Impact of VOC removal by activated carbon on biodegradation rates of diesel,
Syntroleum and biodiesel in contaminated sand

Agota Horel^{1,2} and Silke Schiewer^{2,*}

¹ Institute of Soil Sciences and Agricultural Chemistry, Centre for Agricultural Research, Hungarian
Academy of Sciences, Herman O. 15, Budapest 1022, Hungary

² Civil and Environmental Engineering Department, Water and Environmental Research Center,
University of Alaska Fairbanks, P.O. Box 755900, Fairbanks, AK 99775-5900, USA

* Corresponding author: sschiewer@alaska.edu, Phone: +1-907-474-2620, Fax: +1-907-474-6087

ABSTRACT

The degradation of conventional diesel (D), synthetic diesel (Syntroleum), and pure fish biodiesel (B100) by indigenous microbes was investigated in laboratory microcosms containing contaminated sand. The fate of volatiles and the influence of volatilization on degradation rates were examined by placing activated carbon (AC) in microcosm headspaces to sorb volatiles. Three AC regimes were compared: no activated carbon (NAC), regular weekly AC change (RAC), and frequent AC change (FAC), where the frequency of activated carbon exchange declined from daily to weekly. Generally, the alternative fuels were biodegraded faster than diesel fuel. Hydrocarbon mineralization percentages for the different fuel types over 28 days were between 23% (D) and 48% (B100) in the absence of activated carbon, decreased to 12% (D) – 37% (B100) with weekly AC exchange, and were further reduced to 9-22% for more frequent AC change. Sorption of volatiles to AC lowered their availability as a substrate for microbes, reducing respiration. Volatilization was negligible for the biodiesel. A mass balance for the carbon initially present as hydrocarbons in microcosms with activated carbon in the head space was on average 92% closed, with 45-70% remaining in the soil after 4 weeks, 9-37% mineralized and up to 12% volatilized. Based on nutrient consumption, up to 29 % of the contaminants were likely converted into biomass.

Keywords: Biodegradation; volatilization; biodiesel; degradation rates; activated carbon

1. Introduction

Microorganisms in soils can adapt to changes in the environment such that if a hydrocarbon fuel spill occurs, some are able to use the contaminant as their main carbon source, and natural degradation and contaminant removal begins to take place. Natural biodegradation is one of the cheapest

contaminant removal options; however it may take a long period of time, especially in cold environments (Leahy and Colwell 1990; Horel and Schiewer 2009).

The biodegradation rate of a specific contaminant depends on the surrounding environmental conditions such as temperature, soil moisture content, type of soil (Horel and Schiewer 2009) and the amount of contaminant present (Horel et al. 2015). Measuring the respiration rate as CO₂ production is a well-accepted method for determining microbial activity in aerobic microbial remediation systems. However, not all of the initially present hydrocarbons are converted to carbon dioxide or remain in the soil; Horel and Schiewer (2009) found that 13 to 67% of the initial substrate remained unaccounted for after 17 weeks. Therefore the present study investigated the role of volatilization in overall hydrocarbon removal from soil to better close the carbon mass balance and help understand the different processes during bioremediation of a contaminant. In hydrocarbon degradation experiments, the short-chained alkenes can be highly toxic to many microorganisms; however low molecular weight hydrocarbons can also evaporate fast into the atmosphere (Atlas and Bartha 1998). The most easily degradable hydrocarbons are the relatively shorter chained compounds, which enable fast microbial colony growth and for this reason are an important component of the biodegradation process.

Activated carbon (AC) is commonly used for volatile organic compound (VOC) removal from soil (Gong et al. 2007; Arora et al. 2011), groundwater (Ayotamuno et al. 2006), and air (Ferraz 1988). AC is considered as a good adsorbent due to its large surface area (Khan and Ghoshal 2000), which can be used to remove different types of contaminants from different media. Therefore AC in the head space can be a useful tool to measure the volatilization of light hydrocarbons in laboratory experiments. However, AC adsorbs not only some of the volatile hydrocarbon compounds

but also some moisture (McCallum et al. 1999; Cal et al. 1997), both of which might reduce microbial contaminant degradation in laboratory setups. The absorptive potential of the AC depends on the relative humidity of the surrounding air, and the co-adsorption of water can also influence the adsorption capacity of AC for organic chemicals (Qi et al. 2000). Temperature can influence both the diffusion coefficients and the sorption of contaminants onto AC, especially for granulated activated carbon (Arora et al. 2011).

In the present research, three fuel types were investigated: diesel (D), arctic grade Syntroleum (S), and processed fish biodiesel (B100). The arctic grade Syntroleum fuel used here was produced from natural gas and processed through the Fischer–Tropsch gas-to-liquid technique. Syntroleum fuel is less toxic than conventional diesel due to its minimal content of aromatics, sulfur, and heavy metals (Federal Transit Administration 2007). Syntroleum is comprised of 82% iso-paraffin and 18% n-paraffin; in the absence of aromatics the fuel slows the molecular growth to higher PAHs (Kumaran and Sadr 2012). Initial research (Horel and Schiewer 2009) showed that Syntroleum is more degradable than conventional diesel fuel due to its higher content of straight aliphatics (C_{8–28}), which are typically more easily degradable (Rojo 2009) than branched or cyclic hydrocarbons present in conventional diesel fuel. In prior soil microcosm studies fish biodiesel and its blends showed better biodegradability compared to conventional diesel fuels (Horel and Schiewer 2011; Horel et al. 2015).

The aim of the present study was to investigate the contribution of volatilization to hydrocarbon removal and its impact on respiration by conducting experiments with and without the presence of AC. Microbial hydrocarbon degradation in soils was studied for the three fuel types. The hypotheses of this research were that volatilization could on the one hand reduce hydrocarbon levels

in contaminated matrices by transferring the contaminant to the air. On the other hand, however, sorption of easily degradable volatiles to AC could reduce biodegradation by microorganisms by removing some of these highly degradable substrates. AC addition can be used to study to what extent volatilization to the open atmosphere may occur, and how that affects overall contaminant removal rates. With this comprehensive approach, this study aims to quantify the contribution of different mechanisms to overall hydrocarbon removal from soil and establish a more comprehensive carbon mass balance for contaminant removal from soils and sediments.

2. Experimental section

2.1. Standard experimental setup with or without activated carbon

The rate of hydrocarbon degradation was studied as a function of time for the three different fuel types: arctic-grade Syntroleum (S), conventional diesel (D), or fish biodiesel (B100) under favorable conditions, as determined in previous studies (Schiewer and Niemeyer 2006; Horel and Schiewer 2009). The biodiesel was processed as described by Horel and Schiewer (2011).

Each airtight 2.5 L microcosm setup received 1000 g of previously uncontaminated coarse to very fine sand with less than 0.3% clayey material. The soil properties, determined as previously described by Horel and Schiewer (2009, 2011), were the following: bulk density 1.4-1.6 g/cm³, bulk porosity 40-49%, hydraulic conductivity 2.6 10⁻² cm/s, 0.0054% total nitrogen, 0.165% total carbon, air dried with approximately 1% residual soil moisture, and pH of 7.5 (±0.2).

Quantified amounts (2000 mg kg⁻¹ dry weight soil) of the selected contaminant type (i.e. diesel fuel, Syntroleum or B100) were added to the soil surface, except for the uncontaminated blank for which no hydrocarbons were added. Approximately 2000 mg of soil with known existing microbial cultures from previous experiments that were already adapted to diesel fuel hydrocarbons

was added to provide an inoculum of microbes. The added inoculum contained approximately 1.6×10^4 hydrocarbon degraders per g soil, quantified by using the Most Probable Number method (Wrenn and Venosa 1996; de Man 1983). Fertilizer of the type 20–20–20 (N–P₂O₅–K₂O, where the total nitrogen ingredients were: 20% ammonia N, 30% nitrate N, and 50% urea nitrogen) was added to the microcosms at a resulting concentration of 300 mg N kg⁻¹ to provide sufficient nutrients for microbial growth (Schiewer and Niemeyer 2006; Horel and Schiewer 2009). The soil was not mixed after or during these additions to better represent natural conditions after a spill on the soil surface.

The samples were incubated at 20°C and data were collected during a 28 day period. In order to determine the amount of hydrocarbons remaining in the soil as a function of time, some microcosms were terminated in weekly intervals for subsequent soil analysis by gas chromatography – mass spectrometry (GC/MS, section 2.4). All treatments were prepared in four replicates at the beginning of the experiment. Every seventh day one setup per fuel type was terminated to analyze the amount of hydrocarbons remaining in the soil.

General soil analyses were performed for total nitrogen and carbon and also to determine the total extractable inorganic nitrogen in the soil samples. Using an elemental combustion system (Costech Instruments), the average percent of total nitrogen in the original sediment (before nutrient addition) was less than 0.006 % and the fraction of total carbon was 0.165 %. A Dionex DX 500 ion chromatograph with PeakNet software was used to determine the total extractable ionic nitrogen species.

Three variations of this laboratory setup were used in order to investigate the effect of AC on biodegradation and quantify evaporation of volatiles: i) no activated carbon was used (NAC); ii) regular AC change (RAC) where AC was placed in the jar's headspace and changed in every 7th day;

and iii) frequent AC change (FAC) where the frequency of changing AC was reduced over time, i.e. daily during the first week, every two days during the second week, twice during the third week and once during the 4th week. The exchange frequency was reduced as the release of volatiles was expected to decrease over time. In experimental setups where RAC or FAC were used, 10 g AC was placed at the top of each microcosm's headspace to capture volatile compounds from the fuels. At the time intervals specified above, the activated carbon was replaced by fresh AC. The amount of fuel volatilized was estimated by GC/MS analysis after extraction with methanol, as described in section 2.4.

In a separate 5 week long experiment, mass-based hydrocarbon volatilization for different fuel types was compared by monitoring the weight loss as a function of time from an open jar containing 5000 mg of a specific fuel without soil or activated carbon.

2.2. *Respiration measurements*

Carbon dioxide production was measured as an indicator for contaminant mineralization by aerobic processes. The containers were opened once daily for re-aeration and to measure the evolved CO₂ based on the method described by Andersen (1982).

Though there are some limitations (Horel and Schiewer 2009), CO₂ production can be linked quantitatively to the degradation of hydrocarbons, using the following stoichiometric equation, assuming a C:H ratio of CH_{2.12} for Syntroleum (Bergin 2004) or CH_{2.54} for #2 diesel fuel (Curl and O'Donnell 1977):

2.3. *Modeling*

Since previous research (Horel and Schiewer 2009, 2014) under similar conditions had shown that the first order model, where the degradation rate is proportional to the substrate concentration, fit well if separate rate constants were determined for two or three phases, this approach was used in the present study. The first order model is described by

$$C_t = C_0 e^{-k t} \quad \text{eq. 2}$$

where C_0 is the initial contaminant concentration, k is the rate constant and C_t is the remaining contaminant (i.e. substrate) concentration at any given time t . To calculate C_t , respiration data were used rather than GC/MS measurements, which rely on a destructive sampling method and could not have been obtained on a daily basis. Overall rate constants were determined via linear regression. The lengths of phases were determined by visual identification of sudden changes in the slope of the 1st order plot. The rate constants for each phase were determined by nonlinear fitting, minimizing the root mean square error (RMSE) between model prediction and experimental data as described by Horel and Schiewer (2009).

2.4. *Gas Chromatography – Mass Spectrometry analysis of hydrocarbons in soil*

To evaluate the relationship between substrate use and CO₂ production, GC/MS analysis was performed and compared to respiration data. The soil was extracted with methylene chloride solvent as described by Horel and Schiewer (2011). To determine the diesel range organics (DRO) remaining in the soil samples, a modified method based on the AK 102 (Alaska Department of Environmental Conservation 2002) and EPA 8270 (semi volatile organics by GC–MS) methods was used (Environmental Protection Agency 1996). For the volatile organic compound analysis the EPA

8260C (Volatile Organic Compounds by Gas Chromatography/Mass Spectrometry) method was used. The GC/MS analysis (Agilent Technologies 6890N Network GC System coupled to a 5873 mass selective detector with 30 m by 250 μ m by 0.25 μ m column) used splitless injection with helium carrier gas (pressure: 6.895 kPa; flow: 0.5 mL min⁻¹; average velocity: 26 cm s⁻¹). The oven temperature increased from 40°C to 320°C for the duration of 87 min to ensure no carry-over contamination between different samples. Surrogate (p-terphenyl-d14 with a concentration of 4000 μ g mL⁻¹) was used to evaluate the extraction efficiency and internal standards (Perylene-d12 with a concentration of 5000 μ g mL⁻¹) were used to verify instrument reliability after being in use for a longer time period. Recovery data analysis was performed for each fuel type by adding a known amount of fuel to the soil and extracting immediately. The highest recovered hydrocarbon amounts were between 93% and 104% for each fuel type and these values were used as reference values of 100% for each fuels for the GC-MS data analysis instead of the theoretical value.

2.5. *Statistical analyses*

The statistical analysis used to compare treatments was a two tailed, paired, t-test and single factor ANOVA within the different treatment groups. When significant differences occurred, Tukey's honestly significant difference (HSD) post hoc tests were used to determine significant interactions. The statistical significance in the analysis was defined at $P < 0.05$.

3. Results

3.1. Effect of AC addition and fuel type on daily mineralization rates

Daily CO₂ production for the different fuel types is compared in **Fig. 1** for two AC change regimes (note the lower y-scale for FAC). B100 and Syntroleum had noticeably higher respiration values, compared to the diesel fuel contaminated samples. Overall B100 had the highest cumulative CO₂ production (Fig. 1c). While for RAC the B100 peak occurred shortly after the Syntroleum peak, for FAC the B100 peak occurred much later.

Fig. 2 compares the data with emphasis on the different AC change regimes. Over the course of the experiment, the highest respiration was generally observed in the absence of activated carbon. For Syntroleum and B100 (Fig. 2b and c) the respiration peaks for RAC occurred at a similar height and time, after about one week, as for NAC, with a faster decline and somewhat lower respiration values for RAC (Fig. 2). For each fuel type, with increasing AC change frequency (FAC) daily respiration values decreased noticeably for the first three weeks (Fig. 2), most distinctively in case of B100 treatment (Fig. 2c).

In general, significant differences ($P < 0.05$) in daily CO₂ production were observed when comparing AC change regimes (NAC vs. RAC vs. FAC) for a given fuel. Also significant differences were noted when comparing daily CO₂ production for different fuels for a given AC exchange regime ($P < 0.05$).

3.2. Duration of growth phases and corresponding rate constants

For the first 3-4 days, respiration was low and the fuel type (Fig. 1) or the AC exchange frequency (Fig. 2) had no extensive impact on respiration rates. During this initial phase, respiration rates for

all microcosms were quite similar, averaging around 20 mg CO₂ per day. To identify phase lengths and for determining the overall mineralization rate constants, linearized first-order plots based on equation (2) were used (**Fig. 3**). Fig. 3 shows that substrate degradation was initially slow, which corresponds to a low rate constant according to equation (2). The mineralization rate constants and microbial growth phases based on first-order kinetics are summarized in **Table 1**. The duration of the initial slow phase, which will be called lag phase for simplicity (though some degradation takes place during this period) was around 4 days in most cases (Table 1), after which the slope in Fig. 3 became steeper, indicating the end of the lag phase.

In the subsequent exponential phase, microbial growth and contaminant degradation occur at substantially higher rates than in the lag phase. The length of the exponential phase was shortest (7 d) for Syntroleum with NAC, averaged about 10 days (Table 1), and was longest for B100 NAC, where it extended till the end of the experiment.

For NAC and RAC, three growth phases (lag, exponential, stationary) were generally observed. The experimental time period was long enough for the microorganisms to enter the stationary or even the death phase where respiration rates decreased, in the present study after about 2 weeks, as shown in Fig. 3 by the decreasing slope. Only for B100 with NAC, the exponential phase continued till the end of the experiment (Fig. 3c). Consequently, a two-phase model was applied for B100 with NAC, while for the other NAC and RAC data a three-phase model could be applied. The three phase model fit the data very well as shown by the lines in Fig. 3a, b, c and the low RMSE in Table 1. For comparison, if one overall rate constant was used to describe data over the entire 28 day duration, the modeling error was more than 5 times as high as for the three phase model (last column in Table 1).

For FAC, respiration data showed an unusual microbial phase progression, where a slow initial phase with similar rates as for RAC and NAC (note overlapping data in Fig. 3a-c for the first 5 days) was followed by a faster 2nd phase, a slow 3rd phase and a fast 4th phase as shown in Fig. 3d, which summarizes all FAC data. Though a 4-phase model would have led to a slightly better fit, a 3-phase model was chosen to describe the data in order to be consistent with the RAC and NAC modeling, and it generally fitted very well. To apply a 3 phase model for FAC, the initial lag phase and subsequent faster phase were lumped into one, therefore the seemingly longer duration of the first phase (in this case not a regular lag phase) in Table 1. The FAC rate constants for that initial lumped phase were of similar magnitude but somewhat higher than lag phase rate constants for NAC and RAC.

Phase 2 rate constants increased on average by a factor of 7.4 (± 3.71 SD) from FAC to RAC and by another factor of 1.2 (± 0.43 SD) from RAC to NAC. The low phase 2 rate constants for FAC are in part due to the unusual phase progression, where phase 2 represents a slow phase. However overall rate constants k_o also increased strongly with decreasing AC change frequency. On average, k_o doubled from FAC to RAC, and further increased by 50% from RAC to NAC (Table 1).

3.3. *Fraction of hydrocarbons mineralized*

The mineralization percentage at the end of the experiments was calculated from the cumulative CO₂ production multiplied by a factor of 14 g fuel used per mole of CO₂ produced, based on eq. (1). The calculated mineralization percentage is shown in **Fig. 4a** for different fuel types and AC exchange regimes. Since respiration was very low in uncontaminated blanks (1.6 - 5.6 mg CO₂ day⁻¹, resulting in 92 mg CO₂ per 28 days, compared to 530-3044 mg CO₂ for samples with fuel contamination),

mineralization percentages in Fig. 4a were based on the assumption that all CO₂ released resulted from degradation of the contaminant.

For all fuels, the total mineralization decreased in the order of NAC > RAC > FAC. The percentage of mineralization for FAC was only approximately half or less of that observed for NAC (Fig. 4a). The overall mineralization percentages showed significant differences between AC change regimes ($P < 0.032$). The fraction of hydrocarbons mineralized after 4 weeks was the greatest for B100 NAC with approximately 50% (Fig. 4). The lowest mineralization was observed for diesel fuel with FAC, where less than 10% of cumulative mineralization was observed.

3.4. Nutrient Availability

The percentage of nitrogen depletion from soil was calculated from the sum of recovered extractable nitrogen concentrations (nitrate and nitrite) in the soil, measured as described in section 2.1, and the initial nitrogen concentration of 300 mg/kg according to equation (3).

$$\% \text{ N used} = 100 (N_{\text{initial}} - N_{\text{recovered}}) / N_{\text{initial}} \quad (\text{eq 3})$$

Nitrogen depletion at different AC change intervals for the different fuel types was generally highest for B100 and lowest for diesel (Fig. 4b). Nitrogen depletion more than doubled from FAC to RAC. Nitrogen consumption again approximately doubled from RAC to NAC.

3.5. Volatilization

In past research, the carbon mass balance for the total hydrocarbons present was established based on carbon from cumulative CO₂ production, plus carbon still present in soil based on GC-MS analysis, plus “other”, which was not be accounted for by the previous categories (Horel and Schiewer 2009). This unaccounted amount could be due to volatilization, incomplete degradation, or

incorporation into new microbial biomass; however, 100% recovery cannot be expected due to experimental variability and measurement errors.

Volatilization for diesel and synthetic diesel fuels amounted to 12% and 10%, respectively, after 28 days, while fish biodiesel showed no or negligible mass-based volatilization (**Fig. S1** of supporting information). Diesel volatilization occurred fastest during the first few days; within the first week more than 6% of the total hydrocarbon volatilized, while during the fourth week volatilization increased only by 1.3 percentage points. Syntroleum on the other hand showed a continuous, almost linear volatilization rate over time (Fig. S1).

Based on GC/MS analysis of volatile compounds sorbed by activated carbon in the headspace over the entire 4 week experiment (Fig. S2 of supporting information), volatilization for RAC microcosms reached about 5% for diesel and Syntroleum, while no VOC were recovered for B100 (Fig S2), which is consistent with the results of Fig. S1.

3.6. *Hydrocarbon mass balance*

The carbon mass balance based on microcosms sacrificed for GC/MS analysis in weekly intervals is shown in **Fig. 5** for diesel, Syntroleum and B100 with RAC or FAC. For all fuels, the carbon mineralized to CO₂ more than doubled from week one to week two, and the diesel range organics (DRO) remaining in the soil were reduced by approximately 10 percentage points during the second week (Fig. 5). Smaller changes occurred during later weeks.

DRO remaining in the soil for RAC after 4 weeks was 57% for diesel and noticeably lower (around 46%) for the alternative fuels. This implies that about half of the initially present hydrocarbons had been removed from the soil within 4 weeks.

Volatilization was negligible for B100, but accounted for approx. 10% of diesel and Syntroleum removal after 4 weeks.

4. **Discussion**

4.1. *Effect of AC on degradation rates*

The earlier and higher respiration peaks observed for diesel and Syntroleum with NAC (Figure 2) are likely due to degradation of short chain hydrocarbons, which were not available to the same extent for RAC, due to sorption by activated carbon.

For FAC, respiration around the second and third week was considerably less than for NAC and RAC, with a lower maximum of CO₂ production occurring after approximately one week (Fig. 2). Due to adsorption, lower quantities of easily degradable short chained hydrocarbons were available when AC change occurred frequently. However, after two weeks, when the frequency of

AC change for the FAC setup was reduced (twice per week during the third week and once in the 4th week), the FAC daily respiration rates increased for all fuel types (Fig. 1b) and exceeded that of RAC by the end of the experiment, reaching similar or higher levels as for NAC (Fig. 2). Even though daily soil respiration increased for FAC towards the end of the experiment, it never reached the peak amount measured for NAC or RAC (Fig. 1 and 2). In general, the increase in soil respiration after two weeks can be explained by the lower frequency of AC change in later weeks.

However, this cannot explain the pronounced second maximum on day 26 for fish biodiesel (Fig. 2c), which was not observed for other fuels. The occurrence was likely due to additional degradation by fungal colonies, especially for fish biodiesel. In the present experiment, for FAC and RAC, fungal colony growth could be visually observed in later weeks, most distinctly for fish biodiesel. For diesel fuel on the other hand, no visible fungal colonization was observed. This finding supports the assumption that for biodiesel, degradation by fungi may have contributed to the high CO₂ production in later weeks, when less volatiles and less moisture were removed by activated carbon (Fig. 1b and 2). For biodiesel with RAC, the lower substrate levels in later weeks (Fig. 3c) may be the reason why, in spite of fungal growth, no such increase in respiration was observed as for biodiesel with FAC (Fig. 2c). Fungal colonization was observed earlier for RAC than for FAC.

Fungi are able to utilize volatile compounds as their carbon source; as Qi et al. (2002) observed, fungal species can grow on ceramic support media and use volatile organic compounds as their sole carbon source for energy. This indicates that fungal growth would be lower in the absence of volatiles and lowered air moisture in the experimental headspace. While removal of volatiles may be an explanation for reduced fungal respiration in the present study for Syntroleum-spiked

microcosms with FAC, this is likely less important for B100, where volatilization was negligible, The role of fungi is the subject of a further study by the present authors.

4.2. Durations of growth phases and corresponding rate constants

During microbial degradation of hydrocarbons, commonly four distinctive microbial growth phases can be observed: the lag, exponential, stationary, and death phases. During the bioremediation of petroleum hydrocarbons, the first two phases are especially important, as short adaptation periods and longer exponential phases can mitigate the harmful effects of the contaminant (Horel and Schiewer 2014). Lag times can vary substantially, being greatly influenced by the contaminant type as well as the sediments' physical, chemical, and biological parameters (Hamamura et al. 2006; Horel and Schiewer 2014). Labud et al. (2007) found that in diesel contaminated sandy soils the microbial adaptation periods can be under 20 days depending on soil physical properties, while Mortazavi et al. (2013) observed adaptation periods of less than one day when crude oil contaminated sand was investigated.

The length of the first growth phase with low degradation rates may also depend on the initially present number of hydrocarbon degraders. In the present study, the uncontaminated soil at the beginning of the experiment had a total petroleum hydrocarbon (TPH) degrader microbial density between 1.81×10^3 and 3.62×10^4 per g soil. This number is similar to other studies completed in warmer environments, such as in sediments in the Gulf of Mexico (Horel et al. 2015a). However even in cold environments, a high microbial density can be observed. For example, Coulon et al. (2005) investigated diesel and crude oil degradation in contaminated sub-Antarctic soil samples, where the uncontaminated samples had an estimated 2.2×10^5 MPN g⁻¹ soil hydrocarbon degrading

microorganisms, which is approximately a magnitude higher compared to the current research. After adapting to the contaminant, the density of these different microorganisms is expected to increase substantially based on preferential fractions of hydrocarbons being utilized as carbon sources. In the present study, Syntroleum contaminated sand had between 1.94×10^4 and 4.54×10^5 TPH degraders compared to between 3.39×10^3 and 6.40×10^5 TPH degraders in diesel contaminated samples after a few weeks.

Microbial lag phase durations also depend on the type of carbon source present in the soils. Lisiecki et al. (2014) investigated different microbial abundances and their density with respect to biodiesel blend addition to diesel fuel in saturated media. The authors found that with increasing biodiesel amount, some microbial groups, such as *Achromobacter* and *Comamonadaceae* were suppressed, the abundance of *Citrobacter* increased, while *Alcaligenes* and *Sphingobacterium* sp. density were unaffected by the fuel types (Lisiecki et al. 2014). Bückner et al. (2011) investigated the growth of different types of fungal species (such as *Aspergillus fumigatus*, *Rhodotorula* sp. and *Paecilomyces* sp.) using diesel, pure biodiesel, and their blends as carbon sources, where the authors found that even small amounts of biodiesel in diesel fuel resulted in significant fungal growth compared to pure diesel fuel. Similar to our study, slower microbial adaptation was observed in case of diesel fuel (Bückner et al. 2011) compared to biodiesel fuel.

The trends in the microbial phases were at times unexpected in the present study, especially for FAC, where the data did not follow the typical lag - exponential - stationary phase pattern but showed a second phase with high rates towards the end of the experiment. The main cause for this must have been the changes in the frequency of AC exchange over time for the FAC experiment (initially once daily, at the end once weekly), as that was the only major difference between FAC

and RAC. The observed increase in rates during the last week for FAC apparently occurred either because the frequency of activated carbon exchange was reduced such that more of the lower weight hydrocarbons were available for microbial degradation, which may have contributed to the gradual increase in respiration during the last week for diesel or Syntroleum with FAC, or because fungi were introduced in conjunction with more frequent opening of jars for AC exchange, which appeared to be the main cause for the strong respiration increase for B100. For diesel, no fungal growth had been observed. Fungal growth also might help bacterial mobilization, as found by Kohlmeier et al. (2005), where the authors studied motility of hydrocarbon degrading bacteria species along liquids forming around fungal hyphae.

4.3. *Fraction of hydrocarbons mineralized*

The finding that hydrocarbon mineralization was consistently highest in the NAC setups and lowest in FAC setups (Fig. 4a) implies that the addition of activated carbon, especially when changed frequently, interferes with the contaminant mineralization process. One of the main reasons is likely the removal of easily degradable volatile compounds by adsorption to activated carbon, where they are not available for biodegradation, which consequently reduces CO₂ production.

Additionally, removal of moisture by adsorption to AC could generally contribute to lower microbial activity. Cal et al. (1997) found that water adsorption to AC cloths was directly proportional to water vapor present in the headspace. In the present study however, the final moisture contents of all microcosms were similar or higher than the initial moisture content; for FAC the increase in VWC was approx. one percentage point less than for RAC samples (data not shown), which may be due to lower hydrocarbon mineralization to CO₂ and water for FAC.

4.4. *Nutrient assimilation*

Nitrogen usage (Fig. 4b) correlated well with the cumulative percentage of hydrocarbons mineralized in the soil (Fig. 4a), implying that the more hydrocarbons mineralized, the more extractable nitrogen was depleted from the soil (Fig. 4c). This suggests a biological cause for the nitrogen loss; increased nitrogen consumption due to increased microbial activity and growth can explain this observation. The amount of nitrogen used can be converted to an estimated production of biomass with the overall stoichiometric formula C₅H₇O₂N.

$$\% \text{ C converted to biomass} = \% \text{ N used} \frac{300 \text{ mg } N_{\text{initial}}}{1700 \text{ mg } C_{\text{initial}}} \frac{60 \text{ mg } C_{\text{biomass}}}{14 \text{ mg } N_{\text{biomass}}} = 0.75 \times \% \text{ N used} \quad \text{eq. 4}$$

Based on nitrogen depletion, for RAC 10-17% of the initially added carbon could have been converted to biomass according to eq (4) after 4 weeks, depending on the fuel type (Fig. 4b). Estimated biomass production was lowest for FAC (5-7%) and highest for NAC (23-29%). Comparing the fuel types, carbon converted to biomass was highest for B100 (7-29%) and lowest for diesel (5-23%).

4.5. Volatilization

According to an Energy Laboratories, Inc. report (2005), in conventional diesel the more volatile light carbon compounds are approximately between 8% and 11% of total hydrocarbons. The observed mass based volatilization values of 12% for diesel in the present study (Figure S1 of supporting information) were close to those values. Since these data result from experiments without soil or microbial activity, they represent an upper limit to the amount volatilized in the microcosms, where sorption to soil and biodegradation reduce the amount that can volatilize, as it was indeed noted for volatiles captured by activated carbon in microcosms where volatilization was around 5% (Figure S2 of supporting information).

While both diesel and Syntroleum showed substantial volatilization, the missing volatilization for B100 was noteworthy. The lack of measurable volatilization mainly was due to the fuel's chemical characteristics. Oxidation can lead to a higher-molecular-weight species through oxidative polymerization (Knothe 2006). Fish biodiesel reacts with oxygen when open to the atmosphere and during the oxidation process higher molecular weight compounds could have been produced; however some volatilization still can occur due to volatile oxidation products (Wadumesthrige et al. 2008).

4.6. *Hydrocarbon mass balance*

Weekly changes in the hydrocarbon mass balances are plausible, showing increased mineralization and volatilization over time, in conjunction with a steadily decreasing percentage remaining in the soil based on GC/MS data. As shown in Fig. 5, at the end of the four week experiment, about half of the hydrocarbons (30-55%) were removed from the soil, whereby mineralization (9-37%) contributed more to hydrocarbon removal than volatilization (0.2-12%). For B100 volatilization was minimal, while mineralization was the highest compared to the other fuels.

As discussed above, nitrogen depletion from the soil during bioremediation is likely due to incorporation of nitrogen into new biomass; in the present study 10–17% of the initial carbon could have been converted into biomass after four weeks for RAC.

The carbon that was not accounted for in the mass balance by CO₂, DRO in the soil, biomass production, or volatilization, was on average 12% for diesel fuel, 10% for Syntroleum, and 3% for B100 (Fig. 5). For B100, the mass balance slightly exceeded 100% after 3 weeks, indicating most likely overestimation of either new biomass production or the remaining fuel still present in the soil.

In conclusion, the mass balance was more or less closed, on average adding up to 92% of initially present carbon being recovered. Due to experimental variability, it would be unlikely to achieve complete recovery.

5. **Conclusions**

Activated carbon use in the experimental setups influenced the hydrocarbon degradation process for all fuel types. The highest respiration and the highest overall degradation rate constants were observed when no AC was used in the headspace. When the AC was changed frequently, respiration

peaks were lower and the overall degradation rate constants decreased considerably. The lower mineralization probably resulted from AC adsorbing volatile fractions of the hydrocarbons, making them unavailable as a substrate. Degradation of short-chained hydrocarbons was especially important in the brief exponential phase (~ 2nd week), when easily degradable compounds were quickly consumed. For FAC an unusual phase progression was observed, with a 2nd fast growth phase toward the end of the experiment, which may be due to fungal growth, especially for the biodiesel.

Cumulative CO₂ production increased in the order FAC < RAC < NAC, as did nitrogen consumption and the estimated biomass production. After 28 days, mass based volatilization was 12% for diesel and 10% for synthetic diesel, while for fish biodiesel no volatilization was noted. For microcosms with activated carbon between 46 and 70% of the hydrocarbons remained in the sediment after 28 days. This means up to approximately 50% were removed, mostly due to mineralization, except for diesel, where volatilization was the main removal mechanisms. Based on CO₂ produced, DRO recovered by GC/MS from the soil, biomass production (estimated from nutrient consumption), and mass-based volatilization, the mass balance was on average 92% closed.

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Supporting information: The supporting information provides Figs. S1 and S2 which show the volatilization of all three fuels over time.

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Figure captions:

Fig. 1 Daily respiration (CO_2) values for conventional diesel, Syntroleum, and B100 for a) weekly AC change (RAC) b) frequent activated carbon change (FAC); and c) cumulative CO_2 production for RAC. Values shown are means (n=2 to n=4).

Fig. 2 Daily CO_2 production for a) diesel, b) Syntroleum and c) B100 with varying frequency of AC change. Values shown are means (n=2 to n=4). NAC = no activated carbon, RAC = regular weekly activated carbon change, and FAC = frequent activated carbon change.

Fig. 3 Linearized 1st order rate plots for different fuel types and activated carbon change regimes. Data (means of n=2 to n=4) and model predictions with parameters from Table 1. NAC = no activated carbon, RAC = regular weekly activated carbon change, and FAC = frequent activated carbon change. a) diesel, b) Syntroleum, c) B100 and d) FAC data (no model).

Fig. 4 Percentage of a) cumulative hydrocarbon mineralization, and b) nitrogen conversion. after 28 days for the different fuel types with varying frequency of activated carbon change. Values shown are means $\pm$ SD (n=2). FAC = frequent activated carbon change; RAC = regular activated carbon change; NAC = no activated carbon.

Fig. 5 Weekly carbon mass balance for a) conventional diesel; b) Syntroleum; c) fish biodiesel (B100), with weekly activated carbon changes (RAC); except for samples with * symbols (data highlighted in red color), which represent frequent activated carbon change (FAC). Values shown are means (n=2 to n=4).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Table 1 Mineralization rate constants and growth phase durations for degradation of different fuels in 28 day experiment using either a three phase or overall model (means of n=2 to n=4). Varying frequency of AC change: FAC = frequent activated carbon change, RAC = regular weekly activated carbon change, NAC = no activated carbon

Fuel types		3 phase model						Overall		
		Phase duration ^a		Rate constant k^b			error RMSE	Constant k_o^c (d-1)	R^2	error RMSE
		phase 1 (d)	phase 2 (d)	phase1 (d-1)	phase 2 (d-1)	phase 3 (d-1)				
Diesel	FAC	11	12-24	0.0049	0.0019	0.0041	0.0015	0.0032	0.95	0.0077
	RAC	5	7-15	0.0032	0.0065	0.0038	0.0013	0.0050	0.99	0.0045
	NAC	2	3-17	0.0012	0.0129	0.0067	0.0022	0.0105	0.98	0.0117
Syntroleum	FAC	11	12-23	0.0079	0.0022	0.0054	0.0047	0.0047	0.91	0.0150
	RAC	4	5-12	0.0042	0.0238	0.0058	0.0033	0.0117	0.92	0.0296
	NAC	3	4-10	0.0081	0.0288	0.0120	0.0059	0.0162	0.97	0.0258
B100	FAC	11	12-20	0.0053	0.0036	0.0201	0.0036	0.0077	0.88	0.0269
	RAC	5	6-16	0.0036	0.0266	0.0135	0.0039	0.0193	0.98	0.0293
	NAC	5	6-28	0.0044	0.0279	n/a	0.0035	0.0258	0.99	0.0230
Average	FAC	11		0.0060	0.0026	0.0099		0.0052		
	RAC	4.7		0.0037	0.0190	0.0077		0.0120		
	NAC	3.3		0.0046	0.0232	0.0094		0.0175		
	overall	6.3					0.003			0.019

^a Phase durations were determined by visual inspection of linearized first order plot.

^b Rate constants for the different phases were determined by nonlinear fitting.

^c Overall rate constants were determined from linearized rate plot.

Graphical abstract: