CHEMICAL AND BIOLOGICAL METHODS FOR THE ANALYSIS AND REMEDIATION OF ENVIRONMENTAL CONTAMINANTS FREQUENTLY IDENTIFIED AT SUPERFUND SITES

A Dissertation

by

MELINDA CHRISTINE WILES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Toxicology

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ABSTRACT

Chemical and Biological Methods for the Analysis and Remediation of Environmental Contaminants Frequently Identified at Superfund Sites. (August 2004)

Melinda Christine Wiles, B.A., College of Wooster

Co-Chairs of Advisory Committee: Dr. Timothy Phillips
Dr. Robert Burghardt

Substantial environmental contamination has occurred from coal tar creosote and pentachlorophenol (C_5P) in wood preserving solutions. The present studies focused on the characterization and remediation of these contaminants. The first objective was to delineate a sequence of biological changes caused by chlorinated phenol (CP) exposure. In Clone 9 cells, short-term exposure to 10 μM C_5P decreased pH, GJIC, and GSH, and increased ROS generation. Long-term exposure caused mitochondrial membrane depolarization (25 μM), increased intracellular Ca^{2+} (50 μM), and plasma membrane depolarization (100 μM). Cells were affected similarly by C_5P or 2,3,4,5-C_4P, and similarly by 2,3,5-C_3P or 3,5-C_2P. Endpoints were affected by dose, time, and the number of chlorine substituents on specific congeners. Thus, this information may be used to identify and quantify unknown CPs in a mixture to be remediated.

Due to the toxic effects observed due to CP exposure *in vitro*, the objective of the second study was to develop multi-functional sorbents to remediate CPs and other components of wood preserving waste from groundwater. Cetylpyridinium-exchanged low pH montmorillonite clay (CP-LPHM) was bonded to either sand (CP-LPHM/sand)

or granular activated carbon (CP-LPHM/GAC). Laboratory studies utilizing aqueous solution derived from wood preserving waste indicated that 3:2 CP-LPHM/GAC and CP-LPHM/sand were the most effective formulations. *In situ* elution of oil-water separator effluent indicated that both organoclay-containing composites have a high capacity for contaminants identified in wood preserving waste, in particular high molecular weight and carcinogenic PAHs. Further, GAC did not add substantial sorptive capacity to the composite formulation.

Following water remediation, the final aim of this work was to explore the safety of the parent clay minerals as potential enterosorbents for contaminants ingested in water and food. Calcium montmorillonite and sodium montmorillonite clays were added to the balanced diet of Sprague-Dawley rats throughout pregnancy. Based on evaluations of toxicity and neutron activation analysis of tissues, no significant differences were observed between animals receiving clay supplements and control animals, with the exception of slightly decreased brain Rb in animals ingesting clay. Overall, the results suggest that neither clay mineral, at relatively high dietary concentrations, influences mineral uptake or utilization in the pregnant rat.

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CHAPTER I

INTRODUCTION

In response to public concern over abandoned hazardous waste sites, the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), also known as Superfund, was passed by Congress in 1980 (USDHHS, 1996; USEPA, 2002; USHR, 2002). CERCLA established regulations for closed and/or abandoned hazardous waste sites, provided for the assessment of liability for releases of hazardous waste at these sites, and established a trust fund to cover the costs of remediation if the responsible party is not identified. The most serious of these hazardous waste sites make up the National Priorities List (NPL) of Superfund sites. These sites are targeted by the Environmental Protection Agency (EPA) for long-term federal cleanup that is designed to permanently and significantly reduce the dangers associated with releases or threats of releases of hazardous substances that are serious, but not immediately life threatening. At many of these Superfund sites, groundwater quality has been adversely impacted to some degree.

1.1 Wood preserving waste

Coal tar creosote and C_5P have both been used as wood preservative pesticides in the U.S. for 100 years due to their strong fungicidal and antibacterial activity (USDHHS, 1996, 1999). Coal tar creosote is primarily used as a wood preservative and water-

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This dissertation follows the style of Toxicology and Applied Pharmacology.

proofing agent in log homes, railroad ties, telephone poles, marine pilings, and fence posts, and is a component of roofing pitch, fuel oil, lamp black, and pharmaceutical agents in the treatment of psoriasis. C_5P was once one of the most widely used biocides in the U.S., available for home and commercial use in herbicides, disinfectants, insecticides, algaecides, molluscicides, and as an ingredient in antifouling paint. In the U.S., C_5P is now considered a restricted-use pesticide and use is limited to the wood preservation industry, primarily for the treatment of utility poles. Among other contaminants, creosote has been identified at approximately 46 of 1,613 NPL sites within the U.S. (USDHHS, 2002), and pentachlorophenol (C_5P) at 313 of 1,585 sites (USDHHS, 2001). However, it is unknown how many NPL sites have been evaluated for these substances.

1.2 Coal tar creosote

1.2.1 Sources and chemical composition

The term "creosote" is used to designate a variety of products that are mixtures of many chemicals. Rarely formed in nature, creosotes are created through the high-temperature treatment of beech and other woods (i.e., beechwood creosote), or coal (i.e., coal tar creosote), or from the resin of the creosote bush (i.e., creosote bush resin) (USDHHS, 1996). Specifically, coal tar creosote designates a product created by the distillation of coal tar that consists of a variable mixture of chemicals, with some estimates reporting as many as 10,000 individual components (Pollard et al., 1992). In general, the mixture is composed of approximately 85% polycyclic aromatic

hydrocarbons (PAHs), 10% phenolics, and 5% other N-, S-, and O-heterocyclics (Nestler, 1974; Mueller et al., 1989) and may range in color from yellowish-dark green to brown (USDHHS, 1996).

1.2.2 Governmental regulations and health advisories

Both the U.S. Environmental Protection Agency (EPA) and the International Agency of Research on Cancer (IARC) classify creosote as a probable human carcinogen (Class B1 and Group 2A, respectively), while coal tar is also classified as being carcinogenic to humans by IARC (Group 1) (IRIS, 2004). Further, the National Toxicology Program classifies coal tar including coke oven emissions, coal tar, coal tar pitch, and creosotes, as known human carcinogens (NTP, 1998).

1.3 Polycyclic aromatic hydrocarbons (PAHs)

1.3.1 Sources and chemical composition

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds made up of two or more fused benzene rings. In addition to being a major constituent of creosote, the compounds may be produced as a result of incomplete combustion of wood and fuel, or combustion from motor vehicles and gas-burning engines, wood-burning stoves and furnaces, cigarette smoke (Adams et al., 1987), industrial smoke or soot, and charcoal-broiled foods (Kazerouni et al., 2001). Natural sources of PAHs include volcanoes, forest fires, crude oil, and shale oil (Lee et al., 1977; USDHHS, 1995). These contaminants frequently occur in mixtures and PAHs with greater molecular masses

(>228) are most often found partly or fully sorbed to airborne particulate matter (Lesage et al., 1987).

In general, PAHs are characterized by chemical stability, low to very low water solubility, and low to moderate volatility (USDHHS, 1995). They are relatively resistant to degradation and the half lives vary from 1 week to 2 months for those found in aquatic environments, 2 months to 2 years for soil, and 8 months to 6 years in sediment. The log *n*-octanol/water partition coefficients (log K_{ow}s) range from approximately 3.0 to 7.0 and increase with increasing molecular mass, indicating high hydrophobicity for the high molecular weight PAHs. The 16 priority PAHs identified by the U.S. EPA include: naphthalene, acenaphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene (BaP), indeno[1,2,3-*c,d*]pyrene, dibenz[*a,h*]anthracene, and benzo[*g,h,i*]perylene (Figure 1).

1.3.2 Exposure

Exposure to PAHs occurs primarily through direct inhalation of polluted air and tobacco smoke, dietary intake of smoked and other foodstuffs and polluted water, and dermal contact with soot, tars, and polluted soils (IARC, 1983). Coal tar is contained in products for the treatment of psoriasis and atopic dermatitis, however use of these products is generally limited to relatively short periods of time (Veenhuis et al., 2002). Occupational exposures occur in several industries, most notably those involving the production of aluminum, iron, and steel (Bjoresth and Becher, 1986). In the steel

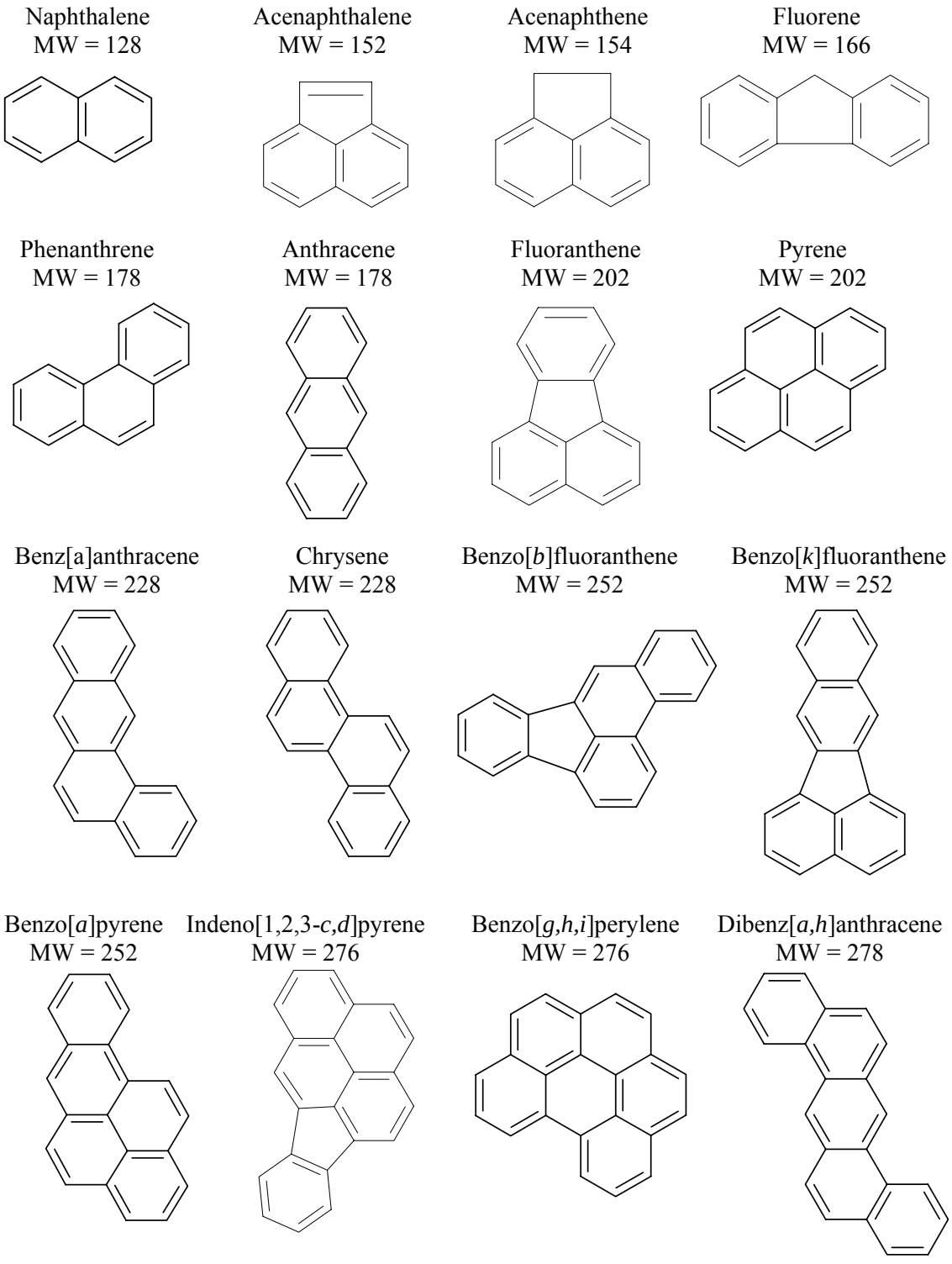


Figure 1. Two-dimensional structure and molecular weight of 16 priority PAHs identified by the U.S. EPA.

industry, coke-oven workers are heavily exposed to PAHs through both inhalation and skin contact (Grimmer et al., 1993; Boffetta et al., 1997; Pyy et al., 1997).

Dietary exposure, estimated at 2 to 3 μg/day, is the most important non-occupational source of PAHs in non-smokers (Hatterman-Frey and Travis, 1994; Mumtaz et al 1996; Vyskocil et al., 2000). Due to its high carcinogenicity and common occurrence in PAH mixtures, BaP is often used as a surrogate marker for the carcinogenic potential of these mixtures (Sun et al., 1982; Naylor et al., 1990; Culp et al., 1998; Singh et al., 1998b; Shimizu et al., 2000; Kazerouni et al., 2001; Saunders et al., 2001). High levels of BaP are found primarily in very-well-done grilled or barbecued steaks, hamburgers, and chicken with skin (4 μg/kg), while in a variety of other food products BaP levels ranging from 0.09 to 30 μg/kg have been reported (Kazerouni et al., 2001). The maximum daily BaP intake from food has been estimated to be 1 μg with the values for pyrene and phenanthrene estimated to be 3 to 10 times higher (Jacob and Seidel, 2002). Levels in drinking water range from 0.1 to 1 ng/L BaP, and correspond to a daily intake of 2 to 3 ng BaP and 10 to 20 times that amount for pyrene.

Concentrations in the atmosphere vary across geographical areas but range from 0.01 to 100 ng/m^3 BaP (Vyskocil et al., 1997) and daily inhalation from ambient air has been estimated to be approximately 15 ng BaP, 200 ng pyrene, and 100 ng phenanthrene (Jacob and Seidel, 2002). In particular, BaP levels in mainstream smoke range from 10 to 50 ng/cigarette, although the concentration in side-stream smoke is approximately 4 times higher than that in main stream smoke (IARC, 1986).

1.3.3 Absorption and metabolism

Inhaled PAHs are absorbed mainly through the bronchial epithelium. Inhalation of 500 μg/L ^{3}H-BaP for 1 h showed high concentrations of radioactivity in the nasal turbinates, trachea, larynx, lungs, tracheobronchial lymph nodes, kidneys, and liver, while lower concentrations were detected in the brain, testes, and spleen of exposed rats (Mitchell, 1982). Individuals using coal tar-containing products for the treatment of dermatological conditions showed dose-dependent excretion of PAH metabolites in urine related to the amount of coal tar applied to the skin and the amount of total skin coverage, and indicated skin absorption was not dependent upon the condition of the epidermal barrier (Veenhuis et al., 2002). Oral absorption in the lobster was assessed with ^{3}H-BaP and showed the highest levels of radioactivity in the hepatopancreas and the muscle, although radioactivity was also detected in the intestine, antennal glands and gonads (James et al., 1995).

PAHs must be activated by metabolic enzymes to produce chemically reactive epoxy- and hydroxy-derivatives in order to exert toxicity. In general, the compounds are metabolized by phase I metabolizing enzymes (i.e., cytochromes P_{450}) to form arene oxides and phenols (Hecht, 1999; Simpson et al., 2000). PAH-inducible cytochromes P_{450} include CYP1A1 (Nebert, 1989), CYP1A2 (Landi et al., 1999), CYP1B1 (Spink et al., 2002), CYP2A (Kimura et al., 1989), CYP2C (Fisslthaler et al., 1999), and CYP2S1 (Rivera et al., 2002). The arene oxides may rearrange spontaneously to form phenols or undergo hydration catalyzed by epoxide hydrolase to form dihydrodiols that may then be further conjugated by phase II metabolizing enzymes in preparation for excretion

(Shimada and Fujii-Kuriyama, 2004). PAH metabolites are predominantly conjugated with glucuronic acid or glutathione by phase II enzymes and excreted as hydroxylated or sulfated metabolites (Jongeneelen, 1997). However, a small proportion is excreted as sulfated products or even unconjugated.

The importance of metabolic activation in BaP carcinogenesis was illustrated with a study showing that both dermal and subcutaneous injection of BaP induced skin tumors in AhR (+/+) mice, but not in AhR (-/-) mice (Shimizu et al., 2000). BaP is metabolically activated by cytochromes P_{450} to form (±)-BaP-7,8-oxides (Shimada and Fujii-Kuriyama, 2004). Microsomal epoxide hydrolase catalyzes the conversion to (±)-BaP-7,8-dihydrodiols which then undergo additional oxidation catalyzed by cytochromes P_{450} and other enzymes to produce (±)-BaP-7,8-dihydrodiol-9,10-oxides. Of the four enantiomers possible in these reactions, (+)-*anti*-BaP-7,8-dihydrodiol-9,10-oxide (BPDE) is formed to the greatest extent and shows the highest carcinogenic activity.

1.3.4 Biological monitoring

The most commonly used biomarkers of PAH exposure are urinary metabolites of PAHs and PAH-DNA adducts. Urinary thioethers may also be used as biomarkers, but this method lacks sensitivity and is not suitable for routine monitoring of levels presently identified in occupational exposure settings (Reuterwall et al., 1991; Ferreira et al., 1994).

Although the composition of PAH mixtures varies, the partial pyrene content is fairly constant, and is therefore considered an indicator of the total PAH contaminant load (Butler and Crossley, 1987; Jongeneelen, 1994). In humans, the principal product of pyrene metabolism is 1-hydroxypyrene (1-OHP), which represents approximately 90% of pyrene metabolites (Jongeneelen et al., 1985, 1987; Levin et al., 1995; Wu et al., 1998). 1-OHP is further conjugated to a glucuronide and eliminated in the urine where it has been used to monitor low level exposure to environmental PAHs, despite being a metabolite of only one particular PAH (van Rooij et al., 1994; Gundel et al., 1996). The elimination half-life of 1-OHP in human urine has been estimated at 4 to 35 h (Jongeneelen et al., 1990; Buchet et al., 1992; Buckley and Lioy, 1992; van Schooten et al., 1995; Viau et al., 1995; Brzeźnicki et al., 1997), and was not affected by the presence of naphthalene or BaP in binary and ternary mixtures at customary exposure levels (Bouchard et al., 1998).

In one study in the Netherlands, 1-OHP in the urine of non-occupationally exposed males was found to be approximately 0.29 μmol/mol creatinine for non-smokers and 0.79 μmol/mol creatinine for smokers (van Rooij et al., 1994). While female residents of an industrial area of Germany showed levels of 0.24 μmol/mol creatinine in non-smokers and 0.76 μmol/mol creatinine in smokers (Gundel et al., 1996). This is the lowest reported 95th percentile levels for non-occupationally exposed individuals.

PAHs are excreted mainly through the feces, with only about 10% eliminated in the urine (Withey et al., 1991, 1992, 1993). In one study, only about 5% of an oral or dermal pyrene exposure dose was excreted as 1-OHP in the urine of human volunteers

(Viau et al., 1995), and in smokers about 1 to 4% of pyrene inhaled in cigarette smoke was excreted in the same manner (Kang et al., 1995). Despite this small percentage, studies have reported a good correlation, with a linear relationship over a large range of doses, between PAHs and urinary 1-OHP in a variety of exposure routes (Ovrebo et al., 1994; Zhao et al., 1995b; Kuljukka et al., 1997; Viau et al., 1999).

A small proportion of PAHs may react with nucleophilic sites in DNA to produce PAH-DNA adducts or with protein to produce PAH-protein adducts. Studies examining PAH exposure through smoking or in occupational settings have shown that at high BaP levels there is good correlation between urinary 1-OHP and DNA adducts (Dor et al., 1999). BaP is often used as a surrogate marker for the carcinogenic potential of PAH mixtures (Gomes and Santella, 1990; Culp et al., 1998) because it has been shown to be the major carcinogen in coal tar (Cook et al., 1933). Oxidative DNA damage is produced when the major metabolite, BPDE, reacts with DNA at the N^2-position of deoxyguanosine (BPDE-N^2-dG) (Weinstein et al., 1976). In addition, individual PAHs have been shown to interact in a synergistic manner (enhancement or inhibition) (Hermann, 1981; Munoz and Tarazona, 1993). For example, co-application of either fluoranthene or pyrene with ^{3}H-BaP to the skin of mice increased ^{3}H-BaP-DNA adducts as compared to application of ^{3}H-BaP alone. In contrast, co-application with phenanthrene decreased ^{3}H-BaP-DNA adduct formation (Rice et al., 1984). In addition to DNA adducts, BaP forms adducts with albumin (Day et al., 1992) and hemoglobin (Naylor et al., 1990), but there is substantial analytical and biological variation and, in

many cases, a clear relationship between adduct formation and PAH exposure is absent (dell'Omo et al., 1993).

1.3.5 Signs and symptoms of exposure

C57BL/6 (Ah+/+) mice exposed to 100 mg/kg of benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, BaP, indeno[1,2,3-*c,d*]pyrene, or dibenz[*a,h*]anthracene alone showed greater than 50% immunosuppression within 12 h of dosing (Silkworth et al., 1995). In another study, female B6C3F1 mice exposed for 14 days to benz[*a*]anthracene, BaP, or dibenz[*a,h*]anthracene showed significant immunosuppression, whereas exposure to anthracene or chrysene did not produce these effects (White et al., 1985). However, much of the research is focused specifically on BaP.

Both the acute and subchronic toxicities of BaP are relatively low, although *in vitro* treatment has been shown to induce cytotoxicity in a variety of cell types including vascular smooth muscle cells (Ou and Ramos, 1992) renal mesangial cells, (Bowes and Ramos, 1994) hepatocytes (Zhao and Ramos, 1995), SY5Y (Tang et al., 2003). When administered as a single gavage dose to F-344 rats, the chemical showed dose-, sex-, and time-dependent effects (Saunders et al., 2001). Within 2 and 4 h after dosing changes were observed in neuromuscular, autonomic, sensorimotor and physiological functions and motor activity was suppressed with doses above 12.5 mg/kg. Males showed greater sensitivity to the compound than females, although all treated animals recovered within 72 h. Acute exposures up to 1000 mg/kg BaP resulted in suppression of white blood

cells and subchronic exposure (90 days) resulted in additional decreases to red blood cells and hematocrit hemoglobin, and caused elevation of blood urea nitrogen and creatinine, indicating immunosuppression (Knuckles et al., 2001).

1.3.6 Phototoxicity

There is evidence that individual PAHs, as well as mixtures, are phototoxic toward microorganisms, plants, cells, and animals, and toxicity can increase up to 100 times with light exposure (Morton et al., 1942; Pelletier et al., 1997). Photooxidation of PAHs leads to production of the respective quinone moieties, ring opening products, or hydroxyl-substituted products (Yu, 2002). Photomutagenicity tested with *Salmonella typhimurium* TA102 and light without metabolic activation indicated that of the 16 priority PAHs anthracene, benz[*a*]anthracene, benzo[*g,h,i*]perylene, benzo[*a*]pyrene, indeno[1,2,3-*c,d*]pyrene, and pyrene were strongly photomutagenic, while acenaphthene, acenaphthalene, benzo[*k*]fluoranthene, chrysene, and fluorene were weakly photomutagenic (Yan et al., 2004). In particular, mammalian cells exposed to BaP and fluorescent light showed 3 to 10 fold increases in 8-oxydeosyguanosine (8-OHdG) adducts than were induced with BaP alone (Mauthe et al., 1995).

1.3.7 Mutagenicity

Vapor escaping from creosote and coal tar tested positive in the taped-plate assay, a modification of the Ames assay designed for detection of volatile mutagens, in *Salmonella typhimurium* strains TA98 and TA100 in the presence of S9 mix (Bos et al.,

1985). In addition, some of the individual PAH components, fluoranthene, phenanthrene, and pyrene have tested positive for mutations with the same assay (Bos et al., 1987, 1988). Fluoranthene, pyrene, and BaP were shown to be highly mutagenic in reverse mutation assays in *Salmonella typhimurium* strains TA97 in the presence of metabolic activation (Sakai et al., 1985; Bos et al., 1988), while dibenz[*a,h*]anthracene, chrysene, indeno[*g,h,i*]perylene benzo[*k*]fluoranthene, and benzo[*b*]fluoranthene have tested positive for reverse mutations in strains TA100 and TA98 (McCann et al., 1975; LaVoie et al., 1979, 1980). Dibenz[*a,h*]anthracene has also induced forward mutations in Chinese hamster embryo cells (Krahn and Heidelberger, 1977) and Syrian hamster embryo cells (Pienta et al., 1977), and BaP has exhibited mutagenicity in *E. coli* (Bernelot-Moens, 1990) and Hep G2 cells (Diamond et al., 1980).

1.3.8 Genotoxicity

Increased chromosome breaks following exposure to PAH mixtures *in vitro* were detected in Chinese hamster cells (Kato et al., 1969). In separate studies using pure compounds, anthracene, fluoranthene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, BaP, indeno[1,2,3-*c,d*]pyrene, benzo[*g,h,i*]perylene, and dibenz[*a,h*]anthracene were shown to be genotoxic in *E. coli* PQ37 in the presence of metabolic activation (Mersch-Sundermann et al., 1992) and anthracene, naphthalene, and phenanthrene were shown to be genotoxic in *Drosophila melanogaster* (Dalgado-Rodriguez et al., 1995). Epidemiologic studies show increased sister chromatid exchanges (Buchet et al., 1995), DNA-strand breaks (Popp et al., 1997),

and PAH-DNA adducts (van Schooten et al., 1995) in lymphocytes of workers occupationally exposed to PAH mixtures.

1.3.9 Carcinogenicity

Evidence demonstrating a clear association between PAH exposure and cancer is difficult to obtain with certainty due to the complexity and inconsistency of the mixtures. *In vitro* analyses of primary or secondary cultures of Syrian hamster embryo cells suggested that PAH exposure produced morphological transformations associated with a capacity to induce tumors when inoculated into adult hamsters (Berwald and Sachs, 1965). In mouse skin, the application of coal tar, creosote or bitumen products led to PAH-DNA adducts in both the skin and lungs (Schoket et al., 1988). A study in newborn female mice exposed to a PAH-enriched exhaust by inhalation over 10 months showed increased lung tumors with a dose-dependent increase in malignant lung tumors (Schulte et al., 1994), and lifetime ingestion studies of coal tar in the diet of B6C3F1 mice showed a dose-dependent increase in tumor incidence in the lung, forestomach, and small intestine (Culp et al., 1998).

In humans, epidemiological information has been used to link PAHs to cancer following inhalation and dermal exposure but data is inadequate in the case of oral exposure, although the carcinogenic potential for this route is anticipated. Occupational exposure to PAHs has been linked to bladder cancer in aluminum plant workers (Tremblay et al., 1995; Romundstad et al., 2000), lung and bladder cancers in coal gasification workers, in particular coal carbonization workers (Doll, 1952; Doll et al.,

1965), and lung cancer in coke production workers (Wu et al., 1988; Costantino et al., 1995) and iron and steel foundry workers (Sorahan et al., 1994). Further, several 5-ring PAHs frequently identified in the mixtures are classified as possible or probable human carcinogens by various U.S. and international agencies. Specifically, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, BaP, indeno[1,2,3-*c,d*]pyrene, and dibenz[*a,h*]anthracene are classified as probable human carcinogens by the U.S. EPA (IRIS, 2004).

Benzo[*b*]fluoranthene, benz[*a*]anthracene, dibenz[*a,h*]anthracene, chrysene, BaP, and indeno[1,2,3-*c,d*]pyrene have shown positive results for initiating activity and complete carcinogenic activity in mouse skin-painting assays (Cook et al., 1933; Wynder and Hoffman, 1959; IARC, 1973; LaVoie et al., 1982; Rice et al., 1985, 1986), while benzo[*k*]fluoranthene yielded positive results only for initiating activity following promoting treatments with croton resin (LaVoie et al., 1982). A dose-response relationship was observed for carcinomas in a lung implantation study in Osborne-Mendel rats following treatment with pure BaP, benzo[*b*]fluoranthene, indeno[1,2,3-*c,d*]pyrene, or benzo[*k*]fluoranthene (Deutsch-Wenzel et al., 1983). Gavage treatment with benz[*a*]anthracene has yielded increased lung tumors (Klein, 1963) and forestomach papillomas (Bock and King, 1959) in mice, and dibenz[*a,h*]anthracene produced lung and mammary carcinomas and lung adenomas in mice with administration in an olive oil vehicle in drinking water (Snell and Stewart, 1962, 1963) or by gavage (Biancifiori and Caschera, 1962). Benzo[*a*]pyrene added to the diet of male and female CFW-Swiss mice or Sprague-Dawley rats has been shown to produce

increased forestomach tumors (Neal and Rigdon, 1967; Brune et al., 1981), and an elevated incidence of respiratory tract and upper digestive tract tumors upon intratracheal instillation or inhalation in guinea pigs, hamsters, and rats (USEPA, 1991).

1.3.10 LOAEL and NOAEL

The lowest-observed-effects-level (LOAEL) and the no-observed-adverse-effects-level (NOAEL) have been determined for the pure forms of individual PAHs not identified as possible human carcinogens in a 90 day subchronic study in CD-1 mice (Table 1) (IRIS, 2004). In addition, the U.S. EPA has used the data to calculate the oral reference dose (RfD), defined as an estimate of the daily oral exposure to the human population over a lifetime that is likely to be without an appreciable risk of deleterious effects even in sensitive populations. However, single contaminant exposure is rare.

Epidemiologic studies have been carried out to determine the LOAEL and NOAEL for specific genotoxic effects in occupationally exposed workers. The earliest biological effects in cokeoven workers in Belgium heavily exposed to PAHs were high-frequency cells and sister chromatid exchanges (HFC-SCE) in lymphocytes (Buchet et al., 1995). The NOAEL in which no increased HFC-SCE were observed in non-smoking workers was found to be 2.7 μg/g creatinine (1.4 μmol/mol). The LOAEL in German cokeoven workers exposed to average BaP concentrations of 1.7 $\mu g/m^3$ was 3.6 μg/g creatinine (1.9 μmol/mol) (Popp et al., 1997). This level was measured as the lowest exposure concentration that showed increased DNA-strand breaks in lymphocytes. An additional study measuring PAH-DNA adducts and urinary 1-OHP

Table 1
LOAEL, NOAEL, RfD, and carcinogenicity characterization for 16 priority PAHs based on available data by the U.S. EPA

	LOAEL[a] (mg/kg/day)	NOAEL[a] (mg/kg/day)	Criteria for NOAEL (mg/kg/day)	RfD[b] (mg/kg/day)	***Weight of evidence characterization***[c]
Naphthalene	200	100	decreased mean terminal body weight in males	$2x10^{-2}$	D
Acenaphthalene					D
Acenaphthene	350	175	hepatoxicity	$6x10^{-2}$	unavailable
Fluorene	250	125	decreased red blood cell packed cell volume and hemoglobin	$4x10^{-2}$	D
Phenanthrene					D
Anthracene	None	1000	no observed effects at highest dose tested	$3x10^{-1}$	D
Fluoranthene	250	125	nephropathy, increased liver weights, hematological alterations, and clinical effects	$4x10^{-2}$	D
Pyrene	125	75	kidney effects including renal tubular pathology, and decreased kidney weights	$3x10^{-2}$	D
Benz[*a*]anthracene					B2
Chrysene					B2
Benzo[*b*]fluoranthene					B2
Benzo[*k*]fluoranthene					B2
Benzo[*a*]pyrene					B2
Indeno[1,2,3-*c,d*]pyrene					B2
Dibenz[*a,h*]anthracene					B2
Benzo[*g,h,i*]perylene					D

[a] The no-observable-adverse-effects levels (NOAEL) and the lowest-observable-adverse-effects levels (LOAEL) were calculated by the U.S. EPA based on a 90 day subchronic oral exposure study in CD-1 mice (USEPA, 1989; IRIS, 2004).
[b] The oral reference dose (RfD) is a calculated estimate of the daily exposure to the human population over a lifetime that is likely to occur without an appreciable risk of deleterious effects (IRIS, 2004).
[c] The U.S. EPA weight of evidence characterization: B2 = probable human carcinogen, D = not enough data to assign carcinogenicity.

determined that the LOAEL for genotoxic effects in primary aluminum plant workers was 3.8 μmol/mol creatinine (van Schooten et al., 1995).

1.3.11 Governmental regulations and health advisories

IARC classifies benz[*a*]anthracene and benzo[*a*]pyrene as probable human carcinogens (Group 2A), benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and indeno[1,2,3-*c,d*]pyrene as possible human carcinogens (Group 2B), and lists anthracene, benzo[*g,h,i*]perylene, chrysene, fluoranthene, fluorene, phenanthrene, and pyrene as having not enough information to assess carcinogenicity (Group 3) (IRIS, 2004). The U.S. EPA classifies benz[*a*]anthracene, indeno[1,2,3-*c,d*]pyrene, dibenz[*a,h*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*a*]pyrene as probable human carcinogens (Group B2) and lists fluoranthene, anthracene, and, like IARC, acenaphthalene as having not enough information to assess carcinogenicity (Group D) (Table 1). Further, the EPA has derived a maximum contaminant level (MCL) in drinking water for selected PAHs. The MCL is 0.1 μg/L for benz[*a*]anthracene, 0.2 μg/L for benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and chrysene, 0.3 μg/L for dibenz[*a,h*]anthracene, and 0.4 μg/L for indeno[1,2,3-*c,d*]pyrene. The European standard for PAHs in drinking is 0.2 μg/L (WHO, 1971, 2001).

1.3.12 Analytical measurement

Analytical methodology has been developed to measure PAHs in soil (Lundstedt et al., 2000; Szolar et al., 2002), marine sediment (Pino et al., 2000), atmospheric particulate matter (Piñeiro-Iglesias et al., 2000), food (Grimmer and Bohnke, 1975; Wang et al., 1999), fly ash (Arditsoglou et al., 2003), biological tissues (Modica et al., 1982; Ali and Cole, 1998), plasma (Singh et al., 1998a), surface waters (Crozier et al., 2001), drinking water (Davis et al., 1999), and urine (Jongeneelen et al., 1987, 1990). Techniques include: solvent extraction (Gay et al., 1980; Modica et al., 1982), Soxhlet extraction (Szolar et al., 2002; Arditsoglou et al., 2003), static subcritical water extraction (McGowin et al., 2001), pressurized liquid extraction (PLE) to extract ketones of PAHs (Zdrahal et al., 2000; Lundstedt et al., 2000), fluidized-bed extraction (Gfrerer et al., 2002), and ultrasonication techniques (Kayali-Sayadi et al., 2000; Rababah and Matsuzawa, 2002).

Newer quantitative methodology utilizing high temperatures and pressures to take advantage of increased analyte solubility and desorption under these conditions can accelerate the extraction procedure, but may also lead to the degradation of some compounds. Accelerated solvent extraction (ASE) uses conventional solvents (Fisher et al., 1997; Tao et al., 2002), whereas supercritical fluid extraction (SFE) replaces these hazardous solvents with CO_2 (Langenfeld et al., 1994, Librando et al., 2004). Microwave solvent extraction (Vázquez-Blanco et al., 2000; Shu et al., 2003), specifically microwave assisted extraction (MAP^{TM}) (Li et al., 1996), reduces solvent usage and shortens extraction times by heating solid samples quickly in a polar solvent

via microwave energy. However, water interferes so samples must be standardized for moisture content (Shu and Lai, 2001).

Solid phase extraction (SPE) may be used for purification of sample extracts (Dabrowska et al., 2003) or extraction of PAHs from aqueous solution (Biziuk et al., 1996; Singh et al., 1998b; Crozier et al., 2001; Ake et al., 2003). SPE media may include C_{18} (Crozier et al., 2001; McGowin et al., 2001), florisil (Singh et al., 1998b), alumina (Modica et al., 1982), silica (Gay et al., 1980), XAD (Biziuk et al., 1996), glass fiber (Urbe and Ruana, 1997), and polystyrene-divinylbenzene (Bernal et al., 1997). Airborne PAHs are frequently collected on XAD resin, while those sorbed to particulate matter are collected on glass fiber filters and desorbed with solvent (Heikkila et al., 1987; Brzeźnicki et al., 1997).

PAH identification and quantification is primarily carried out using gas chromatography – mass spectrometry (GC/MS) (Lee et al., 1977; Gfrerer et al., 2002; Tao et al., 2002; Librando et al., 2004) or high performance liquid chromatography (HPLC) coupled with fluorescence detection (Kayali-Sayadi et al., 2000). However, HPLC separation may also be coupled to UV detection (Pino et al., 2000) and GC separation may be coupled to a flame ionization detector (FID) (Hyotylainen and Oikari, 1999), ion trap mass spectrometry (GC-ITMS) (Crozier et al., 2001) or time of flight mass analysis (GC-TOF-MS) (Davis et al., 1999; Zou et al., 2003). Liquid chromatography (LC) has also been coupled to MS (Dark et al., 1977) or to electrospray ionization mass spectrometry (LC-ESI-MS) (Takino et al., 2001).

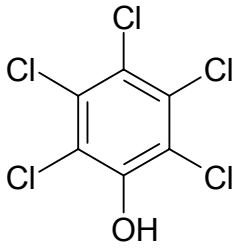
As a measure of oxidative DNA damage, PAH-DNA adducts in blood and tissue are frequently detected using ^{32}P- (Ovrebo et al., 1990; Kondraganti et al., 2003) or ^{35}S- (Baird et al., 1993) postlabeling with radioactivity counting, especially in the case of the larger, bulkier compounds. Coupling to HPLC may increase sensitivity, improve resolution, and allow for quantification of specific adducts (Miege et al., 1999). For specific DNA adducts, enzyme-linked immunosorbent assay (ELISA) is used to detect antibodies in serum bound to BPDE-DNA adducts (Gomes and Santella, 1990) while radioimmunoassay (Hutcheon et al., 1983) and ultrasensitive enzyme radioimmunoassay (USERIA) measure the immune response of BPDE-DNA in the presence of rabbit anti-serum (Ovrebo et al., 1990). Synchronous fluorescence spectroscopy (SFS) measures the physical properties (e.g., fluorescence) of the carcinogen-DNA adduct, as their aromaticity makes PAHs highly fluorescent (Weston et al., 1993). Immunoaffinity solid-phase extraction columns may be used for separation and purification prior to any of these techniques (Miege et al., 1999).

1.4 Pentachlorophenol (C_5P)

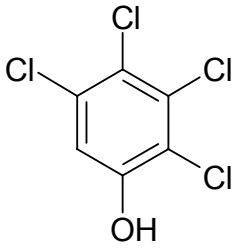
1.4.1 Sources and chemical composition

Chlorinated phenols (CPs) constitute a series of 18 mono-, di-, tri-, and tetra-chlorinated isomers and one penta-chlorinated compound (Figure 2) (Ahlborg and Thunberg, 1980; Seiler, 1991; IARC, 1991; USDHHS, 1999). As a group of industrial chemicals they have been used worldwide as fungicides, bactericides, herbicides, insecticides, and precursors in the synthesis of other pesticides since the early 1930s.

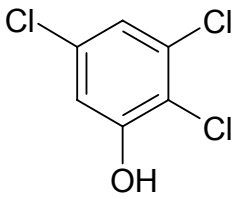
Pentachlorophenol
MW = 264



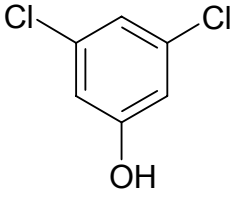
2,3,4,5-Tetrachlorophenol
MW = 230



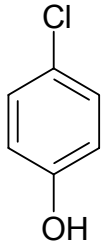
2,3,5-Trichlorophenol
MW = 196



3,5-Dichlorophenol
MW = 162



4-Chlorophenol
MW = 128



Phenol
MW = 94

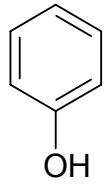


Figure 2. Two-dimensional structure and molecular weight of 5 chlorinated phenols (CPs) selected as representative positional isomers and phenol.

During this period, CP production has lead to well-known industrial hygiene incidents, for example the tragic accident in Seveso, Italy in 1976, but otherwise has not been blamed for major environmental problems. Technical grade pentachlorophenol (C_5P) has been shown to contain a large number of impurities depending on the method of manufacture including various lower chlorinated phenols, polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzo-*p*-furans, chlorinated cyclohexenones, chlorinated cyclohexadienones, chlorinated phenoxyphenols, and hexachlorobenzene, and many of the health effects associated with CP exposure may be attributed to these impurities. However, improved analytical methodology revealing the ubiquitous occurrence of C_5P has led to worldwide concern over the safety of these compounds and their persistence in the environment.

Although CPs containing less than three chlorine substituents are of limited use today, C_5P has been used extensively in the wood preservation industry as a general cytotoxic agent to control discoloration and deterioration of newly cut lumber resulting from mold, mildew, and termite infestation (Ahlborg and Thunberg, 1980). It has been estimated that 97% of C_5P usage in the U.S. is as a wood preservative (Eckerman, 1986), although the compound is now regulated as a restricted-use pesticide and is no longer contained in wood preserving solutions or in insecticides or herbicides available for home and garden use (USDHHS, 2001). The compound is restricted to use in the treatment of utility poles, railroad ties, and wharf pilings. Freshly cut wood is either spray-treated with an aqueous solution of sodium pentachlorophenate, or C_5P dissolved

in organic solvent is applied by high-pressure impregnation in a closed system or by dipping in open vats (Seiler, 1991).

1.4.2 Exposure

Exposure may occur by ingestion of contaminated food and water, inhalation, or percutaneously, either through direct contact with C_5P solutions in occupational settings or through treated goods and bactericidal soaps (Gebefugi et al., 1979; Williams, 1982; Embree et al., 1984). Although the compound is both photo- and microbially-degraded under appropriate conditions (Steiert et al., 1987), C_5P is highly persistent with a reported half-life of up to 5 years. Concern has been focused mainly on chronic poisoning in occupational settings (Thind et al., 1991; Coloso et al., 1993; Dimich-Ward et al., 1996; Hryhorczuk et al., 1998; Walls et al., 1998; Gorman et al., 2001) and in individuals living in C_5P-treated log homes (Cline et al., 1989). However, the food chain, especially fruits, vegetables, and grains, is responsible for 99.8% of human exposure in people not occupationally or intentionally exposed (Newsome et al., 1984; Coad and Newhook, 1992; Hattemer-Frey and Travis, 1989; Fries et al., 2002).

1.4.3 Absorption and metabolism

CPs, in particular C_5P, are readily absorbed through the skin, lungs, and gastrointestinal tract and exhibit strong biological effects. However, studies conducted in the monkey (Braun and Sauerhoff, 1976), Sprague-Dawley rat (Braun et al., 1977; Reigner et al., 1991), male Wistar rat (Meerman et al., 1983), and male B6C3F1 mouse

(Reigner et al., 1992b), indicate that absorption in the gastrointestinal tract is affected by the gavage vehicle. In particular, corn oil has been shown to significantly delay absorption (Chidgey and Caldwell, 1986; Yuan et al., 1991) and can cause erratic absorption (Braun et al., 1977; Reigner et al., 1991).

The biological half-life of C_5P is relatively short and the compound is excreted primarily in the urine. Some studies suggest biphasic elimination kinetics in rats and mice, specifically, an initial quick elimination phase with a short half-life of 6 to 27 h followed by a second slower elimination phase with a half-life of 33 to 374 h (Larsen et al., 1972; Braun et al., 1977). Overall, 90% was eliminated within 168 h. However, other experiments in F-344 rats suggest a monophasic model for absorption and elimination kinetics in plasma (Yuan et al., 1994). Following a single gavage dose, the estimated absorption half-life was 1.3 h while the elimination half-life was approximately 5.6 h in males and 9.5 h in females. Of interest, although the absorption and elimination half-lives were not significantly affected, bioavailability of C_5P was shown to be lower when administered in the diet rather than by gavage.

Studies of rats dosed with ^{14}C-C_5P indicate that the target organ in both mice and rats is the liver, followed by the kidneys and blood (Larsen et al., 1972; Braun et al., 1977; NTP, 1989, 1999). Mice exhibit greater sensitivity than rats to the compound (NTP, 1999; Tsai et al., 2002). Pathological signs following chronic exposure include increased liver and kidney weights (Johnson et al., 1973; Schwetz et al., 1978; Renner et al., 1987; Umemura et al., 1996), pigmentation in the liver and kidneys (Schwetz et al., 1978), and hepatocyte degeneration (Chhabra et al., 1999). In one study, chronic C_5P

ingestion in male Wistar albino rats lead to irreversible dose- and time-dependent neurotoxicity that culminated in the degeneration of myelinated nerve fibers to block nerve conduction and produce morphological nerve damage, and damage to glomerular structure and function in the kidneys (Villena et al., 1992). In blood, C_5P has been shown to reduce hemoglobin content both in the hematocrit and in the number of erythrocytes (Renner et al., 1987).

C_5P is not extensively metabolized in laboratory animals and there is only a slight tendency toward bioaccumulation (Bernard et al., 2002). Metabolism occurs primarily through the action of cytochromes P_{450} to form various quinones which are proposed to be the toxic intermediates in C_5P-induced damage (Ahlborg et al., 1978; van Ommen et al., 1988; Renner and Hopfer, 1990; Lin et al., 1999). Redox cycling between quinones and their semiquinone forms creates reactive intermediates that may covalently bind to macromolecules (Witte et al., 1985; Waidyanatha et al., 1996; van Ommen et al., 1988; Ehrlich, 1990; Lin et al., 1999, 2001a; Bodell and Pathak, 1998) and/or generate reactive oxygen species (O'Brien, 1991; Monks et al., 1992; Monks and Lau, 1992; Bolten et al., 2000) that may lead to oxidative damage to genomic DNA (Jansson and Jansson, 1992; Naito et al., 1994; Dahlhaus et al., 1994, 1995, 1996; Sai-Kato et al., 1995; Umemura et al., 1999; Witte et al., 2000; Lin et al., 2001b). In particular, increased lipid peroxidation was detected in isolated rat hepatocytes following C_5P treatment (Suzuki et al., 1997) and mice exposed to doses ranging from 300 to 1200 mg/kg in the diet for up to 4 weeks showed persistent induction of cell proliferation and increased 8-OHdG formation in liver DNA, indicating oxidative DNA damage

(Umemura et al., 1996). Further, *in vitro* analyses indicate that C_5P interferes with microsomal electron transport between NADPH-P_{450} reductase and cytochromes P_{450} *in vitro,* which may lead to enhanced production of reactive metabolites due to altered detoxification patterns of some P_{450} substrates (Arrhenius et al., 1977a, b).

CPs effectively partition into the phospholipid bilayers of mitochondrial membranes, which increases the permeability of protons to alter proton transport through the membrane. This leads to dissipation of the energy gradient and the uncoupling of oxidative phosphorylation (Weinbach, 1954; Weinbach and Garrbus, 1969; Shannon et al., 1991). Incorporation of inorganic phosphate into ATP is prevented without blocking the electron transport chain, leading to depletion of cellular ATP (Aschman et al., 1989). Thus, cells continue to respire but are quickly depleted of the ATP required for energy utilization. Clinically, this is manifested as a decrease in body weight (Chhabra et al., 1999) and fever resulting from the increased metabolic activity that generates heat (Byard, 1979). Studies of structure-activity relationships among a series of chlorinated phenols showed that this effect increases with an increase in chlorination (Farquharson et al., 1958; Narasimhan et al., 1992). An increase in toxicity with a concomitant increase in chlorine substituents has also been observed in studies utilizing *Hydra attenuata* (Mayura et al., 1991), human embryonic palatal mesenchymal cells (Zhao et al., 1995a), BF-2 cells (Babich and Borenfreund, 1987), bacteria including *Burkholderia* species *Rasc c2* and *Pseudomonas fluorescens* (Boyd et al., 2001), and bovine spermatozoa (Seibert et al., 1989).

Not only do CPs interfere with phase I metabolizing enzymes, but the compounds also inhibit phase II metabolism (Carlsson, 1978; Aschman et al., 1989) including glutathione-*S*-transferase and microsomal epoxide hydroxylase (Moorthy and Randerath, 1996). Specifically, C_5P has been found to strongly inhibit sulfotransferase activity in rat and mouse liver cytosol (Mulder and Scholtens, 1977; Boberg et al., 1983; Meerman et al., 1983), Ca^{2+}-ATPase (Janik and Wolf, 1992), and glycolytic phosphorylation (Weinbach, 1956).

1.4.4 Biological monitoring

In Sprague-Dawley rats, orally administered ^{14}C-C_5P is eliminated in the urine largely unchanged (48%), and, to a lesser extent, as tetrachlorohydroquinone (TCHQ) (10%) and C_5P-glucuronide (6%) (Braun et al., 1977). In humans, the compound is excreted in the urine primarily as C_5P-glucuronide and, to a lesser extent free C_5P (Uhl et al., 1986; Reigner et al., 1992a), whereas experiments in rhesus monkeys show that the entire dose is excreted unchanged (Braun and Sauerhoff, 1976). Based on first order kinetics, the elimination half-life in urine for 4 volunteers receiving a single dose of 3.9, 4.5, 9, or 18.8 mg was calculated at 20 days (Uhl et al., 1986). In a separate case of accidental exposure in which a worker had dipped his hands into a vat of a 0.4% solution of C_5P for 10 min, an elimination half-life in urine of 16 days was calculated (Bevenue et al., 1967).

C_5P is most readily measured in urine or blood, usually by gas chromatography following acid hydrolysis (Jorens and Schepens, 1993). Good correlation exists between

levels in urine and blood. C_5P in the urine of individuals in unexposed populations has been measured at 1 to 12 μg/g creatinine, while those living in log homes ranged from 17 to 190 μg/g creatinine in one study and 1 to 1179 μg/g creatinine in a separate study (Grimm et al., 1986; Cline et al., 1989). Employees exposed as sprayers showed concentrations ranging from 11 to 1260 μg/g creatinine (Jones et al., 1986).

Serum levels, like those in urine, are generally higher in occupationally exposed individuals than in controls but are frequently in the same range as those living in treated log homes. In one study, C_5P levels in serum ranged from 15 to 75 μg/L in control populations and 69 to 1340 μg/L in individuals living in log homes (Cline et al., 1989). The highest serum levels in occupationally exposed workers were found to be in employees involved in chemical packaging, with concentrations ranging from 26 to 84,900 μg/L, almost 700 times higher than person involved in the construction of log homes. Serum levels less than 30 μg/L are generally not associated with outward symptoms of exposure, while those ranging from 30 to 100 μg/L are generally associated with mild symptoms, and those greater than 100 μg/L are indicative of more serious exposure.

1.4.5 Signs and symptoms of exposure

Several cases of acute accidental, suicidal, or occupational C_5P poisoning in humans have been reported. Acute poisoning may present as high fever, profuse sweating, increased heart rate, and difficulty breathing (Weinbach, 1954) as well as restlessness, agitation, muscle twitching, tremors, epigastric tenderness, leg pain, and

increased respiration rate (Haley, 1977). Sudden death may occur as a result of cardiac arrest and victims usually show marked rigor mortis (Wood et al., 1983). Symptoms of chronic poisoning are vague and vary considerably in different reports but can include chloracne, anorexia, weight loss, general weakness, dizziness, obstinate headache, personality changes, or anxiety (Jorens and Schepens, 1993). Occupational exposures to technical grade C_5P has resulted in various skin and mucous membrane disorders including conjuctivitis, chronic sinusitis, and chronic upper respiratory conditions (Klemmer et al., 1980; O'Malley et al., 1990). The incidence of chloracne, although probably due to contaminants present in technical grade C_5P, was shown to be highest among individuals having had direct skin contact with the compound (Mathias, 1988). The minimal lethal dose has been estimated to be 29 mg/kg body weight (Ahlborg and Thornberg, 1980).

1.4.6 Mutagenicity

Mixed results have been obtained with mutagenicity testing of C_5P in bacterial systems, but results largely indicate that the compound is not mutagenic. Positive results for mutagenicity were obtained with *Bacillus subtilis* strains H17 rec^+ versus M45 rec^- (Matsui et al., 1989), following negative results in an earlier study (Shirasu et al., 1976). Although C_5P was not shown to increase the frequency of revertant colonies in either the absence or presence of metabolic activation in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 (Haworth et al., 1983). Additional negative results were

obtained in separate studies by Simmon et al. (1977), Moriya et al. (1983), and McConnell (1991).

1.4.7 Genotoxicity

In vitro studies in mammalian cells suggest that C_5P does not produce point mutations (Hattula and Knuutinen, 1985; Jansson and Jansson, 1986), although the cytotoxicity of this compound may bias conclusions. Weakly positive results in Chinese hamster CHO cells suggest C_5P induces chromosomal aberrations and sister-chromatid exchanges (Galloway et al., 1987), but findings were negative in a separate study in Chinese hamster lung fibroblasts (Ishidate, 1988). Further, two studies report negative results for genotoxicity in *Drosophila melanogaster* (Vogel and Chandler, 1974; Ramel and Magnusson, 1979). *In vivo* studies were positive for chromosomal aberrations in lymphocytes of 22 occupationally exposed workers (Bauchinger et al., 1982; Schmid et al., 1982), but negative in a separate study of 20 workers (Ziemsen et al., 1987) and both studies were negative for sister-chromatid exchanges. Taken together, these data suggest that C_5P exposure may lead to increased chromosomal aberrations but not point mutations or sister chromatid exchanges.

In contrast to C_5P, TCHQ has been shown to be genotoxic (Witte et al., 1985), as the metabolite both binds to DNA and induces DNA strand breakage in mammalian cells (Juhl et al., 1985; Witte et al., 1985; Carstens et al., 1990; Wang and Lin, 1995). In rats, TCHQ depleted glutathione content and induced glutathione conjugate formation, p53 protein accumulation (Wang et al., 1997), protein adduct formation (Lin et al., 1999),

oxidative DNA lesions (Dahlhaus et al., 1996), and lipid peroxidation (Wang et al., 2001). In addition, unlike C_5P-treated cells, TCHQ-treated Hep G2 cells showed significantly decreased CAS gene expression, increased *hsp*-70 gene expression, and a decrease in the bcl-2/bax protein ratio, all factors indicative of apoptosis (Wang et al., 2001). Still, it is unclear as to whether this compound is a major metabolite in humans.

1.4.8 Carcinogenicity

Several carcinogenicity studies have been performed using laboratory animals. While C_5P has been shown to be carcinogenic in both male and female mice (NTP, 1989; McConnell et al., 1991), the chemical failed to induce tumors in rats (Schwetz et al., 1978; NTP, 1999). Mice exposed to technical grade C_5P in the diet (approximately 18, 35, or 116 mg/kg/day) exhibited increased incidence of hepatocellular adenomas and carcinomas, adrenal pheochromocytomas, and hemangiosarcomas of the spleen and liver (NTP, 1989; McConnell et al., 1991). In rats, increased 8-OHdG and direct adducts in hepatic DNA were detected following chronic exposure, but similar conditions did not lead to increases in liver cancer (Lin et al., 2002). Although C_5P has been reported to covalently bind to microsomal protein and DNA, it does not appear to produce real DNA damage (van Ommen et al., 1986). Thus, C_5P is believed to be a promoter, but not an initiator in liver carcinogenesis (NTP, 1989; McConnell, 1991; Sai et al., 1998).

In addition, *in vitro* studies suggest that C_5P interferes with microsomal electron transport between NADPH-P_{450} reductase and cytochromes P_{450}, which may lead to enhanced production of reactive metabolites when detoxification patterns of some P_{450}

substrates are altered (Arrhenius et al., 1977a, b). Because this inhibition would then favor flavin-mediated oxygenation over cytochrome P_{450}-dependent reactions, C_5P theoretically has the potential to increase the toxicity and carcinogenicity of other chemicals.

In humans, epidemiological studies have linked occupational exposure to C_5P with increased occurrence of malignant lymphoma and leukemia (Roberts, 1983; Roberts, 1990), soft tissue sarcomas (Choudhury et al., 1986; Hardell and Sandstrom, 1979), and non-Hodgkin's lymphoma (Pearce et al., 1986; Hardell et al., 1994). In addition, occupational exposure to C_5P has been linked to Hodgkin's disease in 3 siblings and a first cousin, out of 5 family members exposed through contact with wood immersed in C_5P (Greene et al., 1978).

1.4.9 Teratogenicity and embryo/fetotoxicity

C_5P is not teratogenic in rats (Courtney et al., 1976; Schwetz et al., 1974, 1978; Mayura et al., 1991), but is embryo/fetotoxic (Exon and Koller, 1982; Welsh et al., 1987). When ^{14}C-C_5P was administered orally on day 15 of pregnancy, the maximum amount of radiolabel in maternal blood was 1.1% of the dose, and never exceeded 0.3% in the placenta or 0.1% in the fetuses (Larsen et al., 1975). Thus, very little of the compound crosses the placental barrier. However, in later studies it was shown to significantly reduce litter size, survival to weaning, neonatal body weight, and weight at weaning (Schwetz et al., 1978). Further, gavage doses ranging from 0 to 30 mg/kg/day on days 6 to 18 of gestation to pregnant rabbits resulted in reduced maternal body weight

gain at middle and high doses, and transient weight loss and reduced feed consumption at high doses (Bernard et al., 2001). In this study, no effect was observed on embryo/fetal development, thus the compound was not found to be a developmental toxicant.

1.4.10 LOAEL and NOAEL

C_5P has been found to be highly toxic to many species of fish. The 96 h median lethal concentration (LC_{50}) in Chinook (68 ng/mL), rainbow trout (52 ng/mL), fathead minnow (205 ng/mL), channel catfish (68 ng/mL) and bluegill sunfish (32 ng/mL) have been determined (Johnson and Finley, 1980), as well as in the freshwater fish *Heteropneustes fossilis* (0.58 mg/L), *Clarias batrachus* (0.64 mg/L), and *Channa punctatus* (0.77 mg/L) (Farah et al., 2004). The 50% lethal body residue (LBR_{50}), the concentration of a compound to cause 50% mortality in a population over a given time, for 48 h exposure in the midge (*Chironomus riparius* larvae) was 0.15 μmol/g wet weight and 0.45 to 0.66 μmol/g wet weight for the oligochaete worm (*Lumbriculus variegatus*) (Kukkonen, 2002). In laboratory rodents, the acute oral LD_{50} ranged from 117 mg/kg body weight in female mice to 177 mg/kg in males (Borzelleca et al., 1985), and from 27 to 175 mg/kg body weight in rats (Deichmann et al., 1942; Gaines, 1969; Ahlborg and Larsson, 1978).

In rats dosed orally with C_5P, the NOAEL for liver and kidney pathology is 3 mg/kg/day and the LOAEL is 10 mg/kg/day (Schwetz et al., 1978). In pregnant Sprague-Dawley rats dosed by gavage for 10 d with either purified- or commercial-grade

C_5P formulations the LOAEL for developmental effects in pups, as indicated by increased occurrence of delayed skull ossification, was 5 mg/kg/day (Schwetz et al., 1974). However, these studies were conducted using C_5P formulations no longer commercially available. More recent studies in which Sprague-Dawley rats were dosed by gavage from gestational day 6 to day 15 showed the NOAEL for both maternal and developmental toxicity to be 30 mg/kg/day, while the LOAEL for developmental toxicity was 80 mg/kg/day, as indicated by increased resorptions, reduced litter size and fetal body weights, and increased malformations in pups (Bernard and Hoberman, 2001). In a two-generational reproductive study using a more pure form of C_5P, Sprague-Dawley rats showed a LOAEL of 30 mg/kg/day and a NOAEL for both reproductive and general toxicity of 10 mg/kg/day (Bernard et al., 2002). In a related study, pregnant rabbits dosed by gavage from gestational days 6 to 18, the maternal NOAEL was 7.5 mg/kg/day and the developmental NOAEL was 30 mg/kg/day (Bernard et al., 2001). It should be noted that a dose of 10 mg/kg/day is 7,000 to 20,000 times higher than human exposure.

1.4.11 Governmental regulations and health advisories

The U.S. EPA lists C_5P as a probable human carcinogen (Class B2) based on the weight-of-evidence (IRIS, 2004), while IARC considers C_5P possibly carcinogenic to humans (Group 2B) (IARC, 1991). Further, the U.S. EPA has derived a RfD of 0.03 mg/kg/day for C_5P (based on Schwetz et al., 1978), and a MCL in drinking water of 1,000 ng/L for the compound. The European standard for C_5P in drinking water is 9,000

ng/L (WHO, 2001). The minimal risk level to humans for noncancerous end points (MRL) for acute duration oral exposure is 0.005 mg/kg/day (based on Schwetz et al., 1974) while that for intermediate or chronic duration oral exposure is 0.001 mg/kg/day (based on Beard et al., 1997, and Beard and Rawlings, 1998, respectively).

1.4.12 Analytical measurement

Analytical chromatographic methods to measure exposure to CPs in human urine (Kalman, 1984; Kontsas et al., 1995; Lee et al., 1998; Wada et al., 1999), blood serum (Yost et al., 1984; de Ruiter et al., 1990), and plasma (Angerer, 1997), as well as landfill leachate (Ribeiro et al., 2002) and environmental (air, water, and soil) samples (Abrahamsson and Xie, 1983; Turnes et al., 1996; Jáuregui et al., 1997; Bagheri and Saraji, 2001; Nakamura et al., 2001; Tauler et al., 2001; Hanada et al., 2002; Sarrion et al., 2002; Yang and Lee, 2002) have been developed. Immunoassays for specific CP congeners in water (van Emon and Gerlach, 1992; Noguera et al., 2002; Nistor and Emneus, 2003) urine (Galve et al., 2002; Nichkova et al., 2003), and soil (Li et al., 2001) have also been developed.

In the case of C_5P, most methods involve acidification of the sample to convert the compound to the non-ionized form, extraction into an organic solvent, purification, and detection by GC/ECD or GC/MS. In addition, HPLC with fluorescence detection can be used to determine total and free C_5P in urine after fluorescence labeling with 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (Wada et al., 1999). C_5P in air, water, plant material (e.g., sugarcane foliage, cotton foliage, and beans) textile effluent, and

biological samples (e.g., blood and urine) may also be determined using a spectrophotometric method (Agrawal et al., 1998). C_5P is converted to chloranil using concentrated nitric acid which liberates iodine from potassium iodide. The iodine then selectively oxidizes leucocrystal violet to form crystal violet which can be measured spectrophotmetrically.

Of particular interest, solid phase extraction (SPE) using different sorbents (e.g., synthetic resins, C_{18}, or graphitized carbon) allows for the processing of large sample volumes before the final extracts are concentrated to smaller volumes (Turnes et al., 1996; Bagheri and Saraji, 2001). However, this solvent evaporation step may result in the loss of more volatile compounds. Samples may then be analyzed by HPLC or GC, although peaks of more polar compounds may appear broad and exhibit tailing unless the sample is derivatized prior to analysis, most commonly through acetylation (Abrahamsson and Xie, 1983; Hanada et al., 2002). Thus, a selective detection method such as GC/MS or tandem GC/MS/MS is preferred (Turnes et al., 1996).

1.5 Environmental contamination

1.5.1 Sources of environmental contamination

As a result of use in the wood preservation industry, substantial environmental contamination by coal tar creosote and C_5P has occurred (USDHHS, 1996). Excess free product may be released from treated materials during pressure impregnation of wood products and treatment solution components may be subsequently released from the surface of treated wood products over time by oil exudation, leaching from contact with

water, or volatilization (Henningsson, 1983; DeLeon et al., 1988; Hale and Aneiro, 1997). Leaching of spilled wastes from the application sites is not uncommon, although spills may also occur during transport of coal tar materials on barges or as a result of accidents during navigation, docking, or loading and unloading. Atmospheric releases may occur any time within the treatment process and during incineration of scrap treated wood, however, the major source of creosote released to the environment is waste water effluents which can lead to surface water and groundwater contamination (USDA, 1980).

In the past, waste water generated from wood treatment facilities was often discharged into unlined evaporation/settling lagoons (USDHHS, 1996). Settling lagoons are no longer part of appropriate disposal methodology and existing structures are currently being remediated. Over time, a layer of sludge would form and water-soluble components would percolate through the soil to contaminate groundwater reservoirs. However, given the viscous nature of creosote, significant migration into groundwater supplies is rare unless the soil is extremely porous, for example the sandy soil found at the American Creosote NPL site in Pensacola, FL.

In general, a spill released to the soil percolates through the vadose zone (unsaturated zone) by gravity and soil capillarity to the saturated zone where soluble phase components contaminate groundwater (Huling and Weaver, 1995). Percolation through both zones is vertical until the volume is eventually exhausted by the saturation process or until it reaches a zone of low permeability where it will begin to migrate laterally, also by soil capillarity. Water percolating through the vadose zone may leach

soluble phase components referred to as residual saturation, the fraction of hydrocarbon retained by capillary forces in the soil. In addition, residual saturation near the water table is also subject to leaching with the rise and fall of the water table.

1.5.2 Types of organic contaminants

Organic contaminants have limited water solubility, are typically in a liquid or semi-liquid state, and are capable of independent movement through both the vadose and saturated zones of the subsurface (McCaulou et al., 1995). In the scope of water remediation, these compounds are termed non-aqueous-phase-liquids (NAPLs) and include volatile aromatics (e.g., benzene, toluene, styrene, and xylenes), halogenated volatiles (e.g., vinyl chloride and chloroethane), and volatile ketones and furans. Based on the specific gravity with regard to water, NAPLs are made up of both light (LNAPL) and dense (DNAPL) components. LNAPLs commonly include fuels and oils. Creosote is classified as DNAPL in addition to halogenated solvents (e.g., tetrachloroethylene, trichloroethylene, dichloroethylene and carbon tetrachloride), polychlorinated biphenyls (PCBs), pesticides, PAHs, and chlorinated benzenes and phenols.

1.6 Soil and groundwater remediation

1.6.1 General remediation technologies

Leaching of wastewater into soil and subsequently, groundwater, has resulted in a rise in research initiatives to address the need for remediation. The main source of contaminants may be substantially reduced or eliminated by removing free product early

in the remediation process. This is mainly accomplished using either trench or pump-and-treat systems (Huling and Weaver, 1995). Pumping removes components in the aqueous phase from the immiscible (continuous and residual saturation), solid (sorbed to soil), and gaseous phases. If continuous DNAPL can be located in the subsurface in a shallow, impermeable reservoir, recovery wells may be used to pump pure phase product which substantially improves overall recovery. However, these pools are difficult to locate and DNAPL yield is generally poor. Another method, installation of trench systems, involves the horizontal placement of recovery lines on top of the impermeable zone, usually when the DNAPL reservoir is located near the surface. DNAPL flows into the collection trenches and seeps into the recovery lines, which then drain into a collection sump and DNAPL is pumped to the surface. Subsequent *in-situ* and *ex-situ* remediation may then be used to prevent plume migration and remove soluble components.

Installation of physical and non-permeable barriers provides hydraulic control to prevent plume migration in the subsurface by physically containing DNAPL inside treatment cells that are then the focus of further remediation strategies (e.g., soil vacuum extraction (SVE), natural attenuation, bioremediation, and soil flushing) (Huling and Weaver, 1995). Bioremediation (Guerin, 1999; Atagana et al., 2003) may consist of amending soil conditions to improve the growth of indigenous microbial species (e.g., aeration or the addition of nutrients) (Nieman et al., 2001) or the introduction of a specific contaminant-degrading colony (Boonchan et al., 2000). Treatment may be carried out *in situ* or, following excavation, in a bioreactor or on-site land-treatment bed.

The technique is limited because DNAPL is a highly hostile environment for the survival of most microorganisms and the basic requirements for microbial proliferation (i.e., nutrients, osmotic potential, pH, moisture, electron acceptor, etc.) are difficult to maintain in this environment. However, these strategies may used as an effective means to further reduce the mass of contaminants at the site following NAPL recovery by trench or pump-and-treat systems.

Permeable reactive barriers are installed across the path of migration of a contaminant plume and consist of a trench back-filled with barrier material such as granular iron, peat, activated carbon, or zeolite (Rasmussen et al., 2002). As groundwater passes the barrier, pollutants are removed from the water by chemical, physical or biological processes, to protect the surrounding environment.

SVE involves applying a vacuum to vadose zone subsurface strata to induce air flow to volatilize residual saturation or soluble phase contaminants in the vadose zone (Huling and Weaver, 1995). Vapors are then collected and treated, typically with granular activated carbon, catalytic oxidation, or direct combustion. The same strategy can be used to remove DNAPL from the saturation zone, and is especially effective in cases where the precise location of DNAPL is unknown. A closely related strategy, air stripping, involves volatilizing contaminants using an air stream. While SVE is historically used to remove volatile compounds from soil, it has also been shown to enhance aerobic biodegradation of volatile and semivolatile organic compounds by aiding in the supply of oxygen to the vadose zone.

Soil flushing enhances dissolution of adsorbed and dissolved phase contaminants, and displaces free-phase nonaqueous contaminants (Huling and Weaver, 1995). Surfactants and alkali are added to reduce surface tension between DNAPL and water to increase mobility and polymers increase the viscosity of the flushing fluid to maintain hydraulic control and improve efficiency (Makkar and Rockne, 2003; Zhu and Feng, 2003). Thermal methods can be used by injecting hot water or steam to increase volatilization and solubilization which decreases the viscosity and density of mobilized NAPL (Richardson et al., 2002). The mobile phases are then recovered with a second approach such as SVE or pump-and-treat.

Chemical oxidation uses chemical oxidizing agents (e.g., ozone, hydrogen peroxide, chlorine and chlorine dioxide) to destroy volatile and semivolatile organic chemicals and cyanide in groundwater. The use of ultraviolet light (UV) in conjunction with an oxidizing agent provides for more complete destruction, can enhance the destruction of resistant chemicals (e.g., PCBs), and can increase the reaction rate by a factor of 100 to 1,000. Photo-Fenton (Engwall et al., 1999) and ozonolysis (Ottinger et al., 1999; Hong and Zeng, 2002) reactions are advanced oxidation processes based on hydroxyl radical chemistry that can efficiently oxidize organic compounds in aqueous solution. Fenton's reagent, a mixture of ferrous ion (Fe^{2+}) and hydrogen peroxide, produces hydroxyl radicals ($OH\cdot$), as shown in equation 1 (Moraes et al., 2004). In the presence of ultraviolet (UV) radiation, the ferric ions (Fe^{3+}) are converted back to ferrous ions (Fe^{2+}) with the formation of an additional equivalent of hydroxyl radical, represented in equation 2. Hydroxyl radicals then promote the oxidation of organic

species (R) as shown in equation 3. The process is highly influenced by pH, as iron precipitates out of solution as a hydroxide at values higher than pH 4.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \quad (1)$$

$$Fe^{3+} + H_2O \rightarrow Fe^{2+} + H^+ + OH\cdot \quad (2)$$

$$HO\cdot + RH \rightarrow H_2O + R\cdot \quad (3)$$

Under acidic or neutral conditions, ozonolysis proceeds through an electrophilic addition of molecular ozone to electron rich C=C bonds (Ottinger et al., 1999; Kornmuller and Wiesmann, 2003), while ozone decays to form radicals, primarily hydroxyl radicals, which leads to unspecific radical reactions with organic substances under alkaline conditions.

Of particular interest is the use of sorbent materials to sequester contaminants (Mortland et al., 1986; Boyd et al., 1988; Phillips et al., 1995; Clark et al., 1998). Organically-modified clay minerals have been used to effectively sequester PAHs and C_5P (Srinivasan and Fogler, 1990a; 1990b) and both naturally occurring and organically-modified clay minerals have been shown to remove a variety of metals from water (Ake et al., 2001; Lin and Juang, 2002). However, the most commonly used sorbent is granular activated carbon (GAC).

1.6.2 Granular activated carbon (GAC)

Carbons have been used for purification purposes dating back to the times of the early Egyptians (Allen, 1996). Carbonaceous materials are porous and have a high internal surface area (approximately 10 m^2/g). The process of activating these materials greatly increases the surface area and further develops porosity. Activation is a 2-step process that consists of heating the carbon source to 600 ºC in the absence of air. This resultant carbonized char is subsequently activated either by steam at 1000 ºC or by chemical treatment involving acid or acid salts. The properties of each activated carbon may be controlled during the activation process in order to develop specialized sorbent materials with specific affinities. The principal form for activated carbon used for groundwater remediation is granular activated carbon (GAC).

GAC is the most commonly used porous media for the cleanup of contaminated water (Allen, 1996). The sorbent's effectiveness is based mainly on its large surface area (generally 500 to 2,000 m^2/g). Most notably, GAC has been shown to be effective for the filtration of organic compounds including trihalomethanes (e.g., bromoform), pesticides (e.g., molinate and terbutylazine), surfactants (e.g., polyethoxylated nonylphenone and bromo- polyethoxylated nonylphenones), plasticizers (e.g., diethyl phthalate and tri-*n*-butyl phosphate), and halogenated compounds (e.g., trichlorobenzenes and 2-chloropyridine) (Paune et al., 1998). Further, GAC has been shown to effectively reduce patulin, a mycotoxin, in both aqueous samples and naturally contaminated apple juice (Huebner et al., 2000) and apple cider (Sands et al., 1976).

GAC acts by physically separating contaminants from aqueous media and, after exhaustion, spent product can be reactivated, regenerated, incinerated, or disposed (USEPA, 1996). Thermal reactivation, using heat alone or steam, and incineration both destroy most or all adsorbed organic contaminants, although GAC used for metal removal is generally disposed of, frequently as a hazardous waste material. Steam or hot gas regeneration may be used for spent GAC used in air emission control devices but is not appropriate for spent sorbent regeneration following groundwater remediation.

Although GAC is a well-developed and widely utilized technology as part of a successful groundwater remediation strategy, it is most appropriate for use as a secondary polishing agent. The adsorptive capacity of GAC is higher for gas phase than for liquid phase treatment, especially in the case of metal remediation (USEPA, 1996). This may be partly due to the fact that the presence of natural organic matter can drastically decrease GAC capacity and bedlife (Hopman et al., 1994; Knappe et al., 1999). Further, the sorbent is less effective for large organic molecules and competitive sorption of cosolutes results in depressed (antagonistic) sorption (Xu et al., 1997). In contrast, many clay minerals have been shown to strongly sorb contaminants even in the presence of organic matter (Sheng et al., 2001).

1.6.3 Clay minerals

Historically, clays have been used extensively in a variety of applications. Naturally occurring clay minerals have been used for sorption of contaminants to protect water supplies (Kishk et al., 1979; Huebner et al., 1999; Abollino et al., 2003) and as

enterosorbents in animal diets to protect against mycotoxicoses (Smith, 1980, 1984; Phillips et al., 1988, 1995; Kubena et al., 1990a, 1990b; Harvey et al., 1991; Smith et al., 1994; Phillips, 1999). Clays have also played a major role in the human diet, particularly during pregnancy where they have been described as a common food craving (Geissler et al., 1999), and are active ingredients in pharmaceutical formulations including laxatives (e.g., sodium smectites), antidiarrhetics (e.g., kaolinite and polygorskite), dermatological protectors (e.g., kaolinite, smectites, and talc), excipients (e.g., kaolinite, palygorskite, smectites, and talc), and cosmetics (e.g., kaolinite, palygorskite, smectites, and talc) (Carretero, 2002).

Adsorption is particularly favored when clay minerals have high surface areas (e.g., smectites) and contain exchangeable cations with low hydration energies (e.g., K^+ and Cs^+) (Haderlein and Schwarzenbach, 1993). The type and position of substituents on the aromatic ring of the contaminant influences adsorption, as those that are strongly electron-withdrawing enhanced adsorption (Weissmahr et al., 1997).

1.7 Clay technology

1.7.1 Silicate clay minerals

Silicate minerals comprise the bulk of most soils and 90% of the earth's crust. The structural morphology of silicates, including phyllosilicate clays, is based on the SiO_4 tetrahedron which is composed of Si^{4+} surrounded by four O^{2-} (Figure 3) (Schultz, 1989). SiO_4 tetrahedra are then linked together, with each tetrahedron sharing three O^{2-} ions with three adjacent tetrahedra. This arrangement extends in all directions to form a

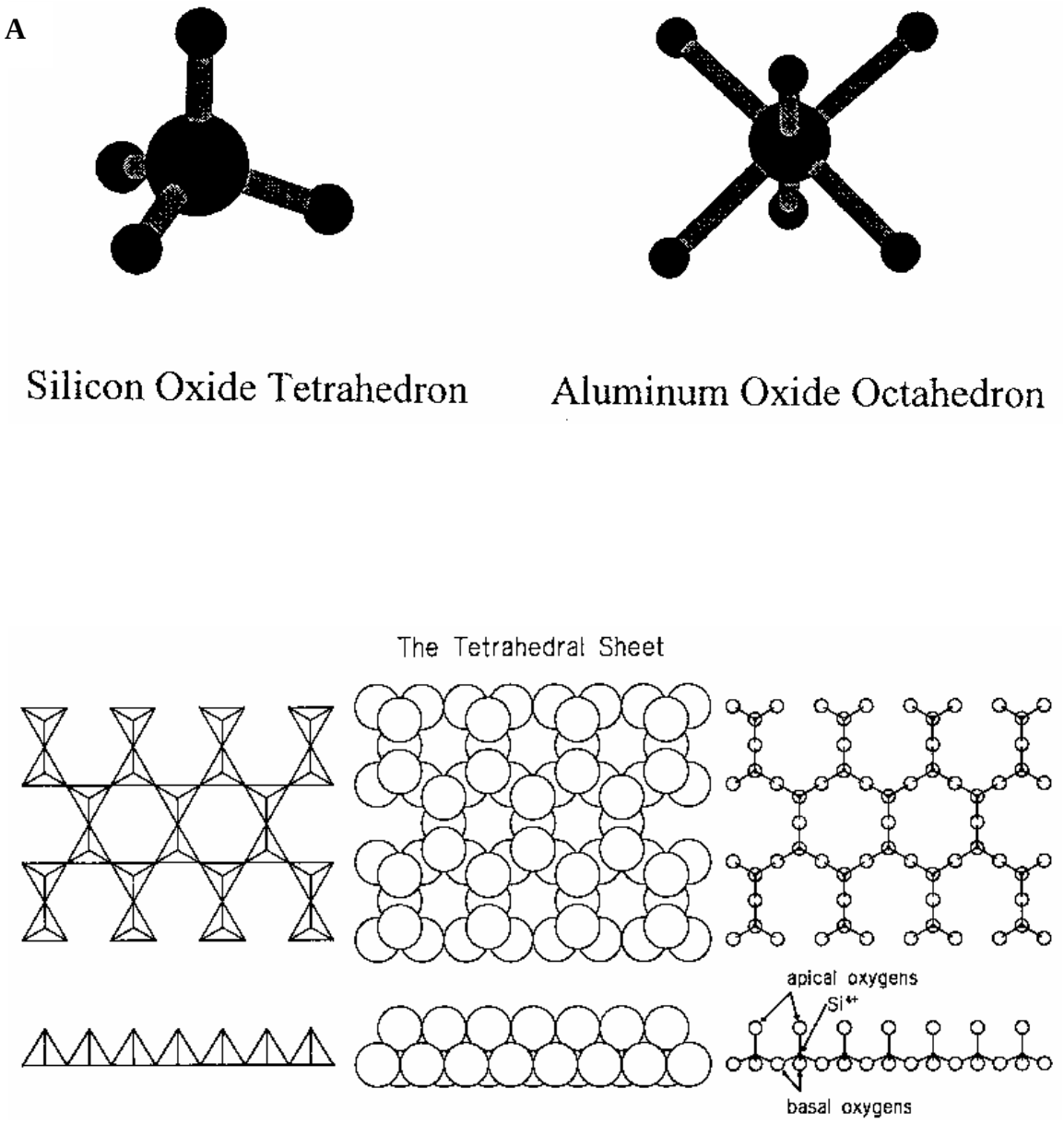


Figure 3. Silicon and aluminum oxides and their arrangement in tetrahedral and octahedral sheets found in phyllosilicate clay minerals. A. Top: ball and stick representation of aluminum and silicon oxide basic structures. Three dimensional structures drawn in HyperChem v 7.0 based on AM1 energy minimized molecules. Bottom: arrangement of silicon oxide and aluminum oxide units to form tetrahedral layers. Adapted from Schultz (1989).

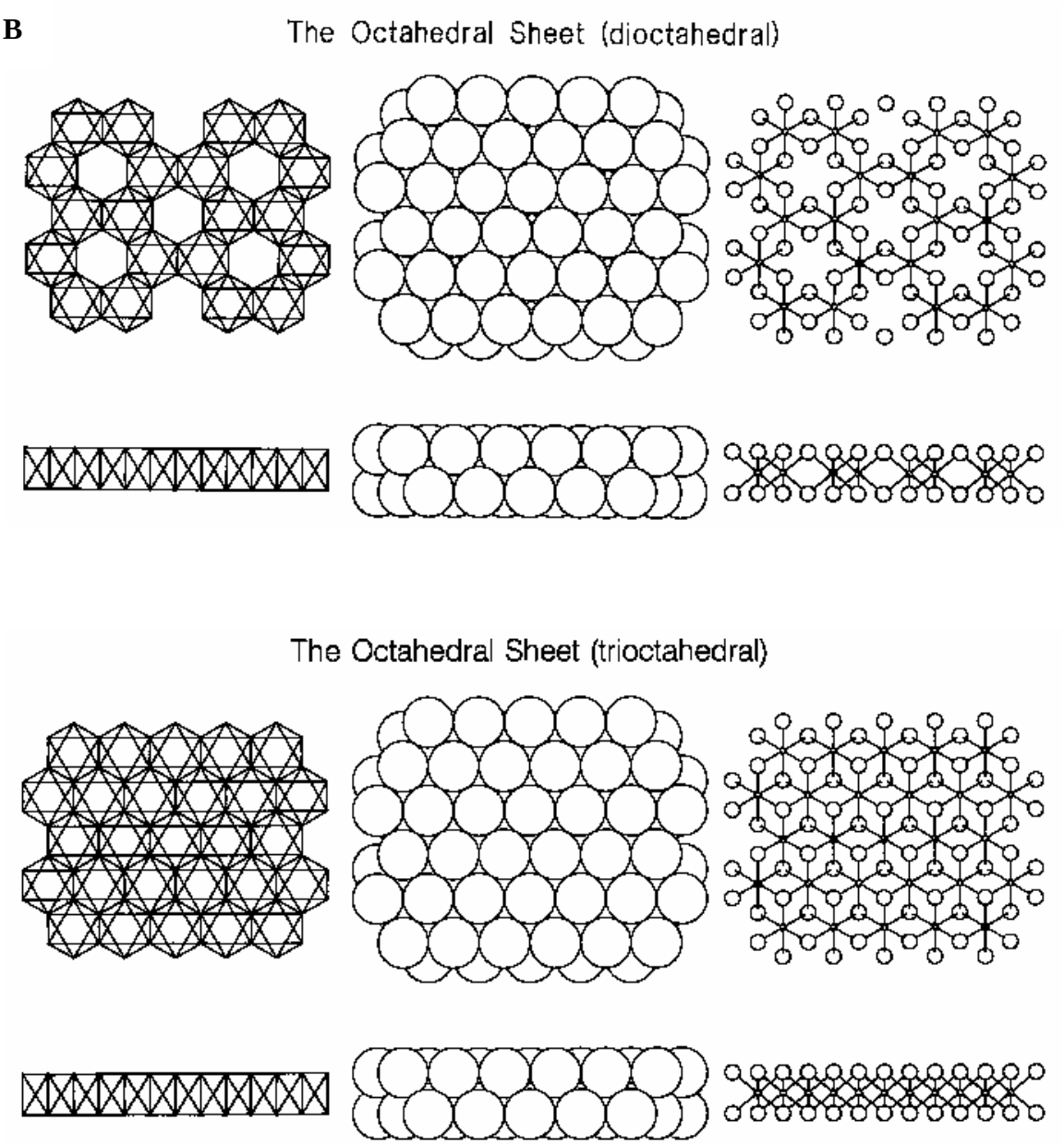


Figure 3. Continued. B. Top: arrangement of silicon oxide and aluminum oxide units to form dioctahedral layers. Bottom: arrangement of silicon oxide and aluminum oxide unites to form trioctahedral layers. Adapted from Schultz (1989).

plane of basal oxygens. The fourth O^{2-} ion, the apical oxygen, is free and available to bind to other elements. Thus, the octahedral sheet is comprised of two planes of OH^- groups that form a hexagonal close packing arrangement. The octahedral sheet may be classified as having either a dioctahedral or trioctahedral arrangement. A trioctahedral arrangement is formed when octahedral layers contain divalent ions (Mg^{2+}, Ca^{2+}) in all possible octahedral sites, specifically, hectorite is a trioctahedral smectite. A dioctahedral arrangement is produced when octahedral layers contain trivalent ions (Al^{3+}) in two of every three possible octahedral sites, specifically, montmorillonite is a dioctahedral smectite.

Phyllosilicate minerals, a class of silicates, dominate the clay fraction of most soils (Schultz et al., 1989). These minerals are formulated in distinctive layer-lattice structures composed of both tetrahedral and octahedral sheets, producing a high surface area characteristic of these minerals. The 1:1 type phyllosilicates are comprised of a series of layers, each containing one octahedrally coordinated silica sheet, while 2:1 type phyllosilicates (Figure 4) are composed of one octahedral alumina sheet coordinated between two tetrahedral silica sheets. Some common 1:1 type phyllosilicates include kaolinite and halloysite, while talc, pyrophyllite, mica, vermiculite, smectites, chlorites, palygorskite, and sepiolite are 2:1 type phyllosilicates (Figure 5).

Phyllosilicates possess a net negative surface charge on their structural layers (Schultz et al., 1989). In 1:1 phyllosilicate clays this is primarily due to the dissociation of H^+ ions from hydroxyl groups attached to Si^{4+} or Al^{3+} atoms at the broken edges of clay layers. However, in the case of 2:1 layer lattice clays, the negative charge is more

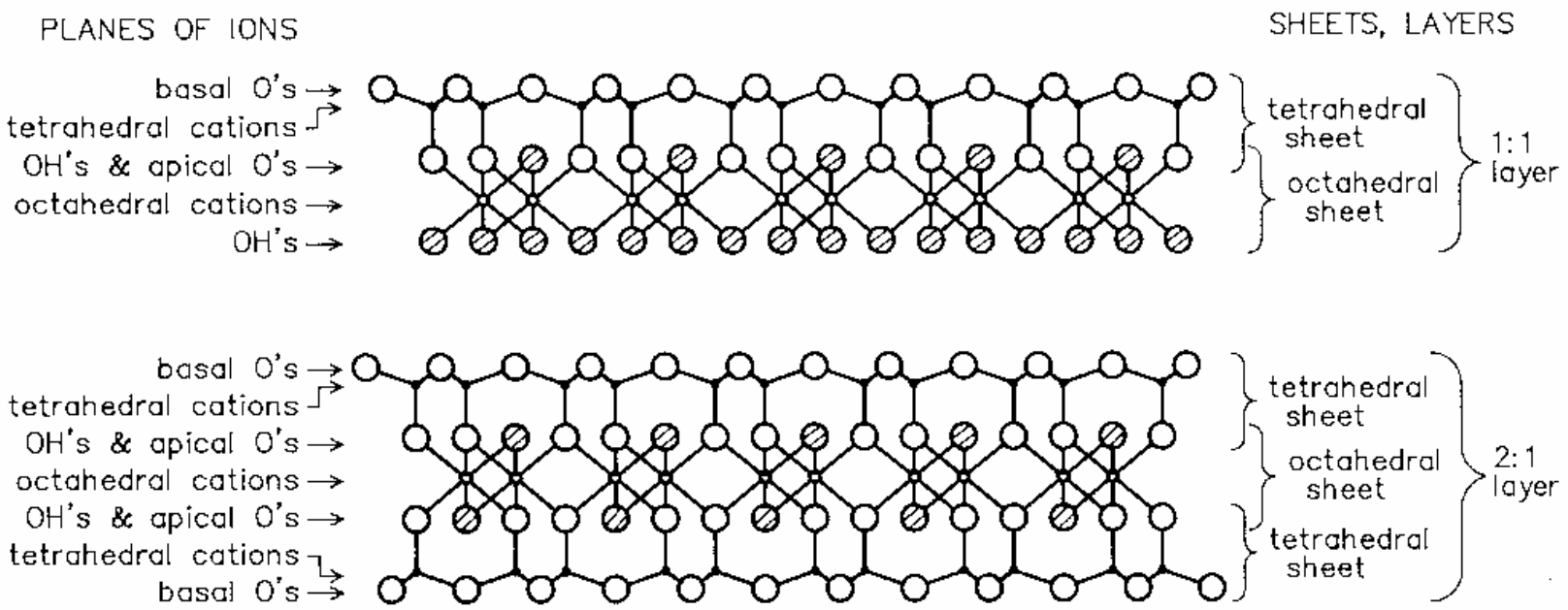


Figure 4. 1:1 and 2:1 layer lattice clay structures. Note the arrangement of basal and apical oxygens, tetrahedral and octahedral cations, and hydroxyl groups illustrated within the clay structure as well as the arrangement of the tetrahedral and octahedral sheets. Adapted from Schultz (1989).

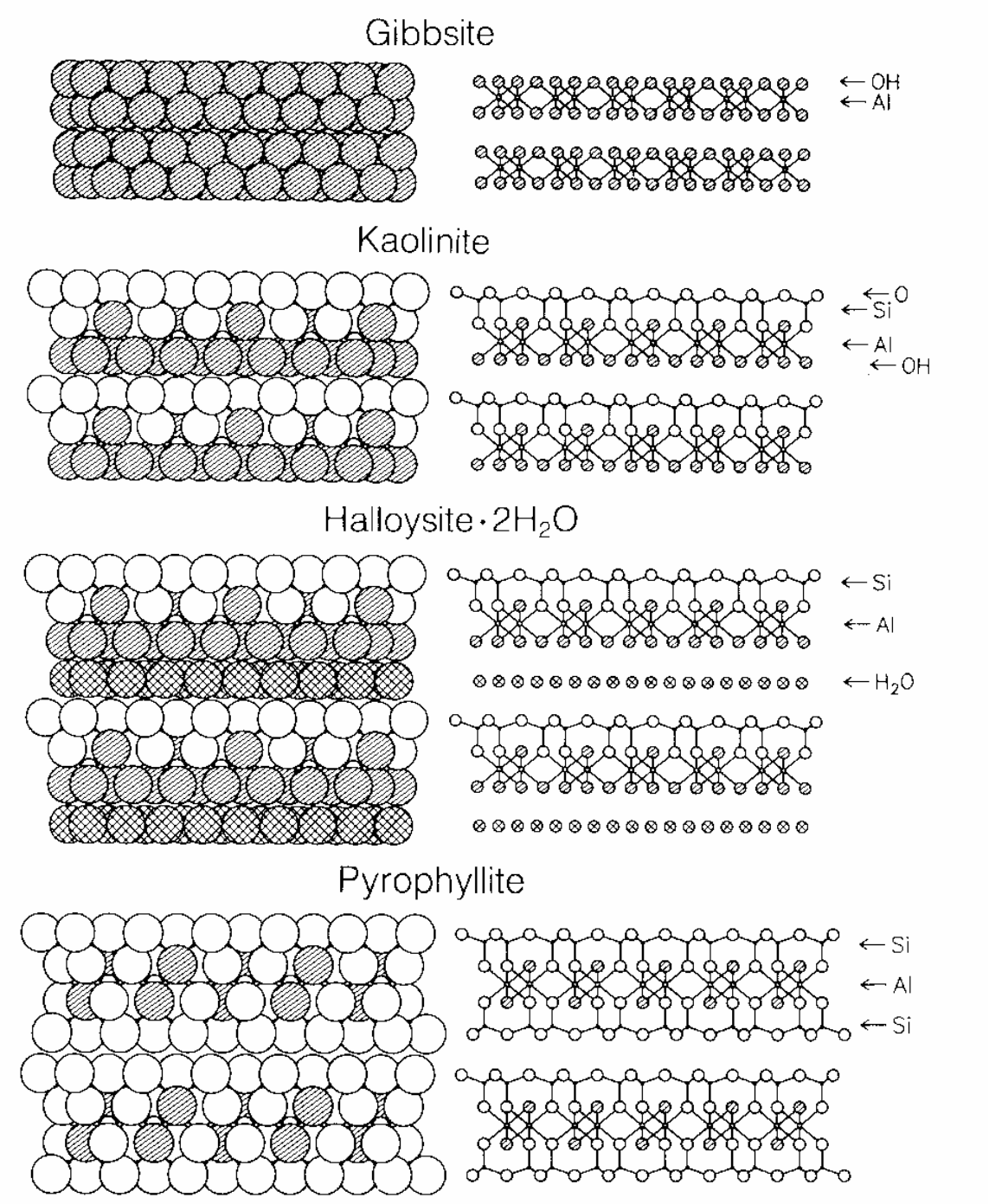
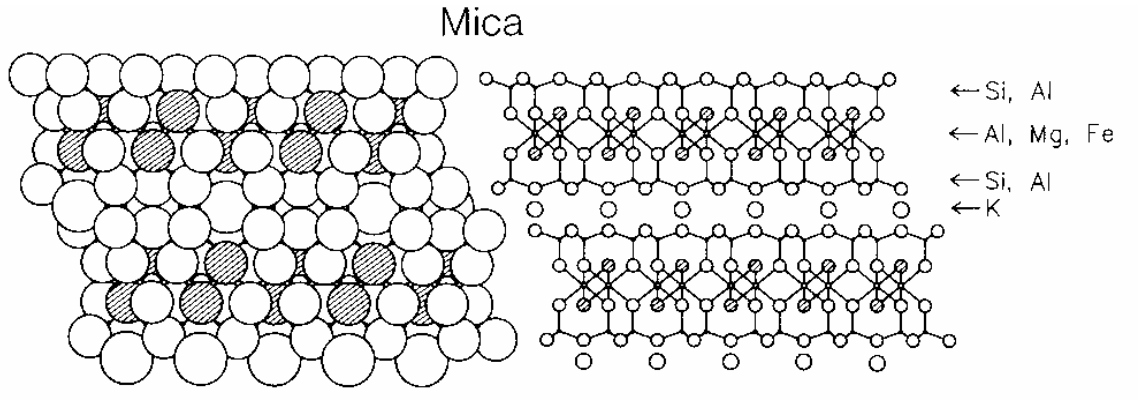


Figure 5. Structural diagrams of major phyllosilicate clays. Adapted from Schultz (1989).



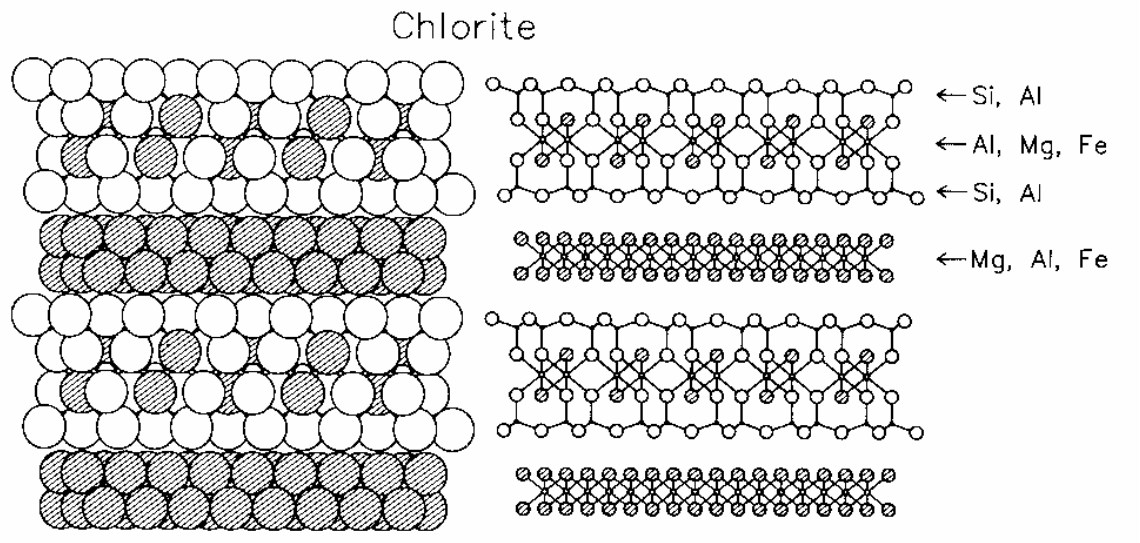


Figure 5. (Continued)

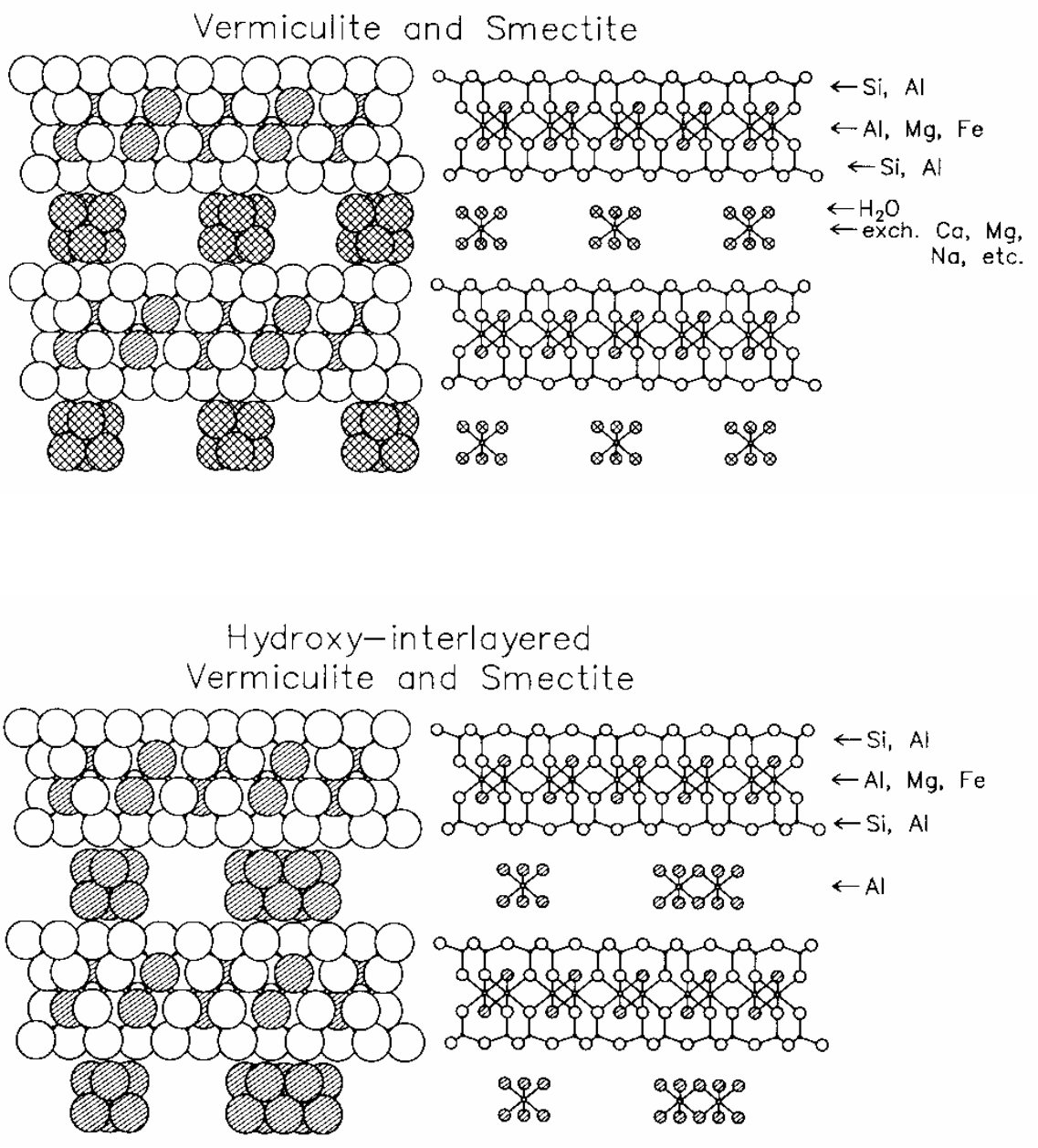


Figure 5. (Continued)

likely due to the natural replacement of Si^{4+} or Al^{3+} with a cation of similar geometry but of lower charge, for example the substitution of Mg^{2+} for Al^{3+} or of Al^{3+} for Si^{4+}. This net negative charge is neutralized by cations attracted to surfaces of the layers. The cations most commonly encountered in the interlayer of naturally occurring clay minerals are Na^{+}, Ca^{2+}, and Mg^{2+} and clays are often identified based on the predominant cation (e.g., sodium montmorillonite). The net negative charge resulting from cation substitutions influences the cation exchange capacity (CEC), the quantity of cations that may be attracted to exchangeable surfaces in a particular clay mineral.

Smectites, a 2:1 type of phyllosilicate clay, have moderately high CEC values, approximately 135 $cmol_{charge}/kg$, and are characterized by their ability to absorb multiple times their weight in water (Borchardt, 1989; Proust et al., 1998). Hydration spheres surrounding interlayer cations interact with basal OH^{-} groups via hydrogen bonding and columbic interactions with the negatively charged clay platelet, although hydrophobic areas of the platelet prevent complete coverage of hydrated cations. Once the cations are hydrated they form pillars to expand interlayer spacing. The size to charge ratio determines the degree of hydration of the cation within the interlayer space, which determines the degree of expansion characteristic to a various type of clay. For example, Ca^{2+} smectites may be hydrated by six or more H_2O molecules. The hydrated Ca^{2+} forces the clay platelets open, but columbic attraction prevents unlimited expansion. In contrast, Na^{2+} smectites are considered "swelling clays" because the columbic attractions between the negatively charged clay platelets are overwhelmed by the extent of Na^{2+} hydration so that unlimited expansion occurs.

1.7.2 Organically modified silicate clay minerals

Although naturally occurring clay minerals have been shown to strongly bind contaminants from water with high capacity, they do not effectively sorb most hydrophobic organic compounds due to preferential adsorption of water around metallic cations (Xu et al., 1997). However, replacement of inorganic interlayer cations with organic cations produces organically-modified clay minerals (i.e., organoclays) with organophilic properties that more effectively sorb hydrophobic contaminants, including PAHs and C_5P (Srinivasan and Fogler, 1990a, 1990b). The hydrophobicity of organoclays is influenced by the size and structure of the R group, the clay type, solution conditions, and the nature of the native exchangeable cation (Xu et al., 1997). The organic ions used most extensively are quaternary ammonium cations of the general form $[(CH_3)_3NR]^+$ or $[(CH_3)_2NRR']^+$ where R is an aromatic or alkyl hydrocarbon. Some of the more widely studied quaternary ammonium cations include cetylpyridinium (CP) (Ake et al., 2003), hexadecyltrimethylammonium (HDTMA) (Sheng et al., 1996), dioctodecyldimethylammonium (DODMA) (Boyd et al., 1988), tetramethylammonium (TMA) and trimethylphenylammonium (Brixie and Boyd, 1994) (Figure 6). Quaternary ammonium moieties have a unique property that part of the molecule is hydrophobic (the aliphatic tail) and part is positively charged and hydrophilic (the quaternary ammonium moiety) (Xu et al., 1997). This allows the surfactant to be exchanged into the interlayer because the positively charged nitrogen interacts by cation exchange with the negatively charged basal surface of the clay platelet, while the aliphatic tails produce a hydrophobic environment in the interlayer space. These

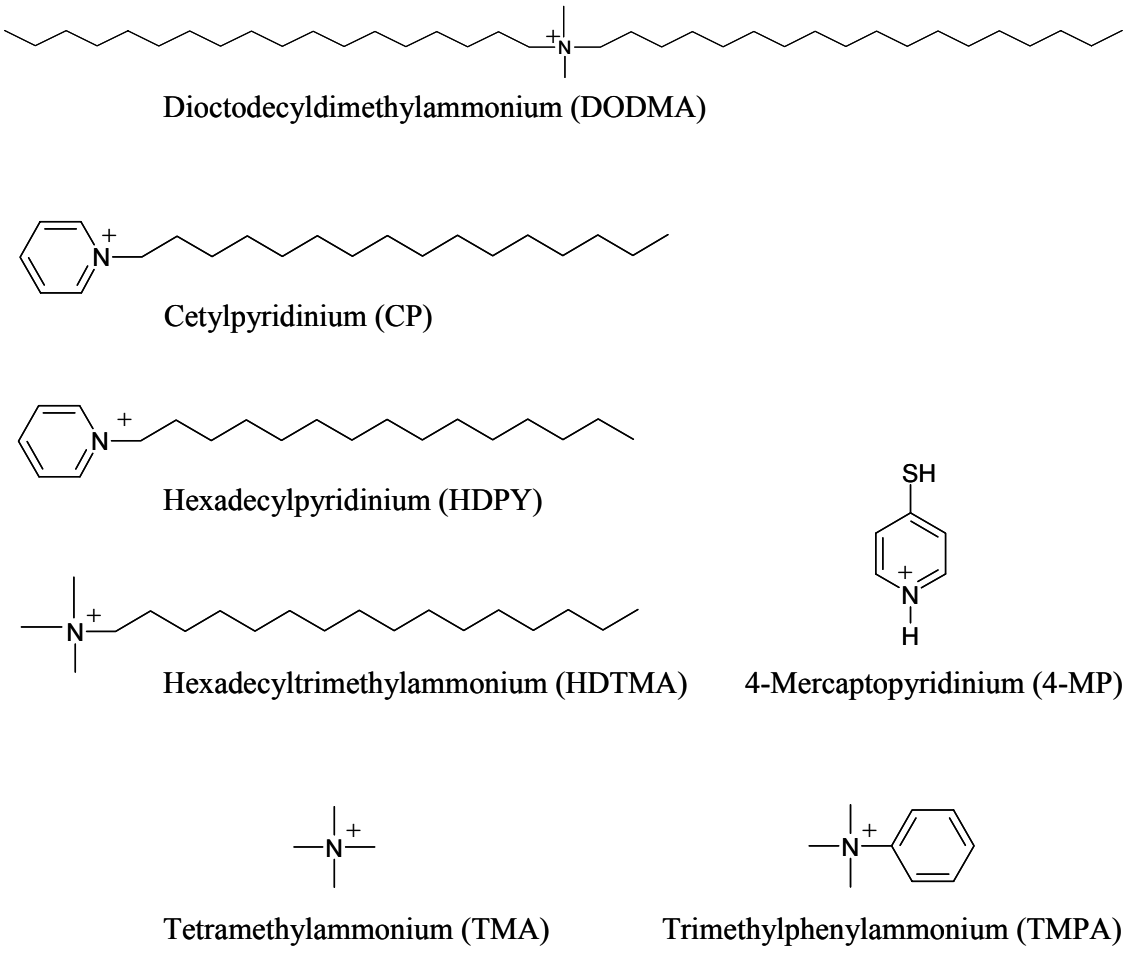


Figure 6. Name, common abbreviation, and structure of organic cations used as exchange surfactants in organoclay production.

nonelectrostatic (nonpolar) interactions may also occur between alkyl moieties of R groups of free organic cations and those exchanged onto the clay surface to increase surfactant adsorption and, therefore, hydrophobicity. Thus, surfactant sorption occurs in a synergistic manner.

The hydrophobicity of the clay platelet's interlayer may be regulated by exchanging surfactant into the clay as a fraction of the native clay's CEC value (Greenland and Quirk, 1960; Zhang et al., 1993; Xu and Boyd, 1995). Specifically, organoclays containing cations that have been exchanged at 25% of the CEC value will be less hydrophobic than those with cations exchanged at 75%. In addition, the amount of surfactant that may be exchanged onto the interlayer surfaces of clay minerals increases with an increase in surfactant alkyl chain length to the extent that when the alkyl chain length is at least 12 to 16 carbons long, exchange may extend past the CEC of the clay. Below the CEC value exchange occurs primarily by cation exchange but also by van der Waals mechanisms. Once the cation exchange capacity is reached, however, further exchange occurs by van der Waals mechanisms only.

In addition to the hydrophobicity, the amount of surfactant also affects the interlayer arrangement and the overall structure of the clay mineral (Xu et al., 1997). Specifically, depending upon the mineral surface charge (the amount of tetrahedral charge and octahedral charge) and the size of the surfactant (e.g., the length of the alkyl chain and the size of the charged head group) the exchanged surfactant may be arranged as a monolayer, bilayer, pseudotrimolecular layer or in a paraffin complex (Figure 7).

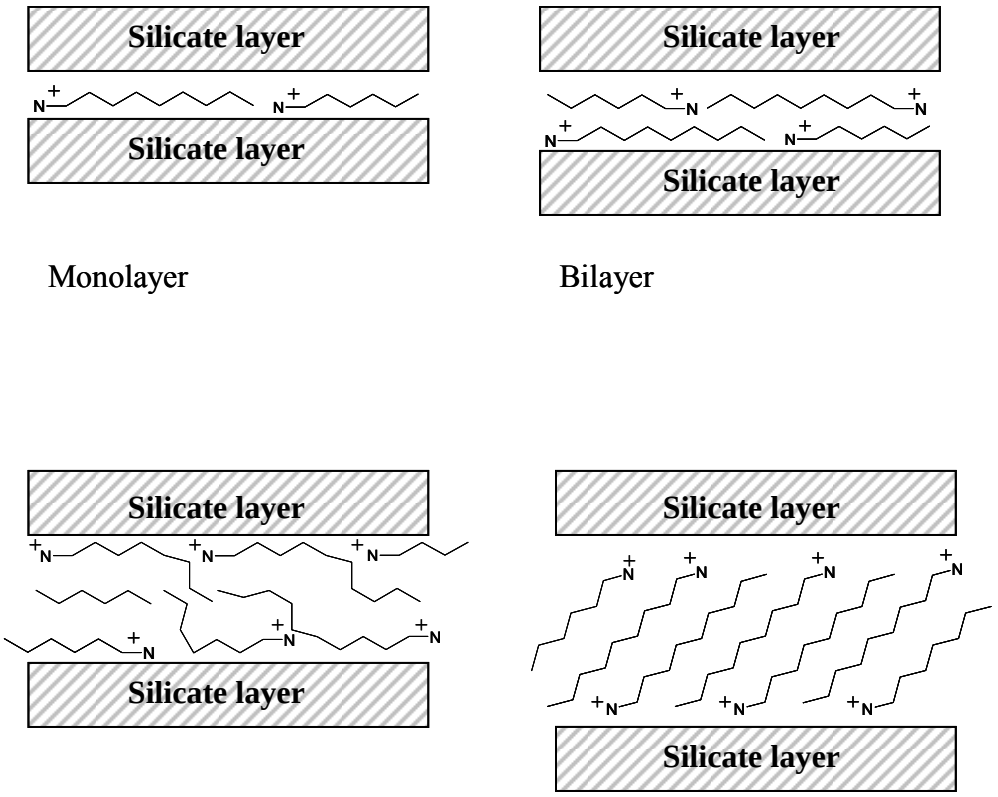


Figure 7. Surfactant arrangements in the interlayer of clay platelets of a representative smectite clay. Note the increase in interlayer spacing associated with increased quantities of surfactant that are exchanged into the interlayer and the corresponding changes in surfactant arrangement. Adapted from Xu et al. (1997).

The arrangement of the surfactant ions in the interlayer determines the van der Waals contact among the alkyl chains and between the adsorbed organic cation and the basal surface of the clay platelet. A flat-laying monolayer is favored because this arrangement maximizes contact area between surfactant ions and the clay surface, resulting in higher van der Waals interactions than in a bilayer arrangement in which only one side of an organic ion contacts the surface directly or a paraffin-type arrangement where there is no direct contact. Therefore, surfactant ions in a monolayer arrangement have a higher affinity than those in a bilayer or paraffin-type arrangement.

Organoclays containing long-chain organic cations have been shown to efficiently remove ionizable organic compounds such as C_5P (Boyd et al., 1988), PAHs (Chen and Zhu, 2001), and various inorganic anions including Pb^{2+} (Lee et al., 2002) and Cr^{4+} (Krishna et al., 2000) from water. Adsorption occurs primarily through partitioning of the neutral species and hydrophobic binding in the hydrophobic interlayer that is produced from tail-tail interactions between surfactant molecules (Boyd et al., 1988; Sheng et al., 1996). As is the case when surfactants are exchanged in the interlayer, sorption of one contaminant may have synergistic effects on the sorption of cosolutes (Xu et al., 1997). Sorption of individual contaminants can cause interlayer expansion that then leads to binding of larger size compounds otherwise unsorbed as a result of steric constraints of the interlayers. Hydrophobic tails of exchanged long-chain cations become more flexible as solute concentrations increase and a solvent-like hydrophobic phase forms in the interlayer to make partitioning the predominant sorption mechanism, although this is not common in complexes containing small organic

moieties (e.g., tetramethylammonium or trimethylphenylammonium) or an inorganic cation (e.g., NH_4^+). In saturated or oversaturated (relative to the CEC) organoclays, adsorption of contaminants may also occur through columbic attraction between the anionic head group of the contaminant and positively charged head groups of organic surfactant cations on the clay surface.

1.7.3 CP-exchanged montmorillonite

CP-exchanged montmorillonite clays have been used to remove a variety of contaminants from aqueous solution (Springman et al., 1999; Herrera et al., 2000; Ake et al., 2003). X-ray crystallography data shows interlayer spacing of either 21 or 42 Å (Greenland and Quirk, 1960; Malik et al., 1972; Xu and Boyd, 1994; 1995). It has been postulated that the 21 Å spacing results from a monolayer arrangement in which CP molecules lie parallel to the basal surface of the clay platelet. The 42 Å spacing is only observed with high exchange capacities and is believed to be the result of either multiple parallel layers or a perpendicular arrangement placing the center of positive charge in the pyridine ring in close proximity to the negatively charged clay surface.

1.7.4 Clay minerals in water remediation

Clay minerals have been extensively studied as sorbents for water remediation. Sodium and calcium montmorillonite clays have been shown to decrease ergotamine (Huebner et al., 1999) and methyl parathion (O,O-dimethyl O-(nitrophenyl) phosphorothioate) (Kishk et al., 1979). In the case of metal remediation, naturally-

occurring sodium montmorillonite has been shown to decrease Cd, Cr, Cu, Mn, Ni, Pb, and Zn in aqueous solution (Ake et al., 2001; Abollino et al., 2003). Sodium dodecylsulfate (SDS)-exchanged montmorillonite has been shown to remove Cu^{2+} and Zn^{2+} (Lin and Juang, 2001), HDTMA-exchanged montmorillonite has been shown to remove Cr^{4+} (Krishna et al., 2000), and CP-exchanged montmorillonite has been used to remove *Salmonella enteritidis* (Herrera et al., 2000) from aqueous solution. Further, CP-exchanged montmorillonite has also been shown to decrease PAHs from groundwater in small-scale field studies (Springman et al., 1999; Ake et al., 2003). However, since these and other organoclays have limited permeability in water they have not been extensively utilized for filtration and remediation of large volumes.

Hydraulic conductivity may be influenced by the organic modification of clay minerals. During organoclay preparation, if surfactants are adsorbed onto the surface of swelling clays at sub-saturation levels (with respect to the CEC) the clay may flocculate (Xu et al., 1997). The binding of small molecules together then forms extensive aggregation networks that increase hydraulic conductivity of the organoclay over that of unmodified clay minerals. However, surfactants adsorbed onto the surface of clay minerals in excess of the CEC can produce a net positive charge on the clay surface that may then dismantle clay aggregates and cause clay dispersion. The disperse clay can then clog pores and reduce hydraulic conductivity of the organoclay.

In order to utilize saturated organoclays for use in water filtration, methods have recently been developed to adhere clay minerals onto the surface of various solid support matrices (Phillips and Sarr, 1999; Ake et al., 2001). This allows for the construction of

column filtration systems made up of composite materials with void volume, increased surface area, and enhanced hydraulic conductivity. In one study, CP-exchanged montmorillonite was bonded to sand and packed into columns to sorb PAHs and C_5P from groundwater (Ake et al., 2003), while in a separate study cetyldimethylethylammonium (CDEA) exchanged montmorillonite was bonded to sand to reduce *Escherichia coli* and *Salmonella enteritidis* in aqueous suspension (Herrera et al., 2004).

1.7.5 Clay minerals as enterosorbents

In addition to their use in water filtration, clay minerals have also been studied extensively as enterosorbents for use *in vivo*. A variety of naturally occurring silicate clay minerals have been added to animal feeds as enterosorbents to bind and reduce the bioavailability of mycotoxins. Phyllosilicate clays (at levels as low as 0.5% w/w in the diet) have been shown to effectively bind aflatoxins and prevent aflatoxicosis in multiple animal species (Phillips et al., 1988; Kubena et al., 1990). In addition, significant reductions in the production of aflatoxin M_1 in the milk of dairy cows (44%) and goats (52%) have been achieved with the addition of 1% clay in the diet (Harvey et al., 1991; Smith et al., 1994). Dietary inclusion of zeolite (Smith, 1980) and spent bleaching clay from canola refining (Smith, 1984) has been shown effective in the reduction of some of the toxic effects of zearalenone and T-2 toxin in rats and immature swine.

Of particular concern, sorbents added to the diet may bind enzymes and other necessary nutritive elements, making prolonged use inadvisable. Several case studies

suggest an association between clay ingestion and profound muscle weakness, anemia, and hypokalemia in humans (Mengel et al., 1964; Gonzalez et al., 1982; Severance et al., 1988). Clays that sorb nonselectively may interact with nutrients, minerals, and other feedborne chemicals to pose significant hidden risks as a result of dietary supplementation. This is especially of concern during prenatal development due to the highly susceptible nature of both the mother and fetus to nutrient deficiency. Specifically, one study showed that rats fed high levels (20%) of kaolin in the diet throughout gestation exhibited significant reductions in hemoglobin, hematocrit, and red blood cell levels, indicating maternal anemia, and pups borne to these rats had lower birth weights (Patterson and Staszak, 1977). In the same study, iron supplementation to the kaolin-diet showed a protective effect for both dams and pups. Other studies have shown that rats fed a zinc-deficient diet supplemented with clay experienced lower mortality rates than rats maintained only on a zinc-deficient diet, suggesting that clay supplementation can be beneficial in some cases of mineral deficiencies (Smith and Halsted, 1970).

Additionally, metals potentially bound to clay minerals may desorb in the gastrointestinal tract and bioaccumulate, leading to a variety of adverse health conditions. Mineralogical data indicates that phyllosilicate clays are important aluminum carriers and many, including smectite, chlorite, and illite, are known to be unstable in acidic environments, such as the stomach (Donner, and Lynn, 1989; Rai and Kittrick, 1989). In particular, *in vitro* studies have shown aluminum silicate-containing bentonite and montmorillonite clays to be cytotoxic to human umbilical vein endothelial

cells but not ROC-1 oligodendroglia cells (Murphy et al., 1993a) and to primary neuronal cells but not differentiated N1E-115 neuroblastoma cells (Murphy et al., 1993b). Separate studies indicated aluminum silicate-containing kaolinite and montmorillonite and the magnesium silicate hectorite were acutely toxic to differentiated neuroblastoma cells (Banin and Meiri, 1990). However, comprehensive *in vivo* studies concerning the release and subsequent accumulation of metals, in particular aluminum, in tissues are lacking. Further, structural analysis of clay platelets suggests that the bioavailability of Al ions is limited due to octahedral coordination with SiO_4 sheets in the center of the platelets (Phillips et al., 2002).

1.8 Research objectives

In response to worldwide concern over the occurrence and persistence of hazardous pesticides and other chemicals identified in groundwater, steps must be taken to ensure the safety of drinking water. In the event of contamination, sensitive analytical methods must be developed in order to identify the chemicals and evaluate toxicity associated with exposure. In addition, the safety of sorbents proposed as dietary supplements must be evaluated to ensure that they do no act nonselectively with nutrients and do not increase the bioavailability of trace metals.

Therefore, the principal goals of this research were:

1) To delineate a sequence of measurable biological changes leading to cellular injury following CP exposure, specifically C_5P, in a sensitive cell line by monitoring endpoints used to evaluate cellular homeostasis. These endpoints include intracellular glutathione

(GSH) and Ca^{2+} concentrations, generation of reactive oxygen species (ROS), mitochondrial membrane potential (MMP), plasma membrane potential (PMP), pH, and gap junctional intracellular communication (GJIC).

2) To develop organoclay-based composite sorbents for both *in vitro* and *in situ* reduction of PAHs and C_5P in groundwater. This was carried out by characterizing the effect of differing amounts of organoclay bonded to a prioritized support matrix, considering the contribution of support matrices to sorption, and optimizing previously developed processing techniques to enhance and strengthen organoclay coverage on the surface of an optimal support matrix.

3) To determine the effects of naturally-occurring clay minerals commonly added to animal feeds in the balanced diet of Sprague-Dawley rats throughout pregnancy. Evaluations of maternal and fetal toxicity (i.e., maternal body weights, maternal feed intakes, litter weights, and embryonic resorptions) and neutron activation analysis of tissues (i.e., liver, kidneys, tibia, brain, uterus, pooled placentas, and pooled embryonic mass) were used to assess the influence of the clay on mineral uptake and utilization in the pregnant rat.

CHAPTER II

DELINEATION OF A SEQUENCE OF CHANGES TO ENDPOINTS INVOLVED IN CELLULAR HOMEOSTASIS FOLLOWING CHLOROPHENOL EXPOSURE IN CLONE 9 CELLS

Chlorinated phenols (CPs) constitute a series of 18 mono-, di-, tri-, and tetra-chlorinated isomers and one penta-chlorinated compound (Figure 1). As a group of industrial chemicals they have been used worldwide as fungicides, bactericides, herbicides, insecticides, and precursors in the synthesis of other pesticides since the early 1930s. Although CPs containing less than three chlorine substituents are of limited use today, pentachlorophenol (C_5P) has been used extensively as a wood preserving agent to control discoloration and deterioration of newly cut lumber (Ahlborg and Thunberg, 1980; Seiler, 1991; IARC, 1991; USDHHS, 1999).

CPs are readily absorbed through the skin, lungs, and gastrointestinal tract and exhibit strong biological effects. Once absorbed, CPs are distributed throughout the body, accumulating in the liver, kidneys, brain, spleen, and fat (Braun et al., 1977). CPs then partition into the phospholipid bilayers of mitochondrial membranes which increases the permeability of protons to alter proton transport through the membrane. This leads to dissipation of the energy gradient and the uncoupling of oxidative phosphorylation (Weinbach, 1954; Shannon et al., 1991), which leads to depletion of cellular ATP (Aschman et al., 1989). CPs have also been found to inhibit cytochrome P_{450} dependent oxidation processes and phase II metabolism (Carlsson, 1978; Aschmann

et al., 1989) including glutathione-*S*-transferase and microsomal epoxide hydroxylase (Moorthy and Randerath, 1996). Specifically, C_5P has been shown to inhibit Ca^{2+}-ATPase activity (Janik and Wolf, 1992) and various sulfation processes (Mulder and Scholtens, 1977) and is able to bind to microsomal protein and DNA (van Ommen, 1986; Waidyanatha et al., 1996; Lin et al., 1999). However, a sequence of specific cellular responses correlating to exposure levels in a sensitive cell line for *in vitro* testing has not been established. This is worthwhile since the extent of these responses upon exposure to environmental samples may provide verification of effective and complete remediation, particularly on Superfund sites.

Previous studies in *Hydra attenuata* have shown a linear relationship between toxicity and the degree of chlorine substitution with C_5P > 2,3,4,5-tetrachlorophenol (2,3,4,5-C_4P) > 2,3,5-trichlorophenol (2,3,5-C_3P) > 3,5-dichlorophenol (3,5-C_2P) > 4-chlorophenol (4-CP) > phenol (Mayura et al., 1991). In addition, the Clone 9 cell line, derived from normal rat liver epithelium, has shown sensitivity to various chemical agents frequently identified in addition to CPs at wood preservation-based Superfund sites, including PAHs (Reeves et al., 2001), in particular BaP (Barhoumi et al., 2000; Barhoumi et al., 2002). In the present study, several endpoints were used to evaluate cellular homeostasis, specifically intracellular glutathione (GSH) and Ca^{2+} concentrations, generation of reactive oxygen species (ROS), mitochondrial membrane potential (MMP), plasma membrane potential (PMP), pH, and gap junctional intracellular communication (GJIC). These parameters were evaluated immediately after dosing and following 24 h in the continued presence of CPs with varying degrees of

chlorination in order to delineate the sequence of measurable biological changes leading to cellular injury caused by CP exposure in the Clone 9 cell line.

2.1 Materials and methods

2.1.1 Chemicals

Culture media, Dulbecco's phosphate buffered saline (PBS), serum, and general chemical reagents were purchased from Sigma (St. Louis, MO). C_5P (98% purity) and phenol were purchased from Aldrich (Milwaukee, WI). 2,3,4,5-C_4P (99.0% purity), 2,3,5-C_3P (99.0% purity), 3,5-C_2P (98.7% purity), and 4-CP (99.9% purity) were purchased from Supelco (Bellefonte, PA). Tissue culture flasks and 96-well plates were purchased from Corning (Oneonta, NY) and Becton Dickinson (Franklin Lakes, NJ). Lab-Tek 2-well 1.0 chamber slides were purchased from Nunc, Inc. (Naperville, IL). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC_4(3)), monochlorobimane (mBCl), Fluo-4, AM, Rhodamine (R123), 2',7'-bis-2(carboxymethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), and 5-carboxyfluorescein diacetate (CFDA) were purchased from Molecular Probes, Inc. (Eugene, OR). Stock solutions of CPs were prepared in 100% ethanol and diluted in PBS. For use in cell cultures, CPs were dissolved in Ham's Nutrient Mixture F-12 culture medium for serial dilution (0.1 to 1000 μM, <1.0% ethanol) immediately prior to each experiment. Fluo-4, AM was prepared with DMSO and diluted with medium to 3.0 μM (0.3% final DMSO concentration) for loading in cultured cells. DiBAC_4(3) was prepared as 20 mM stock in

DMSO and used at 10 μM in medium (0.05% DMSO). Stock of mBCl was prepared as 50 mM stock in ethanol and diluted to 50 μM in PBS (0.1% ethanol). CM-H_2DCFDA was prepared as 5 mM stock in DMSO and used as 5 μM in medium (0.05% DMSO). R123 was prepared as 5 mg/mL stock in ethanol and used as 5 μg/mL in medium. CFDA was prepared as 10 mg/mL and used as 10 μg/mL in DMSO. Stock of BCECF-AM was prepared as 250 μM in DMSO and used as 0.25 μM. Janus green dye was purchased from Sigma (St. Louis, MO) and dissolved in PBS at 1 mg/mL immediately prior to each experiment.

2.1.2 Cell culture

Experiments were conducted using the rat liver cell line Clone 9 (ATCC, CRL 1439, passage 17) between passages 21 to 40. Clone 9 cells were grown in Ham's Nutrient Mixture F-12 containing 10% fetal bovine serum. Analysis of cell viability, intracellular GSH and Ca^{2+}, ROS, pH, MMP, and PMP were conducted on 96-well plates seeded at a density of 20,000 cells/well, while GJIC analysis was conducted using 2-well 1.0 slides seeded at a density of 100,000 cells/well. For long-term analysis cells were cultured for 24 h, dosed with graded concentrations of CPs, and cultured for an additional 24 h prior to analysis, while cells were seeded and cultured for 48 h prior to analysis of short-term treatments.

2.1.3 Assessment of cell viability (IC_{50})

Cell viability was determined using the Janus green spectrophotometric assay as described in Reick et al. (1993). Janus green selectively stains damaged cells blue, presumably by altering membrane integrity, which allows intracellular penetration of the dye. The weakly cationic dye interacts with anionic intracellular organelle membranes through electrostatic and hydrophobic interactions that are subsequently disrupted with the addition of alcohol, allowing extraction of the dye from cells. The concentration of each toxin that causes 50% inhibition of cell proliferation at 24 h (IC_{50}) was determined. Monolayers of cells were grown in 96-well tissue culture plates for 24 h prior to treatment with concentrations ranging from 1 to 1000 μM of each CP at 37 °C for 24 h. Cells were then rinsed in phosphate-buffered saline (PBS) and fixed with ethanol. Janus green (1 mg/mL solution in PBS) was applied for 60 s and excess dye was removed by rinsing with PBS. Janus green in the cells was then extracted with 100% ethanol and diluted in ddH_2O. The relative Janus green concentration was determined by measuring absorbance at 630 nm with a microplate fluorescence reader (Bio-Tek Fl600, Bio-Tek Instruments, Inc., Winooski, VT). This value was correlated to absolute cell number using a standard curve. Experiments were performed in triplicate.

2.1.4 Laser cytometry

The effects of graded concentrations of C_5P (0 to 150 μM), 2,3,4,5-C_4P (0 to 150 μM), 2,3,5-C_3P (0 to 400 μM), 3,5-C_2P (0 to 150 μM), 4-CP (0 to 1000 μM) or phenol (0 to 1000 μM) on Clone 9 cells were monitored. Intracellular GSH and Ca^{2+}

concentrations, pH, ROS, MMP, and PMP were analyzed using a microplate fluorescence reader (Bio-Tek Fl600, Bio-Tek Instruments, Inc., Winooski, VT) while GJIC between cells was monitored using a Meridian Ultima workstation (Meridian Instruments, Okemos, MI). Both methods used to conduct fluorescence measurements are detailed below. It should be noted that to minimize differences in each probe from experiment to experiment, cells were seeded at the same density and each treatment was compared to a separate, concurrently monitored, control.

2.1.5 Analysis of Ca^{2+}, pH, ROS, MMP, and PMP

For microplate fluorescence reader analysis of intracellular Ca^{2+} concentrations, pH, ROS, MMP, and PMP following short-term CP treatment, cells were rinsed with serum- and phenol red-free medium, loaded with fluorescent probe, and incubated at 37 °C. Following loading, cells were again washed with serum and phenol red-free medium and basal measurements were recorded by scanning the wells for 30 to 45 s. Graded concentrations of CPs were then added and changes in fluorescence intensity were recorded every 15 s for 15 min. For analysis of the same parameters following long-term CP treatment cells were rinsed with serum- and phenol red-free medium, loaded with fluorescent probe and incubated at 37 °C. Following loading, cells were washed with serum- and phenol red-free medium and basal measurements were recorded for each well and corrected to reflect cell viability. For intracellular Ca^{2+} concentrations, ROS, MMP, and PMP, all fluorescence measurements were recorded at 485/20 nm excitation and 530/25 nm emission. Fluorescence intensity levels for pH were measured

using a single excitation at 485/20 nm and dual emission at 530/25 and 645/40 nm wavelengths.

Intracellular Ca^{2+} was measured in cells loaded with 3 μM Fluo-4 AM and incubated for 1 h prior to analysis. Fluo-4, AM is a non-ratiometric visible wavelength probe that exhibits approximately a 40-fold enhancement of fluorescence intensity with Ca^{2+} binding (Gee et al., 2000).

PMP measurements were conducted after incubating cells loaded with 10 μM $DiBAC_4(3)$ for 30 min. This slow responding probe enters depolarized cells where it binds to intracellular proteins or membranes to exhibit enhanced fluorescence over that of extracellular $DiBAC_4(3)$ (Apell and Bersch, 1987; Haughland, 2000). The anionic nature of $DiBAC_4(3)$ prevents error associated with nonspecific membrane interaction, as it is unable to cross the mitochondrial membrane. In contrast, hyperpolarization of the plasma membrane is exhibited by decreased fluorescence resulting from dye extrusion. Measurements are conducted in the continual presence of extracellular probe.

ROS was measured in cells loaded with 5 μM CM-H_2DCFDA and incubated for 30 min. CM-H_2DCFDA is an oxidation sensitive fluorescent probe that passively diffuses into cells, where endogenous esterases cleave acetate moieties releasing the corresponding dichlorodihydrofluorescein derivative while the thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Further oxidation yields a stable, fluorescent adduct that is trapped inside the cells, facilitating long-term studies (Haughland, 2000).

MMP was analyzed using R123, a cell-permeant, cationic, fluorescent dye that is widely used as a marker of mitochondria structure and as an indicator of mitochondrial activity (Haughland, 2000). The probe is readily sequestered by active mitochondria, without induction of cytotoxic effects. Cells were loaded with 5 μg/mL R123 and incubated for 15 min before fluorescence intensity levels were measured.

Intracellular pH was measured after incubating cells loaded with 0.25 μM BCECF-AM for 15 min. BCECF is an anionic, dual-emission ratiometric pH indicator (Haughland, 2000) resulting from hydrolysis of the AM ester. The probe is membrane permeant and, upon entering the cell, is hydrolyzed by intracellular esterases to release the fluorescent BCECF-free acid that is retained by the cell (Musgrove et al., 1986).

2.1.6 Analysis of GSH

Cellular GSH was evaluated with mBCl, a cell-permeant, non-fluorescent probe that passively diffuses across the plasma membrane and forms fluorescent adducts with GSH and thiol-containing proteins in a reaction catalyzed by glutathione-*S*-transferase (Shrieve et al., 1988; Haughland, 2000). Kinetic analysis of mBCl-GSH conjugation was used to measure the effects of both short- and long-term treatments. Cells were rinsed with serum-free medium and scanned sequentially for 30 to 45 s to determine basal fluorescence intensity levels of GSH. Cells were then loaded with 50 μM mBCl in the presence or absence of CPs, which were added in concert for short-term analysis. Kinetic analysis of mBCl-GSH conjugation was performed by recording changes in

fluorescence intensity at 15 s intervals for 15 min at 360/40 nm excitation and 460/40 nm emission wavelengths (Barhoumi et al., 1995).

2.1.7 Analysis of gap junctional intracellular communication (GJIC)

For GJIC analysis, cells were rinsed with serum- and phenol red-free medium, loaded for 15 min with 10 μg/mL CFDA. CFDA is a membrane-permeable, nonfluorescent precursor of carboxyfluorescein, which is membrane impermeant and is transferred only between cells that are coupled by gap junctions (Haughland, 2000). After loading, cells were washed and maintained in serum- and phenol red-free medium in the presence of absence of CPs for analysis. GJIC between cells was monitored by dye coupling using the fluorescence recovery after photobleaching (FRAP) technique adapted to determine the rate constant for dye transfer as previously described by Barhoumi et al. (1993).

2.1.8 Statistical analysis

Unless otherwise noted, all values are expressed as means normalized to control cells ± SE. Data were compared statistically using the General Linear Models procedure using the Dunnett method and considered significant if $p<0.05$.

2.2 Results

2.2.1 Cell viability

Cell viability, represented as concentration of CP causing 50% inhibition of cell proliferation at 24 h (IC_{50}), was determined for four of the six CPs investigated in Clone 9 cells exposed to graded doses of CPs. C_5P, 2,3,4,5-C_4P, and 3,5-C_2P exhibited similar IC_{50} values of approximately 150 μM while the IC_{50} value for 2,3,5-C_3P was 400 μM (data not shown). However, cytotoxicity was first detected with 100 μM C_5P, 50 μM 2,3,4,5-C_4P, 25 μM 2,3,5-C_3P, and 50 μM 3,5-C_2P. Phenol and 4-CP did not appear to significantly affect cell viability at concentrations up to, and including, the maximum concentration tested (1 mM). Hence no IC_{50} was determined.

2.2.2 Analysis of GJIC

GJIC was monitored in cells exposed to graded doses of CPs using a fluorescence recovery after photobleaching assay. Immediately after CP exposure, significant suppression of GJIC was detected in cells treated with a minimum of 10 μM C_5P (49.3 ± 4.8%), 10 μM 2,3,4,5-C_4P (71.2 ± 3.3%), 10 μM 2,3,5-C_3P (41.7 ± 2.2%), 100 μM 3,5-C_2P (75.9 ± 4.7%) or 100 μM 4-CP (18.5 ± 5.8%) (Figure 8). Phenol affected cells only at much greater doses (1000 μM). After 24 h, the effect of C_5P, 2,3,4,5-C_4P, and 3,5-C_2P was maintained. However, the effect of 2,3,5-C_3P disappeared at low concentrations and appeared again at 150 μM (38.7 ± 0.8%) (Figure 9). Neither 4-CP nor phenol significantly affected cells at the doses tested over long-term exposure. All of the groups of cells analyzed were viable, based on the absence of dye leakage

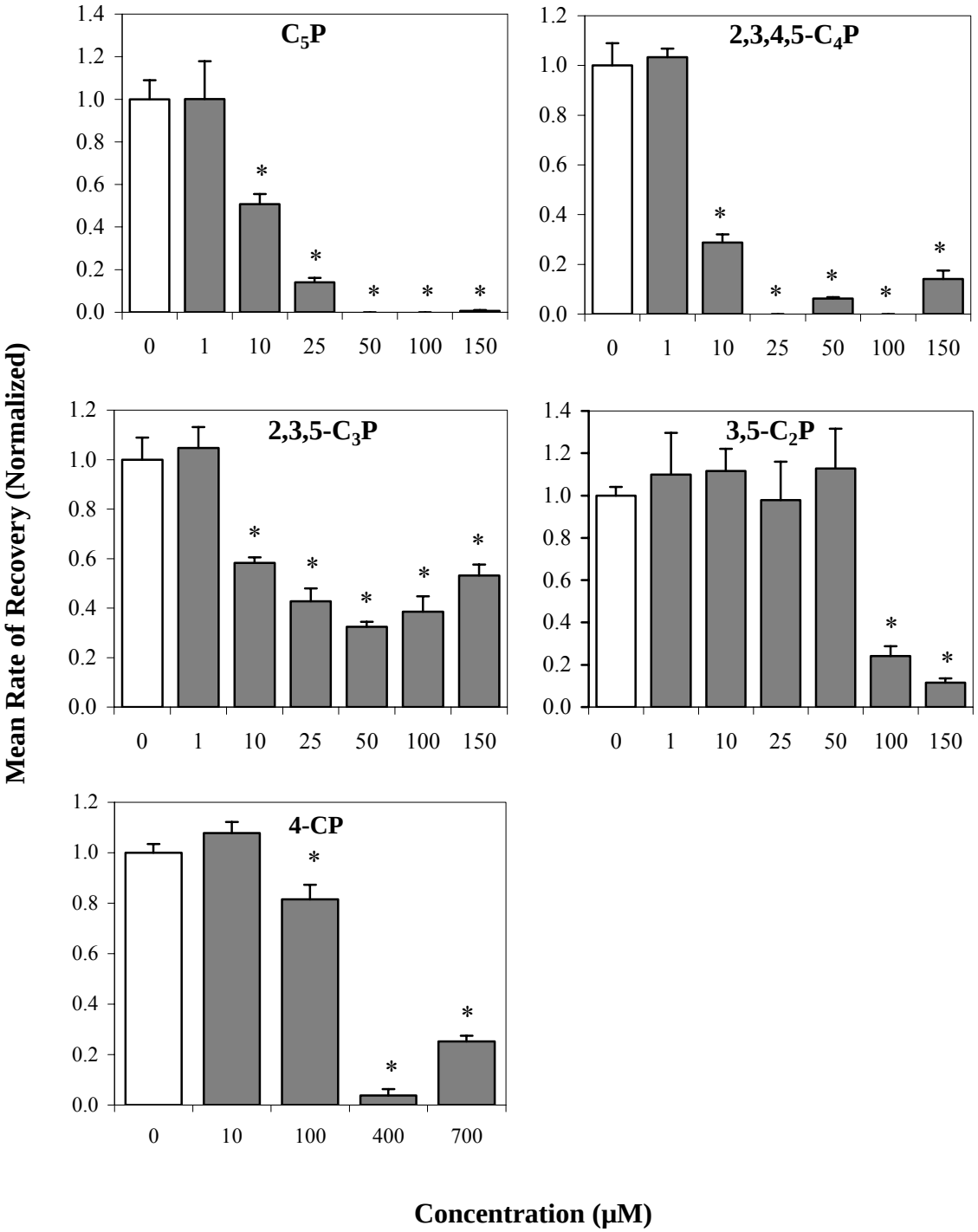


Figure 8. Effect of short-term exposure to CPs on GJIC. Values are the mean rate constant for fluorescence recovery of carboxyfluorescein after photobleaching ± SE for 20 cells and are normalized to control cells. An asterisk indicates a significant change in the endpoint (p<0.5) from untreated control cells.

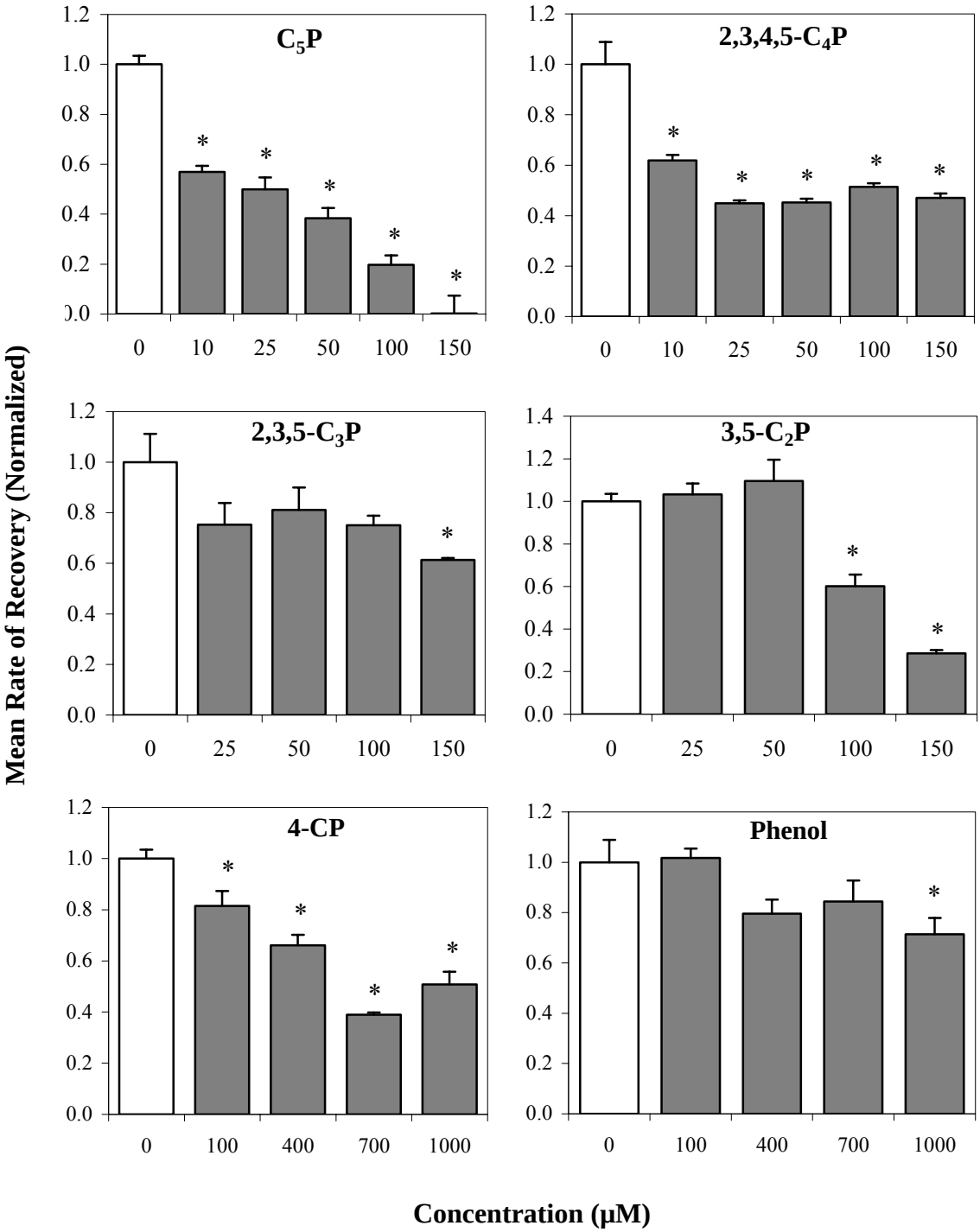


Figure 9. Effect of long-term exposure to CPs on GJIC. Values are the mean rate constant for fluorescence recovery of carboxyfluorescein after photobleaching ± SE for 20 cells and are normalized to control cells. An asterisk indicates a significant change in the endpoint (p<0.5) from untreated control cells.

from the corresponding non-photobleached control cells that would indicate reduced plasma membrane potential.

2.2.3 Analysis of intracellular pH

Although decreased GJIC was a short-term affect of CP exposure, changes to intracellular pH was more meaningful. Initially, exposure to 1 μM C_5P (2.5 ± 0.7%), 1 μM 2,3,4,5-C_4P (4.5 ± 0.3%), 10 μM 2,3,5-C_3P (13.6 ± 0.8%), and 10 μM 3,5-C_2P (2.9 ± 0.3%), and 100 μM 4-CP (2.2 ± 0.3%) decreased intracellular pH, frequently in a dose-dependent manner (Figure 10). Phenol did not appear to significantly affect the pH over short-term treatments up to the maximum dose tested (1000 μM). For long-term treatments, pH changes disappeared at lower CP concentrations and were only maintained at higher treatment levels of 100 μM C_5P (16.8 ± 1.4%), 50 μM 2,3,4,5-C_4P (11.3 ± 1.5%), 25 μM 2,3,5-C_3P (5.6 ± 1.5%), and 25 μM 3,5-C_2P (4.8 ± 2.3%). Minimal pH changes were seen following exposure to lower chlorinated phenols, as 1000 μM 4-CP (12.5 ± 2.4%) and 100 μM phenol (7.4 ± 1.5%) significantly decreased intracellular pH with 24 h of continuous treatment (Figure 11).

2.2.4 Analysis of intracellular GSH

Depletion of GSH occurred simultaneously or directly following decreases in pH. Immediately following exposure, a significant depletion of GSH was apparent with 10 μM C_5P (8.6 ± 1.1%), 10 μM 2,3,4,5-C_4P (5.8 ± 0.3%), 100 μM 2,3,5-C_3P (2.5 ± 0.6%),

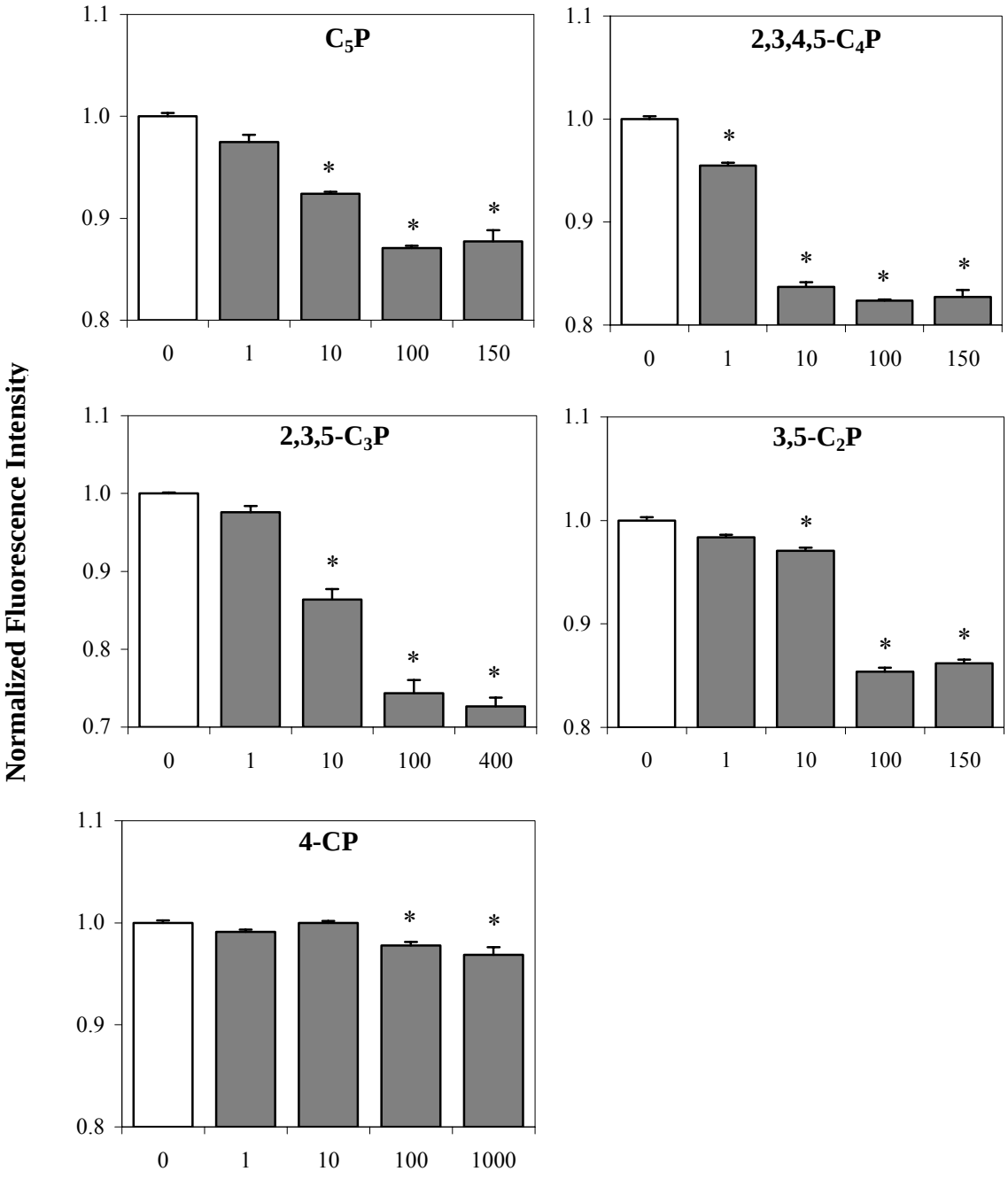


Figure 10. Effect of short-term exposure to CPs on pH. Values are the mean of the ratio of fluorescence intensity at dual emission wavelengths (530/645 nm) and are normalized to control cells ± SE for 3 wells. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

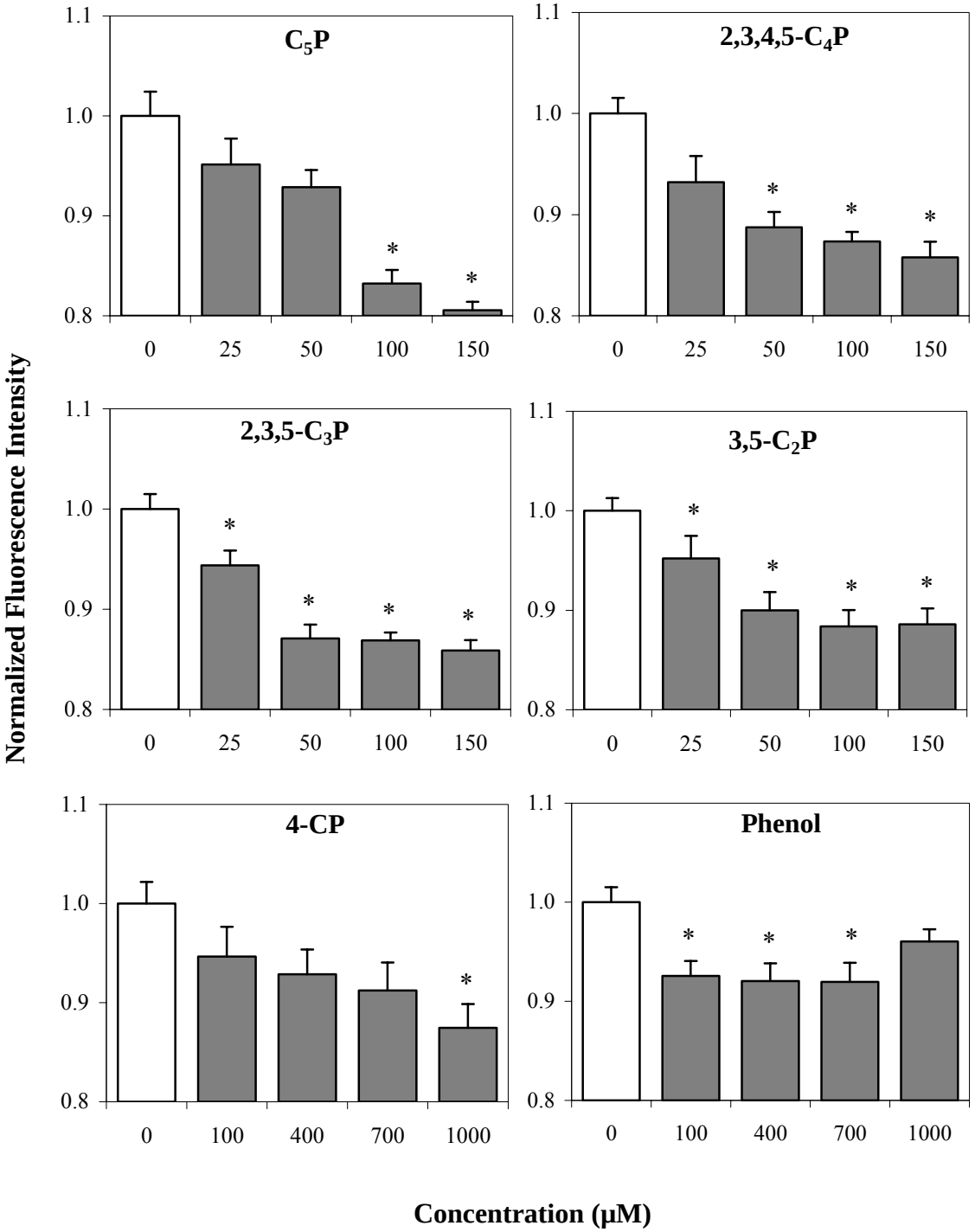


Figure 11. Effect of long-term exposure to CPs on pH. Values are the mean of the ratio of fluorescence intensity at dual emission wavelengths (530/645 nm) and are normalized to control cells ± SE for 3 wells. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

and 100 μM 3,5-C_2P (12.4 ± 0.6%) (Figure 12). Treatments of less than 1000 μM 4-CP (7.0 ± 0.4%) did not significantly affect GSH. Decreased GSH was not maintained over the long-term following CP exposure, except in the case of 4-CP (7.44 ± 0.45%). Fluorescence of the mBCl-GSH conjugate was decreased at a minimum dose of 100 μM C_5P (11.1 ± 0.4%), 50 μM 2,3,4,5-C_4P (4.8 ± 0.5%), 50 μM 2,3,5-C_3P (4.4 ± 0.5%), or 50 μM 3,5-C_2P (1.8 ± 0.7%) (Figure 13).

2.2.5 Analysis of ROS

Immediately following CP exposure, ROS generation was increased in cells treated with 10 μM C_5P (7.3 ± 0.6%), 100 μM 2,3,4,5-C_4P (5.7 ± 0.3%), 100 μM 2,3,5-C_3P(4.7 ± 1.8%), 100 μM 3,5-C_2P (5.6 ± 1.2%), 100 μM 4-CP (20.8 ± 1.9%), and 100 μM phenol (6.2 ± 1.5%) (Figure 14). A dose-dependent increase in ROS generation was apparent in all CPs following long-term exposure beginning at 50 μM 2,3,4,5-C_4P (18.2 ± 5.1%) and 50 μM 2,3,5-C_3P (19.4 ± 1.6%) and at greater doses of other CPs. Further, the effects of 100 μM 3,5-C_2P (12.1 ± 2.1%) and 100 μM 4-CP (14.9 ± 2.1%) were maintained over the 24 h period, although this was not apparent in cells treated with 100 μM C_5P (17.8 ± 2.0%) (Figure 15).

2.2.6 Analysis of PMP

The final parameter to show significant changes immediately following CP exposure in Clone 9 cells was PMP. Treatment with 100 μM C_5P (8.6 ± 0.8%), 100 μM

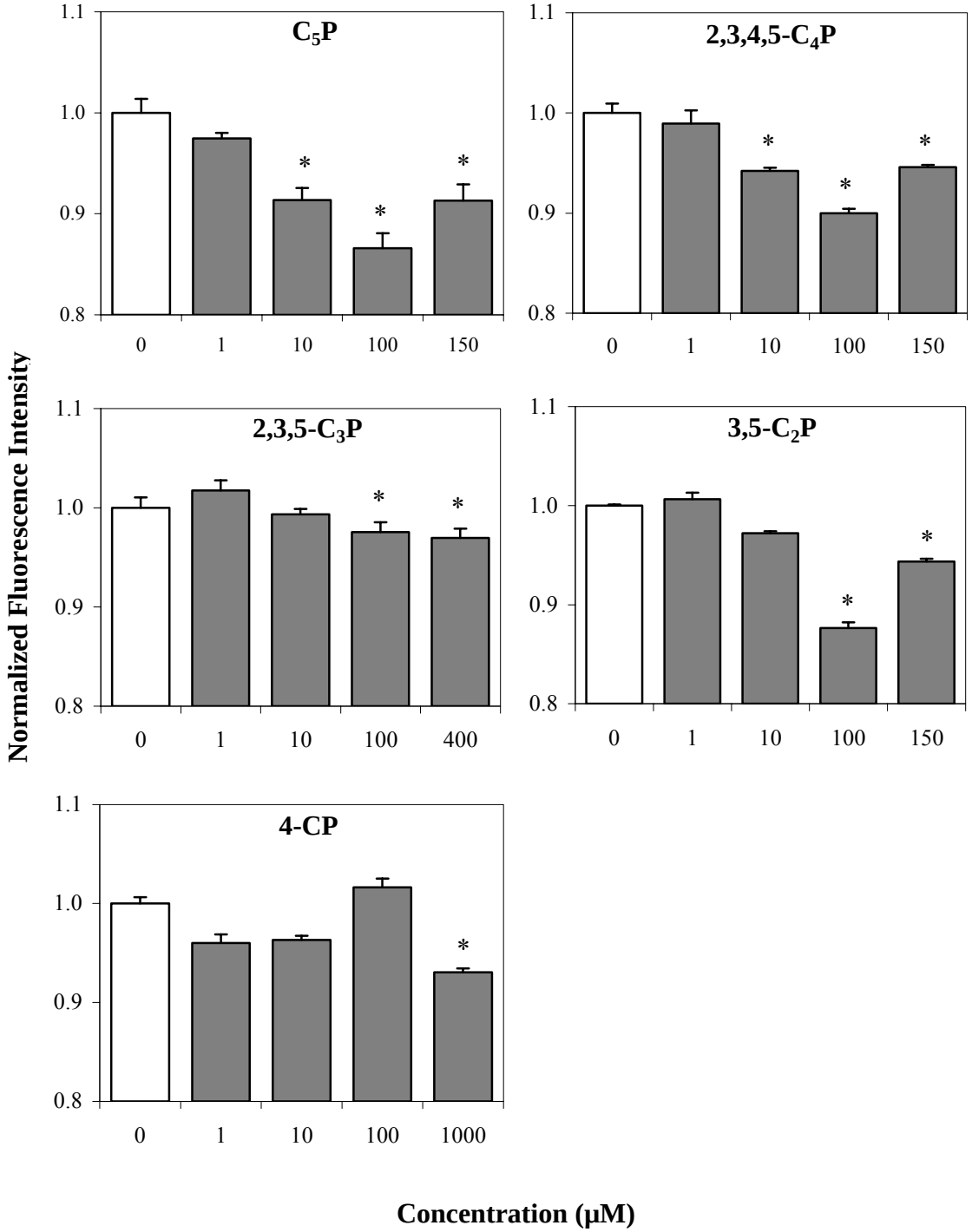


Figure 12. Effect of short-term exposure to CPs on GSH. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

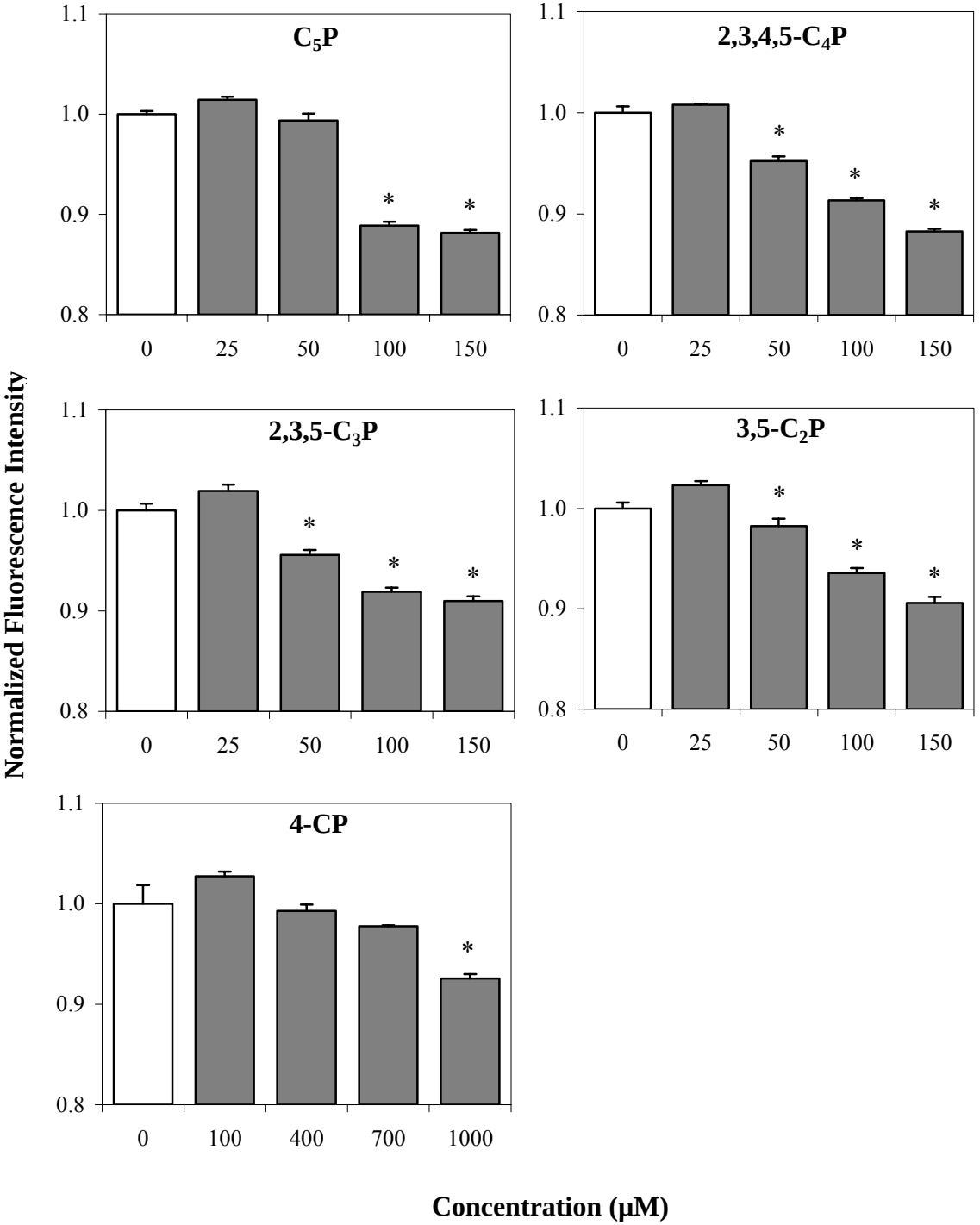


Figure 13. Effect of long-term exposure to CPs on GSH. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

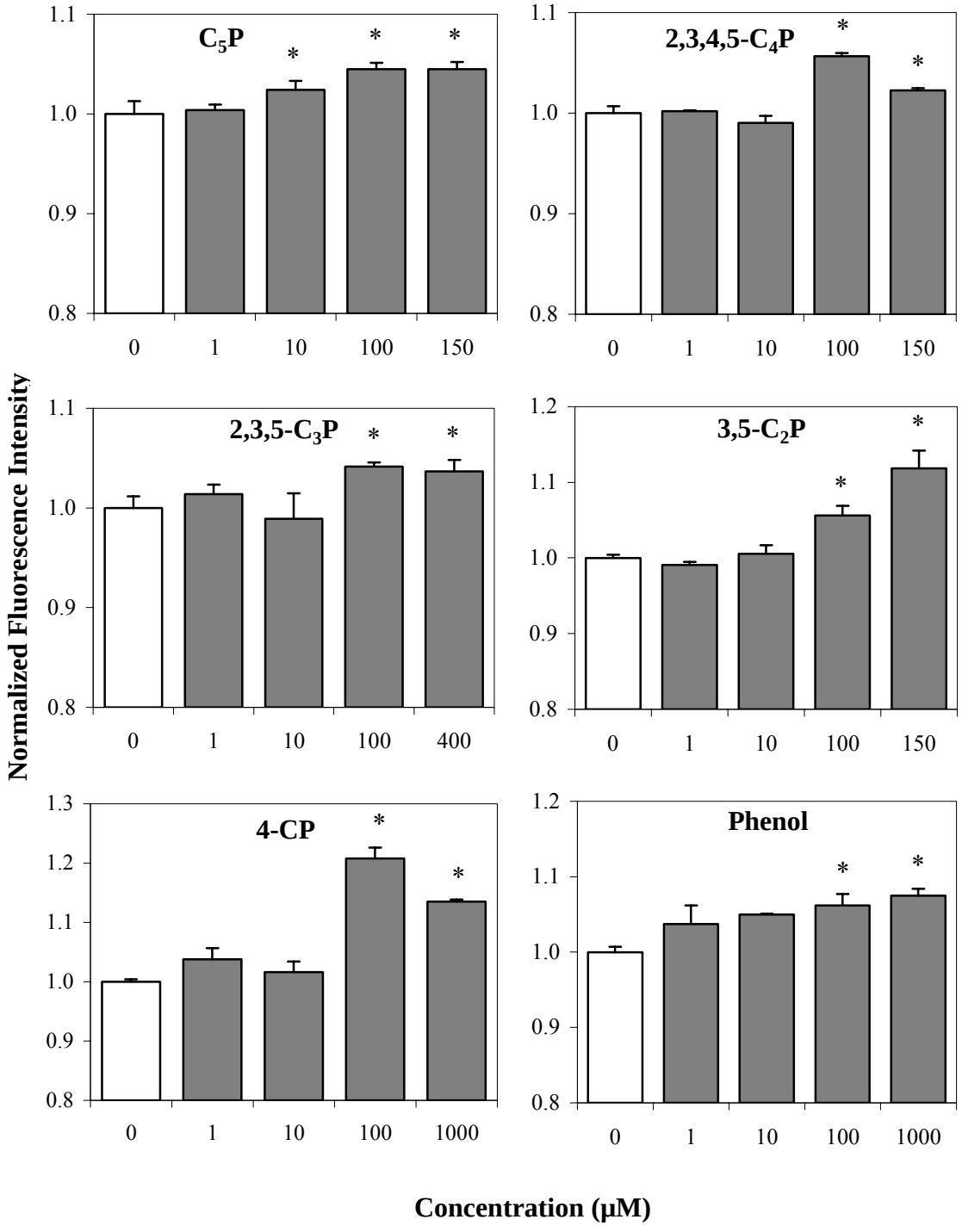


Figure 14. Effect of short-term exposure to CPs on ROS generation. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

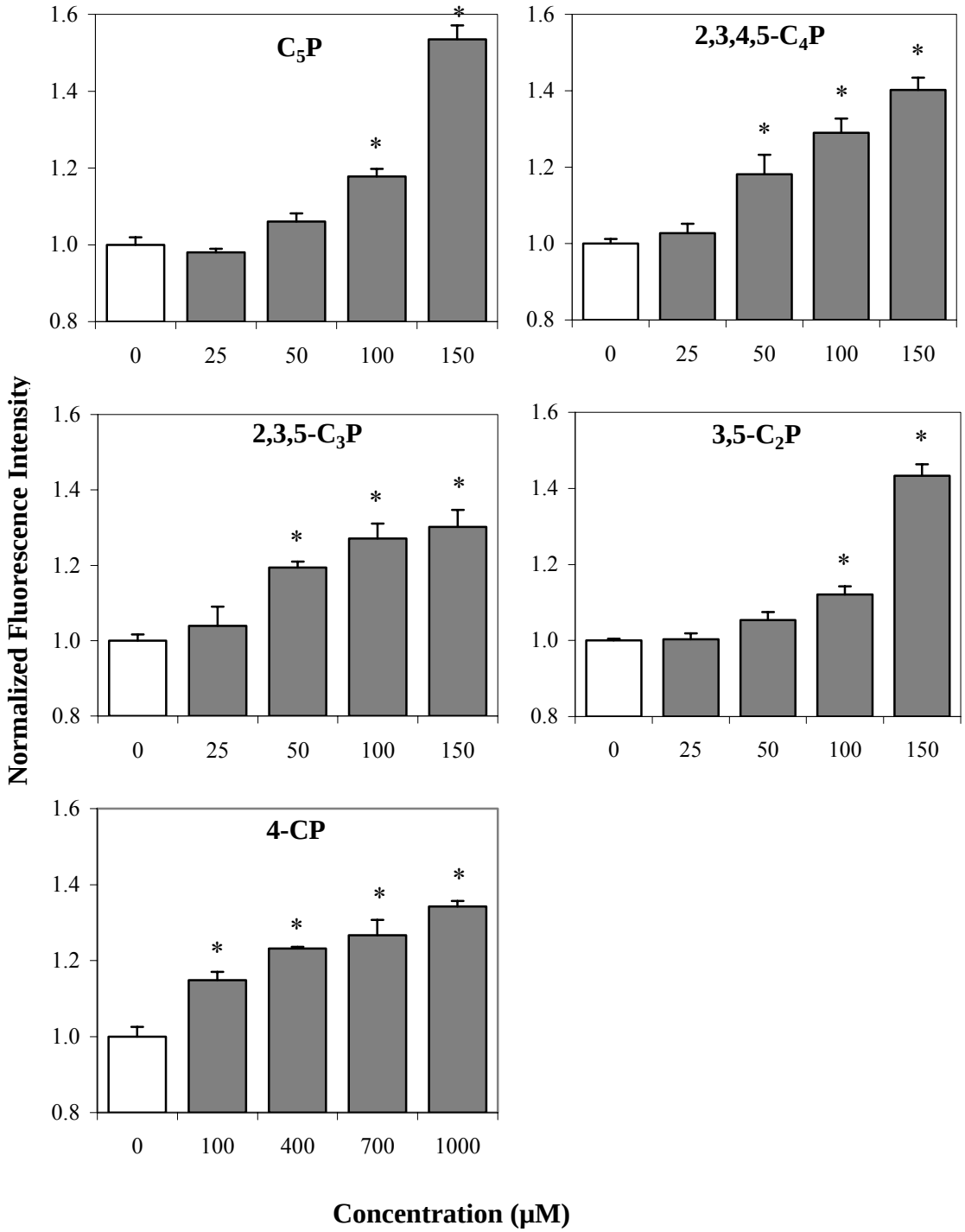


Figure 15. Effect of long-term exposure to CPs on ROS generation. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

2,3,4,5-C_4P (12.7 ± 0.8%), or 100 μM 3,5-C_2P (4.6 ± 0.3%) suppressed DiBAC$_4$(3) fluorescence, indicating plasma membrane hyperpolarization (Figure 16). This effect was observed only at high-dose 400 μM 2,3,5-C_3P (7.8 ± 0.2%), and was not apparent in 4-CP- or phenol-treated cells up to the greatest doses tested (1000 μM). In contrast, long-term exposure to 100 μM C_5P (14.0 ± 4.3%), 100 μM 2,3,4,5-C_4P (6.3 ± 0.4%), 100 μM 2,3,5-C_3P (16.2 ± 2.3%), or 100 μM 3,5-C_2P (10.9 ± 0.8%), caused plasma membrane depolarization, indicated by increased DiBAC$_4$(3) fluorescence (Figure 17).

2.2.7 Analysis of MMP

Significant changes in MMP were not observed for short term CP exposure (data not shown). However, with the continued presence of 25 μM C_5P (8.1 ± 2.4%), 50 μM 2,3,4,5-C_4P (28.4 ± 1.9%), 50 μM 2,3,5-C_3P (22.6 ± 1.9%), 50 μM 3,5-C_2P (14.9 ± 2.8%), and 100 μM phenol (12.7 ± 0.8%) decreased R123 fluorescence, suggesting mitochondrial membrane depolarization (Figure 18). Significant changes in cells dosed with 4-CP were not apparent up to the greatest doses tested (1000 μM).

2.2.8 Analysis of basal intracellular Ca^{2+}

Increased basal intracellular Ca^{2+} levels were detected only with 24 h exposure to CPs and in a dose-dependent manner (Figure 19). Specifically, continuous 24 h exposure to 50 μM C_5P (22.6 ± 2.6%), 50 μM 2,3,4,5-C_4P (17.5 ± 2.5%), 50 μM 2,3,5-

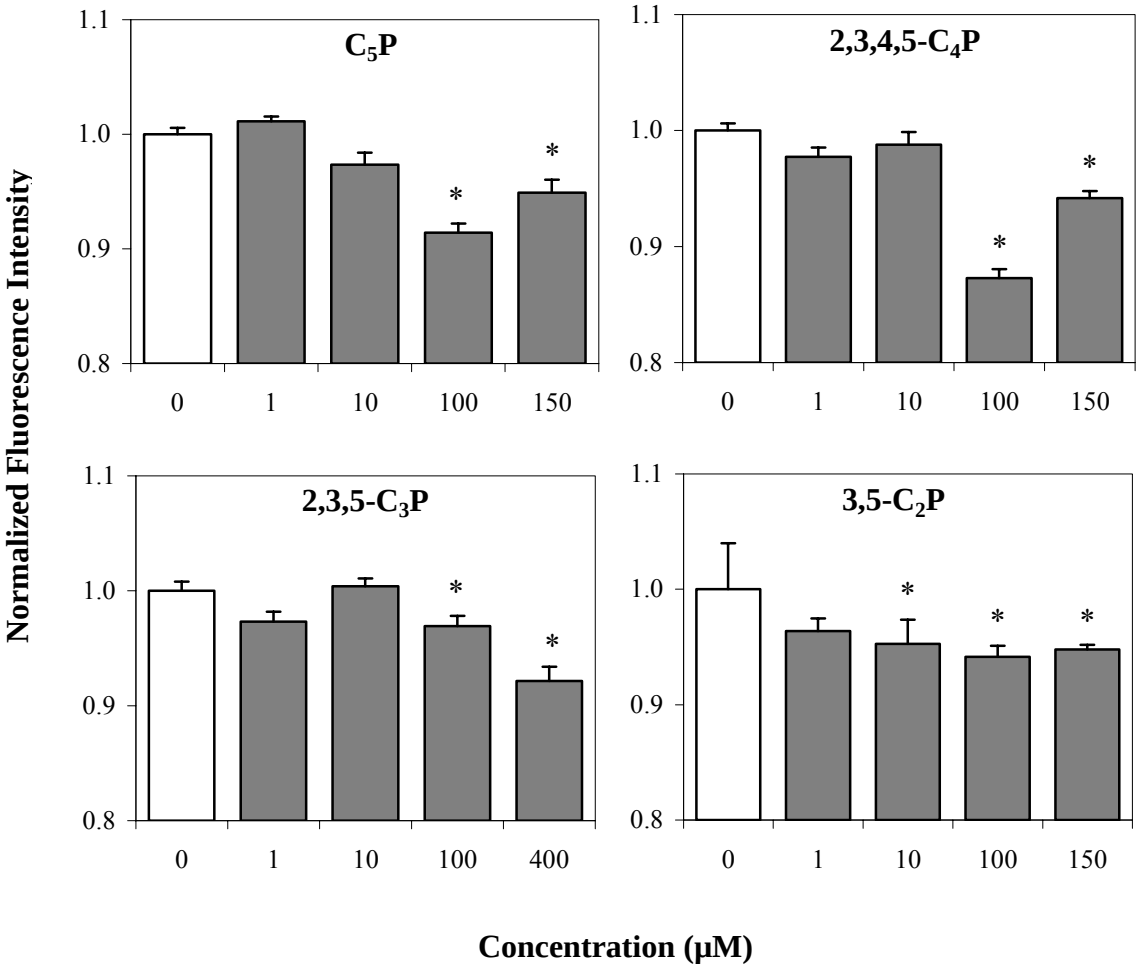


Figure 16. Effect of short-term exposure to CPs on PMP. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

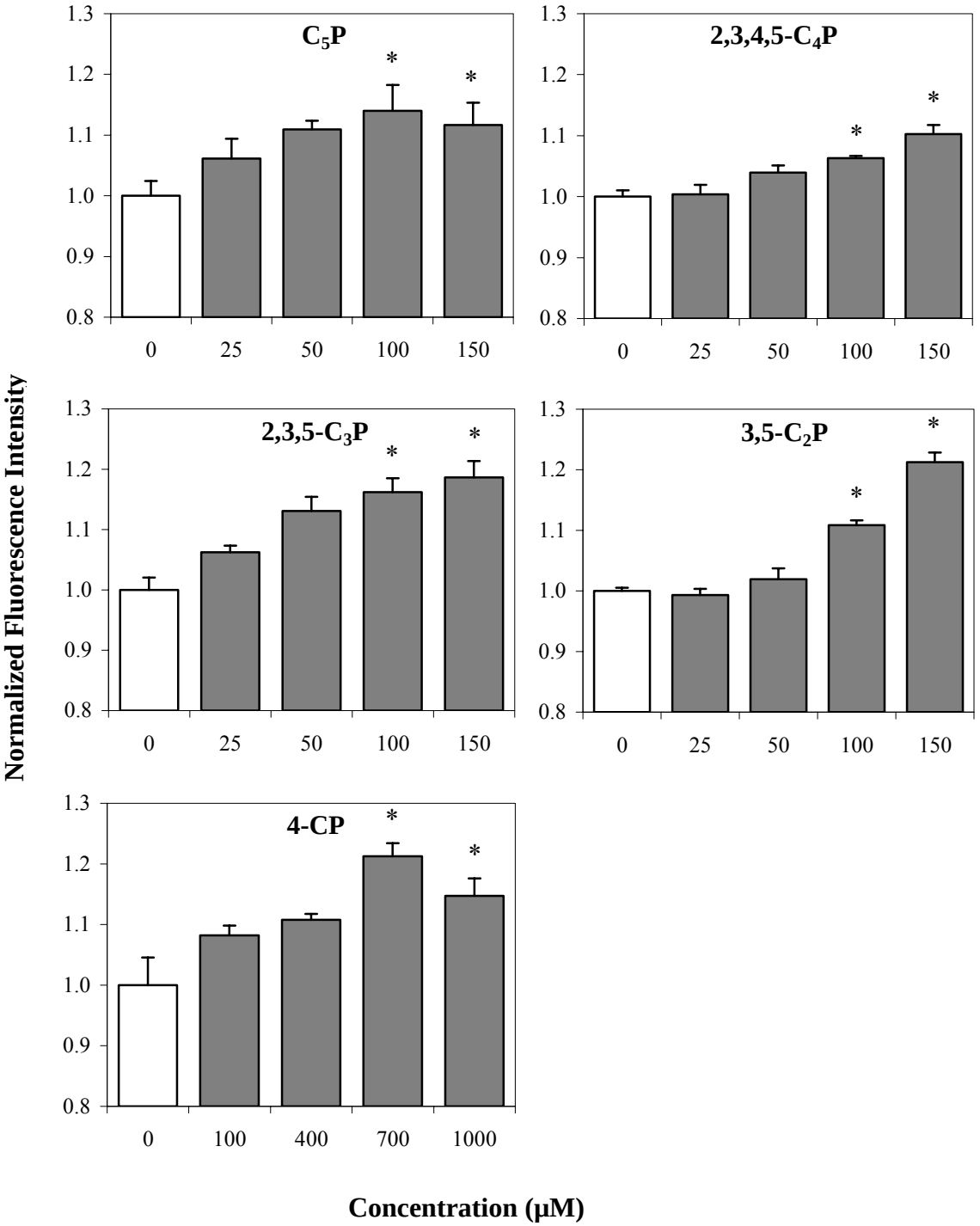


Figure 17. Effect of long-term exposure to CPs on PMP. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

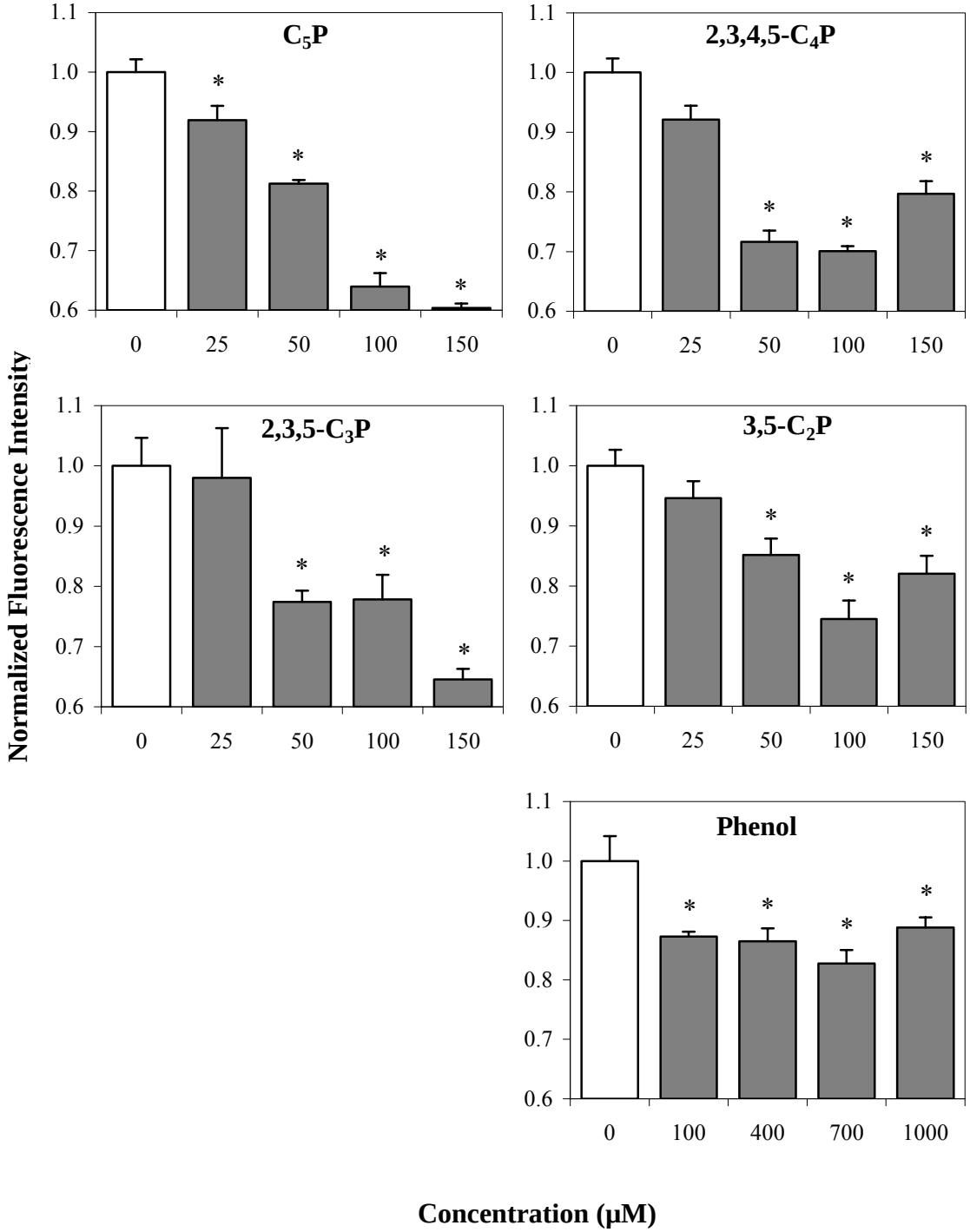


Figure 18. Effect of long-term exposure to CPs on MMP. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

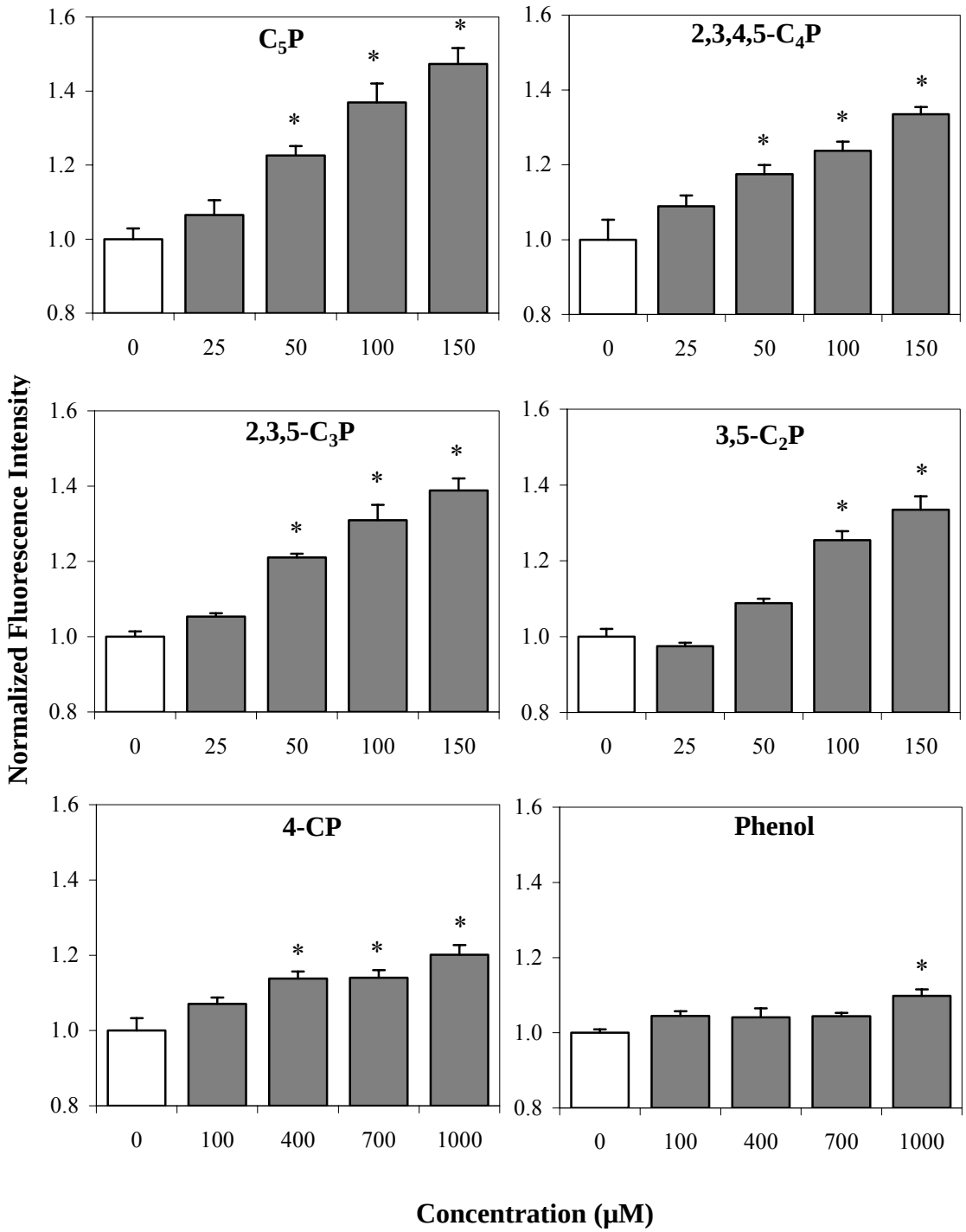


Figure 19. Effect of long-term exposure to CPs on Ca^{2+}. Values are the mean fluorescence intensity for 3 wells normalized to control cells ± SE. An asterisk indicates a significant change in the endpoint from control cells (p<0.5).

C_3P (21.0 ± 1.0%), or 100 μM 3,5-C_2P (25.4 ± 2.4%), but only at greater doses of 400 μM 4-CP (13.8 ± 1.9%) and 1000 μM phenol (9.8 ± 1.7%).

2.3 Discussion

In this study, the effects of CP exposure on selected endpoints cellular homeostasis was evaluated in Clone 9 cells. Of the seven parameters monitored in this study, both GJIC and pH were affected foremost by CP exposure. Although intracellular pH was frequently affected by lower doses, C_5P- and 2,3,4,5-C_4P-treated cells exhibited an apparent recovery from the initial insult, as the dose required to maintain a lowered pH over 24 h (100 μM and 50 μM, respectively) was a great deal higher than that necessary to produce a significant decrease in the parameter initially (1 μM). Further, long-term decreases in pH occurred at concentrations similar to those necessary to initially detect cytotoxicity, (i.e., 100 μM C_5P). This is not surprising as acidosis has been shown to protect against lethal oxidative injury, possibly through inhibition of degradative enzymes that become activated with ATP depletion (Gores et al., 1988; Bond et al., 1991; Bronk and Gores, 1991; Harper et al., 1993).

In contrast to pH, the decrease in GJIC detected with low-dose short-term exposure was maintained over the 24 h period for all CPs except 2,3,5-C_3P. Treatment with 10 μM 2,3,5-C_3P caused an initial decrease in GJIC, but long-term exposure did not significantly alter the parameter at doses below 150 μM. The maintained loss of GJIC may be the result of the effects of CPs on other cellular parameters. In particular, previous research suggests that downregulation of GJIC may be affected by decreased

cellular pH (White et al., 1990; Peracchia et al., 1996), changes to second messengers such as cAMP and Ca^{2+} (Rose and Lowenstein, 1975; Peracchia et al., 2000; Grazul-Bilska et al., 2001), increased intracellular ROS (Kuo et al., 1998), and changes to the phosphorylation states of connexins (Matesic et al., 1994; Guan and Ruch, 1996; Yamasaki et al., 1996; Upham et al., 1997).

In both short- and long-term exposure to either C_5P, 2,3,4,5-C_4P, 2,3,5-C_3P, or 3,5-C_2P, increased ROS generation was apparent at equal or slightly higher doses that those necessary to cause a significant depletion of GSH. ROS generation can increase due to the formation of highly reactive oxygen radicals during redox cycling. For example C_5P is metabolized to tetrachlorohydroquinone (TCHQ), which can be oxidized to the corresponding semiquinone radical (Juhl et al., 1985; Renner and Hopfer, 1990). GSH counteracts the increase in ROS by acting as an electron donor in order to reduce the semiquinone radical back to the semiquinone, in this case TCHQ. Reduction of the radical results in a decrease in the cytosolic GSH pool which puts the cell at risk for further oxidative damage (Wang et al., 1997; Townsend et al., 2003).

In addition to increased ROS generation, it has also been suggested that GSH deficiency may lead to mitochondrial damage (Jain et al., 1991). Previous research specific to C_5P exposure maintains that this occurs when the contaminant uncouples oxidative phosphorylation (Weinbach, 1954; Weinbach and Garbus, 1965), which allows for inhibition of cellular ATP synthesis (Aschman et al., 1989). Immediately following treatment with 10 to 100 μM C_5P, 2,3,4,5-C_4P, 2,3,5-C_3P, or 3,5-C_2P, cells showed diminished intracellular GSH, but no significant effects to mitochondrial membrane

potential were detected. However, the continuous presence of 25 to 50 μM C_5P, 2,3,4,5-C_4P, 2,3,5-C_3P, or 3,5-C_2P over 24 h caused significant mitochondrial membrane depolarization. This indicates that Clone 9 cells were able to recover from the initial CP insult but not from the depletion of intracellular GSH pools, a consequence of prolonged and/or high dose exposure.

Another consequence of prolonged exposure, increased intracellular Ca^{2+} was apparent only following continuous 24 h CP treatment. An uncontrolled and sustained increase in cytosolic Ca^{2+} has been shown to occur following mitochondrial membrane depolarization (Nicotera et al., 1989). Clone 9 cells were able to recover from short-term exposure and thus, both mitochondrial membrane depolarization and increased intracellular Ca^{2+} concentrations were seen only after an extended treatment period. Further, it has been reported that increased cytosolic Ca^{2+} is responsible for the rapid activation of Ca^{2+}-dependent degradative enzymes that damage vital cell components, resulting in critical cell injury or death (Herman et al., 1990). Thus, it is not surprising that plasma membrane depolarization occurred at the highest CP concentrations tested in this study. Although high dose CP treatment lead to acute plasma membrane hyperpolarization (signified by increased $DiBAC_4(3)$ fluorescence), depolarization (signified by decreased $DiBAC_4(3)$ fluorescence) was apparent after 24 h of exposure.

The IC_{50} values for continuous exposure to each compound for 24 h were greater for the more highly chlorinated compounds, indicating an increase in toxicity with an increased number of chlorine substituents. This has been observed in previous studies utilizing *Hydra attenuata* (Mayura et al., 1991), human embryonic palatal mesenchymal

cells (Zhao et al., 1995a), BF-2 cells (Babich and Borenfreund, 1987), bacteria including *Burkholderia* species *Rasc c2* and *Pseudomonas fluorescens* (Boyd et al., 2001), and bovine spermatozoa (Seibert et al., 1989). However, the IC_{50} values observed in this study suggest that the less highly chlorinated 3,5-C_2P (150 μM) may be more toxic than the more highly chlorinated 2,3,5-C_3P (400 μM). Although these congeners are all co-effective developmental hazards, previous research suggests that 3,5-C_2P is also a potential teratogen based on developmental toxicity assays, including *Hydra attenuata* and postimplantation rat whole embryo culture (Mayura et al, 1991).

The endpoints evaluated in this study showed effects resulting from CP exposure that were dependent upon dose, time, and the number of chlorine substituents. Clone 9 cells were affected similarly by exposure to C_5P or 2,3,4,5-C_4P, and similarly by 2,3,5-C_3P or 3,5-C_2P, however, the changes to specific endpoints resulting from treatment with 4-CP or phenol were slight and were observed only upon treatment with high doses and/or prolonged exposures. For example, decreases to GSH occurred following short-term exposure to 10 μM doses of C_5P or 2,3,4,5-C_4P, but at 100 μM doses of 2,3,5-C_3P or 3,5-C_2P, and only at 1000 μM doses of 4-CP. In the case of GJIC, long-term CP exposure led to decreased GJIC at 10 μM doses of C_5P or 2,3,4,5-C_4P, 100 to 150 μM doses of 2,3,5-C_3P and 3,5-C_2P, and 1000 μM doses of 4-CP.

The contribution of time to alterations in specific endpoints was most notable in the evaluation of MMP and intracellular Ca^{2+} concentrations, where significant changes to either parameter were observed only with long-term CP exposures. These parameters also exhibited a clear relationship between the number of chlorine substituents and the

dose-dependent changes to cellular endpoints observed with CP exposure. With long-term exposure, mitochondrial membrane depolarization occurred at 25 μM doses of C_5P, but at 50 μM doses of 2,3,4,5-C_4P, 2,3,5-C_3P, or 3,5-C_2P. In the case of alterations to intracellular Ca^{2+} concentrations, increases occurred after long-term exposure to 50 μM C_5P, 2,3,4,5-C_4P, or 2,3,5-C_3P, 100 μM 3,5-C_2P, 400 μM 4-CP, and 1000 μM phenol. Thus, the greatest toxicity was observed for high-dose, long-term exposure of cells to CPs with the greatest number of chlorine substituents.

The results of the present study demonstrate that the Clone 9 cell line is a sensitive *in vitro* model for CP exposure. Various cellular parameters may be screened to monitor cellular homeostasis and to delineate a sequence of events consistent with cellular injury. In the case of C_5P, this sequence consisted of decreased pH, GJIC, and GSH, increased ROS generation, and plasma membrane hyperpolarization as a result of short-term exposure. Prolonged C_5P exposure (24 h) led mitochondrial membrane depolarization, increased intracellular Ca^{2+} concentrations, and, finally, plasma membrane depolarization. The suggested sequence of events outlined in this study may allow for the estimation of cytotoxicity in the case of low-dose chemical concentrations.

In addition, elucidation of the dose-, time-, and level of chlorine substitution-dependent effects has potential for use in the identification of specific congeners present in a mixture. This knowledge may then allow the researcher to evaluate the extent of remediation efforts or design remediation technologies based on the prevalence of a specific congener in the field. Further, because the Clone 9 cell line has also been used successfully to study PAHs (Reeves et al., 2001), in particular BaP (Barhoumi et al.,

2000; Barhoumi et al., 2002), the methods described here show promise for identification of specific contaminants in environmental samples containing a complex mixture, for example creosote-contaminated water resulting from wood treatment procedures.

CHAPTER III

MATRIX-IMMOBILIZED ORGANOCLAY FOR THE SORPTION OF POLYCYCLIC AROMATIC HYDROCARBONS AND PENTACHLOROPHENOL FROM GROUNDWATER

Polycyclic aromatic hydrocarbons (PAHs), derived from creosote, and pentachlorophenol (C_5P) have been widely used in various formulations of wood preservatives. PAHs make up approximately 85% of the chemical composition of coal tar creosote created by the distillation of coal tar. C_5P is a general cytotoxic agent that historically has been applied to freshly cut wood as an aqueous solution of sodium pentachlorophenate or in organic solvent via spray-treatment, high-pressure impregnation, or by dipping in open vats. Inadvertent releases at wood preserving treatment facilities have contaminated pristine environments with high levels of both PAHs and C_5P. As a result, many of these facilities have been placed on the National Priorities List of Superfund sites and are now in the process of undergoing remediation (Hale and Aneiro, 1997).

In the past, wastewater generated from wood treatment facilities has been frequently released into unlined evaporation and settling lagoons where it may leach into soil, and subsequently groundwater. The main source of contaminants may be substantially reduced or eliminated by removing free product early in the remediation process by utilizing trench systems, excavation, recovery wells, groundwater pump-and-treat methods, enhanced oil-recovery technology, and physical and non-permeable

barriers (Udell et al., 1995). Additional remediation technologies include: air sparging (Hall et al., 2000), soil washing (Khodadoust et al., 1999), in-situ remediation (Tse et al., 2001), natural attenuation (Sharak Genthner et al., 1997) and bioremediation (Mueller et al., 1993), surfactant-enhanced remediation (Chun et al., 2002), hyperfiltration (Middaugh et al., 1994), photo-Fenton reaction chemistry (Engwall et al., 1999), and chemical degradation such as ozonation (Hong and Zeng, 2002).

Of particular interest to this laboratory, is the use of sorbent materials to sequester contaminants (Mortland et al., 1986; Boyd et al., 1988; Phillips et al., 1995; Clark et al., 1998). One of the most common sorbents used in water remediation is granular activated carbon (GAC). GAC has been shown to be effective for the filtration of organic compounds including trihalomethanes, pesticides, surfactants, plasticizers, and halogenated compounds (Paune et al., 1998). However, the presence of natural organic matter in water may drastically decrease both its adsorption rate and capacity (Knappe et al., 1999). In contrast, many clay minerals have been shown to strongly sorb contaminants even in the presence of organic matter (Sheng et al., 2001).

Naturally occurring clay minerals have been shown to strongly bind hydrophilic/polar organic contaminants from water with high capacity, but do not effectively sorb most hydrophobic organic compounds. However, replacement of inorganic interlayer cations with quaternary ammonium cations of the general form $[(CH_3)_3NR]^+$ or $[(CH_3)_2NRR']^+$ where R is an aromatic or alkyl hydrocarbon, yields organically-modified clay minerals with organophilic properties that more effectively sorb hydrophobic contaminants (Srinivasan and Fogler, 1990a; 1990b).

Methods have recently been developed in this laboratory to adhere clays and organically-modified clay minerals onto the surfaces of various solid supports for use in column filtration systems (Phillips and Sarr, 1999). The porous nature of these clay-based composites enhances their hydraulic conductivity and flow characteristics in filtration applications and facilitates the movement of organic contaminants through the matrix (Ake et al., 2001, 2003). Earlier studies suggested that multi-functional composite materials of organoclay bonded to GAC, rather than sand as a solid support, may be more effective for the treatment of contaminated groundwater under field conditions that either GAC or CP-LPHM alone (Ake et al., 2003). In this study, cetylpyridinium exchanged low pH montmorillonite clay (CP-LPHM) was bonded to either sand or GAC using the free acid form of a cellulose polymer, carboxymethylcellulose, as an adhesive. Various formulations of these organoclay-based sorbents were evaluated for PAH and C_5P removal from contaminated water. Initial studies were carried out to delineate the effect of differing amounts of organoclay bonded to a selected solid support matrix (i.e., GAC), followed by evaluations as to the contribution of different solid support matrices (i.e., GAC versus sand) on overall composite performance.

3.1 Materials and methods

3.1.1 Chemicals

For all experiments purified water (ddH_2O) was prepared by processing deionized water through a Milli-Q^{UF+} purification system (Millipore Corp., Bedford,

MA). Low pH montmorillonite (LPHM) clay was obtained from Engelhard Corporation (Cleveland, OH). GAC (–20 to +50 mesh; 297 to 841 μm) was obtained from Alfa Aesar (Ward Hill, MA) and washed to remove ultrafine particles prior to use. Acid-washed sand used for all composite materials was made by washing –50 to + 70 mesh (210 to 297 μm) quartz sand (Sigma Chemical Co., St Louis, MO) in prepared Nochromix® (Aldrich Chemical Co., Milwaukee, WI) and rinsing with ddH_2O to achieve a neutral pH. Cetylpyridinium (CP), carboxymethylcellulose (Na-CMC), and ^{14}C-PCP were purchased from Sigma Chemical Co. (St Louis, MO). Dense non-aqueous phase liquid (DNAPL) was collected from a creosote-contaminated Superfund site in the northwestern United States. Solid phase extraction media (6 mL Porapak® R_{DX} Sep-Pak® cartridges and Sep-Pak® tC18 Environmental cartridges) were obtained from Waters Corp. (Milford, MA).

3.1.2 Preparation of organoclay

The preparation of CP-LPHM has been reported previously (Lemke et al., 1998). Briefly, LPHM was washed in ddH_2O (100 mL/g) for 120 h while shaking, spun down by centrifugation, and the supernatant was discarded. The clay was resuspended in ddH_2O, and CP was added based on the estimated cation exchange capacity (CEC) for LPHM (90 $cmol_{charge}$/kg of clay, obtained from the provider). The clays were allowed to exchange for 24 h, followed by centrifugation and disposal of the supernatant. The exchanged clay was then washed in ddH_2O for an additional 24 h, spun down, dried, ground, and sieved to obtain particles <45 μm.

3.1.3 Preparation of CP-LPHM/sand and CP-LPHM/GAC

The methods for producing sand- and GAC-immobilized CP-LPHM composites were based on those described by Huebner et al. (2000) and Ake et al. (2001). Briefly, a 3% sodium carboxymethylcellulose (Na-CMC) solution was converted to the free acid form (H-CMC) by cation-exchange using a modification of the procedure of Dieckman et al. (1953) which has been previously reported (Ake et al., 2001). Formulations were based on the stoichiometry of active produce (i.e., CP-LPHM) to solid support (i.e., GAC or sand), and were produced by adding 1.5, 3, 6, and 15 g CP-LPHM to a 30 g GAC:30 mL H-CMC slurry to yield a produce of 1:20, 1:10, 1:5, and 1:2 g (w/w), respectively. An additional 3 part to 2 part GAC formulation (3:2 CP-LPHM/GAC) was produced by adding 45 g CP-LPHM to a 30 g GAC:60 mL H-CMC slurry. Sand-immobilized CP-LPHM (CP-LPHM/sand) was produced by adding 45 g CP-LPHM to a 120 g sand:60 mL H-CMC slurry. After thorough mixing, the composites were dried for 24 h at 105 ºC. CP-LPHM/sand and the 3:2 CP-LPHM/GAC composites were then gently washed with ddH_2O until loose CP-LPHM was removed, and subsequently air-dried for 24 h. CP-LPHM/sand was further ground and sieved to a particle size of <1 mm prior to use.

3.1.4 Preparation of reconstituted aqueous phase (RAP)

A contaminated water supply designed to simulate conditions *in situ* was prepared for use in all laboratory experiments. DNAPL was collected from a creosote-contaminated Superfund site in the northwestern U.S. To determine the appropriate

length of time necessary for the solution to equilibrate prior to use, 50 μL DNAPL was suspended in 15 mL ddH_2O, agitated for 10 s, and stored in the absence of light for 1, 2, 4, 16, 24, 48, or 72 h, or 6 days. For analysis, 13 mL of the resulting ddH_2O was passed through an in-line pair of preconditioned Porapak® R_{DX} Sep-Pak® and Sep-Pak® tC18 Environmental cartridges via mild suction. Contaminants were eluted from each cartridge separately using 3 mL of 1:1 CH_3OH:CH_2Cl_2, and eluates were combined for each sample. Combined eluates were then passed through 10 g of pre-dried (400 ºC for 24 h) Na_2SO_4 into graduated conical test tubes, evaporated under N_2 to approximately 0.5 mL, and quantitatively transferred to pre-cleaned amber vials (final volume 2 mL). Analyte concentrations were determined by GC/MS based on retention times and spectra of authentic standards.

For bench filtration experiments, DNAPL was added to ddH_2O in excess and the solution was agitated and allowed to equilibrate for 48 h at room temperature protected from light. The equilibrated solution was filtered through a column of sand by gravity to remove any free phase oil droplets and/or particulate matter prior to use. The filtered solution was then labeled with ^{14}C-C_5P to attain a response of approximately 200 dpm in 0.5 mL diluted with 3.5 mL scintillation fluid as determined with a Tri-Carb 1600 Series Liquid Scintillation Analyzer (Packard, Downers Grove, IL).

3.1.5 Filtration studies for ^{14}C-C_5P sorption

Glass pipets (14.5 mm x 8 mm ID) were packed with silanized glass wool, followed by approximately 400 mg sand, 200 mg GAC or an equivalent volume of test

composite, and topped with approximately 400 mg sand. ^{14}C-labeled RAP was passed through the test columns by gravity at a rate of approximately 2 mL/min. Free radioactivity (0.5 mL eluate diluted with 3.5 mL scintillation fluid) was measured from eluates collected over various time intervals with a Tri-Carb 1600 Series Liquid Scintillation Analyzer (Packard, Downers Grove, IL). Data was used to calculate bedlife and determine the breakthrough characteristics for each composite.

3.1.6 Preparation of columns for field studies and sample collection

Identical glass columns (20 cm x 2.5 cm ID) (BioRad Laboratories, Hercules, CA) were packed with composite sorbents and covered with sand and glass wool to fill the column. In the first experiment, either 10 or 20 g of 1:20, 1:10, or 1:5 CP-LPHM/GAC was added to each column, whereas, the second experiment utilized CP-LPHM/sand and 3:2 CP-LPHM/GAC on an equivolume basis (11 mL; mass equivalence of 4 g 3:2 CP-LPHM/GAC or 13 g CP-LPHM/sand). Control columns used to monitor untreated water were filled with sand and glass wool alone. The sorbent columns were placed in-line using existing sample ports at the effluent of a 10,000 gal oil-water separator (OWS) housed inside a metal building on-site at a Superfund site in the northwestern U.S (Figure 20). Untreated OWS effluent was filtered through the columns by gravity, and eluates were collected in 1 L amber Kimax bottles at various time points over 48 h. Samples were collected continuously each hour for 7 h in the first experiment and at 0.6, 4.3, 10.1, 20.5, 26.4, 34.7, and 47.8 h in the second experiment. Eluate from the control column was collected at each time point and used to determine

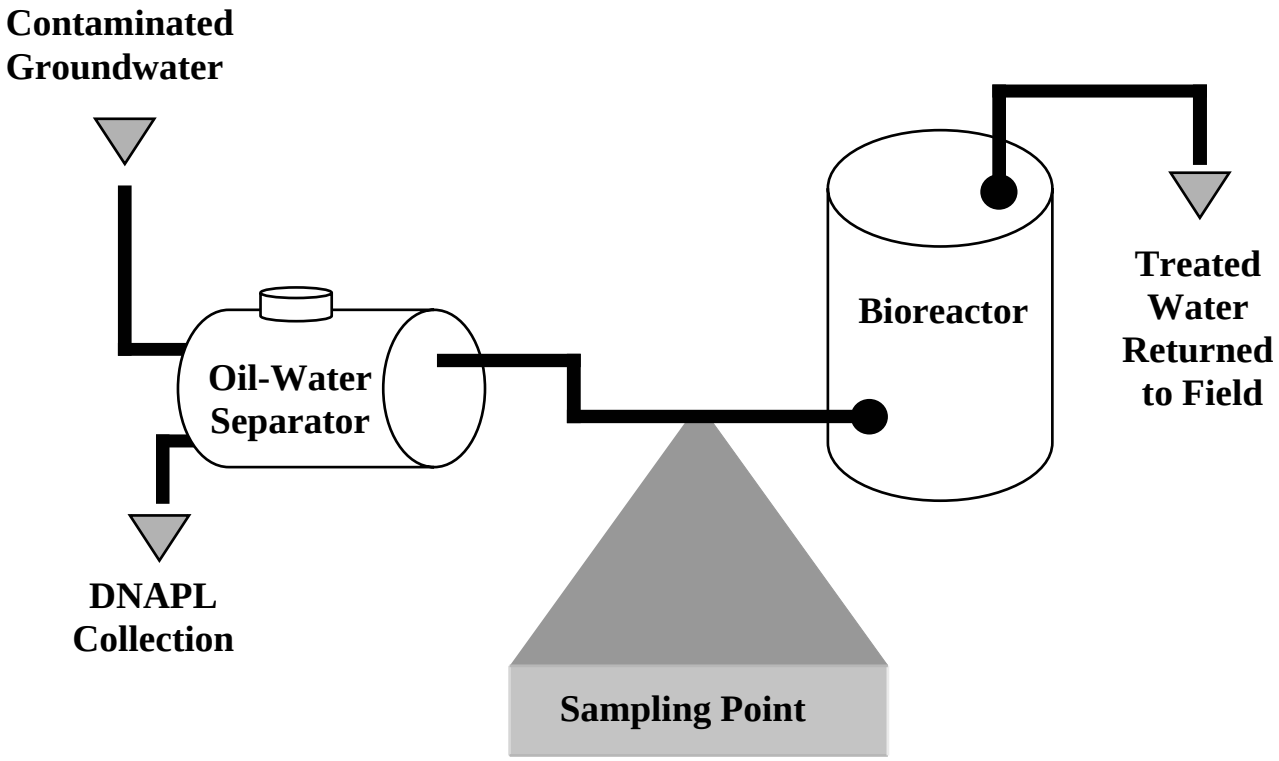


Figure 20. Schematic representation of the groundwater treatment facility used for field research. Groundwater from the aquifer is pumped into the oil-water separator (OWS) (10,000 gal). After separation, DNAPL is removed. The aqueous phase is routed to two, parallel, fixed-film bioreactors (10,000 gal each) and then applied to on-site land treatment units.

the mean contaminant load of each of 40 principal hazardous constituents, including all 16 PAHs identified in EPA Method 550, in untreated OWS effluent. In experiments utilizing CP-LPHM/sand and 3:2 CP-LPHM/GAC, each composite was tested in triplicate (3 columns of equal volume) and the mean concentration of each of 40 principal hazardous constituents was determined at each time point. Only contaminants identified above quantitation limits in untreated OWS effluent were reported.

3.1.7 Sample preparation and analysis

Sample preparation was based on a modification of EPA Method 3535 B Solid Phase Extraction (USEPA, 1997). Specifically, 2 mL of 1 N HCl was added to a 500 mL portion of sample that was then passed through an in-line pair of Porapak® R_{DX} Sep-Pak® and Sep-Pak® tC18 Environmental cartridges under mild vacuum. The cartridges were pre-conditioned with 15 mL CH_3OH, followed by 30 mL ddH_2O. Contaminants were eluted from each cartridge separately using 3 mL of 1:1 CH_3OH:CH_2Cl_2, and eluates were combined for each sample. Combined eluates were then passed through 10 g of pre-dried (400 ºC for 24 h) Na_2SO_4 into graduated conical test tubes, evaporated under N_2 to approximately 2 mL, and quantitatively transferred to pre-cleaned amber vials (final volume 5 mL).

3.1.8 GC/MS analysis

Analyte concentrations were determined quantitatively by GC/MS using a Hewlett Packard 5890 GC equipped with an HP 5972 MS engine. Analytical

methodology was based upon EPA Method 8270B (USEPA, 1997). Samples (2 µl) were introduced via heated injection port (300 ºC) with an HP 7673 autosampler. An HP5-ms bonded stationary phase capillary column (30 m x 0.25 mm I.D.) with a film thickness of 0.25 µm was used. The oven temperature program was as follows: initial oven temperature at 35 ºC for 6 min; to 300 ºC at 5 ºC/min; hold 300 ºC for 20 min. Transfer line temperature was 280 ºC. Helium was used as the carrier gas 0.75 mL/min. A mass selective detector was used in selected ion mode with an ionization energy of 70 eV and an ion source temperature of 180 ºC. Identification and quantitation of analytes was based on retention times and spectra as compared to those of authentic standards (minimum quantitation limit of 10 ng/L). Data were analyzed with HP MS ChemStation Data Acquisition software.

3.2 Results

3.2.1 Development of ^{14}C-labeled RAP

A contaminated water supply was prepared in-house and served to simulate conditions *in situ* for bench experiments. DNAPL was suspended in ddH_2O for time periods ranging from 1 h to 6 days and the resulting reconstituted aqueous phase (RAP) was extracted to determine PAH content. After all time periods, aqueous solution contained high concentrations of PAHs. Therefore 48 h was randomly selected as the equilibration time period used for the bench studies (data not shown). A 500 mL portion of sand-filtered RAP used for all bench experiments (prior to addition of the radiolabel)

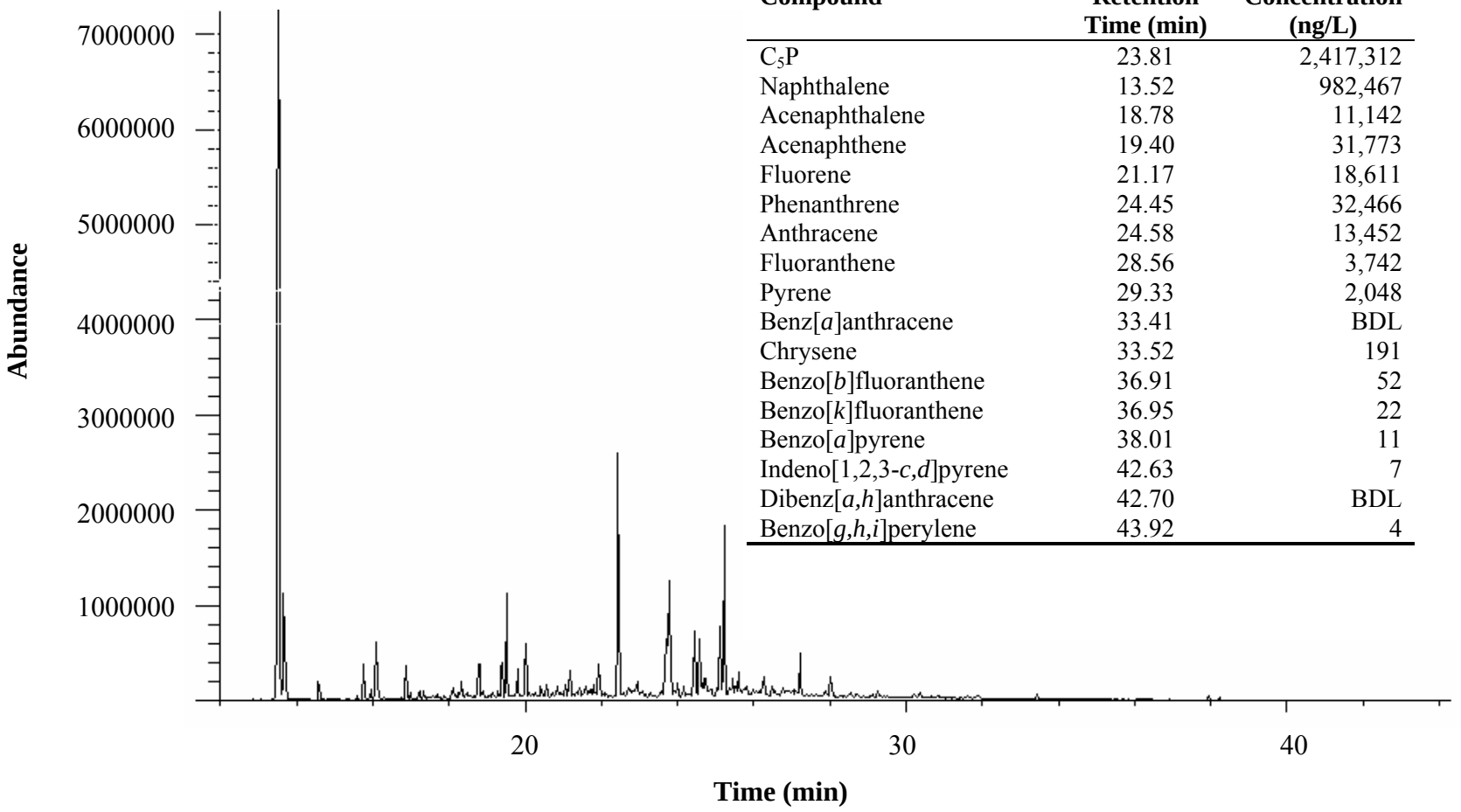
was analyzed by GC/MS to confirm that the solution was representative of on-site conditions where high concentrations of C_5P and PAHs predominate (Figure 21).

3.2.2 Bench studies

Initially, CP-LPHM/sand, and 1:10, 1:2, and 3:2 CP-LPHM/GAC removed >90% of ^{14}C-C_5P from RAP (Figure 22). After continuous contaminant loading throughout the course of the experiment, the removal of ^{14}C-C_5P by both 1:10 and 1:2 CP-LPHM/GAC decreased to 55 and 67%, respectively, whereas both CP-LPHM/sand and 3:2 CP-LPHM/GAC steadily removed 86 and 80% of the contaminant, respectively. Overall, the least effective sorbent was 1:20 CP-LPHM/GAC which removed only 75% initially, achieved 50% breakthrough for the removal of ^{14}C-C_5P after 150 mL of elution, and continued to remove only 35% of the contaminant following continuous elution of 500 mL through a 200 mg volume.

3.2.3 Field studies: effect of amount of organoclay

To test the relationship between the amount of organoclay on a solid support versus contaminant removal, three composites that performed well in the laboratory (i.e., 1:20, 1:10, and 1:5 CP-LPHM/GAC) were field-tested. Flow rates for all composite columns were adjusted with stopcocks at the base of the column and hose clamps on the tubing lines at the top to allow for a total volume of approximately 7 L over 7 h for each column of test material. The average flow rate was calculated to be 16.4 ± 0.9 mL/min. A total of 40 principal hazardous constituents, including all 16 PAHs identified in EPA



Compound	Retention Time (min)	Concentration (ng/L)
C_5P	23.81	2,417,312
Naphthalene	13.52	982,467
Acenaphthalene	18.78	11,142
Acenaphthene	19.40	31,773
Fluorene	21.17	18,611
Phenanthrene	24.45	32,466
Anthracene	24.58	13,452
Fluoranthene	28.56	3,742
Pyrene	29.33	2,048
Benz[*a*]anthracene	33.41	BDL
Chrysene	33.52	191
Benzo[*b*]fluoranthene	36.91	52
Benzo[*k*]fluoranthene	36.95	22
Benzo[*a*]pyrene	38.01	11
Indeno[1,2,3-*c,d*]pyrene	42.63	7
Dibenz[*a,h*]anthracene	42.70	BDL
Benzo[*g,h,i*]perylene	43.92	4

Figure 21. Representative GC/MS chromatogram of the reconstituted aqueous phase (RAP) used in all bench experiments. Inset lists retention times and concentrations of C_5P and PAHs in the chromatogram.

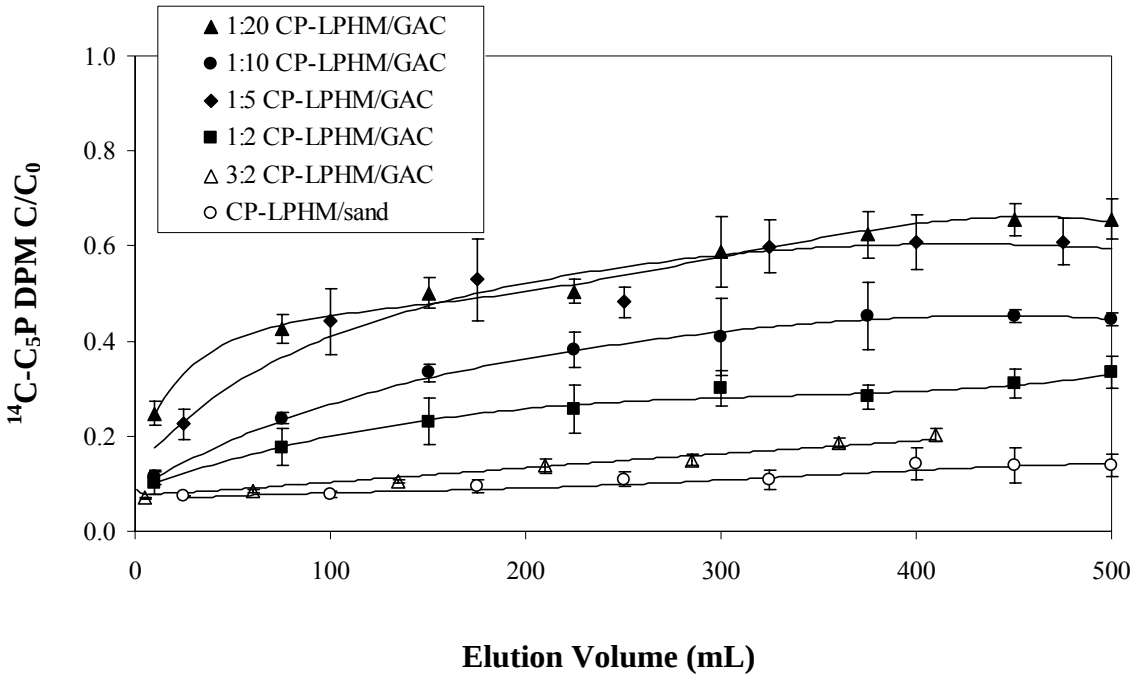


Figure 22. Comparison of various composite sorbents for removal of ^{14}C-C$_5$P from complex aqueous solution. Graph shows ^{14}C-C$_5$P remaining in solution (normalized to untreated control solution) ± SE following elution of aqueous ^{14}C-labeled reconstituted aqueous phase (RAP) through equivalent bed volumes of sorbent (equal to 200 mg GAC).

Method 550, were quantified in both untreated and composite-treated OWS samples. However, only contaminants present above quantitation limits (10 ng/L) in untreated OWS effluent controls have been reported here.

In untreated OWS effluent, GC/MS analysis showed that the contaminant present in the greatest concentration was C_5P (984,211 ± 343,040 ng/L), followed by phenanthrene (786,367 ± 243,369 ng/L), C2-substituted naphthalenes (561,967 ± 161,058 ng/L), C1-substituted naphthalenes (480,733 ± 125,022 ng/L), and naphthalene 477,500 ± 86,567 ng/L) (Tables 2 to 7). Other contaminants present in substantial amounts were C3-substituted naphthalenes (394,900 ± 114,806 ng/L), acenaphthene (304,567 ± 68,741 ng/L), C1-substituted phenanthrene/anthracenes (258,900 ± 74,852 ng/L), fluorene (249,967 ± 65,828 ng/L), fluoranthene (244,100 ± 74,606 ng/L), C4-substituted naphthalenes (235,533 ± 68,838 ng/L), and anthracene (232,733 ± 65,232 ng/L). The total contaminant load of carcinogenic PAHs (benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*c,d*]pyrene, and dibenz[*a,h*]anthracene) was 130,178 ± 45,555 ng/L, while the total PAH contaminant load was 5,919,528 ± 1,651,969 ng/L.

Effluent that was eluted through each composite sorbent was collected over every hour for 7 h throughout this portion of the study. Breakthrough was not achieved for any contaminant investigated, therefore, for clarity of data, only the results for those samples collected during the final time interval have been presented (Tables 2 to 7). Elution through either 10 or 20 g of 1:20, 1:10, or 1:5 CP-LPHM/GAC reduced almost all

Table 2
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 7 h of continuous elution through 10 g of 1:20 CP-LPHM/GAC

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	984,211 ± 343,040	35,100	96
Naphthalene	477,500 ± 86,567	BDL	>99
C1-naphthalenes	480,733 ± 125,022	BDL	>99
C2-naphthalenes	561,967 ± 161,058	BDL	>99
C3-naphthalenes	394,900 ± 114,806	BDL	>99
C4-naphthalenes	235,533 ± 68,838	BDL	>99
1,1'-Biphenyl	95,767 ± 23,748	BDL	>99
Acenaphthalene	2,823 ± 575	BDL	>99
Acenaphthene	304,567 ± 68,741	BDL	>99
Fluorene	249,967 ± 65,828	BDL	>99
C1-fluorenes	100,433 ± 27,824	BDL	>99
C2-fluorenes	113,467 ± 29,927	BDL	>99
Anthracene	232,733 ± 65,232	101	>99
Phenanthrene	786,367 ± 243,369	22	>99
C1-phenanthrene/anthracene	258,900 ± 74,852	BDL	>99
C2-phenanthrene/anthracene	150,233 ± 43,164	BDL	>99
Dibenzothiophene	113,900 ± 31,141	BDL	>99
Fluoranthene	244,100 ± 74,606	16	>99
Pyrene	181,967 ± 58,329	21	>99
C1-fluoranthenes/pyrenes	59,333 ± 18,562	63	>99
Benz[*a*]anthracene	35,400 ± 11,405	BDL	>99
Chrysene	29,033 ± 9,153	BDL	>99
C1-chrysenes	13,297 ± 3,919	BDL	>99
Benzo[*b*]fluoranthene	21,300 ± 7,617	BDL	>99
Benzo[*k*]fluoranthene	20,833 ± 7,670	BDL	>99
Benzo[*e*]pyrene	8,573 ± 3,257	BDL	>99
Benzo[*a*]pyrene	17,743 ± 6,959	BDL	>99
Perylene	3,230 ± 1,296	BDL	>99
Indeno[1,2,3-*c,d*]pyrene	4,403 ± 2,017	BDL	>99
Dibenz[*a,h*]anthracene	1,464 ± 2,017	BDL	>99
Benzo[*g,h,i*]perylene	2,530 ± 1,259	BDL	>99
Total PAHs	5,919,528 ± 1,651,969	222	>99

[a] Values represent average contaminant concentrations ± SE of 3-500 mL samples obtained at regular intervals during continuous elution over the course of 7 h.
[b] Values represent contaminant concentrations of 500 mL after 7 h of continuous elution.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 3
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 7 h of continuous elution through 20 g of 1:20 CP-LPHM/GAC

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	984,211 ± 343,040	35,400	96
Naphthalene	477,500 ± 86,567	BDL	>99
C1-naphthalenes	480,733 ± 125,022	BDL	>99
C2-naphthalenes	561,967 ± 161,058	BDL	>99
C3-naphthalenes	394,900 ± 114,806	BDL	>99
C4-naphthalenes	235,533 ± 68,838	BDL	>99
1,1'-Biphenyl	95,767 ± 23,748	BDL	>99
Acenaphthalene	2,823 ± 575	BDL	>99
Acenaphthene	304,567 ± 68,741	BDL	>99
Fluorene	249,967 ± 65,828	BDL	>99
C1-fluorenes	100,433 ± 27,824	BDL	>99
C2-fluorenes	113,467 ± 29,927	BDL	>99
Anthracene	232,733 ± 65,232	101	>99
Phenanthrene	786,367 ± 243,369	30	>99
C1-phenanthrene/anthracene	258,900 ± 74,852	BDL	>99
C2-phenanthrene/anthracene	150,233 ± 43,164	BDL	>99
Dibenzothiophene	113,900 ± 31,141	BDL	>99
Fluoranthene	244,100 ± 74,606	24	>99
Pyrene	181,967 ± 58,329	54	>99
C1-fluoranthenes/pyrenes	59,333 ± 18,562	55	>99
Benz[*a*]anthracene	35,400 ± 11,405	13	>99
Chrysene	29,033 ± 9,153	23	>99
C1-chrysenes	13,297 ± 3,919	BDL	>99
Benzo[*b*]fluoranthene	21,300 ± 7,617	BDL	>99
Benzo[*k*]fluoranthene	20,833 ± 7,670	BDL	>99
Benzo[*e*]pyrene	8,573 ± 3,257	BDL	>99
Benzo[*a*]pyrene	17,743 ± 6,959	BDL	>99
Perylene	3,230 ± 1,296	BDL	>99
Indeno[1,2,3-*c,d*]pyrene	4,403 ± 2,017	BDL	>99
Dibenz[*a,h*]anthracene	1,464 ± 2,017	BDL	>99
Benzo[*g,h,i*]perylene	2,530 ± 1,259	BDL	>99
Total PAHs	5,919,528 ± 1,651,969	298	>99

[a] Values represent average contaminant concentrations ± SE of 3-500 mL samples obtained at regular intervals during continuous elution over the course of 7 h.
[b] Values represent contaminant concentrations of 500 mL after 7 h of continuous elution.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 4
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 7 h of continuous elution through 10 g of 1:10 CP-LPHM/GAC

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	984,211 ± 343,040	231,000	77
Naphthalene	477,500 ± 86,567	BDL	>99
C1-naphthalenes	480,733 ± 125,022	BDL	>99
C2-naphthalenes	561,967 ± 161,058	BDL	>99
C3-naphthalenes	394,900 ± 114,806	BDL	>99
C4-naphthalenes	235,533 ± 68,838	BDL	>99
1,1'-Biphenyl	95,767 ± 23,748	23	>99
Acenaphthalene	2,823 ± 575	272	90
Acenaphthene	304,567 ± 68,741	3,520	99
Fluorene	249,967 ± 65,828	203	>99
C1-fluorenes	100,433 ± 27,824	567	>99
C2-fluorenes	113,467 ± 29,927	BDL	>99
Anthracene	232,733 ± 65,232	1,470	>99
Phenanthrene	786,367 ± 243,369	53	>99
C1-phenanthrene/anthracene	258,900 ± 74,852	1,120	>99
C2-phenanthrene/anthracene	150,233 ± 43,164	1,210	>99
Dibenzothiophene	113,900 ± 31,141	301	>99
Fluoranthene	244,100 ± 74,606	2,560	99
Pyrene	181,967 ± 58,329	1,770	99
C1-fluoranthenes/pyrenes	59,333 ± 18,562	656	99
Benz[*a*]anthracene	35,400 ± 11,405	391	99
Chrysene	29,033 ± 9,153	296	99
C1-chrysenes	13,297 ± 3,919	BDL	>99
Benzo[*b*]fluoranthene	21,300 ± 7,617	BDL	>99
Benzo[*k*]fluoranthene	20,833 ± 7,670	BDL	>99
Benzo[*e*]pyrene	8,573 ± 3,257	BDL	>99
Benzo[*a*]pyrene	17,743 ± 6,959	BDL	>99
Perylene	3,230 ± 1,296	BDL	>99
Indeno[1,2,3-*c,d*]pyrene	4,403 ± 2,017	BDL	>99
Dibenz[*a,h*]anthracene	1,464 ± 2,017	BDL	>99
Benzo[*g,h,i*]perylene	2,530 ± 1,259	BDL	>99
Total PAHs	5,919,528 ± 1,651,969	17,800	>99

[a] Values represent average contaminant concentrations ± SE of 3-500 mL samples obtained at regular intervals during continuous elution over the course of 7 h.
[b] Values represent contaminant concentrations of 500 mL after 7 h of continuous elution.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 5
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 7 h of continuous elution through 20 g of 1:10 CP-LPHM/GAC

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	984,211 ± 343,040	12,600	99
Naphthalene	477,500 ± 86,567	6,330	99
C1-naphthalenes	480,733 ± 125,022	1,250	>99
C2-naphthalenes	561,967 ± 161,058	966	>99
C3-naphthalenes	394,900 ± 114,806	1,120	>99
C4-naphthalenes	235,533 ± 68,838	968	>99
1,1'-Biphenyl	95,767 ± 23,748	94	>99
Acenaphthalene	2,823 ± 575	BDL	>99
Acenaphthene	304,567 ± 68,741	464	>99
Fluorene	249,967 ± 65,828	374	>99
C1-fluorenes	100,433 ± 27,824	BDL	>99
C2-fluorenes	113,467 ± 29,927	BDL	>99
Anthracene	232,733 ± 65,232	602	>99
Phenanthrene	786,367 ± 243,369	1,510	>99
C1-phenanthrene/anthracene	258,900 ± 74,852	392	>99
C2-phenanthrene/anthracene	150,233 ± 43,164	439	>99
Dibenzothiophene	113,900 ± 31,141	258	>99
Fluoranthene	244,100 ± 74,606	342	>99
Pyrene	181,967 ± 58,329	1,060	>99
C1-fluoranthenes/pyrenes	59,333 ± 18,562	409	>99
Benz[*a*]anthracene	35,400 ± 11,405	97	>99
Chrysene	29,033 ± 9,153	142	>99
C1-chrysenes	13,297 ± 3,919	BDL	>99
Benzo[*b*]fluoranthene	21,300 ± 7,617	BDL	>99
Benzo[*k*]fluoranthene	20,833 ± 7,670	BDL	>99
Benzo[*e*]pyrene	8,573 ± 3,257	BDL	>99
Benzo[*a*]pyrene	17,743 ± 6,959	BDL	>99
Perylene	3,230 ± 1,296	BDL	>99
Indeno[1,2,3-*c,d*]pyrene	4,403 ± 2,017	BDL	>99
Dibenz[*a,h*]anthracene	1,464 ± 2,017	BDL	>99
Benzo[*g,h,i*]perylene	2,530 ± 1,259	BDL	>99
Total PAHs	5,919,528 ± 1,651,969	17,640	>99

[a] Values represent average contaminant concentrations ± SE of 3-500 mL samples obtained at regular intervals during continuous elution over the course of 7 h.
[b] Values represent contaminant concentrations of 500 mL after 7 h of continuous elution.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 6
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 7 h of continuous elution through 10 g of 1:5 CP-LPHM/GAC

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	984,211 ± 343,040	68,900	93
Naphthalene	477,500 ± 86,567	295	>99
C1-naphthalenes	480,733 ± 125,022	203	>99
C2-naphthalenes	561,967 ± 161,058	1010	>99
C3-naphthalenes	394,900 ± 114,806	2190	>99
C4-naphthalenes	235,533 ± 68,838	1590	>99
1,1'-Biphenyl	95,767 ± 23,748	38	>99
Acenaphthalene	2,823 ± 575	145	95
Acenaphthene	304,567 ± 68,741	1540	>99
Fluorene	249,967 ± 65,828	120	>99
C1-fluorenes	100,433 ± 27,824	218	>99
C2-fluorenes	113,467 ± 29,927	375	>99
Anthracene	232,733 ± 65,232	626	>99
Phenanthrene	786,367 ± 243,369	329	>99
C1-phenanthrene/anthracene	258,900 ± 74,852	655	>99
C2-phenanthrene/anthracene	150,233 ± 43,164	601	>99
Dibenzothiophene	113,900 ± 31,141	26	>99
Fluoranthene	244,100 ± 74,606	1350	>99
Pyrene	181,967 ± 58,329	888	>99
C1-fluoranthenes/pyrenes	59,333 ± 18,562	320	>99
Benz[*a*]anthracene	35,400 ± 11,405	200	>99
Chrysene	29,033 ± 9,153	184	>99
C1-chrysenes	13,297 ± 3,919	BDL	>99
Benzo[*b*]fluoranthene	21,300 ± 7,617	BDL	>99
Benzo[*k*]fluoranthene	20,833 ± 7,670	BDL	>99
Benzo[*e*]pyrene	8,573 ± 3,257	BDL	>99
Benzo[*a*]pyrene	17,743 ± 6,959	BDL	>99
Perylene	3,230 ± 1,296	BDL	>99
Indeno[1,2,3-*c,d*]pyrene	4,403 ± 2,017	BDL	>99
Dibenz[*a,h*]anthracene	1,464 ± 2,017	BDL	>99
Benzo[*g,h,i*]perylene	2,530 ± 1,259	BDL	>99
Total PAHs	5,919,528 ± 1,651,969	14,900	>99

[a] Values represent average contaminant concentrations ± SE of 3-500 mL samples obtained at regular intervals during continuous elution over the course of 7 h.
[b] Values represent contaminant concentrations of 500 mL after 7 h of continuous elution.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 7
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 7 h of continuous elution through 20 g of 1:5 CP-LPHM/GAC

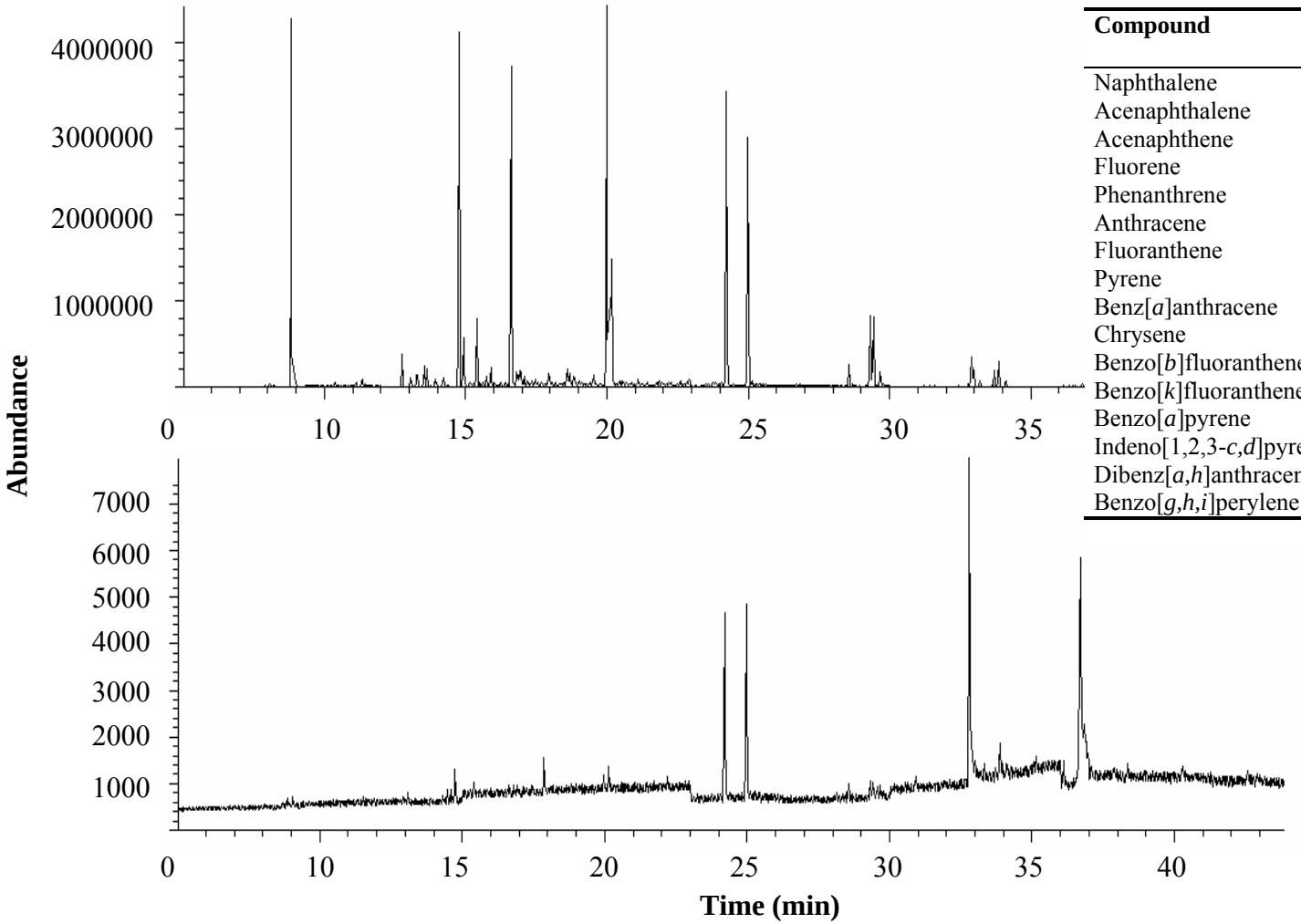
PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	984,211 ± 343,040	BDL	>99
Naphthalene	477,500 ± 86,567	BDL	>99
C1-naphthalenes	480,733 ± 125,022	BDL	>99
C2-naphthalenes	561,967 ± 161,058	BDL	>99
C3-naphthalenes	394,900 ± 114,806	BDL	>99
C4-naphthalenes	235,533 ± 68,838	BDL	>99
1,1'-Biphenyl	95,767 ± 23,748	BDL	>99
Acenaphthalene	2,823 ± 575	BDL	>99
Acenaphthene	304,567 ± 68,741	BDL	>99
Fluorene	249,967 ± 65,828	BDL	>99
C1-fluorenes	100,433 ± 27,824	BDL	>99
C2-fluorenes	113,467 ± 29,927	BDL	>99
Anthracene	232,733 ± 65,232	BDL	>99
Phenanthrene	786,367 ± 243,369	BDL	>99
C1-phenanthrene/anthracene	258,900 ± 74,852	BDL	>99
C2-phenanthrene/anthracene	150,233 ± 43,164	BDL	>99
Dibenzothiophene	113,900 ± 31,141	BDL	>99
Fluoranthene	244,100 ± 74,606	172	>99
Pyrene	181,967 ± 58,329	471	>99
C1-fluoranthenes/pyrenes	59,333 ± 18,562	126	>99
Benz[*a*]anthracene	35,400 ± 11,405	BDL	>99
Chrysene	29,033 ± 9,153	BDL	>99
C1-chrysenes	13,297 ± 3,919	BDL	>99
Benzo[*b*]fluoranthene	21,300 ± 7,617	BDL	>99
Benzo[*k*]fluoranthene	20,833 ± 7,670	BDL	>99
Benzo[*e*]pyrene	8,573 ± 3,257	BDL	>99
Benzo[*a*]pyrene	17,743 ± 6,959	BDL	>99
Perylene	3,230 ± 1,296	BDL	>99
Indeno[1,2,3-*c,d*]pyrene	4,403 ± 2,017	BDL	>99
Dibenz[*a,h*]anthracene	1,464 ± 2,017	BDL	>99
Benzo[*g,h,i*]perylene	2,530 ± 1,259	BDL	>99
Total PAHs	5,919,528 ± 1,651,969	769	>99

[a] Values represent average contaminant concentrations ± SE of 3-500 mL samples obtained at regular intervals during continuous elution over the course of 7 h.
[b] Values represent contaminant concentrations of 500 mL after 7 h of continuous elution.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

contaminants investigated in this study by ≥99%, including naphthalene, phenanthrene, anthracene, pyrene, fluorene, fluoranthene, and benzo[a]pyrene. Many contaminants were below the limits of detection for the method and the total PAH contaminant load was reduced by ≥99% in all trials. The only contaminant that was routinely detected in solution was C_5P. C_5P was reduced by ≥90%, frequently by ≥99%, for all sorbents with the exception of 10 g of 1:10 CP-LPHM/GAC. This composite formulation showed only a 77% reduction in C_5P following 7 h of continuous elution (Table 4). The extent of contaminant removal in the treated OWS effluent is further illustrated by representative GC/MS chromatograms of both untreated OWS effluent and eluate from a column of 10 g of 1:10 CP-LPHM/GAC (Figure 23).

3.2.4 Field studies: effect of solid support matrix

To test the contribution of the solid support matrix, two composites containing a maximum amount of organoclay in equivolume amounts were field-tested, 3:2 CP-LPHM/GAC and CP-LPHM/sand. In this study, contaminants present in the greatest concentrations in untreated OWS effluent C_5P (2,332,529 ± 174,708 ng/L), followed by naphthalene (367,457 ± 53,400 ng/L), and C1-substituted naphthalenes (126,157 ± 16,694 ng/L) (Tables 8 to 11). Other contaminants present in substantial amounts were benzothiophene (91,843 ± 11,592 ng/L), carbazole (57,057 ± 6,688 ng/L), acenaphthene (45,886 ± 6,043 ng/L), C2-substituted naphthalenes (31,886 ± 4,772 ng/L), C1-substituted benzothiophenes (25,471 ± 3,520 ng/L), and dibenzofuran (25,340 ± 3,612



Compound	Retention Time (min)
Naphthalene	8.87
Acenaphthalene	14.15
Acenaphthene	14.78
Fluorene	16.62
Phenanthrene	19.99
Anthracene	20.18
Fluoranthene	24.24
Pyrene	25.01
Benz[*a*]anthracene	29.35
Chrysene	29.47
Benzo[*b*]fluoranthene	32.93
Benzo[*k*]fluoranthene	33.02
Benzo[*a*]pyrene	33.90
Indeno[1,2,3-*c,d*]pyrene	37.13
Dibenz[*a,h*]anthracene	37.24
Benzo[*g,h,i*]perylene	37.92

Figure 23. Representative GC/MS chromatograms of untreated OWS effluent and OWS effluent eluted through 10 g of 1:10 CP-LPHM/GAC. Top: untreated OWS effluent. Bottom: OWS effluent eluted through 10 g of 1:10 CP-LPHM/GAC. Inset lists PAHs and their approximate retention times in the chromatograms.

Table 8
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent eluted through 3:2 CP-LPHM/GAC

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	Average PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	2,332,529 ± 174,708	2,150 ± 618	>99
Naphthalene	367,457 ± 53,400	143,333 ± 39,751	61
C1-naphthalenes	126,157 ± 16,694	7,660 ± 717	94
C2-naphthalenes	31,886 ± 4,772	1,127 ± 1,127	96
Benzothiophene	91,843 ± 11,592	23,233 ± 2,326	75
C1-Benzothiophene	25,471 ± 3,520	3,823 ± 301	85
C2-Benzothiophene	14,943 ± 1,985	2,593 ± 245	83
C3-Benzothiophene	4,711 ± 773	1,150 ± 86	76
Biphenyl	16,885 ± 2,404	467 ± 46	97
Acenaphthalene	2,047 ± 472	601 ± 268	71
Acenaphthene	45,886 ± 6,043	2,037 ± 158	96
Dibenzofuran	25,340 ± 3,612	326 ± 51	99
Fluorene	21,353 ± 3,129	208 ± 23	99
Carbazole	57,057 ± 6,688	403 ± 96	99
Anthracene	6,250 ± 936	248 ± 22	96
Phenanthrene	16,731 ± 3,268	76 ± 31	>99
C1-phenanthrenes/ Anthracenes	1,621 ± 443	24 ± 16	99
Dibenzothiophene	3,034 ± 797	154 ± 14	95
Fluoranthene	1,280 ± 489	25 ± 15	98
Pyrene	886 ± 416	23 ± 13	97
C1-fluoranthenes/ Pyrenes	206 ± 128	<13	>99
Benz[*a*]anthracene	114 ± 83	3 ± 2	97
Chrysene	104 ± 87	4 ± 2	96
Total PAHs	3,201,515 ± 254,840	189,668 ± 43,137	94

[a] Values represent average contaminant concentrations ± SE of 7-500 mL samples obtained at regular intervals of continuous elution over the course of 48 h.
[b] Values represent average contaminant concentrations ± SE of 3-500 mL collected at the initial time point (1 h) of the experiment.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 9
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 48 h of continuous elution through 3:2 CP-LPHM/GAC

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	Average PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	2,332,529 ± 174,708	2,568,600 ± 162,243	<1
Naphthalene	367,457 ± 53,400	480,300 ± 56,458	<1
C1-naphthalenes	126,157 ± 16,694	111,533 ± 4,259	12
C2-naphthalenes	31,886 ± 4,772	19,933 ± 1,122	37
Benzothiophene	91,843 ± 11,592	85,400 ± 7,649	7
C1-Benzothiophene	25,471 ± 3,520	12,488 ± 6,114	51
C2-Benzothiophene	14,943 ± 1,985	9,760 ± 170	35
C3-Benzothiophene	4,711 ± 773	2,453 ± 141	48
Biphenyl	16,885 ± 2,404	11,567 ± 491	31
Acenaphthalene	2,047 ± 472	1,979 ± 646	3
Acenaphthene	45,886 ± 6,043	35,300 ± 1,069	23
Dibenzofuran	25,340 ± 3,612	14,467 ± 470	43
Fluorene	21,353 ± 3,129	10,030 ± 589	53
Carbazole	57,057 ± 6,688	24,200 ± 2,751	58
Anthracene	6,250 ± 936	2,017 ± 135	68
Phenanthrene	16,731 ± 3,268	1,153 ± 239	93
C1-phenanthrenes/ anthracenes	1,621 ± 443	215 ± 417	87
Dibenzothiophene	3,034 ± 797	1,044 ± 173	66
Fluoranthene	1,280 ± 489	26 ± 6	98
Pyrene	886 ± 416	42 ± 21	95
C1-fluoranthenes/ pyrenes	206 ± 128	11 ± 11	95
Benz[*a*]anthracene	114 ± 83	7 ± 1	94
Chrysene	104 ± 87	5 ± 1	95
Total PAHs	3,201,515 ± 254,840	3,392,631 ± 216,713	<1

[a] Values represent average contaminant concentrations ± SE of 7-500 mL samples obtained at regular intervals over the course of 48 h.
[b] Values represent average contaminant concentrations ± SE of 3-500 mL samples collected at the final time point (48 h) of the experiment.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 10
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent eluted through CP-LPHM/sand

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	Average PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	2,332,529 ± 174,708	244,033 ± 148,338	90
Naphthalene	367,457 ± 53,400	108,967 ± 55,745	70
C1-naphthalenes	126,157 ± 16,694	21,230 ± 7,412	83
C2-naphthalenes	31,886 ± 4,772	4,090 ± 1,205	87
Benzothiophene	91,843 ± 11,592	37,967 ± 10,177	89
C1-Benzothiophene	25,471 ± 3,520	7,167 ± 1,511	72
C2-Benzothiophene	14,943 ± 1,985	3,483 ± 969	77
C3-Benzothiophene	4,711 ± 773	827 ± 263	82
Biphenyl	16,885 ± 2,404	1,906 ± 674	89
Acenaphthalene	2,047 ± 472	2,900 ± 617	<1
Acenaphthene	45,886 ± 6,043	6,943 ± 2,072	85
Dibenzofuran	25,340 ± 3,612	1,708 ± 713	93
Fluorene	21,353 ± 3,129	1,286 ± 527	94
Carbazole	57,057 ± 6,688	5,963 ± 2,641	90
Anthracene	6,250 ± 936	1,208 ± 570	81
Phenanthrene	16,731 ± 3,268	756 ± 369	95
C1-phenanthrenes/ anthracenes	1,621 ± 443	201 ± 77	88
Dibenzothiophene	3,034 ± 797	222 ± 70	93
Fluoranthene	1,280 ± 489	74 ± 29	94
Pyrene	886 ± 416	61 ± 23	93
C1-fluoranthenes/ pyrenes	206 ± 128	31 ± 12	85
Benz[*a*]anthracene	114 ± 83	9 ± 3	92
Chrysene	104 ± 87	6 ± 2	94
Total PAHs	3,201,515 ± 254,840	455,871 ± 230,996	86

[a] Values represent average contaminant concentrations ± SE of 7-500 mL samples obtained at regular intervals over the course of 48 h.
[b] Values represent average contaminant concentrations ± SE of 3-500 mL samples collected at the initial time point (1 h) of the experiment.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

Table 11
Principal hazardous constituent (PHC) concentrations in untreated OWS effluent and OWS effluent after 48 h of continuous elution through CP-LPHM/sand

PHCs	Average PHC concentration in untreated OWS effluent (ng/L)[a]	Average PHC concentration in treated OWS effluent (ng/L)[b]	Percent reduction of PHCs[c]
C_5P	2,332,529 ± 174,708	2,033,733 ± 33,361	13
Naphthalene	367,457 ± 53,400	422,300 ± 112,856	<1
C1-naphthalenes	126,157 ± 16,694	68,200 ± 5,543	46
C2-naphthalenes	31,886 ± 4,772	13,550 ± 1,899	58
Benzothiophene	91,843 ± 11,592	65,067 ± 9,947	29
C1-Benzothiophene	25,471 ± 3,520	14,000 ± 1,266	45
C2-Benzothiophene	14,943 ± 1,985	6,737 ± 282	55
C3-Benzothiophene	4,711 ± 773	2,503 166	47
Biphenyl	16,885 ± 2,404	7,093 ± 794	58
Acenaphthalene	2,047 ± 472	2,195 ± 807	<1
Acenaphthene	45,886 ± 6,043	24,000 ± 1,724	48
Dibenzofuran	25,340 ± 3,612	9,070 ± 1,252	64
Fluorene	21,353 ± 3,129	7,450 ± 1,506	65
Carbazole	57,057 ± 6,688	16,900 ± 2,743	70
Anthracene	6,250 ± 936	2,473 ± 444	60
Phenanthrene	16,731 ± 3,268	4,232 ± 2,579	75
C1-phenanthrenes/ anthracenes	1,621 ± 443	513 ± 294	68
Dibenzothiophene	3,034 ± 797	334 ± 207	89
Fluoranthene	1,280 ± 489	323 ± 266	75
Pyrene	886 ± 416	237 ± 199	73
C1-fluoranthenes/ pyrenes	206 ± 128	49 ± 49	76
Benz[*a*]anthracene	114 ± 83	27 ± 19	76
Chrysene	104 ± 87	23 ± 15	78
Total PAHs	3,201,515 ± 254,840	2,702,032 ± 152,839	16

[a] Values represent average contaminant concentrations ± SE of 7-500 mL samples obtained at regular intervals over the course of 48 h.
[b] Values represent average contaminant concentrations ± SE of 3-500 mL samples collected at the final time point (48 h) of the experiment.
[c] Values represent the percent reduction of contaminant concentration in treated effluent as compared to untreated effluent.

ng/L). The total contaminant load of carcinogenic PAHs (benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*c,d*]pyrene, and dibenz[*a,h*]anthracene) was 49.8 ± 8.4 ng/L, although benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*c,d*]pyrene, and dibenz[*a,h*]anthracene were detected below quantitation limits. The total PAH contaminant load was 3,201,515 ± 254,840 ng/L.

Although samples were collected at 7 time points throughout the study, to illustrate the effectiveness of these composite materials complete data for the initial (1 h) and final (48 h) time points has been presented. Elution through 3:2 CP-LPHM/GAC initially reduced many contaminants by ≥90%, including biphenyl (467 ± 46 ng/L), acenaphthene (2,037 ± 158 ng/L), anthracene (248 ± 22 ng/L), dibenzothiophene (154 ± 14 ng/L), fluoranthene (25 ± 15 ng/L), pyrene (23 ± 13 ng/L), benz[*a*]anthracene (3 ± 2 ng/L), and chrysene (4 ± 2 ng/L), while several were reduced by ≥99%, including C_5P (2,150 ± 618 ng/L), dibenzofuran (326 ± 51 ng/L), fluorene (208 ± 23 ng/L), carbazole (403 ± 96 ng/L), and phenanthrene (76 ± 31 ng/L) (Table 8). After 48 h, some contaminants remained in column eluates, including naphthalene (143,333 ± 39,751 ng/L), benzothiophene (23,233 ± 2,326 ng/L), and acenaphthalene (601 ± 268 ng/L). The total PAH contaminant load was reduced by 94% (189,668 ± 43,137 ng/L) (Table 9).

Columns filled with CP-LPHM/sand were similarly effective, as elution through this composite reduced many of the same contaminants by ≥90%, including C_5P

(244,033 ± 148,338 ng/L), dibenzofuran (1,708 ± 713 ng/L), fluorene (1,286 ± 527 ng/L), carbazole (5,963 ± 2,641 ng/L), phenanthrene (756 ± 369 ng/L), dibenzothiophene (222 ± 70 ng/L), fluoranthene (74 ± 30 ng/L), pyrene (61 ± 23 ng/L), benz[*a*]anthracene (9 ± 3 ng/L), and chrysene (6 ± 2 ng/L) (Table 10). Reduction of naphthalene (108,967 ± 55,745 ng/L), benzothiophene (37,967 ± 10,177 ng/L), biphenyl (1,906 ± 674 ng/L), acenaphthene (6,943 ± 2,072 ng/L), and anthracene (1,208 ± 570 ng/L) was not as great. The total PAH contaminant load was reduced by 86% (455,871 ± 230,996 ng/L).

Complete breakthrough of naphthalene occurred following approximately 15 h of elution through 3:2 CP-LPHM/GAC (Figure 24) and 22 h through CP-LPHM/sand (Figure 25). C_5P exhibited breakthrough following 18 h of elution through 3:2 CP-LPHM/GAC (Figure 24) and 26 h through CP-LPHM/sand (Figure 25). In contrast, higher molecular weight PAHs such as fluorene, phenanthrene, and pyrene, failed to attain even 50% breakthrough in either of the composite materials after 48 h (Tables 9 and 11). While both composites remained effective for reduction of higher molecular weight PAHs, they were less effective for removing those of lower molecular weight after 48 h of continuous elution. At the end of the elution period, 3:2 CP-LPHM/GAC continued to show ≥90% reduction of fluoranthene (26 ± 6 ng/L), pyrene (42 ± 21 ng/L), benz[*a*]anthracene (7 ± 1 ng/L), and chrysene (5 ± 1 ng/L) (Table 9). These same contaminants were reduced by 73 to 80% after 48 h of elution through CP-LPHM/sand (Table 11). The extent of contamination that has been remediated in the treated OWS effluent can be further illustrated by GC/MS chromatograms of a representative

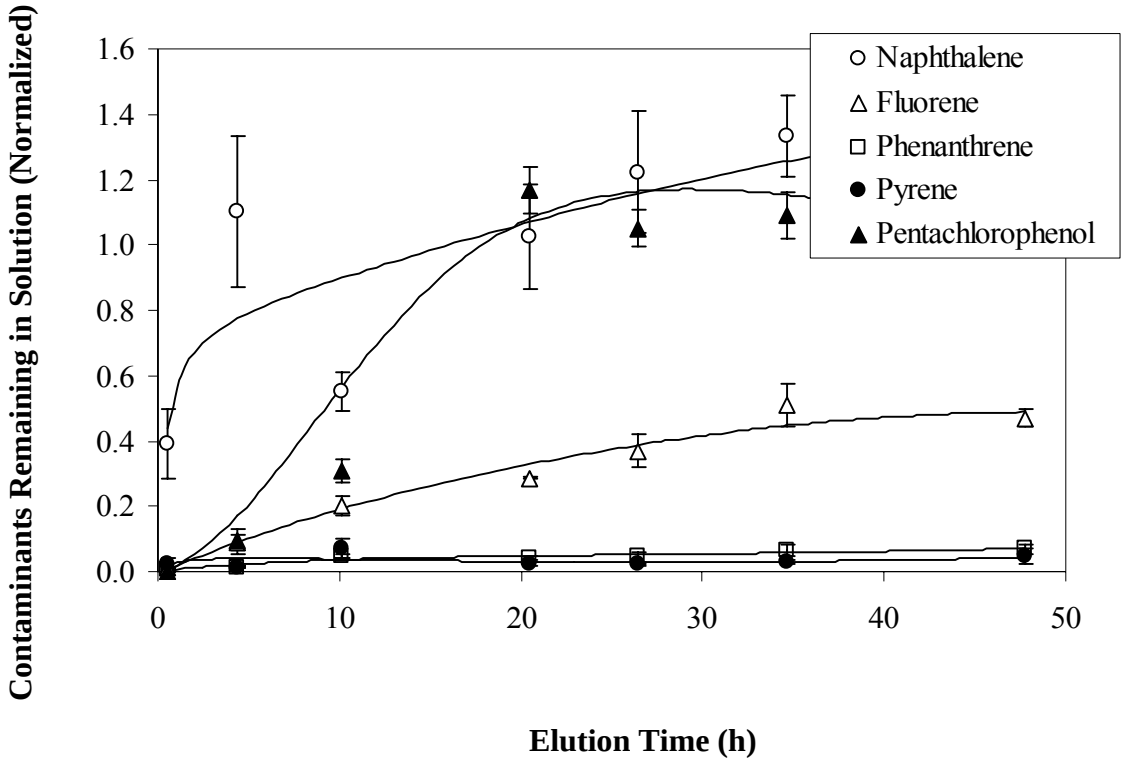


Figure 24. Contaminants remaining in solution at selected time points over 48 h of continuous elution of OWS effluent through 3:2 CP-LPHM/GAC. Values represent mean concentrations for 3 separate composite columns (normalized to untreated OWS effluent) ± SE.

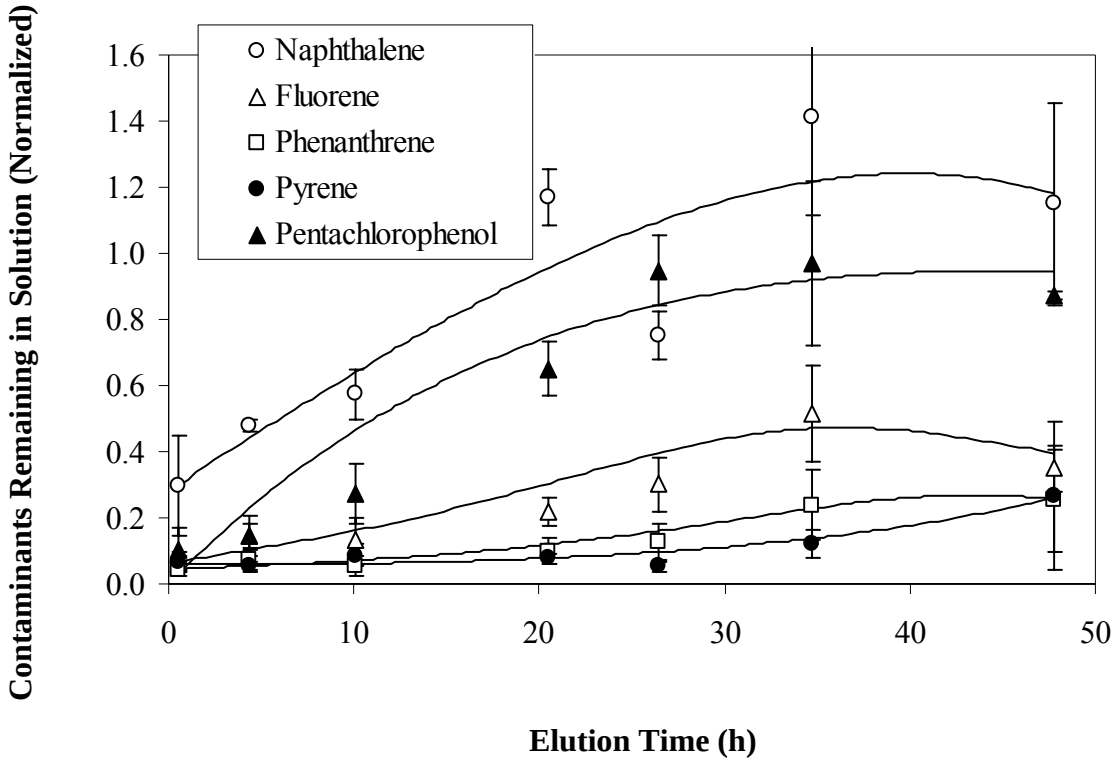
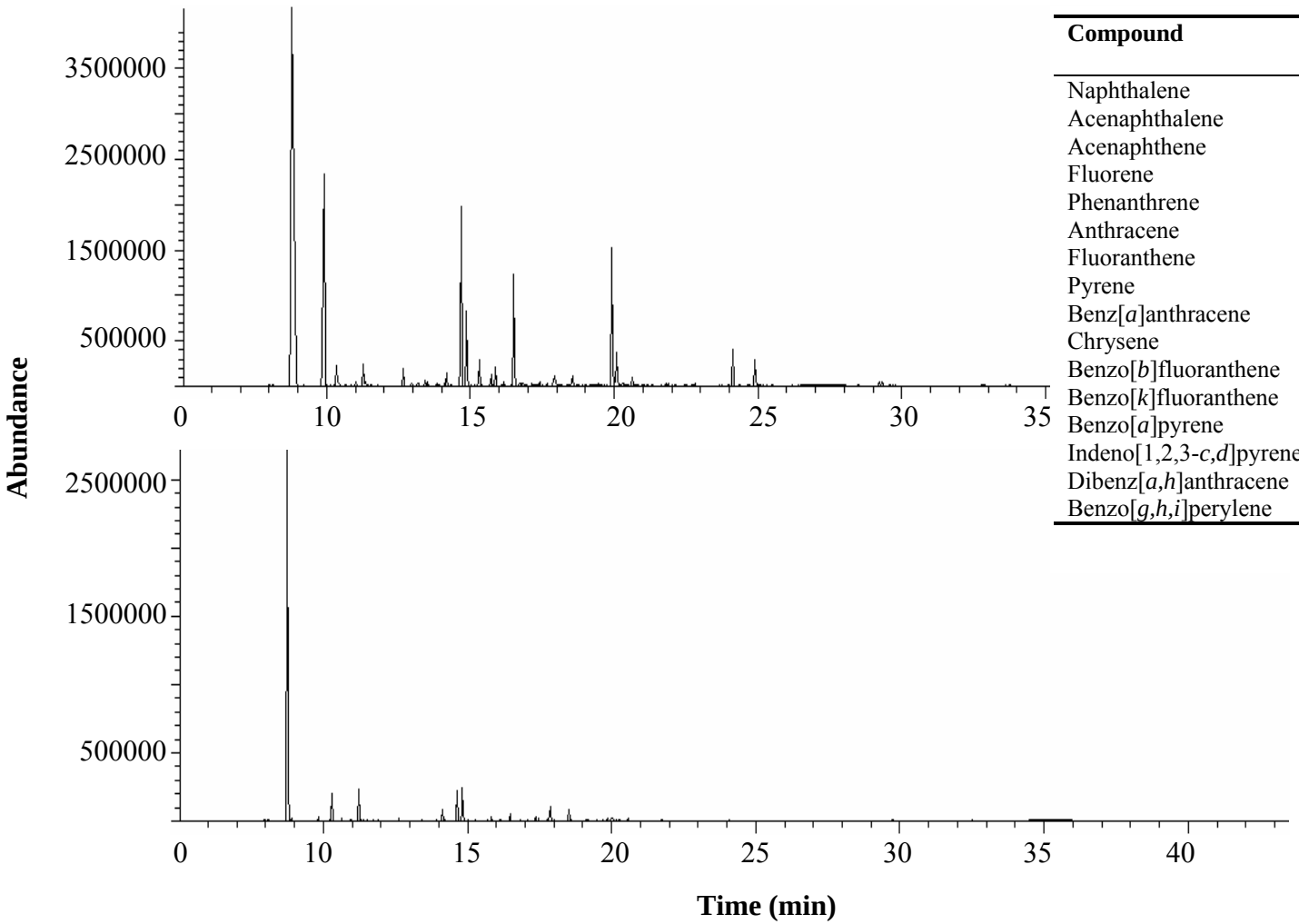
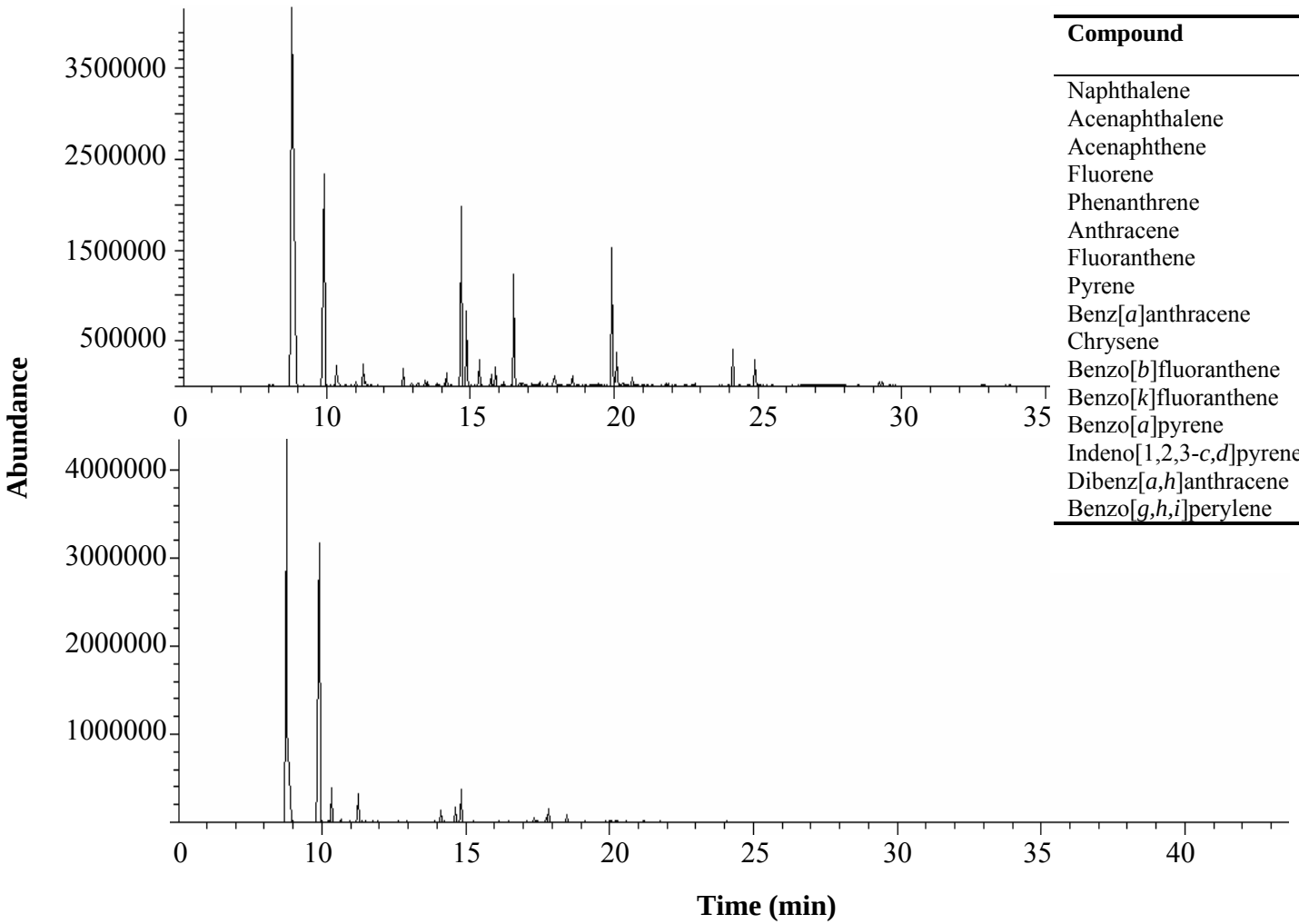


Figure 25. Contaminants remaining in solution at selected time points over 48 h of continuous elution of OWS effluent through CP-LPHM/sand. Values represent mean concentrations for 3 separate composite columns (normalized to untreated OWS effluent) ± SE.



Compound	Retention Time (min)
Naphthalene	8.87
Acenaphthalene	14.15
Acenaphthene	14.78
Fluorene	16.62
Phenanthrene	19.99
Anthracene	20.18
Fluoranthene	24.24
Pyrene	25.01
Benz[*a*]anthracene	29.35
Chrysene	29.47
Benzo[*b*]fluoranthene	32.93
Benzo[*k*]fluoranthene	33.02
Benzo[*a*]pyrene	33.90
Indeno[1,2,3-*c,d*]pyrene	37.13
Dibenz[*a,h*]anthracene	37.24
Benzo[*g,h,i*]perylene	37.92

Figure 26. Representative GC/MS chromatograms of untreated OWS effluent and OWS effluent eluted through 3:2 CP-LPHM/GAC. Top: untreated OWS effluent. Bottom: OWS effluent eluted through 3:2 CP-LPHM/GAC. Inset lists PAHs and their approximate retention times in the chromatograms.



Compound	Retention Time (min)
Naphthalene	8.87
Acenaphthalene	14.15
Acenaphthene	14.78
Fluorene	16.62
Phenanthrene	19.99
Anthracene	20.18
Fluoranthene	24.24
Pyrene	25.01
Benz[*a*]anthracene	29.35
Chrysene	29.47
Benzo[*b*]fluoranthene	32.93
Benzo[*k*]fluoranthene	33.02
Benzo[*a*]pyrene	33.90
Indeno[1,2,3-*c*,*d*]pyrene	37.13
Dibenz[*a*,*h*]anthracene	37.24
Benzo[*g*,*h*,*i*]perylene	37.92

Figure 27. Representative GC/MS chromatograms of untreated OWS effluent and OWS effluent eluted through CP-LPHM/sand. Top: untreated OWS effluent. Bottom: OWS effluent eluted through CP-LPHM/sand. Inset lists PAHs and their approximate retention times in the chromatogram.

untreated OWS effluent sample versus eluate from a column of 3:2 CP-LPHM (Figure 26) and from a column of CP-LPHM/sand (Figure 27).

The volume of composite-treated effluent was calculated to be 48.0 ± 2.9 L by CP-LPHM/sand and 51.8 ± 2.3 L by 3:2 CP-LPHM/GAC over the course of 48 h. The average flow rate of OWS effluent was 20.4 ± 1.7 mL/min through CP-LPHM/sand, 22.0 ± 1.4 mL/min through 3:2 CP-LPHM/GAC, and 21.3 ± 2.2 mL/min through control columns filled with sand and glass wool alone.

3.3 Discussion

This research focused on characterizing the effectiveness of various formulations of organoclay composite sorbents for PAH and C_5P removal from creosote contaminated water. Following preliminary laboratory characterization, field trials were carried out to further evaluate various factors in formulation on the overall composite performance. Initial controlled laboratory studies provided a rapid, small-scale indication of the large-scale performance of each composite sorbent. These studies utilized a ^{14}C-labeled reconstituted aqueous phase (RAP) made from DNAPL that was collected from the same Superfund site where subsequent field studies were conducted. Although the methods offered an indication of field performance, complete breakthrough of the contaminant was not achieved and analysis was limited to C_5P alone. Subsequent field studies, in which creosote-derived pollutants were filtered through fixed-bed columns by continuous gravity-flow elution, were selected to as the practical model of waste treatment conditions in the field (Jusoh et al., 2002).

Small-scale bench studies indicated that composite sorbents ranging from 1:20 to 3:2 g organoclay:GAC were effective to some extent in removing aqueous ^{14}C-C_5P from the complex RAP solution (Figure 22). There was a positive relationship between the amount of organoclay bonded to GAC and the effectiveness of the composite to remove C_5P from solution for all sorbents except 1:5 CP-LPHM/GAC, which was no more effective than 1:20 CP-LPHM/GAC. Overall, CP-LPHM/sand and 3:2 CP-LPHM/GAC were shown to be the most effective sorbents.

In a comparative study, the effect of using GAC or sand as a solid support in organoclay composites was evaluated. Both of these composites were formulated to contain a maximum yet equal amount of organoclay per unit volume. GAC is the most commonly used porous media for the cleanup of contaminated water, notably for certain volatile organic compounds, pesticides, surfactants, and chlorinated compounds (Paune et al., 1998). However, in the presence of natural organic matter GAC capacity and bedlife can be drastically decreased (Hopman et al., 1994). Thus, multi-functional, porous composites of organoclay bonded to either GAC or inert sand as a solid support were designed to overcome these limitations. Although 3:2 CP-LPHM/GAC was somewhat more effective than CP-LPHM/sand for the removal of higher molecular weight PAHs, the use of GAC as a solid support did not appear to provide a substantial added sorptive capacity over that of sand in either bench or field studies.

In field studies, a total of 40 principal hazardous constituents, including all 16 priority PAHs identified by the U.S. EPA, were quantified in both untreated and composite-treated OWS samples. Untreated control samples were collected by passing

OWS effluent through columns filled with sand and glass wool to filter out particulate matter and free phase oil droplets. All sorbents outlined in this study were effective in the removal of C_5P and PAHs from OWS effluent. These findings were consistent with previous research which reported that organoclay-based composites have a high capacity for contaminants found in wood preserving waste (Ake et al., 2003). Results showed that 1:20, 1:10 or 1:5 CP-LPHM/GAC frequently reduced contaminant concentrations in OWS effluent to levels below the limits of detection where they remained at the end of the experiment. However, these sorbents were tested at 10 and 20 g amounts and for only 7 h of elution (Tables 2 to 7). In contrast, 4 g of 3:2 CP-LPHM/GAC and 13 g of CP-LPHM/sand, designed to contain equal amounts of CP-LPHM per unit volume and packed into columns on an equivolume basis, remained quite effective for many contaminants following 48 h of continuous elution (Tables 9 and 11). It should be noted that only contaminants present above the quantitation limit (10 ng/L) in concurrently collected untreated OWS effluent controls have been reported.

Complete breakthrough of both naphthalene and C_5P occurred during the 48 h study of 3:2 CP-LPHM/GAC and CP-LPHM/sand (Figures 24 and 25). In contrast, both sorbents were effective in preventing even 50% breakthrough of higher molecular weight PAHs such as fluorene, phenanthrene, and pyrene over the same time period (Tables 9 and 11). Thus, both composites decreased higher molecular weight PAHs with higher numbers of aromatic rings more readily than those with lower molecular weights. This is of particular importance as those PAHs that have been indicated to be carcinogenic (benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene,

benzo[*a*]pyrene, indeno[1,2,3-*c,d*]pyrene, and dibenz[*a,h*]anthracene) are also of high molecular weight. However, many of these contaminants were present only in small amounts and were frequently below quantitation limits (10 ng/L) during field evaluation of CP-LPHM/sand and 3:2 CP-LPHM/GAC where breakthrough was most apparent.

The average flow rate of OWS effluent through 1:20, 1:10, and 1:5 CP-LPHM/GAC was 16.4 ± 0.9 mL/min, and was 20.4 ± 1.7 mL/min through 3:2 CP-LPHM/GAC and CP-LPHM/sand. In previous studies utilizing 100 g of composite, flow rates were held much lower at approximately 3.3 mL/min (Ake et al., 2003). This could affect the removal of contaminants because sorption effects are, at least partly, a function of time and a slower flow rate allows for greater contact time between sorbent materials and contaminants leading, presumably, to greater sorption of contaminants (Hwang and Cutright, 2002).

It is important to note the extremely high concentrations of C_5P and PAHs present in the OWS effluent at this particular site, as well as the highly dynamic nature of the concentration of these pollutants in water as a function of time. The field studies were conducted in two phases. In the first phase, which tested the effectiveness of different amounts of organoclay, the concentration of C_5P in untreated OWS effluent was 984,211 ± 343,040 ng/L, phenanthrene was 786,367 ± 243,369 ng/L, and benzo[*a*]pyrene was 17,743 ± 6,959 ng/L (Tables 2 to 7). However, in the second phase, which delineated the contribution of different solid supports, the concentration of C_5P had doubled to 2,332,529 ± 174,708 ng/L and the concentrations for the higher molecular weight and carcinogenic PAHs were much lower. For example, phenanthrene decreased

to 16,731 ± 3,268 ng/L and benzo[*a*]pyrene was not measured above detection limits in untreated OWS effluent (Tables 8 to 11). Of particular interest, C_5P has been designated a priority pollutant and the maximum contaminant level (MCL) set by the U.S. EPA is 1,000 ng/L in drinking water, well below the maximum measured in this study (USEPA, 2002; IRIS, 2004). Thus, small amounts of these composite materials have been challenged with a tremendous contaminant load.

The use of either GAC or organoclays can be an effective technique in wastewater remediation. However, GAC is less effective for large organic molecules and competitive sorption of cosolutes results in depressed (antagonistic) sorption whereas, in the case of organoclays, sorption of one contaminant can have synergistic effects on the sorption of cosolutes. Sorption of individual contaminants may cause interlayer expansion, leading to the binding of larger size compounds previously unsorbed due to steric constraints of the interlayers (Xu et al., 1997). In addition, hydrophobic tails of exchanged long-chain cations become more flexible as solute concentrations increase and a solvent-like hydrophobic phase forms in the interlayer to make partitioning the predominant sorption mechanism in organoclays (Boyd et al., 1988; Sheng et al., 1996). Likewise, in this study, the high exchangeable surface area of LPHM clay allows for a much greater capacity of CP per volume of sorbent thus greatly enhancing the removal of hydrophobic contaminants from OWS effluent. With this in mind, the organoclay-based composites outlined in this paper, as a pre-polishing step to GAC, are expected to more effectively reduce the total contaminant load of PAHs, in particular carcinogenic PAHs, by a greater percentage than GAC treatment alone.

One important issue concerning the use of organoclays is the potential for the quaternary amine to leach from the clay with time. The leached quaternary amine may cause toxicity to microbial flora in a remediation approach that includes a biodegradation step subsequent to filtration. Thus, future studies should explore ways to capture desorbed quaternary amine as well as methods to monitor and prevent leaching. In addition, the reported studies were carried out using small amounts of composite in order to attain breakthrough necessary to determine sorbent capacity and bedlife. Field-scale applications that require larger bed volumes for filtration of greater elution volumes should also be performed to explore possible co-solvent effects and contaminant interactions over time.

CHAPTER IV

TOXICOLOGICAL EVALUATION AND METAL BIOAVAILABILITY IN PREGNANT SPRAGUE-DAWLEY RATS FOLLOWING EXPOSURE TO CLAY MINERALS IN THE DIET*

A variety of silicate clays are frequently added to animal feeds as enterosorbents to bind and reduce the bioavailability of mycotoxins. In the past, phyllosilicate clays have been successfully added to animal feeds to bind aflatoxins in the gastrointestinal tract and subsequently prevent aflatoxicosis in farm animals, including chickens, turkey poults, goats, pigs, and mink (Phillips et al., 1995; Phillips, 1999). Historically, clays have also played a major role in the human diet, particularly during pregnancy where they have been described as a common food craving (Geissler et al., 1999). In addition, polygorskite and kaolinite have been used as to sorb toxins, bacteria, and viruses and as anti-diarrhetics, while sodium smectites have been used therapeutically as laxatives (Carretero, 2002). However, these sorbents may also bind enzymes and other necessary nutritive elements, making prolonged use inadvisable. For instance, several case studies suggest an association between clay ingestion and profound muscle weakness, anemia, and hypokalemia in humans (Mengel et al., 1964; Gonzalez et al., 1982; Severance et al., 1988).

Clays that sorb nonselectively may interact with nutrients, minerals, and other feedborne chemicals to pose significant hidden risks as a result of dietary supplementation. This is especially of concern during prenatal development due to the highly susceptible nature of both the mother and fetus to nutrient deficiency. In previous research, rats fed high levels (20%) of kaolin in the diet throughout gestation exhibited significant reductions in hemoglobin, hematocrit, and red blood cell levels, indicating maternal anemia, and pups borne to these rats had lower birth weights (Patterson and Staszak, 1977). In the study, iron supplementation to the kaolin-diet showed a protective effect for both dams and pups. Conversely, other studies have shown that rats fed a zinc-deficient diet supplemented with clay experienced lower mortality rates than rats maintained only on a zinc-deficient diet, suggesting that clay supplementation can be beneficial in some cases of mineral deficiencies (Smith and Halsted, 1970).

Additionally, heavy metals from environmental and food sources may accumulate in the body, leading to a variety of adverse health conditions. Chronic ingestion of arsenic, a known human carcinogen, has been correlated with an increased incidence of skin, urinary bladder, liver, and kidney cancers (Golub et al., 1998; Abernathy et al., 1999; Bernstam and Nriagu, 2000; Hughes, 2002). *In vitro* studies have shown aluminum silicate-containing bentonite and montmorillonite clays to be cytotoxic to human umbilical vein endothelial cells but not ROC-1 oligodendroglia cells (Murphy et al., 1993a) and to primary neuronal cells but not differentiated N1E-115 neuroblastoma cells (Murphy et al., 1993b). However, separate studies indicated aluminum silicate-

containing kaolinite and montmorillonite and the magnesium silicate hectorite were acutely toxic to differentiated neuroblastoma cells (Banin and Meiri, 1990).

Mineralogical data indicates that phyllosilicate clays are important aluminum carriers and many including smectite, chlorite, and illite, are known to be unstable in acidic environments, such as the stomach (Donner, and Lynn, 1989; Rai and Kittrick, 1989). However, comprehensive *in vivo* studies concerning the release and subsequent accumulation of aluminum in tissues are lacking. In this study, the effects of two common clay minerals, a calcium montmorillonite clay (*NOVASIL PLUS*™, NSP) and a sodium montmorillonite clay (Swy-2) were examined for maternal and fetal toxicity as well as their influence on mineral uptake and utilization in the pregnant rat.

4.1 Materials and methods

4.1.1 Chemicals

For all experiments purified water (ddH_2O) was prepared by processing deionized water through a Milli-Q^{UF+} purification system (Millipore Corp., Benford, MA). NSP was obtained from Engelhard Corporation (Cleveland, Ohio). Wyoming sodium montmorillonite (Swy-2) was obtained from the Clay Minerals Repository (University of Missouri, Columbia).

4.1.2 Experimental animals

Treatment of experimental animals was based on methods previously reported in Mayura et al. (1998). Ten week old, sexually mature, virgin Sprague-Dawley female and

mature male rats (Harlan, Sprague-Dawley Inc., Houston, TX) were maintained on feed and water *ad libitum* at Texas A&M University Laboratory Animal Resources and Research Facility (LARR). After an acclimation period of 3 to 4 days, females were paired with males overnight in filter-top polycarbonate cages housed in a temperature-controlled and artificially illuminated room (12 h dark/12 h light cycle) free from any sources of chemical contamination. Successful mating was determined by the presence of vaginal copulatory plugs in the cages and was designated as day 0 of pregnancy.

4.1.3 Experimental design

Three diets were prepared from a balanced powdered rodent feed (Tekland rodent diet 8604, Harlan feeds, Madison, WI) and included: 1) basal feed 2) basal feed with 2% NSP (w/w) and 3) basal feed with 2% Swy-2 (w/w). The pregnant animals were distributed into one of 3 treatment groups receiving one of the 3 diets. Each treatment group had 6 animals maintained on their respective diets through the experimental period (from gestation days 0 to 16). Treatment groups were exposed to clays in the feed as well as by gavage. Untreated animals were fed only the basal diet in the absence of clay. To ensure a continuous presence of clay in the stomach, animals maintained on diets containing clay were gavaged once per day midway through the fasting period (12 h light cycle) with the respective clay between gestation days 1 to 15. The gavage dose (0.25% w/v aqueous suspension) was selected as the maximum amount of clay that could be suspended in 1 mL without increasing the viscosity beyond the ability to deliver the dose through the gavage needle. Untreated animals were gavaged with 1 mL vehicle alone.

Stock clay suspensions were made by shaking 25 g clay in 400 mL ddH_2O overnight. Dosing volumes were 1 mL in all treatment groups. Feed intake of animals was recorded daily, and maternal body weights were recorded on days 1, 8, and 16 of gestation.

4.1.4 Assessment of toxicity

Dams were euthanized by carbon dioxide asphyxiation on day 16 of gestation and cesarean sections were performed. An abdominal incision was made followed by exposing the uterine horns and the litter weights (uterus plus pups) were recorded. The uterine wall was cut open and the number of implants, resorptions, dead and live fetuses were counted. In addition, maternal liver, kidneys, tibia, brain, uterus, pooled placentae, and pooled embryonic mass were collected, weighed, and frozen prior to elemental analysis.

4.1.5 Neutron activation analysis (NAA)

Representative samples (100 mg) of NSP and Swy-2 were analyzed in addition to tissues. Collected tissues were lyophilized and 250 mg of dried material was added to pre-cleaned polyethylene vials. Irradiations for neutron activation analysis (NAA) were performed at Texas A&M University's 1 MW research reactor. Short-lived isotope samples were irradiated in a pneumatic facility for 1 min (flux approximately 1 x 10^{13} n/cm/s) and counted for 500 s on a high-resolution germanium gamma-ray detector after a decay period of a few minutes. Standards and quality control material were irradiated and counted in the same manner. For those elements that produce longer-lived species

upon irradiation, samples, standards and quality control material were irradiated together in a rotisserie position for 14 h. After decays of approximately 1 and 3 weeks, additional counts of 30 and 60 min, respectively, were made. Peak areas for characteristic gamma-rays for isotopes of elements of interest were compared between the standards and samples to compute the concentrations. Spectra were evaluated and concentrations were computed using Canberra Industries' Genie ESP Software on a DEC workstation running an alpha processor using Open VMS operating system. Detection limits varied by tissue and element and were calculated based on concurrently analyzed multi-element standards however, many were not determined due to high levels of Na in many of the samples.

4.1.6 Inductively coupled plasma-mass spectroscopy (ICP-MS)

Aluminum concentrations were determined on a subset of brain tissue samples in order to achieve lower detection limits. Wet tissue samples were digested with ultrapure nitric acid and hydrogen peroxide and diluted to volume with deionized water. ^{27}Al was monitored on a Perkin-Elmer/Sciex DRC 2 in peak hopping mode, using external calibration with commercial standards and ^{71}Ga as an internal standard (Inorganic Ventures, Lakewood, NJ). Calibration was checked using NIST SRM 1640. CRM DOLT-2 was processed with the samples and showed 93% recovery of its certified value. Recoveries of a laboratory control sample and a matrix spike were 101% and 107%, respectively.

4.1.7 Statistical analysis

Unless otherwise noted, all values are expressed as means ± SE. Mean values for body and organ weights, feed intake, and developmental toxicity parameters were calculated from 6 rats in each treatment group. From those 6 rats, 3 were randomly selected for determination of elemental content via neutron activation analysis. Data were compared statistically using the General Linear Models procedure with Tukey's test and considered significant if $p<0.05$.

4.2 Results

4.2.1 NAA of elemental content of clay minerals

The two clay minerals, NSP and Swy-2, which were supplemented in the diet of pregnant Sprague-Dawley rats, were subjected to NAA for the determination of their elemental composition (Table 12). Predominant metal constituents for both clay minerals included Al, Fe, Na, Mg, Ba, Ce, Mn, Sr, Zn, and Zr. The most prevalent metals for NSP were Al (9.34%) and Fe (3.55%), followed by Mg (0.69%), Na (0.19%), and Sr (0.17%). Swy-2 was also composed primarily of Al (9.97%) and Fe (2.52%), followed by Na (1.04%), Mg (0.58%), and Sr (0.03%). In addition, a variety of metals were identified but were found to be present below 0.01% including: As, Co, Cr, Cs, Dy, Eu, Hf, La, Lu, Nd, Ni, Rb, Sb, Sc, Sm, Ta, Tb, Th, U, and Yb. Metals present below the detection limits for this method (based on concurrently analyzed multi-element standards) included Cu, Mo, S, Te, Ti, Tl, and V.

Table 12
Analysis and percent abundance of selected metals in Swy-2 and NSP

Metal	Analysis of metals in Swy-2 (mg/kg)[a]	Percent abundance in Swy-2	Analysis of metals in NSP (mg/kg)[a]	Percent abundance in NSP
Al	93,381.6 ± 0.9	9.97	99,711.1 ± 1.3	9.34
Ba	174.9 ± 8.1	0.03	340.7 ± 5.1	0.02
Ce	251.1 ± 1.3	0.01	87.1 ± 4.0	0.03
Fe	35,524.3 ± 0.8	2.52	25,239.7 ± 3.5	3.55
Mg	6,854.3 ± 2.8	0.58	5,786.0 ± 5.9	0.69
Mn	261.9 ± 1.3	0.02	195.7 ± 5.2	0.03
Na	1,945.5 ± 3.9	1.04	10,369.6 ± 0.5	0.19
Sr	1,660.2 ± 0.5	0.03	264.2 ± 5.5	0.17
Zn	131.8 ± 9.6	0.01	75.4 ± 3.7	0.01
Zr	450.3 ± 0.4	0.02	215.0 ± 2.7	0.05

[a] Analysis of metals in Swy-2 and NSP is based on mean values for 3 rats per treatment group ± SD. Metals detected at or below 0.01% abundance in both clays include: As, Co, Cr, Cs, Dy, Eu, Hf, La, Lu, Nd, Ni, Rb, Sb, Sc, Sm, Ta, Tb, Th, U, Yb.

4.2.2 Assessment of toxicity

Evaluations of maternal and fetal toxicity included maternal body weights, maternal feed intakes, litter weights, and embryonic resorptions. Upon dissection, no significant differences were observed for total implantations, resorptions, or embryos in control rats versus those maintained on either clay-based diet (Table 13). In addition, no statistically significant differences were observed between tissue weights (liver, kidneys, tibia, brain, uterus, pooled placentae, and pooled embryonic mass) in control rats as compared to those fed clay-based diets (Table 14). Further, maternal body weight gain, litter weights, and feed intake were not significantly affected by addition of clay to the diets (Table 15).

4.2.3 NAA of elemental content of tissues

Results of NAA analysis performed on maternal liver, kidneys, tibia, brain, uterus, pooled placentae, and pooled embryonic mass, specifically, differences between control and treated animals, are shown in Figure 28. In maternal brain tissue, Rb levels were somewhat lowered in rats consuming either clay as compared to control rats (averages of raw Rb concentrations were 11.3 ± 0.1 mg/kg for control, 10.8 ± 0.2 mg/kg for NSP, and 10.7 ± 0.1 mg/kg for Swy-2). This was the only statistically significant difference observed upon analysis of the selected tissues in this study, although several tendencies were apparent. Rats maintained on either NSP or Swy-2-supplemented diets exhibited decreased As in the uterus, placentae, and kidneys, however a slight increase was observed in brain tissue. In addition, a tendency toward increased Co was apparent

Table 13
Effects of clay-based diets on implantations, resorptions, and embryos in the pregnant rat

	Control diet[a]	2% Swy-2 diet[a]	2% NSP diet[a]
Implantations	17 ± 1	15 ± 1	17 ± 1
Embryos	15 ± 1	14 ± 1	15 ± 1
Resorptions	1 ± 1	1 ± 0	2 ± 1

[a] Clays (NSP and Swy-2) were added to a powdered basal diet at a level of 2% (w/w) and fed to the rats between days 1 to 15 of pregnancy. Test animals were dosed orally with 0.25% (w/v) clay in 1 mL aqueous suspension once per day (midway through the fasting period). Animals fed the control diet were dosed orally with 1 mL aqueous vehicle alone. All data is expressed as mean values for 6 rats per treatment group ± SD. Mean values for rats maintained on Swy-2 and NSP diets were not statistically different ($p \leq 0.05$) from controls.

Table 14
Effects of clay-based diets on tissue weights in the pregnant rat

Tissues[a]	Weight of tissues from rats fed control diet[b] (g)	Weight of tissues from rats fed 2% Swy-2 diet[b] (g)	Weight of tissues from rats fed 2% NSP diet[b] (g)
Liver	12.6 ± 0.7	12.5 ± 0.4	12.7 ± 1.0
Right kidney	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
Left kidney	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1
Brain	1.7 ± 0.0	1.6 ± 0.1	1.7 ± 0.0
Right tibia	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1
Left tibia	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
Uterus	4.6 ± 0.4	4.4 ± 0.6	4.7 ± 0.3
Pooled placentae	5.1 ± 0.8	4.8 ± 1.1	5.0 ± 0.8
Pooled embryonic mass	7.3 ± 0.7	6.7 ± 1.6	7.2 ± 0.9

[a] Tissues were collected from pregnant rats at gestation day 16.
[b] Clays (NSP and Swy-2) were added to a powdered basal diet at a level of 2% (w/w) and fed to the rats between days 1 to 15 of pregnancy. Test animals were dosed orally with 0.25% (w/v) clay in 1 mL aqueous suspension once per day (midway through the fasting period). Animals fed the control diet were dosed orally with 1 mL aqueous vehicle alone. All data is expressed as mean values for 6 rats per treatment group ± SD. Mean weight of tissues for rats maintained on Swy-2 and NSP diets were not statistically different ($p \leq 0.05$) from controls.

Table 15

Effects of clay-based diets on gain in maternal body weights, litter weights, and feed intake in the pregnant rat

Diet[a]	Maternal body weight at day 1 (g)[b]	Maternal body weight at day 16 (g)[b]	Gain in maternal body weight (g)[b]	Litter weight (g)[b]	Total feed intake (g)[b]
Control	241 ± 11.8	309 ± 7.4	68.0 ± 5.7	28.9 ± 1.1	330 ± 13
2% Swy-2	237 ± 9.1	308 ± 8.7	71.0 ± 3.5	27.0 ± 2.1	352 ± 7
2% NSP	240 ± 7.4	309 ± 8.1	69.3 ± 3.5	29.2 ± 1.4	345 ± 7

[a] Clays (NSP and Swy-2) were added to a powdered basal feed at a level of 2% (w/w) and fed to the rats between days 1 to 15 of pregnancy. Test animals were dosed orally with 0.25% (w/v) clay in 1 mL aqueous suspension once per day (midway through the fasting period). Animals fed the control diet were dosed orally with 1 mL aqueous vehicle alone.

[b] Data is expressed as mean values for 6 rats per treatment group ± SE. Mean values for rats maintained on Swy-2 and NSP diets were not statistically different ($p \leq 0.05$) from controls.

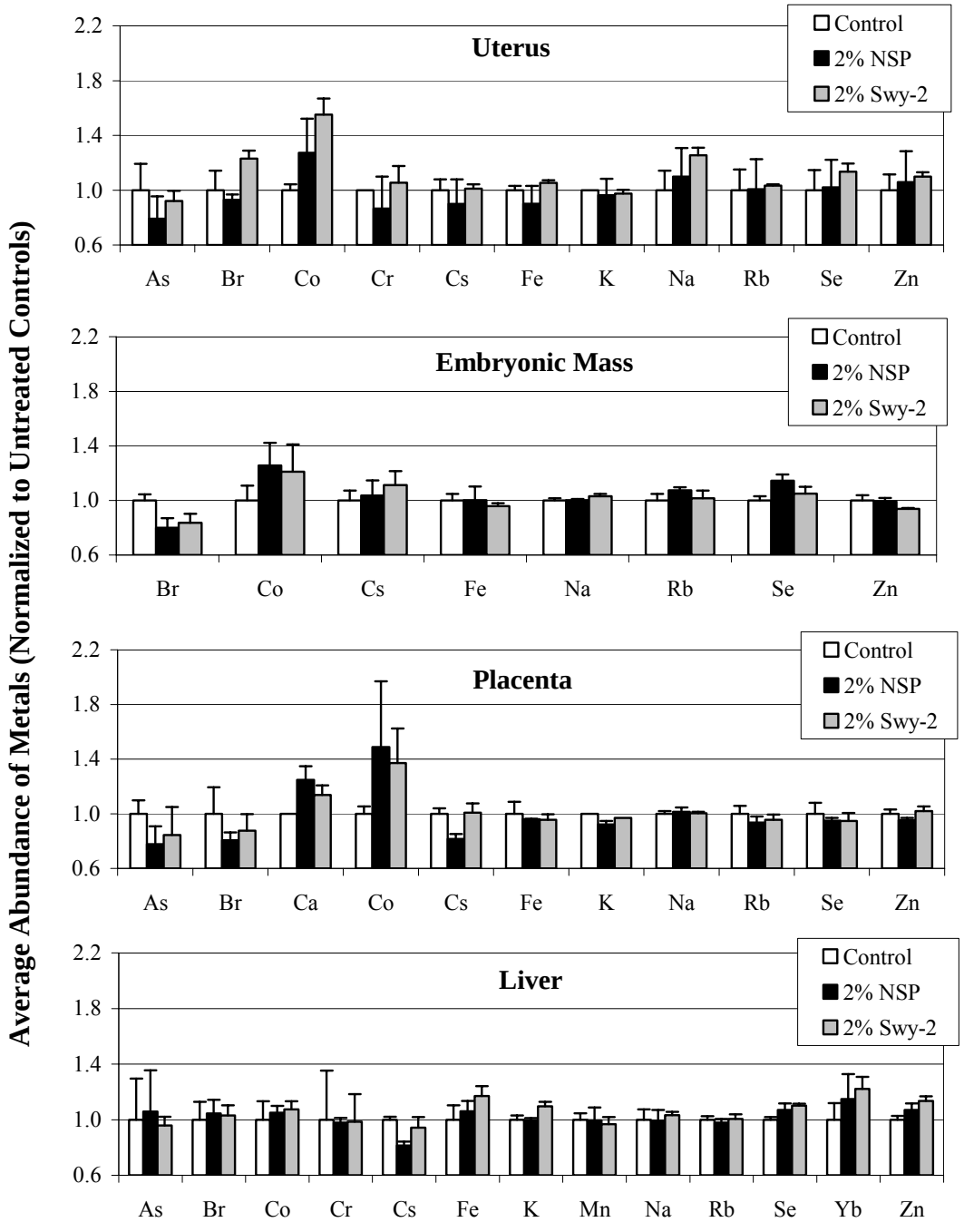


Figure 28. Metal abundance in uterus, embryonic mass, placenta, liver, kidney, brain, and tibia of rats fed control and clay-based diets. Values represent data for 3 randomly selected rats per treatment group (normalized to untreated controls) ± SE. Data is shown only for those metals present above detection limits for all rats in all treatment groups.

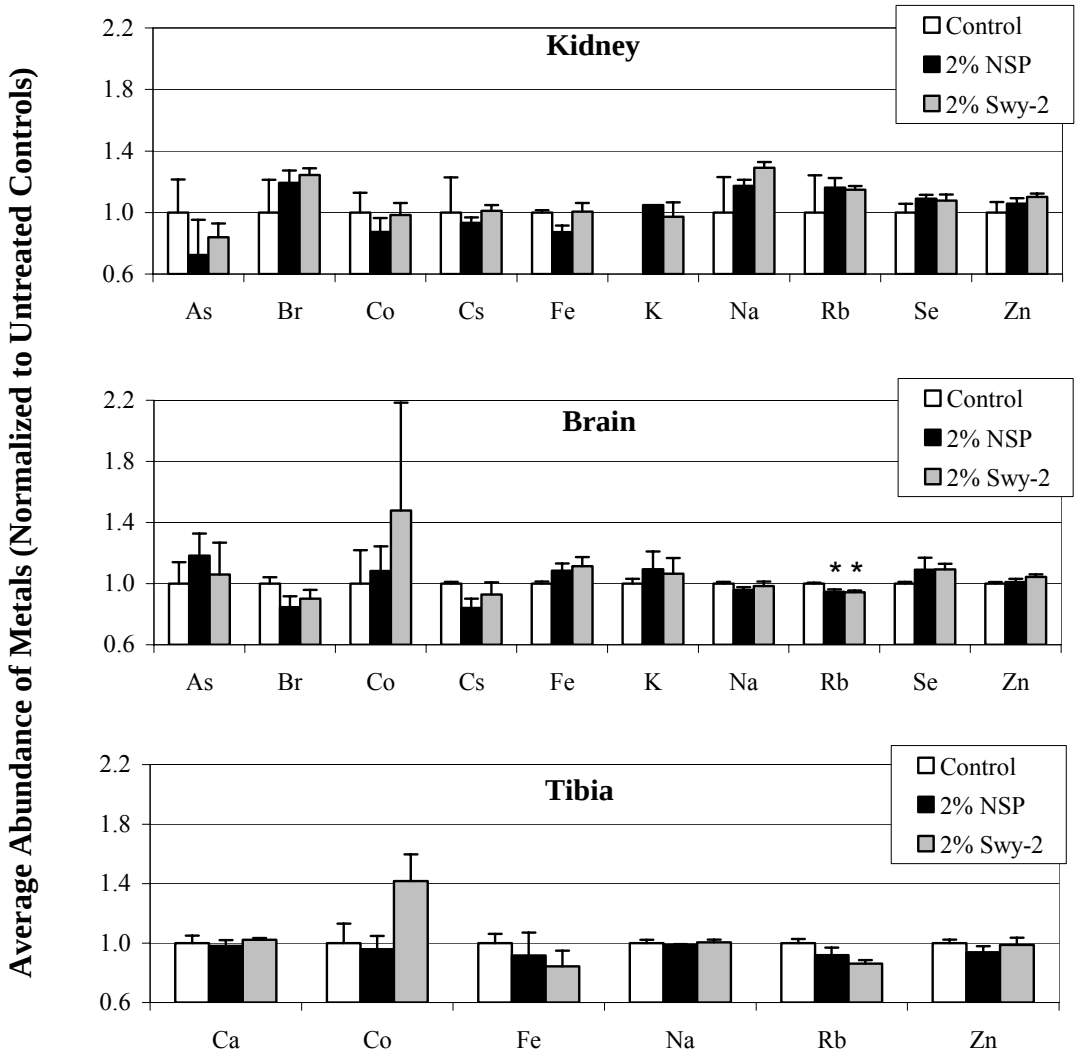


Figure 28. (Continued).

with either clay-supplemented diet in the uterus, embryonic mass, placentae, liver, and brain, as well as in the tibia in the case of the Swy-2-supplemented diet.

Additional tendencies were seen in rats fed an NSP-supplemented diet where decreased Cs was observed in the uterus, placentae, liver, brain, and kidneys, although the metal was increased in the embryonic mass. Increased Na was apparent in both the uterus and kidneys of rats fed either clay-supplemented diet and Fe was increased in the liver and brain, but decreased in the tibia, of these same rats. Br was increased in the kidneys and decreased in the embryonic mass, placentae, and brain tissue of rats fed either clay-supplemented diet. However, rats supplemented with Swy-2, but not NSP, showed increased Br in the uterus. Zn and Se were increased in the uterus, liver, brain, and kidneys and Yb was increased in the liver of rats fed either clay-supplemented diet.

4.2.4 ICP-MS of tissues

Aluminum was determined to be below the detection limits for all tissues investigated based on NAA. For further confirmation of Al levels in the brain, tissue samples from three rats in each treatment group were also analyzed by ICP-MS. This procedure supported the NAA results, as Al was below detection limits for ICP-MS (< 0.5 mg/kg) in all samples analyzed (data not shown). Other metals analyzed for by NAA, but found to be present below the detection limits in the tissue samples, included: Cu, Dy, Eu, Hf, La, Lu, Mo, Nd, S, Sb, Sm, Tb, Te, Th, Ti, Tl, U, V, and Zr.

4.3 Discussion

In this study, maternal and fetal toxicity of two common clay minerals supplemented in the diet of pregnant Sprague-Dawley rats was assessed. NAA showed that the major metal components of the two clays, a calcium montmorillonite (NSP) and a sodium montmorillonite (Swy-2), were similar (Table 1). However, these clays can be differentiated based on their Na content, as Swy-2 contains mainly Na ions in the interlayer making these ions more prevalent in the overall composition (1.04% abundance in Swy-2; 0.19% abundance in NSP).

In addition to Na, both clays contained relatively large amounts of Al, Fe, Mg, and Sr. However, of the elements analyzed in this study the major component of both clays was Al (9.97% abundance in Swy-2; 9.34% abundance in NSP). Further, mineralogical data indicates that phyllosilicate clays are important aluminum carriers and many are known to be unstable in acidic environments, such as the stomach (Donner and Lynn, 1989; Rai and Kittrick, 1989). Interestingly, although Al was greater than 9% abundance in both clay minerals in this study, the metal was not identified above NAA detection limits in any of the 7 tissues. In this method, the limit of detection of Al in brain tissue was 300 mg/kg. ICP-MS provided a more sensitive measure of Al and confirmed the absence of Al deposits (<0.5 mg/kg) in brain tissues from rats in all treatment groups. The absence of any significant Al leachate may be partially due to the structural morphology of 2:1 layer-lattice dioctahedral montmorillonite clays. In these clays, SiO_4 tetrahedra are linked together, with each tetrahedron sharing three O^{2-} ions with three adjacent tetrahedra. This arrangement extends in all directions to form a plane

of basal oxygens. Al ions are octahedrally coordinated with SiO_4 sheets, in the center of platelets, which limits bioavailability (Schultz, 1989; Phillips et al., 2002). In addition, recent studies in both rats and humans have implicated dietary Si as a complexing agent that may reduce gastrointestinal absorption and increase renal excretion of Al (Edwardson et al., 1993; Roberts et al., 1998; Yokel et al., 1996).

The only statistically significant difference observed upon analysis of the selected tissues in this study was decreased Rb in the brain of rats fed either NSP or Swy-2 as compared to controls. The essentiality of Rb has been tested in goats, which exhibited decreased food intake, growth, and life expectancy, and increased spontaneous abortions following ingestion of Rb-deficient diets (Anke et al., 1997). However, additional studies are lacking and animal and human data for this ultra trace element are both limited and controversial, making extrapolations with regard to the decreased Rb observed here inappropriate.

Aside from Rb, the clays utilized in this study did not significantly bind or leach any of the elements analyzed, although several tendencies (not statistically significant) were observed. Rats maintained on clay-supplemented diets in this study exhibited decreased As in the uterus, placentae, and kidneys, but the metal was increased in the brain. This is of interest given that As is toxic and carcinogenic, especially in the inorganic form (Golub et al., 1998; Abernathy et al., 1999; Bernstam and Nriagu, 2000; Hughes, 2002), although nutritional studies have provided circumstantial evidence that As is also an essential nutrient in animals, possibly playing a role in methionine metabolism (Nielsen, 1991). A tendency toward increased Co versus basal levels was

also noted in some tissues, but with a high degree of variation. Although Co is required for the formation of vitamin B_{12}, excessive administration can result in goiter and reduced thyroid activity (Barceloux, 1999), and chronic dietary exposure has been associated with severe cardiac insufficiency (Alexander, 1972). In one study, chronic oral ingestion of Co supplemented in the diets of rats over 24 weeks resulted in decreased manganese superoxide dismutase activity, a decrease in mitochondrial ATP production, and a general reduction in the capacity of the respiratory chain (Clyne et al., 2001). In regard to a tendency for decreased Br observed in some tissues following clay-supplementation, a decrease in this element has been reported to depress growth, fertility, hematocrit, hemoglobin, and life expectancy, and to increase milk fat and abortions in goats, but evidence of both its essentiality and toxicity is limited (Nielsen, 2000).

It's important to note that greater than normal intake of any nutrient can interfere with absorption and availability of others. For example, moderately high intake of Zn has been shown to interfere with Cu utilization (Penland et al., 2000). However, even at the elevated levels reported here (≥2%), neither NSP nor Swy-2 adversely affected maternal body weights, maternal feed intakes, litter sizes, litter weights, tissue weights, or significantly altered embryonic resorptions in the pregnant rat (Tables 2, 3, and 4). In previous studies similar phyllosilicate clays (at levels as low as 0.5% w/w in the diet) have been shown to effectively bind aflatoxins and prevent aflatoxicosis in multiple animal species (Kubena et al., 1990a, 1990b; Phillips et al., 1988). In addition, significant reductions in the production of aflatoxin M_1 in the milk of dairy cows (44%) and goats (52%) have been achieved with the addition of 1% clay in the diet (Harvey et

al., 1991; Smith et al., 1994). In this study, pregnant rats consuming more than twice the level of clay shown to be effective as an enterosorbent for aflatoxin, displayed no noticeable maternal or fetal toxicity.

Nonselective interaction between any food additive and nutrients or minerals already present in the diet may pose significant hidden risks, especially during prenatal development. To guard against this, the safety of any product added to animal feed or to the human diet must be thoroughly investigated prior to use. In this study, neither of two clay minerals commonly added to animal feeds were shown, even at high concentrations, to produce maternal or fetal toxicity in pregnant rats, nor significantly affect the bioavailability of selected trace elements in a variety of tissues. Additional studies are warranted to characterize the safety and health effects of chronic ingestion of clay minerals in the diet.

CHAPTER V

SUMMARY AND CONCLUSIONS

Substantial environmental contamination has occurred as a result of the widespread use of coal tar creosote and pentachlorophenol (C_5P) as components in wood preserving solutions. Accidental spills, improper waste disposal, volatilization, and leaching of the components into soil during wood treatment procedures or from treated wood products has lead to extensive groundwater contamination. The adverse microbial environment created by much of this large-scale industrial contamination discourages natural degradation and, as a consequence, the chemical components of wood preserving waste are highly persistent in both soil and groundwater. In response to worldwide concern over the safety of drinking water supplies, research initiatives have focused on the development of both sensitive methodologies for analytical measurement and effective remediation technologies to contain, decrease, or eliminate contamination. With this in mind, the present studies were divided into three distinct phases, ultimately focused on characterizing the contaminants of wood preserving waste and developing appropriate strategies for successful remediation.

In the event of contamination, sensitive analytical methodology must be developed in order to evaluate the potential for toxicity associated with exposure. Thus, the objective of the first study was to outline a sequence of measurable biological changes in a sensitive *in vitro* model that occur as a consequence of exposure to various chlorinated phenols (CPs). The Clone 9 cell line, derived from normal rat liver

epithelium, has shown sensitivity to various chemical agents frequently identified in addition to CPs at wood preservation-based Superfund sites (Reeves et al., 2001). Endpoints used to evaluate cellular homeostasis were analyzed immediately after dosing and following 24 h in the continued presence of CPs with varying degrees of chlorination. In the case of C_5P treatment, low-dose exposure caused decreased intracellular pH (10 μM), GJIC (10 μM), and GSH (10 μM), increased ROS generation (10 μM), and plasma membrane hyperpolarization (100 μM). Prolonged exposure led to mitochondrial membrane depolarization (25 μM), followed by increased intracellular Ca^{2+} (50 μM), and, finally, plasma membrane depolarization (100 μM). The effects to specific endpoints were dependent upon dose, time, and the number of chlorine substituents. Similar effects were observed following exposure to either C_5P or 2,3,4,5-C_4P, and to either 2,3,5-C_3P or 3,5-C_2P. Effects resulting from 4-CP or phenol were slight and frequently observed only with high dose and/or prolonged exposure. For example, long-term CP exposure led to decreased GJIC at 10 μM doses of C_5P or 2,3,4,5-C_4P, 100 to 150 μM doses of 2,3,5-C_3P and 3,5-C_2P, and 1000 μM doses of 4-CP. Thus, the dose-, time-, and level of chlorine substitution-dependent differences allow for the delineation of specific congeners from a mixture. This knowledge may then allow the researcher to evaluate the extent of remediation efforts or design remediation technologies based on the prevalence of a specific congener in the field.

The results of this study demonstrate that the Clone 9 cell line is a sensitive *in vitro* model for CP exposure, especially in the case of the more highly chlorinated congeners. Assessment of selected markers of cellular homeostasis, for example

intracellular pH or GJIC, may be a more useful measure of the overall toxicity of a contaminant mixture than conventional analytical methods, which primarily involve quantification of individual components in the absence of complete knowledge of potential additive or synergistic interactions. Because the strength of this model with respect to complex chemical mixtures remains unclear, future research should focus on delineating the sequence of events that follow exposure to multi-contaminant mixtures and to determine the similarity to, or divergence from, the sequences established here in CP-treated cells. In particular, the model should be tested with wood preserving waste, which is composed largely of PAHs, as the cell line used in this study has also shown sensitivity to these compounds (Reeves et al., 2001). In addition, future development of this model shows promise not only for analysis of the toxicity of the contaminants, but also to evaluate the extent of remediation at facilities contaminated with wood preserving waste. For instance, a noticeable decrease in pH or GSH in Clone 9 cells exposed to a particular mixture may suggest a need for continued remediation, even though levels of a particular model chemical, such as C_5P, may be deemed sufficiently low by accepted analytical standards.

In response to worldwide concern over the presence of persistent chemicals in groundwater and the toxic biological effects observed in *in vitro* model systems, it becomes necessary to focus on applied research in order to ensure the safety of drinking water supplies heavily relied upon by the general public. The results of the first objective suggest the use of *in vitro* methodology to identify specific CP congeners in a complex mixture in order to either evaluate the extent of remediation efforts or design

remediation technology in the field. However, this method is neither sufficiently developed for the resources available at most on-site facilities nor is it economically feasible to continuously ship samples to a laboratory for analysis. Thus, the objective of the second study was to develop multi-functional sorbents for the comprehensive remediation of wood preserving waste contaminants from groundwater. One of the most common sorbents used in water remediation is granular activated carbon (GAC). Although GAC technology is a well-developed and widely utilized as part of a successful groundwater remediation strategy, it is most appropriate for use as a secondary polishing agent (USEPA, 1996). This is primarily because the adsorptive capacity and bedlife of GAC may be drastically decreased in the presence of organic matter in water (Hopman et al., 1994; Knappe et al., 1999). In contrast, many clay minerals have been shown to strongly sorb contaminants even in the presence of organic matter (Sheng et al., 2001). However, although naturally occurring clay minerals have been shown to strongly bind hydrophilic/polar organic contaminants from water with high capacity (Kishk et al., 1979; Huebner et al., 1999; Abollino et al., 2003), they do not effectively sorb most hydrophobic organic compounds like those present in wood preserving waste (Srinivasan and Fogler, 1990a; 1990b). Thus, interlayer cations in low pH montmorillonite clay were exchanged with cetylpyridinium to produce an organoclay (CP-LPHM) that would more effectively sorb hydrophobic contaminants, including PAHs and C_5P. In order to optimize hydraulic conductivity various amounts of CP-LPHM were bonded to either GAC or sand as a solid support using a carboxymethylcellulose adhesive and

characterized for their ability to effectively decrease contaminants in water both *in vitro* and *in situ*.

Laboratory studies utilizing a ^{14}C-labeled reconstituted aqueous phase (RAP) made from DNAPL collected from a creosote-contaminated Superfund site suggested that CP-LPHM/GAC composites formulated with the highest ratio of organoclay to GAC (3:2 CP-LPHM/GAC) showed the most promise for successful *in situ* remediation. A comparable sand composite (CP-LPHM/sand) was constructed to contain an equal amount of organoclay per unit volume to assess the contribution of the solid support matrix. Subsequent on-site elution of oil-water separator effluent through equal bed volumes of composite sorbents demonstrated that both 3:2 CP-LPHM/GAC and CP-LPHM/sand have high capacities for contaminants found in wood preserving waste, most notably higher molecular weight and carcinogenic PAHs. Further, studies testing the effects of different solid support matrices indicated that, although 3:2 CP-LPHM/GAC was somewhat more effective than CP-LPHM/sand for the removal of higher molecular weight PAHs, the use of GAC did not appear to provide a substantial added sorptive capacity over that of sand for composite formulation. This is an important consideration when developing a remediation strategy, as sand is more economically feasible.

Overall, both organoclay-based composites outlined in the study, as a pre-polishing step to GAC, may more effectively reduce the total contaminant load of PAHs, in particular carcinogenic PAHs, than GAC treatment alone. Future studies should examine the effectiveness of these sorbents in relation to those currently available for water remediation both for large-scale operations at restricted Superfund sites, but also

those commercially available for purchase by consumers. In this study, small amounts of composite materials were challenged with a tremendous contaminant load in order to attain breakthrough data necessary to determine sorbent capacity and bedlife. C_5P was present at a maximum of 2,332,529 ng/L, well above the maximum contaminant level (MCL) of 1,000 ng/L set by the U.S. EPA for drinking water (USEPA, 2002; IRIS, 2004). Studies should compare the effectiveness, capacity, and bedlife of commercially available carbon-based sorbents distributed for home use with a more economically feasible CP-LPHM/sand sorbent. In addition to smaller scale studies, large-scale applications that require larger bed volumes for filtration of greater elution volumes should also be conducted to explore possible co-solvent effects and contaminant interactions in a field-practical setting.

One important consideration concerning the use of organoclays is the potential for the quaternary amine to leach from the clay with time. The leached quaternary amine may exert toxicity upon microbial flora in a remediation approach that includes a biodegradation step subsequent to filtration. Thus, future studies should also explore ways to capture desorbed quaternary amine, as well as methods to monitor and prevent leaching. In particular, an in-line system consisting of a column of sand to separate particulate matter, followed by CP-LPHM/sand to sorb contaminants, then parent LPHM clay bonded to sand in order to regenerate the CP-LPHM/sand composite as CP is leached, and GAC as a final polishing step should be tested. This system would utilize the more economically feasible sand-based sorbent to remove the majority of contaminants, making it possible to limit the amount of GAC while building on the

sorbents strength as a secondary polishing agent. Additionally, the quality of drinking water would be protected with a column of parent clay bonded to sand to catch and re-exchange leached surfactant, while allowing the depleted organoclay-based sorbent to be replaced with new sorbent produced from that leached surfactant.

In addition to their use in water remediation by filtration, future studies concerning clay minerals will explore their inclusion in the diet for the sorption of contaminants ingested with food and water. Although PAHs are a major constituent of coal tar creosote and present in areas heavily polluted with wood-preserving waste, dietary exposure, estimated at 2 to 3 μg/day, is the most important non-occupational source of PAHs in non-smokers (Hatterman-Frey and Travis, 1994; Mumtaz et al 1996; Vyskocil et al., 2000). In particular, high levels of BaP are found primarily in very-well-done grilled or barbecued steaks, hamburgers, and chicken with skin (4 μg/kg), while in a variety of other food products BaP levels ranging from 0.09 to 30 μg/kg have been reported (Kazerouni et al., 2001).

A variety of naturally occurring silicate clay minerals have been added to animal feeds as enterosorbents in order to bind and reduce the bioavailability of mycotoxins (Smith, 1980, 1984; Phillips et al., 1995; Phillips, 1999). Phyllosilicate clays (at levels as low as 0.5% w/w in the diet) have been shown to effectively bind aflatoxins and prevent aflatoxicosis in multiple animal species (Phillips et al., 1988; Kubena et al., 1990). In addition, significant reductions in the production of aflatoxin M_1 in the milk of dairy cows (44%) and goats (52%) have been achieved with the addition of 1% clay in the diet (Harvey et al., 1991; Smith et al., 1994). Historically, clays have played a major

role in the human diet, particularly during pregnancy where they have been described as a common food craving (Geissler et al., 1999). However, several case studies suggest an association between clay ingestion and profound muscle weakness, anemia, and hypokalemia (Mengel et al., 1964; Gonzalez et al., 1982; Severance et al., 1988). It is important to evaluate the safety of clay minerals proposed for dietary addition as clays that sorb nonselectively may interact with nutrients, minerals, and other food borne chemicals to pose significant hidden risks, especially during prenatal development. Thus, the first step is to evaluate the safety of the parent clays themselves to ensure the absence of nonselective nutrient interactions or alterations to trace metal bioavailability prior to further studies utilizing organoclays.

In this study, a calcium montmorillonite clay (*NOVASIL PLUS*™, NSP) and a sodium montmorillonite clay (Swy-2) were supplemented into the balanced diet of Sprague-Dawley rats during pregnancy at a level of 2% (w/w), nearly 4 times that necessary to sorb aflatoxins and prevent aflatoxicosis in previous studies (Phillips et al., 1988; Kubena et al., 1990). Animals supplemented with either clay mineral were similar to controls with respect to toxicity evaluations (i.e., maternal body weights, maternal feed intakes, litter weights, and embryonic resorptions) and metal analysis of selected tissues (i.e., liver, kidneys, tibia, brain, uterus, pooled placentas, and pooled embryonic mass), with the exception of decreased brain Rb among rats consuming clay. Averages of raw Rb concentrations were 11.3 ± 0.1 mg/kg for control, 10.8 ± 0.2 mg/kg for NSP, and 10.7 ± 0.1 mg/kg for Swy-2. The essentiality of Rb has been tested in goats, which exhibited decreased food intake, growth, and life expectancy, and increased spontaneous

abortions following ingestion of Rb-deficient diets (Anke et al., 1997). However, additional studies are lacking and animal and human data concerning this ultra trace element are both limited and controversial, making extrapolations with regard to the decreased Rb observed here inappropriate.

Overall, the results of this study suggest that neither NSP nor Swy-2, at relatively high dietary concentrations and based on current knowledge, influence mineral uptake or utilization in the pregnant rat. However, future studies should be carried out in order to confirm the present findings and determine the effects of chronic ingestion, especially in the case of brain Rb. In addition, the effect on specific vitamins and minerals as well as interactions with the function of critical enzymes and hormones should be evaluated. For instance, the surfaces of kaolinite and illite and the interlayer spaces of montmorillonite have been shown to sorb 17β-estradiol from aqueous solution (van Emmerik et al., 2003). Those clays that do not show significant nonselective nutrient interactions or exert overt toxicity in *in vivo* models may be further evaluated for safety and the potential for contaminant sorption from both food and water as a component of the human diet.

In an extension of the same study, both NSP and Swy-2 were tested for their effectiveness to sorb Pb^{2+} *in vivo*. Previous research has shown naturally-occurring sodium montmorillonite to decrease Cd, Cr, Cu, Mn, Ni, Pb, and Zn in aqueous solution (Ake et al., 2001; Abollino et al., 2003). Studies specific to Pb^{2+} indicated that, of a variety of clay minerals screened, Swy-2 showed the highest average sorption from aqueous solution (Ake et al., 2001). In order to test this hypothesis *in vivo*, clay minerals

were added to the diets of Sprague-Dawley rats at 2% (w/w). After confirmation of successful mating (day 0), rats were also gavaged with aqueous solution containing either 0.25% clay + sodium acetate, 0.25% clay + lead acetate, sodium acetate alone, or lead acetate alone once per day from gestation days 1 to 15. On day 16 of pregnancy rats were euthanized, and tissues were collected. Additionally, blood was collected on days 1 and 16. Pb^{2+} was quantified in blood samples by atomic absorption but, the results were largely inconclusive. Therefore, in the interest of avoiding the inclusion of incomplete or unclear data, this study has not been presented. Further studies are warranted to provide more conclusive results as to the effectiveness of Swy-2 for metal sorption *in vivo*.

Once the parent clay minerals are evaluated for safety and effectiveness *in vivo*, organoclay minerals that may more effectively sorb organophilic compounds, including PAHs, should be tested. Organophilic compounds such as PAHs are more effectively sorbed into a hydrophobic interlayer produced by exchanging naturally occurring hydrated metal ions with organic cations (Srinivasan and Fogler, 1990a, 1990b). The hydrophobicity of the clay platelet's interlayer may be regulated by exchanging surfactant into the clay as a fraction of the native clay's CEC value (Greenland and Quirk, 1960; Zhang et al., 1993; Xu and Boyd, 1995). However, previous studies have shown that selected organoclays made by exchanging the surfactants at levels equal to or greater than the maximum CEC were very effective in *in vitro* isothermal analysis experiments but toxic to *Hydra attenuata* in an Adult Hydra Bioassay (Afriyie-Gyawu et al., 2004). Therefore, future studies should determine the fraction of the CEC necessary to promote sorption of the desired contaminant but not alter the relative safety of the

ingested mineral. In addition, the structure of the specific surfactant molecule may play a role in hydrophobicity and toxicity, thus, different surfactants should also be evaluated.

The presence and persistence of pesticides and other chemicals in drinking water justifies the need for comprehensive analytical methodology that can be used to evaluate the toxicity of complex mixtures, especially when the interactions of individual components are unclear. In addition, the effectiveness of sorbents used for water filtration and the safety of those proposed as dietary supplements must be ensured. This research proposes an economically feasible water remediation media and a method with which to determine both the extent of contamination prior to remediation and to verify its completion. Further, the first step in studies designed to evaluate the addition of clay-based sorbents to the diet in order to protect from ingested contaminants without altering mineral uptake or utilization has been conducted in test animals.

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Related Publications

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