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Toxicology of Biodiesel Combustion Products

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1. Introduction

The toxicology of combusted biodiesel is an emerging field. Much of the current knowledge about biological responses and health effects stems from studies of exposures to other fuel sources (typically petroleum diesel, gasoline, and wood) incompletely combusted. The ultimate aim of toxicology studies is to identify possible health effects induced by exposure of both the general population as well as sensitive or susceptible populations, including determination of the exposure threshold level needed to induce health effects. The threshold should include not only a concentration but a duration metric, which could be acute or repeated exposures. From such information on sensitive groups and pollutant concentrations needed to induce effects, strategies can be put in place if deemed needed to improve public health. Because possible health effects may take years of exposure to discern, e.g., lung cancer, fibrosis, emphysema, mitigation of the exposure and/or effects may be too late for an individual. Typically markers and biological responses believed to be an early step leading to a clinical disease are measured as a surrogate of the health effect. A biological marker, or “biomarker”, indicates a homeostatic change in an organism or a part of the organism (ranging from organ systems to the biochemicals within cells), that will ultimately lead to a disease induced by exposure to a pollutant (Madden and Gallagher, 1999). So with the previous example of lung cancer, damage to lung DNA induced by an exposure would substitute as the biomarker of effect, or possibly examination of the mutagenic potential of the combustion products through an Ames assay using bacterial strains.

For brevity, this chapter will primarily examine human responses to combustion products though an extensive literature exists on nonhuman animal effects. Discussion of nonhuman animal findings will be used to present findings where human data are sparse or nonexistent, and to provide information on health effects mechanisms. Much of the nonhuman findings fill in data gaps concerning extrapulmonary effects of combustion emissions, particularly cardiac and vascular effects.

2. Combustion emissions composition

Products of incomplete fuel combustion from various sources have some similarities, including some of the same substances and induction of related biological responses. Identification of the compounds, and quantities of the compounds, of the emissions from
various combustion sources may allow a prediction of the biological responses that occur in exposed people. Additionally, examination of the compounds could indicate unique markers that would serve as an indicator of exposure to that source, as well as raising unique biological responses. For example, levoglucosan is a unique marker of woodsmoke combustion and can be used to determine an individual’s exposure to fireplace emissions. A fairly comprehensive list of the chemical species in onroad emissions in California, U.S. derived primarily from gasoline and petroleum diesel powered engines is given in the report by Gertler et al (2002). It is not the focus of the chapter to comprehensively list all emission species; however briefly, the types of components in the gas and particulate matter (PM) phases include single aromatic and polyaromatic hydrocarbons (PAHs) and related compounds (e.g., alkylbenzenes, oxy- and nitro-PAHs), metals, alkanes, alkenes, carbonyls, NOx, CO and CO2 inorganic ions (e.g., sulfates, carbonates), among other chemicals. Woodsmoke particles tend to be relatively rich in certain metals, including iron, magnesium, aluminum, zinc, chromium, nickel, and copper (Ghio et al., 2011).

Biodiesel combustion produces gaseous and PM phases. Compared to other petroleum diesel fuels, biodiesel combustion in “modern” engines generally tends to produce lower concentrations of PAHs, PM, sulfur compounds, and carbon monoxide (CO) ((McDonald and Spears 1997; Sharp, Howell et al. 2000; Graboski, McCormick et al. 2003). There are conflicting reports of whether nitrogen dioxide (NO2) levels are decreased (Swanson et al., 2007). Regarding biodiesel PM, the soluble organic fraction of the biodiesel PM is commonly a greater percentage of biodiesel exhaust emissions, but a smaller percentage of organic insoluble mass is present relative to petroleum diesel soot (Durbin, Collins et al. 1999). A decreased production of biodiesel PM but coupled with a greater concentration of soluble organic material may impact the biological effects of biodiesel exhaust PM. Combusted biodiesel PM is lower in metal content than ambient air PM. Combustion of gasoline generally tends to produce less PM but more gas phase amounts than petroleum diesel combustion.

Gas phase components of biodiesel exhaust have been studied. A U.S. Environmental Protection Agency report (EPA420-P-02-001) comparing standard petroleum diesel and biodiesel emissions of specific compounds termed Mobile Source Air Toxics (e.g., volatile substances such as acrolein, xylene, toluene, etc) concluded that while the total hydrocarbon (THC) measurement decreased from biodiesel emissions, there was a shift in the composition towards more unregulated pollutants. (U.S. EPA, 2002a). However the shift was too small to increase total air toxics compared to petroleum diesel emissions. Biodiesel fuel with a high glycerol content (indicative of poor post-transesterification refining) produces greater acrolein emissions (Graboski and McCormick 1998). Ethanol and methanol are used in biodiesel production to provide ethyl and methyl esters, respectively. These alcohols are aldehyde precursors if not removed from the biodiesel and lead to increased formaldehyde and acetaldehyde formation. Biodiesel combustion leads to fatty acid fragments of the starting material (i.e., methylated fatty acids, or FAMEs). The gas phase exhaust of 2002 Cummins heavy duty engine operated under a wide range of operating conditions was reported to produce methyl acrylate and methyl 3-butanoate (Ratcliff et al, 2010); these compounds are believed to be unique markers for biodiesel combustion. It is unclear whether intact FAMES are emitted in the exhaust due to incomplete and/or poor combustion, but the possibility has implications for toxicity. Intact FAMES from biodiesel fuel can be released into the environment via 1) spills such as in the Black Warrior River in Alabama, USA (New York Times, 2008) and 2) the introduction of the fuel into lubrication
Plant oils are utilized in biodiesel production on a commercial scale in the United States, though some biodiesel fuel can be produced from animal fats. At present, the main plant oil feedstocks for the United States and Europe are soybean oil and rapeseed oil, respectively (Swanson et al, 2007). Other sources globally potentially include switchgrass, jatropha, and palm oil. Algal feedstocks potentially can produce more energy per volume due to their increased fatty acid content. It is unclear if the fatty acid composition is significantly different among the feedstocks, or within feedstocks grown under different conditions.

3. Human health effects

3.1 Nonbiodiesel combustion sources

Identification of health effects observed in humans exposed either acutely or repeatedly to combustion sources other than biodiesel provides guidance for which effects, or surrogate biomarkers of the effects, to examine with combusted biodiesel exposures. Although the epidemiological studies linking biofuel exhausts and impaired human health have not yet surfaced, diesel exhausts, biomass burning, forest fires, and coal burning have been strongly associated with adverse effects and mortality. Recently increases in emergency room visits for asthma symptoms, chronic obstructive pulmonary disease, acute bronchitis, pneumonia, heart failure, and other cardiopulmonary symptoms were noted for people exposed to a peat fire in eastern North Carolina, USA (Rappold, Stone, et al., 2011). These studies are supported by the further evidence of increases in blood pressure in near-road residents (diesel exhaust can be the primary contributor of near road PM in certain locations) (Auchincloss, Diez Roux et al. 2008) and add into consistency of evidence that can be linked to emissions from biologically based and fossil fuels. A number of clinical studies have similarly shown vasoconstrictive and hypertensive effects with petroleum diesel exhaust (PDE) (Peretz, Sullivan et al. 2008) including a decrease in brachial artery diameter in humans. These human studies supporting evidence of adverse cardiovascular impairments have been concurrently proved to be true with animal toxicological studies. However, the mechanism of these apparent cardiovascular impairments without pulmonary health effects are not understood due to inherent variability in the chemical nature of exhaust PM examined and varied exposure scenarios and the variable responsiveness of animal models. Moreover, the physiological relationship between vasoconstrictive effect and change in blood pressure are not understood. PDE have been long studied for their immunological and carcinogenic effects on the lung, however more recent evidence also points to the effects on cardiovascular system.

3.1.1 Lung cancer

With PDE exposures, lung cancer is of concern. The International Agency for Research on Cancer (IARC), the U.S. EPA, the U.S. National Institute for Occupational Safety and Health (NIOSH), and the National Toxicology Program (NTP) have classified PDE as a probable carcinogen, likely carcinogen, potential occupational carcinogen, and reasonably anticipated to be a human carcinogen, respectively, regarding human exposures. There is some question of PDE as a carcinogen due to confounding variables and uncertainties related to exposure levels in some of the epidemiological studies. The increased risk for lung cancer associated with diesel exhaust exposure are derived primarily from epidemiological findings.
performed prior to 2000. A recently published study involved trucking industry workers regularly exposed to diesel exhaust and the development of lung cancer (Garshick, 2008). The findings showed an elevated risk for the development of lung cancers in those with greater exposure compared to workers (e.g., office workers) with a lower exposure.

3.1.2 Lung inflammation and immune system
Controlled exposures of humans to whole PDE typically results in lung inflammation as shown with neutrophils entering the lungs; these studies are generally 1-2 hr at approximately 100-300 µg/m³ with healthy adults (Holgate 2003). In these same exposures, several soluble substances which mediate inflammation, e.g., interleukin-8 (IL-8) were shown to be increased by use of lung lavage or inducing sputum production to recover airways secretions. PDE PM induced an adjuvancy effect using nasal instillations of 300 µg particles in allergic subjects as common biomarkers of allergy (e.g., increased IgE production and histamine release) increased in nasal secretions (Diaz-Sanchez et al, 1997). Neutrophil influx into the lungs of healthy volunteers exposed to nearly 500 µg/m³ woodsmoke for 2 hr was observed (Ghio et al, 2011) suggesting a common outcome from different combusted fuel sources. There are no studies of human volunteers exposed in a controlled manner to gasoline exhaust.

3.1.3 Cardiac physiology
Biomass, wood smoke and PDE have been linked to increased blood pressure in humans (Sarnat, Marmur et al. 2008). More mechanistic understanding of combustion induced effects have been derived from studies in nonhuman animal models. Animal toxicology studies have provided some understanding of how diesel exhausts inhalation, while producing small effects in the lung, could have profound effects on the vasculature and myocardium. A few studies have considered the balance of sympathetic and parasympathetic tone, and how these may be altered by PDE. In early high concentration PM studies, classical arrhythmias were apparent, along with heart rate changes, but, when doses fell to more relevant levels, these effects became more difficult to discern (Watkinson, Campen et al. 1998). Increased arrhythmogenicity after aconitine challenge has been noted following environmentally relevant low concentrations of PDE in rats, suggesting that prior air pollution exposure increases the susceptibility to develop arrhythmia in response to severe cardiac insult (Hazari et al., 2011). This increased arrhythmogenic effect of PDE has been postulated to occur as a result of increased intracellular calcium flux. It is not known if preexistent arrhythmogenic status might result in mortality following subsequent air pollution exposure. Thus, PDE exposures, together with compromised cardiac function (especially ischemia), myocardial infarction, hypertension, or heart failure, likely cause arrhythmogenicity in susceptible humans. Biodiesel exhaust might have similar effect on cardiac performance but these studies are needed to understand the influence of compositional similarities and differences in PDE and BDE-induced cardiac injuries.

The lack of cardiac inflammation, myocardial cell injury, or mitochondrial damage despite cardiac physiological impact in many studies (Campen et al., 2005; Cascio et al., 2007; Hansen et al., 2007; Sun et al., 2008; Toda et al., 2001), supports the findings that PDE induces physiological transcriptome response without altering pathological abnormalities in short-term exposure scenarios (Gottipolu et al., 2009).
3.1.4 Systemic thrombogenic effects
While some clinical studies provide negative evidence of systemic thrombogenic effects of PDE, most clinical studies are consistent with increased systemic thrombus formation (Lucking et al. 2011) in humans. Animal studies have shown fairly consistent results in regards to increased vascular thrombogenicity of PDE. Exacerbation of systemic thrombus formation in response to UV-induced vascular injury in hamsters and mice exposed to PDE has been known for few years (Nemmar, Nemery et al. 2002; Nemmar, Nemery et al. 2003). The increase in intravascular thrombosis in these earlier studies coincided with inflammation and mast cell degranulation. In hamsters, the thrombogenic effect of PDE was diminished by pretreatment with the anti-inflammatory agents dexamethasone or mast cell stabilizing sodium cromoglycate, implicating the role of inflammatory cells—specifically mast cells (Nemmar, Nemery et al. 2003; Nemmar, Hoet et al. 2004). Pulmonary injury was postulated to cause procoagulant changes and the systemic vascular response to PDE. A number of studies since then have shown prothrombotic effects of PDE exposure in the thoracic aorta of mice and rats (Kodavanti et al., 2011). The precise mechanisms of how PDE or other biodiesel particles might induce thrombogenic effects and the role of pulmonary versus systemic vasculature are now well understood. The evidence supports the role of pulmonary injury/inflammation in eliciting this vascular effect.

3.1.5 Vascular physiology and inflammation
Human clinical and animal studies have provided the evidence that inhalation of PDE and woodsmoke results in peripheral vasoconstriction and increased prothrombotic effects (Mills et al., 2007; Peretz et al., 2008; Lucking et al., 2008; Laumbach et al., 2009; Törnvqvist et al., 2007; Campen et al., 2005; Knuckles et al., 2008; Barregard et al, 2006). Vasoconstrictive effects of PDE have been noted even at environmentally relevant inhalation concentrations (Peretz et al., 2008; Brook, 2007). A reproducible decrease in vasodilation in response to various agonists for about 2-24 hr after petroleum diesel exposure has been demonstrated (Mills et al, 2005). Healthy and compromised animal models show alterations in the NO-mediated vasorelaxation and endothelin-mediated vasoconstriction (Nemmar et al., 2003; Knuckles et al., 2008; Lund et al., 2009). PDE-included vasoconstrictive response has been thought to involve impairment of vasodilation due to decreased availability of NO (Mills et al., 2007). Newer studies suggest that vascular effects of PDE and gasoline exhausts might be primarily due to gaseous components such as carbon monoxide and nitrogen oxides. Numerous studies done using PDE and gasoline exhausts have used ApoE-/- mouse model of atherosclerosis and shown that PDE and gasoline exhausts exacerbate lesion development and molecular changes associated with atherogenic susceptibility of ApoE-/- mice. An array of plasma markers, including cytokines; biomarkers of coagulation and thrombosis; antioxidants; adhesion molecules; and acute phase proteins have been evaluated in a number of studies where animals or humans are exposed to PDE. Although a number of effects have been reported, the results from systemic biomarker studies lack consistency in terms of a similar effect on a given biomarker regardless of some differences in the protocols; in one study, one marker might be increased, whereas, in the other, a different marker may be affected. For example, in one study, PDE exposure has been shown to increase IL-6 (Tamagawa, Bai et al. 2008), whereas, in another, it may show no effect (Inoue, Takano et al. 2006). This discrepancy could result from a small magnitude of effects with a limited sample size; insensitivity of the methods, difficulty in controlling human behavior variables among sequential testing; variable composition of PDE; low exposure

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concentrations; and, perhaps more importantly, the overwhelming variability in individual host factors. Owing to the fact that biodiesel exhaust might contain more gas-phase components, the systemic biomarkers might respond differently.

3.1.6 Other organ systems
Common symptoms of combustion emissions exposures typically reported include nausea, headache, eye and throat irritation, and dizziness (US EPA, 2002). Other possible biological responses and health effects induced by PDE have been initially investigated by use of epidemiological approaches and rodent models. These endpoints are typically difficult to be examined in controlled exposure studies with humans. For instance, rodent spermatogenesis decreased with exposure in utero (Watanabe et al, 2005), and atrial defects (odds ratio of 2.27) was observed in newborns in seven Texas (USA) counties (Gilboa et al, 2005) and were associated with PM and CO concentrations. These findings of reproductive and in utero atrial defects and the initial observations of decreased spermatogenesis need to be followed up for reproducibility of the findings.

3.2 Biodiesel combustion products
Mutagenicity of substances is typically assessed in bacterial or cellular mutagenicity assays. The vast majority of mutagens are also carcinogenic. Studies indicate that petroleum diesel is more mutagenic than biodiesel. The soluble organic fraction of PDE had more mutagenic potential than biodiesel originated from rapeseed in a mutagenicity assay using cultured rat hepatocytes. Similar results were found with PDE using bacterial culture in the Ames assay. (Eckl et al 1997) Soluble organic fraction of PDE regardless of the various engine cycle combustion conditions still induces more bacterial mutagenesis when compared to biodiesel (Rapeseed methyl ester). (Bunger et al 1998) The same organic extracts were tested for potency of mutagenesis after incubation with enzymes extracted from the S9 fraction, and produced the same results indicting PDE is more mutagenic even after liver detoxification. Comparison of PDE from high sulfur and low sulfur content fuel results in more mutagenic activity from high sulfur fuel exhaust regardless of engine mode and incubation with liver metabolic enzymes. (Kado and Kuzmicky 2003) Similar studies with combusted vegetable oils including sunflower seed, cotton seed, soybean and peanut all indicated the soluble extract was less mutagenic than PD extract. (Jacobus et al 1983) However recent regulations have shifted PD over to low sulfur diesel and some have reported biodiesel extracts to be more mutagenic than the new low sulfur PD combustion extracts. Biodiesel exhaust extract from methylated feedstocks of soy, canola, and beef tallow were found to be more mutagenic than Philips Petroleum-certified PD. (Bunger et al 2000a AND Burger et al 2000b) In the same study they combusted non-methylated rapeseed oil along with rapeseed methyl esters and found the non-methylated to be more mutagenic than either the methylated or PD. Additionally the gas phase components were collected by cooling and extraction into a solvent. The condensates of the gas phase showed little difference between the combusted PD and biodiesel mutagenicity. The BD and PD extracts have recently been used in in vitro toxicity testing. Exposure of PD and BD (soy methyl and ethyl) soluble organic extracts to cultured human airway epithelial cells (BEAS-2B) resulted in elevated cytokine production (IL-6, IL-8) from BD after 24hr exposure. (Swanson et al 2009) An immortal lung epithelial cell line (A549) after exposure to PM from both biodiesel and PD revealed cell morphological changes. The control (unexposed cells) had baseline of
7% multinucleated cells, whereas exposure to Biodiesel blend of 80% increased multinucleated cells to 16%. Biodiesel blend of 20% (80% petroleum) increased the multinucleation rate up to 52%. (Ackland et al 2007) Cultured mouse fibroblast cells also indicate BD exhaust soluble extract to be more cytotoxic relative to the PD extracts. (Bunger et al 2000b) Some speculation as to components driving this shift toward increased mutagenicity in biodiesel indicate the increased carbon and carbonyl content in the biodiesel to interfere with cells for longer lengths before the components can be metabolized. The variability of responses can be due to the contents of the soluble extract based on type of solvent and combustion conditions or to the robustness of the cell line.

Animal exposure studies eliminate some of the in vitro variability. Rats exposed to filtered air, PD, B50, and 100% BD (soy ethyl ester) for 1 hr were analyzed for lung inflammation. Results indicate lung lavage to have increase in total cell count in the three treatment groups but non were statistically greater in cell count indicating one PM doesn’t cause more inflammation. The lung parenchymal tissue was analyzed for inflammation and also resulted positive for inflammation but none of the PM types induced significantly elevated levels. (Brito et al 2010) A second study utilized intratracheal instillation of exhaust PM collected as water aerosol from PD, gasoline, and Biodiesel powered engines (without oxidation catalyst). The aerosols were instilled into mice and the lungs were examined 24 hrs later for inflammatory response. The instillation from the gasoline and diesel engines were the most potent to induce an increased neutrophill influx into lungs (inflammatory response), relative to saline control mice. (Tzamkiozis et al 2010)

Chronic exposure with BD and PD produce similar results however the extent of the inflammation may vary. Particle laden alveolar macrophages, lung neutrophilia and fibrosis are detectable in BD exposed rats however the difference from PD an BD exposure was not statistically significant. (Finch et al 2002, Mauderly 1994, Hobbs et al 2002). Human exposure to delivery truck workers, road maintenance workers, and industrial fork lift truck drivers all exposed to BDE or PDE occupationally were asked to report their symptoms in a questionnaire. The results of the questionnaire indicate dose related respiratory effects but nothing to indicated significant differences between the combustion of different fuels.

3.3 Summary

Based on the literature available at present, biodiesel exhaust can have more, less, or the same potency in inducing biological responses and health effects as PDE. This may be due to the chemical mix of exhausts and the differences between various types of exhaust emissions. Better reproducibility of design from study to study in the future would assist in the assessment of whether biodiesel exhaust induced the same biological responses. The designs should try to narrow down the fuel type utilized, minimize fuel impurities, utilize an engine commonly available and in use, standardize the run conditions (load, ambient temperature of intake air, etc), so that emissions used in biological test system are fairly similar.

4. Components found in biodiesel combustion with known health effects

Some compounds present in combusted biodiesel exhaust can induce known toxicity in exposed human populations down to cellular effects. The literature on these components may allow a research strategy to determine if these substances exist in great enough concentrations to induce health effects in humans, and if so, how to attenuate the effects
through management of the emissions quantities. Additionally, examination of whether the gas and/or the PM phase is primarily responsible for the induction of any observed effects could also be utilized relative to decreasing biologically active substances.

4.1 Filtered particle exhaust studies

PDE studies provide preliminary information for predicting BDE toxicity specifically the studies can give insight on the potency of gas phase and PM. The removal of particles from petroleum diesel exhaust can attenuate the adverse effects caused by inhalation of diesel exhaust. The exhaust can be filtered to completely remove particles or minimize the amount. Controlled human studies conducted in exhaust chambers fitted with ceramic filters (temperature maintained to eliminate PM nucleation) to capture the particulate successfully reduced the PM by 25%. In this study, the exposures without particles significant increased activated immune response cells (CD3-labeled T lymphocytes) more than particle laden exposures. (Rudell, Blomberg et al. 1999) A lung lavage sample from each exposure indicated no changes in total cell number indicating no significant inflammatory responses. However there was a noticeable decrease in the number of macrophages collected from the bronchial location of the lungs in individuals exposed to the filtered exhaust. A number of explanations for the lack of sentinel macrophages can be concluded, including the filtered exhaust was eliminating larger PM which removes interference from PM deposition and the immediate immune response resulting in two completely different immune responses. However not all studies with PDE indicate gas phase to have more potency. In a mouse exposure study with particle (3.3mg/m3 ) and filtered PDE (PM < 0.1mg/m3) followed by immediate challenge with pollen, results indicate similar increases in IgE and IgG2 serum for the mice exposed to both the filtered and non-filtered exposures. (Maejima, Tamura et al. 2001) However, there was no detectable dose dependent increase to the pollen in only the group exposed to the diesel exhaust gas components. This study proposes an allergic challenge is attenuated after exposure to filtered PDE or PDE with particles, increasing the confidence that each exposure is unique. The use of low sulfur diesel fuel has been indicted to reduce the PM by reducing the soot nucleation rate. (Karavalakis, Bakeas et al. 2010) A study using both low sulfur fuel and a particle trap to reduce the emissions was successful in reducing the toxic health effects relative to regular emissions. (McDonald, Harrod et al. 2004) In this study mice were exposed to the two exhaust types and results indicate with reduced emissions there is significant reduction in the number of potentially toxic inflammatory responses and reactive oxygen species generation. Lung toxicity measured with IL-6, interferon-γ and tumor necrosis factor-α (TNF-α) and antioxidant enzymes (heme oxygenase-1) were all reduced after exposure to reduced PM. The study measured inflammatory response in the mice after a seven day exposure. The study concluded most components of both exhausts were in the range of background air however the responses indicate particles have substantial roles in inflammation and oxidative stress. Not all endpoints of injury indicate filtered exhaust to be less harmful. In an experiment with healthy male subjects who were exposed to both filtered and unfiltered diesel exhaust exposure indicate a reduced response of vasomotor function in subjects exposed to diluted diesel exhaust. Lucking, Lundbäck et al, 2011). Specifically there was reduced vasodilatation even with agonists to promote constriction after exposure to dilute diesel exhaust but not with filtered exhaust. In this study the effects of pure carbon nanoparticles was utilized as a control for the particles however there was no significant alterations of vasoconstriction abilities inhibited by the pure nanoparticles. The particles of diesel exhaust consist of surface bound hydrocarbons or other charged components and are likely interfering with the localized cellular response.
4.2 Carbonyls

Carbonyls (aldehydes and ketones) are common components of fossil fuel combustion. Common species in combustion exhaust are short chain aldehydes such as acetaldehyde and formaldehyde. The use of catalyzed diesel particle filter and plant based fuel reduces carbonyl emissions; however with biodiesel blends there noticeable increases in formaldehyde and acetaldehyde emissions from diesel alone. (Ratcliff, Dane et al. 2010; Jayaram, Agrawal et al. 2011) Petroleum diesel combustion also releases formaldehyde and acetaldehyde with a larger percentage of total carbonyl release being acrolein. Acrolein is a highly reactive aldehyde which creates adducts leading to various degrees of toxicity. Inhalation of acrolein can lead to onset of pulmonary edema, respiratory disturbance and asthma like symptoms. New research indicates acrolein may initiate platelet activation, an event both beneficial and detrimental if induces plaque buildup. Due to the nature of the highly reactive acrolein, specific measures were taken to identify acrolein adducts were not the primary cause of platelet activation but acrolein works directly on platelets as it forms covalent adducts. (Sithu, Srivastava et al. 2010) The study conducted by Sithu et al, utilized fresh mice platelets and vaporized acrolein to conduct exposures. Removal of the blood and isolation of the platelets also found increases in activation proteins like fibrinogen and platelet derived growth factor and platelet factor 4 with exposure to acrolein alone. The observed events were not inflammatory responses because the study measured mRNA expression of pro-inflammatory cytokines and found none were increased above control. Recent studies have indicated increased release of formaldehyde from the combustion process of soy based biodiesel. (Ratcliff, Dane, et al. 2010; Karavalakis, Bakeas et al. 2010) Recently formaldehyde has been classified as a carcinogen. Many studies have addressed the mutagenic characteristics of formaldehyde. In a study of formaldehyde exposure to rat nasal epithelial cells, multiple toxic end points were increased. The study measured the frequencies of micronuclei formations, un-regulated cell proliferation, and pathological changes. Exposure doses larger than 2ppm resulted in site specific increase in cell proliferation. Additionally lesions and metaplastic changes were observed in only the formaldehyde exposed. Histopathology of the nasal regions indicated increases in leukocytes, indicating inflammatory response. Epithelial cell were sloughing off as well as abundant indications of squamous cell metaplasia and the nasopharyngeal duct displayed transitional cell metaplasia. (Speit, Schutz et al. 2011) Basic cellular observations of increased aldehydes released by biodiesel combustion needs to be better understood for any adverse health effects.

4.3 Fatty acids and derivatives

Biodiesel fuel is created with trans-esterification of fatty acids. The composition of BDE has found a number of methyl esters, cyclic fatty acids and nitro fatty acids. Fatty acids including palmitic acid, oleic acid, and stearic acids are considered pulmonary irritants. Fatty acids can be simply classified as unsaturated or saturated and the complexity increases with the types of functional groups bound. Some of the more complex components derived from fatty acids are created with enzymatic reactions others are not. A characteristic of fatty acid derived structures, specifically lipids, is their ability to have dual polarity. Phospholipids create the barriers established in all cell membranes. Normally the fatty acid tail is the hydrophobic region and the carboxyl head is hydrophilic. Tampering with the membrane structures can lead to cell death. Fatty acids play a crucial role in maintaining the pliability of surfaces. Lungs are an important location of fatty acid mediated flexibility, as the lungs fill up with air there is limited distension however when the air is exhaled the ability to expand should remain unaffected. A component of the lungs that allows for this rapid intermittent expansion and contraction is surfactant. Surfactant is a complex mixture
of proteins and lipids that is largely dipalmitoyl phosphatidylcholine and its purpose can be adversely affected with intrusion by other fatty acids. Many studies have been conducted on the disruption of surfactant by an increase of a type of lipid, oleic acid. (Hall, Lu et al. 1992) One set of experiments conducted was able to measure the surface pressure changes created with the addition of oleic acid. Surface pressure and the created tension by the lipid molecules, can be inferred with correlation to the absorption of surface pressure. In this study the excised calf lung were lavaged with saline instead of air and the lungs were either treated with the oleic acid or control. Overall results indicated oleic acid disruption of the dynamic lung compression and expansion model can’t be correlated directly to an absolute concentration, however inhibition occurs when oleic acid is relatively higher than surfactant concentrations. The incorporation of the oleic acid prevented the spreading of the surfactant film to occur during contraction of a simulated compression. The ability of the lungs to maintain elasticity weakens as the repetitive cycles increased. General observations of extraneous lipid incorporation include disruption of the surfactant films created for lung flexibility and can cause harm to the mechanical physiology of the lung.

4.4 Transition metals
Metals are more abundant in petroleum diesel combustion exhaust than biodiesel. The metals originate from multiple sources including the fuel. Metal particles can be emitted from engine components. Several studies indicate there is a decrease in the concentration of transition metals in biodiesel combustion exhaust. (Brito, Luciano Belotti et al. 2010) Biodiesel blends result in increases in the transition metals Cu, Fe, and Zn in soy based B50 compared to B100. (Brito, Luciano Belotti et al. 2010) Transition metals are highly oxidative species and can lead to intracellular redox cycling. Metals have the ability to generate radicals which likely lead to depletion of antioxidants and increases in DNA, and protein adducts. Both biodiesel and diesel exhaust particles analyzed for elemental metal composition, were found to have metals bound to the carbon core. Several studies have observed the decrease in DNA adduct formation with the pre-treatment of particles with a metal chelator. One study was also able to develop a method to measure the indirect products of ROS and they concluded, diesel exhaust particles treated with a diethylenetriamine pentaacetic acid (DTPA) generate fewer ROS products. The study utilized the same method to measure the amount of 2,3- and 2,5- dihydroxybenzoate (DHBA) generated in the presence of known amounts of Cu and Fe; both are toxic and highly reactive metals. (DiStefano, Figueroa-Fernandez et al. 2009) Other studies were able to study the inflammatory effects residual oil fly ash (ROFA), a dust rich in transitions metals especially V, Ni, and Fe, alone and with pre-treatment with a metal chelator. Similar results were found indicating cytokine induction and depletion of antioxidants is partly due to the metals that are bound to the various particles. (Carter JD, Ghio AJ et al. 1997) Metals are essential elements within cells however too much metals can cause harm to cellular homeostasis and induce cellular toxicity.

4.5 PAH and PAH-related compounds
Polyaromatic hydrocarbons classified by the functional group attachments most prevalent in combustion byproducts are nitro- and oxy- species. They can also vary in reactivity based on their molecular weight. Biodiesel blends up to 50% are analyzed to have large decreases in PAH emissions when compared to diesel fuel combustion. (Brito, Luciano Belotti et al. 2010) A commonly measured sample PAH released during diesel combustion is...
phenanthraquinone (PQ). PQ can be reduced by flavin enzymes including NADPH located in the mitochondria and along energy transport membranes. The reduction leads to generations of semiquinone radicals, oxidative stress and DNA damage followed by cytotoxicity. Experiments with PQ exposure to human pulmonary epithelial cells have observed increases in toxic byproducts of ROS generation. Some increases measured are increase in protein carbonyl formation, increased levels of superoxide dismutase (Cu/Zn SOD) and heme oxygenase (HO-1). (Rika Sugimotoa, Yoshito Kumagaia et al. 2005) Protection from the damaging consequences of protein carbonyl formation originated from both the use of iron chelators and antioxidants. High emission of NO\textsubscript{2} lead to the nitration of the available PAH’s forming nitro-PAH’s. Using soy based biodiesel, species identified included few volatile nitro compounds that were more abundant in B100 as opposed to the B20, however the overall trend was a decrease in the nitro-PAH emission when biodiesel was combusted. Detailed analysis of 7 nitro PAH emission concluded in several products decreased by more than 50% with the blending of B20 into the petroleum diesel and further decreases with B100. (Ratcliff, Dane et al. 2010) Naphthalenepec is a larger percent of the combustion emissions, in both biodiesel and diesel fuel engines. (Ratcliff, Dane et al. 2010; Jayaram, Agrawal et al. 2011) Naphthalene vapors are toxic and are commonly used as pesticides. There are several signaling pathways that have been identified which are initiated with the binding of PAH’s to the acryl hydrocarbon receptor, however many PAH’s have not been identified as ligands. Other indirect increases in cellular toxicity from PAH’s involves thiol generation which inactivate proteins with sulfhydryl groups. Quinones generally are not alkylating agents but they can generate redox cycling which generates thiol oxidants including hydrogen peroxides. PAHs will cause cellular and regional increases in ROS generation and further deplete antioxidants while repair processes work to increase antioxidant defenses.

4.6 Other hydrocarbons
Toluene is a common aromatic hydrocarbon emitted with the combustion of fossil fuels. Diesel emissions contain detectable amounts of toluene in both the vapor phase and particle. Toluene is more reactive than benzene due to the methyl group and is easily nitrated in the presence of increases NO\textsubscript{2}. Toluene has been found to interact with the aryl hydrocarbon receptor in cells. In a study conducted using Drosophila flies to study genotoxicity and apoptosis, toluene exposure produced large amounts of cell death. (Singh, Mishra et al. 2011) The study also measured the amount of apoptosis after treating the cells with a known aryl hydrocarbon receptor blocker before exposure to toluene. The results of the study with AHR blocker producing less toxicity can justify the observations indicating toluene works via the AHR. Activation of the AHR can increase transcription of antioxidants. (Singh, Mishra et al. 2011) Apoptosis increased with toluene exposure was measured with TUNEL assay. Previous research with other aromatic hydrocarbons has observed increases in inflammation and increased activation of T-lymphocytes and eosinophils. Hydrocarbons like toluene, with reactive functional groups are likely to enter into the cell and cause cellular apoptosis as they are to accumulate in tissue and cause regional inflammation.

4.7 Carbon monoxide and nitrogen dioxide
Primary concern of carbon monoxide (CO) poisoning involves the ability of CO to bind to hemoglobin in the blood and inhibit binding oxygen molecules to hemoglobin. Cardiac compromised patients, such as ones with angina, are a sensitive population to the effects of
Nitrogen dioxide (NO$_2$) is well known to cause lung function decrements and increase airways hyperresponsiveness, especially in asthmatic individuals. Comprehensive reviews of CO and NO$_2$ toxicity have been published by the U.S. EPA (U.S. EPA 2000; U.S. EPA 2008).

5. Sensitive and susceptible populations

Human responses to air pollutants are heterogeneous. Certain factors can make an individual sensitive or resistant. Some factors identified that affect the type of response as well as the magnitude of a response include age, genetics (i.e., genotypes), diet, medication, body mass index (BMI), and disease status. Lung function decrements (e.g., the forced expiratory volume exhaled in 1 sec, or FEV$_1$) induced by ozone inhalation are dependent on age in normal healthy individuals (McDonnell et al, 2007); smaller decrements are observed in older individuals compared to adolescents. Nonsteroidal anti-inflammatory medications such as ibuprofen have been shown to attenuate ozone-induced FEV$_1$ decrements, but not lung neutrophil influx (Hazucha et al, 1996). Women with BMI > 25 had greater lung function decrements to ozone exposure (Bennett et al, 2007). Individuals placed on high antioxidant intake had smaller FEV$_1$ decrements than those on placebo regimens (Samet et al, 2001) with less indication of ozone-induced oxidative stress to lung tissue (Sawyer et al, 2008). Individuals with the specific genotypes of glutathione-S-transferase, e.g., the M1 and P1 null types, had augmented nasal ragweed allergic responses such as increased histamine production when PDE PM was instilled in the nose (Gilliland et al, 2004). Diabetics had more hospital admissions for cardiopulmonary illnesses associated with ambient levels of carbon monoxide and coarse ambient PM (PM between 2.5 and 10 um) in Los Angeles (Linn et al, 2000). These are select examples of some factors associated with exaggerated responses of sensitive individuals to certain air pollutants and are not intended as a comprehensive review of susceptible populations.

It is unclear at present which human population may be more sensitive to biodiesel combustion emissions. What is known from sensitivity factors for other air pollutants will assist in designs for examining potentially susceptible groups. Potentially if fatty acids and/or fatty acid fragments are emitted from biodiesel combustion and are deposited in the lung, these substances may induce greater responses and health effects in those individuals with defects in fatty acid metabolism.

6. Future issues and challenges

6.1 Fuel additives

Biodiesel fuel has several classes of substances intentionally added to cover several purposes. Antimicrobials, cold-flow improvers, detergents, corrosion inhibitors, and fuel stabilizers are blended into fuel depending on the need, e.g., storage duration, ambient temperature, etc. Additionally, the possibility of pesticides being unintentionally present in fuel due to residues in the fuel stock being carried through the production process has not been confirmed as well as the possible health implications addressed. As previously mentioned, poor quality fuels have alcohols and/or glycerol present. Hence biodiesel is not solely FAMEs. The combustion of the substances non-FAME components likely contributes to the emissions, but it is unclear whether the combustion products contribute to the toxicity, or modify the toxicity (negatively, additively, synergistically) of the FAME combustion products.
6.2 Fuel blends
Currently biodiesel is primarily used commercially as a 20% blend with petroleum diesel fuel in the United States. It is unclear whether this ratio of biodiesel to petroleum fuel will increase and to what extent. Some vehicles will continue to operate on 100% biodiesel. A potential problem in assessment of biodiesel toxicity is that changing the proportion of biodiesel in blends can alter the amounts of some combustion products emitted in a nonlinear manner. For instance, in changing from 100% to 50% to 0% petroleum diesel fuel (make up fuel being biodiesel), metals changed in the exhaust fairly linearly and predictably, i.e., from 1.0 to 0.9 (i.e., a 10% decrease) to 0.8 (a 20% decrease) relative concentration units, respectively (Brito et al, 2010). However CO and black carbon changed in a nonlinear (concave shaped) fashion, i.e., for CO, from 1.0 to 1.6 to 0.7 relative concentration units, respectively, while volatile organic compounds (VOCs) and PAHs changed in a nonlinear (convex shaped) fashion, i.e., for VOCs, from 1.0 to 0.2 to 0.6 relative concentration units, respectively. Such nonlinear changes in emissions from blended biodiesel make prediction of the combustion product concentrations more difficult, and hence prediction of human responses or health effects harder to characterize if the products affect the toxicity. The potential shapes of the changes in an emission component are presented in Figure 1 below.

Fig. 1. Theoretical changes in an emission component as the proportion of biodiesel changes from 0 (B0) to 100 (B100) %. Linear changes are reflected by straight lines and diamonds, concave and convex changes are reflected by dashed lines and circles, and sigmoidal changes are reflected by dashed lines and rectangles.
6.3 Evolving fuel standards and engine technology
As fuels evolve, emissions will also change. For instance, petroleum diesel fuel sulfur content for onroad use has decreased in most countries, resulting in lower PM exhaust concentrations. As mentioned previously, petroleum diesel is currently blended with biodiesel in the U.S and other countries; hence changes in fuel components will likely affect emission components. This constantly changing fuel composition will be driven by requirements for meeting specific standards. The first national biodiesel specification in the USA was the ASTM standard D 6751, “Standard Specification for Biodiesel Fuel (B100) Blend Stock for Distillate Fuels”, adopted in 2002. Findings from toxicology studies using fuels created before current standards in affected countries will likely have different emissions, and possibly different health effects and responses. For instance, Brito et al, 2010 used petroleum diesel fuel containing ethylated (not methylated) fatty acids and relatively high sulfur content (500 ppm) in their studies. This is likely due to the fuel being produced in Brazil with abundant ethanol production and standards allowing higher sulfur content in petroleum diesel. A potential major issue is whether fuels derived from 3rd generation feedstock, such as algae, produce a different fuel than those of 1st or 2nd generation feedstocks, and if so, do the emissions change considerably along with biological responses being altered.

6.4 Risk assessment
The attractiveness of biodiesel in part stems from lower emissions of some pollutants such as PM and CO, and additionally lower mutagenic potential associated with the PM phase relative to petroleum diesel emissions. However some studies report increased inflammatory mediator release (Swanson et al, 2009), and increased cell death (Bunger et al, 2000). These health effects need to be examined in the context of the amount of pollutant emitted per mile or unit work as biodiesel replaces petroleum diesel. Health effects may need prioritization based on the degree of adversity, reversibility of the effect, and proportion of the general population and also potentially sensitive population(s) exposed to biodiesel exhaust.

7. Challenges
There are several challenges ahead for assessing the implications of increased biodiesel end use. The toxicology of what is emitted from combusted biofuels needs more establishment. This establishment would be aided if reproducible study designs could be established. In part, experiments could be fairly similar with exposures using the same atmospheres. Hence, standardized biodiesel fuels, use of engines with a large market penetration to simulate what most individuals may be exposed to, and several similar endpoints (e.g., mutagenicity, lung inflammation, vascular and cardiac changes) should be incorporated. The appropriateness of whole animal and cultured cells models to human exposures and effects will need to be established, as is currently being determined in the field of PDE toxicology. Included in the validation of nonhuman models would be extrapolation of effects are relatively high doses to low dose exposures of humans, especially if sensitive human populations to biodiesel exhaust are identified. The toxicology of PDE has been advanced to some extent with the creation of some standardized PDE particles to use as an internal control condition, such as those Standard Reference Materials (SRMs)
at the National Institutes of Standards and Technology in Gaitherburg, MD (http://www.nist.gov/). The bioactivities of the biodiesel gas phase and PM phase are still largely unknown, so research effort at present must be put into both phases in order to eventually determine if adverse health effects exist, and if so, which phase to manipulate to effect fewer effects. Studies are still scant where health effects and biological responses have been measured when individuals are exposed to whole biodiesel exhaust. Only one study is currently published, though a few are underway currently, or have finished and are awaiting publication. Now would be an opportunistic time to design and implement studies, especially in an occupational setting, as biodiesel fuels replace petroleum based fuels. Data can be collected from workers in regards to possible adverse symptoms and other health effects induced by PDE, and similar endpoints at a later time after biodiesel is introduced into the workplace. A final challenge ultimately will be to incorporate the knowledge of human health effects induced by exposures to combusted biodiesel emissions into a comprehensive strategy for management of 1) issues related to increased biodiesel production (soil use, production, transport and distribution) and 2) issues related to future energy production in general, such as how well biodiesel measures up to other fuel alternatives (ethanol, butanol, wind, solar, nuclear, etc) in terms of feasibility and public health impacts.

8. Disclaimer

This manuscript has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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10. References


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This book entitled “Biodiesel: Quality, Emissions and By-products” covers topics related to biodiesel quality, performance of combustion engines that use biodiesel and the emissions they generate. New routes to determinate biodiesel properties are proposed and the process how the raw material source, impurities and production practices can affect the quality of the biodiesel is analyzed. In relation to the utilization of biofuel, the performance of combustion engines fuelled by biodiesel and biodiesels blends are evaluated. The applications of glycerol, a byproduct of the biodiesel production process as a feedstock for biotechnological processes, and a key compound of the biorefinery of the future is also emphasized.

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