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Defined inoculum for the investigation of microbial contaminations of liquid fuels

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ABSTRACT

Microbial contamination of hydrocarbons, especially fuels, has been intensively investigated, as the contamination leads to quality losses of the fuel or damage and even destruction of storage equipment. Researchers use different microbes for these investigations. Alternative inocula include pure cultures, low diversity mixed cultures or samples from (contaminated) storage tanks. In contrast to chemical investigations, no standards exist regarding the composition and use of the organisms; hence, the results may differ widely. Here, a defined mixture for the investigation of microbial contamination of stored fuels, especially middle distillates under standardized conditions, is presented. The organisms represent genera and species commonly found in fuel storage systems. The mixture includes 27 individual species, consisting of bacteria, yeast, and molds. The microbes use heating oil as well as biodiesel as sole carbon source and produce acids and surfactants during growth. The defined mixture is diverse enough to cover all aspects of fuel contamination but defined enough for easy handling during experiments including analytics. The defined microbe mix could contribute to greater reproducibility of experiments, resulting in faster development of technical solutions to minimize or avoid microbial contamination and its negative results during fuel storage.

1. Introduction

The ability of microorganisms to adapt to nearly all conditions occurring on Earth leads to their appearance in nearly every environment (Madigan et al., 2010). With an estimated total carbon content of 350–550 Pg for prokaryotes alone, microorganisms are presumed to contain more biomass than plants (Whitman et al., 1998). In addition to their enormous occurrence and diversity, the metabolic pathways used by microbes are highly diverse (Caspi et al., 2006; Kanehisa and Goto, 2000). Among these pathways, microorganisms are able to degrade different types of hydrocarbons (Fathepure, 2014). Hydrocarbons are present in great amounts in crude oil (Benassi et al., 2013) and in derived products such as fuels.

The microbial ability to tolerate and degrade hydrocarbons, on the one hand, is wanted, for example, to clean up oil spills (King et al., 2015). On the other hand, microbial growth in stored hydrocarbons, such as fuel for engines or heating, must be prevented to avoid loss in quality or functionality (Gaylarde et al., 1999). This holds true for oil fields as well, which also suffer from microbial degradation as the organisms can decompose the oil nearly completely, leaving behind only inferior-quality products such as bitumen (Rubinstein et al., 1977). Many different microorganisms have been described that consume fuels (Itah et al., 2009; Salleh et al., 2003; Yemashova et al., 2006), but most of the identified organisms have not been subject to further investigation. If so, very often, pure cultures or low diversity mixed cultures of the most dominant species were analyzed, even with the knowledge that higher degradation rates are achieved by using mixed consortia (Ameen et al., 2016; Gassen et al., 2015; Jung et al., 2002). However, little is known about microbial synergy that occurs during fuel degradation.

The topic of biofouling of fuels is not new (Hettige and Sheridan, 1989; Reisfeld et al., 1972), but research groups around the world must address it anew every few years (Bento et al., 2005; Klofutar

Abbreviations: B0, domestic heating oil; B20, Domestic heating oil + 20% (v/v) biodiesel; DHO, Domestic heating oil; FAME, Fatty acid methyl ester.

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and Golob, 2007; Leahy and Colwell, 1990; Pitcher, 1989) because the fuels change over time, especially within recent years as a result of the blending of fossil fuels with biofuels (Pasqualino et al., 2006; Passman, 2013; Sorensen et al., 2011). The number of biofuels will likely increase (Hoppe et al., 2016), and as a result, research into blends will become increasingly complex, covering physico-chemical as well as biological aspects. To be able to compare results, standardized tests must be established. This is the case for many engineering questions (at least 29 DIN or ASTM methods are used for testing diesel parameters and an additional 15 are used for testing biodiesel parameters). In contrast, research in the field of biodeterioration and biofouling may use several standards such as, for example, the defined mineral medium Bushnell-Haas (Bushnell and Haas, 1941). Nevertheless, many experimental parameters are still not standardized, leading to experiments that use unknown, or even worse, unreproducible starting conditions. The most dominant of these unknown factors is the microorganisms that are used. It is written in the ASTM E1295-16 that "contaminated fuel system microbial communities can be quite diverse and contain >50 different taxa. Consequently, […] multi-taxa inocula provide a more realistic challenge population than either single or commonly used, three taxa inocula" and "The use of standardized cultures to prepare microcosm inocula facilitates corroborative testing". Hence, a defined mixture representing organisms that are present under hydrocarbon-degrading conditions would be desirable to standardize experiments. This culture should be able to behave in previously described ways, i.e. by forming biofilms and producing acids and surfactants (Gaylarde et al., 1999). This unwanted microbial growth in storage tanks leads to well known effects, ranging from the blockage of filters and pipes as a result of biofilm formation to the corrosion of metal parts resulting from the production of acids (Itah et al., 2009). The production of surfactants which are used by the organisms to degrade hydrocarbons is also well described (Ganesh and Lin, 2009).

The enormous number of identified organisms as well as their diversity indicates the ability of many organisms to contaminate fuels. This high number of organisms is one reason to use a defined mixture. Some reviews list over 100 different microorganisms isolated from different fuel sources (Gaylarde et al., 1999). Hence, using all the identified organisms in fuel contamination research is impractical. The alternative, using only single cultures or mixed cultures with only a few different organisms circumvents this problem, but it does not well reflect contaminated fuel. The use of a "readily mixed population," i.e. organisms taken from a fuel storage system, is also not recommended, as the biocenosis may change over time or as a result of altered fuel properties (Kleinsteuber et al., 2006; Militon et al., 2010). To be able to compare results independent of time and location all parameters including the microorganisms used should remain identical.

In this study, a mixture of microbes for the standardization of experiments was established and characterized. This defined mixture can be used for fuel storage and contamination experiments. .

2. Materials and methods

2.1. Sampling of domestic heating oil

From September 2010 to November 2011, ten different domestic heating oil (DHO) tanks in Aachen and Hamburg, Germany, were sampled. Except for one, all the tanks contained heating oil with 10% or 20% (v/v) biodiesel (fatty acid methyl ester (FAME); rapeseed methyl ester (RME)). Samples were taken aseptically using a self-constructed sampling device (Fig. 1) which was, including all bottles and hoses, autoclaved before sampling. From every tank,

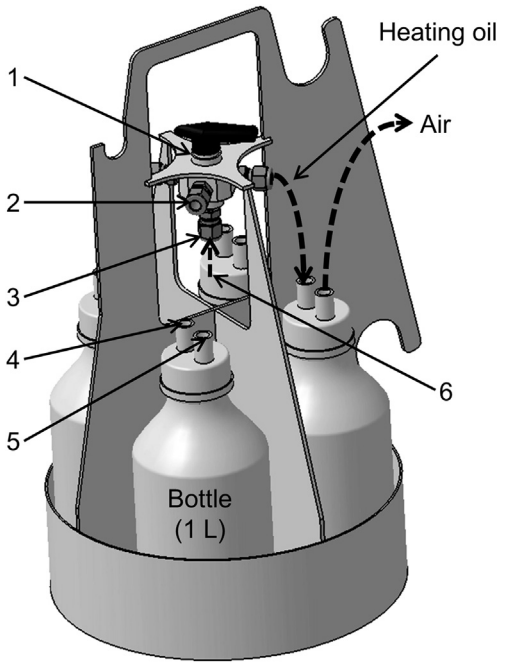

Fig. 1. Device for heating oil sampling. 1: Valve; 2: Connection port to connect a 1 L bottle to the valve; 3: Central entrance of the hose immersed in the tank; 4: Filling port of the bottle; 5: Evacuation port of the bottle; 6: Flow direction of the heating oil from the storage tank. The device can be autoclaved. Samples were taken by evacuating a bottle and sucking in the sample, distributing it through the central valve to one bottle.

samples were taken from the top (directly beneath the surface), middle (middle of filling level), and bottom phase (directly above the bottom) by immersing a hose attached to the device at the desired position. Prior to sampling a new phase, several pipe volumes were rinsed through the sampling device to minimize cross-contamination. All samples were stored at 4 °C until further processing.

2.2. Identification of microorganisms present within the fuel

The identification of the microbiome was conducted by cultivating the organisms present in the fuel prior to total DNA extraction and subsequent sequencing of the 16S/18S rDNA using next-generation sequencing (Leuchtle et al., 2015).

2.3. Isolation of pure cultures from DHO samples

To obtain pure cultures, 100 μL fuel sample were plated on rich medium (Standard I (Std I, Carl Roth, Karlsruhe, Germany) or YPD [10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose]) and incubated for one to four days at 30 °C. From the grown organisms, one inoculating loop of cell material was taken and separated using a streak plate method. Single colonies were picked and enriched in rich medium. Identification was conducted via colony PCR or after DNA isolation by amplification and subsequent sequencing of the 16S/18S rDNA gene using the primer pair B27F (AGAGTTTGATCMTGGCTCAG) and L1492 (GGTTACCTTGTTACGACTT) for prokaryotes and NS1 (GTAGTCATATGCTTGTCTC) and NS8 (TCCGCAGGTTCACCTACGGA) for eukaryotes (Dadrasnia and Ismail, 2015; Marshall et al., 2003).

2.4. Organisms used

The organisms used to build up the defined mixture were

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