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Effects of Creosote-Contaminated Sediment Extracts on Mouse Macrophage Physiology and Function

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EFFECTS OF CREOSOTE-CONTAMINATED SEDIMENT EXTRACTS ON MOUSE
MACROPHAGE PHYSIOLOGY AND FUNCTION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

by
Rayna A. Silva
May 2014

Accepted by:
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ABSTRACT

The southern branch of the Elizabeth River, at Portsmouth Virginia is one of the most polluted systems in North America. This harbor estuary system is also home to the Atlantic Wood Superfund site, which is heavily contaminated with creosote from the Atlantic Wood (AW) preservative company that ceased production in the later 1990s. Creosote is a mixture of polyaromatic hydrocarbons (PAHs), metals, and numerous aliphatic hydrocarbons, and well known to be carcinogenic. The toxicity of sediments and pore waters from the AW site has been studied extensively using *Fundulus heteroclitus*, also known as the mummichog, or Atlantic killifish. Most adult killifish from AW have hepatic lesions, ranging from focal hyperplasia to highly malignant tumors. As embryos and juveniles, these fish are resistant to developmental toxicity of PAHs relative to a reference population on King's Creek (KC) located near Mobjack Bay on the York River. Moreover, the resistance of AW fish to developmental toxicity is associated with altered aryl-hydrocarbon receptor (AhR) functions and signaling, leading to recalcitrance in CYP1A induction by PAHs. To determine the potential of AW sediment pore water to modulate immune functions in mammals, this study used the mouse macrophage cell line RAW264.7 as a model for pro-inflammatory functions. Compared to KC sediment pore water, AW pore water induces more iNOS and COX-2 protein expression, nitric oxide and IL-6 secretion, in RAW264.7 cells. This enhanced pro-inflammatory property of AW extracts is due, in large part, to high levels of endotoxin, as polymyxin-B ameliorates this property. Moreover, AW sediment extracts have quantitatively higher endotoxin as measures by the limulus lysate assay.

DEDICATION

I dedicate this work to you, Mom.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Charles D. Rice for all the advice and guidance he has given throughout my research. I would also like to thank my lab members who have helped and supported me throughout my time in lab.

Finally, I would like to thank my friends and family. Without your love and support, I would not be where I am today. In particular, I would like to thank my parents for caring and allowing me to pursue everything I want out of life.

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CHAPTER ONE
LITERATURE REVIEW

CREOSOTE

The Elizabeth River, an estuary of the Chesapeake Bay near Portsmouth, VA is known for the EPA superfund site, Atlantic Woods Industries. Atlantic Woods Industries is a former wood treatment plant located on the southern most branch of the river. In operation from 1926 to 1992, it is known for its use of creosote in the wood-preserving process. Creosote consists of a mixture of more than 200 chemical compounds, 85% of which are polycyclic aromatic hydrocarbons (PAHs) (Mueller, *et al.* 1989). Since there are no known sources of creosote in nature, contamination is a direct consequence of anthropogenic actions. Use of creosote in wood-preserving processes inevitably exposes the environment to hazardous chemical contamination. Treatment processes, wastewater effluents, improper disposal of waste material, chemical mismanagement, and accidental releases are just some examples of releases into the environment. For these reasons, creosote released into the Elizabeth River from the Atlantic Wood superfund site results in one of the highest levels of PAHs in the U.S. (up to 2200 mg/kg dry weight) (Bieri, *et al.* 1986). Once released, hazardous waste sludge can form at sediments in contaminated waters. It has been estimated that functioning wood-treatment plants produced 1,000 metric sludge/year in the 1990s (Davis, *et al.* 1993, Mueller, *et al.* 1989). In particular, Atlantic Wood sediments are highly toxic, which is demonstrated by Ownby *et al.*, where 100% of reference population fish died when exposed to as low as 25% of sediment extracts (Ownby, *et al.* 2002).

With creosote being released into the environment, the health effects have been extensively studied since the 1920s. One of the first studies published was a case study by A.H. Cookson, commenting on the tumor-inducing effects of prolonged occupational exposure to creosote (H. A. Cookson 1924). Numerous other case studies describe cancer development after chronic exposure to creosote (Carlsten, *et al.* 2005, Karlehagen, *et al.* 1992, Moulin, *et al.* 1993, Shimauchi, *et al.* 2000). Exposure routes for humans include inhalation and dermal exposure, increasing occupational hazards for individuals working with creosote. Studies by Van Rooij *et al.*, Klinger and McCorkle, and Malkin *et al.*, determined dermal exposure results in an increased creosote body burden, compared to inhalation exposures (Klingner and McCorkle 1994, Malkin, *et al.* 1996, Van Rooij, *et al.* 1993). More recently, studies have used 1-hydroxypyrene, a metabolite of the PAH pyrene, as a biomarker for PAH, and more generally, creosote exposures (Elovaara, *et al.* 1995, Zhang, *et al.* 2001, Zhao, *et al.* 1990). This allows employers to monitor employee health; especially in occupations where individuals are most at risk of creosote and PAH exposure. In addition to cancer-causing effects in humans, several studies have characterized the carcinogenic properties of creosote in mouse and other small rodent models (Boutwell and Bosch 1958, Kammer and Poel 1957, Lijinsky, *et al.* 1957).

ATLANTIC WOOD TOXICITY AND MARINE ORGANISMS

Since the closure of the Atlantic Wood Industries in 1992, various studies focused on the effects of creosote and PAH contamination on marine organisms. One such model organism is the estuarine killifish, *Fundulus heteroclitus*, found along the Atlantic coast

from Newfoundland, Canada to northern Florida. Also known as the mummichog, *F. heteroclitus* has high home-range fidelity, remaining in the same area for the duration of its life cycle. Thus, environmental conditions can be assessed through mummichog toxicity studies. Several studies have characterized behavioral responses in killifish after contaminant exposure (Fingerman and Russell 1980, Timme-Laragy, *et al.* 2006, Weis, *et al.* 2001, Weis, *et al.* 2011). In particular, mummichogs exhibit decreased predation, changes in diet, and altered activity and locomotion, yet still remain reproductively viable. Tolerance studies revealed *F. heteroclitus* is able to acclimate and thrive in environments contaminated with PAHs (Meyer, *et al.* 2002, Ownby, *et al.* 2002, Van Veld and Westbrook 1995), polychlorinated biphenyls (PCBs) (Nacci, *et al.* 1999, Nacci, *et al.* 2002), heavy metals (Weis and Weis 1989), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) (Prince and Cooper 1995a, Prince and Cooper 1995b).

However, studies have shown fish exposed to contaminants have increased liver lesions (Vogelbein, *et al.* 1990a) and altered enzymatic activities. Specifically, killifish from the Atlantic Wood site have increased glutathione reductase and glutathione peroxidase activities compared to reference populations (Bacanskas, *et al.* 2004). Cytochrome P450 1A (CYP1A), which is part of the AhR pathway and involved in the breakdown of many harmful contaminants, has been shown to decrease in populations from highly contaminated sites (Meyer, *et al.* 2002, Meyer, *et al.* 2003b, Van Veld and Westbrook 1995). Fish exposed to the sediments from the Elizabeth River also exhibited increased glutathione concentrations and glutathione reductase levels, as well as

increased antioxidant concentrations in larval fish, all indicative of immediate and heritable responses in sediment-exposed fish (Meyer, *et al.* 2003a). Other studies investigated maternal effects and genotoxicity resulting from sediment exposure (Jung, *et al.* 2011, Nye, *et al.* 2007).

Numerous studies have characterized the effects of PAHs on fish immunity and immune responses (Bols, *et al.* 2001, Carlson, *et al.* 2004a, Carlson, *et al.* 2004b, Reynaud and Deschaux 2006, Rice and Arkoosh 2002, White and Holsapple 1984). In Atlantic Wood populations, Kelly-Reay *et al.* found that macrophages produced higher levels of reactive oxygen intermediates (ROIs) compared to reference fish, and production of ROIs increased when stimulated by synthetic chemicals targeted to induce oxidation (Kelly-Reay and Weeks-Perkins 1994). Weeks and Warinner found decreased phagocytic ability of macrophages collected from two species of fish exposed to the Elizabeth River compared to reference populations (Weeks and Warinner 1984). A more recent study described immune specific changes in Atlantic Wood-exposed mummichogs, including reduced levels of circulating total and bacteria-specific IgM, increased levels of circulating lysozyme, even higher levels of lymphoid lysozyme, and elevated cyclooxygenase-2 (COX-2) expression (Frederick, *et al.* 2007).

INFLAMMATION AND IMMUNE RESPONSES

Inflammation is a key indicator of immune system activation and responses. Initial stages include recruitment of immune cells, such as neutrophils and macrophages, to the site of injury and initiation of inflammatory indicators (pain, swelling, redness, and

heat). Macrophages are differentiated tissue-specific immune cells derived from blood monocytes, and are able to phagocytize foreign material. These cells secrete cytokines, protein molecules with various immune functions, in response to stimuli. Pro-inflammatory cytokines such as IL-1, IL-6, TNF- α , IL-8, and IL-12 are continuously released during an immune response. Studies have shown anti-inflammatory cytokines such as IL-10 inhibit pro-inflammatory markers from inducing inflammation (Fiorentino, *et al.* 1991, Moore, *et al.* 2001, Niiro, *et al.* 1995, Niiro, *et al.* 1994). While acute inflammation is beneficial to host systems, chronic inflammation has proven to be detrimental to the body. Studies have linked chronic IL-6 secretion and inflammation to several types of cancer (Chung, *et al.* 1999, Chung and Chang 2003, KozÅ,owski, *et al.* 2003, Lou, *et al.* 2000), Crohn's disease (Atreya, *et al.* 2000, Bross, *et al.* 1996, Gross, *et al.* 1992, Niederau, *et al.* 1997), and rheumatoid arthritis (Cronstein 2007, Partsch, *et al.* 1997, Ueki, *et al.* 1996).

Another pro-inflammatory bio-indicator is cyclooxygenase-2 (COX-2) expression. COX-2 is an inducible enzyme responsible for converting arachidonic acid into prostaglandin H₂ (PGH₂). The resulting prostaglandins (D₂, E₂, F_{2a}, I₂, and TXA₂), involved in various normal cellular and physiological functions, are also associated with inflammatory responses such as pain, swelling, and fever (Greenhough, *et al.* 2009). Specifically, prostaglandin E₂ (PGE₂) is linked to inflammatory responses (Funk 2001). Yao *et al.* determined PGE₂ is involved in inflammation by promoting helper T cell differentiation (Yao, *et al.* 2009) and other studies have shown mast cell activation by PGE₂ (Nakayama, *et al.* 2006, Wang and Lau 2006). Interestingly, PGE₂ has also been

shown to secrete IL-10, exhibiting anti-inflammatory properties as well (Kalinski 2012, Wang, *et al.* 2007).

As part of an immune response to stimuli, activated macrophages also secrete higher than basal levels of nitric oxide through the inducible nitric oxide synthase (iNOS) pathway. iNOS converts L-arginine into the intermediate OH-L-arginine, which is further oxidized into NO and L-citrulline. Since iNOS is not constitutively expressed, it is only present in cells responding to stimuli, stress, or other cytokines. Studies have shown increased iNOS expression in a variety of cancers, including breast cancer (Vakkala, *et al.* 2000), lung cancer (Marrogi, *et al.* 2000), and prostate cancer (Aaltoma, *et al.* 2001, Uotila, *et al.* 2001).

LIPOPOLYSACCHARIDE

IL-6, iNOS, and COX-2 levels are all produced in response to external stimuli. One such stimulus is endotoxin (lipopolysaccharide, LPS), a component of gram-negative bacterial cell walls. LPS exerts its effect through the MD-2 and TLR4 pathway. LPS binds to LPS binding protein, which facilitates the binding of LPS to CD14. The LPS-LBP-CD14 complex then binds to the Toll like receptor 4 (TLR4) and MD-2 receptors on cell surfaces. Binding of LPS to TLR4-MD-2 receptor complex enables cell identification of LPS, which initiates signaling transduction pathways utilizing signal transduction adapter proteins (i.e. MyD88, TIRAP, TRAM, TRIF, SARM) (Lu, *et al.* 2008). Ultimately, the transcription factors NF- κ B and MAP kinases produce pro-inflammatory cytokines IL-1, TNF- α , IL-6, IL-12, and IL-8, concomitant with iNOS and

COX-2 induction (Chen, *et al.* 2013). Finally, studies have shown that LPS induced inflammatory responses can be inhibited through the use of the antibiotic, polymyxin-B (PMB) (Cavaillon and Haeffner-Cavaillon 1986, Mares, *et al.* 2009).

This study focuses on the immunological effects of Atlantic Wood pore water sediment extracts on the mammalian system. In particular, RAW264.7 cells were used to test the hypothesis that AW sediment extracts increase induction of innate immune indicators, such as iNOS, COX-2 expression, and IL-6 secretion, compared to cells treated with reference sediment extracts from King's Creek, VA. Immunological responses resulting from sediment extract treatment were compared to those produced when cells were treated with a known inflammatory stimulator, LPS. Outcomes from this study can potentially help characterize innate immunity effects on mammalian systems exposed to water and sediments from the contaminated Atlantic Wood site.

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CHAPTER TWO

EFFECTS OF CREOSOTE-CONTAMINATED SEDIMENT EXTRACTS ON MOUSE

MACROPHAGE PHYSIOLOGY AND FUNCTIONS

INTRODUCTION

Most harbor estuaries are heavily polluted from a long history of industrialization, military activity, and intense recreational use. One such estuary is the Elizabeth River near Portsmouth, Virginia, and the southern branch of the river is heavily contaminated with polycyclic aromatic hydrocarbons (PAHs), metals, organotins, and pentachlorophenol (PCP), stemming from several sources during the 1900s (Walker and Dickhut 2001). Non-point sources of pollution in the Elizabeth River come from the entire watershed, including the cities of Chesapeake, Norfolk, Portsmouth, and Virginia Beach (Conrad, *et al.* 2007).

One of the more polluted sites on the Elizabeth River is where Atlantic Wood, a creosote wood-preserving company, is located. It operated from 1926 to 1992, and is now a US EPA superfund site (EPA 2014). During the years in operation, creosote was released directly at the Atlantic Wood site and nearby habitats. Creosote consists of a mixture of over 200 chemical compounds with about 85% being PAHs, several of which are on the US EPA National Priority List (Mueller, *et al.* 1989) (World Health Organization, 2004). As a result of the creosote use at the Atlantic Wood site, PAH concentrations are as high as 2200 mg/kg dry weight, the highest levels in the US (Bieri, *et al.* 1986, Walker, *et al.* 2004). Many PAHs, and especially those on the US EPA NPL, are highly teratogenic, mutagenic, carcinogenic, and immunotoxic (Dix and Marnett,

1983), therefore sediments at the Atlantic Wood site are highly toxic, as was demonstrated by Ownby *et al* (2002). In their study, Ownby *et al* showed that Atlantic Wood sediments as low as 25% in mixture with reference sediments were lethal to 100 % of non-resident fish (reference population).

The toxicity of sediments and/or compounds therein from the Atlantic Wood site has been studied extensively using the Atlantic killifish, *Fundulus heteroclitus* (a.k.a. the mummichog), an estuarine killifish found along the Atlantic coast of the U.S. from Newfoundland, Canada to northern Florida. These high marsh fish have a high home-range fidelity, thus remaining in the same general area throughout the entire life cycle, and serve as key sentinels for environmental quality (Kneib 1986, Lotrich 1975). Several studies show that mummichogs are able to acclimate and thrive in estuarine waters highly contaminated with polychlorinated biphenyls (PCBs) (Nacci, *et al.* 1999, Nacci, *et al.* 2002), PAHs (Meyer, *et al.* 2002, Ownby, *et al.* 2002, Van Veld and Westbrook 1995), heavy metals (Weis and Weis 1989), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) (Prince and Cooper 1995a, Prince and Cooper 1995b). Although reproductively viable, mummichogs exposed to the Elizabeth River sediments *in situ* have a high incidence of liver lesions and tumors (Vogelbein, *et al.* 1990b). Furthermore, killifish from the Atlantic Wood site have exhibited increased glutathione reductase and glutathione peroxidase activities (Bacanskas, *et al.* 2004), as well as decreased cytochrome P450 1a (CYP1A) induction (Meyer, *et al.* 2003b, Van Veld and Westbrook 1995) compared to reference fish, indicating a level of acquired resistance or adaptation in creosote-exposed fish. One study in particular showed that

embryonic killifish from Atlantic Wood site were completely resistant to the teratogenic effects of sediments from that site, while reference fish were 100 % susceptible at only 25% sediment in mixtures (Ownby, *et al.* 2002).

The immune system of both fish and mammals is particularly sensitive to PAHs (Bols, *et al.* 2001, Carlson, *et al.* 2004a, Carlson, *et al.* 2004b, Reynaud and Deschaux 2006, Rice and Arkoosh 2002, White and Holsapple 1984), yet surprisingly little is known about the immune system of fish exposed to sediments from the Atlantic Wood superfund site. One study found that killifish from this site had a higher than basal level of macrophage produced reactive oxygen intermediates (ROI), and produced more ROI when stimulated with pharmacological initiators of the oxidative burst event associated with NADPH-oxidase system (Kelly-Reay and Weeks-Perkins 1994). A more recent study found decreased levels of circulating total and bacteria-specific IgM, increased levels of circulating lysozyme, higher levels of lymphoid lysozyme, and elevated cyclooxygenase-2 (COX-2) expression in mummichogs collected from the Elizabeth River (Frederick, *et al.* 2007). Taken together, these two studies suggest that innate immune functions in killifish from the Atlantic Wood site are elevated compared to reference fish, and perhaps that the ability to maintain antibody responses is compromised.

To date, however, the sediments from the Atlantic Wood site have not been examined in a mammalian system. Toxic PAHs found in Atlantic Wood sediments, like benzo-*a*-pyrene (BAP) and others on the US EPA NPL, are well known immunotoxic compounds in rodent models. In mice, PAHs have been shown to suppress antibody

responses, decrease natural killer cell activity, and inhibit tumor suppression (Dean, *et al.* 1986). Furthermore, Burchiel *et al* and Davis *et al* showed PAH immunotoxicity in mouse spleen cells (Burchiel, *et al.* 1988, Davis, *et al.* 1991). Finally, studies have shown increased apoptosis in murine macrophage cell line, RAW264.7, exposed to PAHs (Kafoury 2013). Thus, previous studies have shown murine cell lines, including RAW264.7 cells, respond to PAHs from an immune system perspective.

Based on observations from killifish at the Atlantic site, this study tests the hypothesis that sediment pore waters from this site are immunotoxic in a mammalian system. Sediment pore water extract studies can provide insight to the toxicity and bioavailability of contaminants at a particular site. Since previous studies show that fish from the Atlantic Wood site have elevated markers of innate immunity, the study described herein utilizes the mouse macrophage cell line RAW264.7, a common model for studying macrophage physiology and functions, including immunopharmacology (Babcock, *et al.* 2013, Denlinger, *et al.* 1996). Because of their wide tissue distribution, macrophages are strategically located to provide an immediate defense against not only pathogens, but damaged tissues as well, which can lead to inflammatory responses to endogenous danger signals. RAW264.7 cells respond well to TLR ligands, express high levels of lysozyme, are phagocytic, and secrete a cytokine profile comparable to freshly isolated mouse macrophages (Kurasawa, *et al.* 1996, Raschke, *et al.* 1978). These cells are especially responsive to lipopolysaccharide (LPS), a gram-negative bacterial cell wall product that induces classical pro-inflammatory profiles mediated through TLR-4 (Akira, *et al.* 2001, Kawai, *et al.* 1999, Ulevitch and Tobias 1999). Free LPS binds to the CD14

receptor, creating a LPS-CD14 complex, which then binds to toll like receptor 4 (TLR-4) and MD-2, with subsequent activation of intracellular signaling. The ultimate signaling transcription factor responding through TLR-4 is NF- κ B (RelA, p65) and MAP-kinases (Chen, *et al.* 2013), with high levels of IL-1, TNF- α , IL-6, IL-12, and IL-8 being the classical pro-inflammatory cytokines, concomitant with iNOS and COX-2 induction.

Inducible nitric oxide synthase (iNOS) converts L-arginine into the intermediate OH-L-arginine, which is further oxidized into NO and L-citrulline (Marletta 1994).

Nitric oxide is an especially anti-microbial ROI in combination with superoxide anion also produced by activated phagocytes (Nathan and Hibbs Jr 1991). The byproduct peroxynitrite is more potent than superoxide anion and nitric oxide alone (Jourdeuil, *et al.* 2001). COX-2 is an inducible enzyme involved in innate immune responses of all vertebrates by being the prostaglandin H₂ synthase (PGH₂), which is further metabolized to several other prostaglandins (Chen, *et al.* 2013, Griswold and Adams 1996).

Prostaglandins and their metabolites have numerous functions, including vasodilatation and pain signaling (Chen, *et al.* 2013). Thus, both iNOS and COX-2 are considered to be bio-indicators of inflammatory responses in macrophages.

In addition to COX-2 and iNOS, cytokines such as IL-6 and IL-10 act as regulators of inflammation. IL-6 is typically known as a pro-inflammatory cytokine, produced by numerous cells types, including macrophages and monocytes (Gabay 2006). On the other hand, IL-10, usually considered an anti-inflammatory cytokine, inhibits inflammatory responses and macrophage functions such as cytokine production and NO production (Moore, *et al.* 2001). Specifically, IL-10 reduces IL-6 secretion and down

regulates COX-2 expression (Fiorentino, *et al.* 1991, Moore, *et al.* 2001, Niiro, *et al.* 1995, Niiro, *et al.* 1994).

This study examines the potential immunotoxicity of Atlantic Wood sediment extracts using RAW264.7 cells as an *in vitro* model. To date, no other study has looked at the effects of Atlantic Wood sediment extracts on mammalian cells, and especially in terms of any potential anti- or pro-inflammatory properties

MATERIALS AND METHODS

Cell culture.

Murine macrophage cell line RAW 264.7 was obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; CellGro Mediatech) supplemented with 10% iron-supplemented fetal calf serum (FCS; HyClone Thermo), 20 mM HEPES (pH 7.5), 10 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1.5 g/l NaHCO₃ (each from Sigma unless otherwise noted), and 1% non-essential amino acids (100x stock; HyClone Thermo). Hepa1c1c7 murine hepatoma cells were used to study CYP1A1 induction and the AhR pathway. Cells were obtained by ATCC (Manassas, VA, USA) and cultured in α -Minimum Essential Medium (MEM; CellGro Mediatech) supplemented with the same components as listed above.

Sediment extracts.

Sediments were a generous gift from Dr. Rich Di Giulio at Duke University, and prepared as previously described (Clark *et al.*, 2013). In brief, freshly collected sediments from the Atlantic Wood superfund site and from King's Creek on the York River near Mobjack bay were collected at low tide, and consisted of composite-pooling of several samples that were then mixed with deionized water (1:1 v/v, typically 25 ml of each), and shaken vigorously for about 1 min, followed by centrifugation at 10,000 x g. The overlying water was filter sterilized through a 0.45 µM filter, then again through a

0.20 μM sterile filter. The salinity of the mixture was the same as for tissue culture media. AW biofilm extract was also prepared in this manner.

Cell treatments

All cells were treated with dilutions of Atlantic Wood sediment extract (AWSE; 10%, 2.5%, 0.625%), dilutions of Kings Creek sediment extract (KCSE; 10%, 2.5%, 0.625%), 10 μM benzo-*a*-pyrene (BAP), and/or 0.1 $\mu\text{g}/\text{ml}$ LPS (UltraPure TLR-4 Ligand, Alexis Chemicals). LPS is a TLR-4 ligand on macrophages that specifically binds LPS to stimulate macrophage activation. The following combinations, AWSE or KCSE (10%, 2.5%, 0.625%) and LPS, AWSE or KCSE (10%, 2.5%, 0.625%) and BAP, LPS + BAP, unless otherwise noted. BAP treatments were used as an internal PAH for comparison to sediment extracts. Media alone without the above treatments served as the control.

Viability assay.

Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Cells were grown and plated to a concentration of 1×10^6 cells/ml in 96-well plates. Cells were treated with 0.1 $\mu\text{g}/\text{ml}$ LPS, and 20%, 10%, and 5% each of AWSE and KCSE. After 20 h, 20 μl MTT was added to all wells and incubated for four hours at 37°C with 5% CO_2 . After incubation, supernatants were aspirated off and 100 μl of acidified isopropanol (400 mL of 70% isopropyl alcohol, 1.4 ml of 12N hydrochloric acid) was added to each well. Cells and acidified isopropanol

were mixed with a pipette and shaken for 1 minute on plate reader. Plates were read at 550 nm.

iNOS assay.

iNOS assays were conducted to measure nitrite concentrations using a 1:1 ratio of NED (*N*-(1-naphthyl)ethylenediamine dihydrochloride; 0.1 g NED (Sigma) in 100 ml of dH₂O) and sulfanilamide (1.0 g sulfanilamide (Sigma) in 100 ml of 5% phosphoric acid). Cells were cultured in phenol red-free minimum essential media (MEM; Gibco) supplemented with 10% iron-supplemented fetal calf serum (FCS; HyClone Thermo), 20 mM HEPES (pH 7.5), 10 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1.5 g/l NaHCO₃ (each from Sigma), and 1% non-essential amino acids (100x stock; HyClone Thermo) and plated at 1 x 10⁶ cells/ml concentrations in 96-well plates. After treating with appropriate contaminants, cells were incubated for 24 hours in a 37°C with 5% CO₂ incubator. At 24 h, 100 µl of supernatants were pipetted into a new 96-well plate and 100 µl of the 1:1 NED-sulfanilamide solution was added to all supernatants. A 200 µM serial dilution of NaNO₂ was used as a standard. After 15 minutes incubation the plates were read at 550 nm.

ELISA Assays.

Elisa assays were performed using ELISA MAXTM Deluxe Sets mouse IL-10 (BioLegend) and Mouse IL-6 ELISA Ready-SET-Go!® (eBioscience) kits. Protocols were followed as described in the kits. Briefly, 96-well plates were coated overnight with

capture antibody at 4°C, washed 4x with wash buffer (PBS; 0.05% Tween-20), incubated with 1x Assay Diluent A for one hour at room temperature (RT) with shaking, washed 4x with wash buffer, incubated with treatment supernatants and standards overnight at 4°C, washed 4x with wash buffer, incubated with detection antibody for one hour at RT with shaking, washed 4x with wash buffer, incubated with Avidin-HRP for 30 minutes at RT with shaking, washed 4x with wash buffer, incubated with TMB substrate solution for 20 minutes at RT in the dark (IL-10) or 15 minutes (IL-6), and stopped with a 2N H₂SO₄ stop solution. Plates were read at 450 nm and 570 nm.

Protein determinations.

Cells were cultured in phenol red-free MEM, plated to a concentration of 1 x 10⁶ cells/mL in 6-well plates, and treated for 24 h. Supernatants were then collected and stored at -20°C for future experiments. Cells were lysed using 300 µl RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with a cocktail of protease inhibitors (2 µg/ml aprotin; 2 µg/ml leupeptin; 1 µg/ml pepstatin; 100 µg/ml phenylmethylsulfonyl fluoride, PMSF; 1:1000 chloromethyl HCl). Samples were centrifuged at 14,000 rpm for 10 minutes. Supernatants were collected and protein concentrations determined using Pierce® BCA Protein Assay Kit.

Immunoblotting.

Samples were normalized for protein concentration and subjected to SDS-PAGE, then transferred overnight (35 V at 4°C) onto a PVDF membrane. The membrane was

blocked for 1 hour using 10% FBS in PBS, incubated in primary antibody for 1 h, washed (3x, 5 minutes) with 0.05% Tween-20 in PBS, and incubated with secondary antibody for 1 h. After washing (3x, 5 minutes) with 0.05% Tween-20 in PBS again, alkaline phosphatase activity was visualized using a substrate solution of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt). Primary antibodies used were goat anti-COX-2 antibody (C-20; Santa Cruz Biotechnology) at 1:750, rabbit anti-mouse iNOS (#610333; BD Transduction Labs) at 1:500, and anti-mouse β -actin (mAb AC-74; Sigma Chemical Company). Secondary antibodies were rabbit anti-goat IgGm, goat anti-rabbit IgG, and goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (SouthernBiotech) diluted 1:1000 in PBS-tw20 containing 1% FCS.

Oxidative stress determination.

RAW264.7 and HEPA1c1c7 were compared for their relative sensitivity to potential oxidative stress associated with exposure to the treatments. Cells were seeded to 10^5 cells/well and 5×10^4 cells/well, respectively, in black/clear bottom 96-well plates, (CostarTM). Cells were then treated for 23.5 h and the general oxidative stress (via H_2O_2 production) probe carboxy- H_2DCFDA was added to all treated wells, at a final concentration of 10 μM . After 30 minutes (total of 24 h exposure with treatments), plates were read at 494 nm excitation and 522 nm emission wavelengths.

Phagocytosis assay.

RAW264.7 cells were seeded to 5×10^5 cells/ml in 96-well black/clear bottom plates (Costa™) and treated for 24 h as previously described (Babcock, *et al.* 2013). Briefly, at 24 h treatment, 1 μm Fluoresbrite® YG microspheres (Polysciences) were diluted in 50 mM Na_2HPO_4 and added to the cells at a ratio of 100:1 (beads:cells) for a total well volume of 200 μl . Beads and cells were incubated for 90 minutes at 37°C. Phagocytosis was stopped with 3 washes of 100 μl a cold PBS solution (PBS, 1 mM CaCl_2 , 1 mM MgCl_2). One hundred μl trypan blue solution (2 mg/ml trypan blue, 20 mM citrate, 150 mM NaCl; pH 4.5) were added to each well to quench extracellular fluorescence. Plates were read at an excitation and emission wavelength of 441 nm and 486 nm.

Photographic images.

Cells were seeded in 96-well plates at 5×10^5 cells/ml and treated for 24 h. Images were taken using an Olympus CK2 microscope and Canon EOS Rebel T2i camera under 200x and 100x magnification for RAW264.7 cells and Hepa1c1c7 cells, respectively.

NO production via AW biofilm exposures.

RAW264.7 cells were cultured in phenol red-free MEM, seeded in 96-well plates at 1×10^6 cells/ml, and treated with a serial dilution of AW biofilm (20% to 0.3125%; 0% acts as a control) for 24 h. An iNOS assay was carried out as described above. Briefly, a

1:1 ratio of NED:sulfanilamide (100 µl) was added to 100 µl of supernatants from treated RAW264.7. A 200 µM serial dilution of NaNO₂ was used as a standard. After 15 minutes incubation the plates were read at 550 nm.

Endotoxin quantitation via Limulus Amebocyte Lysate (LAL) assay.

Endotoxin levels in AWSE and KCSE were quantitated using Pierce ® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) following directions provided by the manufacturer. Briefly, 50 µl of sample and standard was added to a 96-well plate and incubated at 37°C for 10 minutes. LAL and a chromogenic peptide substrate were added to each well and samples were read using a spectrophotometer at 405 nm. *E. coli* endotoxin (derived from *E. coli* strain O111:B4) was used as a standard. Samples and standards were done in duplicate.

RESULTS

Cell images and viability assay.

Viability of treated RAW264.7 and Hepa1c1c7 cells was assessed through the MTT viability assay. There were no significant differences found between untreated RAW264.7 cells and cells treated with any treatment (Figure 1A). There was a significant increase in viability in Hepa1c1c7 treated with 20% AWSE and 10% AWSE compared to KCSE (Figure 2A). Images were taken of RAW264.7 and Hepa1c1c7 cells treated for 24 h with BAP, sediment extracts (10%, 0.625%) and sediment extracts with BAP (10%, 0.625%) (Figures 1B and 2B, respectively).

iNOS assay and NO production.

Macrophage activation in the presence or absence of LPS was studied by examining NO production through the Griess-reagent assay. Results demonstrate that AWSE contain more iNOS inducing stimulants than do KCSE, and this can be seen very clearly at the lower concentrations of extracts. However, both sediment preparations contain stimulants to the point that at higher concentrations the responses are the same. The addition of BAP did not change the results, as 2.5% AWSE+BAP and 0.625% AWSE+BAP were also significantly increased compared to reference samples (Figure 3). Of particular note, the levels of NO produced by cells exposed to extracts at higher concentrations were comparable to cells treated with LPS, the model TLR-4 ligand.

Figure 1

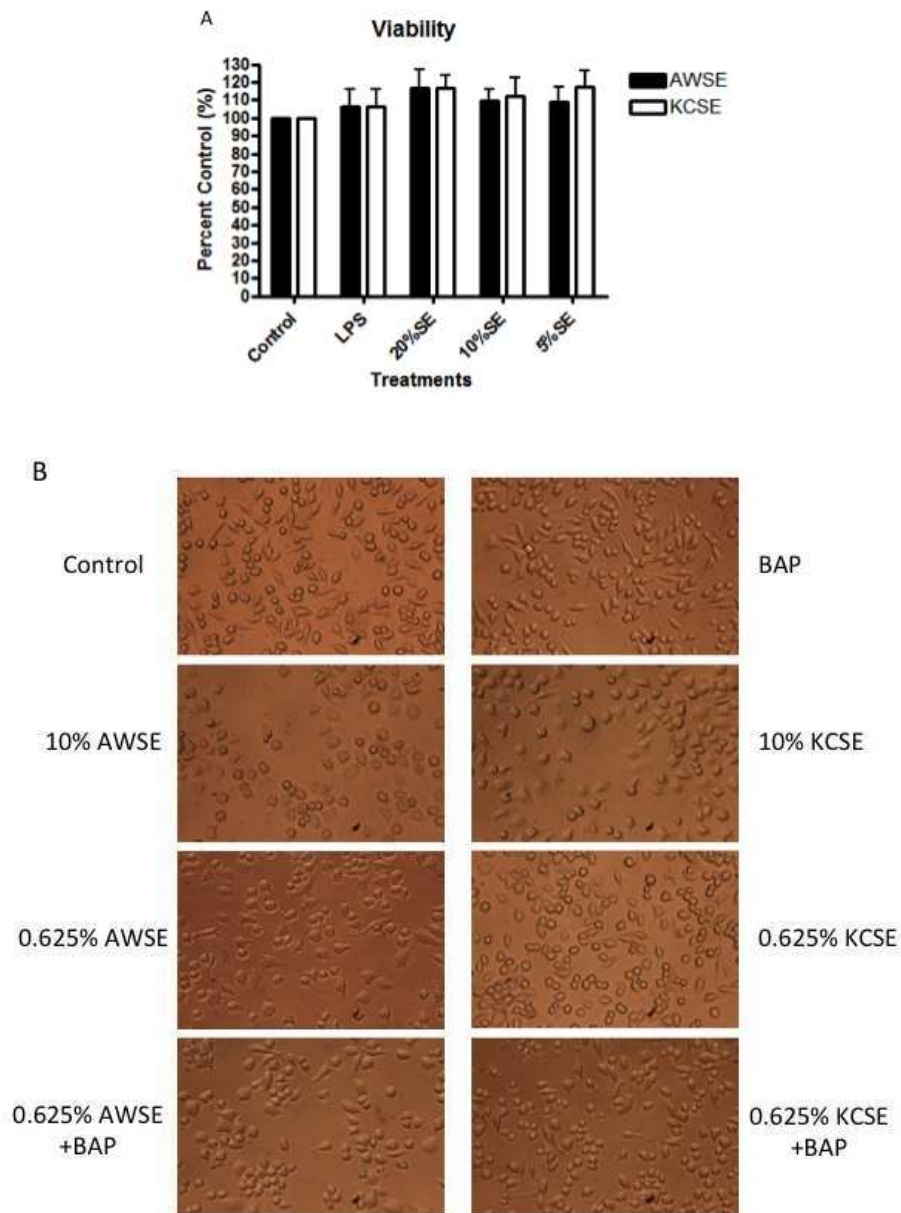


Figure 1. Cell images and viability assessment. A. Viability of 24 h AWSE-, KCSE-, and LPS-treated RAW264.7 cells. Data represents mean \pm standard error of $n=3$ individual experiments (no significance observed). B. Cell images of treated RAW264.7 cells at 200x magnification.

Figure 2

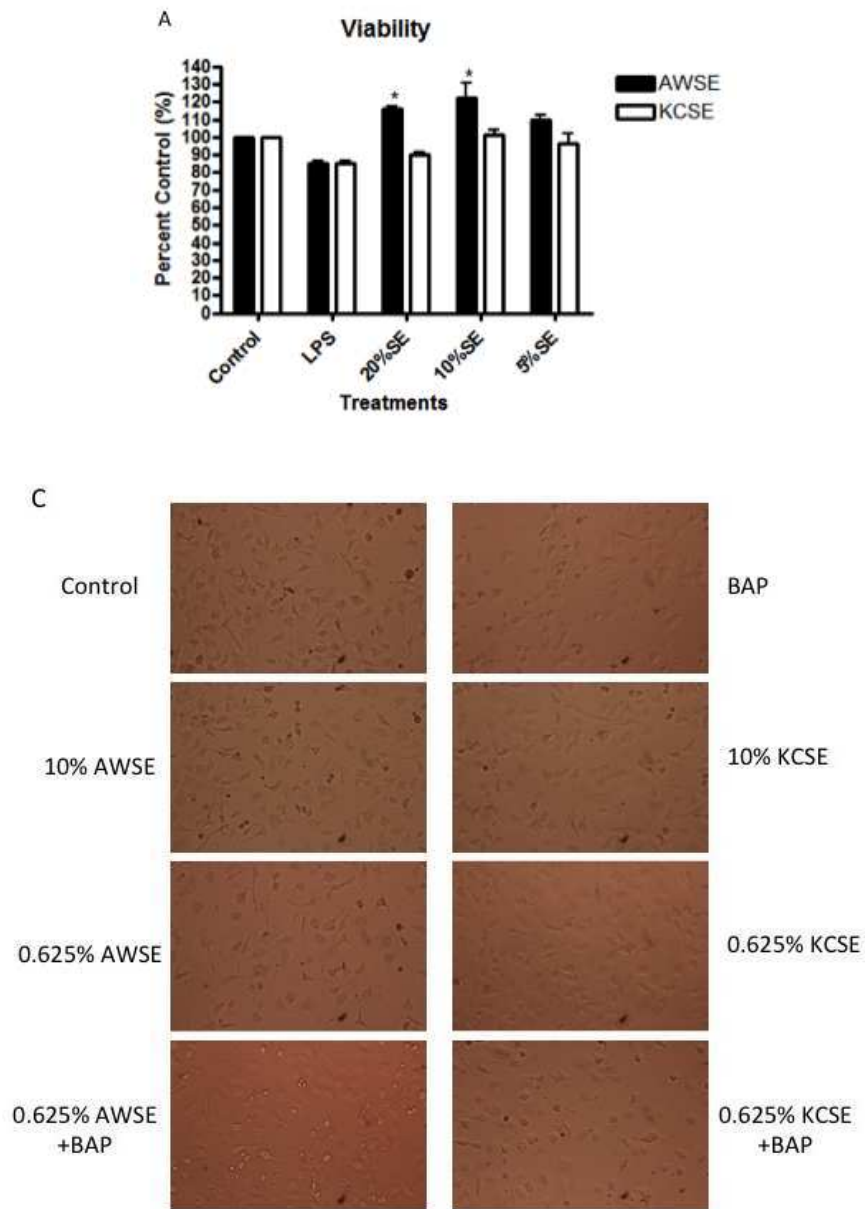


Figure 2. Cell images and viability assessment. A. Viability of 24 h AWSE-, KCSE-, and LPS-treated Hepa1c1c7 cells. Data represents mean \pm standard error of n=3 individual experiments (*represents significance $p < 0.05$; represents differences between SE exposures). B. Cell images of treated Hepa1c1c7 cells at 100x magnification.

Figure 3

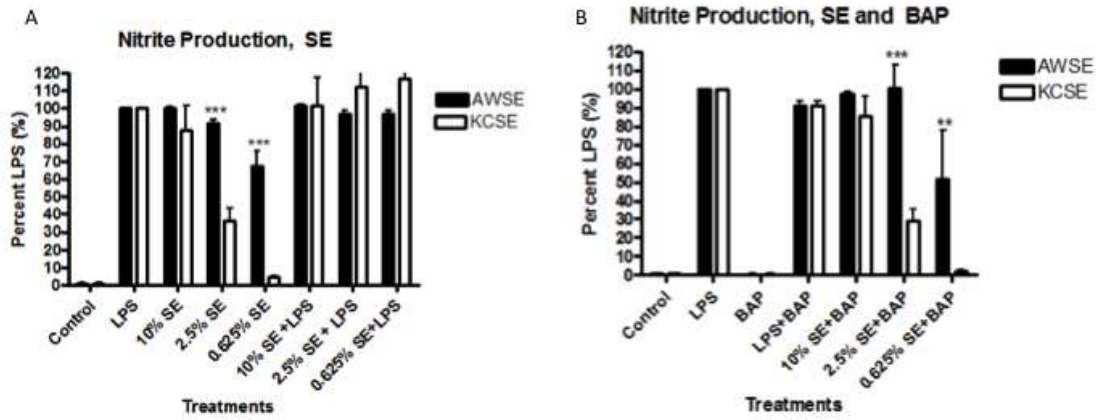


Figure 3. Nitric oxide production as a percentage of LPS. A. RAW264.7 macrophages treated for 24 h with AWSE, KCSE, LPS, and SE+LPS. B. AWSE-, KCSE-, LPS-, BAP-, LPS+BAP-, and SE+BAP-treated RAW264.7 cells. Data represent mean \pm standard error of n=3 individual experiments (** represents significance $p < 0.01$; *** represents significance $p < 0.001$; represents differences between SE exposures).

Protein expression and immunoblotting.

The effects on pro-inflammatory bioindicators iNOS and COX-2 protein expression were examined through SDS-PAGE/immunoblotting analysis (Figures 4 and 5). The levels of iNOS protein reflect the amount of NO produced as shown in Figure 2. iNOS protein expression in cells treated with the lowest concentration of KCSE was virtually absent, but at this same concentration AWSE still induced this protein. BAP treatments had no effect on the outcome (Figure 4). The expression of COX-2 protein following treatments with LPS, sediment extracts, and BAP was the same as seen for iNOS (Figure 5)

IL-6 and IL-10 cytokine secretion.

To determine if cytokines indicative of immune responses were being secreted in response to LPS and sediment extract treatments, ELISA assays specific for IL-6 and IL-10 secretion were conducted. As expected, LPS stimulated RAW cells produce high levels of IL-6, and only modest levels of IL-10 (Figure 6). Unexpectedly, AWSE was very potent in inducing IL-6 secretion, and much more so than KCSE, which stimulated secretion of IL-6 comparable to that of LPS alone. Likewise, IL-10 secretion was stimulated by AWSE (only 10% dilution) above what was measured in LPS-treated cells. BAP treatments did not affect cytokine secretion.

Figure 4

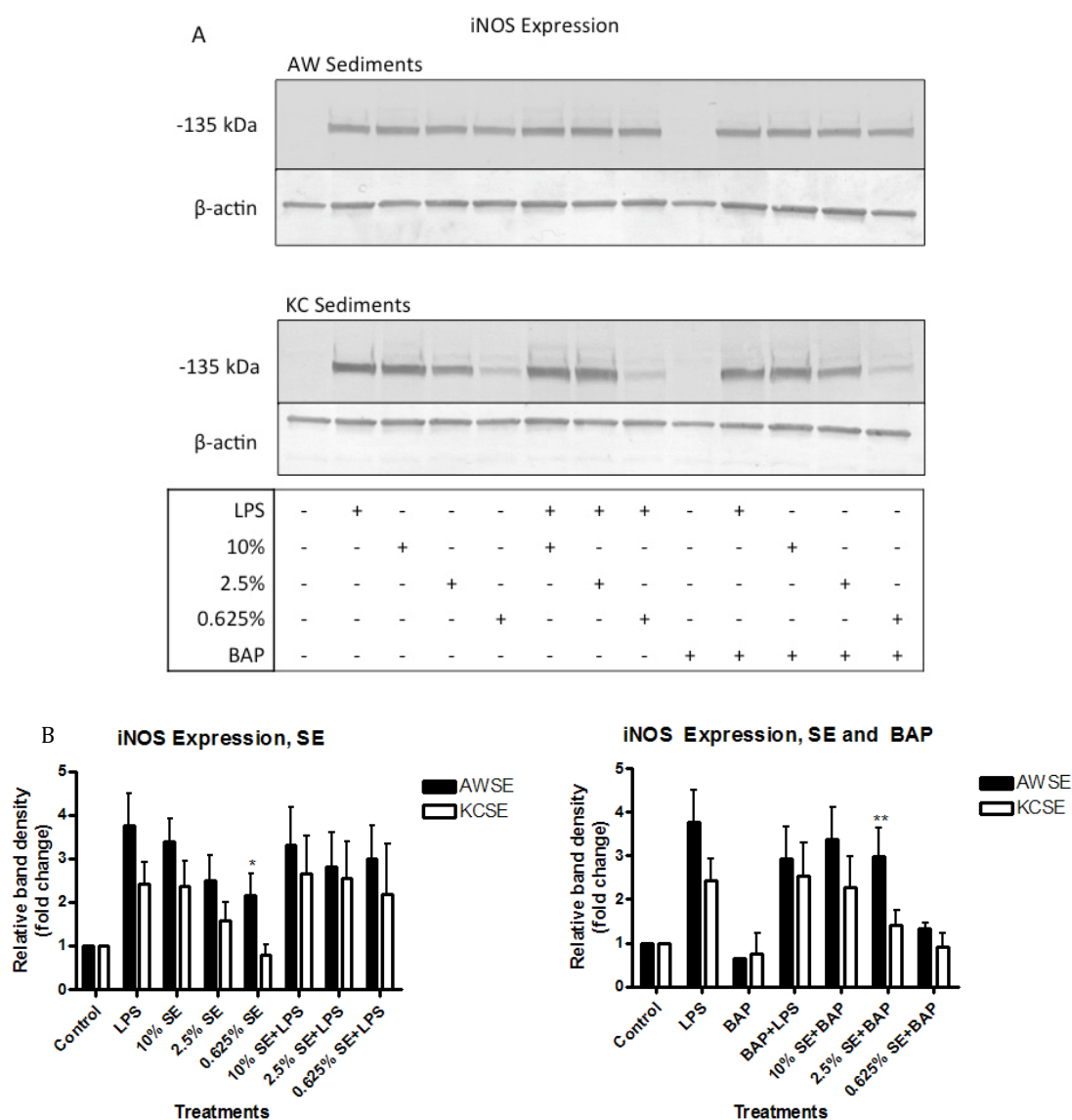


Figure 4. Immunoblotting analysis of iNOS expression in RAW264.7 cells treated for 24 h with AWSE, KCSE, LPS, BAP, SE+LPS, and SE+BAP. A. Representative immunoblots showing iNOS and β -actin (loading control). B. Data represent mean band density \pm standard error of $n=3$ individual experiments (*represents significance $p < 0.05$; ** represents significance $p < 0.01$; represents difference between SE exposures).

Figure 5

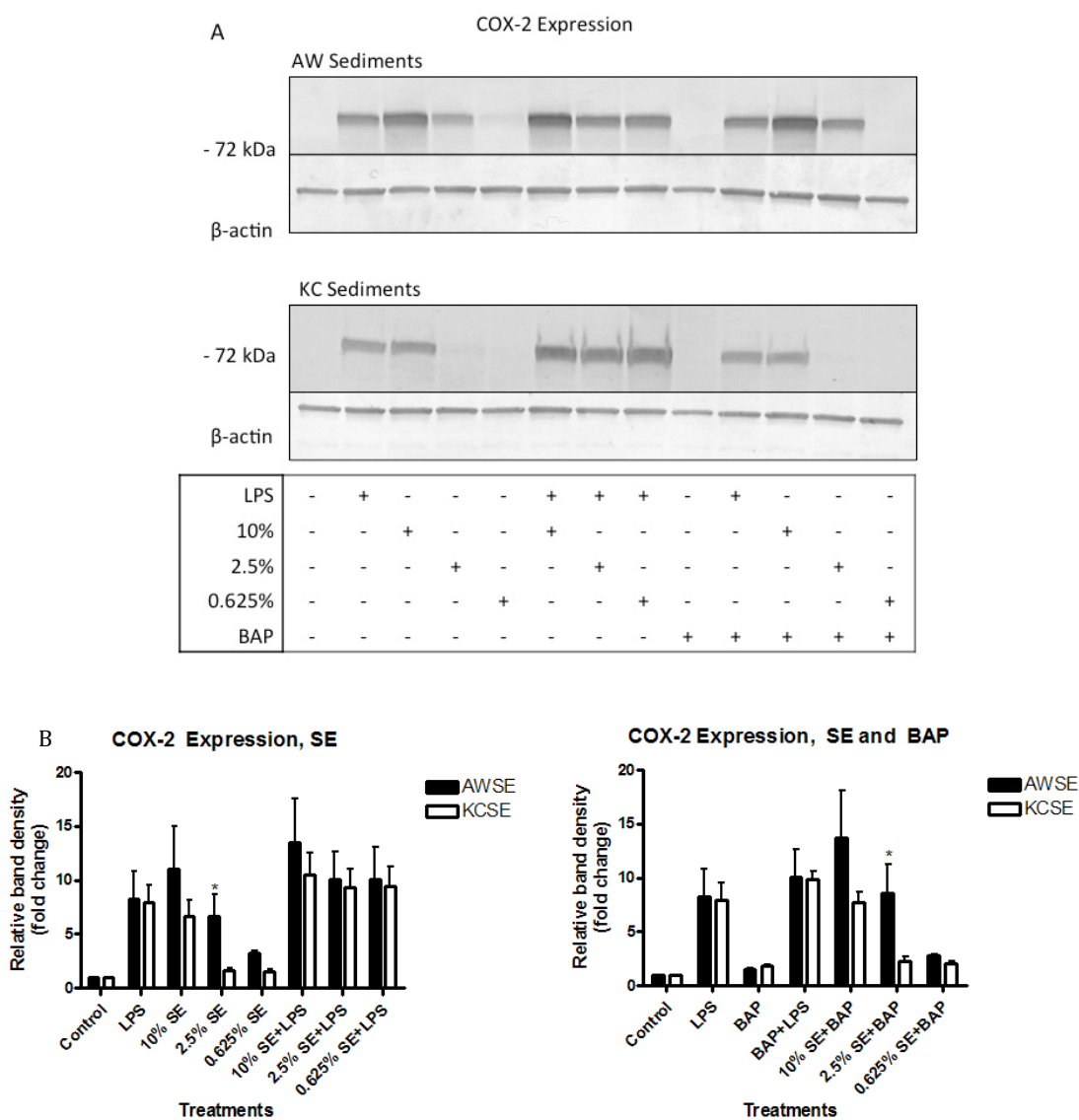


Figure 5. COX-2 expression of AWSE, KCSE, LPS, BAP, SE+LPS, and SE+BAP treated RAW264.7 via immunoblotting. A. Representative immunoblots depicting COX-2 and β -actin (loading control). B. Data represent mean relative band density \pm standard error of $n=3$ individual experiments (*represents significance $p < 0.05$; represents difference between SE exposures).

Figure 6

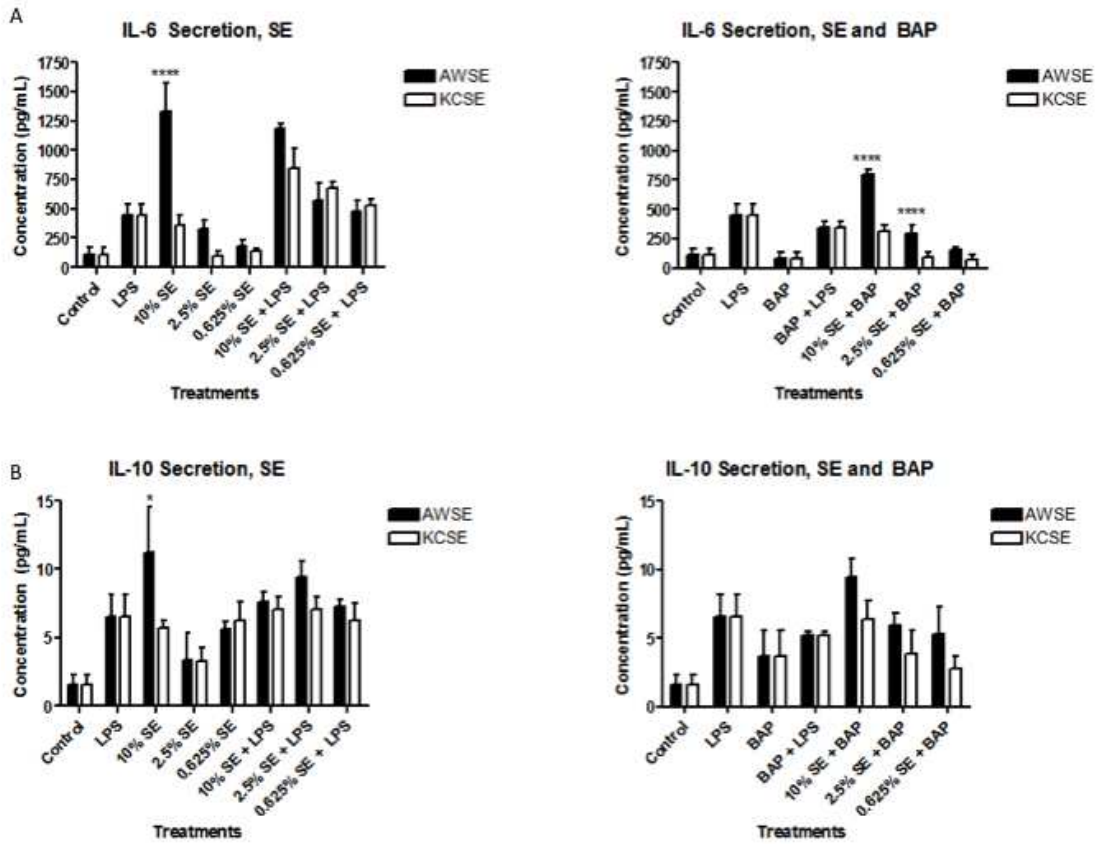


Figure 6. Levels of IL-6 and IL-10 cytokine secretion from 24 h AWSE, KCSE, LPS, BAP, SE+LPS, and SE+BAP treated RAW264.7 cells. A. IL-6 secretion from treated RAW264.7 cells. B. IL-10 secretion from treated RAW264.7 cells. Data represents mean \pm standard error of n=3 individual experiments (* represents significance $p < 0.05$; **** represents significance $p < 0.0001$; represents differences between SE exposures).

Effect of Polymyxin B on NO production.

To test whether the immune responses seen thus far are due to LPS in the sediment extracts, polymyxin B (PMB) was added as a treatment for RAW264.7 cells. Polymyxin B is a known antibiotic used to treat gram-negative bacterial infections via binding to the LPS component in the cell walls (therefore, PMB binds and chelates LPS) (Zavascki, *et al.* 2007). Results indicate PMB significantly decreases nitric oxide production in RAW264.7 cells treated with AWSE at all dilutions (Figure 7A). PMB has no significant effect on NO production from KCSE-treated RAW264.7 cells (Figure 7B).

ROS assay.

To determine if cells exposed to sediment extract, LPS, BAP, or combinations are under general oxidative stress, RAW264.7 macrophages were compared to Hepa1c1c7 liver cells for their relative sensitivity. With the presence of LPS, neither cell line was under oxidative duress (Figures 8A and 9A). However, in the presence of BAP, macrophages were more stressed with combinations of AWSE extracts at high concentrations (Figure 8B and 9B).

Phagocytosis assay.

The ability of macrophages to phagocytize foreign particles was determined using fluorescent beads. Neither of the treatments affected phagocytosis (Figure 10).

Figure 7

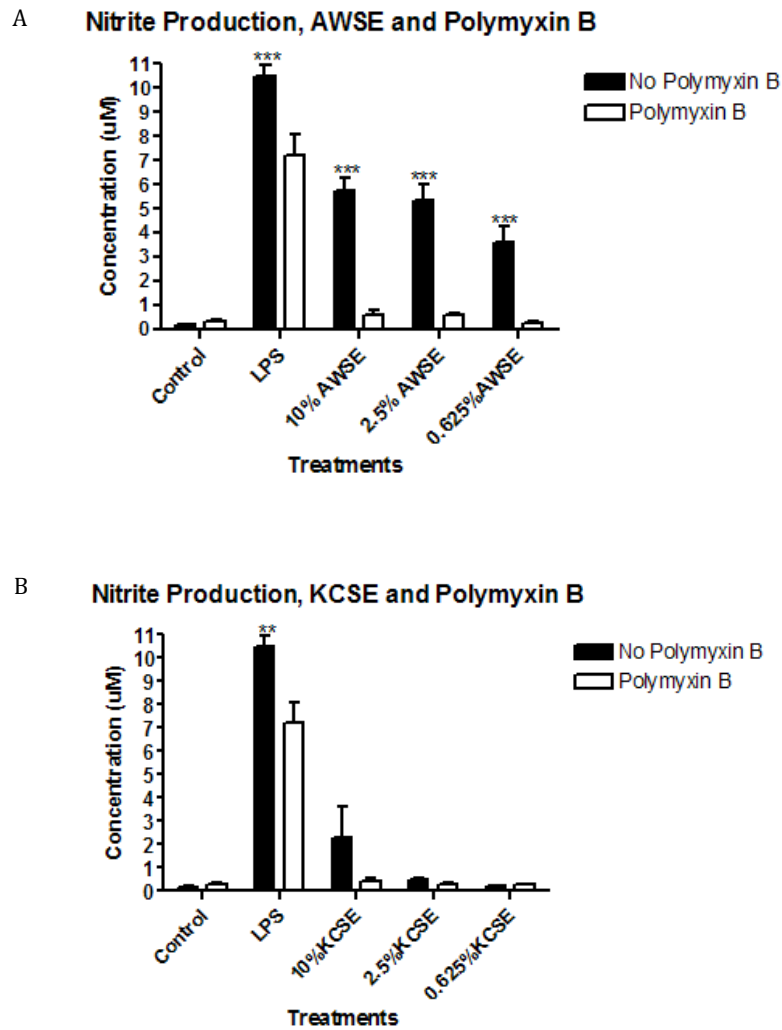


Figure 7. Nitric oxide production in sediment extract and polymyxin B treated RAW264.7 cells. A. RAW264.7 macrophages treated for 24 h with LPS and AWSE, with and without PMB. B. KCSE- and LPS-treated RAW264.7 cells with and without PMB treatments. Data represent mean concentration \pm standard error of $n=3$ individual experiments (** represents significance $p < 0.01$; *** represents significance $p < 0.001$; represents differences between treatment with and without PMB).

Figure 8

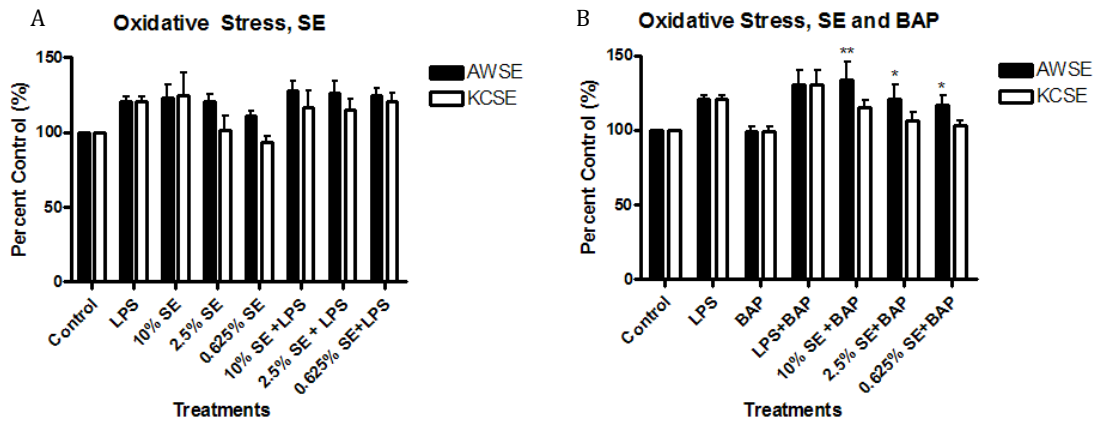


Figure 8. Effects of 24 h AWSE, KCSE, LPS, BAP, LPS+BAP, SE+LPS, and SE+BAP treatment on RAW264.7 cells on general reactive oxygen species production. A. AWSE, KCSE, LPS, and SE+LPS treatment on RAW264.7 cells. B. AWSE, KCSE, LPS, BAP, LPS+BAP, SE+BAP treatment on RAW264.7 cells. Data represents mean \pm standard error of $n=3$ individual experiments (* represents significance $p < 0.05$; ** represents significance $p < 0.01$; represents differences between SE exposures).

Figure 9

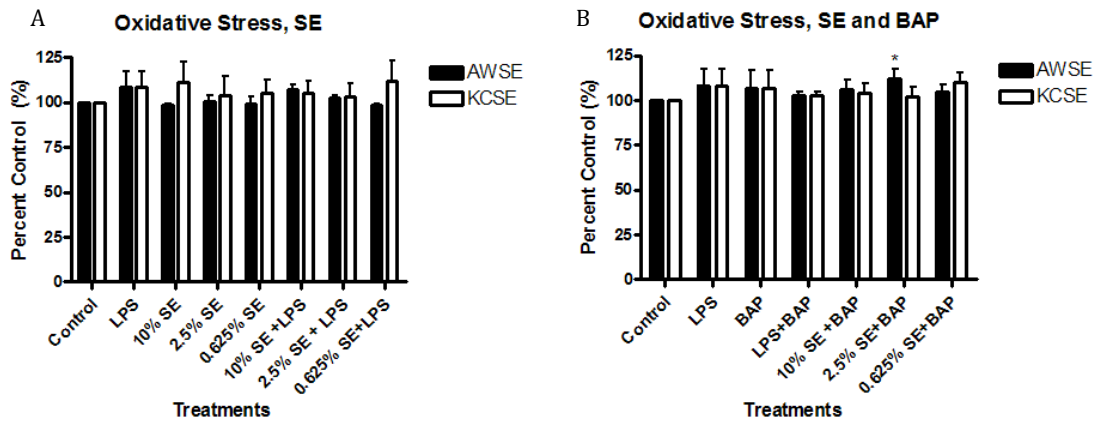


Figure 9. General oxidative stress in 24 h AWSE-, KCSE-, LPS-, BAP-, SE+LPS-, and SE+BAP-treated Hepa1c1c7 cells. A. AWSE, KCSE, LPS, and SE+LPS treatment on Hepa1c1c7 cells. B. AWSE, KCSE, LPS, BAP, LPS+BAP, SE+BAP treatment on Hepa1c1c7 cells. Data represents mean \pm standard error of n=3 individual experiments (* represents significance $p < 0.05$; represents differences between SE exposures).

Figure 10

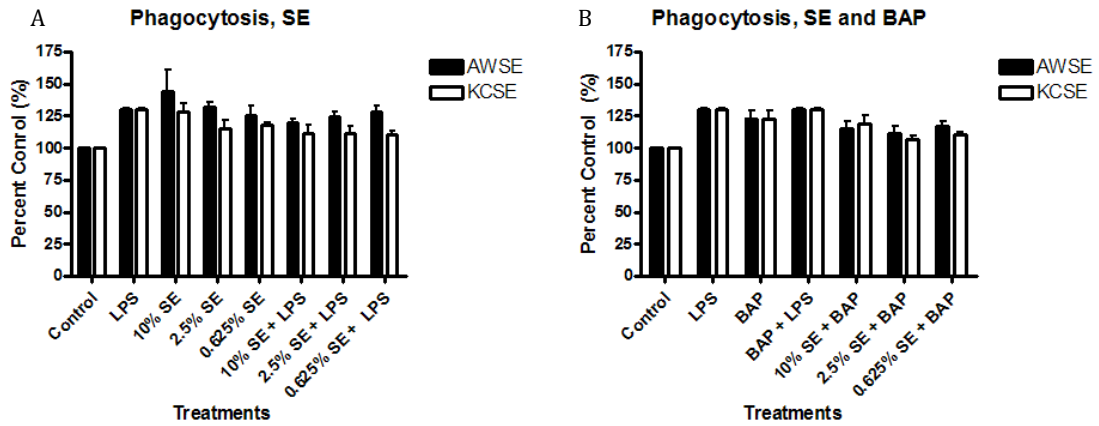


Figure 10. Phagocytic capability of RAW264.7 cells treated with AWSE, KCSE, LPS, BAP, SE+LPS, and SE+BAP for 24 h. A. AWSE, KCSE, LPS, and SE+LPS treatment on RAW264.7 cells. B. AWSE, KCSE, LPS, BAP, LPS+BAP, SE+BAP treatment on RAW264.7 cells. Data represents mean \pm standard error of n=3 individual experiments (no significance observed).

NO production via AW Biofilm exposure.

To investigate the role of a biofilm found at the AW site on the immune responses seen in the results of this study, the AW biofilm was treated in the same manner as the AWSE and KCSE samples. RAW264.7 cells were treated with a serial dilution of biofilm extract, with- and without-PMB, and NO production was examined through the iNOS assay. No significant difference in NO production was seen between treatment groups at any biofilm dilution (Figure 11).

Endotoxin levels in sediment extracts

Endotoxin levels were examined in sediment extracts to determine if the immunological responses seen in AWSE are due to endotoxin present in the sediment extracts. Levels were quantified using the Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific; Figure 12).

Figure 11

Nitrite Production, Biofilm and Polymyxin B

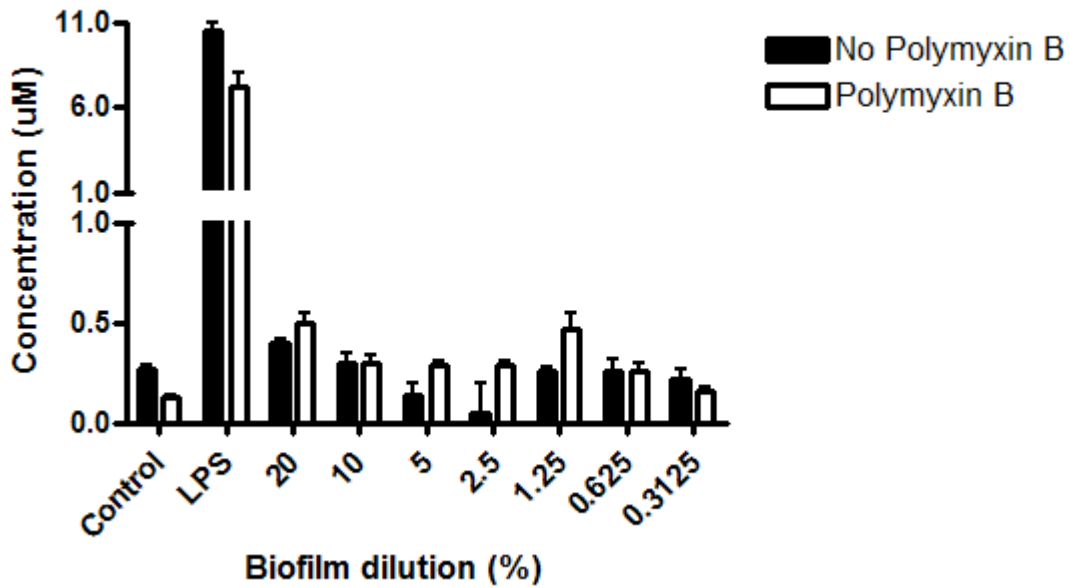


Figure 11. Nitric oxide production from RAW246.7 cells treated with a biofilm found at the AW site. Cells were treated with a serial dilution of biofilm (from 20% to 0.3125%) in the absence and presence of polymyxin B. Data represents mean concentration \pm standard error of $n=3$ individual experiments (no significance observed between PMB and non-PMB treatments). Biofilm did not induce iNOS activity.

Figure 12

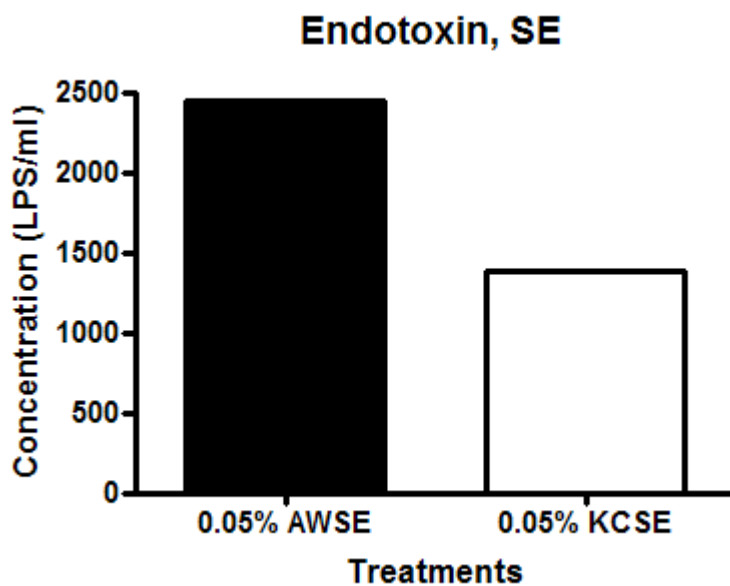


Figure 12. Levels of endotoxin (LPS) in AW and KC sediment extracts. Endotoxin was measured using the Pierce ® Limulus Amebocyte Lysate (LAL). KCSE has a lower endotoxin concentration (LPS/ml) compared to AWSE.

DISCUSSION

This study examined the effects of pore water extracts from a highly contaminated US EPA superfund site (Atlantic Wood) on the Elizabeth River, VA on pro-inflammatory functions in murine macrophage RAW264.7 cells. It was found that AW extracts induce nitric oxide production, iNOS and COX-2 protein expression, and IL-6 secretion in treated macrophages compared to the King's Creek pore water extracts. Only a few studies have examined the effects of contaminants from the Atlantic Wood on organisms, and these have been limited to the estuarine killifish, *Fundulus heteroclitus* (Kelly-Reay and Weeks-Perkins, 1994; Frederick *et al.*, 2007). In general, those PAHs found at the Atlantic Wood site should be immunotoxic to both innate and specific immunity in fish, at least based on experimental data (Karrow *et al.*, 1999; Karrow *et al.*, 2001; Carlson *et al.*, 2002; Carlson *et al.*, 2004). However, the above studies using killifish collected from the Atlantic Wood site indicate that innate parameters of the immune system are elevated compared to reference fish, and that specific immunity is more susceptible. Unfortunately, there are no killifish cell lines available to tease apart some the possible mechanisms associated with heightened innate macrophage responses *in vitro*, and to carry out comparative studies with mammalian systems. To that end, using a mouse macrophage cell line, such as the RAW264.7 cells used in this study, is an ideal model macrophage line for understanding basic pro-inflammatory (heightened) responses in mammalian systems.

The downside of using RAW264.7 cells is that they seem to lack functional AhR signaling, therefore the usual markers of exposure to PAHs like BAP, including CYP1A1

and CYP1B1 induction, are not easily followed. Nonetheless, RAW264.7 cells respond well to TLR-ligands, such as endotoxin or LPS, in that they produce large amounts of inducible nitric oxide, COX-2, and cytokines. As expected, ultra-pure LPS easily induced iNOS and COX-2, and induced the expression and secretion of IL-6, the prototypical inflammatory cytokine responsible for both local and systemic responses to inflammation (Gabay, 2006). Treatment with LPS also induced IL-10 secretion, an anti-inflammatory cytokine considered to be regulatory by dampening strong responses to TLR-ligands (Asadullah *et al.*, 2003).

Contaminant levels in the sediments at the Atlantic Wood site are some of the highest in the country (Bieri *et al.*, 1986), yet pore water extracts were relatively non-toxic to macrophages used in this study, in that viability and cell morphology was unchanged during *in vitro* exposures. Mouse hepatoma cell line hepa1c1c7, which is a common model in toxicology, was used to compare with mouse macrophages. It was found there was a similar lack of toxicity and lack of effect on cell morphology with hepa1c1c7. However, when using the nitric oxide induction model, it was evident early on that sediment extracts from the Atlantic Wood site were stimulatory, and comparable to that of LPS. Sediments from the historical reference site (King's Creek) were also stimulatory, but to a much lesser extent. Throughout the various assays of pro-inflammatory functions, including iNOS and COX-2 induction and expression and IL-6/IL-10 secretion, AWSE were very potent in stimulating macrophages. Of particular note, IL-6 secretion in response to AWSE was higher than previously published studies from RAW264.7 cells, and even in response to LPS.

Mechanistically, iNOS and COX-2 induction, as well as IL-6 and IL-10 secretion in response to LPS is due to TLR-4 activation and subsequent release of NF- κ B, the major transcription factor for LPS-induced activation. Specifically, this pathway uses RelA (p65), the canonical pathway for NF- κ B signaling, and not the non-canonical pathway of RelB, which is more typical during development of lymphoid tissues and B-cell development (Karin *et al.*, 2004; Miller *et al.*, 2010). TLR-4 signaling also involves p38 α (MAPKinase) activity which together with p65 drives inducible pro-inflammatory processes and activities (Hoesel and Schmid, 2013). Just how contaminants in AWSE, and, to a lesser degree, KCSE, stimulate inflammation profiles in a manner similar to LPS is unknown at this point. Oxidative stress can lead to NF- κ B activation, but oxidative stress was not observed under the conditions of this study. On the other hand, some AhR ligands are known to polarize lymphocytes towards a pro-inflammatory phenotype, and dampening anti-inflammatory regulatory cells (Ho and Steinman, 2008; Lawrence *et al.*, 2008; Kiss *et al.*, 2011).

Another avenue explored for such potent stimulatory properties of sediments (from both sampling sites) was the idea that sediments have a large microbial community, including marine LPS-producing Gram-negative bacteria, and especially after the brisk agitation during sediment extraction in water. For example, marine *Vibrio* species are ubiquitous, including *V. vulnificus*, *V. anguillarum*, and *V. cholera* (Tao *et al.*, 2011; Tao *et al.*, 2012; Froelich *et al.*, 2013), and these bacteria are at the Atlantic Wood site because even killifish there are sero-positive (Frederick *et al.*, 2007). The argument for a biological rather than chemical source of immune-stimulation properties of sediment

extracts is further supported by the observation that sediments at Atlantic Wood site are rich in a dark green/black biofilm of what appears to be algal-microbial mats. This biofilm is not observed at the reference site, but killifish feed heavily on this energy source (observed in situ). In an effort to characterize the biofilm at the AW site, nitric oxide production was measured in macrophages treated with the biofilm, in the presence and absence of polymyxin B (Figure 11). Results showed no significant differences between PMB- and non-PMB-treated cells, indicating biofilm presence has no effect on the immune responses previously described in this study. Furthermore, and to my knowledge, this is the first study to show that sediment extracts from the AW site contain nearly twice as much endotoxin as does the reference sediment extracts from KC, VA.

The results of this study using mammalian cell lines indicate exposure to the Atlantic Wood pore water extracts induces an immune response at dilutions as low as 0.625% in RAW264.7 cells after only 24 hours of treatment. In particular, pro-inflammatory indicators, such as IL-6 secretion and iNOS and COX-2 expression, were induced in AWSE treated cells. While acute inflammatory responses aid in the removal of pathogens and provide organismal protection, chronic inflammation has led to several health issues and concerns, including cancer (Chung, *et al.* 1999, Chung and Chang 2003, Kozłowski, *et al.* 2003, Lou, *et al.* 2000), Crohn's disease (Atreya, *et al.* 2000, Bross, *et al.* 1996, Gross, *et al.* 1992, Niederau, *et al.* 1997), and rheumatoid arthritis (Cronstein 2007, Partsch, *et al.* 1997, Ueki, *et al.* 1996). This study provides evidence that higher organisms may be affected by exposure to the Atlantic Wood site on the Elizabeth River. Since the area is known for its high recreation, military, and industrial use, it would be

interesting to study immune responses after longer exposure to AWSE compared to KCSE treatments. Other future studies should focus on determining if sediment extracts from the Atlantic Wood site and reference site at Kings Creek, VA activate the Ahr pathway in RAW264.7 cells using reporter assays, or even using the hepa1c1c7 hepatoma cell line.

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