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Fuel biodegradation and molecular characterization of microbial biofilms in stored diesel/biodiesel blend B10 and the effect of biocide

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ABSTRACT

Microbial biofilms are formed at oil/water interfaces in storage tanks containing diesel–biodiesel blends, decreasing fuel quality and increasing economic and environmental losses. Biocides may suppress the microorganisms responsible for the damage, but they are not used in all parts of the world. A B10 diesel–biodiesel blend (oil as received with or without an inoculum derived from diesel sludge) was incubated with or without 3,3′-methylene bis(5-methyloxazolidine, MBO) – 100% and 50% formulations at 1000, 500, and 0 ppm – over 60 days. The biofilms formed at the oil–water interface were collected for extraction of genomic DNA followed by amplification, purification, and Illumina HiSeq sequencing of the 16S rRNA gene. The prevalent genera in the control fuel (as-received and inoculated) were similar at 28 days (*Pseudomonas*, *Comamonas*, and *Burkholderia*); by the 60th day, the microbial community had changed only in the as-received fuel, where the prevalent genera were *Comamonas*, *Klebsiella*, and *Tolumonas*. Archea were detected in samples at 28 and 60 days. 500 ppm (as supplied) MBO 50% did not control the microbial growth and an interfacial biofilm was formed. After 28 days of incubation, taxonomic diversity in the as-received fuel and inoculated fuel decreased by 99.7% and 80.9%, respectively. The analysis also revealed that Firmicutes dominated the communities in the treatments with 500 ppm (as supplied) MBO 50%, followed by Proteobacteria, except in the 60 days sample from the as-received fuel, where Proteobacteria dominated, followed by Firmicutes. Inoculation increased degradation of the fuel.

1. Introduction

Microbial contamination of fuel can occur at various points in the distribution system, but it is particularly noticeable during storage, especially if there is free water in the tank. Biodeterioration of the fuel is shown by the accumulation of biomass and degradation of fuel components (hydrocarbons, fatty acid esters, etc.) and can result in blockage of filters, damage to the injection system, and reduced product quality (Gaylarde et al., 1999; Bento et al., 2005; Passman and Dobranic, 2005; Bücker et al., 2011; Passman, 2013; Zimmer et al., 2013). It is accepted that problems in diesel oil are chronic, but recent changes in the composition of this fuel, the introduction of biodiesel mixtures, reduction of sulfur levels, and increase in the use of additives, have increased the biodeterioration risks (Passman, 2013). Various studies have demonstrated that diesel/biodiesel blends are more susceptible to biodegradation and biomass formation during storage (Passman and Dobranic, 2005; Bücker et al., 2011; Sørensen et al., 2011; Silva et al., 2012; Passman, 2013).

The removal of bottom water and/or chemical control with biocides are the preventive treatments most frequently recommended (Gaylarde et al., 1999; Bento and Gaylarde, 2001; ABNT 15512, 2008; Passman, 2013). The free water formed in the tank bottom by condensation from humid air stimulates the production

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of microbial biofilms (Gaylarde et al., 1999; Bento and Gaylarde, 2001; Passman, 2013). Biocide use in fuel is allowed in the U.S., following regulations of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (Passman, 2013). In Brazil, this does not happen (for diesel B). One potential antimicrobial compound for use in fuels is 3,3-methylenebis (5-methyloxazolidine – MBO), which has been used principally for aviation fuel (Passman, 2013).

Studies on microbial contamination in fuel storage tanks traditionally use isolation and cultivation techniques. These are the methods recommended in the Standard Guide for Microbial Contamination in Fuels and Fuel Systems (ASTM 6469-11). However, such techniques can underestimate the microbial diversity, being unable to identify the greater part of the species in the population. White et al. (2011) showed that traditional culture methods identified the predominance of bacteria of the genera *Pseudomonas*, *Burkholderia*, and *Bacillus* in contaminated diesel, while culture-independent techniques (pyrosequencing of the 16S rRNA gene) indicated that the genera *Marinobacter*, *Achromobacter*, *Burkholderia*, and *Halomonas* were the most common. It has been shown that the addition of biodiesel to diesel oil makes the fuel more susceptible to microbial contamination (Bücker et al., 2011; Sørensen et al., 2011; Silva et al., 2012). Lee et al. (2010) and Sørensen et al. (2011), using DGGE, showed that the microbial communities in fuel blends varied according to the proportion of biodiesel present. This and other molecular techniques are important to identify previously undetected contaminants, discover the dynamics of growth in the fuel, and determine populations resistant or tolerant to antimicrobial treatments.

We applied Illumina high throughput sequencing to study the diversity of microbial communities in microcosms simulating B10 storage with and without MBO in pure form (MBO 100%) and as an additive package (MBO 50%).

2. Materials and methods

2.1. Fuel

Fuels used were: low sulfur content (50 ppm) diesel oil (Diesel A), and biodiesel (80% soy and 20% tallow); these were provided by Ipiranga Produtos de Petróleo S/A (Canoas, Rio Grande do Sul, Brazil). The B10 blend was prepared in the laboratory by aseptically mixing 10% biodiesel and 90% diesel A. The fuels were used in as-received condition, without sterilization.

2.2. Biocide test

The 3,3′-methylene bis(5-methyloxazolidine) (MBO) was tested in the pure form (MBO 100%) and as an additive formulation containing 50% biocide (MBO 50%) for its ability to inhibit microbial growth. Final concentrations of 1000, 500, and 0 ppm (control) of either were added to the oily phase and homogenized before adding the flasks with water phase sterilized (mineral medium). The MBO concentrations (500 and 1000 ppm) are presented as supplied (a.s.) or active ingredient (a.i.).

2.3. Simulated storage

Sixty milliliters of Bushnell–Haas mineral medium (Bushnell and Haas, 1941) were sterilized in 200-ml glass flasks. Twenty milliliters of B10 blend was added and the flasks were incubated at 28 °C for 60 days. Destructive (microcosm) sampling was carried out in quintuplicate every 14 days. Measures for analyzing the estimation of microorganisms in the aqueous phase, in the oily phase, and in the biomass formed at the oil–water interface were performed in triplicate. The other two replicates were used for DNA extraction of biomass formed at the oil–water interface (just at 28 and 60 days). From each repetition a sample of extracted DNA was assembled in a single tube.

2.4. Inoculation

Two levels of fuel contamination were used: fuel as received from the distributor and the same fuel inoculated with a mixture of microorganisms prepared according to ASTM E1259 (ASTM, 2010). For the latter, an Erlenmeyer flask containing 100 ml of Bushnell–Haas broth (Bushnell and Haas, 1941) supplemented with 2% sterile B10 blended fuel was inoculated with 5 ml of microbial sludge mix previously obtained from various fuels (diesel, biodiesel, and diesel/biodiesel blends B4 and B5) and incubated at 28 °C and 200 rpm for 7 days. In order to obtain the media microbial challenge the inoculum prepared as described above was then diluted 1:10 to produce the medium challenge (1×10^6 CFU/ml to bacteria and fungi) using Bushnell–Haas broth.

2.5. Analysis

2.5.1. Microbial growth

The aqueous phase from the microcosms was periodically diluted in sterile saline and plated on plate count agar (PCA – Himedia) for heterotrophic bacteria. Fungi were enumerated on potato dextrose agar (PDA – Himedia). Plates were incubated at 28 °C for 48 h in the case of PCA and 7 days for PDA. The enumeration of microorganisms from the oil phase was performed according to the French Standard AFNOR-M 07-070/92 (Hill, 1998). Five milliliters of B10 blend was collected, diluted in an aqueous solution of 0.1% Tween 80, and plated on agar (PDA) for fungi and for heterotrophic bacteria (PCA).

After 28 and 60 days, the interfacial biomass was filtered through previously weighed filter paper. To remove adhered fuel from the biomass, discs were filter-washed with 4 ml of hexane. They were then placed at 30 °C for 48 h and transferred to a dehydrating chamber for 24 h to remove water, and the dry weight was recorded. Biomass weight was calculated as final weight minus initial weight (in milligrams); triplicate values were averaged.

2.5.2. Oil–water interface

2.5.2.1. DNA extraction. The biomass formed at the oil–water interface from two replicates (microcosms destructives) after 28 and 60 days was centrifuged at 6000 rpm for 10 min in sterile 15-ml tubes containing 5 ml Mili Q water to remove excess oil. The supernatant was discarded and 300 mg of the pellet was used for DNA extraction using the PowerSoil DNA Isolation Kit (MOBIO, Inc., Laboratories, USA), according to the manufacturer's instructions, modified as described by Silva et al. (2012). Separate extractions for each replicate were done and a sample of extracted DNA was assembled in a single tube for each time (28 and 60 days), from two replicates.

2.5.2.2. Analysis of microbial population by Illumina HiSeq – high throughput sequencing of the 16S rRNA gene. The extracted genomic DNA was quantified and standardized prior to amplification of the V4 region of the 16S rDNA gene by PCR. Primers used were 515F and 806R (Caporaso et al., 2010). The DNA was denatured at 94 °C for 3 min, followed by 20 cycles of 94 °C for 45 s, 53 °C for 30 s, and 65 °C for 90 s. Final elongation was at 65 °C for 10 min. The PCR products were purified using the Qiagen™ PCR purification kit, following the manufacturer's protocol (Qiagen, Valencia, CA, USA), apart from the elution step, when sterile water was used. The final product was quantified by fluorimetry in a Qubit 2.0 Fluorimeter (Invitrogen, NY, USA).