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Effect of biodiesel addition on microbial community structure in a simulated fuel storage system

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HIGHLIGHTS

- A microbial community structure was studied in a simulated fuel storage tank.
- CLPP analysis reveals that biodiesel influences carbon consumption patterns.
- 16s rDNA proved that communities growing in biodiesel and diesel were different.
- Pure biodiesel communities were able acidify the culture media.

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ABSTRACT

Understanding changes in microbial structure due to biodiesel storage is important both for protecting integrity of storage systems and fuel quality management. In this work a simulated storage system was used to study the effect of biodiesel (0%, 25%, 50%, 75% and 100%) on a microbial population, which was followed by community level physiological profiling (CLPP), 16s rDNA analysis and plating in selective media. Results proved that structure and functionality were affected by biodiesel. CLPP showed at least three populations: one corresponding to diesel, one to biodiesel and one to blends of diesel and biodiesel. Analysis of 16s rDNA revealed that microbial composition was different for populations growing in diesel and biodiesel. Genera identified are known for degradation of hydrocarbons and emulsifier production. Maximum growth was obtained in biodiesel; however, microbial counts in standard media were lower for this samples. Acidification of culture media was observed at high biodiesel concentration.

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1. Introduction

Biodiesel is an alternative fuel that can replace diesel partially or completely. It is produced by trans esterification of fatty acids with an alcohol (usually methanol) in the presence of a catalyst. A recent report indicated that its production increased 169% going from 326 to 878 million gallons between 2009 and 2011 in the United States alone (U.S. Energy Information Administration, 2012). This increase in production has been driven by growing concerns on the stability of petroleum supply as well as by volatility of the price of crude oil. This trend has led to a transition for the diesel industry from 100% diesel to blends at different ratios with biodiesel. In Europe, the objective is to reach a 10% replacement by 2020 (Sørensen et al., 2011) and in Canada there is already a requirement for a 2% blend.

Research on the effect of biodiesel on microbial population structure has been mainly focused in biodegradation of biodiesel, with an emphasis on bioremediation (Mariano et al., 2008; Owsianiak et al., 2009; Silva et al., 2012). However, there is little reported research on the effects that biodiesel may have on the microbial populations developed in storage and transport infrastructure. The few studies available do not present conclusive evidence on the effect of biodiesel over microbial community structure (Klofutar and Golob, 2007; Chao et al., 2010; Cyplik et al., 2011; Sørensen et al., 2011). On the one hand both Sørensen et al. (2011) and Klofutar and Golob (2007) found respectively by denaturing gradient gel electrophoresis (DGGE) and plating techniques that the dominant groups of microorganism in a fuel system were influenced by biodiesel addition, on the other hand Cyplik et al. (2011) found by quantitative PCR of selected groups that a consortium isolated from soil remains stable independently of the biodiesel concentration used as carbon source. This lack of agreement in results can be explained if it is considered that the source of the communities used in these studies was different, while the first one used a community obtained from a diesel storage tank, the second one used a more diverse soil community. Although the results can be community dependent the question

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for the effect of biodiesel on microbial structure remains open for discussion and more research is necessary in order to gain a deep insight of the problem.

Microbial communities are usually developed at the bottom of fuel storage tanks due to the presence of water, which accumulates as a product of condensed environmental moisture when temperature drops (Klofutar and Golob, 2007). This microenvironment is well suited for growth of microorganisms able to use either olefins or aromatics as carbon sources. Presence of biodiesel in storage tanks may enhance microbial activity and lead to a change in ecosystem composition. An increase in microbial activity once biodiesel is added is expected because its higher bioavailability and hygroscopicity (Sørensen et al., 2011). An impact on the kind of microbial populations is also expected because blending of diesel and biodiesel changes the chemical nature of the fuel.

Typically, microbial communities can be studied by using three different strategies: culturing of microorganisms in selective media, analysis of community level physiological profiles (CLPP) or thirdly, the analysis of data from the 16/18 sRNA gene generated by PCR using universal primers. Culture in selective media is a classic approach; however, it has the limitation that only 1 5% of the microorganisms present in the environment can growth in synthetic culture media (Head et al., 1998), and so the results obtained are skewed to microorganisms able to grow in the culture media used. CLPP is a technique that uses a microplate containing a number of different carbon sources, microbial communities are inoculated directly in the plate and differences between them are determined based in the pattern of carbon utilization. Although CLPP is also skewed for microorganisms able to growth in culture it has proved to be very effective for studying shifts in microbial populations as a result of ecological perturbations (Weber et al., 2008); however, the technique does not give an indication of the kind of microorganisms that are present in the population. Finally, amplification of 16 sRNA sequence has been used as a strategy to identify the kind of microorganisms present in a population. PCR products can be sequenced or run in a denaturing gel to generate a fingerprinting. This approach is very labour intensive nevertheless it is useful when the identities of the microorganisms are required. Tracking changes in fuel storage tanks will require the simultaneous use of these techniques in such a way that the maximum amount of metabolic and genetic information is obtained and relevant conclusions can be made both in for the composition and metabolic capabilities of the community under study.

In this work a simulated fuel storage tank (mesocosm) is used to study the effects of biodiesel addition on microbial structure and function in a community obtained from a diesel storage facility. Changes and evolution of the microbial population were tracked with a combined strategy using the three approaches described above: community level physiological profiling, construction of libraries for the 16s RNA gene and culturing in selective media. Growth and pH were also followed during the course of the experiment.

2. Methods

2.1. Experimental set up

A mesocosm was designed to simulate the bottom of a fuel storage tank in which an excess of water was accumulated as product of condensation. An Erlenmeyer flask (500 ml) was prepared containing 50 ml of Richard and Vogel's culture media (All chemicals were obtained from Sigma Aldrich, St. Louis, USA) at pH 7 (Richard and Vogel, 1999), 50 ml of a water sample collected from the bottom of a tank used for diesel storage (2 L of water were collected during the spring of 2012 from Imperial Oil Ltd. at their operational facilities in Sarnia, ON) and 100 ml of a diesel/biodiesel blend. Two immiscible phases were developed, one containing water and hydrophilic compounds, with the top phase containing the less dense fuel. Experimental units with 0%, 25%, 50%, 75%, 100% (v/v) of biodiesel were evaluated. Experiments were carried out for 200 days continuously and microbial growth with composition was analyzed. Samples were prepared in triplicate.

The Richard and Vogel's media is a mixture of mineral salts that fulfill minimum requirements of nitrogen, phosphate and microelements; the only carbon source available was the fuel blend that diffuses to the water layer. The water sample that was added contained an inoculum with a real microbial population metabolically adapted for the use of diesel as a carbon source. The system was kept in darkness at 25 °C without aeration; however, oxygen diffusion was allowed. Fuels were sterilized by means of a 0.2 μm filter (Millipore, Billerica, USA,), and the culture media was sterilized at 15 psig and 121 °C for 15 min.

2.2. Properties of the broth

Microbial growth was measured as an increase in the optical density at 590 nm of the water layer. Tests were performed with a plate reader (Infinite M200 pro, Tecan Group Ltd., Seestrasse, Switzerland) at 0, 50, 75, 100 and 200 days of storage. Acidity of the broth was determined with a pH meter (Phi 40 pH meter, Beckman Coulter Inc., Indianapolis, USA) at 50, 75, 100 and 200 days. Statistical analysis was performed using a mixed model (included analysis of fixed and random effects) using the statistical package SAS Version 9.3 (SAS Institute INC., Cary, USA). The structure of variance was chosen among a compound symmetric, unstructured or autoregressive by using the bayesian information criteria (BIC). An ANOVA for the treatments was performed to evaluate the significance of both simple effects and interactions. A t test ($p < 0.01$) was used for comparison between treatments.

2.3. Microbial counts

Colony forming units were determined for bacteria and anaerobes in selective culture media. Dilutions of the communities were prepared in peptone water (0.1 g/L) pH 7. Bacterial counts were determined by plating in agar plate count pH 7 (Sigma Aldrich, St. Louis, USA) after incubation at 30 °C during 48 h. Anaerobes were determined by plating in agar Wilkin Chalgren pH 7.1 (Hmedia, Mumbai, India) after incubation at 25 °C for 120 h (Wilkins and Chalgren, 1976); anaerobic conditions were obtained by means of oxygen capture bags in an sealed jar. Culture medium was sterilized by autoclaving at 121 °C, 15 psig for 15 min. Statistical analysis was performed as described in the preceding section.

2.4. Community level physiological profiling (CLPP)

CLPP was performed every 25 days during the first 100 days of the experiment for communities developed in the water layer. For the experiment 96 well plates (ECOPLATE, Biolog Inc., Hayward, USA) were used. These plates contain 31 different carbon sources and a blank. Each well was inoculated with 150 μL of the undiluted community (after incubating for 0, 25 and 50 days) or a 1/5 dilution of the community (after incubating for 75, and 100 days). The dilution at higher storage times was necessary to avoid color development in the blank. Plates were incubated at 25 °C and absorbance readings at 590 nm were performed approximately every 8 h over a 96 h period in a microplate reader (Tecan Group Ltd., Seestrasse, Switzerland).

Data were transformed for principal component analysis using Taylor power law in order to improve the normality and homogeneity of the variance (Weber et al., 2007). The "b" value in the

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