Dichlorobiphenyl
4,4′DC3BP	4,4′-Dichloro-3-biphenylol
DMSO	Dimethyl Sulfoxide
ECD	Electron Capture Detector
FBS	Fetal Bovine Serum
FOC	Fraction of the Control
GC/ECD	Gas Chromatography/Electron Capture Detection
GJIC	Gap Junctional Intercellular Communication
HBA	Hydroxylated Benzoic Acid
4-HBA	4-Hydroxybenzoic Acid
MB	Methylene Blue

MeOH	Methanol
OH-BP	Hydroxylated Biphenyl
OH-PCB	Hydroxylated Polychlorinated Biphenyl
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyl
SD	Standard Deviation
SL/DT	Scrape-Loading/Dye Transfer
TLC	Thin-Layer Chromatography

Chapter 1

Introduction

1.1 Background and Environmental Significance

Polychlorinated biphenyls (PCBs) are synthetic organic compounds with a biphenyl backbone consisting of two hexagonal "rings" of carbon atoms connected by carbon-carbon bonds. Polychlorinated biphenyls have between 1 and 10 chlorine atoms substituted for hydrogen atoms on the biphenyl rings and, therefore, are part of a larger group of chemicals known as halogenated aromatic compounds. The combinations of the chlorine atoms on the biphenyl molecule result in 209 possible chemical structures known as congeners. Chemical properties, such as nonflammability, chemical and thermal stability, low reactivity, miscibility with organic compounds, and excellent dielectric properties, made PCBs ideal for use in numerous commercial and industrial products, such as transformers, capacitors, light ballasts, paints, adhesives, dyes, plastics, hydraulic fluids, flame retardants, and lubricants (*1, 2*). PCBs are moderately volatile from water and soil, have melting points of 340 °C to 375 °C, and are non-polar.

PCBs were manufactured in the United States from 1929 to 1977. The decision to ban commercial production, processing, and distribution of all PCBs in the United States was reached in 1976, following a series of events which alerted worldwide concern about the potential health effects of PCBs. Manufacture of PCBs in the United States ceased in 1977 under the Toxic Substances Control Act. PCBs were banned by many countries in the late 1970's and a global ban occurred in 2001 under the Stockholm Convention on persistent organic pollutants.

PCB-contaminated soils are classified into 3 groups based on the levels of contamination and in the order of decreasing severity of disposal restrictions: (i) greater than 500 ppm as PCB material, (ii) 50 to 500 ppm as PCB-contaminated material, and (iii) less than 50 ppm as waste material (*3*). Due to the severity of disposal restrictions applied to materials with high levels of PCB contamination, an incentive exists for PCB waste generators to develop methods to reduce the PCB level to the range where the wastes can be disposed of under less severe restrictions. Drinking water standards for chemicals that are implicated as causing health problems were developed as a result of the Safe Drinking Water Act. Maximum Contaminant Level Goals (MCLG) are non-enforceable levels based solely on possible health risks and exposure. The MCLG for PCBs has been set at zero because the EPA believes this level of protection would not cause any potential health problems. The EPA has set an enforceable standard, based on the MCLG, called the Maximum Contaminant Level (MCL), which is set as close to the MCLGs as possible, considering the ability of public water systems to detect and remove contaminants using suitable treatment technologies. The MCL for PCBs is set at 0.5 parts per billion (ppb) (*4*).

Although production of PCBs has been discontinued and disposal of PCBs from existing sources has been highly regulated, PCBs continue to be a concern due to their persistence in the environment, slow rate of biodegradation, ability to bioaccumulate, and the potential impact on humans and the environment. PCBs have been found to exist in almost every component of the global ecosystem including air, water, sediments, and soils (*1*). Environmental cycling of PCBs previously introduced into the environment can occur by volatilization from ground surfaces (water, soil) into the atmosphere, removal from the atmosphere via wet/dry deposition, and revolatilization (*4*). Potential environmental sources of PCBs include past open uncontrolled uses, past disposal practices, illegal disposal, and accidental releases (*5, 6*). Humans and wildlife can be exposed to PCBs either directly from contact with contaminated air, sediments, or water or indirectly through food. The non-polar, lipophilic physical properties of PCBs, as well as their resistance to biochemical degradation, tend to cause their accumulation in fatty tissues of humans and wildlife (*2*). Numerous human health effects, including reproductive disorders, embryotoxicity, oncogenicity, estrogenic endocrine disruption, wasting syndrome, reduced body weight, immunotoxicity, vitamin A deficiency, thyroid deficiency, and reduced birth weight, have been attributed to exposure to PCBs (*5, 7, 2*). The EPA has classified all PCBs as Group B2, probable human carcinogens (*8*).

A number of PCBs are potent inducers of hepatic and extrahepatic xenobiotic metabolizing enzymes and the activity of the individual compounds are remarkably dependent on structure. They can be divided schematically into three groups. Group 1 consists of Phenobarbital (PB)-type inducers, Group 2 consists of 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-type inducers, and Group 3 consists of inducers of both Groups 1 and 2 and is called mixed-type inducers. The most toxic congeners, 3,4,3′4′-tetrachlorobiphenyl, 3,4,5,3′4′-pentachlorobiphenyl, and 3,4,5,3′4′5′-hexachlorobiphenyl are lacking chlorosubstitution in the ortho positions. These congeners might form coplanar conformation and are thereby approximate isostereomers of 2,3,7,8-trichlorodibenzodioxin (TCDD). They also elicit biological effects comparable to those reported for TCDD, including enzyme induction pattern of the MC-type, both in vitro and in vivo. Therefore, the main structural characteristics are chloro substituents at both para positions, chloro substitution in at least one meta position of both phenyl rings and no ortho substituents. TCDD has been shown to not inhibit gap junction intercellular communication (measured as metabolic cooperation between Chinese Hamster V-79 cells originally derived from lung tissue). TCDD-like PCBs and other PCBs lacking ortho substituted chlorine atoms, have a coplanar conformation and also do not inhibit gap junction intercellular communication (*9*).

Introduction of one or more chlorosubstituents in the ortho position results in a decreased degree of coplanarity between the two phenyl rings due to steric interactions. Mono- and diortho-chloro substitutions of the three most toxic congeners give toxic effects that resemble the toxicity of TCDD, qualitatively. However, the liver enzyme induction pattern is often of the mixed type. Gap junction intercellular communication was totally inhibited by all PCBs containing at least one chloro substituent in the ortho position with the exception of one highly chlorinated congener, 2,3,4,5,3′,4′,5′-heptachlorobiphenyl. One hypothesis for why 2,3,4,5,3′,4′,5′-heptachlorobiphenyl did not totally inhibit gap junction intercellular communication is that highly chlorinated congeners require more ortho substituents to inhibit gap junction intercellular communication. The potential for inhibition of gap junction intercellular communication generally increased with increasing numbers of ortho-substituted chlorine atoms (*9*). Results show that substitution in the ortho position from the carbon bridge is essential and at least one chloro substituent in the ortho position is necessary for the ability to inhibit gap junction intercellular communication (*9*). However, the total number of substitutions may not be crucial for the ability to inhibit gap junction intercellular communication (*9*). The steric interactions between more than two ortho substituents would markedly inhibit biphenyl ring coplanarity (thus, making these compounds noncoplanar). Many compounds of this group have been found to be PB-type inducers (*9*).

Brown et al. (*10*) studied the ability of polychlorinated biphenyls (PCBs) to stimulate polymorphonuclear leukocytes (neutrophils) in vitro in Sprague-Dawley rats. Congeners which are noncoplanar stimulated neutrophil O_2^- (superoxide) production, whereas coplanar congeners with high affinity for the arylhydrocarbon receptor (AhR) do not and might even inhibit this response.

1.2 Remediation of Polychlorinated Biphenyls Using Fenton's Reagent

A variety of physical, chemical, and biological processes have been investigated for the remediation of PCBs. Chemical oxidation could be used as a pretreatment step to enhance the biodegradability of PCBs or as a primary method of remediation. One form of chemical oxidation, Fenton's reagent, has been found to be an effective method of remediating PCB contaminated soils and aqueous solutions through oxidation by hydroxyl radicals (*3, 11*). In the Fenton's reagent process, highly reactive hydroxyl radicals are produced by the oxidation of ferrous iron and the reduction of hydrogen peroxide (*3*). The hydroxyl radical is one of the most reactive chemical species known and is second only to elemental fluorine in its relative oxidation power. The classical procedure of the Fenton's reagent treatment consists of the addition of hydrogen peroxide to a solution or suspension of compounds (reactive chemicals) in the presence of ferrous iron (*12*) at pH 2-3. Numerous reactions might be involved with Fenton's reagent, depending on the nature of the reacting substrates. One potential pathway, presented by

Trapido et al. (*12*), involving the abstraction of a hydrogen atom and initiating a radical chain oxidation is shown below:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{-} + \bullet OH \qquad (1)$$

$$RH + \bullet OH \rightarrow R\bullet + H_2O \qquad (2)$$

$$R\bullet + H_2O_2 \rightarrow ROH + \bullet OH \qquad (3)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \qquad (4)$$

The oxidation efficiency of the Fenton's type reactions depends on the $Fe^{2+}:H_2O_2$ ratio and the pH value. The optimal pH for the Fenton's reagent reaction efficiency has been shown to be between pH 3 and 5. At more basic pH values, the iron is converted from a hydrated ferrous form to a colloidal ferric form, thereby causing a decrease in the effectiveness of the reaction and the formation of ferric hydroxide (*13-15*).

Advantages of Fenton's reagent remediation are as follows:

a) The hydroxyl radical is a powerful oxidant. It is twice as reactive as chlorine and second only to fluorine among common oxidants in the oxidation potential series.

b) Fenton's remediation can detoxify a broad range of organic wastes and is effective over a wide range of contaminant concentrations (*13*).

c) Hydrogen peroxide, unlike chlorine, does not require large cylinders for storage and does not pose the dangers of chlorine gas. It can be stored on-site in quantities appropriate to user requirements.

d) Both iron and hydrogen peroxide are cheap and environmentally friendly. Unreacted hydrogen peroxide degrades in the environment to oxygen and water.

e) Fenton's remediation is effective as a pretreatment step that increases the rate and efficiency of biological degradation of contaminants. Partial chemical oxidation enhances water solubility of the organic compounds and increases biosusceptibility, facilitates microbial action, and increases the biodegradation rate. (*16*)

Disadvantages of Fenton's reagent remediation are as follows:

a) Since the reaction requires acidic conditions, the method of Fenton's oxidation might not be applicable to alkaline solutions or sludges with high buffering capacities (*17*).
b) Production of iron sludge, which must be disposed of.
c) Hydroxyl radicals are non-specific in nature and might react with non-pollutant species present at a higher concentration. (*13*)
d) Effectiveness of the reaction is influenced by pH, iron complexation, iron solubility, the Fe^{2+}:H_2O_2 ratio, and iron redox cycling between the +2 and +3 states.
e) Potential production of remediation byproducts with equal or greater toxicity than the original contaminant.
f) In some situations a rise in temperature might occur as the reaction proceeds.
g) Hydrogen peroxide is highly reactive. The reaction can be vigorous, with the rapid evolution of oxygen, steam, and carbon dioxide. Allowances should be made to ensure adequate venting of these gases.

When applied to PCBs, this process would result in the addition of a hydroxyl group to nonhalogenated sites and the production of hydroxylated polychlorinated biphenyls (*11*). Sedlak et al. (*11*) reported that dechlorination reactions are insignificant relative to hydroxylation reactions in the initial attack of hydroxyl radicals on PCBs; however, further reactions might occur, resulting in dechlorination and production of hydroxylated biphenyls. Basu et al. (*18*) showed that reactions of Fenton's reagent with aqueous phase 2,4,6-trichlorophenol are also associated with the immediate release of chlorine atoms into free chloride ions in solution. In a study on Fenton's oxidation of 2-chlorobiphenyl in aqueous solution, Sedlak et al. (*11*) suggested 5-hydroxy-2-chlorobiphenyl (or 2-chloro-5-biphenylol) as one of the byproducts.

Current remediation practices often emphasize the disappearance of the parent compound to at or below the regulatory limits, but often disregard the importance of reducing the overall toxicity. Since remediation byproducts can exhibit equal or greater toxicity than the parent compound, it is important to consider in a remediation process not only the removal of the parent compound, but also the toxicological impact of the remediation byproducts (*19*). Although the reaction of Fenton's reagent with various PCBs has been investigated (*11, 3*), the subsequent toxicity and characterization of remediation byproducts have not been extensively studied.

1.3 Toxicity of Hydroxylated Polychlorinated Biphenyls (OH-PCBs)

Within organisms, PCBs are metabolized by a diverse enzyme system, the cytochrome P450 monooxygenases, into hydroxylated polychlorinated biphenyls (OH-PCBs) (*20*). The metabolite formed is dependent upon the chlorine arrangement in the PCB and the type of enzyme involved (*21*). Less halogenated PCBs are readily metabolized to monohydroxy derivatives, which might be further metabolized to ortho- or para-dihydroxy metabolites and quinones (*22*). OH-PCBs have been detected in human organs, blood, fatty tissue, and milk, as well as in fish and wildlife (*23*). OH-PCBs have been shown to be transferred by placental transfer to the fetus both in humans and in animals (*24*). Several high molecular weight hydroxylated PCB metabolites have been found to be strongly and selectively accumulated in mammalian tissues, including human blood (*22*). Although lower molecular weight OH-PCBs have been considered to be nonpersistent, they may occur transiently during episodic exposures to PCBs (*22*). Some OH-PCBs might have a greater potential to disrupt biological systems than their parent compound. (*21*). OH-PCBs have been shown to have estrogenic and anti-estrogenic effects, can disrupt thyroxin and vitamin A transport, and might lead to adverse neurodevelopmental effects (*20, 25*). Since OH-PCBs are relatively stable in mammalian systems, they are eliminated mainly by excretion in urine and feces and are detected as residues in the environment (*25*). Based upon 1996 amendments to the Safe Drinking Water and Food Quality Protection Act, OH-PCBs are among the pollutants requiring monitoring as estrogenic substances in drinking water (*25*). The OH-PCBs produced from PCB remediation processes, such as the Fenton's reagent procedure, are a potential environmental concern, thereby justifying toxicological evaluation.

Few studies have been performed regarding the effects of OH-PCBs on GJIC and none have investigated the OH-PCBs examined in this dissertation. Machala et al. (*22*) observed inhibition of GJIC for a selected group of OH-PCBs, with the strongest inhibition for OH-PCBs with the highest molecular weight. The majority of the mono- and dihydroxy-PCBs studied inhibited GJIC in vitro in micromolar concentrations. No cytotoxic effects of the tested compounds were observed with 30 minutes and 24 hours of exposure at concentrations up to 100 μM.

1.4 Gap Junctional Intercellular Communication (GJIC) as a Marker of Toxicity

When considering the toxicity of a chemical, the potential of that chemical to cause cancer is a major concern. Three means exist by which toxic chemicals can alter the functioning of cells. "Genotoxicity" or mutagenesis is when a toxic chemical alters the genetic information of a cell (the DNA code) or the number or structure of the chromosomes. Cytotoxicity is when a toxic chemical kills a cell by a necrotic mechanism (cell basically dies because of membrane damage, critical enzymes or proteins are destroyed) or an "apoptotic" mechanism (a chemical causes the cell to commit suicide (programmed cell death)). Epigenetic toxicity is when a nonmutagenic and noncytotoxic chemical alters the expression of normal genes causing inappropriate "turning on or off" of genes in a cell at the wrong place at the wrong time (*26*). The alteration of the expression of genes can occur at the transcriptional, translational, or posttranslational levels (*19*). The ability to disrupt gap junctional intercellular communication (GJIC) is indicative of one form of a chemical's epigenetic toxicity. Epigenetic toxicity can be evaluated using the GJIC assay, a nongenotoxic assay that measures the level of cell-cell communication (or GJIC).

Gap junctions are organized collections of protein channels in the cell membrane that allow ions and small molecules ($\leq$ 1200 Da) to traverse passively between the cells that they connect (*27*). GJIC, which is required for cellular growth control, developmental and differentiation processes, synchronization, and metabolic regulation, results from this passive transfer of ions and small molecules through the gap junctions (*28*). Gap junctions exist in all metazoans (multi-cellular organisms) and in almost all cell types in these organisms. Some cells that lack gap junctions include skeletal muscle, red blood cells, and free standing cells such as circulating lymphocytes (*27*). The majority of cancer cells have dysfunctional gap junctional intercellular communication (*29*). Also, many non-mutagenic but toxic chemicals have been shown to inhibit GJIC and to promote the growth of premalignant tumor cells (*29*). It has been suggested by Trosko et al. (*30*) that the reversible inhibition of GJIC by endogenous or exogenous tumor promoters is responsible for the tumor promotion phase of carcinogenesis. Some PCBs have been shown to be epigenetically toxic by being capable of disrupting GJIC (*1*, *9*, *31*, *32*). PCBs with coplanar conformations appear to be less likely to inhibit GJIC, whereas decreased coplanarity of the PCB increases the chances of inhibition of GJIC (*9*, *31*, *32*). No literature could be found regarding the effects of hydroxylated biphenyls (OH-BPs) on GJIC. By performing bioassays that measure the effect of chemical exposure on GJIC, an evaluation of the potential cancer risk is possible.

1.5 Hypotheses and Objectives

The dissertation research was performed with the hypothesis that Fenton's remediation of the parent PCB, 4,4′-dichlorobiphenyl (4,4′DCBP), under a set of selected conditions would result in a final remediation solution that indicates a decrease in the concentration of the parent PCB, is more toxic than the parent PCB, and exhibits some of the toxicological properties demonstrated by the selected potential remediation byproduct standards (particularly 4,4′-dichloro-3-biphenylol).

The dissertation research was performed with the following objectives:

a) To determine whether there is any correlation between the chemical and structural properties of OH-PCBs and OH-BPs, as potential byproducts of remediation of PCBs with Fenton's reagent, and their observed epigenetic toxicity and cytotoxicity.

b) To evaluate the toxicity of the parent PCB, 4,4′-dichlorobiphenyl in rat liver epithelial cells using a nongenotoxic bioassay that determines the in vitro modulation of GJIC as a measure of the epigenetic toxicity.

c) To develop a new test that qualitatively indicates the presence of hydroxyl radicals in aqueous samples and evaluate its applicability for the detection of hydroxyl radicals in a Fenton's reaction aqueous solution.

d) To verify that the reaction of Fenton's reagent with the solvent for remediation of the parent PCB does not result in any toxic byproducts, and select a suitable solvent for the remediation of the parent PCB.

e) To develop a procedure for Fenton's remediation of the parent PCB.

f) To perform Fenton's remediation of 4,4′DCBP in the selected solvent, followed by examination of the toxicity of the final Fenton's remediation solution and disappearance of 4,4′DCBP.

g) To compare the toxicity of the final Fenton's remediation solution to the toxicity observed for the potential remediation byproducts and parent PCB.

h) To analyze the gas chromatograms from the final Fenton's remediation solution for the presence of any remediation byproduct peaks.

i) To determine the retention times for the chlorinated potential remediation byproducts previously investigated in the toxicology studies and compare the retention patterns to those of the extracted samples.

1.6 Organization of Dissertation

The research performed for the dissertation was separated into four main divisions. The first division, comprised of Chapters 2 and 3, involved toxicology studies of six selected potential Fenton's remediation byproducts and the selected parent PCB (4,4′-dichlorobiphenyl). The objective of the research described in Chapter 2 was to determine whether there is any correlation between the chemical properties (including structure) of OH-PCBs and OH-BPs and their observed epigenetic toxicity and cytotoxicity. Six commercially available chemicals considered to be representative of OH-BPs and OH-PCBs, which are potential byproducts of remediation of PCBs with Fenton's reagent, were selected for study. These six chemicals were 2-biphenylol (2BP), 3-biphenylol (3BP), 2,2′-biphenyldiol (2,2′BP), 3,3′-biphenyldiol (3,3′BP), 3-chloro-2-biphenylol (3C2BP), and 4,4′-dichloro-3-biphenylol (4,4′DC3BP). The toxicity of these chemicals was evaluated in rat liver epithelial cells using a nongenotoxic bioassay that determines the in vitro modulation of GJIC as a measure of the epigenetic toxicity. For each chemical the dose-response, time-response, time of recovery, and cytotoxicity were investigated. In view of the cytotoxicity and GJIC inhibitory effects observed for 4,4′-dichloro-3-biphenylol (4,4′DC3BP), the PCB congener 4,4′-dichlorobiphenyl (4,4′DCBP) was selected for Fenton's reagent remediation studies. In Chapter 3, the toxicity of the parent PCB (4,4′DCBP) for the Fenton's remediation studies was evaluated in rat liver epithelial cells using a nongenotoxic bioassay that determines the in vitro modulation of GJIC as a measure of the epigenetic toxicity.

The methylene blue (MB) dye test was developed as a new test that qualitatively indicates the presence of hydroxyl radicals through an immediate, distinct bleaching of the MB dye on a paper test strip after applying an aqueous sample containing hydroxyl radicals. The second division, comprised of Chapter 4, tested the applicability of the MB dye test for the detection of hydroxyl radicals in a Fenton's reaction aqueous solution and verified the results by benzoic acid chemical probe hydroxyl radical detection methods using thin layer chromatography and spectrophotometric wavelength scans. For Fenton's remediation experiments throughout the dissertation research, MB dye tests were performed during the Fenton's reaction to verify the formation of hydroxyl radicals and during the quenching process to verify completion of quenching (absence of hydroxyl radicals).

The third division, comprised of Chapters 5 and 6, verified that the reaction of Fenton's reagent with the solvents (Milli-Q H_2O alone, 80/20 Milli-Q H_2O/ACN (by volume), and 50/50 Milli-Q H_2O/ACN (by volume)) in the absence of 4,4'DCBP, does not result in any toxic byproducts. If no toxic response occurs as a result of a reaction of Fenton's reagent with the solvent, any toxicity resulting from the Fenton's reagent remediation of 4,4'DCBP can be assumed to be independent of the solvent. Additionally, this series of experiments allowed for the development of a remediation procedure that later would be applied to 4,4'DCBP. Evaluation of whether Fenton's reagent remediation in water alone resulted in any toxicity is discussed in Chapter 5. Although 4,4'DCBP is insoluble in water alone, since water constituted some part of the solvent used for dissolving 4,4'DCBP in remediation, it was important to investigate whether Fenton's reagent remediation with water alone resulted in any toxicity. Three molar Fe^{2+}:H_2O_2 ratios, 1:5, 1:20, and 1:40, were investigated in this section of research to determine the ratio that would be used for Fenton's remediation of 4,4'DCBP. In Chapter 6, Fenton's remediation of the solvents 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN (by volume) were investigated to determine the toxicological effect of ACN in combination with water as a solvent in remediation. Based on the results of Fenton's remediation in Milli-Q water, discussed in detail in Chapter 5, an Fe^{2+}:H_2O_2 ratio of 1:20 was selected for each Fenton's remediation experiment in Chapter 6. In both Chapters 5 and 6, the toxicity of the resulting final Fenton's remediation solutions was evaluated in rat liver epithelial cells, using a nongenotoxic bioassay that determines the in vitro modulation of gap junctional intercellular communication (GJIC) as a measure of the epigenetic toxicity.

The fourth division, comprised of Chapters 7 and 8, involved Fenton's remediation of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN, followed by examination of the toxicity of the final Fenton's remediation solution and disappearance of 4,4′DCBP as a result of Fenton's remediation. The Fenton's reaction conditions were pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM. The pre-remediation concentration of 4,4′DCBP in the reaction mixture was 2 mM (or 446 ppm). After a 60 minute remediation reaction period, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v). Following quenching with 10% Na_2SO_3, the Fenton's reaction mixture pH was adjusted to 9.0 (unless the pH was already greater than or equal to pH 9.0) and the temperature remained at 23.0 °C. The reaction mixture was then filtered through a 1.0 μm glass fiber filter and the filtrate was adjusted to pH 7.0. In Chapter 7, the toxicity of the resulting final Fenton's remediation solution was evaluated in rat liver epithelial cells, using a nongenotoxic bioassay that determines the in vitro modulation of gap junctional intercellular communication (GJIC) as a measure of the epigenetic toxicity. The toxicity of the final Fenton's remediation solution was compared to the toxicity observed for the potential remediation byproducts and 4,4′DCBP studied in Chapters 2 and 3, respectively. Chapter 8 determined the disappearance of 4,4′DCBP throughout the Fenton's remediation process for the reaction times of 0, 15, 30, and 60 minutes. Samples of the Fenton's remediation mixture extracted with an isooctane liquid-liquid extraction method were analyzed by gas chromatography/electron capture detection (GC/ECD) to quantitate the disappearance of the parent PCB, 4,4′DCBP, over the period of remediation. The gas chromatograms were analyzed for the presence of any remediation byproduct peaks. In addition, retention times were determined for the chlorinated potential remediation byproducts previously investigated in toxicology studies (Chapter 2) and the retention patterns were compared to those of the extracted samples.

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Chapter 2

Epigenetic Toxicity of Hydroxylated Biphenyls and Hydroxylated Polychlorinated Biphenyls as Potential Remediation Byproducts

2.1 Introduction

An article (*1*) based on this chapter was published in *Environmental Science and Technology*.

Exposure to polychlorinated biphenyls (PCBs) is known to have numerous human health effects including reproductive disorders, embryotoxicity, oncogenicity, and estrogenic endocrine disruption, as well as probable human carcinogenicity (*2-4*). Within organisms, PCBs are metabolized into hydroxylated polychlorinated biphenyls (OH-PCBs) (*5*). OH-PCBs, which have been detected in human organs, blood, fatty tissue, and milk, as well as in fish and wildlife, have estrogenic and anti-estrogenic effects, can disrupt thyroxin and vitamin A transport, and might lead to adverse neurodevelopmental effects (*5-7*). Since OH-PCBs are relatively stable in mammalian systems, they are eliminated mainly by excretion in urine and feces and are detected as residues in the environment (*6*). Hydroxylated PCBs can also be formed during Fenton's oxidation, which has been found to be an effective method of remediating PCB contaminated soils and aqueous solutions (*8, 9*). Fenton's oxidation of PCBs would result in the addition of a hydroxyl group to nonhalogenated sites and the production of hydroxylated polychlorinated biphenyls, and further reactions might occur resulting in dechlorination (*8*).

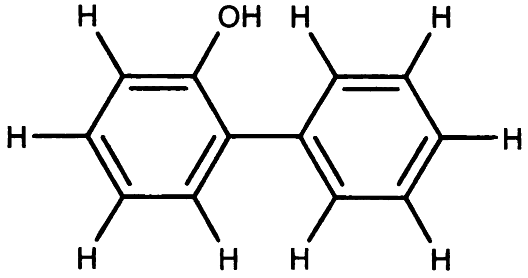
The ability to disrupt gap junctional intercellular communication (GJIC) is indicative of one form of a chemical's epigenetic toxicity (*10*). Epigenetic toxicity is but one of three means by which toxic chemicals can alter the functioning of cells: (a) "genotoxicity" or mutagenesis is when a toxic chemical alters the genetic information of a cell (the DNA code) or the number or structure of the chromosomes; (b) cytotoxicity is when a toxic chemical kills a cell by a necrotic mechanism (cell basically dies because of membrane damage, critical enzymes or proteins are destroyed) or an "apoptotic" mechanism (a chemical causes the cell to commit suicide (programmed cell death)); or (c) epigenetic toxicity is when a non-mutagenic and non-cytotoxic chemical alters the expression of normal genes causing inappropriate "turning on or off" of genes in a cell at the wrong place at the wrong time (*11*). The alteration of the expression of genes can occur at the transcriptional, translational, or posttranslational levels (*12*). Epigenetic toxicity can be evaluated using the GJIC assay, a nongenotoxic assay that measures the level of cell-cell communication. Gap junctional intercellular communication, which is required for cellular growth control, developmental and differentiation processes, synchronization of cellular functions, and metabolic regulation, results from the transfer of ions and small molecules ($\leq$ 1200 Da) between cells through membrane channels called gap junctions (*13*). It has been suggested by Trosko et al. (*14*) that the reversible inhibition of GJIC by endogenous or exogenous tumor promoters is responsible for the tumor promotion phase of carcinogenesis. Some PCBs have been shown to have epigenetic toxicity by being capable of disrupting GJIC (*15, 16*). PCBs with coplanar conformations appear less likely to inhibit GJIC, whereas decreased coplanarity of the PCB increases the chances of inhibition of GJIC (*17-19*). Few studies have been performed to determine the effects of OH-PCBs on GJIC and none have investigated the OH-PCBs examined in this chapter. Machala et al. (*20*) observed inhibition of GJIC for a selected group of OH-PCBs, with the strongest inhibition for OH-PCBs with the highest molecular weight. The majority of the mono- and dihydroxy-PCBs studied inhibited GJIC in vitro in micromolar concentrations. No cytotoxic effects of the tested compounds were observed with 30 minutes and 24 hours of exposure at concentrations up to 100 μM. No literature could be found regarding the effects of hydroxylated biphenyls (OH-BPs) on GJIC.

The objective of this study was to determine whether there is any correlation between the chemical properties (including structure) of OH-PCBs and OH-BPs and their observed epigenetic toxicity and cytotoxicity. Six commercially available chemicals considered to be representative of OH-BPs and OH-PCBs, which are potential byproducts of the remediation of PCBs with Fenton's reagent, were selected for study. These six chemicals were 2-biphenylol (2BP), 3-biphenylol (3BP), 2,2′-biphenyldiol (2,2′BP), 3,3′-biphenyldiol (3,3′BP), 3-chloro-2-biphenylol (3C2BP), and 4,4′-dichloro-3-biphenylol (4,4′DC3BP). The toxicity of these chemicals was evaluated in rat liver epithelial cells using a nongenotoxic bioassay that determines the in vitro modulation of GJIC as a measure of the epigenetic toxicity. For each chemical the dose-response, time-response, time of recovery, and cytotoxicity were investigated.

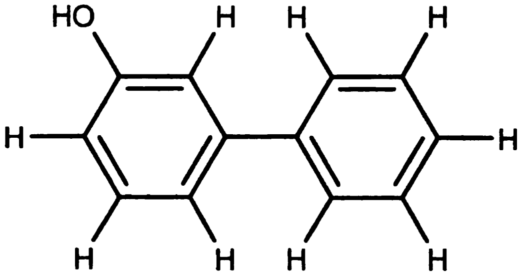
2.2 Experimental Section

2.2.1 Chemicals

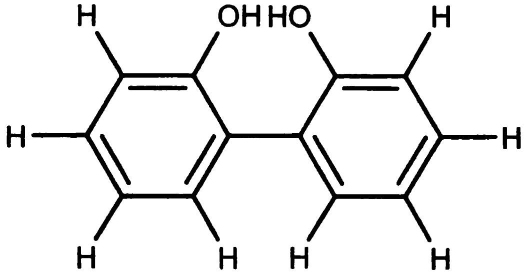
2-Biphenylol, 3-biphenylol, 2,2′-biphenyldiol, 3,3′-biphenyldiol, 3-chloro-2-biphenylol, and 4,4′-dichloro-3-biphenylol were all purchased from Chem Service Inc. (West Chester, PA) (Figure 2.1). All chemicals used as toxicants in the study had a



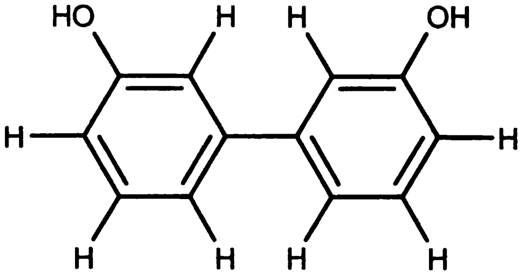
2-Biphenylol (2BP)



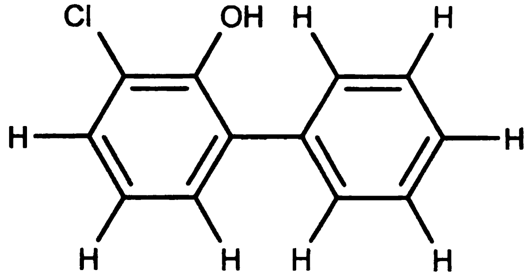
3-Biphenylol (3BP)



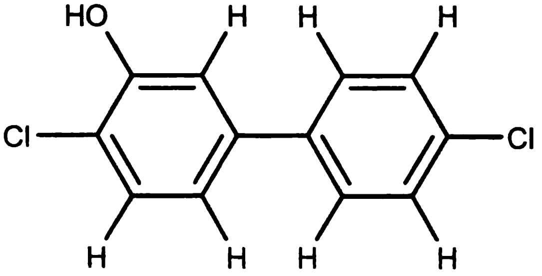
2,2′-Biphenyldiol (2,2′BP)



3,3′-Biphenyldiol (3,3′BP)



3-Chloro-2-biphenylol (3C2BP)



4,4′-Dichloro-3-biphenylol (4,4′DC3BP)

Figure 2.1 Chemical structures of the six chemicals selected as potential byproducts of remediation of PCBs with Fenton's reagent and evaluated for toxicity.

purity of 95% or greater. Acetonitrile (99.8% purity) was purchased from EM Science (Gibbstown, NJ). For cell culture, D-medium (Formula No. 78-5470 EG), Fetal Bovine Serum (FBS), and Gentamicin were purchased from GIBCO Laboratories (Grand Island, NY). Lucifer Yellow CH, dilithium salt, was purchased from Molecular Probes Inc. (Eugene, OR) and ICN Biomedicals Inc. (Aurora, OH). Neutral red dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) for the cytotoxicity bioassays was purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid (99.7 %) was purchased from EMD Chemicals Inc. (Gibbstown, NJ). Ethanol (95% purity) was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY). Formaldehyde solution (37%) for the GJIC bioassays was purchased from J.T. Baker (Phillipsburg, NJ).

2.2.2 Methods

2.2.2.1 Toxicological Evaluation

Stock solutions of the purchased OH-BPs and OH-PCBs were prepared in acetonitrile (ACN). ACN was chosen as the solvent for the stock solutions because it has minimal effect on GJIC at a final ACN concentration of up to 1.5% in the cell culture medium as determined by an ACN dose-response test. Since slight inhibition of GJIC occurs at a final ACN concentration of 2.0% in the cell culture medium (corresponding to 40 μL of ACN), all experiments were conducted at final ACN concentrations of 1.5% or less (corresponding to 30 μL of ACN or less) (*21*).

2.2.2.2 Cell Culture Techniques

WB-F344 rat liver epithelial cells were obtained from Dr. J. W. Grisham and Dr. M. S. Tsao of the University of North Carolina (Chapel Hill, NC) (*12*). This cell line was selected because it is a diploid, nontumorigenic cell line originating from a strain of rat that has been used for toxicological/cancer studies of numerous chemicals, thereby allowing for a source of comparison (*12*). Since 70% of the chemicals that are carcinogens are liver carcinogens and the liver is the "first pass" organ for ingested toxins, liver cells are important for toxicological/cancer studies (*22*). Furthermore, the WB-F344 cell line was designed for in vitro assays to match the many in vivo tumor promotion assays that had been done in rat liver, specifically, in the Fischer 344 rat.

The cell culture techniques performed were similar to those described by Herner et al. (*12*) and Luster-Teasley et al. (*23*). Cells were cultured in 150 cm^2 sterile, treated, polystyrene cell culture flasks (Corning Inc., Corning, NY) in 25 mL of D-medium containing 5% Fetal Bovine Serum (FBS) and 0.2% Gentamicin. The cells were incubated at 37 °C in a water-jacketed IR Autoflow Automatic CO_2 incubator (NUAIRE, Inc., Plymouth, MN) in a humidified atmosphere with 5% CO_2 and 95% air. The time required for cell growth confluency was about two days. The confluent culture was split and transferred every other day into a new 150 cm^2 culture flask with new medium mixture. In addition, from 150 cm^2 flasks of confluent cells, cultures were prepared for the bioassays in 35 mm diameter, sterile, treated polystyrene cell culture dishes (Corning Inc., Corning, NY) with 2 mL of D-medium supplemented with 5% FBS. The cultures for the bioassays were incubated under the same conditions as the aforementioned flasks.

2.2.2.3 In Vitro Bioassay for GJIC

The bioassays of GJIC were performed on confluent cell cultures (usually 2 days of growth) grown in 35 mm diameter culture dishes (as described in the preceding section). The scrape-loading/dye transfer (SL/DT) procedure for determining the GJIC was adapted from the method described by El-Fouly et al. (*24*) and is described in detail by Herner et al. (*12*). A detailed description of the spread of Lucifer yellow dye from the scrape to neighboring cells can be found in Wilson et al. (*25*). Chemical treatments, controls (no dose), and vehicle controls (ACN only) were performed in triplicates. Doses were applied directly to the dishes of confluent cell cultures. Vehicle controls were dosed with a volume of ACN corresponding to the largest volume of chemical dose tested in the treatment dishes. Chemical treatments were performed at noncytotoxic levels as determined by the neutral red uptake assay (cytotoxicity bioassay) (*26*). Measurement of GJIC has to be done at concentrations that do not cause cytotoxicity, since the objective is to determine if a potential toxicant can bring about its physiological toxic endpoint without tissue destruction but by noncytotoxic mechanisms.

The specific GJIC bioassays that were performed included dose-response, time-response, and time of recovery. All culture dishes were examined within 24 hours of the experiment completion. Each culture dish of cells was digitally photographed such that the observed scrape spanned the full horizontal width of the picture. A COHU High Performance Color CCD Camera (Cohu, Inc., San Diego, CA) with a magnification of 200× under a Nikon Diaphot-TMD epifluorescence phase-contrast microscope (Nikon Corp., Japan) illuminated with a Nikon HB-10101AF Super High Pressure Mercury 100W lamp (Nikon Corp., Japan), or a Nikon TE300 Eclipse Inverted Microscope (Nikon

Corp., Japan) with a Nikon HB-10103AF Super High Pressure Mercury 100W lamp (Nikon Corp., Japan) was used.

The fluorescence of the Lucifer yellow dye was used to determine the distance the dye traveled perpendicular to the scrape. This distance of dye travel was indicative of the level of GJIC within the culture. Quantitative analysis of the distance of dye spread was performed using NucleoTech GelExpert software (NucleoTech Corp., Hayward, CA). The distance of dye spread was measured in terms of the area of dye spread, by tracing manually via free object quantification the area of farthest visible fluorescence. Since the width of the photographed section was the same for every culture dish, measuring the area of the dye spread was equivalent to measuring the distance of dye spread perpendicular to the scrape. The area of dye spread for each chemical treatment dish was compared to a control group of cells that were exposed to ACN only (vehicle controls) under the same assay as the treated cells. For each chemically treated dish, the fraction of the control was calculated as the area of dye spread in the treated dish divided by the average area of dye spread in the triplicate set of vehicle control dishes. The results for each set of chemically treated triplicates were reported as an average fraction of the control (FOC) ± standard deviation (SD) determined at the 95% confidence interval (CI).

The level of GJIC in cells exposed to the chemical was assessed by the decrease in communication of the cells as compared to the vehicle control groups, exposed to ACN only. A decrease in FOC corresponds directly to a decrease in GJIC (where the doses are not cytotoxic). Interpretations of GJIC results are consistent with Luster-Teasley et al. (*23*) and Herner et al. (*12*). Complete communication (100%) between the cells is identified as a FOC value of 1.0 as seen in the vehicle control. A FOC value greater than 0.9 is difficult to statistically distinguish from the vehicle controls. FOC values between 0.9 and 0.5 indicate partial inhibition of GJIC. A FOC value less than or equal to 0.5 is indicative of a significant amount of inhibition of GJIC, since this would be representative of communication levels that are 50% or less than the normal communication levels. FOC values between 0.3 and 0.0 are representative of complete inhibition of GJIC. A FOC value of 0.3 is usually used to represent complete inhibition, as it corresponds to the width of a single row of cells with no dye spreading beyond its boundaries (*12*). Controls, which received no dose of chemical or solvent (ACN), were performed for each experiment as a means of evaluating a normal level of GJIC and the overall "health" of the cells. By performing a t-test for each experiment, it was found that the areas of dye spread for the control dishes (no solvent or chemical) did not vary significantly from the areas for the vehicle controls (only ACN) at a 95% CI. Therefore, it could be concluded that the solvent, at the volume tested, was not a significant source of inhibition in the experiments. Statistical analyses were performed by means of the t-test and One Way Analysis of Variance (ANOVA) to compare the chemical treatment results and vehicle control results.

2.2.2.4 In Vitro Bioassay for Cytotoxicity

The procedure for determining cytotoxicity using the neutral red uptake assay was adapted from the method of Borenfreund and Puerner (*26*) and the method described by Weis et al. (*27*). The WB-F344 rat liver epithelial cells were cultured in the same manner as that used for the GJIC bioassays. Dishes with a confluent growth of cells of similar density were used for both the dye incubation test and cytotoxicity bioassays.

Chemical treatments, controls (no dose) and vehicle controls (ACN only) were performed in triplicate. A solution of neutral red dye (0.08%) in D-medium supplemented with 5% FBS was incubated at 37 °C for approximately 2 hours. The neutral red dye solution was centrifuged at 1200 rpm for 10 minutes and the supernatant was filtered through a 0.22 μm hydrophilic polysulfone membrane filter.

To determine the amount of time necessary for incubation of the dishes with the neutral red dye solution, a dye incubation test was performed. A set of 8 control dishes (not chemically treated) were each incubated with 2 mL of the neutral red dye solution at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. At 5, 10, 15, 20, 25, 30, 40, and 60 minutes, a single dish was removed from the incubator, rinsed five times with Ca^{2+}/Mg^{2+} PBS (Phosphate Buffered Saline), and 1 mL of neutral red solubilizer (an aqueous solution of 1% acetic acid, 50% ethanol, and 49% R.O. water) was added and allowed to lyse the cells for 15 minutes at room temperature. The absorbance of the solubilized dye was measured in cuvettes (polystyrene, optical pathlength of 10 mm) at 540.0 nm on a Beckman DU 7400 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). A time of dye incubation was selected which gave an absorbance measurement close to 1.5 at 540.0 nm. If all incubation times yielded an absorbance greater than 2.0 at 540.0 nm, then the samples of solubilized dye were reanalyzed following dilution with neutral red solubilizer.

To test the cytotoxicity of a chemical toxicant, after the cells were exposed to the chemical for the desired incubation time, the cells were rinsed five times with Ca^{2+}/Mg^{2+} PBS, and 2 mL of the neutral red dye solution was added to each dish. The cells were then incubated for the predetermined dye incubation time at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. After incubation, the dishes were rinsed five times with Ca^{2+}/Mg^{2+} PBS, and 1 mL of neutral red solubilizer was added to each dish and allowed to lyse the cells for 15 minutes at room temperature. If dilution was found to be necessary in the dye incubation test, the solubilized dye solutions were diluted as previously mentioned. The absorbance of the solubilized dye was measured as aforementioned at 540.0 nm and 630.0 nm. The background absorbance at 630.0 nm was subtracted from the absorbance measured at 540.0 nm to obtain a true absorbance. For each chemically treated dish, the fraction of the control was calculated as the true absorbance in the treated dish divided by the average true absorbance in the triplicate set of vehicle control dishes. The results for each set of chemically treated triplicates were reported as an average fraction of the control (FOC) ± standard deviation (SD) determined at the 95% confidence interval.

A cytotoxicity FOC of about 1.0 indicates that the neutral red uptake was approximately equivalent to that of the vehicle control (a noncytotoxic response). A FOC value greater than or equal to 0.8 is considered indicative of a noncytotoxic response. A FOC value of less than 0.8 indicates that significantly less neutral red dye solution was retained by the cells than within vehicle control cells; therefore, the chemical is cytotoxic at that dose (*28*). Statistical analyses equivalent to those performed for the GJIC bioassays were performed.

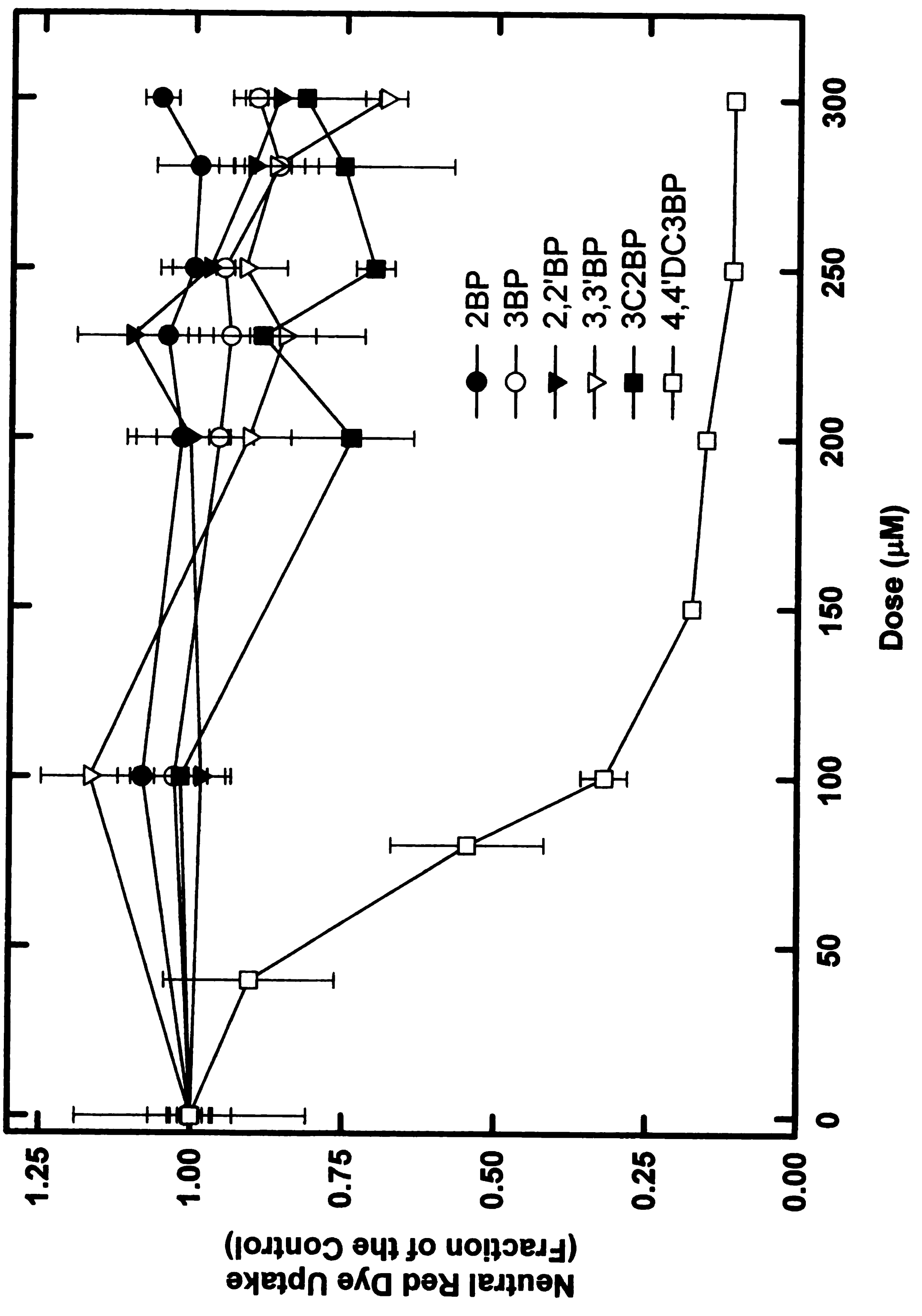
2.3 Results and Discussion

2.3.1 30-Minute Cytotoxicity Assay

It is important to use noncytotoxic levels of chemical toxicants in the GJIC bioassays since if a cytotoxic dose were used, it would be impossible to distinguish between decreased intercellular communication due to cell death and decreased intercellular communication due to inhibition of GJIC by closure or constriction of the gap junctions. The concept behind the neutral red uptake assay is that viable cells will incorporate the dye by active transport, whereas nonviable cells will not. A change in the number of cells or their physiological state will result in a direct change in the amount of dye the cells incorporate and, hence, the degree of cytotoxicity indicated. For the 30 minute cytotoxicity experiments, confluent growths of cells of similar density to those used in the bioassays for GJIC were exposed to varying chemical doses and allowed to incubate for 30 minutes before assaying for cytotoxicity. As shown in Figure 2.2, each chemical was tested at a dose range of 0 to 300 μM.

Four of the chemicals examined (2BP, 3BP, 2,2′BP, and 3C2BP) were not cytotoxic within the dose range of 0 to 300 μM. 3C2BP produced FOC values that oscillated around 0.8 (range 0.70 ± 0.03 to 0.89 ± 0.17) for doses between 200 and 300 μM. Typically when cytotoxicity occurs, the FOC will continue to decrease following an initial downward trend, rather than oscillate about a value. In addition, using one way analysis of variance to compare the cytotoxicity results within this region of oscillation indicated that the oscillation about the FOC of 0.8 is not statistically significant. One could consequently assume that the results for 3C2BP are not indicative of cytotoxicity.

Figure 2.2 Cytotoxicity results using the neutral red uptake assay for each of the six selected potential remediation byproducts. Cell cultures were exposed to the chemicals for 30 minutes. Each chemical was tested for a dose range of 0 to 300 μM. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.


Neutral Red Dye Uptake
(Fraction of the Control)
0.00
0.25
0.50
0.75
1.00
1.25
0
50
100
150
200
250
300
Dose (µM)
2BP
3BP
2,2'BP
3,3'BP
3C2BP
4,4'DC3BP

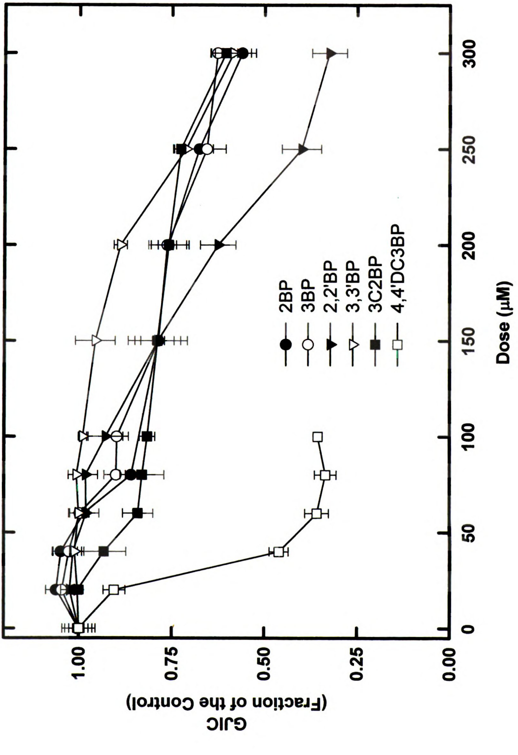
3,3′BP and 4,4′DC3BP were the only two chemicals found to be cytotoxic. Between doses of 0 to 280 μM, no cytotoxicity was observed for 3,3′BP. At a dose of 300 μM; however, 3,3′BP was found to be cytotoxic (FOC = 0.69 ± 0.03). Cytotoxicity was observed for 4,4′DC3BP at doses greater than 40 μM. The FOC rapidly declined as doses of 4,4′DC3BP increased above 40 μM.

2.3.2 Dose-Response Bioassay

For the dose-response experiments, confluent cells were exposed to varying chemical doses and allowed to incubate for 30 minutes before assaying for GJIC. Each chemical was tested over a dose range of 0 to 300 μM. Chemical doses greater than 300 μM were not tested in order to ensure that ACN final concentrations were not greater than 1.5 % (discussed in detail in the Experimental Section) and that doses were below the solubility of the chemical in the cell culture media. Figure 2.3 presents a comparison of the dose-response curves for the six chemicals tested.

The maximum levels of inhibition occurred for 2,2′BP at 300 μM (FOC = 0.33 ± 0.05) and 4,4′DC3BP at 40 μM (FOC = 0.46 ± 0.03). 2,2′BP and 4,4′DC3BP, the two chemicals that exhibited a steep decline in GJIC with an increase in dose, attained a significant amount of inhibition of GJIC (FOC ≤ 0.5). 2,2′BP was the only chemical to achieve complete inhibition (this occurs at 300 μM). Since 300 μM was not found to be a cytotoxic dose for 2,2′BP, the complete inhibition of GJIC observed is not likely to be due to cytotoxicity, but rather a consequence of chemical inhibition. Although a continued decline in communication was observed for 4,4′DC3BP at doses greater than

Figure 2.3 Dose-response results for each of the six selected potential remediation byproducts. Cell cultures were exposed to the chemicals for 30 minutes. Each chemical was tested for a dose range of 0 to 300 µM. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

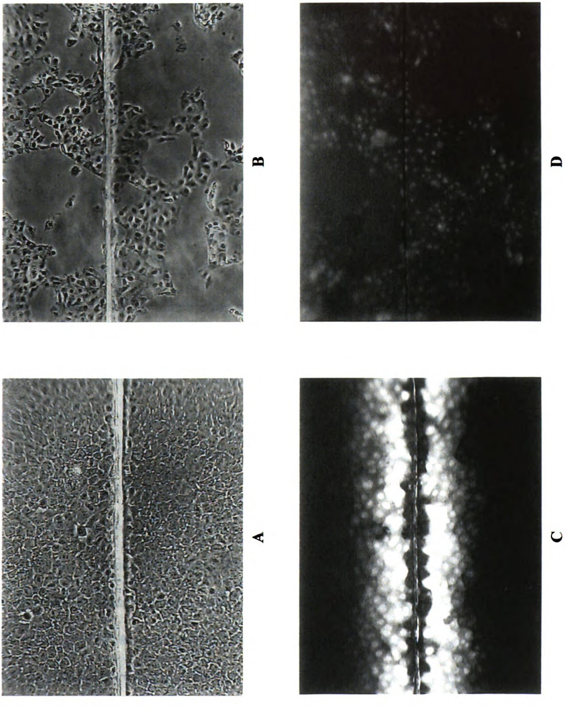

GJIC
(Fraction of the Control)
0.00
0.25
0.50
0.75
1.00
Dose (μM)
0
50
100
150
200
250
300
● 2BP
○ 3BP
▶ 2,2'BP
▷ 3,3'BP
■ 3C2BP
□ 4,4'DC3BP

40 μM, this lack of communication can be attributed to cytotoxicity rather than inhibition of GJIC.

No dose-response data were obtained for 4,4'DC3BP at doses above 100 μM, since at concentrations of 150 μM and greater it became impossible to quantitate a level of dye spread. When observed under visible light (phase contrast) at these higher doses, detachment of cells from the surface of the dish became apparent as sections of missing cells in the otherwise confluent layer. When observed under ultraviolet (UV) light at these same doses, there was no apparent front of dye spread, but rather a fluorescing of random or remaining cells on the dish. Figure 2.4 depicts phase contrast and UV epifluorescent images, which compare 0 and 250 μM doses of 4,4'DC3BP. The detachment of cells from the surface of the dish might be attributed to the chemical's effect on the cell adhesion proteins, which adhere the cells to the treated dish surface, resulting in subsequent cell death. Fluorescing of random or remaining cells on the dish is indicative of perforation of the cell membranes by chemical damage, allowing the dye to enter the cell freely rather than passing through the gap junctions from neighboring cells. As shown in Figure 2.4B and D, the presence of isolated fluorescing cells, lacking direct contact to neighboring cells, is further indication of perforation of the cell membranes, since dye could not reach these cells via passage through gap junctions. Both the loss of adhesion of the cells and perforation of the cell membranes are consistent with 30 minute cytotoxicity results.

2BP, 3BP, 3,3'BP, and 3C2BP exhibited a gradual decline in GJIC with an increase in dose. Only partial inhibition of GJIC (FOC between 0.9 and 0.5) was observed for these chemicals. In terms of increasing maximum inhibition at noncytotoxic

Figure 2.4 Phase contrast (visible light) and UV epifluorescent photomicrographs at 200× magnification comparing 0 and 250 μM doses of 4,4′DC3BP. A treatment of 0 μM 4,4′DC3BP for 30 minutes was a vehicle control treatment with 25 μL of ACN for 30 minutes. (A) Phase contrast and (C) UV epifluorescent photomicrographs of cell cultures treated with 0 μM 4,4′DC3BP for 30 minutes indicate a confluent layer of healthy cells with complete communication. (B) Phase contrast and (D) UV epifluorescent photomicrographs of cell cultures treated with 250 μM 4,4′DC3BP for 30 minutes indicate detachment of cells from the cell monolayer and a fluorescing of the remaining cells.


A
B
C
D

levels, the order of these chemicals was 3,3′BP (FOC = 0.71 ± 0.03 at 250 μM), 3BP (FOC = 0.63 ± 0.02 at 300 μM), 3C2BP (FOC = 0.61 ± 0.03 at 300 μM), and 2BP (FOC = 0.56 ± 0.02 at 300 μM). On the basis of the results of the 30 minute cytotoxicity assay, the reduction in communication observed at 300 μM for 3,3′BP is a consequence of cytotoxicity rather than chemical inhibition of GJIC.

2.3.3 24-Hour Cytotoxicity Assay

A 24 hour cytotoxicity assay was performed to aid in the selection of the dose to be used for the time-response experiment. To determine whether a reduction in intercellular communication at 24 hours of toxicant exposure is due to chemical inhibition by closure or constriction of the gap junctions rather than cytotoxicity, it is essential that the dose selected is not cytotoxic at 24 hours of toxicant exposure. The only variation of the 24 hour cytotoxicity assay from the 30 minute cytotoxicity assay was the 24 hour exposure period. In addition, the cells were evaluated visually (under a microscope) after 24 hours for indications of cell stress or detachment of cells from the dish surface.

Table 2.1 presents the 24 hour cytotoxicity results for the selected time-response dose for each chemical evaluated. None of the six chemicals studied were cytotoxic after 24 hours of toxicant exposure at the doses used for the time-response experiments. Hence, it can be assumed that any inhibition observed at these doses for up to 24 hours of toxicant exposure could be attributed to chemical inhibition by closure or constriction of the gap junctions.

Table 2.1 24 Hour Cytotoxicity at the Doses Used for the Time-Response Bioassays

Compound	**Time-Response Dose (μM)**	**24 hr Neutral Red Dye Uptake (FOC ± SD)**
2-biphenylol	230	1.04 ± 0.10
3-biphenylol	150	0.93 ± 0.07
2,2′-biphenyldiol	250	0.85 ± 0.11
3,3′-biphenyldiol	250	1.13 ± 0.16
3-chloro-2-biphenylol	100	1.25 ± 0.11
4,4′-dichloro-3-biphenylol	40	0.93 ± 0.03

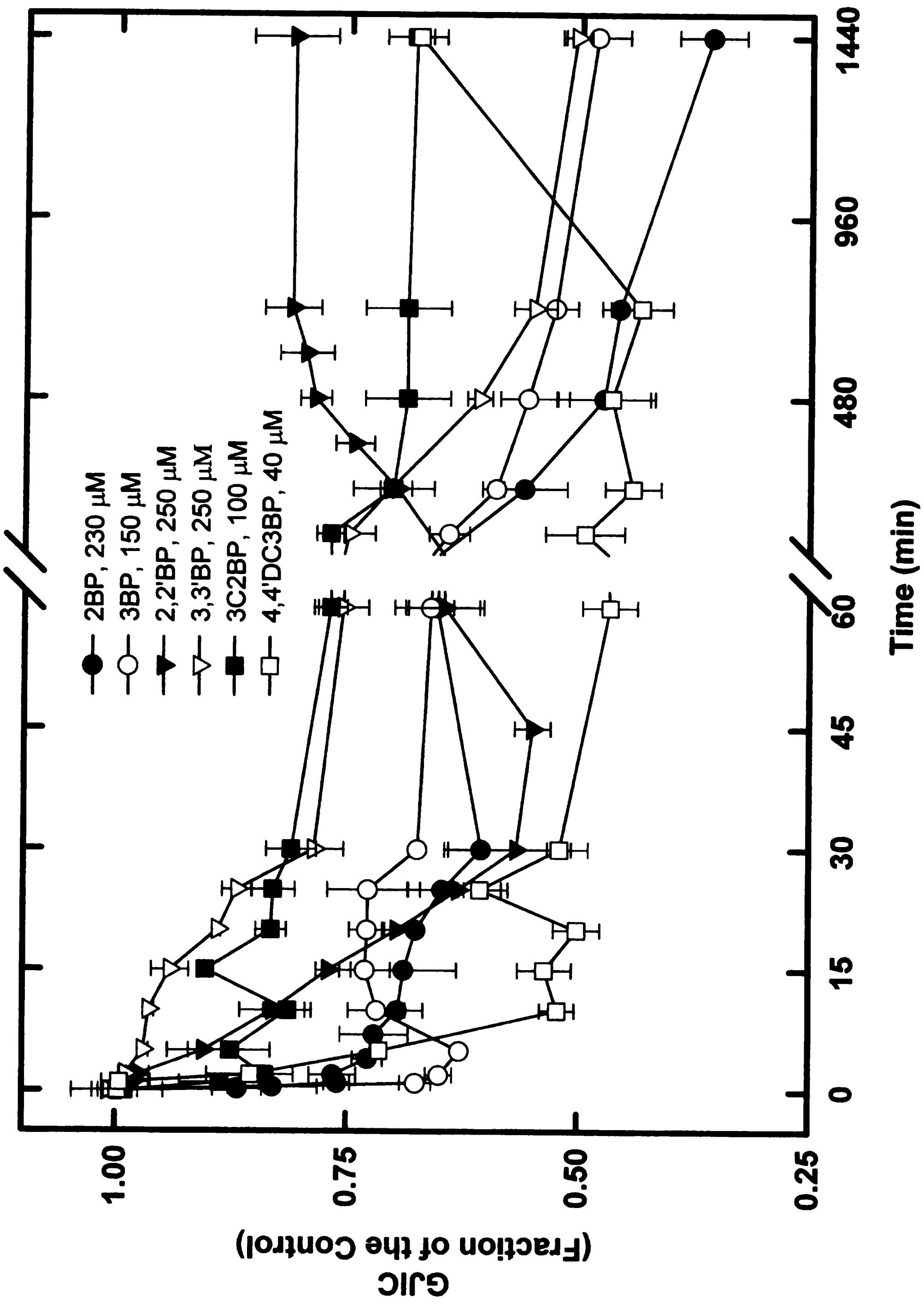
2.3.4 Time-Response Bioassay

The time-response assay was used to determine the effect of toxicant exposure time on intercellular communication. For the time-response experiments, confluent cultures of cells were exposed to a fixed dose of toxicant for varying amounts of time ranging from 0 to 24 hours (1440 minutes) followed by GJIC bioassays. The dose for the time-response experiment for each chemical was selected as the highest dose that is not cytotoxic at 24 hours of toxicant exposure, but causes a substantial amount of inhibition at 30 minutes of toxicant exposure. Figure 2.5 is a comparison of the time-response curves for the six chemicals tested. The dose selected for the time-response experiment for each chemical is indicated in the legend of this figure.

2BP and 3BP both rapidly inhibited GJIC. Within 30 minutes of toxicant exposure, GJIC was reduced to a FOC of 0.60 ± 0.04 for 2BP and a FOC of 0.67 ± 0.01 for 3BP. This rapid rate of inhibition suggests that inhibition was a consequence of the parent compound (2BP or 3BP), rather than a metabolite, which would require more time to form. 2BP was the only chemical to achieve almost complete inhibition (FOC = 0.36 ±0.04) within 24 hours of toxicant exposure. A maximum level of inhibition for 3BP (FOC = 0.48 ±0.03) was attained at 24 hours of toxicant exposure. For 3BP, between 5 minutes and 30 minutes, a slight increase and then decrease of FOC was observed, which might indicate a brief period of adaptation followed by resumed inhibition.

The inhibition of GJIC by 2,2′BP and 4,4′DC3BP occurred rapidly. 2,2′BP achieved maximum inhibition at 45 minutes (FOC = 0.55 ± 0.02), while 4,4′DC3BP achieved a FOC of 0.52 ± 0.02 at 10 minutes and then maintained an average FOC of 0.50 until 720 minutes (12 hours). A partial recovery of communication without removal

Figure 2.5 Time-response results for each of the six selected potential remediation byproducts. The legend in the figure indicates the dose used for each of the chemicals. Time of exposure varied from 0 minutes to 1440 minutes (24 hours). Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval. Data points after the break correspond to 120 minutes, 240 minutes, 360 minutes, 480 minutes, 600 minutes, 720 minutes, and 1440 minutes of chemical exposure, respectively.


2BP, 230 μM
3BP, 150 μM
2,2'BP, 250 μM
3,3'BP, 250 μM
3C2BP, 100 μM
4,4'DC3BP, 40 μM
GJIC
(Fraction of the Control)
1.00
0.75
0.50
0.25
0
15
30
45
60
480
960
1440
Time (min)

of the chemical was exhibited for both chemicals. For 2,2′BP, partial recovery occurred between 45 and 720 minutes (a period of 675 minutes) with a FOC of 0.81 ± 0.03 as the maximum recovery. This level of maximum recovery was maintained through 1440 minutes. For 4,4′DC3BP, partial recovery occurred between 720 minutes and 1440 minutes (a period of 720 minutes) with a FOC of 0.68 ± 0.01 as the maximum recovery. Although GJIC was used as a screening "biomarker" and not for the purpose of studying biochemical mechanisms, one possible speculative explanation for this partial recovery without removal of the chemical might be the activation of an autoregulatory pathway. In this case, the chemical would activate a pathway that would act to inhibit GJIC and then, in an autoregulatory fashion, another pathway would be activated which would reestablish GJIC. This partial recovery might also be attributed to the cell's ability to adapt to the change in conditions (such as cell homeostasis) due to toxicant exposure that resulted in the initial inhibition. Finally, partial recovery without the removal of the chemical might be a consequence of the cells metabolizing the chemical into less toxic metabolites.

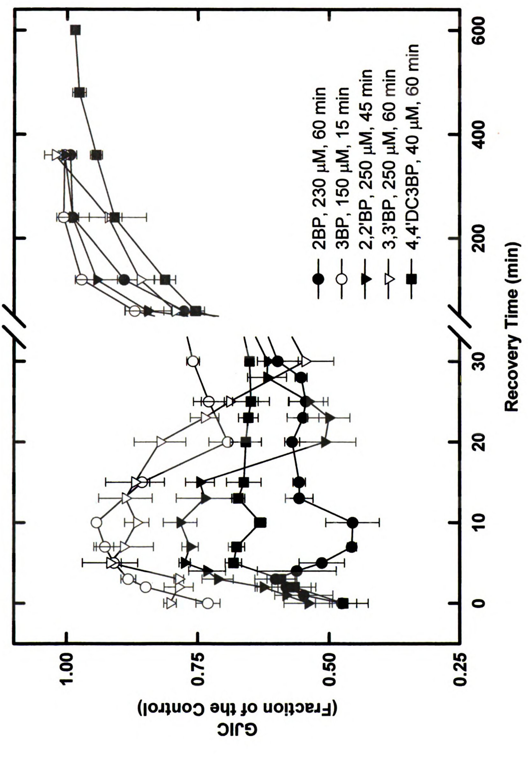
The inhibition of GJIC by 3,3′BP and 3C2BP occurred slowly. Within 30 minutes of toxicant exposure, 3,3′BP achieved a FOC of 0.79 ± 0.03, while 3C2BP achieved a FOC of 0.81 ± 0.03. The decline in communication for 3,3′BP continued to a maximum level of inhibition of a FOC of 0.51 ± 0.02 at 24 hours (1440 minutes). This slow rate of inhibition observed for 3,3′BP might be a consequence of the cells slowly metabolizing this chemical into a toxic metabolite. No significant level of inhibition was observed for 3C2BP during the 24 hours of exposure time. The maximum level of inhibition for 3C2BP was only a FOC of 0.68 ± 0.03 at 24 hours, which is equivalent to the amount of inhibition obtained for 3BP at 30 minutes.

2.3.5 Time of Recovery Bioassay

The time of recovery assay evaluated the ability of toxicant exposed cells to recover complete communication following the removal of the toxicant and replacement with new medium. From the results of the time-response bioassay, an incubation time for each chemical was selected that resulted in either a significant amount of inhibition or an amount of inhibition of interest. For each chemical, cells were exposed to a fixed dose of toxicant identical to that selected for the time-response experiment and were incubated for the selected incubation time. Following incubation, the cells were rinsed with Ca^{2+}/Mg^{2+} PBS, new medium was added, and the cells were returned to the incubator for varying amounts of recovery time followed by GJIC bioassays. Figure 2.6 is a comparison of the time of recovery curves for five of the chemicals tested. Since no significant level of inhibition was observed for 3C2BP during the time-response experiment, no time of recovery experiment was performed for this chemical. The dose and incubation time selected for the time of recovery experiment for each chemical is indicated in the legend of this figure. Complete recovery is attained at a FOC value of 0.97 and greater.

For all of the chemicals tested, cell cultures completely recovered communication following removal of the toxicant from the cell cultures. The times required to achieve complete recovery after removal of 2BP, 3BP, 2,2′BP, 3,3′BP, and 4,4′DC3BP were 240 minutes, 120 minutes, 240 minutes, 360 minutes, and 480 minutes, respectively. Trosko

Figure 2.6 Time of recovery results for five of the six selected potential remediation byproducts. Since no significant level of inhibition was observed in the time-response results for 3C2BP, no time of recovery experiment was performed for this chemical. The legend in the figure indicates the dose and incubation time selected for the time of recovery experiment for each chemical. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval. Data points after the break correspond to 60 minutes, 120 minutes, 240 minutes, 360 minutes, 480 minutes, and 600 minutes of recovery, respectively.


GJIC
(Fraction of the Control)
1.00
0.75
0.50
0.25
0
10
20
30
200
400
600
Recovery Time (min)
2BP, 230 μM, 60 min
3BP, 150 μM, 15 min
2,2'BP, 250 μM, 45 min
3,3'BP, 250 μM, 60 min
4,4'DC3BP, 40 μM, 60 min

et al. (*13*) hypothesized that most, if not all, tumor promoting chemicals reversibly inhibit GJIC. One could therefore hypothesize that the reversible inhibition of GJIC observed for these five chemicals in vitro is predictive of their potential to be tumor promoters in vivo as well as other disease endpoints (*29*). In addition, based on similar results from time of recovery experiments performed with fluoranthene and pyrene, Upham et al. (*21*) suggested that the ability of cells to completely recover from a substantial level of inhibition indicated that the inhibition was probably not at the transcriptional or gene level but was rather a consequence of a post-translational biochemical change. Furthermore, the complete recovery of communication indicates indirectly that the chemicals tested were not cytotoxic at the inhibitory doses used for the time of recovery experiment (*12*).

A cyclic or oscillatory behavior of GJIC FOC was observed during the first 30 minutes of recovery time for all the chemicals tested. This phenomenon might be indicative of a period of adaptation following the removal of the chemical and might entail the re-opening of existing, closed channels followed by the synthesis of new channels. At recovery times 60 minutes and longer, there was a smooth increase in GJIC until complete recovery was achieved.

As presented in the time-response section, 2,2′BP and 4,4′DC3BP both exhibited partial recovery without removal of the chemical. In the time-response experiment, in which 2,2′BP was not removed from the cell culture, partial recovery (FOC = 0.81 ± 0.03) occurred 675 minutes (11 hours 15 minutes) after inhibition reached a maximum. In the time of recovery experiment, however, complete recovery occurred 240 minutes (4 hours) after removal of 2,2′BP. Similarly, in the time-response experiment, in which

4,4′DC3BP was not removed from the cell culture, partial recovery (FOC = 0.68 ± 0.01) occurred 720 minutes (12 hours) after inhibition reached a maximum. In the time of recovery experiment, however, complete recovery occurred 480 minutes (8 hours) after removal of the chemical. For both chemicals, there was a greater extent and faster rate of recovery of communication after removal of the chemical than without removal of the chemical for doses studied.

2.3.6 Estimation of Octanol/Water Partition Coefficients and Solubility in Water

To correlate the observed toxicity to the chemical properties, the octanol/water partition coefficient (K_{OW}) and the solubility in water (S) were estimated for each chemical studied. K_{OW} values have been shown to be useful in associating structural changes in drug chemicals with observed changes in various biological, biochemical, or toxic effects (*30*). K_{OW} values were estimated using Leo's Fragment Constant Method (*30*). The estimations of K_{OW} for each of the chemicals studied were based on the log K_{OW} for 2,4,5,2′,5′- PCB (*31*). The solubility in water for each chemical was estimated using a regression equation for the estimation of S (Eqn. 1) representative of aromatics and chlorinated hydrocarbons (*30*).

$$\log S = -1.37 \log K_{OW} + 7.26 \qquad \text{(Eqn.1)}$$

Table 2.2 presents the estimated log K_{OW} and S values for the chemicals studied, where S is represented as units of mol/L.

On the basis of the estimations of log K_{OW} and S, it appears that OH-BPs with only one OH group (2BP and 3BP) were more hydrophobic than OH-BPs with two OH groups (2,2′BP and 3,3′BP). This is consistent with the observation that functional

Table 2.2 Estimated Octanol/Water Partition Coefficients and Water Solubility

Compound	**log K_{OW}**	**S (mol/L)**
2-biphenylol	1.89	0.047
3-biphenylol	1.89	0.047
2,2′-biphenyldiol	1.22	0.388
3,3′-biphenyldiol	1.22	0.388
3-chloro-2-biphenylol	2.60	4.99×10^{-3}
4,4′-dichloro-3-biphenylol	3.31	5.31×10^{-4}

groups containing oxygen tend to be involved in polar and hydrogen-bonding interactions and act to pull the rest of the molecule in which they are contained into the polar aqueous phase (*32*). OH-PCBs appear to be more hydrophobic than OH-BPs. Within the category of OH-PCBs, solubility in water appears to decrease with an increase in the number of chlorine functional groups.

4,4'DC3BP exhibited the highest estimated log K_{OW} and lowest estimated S. The aforementioned cytotoxicity and GJIC inhibitory effects observed for 4,4'DC3BP might be, although not exclusively, a consequence of the lipophilic nature of this chemical, which would permit diffusion across the phospholipid bilayer of the cellular membrane.

2.3.7 Structure-Toxicity Relationships

Structure might be influential in determining the toxicity observed for the OH-BPs and OH-PCBs studied. Biphenyls are remarkable in that a rotational degree of freedom is present in the phenyl-phenyl linkage that is absent in most polycyclic aromatic compounds (*33*). Since chlorines are bulky atoms, it has been shown that substitution of chlorines at various positions, especially at ortho positions, of the biphenyl can result in constraints on the rotational freedom of the phenyl-phenyl linkage (*34*). PCBs lacking ortho-substituted chlorine atoms may have a coplanar conformation, whereas an increased number of chloro substituents in ortho positions has been shown to result in decreased coplanarity (*19*). Similarly, due to their bulky nature, substitutions by hydroxyl functional groups on a biphenyl can be considered to impart constraints on the rotational freedom of the phenyl-phenyl linkage. The rotational degree of freedom in the biphenyl molecule can also be influenced by attractive forces or bonding between functional groups. Dr. H. Rosenkranz, using a modeling system based on multiple chemical attributes, has come to the conclusion that GJIC is a very reliable biomarker for chemical teratogens, tumor promoters, immunotoxicants, reproductive toxicants, and neurotoxicants (*29*).

Although 2,2′BP and 3,3′BP both possess two hydroxyl functional groups, 2,2′BP was found to be more inhibitory to GJIC. The dissimilarity between the toxicity results for these two chemicals might be explained by their structural differences. For 2,2′BP, the presence of hydroxyl groups at two ortho positions of adjacent aromatic rings would be expected to result in steric hindrance, thereby increasing the average dihedral (or twist) angle between the aromatic rings. In addition, significant hydrogen bonding would be expected to occur between the hydroxyl functionalities on the adjacent aromatic rings of 2,2′BP. This intramolecular hydrogen bonding causes the protic hydrogen of the free hydroxyl group to become more favorable for deprotonation, in comparison to the hydroxyl groups of 3,3′BP (*35*). The recovery of GJIC without the removal of the chemical exhibited in the time-response bioassay results for 2,2′BP might be a consequence of metabolism, in which such deprotonation results in a less toxic metabolite. In the case of 2,2′BP conformation, it can be expected that steric congestion factors will predominate over hydrogen bonding, resulting in greater noncoplanarity than 3,3′BP, which lacks steric hindrance and hydrogen bonding. The more noncoplanar conformation of 2,2′BP might explain the more inhibitory behavior of 2,2′BP to GJIC in comparison to 3,3′BP. Hemming et al. speculated that a decreased coplanarity increases the affinity for a cellular target site that is critical for inhibition of GJIC (*19*).

2BP and 3BP both possess one hydroxyl group; however, 2BP was observed to be slightly more inhibitory to GJIC. In the structure of 2BP, the introduction of an ortho-hydroxyl group to the biphenyl brings the oxygen and hydrogen atoms within the van der Waals radii (*36*). Similar to 2,2′BP, steric hindrance would occur within the 2BP molecule due to the presence of a bulky hydroxyl group. In the case of 2BP conformation, it can be expected that steric congestion factors will predominate over any van der Waals forces of attraction, increasing the average dihedral angle between the aromatic rings and increasing the noncoplanarity of the molecule over that of 3BP. The more noncoplanar conformation of 2BP might explain the more inhibitory behavior of 2BP to GJIC in comparison to 3BP.

For 3C2BP, as previously mentioned for 2BP, the presence of an ortho-hydroxyl group on the biphenyl structure would be expected to bring the oxygen and hydrogen atoms within the van der Waals radii. One would expect the same steric hindrance in the 3C2BP molecule as was observed in 2BP, increasing the average dihedral angle between the aromatic rings and increasing the noncoplanarity of the molecule. The observation that 3C2BP was less inhibitory to GJIC than 2BP might be explained by the presence of the chlorine functional group, which might act to attenuate the toxic effect of the ortho-hydroxyl group.

Since for 4,4′DC3BP there is an absence of any ortho-hydroxyl groups, no steric hindrance, hydrogen bonding, or attractive forces are expected. The conformation of the molecule is not expected to be as noncoplanar as 2,2′BP, 2BP, and 3C2BP. Introduction of meta-chloro substituents into a 4,4′-dichlorobiphenyl molecule has been shown to have significant effects on the resultant toxicity of the compound (*37*). In the case of

4,4′DC3BP, the introduction of a meta-hydroxyl substituent into a 4,4′-dichlorobiphenyl molecule might have a similar toxic effect as illustrated by the observed cytotoxicity and inhibition of GJIC. One possible explanation for the significant amount of inhibition of GJIC observed for 4,4′DC3BP is hindrance of the rotation of the biphenyl rings by the hydroxyl group, despite the absence of ortho-substitution, resulting in a noncoplanar (twisted) conformation of the chemical structure (*38*). Alternatively, the hydroxyl group may create a bay-like region on the molecule, which has been shown to result in inhibition of GJIC and activation of MAPK (mitogen-activated protein kinase) (*38, 39*).

2.4 Conclusions

2-Biphenylol, 3-biphenylol, 2,2′-biphenyldiol, 3,3′-biphenyldiol, 3-chloro-2-biphenylol, and 4,4′-dichloro-3-biphenylol were evaluated using the scrape-loading/dye transfer (SL/DT) technique to determine the in vitro modulation of gap junctional intercellular communication (GJIC) in a normal rat liver epithelial cell line as a measure of the epigenetic toxicity. Cytotoxicity was determined using the neutral red uptake assay. A dose range of 0 to 300 μM was examined. Only 3,3′-biphenyldiol and 4,4′-dichloro-3-biphenylol induced cytotoxicity within the tested dose ranges. Noncytotoxic doses were selected for evaluation of epigenetic toxicity. 4,4′-Dichloro-3-biphenylol was most inhibitory to GJIC at the lowest dose. The cytotoxicity and GJIC inhibitory effects observed for 4,4′-dichloro-3-biphenylol might be, although not exclusively, a consequence of the lipophilic nature of this chemical. Alternative explanations for the significant amount of inhibition of GJIC observed for 4,4′-dichloro-3-biphenylol are (1) hinderance of the rotation of the biphenyl rings by the hydroxyl group resulting in a noncoplanar conformation and (2) creation of a bay-like region on the molecule by the hydroxyl group. 3-Chloro-2-biphenylol was least inhibitory to GJIC. 3-Chloro-2-biphenylol was less inhibitory to GJIC than 2-biphenylol because of the presence of the chlorine functional group, which appears to attenuate the toxic effect of the ortho-hydroxyl group. Although cells were capable of complete recovery of GJIC after removal of each of the chemicals, only with 2,2′-biphenyldiol and 4,4′-dichloro-3-biphenylol did the cells demonstrate partial recovery without removal of the chemical. The more noncoplanar conformation of 2,2′-biphenyldiol and 2-biphenylol might explain their more inhibitory behavior in comparison to 3,3′-biphenyldiol and 3-biphenylol, respectively.

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Chapter 3

Toxicity of Parent Polychlorinated Biphenyl (4,4′-Dichlorobiphenyl)

3.1 Introduction

Although polychlorinated biphenyl (PCB) contamination is typically encountered as site-specific mixtures of PCB congeners, toxicity tests on individual PCB congeners are important to conduct so that the toxicity of a particular mixture can be extrapolated, based on the toxicity of its component congeners (*1*). In view of the cytotoxicity and GJIC inhibitory effects observed for 4,4′-dichloro-3-biphenylol (4,4′DC3BP), the PCB congener 4,4′-dichlorobiphenyl (4,4′DCBP) was selected for Fenton's reagent remediation studies. The biological significance of 4,4′DCBP is further indicated by the results of metabolism studies in various organisms. In a study performed by Tulp et al. (*2*), 4,4′DC3BP was found to be excreted as a major metabolite following the metabolism of 4,4′DCBP by rats, rabbits, goats, frogs, and fungi. Further studies on the metabolism of 4,4′DCBP in frogs revealed that both 4,4′DC3BP and 4,4′DCBP became "trapped" in tissues (*2*). In studies of 4,4′DCBP in goats, the only urinary metabolite was identified as 4,4′DC3BP; however, this metabolite was absent in the feces and several organs (*3*). In rats, the metabolites of 4,4′DCBP excreted in bile were identified as 3,4′-dichloro-4-biphenylol and 4,4′DC3BP (*4*). In studies of white-rot fungi *Phanerochaete chrysosporium* and *Phanerochaete* sp. MZ142, 4,4′DCBP was metabolized to various hydroxylated PCBs as a key step in the PCB degradation process (*5*).

Experimental results show that substitution in the ortho position from the carbon bridge of the biphenyl is essential and at least one chloro substituent in the ortho position is necessary for the ability to inhibit gap junctional intercellular communication (GJIC) (*6*). PCBs lacking ortho-substituted chlorine atoms may have a coplanar conformation, whereas an increased number of chloro substituents in ortho positions has been shown to result in decreased coplanarity (*6*). Although PCBs with coplanar conformations appear less likely to inhibit GJIC, decreased coplanarity of the PCB increases the chances of inhibition of GJIC (*6-8*). Since 4,4′DCBP lacks ortho-substituted chlorine atoms, a coplanar conformation can be expected.

In this section of research, the toxicity of the parent PCB (4,4′DCBP) for the Fenton's remediation studies was evaluated in rat liver epithelial cells using a nongenotoxic bioassay that determines the in vitro modulation of GJIC as a measure of the epigenetic toxicity.

3.2 Experimental Section

3.2.1 Chemicals

4,4′-dichlorobiphenyl (99.4% purity) was purchased from Chem Service Inc. (West Chester, PA). Acetonitrile (ACN) (99.8% purity) was purchased from EM Science (Gibbstown, NJ). Dimethyl sulfoxide (DMSO) (99.7% purity) and fatty acid free bovine serum albumin (BSA) (96% purity) were purchased from Sigma Chemical Co. (St. Louis, MO). Acetone (99.8% purity) was purchased from J.T. Baker (Phillipsburg, NJ). For cell culture, D-medium (Formula No. 78-5470 EG), Fetal Bovine Serum (FBS), and Gentamicin were purchased from GIBCO Laboratories (Grand Island, NY). Lucifer Yellow CH, dilithium salt, was purchased from Molecular Probes Inc. (Eugene , OR), ICN Biomedicals Inc. (Aurora, OH), and Sigma Chemical Co. (St. Louis, MO).

Formaldehyde solution (37%) for the GJIC bioassays was purchased from J.T. Baker (Phillipsburg, NJ).

3.2.2 Methods

3.2.2.1 Cell Culture Techniques

The cell culture techniques performed were identical to those described in Chapter 2.2.2.2 for epigenetic toxicity studies of hydroxylated biphenyls and hydroxylated polychlorinated biphenyls as potential remediation byproducts.

3.2.2.2 Solvent Evaluation for GJIC Bioassay

In the Chapter 2 toxicology studies, solutions of the six potential remediation byproducts were prepared with 100% acetonitrile (ACN) as the solvent. Although 4,4′DCBP completely dissolved in the ACN solvent, when the solution was added to the media in the cell culture dishes, an oily film and/or cloudiness appeared. Therefore, ACN proved to be an inappropriate solvent for 4,4′DCBP due to the insolubility of the 4,4′DCBP upon application of the solution to the media in the cell culture dishes, thereby hindering exposure of the cells to the toxicant. In experiments evaluating the effects of various PCBs on GJIC, Hemming et al. (*6*) used dimethyl sulfoxide (DMSO) or DMSO-acetone (1:1 by volume) as solvents for the preparation of test solutions. To determine the best solvent for 4,4′DCBP for the toxicology studies, four solvents were tested: 100% ACN, DMSO-ACN (1:1 by volume), DMSO, and DMSO-acetone (1:1 by volume). A 20 mM test solution of 4,4′DCBP was prepared in each solvent and mixed thoroughly (by sonication). Test volumes of 15 or 30 μL of 4,4′DCBP test solution (corresponding with

150 μM and 300 μM doses, respectively) were then applied to 2 mL of D-medium supplemented with 5% FBS in 35 mm diameter culture dishes lacking cell cultures. Each solvent was evaluated based on the ability to produce a test solution in which 4,4'DCBP dissolves completely and remains in solution when added to the media in the culture dishes.

3.2.2.3 PCB "Transport" Using DMSO/BSA/PBS Solvent for GJIC Bioassay

One possible option to solving the insolubility problem of 4,4'DCBP for the toxicology studies was to find a means of "transporting" the PCB to the cells attached to the bottom of the culture dish. Most proteins are comprised of hydrophilic regions and a hydrophobic domain to transport hydrophobic compounds to specific biological sites (*9*). In the body, hydrophobic foreign compounds, such as PCBs, are often transported in the blood and to the tissues by binding with plasma proteins such as albumin (*10, 11*). Likewise, serum albumin would be useful for "transporting" 4,4'DCBP to the cells in the bioassays. Since serum albumin carries large quantities of fatty acid (*12*), which would cause inhibition of GJIC (*13*), fatty acid free bovine serum albumin (BSA) was selected for use in 4,4'DCBP toxicology studies. Two solutions were combined to produce the final 4,4'DCBP test solution: (1) 1 mL of 20 mM 4,4'DCBP in DMSO and (2) 9 mL of 2% BSA in phosphate buffered saline (PBS) (w/v). A 20 mM solution of 4,4'DCBP was prepared in DMSO and mixed thoroughly (by sonication). In a 25 mL Erlenmeyer flask, the 20 mM 4,4'DCBP/DMSO solution was slowly added to the 2% BSA/PBS solution. Using a stir bar, the test solution was mixed thoroughly throughout the 4,4'DCBP/DMSO solution addition to create a solution of white milky appearance (indicative of the microparticles responsible for "transporting" the 4,4′DCBP to the cells). The final test solution concentration of 4,4′DCBP in DMSO/BSA/PBS was 2 mM.

3.2.2.4 Vehicle Tolerance Test for DMSO/BSA/PBS

The vehicle tolerance test (dose-response) was performed to determine the largest volume of the solvent DMSO/BSA/PBS that could be applied to cell cultures without causing inhibition of gap junctional intercellular communication (GJIC). For the DMSO/BSA/PBS vehicle tolerance test, two solutions were combined to produce the final vehicle test solution: (1) 1 mL of DMSO and (2) 9 mL of 2% BSA in PBS (w/v). In a 25 mL Erlenmeyer flask containing a stir bar, DMSO was slowly added to the 2% BSA/PBS solution and mixed thoroughly to produce the vehicle test solution. Bioassays of GJIC were performed on confluent cell cultures (usually 2 days of growth) grown in 35 mm diameter culture dishes containing 2 mL of D-medium supplemented with 5% FBS (as described in Chapter 2.2.2.2). The scrape-loading/dye transfer (SL/DT) procedure for determining the GJIC was adapted from the method described by El-Fouly et al. (*14*) and is described in detail by Herner et al. (*15*). Treatments with the DMSO/BSA/PBS vehicle test solution and controls (no dose) were performed in triplicates. Doses were applied directly to the dishes of confluent cell cultures and allowed to incubate for 30 minutes (at 37 °C in a water-jacketed IR Autoflow Automatic CO_2 incubator (NUAIRE, Inc., Plymouth, MN) in a humidified atmosphere with 5% CO_2 and 95% air) before assaying for GJIC. The vehicle test solution was evaluated over a volume range of 0 to 350 μL.

All culture dishes were examined within 24 hours of the experiment completion. Each culture dish of cells was digitally photographed such that the observed scrape spanned the full horizontal width of the picture. A COHU High Performance Color CCD Camera (Cohu, Inc., San Diego, CA) with a magnification of 200× under a Nikon Diaphot-TMD epifluorescence phase-contrast microscope (Nikon Corp., Japan) illuminated with a Nikon HB-10101AF Super High Pressure Mercury 100W lamp (Nikon Corp., Japan), or a Nikon TE300 Eclipse Inverted Microscope (Nikon Corp., Japan) with a Nikon HB-10103AF Super High Pressure Mercury 100W lamp (Nikon Corp., Japan) was used.

The fluorescence of the Lucifer yellow dye was used to determine the distance the dye traveled perpendicular to the scrape. This distance of dye travel was indicative of the level of GJIC within the culture. Quantitative analysis of the distance of dye spread was performed using NucleoTech GelExpert software (NucleoTech Corp., Hayward, CA). The distance of dye spread was measured in terms of the area of dye spread, by tracing manually via free object quantification the area of farthest visible fluorescence. Since the width of the photographed section was the same for every culture dish, measuring the area of the dye spread was equivalent to measuring the distance of dye spread perpendicular to the scrape. The area of dye spread for each vehicle test solution treatment dish was compared to a group of cells that were exposed to no dose (controls) under the same assay as the treated cells. For each dish treated with the vehicle test solution, the fraction of the control was calculated as the area of dye spread in the treated dish divided by the average area of dye spread in the triplicate set of control (no dose) dishes. The results for each set of vehicle test solution treated triplicates were reported as an average fraction of the control (FOC) ± standard deviation (SD) determined at the 95% confidence interval (CI).

The level of GJIC in cells exposed to the vehicle test solution was assessed by the decrease in communication of the cells as compared to the control group, which was exposed to no dose of chemical. A decrease in FOC corresponds directly to a decrease in GJIC (where the doses are not cytotoxic). Interpretations of GJIC results are consistent with those of Luster-Teasley et al. (*16*) and Herner et al. (*15*). Complete communication (100%) between the cells is identified as a FOC value of 1.0 as seen in the control. A FOC value greater than 0.9 is difficult to statistically distinguish from the control. FOC values between 0.9 and 0.5 indicate partial inhibition of GJIC. A FOC value less than or equal to 0.5 is indicative of a significant amount of inhibition of GJIC, since this would be representative of communication levels that are 50% or less than the normal communication levels. FOC values between 0.3 and 0.0 are representative of complete inhibition of GJIC. A FOC value of 0.3 is usually used to represent complete inhibition, as it corresponds to the width of a single row of cells with no dye spreading beyond its boundaries (*15*). Controls, which received no dose of vehicle test solution, were also performed as a means of evaluating a normal level of GJIC and the overall "health" of the cells. Statistical analyses were performed by means of the t-test and One Way Analysis of Variance (ANOVA) to compare the vehicle test solution results and control results.

3.2.2.5 Dose-Response Bioassay

Bioassays of GJIC were performed on confluent cell cultures (usually 2 days of growth) grown in 35 mm diameter culture dishes containing 2 mL of D-medium supplemented with 5% FBS (as described in Chapter 2.2.2.2). The scrape-loading/dye transfer (SL/DT) procedure for determining the GJIC was adapted from the method described by El-Fouly et al. (*14*) and is described in detail by Herner et al. (*15*). A detailed description of the spread of Lucifer yellow dye from the scrape to neighboring cells can be found in Wilson et al. (*17*).

For reasons to be discussed later in this chapter, which determined that further toxicology studies were unnecessary, only dose-response GJIC bioassays were performed. Test solutions of 2 mM 4,4′DCBP in DMSO/BSA/PBS (4,4′DCBP/DMSO/BSA/PBS) were prepared as described in Section 3.2.2.3. The vehicle control solution (DMSO/BSA/PBS) was prepared as described in Section 3.2.2.4. Chemical treatments (4,4′DCBP/DMSO/BSA/PBS), controls (no dose), and vehicle controls (DMSO/BSA/PBS) were performed in triplicates. Doses were applied directly to the dishes of confluent cell cultures and allowed to incubate for 30 minutes, 2 hours, 6 hours, and 24 hours (at 37 °C in a water-jacketed IR Autoflow Automatic CO_2 incubator (NUAIRE, Inc., Plymouth, MN) in a humidified atmosphere with 5% CO_2 and 95% air) before assaying for GJIC. Vehicle controls were dosed with a volume of DMSO/BSA/PBS corresponding to the largest volume of chemical dose tested in the treatment dishes (300 μL). Since the volume change caused by the addition of the chemical dose in each culture dish was significant (greater than 2%), the observed or "true" dose was calculated by adjusting for the added dose volume. A "true" dose range of 0 to 260.87 μM of 4,4′DCBP was tested and is reflected in the figures.

All culture dishes were examined within 24 hours of the experiment completion. Each culture dish of cells was digitally photographed and analyzed by the methods described previously in Section 3.2.2.4. The area of dye spread for each chemical treatment dish was compared to a control group of cells that were exposed to DMSO/BSA/PBS only (vehicle controls) under the same assay as the treated cells. For each chemically treated dish, the fraction of the control was calculated as the area of dye spread in the treated dish divided by the average area of dye spread in the triplicate set of vehicle control dishes. The results for each set of chemically treated triplicates were reported as an average fraction of the control (FOC) ± standard deviation (SD) determined at the 95% confidence interval (CI).

The level of GJIC in cells exposed to the chemical was assessed by the decrease in communication of the cells as compared to the vehicle control groups, exposed to DMSO/BSA/PBS only. A decrease in FOC corresponds directly to a decrease in GJIC (where the doses are not cytotoxic). Interpretations of GJIC results are consistent with Luster-Teasley et al. (*16*) and Herner et al. (*15*). Complete communication (100%) between the cells is identified as a FOC value of 1.0 as seen in the vehicle control. A FOC value greater than 0.9 is difficult to statistically distinguish from the vehicle controls. FOC values between 0.9 and 0.5 indicate partial inhibition of GJIC. A FOC value less than or equal to 0.5 is indicative of a significant amount of inhibition of GJIC, since this would be representative of communication levels that are 50% or less than the normal communication levels. FOC values between 0.3 and 0.0 are representative of complete inhibition of GJIC. A FOC value of 0.3 is usually used to represent complete inhibition, as it corresponds to the width of a single row of cells with no dye spreading beyond its boundaries (*15*). Controls, which received no dose of chemical or solvent (DMSO/BSA/PBS), were performed for each experiment as a means of evaluating a normal level of GJIC and the overall "health" of the cells. By performing a t-test for each experiment with incubation times of 30 minutes, 2 hours, 6 hours, and 24 hours, it was found that the areas of dye spread for the control dishes (no solvent or chemical) did not vary significantly from the areas for the vehicle controls (only DMSO/BSA/PBS) at a 95% CI. Therefore, it could be concluded that the solvent (DMSO/BSA/PBS), at the volume tested and time of incubation, was not a significant source of inhibition in the experiments. Statistical analyses were performed by means of the t-test and One Way Analysis of Variance (ANOVA) to compare the chemical treatment results and vehicle control results.

3.3 Results and Discussion

3.3.1 Solvent Evaluation for GJIC Bioassay

4,4′DCBP was determined to be completely soluble in all four solvents tested (100% ACN, DMSO-ACN (1:1 by volume), DMSO, and DMSO-acetone (1:1 by volume) in preparation of the 20 mM test solutions. The test solutions were clear and colorless with no oily film or cloudiness. However, when 15 or 30 μL of the 20 mM 4,4′DCBP test solutions (corresponding with 150 μM and 300 μM doses, respectively) were added to the culture dishes containing 2 mL of D-medium supplemented with 5% FBS, an oily film on the surface of the media and/or precipitate/cloudiness was observed. If these culture dishes had contained cell cultures, chemical contact with the cells would have been hindered, since the cells would have been attached to the bottom surface of the dish. Therefore, none of the solvents tested were appropriate for the toxicology studies with 4,4′DCBP.

3.3.2 Vehicle Tolerance Test for DMSO/BSA/PBS

The DMSO/BSA/PBS vehicle test solution was clear and colorless with no oily film, cloudiness, or milky appearance. When doses were added to the dishes of confluent cell cultures, the media remained clear and colorless. As shown in Figure 3.1, the vehicle tolerance test GJIC bioassay results with 30 minutes of incubation indicated no inhibition of GJIC for a volume range of 0 to 350 μL of DMSO/BSA/PBS. It can be assumed, therefore, that any inhibition of GJIC observed for doses of 4,4′DCBP containing no more than 350 μL of the vehicle (DMSO/BSA/PBS) is not attributable to the vehicle, but rather an effect of the presence of 4,4′DCBP. Volumes of DMSO/BSA/PBS greater than 350 μL were not investigated, since the maximum test volume of 2 mM 4,4′DCBP/DMSO/BSA/PBS was 300 μL.

3.3.3 Dose-Response Bioassay

The test solution of 2 mM 4,4′DCBP in DMSO/BSA/PBS had a white milky appearance, while the vehicle control solution (DMSO/BSA/PBS) was clear and colorless with no oily film, cloudiness, or milky appearance. When the vehicle control solution was added to the dishes of confluent cell cultures, the media remained clear and colorless. Although the media in the dishes of confluent cell cultures remained clear and colorless for 4,4′DCBP at doses up to 76.92 μM, at higher doses the media became more milky in appearance.

As shown in Figures 3.2, 3.3, and 3.4, no significant level of inhibition of GJIC was observed for 4,4′DCBP for a dose range of 0 to 260.87 μM for incubation times of 30 minutes, 2 hours, and 6 hours, respectively. The maximum levels of inhibition of

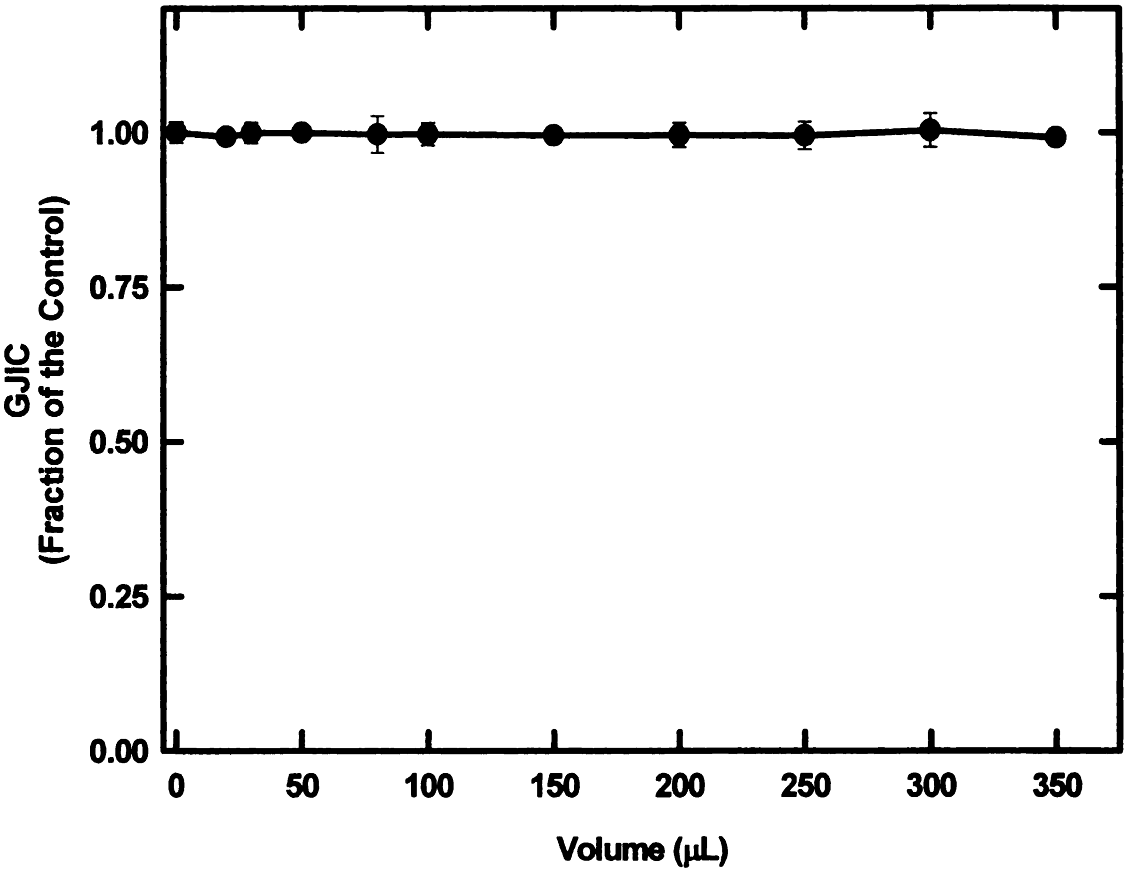

Figure 3.1 Vehicle tolerance test GJIC bioassay results with a 30 minute incubation time for a volume range of 0 to 350 µL of DMSO/BSA/PBS. Each data point is representative of the results for a set of DMSO/BSA/PBS treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

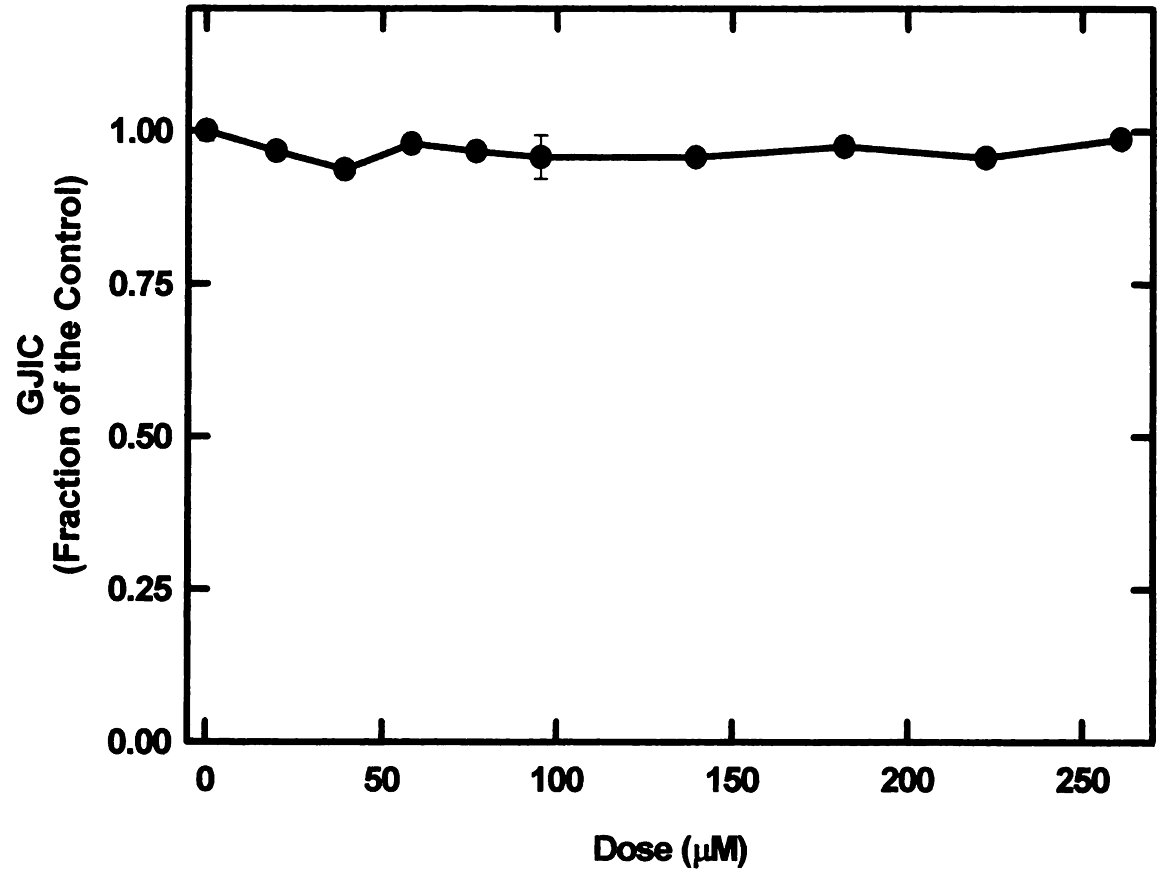

Figure 3.2 Dose-response bioassay results for 4,4′-dichlorobiphenyl (DMSO/BSA/PBS solvent) with 30 minutes of incubation time for a dose range of 0 to 260.87 μM. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

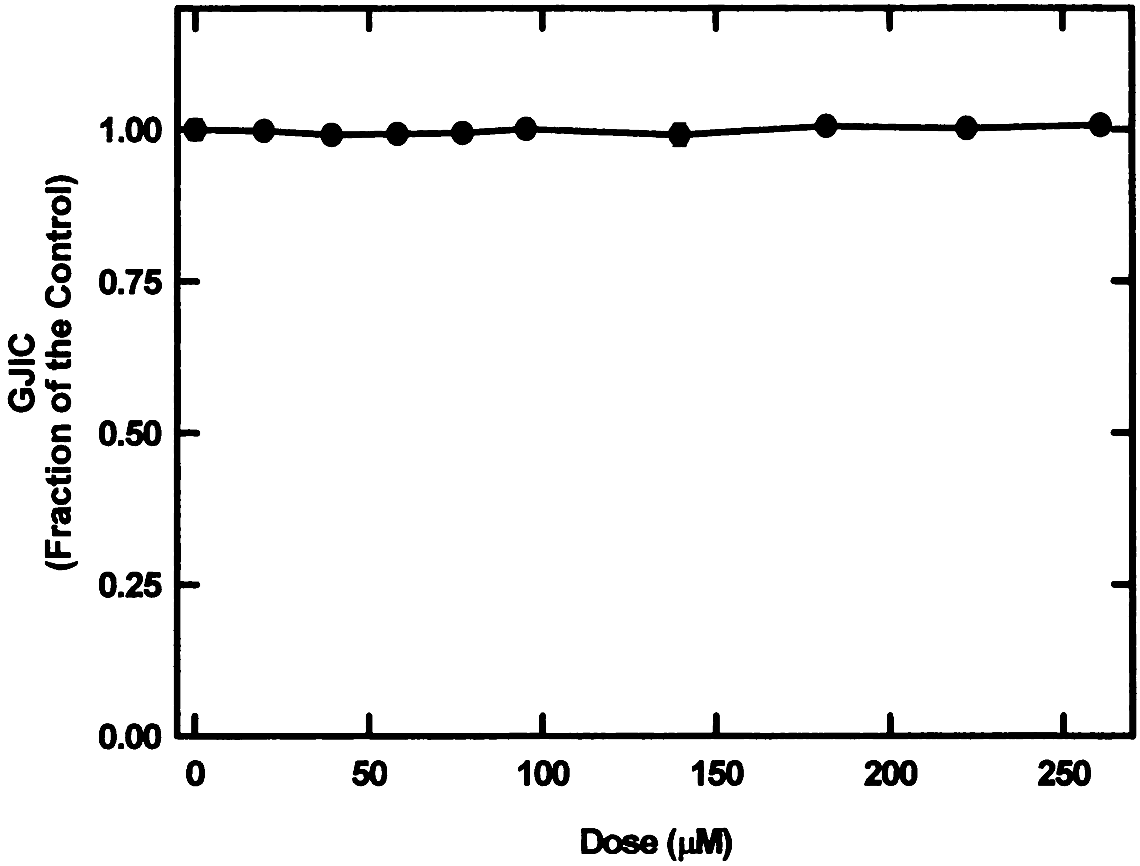

Figure 3.3 Dose-response bioassay results for 4,4′-dichlorobiphenyl (DMSO/BSA/PBS solvent) with 2 hours of incubation time for a dose range of 0 to 260.87 μM. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

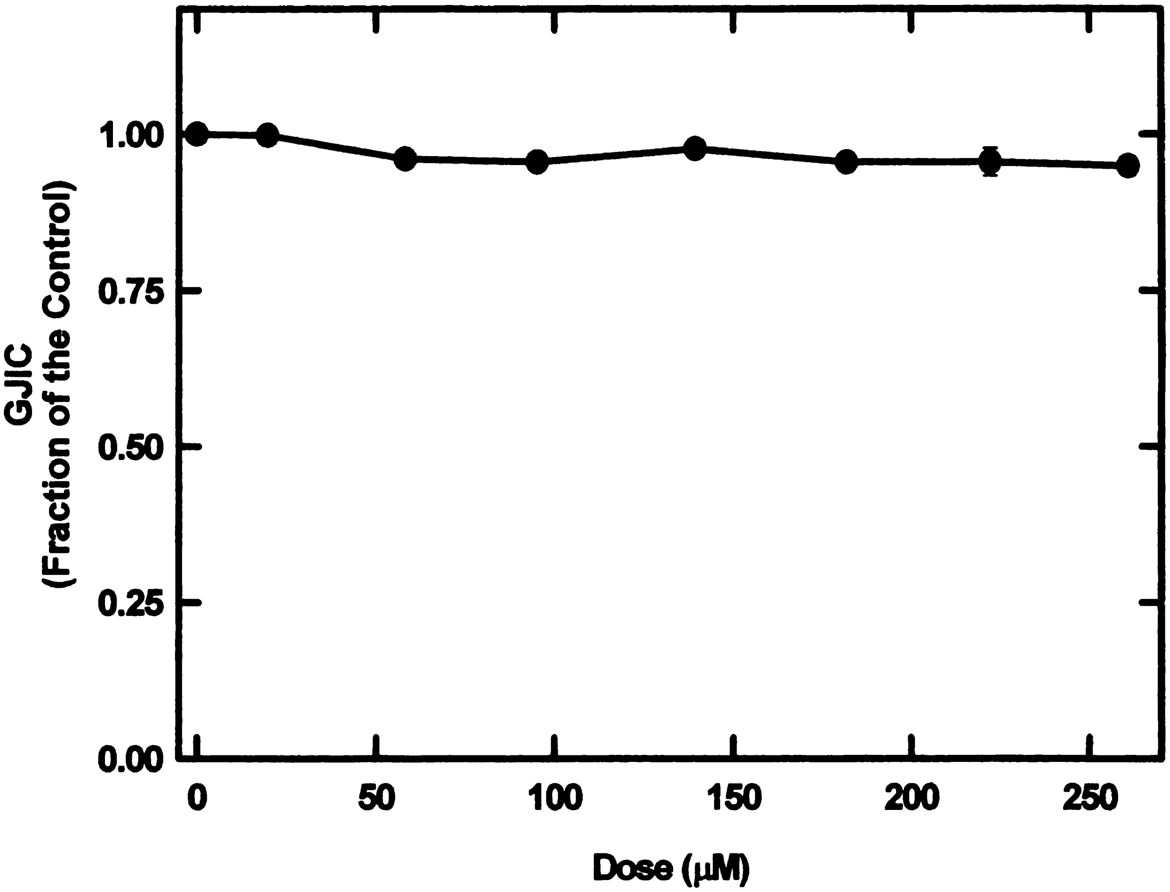

Figure 3.4 Dose-response bioassay results for 4,4′-dichlorobiphenyl (DMSO/BSA/PBS solvent) with 6 hours of incubation time for a dose range of 0 to 260.87 µM. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

GJIC observed for 30 minutes, 2 hours, and 6 hours of incubation were FOC values of 0.94 ± 0.01 at 39.22 μM, 0.99 ± 0.02 at 139.53 μM, and 0.95 ± 0.02 at 260.87 μM, respectively. Figure 3.5 presents the dose-response GJIC bioassay results for 4,4′DCBP for a dose range of 0 to 260.87 μM with an incubation time of 24 hours. Very slight inhibition of GJIC occurred between doses of 0 to 95.24 μM of 4,4′DCBP with 24 hours of incubation. The maximum level of inhibition of GJIC for 24 hours of incubation was exhibited at a dose of 19.80 μM (FOC = 0.86 ± 0.02). No significant level of inhibition of GJIC was observed over a dose range of 139.53 to 260.87 μM for 24 hours of incubation.

3.4 Conclusions

The dose-response results for 4,4′DCBP are consistent with observations that PCBs with coplanar conformations appear less likely to inhibit GJIC (*6-8*). Although 4,4′DCBP was not observed to be significantly inhibitory to GJIC, Fenton's remediation of this chemical has the potential of generating byproducts which are inhibitory to GJIC and/or cytotoxic (based on the potential of forming byproducts such as 4,4′DC3BP). Likewise, Fenton's remediation of a mixture of PCBs which included 4,4′DCBP might be a matter of concern. Since very slight to no inhibition of GJIC was observed for 4,4′DCBP for the dose-response experiments for incubation times of 30 minutes, 2 hours, 6 hours, and 24 hours, no further toxicity studies were performed.

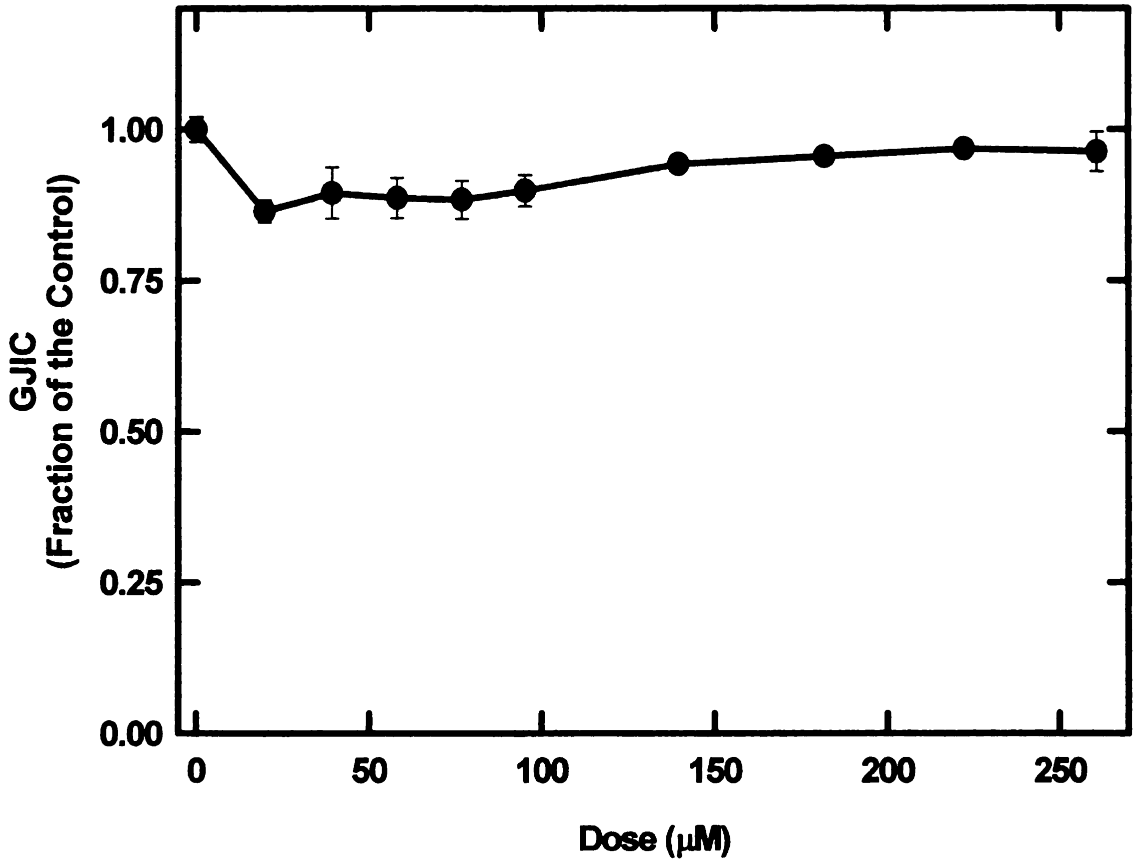

Figure 3.5 Dose-response bioassay results for 4,4′-dichlorobiphenyl (DMSO/BSA/PBS solvent) with 24 hours of incubation time for a dose range of 0 to 260.87 μM. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

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Chapter 4

Methylene Blue Dye Test for Rapid Qualitative Detection of Hydroxyl Radicals Formed in a Fenton's Reaction Aqueous Solution

4.1 Introduction

An article (*1*) based on this chapter was published in *Environmental Science and Technology* and a U.S. Patent Application, Serial No. 11/478,959, has been filed.

Several methods have been developed to detect hydroxyl radicals; however, none can rapidly and qualitatively determine hydroxyl radicals by a simple procedure requiring inexpensive materials and without interferences with the reaction. Previous methods developed for the quantitation/detection of hydroxyl radicals often depend on the addition of chemical probes, such as benzoic acid, 1-propanol, or salicylic acid, and the hydroxylated reaction products have been used as an indirect measurement of the presence of hydroxyl radicals (*2-4*). These methods involve complicated, time-consuming procedures, are non-specific for the hydroxyl radical, and were often developed for a specific experimental study or system. Furthermore, in the presence of other hydroxyl radical scavenging/reacting chemicals, the addition of a chemical probe results in competition for the hydroxyl radicals and the possibility of side reactions. The numerous hydroxylated products formed often make the quantitative detection of hydroxyl radicals complicated. Another commonly used method, electron spin resonance (ESR), capable of detecting hydroxyl radicals and superoxide anion radicals, requires expensive instrumentation, is laborious, and may involve a number of transient radicals (*5*). Although in iron/hydrogen peroxide (Fenton's reagent) systems, some researchers have measured the loss of hydrogen peroxide as an indicator for hydroxyl radical formation, only a fraction of the peroxide degraded is converted to hydroxyl radicals (as the complexity of the reaction leads to the formation of a variety of species) (*2*).

The methylene blue (MB) dye test is a new test that qualitatively indicates the presence of hydroxyl radicals through an immediate, distinct bleaching of the MB dye on a paper test strip after applying an aqueous sample containing hydroxyl radicals. This method is simple, requires inexpensive materials, and does not suffer from interferences resulting from the addition of probe chemicals. Methylene blue (3,7-bis(dimethylamino)phenothiazin-5-ium chloride) is a basic dye of the thiazine series used extensively for dyeing and printing cloth and for medicinal purposes (based on its antiseptic properties) (*6*). The structure of methylene blue is shown in Figure 4.1 (*7*).

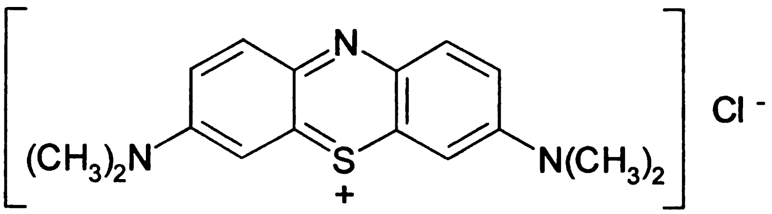


Figure 4.1 Structure of Methylene Blue

In the MB dye test, the hydroxyl radical reacts with the MB cation to produce a hydroxide ion and a MB radical cation (*8*). Since the MB cation is dark blue in color and the MB radical cation is colorless, application of a sample containing hydroxyl radicals to MB dye results in a change of color from dark blue to colorless. Hydrogen peroxide (3%) does not cause bleaching of MB dye.

A Fenton system was used as the source of hydroxyl radicals to test the applicability of the MB dye test for the detection of hydroxyl radicals. Highly reactive hydroxyl radicals are produced by the oxidation of ferrous iron and the reduction of hydrogen peroxide (*9*). Hydrogen peroxide is added in the presence of ferrous iron to a solution or suspension of compounds to be treated (*10*). The oxidation efficiency of the Fenton's type reactions depends on the Fe^{2+}:H_2O_2 ratio and the pH (*11*). The optimal pH for the Fenton's reaction efficiency has been shown to be between pH 3 and 5 (*12*). At more basic pH values, the iron is converted from a hydrated ferrous form to a colloidal ferric form, thereby causing a decrease in the effectiveness of the reaction (*12*). Fenton's reagent is an effective method of remediating contaminated soils and aqueous solutions through the oxidation by hydroxyl radicals, which readily degrade a wide variety of organic pollutants (*9*). Rapid, inexpensive detection of the presence of hydroxyl radicals would allow for the immediate monitoring of the Fenton's remediation process and aid in optimizing the degradation efficiency.

The objective of this study was to test the applicability of the MB dye test for the detection of hydroxyl radicals in a Fenton's reaction aqueous solution and verify the results by benzoic acid chemical probe hydroxyl radical detection methods using thin layer chromatography and spectrophotometric wavelength scans.

4.2 Experimental Section

4.2.1 Chemicals

Methylene blue dye (3,7-bis(dimethylamino)phenothiazin-5-ium chloride) (97% purity) was purchased from Fluka (Buchs, Switzerland). Thirty-percent hydrogen peroxide (H_2O_2) (unstabilized), iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) (99% purity), sodium sulfite (Na_2SO_3) (anhydrous, 98% purity), benzoic acid (BA) (99.5% purity), and 4-hydroxybenzoic acid (4-HBA) (99+ % purity) were purchased from

Sigma–Aldrich (St. Louis, MO). Sulfuric acid (H_2SO_4) (96% purity), sodium hydroxide (NaOH) pellets (99% purity), methanol (MeOH) (100%), and chloroform (100%) were purchased from J.T. Baker (Phillipsburg, NJ). Throughout this chapter and dissertation, Milli-Q water was obtained from a Milli-Q Ultrapure Water Purification System (System Type ZMQS6VFOY) purchased from Millipore Corp. (Bedford, MA).

4.2.2 Methods

4.2.2.1 Methylene Blue Dye Test

Prior to the development of the methylene blue dye test strip, a liquid methylene blue dye test was investigated. In the liquid methylene blue dye test, 60 μL of a liquid test sample was added to a 0.2 mL PCR vial containing 100 μL of 0.1 mM methylene blue dye solution. The solution was then completely mixed by inverting the closed vial. This liquid methylene blue dye test was later abandoned; however, since the color change of the liquid methylene blue dye due to the presence of hydroxyl radicals occurred only after a lengthy period of time, approximately 40 minutes. These results are consistent with those observed by Dutta et al. (*6*), where oxidation of methylene blue in aqueous solution by hydroxyl radicals required 1 hour to achieve 98% discoloration of the dye. The methylene blue dye test strip was developed as an alternative to this liquid method and allowed for the immediate detection of hydroxyl radicals. In a liquid phase reaction, such as the liquid methylene blue dye test, where molecules are allowed to collide in 3-dimensional space, the reaction is dependent on the concentration of the colliding species. In the dye test strip, since the methylene blue dye is soaked into and onto the filter paper fibers, the oxidation of methylene blue by the hydroxyl radicals is aided by the greater surface area available for the reaction and a higher concentration, resulting in a higher rate of reaction.

To standardize the MB dye test, test strips were developed such that each had a consistent, uniform MB dyed section on which samples could be applied. A 1.0 mM MB dye solution was prepared in Milli-Q water from a 10 mM stock solution prepared with methanol. Qualitative filter paper (Grade 1, 70 mm diameter circles, medium porosity) (Fisher Scientific, Hanover Park, IL) was cut into two rectangular test strips approximately 2 cm by 6 cm in size. Using a black, fine point, industrial strength, permanent marker, a horizontal line was made on both sides of the test strip about 1.5 cm from the bottom and allowed to dry. This marker line serves as a hydrophobic barrier that prevents the MB dye from spreading above this line during the dipping process. The bottom of the test strip was then dipped 10 times into 1.0 mM MB dye solution to the level of the marker line. The dipped test strip was then placed onto a paper towel-lined tray and allowed to completely dry in the dark. Dried strips stored for 24 hours were used in this study; however, strips can be stored in a sealed, dark plastic bag for up to 33 days without adversely affecting test results.

The MB dye test was performed during the Fenton's reaction to verify the formation of hydroxyl radicals and during the quenching process to verify completion of quenching. Forty microliters of a liquid sample were placed dropwise onto the center of the MB dyed section of a test strip, allowing for absorption between drops. All MB dye tests were compared against a test strip tested with Milli-Q water. The absence of bleaching of the MB dye indicated that no hydroxyl radicals were present to the extent detectable by this qualitative test. In the quenching process of the Fenton's reaction, the absence of bleaching signified that quenching was complete. Bleaching of the MB dye, due to the presence of hydroxyl radicals in a sample, was indicated by an immediate discoloration of the MB dye from a dark blue color to an almost white color, concentrated at the point of application, with a dark blue outline.

4.2.2.2 Fenton's Reaction in Milli-Q Water

A Fenton's reaction was performed to generate hydroxyl radicals in aqueous samples. All experiments were performed in Milli-Q water. For the Fenton's reaction, a Fe^{2+}:H_2O_2 molar ratio of 1:20 was used. The initial concentrations of Fe^{2+} and H_2O_2 in the reaction mixture were 0.15 mM and 3 mM, respectively, which are within the ranges previously used by Trapido et al. (*10*). For optimal Fenton's reaction efficiency, the $FeSO_4$ solution was adjusted to pH 3 with 0.5 M H_2SO_4 and/or 1M NaOH. The pH was monitored using a 720A plus pH/ISE meter with an 8102 BNU Ross Ultra Combination pH electrode (ThermoOrion, Beverly, MA).

The Fenton's reaction was initiated by the addition of 3% H_2O_2, prepared from 30% unstabilized H_2O_2 and Milli-Q water, to the reaction mixture to obtain an initial concentration of 3 mM H_2O_2. Since stabilizing agents (hydroxyl radical scavengers) in commercial H_2O_2 might affect the results (*13*), only H_2O_2 devoid of stabilizing agents was used. In order to prevent localized reactions (that might occur when a small volume of very concentrated solution is added to a reaction mixture), 3% H_2O_2 rather than 30% H_2O_2 was used to initiate the reaction. To test, qualitatively, the production of hydroxyl radicals during the reaction, MB dye tests were performed on the unquenched reaction mixture at 15, 30, and/or 60 minutes of reaction.

After 60 minutes, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v), prepared from Na_2SO_3 and Milli-Q water. Trapido et al. (*10*, *14*) recommended quenching by the addition of 2 to 3 drops of 10% Na_2SO_3 solution for every 10 mL of reaction mixture. To verify, qualitatively, that quenching was complete, 5 minutes after the addition of the 10% Na_2SO_3 solution, a MB dye test was performed. Additional 10% Na_2SO_3 solution was added to the reaction mixture and the MB dye test was repeated until no discoloration of the MB dye was observed (quenching was complete).

4.2.2.3 Hydroxyl Radical Detection by Benzoic Acid

To verify the ability of the MB dye test to detect the presence of hydroxyl radicals in a Fenton's reaction, experiments were performed using benzoic acid (BA) as a chemical probe. BA reacts with hydroxyl radicals to form *o*-, *m*-, and *p*-hydroxybenzoic acids as well as other products (*2*, *15*). The presence of hydroxyl radicals can be indirectly determined through the detection of these hydroxylated benzoic acids (HBAs) using thin layer chromatography (TLC) and spectrophotometric wavelength scans.

Two Fenton's reaction experiments were performed with the addition of BA. Based on studies performed by Lindsey et al. (*2*), finely ground BA was added to the Fenton's reaction solution to obtain a final concentration of 9 mM BA. The experiment, "Benzoic Acid in an Unquenched Fenton's Reaction Mixture," was similar to the procedure of the "Fenton's Reaction in Milli-Q Water" prior to quenching, except that BA was added immediately preceding the initiation of the Fenton's reaction. Following initiation, the reaction was monitored using TLC and spectrophotometric wavelength scans at various times throughout the reaction period (120 minutes). The objective was to verify the presence of hydroxyl radicals in the Fenton's reaction mixture, as was previously detected by the MB dye test. The second experiment, "Benzoic Acid in a Quenched Fenton's Reaction Mixture," was identical to the procedure of the "Fenton's Reaction in Milli-Q Water," except that BA was added following the completion of quenching. TLC and spectrophotometric wavelength scans were performed at various times until 90 minutes had elapsed. A wavelength scan of the reaction mixture was also performed immediately preceding the addition of BA. The objective was to verify the absence of hydroxyl radicals in the Fenton's reaction mixture following the completion of quenching, as was previously detected by the MB dye test.

4.2.2.4 Thin-Layer Chromatography (TLC)

TLC was performed by the procedure described in Skoog et al. (*16*). TLC plates were spotted using Drummond 1 μL "Microcap" micropipettes (Fisher Scientific, Hanover Park, IL) onto 2.5 x 7.5 cm silica gel 60 F_{254} precoated TLC plates with acid-stable fluorescent indicator (Fisher Scientific, Hanover Park, IL). The left side of the TLC plate was spotted with one of three selected standard solutions: 2 μL of 9 mM BA/MeOH, 1 μL of 9 mM 4-HBA/MeOH, or 2 μL of a mixed standard consisting of 50 μL 9 mM BA/MeOH and 25 μL 9 mM 4-HBA/MeOH. A volume of 3 μL of Fenton's reaction sample was spotted on the right side of the TLC plates. The origin, where the standard and sample were spotted, was marked near the bottom of the plate by two horizontal lines on either side of the plate. Although 12 different eluting solvents (Table 4.1) were investigated for developing the TLC plates, a solution of methanol:chloroform at a volumetric ratio of 2:10 was selected as the eluting solvent, since it resulted in the best separation of BA and 4-HBA.

Table 4.1 Potential Eluting Solvents Investigated for TLC Plate Development

Eluting Solvent	Volumetric Ratio
Butanol:Water:Acetic Acid	60:25:15
Methanol:Water	50:50
Ethanol:Water	50:50
Isopropanol:Water	50:50
Butanol:Water	50:50
Methanol:Chloroform	1:10
Dichloromethane:Methanol	10:1
Ethyl Acetate:n-Hexane	50:50
n-Hexane:Ethanol:Ethyl Acetate	80:10:10
Methanol:Chloroform	1:12
Methanol:Chloroform	1:40
Methanol:Chloroform	2:10

TLC plates were examined under ultraviolet light (254 nm) and the resulting "spots" were carefully traced with a pencil with a dot marked in the center of maximum intensity. Retention factors (R_f) for each separated compound were calculated as the distance traveled by the compound measured to the point of maximum intensity divided by the distance traveled by the solvent front. The R_f value of each compound separated from the Fenton's reaction sample was compared with the R_f value of the standard on the same plate (*17*). An absolute R_f difference of greater than 0.05 was considered to be a significant difference and indicated that the compound was different from the standard.

An additional experiment, "TLC of an Unquenched Fenton's Reaction Mixture without BA Addition," was similar to the procedure of "Fenton's Reaction in Milli-Q Water" prior to quenching. Following initiation, the reaction was monitored by performing TLC at various times. The objective was to verify that the TLC results of "Benzoic Acid in an Unquenched Fenton's Reaction Mixture" were due to the reaction of BA with hydroxyl radicals.

4.2.2.5 Spectrophotometric Wavelength Scans

Fenton's reaction samples were scanned spectrophotometrically in cuvettes (polystyrene, optical pathlength of 10 mm) on a Beckman DU7400 spectrophotometer (Beckman Instruments, Fullerton, CA). Milli-Q water was used as a blank.

4.3 Results and Discussion

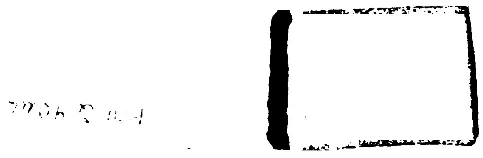
4.3.1 Fenton's Reaction in Milli-Q Water (No Benzoic Acid Addition)

The MB dye test was performed to determine the presence of hydroxyl radicals generated by the Fenton's reaction. As shown in Figure 4.2A, the MB dye test control strip (no sample added) was homogeneously dark blue in color. When the MB dye test was performed with Milli-Q water (Figure 4.2B), no bleaching or discoloration was observed. During the Fenton's reaction experiment prior to quenching, no significant change in solution pH (~ pH 3.0) and temperature (23.0 °C) occurred during the 60 minute reaction period, and the solution remained clear and colorless in appearance. As shown in Figures 4.2C and 4.2D, strips tested with unquenched Fenton's reaction mixture at 15 and 30 minutes, respectively, indicated the presence of hydroxyl radicals by immediate bleaching.

The Fenton's reaction was quenched after 60 minutes. The pH of the reaction mixture increased from 3.1 to 7.7. The temperature was 22.5 °C. The color of the

Figure 4.2 Methylene blue dye test results for Fenton's reaction in Milli-Q water. For test strips (A) control (no sample added) and (B) 40 μL of Milli-Q water, no bleaching or discoloration of the methylene blue dye is observed. Test strips with 40 μL of unquenched Fenton's reaction mixture at (C) 15 minutes and (D) 30 minutes of reaction indicate the presence of hydroxyl radicals by bleaching of the methylene blue dye from dark blue to an almost white color with a dark blue outline. Test strips with 40 μL of Fenton's reaction mixture quenched with (E) 30 drops and (F) 35 drops of 10% Na_2SO_3 indicate the incomplete quenching and absence of hydroxyl radicals by very slight bleaching and no bleaching, respectively.

A

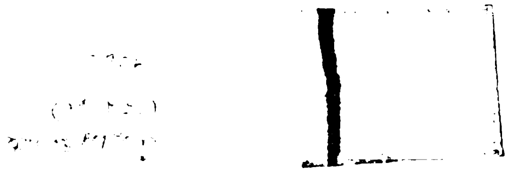


B

C

D

E



F

reaction mixture changed from colorless to light orange, which can be attributed to the more basic pH of the reaction mixture during the quenching process, resulting in the conversion of iron from a hydrated ferrous form to a colloidal ferric form and the formation of ferric hydroxide (*2*, *18*, *19*). As shown in Figure 4.2E, a MB dye test of the Fenton's reaction mixture quenched with 30 drops (1.23 mL) of quenching agent (10% Na_2SO_3) produced very slight bleaching as indicated by light blue discoloration with no dark blue outline. As shown in Figure 4.2F, the addition of 5 more drops (0.21 mL) of 10% Na_2SO_3 to the reaction mixture resulted in a complete absence of bleaching, indicating that quenching was complete and no hydroxyl radicals remained.

4.3.2 Benzoic Acid in an Unquenched Fenton's Reaction Mixture

An experiment involving Fenton's reaction and the addition of BA was performed to verify the presence of hydroxyl radicals. Since BA is only slightly soluble in water at pH 3, the remaining BA was allowed to remain floating on the liquid surface or suspended in solution. The addition of BA to the reaction mixture did not significantly alter the pH. Following initiation of the Fenton's reaction by addition of 3% H_2O_2, the reaction mixture appearance changed from colorless to a light pink color. No significant change in solution pH (~ pH 3.0) and temperature (23.0 °C) occurred during the 120 minute reaction period.

The reaction mixture continued to darken in color to violet (at 30 minutes) and eventually dark violet (at 60 minutes), corresponding to the gradual dissolution of BA. Complete dissolution of BA occurred after 90 minutes through the reaction with hydroxyl radicals to form more soluble HBAs. One possible explanation for the change in reaction mixture color to dark violet is the hydroxylation of BA by hydroxyl radicals to form salicylic acid (2-hydroxybenzoic acid), followed by the formation of a tetraaquosalicylatroiron (III) complex with Fe^{3+} (*20*). This violet complex is formed under an acidic pH (*21*) and is characterized by peak absorption at a wavelength of 520 nm (*22*).

Following the addition of 3% H_2O_2 to the reaction mixture, the reaction was monitored using TLC, as presented in Figure 4.3. The first two TLC plates, Figures 4.3A and 4.3B, were spotted with the standards 9 mM BA/MeOH and 9 mM 4-HBA/MeOH, respectively. Based on the results of these plates, the BA can be distinguished from the 4-HBA. The remainder of the TLC plates (Figures 4.3C-4.3F) was spotted with the mixed standard. For the TLC plates in Figures 4.3A to 4.3C, the Fenton's sample was obtained after 30 minutes of reaction, while for Figures 4.3D, 4.3E, and 4.3F, the sampling times were 60, 90, and 120 minutes, respectively. The elution time for each TLC plate was approximately 10 minutes.

The BA consistently traveled further on the plate than the 4-HBA, which was darker in intensity than the BA. The products present in the reaction mixture changed as the reaction proceeded. The TLC plates at 30 minutes and 60 minutes, Figures 4.3C and 4.3D, respectively, indicated one major product in a larger amount and numerous products present in smaller amounts (a series of smaller connected spots). However, at 90 minutes, Figure 4.3E, one major product in a larger amount, numerous products in smaller amounts, and two secondary products were observed. At 120 minutes, Figure 4.3F, two major products were observed with numerous products present in smaller amounts.

Figure 4.3 Thin layer chromatography results from "Benzoic Acid in an Unquenched Fenton's Reaction Mixture." The left side of each TLC plate was spotted with samples of standards. Figures 4.3A and 4.3B, were spotted with the standards 9 mM benzoic acid (BA) in methanol (MeOH) and 9 mM 4-hydroxybenzoic acid (HBA) in methanol, respectively. The remainder of the TLC plates (Figures 4.3C-4.3F) was spotted with 2 μL of a mixed standard consisting of 50 μL 9 mM BA/MeOH and 25 μL 9 mM HBA/MeOH. The right side of each TLC plate was spotted with a 3 μL sample of Fenton's reaction mixture from a particular reaction time. For Figures 4.3A to 4.3C, the Fenton's sample was from 30 minutes of reaction, while for Figures 4.3D, 4.3E, and 4.3F, the sampling times were 60, 90, and 120 minutes, respectively.

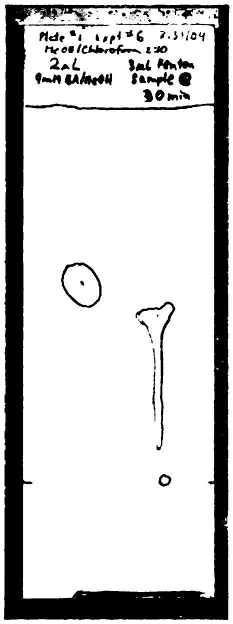


Plate #1 Expt #6 7.31/04
MeOH/Chloroform 2:10
2µL
9mM BA/MeOH
3µL Fenton Sample @ 30min

A

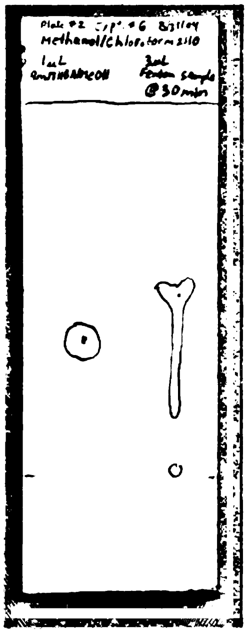


Plate #2 Expt. #6 8/31/04
Methanol/Chloroform 2:10
1µL
9mM BA/MeOH
3µL Fenton sample @30min

B

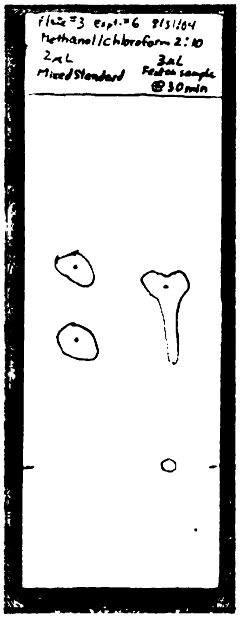


Plate #3 Expt. #6 8/31/04
Methanol/Chloroform 2:10
2µL
Mixed Standard
3µL Fenton sample @30min

C

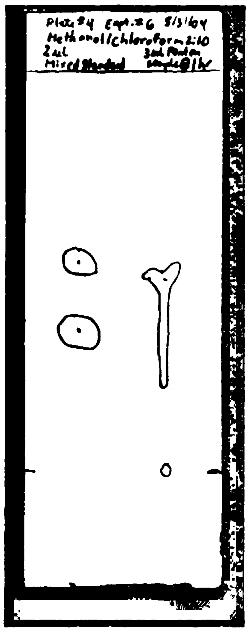


Plate #4 Expt. #6 8/31/04
Methanol/Chloroform 2:10
2µL
Mixed Standard
3µL Fenton sample @1hr

D

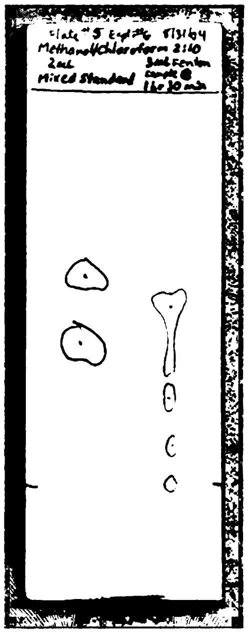


Plate #5 Expt #6 8/31/04
Methanol/Chloroform 2:10
2µL
Mixed Standard
3µL Fenton sample @ 1hr 30 min

E

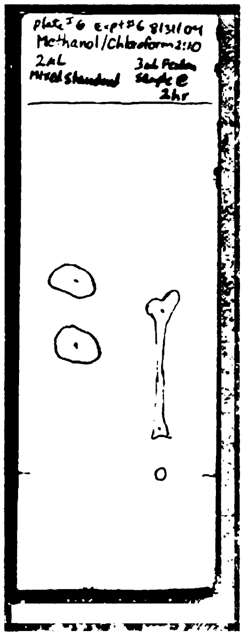


Plate #6 Expt #6 8/31/04
Methanol/Chloroform 2:10
2µL
Mixed Standard
3µL Fenton Sample @ 2hr

F

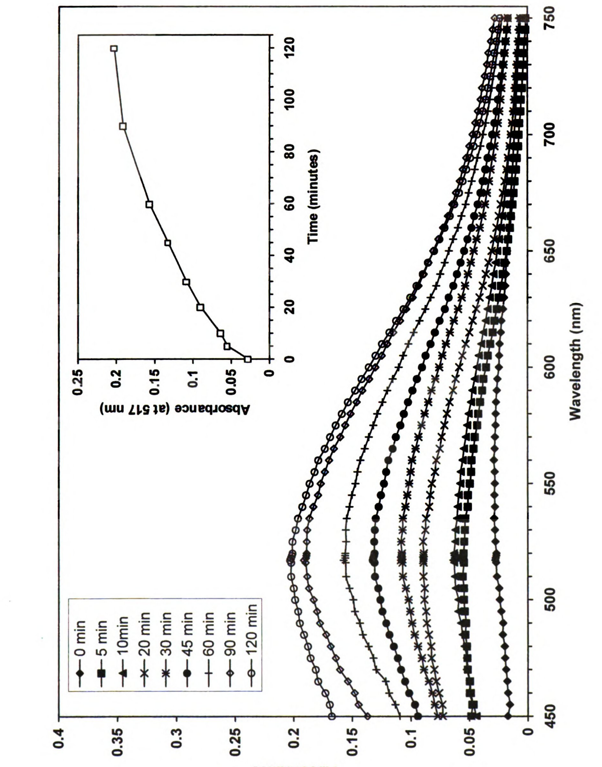
For all the Fenton's samples, none of the major spots correlated with either the BA or 4-HBA standards, suggesting that the major spots are more likely HBA products other than 4-HBA. Retention factor (R_f) values were only calculated for major spots and are presented in Table 4.2 (Plates A-F). The spots were numbered with the spot of farthest migration labeled as 1 and the remaining spots numbered consecutively. For all the Fenton's samples, the R_f values of the major spots were significantly different (> 0.05) from the R_f values calculated for BA and 4-HBA standards on the same plate. However, the visual correspondence between the numerous products present in smaller amounts and the 4-HBA standard spot suggests that 4-HBA was produced in a small quantity. The presence of HBAs on the developed TLC plate for the Fenton's reaction mixture sample verified that hydroxyl radicals were present in the reaction solution to react with the added BA. Comparing TLC plates, based on the absolute difference in the R_f values, spot 1 of the Fenton's samples from 30, 60, 90, and 120 minutes of reaction time are most likely the same compound. Furthermore, spot 2 of the Fenton's sample from 120 minutes is most likely the same compound as spot 3 of the Fenton's sample from 90 minutes.

The change in color of the Fenton's reaction mixture to dark violet was monitored by spectrophotometric wavelength scans. Figure 4.4 presents the wavelength scans over a range of 450 nm to 750 nm on 1000 μL samples of unquenched Fenton's reaction mixture containing BA at 0 minutes to 120 minutes of elapsed reaction time. As the reaction time increased, there was an increase in the absorbance value for each wavelength correlating with the increase in the intensity of the violet color of the reaction mixture. The maximum absorbance value (peak absorbance) occurred at a wavelength of

Table 4.2 Retention Factor (R_f) Results for Benzoic Acid Chemical Probe in an Unquenched (Plates A-F) and Quenched (Plates G-J) Fenton's Reaction Mixture

Plate #	**Standard Type**		**Standard R_f**		**ReactionTime (minutes)**	**Fenton Sample R_f**		
	Single	**Mixed**	**BA**	**HBA**		**Spot 1**	**Spot 2**	**Spot 3**
A	●		0.54		30	0.46		
B	●			0.35	30	0.48		
C		●	0.54	0.33	30	0.48		
D		●	0.53	0.35	60	0.47		
E		●	0.52	0.36	90	0.46	0.22	0.10
F		●	0.52	0.35	120	0.44	0.13	
G	●		0.51		30	0.49		
H	●			0.32	30	0.51		
I		●	0.50	0.34	30	0.52		
J		●	0.47	0.33	90	0.51		

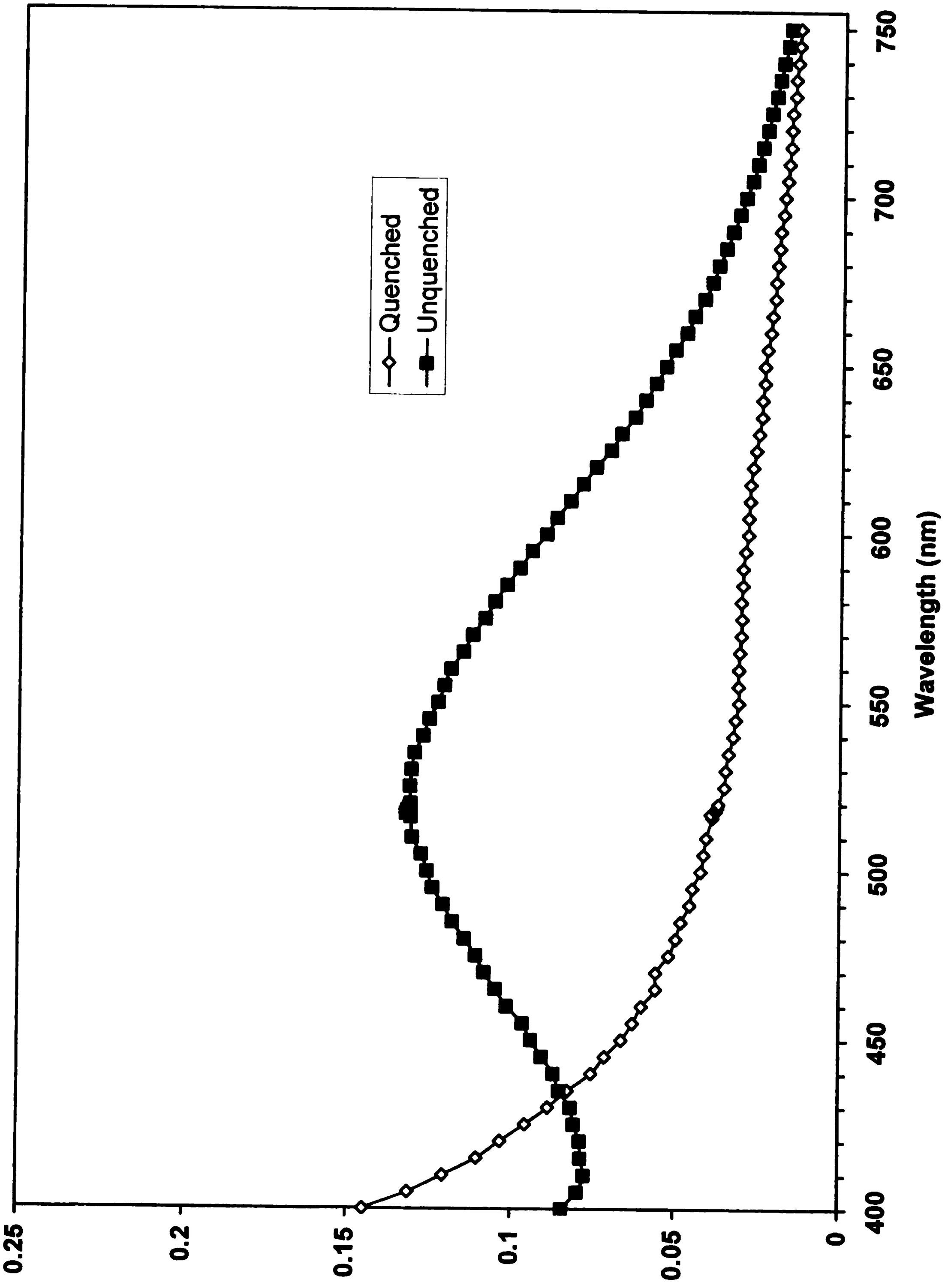
Figure 4.4 Wavelength scans (absorption spectra) of unquenched Fenton's reaction mixture with a benzoic acid chemical probe. Wavelength scans were performed over a wavelength range of 450 nm to 750 nm. Each wavelength scan represents a Fenton's reaction mixture sample at a different reaction time ranging from 0 minutes to 120 minutes after initiation of the Fenton's reaction. Absorbance vs. reaction time of unquenched Fenton's reaction mixture with a benzoic acid chemical probe at the wavelength of 517 nm is shown in the inset. Each data point is representative of the absorbance at 517 nm obtained from the wavelength scan of the Fenton's reaction mixture at a particular reaction time. Polystyrene cuvettes with an optical pathlength of 10 mm were used.


Absorbance
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
450
500
550
600
650
700
750
Wavelength (nm)
0 min
5 min
10min
20 min
30 min
45 min
60 min
90 min
120 min
Absorbance (at 517 nm)
0.25
0.2
0.15
0.1
0.05
0
0
20
40
60
80
100
120
Time (minutes)

517 nm as the tetraaquosalicylatroiron (III) complex was generated. The violet color of the reaction mixture and the peak absorbance at a wavelength close to 520 nm are indicative of the formation of HBA products and verify the presence of hydroxyl radicals. The Figure 4.4 inset illustrates the increase in absorbance at 517 nm with an increase in the reaction time.

After performing a wavelength scan on a 1000 μL sample of the unquenched Fenton's reaction mixture containing BA at 45 minutes reaction time, 25 μL of 10% Na_2SO_3 was mixed into the sample in the cuvette. The violet color of the solution immediately changed to light yellow. A wavelength scan was then repeated over a range of 400 nm to 750 nm. Figure 4.5 presents the wavelength scan of the unquenched Fenton's reaction mixture containing BA after 45 minutes of reaction time alongside the wavelength scan of the same sample following quenching with 10% Na_2SO_3. Unlike the unquenched sample scan for which a peak absorbance occurred at 517 nm, for the quenched sample there was a loss of this peak corresponding with the disappearance of the violet color. The disappearance of the violet color can be explained as the reduction of Fe^{3+} to Fe^{2+} by Na_2SO_3 (reducing agent), resulting in loss of the tetraaquosalicylatroiron (III) complex. In addition, the Na_2SO_3 quenched the hydroxyl radicals in the reaction mixture, thereby preventing further formation of salicylic acid by the hydroxylation of BA. The light yellow color can be attributed to the more basic pH of the reaction mixture during the quenching process as previously described. Since the formation of the tetraaquosalicylatroiron (III) complex requires acidic conditions (*21*), the presence of Fe^{3+} in the quenched reaction mixture does not result in the reforming of the complex.

Figure 4.5 Wavelength scans of the effect of quenching an unquenched Fenton's reaction mixture containing a benzoic acid chemical probe at 45 minutes of reaction time. Wavelength scans were performed over a wavelength range of 400 nm to 750 nm. After performing a wavelength scan on a 1000 μL sample of unquenched Fenton's reaction mixture from 45 minutes of reaction time, 25 μL of 10% sodium sulfite quencher was added to the sample. Following mixing, a wavelength scan was performed on the quenched sample. Polystyrene cuvettes with an optical pathlength of 10 mm were used.



Absorbance
0.25
0.2
0.15
0.1
0.05
0
Wavelength (nm)
400
450
500
550
600
650
700
750
Quenched
Unquenched

4.3.3 TLC of an Unquenched Fenton's Reaction Mixture without BA Addition

To verify that the unquenched TLC results of the preceding section were due to the reaction of BA with hydroxyl radicals, the same experiment was repeated without the addition of BA. No significant change in solution pH (~ pH 3.0) and temperature (22.5 °C) occurred throughout the 60 minute reaction period. The reaction mixture remained clear and colorless following initiation of the Fenton's reaction, indicating that the change in color to violet depends on the presence of both BA and hydroxyl radicals. This observation was further supported by the "Benzoic Acid in an Unquenched Fenton's Reaction Mixture" experiment, in which the pink color did not form in the presence of BA until after the addition of 3% H_2O_2, resulting in the generation of hydroxyl radicals. No spots appeared on the TLC plate for the Fenton's reaction mixture sample at 30 and 60 minutes of reaction time in the absence of BA, indicating that the spots observed above the origin on the developed TLC plates for the "Benzoic Acid in an Unquenched Fenton's Reaction Mixture" experiment were a result of the reaction of BA with hydroxyl radicals.

4.3.4 Benzoic Acid in a Quenched Fenton's Reaction Mixture

An experiment, involving Fenton's reaction and the addition of BA following the completion of quenching, was performed to verify the absence of hydroxyl radicals in the reaction mixture. In the reaction prior to quenching, the pH (~ pH 3.0) and temperature (22.0 °C) remained constant, and the reaction mixture remained clear and colorless. After 60 minutes of reaction time, the Fenton's reaction was quenched, and the reaction mixture changed to light orange, attributable to the more basic pH of the reaction mixture as a result of the quenching process. The pH ranged from 7.5 to 7.6 and the temperature remained at 21.0 °C. MB dye tests were performed to determine the completion of quenching, which occurred after the addition of 40 drops (1.76 mL) of 10% Na_2SO_3.

Following the addition of BA to the reaction mixture, the pH slowly decreased from 7.6 to 4.6 after 90 minutes, and the temperature remained constant at 21.0 °C. The BA dissolved in the reaction mixture slightly faster than what was observed in the "Benzoic Acid in an Unquenched Fenton's Reaction Mixture" experiment, achieving complete dissolution in 60 minutes. The more rapid dissolution of BA can be explained by the higher solubility of BA at a pH of 7.6 versus that at a pH of 3. The reaction solution remained light orange following the addition of BA after quenching, further supporting that the pink to violet color change is dependent on the presence of both BA and hydroxyl radicals. In the absence of hydroxyl radicals and at a basic pH, salicylic acid, and ultimately the tetraaquosalicylatroiron (III) complex, cannot form.

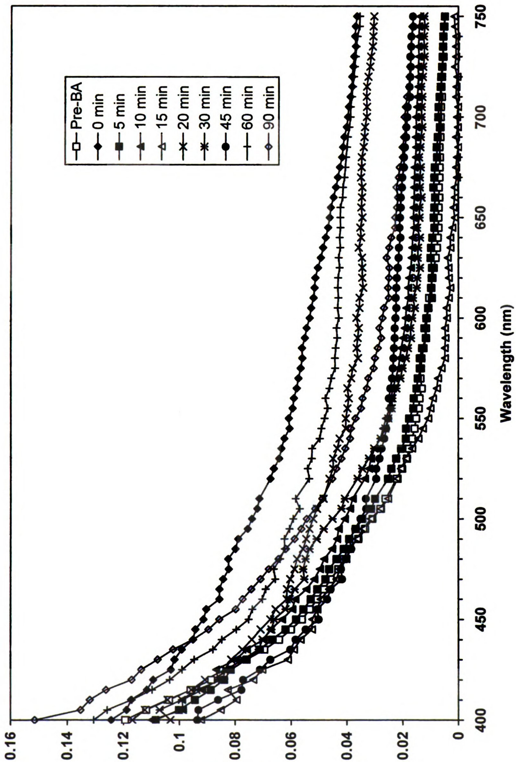
Following the addition of BA, the reaction was monitored using TLC. For all Fenton's reaction mixture samples, only one spot appeared on the TLC plate above the origin and correlated with BA, as supported by the R_f calculations presented in Table 4.2 (Plates G-J). The Fenton's reaction mixture spot had an R_f range of 0.49 to 0.52. Since the maximum absolute R_f difference between the Fenton's reaction mixture spot and the BA standard spot on the same plate was 0.04, these spots are assumed to be the same compound. The absence of HBAs on the TLC plate results for the Fenton's reaction mixture sample verified that quenching was complete, and no hydroxyl radicals were present in the solution when BA was added.

The absence of hydroxyl radicals following quenching was also assessed by spectrophotometric wavelength scans over a wavelength range from 400 nm to 750 nm. Prior to the BA addition but after completion of quenching, a wavelength scan was performed on the reaction mixture to compare to the effect of BA. The results of this wavelength scan, identified as "Pre-BA," as well as scans performed on 1000 μL samples of quenched Fenton's reaction mixture from 0 to 90 minutes after the addition of BA, are presented in Figure 4.6. The wavelength scan results, following the addition of BA, did not vary significantly from that observed for "Pre-BA." The absence of peak absorbance at close to 520 nm was an indication of the lack of HBA products in the reaction mixture and suggests the absence of hydroxyl radicals. Since no hydroxyl radicals were present in the quenched reaction mixture to react with the added BA, the tetraaquosalicylatroiron (III) complex did not form.

4.3.5 Methylene Blue Dye Test Strip Studies: Age, 3% H_2O_2, and Influence of pH

Three additional experiments were performed (Figure 4.7) to test the performance of the methylene blue dye test strips. In the first experiment (Figure 4.7A), the effect of age of the methylene blue dye test strip on test results was evaluated by applying 40 μL of the same unquenched Fenton's reaction solution (30 minutes of reaction time) to strips of different ages (4 days and 33 days old). The unquenched Fenton's reaction solution was prepared as described previously in Section 4.2.2.2 ("Fenton's Reaction in Milli-Q Water"). Since there was no significant difference between the degree of bleaching for the 4 day and 33 day old test strips, age of the test strip does not appear to affect the performance of the methylene blue dye test. It should also be noted that the degree of

Figure 4.6 Wavelength scans (absorption spectra) of quenched Fenton's reaction mixture. Wavelength scans were performed over a wavelength range of 400 nm to 750 nm. The "Pre-BA" wavelength scan represents a sample of the Fenton's reaction mixture prior to the benzoic acid chemical probe addition, but after completion of quenching. The remainder of the wavelength scans represents Fenton's reaction mixture samples at 0 to 90 minutes following the addition of benzoic acid after the completion of quenching. Polystyrene cuvettes with an optical pathlength of 10 mm were used.


Absorbance
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
400
450
500
550
600
650
700
750
Wavelength (nm)
Pre-BA
0 min
5 min
10 min
15 min
20 min
30 min
45 min
60 min
90 min

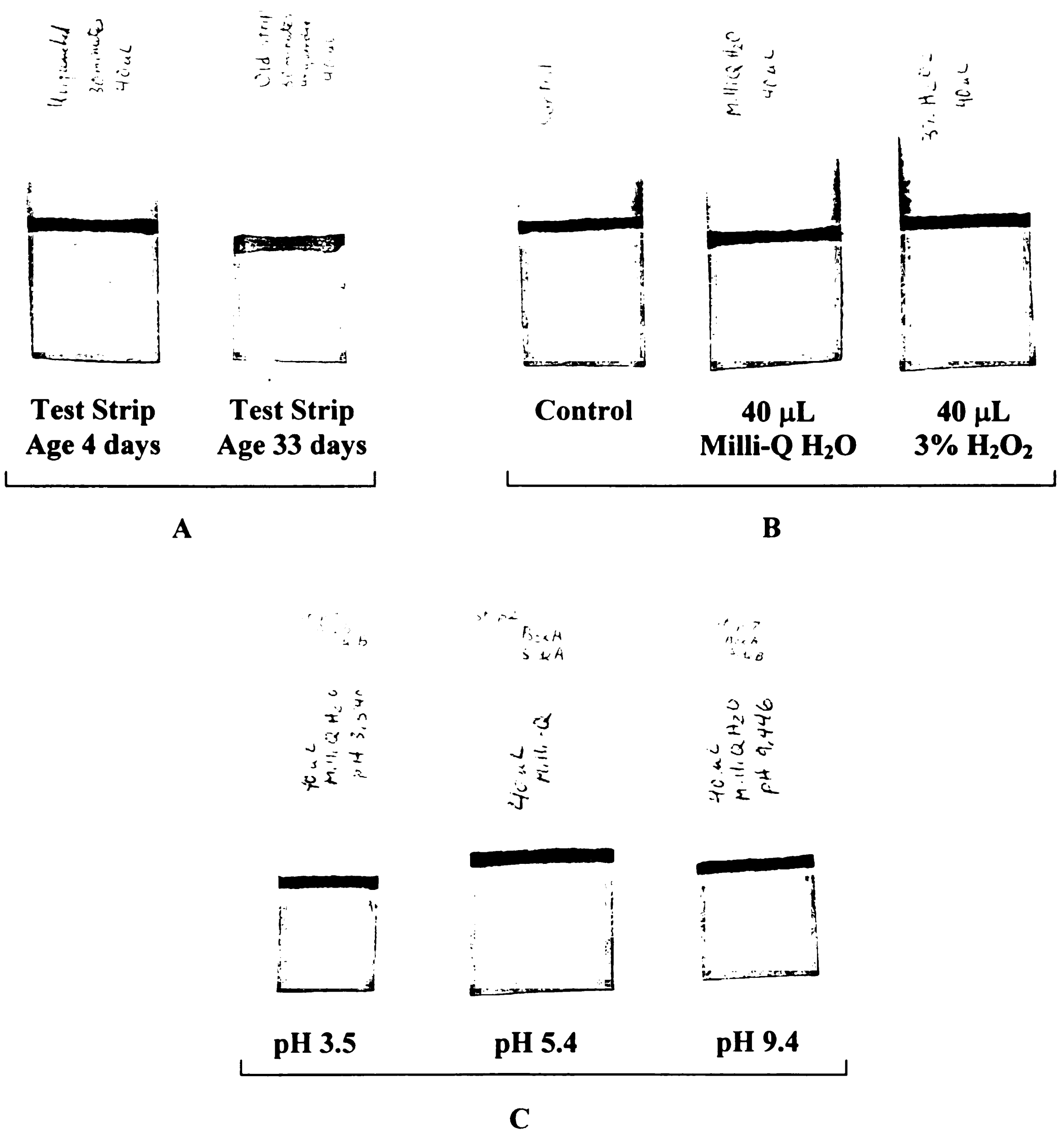


Figure 4.7 Methylene blue dye test results evaluating (A) the affect of age of the methylene blue dye test strip on test results by applying identical samples of unquenched Fenton's reaction solution to test strips aged 4 and 33 days, (B) the ability of 3% H_2O_2 to cause bleaching of the methylene blue dye as compared to Milli-Q H_2O and control (no sample added) test strips, and (C) the affect of Milli-Q H_2O pH on the methylene blue dye test.

bleaching of these two test strips was not significantly different from the 24 hour old strip tested with 40 µL of unquenched Fenton's reaction solution at 30 minutes of reaction time (Figure 4.2D). In the second experiment (Figure 4.7B), 40 µL of 3% H_2O_2 was tested for its ability to cause discoloration/bleaching in the methylene blue dye test as compared with strips tested with 40 µL of Milli-Q H_2O and no sample (control). Since 3% H_2O_2 did not appear to cause any discoloration/bleaching of the methylene blue dye, the reaction that causes the discoloration/bleaching of the methylene blue dye is independent of H_2O_2. It can therefore be assumed that the bleaching observed for the methylene blue dye tests with unquenched Fenton's reaction solutions was a result of the presence of hydroxyl radicals rather than unreacted H_2O_2. In the third experiment (Figure 4.7C), the effect of Milli-Q H_2O pH on the methylene blue dye test was evaluated by testing 40 µL samples of Milli-Q H_2O adjusted to pH 3.5, 5.4, and 9.4 by the addition of 0.5 M H_2SO_4 and/or 1M NaOH. Since no discoloration of methylene blue dye was observed for Milli-Q H_2O at any pH tested, methylene blue dye test results are not influenced by sample pH. The bleaching observed for the methylene blue dye tests with unquenched Fenton's reaction solutions was a result of the presence of hydroxyl radicals rather than an effect of an acidic pH.

4.4 Conclusions

A new procedure, the methylene blue dye test, qualitatively indicates the presence of hydroxyl radicals through the immediate, distinct bleaching of methylene blue dye on a paper test strip. This method employs a simple procedure requiring inexpensive materials, without the addition of competitive probe chemicals that potentially can interfere with the reaction. A Fenton's reaction with an Fe^{2+}:H_2O_2 molar ratio of 1:20 generated hydroxyl radicals in Milli-Q water. The presence and absence of hydroxyl radicals were determined prior to and following quenching of the Fenton's reaction with 10% sodium sulfite, respectively. Bleaching of methylene blue dye, due to the presence of hydroxyl radicals in a sample, was indicated by a discoloration from a dark blue color to an almost white color, concentrated at the point of application, with a dark blue outline. A lack of bleaching indicated the absence of hydroxyl radicals in the sample. The presence of hydroxyl radicals was verified by benzoic acid chemical probe experiments with thin layer chromatography (TLC) and spectrophotometric wavelength scans. The presence of hydroxyl radicals was indirectly determined by detection of hydroxylated benzoic acids on TLC plates and a violet solution color with a peak absorbance at a wavelength close to 520 nm. In experiments to test the performance of the methylene blue dye test strips, age of the test strip, presence of H_2O_2, and sample pH were determined to have no significant effect on the methylene blue dye test results.

4.5 References

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Chapter 5

Toxicology Studies of Fenton's Remediation in Milli-Q Water

5.1 Introduction

As a first step in evaluating Fenton's reagent as a remediation process, it is important to verify that the reaction of Fenton's reagent with the solvent (in the absence of 4,4′-dichlorobiphenyl (4,4′DCBP)) does not result in any toxic byproducts. If no toxic response occurs as a result of a reaction of Fenton's reagent with the solvent, any toxicity resulting from the Fenton's reagent remediation of 4,4′DCBP can be assumed to be independent of the solvent. Additionally, this series of experiments allowed for the development of a remediation procedure that later would be applied to 4,4′DCBP. The three solvents investigated were Milli-Q H_2O alone, 80/20 Milli-Q H_2O/ACN (by volume), and 50/50 Milli-Q H_2O/ACN (by volume). In this chapter only Fenton's reaction in Milli-Q H_2O is presented. Reactions in the solvents 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN are discussed in Chapter 6. Although 4,4′DCBP is insoluble in water alone, since water constituted some part of the solvent used for dissolving 4,4′DCBP in remediation, it was important to investigate whether Fenton's reagent remediation with water alone resulted in any toxicity. Three molar Fe^{2+}:H_2O_2 ratios, 1:5, 1:20, and 1:40, were investigated in this section of research to determine the ratio that would be used for Fenton's remediation of 4,4′DCBP. The toxicity of the resulting final Fenton's remediation solutions was evaluated in rat liver epithelial cells using a nongenotoxic bioassay that determines the in vitro modulation of gap junctional intercellular communication (GJIC) as a measure of the epigenetic toxicity.

5.2 Experimental Section

5.2.1 Chemicals

For the Fenton's remediation portion of this section of research the following chemicals were used. Methylene blue dye (3,7-bis(dimethylamino)phenothiazin-5-ium chloride) (97% purity) was purchased from Fluka (Buchs, Switzerland). Thirty-percent hydrogen peroxide (H_2O_2) (unstabilized), iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (99% purity), and sodium sulfite (Na_2SO_3) (anhydrous, 98% purity) were purchased from Sigma–Aldrich (St. Louis, MO). Sulfuric acid (H_2SO_4) (96% purity) and sodium hydroxide (NaOH) pellets (99% purity) were purchased from J.T. Baker (Phillipsburg, NJ). Throughout this chapter and dissertation, Milli-Q water was obtained from a Milli-Q Ultrapure Water Purification System (System Type ZMQS6VFOY) purchased from Millipore Corp. (Bedford, MA).

For the toxicology portion of this section of research the following chemicals were used. For cell culture, D-medium (Formula No. 78-5470 EG), Fetal Bovine Serum (FBS), and Gentamicin were purchased from GIBCO Laboratories (Grand Island, NY). Lucifer yellow CH, dilithium salt, was purchased from Molecular Probes Inc. (Eugene, OR), ICN Biomedicals Inc. (Aurora, OH), and Sigma Chemical Co. (St. Louis, MO). Formaldehyde solution (37%) for the GJIC bioassays was purchased from J.T. Baker (Phillipsburg, NJ).

5.2.2 Methods

5.2.2.1 Fenton's Remediation in Milli-Q Water

For each experiment, the initial concentration of H_2O_2 in the reaction mixture was 3 mM, which is within the range used by Trapido et al. (*1*) (1.0 to 10.0 mM). The initial Fe^{2+} concentrations required for the Fe^{2+}:H_2O_2 ratios 1:5, 1:20, and 1:40 were 0.6 mM, 0.15 mM, and 0.075 mM, respectively. These Fe^{2+} concentrations are within the range used by Trapido et al. (*1*) (0.004 mM to1.0 mM). Throughout the remediation process, the pH was adjusted/monitored using a 720A plus pH/ISE meter with an 8102 BNU Ross Ultra Combination pH electrode (ThermoOrion, Beverly, MA).

The methylene blue (MB) dye test was performed during the Fenton's reaction to verify the formation of hydroxyl radicals and during the quenching process to verify the completion of quenching. MB dye test strips were prepared by the method of Satoh et al. (*2*) as described in detail in Chapter 4. Forty microliters of a liquid sample were placed dropwise onto the center of the MB dyed section of a test strip, allowing for absorption between drops. All MB dye tests were compared against a test strip tested with Milli-Q water. The absence of bleaching of the MB dye indicated that no hydroxyl radicals were present to the extent detectable by this qualitative test. In the quenching process of the Fenton's reaction, the absence of bleaching signified that quenching was complete. Bleaching of the MB dye, due to the presence of hydroxyl radicals in a sample, was indicated by an immediate discoloration of the MB dye from a dark blue color to an almost white color, concentrated at the point of application, with a dark blue outline.

For each Fenton's remediation experiment a 5.0 mM $FeSO_4$ stock solution was prepared from $FeSO_4{\cdot}7H_2O$ and Milli-Q water. The 5.0 mM $FeSO_4$ stock solution was then used to prepare 100 mL of a $FeSO_4$ reaction solution with the pre-determined Fe^{2+} concentration required for the selected $Fe^{2+}:H_2O_2$ ratio. A 150 mL glass beaker with a stir bar was used as the reaction vessel. For optimal Fenton's reaction efficiency, the $FeSO_4$ reaction solution was adjusted to pH 3 with 0.5 M H_2SO_4 and/or 1M NaOH. The Fenton's reaction was initiated by the addition of 3% H_2O_2, prepared from 30% unstabilized H_2O_2 and Milli-Q water, to the reaction mixture to obtain an initial concentration of 3 mM H_2O_2. Since stabilizing agents (hydroxyl radical scavengers) in commercial H_2O_2 might affect the results (*3*), only H_2O_2 devoid of stabilizing agents was used. In order to prevent localized reactions (that might occur when a small volume of very concentrated solution is added to a reaction mixture), 3% H_2O_2 rather than 30% H_2O_2 was used to initiate the reaction. To test, qualitatively, the production of hydroxyl radicals during the reaction, MB dye tests were performed on the unquenched reaction mixture at 15, 30, and/or 60 minutes of reaction.

After 60 minutes, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v), prepared from Na_2SO_3 and Milli-Q water. Trapido et al. (*1*, *4*) recommended quenching by the addition of 2 to 3 drops of 10% Na_2SO_3 solution for every 10 mL of reaction mixture. A 0.5 mL glass pipette was used to administer the drops to the reaction mixture. To verify, qualitatively, that quenching was complete, 5 minutes after the addition of the 10% Na_2SO_3 solution, a MB dye test was performed. An additional aliquot of 10% Na_2SO_3 solution was added to the reaction mixture, and the MB dye test was repeated until no discoloration of the MB dye was observed (quenching was complete). To aid in the removal of iron from the reaction mixture by precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to pH 9 with 0.5 M

H_2SO_4 and/or 1M NaOH. If a large amount of precipitate in the reaction mixture was observed, the solution was centrifuged at 1200 rpm for 10 minutes and the supernatant was retained for filtration. The reaction mixture or supernatant was then filtered through a 1.0 μm glass fiber filter, using a vacuum filtration unit, into a 1000 mL Erlenmeyer side-arm filtration flask. Milli-Q water was used for rinsing during filtration only if the reaction mixture touched the sides of the funnel or there was residue in the reaction vessel. The filtered reaction mixture was transferred to a clean 150 mL glass beaker containing a stir bar. To neutralize the reaction mixture pH for the cells in the toxicology bioassays, the filtered solution was adjusted to pH 7 with 0.5 M H_2SO_4 and/or 1M NaOH. This final solution was transferred to an amber, 120 mL Boston round bottle with a TFE closure and was refrigerated at 4 °C until toxicology bioassays were performed.

5.2.2.2 Cell Culture Techniques

WB-F344 rat liver epithelial cells were obtained from Dr. J. W. Grisham and Dr. M. S. Tsao of the University of North Carolina (Chapel Hill, NC) (*5*). This cell line was selected because it is a diploid, nontumorigenic cell line originating from a strain of rat that has been used for toxicological/cancer studies of numerous chemicals, thereby allowing for a source of comparison (*5*). Since 70% of the chemicals that are carcinogens are liver carcinogens and the liver is the “first pass” organ for ingested toxins, liver cells are important for toxicological/cancer studies (*6*). Furthermore, the WB-F344 cell line was designed for in vitro assays to match the many in vivo tumor promotion assays that had been done in rat liver, specifically, in the Fischer 344 rat.

The cell culture techniques performed were similar to those described by Herner et al. (*5*) and Luster-Teasley et al. (*7*). Cells were cultured in 150 cm^2 sterile, treated, polystyrene cell culture flasks (Corning Inc., Corning, NY) in 25 mL of D-medium containing 5% Fetal Bovine Serum (FBS) and 0.2% Gentamicin. The cells were incubated at 37 °C in a water-jacketed IR Autoflow Automatic CO_2 incubator (NUAIRE, Inc., Plymouth, MN) in a humidified atmosphere with 5% CO_2 and 95% air. The time required for cell growth confluency was about two days. The confluent culture was split and transferred every other day into a new 150 cm^2 culture flask with new medium mixture. In addition, from 150 cm^2 flasks of confluent cells, cultures were prepared for the bioassays in 35 mm diameter, sterile, treated polystyrene cell culture dishes (Corning Inc., Corning, NY) with 2 mL of D-medium supplemented with 5% FBS. The cultures for the bioassays were incubated under the same conditions as the aforementioned flasks.

5.2.2.3 In Vitro Bioassay for GJIC

The bioassays of GJIC were performed on confluent cell cultures (usually 2 days of growth) grown in 35 mm diameter culture dishes (as described in the preceding section). The scrape-loading/dye transfer (SL/DT) procedure for determining the GJIC was adapted from the method described by El-Fouly et al. (*8*) and is described in detail by Herner et al. (*5*). A detailed description of the spread of Lucifer yellow dye from the scrape to neighboring cells can be found in Wilson et al. (*9*).

For reasons to be discussed later in this chapter, which determined that further toxicology studies were unnecessary, only dose-response GJIC bioassays were performed. Chemical treatments (reaction mixture solution), controls (no dose), and vehicle controls were performed in triplicates. Doses were applied directly to the dishes of confluent cell cultures. The final reaction mixture solution, that was stored at 4 °C, was warmed to room temperature prior to use in the bioassay. All GJIC bioassay results assumed that the filtration process performed during the Fenton's remediation experiment removed no toxic chemicals generated during the reaction. Since the exact molecular weight and concentrations of the reaction mixture solutions were unknown, GJIC bioassay doses were investigated in terms of volumes rather than concentrations. For the dose-response experiments, confluent cells were exposed to dose volumes of 100, 150, 200, 250, and 300 μL of reaction mixture solution with incubation times of 30 minutes (for each volume) and 2 hours (for the highest tested volume). Vehicle controls for the GJIC bioassays were performed using Milli-Q water and were incubated for the same incubation times. Vehicle controls were dosed with a volume corresponding to the largest volume of reaction mixture tested in the treatment dishes. Following incubation, the GJIC was determined by the SL/DT procedure. All culture dishes were examined within 24 hours of completion of the experiment. Each culture dish of cells was digitally photographed such that the observed scrape spanned the full horizontal width of the picture. A COHU High Performance Color CCD camera (Cohu, Inc., San Diego, CA) with a magnification of 200× under a Nikon TE300 Eclipse Inverted Microscope (Nikon Corp., Japan) with a Nikon HB-10103AF Super High Pressure Mercury 100W lamp (Nikon Corp., Japan) was used.

The fluorescence of the Lucifer yellow dye was used to determine the distance the dye traveled perpendicular to the scrape. The distance of dye travel was indicative of the level of GJIC within the culture. Quantitative analysis of the distance of dye spread was performed using NucleoTech GelExpert software (Nucleotech Corp., Hayward, CA). The distance of dye spread was measured in terms of the area of dye spread by tracing manually via free object quantification the area of farthest visible fluorescence. Since the width of the photographed section was the same for every culture dish, measuring the area of dye spread was equivalent to measuring the distance of dye spread perpendicular to the scrape. The area of dye spread for each chemical treatment dish was compared to a control group of cells that were exposed to Milli-Q water only (vehicle controls) under the same assay as the treated cells. For each chemically treated dish, the fraction of the control was calculated as the area of dye spread in the treated dish divided by the average area of dye spread in the triplicate set of vehicle control dishes. The results for each set of chemically treated triplicates were reported as an average fraction of the control (FOC) ± standard deviation (SD) determined at the 95% confidence interval (CI).

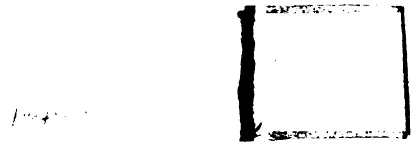
The level of GJIC in cells exposed to the chemical treatment was assessed by the decrease in communication of the cells as compared to the vehicle control groups. A decrease in FOC corresponds directly to a decrease in GJIC (where the doses are not cytotoxic). Interpretations of GJIC results were consistent with Luster-Teasley et al (*7*) and Herner et al. (*5*). Complete communication (100%) between the cells is identified as a FOC value of 1.0 as seen in the vehicle control. A FOC value greater than 0.9 is difficult to statistically distinguish from the vehicle controls. FOC values between 0.9 and 0.5 indicate partial inhibition of GJIC. A FOC value less than or equal to 0.5 is indicative of a significant amount of inhibition of GJIC, since this would be representative of communication levels that are 50% or less than the normal communication levels. FOC values between 0.3 and 0.0 are representative of complete inhibition of GJIC. A FOC value of 0.3 is usually used to represent complete inhibition, as it corresponds to the width of a single row of cells with no dye spreading beyond its boundaries (*5*). Controls, which received no dose of chemical or Milli-Q water, were performed for each experiment as a means of evaluating a normal level of GJIC and the overall "health" of the cells. By performing a *t*-test for each experiment, it was found that the areas of dye spread for the control dishes did not vary significantly from the areas for the vehicle controls at a 95% CI. Therefore, it could be concluded that the Milli-Q water, at the volume tested, was not a significant source of inhibition in the experiments. Statistical analyses were performed by means of the *t*-test and one-way analysis of variance (ANOVA) to compare the chemical treatment results and vehicle control results.

5.3 Results and Discussion

5.3.1 Fenton's Remediation in Milli-Q Water

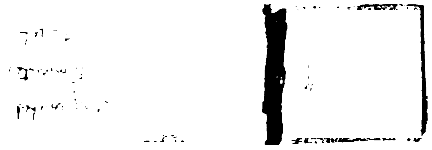
For each molar $Fe^{2+}:H_2O_2$ ratio investigated, during the Fenton's remediation experiments, prior to quenching, no significant change in solution pH (~ 3.0) and temperature (23.0 °C) occurred during the 60 minute reaction period. Although for the $Fe^{2+}:H_2O_2$ ratios 1:20 and 1:40 the unquenched Fenton's reaction mixture remained clear and colorless in appearance during the reaction period, for the $Fe^{2+}:H_2O_2$ ratio 1:5 the solution appeared very light orange and slightly cloudy. Figures 5.1 and 5.2 present the MB dye test results for Fenton's remediation in Milli-Q water with $Fe^{2+}:H_2O_2$ ratios 1:20 and 1:40, respectively. For each of the $Fe^{2+}:H_2O_2$ ratios, MB dye tests indicated the presence of hydroxyl radicals during the reaction period (unquenched) by an immediate bleaching of the MB dye. Similar degrees of bleaching were observed for the reaction

Figure 5.1 Fenton's remediation in Milli-Q water with a Fe^{2+}:H_2O_2 ratio of 1:20. Methylene blue dye test results for (A) control (no sample added); (B) Milli-Q water; unquenched Fenton's reaction mixture at (C) 15 minutes and (D) 30 minutes of reaction; and Fenton's reaction mixture quenched with (E) 30 drops and (F) 35 drops of 10% Na_2SO_3. All methylene blue dye tests were performed using 40 μL samples.



A

B



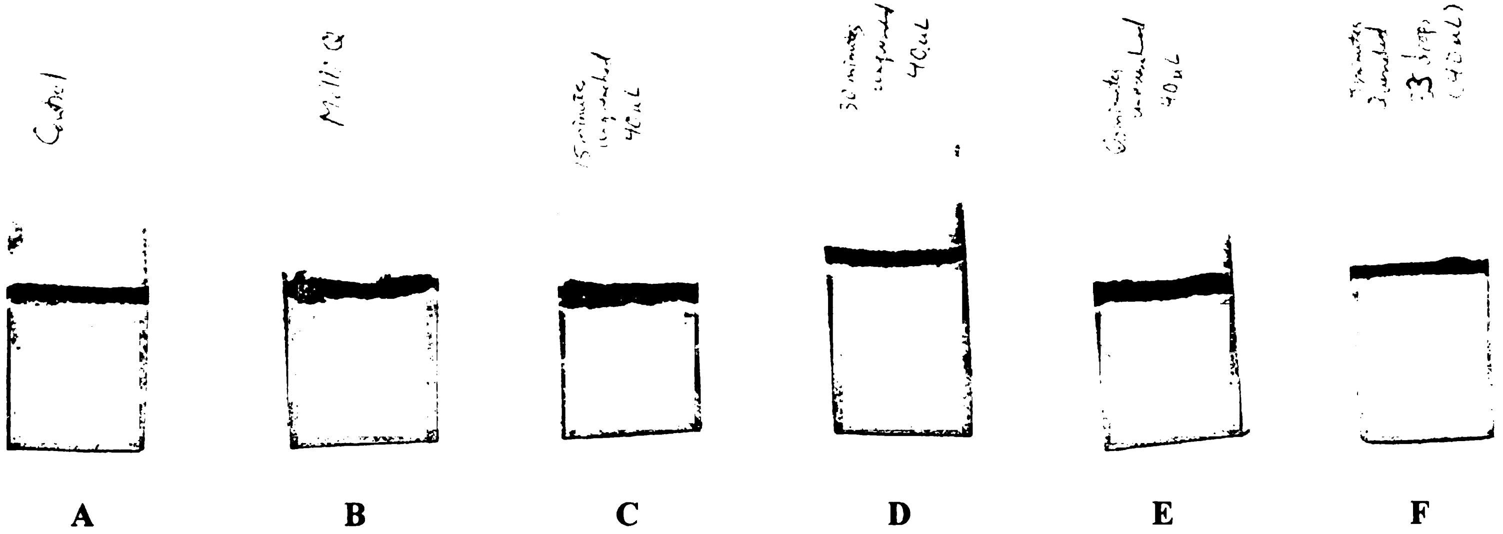
C

D

E

F

Figure 5.2 Fenton's remediation in Milli-Q water with a Fe^{2+}:H_2O_2 ratio of 1:40. Methylene blue dye test results for (A) control (no sample added); (B) Milli-Q water; unquenched Fenton's reaction mixture at (C) 15 minutes, (D) 30 minutes, and (E) 60 minutes of reaction; and Fenton's reaction mixture quenched with (F) 33 drops of 10% Na_2SO_3. All methylene blue dye tests were performed using 40 μL samples.

Control
MilliQ
15 minutes
40uL
30 minutes
40uL
60 minutes
40uL
3 steps
(40uL)
A
B
C
D
E
F

times tested for each of the Fe^{2+}:H_2O_2 ratios. Although the bleaching appeared to be similar for Fe^{2+}:H_2O_2 ratios of 1:5 and 1:20, the bleaching appeared to be significantly greater for the Fe^{2+}:H_2O_2 ratio of 1:20 (Figures 5.1C and D) than for 1:40 (Figures 5.2C, D, and E), therefore suggesting a greater production of hydroxyl radicals with 1:5 and 1:20 than 1:40. One possible explanation for this observation is that the amount of hydroxyl radicals produced will vary with the Fe^{2+}:H_2O_2 ratio, because the chemical species present will vary. In addition, since Fe^{2+} acts as a catalyst in the Fenton's reaction, the rate of hydroxyl radical production will also be affected by the Fe^{2+}:H_2O_2 ratio. For all the experiments, the MB dye test control strip (no sample added) was homogeneously dark blue in color (Figures 5.1A and 5.2A) and no bleaching or discoloration was observed for the MB dye test performed with Milli-Q water (Figures 5.1B and 5.2B).

The Fenton's reaction was quenched after 60 minutes. For each Fe^{2+}:H_2O_2 ratio, the pH of the reaction mixture increased from approximately 3.0 to 7.7. The temperature remained constant at approximately 23.0 °C. The color of the reaction mixture changed from colorless to varying intensities of orange dependent on the Fe^{2+}:H_2O_2 ratio. For the Fe^{2+}:H_2O_2 ratios of 1:5, 1:20, and 1:40, the color changed to dark orange (rust-like), light orange, and very light orange, respectively. This color change to orange can be attributed to the more basic pH of the reaction mixture during the quenching process, resulting in the conversion of iron from a hydrated ferrous form to a colloidal ferric form and the formation of ferric hydroxide (*10*, *11*, *12*). To verify, qualitatively, that quenching was complete, a MB dye test was performed. Additional 10% Na_2SO_3 solution was added to the reaction mixture and the MB dye test was repeated until no discoloration of the MB dye was observed (quenching was complete). For the Fe^{2+}:H_2O_2 ratios of 1:20 and 1:40, the MB dye test results of the quenched Fenton's reaction mixtures are shown in Figures 5.1 E and F, and Figure 5.2F, respectively. Complete quenching of the reaction was achieved for the Fe^{2+}:H_2O_2 ratios of 1:5, 1:20, and 1:40 by the addition of a total of 35 drops (1.55 mL), 35 drops (1.44 mL), and 33 drops (1.27 mL) of 10% Na_2SO_3 solution, respectively.

To aid in the removal of iron from the reaction mixtures by precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to approximately pH 9. Following this pH adjustment, the solution remained clear and colorless with very fine rust colored (light orange) particles in suspension. The amount of precipitate for the Fe^{2+}:H_2O_2 ratio 1:20 was slightly greater than that observed for 1:40, but the amount of precipitate was not great enough for either solution to require centrifugation. However, since the amount of precipitate observed for 1:5 was significantly greater than observed for either 1:20 or 1:40, this solution was centrifuged at 1200 rpm for 10 minutes and the supernatant was retained for filtration. The reaction mixture or supernatant was then filtered and 5 mL of Milli-Q water was used for rinsing during the filtration process. For each solution, the filtrate appeared clear and colorless with no precipitate. To neutralize the reaction mixture pH for the cells in the toxicology bioassays, each filtered solution was adjusted to pH 7. Following adjustment of the filtrate to pH 7, each solution remained clear and colorless with no precipitate. These final reaction mixture solutions were stored at 4 °C.

5.3.2 In Vitro Bioassay for GJIC

Prior to and following warming to room temperature, the final reaction mixture solutions were clear and colorless with no precipitate. Figures 5.3, 5.4, and 5.5 present 30 minute incubation dose-response GJIC bioassay results for a volume range of 0 to 300 μL of final reaction mixture solutions corresponding to the $Fe^{2+}:H_2O_2$ ratios 1:5, 1:20, and 1:40, respectively. For each of the $Fe^{2+}:H_2O_2$ ratios, no inhibition of GJIC was observed after 30 minutes of incubation time. As shown in Table 5.1, for the highest tested volume (300 μL), no inhibition of GJIC was observed at 30 minutes and 2 hours of incubation.

Table 5.1 300 μL GJIC assay results for 30 minutes and 2 hour incubation times

$Fe^{2+}:H_2O_2$ Ratio	30 minutes of incubation (FOC±SD)	2 hours of incubation (FOC±SD)
1:5	1.01±0.02	1.02±0.01
1:20	1.00±0.02	1.00±0.02
1:40	1.01±0.03	1.00±0.01

Since no inhibition of GJIC was observed for the dose-response GJIC bioassay results for incubation times of 30 minutes (0 to 300 μL) and 2 hours (300 μL), it was decided that cytoxicity, time-response, and time of recovery bioassays were not required. In addition, since the final reaction mixture solutions produced from a 1 hour Fenton's reagent reaction (for the selected $Fe^{2+}:H_2O_2$ ratios) did not produce any inhibition of GJIC in the dose-response bioassays, it was decided that the investigation of longer reaction times (5 hours and 24 hours) were unnecessary. Although some researchers

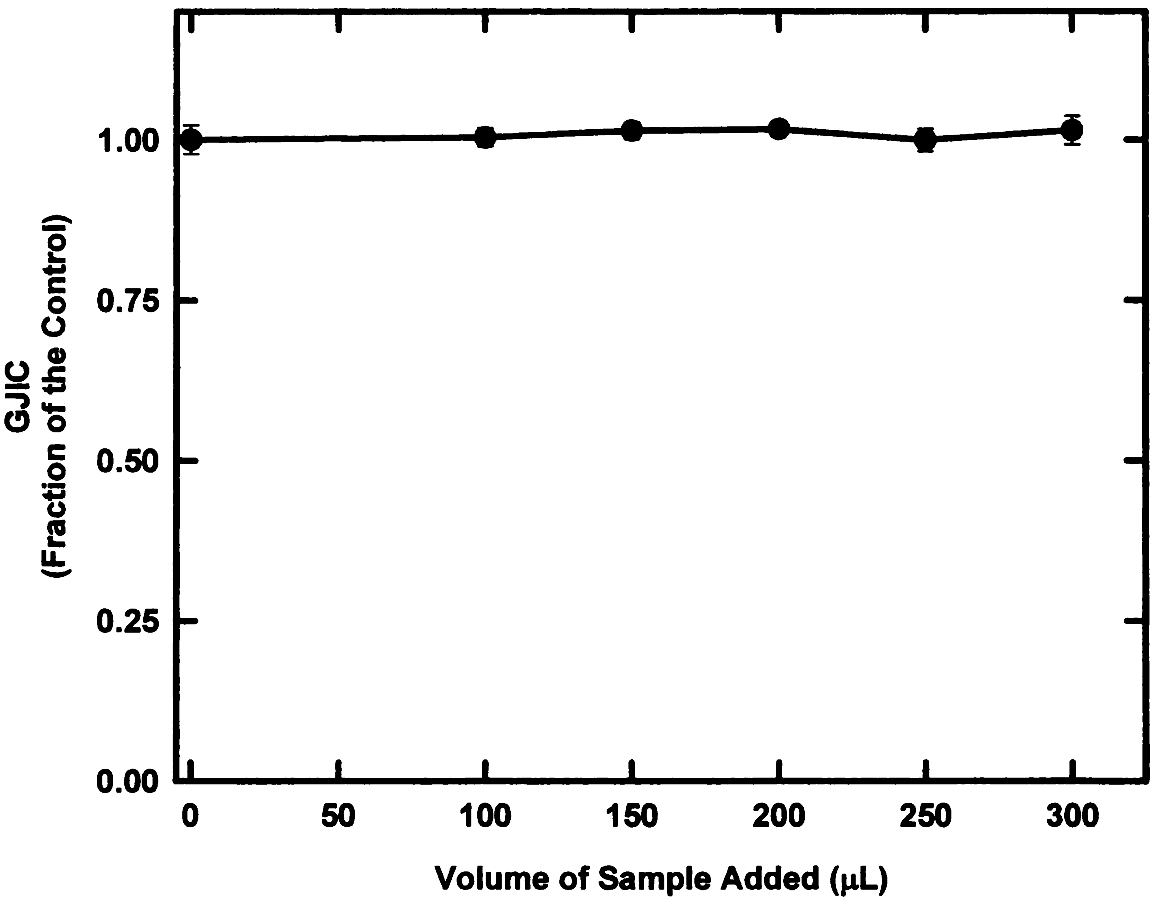


Figure 5.3 Dose-response GJIC bioassay results after a 30 minute incubation time for a volume range of 0 to 300 µL of a solution resulting from Fenton's reagent remediation with only Milli-Q water solvent (no PCB), a Fe^{2+}:H_2O_2 ratio of 1:5, and 1 hour reaction time. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

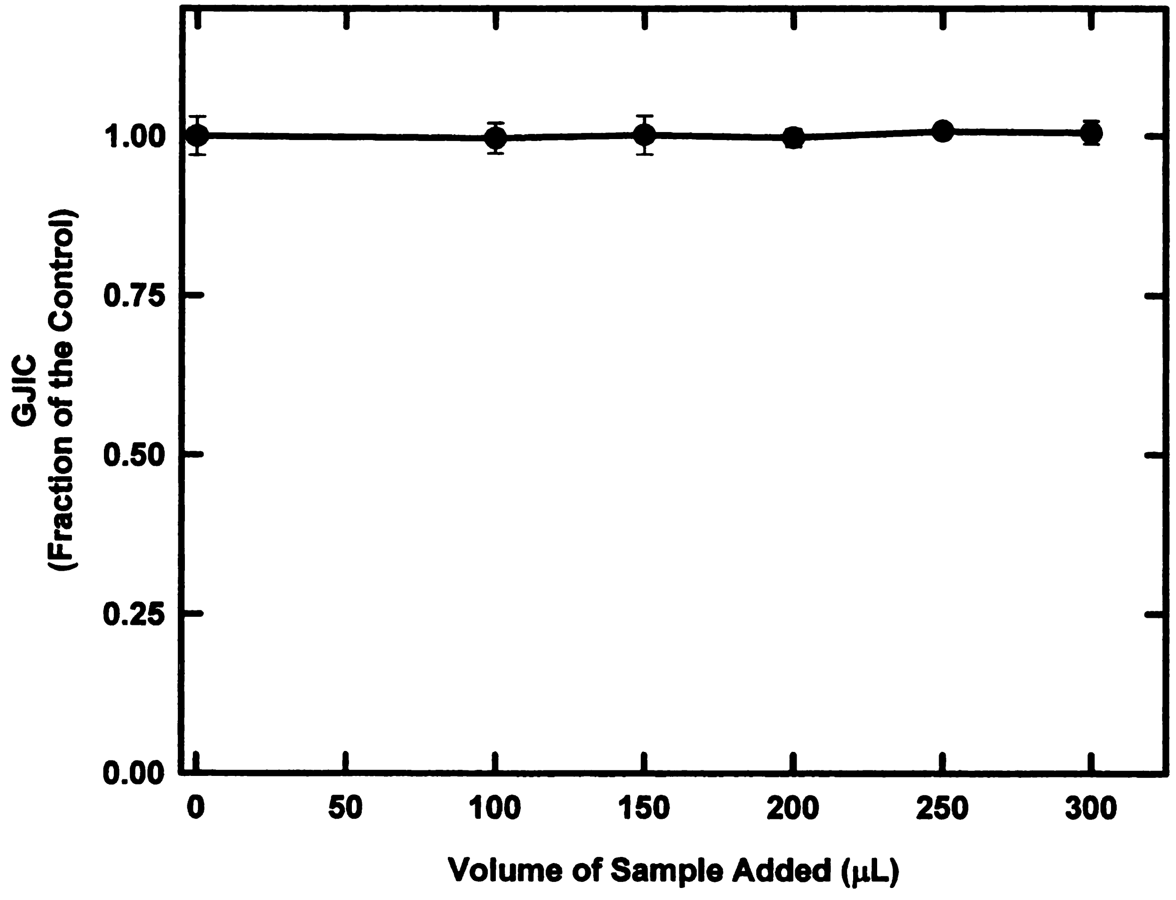


Figure 5.4 Dose-response GJIC bioassay results after a 30 minute incubation time for a volume range of 0 to 300 μL of a solution resulting from Fenton's reagent remediation with only Milli-Q water solvent (no PCB), a Fe^{2+}:H_2O_2 ratio of 1:20, and 1 hour reaction time. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

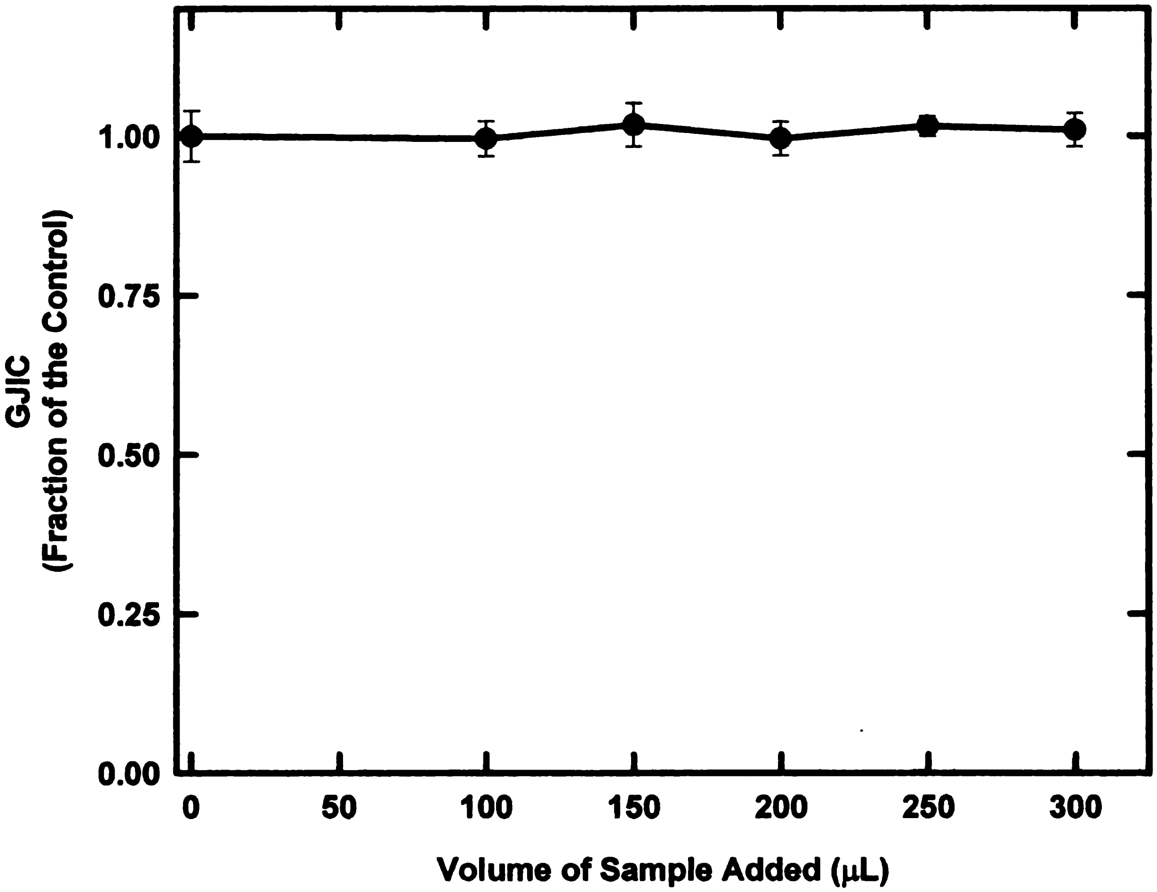


Figure 5.5 Dose-response GJIC bioassay results after a 30 minute incubation time for a volume range of 0 to 300 μL of a solution resulting from Fenton's reagent remediation with only Milli-Q water solvent (no PCB), a Fe^{2+}:H_2O_2 ratio of 1:40, and 1 hour reaction time. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

have used reaction times of up to 1 week (*13*, *14*), a 1 hour (3600 seconds) reaction time is consistent with that used by Trapido et al. (*1*).

5.4 Conclusions

Although 4,4'DCBP is insoluble in water alone, since water (Milli-Q) constituted some part of the solvent used for dissolving 4,4'DCBP in remediation, it was important to investigate whether Fenton's reagent remediation with water alone resulted in any toxicity. For the final reaction mixture solutions corresponding to the Fe^{2+}:H_2O_2 ratios 1:5, 1:20, and 1:40, no inhibition of GJIC was observed for the dose-response GJIC bioassay for incubation times of 30 minutes (0 to 300 μL) and 2 hours (300 μL). Hence, Fenton's reagent remediation with water alone can be expected to not result in any toxicity, and any toxicity resulting from the Fenton's reagent remediation of 4,4'DCBP can be assumed to be independent of the influence of water. The remediation procedure developed in this series of experiments was later applied to the Fenton's remediation of 4,4'DCBP. Based on the results of Fenton's remediation in Milli-Q water, an Fe^{2+}:H_2O_2 ratio of 1:20 was selected for Fenton's remediation of 4,4'DCBP, since it appeared to result in greater production of hydroxyl radicals than 1:40 and resulted in less Fe^{3+} precipitate prior to filtration than 1:5. This selected Fe^{2+}:H_2O_2 ratio is consistent with the optimal ratio for remediation recommended by Trapido et al. (*1*). In addition, it is reported that the formation of hydroxyl radicals by the Fenton's reagent reaction occurs best when the molar ratio of Fe^{2+}:H_2O_2 is kept at a value of 1:15 at a pH of 3 (*15*), which is close to 1:20.

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Chapter 6

Toxicology Studies of Fenton's Remediation of Potential Solvents for 4,4′-Dichlorobiphenyl

6.1 Introduction

A combination of Milli-Q H_2O and acetonitrile (ACN) was selected as the solvent for dissolving 4,4′-dichlorobiphenyl (4,4′DCBP) for use in the Fenton's remediation experiments. Evaluation of whether Fenton's reagent remediation in water alone resulted in any toxicity is discussed in Chapter 5. Acetonitrile was added as part of the solvent to increase the solubility of the 4,4′DCBP. It has been shown that the presence of the acetonitrile does not inhibit the Fenton's reaction, as it is not appreciably reactive with hydroxyl radicals (*1*), and in toxicology studies it does not affect GJIC at low concentrations (*2*). In this chapter, Fenton's remediation of the solvents 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN (by volume) were investigated to determine the toxicological effect of ACN in combination with water as a solvent in remediation. If no toxic response occurs as a result of a reaction of Fenton's reagent with the solvent, any toxicity resulting from the Fenton's reagent remediation of 4,4′DCBP can be assumed to be independent of the solvent.

Based on the results of Fenton's remediation in Milli-Q water, discussed in detail in Chapter 5, an Fe^{2+}:H_2O_2 ratio of 1:20 was selected for each Fenton's remediation experiment. Since Chapter 4 evaluated the use of the Methylene Blue (MB) Dye Test for Fenton's reactions in Milli-Q water only, further experiments were performed to study the MB dye test in the presence of ACN and Milli-Q H_2O/ACN. The toxicity of the resulting final Fenton's remediation solutions was evaluated in rat liver epithelial cells using a nongenotoxic bioassay that determines the in vitro modulation of gap junctional intercellular communication (GJIC) as a measure of the epigenetic toxicity.

6.2 Experimental Section

6.2.1 Chemicals

For the Fenton's remediation portion of this section of research the following chemicals were used. Methylene blue dye (3,7-bis(dimethylamino)phenothiazin-5-ium chloride) (97% purity) was purchased from Fluka (Buchs, Switzerland). Acetonitrile (99.8% purity) was purchased from EM Science (Gibbstown, NJ). Thirty-percent hydrogen peroxide (H_2O_2) (unstabilized), iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) (99% purity), and sodium sulfite (Na_2SO_3) (anhydrous, 98% purity) were purchased from Sigma–Aldrich (St. Louis, MO). Sulfuric acid (H_2SO_4) (96% purity) and sodium hydroxide (NaOH) pellets (99% purity) were purchased from J.T. Baker (Phillipsburg, NJ). Throughout this chapter and dissertation, Milli-Q water was obtained from a Milli-Q Ultrapure Water Purification System (System Type ZMQS6VFOY) purchased from Millipore Corp. (Bedford, MA).

For the toxicology portion of this section of research the following chemicals were used. For cell culture, D-medium (Formula No. 78-5470 EG), Fetal Bovine Serum (FBS), and Gentamicin were purchased from GIBCO Laboratories (Grand Island, NY). Lucifer yellow CH, dilithium salt, was purchased from Molecular Probes Inc. (Eugene, OR), ICN Biomedicals Inc. (Aurora, OH), and Sigma Chemical Co. (St. Louis, MO). Formaldehyde solution (37%) for the GJIC bioassays was purchased from J.T. Baker (Phillipsburg, NJ).

6.2.2 Methods

6.2.2.1 Preliminary Methylene Blue Dye Tests

Two experiments were performed to evaluate the MB dye test in the presence of ACN and Milli-Q H_2O/ACN. MB dye test strips were prepared by the method of Satoh et al. (*3*) as described in detail in Chapter 4. Liquid sample was placed dropwise onto the center of the MB dyed section of a test strip, allowing for absorption between drops. In the first experiment, 40 μL of 100% ACN, 80/20 Milli-Q H_2O/ACN solvent, and 50/50 Milli-Q H_2O/ACN were tested on separate test strips for their ability to cause discoloration/bleaching in the MB dye test as compared with strips tested with 40 μL of Milli-Q H_2O and no sample (control). In the second experiment, the effect of the pH of the 80/20 Milli-Q H_2O/ACN solvent on the MB dye test was evaluated by testing 40 μL samples of 80/20 Milli-Q H_2O/ACN solvent adjusted to a variety of pH values by the addition of 0.5 M H_2SO_4 and/or 1M NaOH.

6.2.2.2 Fenton's Remediation of 80/20 and 50/50 Milli-Q Water/Acetonitrile

Based on the results of Fenton's remediation in Milli-Q water (Chapter 5), an Fe^{2+}:H_2O_2 molar ratio of 1:20 was selected for the Fenton's remediation of 4,4′-dichlorobiphenyl (4,4′DCBP) and therefore Fenton's remediation of the solvents 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN. This selected Fe^{2+}:H_2O_2 ratio is consistent with the optimal ratio for remediation recommended by Trapido et al. (*4*). Furthermore, it is reported that the formation of hydroxyl radicals by the Fenton's reagent reaction occurs best when the molar ratio of Fe^{2+}:H_2O_2 is kept at a value of 1:15 at a pH of 3 (*5*), which is close to 1:20. The initial concentrations of Fe^{2+} and H_2O_2 in the reaction mixtures were 0.15 mM and 3 mM, respectively, which are within the ranges previously used by Trapido et al. (*4*). The volume of 5.0 mM $FeSO_4$ and 3% H_2O_2 added to the reaction mixture was calculated to reflect the assumption that this addition caused a significant change in the overall reaction mixture volume.

The amount of water added by the addition of 5.0 mM $FeSO_4$, pH adjustment, addition of 3% H_2O_2, quenching, and rinsing of the filtration funnel was monitored throughout the experiment, and an adjusted total H_2O/ACN ratio was calculated at the completion of the experiment. In this calculation, the volumes of 5.0 mM $FeSO_4$, 3% H_2O_2, 10% Na_2SO_3, 0.5 M H_2SO_4, and 1M NaOH added were assumed to be 100% water. The total H_2O/ACN ratio suggested the degree of dilution of the sample that occurred during the experiment. In each experiment, this ratio was maintained as constant as possible to allow for comparison of the results. The pH and temperature were monitored throughout the experiments.

MB dye tests were performed during the Fenton's reaction to verify the formation of hydroxyl radicals and during the quenching process to verify the completion of quenching (absence of hydroxyl radicals). Forty microliters of liquid sample were placed dropwise onto the center of the MB dyed section of a test strip, allowing for absorption between drops. For the remediation of each solvent investigated, MB dye tests were compared against a test strip tested with 80/20 Milli-Q H_2O/ACN or 50/50 Milli-Q H_2O/ACN, respectively. The presence of hydroxyl radicals in a reaction mixture was indicated in the MB dye test by an immediate concentrated white discoloration (bleaching) characteristic to the solvent being used. MB dye tests indicating the absence of hydroxyl radicals in a reaction mixture were identified by a resemblance to the MB dye test results for the respective Milli-Q H_2O/ACN solvent alone and a lack of bleaching.

For each Fenton's remediation experiment, the procedure started with the preparation of either the 80/20 Milli-Q H_2O/ACN or 50/50 Milli-Q H_2O/ACN solvent. The volume of solvent prepared as the initial reaction mixture was calculated as the predicted initial reaction mixture volume for Fenton's remediation of 4,4′DCBP. Since in previous toxicology studies (Chapter 3) the final concentration of the test solution was 2 mM 4,4′DCBP, the initial reaction mixture volume for Fenton's remediation of 4,4′DCBP was predicted (based on calculations with 6 mg of 4,4′DCBP) to be close to 20 mL. Therefore, for Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN solvent, the initial reaction mixture consisted of 20 mL of the respective solvent. A 50 mL centrifuge tube with a vaned tube stir bar was used as the reaction vessel. A 5.0 mM $FeSO_4$ stock solution was prepared from $FeSO_4 \cdot 7H_2O$ and Milli-Q water. The 5.0 mM $FeSO_4$ stock solution was then added to the reaction mixture at the calculated volume to achieve a final concentration of 0.15 mM Fe^{2+}. For optimal Fenton's reaction efficiency, the reaction mixture was adjusted to pH 3 with 0.5 M H_2SO_4 and/or 1M NaOH. The pH was adjusted/monitored using a 720A plus pH/ISE meter with an 8102 BNU Ross Ultra Combination pH electrode (ThermoOrion, Beverly, MA).

The Fenton's reaction was initiated by the addition of 3% H_2O_2, prepared from 30% unstabilized H_2O_2 and Milli-Q water, to the reaction mixture to obtain an initial concentration of 3 mM H_2O_2. Since stabilizing agents (hydroxyl radical scavengers) in commercial H_2O_2 might affect the results (*6*), only H_2O_2 devoid of stabilizing agents was used. In order to prevent localized reactions (that might occur when a small volume of very concentrated solution is added to a reaction mixture), 3% H_2O_2 rather than 30% H_2O_2 was used to initiate the reaction. To test, qualitatively, the production of hydroxyl radicals during the reaction, MB dye tests were performed on the unquenched reaction mixture at 15, 30, and 60 minutes of reaction. In addition, MB dye tests were performed on 40 μL samples of Milli-Q water, and 80/20 Milli-Q H_2O/ACN or 50/50 Milli-Q H_2O/ACN solvent.

After 60 minutes, the Fenton's reaction was quenched with a 10% aqueous solution of Na_2SO_3. Trapido et al. (*4, 7*) recommended quenching by the addition of 2 to 3 drops of 10% Na_2SO_3 solution for every 10 mL of reaction mixture. A 0.5 mL glass pipette was used to administer the drops to the reaction mixture. To verify, qualitatively, that quenching was complete, 5 minutes after the addition of the 10% Na_2SO_3 solution, a MB dye test was performed. An additional aliquot of 10% Na_2SO_3 solution was added to the reaction mixture, and the MB dye test was repeated until an absence of hydroxyl radicals in the reaction mixture was indicated (quenching was complete). To aid in the removal of iron from the reaction mixture by precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to pH 9 with 0.5 M H_2SO_4 and/or 1 M NaOH. If a large amount of precipitation in the reaction mixture was observed, the solution was centrifuged at 1200 rpm for 10 minutes and the supernatant was retained for filtration. The reaction mixture or supernatant was then filtered through a 1.0 μm glass fiber filter, using a vacuum filtration unit, into a large culture tube inserted into a 1000 mL Erlenmeyer side-arm filtration flask. Milli-Q water was used for rinsing during filtration only if the reaction mixture touched the sides of the funnel or there was residue in the centrifuge tube. The filtered reaction mixture was transferred to a clean 50 mL centrifuge tube containing a vaned tube stir bar. To neutralize the reaction mixture pH for the cells in the toxicology bioassays, the filtered solution was adjusted to pH 7 with 0.5 M H_2SO_4 and/or 1M NaOH. This final solution was transferred to an amber, 60 mL Boston round bottle with a TFE closure and was refrigerated at 4 °C until the toxicology bioassays were performed.

6.2.2.3 Cell Culture Techniques

The cell culture techniques performed were identical to those described in Chapter 5.2.2.2 for toxicology studies of Fenton's remediation in Milli-Q water.

6.2.2.4 In Vitro Bioassay for GJIC

For reasons to be discussed later in this chapter, based on results which indicated that further toxicology studies were unnecessary, only dose-response GJIC bioassays were performed. Dose-response GJIC bioassays were performed in a manner similar to that used for the resulting solution from Fenton's remediation in Milli-Q water (Chapter 5.2.2.3). The GJIC bioassays were performed on confluent WB-F344 cell cultures (usually 2 days of growth) grown in 35 mm diameter culture dishes (as described in Chapter 5.2.2.2) with 2 mL of D-medium supplemented with 5% FBS. Chemical treatments (reaction mixture solution), controls (no dose), and vehicle controls were performed in triplicates. All GJIC bioassay results assumed that the filtration process performed during the Fenton's remediation experiment removed no toxic chemicals generated during the reaction. Since the exact molecular weight and concentration of the reaction mixture solutions were unknown, GJIC bioassay doses were investigated in terms of volumes rather than concentrations. Since a slight inhibition of GJIC occurs at a final ACN concentration of 2.0% in the cell culture medium (corresponding to 40 μL of ACN), all GJIC bioassays were conducted with a final ACN concentration of 1.5% or less (corresponding to 30 μL of ACN or less) (*2*). In the dose-response bioassay, if the same maximum volume (300 μL) of the resulting solution from Fenton's remediation in Milli-Q water was used for the resulting solutions from Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN, this would represent dose volumes of 60 μL and 150 μL of ACN, respectively. Therefore, the maximum dose volumes that could be tested for the resulting solutions from Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN were 150 μL and 60 μL, respectively. The volumes tested in the dose-response GJIC bioassay for the resulting solution from Fenton's remediation of 80/20 Milli-Q H_2O/ACN were 20, 40, 80, 100, and 150 μL. The volumes tested in the dose-response GJIC bioassay for the resulting solution from Fenton's remediation of 50/50 Milli-Q H_2O/ACN were 10, 20, 30, 40, 50, and 60 μL. For each of the solutions investigated, the bioassays were incubated for 30 minutes for each volume tested and for 2 hours for the highest volume tested.

Vehicle controls for the GJIC bioassays were performed using the respective Milli-Q H_2O/ACN solvent and were incubated for the same incubation times. To be conservative, either the 80/20 or 50/50 Milli-Q H_2O/ACN solvent was used for the vehicle controls instead of a solution based on the adjusted total H_2O/ACN ratio calculated at the completion of the Fenton's remediation. Vehicle controls were dosed with a volume corresponding to the largest volume of reaction mixture tested in the treatment dishes. Controls, which received no dose of chemical or Milli-Q H_2O/ACN solvent, were performed for each experiment as a means of evaluating a normal level of GJIC and the overall "health" of the cells. By performing a *t*-test for each experiment, it was found that the areas of dye spread for the control dishes did not vary significantly from the areas for the vehicle controls at a 95% CI. Therefore, it could be concluded that the 80/20 and 50/50 Milli-Q H_2O/ACN solvents, at the volumes tested, were not a significant source of inhibition in the experiments. Statistical analyses were performed by means of the *t*-test and one-way analysis of variance (ANOVA) to compare the chemical treatment results and vehicle control results.

6.3 Results and Discussion

6.3.1 Preliminary Methylene Blue Dye Tests

Figure 6.1 compares the MB dye test results from the investigations of 100% ACN, 80/20 Milli-Q H_2O/ACN solvent, and 50/50 Milli-Q H_2O/ACN solvent with strips tested with Milli-Q H_2O and no sample (control). As shown in Figure 6.1A, the MB dye test control strip (no sample added) was homogeneously dark blue in color. When the MB dye test was performed with Milli-Q water (Figure 6.1B), no bleaching or discoloration was observed. Similarly, when the MB dye test was performed with 100% ACN (Figure 6.1C), no bleaching or discoloration was observed. Although neither Milli-Q water nor 100% ACN alone resulted in bleaching or discoloration of the MB, Milli-Q H_2O/ACN solvent resulted in discoloration of the MB unique to the 80/20 and 50/50 volume ratios examined. When the MB dye test was performed with 80/20 Milli-Q H_2O/ACN solvent (Figure 6.1D), very slight diffuse discoloration of the MB to light blue appeared with no dark blue outline. As shown in Figure 6.1E, a MB dye test of 50/50

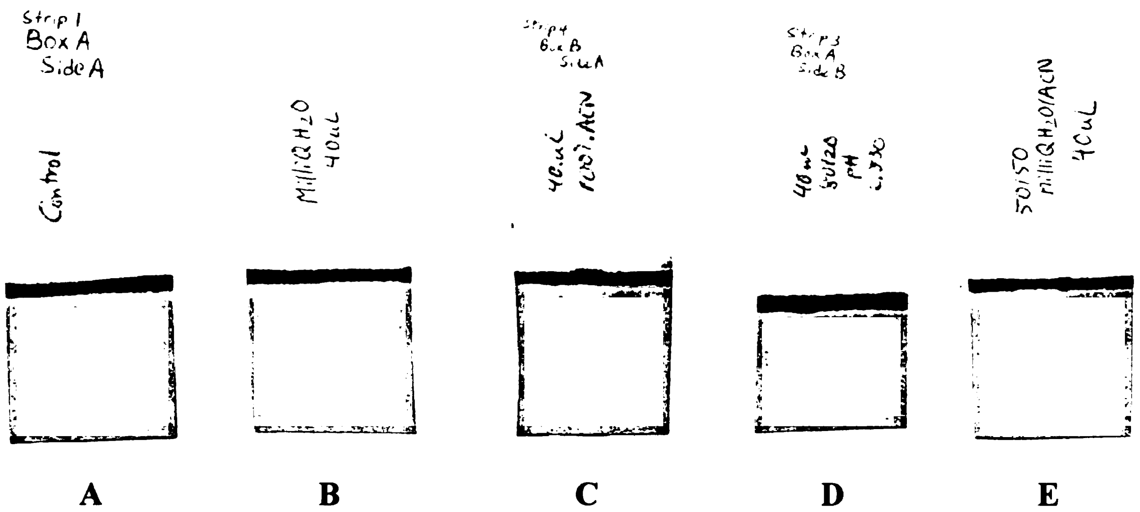


Figure 6.1 Methylene blue dye test results for (A) control (no sample added), (B) 40 μL of Milli-Q H_2O, (C) 40 μL of 100% acetonitrile (ACN), (D) 40 μL of 80/20 Milli-Q H_2O/ACN, and (E) 40 μL of 50/50 Milli-Q H_2O/ACN.

Milli-Q H_2O/ACN solvent produced diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discoloration. The results of the 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN MB dye tests illustrate the importance of conducting MB dye tests of the solvents to be used in performing experiments in which the presence of hydroxyl radicals will be determined. Only by recognizing the appearance of the MB dye test results with the solvents can the presence of hydroxyl radicals be identified in experiments.

Figure 6.2 compares the MB dye test results of 80/20 Milli-Q H_2O/ACN solvent adjusted to pH 3.6, 6.8, 8.3, and 10.0. For each pH tested, almost identical very slight diffuse discoloration of the MB to light blue appeared with no dark blue outline. As was observed for Milli-Q H_2O with varying pH values (Chapter 4.3.5), the MB dye test results for 80/20 Milli-Q H_2O/ACN solvent are not influenced by sample pH.

6.3.2 Fenton's Remediation of 80/20 and 50/50 Milli-Q Water/Acetonitrile

During the Fenton's remediation of 80/20 Milli-Q H_2O/ACN solvent, prior to quenching, no significant change in solution pH (~ 3.0) and temperature (22.5 °C) occurred during the 60 minute reaction period. The unquenched Fenton's reaction mixture remained clear and colorless in appearance throughout the reaction period. Figure 6.3 presents the MB dye test results for Fenton's remediation of 80/20 Milli-Q H_2O/ACN solvent. The MB dye test control strip (no sample added) was homogeneously dark blue in color (Figure 6.3A) and no bleaching or discoloration was observed for the MB dye test performed with Milli-Q water (Figure 6.3B). When the MB dye test was performed with 80/20 Milli-Q H_2O/ACN solvent (Figure 6.3C), very slight diffuse

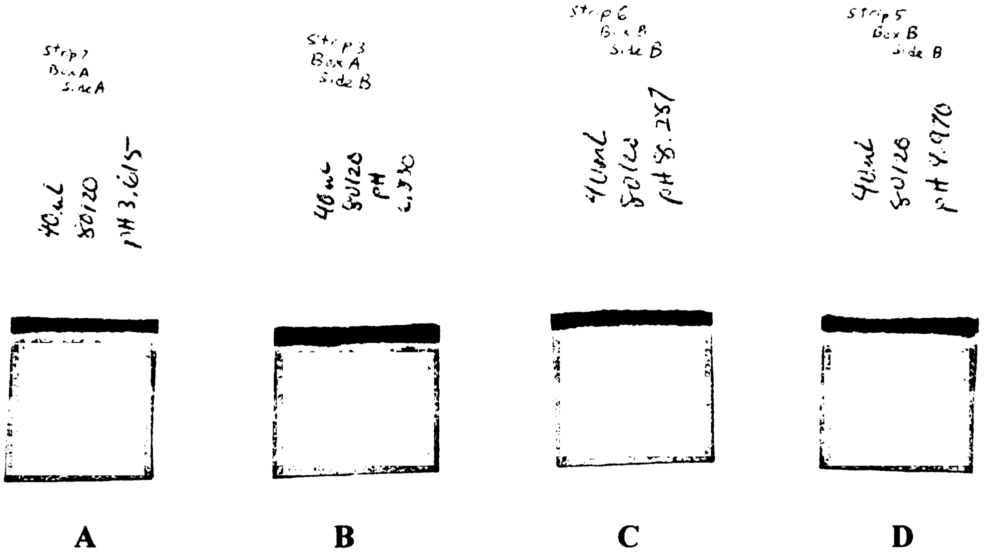


Figure 6.2 Methylene blue dye test results evaluating the effect of 80/20 Milli-Q H_2O/ACN solvent at pH values (A) 3.6, (B) 6.8, (C) 8.3, and (D) 10.0. All methylene blue dye tests were performed using 40 μL samples.

Figure 6.3 Fenton's remediation of 80/20 Milli-Q H_2O/ACN solvent with a Fe^{2+}:H_2O_2 molar ratio of 1:20. Methylene blue dye test results for (A) control (no sample added); (B) Milli-Q water; (C) 80/20 Milli-Q H_2O/ACN; unquenched Fenton's reaction mixture at (D) 15 minutes, (E) 30 minutes, and (F) 60 minutes; and Fenton's reaction mixture quenched with (G) 6 drops and (H) 11 drops of 10% Na_2SO_3. All methylene blue dye tests were performed using 40 μL samples.


Control

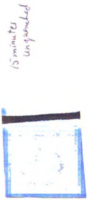
A


Milli-Q H2O

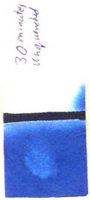
B


48ul
80/20

C


15 minutes
unquenched

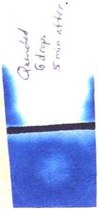
D


30 minutes
unquenched

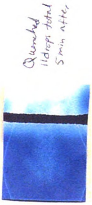
E


60 minutes
unquenched

F


Quenched
6 drops
5 min after

G


Quenched
11 drops total
5 min after

H

discoloration of the MB to light blue appeared with no dark blue outline. As shown in Figures 6.3D, E, and F, MB dye tests indicated the presence of hydroxyl radicals during the reaction period (unquenched) by immediate concentrated light blue/almost white discoloration (bleaching) with a dark blue outline at 15, 30, and 60 minutes, respectively. Similar degrees of bleaching were observed at each of the reaction times. The very slight diffuse discoloration observed for the MB dye test performed with 80/20 Milli-Q H_2O/ACN solvent might have occurred in the background of the unquenched MB dye tests; however, the major discoloration can be attributed to the presence of hydroxyl radicals due to the concentrated nature of the bleaching. Since hydroxyl radicals appeared to be produced throughout the reaction period, the ACN does not appear to have a scavenging effect on the hydroxyl radicals to the extent detectable by the MB dye test. The bleaching observed for Fenton's remediation of 80/20 Milli-Q H_2O/ACN solvent was similar in appearance to that observed for Fenton's remediation in Milli-Q water (Chapter 5).

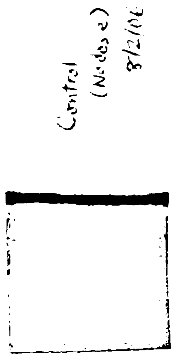
The Fenton's reaction was quenched after 60 minutes. The pH of the reaction mixture increased from approximately 3.0 to 8.4. The temperature remained constant at approximately 22.5 °C. The color of the reaction mixture changed from colorless to light rust orange, attributable to the more basic pH of the reaction mixture during the quenching process, which results in the conversion of iron from a hydrated ferrous form to a colloidal ferric form and the formation of ferric hydroxide (*8*, *9*, *10*). To verify, qualitatively, that quenching was complete, a MB dye test was performed. Additional 10% Na_2SO_3 solution was added to the reaction mixture and the MB dye test was repeated until the results resembled the MB dye test result for 80/20 Milli-Q H_2O/ACN solvent alone, indicating an absence of hydroxyl radicals. The MB dye test results of the quenched Fenton's reaction mixtures are shown in Figures 6.3G and H. The very slight white film, observed over the surface of the test area and most evident as a faint white line at the edges of the test strip, can be attributed to excess 10% Na_2SO_3. Complete quenching of the reaction was achieved by the addition of a total of 11 drops (0.42 mL) of 10% Na_2SO_3 solution. As shown in Figure 6.3H, the MB dye test of the Fenton's reaction mixture, quenched with a total of 11 drops of 10% Na_2SO_3, produced results that closely resemble the MB dye test result indicated for 80/20 Milli-Q H_2O/ACN (Figure 6.3C) and lack bleaching, thereby verifying that quenching was complete.

To aid in the removal of iron from the reaction mixture by precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to approximately pH 9. Following this pH adjustment, the solution was a light rust orange color with very fine light orange particles suspended in solution. Since the reaction mixture was not highly concentrated with precipitate, centrifugation was not performed prior to filtration. The reaction mixture was filtered and 2 mL of Milli-Q water was used for rinsing during the filtration process. The filtrate appeared clear and colorless with no precipitate. Following adjustment of the filtrate to pH 7, the solution remained clear and colorless with no precipitate. The total H_2O/ACN ratio (by volume) at the start of the remediation experiment was 80/20 or 4.00. The adjusted total H_2O/ACN ratio was calculated at the completion of the experiment to be 4.99.

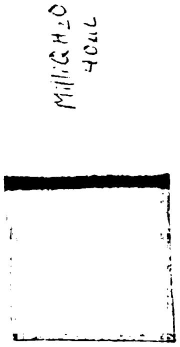
During the Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent, prior to quenching, no significant change in solution pH (~ 3.0) and temperature (23.5 °C) occurred during the 60 minute reaction period. The unquenched Fenton's reaction mixture remained clear and colorless in appearance throughout the reaction period. Figure 6.4 presents the MB dye test results for Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent. The MB dye test control strip (no sample added) was homogeneously dark blue in color (Figure 6.4A) and no bleaching or discoloration was observed for the MB dye test performed with Milli-Q water (Figure 6.4B). The MB dye test performed with 50/50 Milli-Q H_2O/ACN solvent (Figure 6.4C) produced diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discoloration. As shown in Figures 6.4D, E, and F, MB dye test strips tested with unquenched Fenton's reaction mixture at 15, 30, and 60 minutes, respectively, similarly indicated the presence of hydroxyl radicals by an immediate concentrated white discoloration (bleaching) at the point of application surrounded by diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discolored area. Since hydroxyl radicals appeared to be produced throughout the reaction period, the ACN does not appear to have a scavenging effect on the hydroxyl radicals to the extent detectable by the MB dye test.

The diffuse discoloration surrounding the area of bleaching closely resembled the diffuse discoloration observed for the MB dye test of 50/50 Milli-Q H_2O/ACN solvent (Figure 6.4C), and might be explained by the effect of 50/50 Milli-Q H_2O/ACN solvent occurring in the background of the hydroxyl radical bleaching of the MB. Unlike the unquenched Fenton's reaction mixture for 80/20 Milli-Q H_2O/ACN, an area of diffuse discoloration for the unquenched Fenton's reaction mixture for 50/50 Milli-Q H_2O/ACN appeared to spread beyond the area of bleaching. Since the diffuse discoloration of the

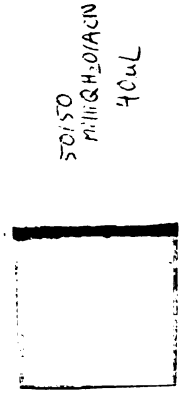
Figure 6.4 Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent with a Fe^{2+}:H_2O_2 molar ratio of 1:20. Methylene blue dye test results for (A) control (no sample added); (B) Milli-Q water; (C) 50/50 Milli-Q H_2O/ACN; unquenched Fenton's reaction mixture at (D) 15 minutes, (E) 30 minutes, and (F) 60 minutes; and Fenton's reaction mixture quenched with (G) 6 drops and (H) 8 drops of 10% Na_2SO_3. All methylene blue dye tests were performed using 40 μL samples.


Control
(No dose)
8/2/06

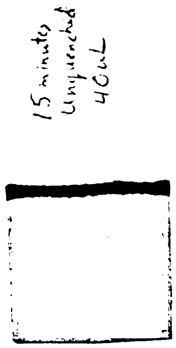
A


MilliQ H2O
40uL

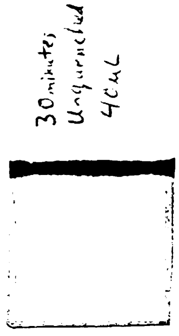
B


50:50
MilliQ H2O/ACN
40uL

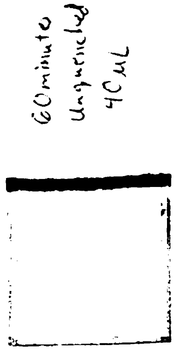
C


15 minutes
Unquenched
40uL

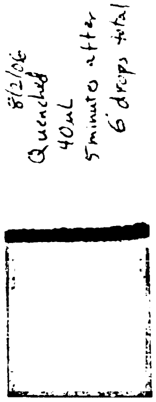
D


30 minutes
Unquenched
40uL

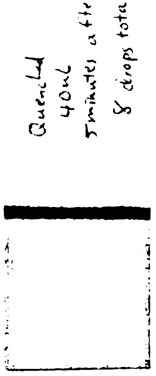
E


60 minute
Unquenched
40uL

F


8/2/06
Quenched
40uL
5 minutes after
6 drops total

G


Quenched
40uL
5 minutes after
8 drops total

H

80/20 Milli-Q H_2O/ACN solvent (Figure 6.3C) was less pronounced than that observed for 50/50 Milli-Q H_2O/ACN solvent (Figure 6.4C), the background effect of the 80/20 Milli-Q H_2O/ACN solvent might have been concealed by the bleaching that occurred for the unquenched Fenton's reaction mixture for 80/20 Milli-Q H_2O/ACN. Furthermore, the dark blue band surrounding the discolored area of the MB dye test performed with the unquenched Fenton's reaction mixture for 50/50 Milli-Q H_2O/ACN closely resembled the dark blue band observed for the MB dye test of 50/50 Milli-Q H_2O/ACN solvent (Figure 6.4C). In addition, the dark blue band was dissimilar from the dark blue outline observed for the unquenched Fenton's reaction mixture for 80/20 Milli-Q H_2O/ACN, which closely followed the contour of the area of bleaching. The results of the unquenched Fenton's reaction mixtures for 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN MB dye tests further emphasize the aforementioned importance of conducting MB dye tests of the solvents to be used in performing experiments in which the presence of hydroxyl radicals will be determined.

The Fenton's reaction was quenched after 60 minutes. The pH of the reaction mixture increased from approximately 3.0 to 8.8. No significant change occurred in the temperature of the reaction mixture. Following quenching, very few fine rust colored particles appeared in the reaction mixture, attributable to the more basic pH of the reaction mixture during the quenching process, which results in conversion of iron from a hydrated ferrous form to a colloidal ferric form and the formation of ferric hydroxide (*8*, *9*, *10*). To verify, qualitatively, that quenching was complete, a MB dye test was performed. Additional 10% Na_2SO_3 solution was added to the reaction mixture and the MB dye test was repeated until the results resembled the MB dye test result for the 50/50

Milli-Q H_2O/ACN solvent alone, indicating an absence of hydroxyl radicals. The MB dye test results of the quenched Fenton's reaction mixtures are shown in Figures 6.4G and H. Complete quenching of the reaction was achieved by the addition of a total of 8 drops (0.36 mL) of 10% Na_2SO_3 solution. As shown in Figure 6.4H, the MB dye test of the Fenton's reaction mixture, quenched with a total of 8 drops of 10% Na_2SO_3, produced results that closely resemble the MB dye test result indicated for 50/50 Milli-Q H_2O/ACN (Figure 6.4C) and lack concentrated white discoloration (bleaching), thereby verifying that quenching was complete.

To aid in the removal of iron from the reaction mixture by the precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to approximately pH 9. Following this pH adjustment, the solution was clear with very few fine rust colored particles in suspension. Since the reaction mixture was not highly concentrated with precipitate, no centrifugation was performed prior to filtration. The reaction mixture was filtered and 1 mL of Milli-Q water was used for rinsing during the filtration process. The filtrate appeared clear and colorless with no precipitate. Following adjustment of the filtrate to pH 7, the solution remained clear and colorless with no precipitate. The total H_2O/ACN ratio (by volume) at the start of the remediation experiment was 50/50 or 1.00. The adjusted total H_2O/ACN ratio was calculated at the completion of the experiment to be 1.22.

6.3.3 In Vitro Bioassay for GJIC

Prior to and following warming to room temperature, the final reaction mixture solutions from Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q

H_2O/ACN solvents were clear and colorless with no precipitate. Figure 6.5 presents the 30 minute incubation dose-response GJIC bioassay results for a volume range of 0 to 150 µL of final reaction mixture solution from the Fenton's remediation of 80/20 Milli-Q H_2O/ACN solvent. Figure 6.6 presents the 30 minute incubation dose-response GJIC bioassay results for a volume range of 0 to 60 µL of final reaction mixture solution from Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent. For each of the final reaction mixture solutions from Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN solvents, no inhibition of GJIC was observed with 30 minutes of incubation time. For the highest tested volume (150 µL) of the final reaction mixture solution from the Fenton's remediation of 80/20 Milli-Q H_2O/ACN solvent, no inhibition of GJIC was observed at 30 minutes (FOC = 1.02 ± 0.03) and 2 hours (FOC = 1.01 ± 0.02) of incubation. Likewise, for the highest tested volume (60 µL) of final reaction mixture solution from Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent, no inhibition of GJIC was observed at 30 minutes (FOC = 1.01 ± 0.03) and 2 hours (FOC = 1.02 ± 0.02) of incubation.

Since no inhibition of GJIC was observed for the dose-response GJIC bioassay results for each of the final reaction mixture solutions from the Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN solvents for incubation times of 30 minutes and 2 hours, it was decided that cytotoxicity, time-response, and time of recovery bioassays were not required. In addition, since the final reaction mixture solutions produced from a 60 minute Fenton's reagent reaction did not produce any inhibition of GJIC in the dose-response bioassays, it was decided that the investigation of longer reaction times (5 hours and 24 hours) were also unnecessary. Although some

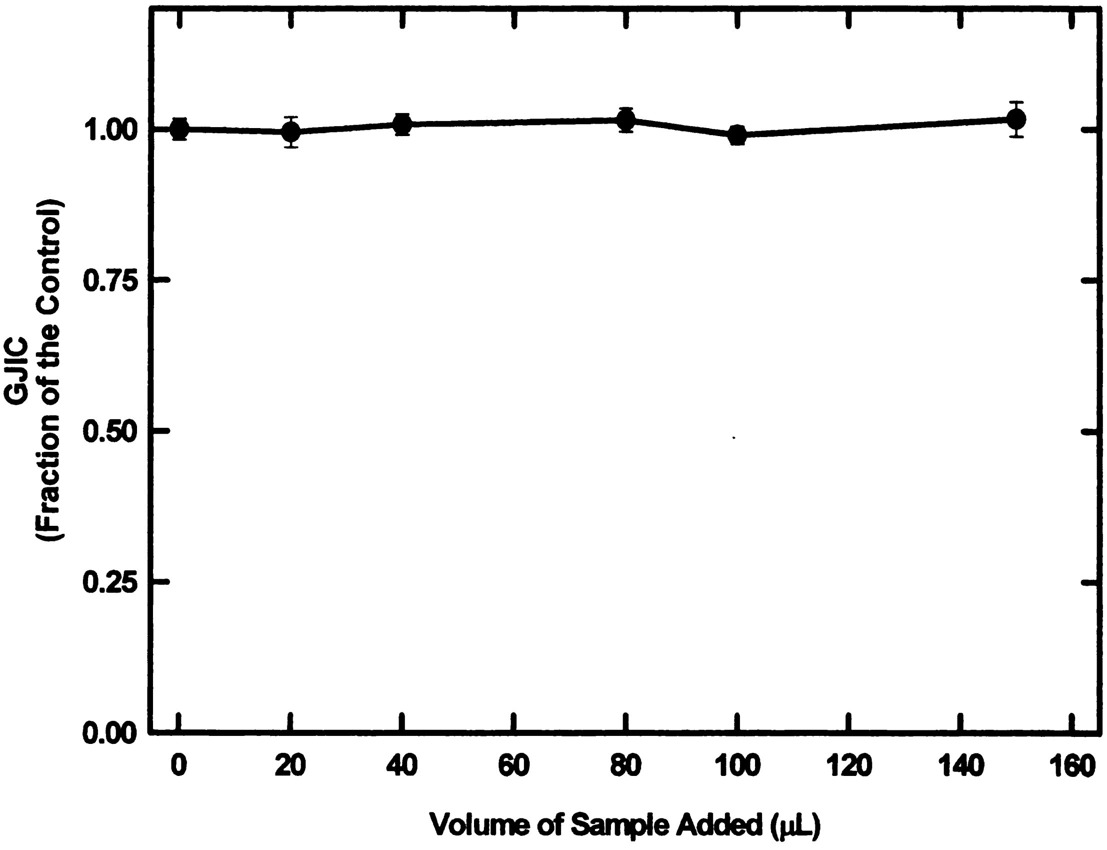


Figure 6.5 Dose-response GJIC bioassay results with a 30 minute incubation time for a volume range of 0 to 150 μL of a solution resulting from Fenton's reagent remediation with only 80/20 Milli-Q water/ACN solvent (no PCB), a Fe^{2+}:H_2O_2 ratio of 1:20, and 60 minutes reaction time. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

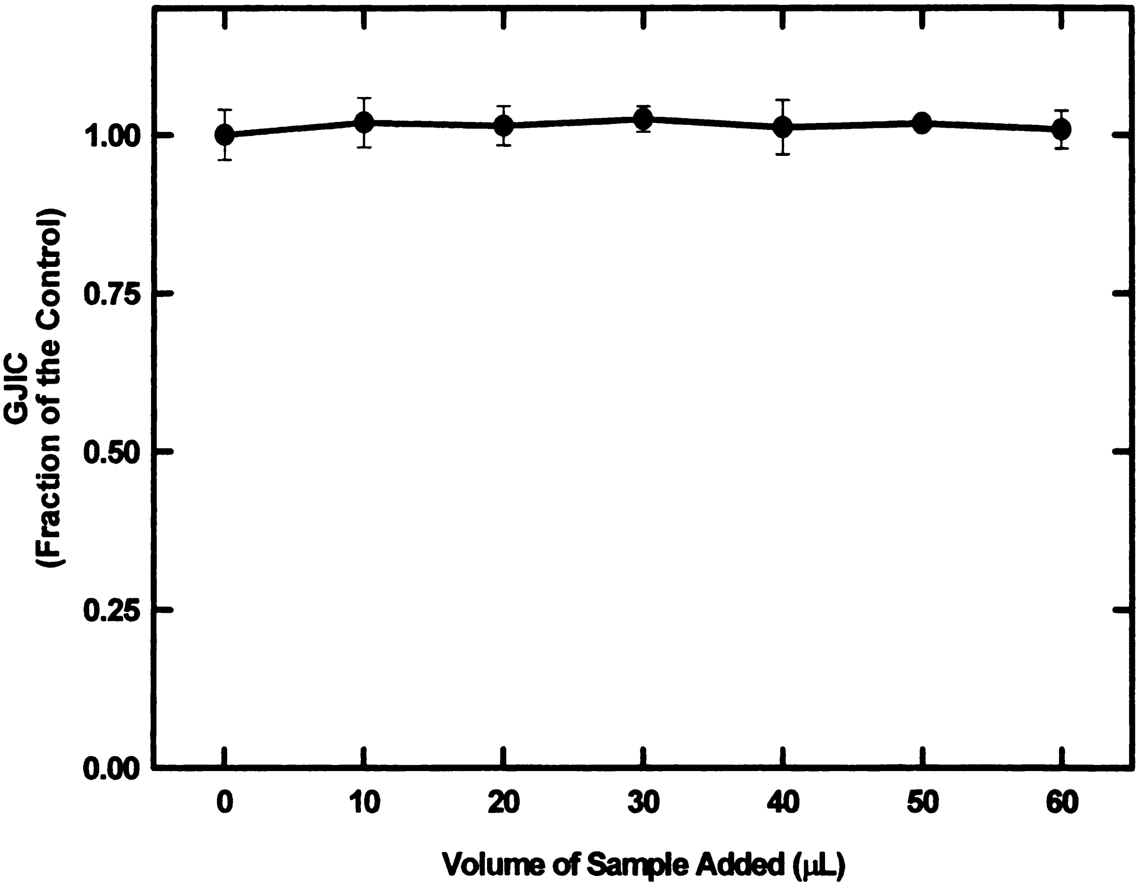


Figure 6.6 Dose-response GJIC bioassay results with a 30 minute incubation time for a volume range of 0 to 60 µL of a solution resulting from Fenton's reagent remediation with only 50/50 Milli-Q water/ACN solvent (no PCB), a Fe^{2+}:H_2O_2 ratio of 1:20, and 60 minutes reaction time. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval.

researchers have used reaction times of up to 1 week (*11*, *12*), a 60 minute reaction time is consistent with that used by Trapido et al. (*4*).

6.4 Conclusions

Fenton's remediation of the solvents 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN (by volume) were investigated to determine the toxicological effect of ACN in combination with water as a solvent. In addition, the use of the MB dye test for Fenton's reaction in Milli-Q H_2O/ACN was examined. Although neither Milli-Q water nor 100% ACN alone resulted in bleaching or discoloration of the MB, Milli-Q H_2O/ACN solvent resulted in the discoloration of the MB unique to the 80/20 and 50/50 volume ratios examined. The results of the 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN MB dye tests illustrated the importance of conducting MB dye tests of the solvents to be used in performing experiments, in which the presence of hydroxyl radicals will be determined. Only by recognizing the appearance of the MB dye test results with the solvents can the presence of hydroxyl radicals be identified in these experiments. As was observed for Milli-Q H_2O with varying pH values (Chapter 4.3.5), the MB dye test results for 80/20 Milli-Q H_2O/ACN solvent were not influenced by the sample pH. The presence of hydroxyl radicals in a reaction mixture was indicated in the MB dye test by an immediate concentrated white discoloration (bleaching) characteristic to the solvent being used. MB dye tests indicating the absence of hydroxyl radicals in a reaction mixture were identified by a resemblance to the MB dye test results for the respective Milli-Q H_2O/ACN solvent alone and a lack of bleaching. In the MB dye tests of unquenched Fenton's reaction mixtures, depending on the solvent, the discoloration effect of the solvent was observed to occur in the background of the hydroxyl radical bleaching of the MB. Since hydroxyl radicals appeared to be produced throughout the reaction period, the ACN does not appear to have a scavenging effect on the hydroxyl radicals to the extent detectable by the MB dye test.

For each of the final reaction mixture solutions from the Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN solvents, no inhibition of GJIC was observed with 30 minutes of incubation time for volume ranges of 0 to 150 μL and 0 to 60 μL, respectively. Furthermore, for the highest tested volumes of the final reaction mixture solutions, no inhibition of GJIC was observed at 2 hours of incubation. Hence, Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN solvents alone cannot be expected to result in any toxicity, and any toxicity resulting from the Fenton's remediation of 4,4′DCBP in either of these solvents can be assumed to be independent of the influence of these solvents. The remediation procedure further developed in this series of experiments was later applied to the Fenton's remediation of 4,4′DCBP.

6.5 References

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Chapter 7

Fenton's Remediation of 4,4′-Dichlorobiphenyl in 50/50 Milli-Q Water/Acetonitrile and Toxicity of Remediation Mixture

7.1 Introduction

Current remediation practices often emphasize the disappearance of the parent compound to at or below regulatory limits, but often disregard the importance of reducing the overall toxicity. Since remediation byproducts can exhibit equal or greater toxicity than the parent compound, it is important to consider in a remediation process not only the removal of the parent compound, but also the toxicological impact of the remediation byproducts (*1*). Although the reaction of Fenton's reagent with various PCBs has been investigated (*2*, *3*), the subsequent toxicity and characterization of remediation byproducts have not been extensively studied. This chapter investigates the toxicological analyses of the solution resulting from Fenton's remediation of 4,4′-dichlorobiphenyl (4,4′DCBP). The disappearance of 4,4′-dichlorobiphenyl, as a result of Fenton's remediation, is presented in Chapter 8.

A combination of Milli-Q H_2O and acetonitrile was selected as the solvent for dissolving 4,4′DCBP in the Fenton's remediation experiments. Acetonitrile (ACN) was added as part of the solvent to increase the solubility of the 4,4′DCBP. It has been shown that the presence of the acetonitrile does not inhibit the Fenton's reaction, as it is not appreciably reactive with hydroxyl radicals (*4*), and, in toxicology studies, it does not affect gap junctional intercellular communication (GJIC) at low concentrations (*5*). In Chapter 6, Fenton's remediation of the solvents 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN (by volume) were investigated to determine the toxicological effect of

ACN in combination with water as a solvent in remediation. Since neither solvent resulted in the inhibition of GJIC for the volume ranges and incubation times tested, any toxicity resulting from the Fenton's remediation of 4,4′DCBP in either of these solvents can be assumed to be independent of the influence of these solvents.

Fenton's remediation of a solution of 4,4′DCBP in 80/20 Milli-Q H_2O/ACN was performed for 60 minutes (with reaction conditions pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM); however, 80/20 Milli-Q H_2O/ACN was determined to be an inappropriate solvent for 4,4′DCBP because of the insolubility of 4,4′DCBP. Throughout the remediation process, small white particles were observed in the reaction mixture and white residue adhered to the surface of the centrifuge tube and thermometer. No decrease in particles or residue occurred as the reaction progressed. Although the final Fenton's remediation solution was clear and colorless, since the white particles were removed by filtration and the white residue remained on the surface of the centrifuge tube and thermometer, toxic chemicals generated during the reaction might have been lost. For Fenton's remediation of 4,4′-dichlorobiphenyl, 50/50 Milli-Q H_2O/ACN was therefore selected as the solvent.

7.2 Experimental Section

7.2.1 Chemicals

For the Fenton's remediation portion of this section of research the following chemicals were used. 4,4′-Dichlorobiphenyl (99% purity) was purchased from Chem Service Inc. (West Chester, PA). Acetonitrile (99.8% purity) was purchased from EM

Science (Gibbstown, NJ). Methylene blue dye (3,7-bis(dimethylamino)-phenothiazin-5-ium chloride) (97% purity) was purchased from Fluka (Buchs, Switzerland). Thirty-percent hydrogen peroxide (H_2O_2) (unstabilized), iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (99% purity), and sodium sulfite (Na_2SO_3) (anhydrous, 98% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Sulfuric acid (H_2SO_4) (96% purity) and sodium hydroxide (NaOH) pellets (99% purity) were purchased from J.T. Baker (Phillipsburg, NJ). Throughout this chapter and dissertation, Milli-Q water was obtained from a Milli-Q Ultrapure Water Purification System (System Type ZMQS6VFOY) purchased from Millipore Corp. (Bedford, MA).

For the toxicology portion of this section of research the following chemicals were used. For cell culture, D-medium (Formula No. 78-5470 EG), Fetal Bovine Serum (FBS), and Gentamicin were purchased from GIBCO Laboratories (Grand Island, NY). Lucifer yellow CH, dilithium salt, was purchased from Sigma Chemical Co. (St. Louis, MO). Formaldehyde solution (37%) for the GJIC bioassays was purchased from J.T. Baker (Phillipsburg, NJ).

7.2.2 Methods

7.2.2.1 Fenton's Remediation of 4,4'-Dichlorobiphenyl in 50/50 Milli-Q H_2O/ACN

Fenton's remediation of a solution of 4,4'DCBP in 50/50 Milli-Q H_2O/ACN (by volume) was performed for 60 minutes. The initiation of remediation was defined as the moment 3% H_2O_2 was added. Based on the results of Fenton's remediation in Milli-Q water (Chapter 5), an Fe^{2+}:H_2O_2 molar ratio of 1:20 was used for the Fenton's reaction. The initial concentrations of Fe^{2+} and H_2O_2 in the reaction mixture were 0.15 mM and 3 mM, respectively, which are within the ranges previously used by Trapido et al. (*6*). The volume of 5.0 mM $FeSO_4$ and 3% H_2O_2 added to the reaction mixture was calculated to reflect the assumption that this addition caused a significant change in the overall reaction mixture volume. The amount of water added by addition of 5.0 mM $FeSO_4$, pH adjustment, addition of 3% H_2O_2, quenching, and rinsing of the filtration funnel was monitored throughout the experiment, and an adjusted total H_2O/ACN ratio was calculated at the completion of the experiment. In this calculation, the volumes of 5.0 mM $FeSO_4$, 3% H_2O_2, 10% Na_2SO_3, 0.5 M H_2SO_4, and 1M NaOH added were assumed to be 100% water. The total H_2O/ACN ratio suggested the degree of dilution of the sample that occurred during the experiment. In each experiment, this ratio was maintained as constant as possible to allow for comparison of the results. The pH and temperature were monitored throughout the experiments.

Methylene blue (MB) dye tests were performed during the Fenton's reaction to verify the formation of hydroxyl radicals and during the quenching process to verify the completion of quenching (absence of hydroxyl radicals). MB dye test strips were prepared by the method of Satoh et al. (*7*) as described in detail in Chapter 4. Forty microliters of liquid sample were placed dropwise onto the center of the MB dyed section of a test strip, allowing for absorption between drops. The MB dye tests were compared against the test strip tested with 50/50 Milli-Q H_2O/ACN solvent, which is characterized by a diffuse discoloration of the MB, spreading out/emanating from the point of application, with a dark blue band surrounding the area of discoloration. MB dye tests indicating the absence of hydroxyl radicals in a reaction mixture with a 50/50 Milli-Q H_2O/ACN solvent were identified by a resemblance to the MB dye test results for 50/50

Milli-Q H_2O/ACN solvent alone. Discoloration of the MB dye, due to the presence of hydroxyl radicals in a reaction mixture with a 50/50 Milli-Q H_2O/ACN solvent, was indicated by an immediate concentrated white discoloration (bleaching) at the point of application surrounded by diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discolored area.

This experiment involved the remediation of 8.2 mg of 4,4′DCBP. A 50 mL centrifuge tube with a vaned tube stir bar was used as the reaction vessel. The 4,4′DCBP was dissolved in 50/50 Milli-Q H_2O/ACN (by volume), in a step-wise procedure, to obtain a final concentration of 2 mM 4,4′DCBP (the final concentration of the test solution used in the 4,4′DCBP toxicology study, Chapter 3). To improve the dissolution of 4,4′DCBP, it was first dissolved in the ACN portion of the 50/50 Milli-Q H_2O/ACN solvent, and then the Milli-Q H_2O portion was added in 1 mL increments. The reaction mixture was sonicated following each addition of the solvent. A 5.0 mM $FeSO_4$ stock solution was prepared from $FeSO_4 \cdot 7H_2O$ and Milli-Q water. The 5.0 mM $FeSO_4$ stock solution was then added to the reaction mixture at the calculated volume to achieve a final concentration of 0.15 mM Fe^{2+}. For optimal Fenton's reaction efficiency, the reaction mixture was adjusted to pH 3 with 0.5 M H_2SO_4 and/or 1 M NaOH. The pH was monitored using a 720A plus pH/ISE meter with an 8102 BNU Ross Ultra Combination pH electrode (ThermoOrion, Beverly, MA).

Following the adjustment of the reaction mixture to pH 3, the Fenton's reaction was initiated by the addition of 3% H_2O_2, prepared from 30% unstabilized H_2O_2 and Milli-Q water, to the reaction mixture to obtain an initial concentration of 3 mM H_2O_2.

Since stabilizing agents (hydroxyl radical scavengers) in commercial H_2O_2 might affect the results (*8*), only H_2O_2 devoid of stabilizing agents was used. In order to prevent localized reactions (that might occur when a small volume of very concentrated solution is added to a reaction mixture), 3% H_2O_2 rather than 30% H_2O_2 was used to initiate the reaction. The remediation reaction was allowed to occur for 60 minutes with occasional sonication of the reaction mixture. To test, qualitatively, the production of hydroxyl radicals during the reaction, MB dye tests were performed on the unquenched reaction mixture at varying times during the remediation. In addition, MB dye tests were performed on 40 μL samples of Milli-Q water and 50/50 Milli-Q H_2O/ACN solvent.

After the 60 minute remediation reaction period, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v), prepared from Na_2SO_3 and Milli-Q water. As recommended by Trapido et al. (*6, 9*), the reaction was quenched by the addition of 2 to 3 drops of 10% Na_2SO_3 solution for every 10 mL of reaction mixture. A 0.5 mL glass pipette was used to administer the drops to the reaction mixture. To verify, qualitatively, that quenching was complete, 5 minutes after the addition of the 10% Na_2SO_3 solution, a MB dye test was performed. An additional aliquot of 10% Na_2SO_3 solution was added to the reaction mixture, and the MB dye test was repeated until an absence of hydroxyl radicals in the reaction mixture was indicated (quenching was complete). To aid in the removal of iron from the reaction mixture by precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to pH 9 with 0.5 M H_2SO_4 and/or 1 M NaOH. If a large amount of precipitation in the reaction mixture was observed, the solution was centrifuged at 1200 rpm for 10 minutes and the supernatant was retained for filtration. The reaction mixture or supernatant was then filtered through a 1.0 μm glass fiber filter, using a vacuum filtration unit, into a large culture tube inserted into a 1000 mL Erlenmeyer side-arm filtration flask. Milli-Q water was used for rinsing during filtration only if the reaction mixture touched the sides of the funnel or there was residue in the centrifuge tube. The filtered reaction mixture was transferred to a clean 50 mL centrifuge tube containing a vaned tube stir bar. The filtered solution was then adjusted to pH 7 with 0.5 M H_2SO_4 and/or 1 M NaOH to neutralize the reaction mixture pH for the cells in the GJIC bioassays. This final Fenton's remediation solution was transferred to an amber, 60 mL Boston round bottle with a TFE closure and was refrigerated at 4 °C until toxicology bioassays were performed.

7.2.2.2 Cell Culture Techniques

WB-F344 rat liver epithelial cells were obtained from Dr. J. W. Grisham and Dr. M. S. Tsao of the University of North Carolina (Chapel Hill, NC) (*1*). This cell line was selected because it is a diploid, nontumorigenic cell line originating from a strain of rat that has been used for toxicological/cancer studies of numerous chemicals, thereby allowing for a source of comparison (*1*). Since 70% of the chemicals that are carcinogens are liver carcinogens and the liver is the "first pass" organ for ingested toxins, liver cells are important for toxicological/cancer studies (*10*). Furthermore, the WB-F344 cell line was designed for in vitro assays to match the many in vivo tumor promotion assays that had been done in rat liver, specifically, in the Fischer 344 rat.

The cell culture techniques performed were similar to those described by Herner et al. (*1*) and Luster-Teasley et al. (*11*). Cells were cultured in 150 cm^2 sterile, treated, polystyrene cell culture flasks (Corning Inc., Corning, NY) in 25 mL of D-medium containing 5% Fetal Bovine Serum (FBS) and 0.2% Gentamicin. The cells were incubated at 37 °C in a water-jacketed IR Autoflow Automatic CO_2 incubator (NUAIRE, Inc., Plymouth, MN) in a humidified atmosphere with 5% CO_2 and 95% air. The time required for cell growth confluency was about two days. The confluent culture was split and transferred every other day into a new 150 cm^2 culture flask with new medium mixture. In addition, from 150 cm^2 flasks of confluent cells, cultures were prepared for the bioassays in 35 mm diameter, sterile, treated polystyrene cell culture dishes (Corning Inc., Corning, NY) with 2 mL of D-medium supplemented with 5% FBS. The cultures for the bioassays were incubated under the same conditions as the aforementioned flasks.

7.2.2.3 In Vitro Bioassay for GJIC

The GJIC bioassays were performed on confluent WB-F344 cell cultures (usually 2 days of growth) grown in 35 mm diameter culture dishes as described in the preceding section. The scrape-loading/dye transfer (SL/DT) procedure for determining the GJIC was adapted from the method described by El-Fouly et al. (*12*) and is described in detail by Herner et al. (*1*). A detailed description of the spread of Lucifer yellow dye from the scrape to neighboring cells can be found in Wilson et al. (*13*). Chemical treatments (final Fenton's remediation solution), controls (no dose), and vehicle controls (50/50 Milli-Q H_2O/ACN solvent) were performed in triplicates. All GJIC bioassay results assumed that the filtration process performed during the Fenton's remediation experiment removed no toxic chemicals generated during the reaction. Since the exact molecular weight and concentration of the reaction mixture solution were unknown, GJIC bioassay doses were investigated in terms of volumes rather than concentrations. Since slight inhibition of

GJIC occurs at a final ACN concentration of 2.0% in the cell culture medium (corresponding to 40 μL of ACN), all GJIC bioassays were conducted with a final ACN concentration of 1.5% or less (corresponding to 30 μL of ACN or less) (*5*). Therefore, the maximum dose volume that could be tested for the resulting solution from Fenton's remediation of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN was 60 μL.

Doses were applied directly to the dishes of confluent cell cultures. Vehicle controls for the GJIC bioassays were performed using 50/50 Milli-Q H_2O/ACN solvent and were incubated for the same incubation times as chemical treatments. To be conservative, the 50/50 Milli-Q H_2O/ACN solvent was used for the vehicle controls instead of a solution based on the adjusted total H_2O/ACN ratio calculated at the completion of the Fenton's remediation. Vehicle controls were dosed with a volume corresponding to the largest volume of final Fenton's remediation solution tested in the treatment dishes. Controls, which received no dose of final Fenton's remediation solution or 50/50 Milli-Q H_2O/ACN solvent, were performed for each experiment as a means of evaluating a normal level of GJIC and the overall "health" of the cells.

For reasons to be discussed later in this chapter, based on results which indicated that further toxicology studies were unnecessary, only dose-response and time-response GJIC bioassays were performed. The volumes tested in the dose-response GJIC bioassay for the final Fenton's remediation solution were 0, 10, 20, 30, 40, 50, and 60 μL. Confluent cells were exposed to the test volumes and allowed to incubate for 30 minutes before assaying for GJIC. The time-response GJIC bioassay was used to determine the effect of chemical exposure time on intercellular communication. For the time-response experiments, confluent cultures of cells were exposed to a fixed volume of final Fenton's remediation solution for varying amounts of time ranging from 0 to 24 hours (1440 minutes) followed by GJIC bioassays. The test volume selected for the time-response experiment was selected as the highest volume that causes a substantial amount of inhibition at 30 minutes of exposure.

All culture dishes were examined within 24 hours of completion of the experiment. Each culture dish of cells was digitally photographed such that the observed scrape spanned the full horizontal width of the picture. A COHU High Performance Color CCD Camera (Cohu, Inc., San Diego, CA) with a magnification of 200× under a Nikon TE300 Eclipse Inverted Microscope (Nikon Corp., Japan) with a Nikon HB-10103AF Super High Pressure Mercury 100W lamp (Nikon Corp., Japan) was used.

The fluorescence of the Lucifer yellow dye was used to determine the distance the dye traveled perpendicular to the scrape. This distance of dye travel was indicative of the level of GJIC within the culture. Quantitative analysis of the distance of dye spread was performed using NucleoTech GelExpert software (NucleoTech Corp., Hayward, CA). The distance of dye spread was measured in terms of the area of dye spread, by tracing manually via free object quantification the area of farthest visible fluorescence. Since the width of the photographed section was the same for every culture dish, measuring the area of the dye spread was equivalent to measuring the distance of dye spread perpendicular to the scrape. The area of dye spread for each chemical treatment (final Fenton's remediation solution) dish was compared to a control group of cells that were exposed to 50/50 Milli-Q H_2O/ACN solvent only (vehicle controls) under the same assay as the treated cells. For each chemically treated dish, the fraction of the control was calculated as the area of dye spread in the treated dish divided by the average area of dye spread in the triplicate set of vehicle control dishes. The results for each set of chemically treated triplicates were reported as an average fraction of the control (FOC) ± standard deviation (SD) determined at the 95% confidence interval (CI).

The level of GJIC in cells exposed to the chemical treatment was assessed by the decrease in communication of the cells as compared to the vehicle control groups. A decrease in FOC corresponds directly to a decrease in GJIC (where the doses are not cytotoxic). Interpretations of GJIC results are consistent with Luster-Teasley et al. (*11*) and Herner et al. (*1*). Complete communication (100%) between the cells is identified as a FOC value of 1.0 as seen in the vehicle control. A FOC value greater than 0.9 is difficult to statistically distinguish from the vehicle controls. FOC values between 0.9 and 0.5 indicate partial inhibition of GJIC. A FOC value less than or equal to 0.5 is indicative of a significant amount of inhibition of GJIC, since this would be representative of communication levels that are 50% or less than the normal communication levels. FOC values between 0.3 and 0.0 are representative of complete inhibition of GJIC. A FOC value of 0.3 is usually used to represent complete inhibition, as it corresponds to the width of a single row of cells with no dye spreading beyond its boundaries (*1*). By performing a t-test for each experiment, it was found that the areas of dye spread for the control dishes did not vary significantly from the areas for the vehicle controls at a 95% CI. Therefore, it could be concluded that the 50/50 Milli-Q H_2O/ACN solvent, at the volume tested, was not a significant source of inhibition in the experiments. Statistical analyses were performed by means of the t-test and One Way Analysis of Variance (ANOVA) to compare the chemical treatment results and vehicle control results.

7.3 Results and Discussion

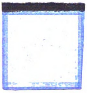
7.3.1 Fenton's Remediation of 4,4′-Dichlorobiphenyl in 50/50 Milli-Q H_2O/ACN

Fenton's remediation of a solution of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN (by volume) was performed for 60 minutes. To improve the dissolution of 4,4′DCBP, it was dissolved in 50/50 Milli-Q H_2O/ACN in a step-wise procedure to obtain the final concentration of 2 mM 4,4′DCBP. When the 4,4′DCBP was first dissolved in the ACN portion of the 50/50 Milli-Q H_2O/ACN solvent, the resulting solution was clear and colorless with no precipitate or oily residue. The solution appearance remained unchanged during the addition of the Milli-Q H_2O portion of the 50/50 Milli-Q H_2O/ACN solvent until 5.19 mL of Milli-Q H_2O had been added. Further addition of Milli-Q H_2O resulted in a light milky white solution. As the remaining Milli-Q H_2O portion was added in 1 mL increments, increasing the length of time the sample was sonicated was necessary in order to ensure complete dissolution. Following the addition of 5.0 mM $FeSO_4$, pH adjustment, and sonication, the reaction mixture was clear and colorless with very fine white particles in suspension. For the Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent (discussed in Chapter 6), the reaction mixture remained clear and colorless with no precipitate or milky appearance. Therefore, the light milky white appearance and very fine white particles observed for Fenton's remediation of a solution of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN can be attributed to the presence of 4,4′DCBP in the solvent and is not a property of the solvent itself.

Following the adjustment of the reaction mixture to pH 3, the Fenton's reaction was initiated by the addition of 3% H_2O_2. The remediation reaction was allowed to occur for 60 minutes with occasional sonication of the reaction mixture. During the Fenton's reaction remediation period, no significant change occurred in the solution pH (~ 3.0) and temperature (23.0 °C). The solution remained clear and colorless with the amount of very fine white particles in suspension decreasing as the reaction progressed, until no precipitate was apparent, after about 5 minutes. No white residue adhered to the surface of the centrifuge tube and thermometer.

In addition to performing MB dye tests on 40 μL samples of Milli-Q water and 50/50 Milli-Q H_2O/ACN, MB dye tests were also performed on 40 μL samples of the unquenched reaction mixture to verify the production of hydroxyl radicals during the reaction. Figure 7.1 presents the MB dye test results for Fenton's remediation of 4,4'DCBP in 50/50 Milli-Q H_2O/ACN. As shown in Figure 7.1A, the MB dye test control strip (no sample added) was homogeneously dark blue in color. When the MB dye test was performed with Milli-Q water (Figure 7.1B), no bleaching or discoloration was observed. As shown in Figure 7.1C, a MB dye test of 50/50 Milli-Q H_2O/ACN solvent produced diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discoloration. As shown in Figures 7.1D, E, and F, MB dye test strips tested with unquenched Fenton's reaction mixture at 15, 30, and 60 minutes, respectively, similarly indicated the presence of hydroxyl radicals by an immediate concentrated white discoloration (bleaching) at the point of application surrounded by diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discolored area. The diffuse discoloration surrounding the area of bleaching closely resembled the diffuse discoloration observed for the MB dye test of 50/50 Milli-Q H_2O/ACN solvent (Figure 7.1C), and might be explained by the effect of 50/50 Milli-Q

Figure 7.1 Fenton's remediation of a solution of 4,4′-dichlorobiphenyl in 50/50 Milli-Q H_2O/ACN. Methylene blue dye test results for (A) control (no sample added); (B) Milli-Q water; (C) 50/50 Milli-Q H_2O/ACN solvent; unquenched Fenton's reaction mixture at (D) 15 minutes, (E) 30 minutes, and (F) 60 minutes; and Fenton's reaction mixture quenched with (G) 6 drops and (H) 8 drops of 10% Na_2SO_3. All methylene blue dye tests were performed using 40 μL samples. The Fenton's reaction conditions were pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM. After a 60 minute remediation reaction period, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v). Following quenching with 10% Na_2SO_3, the Fenton's reaction mixture pH increased to 9.0 and the temperature remained at 23.0 °C.



Control

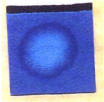
5/16/06

A

40 μL MilliQ H_2O

5/16/06

B



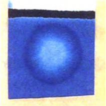
40 μL 50/50 #1

5/16/06

C

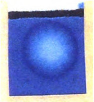
15 minutes
Unquenched
40 μL
5/16/06

D



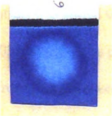
30 minutes
Unquenched
40 μL
5/16/06

E



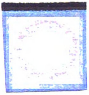
60 minutes
Unquenched
40 μL
5/16/06

F



Quenched
40 μL
6 drops total 10% Na_2SO_3
5 minutes after
5/16/06

G



Quenched
40 μL
8 drops total 10% Na_2SO_3
5 minutes after
5/16/06

H

H_2O/ACN solvent occurring in the background of the hydroxyl radical bleaching of the MB.

After the 60 minute remediation reaction period, the Fenton's reaction was quenched with 6 drops (0.27 mL) of 10% Na_2SO_3 solution. Immediately following the addition of the 10% Na_2SO_3, the pH of the reaction mixture increased from approximately 3.0 to 9.0. No significant change occurred in the temperature of the reaction mixture. The addition of 10% Na_2SO_3 resulted in a change in reaction mixture appearance to clear and colorless with very fine rust colored (light orange) particles in suspension. The appearance of rust colored particles can be attributed to the more basic pH of the reaction mixture during the quenching process, resulting in the conversion of iron from a hydrated ferrous form to a colloidal ferric form and the formation of ferric hydroxide (*14 -16*). As shown in Figure 7.1G, the MB dye test of the Fenton's reaction mixture quenched with 6 drops of 10% Na_2SO_3 solution produced results that closely resembled the MB result indicated for 50/50 Milli-Q H_2O/ACN solvent. The similarity of the MB dye test results for the quenched reaction mixture and 50/50 Milli-Q H_2O/ACN solvent, as well as the absence of an immediate concentrated white discoloration (bleaching) at the point of application, indicated that quenching was complete and no hydroxyl radicals remained.

As a precautionary measure, an additional 2 more drops (0.10 mL) of 10% Na_2SO_3 solution was added to the reaction mixture. Following the addition of the additional 10% Na_2SO_3, the pH and appearance of the reaction mixture remained unchanged from that observed following the initial addition of 10% Na_2SO_3. As shown in Figure 7.1H, the MB dye test of the Fenton's reaction mixture quenched with an additional 2 drops of 10% Na_2SO_3 solution (a total of 8 drops) produced results that closely resembled the MB results indicated for 50/50 Milli-Q H_2O/ACN solvent and the Fenton's reaction mixture quenched with 6 drops of 10% Na_2SO_3 solution, thereby verifying that quenching was complete.

Since the pH of the reaction mixture was already 9.0, no adjustment of the pH was required to aid in the removal of iron from the reaction mixture. Since the reaction mixture was not highly concentrated with precipitate, no centrifugation was performed prior to filtration. No rinsing of the filtration funnel or reaction vessel with Milli-Q water was necessary for the filtration of the reaction mixture. The filter was a uniform light rust color following filtration. The filtrate appeared clear and colorless with no precipitate. Following adjustment of the filtrate (final Fenton's remediation solution) to pH 7, the solution remained clear and colorless with no precipitate.

Throughout the Fenton's remediation experiment, the amount of water added by addition of 5.0 mM $FeSO_4$, pH adjustment, addition of 3% H_2O_2, quenching, and rinsing of the filtration funnel was monitored, and an adjusted total H_2O/ACN ratio by volume was calculated at the completion of the experiment. The total H_2O/ACN ratio suggested the degree of dilution of the sample that occurred during the experiment and was maintained as constant as possible between the remediation experiments to allow for comparison of the results. The original H_2O/ACN ratio at the start of the remediation experiment, based on the solution used to dissolve the 4,4'DCBP, was 50/50 or 1.00. At the completion of the experiment, the calculated adjusted total H_2O/ACN ratio for 60 minutes of remediation was 1.12.

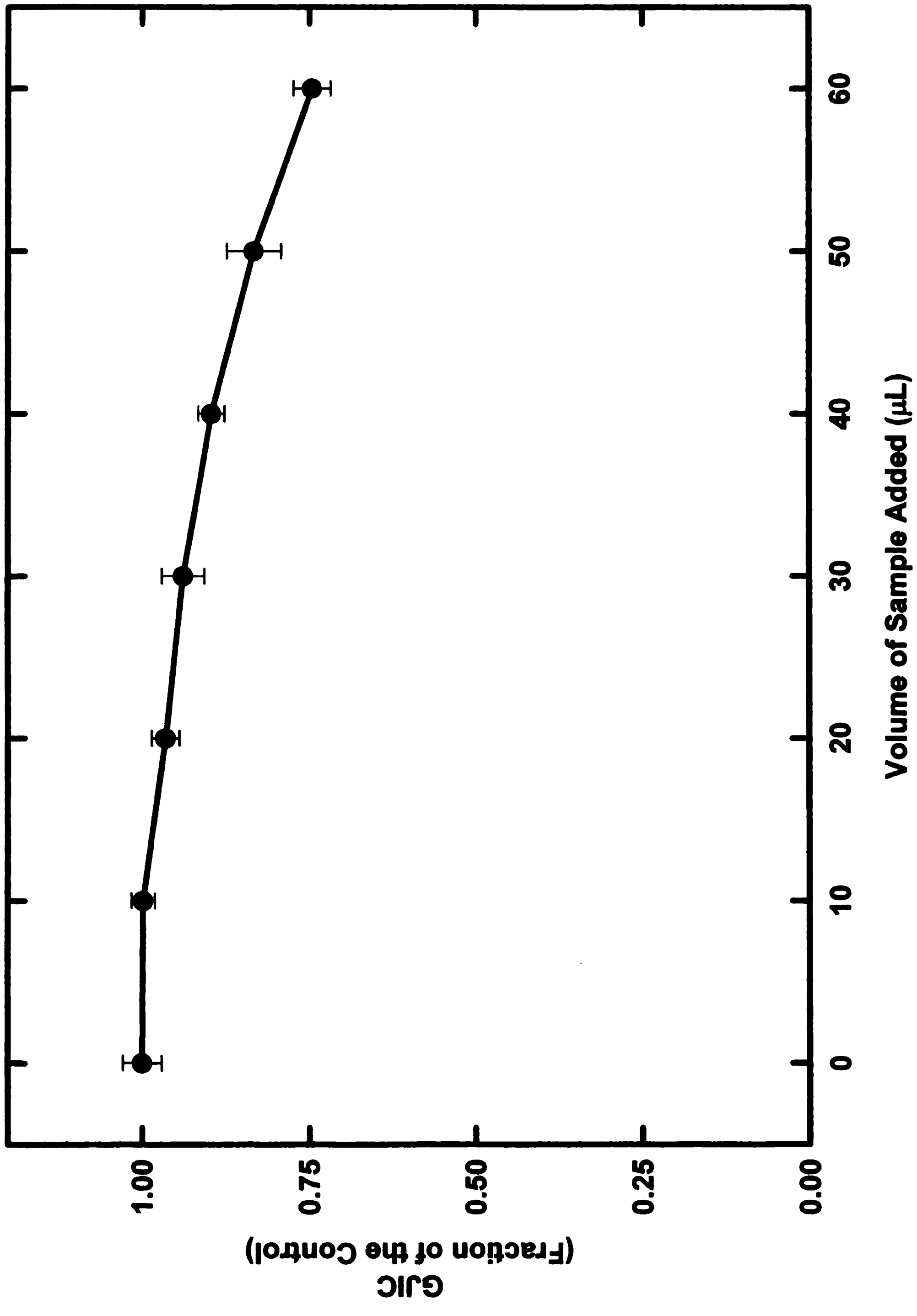
7.3.2 Dose-Response Bioassay

The final Fenton's remediation solution was refrigerated at 4 °C until toxicology bioassays were performed. When the final Fenton's remediation solution was removed from the refrigerator, the solution appeared clear and colorless with very small white crystals. The solution was warmed to room temperature (~ 23.0 °C) and sonicated to achieve complete dissolution of the precipitate prior to use in the bioassay. When test volumes of the final Fenton's remediation solution were added to the dishes of confluent cell cultures, the medium remained clear and colorless with no oily film, precipitate, or cloudiness. Figure 7.2 presents the dose-response GJIC bioassay results for a volume range of 0 to 60 μL of final Fenton's remediation solution and 30 minutes of incubation. A gradual decline in GJIC was observed with an increase in test volume. Partial inhibition of GJIC (FOC between 0.9 and 0.5) occurred for test volumes greater than 40 μL, and a maximum level of inhibition was attained at 60 μL (FOC = 0.75 ± 0.03).

7.3.3 Time-Response Bioassay

The final Fenton's remediation solution was refrigerated at 4 °C until toxicology bioassays were performed. When the final Fenton's remediation solution was removed from the refrigerator, the solution appeared clear and colorless with very small white crystals. The solution was warmed to room temperature (~ 23.0 °C) and sonicated to achieve complete dissolution of the precipitate prior to use in the bioassay. A 60 μL test volume was selected for the time-response experiment since it was the highest volume that caused a substantial amount of inhibition at 30 minutes of chemical exposure. When test volumes of the final Fenton's remediation solution were added to the dishes of

Figure 7.2 Dose-response GJIC bioassay results with a 30 minute incubation time for a volume range of 0 to 60 μL of a solution resulting from Fenton's remediation of 4,4′-dichlorobiphenyl in 50/50 Milli-Q H_2O/ACN solvent. Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval. The Fenton's reaction conditions were pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM. After a 60 minute remediation reaction period, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v). Following quenching with 10% Na_2SO_3, the Fenton's reaction mixture pH increased to 9.0 and the temperature remained at 23.0 °C. The reaction mixture was then filtered through a 1.0 μm glass fiber filter and the filtrate was adjusted to pH 7.


GJIC
(Fraction of the Control)
0.00
0.25
0.50
0.75
1.00
0
10
20
30
40
50
60
Volume of Sample Added (μL)

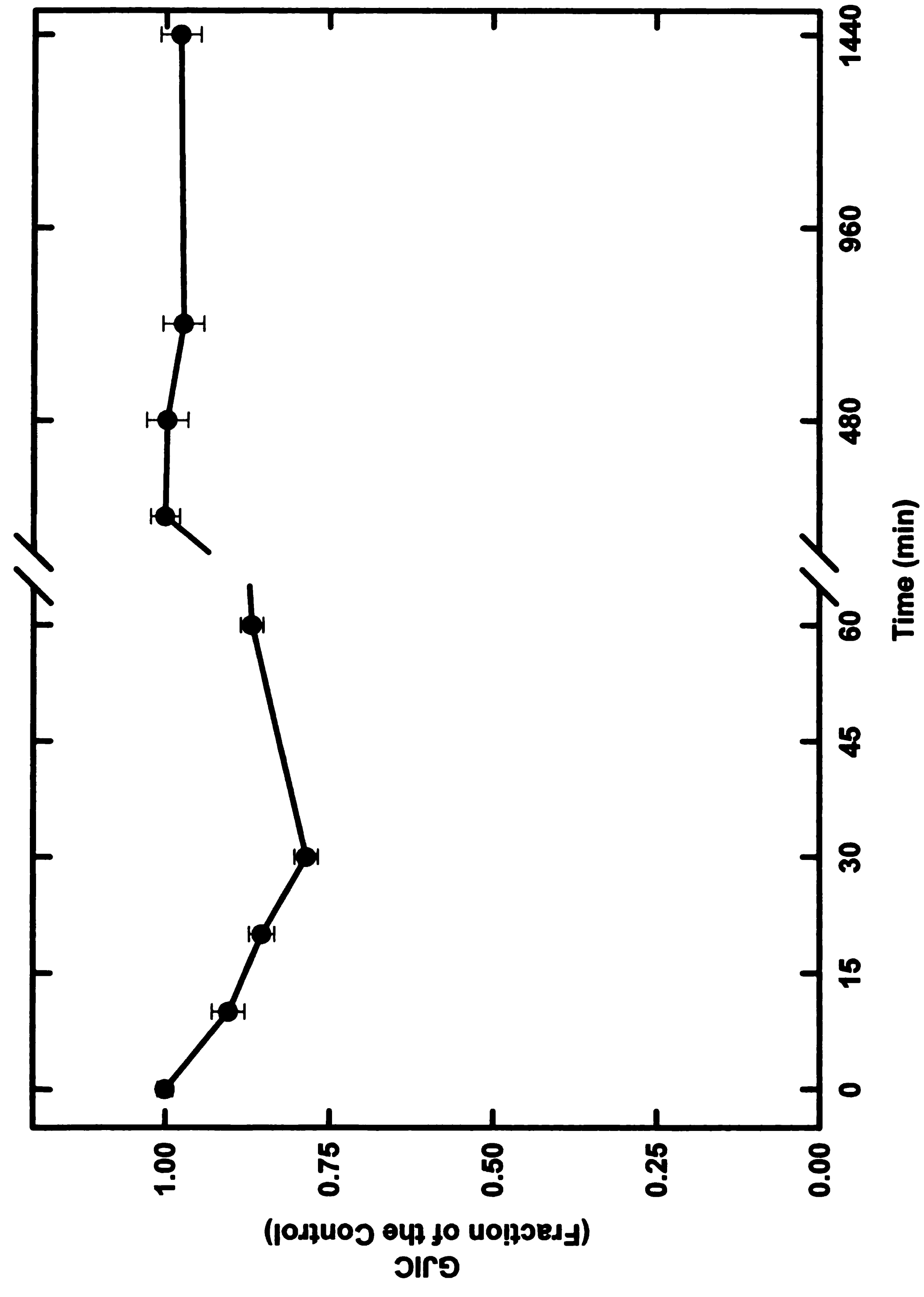
confluent cell cultures, the medium remained clear and colorless with no oily film, precipitate, or cloudiness. The cell cultures were exposed to the final Fenton's remediation solution test volume for varying amounts of time ranging from 0 minutes to 1440 minutes (24 hours) followed by GJIC bioassays.

As shown in Figure 7.3, the time-response results for the final Fenton's remediation solution indicated partial inhibition of GJIC followed by complete recovery with an increase in chemical exposure time. Complete recovery of GJIC was assumed to be attained at a FOC value of 0.97 and greater. A decrease in GJIC was initially observed with partial inhibition achieved at 20 minutes (FOC = 0.85 ± 0.02) and a maximum level of inhibition at 30 minutes (FOC = 0.78 ± 0.02). Between 30 and 240 minutes (a period of 210 minutes) recovery of communication without removal of the chemical was exhibited. Complete recovery of GJIC occurred at 240 minutes (FOC = 1.00 ± 0.02) and was maintained through 1440 minutes (FOC = 0.98 ± 0.03).

One possible explanation for this partial recovery without removal of the chemical might be the activation of an autoregulatory pathway. In this case, the chemical would activate a pathway that would act to inhibit GJIC and then, in an autoregulatory fashion, another pathway would be activated which would reestablish GJIC. This partial recovery might also be attributed to the cell's ability to adapt to the change in conditions (such as cell homeostasis) due to toxicant exposure that resulted in the initial inhibition. Finally, partial recovery without removal of the chemical might be a consequence of the cells metabolizing the chemical into less toxic metabolites.

Since in the time-response experiment, complete recovery of GJIC without removal of the chemical occurred for the highest test volume that resulted in maximum

Figure 7.3 Time-response GJIC bioassay results for a 60 μL test volume of a solution resulting from Fenton's remediation of 4,4′-dichlorobiphenyl in 50/50 Milli-Q H_2O/ACN solvent. Time of chemical exposure varied from 0 minutes to 1440 minutes (24 hours). Each data point is representative of the results for a set of chemically treated triplicates reported as an average FOC ± standard deviation determined at the 95% confidence interval. Data points after the break correspond to 240 minutes, 480 minutes, 720 minutes, and 1440 minutes of chemical exposure time, respectively. The Fenton's reaction conditions were pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM. After a 60 minute remediation reaction period, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v). Following quenching with 10% Na_2SO_3, the Fenton's reaction mixture pH increased to 9.0 and the temperature remained at 23.0 °C. The reaction mixture was then filtered through a 1.0 μm glass fiber filter and the filtrate was adjusted to pH 7.


GJIC
(Fraction of the Control)
0.00
0.25
0.50
0.75
1.00
Time (min)
0
15
30
45
60
480
960
1440

inhibition of GJIC in the 30 minute does-response bioassay, it was decided that cytotoxicity and time of recovery bioassays were not required. Complete recovery of GJIC without removal of the chemical is an indication that the final Fenton's remediation solution did not have a cytotoxic effect. Since complete recovery was achieved without removal of the chemical, time of recovery experiments involving recovery following removal of the chemical were unnecessary.

7.4 Conclusions

Fenton's remediation of a solution of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN (by volume) was performed. Methylene blue (MB) dye tests were performed during the Fenton's reaction (unquenched) to verify the formation of hydroxyl radicals and during the quenching process to verify completion of quenching (absence of hydroxyl radicals). The quenched and unquenched MB dye test results for Fenton's remediation of a solution of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN (Figure 7.1) were identical in appearance to those observed for Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent (Figure 6.4). It can therefore be concluded that the ability of the MB dye test to indicate the presence of hydroxyl radicals does not appear to be influenced by the presence of 4,4′DCBP.

For the 30 minute dose-response GJIC bioassay of the final Fenton's remediation solution, a gradual decline in GJIC was observed with an increase in test volume. Partial inhibition of GJIC occurred for test volumes greater than 40 μL, and a maximum level of inhibition was attained at 60 μL (FOC = 0.75 ± 0.03). For the time-response GJIC bioassay of the final Fenton's remediation solution (60 μL test volume) a maximum level of inhibition was attained at 30 minutes (FOC = 0.78 ± 0.02) of chemical exposure followed by complete recovery of GJIC without removal of the chemical by 240 minutes of chemical exposure. The complete recovery of GJIC without removal of the chemical observed for the final Fenton's remediation solution was similar to the partial recovery of GJIC without removal of the chemical observed for the potential remediation byproducts 2,2′-biphenyldiol (2,2′BP) and 4,4′-dichloro-3-biphenylol (4,4′DC3BP) studied in Chapter 2.3.4. Since Fenton's remediation of 4,4′DCBP would result in the addition of a hydroxyl group to nonhalogenated sites and dechlorination reactions are less likely to occur (*2*), 4,4′DC3BP is a more favorable remediation byproduct than 2,2′BP. As discussed in detail in Chapter 2, among the potential remediation byproducts investigated, 4,4′DC3BP was most inhibitory to GJIC at the lowest dose (FOC = 0.46 ± 0.03 at 40 μM) and exhibited cytotoxicity at doses greater than 40 μM. Since the concentrations of the remaining 4,4′DCBP and remediation byproducts in the final Fenton's remediation solution were unknown, only the toxicity of the solution as a whole is considered here. However, the presence of 4,4′DC3BP as a remediation byproduct might have contributed to the inhibitory effects and complete recovery observed for the final Fenton's remediation solution. The inhibitory effects of 4,4′DC3BP previously observed in Chapter 2 might have been attenuated by its presence at a lower concentration as a component of the final Fenton's remediation solution. Whereas only partial recovery of GJIC without removal of the chemical was achieved for 4,4′DC3BP in the potential remediation byproduct studies of Chapter 2, complete recovery of GJIC without removal of the chemical could have been attained at lower concentrations of 4,4′DC3BP in the final Fenton's remediation solution. The final Fenton's remediation solution was determined to be more toxic (inhibitory to GJIC) than the parent PCB, 4,4′DCBP, which exhibited very slight to no inhibition of GJIC for the dose-response experiments for incubation times of 30 minutes, 2 hours, 6 hours, and 24 hours in Chapter 3.

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Chapter 8

Time Course Studies: Fenton's Remediation of 4,4′-Dichlorobiphenyl in 50/50 Milli-Q Water/Acetonitrile and Disappearance of Parent PCB

8.1 Introduction

One method for evaluating the effectiveness of a remediation process is to measure the disappearance of the parent compound. Current remediation practices often emphasize the disappearance of the parent compound to at or below regulatory limits, but often disregard the importance of reducing the overall toxicity. Since remediation byproducts can exhibit equal or greater toxicity than the parent compound, it is important to consider in a remediation process not only the removal of the parent compound, but also the toxicological impact of the remediation byproducts (*1*). This chapter discusses the disappearance of 4,4′-dichlorobiphenyl as a result of Fenton's remediation. Toxicological analyses of the solution resulting from Fenton's remediation of 4,4′-dichlorobiphenyl are presented in Chapter 7.

In environmental applications, PCB concentrations have often been estimated by comparing retention patterns and gas chromatography/electron capture detection (GC/ECD) response factors from approximated mixtures of Aroclor standards or PCB congeners (*2*, *3*). The electron capture detector (ECD) is highly sensitive to molecules containing electronegative functional groups such as halogens, peroxides, quinones, and nitro groups, but insensitive to functional groups such as amines, alcohols, and hydrocarbons (*4*). The selective nature of the ECD response towards halogen containing compounds has made it one of the most widely used detectors for environmental samples containing chlorinated contaminants, such as chlorinated insecticides and polychlorinated biphenyls (*4*). The more halogenated the molecule, the more sensitive the ECD is to detecting that compound. Therefore, the ECD is more sensitive to detecting highly chlorinated compounds. However, the ECD is capable of detecting compounds with fewer chlorines, if a sufficiently high concentration of the compound is present.

In this section of research, Fenton's remediation of solutions of 4,4′-dichlorobiphenyl (4,4′DCBP) in 50/50 Milli-Q H_2O/ACN was performed for 0, 15, 30, and 60 minutes. Acetonitrile (ACN) was added as part of the solvent to increase the solubility of the 4,4′DCBP. It has been shown that the presence of the acetonitrile does not inhibit the Fenton's reaction, as it is not appreciably reactive with hydroxyl radicals (*5*), and in toxicology studies it does not affect GJIC at low concentrations (*6*). To prepare the remediation mixtures for GC/ECD analysis, an isooctane liquid-liquid extraction method was developed that resulted in greater than ninety percent efficiency of recovery of 4,4′DCBP. The extracted samples were analyzed by GC/ECD to quantitate the disappearance of the parent PCB, 4,4′DCBP, over the period of remediation. In addition, retention times were determined for the chlorinated potential remediation byproducts previously investigated in toxicology studies (Chapter 2) and compared to the retention patterns of the extracted samples.

8.2 Experimental Section

8.2.1 Chemicals

The parent PCB, 4,4′-dichlorobiphenyl (99% purity), and chlorinated potential remediation byproducts, 3-chloro-2-biphenylol (95% purity) and 4,4′-dichloro-3-biphenylol (95% purity), were all purchased from Chem Service Inc. (West Chester, PA).

Acetonitrile (99.8% purity) was purchased from EM Science (Gibbstown, NJ). Isooctane (99.7% purity) was purchased from Burdick & Jackson (Muskegon, MI). Methylene blue dye (3,7-bis(dimethylamino)-phenothiazin-5-ium chloride) (97% purity) was purchased from Fluka (Buchs, Switzerland). Thirty-percent hydrogen peroxide (H_2O_2) (unstabilized), iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (99% purity), and sodium sulfite (Na_2SO_3) (anhydrous, 98% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Sulfuric acid (H_2SO_4) (96% purity) and sodium hydroxide (NaOH) pellets (99% purity) were purchased from J.T. Baker (Phillipsburg, NJ). Throughout this chapter and dissertation, Milli-Q water was obtained from a Milli-Q Ultrapure Water Purification System (System Type ZMQS6VFOY) purchased from Millipore Corp. (Bedford, MA).

8.2.2 Methods

8.2.2.1 Gas Chromatography/Electron Capture Detector (GC/ECD)

GC/ECD was performed using a Perkin-Elmer AutoSystem Gas Chromatograph (GC) equipped with an electron capture detector (ECD) (Norwalk, CT). A DB5 (30 m length x 0.25 mm i.d. x 0.25 μm film thickness) fused silica capillary column (J&W Scientific, Folsom, CA) with phase composition cross linked/surface bonded 5% phenyl, 95% methylpolysiloxane was used for the GC separation. The autosampler was set to inject a sample volume of 1.0 μL. A splitless injector was used with a column head pressure of 15 psi using nitrogen as the carrier gas, producing a flow rate of 1 mL/min. The initial column temperature was held for 1 minute at 50 °C and then ramped at 10 °C/minute to 250 °C for a total run time of 21.00 minutes. Isooctane was used as a blank and was run in the GC/ECD prior to each set of samples.

8.2.2.2 Calibration Curve

For each set of GC experiments, a calibration curve was determined for 4,4'DCBP/isooctane to develop a mathematical relationship between the concentration (ppm) and the area under the curve (μV*sec). Calibration standards, 20, 40, 80, and 160 ppm 4,4'DCBP/isooctane, were prepared and GC/ECD was performed. Using the GC/ECD results, a graph of area vs. prepared concentration was prepared and the calibration curve equation was determined by linear regression. Using the areas provided by the GC/ECD for liquid-liquid isooctane extraction samples of the Fenton's remediation mixtures, this equation was used to determine the concentrations of 4,4'DCBP/isooctane.

8.2.2.3 Percent Efficiency of Recovery by Isooctane Extraction

The percent efficiency of recovery of 4,4'DCBP by isooctane extraction was studied to (1) ensure the isooctane liquid-liquid extraction method resulted in greater than ninety percent efficiency of recovery of 4,4'DCBP and (2) determine the maximum allowable percent of ACN in the dilution water during the extraction procedure without adversely affecting the efficiency. The extraction procedure involved a combination of Milli-Q water and isooctane. Milli-Q water was added to dilute the acetonitrile in the Fenton's remediation mixture. Consistent with EPA Method 8082 (*3*), isooctane was selected as the extraction solvent. In the extraction mixture, the ACN and Milli-Q water formed the bottom layer, while the 4,4'DCBP and isooctane formed the top layer (extracted sample). Although numerous combinations of Milli-Q water and isooctane were evaluated for potential extraction procedures, the efficiency of the extraction procedure finally selected for studying the disappearance of the parent PCB is primarily discussed here.

A 415 ppm (1.86 mM) stock solution of 4,4'DCBP/ACN was prepared as a sample for extraction. Although the extraction procedure was conducted in a 60 mL borosilicate extraction vial, the total volume of the extraction mixture was 63 mL. By utilizing the narrow neck of the vial for the excess volume beyond 60 mL, the isooctane extraction layer could be clearly identified and easily removed. In separate vials, 800, 400, 200, and 100 μL of the 4,4'DCBP/ACN stock solution was added corresponding to the calculated expected extraction concentrations of 166, 83, 41.5, and 20.75 ppm 4,4'DCBP/isooctane, which assumed 100% efficiency of recovery by isooctane extraction. The extraction process was performed in two parts, each part involving the addition of Milli-Q water to dilute the ACN from the sample, the addition of isooctane to extract the 4,4'DCBP from the sample, and shaking/vortexing of the sample. The first part of the extraction process involved the addition of 31 mL of Milli-Q water followed by shaking/vortexing, and then the addition of 1 mL of isooctane followed by shaking/vortexing. The second part of the extraction process involved the addition of 30 mL of Milli-Q water followed by shaking/vortexing, and then the addition of 1 mL of isooctane followed by shaking/vortexing. The extraction mixture was then allowed to rest until a distinct clear top and bottom layer were visible. When removing the extracted sample for the GC/ECD, it was important to ensure that it contained no ACN or water. To improve visualization of the top layer of the extraction mixture, consisting of the extracted 4,4'DCBP and isooctane, this layer was carefully transferred by micropipette to a 4 mL borosilicate culture tube. Approximately 1 mL was transferred from the surface of the solution in the culture tube to a GC sample vial. GC/ECD was performed for each extracted sample using the procedure described previously.

From the areas determined by the GC/ECD, the true extraction concentrations of 4,4'DCBP/isooctane were calculated from the calibration curve. To calculate the percent efficiency of recovery by isooctane extraction, the true extraction concentration of 4,4'DCBP/isooctane was divided by the expected extraction concentration of 4,4'DCBP/isooctane and then multiplied by 100. An efficiency of recovery greater than ninety percent was preferred, since this would mean that greater than ninety percent of the 4,4'DCBP was extracted by the isooctane and no mathematical adjustment of the true extraction concentration would be required. Assuming the volume of 4,4'DCBP/ACN stock solution was primarily ACN and was not significant compared to the volume of dilution water, the percent of ACN in the dilution water was calculated as the volume of stock solution added to the vial divided by the total volume of Milli-Q water added to dilute the ACN (61 mL) and then multiplied by 100.

8.2.2.4 Fenton's Remediation with Varying Reaction Times

Fenton's remediation of solutions of 4,4′-dichlorobiphenyl (4,4'DCBP) in 50/50 Milli-Q H_2O/ACN (by volume) was performed for 0, 15, 30, and 60 minutes. The initiation of remediation was defined as the moment 3% H_2O_2 was added. Since it is impossible to obtain a true zero time of remediation, as this would require instantaneously quenching the reaction following its initiation, "0 minutes" of remediation was interpreted as the remediation procedure just prior to the addition of 3% H_2O_2. For each of the non-zero remediation times, an Fe^{2+}:H_2O_2 molar ratio of 1:20 was used for the Fenton's reaction. The initial concentrations of Fe^{2+} and H_2O_2 in the reaction mixture were 0.15 mM and 3 mM, respectively, which are within the ranges previously used by Trapido et al. (*7*). The volume of 5.0 mM $FeSO_4$ and 3% H_2O_2 added to the reaction mixture was calculated to reflect the assumption that this addition caused a significant change in the overall reaction mixture volume. The amount of water added by addition of 5.0 mM $FeSO_4$, pH adjustment, addition of 3% H_2O_2, quenching, and rinsing of the filtration funnel was monitored throughout the experiment, and an adjusted total H_2O/ACN ratio was calculated at the completion of the experiment. In this calculation, the volumes of 5.0 mM $FeSO_4$, 3% H_2O_2, 10% Na_2SO_3, 0.5 M H_2SO_4, and 1M NaOH added were assumed to be 100% water. The total H_2O/ACN ratio suggested the degree of dilution of the sample that occurred during the experiment. In each experiment, this ratio was maintained as constant as possible to allow for comparison of the results. The pH and temperature were monitored throughout the experiments.

Methylene blue (MB) dye tests were performed during the Fenton's reaction to verify the formation of hydroxyl radicals, during the quenching process to verify completion of quenching (absence of hydroxyl radicals), and during the "0 minutes" of remediation experiment to verify the absence of hydroxyl radicals. Forty microliters of liquid sample was placed dropwise onto the center of the MB dyed section of a test strip, allowing for absorption between drops. The MB dye tests were compared against the test strip tested with 50/50 Milli-Q H_2O/ACN solvent, which is characterized by a diffuse discoloration of the MB, spreading out/emanating from the point of application, with a dark blue band surrounding the area of discoloration. MB dye tests indicating the absence of hydroxyl radicals in a reaction mixture with a 50/50 Milli-Q H_2O/ACN solvent were identified by a resemblance to the MB dye test results for 50/50 Milli-Q H_2O/ACN solvent alone. Discoloration of the MB dye, due to the presence of hydroxyl radicals in a reaction mixture with a 50/50 Milli-Q H_2O/ACN solvent, was indicated by an immediate concentrated white discoloration (bleaching) at the point of application surrounded by diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discolored area.

Each experiment involved the remediation of approximately 8.0 mg 4,4'DCBP. A 50 mL centrifuge tube with a vaned tube stir bar was used as the reaction vessel. The 4,4'DCBP was dissolved in 50/50 Milli-Q H_2O/ACN (by volume), in a step-wise procedure, to obtain a final concentration of 2 mM 4,4'DCBP (the final concentration of the test solution used in the 4,4'DCBP toxicology study). To improve the dissolution of 4,4'DCBP, it was first dissolved in the ACN portion of the 50/50 Milli-Q H_2O/ACN solvent, and then the Milli-Q H_2O portion was added in 1 mL increments. The reaction mixture was sonicated following each addition of the solvent. A 5.0 mM $FeSO_4$ stock solution was prepared from $FeSO_4 \cdot 7H_2O$ and Milli-Q water. The 5.0 mM $FeSO_4$ stock solution was then added to the reaction mixture at the calculated volume to achieve a final concentration of 0.15 mM Fe^{2+}. For optimal Fenton's reaction efficiency, the reaction mixture was adjusted to pH 3 with 0.5 M H_2SO_4 and/or 1 M NaOH. The pH was adjusted/monitored using a 720A plus pH/ISE meter with an 8102 BNU Ross Ultra Combination pH electrode (ThermoOrion, Beverly, MA).

For "0 minutes" of remediation, there was no addition of 3% H_2O_2; however, methylene blue (MB) dye tests were performed on 40 μL samples of the reaction mixture, Milli-Q water, and 50/50 Milli-Q H_2O/ACN solvent. The reaction mixture was then filtered through a 1.0 μm glass fiber filter, using a vacuum filtration unit, into a large culture tube inserted into a 1000 mL Erlenmeyer side-arm filtration flask. Milli-Q water was used for rinsing during filtration only if the reaction mixture touched the sides of the funnel or there was residue in the culture tube. To maintain the conditions and chemical species similar to what would exist prior to remediation initiation, the filtered reaction mixture was not readjusted to pH 7. The filtered reaction mixture was transferred to an amber, 60 mL Boston round bottle with a TFE closure and kept at room temperature for the isooctane extraction procedure.

For each of the non-zero remediation times, following the adjustment of the reaction mixture to pH 3, the Fenton's reaction was initiated by the addition of 3% H_2O_2, prepared from 30% unstabilized H_2O_2 and Milli-Q water, to the reaction mixture to obtain an initial concentration of 3 mM H_2O_2. Since stabilizing agents (hydroxyl radical scavengers) in commercial H_2O_2 might affect the results (*8*), only H_2O_2 devoid of stabilizing agents was used. In order to prevent localized reactions (that might occur when a small volume of very concentrated solution is added to a reaction mixture), 3% H_2O_2 rather than 30% H_2O_2 was used to initiate the reaction. The remediation reaction was allowed to occur for 15, 30, or 60 minutes with occasional sonication of the reaction mixture. To test, qualitatively, the production of hydroxyl radicals during the reaction, MB dye tests were performed on the unquenched reaction mixture at varying times during the remediation. In addition, MB dye tests were performed on 40 μL samples of Milli-Q water and 50/50 Milli-Q H_2O/ACN solvent.

After the specified remediation reaction period, the Fenton's reaction was quenched with a 10% Na_2SO_3 solution (w/v), prepared from Na_2SO_3 and Milli-Q water.

Trapido et al. (*7, 9*) recommended quenching by the addition of 2 to 3 drops of 10% Na_2SO_3 solution for every 10 mL of reaction mixture. A 0.5 mL glass pipette was used to administer the drops to the reaction mixture. To verify, qualitatively, that quenching was complete, 5 minutes after the addition of the 10% Na_2SO_3 solution, a MB dye test was performed. An additional aliquot of 10% Na_2SO_3 solution was added to the reaction mixture, and the MB dye test was repeated until an absence of hydroxyl radicals in the reaction mixture was indicated (quenching was complete). To aid in the removal of iron from the reaction mixture by precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to pH 9 with 0.5 M H_2SO_4 and/or 1 M NaOH. If a large amount of precipitation in the reaction mixture was observed, the solution was centrifuged at 1200 rpm for 10 minutes and the supernatant was retained for filtration. The reaction mixture or supernatant was then filtered through a 1.0 μm glass fiber filter, using a vacuum filtration unit, into a large culture tube inserted into a 1000 mL Erlenmeyer side-arm filtration flask. Milli-Q water was used for rinsing during filtration only if the reaction mixture touched the sides of the funnel or there was residue in the centrifuge tube. The filtered reaction mixture was transferred to a clean 50 mL centrifuge tube containing a vaned tube stir bar. To parallel the Fenton's remediation toxicology studies (in which the reaction mixture pH was neutralized for the cells in the GJIC bioassays), and maintain similar conditions and chemical species, the filtered solution was then adjusted to pH 7 with 0.5 M H_2SO_4 and/or 1 M NaOH. This final solution was transferred to an amber, 60 mL Boston round bottle with a TFE closure and kept at room temperature for the isooctane extraction procedure. The assumption was made that the filtration process performed during the Fenton's remediation experiment did not remove remaining 4,4'DCBP and byproducts generated during the reaction.

8.2.2.5 Isooctane Extraction Procedure

For GC/ECD analysis, two solutions of different concentrations were prepared by isooctane extraction from each of the final Fenton's remediation solutions stored in the Boston round bottles. Since it is unknown prior to GC/ECD analysis what the true concentration of 4,4'DCBP is in each of the final Fenton's remediation solutions, for the purpose of calculations the assumption was made that no remediation had occurred and the concentration of 4,4'DCBP was the same as at the start of the Fenton's reaction (2 mM or 446 ppm). This assumption also prevented the "overloading" of the GC column, which can result from the injection of a sample that is too concentrated. In addition, for the purpose of calculations, the assumption was made that there would be 100% efficiency of recovery by isooctane extraction. Based on these assumptions, the two calculated "concentrations" of 4,4'DCBP in the prepared isooctane extraction solutions were 156.1 and 78.05 ppm (the quotes indicating that these are not the true concentrations, but rather calculated concentrations that would be expected based on the assumptions mentioned above). For each extraction pair, 700 µL and 350 µL of the final Fenton's remediation solution were placed in separate 60 mL borosilicate extraction vials, corresponding to the isooctane extraction solution "concentrations" 156.1 ppm and 78.05 ppm, respectively.

The extraction process was performed in two parts, each part involving the addition of Milli-Q water to dilute the ACN from the sample, the addition of isooctane to extract the 4,4'DCBP from the sample, and shaking/vortexing of the sample. The first part of the extraction process involved the addition of 31 mL of Milli-Q water followed by shaking/vortexing, and then the addition of 1 mL of isooctane followed by shaking/vortexing. The second part of the extraction process involved the addition of 30 mL of Milli-Q water followed by shaking/vortexing, and then the addition of 1 mL of isooctane followed by shaking/vortexing. The extraction mixture was then allowed to rest until a distinct clear top and bottom layer were visible. In the extraction mixture, the ACN and Milli-Q water formed the bottom layer, while the 4,4'DCBP and isooctane formed the top layer (extracted sample). When removing the extracted sample for the GC/ECD, it was important to ensure that it contained no ACN or water. To improve visualization of the top layer of the extraction mixture, consisting of the extracted 4,4'DCBP and isooctane, this layer was carefully transferred by micropipette to a 4 mL borosilicate culture tube. Approximately 1 mL was transferred from the surface of the solution in the culture tube to a GC sample vial. GC/ECD analysis was performed in triplicate for each extracted sample using the procedure described previously.

8.2.2.6 Fenton's Remediation Solution Concentration by Back-Calculation

The concentrations of the final Fenton's remediation solutions were determined through a process of back-calculation from the GC/ECD results. From the areas determined by the GC/ECD, the true extraction solution concentrations of 4,4'DCBP/isooctane (ppm) were calculated from the calibration curve. Since ppm can be defined as μg divided by mL, assuming 100% isooctane extraction efficiency, the mass (μg) of 4,4'DCBP extracted from the sample of final Fenton's remediation solution placed in the extraction vial was determined by multiplying the true extraction solution concentration of 4,4'DCBP/isooctane (ppm) by the 2 mL of isooctane used for extraction. The concentration of the final Fenton's remediation solution was then determined by dividing the extracted mass (μg) of 4,4'DCBP by the volume of final Fenton's remediation solution placed in the extraction vial (mL).

Since these calculations assumed 100% isooctane extraction efficiency, the actual percent isooctane extraction efficiency was determined from the percent of ACN in the dilution water and the results of the "Percent Efficiency of Recovery by Isooctane Extraction" experiment. The solvent used in the Fenton's remediation of 4,4'DCBP was 50/50 Milli-Q H_2O/ACN by volume. Assuming that the volume of ACN was not changed significantly by the remediation process, and the total volume of solvent in the sample of final Fenton's remediation solution was not significant compared to the volume of dilution water in the extraction process, the percent of ACN in the dilution water was calculated as the volume (mL) of ACN added to the extraction vial (50% of the volume of final Fenton's remediation solution placed in the extraction vial) divided by the total volume of Milli-Q water added to dilute the ACN during the extraction process (61 mL) and multiplied by 100. From the "Percent Efficiency of Recovery by Isooctane Extraction" experiment, a mathematical relationship was developed between the percent of ACN in the dilution water and the isooctane extraction efficiency. Using this mathematical relationship, the isooctane extraction efficiency was determined from the percent of ACN in the dilution water in the isooctane extraction of the final Fenton's remediation solutions. If the actual percent isooctane extraction efficiency was greater than ninety percent, no mathematical adjustment of the true extraction solution concentration (and hence the final Fenton's remediation solution concentration) of 4,4'DCBP was required.

Since two solutions of different concentrations were prepared by isooctane extraction from each of the final Fenton's remediation solutions and GC/ECD analysis was performed in triplicate for each extracted sample, a total of six calculated concentrations were determined for each of the final Fenton's remediation solutions. From these six concentrations, the average concentration and standard deviation for each of the final Fenton's remediation solutions were determined. Based on the concentration of 4,4'DCBP at the start of the Fenton's reaction (446 ppm) and the average concentration of the final Fenton's remediation solutions, the average percent of 4,4'DCBP remaining in the final Fenton's remediation solutions were determined.

8.2.2.7 Retention Times for Chlorinated Potential Remediation Byproducts

In chapter 2, the toxicity of six characteristic potential remediation byproducts resulting from the Fenton's remediation of PCBs were examined. The potential remediation byproducts, hydroxylated biphenyls and hydroxylated polychlorinated biphenyls, included 2-biphenylol, 3-biphenylol, 2,2′-biphenyldiol, 3,3′-biphenyldiol, 3-chloro-2-biphenylol, and 4,4′-dichloro-3-biphenylol. In order to attempt to identify the presence of these potential remediation byproducts in the final Fenton's remediation solutions, GC/ECD was performed on stock solutions to determine the retention times for the potential remediation byproducts. Since the ECD response is selective towards halogen containing compounds, only chlorinated remediation byproducts could be detected. Therefore, the retention times were determined for only 3-chloro-2-biphenylol and 4,4′-dichloro-3-biphenylol. Two stock solutions, 220 ppm 3-chloro-2-biphenylol/isooctane and 200 ppm 4,4′-dichloro-3-biphenylol/isooctane, were prepared and approximately 1000 μL of each was transferred to GC sample vials. GC/ECD analysis was performed for each stock solution sample using the procedure described previously. The retention times of 3-chloro-2-biphenylol and 4,4′-dichloro-3-biphenylol were determined from the resultant gas chromatograms.

8.3 Results and Discussion

8.3.1 Retention Times for Chlorinated Potential Remediation Byproducts

Although toxicology studies were performed for six potential remediation byproducts, the retention times were determined for only 3-chloro-2-biphenylol and 4,4′-dichloro-3-biphenylol, since only the chlorinated compounds could be detected by the ECD. As shown in Figure 8.1, the gas chromatogram for the blank (isooctane) revealed no significant peaks, except for small peaks of impurities. The gas chromatograms for the 220 ppm 3-chloro-2-biphenylol/isooctane and 200 ppm 4,4′-dichloro-3-biphenylol/isooctane stock solutions are shown in Figures 8.2 and 8.3, respectively. The retention times for 3-chloro-2-biphenylol and 4,4′-dichloro-3-biphenylol were 14.21 minutes and 17.03 minutes, respectively.

8.3.2 Percent Efficiency of Recovery by Isooctane Extraction

As described previously, four different volumes of 4,4′DCBP/ACN stock solution were extracted through a process involving Milli-Q water and isooctane. Immediately following the extraction process, the extraction mixture was a white emulsion. Following

Figure 8.1 Gas chromatogram for the blank (isooctane).

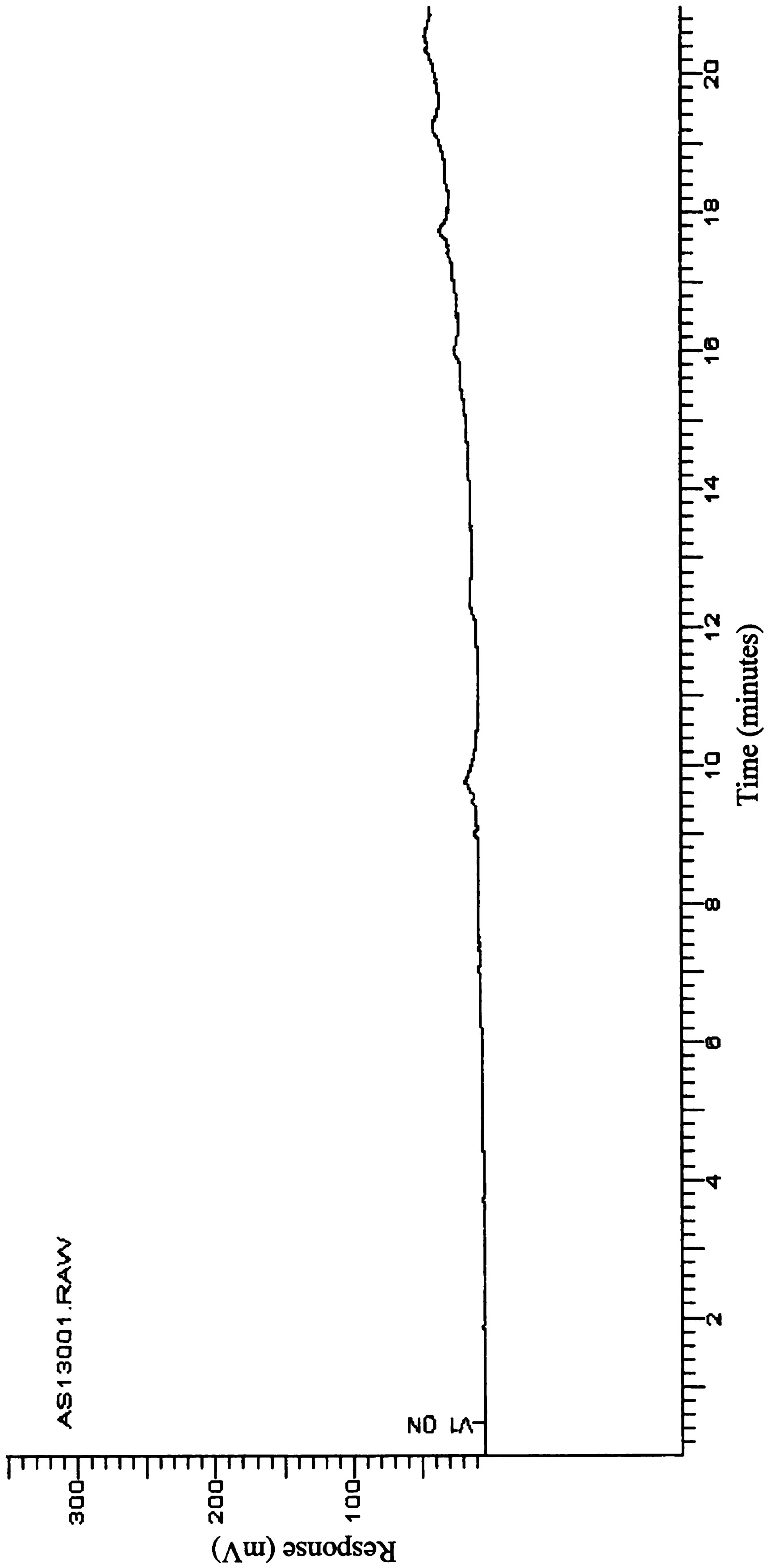

AS13001.RAW
V1 ON
Response (mV)
100
200
300
Time (minutes)
2
4
6
8
10
12
14
16
18
20

Figure 8.2 Gas chromatogram for 220 ppm 3-chloro-2-biphenylol/isooctane.

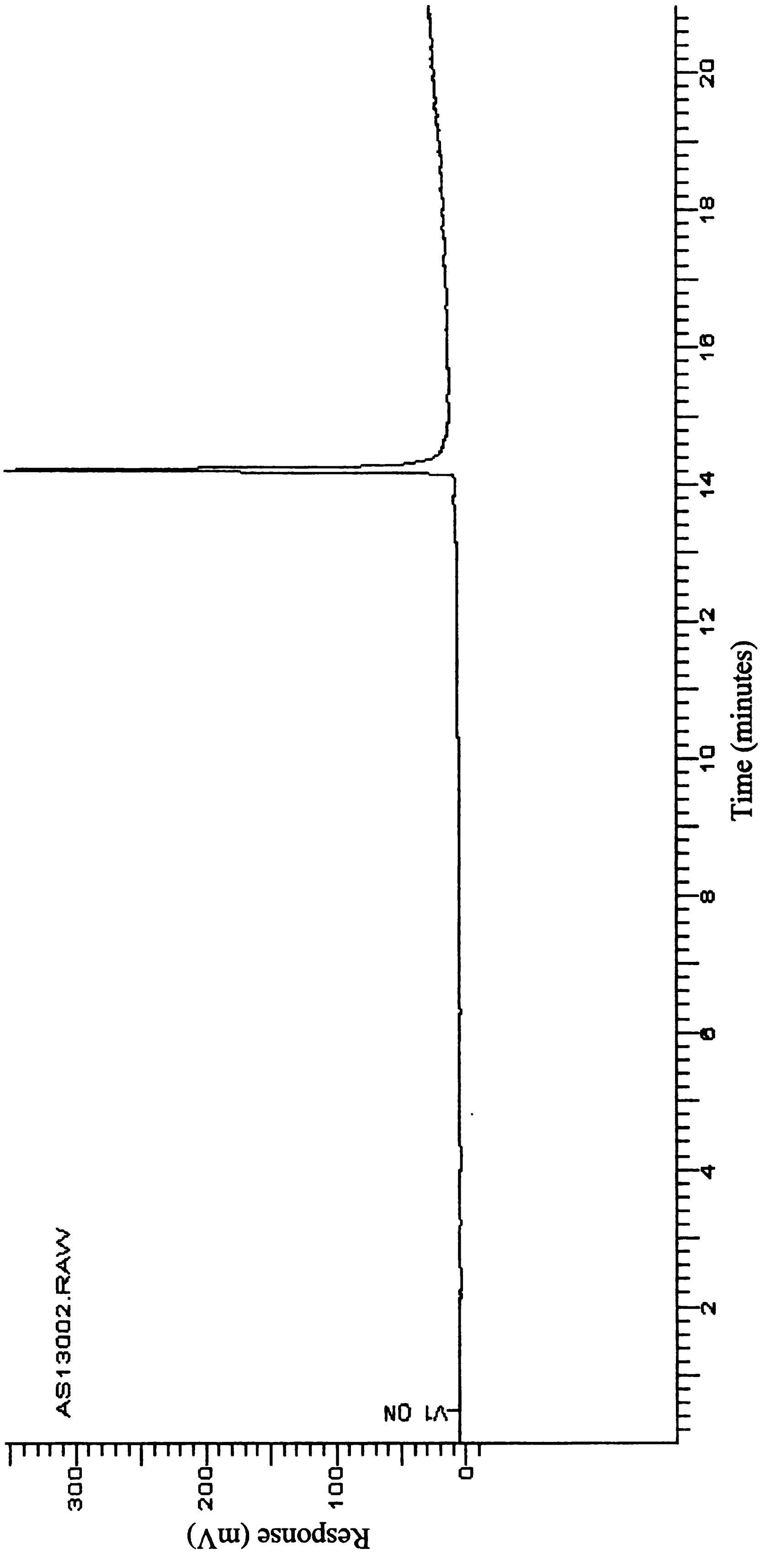
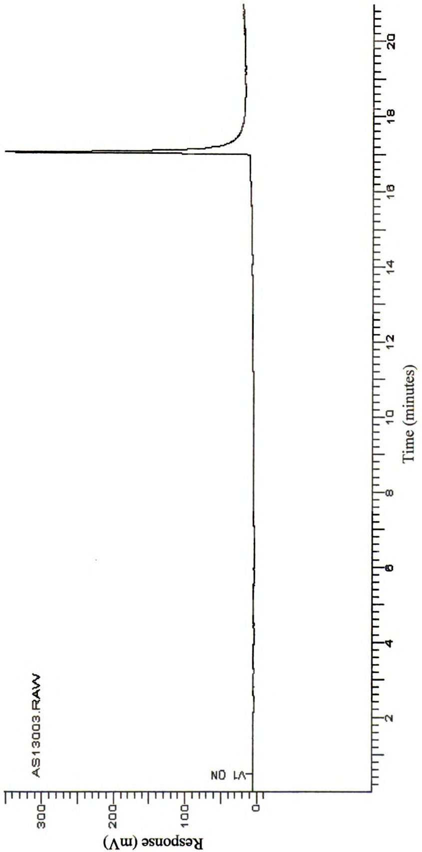

AS13002.RAW
V1 ON
Response (mV)
0
100
200
300
Time (minutes)
2
4
6
8
10
12
14
16
18
20

Figure 8.3 Gas chromatogram for 200 ppm 4,4′-dichloro-3-biphenylol/isooctane.

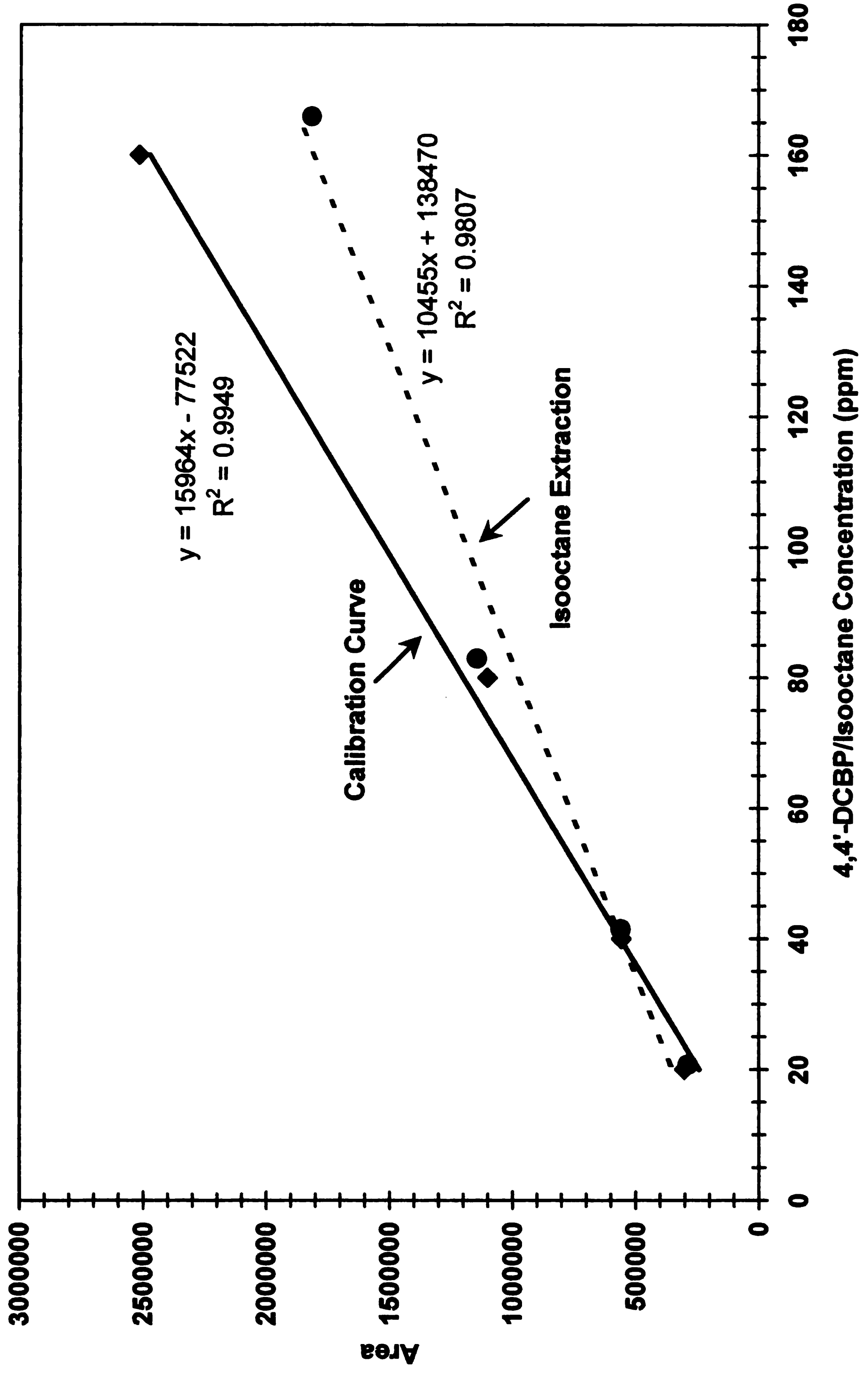

AS13003.RAW
V1 ON
Response (mV)
0
100
200
300
Time (minutes)
2
4
6
8
10
12
14
16
18
20

a rest period, a distinct clear top and bottom layer partially separated by a thin, white ring of froth, along the perimeter of the extraction vial, were visible in the extraction mixture. The top layer dipped through the center of the froth ring, but remained distinct from the bottom layer. In the extraction mixture, the ACN and Milli-Q water formed the bottom layer, while the 4,4'DCBP and isooctane formed the top layer (extracted sample).

The gas chromatogram for the blank (isooctane) revealed no significant peaks, except for small peaks of impurities. From the gas chromatograms for the calibration curve, the retention time (average ± standard deviation) for 4,4'DCBP was determined to be 15.30 ± 0.05 minutes. Similarly, the gas chromatograms for the extracted samples indicated a retention time (average ± standard deviation) of 15.30 ± 0.09 minutes for the 4,4'DCBP peak. For each of the gas chromatograms a small peak was also evident at a retention time close to 12 minutes; however, since this peak also was present in the gas chromatogram for the blank (isooctane), it can be attributed to impurities in the isooctane.

Figure 8.4 is a comparison of the isooctane extraction GC/ECD results, based on the expected extraction concentrations, and the calibration curve. The calibration curve equation determined by linear regression was $y = 15964x - 77522$ with an $R^2 = 0.9949$. The linear regression equation of the relationship of the isooctane extraction GC/ECD results and the expected extraction concentrations was $y = 10455x + 138470$ with an $R^2 = 0.9807$. For the isooctane extraction, the areas corresponding to the expected extraction concentrations 20.75, 41.5, and 83 ppm 4,4'-DCBP/isooctane closely corresponded with the calibration curve. However, the area corresponding to the expected extraction concentration of 166 ppm 4,4'DCBP/isooctane differed significantly from the calibration curve, indicating a lower percent efficiency of recovery. From the GC/ECD isooctane

Figure 8.4 Comparison of the isooctane extraction GC/ECD results for efficiency of recovery of 4,4′-dichlorobiphenyl and the calibration curve for 4,4′DCBP/isooctane. The isooctane extraction GC/ECD results were graphed based on the expected extraction concentrations.

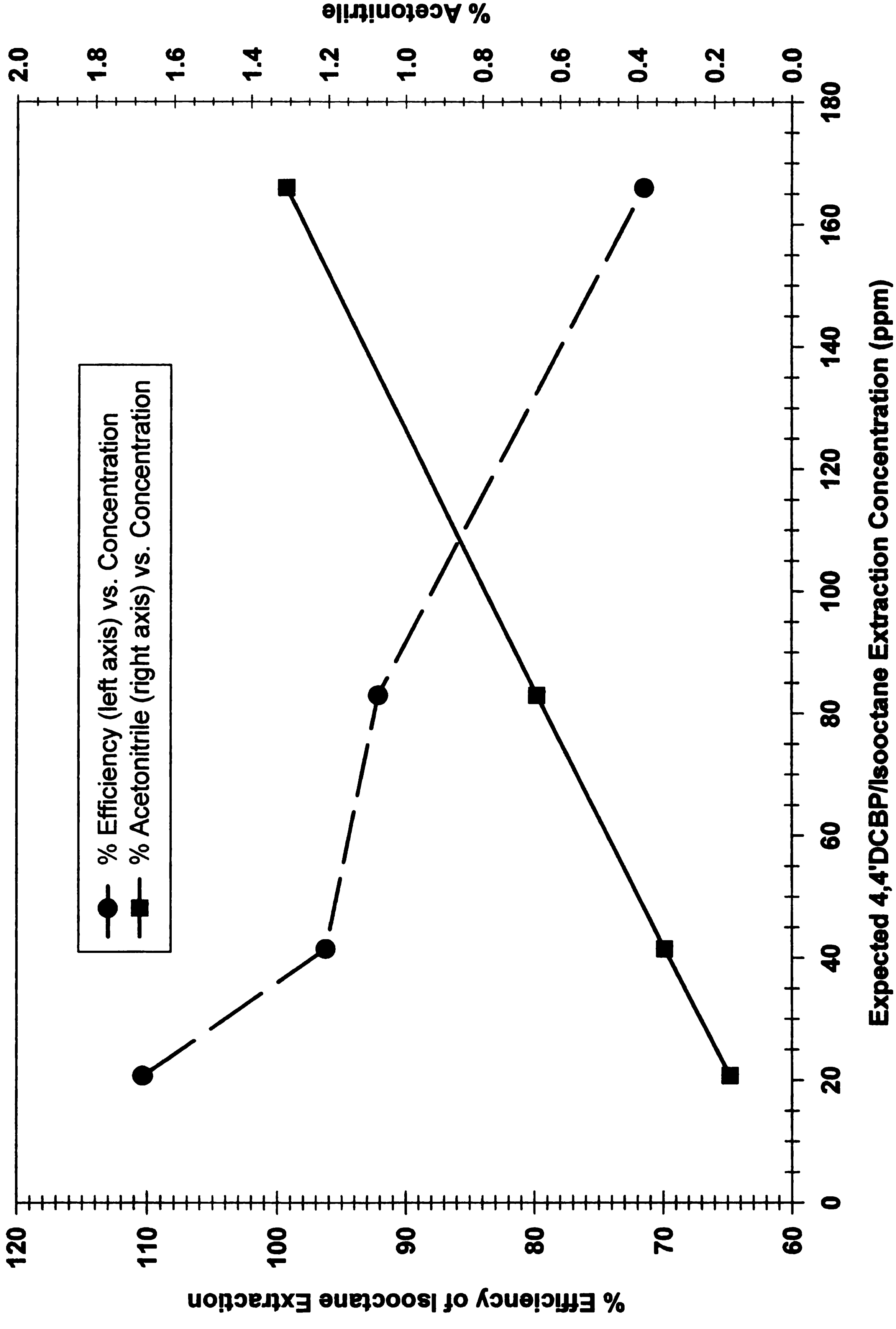

Area
3000000
2500000
2000000
1500000
1000000
500000
0
0
20
40
60
80
100
120
140
160
180
4,4'-DCBP/Isooctane Concentration (ppm)
y = 15964x - 77522
R² = 0.9949
y = 10455x + 138470
R² = 0.9807
Calibration Curve
Isooctane Extraction

extraction areas, the true extraction concentrations of 4,4'DCBP/isooctane were calculated from the calibration curve linear regression equation. The true extraction concentrations were then compared to the expected extraction concentrations to determine the percent efficiency of recovery by isooctane extraction.

Figure 8.5 compares the relationships between the percent efficiency of recovery of 4,4'DCBP by isooctane extraction, the calculated expected extraction concentrations based on the original volumes of 4,4'DCBP/ACN stock solution prepared for extraction, and the percent of ACN in the dilution water of the extraction process. Since higher calculated expected extraction concentrations resulted from higher volumes of 4,4'DCBP/ACN stock solution prepared for extraction, the percent of ACN in the dilution water of the extraction process increased with an increase in the expected extraction concentration. As the percent of ACN in the dilution water of the extraction process increased, there was a coinciding decrease in the percent efficiency of recovery of 4,4'DCBP by isooctane extraction. Greater than ninety percent efficiency of recovery of 4,4'DCBP by isooctane extraction was achieved when the percent of ACN in the dilution water during the extraction procedure was less than 0.66%. At a level of 1.31% ACN in the dilution water, corresponding to an expected extraction concentration of 166 ppm 4,4'DCBP/isooctane, the efficiency of recovery was only 71.50%.

The detrimental effect of a high percent of ACN in the dilution water on the percent efficiency of recovery was further emphasized by previous experiments (results not shown) in which only 39 mL of Milli-Q water was added during the extraction process rather than 61 mL. In contrast to the results from the addition of 61 mL of Milli-Q water during the extraction process to dilute the ACN, when 39 mL of Milli-Q water

Figure 8.5 Comparison of the relationships between the percent efficiency of recovery of 4,4'DCBP by isooctane extraction, the calculated expected extraction concentrations based on the original volumes of 4,4'DCBP/ACN stock solution prepared for extraction, and the percent of ACN in the dilution water of the extraction process.


% Efficiency (left axis) vs. Concentration
% Acetonitrile (right axis) vs. Concentration
% Efficiency of Isooctane Extraction
120
110
100
90
80
70
60
% Acetonitrile
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Expected 4,4'DCBP/Isooctane Extraction Concentration (ppm)
0
20
40
60
80
100
120
140
160
180

was added instead, therefore resulting in a higher percent of ACN in the dilution water, the efficiency of recovery was less than ninety percent for the same expected extraction concentrations at which greater than ninety percent efficiency had been observed. Hence, the addition of Milli-Q water as a means of diluting the ACN in the extraction process, thereby decreasing the percent of ACN in the dilution water, is essential to obtaining an acceptable efficiency of recovery of 4,4'DCBP by isooctane extraction.

8.3.3 Fenton's Remediation of 4,4'-Dichlorobiphenyl and Extraction

Fenton's remediation of solutions of 4,4'DCBP in 50/50 Milli-Q H_2O/ACN (by volume) was performed for "0", 15, 30, and 60 minutes. To improve the dissolution of 4,4'DCBP, for each remediation, the 4,4'DCBP was dissolved in 50/50 Milli-Q H_2O/ACN (by volume) in a step-wise procedure to obtain the final concentration. When the 4,4'DCBP was first dissolved in the ACN portion of the 50/50 Milli-Q H_2O/ACN solvent, the resulting solution was clear and colorless with no precipitate. The solution appearance remained unchanged during the addition of the Milli-Q H_2O portion of the 50/50 Milli-Q H_2O/ACN solvent until approximately 5.0 mL of Milli-Q H_2O had been added. Further addition of Milli-Q H_2O resulted in a light milky white solution. As the remaining Milli-Q H_2O portion was added in 1000 μL increments, increasing amounts of sonication were necessary in order to maintain complete dissolution. Following the addition of 5.0 mM $FeSO_4$ and pH adjustment, the reaction mixture was clear and colorless with very fine white particles in suspension.

For "0 minutes" of remediation, there was no addition of 3% H_2O_2; however, MB dye tests were performed on 40 μL samples of the reaction mixture, Milli-Q water, and

50/50 Milli-Q H_2O/ACN solvent. As shown in Figure 8.6A, the MB dye test control strip (no sample added) was homogeneously dark blue in color. When the MB dye test was performed with Milli-Q water (Figure 8.6B), no bleaching or discoloration was observed. As shown in Figure 8.6C, a MB dye test of 50/50 Milli-Q H_2O/ACN solvent produced diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discoloration. As shown in Figure 8.6D, when the MB dye test was performed with the reaction mixture from "0 minutes" of remediation, the result closely resembled the MB result indicated for 50/50 Milli-Q H_2O/ACN solvent with only slightly more white diffuse discoloration. The similarity of the reaction mixture and 50/50 Milli-Q H_2O/ACN solvent MB dye test results verified the absence of hydroxyl radicals in the "0 minutes" reaction mixture.

To maintain the conditions and chemical species similar to what would exist prior to remediation initiation, the reaction mixture was neither adjusted to pH 9 prior to filtration nor adjusted to pH 7 following filtration. Prior to filtration of the reaction mixture from "0 minutes" of remediation, sonication was performed; however, the reaction mixture remained clear and colorless with very fine white particles in suspension. Following filtration, the reaction mixture appeared clear and colorless with no precipitate.

For each of the non-zero remediation times, following the adjustment of the reaction mixture to pH 3, the Fenton's reaction was initiated by the addition of 3% H_2O_2. The remediation reaction was allowed to occur for 15, 30, or 60 minutes with occasional sonication of the reaction mixture. During the Fenton's reaction remediation period, no significant change occurred in the solution pH and temperature. The solution remained

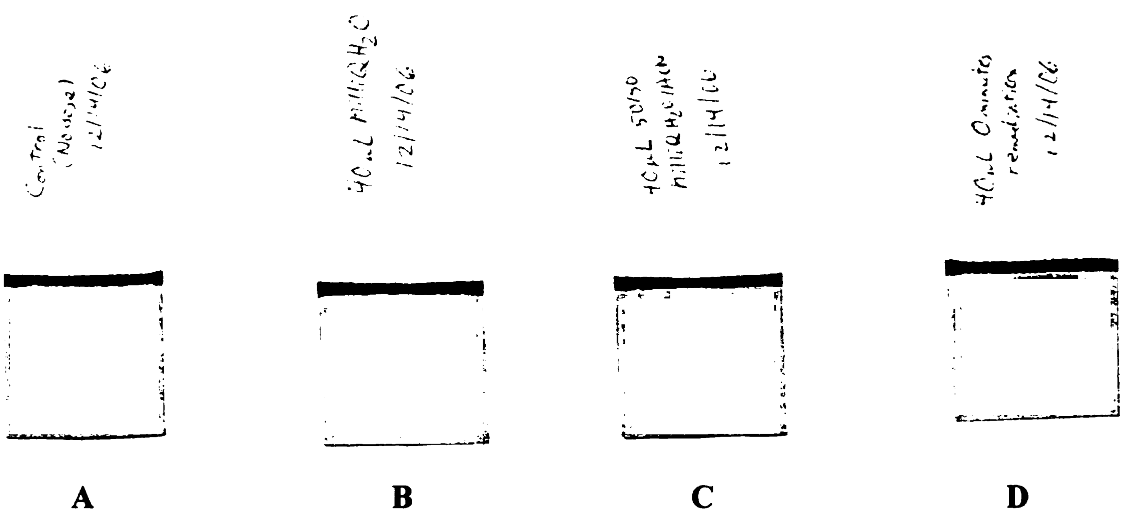


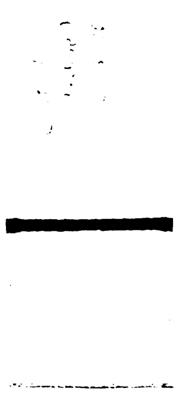
Figure 8.6 Fenton's remediation of a solution of 4,4′-dichlorobiphenyl in 50/50 Milli-Q H_2O/ACN. Methylene blue dye test results for (A) control (no sample added), (B) 40 µL of Milli-Q water, (C) 40 µL of 50/50 Milli-Q H_2O/ACN solvent, and (D) 40 µL of the reaction mixture from "0 minutes" of remediation.

clear and colorless with the amount of very fine white particles in suspension decreasing as the reaction progressed, until no precipitate was apparent, after about 5 minutes.

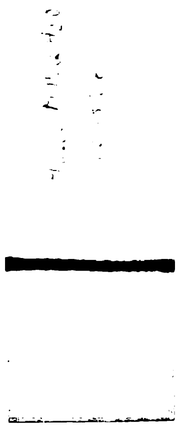
In addition to performing MB dye tests on 40 μL samples of Milli-Q water and 50/50 Milli-Q H_2O/ACN solvent, MB dye tests were also performed on 40 μL samples of the unquenched reaction mixture to verify the production of hydroxyl radicals during the reaction. Figure 8.7 presents the MB dye test results for 60 minutes of remediation; however, the results are representative of those observed for the shorter periods of remediation studied. As shown in Figure 8.7A, the MB dye test control strip (no sample added) was homogeneously dark blue in color. When the MB dye test was performed with Milli-Q water (Figure 8.7B), no bleaching or discoloration was observed. As shown in Figure 8.7C, a MB dye test of 50/50 Milli-Q H_2O/ACN solvent produced diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discoloration. As shown in Figures 8.7D, E, and F, MB dye test strips tested with unquenched Fenton's reaction mixture at 15, 30, and 60 minutes, respectively, similarly indicated the presence of hydroxyl radicals by an immediate concentrated white discoloration (bleaching) at the point of application surrounded by diffuse discoloration of the MB to light blue, spreading out/emanating from the point of application, with a dark blue band surrounding the discolored area.

After the specified remediation reaction period, each Fenton's reaction was quenched with 6 drops (approximately 0.24 mL) of 10% Na_2SO_3 solution. Immediately following the addition of the 10% Na_2SO_3, the pH of the reaction mixture increased from approximately 3 to 9. No significant change occurred in the temperature of the reaction mixture. For each remediation, the addition of 10% Na_2SO_3 resulted in a change in

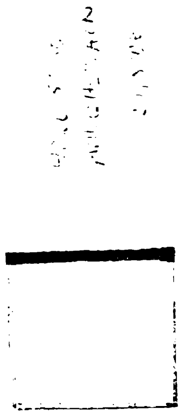
Figure 8.7 Fenton's remediation of a solution of 4,4′-dichlorobiphenyl in 50/50 Milli-Q H_2O/ACN. Methylene blue dye test results for (A) control (no sample added); (B) Milli-Q water; (C) 50/50 Milli-Q H_2O/ACN solvent; unquenched Fenton's reaction mixture at (D) 15 minutes, (E) 30 minutes, and (F) 60 minutes; and Fenton's reaction mixture quenched with (G) 6 drops and (H) 8 drops of 10% Na_2SO_3. All methylene blue dye tests were performed using 40 μL samples.



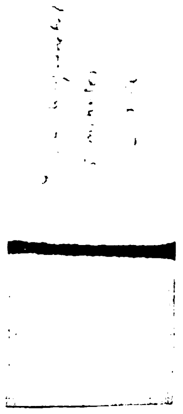
A



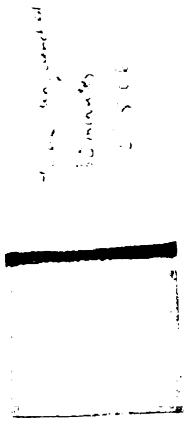
B



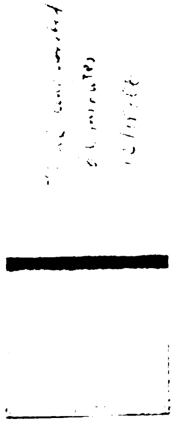
C



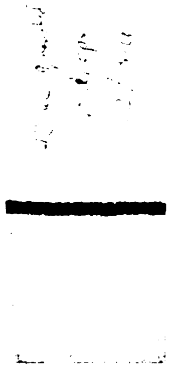
D



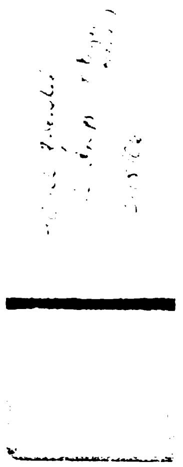
E



F



G



H

reaction mixture appearance to clear and colorless with very fine rust colored (light orange) particles in suspension. The appearance of rust colored particles can be attributed to the more basic pH of the reaction mixture during the quenching process, resulting in the conversion of iron from a hydrated ferrous form to a colloidal ferric form and the formation of ferric hydroxide (*10 -12*). As shown in Figure 8.7G, the MB dye test of the Fenton's reaction mixture quenched with 6 drops of 10% Na_2SO_3 solution produced results that closely resembled the MB result indicated for 50/50 Milli-Q H_2O/ACN solvent. The similarity of the MB dye test results for the quenched reaction mixture and 50/50 Milli-Q H_2O/ACN solvent, as well as the absence of an immediate concentrated white discoloration (bleaching) at the point of application, indicated that quenching was complete and no hydroxyl radicals remained.

As a precautionary measure, an additional 2 more drops (approximately 0.09 mL) of 10% Na_2SO_3 solution was added to each reaction mixture. Following the addition of the additional 10% Na_2SO_3, the pH and appearance of the reaction mixture remained unchanged from that observed following the initial addition of 10% Na_2SO_3. As shown in Figure 8.7H, the MB dye test of the Fenton's reaction mixture quenched with an additional 2 drops of 10% Na_2SO_3 solution (a total of 8 drops) produced results that closely resembled the MB results indicated for 50/50 Milli-Q H_2O/ACN solvent and the Fenton's reaction mixture quenched with 6 drops of 10% Na_2SO_3 solution, thereby verifying that quenching was complete. For the MB dye test of the Fenton's reaction mixture quenched with a total of 8 drops of 10% Na_2SO_3 solution, the slight white film observed over the surface of the test area and spreading beyond the dark blue band surrounding the area of diffuse discoloration can be attributed to excess 10% Na_2SO_3.

To aid in the removal of iron from the reaction mixture by precipitation of Fe^{3+}, following quenching, the reaction mixture was adjusted to pH 9 (unless the pH was already greater than or equal to pH 9). Following this pH adjustment, the solution remained clear and colorless with very fine rust colored (light orange) particles in suspension. Since none of the reaction mixtures were highly concentrated with precipitate, no centrifugation was performed prior to filtration. No rinsing of the filtration funnel with Milli-Q water was necessary for the filtration of any of the reaction mixtures. The filter paper was uniform light rust in color following filtration. The filtrate appeared clear and colorless with no precipitate. Following adjustment of the filtrate to pH 7, the solution remained clear and colorless with no precipitate.

Throughout each of the Fenton's remediation experiments, the amount of water added by addition of 5.0 mM $FeSO_4$, pH adjustment, addition of 3% H_2O_2, quenching, and rinsing of the filtration funnel was monitored, and an adjusted total H_2O/ACN ratio by volume was calculated at the completion of the experiment. The total H_2O/ACN ratio suggested the degree of dilution of the sample that occurred during the experiment and was maintained as constant as possible between the remediation experiments to allow for comparison of the results. The original H_2O/ACN ratio at the start of the remediation experiment, based on the solution used to dissolve the 4,4'DCBP, was 50/50 or 1.00. At the completion of the experiments, the calculated adjusted total H_2O/ACN ratios for "0", 15, 30, and 60 minutes of remediation were 1.07, 1.11, 1.12, and 1.11, respectively. Since these H_2O/ACN ratios result in an average ± standard deviation of 1.10 ± 0.02, the coefficient of variation is only 2.01%, which is within the allowable pipetting error for dispensing liquids. This indicates that the ratio of H_2O/ACN did not significantly change between the remediation experiments.

As described previously, two solutions of different expected "concentrations" (156.1 and 78.05 ppm 4,4'DCBP/isooctane) were prepared by isooctane extraction from each of the final Fenton's remediation solutions. Immediately following the extraction process, the extraction mixture was a white emulsion. Following a rest period, a distinct clear top and bottom layer partially separated by a white ring of froth, along the perimeter of the extraction vial, were visible in the extraction mixture. The top layer dipped through the center of the froth ring, but remained distinct from the bottom layer. The top extraction layer, containing 4,4'DCBP and isooctane, was carefully removed for GC/ECD analysis.

8.3.4 GC/ECD Analysis and Disappearance of Parent PCB

The gas chromatogram for the blank (isooctane) revealed no significant peaks, except for small peaks of impurities. From the gas chromatograms for the calibration curve, the retention time (average ± standard deviation) for 4,4'DCBP was determined to be 15.20 ± 0.04 minutes with a range of 15.16 to 15.27 minutes. Calibration curve gas chromatograms for 40, 80, and 160 ppm 4,4'DCBP/isooctane are shown in Figures 8.8, 8.9, and 8.10, respectively. These chromatograms are representative of those observed for the calibration curve determination. Two very small peaks with retention times of approximately 8 and 12 minutes were also observed on the calibration curve gas chromatograms; however, since these peaks were also observed on the gas chromatogram for the blank (isooctane), they can be attributed to impurities in the isooctane. These

Figure 8.8 Representative calibration curve gas chromatogram for 40 ppm 4,4'DCBP/isooctane.

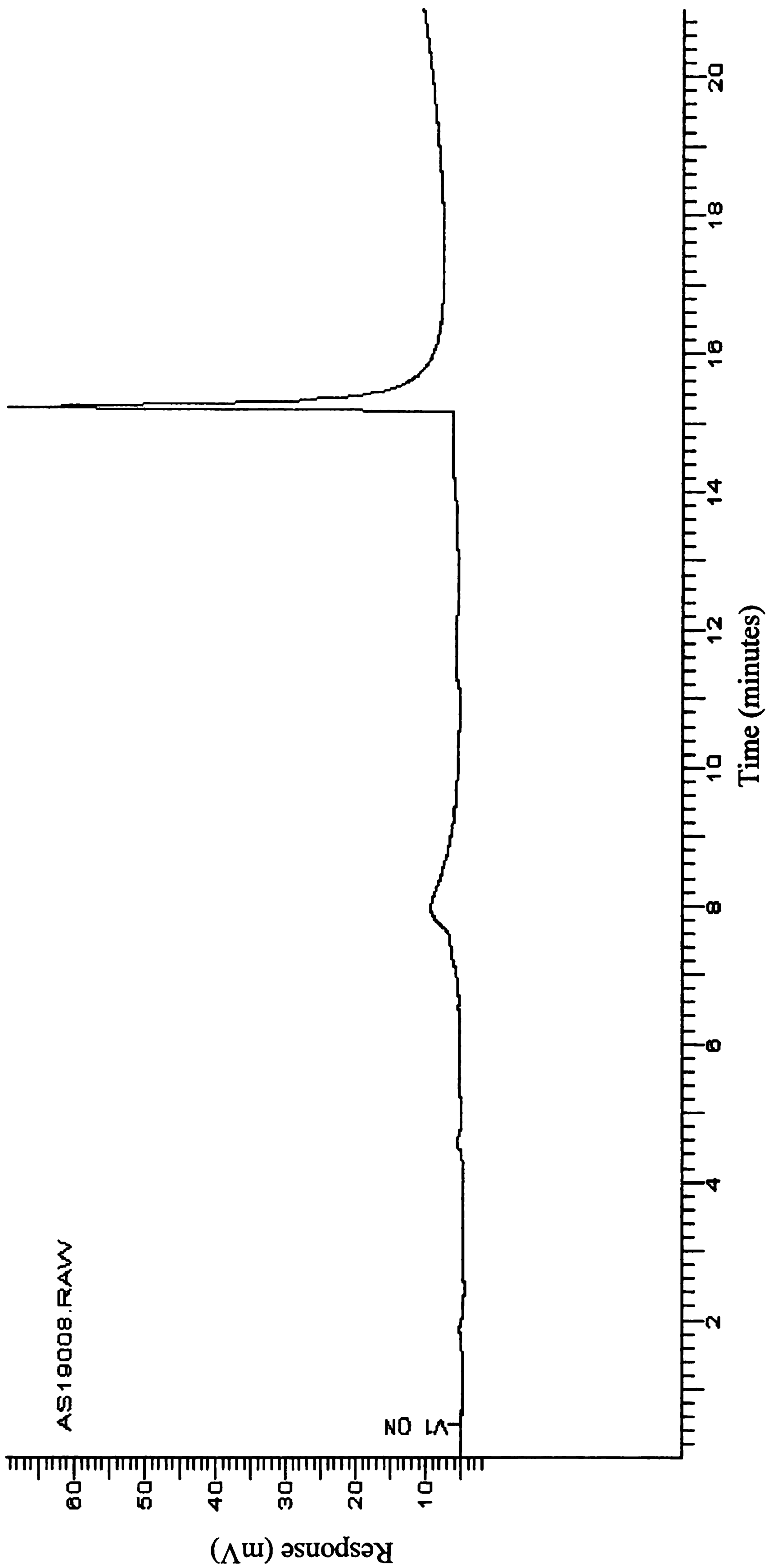

AS19008.RAW
V1 ON
Response (mV)
60
50
40
30
20
10
Time (minutes)
2
4
6
8
10
12
14
16
18
20

Figure 8.9 Representative calibration curve gas chromatogram for 80 ppm 4,4'DCBP/isooctane.

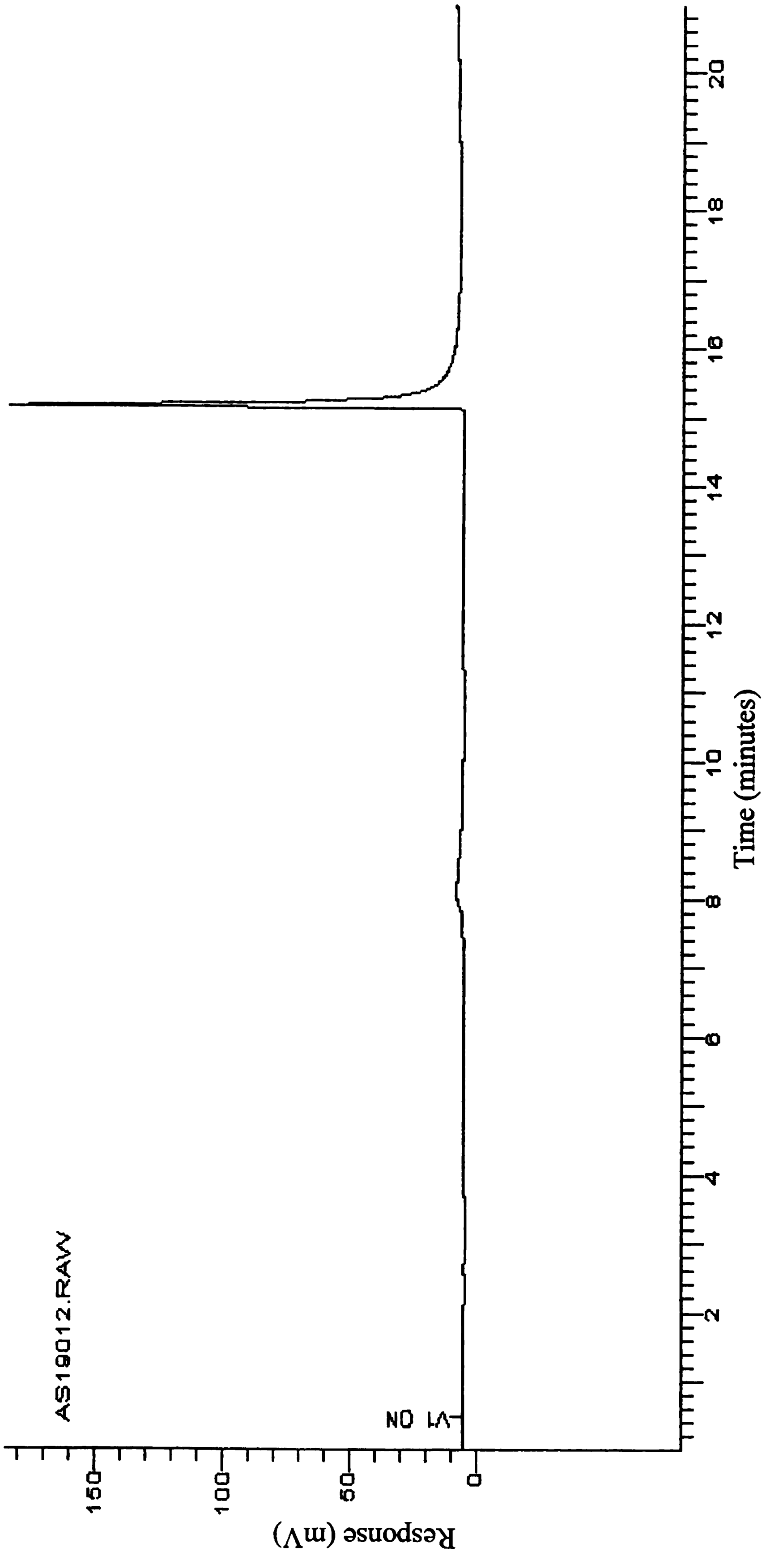
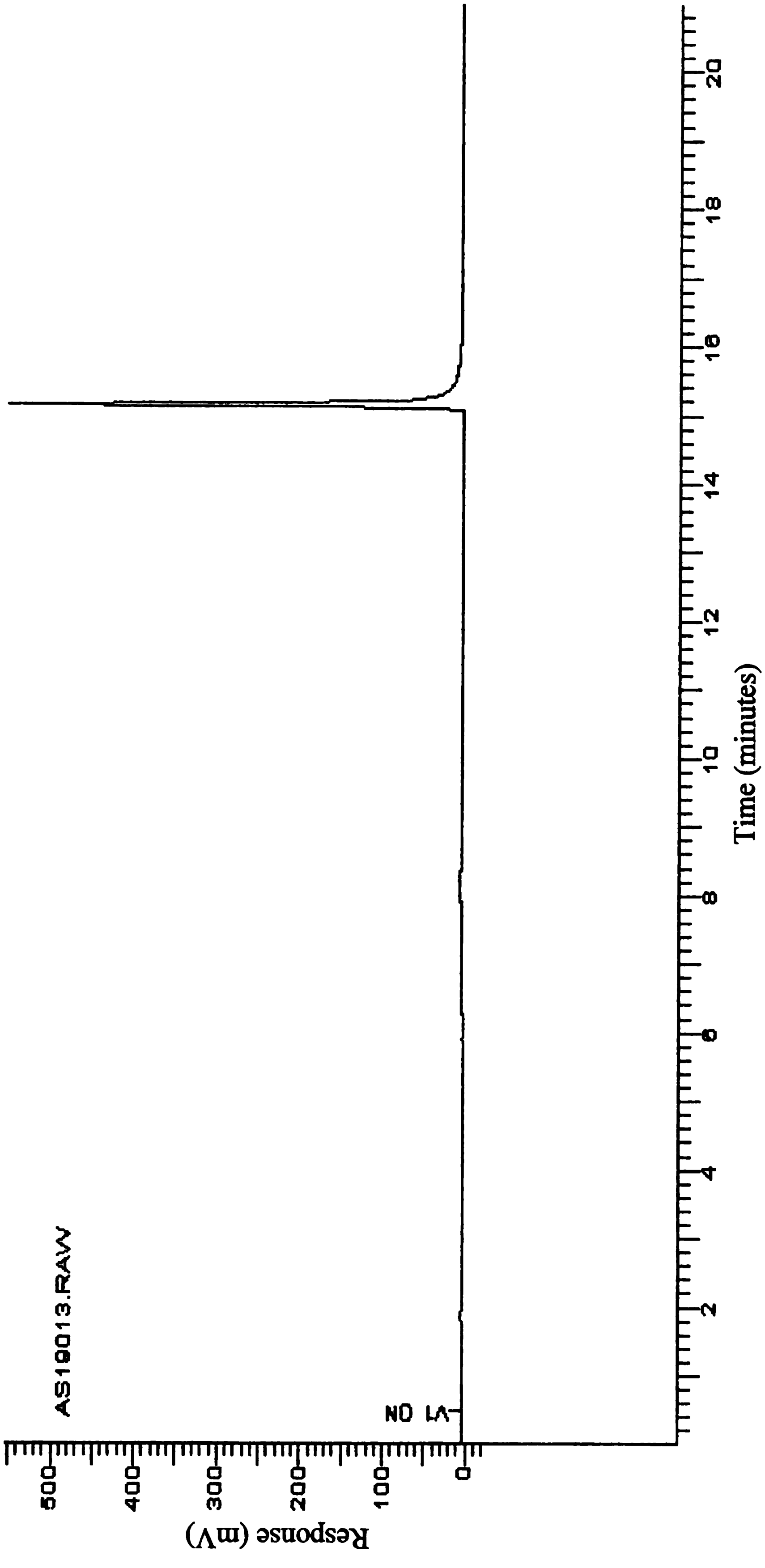

AS19012.RAW
V1 ON
Response (mV)
150
100
50
0
Time (minutes)
2
4
6
8
10
12
14
16
18
20

Figure 8.10 Representative calibration curve gas chromatogram for 160 ppm 4,4'DCBP/isooctane.


AS19013.RAW
V1 ON
Response (mV)
0
100
200
300
400
500
Time (minutes)
2
4
6
8
10
12
14
16
18
20

impurity peaks were most evident on the gas chromatograms plotted with a more limited scale on the response (vertical) axis, such as Figure 8.8. The equation determined by linear regression for the calibration curve (Figure 8.11) was $y = 17018x - 58658$ with an $R^2 = 0.9969$.

The gas chromatograms for the extracted Fenton's remediation solutions indicated only a single major peak, the parent PCB, 4,4′DCBP, with a retention time (average ± standard deviation) of 15.24 ± 0.05 minutes with a range of 15.17 to 15.31 minutes. This retention time correlates closely with that found for the 4,4′DCBP/isooctane standards used for the calibration curve. Gas chromatograms for the "78.05 ppm" and "156.1 ppm" 4,4′DCBP/isooctane extraction samples from the Fenton's remediation solution reacted for 60 minutes are shown in Figures 8.12 and 8.13, respectively. These chromatograms are representative of those observed for the extraction sample "concentrations" prepared from Fenton's remediation solutions reacted for "0", 15, 30, and 60 minutes. As discussed previously, two very small peaks with retention times of approximately 8 and 12 minutes were also observed on the gas chromatograms from the extraction samples, but are attributable to impurities in the isooctane.

No remediation byproduct peaks were observed for any of the extraction samples from the Fenton's remediation solutions. The peaks observed for the potential remediation byproducts, 3-chloro-2-biphenylol (retention time 14.21 minutes) and 4,4′-dichloro-3-biphenylol (retention time 17.03 minutes), were also not observed. One possible explanation for the absence of remediation byproduct peaks might be the production of remediation byproducts in concentrations too small to be detected with the ECD. The ECD is more sensitive to detecting highly chlorinated compounds. If a

Figure 8.11 Calibration curve for 4,4′DCBP/isooctane.

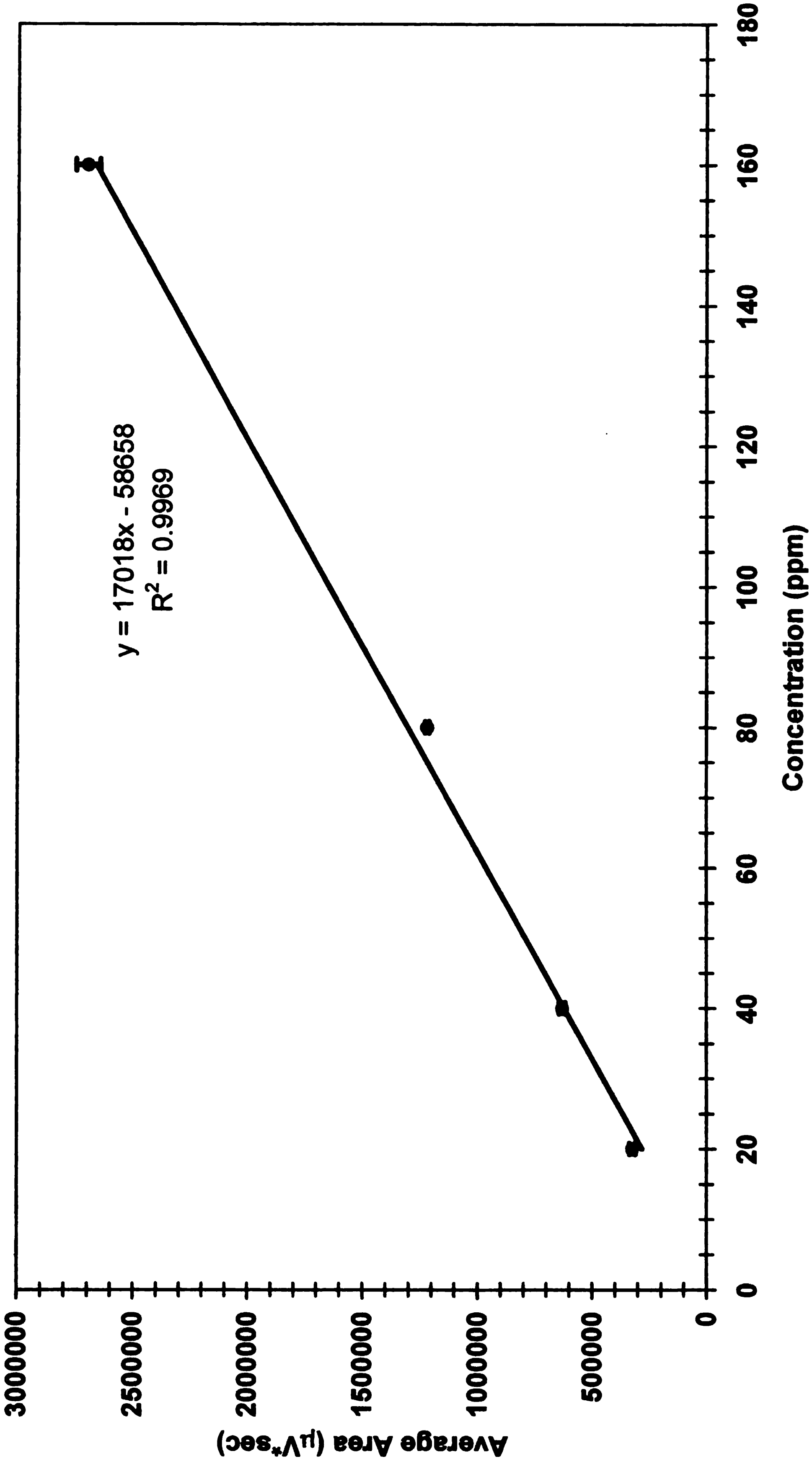

y = 17018x - 58658
R^2 = 0.9969
Average Area (µV*sec)
0
500000
1000000
1500000
2000000
2500000
3000000
Concentration (ppm)
0
20
40
60
80
100
120
140
160
180

Figure 8.12 Gas chromatogram for the “78.05 ppm” 4,4'DCBP/isooctane extraction sample from the Fenton’s remediation solution reacted for 60 minutes (pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM).

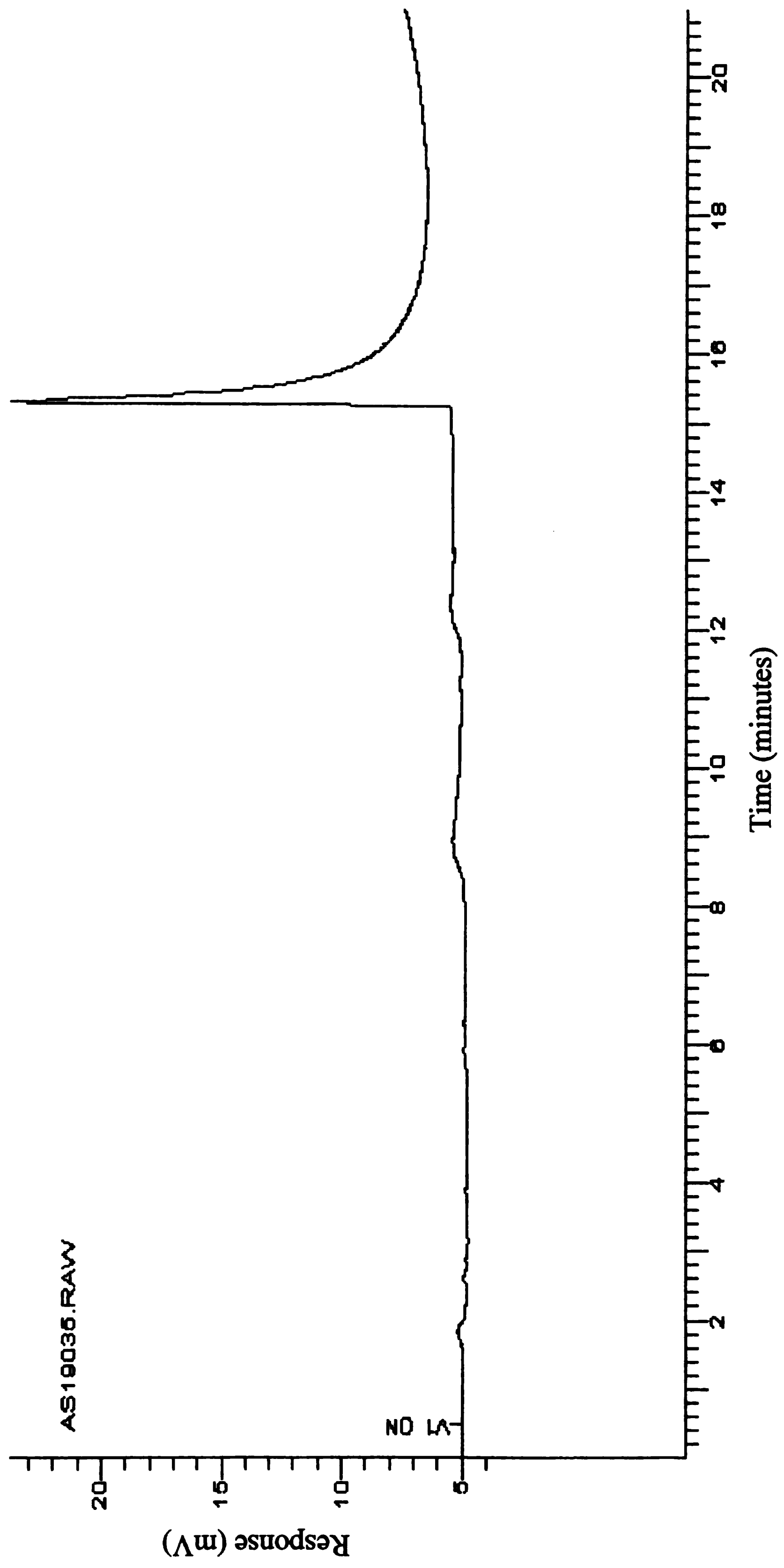
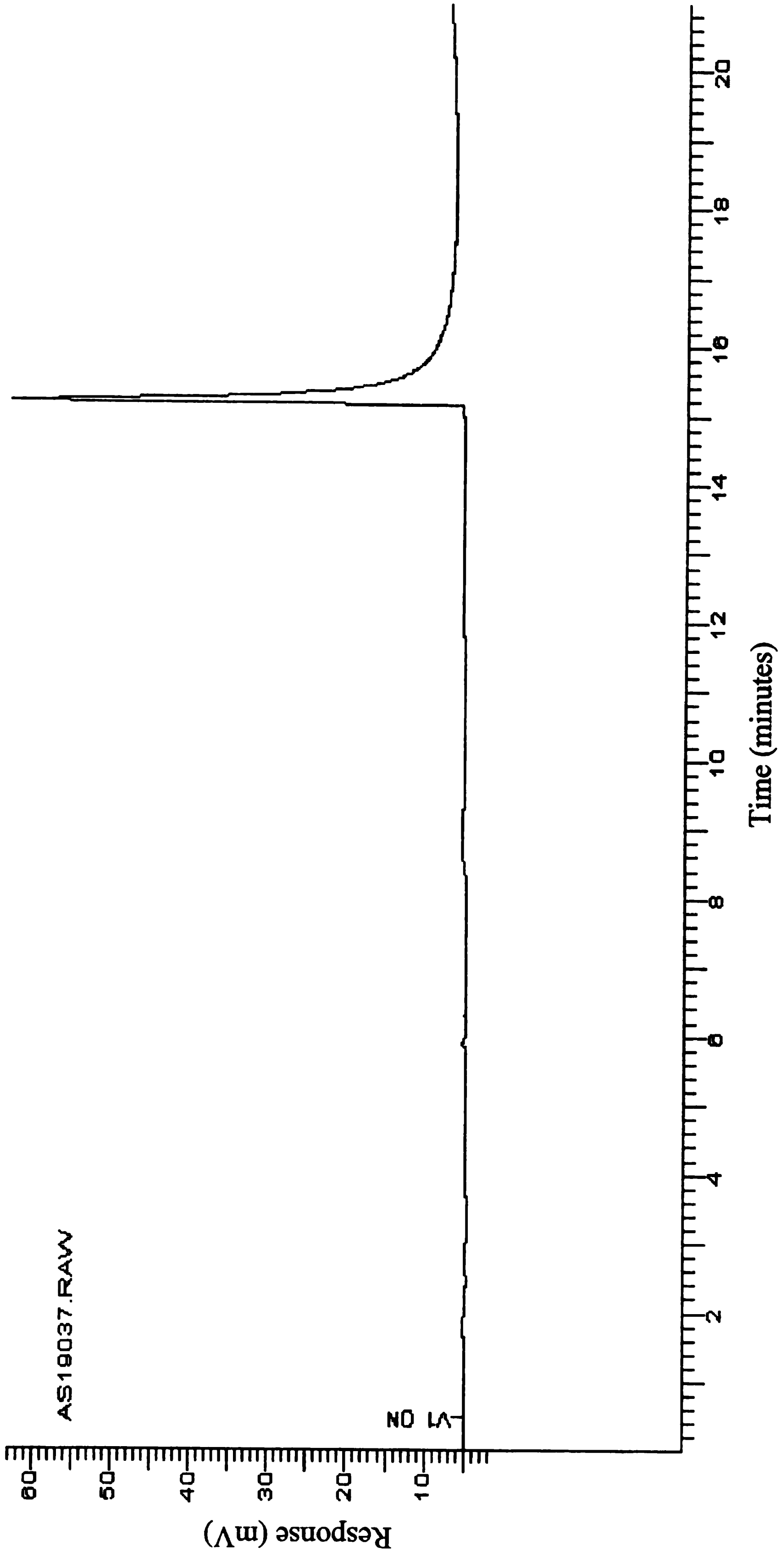

AS19035.RAW
V1 ON
Response (mV)
5
10
15
20
Time (minutes)
2
4
6
8
10
12
14
16
18
20

Figure 8.13 Gas chromatogram for the “156.1 ppm” 4,4'DCBP/isooctane extraction sample from the Fenton’s remediation solution reacted for 60 minutes (pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM).


AS19037.RAW
V1 ON
Response (mV)
60
50
40
30
20
10
Time (minutes)
2
4
6
8
10
12
14
16
18
20

remediation byproduct contains only a few chlorines, a higher concentration might be required for the ECD to detect it. Since the parent compound, 4,4'DCBP, contains only two chlorines, remediation might have resulted in byproducts that are not easily detectable by the ECD. Absence of remediation byproduct peaks might also be attributed to further remediation of remediation byproducts, resulting in the production of secondary and tertiary byproducts. Instead of large concentrations of primary byproducts, further remediation could result in numerous compounds in smaller concentrations, possibly below detectable levels. Finally, the absence of remediation byproduct peaks might be a consequence of the generation of dechlorinated byproducts, which are undetectable by ECD.

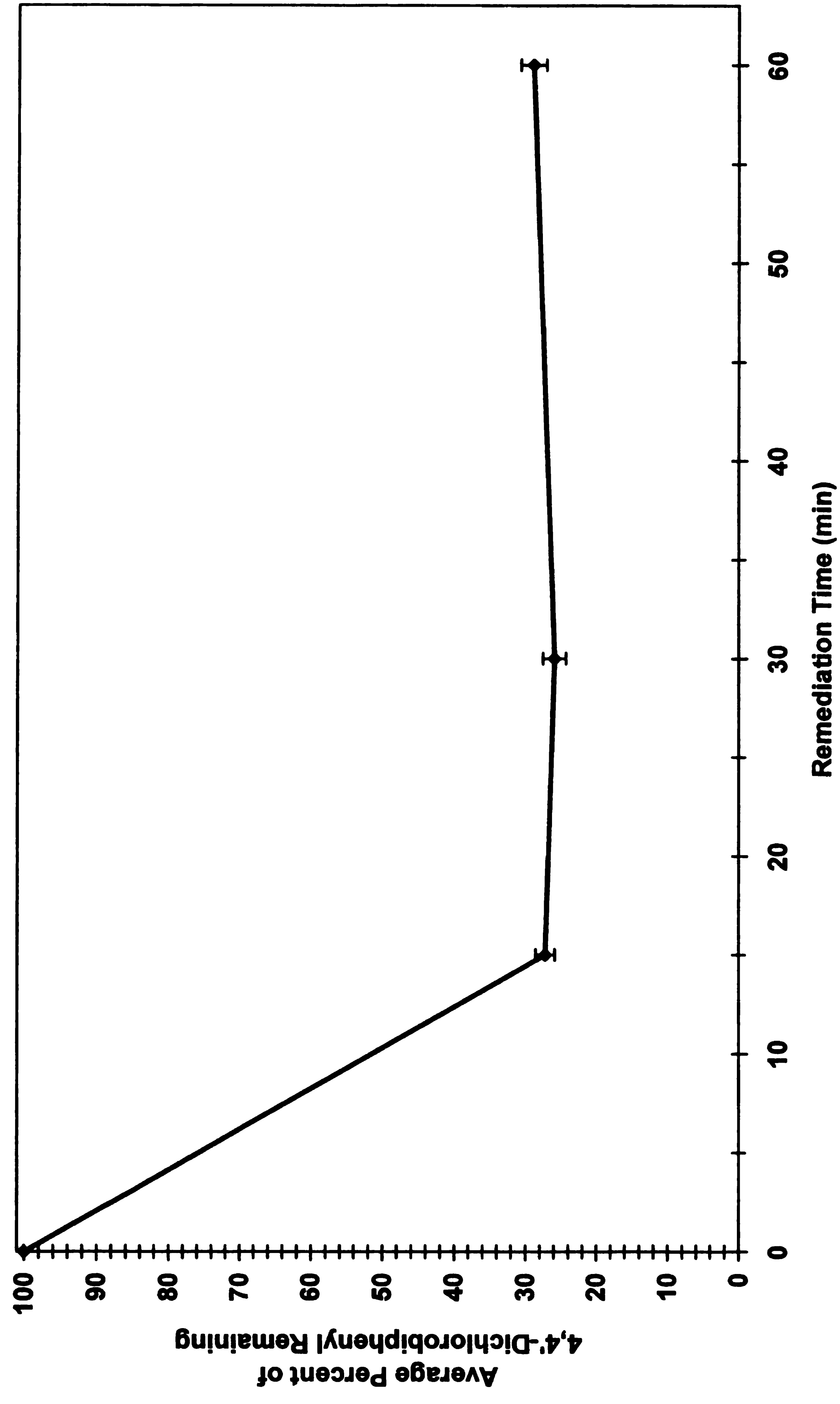
For "0 minutes" of remediation, the concentration of the final Fenton's remediation solution (average ± standard deviation) was determined to be 150.77 ± 10.44 ppm 4,4'DCBP, which is equivalent to removal of 66.19% of the original 446 ppm 4,4'DCBP (33.81% 4,4'DCBP remaining). Since "0 minutes" of remediation was defined as the remediation procedure just prior to the initiation of the Fenton's reaction by the addition of 3% H_2O_2, no reaction should have occurred and the concentration should have remained at 446 ppm 4,4'DCBP. The results of the Fenton's remediation of 4,4'DCBP indicated that initiation of the Fenton's reaction by the addition of 3% H_2O_2 was necessary to solubilize the 4,4'DCBP. Despite repeated sonication, prior to the initiation of the Fenton's reaction the reaction mixture was clear and colorless with very fine white particles in suspension. Following initiation of the Fenton's reaction, the amount of very fine white particles in suspension decreased as the reaction progressed, until no precipitate was apparent, after about 5 minutes. Since there was no initiation of the

Fenton's reaction for "0 minutes" of remediation, any undissolved 4,4'DCBP would have been removed from the solution during the filtration process. Hence, the removal of 4,4'DCBP during the "0 minutes" of remediation experiment can be attributed to loss via the filtration process. Since it is impossible to obtain a true zero time of remediation, as this would require instantaneously quenching the reaction following its initiation, and complete dissolution of 4,4'DCBP requires initiation of the reaction, the decision was made to consider the original concentration of 4,4'DCBP (446 ppm) to be the 0 minutes of remediation concentration. The possibility of losses of 4,4'DCBP during the filtration process for the 15, 30, and 60 minutes of remediation experiments were not evaluated; however, these could constitute potential further studies.

As described previously, the concentrations of the final Fenton's remediation solutions were determined through a process of back-calculation from the GC/ECD results. Since these calculations assumed 100% isooctane extraction efficiency, the actual percent isooctane extraction efficiency was determined from the percent of ACN in the dilution water and the results of the "Percent Efficiency of Recovery by Isooctane Extraction" experiment. The percents of ACN in the dilution water for the "156.1 ppm" and "78.05 ppm" 4,4'DCBP/isooctane extraction samples were 0.57% and 0.29%, respectively. From the "Percent Efficiency of Recovery by Isooctane Extraction" experiment, a mathematical relationship was developed between the percent of ACN in the dilution water and the isooctane extraction efficiency. By applying linear regression to the results corresponding to 0.33% and 0.66% ACN, which are closest to the percent of ACN of interest for the 4,4'DCBP/isooctane extraction samples, the equation $y = 100.33 - 12.45x$ with an $R^2 = 1.00$ was determined where "x" is the percent of ACN and "y" is the percent efficiency of isooctane extraction. By applying this equation to the percent of ACN in the dilution water for the "156.1 ppm" and "78.05 ppm" 4,4′DCBP/isooctane extraction samples, the actual percent isooctane extraction efficiencies were calculated to be 93.23% and 96.72%, respectively. Since both actual percent isooctane extraction efficiencies were greater than ninety percent, no mathematical adjustment of the final Fenton's remediation solution concentrations of 4,4′DCBP was required.

Figure 8.14 presents a graph of the average percent of 4,4′DCBP remaining in the final Fenton's remediation solution versus the remediation time. The original concentration of 4,4′DCBP (446 ppm) was assumed to be the 0 minutes of remediation concentration, corresponding to 100% for an average percent of 4,4′DCBP remaining. By 15 minutes of remediation, a decrease in the average concentration of the final Fenton's remediation solution occurred, resulting in 27.22 ± 1.32% (average ± standard deviation) of 4,4′DCBP remaining (equivalent to a removal of 72.78 ± 1.32% of the 4,4′DCBP). No further significant decrease in the average concentration of the final Fenton's remediation solution occurred with further remediation up to 60 minutes. Using Kruskal-Wallis One Way Analysis of Variance on Ranks, there was no statistically significant difference between the average percents of 4,4′DCBP remaining in the final Fenton's remediation solution remediated for 15, 30, and 60 minutes. It is unlikely that removal of the 4,4′DCBP is a result of sorption onto the reaction vessel walls, since the amount of 4,4′DCBP is small compared to the large surface area of the reaction vessel walls, which would have resulted in almost complete removal of the 4,4′DCBP. Furthermore, according to Sedlak et al. (*13*), the rapid kinetics of the hydroxyl reactions with PCBs relative to adsorption to container walls assures that the reaction of hydroxyl

Figure 8.14 Graph of the average percent of 4,4′-dichlorobiphenyl remaining following Fenton's remediation vs. the remediation time.


Average Percent of
4,4'-Dichlorobiphenyl Remaining
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Remediation Time (min)

radicals with PCBs would be complete before any PCBs were adsorbed to the container walls.

The incomplete removal of 4,4'DCBP can be attributed to multiple factors. One explanation for the incomplete removal observed for the Fenton's remediation of 4,4'DCBP is that the H_2O_2 was the limiting compound. The H_2O_2 could have been depleted during the remediation of the 4,4'DCBP, so that no further removal of 4,4'DCBP occurred after that observed at 15 minutes. Similar results were observed by Trapido et al. (*7*), where incomplete degradation of nitrophenols by Fenton's reagent occurred for a variety of Fe^{2+}:H_2O_2 ratios, including 1:20. H_2O_2 was concluded to be the limiting compound causing the remediation of nitrophenols to become restrained such that no further degradation took place. Likewise, experiments performed by Kuo et al., (*14*) involving the remediation of aqueous 4-chlorobiphenyl and 4,4'-dichlorobiphenyl by Fenton's reaction, resulted in a constant concentration of chlorobiphenyls indicative of incomplete degradation.

As future studies, a variety of experiments could be performed to verify that H_2O_2 is the limiting compound responsible for the incomplete removal of 4,4'DCBP under the conditions investigated. One experiment could involve increasing the initial concentration of H_2O_2 to a value greater than 3 mM while keeping the initial Fe^{2+} concentration at 0.15 mM. Alternatively, the Fenton's reaction could be conducted under the same conditions previously investigated; however, additional H_2O_2 could be added to the reaction mixture after an initial reaction period of 15 minutes. In either experiment, greater removal of 4,4'DCBP than previously observed would indicate that H_2O_2 is the limiting compound. An alternative experiment would be to measure the residual concentration of H_2O_2 after varying Fenton's remediation times. If the H_2O_2 has been depleted when no further removal of 4,4'DCBP occurs, H_2O_2 will be verified as the limiting compound. One procedure for measuring the residual concentration of H_2O_2 is the photometric method for the determination of low concentrations of H_2O_2 by the peroxidase catalyzed oxidation of N,N-diethyl-*p*-phenylenediamine (DPD) described by Bader et al. (*15*)

Although incomplete removal of 4,4'DCBP occurred, hydroxyl radicals were still observed by the MB dye test through 60 minutes of Fenton's remediation. Despite the apparent depletion of H_2O_2, hydroxyl radicals might have continued to be generated (although perhaps at a lower concentration) because of the persisting radical chain reaction. Since the MB dye test only qualitatively detects the presence of hydroxyl radicals, it is not possible to determine the quantity of hydroxyl radicals present at each of the Fenton's remediation times. Preferential reaction of the hydroxyl radicals with the byproducts rather than the 4,4'DCBP would result in no further removal of 4,4'DCBP.

8.4 Conclusions

Fenton's remediation of 4,4'DCBP in 50/50 Milli-Q H_2O/ACN was performed for 0, 15, 30, and 60 minutes. To prepare the remediation mixtures for GC/ECD analysis, an isooctane liquid-liquid extraction method was developed that resulted in greater than ninety percent efficiency of recovery of 4,4'DCBP when the percent of ACN in the dilution water during the extraction procedure was less than 0.66%. The utilization of Milli-Q H_2O as a means of diluting the ACN in the extraction process was essential to obtaining an acceptable efficiency of recovery of 4,4'DCBP by isooctane extraction. The gas chromatograms for the extracted Fenton's remediation solutions indicated only a single major peak, the parent PCB, 4,4'DCBP, with a retention time (average ± standard deviation) of 15.24 ± 0.05 minutes. No remediation byproduct peaks or the peaks observed for the potential remediation byproducts, 3-chloro-2-biphenylol (retention time 14.21 minutes) and 4,4'-dichloro-3-biphenylol (retention time 17.03 minutes), were observed for any of the extraction samples from the Fenton's remediation solutions. Since complete dissolution of 4,4'DCBP required initiation of the reaction, the original concentration of 4,4'DCBP (446 ppm) was assumed to be the 0 minutes of remediation concentration. A decrease in the average concentration of the final Fenton's remediation solution occurred by 15 minutes of remediation, resulting in 27.22 ± 1.32% (average ± standard deviation) of 4,4'DCBP remaining (equivalent to a removal of 72.78 ± 1.32% of the 4,4'DCBP). No further significant decrease in the average concentration of the final Fenton's remediation solution occurred with further remediation up to 60 minutes.

8.5 References

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Chapter 9

Summary and Recommendations

9.1 Summary

The primary goal of this research was to perform Fenton's remediation of 4,4′-dichlorobiphenyl (4,4′DCBP) under a set of selected conditions, followed by an examination of the toxicity of the final Fenton's remediation solution and disappearance of 4,4′DCBP. The first division of research involved toxicology studies of potential Fenton's remediation byproducts and the selected parent PCB (4,4′-dichlorobiphenyl). Six commercially available chemicals considered to be representative of OH-BPs (hydroxylated biphenyls) and OH-PCBs (hydroxylated polychlorinated biphenyls), which are potential byproducts of remediation of PCBs with Fenton's reagent, were selected for study of the correlation between their chemical properties (including structure) and observed epigenetic toxicity and cytotoxicity. These six chemicals were 2-biphenylol (2BP), 3-biphenylol (3BP), 2,2′-biphenyldiol (2,2′BP), 3,3′-biphenyldiol (3,3′BP), 3-chloro-2-biphenylol (3C2BP), and 4,4′-dichloro-3-biphenylol (4,4′DC3BP). Only 3,3′-biphenyldiol and 4,4′-dichloro-3-biphenylol induced cytotoxicity within a dose range of 0 to 300 μM. Noncytotoxic doses were selected for evaluation of epigenetic toxicity. 4,4′-Dichloro-3-biphenylol was most inhibitory to GJIC at the lowest dose. 3-Chloro-2-biphenylol was least inhibitory to GJIC. 3-Chloro-2-biphenylol was less inhibitory to GJIC than 2-biphenylol because of the presence of the chlorine functional group, which appears to attenuate the toxic effect of the ortho-hydroxyl group. Although cells were capable of complete recovery of GJIC after removal of each of the chemicals, only with

2,2′-biphenyldiol and 4,4′-dichloro-3-biphenylol did the cells demonstrate partial recovery without the removal of the chemical. The dose-response results for the selected parent PCB, 4,4′DCBP, were consistent with observations that PCBs with coplanar conformations appear less likely to inhibit GJIC. For a dose range of 0 to 260.87 μM, very slight to no inhibition of GJIC was observed for 4,4′DCBP for the dose-response experiments for incubation times of 30 minutes, 2 hours, 6 hours, and 24 hours. Although 4,4′DCBP was not observed to be significantly inhibitory to GJIC, Fenton's remediation of this chemical has the potential of generating byproducts which are inhibitory to GJIC and/or cytotoxic (based on the potential of forming byproducts such as 4,4′DC3BP).

In the second division of research, the methylene blue (MB) dye test was developed as a new test that qualitatively indicates the presence of hydroxyl radicals through an immediate, distinct bleaching of the MB dye on a paper test strip after applying an aqueous sample containing hydroxyl radicals. When applied to Fenton's remediation, MB dye tests were capable of indicating the formation of hydroxyl radicals during the Fenton's reaction and indicating the completion of quenching (absence of hydroxyl radicals) during the quenching process. The presence of hydroxyl radicals was verified by benzoic acid chemical probe hydroxyl radical detection methods using thin layer chromatography (TLC) and spectrophotometric wavelength scans. The presence of hydroxyl radicals was indirectly determined by the detection of hydroxylated benzoic acids on TLC plates and a violet colored solution with a peak absorbance at a wavelength close to 520 nm. In experiments to test the performance of the methylene blue dye test strips, the age of the test strip, the presence of H_2O_2, and sample pH were determined to have no significant effect on the methylene blue dye test results.

In the third division of research, it was verified that the reaction of Fenton's reagent with the solvents (Milli-Q H_2O alone, 80/20 Milli-Q H_2O/ACN (by volume), and 50/50 Milli-Q H_2O/ACN (by volume)) in the absence of 4,4'DCBP, does not result in any toxic byproducts. For the final reaction mixture solutions of Fenton's remediation in Milli-Q H_2O alone, corresponding to the Fe^{2+}:H_2O_2 ratios 1:5, 1:20, and 1:40, no inhibition of GJIC was observed for the dose-response GJIC bioassay for incubation times of 30 minutes (0 to 300 μL) and 2 hours (300 μL). Hence, Fenton's reagent remediation with water alone is not expected to result in toxicity, and any toxicity resulting from the Fenton's reagent remediation of 4,4'DCBP can be assumed to be independent of the influence of water. The remediation procedure developed in this series of experiments was later applied to the Fenton's remediation of 4,4'DCBP. Based on the results of Fenton's remediation in Milli-Q water, an Fe^{2+}:H_2O_2 ratio of 1:20 was selected for the Fenton's remediation of 4,4'DCBP, since it appeared to result in greater production of hydroxyl radicals than 1:40 and resulted in less Fe^{3+} precipitate prior to filtration than that observed with the ratio 1:5.

Fenton's remediation of the solvents 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN (by volume) were investigated to determine the toxicological effect of ACN in combination with water as a solvent in remediation. In addition, the application of the MB dye test for Fenton's reaction in Milli-Q H_2O/ACN was examined. Although neither Milli-Q water nor 100% ACN alone resulted in bleaching or discoloration of the MB, Milli-Q H_2O/ACN solvent resulted in the discoloration of the MB unique to the 80/20 and 50/50 volume ratios examined. As was observed for Milli-Q H_2O with varying pH values, the MB dye test results for 80/20 Milli-Q H_2O/ACN solvent were not influenced by sample pH. Since hydroxyl radicals appeared to be produced throughout the reaction period, the ACN does not appear to have a scavenging effect on the hydroxyl radicals to the extent detectable by the MB dye test. For each of the final reaction mixture solutions from Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN solvents, no inhibition of GJIC was observed with 30 minutes of incubation time for volume ranges of 0 to 150 μL and 0 to 60 μL, respectively. Furthermore, for the highest tested volumes of the final reaction mixture solutions, no inhibition of GJIC was observed at 2 hours of incubation. Hence, Fenton's remediation of 80/20 Milli-Q H_2O/ACN and 50/50 Milli-Q H_2O/ACN solvents alone did not result in any toxicity, and any toxicity resulting from the Fenton's remediation of 4,4′DCBP in either of these solvents can be assumed to be independent of the influence of these solvents. The remediation procedure further developed in this series of experiments was later applied to the Fenton's remediation of 4,4′DCBP.

The fourth division, involved Fenton's remediation of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN, followed by the examination of the toxicity of the final Fenton's remediation solution and disappearance of 4,4′DCBP as a result of Fenton's remediation. The Fenton's reaction conditions were pH 3.0, temperature 23.0 °C, Fe^{2+}:H_2O_2 molar ratio 1:20, initial Fe^{2+} concentration 0.15 mM, and initial H_2O_2 concentration 3 mM. The pre-remediation concentration of 4,4′DCBP in the reaction mixture was 2 mM (or 446 ppm). The quenched and unquenched MB dye test results for Fenton's remediation of a solution of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN were identical in appearance to those observed for Fenton's remediation of 50/50 Milli-Q H_2O/ACN solvent. It can therefore be concluded that the ability of the MB dye test to indicate the presence of hydroxyl radicals does not appear to be influenced by the presence of 4,4′DCBP.

For the 30 minute dose-response GJIC bioassay of the final Fenton's remediation solution, a gradual decline in GJIC was observed with an increase in test volume. Partial inhibition of GJIC occurred for test volumes greater than 40 μL, and a maximum level of inhibition was attained at 60 μL (FOC = 0.75 ± 0.03). For the time-response GJIC bioassay of the final Fenton's remediation solution (60 μL test volume) a maximum level of inhibition was attained at 30 minutes (FOC = 0.78 ± 0.02) of chemical exposure followed by complete recovery of GJIC without removal of the chemical by 240 minutes of chemical exposure. The complete recovery of GJIC without removal of the chemical observed for the final Fenton's remediation solution was similar to the partial recovery of GJIC without removal of the chemical observed for the potential remediation byproduct 4,4′-dichloro-3-biphenylol (4,4′DC3BP). The inhibitory effects of 4,4′DC3BP previously observed might have been attenuated by its presence at a lower concentration as a component of the final Fenton's remediation solution. The final Fenton's remediation solution was determined to be more toxic (inhibitory to GJIC) than the parent PCB, 4,4′DCBP, which exhibited very slight to no inhibition of GJIC for the dose-response experiments for incubation times of 30 minutes, 2 hours, 6 hours, and 24 hours.

Fenton's remediation of 4,4′DCBP in 50/50 Milli-Q H_2O/ACN was performed for 0, 15, 30, and 60 minutes. Isooctane extracted Fenton's remediation solution samples were analyzed by GC/ECD to quantitate the disappearance of the parent PCB, 4,4′DCBP, over the period of remediation. The gas chromatograms for the extracted Fenton's remediation solutions indicated only a single major peak, the parent PCB, 4,4'DCBP, with a retention time (average ± standard deviation) of 15.24 ± 0.05 minutes. No remediation byproduct peaks or the peaks observed for the chlorinated potential remediation byproducts, 3-chloro-2-biphenylol (retention time 14.21 minutes) and 4,4'-dichloro-3-biphenylol (retention time 17.03 minutes), were observed for any of the extraction samples from the Fenton's remediation solutions. Although the final Fenton's remediation solution was determined to be more toxic (inhibitory to GJIC) than the parent PCB and the inhibitory effects were similar to those observed for the potential remediation byproduct 4,4'DC3BP, the byproducts responsible for the toxicity observed could not be ascertained by the detection methods employed. A decrease in the average concentration of the final Fenton's remediation solution occurred by 15 minutes of remediation, resulting in 27.22 ± 1.32% (average ± standard deviation) of 4,4'DCBP remaining (equivalent to a removal of 72.78 ± 1.32% of the 4,4'DCBP). No further significant decrease in the average concentration of the final Fenton's remediation solution occurred with further remediation up to 60 minutes.

9.2 Recommendations

In view of the results obtained in this research, a series of possible further studies might be conducted. For the Fenton's remediation of 4,4'DCBP, a decrease in the average concentration of 4,4'DCBP in the final Fenton's remediation solution occurred by 15 minutes of remediation with no further decrease with further remediation up to 60 minutes. One explanation for the incomplete removal observed for the Fenton's remediation of 4,4'DCBP is that the H_2O_2 was the limiting compound. The H_2O_2 could have been depleted during the remediation of the 4,4'DCBP, so that no further removal of 4,4'DCBP occurred after that observed at 15 minutes. In future studies, a variety of experiments could be performed to verify that H_2O_2 is the limiting compound responsible for the incomplete removal of 4,4'DCBP under the conditions investigated. One experiment could involve increasing the initial concentration of H_2O_2 to a value greater than 3 mM while keeping the initial Fe^{2+} concentration at 0.15 mM. Alternatively, the Fenton's reaction could be conducted under the same conditions previously investigated; however, additional H_2O_2 could be added to the reaction mixture after an initial reaction period of 15 minutes. In either experiment, greater removal of 4,4'DCBP than previously observed would indicate that H_2O_2 is the limiting compound. An alternative experiment would be to measure the residual concentration of H_2O_2 after varying Fenton's remediation times. If the H_2O_2 has been depleted when no further removal of 4,4'DCBP occurs, H_2O_2 will be verified as the limiting compound. One procedure for measuring the residual concentration of H_2O_2 is the photometric method for the determination of low concentrations of H_2O_2 by the peroxidase catalyzed oxidation of N,N-diethyl-*p*-phenylenediamine (DPD).

For the Fenton's remediation of 4,4'DCBP, since the byproducts responsible for the toxicity observed for the final Fenton's remediation solution could not be ascertained by the detection methods employed, despite a decrease in the average concentration of 4,4'DCBP in the final Fenton's remediation solution, alternative methods could be performed to detect the byproducts. The byproducts might be detectable by gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). An alternative series of experiments could involve fractionation of the final Fenton's remediation solution by high-performance liquid chromatography (HPLC) followed by identification of the fractions by nuclear magnetic resonance (NMR) and GC/MS. In addition, toxicology studies could be performed on the fractions to identify which compounds are associated with the toxicity of the final Fenton's remediation solution. These results could also be compared to the toxicology and structures of the six selected potential remediation byproducts, the parent PCB, and other OH-PCB/OH-BP standards.