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# Quantification of microbial load in diesel storage tanks using culture- and qPCR-based approaches

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ABSTRACT

Microbial contamination of fuels, associated with a wide variety of bacteria and fungi, leads to decreased product quality and can compromise equipment performance by biofouling and microbiologically influenced corrosion of pipelines and storage tanks. Detection and quantification of biomass are critical in monitoring fuel systems for an early detection of microbial outbreaks. The aims of this study are (i) to quantify bacterial and fungal contamination in samples from diesel storage tanks of petrol stations, using both culture dependent- and culture independent (qPCR) approaches, and (ii) to analyse the diversity of cultivable diesel-contaminating microorganisms with the purpose to create a strain collection for further use in biodeterioration experiments.

Both methodological approaches revealed a high microbial contamination in all studied samples, with the bacterial load being much higher than the fungal load. The diversity of cultivable microorganisms was rather low. Based on criteria of abundance and fuel degradation potential, the most relevant microorganisms were identified as bacteria of genera *Bacillus*, *Citrobacter*, *Burkholderia* and *Acetobacter*, the filamentous fungi *Paecilomyces variotii* and *Pseudallescheria boydii*, and a Dipodascaceae yeast. Furthermore the validity and utility of qPCR-based methods are discussed.



## 1. Introduction

Microbial contamination of fuels leads to decreased product quality and can compromise equipment performance by increasing the risk of biofouling and microbiologically influenced corrosion (MIC) of pipelines and storage tank materials (Passman, 2013). Fuel biodeterioration becomes ever more relevant as biodiesel-containing fuel formulations are increasingly used (Schleicher et al., 2009; Passman, 2013). Admixtures of hygroscopic biodiesel further increase the tendency of fuels to absorb moisture from the atmosphere, thus making these materials even more conducive to microbial contamination (Sorensen et al., 2011; Suflita et al., 2012; Passman, 2013; Soriano et al., 2015). These long-term and microbially-induced consequences become especially relevant for stored automobile fuels (Rodriguez-Rodriguez et al., 2009; 2010). Among other fuel facilities, petrol stations commonly show different problems associated with microbial contamination, such as deposit formation in storage tanks and filter clogging (Ludzay and Weyandt, 2009).

One of the microbes frequently isolated from fuels is the fungus *Hormoconis resinae*, also called 'kerosene fungus' or 'diesel bug'. Several studies described its abundance in fuel systems, and its ability to degrade fuels and cause corrosion of aluminium storage tanks (Hettige and Sheridan, 1989; Gaylarde et al., 1999; Bento and Gaylarde, 2001; Bento et al., 2005; Rosales and Iannuzzi, 2008). However, recent studies demonstrated that a wide variety of bacteria and fungi, including moulds and yeasts, colonise fuels, and many organisms are able to degrade diesel, biodiesel, kerosene, etc. (Gaylarde et al., 1999; McNamara et al., 2005; Yemashova et al., 2007; Ludzay and Weyandt, 2009; Rodriguez-Rodriguez et al., 2009; 2010; Sorensen et al., 2011; Suflita et al., 2012). Yemashova et al. (2007) reviewed conditions necessary for biodeterioration of fuels and provided a list of frequently found fungi and bacteria. Most prominent fungi found in fuels systems are filamentous fungi belonging to the genera *Penicillium*, *Aspergillus*, *Cladosporium* and *Paecilomyces*, and yeasts of the genera *Candida*, *Rhodotorula* and *Aureobasidium*. Bacteria contaminating fuel are recurrently aerobic

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bacteria such as *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Micrococcus*, *Enterobacter*, and *Staphylococcus*, but also anaerobes like *Desulfovibrio* and *Clostridium* are reported. In fact, during fuel colonisation a shift of the community structure from aerobic to anaerobic organisms, like sulphate reducing bacteria (SRB), can be observed. Therefore one can assume that the aerobic fuel-contaminating microbial community leads to a higher risk of microbial corrosion processes by SRB (Melo et al., 2011; Santana et al., 2012; Soriano et al., 2015; Williamson et al., 2015).

Our understanding of microbial processes in a particular environment significantly improves when research is able (i) to provide reliable information on the measurable extent (quantification) of the phenomena as well as (ii) to prove by experiment that the organisms in question induce a significant/substantial change in fuel quality. As in recent years the notion of the ubiquity of microbially-induced processes in virtually any material system has steadily emerged, new methodological approaches are gaining ground in material testing. Addressing microbial contamination requires reliable information about the identity of microorganisms that cause such problems. Methods need to be developed which allow an early quantification/characterisation of biological contamination to prevent mass development.

In this work we intend to follow two of the key questions on microbial contamination of fuels that were recently postulated by Suflita et al. (2012): Does the presence of microorganisms represent a substantive threat to fuel use? How should the microbiological status of fuels be monitored? To pursue the experimentally confirmed answers to these questions, we aimed to study whether the main fuel-colonising biomass is formed by fungi or bacteria. In addition we intended to isolate fuel inhabitants and thus create a collection of relevant reference strains that can be further used in lab experiments to quantify deterioration rates and important metabolic products as well as microbially-induced fuel changes.

Detection and quantification of microbial biomass are critical in monitoring fuels and fuel systems. Such methods have to be fast and reliable to ensure their broad application for an early detection of microbial biomass and implementation into corresponding control strategies. Several methods can be used to characterise the microbial contamination of fuels. Most common is the cultivation of bacteria and fungi from contaminated samples (Rauch et al., 2006; Itah et al., 2009), with the known problems regarding cultivable microorganisms. Furthermore methods like denaturing gradient gel electrophoresis (DGGE) (Sorensen et al., 2011; Soriano et al., 2015) and restriction fragment length analysis (Raikos et al., 2011) are used to analyse such contaminations but deliver just descriptive results. Finally sequencing of the microbiome can be used to unravel the community structure inside fuel samples (Stevenson et al., 2011; Suflita et al., 2012), providing a deep insight the community structure but is not applicable for routine measurements. White et al. (2011) compared different methodological approaches, aerobic cultivation, DGGE and pyrosequencing, to assess bacterial fuel contamination, and concluded that cultivation approach recovered isolates broadly representative of the taxa present but lacked accuracy by overrepresenting certain groups.

All methods mentioned can be used to determine the qualitative structure of the microbiome, however, determination of absolute contamination rates are difficult to realize. In this sense, the real-time quantitative polymerase chain reaction (qPCR) is considered the touchstone for nucleic acid quantification and determination of biomass equivalents. After its firm establishment as a research tool, many qPCR-based applications have been developed for diagnostics and microbial quantification in medicine, food industry, agriculture and environmental sciences (Zhang and Fang, 2006; Bustin et al., 2009; Biassoni and Raso, 2014), including studies focused on microbial communities involved in biodegradation and bioremediation of fuels in different environments (Cyplik et al., 2011; Bell et al., 2013; Richardson et al., 2015). However, to the best of our knowledge, just a few studies use qPCR to quantify microbial contamination in fuel systems (Stevenson et al., 2011; Suflita et al., 2012; Galvão and Lutterbach, 2014; Martin-Sanchez et al., in press).

The aims of this study are (i) to quantify bacterial and fungal contamination in samples from diesel storage tanks of petrol stations in Spain, using both culture dependent and culture independent (qPCR) approaches, and (ii) to analyse the diversity of cultivable diesel-contaminating bacteria and fungi.

## 2. Material and methods

### 2.1. Samples

A total of six ultra-low-sulfur diesel (ULSD) samples (A-F) from storage tanks of Spanish petrol stations were included in this study (Fig. 1a, b, Supplementary Table S1). They were collected from the bottom of different contaminated tanks in several years (2012–2014) and kept at 4 °C until shipment to BAM laboratories in May 2014. Upon their arrival, all samples showed abundant aqueous phase and evidence of intense microbial contamination according to their appearance (Fig. 1a,b).

Taking into account that presence of microbes is always associated with the water content, subsamples of aqueous phases were selected to perform this study (Fig. 1c). After homogenisation of flasks by vigorous shaking, when the oil-water interface was again established, subsamples were taken after an additional mixing of water phase using an electronic pipet. One milliliter was used for culture-based analyses, counting and isolation of cultivable microorganisms, and 20 ml were used for DNA extraction and subsequent molecular analyses by qPCR methods.

### 2.2. Counting and isolation of cultivable microorganisms

The total viable counts for bacteria and fungi were determined by plating in triplicate 100 μl aliquots of aqueous subsamples, and four decimal serial dilutions from them, on trypticase soy agar plus 25 mg $l^{-1}$ cycloheximide (TSAcy) for bacteria, and malt extract agar plus 50 mg $l^{-1}$ chloramphenicol (MEAc) for fungi. Culture plates were incubated at 25 °C in dark for one month and periodically checked. The colony forming units (cfu) for both bacteria and fungi were counted after one week. In addition, to enhance the isolation of diesel-degrading microorganisms, flasks containing 50 ml of Bushnell-Haas salt solution and 10 ml of diesel (sterilised by filtration) as sole carbon source (BHD), were inoculated with 100 μl of aqueous subsamples and incubated for one week at 25 °C in dark and shaking at 100 rpm. After that, 100 μl aliquots from the aqueous phase of such cultures, and three serial decimal dilutions from them, were plated on Bushnell-Haas salt solution with 15 g $l^{-1}$ agar and 5 ml $l^{-1}$ diesel sterilised by filtration (BHDA), and incubated as detailed above. Based on colony morphology, the most abundant cultivable bacteria and fungi, as well as some further representative strains, were isolated in pure cultures on the same culture media. Bacterial suspensions in 30% glycerol were stored at −80 °C, and fungal strains were grown on MEA slants and stored at 4 °C until subsequent analyses.

### 2.3. Identification of cultivable microorganisms

Molecular identification of isolated strains was performed by PCR and sequencing of ribosomal markers, 16S rRNA gene (16S) for bacteria, and rDNA internal transcribed spacers (ITS) or 18S rRNA gene for fungi (18S). The 18S marker was only analysed if ITS

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