Fuel 93 (2012) 559–566

Contents lists available at SciVerse ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Water content, temperature and biocide effects on the growth kinetics of bacteria isolated from JP-8 aviation fuel storage tanks

Vassilios Raikos [a,*], Sotiris S. Vamvakas [a], Dimitrios Sevastos [a], John Kapolos [b], George Karaiskakis [a], Athanasia Koliadima [a]

[a] Department of Chemistry, University of Patras, 26504 Patras, Greece
[b] Department of Food Technology, Technological Educational Institute of Kalamata, 24100 Kalamata, Greece

ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 10 October 2011
Accepted 17 October 2011
Available online 31 October 2011

Keywords:
Fuel
JP-8
Microbial growth
Kinetics
Temperature

ABSTRACT

Three bacterial strains isolated from JP-8 aviation fuel storage tanks were used to examine their ability to utilize the fuel as their sole source of carbon and energy. The isolates were *Staphylococcus epidermidis*, *Agrobacterium tumefaciens* and *Ralstonia picketii*. The purpose of this study was to determine the effect of temperature and water content on the growth kinetics of the bacterial isolates. Growth measurements were carried out by using light microscopy to enumerate bacterial cells. Growth phases of the bacterial cell cycle were determined under different experimental conditions and rate constants were calculated for the growth phase from the slopes of the graphical representations of ln(cells/ml) vs. time, assuming that bacterial growth follows a first order process. JP-8 fuel was found to support microbial growth, with increasing temperature and water content having a positive impact on the growth profiles of the bacterial strains. Biocidal activity of an isothiazolone biocide (Kathon FP 1.5) was also examined. The results indicate that the biocide can suppress the growth of *S. epidermidis* by 73%, *A. tumefaciens* by 77% and *R. picketii* by 81%, when used at the appropriate concentration. A mathematical model which correlates the number of cells and biocide concentration for each bacterial species was also developed.

1. Introduction

Fuels are highly favorable media for microbial growth. Microbial contamination of petroleum fuels has been recognized as a problem for at least 5 decades [1–4]. In several cases, microbial contamination occurs during storage in above ground storage tanks, where fuels may spend from days to several weeks, depending on flight frequency. Microorganisms enter fuel tanks from airborne contaminants, through water that enters the tank and from non-sterile fuel [5]. In particular, it is very difficult to avoid water penetration into fuel systems as the main reason for its occurrence, the air condensation phenomenon under conditions of changing temperatures, cannot be easily controlled [6]. The existence of water in fuel systems, not only supports the growth of microorganisms and accelerates biofouling, but may affect the oil properties by altering the viscosity or cause mechanical damage to the oil pumps. Biodeterioration of kerosene has been linked to several airplane crashes during the last decades [7,8]. Microorganisms usually grow in the water settled at the bottom of storage tanks and their populations tends to be large at the water–fuel interface, particularly in tank bottoms which are not regularly drained from fuel storage tanks. A diverse community of bacteria and fungi has been isolated from fuel tanks, depending on, among other factors, the geographic location and the climate of the area of sampling. The fungus *Hormoconis resinae* was the most common corrosive organism contaminating the fuel storage tanks [9,10]. Nevertheless, research in recent years indicates a shift in the microbial community that contaminates particularly jet fuels, from *H. resinae* to bacteria [5]. Problems associated with the undesirable accumulation of microbial populations in aircraft fuel systems include filter plugging, fuel tank and pump corrosion, and engine component damage [11]. In addition, the generation of products of microbial metabolism worsens the physical and chemical properties of oils and fuels and results in fuel deterioration [12,13]. Effective measures employed to suppress the microbial growth in jet fuel storage tanks include physico-mechanical methods such as good housekeeping practices and chemical methods such as the addition of biocides which prevent microbial growth [14].

Numerous studies on the microbiology of petroleum hydrocarbon, which include the growth profile of various microorganisms under a wide range of conditions, have been documented [15–18]. In this study, three bacterial genera (*Staphylococcus epidermidis*, *Agrobacterium tumefaciens* and

* Corresponding author. AddressDepartment of Chemistry, University of Patras, Laboratory of Physical Chemistry, 26504 Patras, Greece. Tel.: +30 2610 997109; fax: +30 2610 997144.
E-mail address: vas.raikos@gmail.com (V. Raikos).

0016-2361/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fuel.2011.10.028

Ralstonia picketii) which were isolated from JP-8 aviation fuel storage tanks and were identified using 16S rRNA gene sequencing, were used to determine their ability to utilize hydrocarbons to support their growth [19]. Furthermore, the need to quantify the influence of environmental factors such as temperature and water content on the growth kinetics of the bacterial species was also taken into account.

The main objective of the present study was to determine the major factors affecting the growth phases of the cell cycle of the above microorganisms in aviation fuel under controlled experimental conditions. The bacteria life cycle consists of the lag phase (cells are freshly inoculated into the medium and no increase in cell number is observed), the log or exponential phase (cells are actively metabolizing and cell division takes place), the stationary phase (metabolism slows and rapid cell division ceases) and the death phase (depletion of nutrients results in cell death and decrease in cell numbers). Factors that influence bacterial growth bear heavily on this cycle [20,21]. Temperature, pH, energy sources and the presence of oxygen, nitrogen, minerals and water all affect bacterial growth, thus affecting the bacteria life cycle [22]. Optimal growing conditions depend on the bacteria species.

The experimental conditions were chosen to mimic the temperature and water content which were identified in the JP-8 storage tanks from which the bacterial genera were initially isolated [19]. This study aims to identify approaches for controlling problems arising from contamination of jet fuel storage tanks. Furthermore, the effect of a commercially-available biocide (Kathon FP 1.5) on the growth profile of the microorganisms isolated from fuel storage tanks was also studied. Up to date the authors are not aware of studies which aim to present the growth profile of *S. epidermidis*, *A. tumefaciens* and *R. picketii*, under controlled environmental conditions, in mineral salt medium containing aviation fuel as sole source of carbon and energy.

2. Materials and methods

2.1. Sources of microorganisms

Operational aviation fuel samples (JP-8, with standard additives) were collected using a sterile sample collector from the lowest accessible point of below-ground storage tanks into sterile glass bottles. Samples were collected from six different airport bases in Greece in a period of 9 months (November 2008–July 2009). Although the Greek climate is predominantly mediterranean, Greece has a remarkable range of micro-climates and local variations. The geographic location (mainland and islands) of each airport base was chosen in order to meet different climate criteria. The temperatures chosen (10–30 °C) are representative of the fuel storage temperatures during the sampling period and the biocide concentrations were chosen according to the suppliers recommendation. Furthermore, the efficiency of the biocide was tested outside the optimum range (100 ppm).

Bacterial isolates were cultured using the pour plate technique from samples collected from JP-8 aviation fuel storage tanks. The solid media used for enrichment culturing included trypticase soy broth agar (TSA): 15 g peptone from casein, 5 g peptone from soy meal, 5 g sodium chloride, 15 g agar, 2.5 g glucose per liter and brain heart infusion (BHI): 40 g blood agar (Fluka Analytical, Devon, UK), 37 g brain heart broth (Fluka Analytical, Devon, UK) per liter. TSA plates were incubated at 37 °C for 48 h, whereas BHI plates were incubated at 37 °C in an anaerobic jar (AnaeroJar, Oxoid Limited, Cambridge, UK) for 72 h. When obvious growth appeared on plates, colonies were used to inoculate liquid growth medium and inoculums were left to incubate for 24 h under appropriate conditions (temperature, aerobic/anaerobic) using stirring conditions (150 rpm). The inoculums were then streaked onto new plates and incubated for either 48 h (TSA) or 72 h (BHI) to obtain a new culture from each individual colony appearing on the initial plate. A portion of the 16S rDNA gene was amplified using PCR reaction [23] and three bacterial genera were identified by DNA sequencing of the amplified DNA portion including the following isolates (data not shown): *A. tumefaciens* strain M5, *S. epidermidis* strain EIV-9 and *R. picketii* strain 12D.

2.2. Bacterial growth on jet-fuel

JP-8 aviation fuel was sterilized by filtration through filter membranes of 0.22 μm pore size (Whatman Ltd, UK). 9 ml of sterile JP-8 aviation fuel was placed in Falcon™ tubes (15 ml). Fresh liquid cultures of each bacterial species were prepared 24 h prior to jet fuel inoculation. Direct enumeration was performed in Petroff–Hausser counting chambers [24–26]. Bacterial cells were observed through immersion under a standard light microscope (Primo Star, Carl Zeiss Microimaging, Jena, Germany) at a magnification of 1000. To achieve an acceptable level of accuracy, enumeration was performed on 10 fields chosen randomly on the slide. In general, cell counting by heamocytometer is the most preferred method, compared to optical density or plate counting, because it is less intrusive and is more rapid [27]. Cell numbers were used to determine the growth profiles of the bacterial cells. 10^5 cells of each bacterial species (~5 μl) were used to inoculate the sterile fuel. To determine the effects of water content on the microbial growth, different amounts of minimal salt medium (2, 4 and 6% v/v) was added to the oil samples. The salt solution (MSM) contained 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NH_4NO_3, 0.1% $MgSO_4.6H_2O$, 0.01% $CaSO_4.H_2O$, 0.001% $Fe_2(SO4)_3.6H2O$, 0.001% $ZnSO_4$ and 0.001% $MnSO_4.H_2O$. Inoculated Falcon™ tubes were incubated at 30 °C (Medline, UK). At certain time intervals (twice a week), three Falcon™ tubes, inoculated with each of the three bacteria, were removed from the incubator and the aqueous phase at the bottom of the tube in addition to a small amount of oil was carefully pipetted into a sterile eppendorf tube. The oil was included to exclude the possibility of discarding microbes colonizing the oil/water interface. The eppendorf tubes were centrifuged at 12,000 rpm for 2 min using a micro-centrifuge (Irmeco, Germany) and the oil phase was discarded. The eppendorf tubes were centrifuged at 12,000 rpm for another 2 min and the supernatant was discarded. 50 μl of sterile water was added and the pellets were redissolved after vigorous mixing using an orbital shaker (Medline, UK) for 5 min to ensure complete solubilisation. Bacterial counts (cells/ml) were performed using a heamocytometer under the optical microscope. Three replicates were prepared for each treatment and standard statistical calculations were performed in order to estimate the standard error for each measurement. Measurements were carried out for a period of 45 days. To determine the effects of temperature on the microbial growth, the inoculated oil samples containing a specified amount of water (6% v/v), were incubated at three different temperatures (10 °C, 20 °C and 30 °C). Samples incubated at 10 °C were cell-counted for a period of 78 days, as microbial growth was relatively slow, whereas samples incubated at 20 °C and 30 °C were cell-counted for a period of 45 days. The protocol used for cell enumeration was the same with the one employed to determine the effect of water content on the microbial growth. Furthermore, the reaction rate constants (k) during the growth phase of each bacterium species for the different water content levels and temperatures were calculated.

2.3. Effect of Kathon FP 1.5 concentration on the bacterial growth

Ten Falcon™ tubes containing 9 ml sterile JP-8 and a specified amount of water (6% (v/v)) were inoculated with each one of the