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Biodeterioration of stored diesel oil: studies in Brazil

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Abstract

The problems of hydrocarbon fuel storage in Brazil are particularly acute for diesel fuel. Visits to bus depots showed that many foremen did not understand the importance of draining water bottoms regularly and most systems were microbially contaminated. Common fungal isolates from refineries and distribution systems, *Hormoconis resinae*, *Aspergillus niger*, *Aspergillus fumigatus*, *Paecilomyces variotii*, and *Candida silvicola*, grew equally well in laboratory diesel/water systems with or without a chemical additive mixture, showing that this package of compounds neither promoted nor retarded fungal growth. Non-sterilised diesel was stored for 450 days over a water bottom, with or without an isothiazolone biocide, in the laboratory. The fungi most frequently detected in the non-biocide treated systems were *H. resinae*, *A fumigatus*, *P. variotii*, a *Penicillium* sp., and the yeasts, *Rhodotorula glutinis* and *Candida silvicola*. Bacterial isolates included oxidative Gram negative rods, sulphate-reducing bacteria and a *Micrococcus* sp. Biocide at 0.1 ppm maintained the systems clean for up to 30 days, and at 1 or 10 ppm for 400 days. After 400 days, the biomass (dry weight) from non-additive-containing diesel in control, 1 and 10 ppm biocide-containing systems was 24.6, 4.6 and 3.3 mg, respectively. The system treated with 0.1 ppm biocide yielded 38.2 mg biomass, indicating that sub-effective doses may lead to increased microbial growth. Within 24 h of addition of 10 ppm biocide to a highly contaminated control flask (145 days storage) there was a 2-log reduction in total aerobic bacterial and yeast population and the filamentous fungal count was > 5 ml^{-1}.

Keywords: Biodeterioration; Diesel oil; Biocide

1. Introduction

Many studies have indicated the importance of microbial contamination of stored hydrocarbon fuels, which can lead to blocking of pipelines and filters, reduced fuel quality and corrosion of storage tanks (Gaylarde et al., 1999). The problems in Brazil are particularly acute for diesel fuel, which frequently presents a substantial biomass at the fuel/water interface. The major bacteria involved in diesel storage tank corrosion are the anaerobic sulphate reducing bacteria (SRB), although aerobic bacteria and fungi may also participate in the process (Gaylarde, 1989; Videla, 1981). Fuel storage tanks in-ground are especially prone to contamination problems, because of the difficulties of drainage. In Brazil, many petrol stations are replacing in-ground tanks by aerial ones, with resulting decrease in microbial problems. There are, of course, other hazards associated with aerial tanks, such as increased risk of physical and fire damage. A recent regulation in the USA requires in-ground tanks to be protected against corrosion by a variety of mechanisms (coatings and cathodic protection), involving considerable expenditure (Uller, 1996). An alternative strategy is the use of biocides (Shennan, 1988).

Bento and Gaylarde (1996) tested four water-soluble biocides for their activity in oil/water systems against planktonic bacteria and fungi isolated from diesel storage tanks in Brazil. An isothiazolone mixture and a quaternary ammonium compound were the most effective, glutaraldehyde and a formaldehyde-releasing agent being active only at relatively high concentrations, if at all. The same isothiazolone mixture was active against biofilms of *H. resinae* on a metal surface at 50 ppm (Guiamet and Gaylarde, 1996) and a slightly different formulation at 2.5 ppm against single species biofilms of various fungi and bacteria on PVC (Elvers et al., 1997).

Biocides are not used to control microbial growth in fuels in Brazil, although there exist additive packages, containing surfactants, corrosion inhibitors, improvers of cetane number, etc. (Batts and Fathoni, 1991), that claim to improve diesel quality. In Brazil, refineries and fuel distribution

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stations rely mainly on good housekeeping techniques, avoiding the use of biocides. We investigated the results of this strategy in a survey of storage tanks in Brazil and followed this up with laboratory and simulation studies of efficacy of the isothiazolone biocide in diesel fuel.

2. Materials and methods

2.1. Survey of microbial contamination in storage tanks

Samples of diesel oil from refinery storage tanks were collected in sterile containers. The main microorganisms present were detected by filtration (0.45 μm Millipore filter) followed by plating on malt or nutrient agar, and by direct plating on these media of any sediments present in the samples. Sediment was also inoculated into Postgate's medium B (Postgate, 1984) for detection of SRB. The most frequently occurring microorganisms detected were purified and identified by the traditional microbiological methods (biochemical and morphological tests for bacteria and yeasts, morphology for filamentous fungi) (Conn et al., 1957; Collins and Lyne, 1985).

A survey of bus companies in the south, south-east and central-west of Brazil, where an additive package had recently been introduced into the diesel, was undertaken on behalf of a company manufacturing engine parts. Increased wear and breakages were occurring in the vehicle engines. The companies were visited and details of routine maintenance procedures and perceived problems noted. Samples of fuel were collected from representative parts of the system for microbiological analysis. The samples were collected into sterile glass containers from pre-filtration tanks, from the petrol pump post-filtration, from the vehicle injection pump and from the vehicle fuel tank. Major contaminants were isolated and identified as previously stated.

Representative isolates were tested for their ability to grow in the laboratory as pure cultures in diesel/water systems, by inoculating them into flasks containing 200 ml filter-sterilised diesel (passed three times through a 0.22 μm Millipore filter) and 100 ml Bushnell–Haas mineral medium (Bushnell and Haas, 1941). The flasks were incubated statically at room temperature for up to four weeks.

2.2. Effect of additive on microbial growth

Since it had been suggested that the introduction of the chemical package for addition to metropolitan diesel was responsible for the increased problems seen in bus companies, the effect of this additive on growth in diesel of fungal isolates was tested. *Hormoconis resinae*, *Aspergillus niger*, *Aspergillus fumigatus*, *Paecilomyces variotii* and *Candida silvicola*, all isolated from metropolitan diesel storage tanks, were inoculated at a final fungal spore concentration of 10^5 ml^{-1}, or yeast cell concentration of 10^{-5} ml^{-1}, into flasks containing Bushnell–Haas medium and diesel with or without additive. The oil was sterilised by filtration, as previously described, and was used in the systems at a ratio of two parts oil to one part aqueous phase. The flasks were incubated, without shaking, for up to 49 days at room temperature ($28^\circ C \pm 3^\circ C$). Five replicates were set up for each fungus and for a mixture of all four filamentous fungal species.

Growth was determined qualitatively by visual inspection and plating a loop of the aqueous phase on malt extract agar, and quantitatively by measurement of dry weight of the biomass or viable plate count (Spiral Plater System) for the yeast.

2.3. Ability of a biocide to control microbial contamination

An isothiazolone biocide formulated for use in fuels was tested for its ability to control microbial contamination in metropolitan diesel with or without additive. The biocide (Kathon FP1.5) was a gift of Rohm & Haas, São Paulo.

Flasks containing Bushnell–Haas medium (100 ml) and additive or non-additive-containing non-sterilised diesel oil (2 : 1 oil:aqueous phase) were maintained at room temperature (16–32°C) for 450 days. To some flasks, at the beginning of this period, the isothiazolone mixture (5-chloro-2-methyl-4-isothiazolin-2-one plus 2-methyl-4-isothiazolin-3-one) was added to give final concentrations in the aqueous phase of 0.01, 0.1, 1.0, or 10 ppm (active ingredient). All treatments were carried out with five replicates. At various time intervals (see results), the flasks were examined visually for the appearance of turbidity and the aqueous phase was plated on solid growth media to detect viable bacteria and fungi. After 400 days, systems exhibiting an interfacial biomass were sampled for the presence of SRB, as previously described, and the dry weight of the total biomass was determined after filtration through a 0.45 μm membrane filter. Main types of fungal and bacterial colonies growing on solid media were purified by repeated subculture and identified as previously described.

After 145 days of incubation, two control flasks from the non-additive containing diesel, with well-developed microbial populations, were selected and one treated with a single dose of 10 ppm biocide. Total aerobic bacterial and yeast numbers were monitored at 3, 8, 24, 48, 72, 96 and 120 h and 7 days of incubation using the Spiral Plater. Filamentous fungi were not enumerated, but live cells were detected in the samples at the stated time intervals by inoculating 200 μl into malt extract broth.

3. Results and discussion

3.1. Microbial contaminants in storage tanks

The main microbial contaminants detected in the diesel fuel in various systems are listed in Table 1. All of the organisms detected have previously been stated in the

Table 1
Microorganisms detected in diesel oil systems in Brazil

Microorganism	Detected in:			
	Refinery storage tanks	Petrol station storage tanks before filtration	Petrol station pumps	Vehicle injection pumps
Aspergillus fumigatus	+	+	+	+
Aspergillus niger	+	+		
Aspergillus flavus	+			
Paecilomyces variotii	+	+	+	+
Hormoconis resinae	+	+	+	+
Penicillium sp.		+	+	+
Penicillium citrinium		+		
Alternaria sp.		+		+
Alternaria alternata	+			
Cochliobolus lunatus	+			
Candida silvicola			+	
Rhodotorula glutinis			+	
Bacillus sp.	+	+		
Bacillus cereus				+
Bacillus subtilis				+
Bacillus firmus				+
Gram negative rods		+	+	+
Pseudomonas sp.	+			
SRB	+	+	+	

literature to be found in stored fuels (Smith, 1991; Gaylarde et al., 1999).

To assess the importance of particular microorganisms in fuel deterioration, it is essential to determine their ability to grow, rather than merely exist, in these systems. The fungi *H. resinae*, *A. niger*, *A. fumigatus* and *C. silvicola*, and the bacteria *Bacillus* sp. and *Pseudomonas* sp. isolated from the diesel systems were all able to grow well in Bushnell–Haas mineral medium plus diesel as sole carbon source, whilst *P. variotii* grew very poorly. Hettige and Sheridan (1989) showed that *H. resinae*, *P. corylophilum* and *P. variotii* all grew in laboratory diesel/water systems when inoculated separately, while in mixed cultures of *H. resinae* and *P. corylophilum*, only *H. resinae* remained viable after 6 weeks. They showed that *P. corylophilum* grew in spent Bushnell–Haas/fuel medium after growth of *H. resinae*, showing that it was not inhibited by *H. resinae* metabolites. The lack of growth of *P. variotii* in our systems could be due to the different strain of fungus. Growth in fuels can vary with microbial strain, as well as with time of storage on laboratory media (Rosales et al., 1994) in our case, the fungus had been stored on malt agar before being tested. More work is needed to define those microorganisms reported as isolated from fuel (Gaylarde et al., 1999) which are able to utilise the hydrocarbon chains, as opposed to those that grow on other fuel components.

3.2. Effect of additive on microbial growth

Fig. 1 shows that the presence of the additive in metropolitan diesel did not influence microbial growth. None of the fungi tested grew differently in diesel with additive than in diesel without additive (some results not shown). It has been suggested that microbial growth in gasoline occurs only at the expense of additives such as vegetable oil phosphatides, and that large-scale microbial contamination is seen only in the presence of organic additives (Davis, 1967). The presence of various additives in diesel oil has also been held responsible for increased microbial growth problems, but our results show that the additive package tested had no such effect on our isolates.

The increased wear reported in vehicles using the diesel with additive was almost certainly not due to increased microbial growth and other causes must be sought if, indeed, it can be confirmed that increased wear is occurring. One of the functions of the additive package is to clean the system. Surfactants in the additive mixture could cause the removal of scale or biofilms already present, leading to an increase in suspended solids in the fuel and a corresponding increase in wear of finely machined parts. The possibility that additive-containing diesel should not be recommended for use in systems known to be highly contaminated should be considered.

The survey made during visits to the bus companies showed that many of the foremen did not understand the importance of draining the water from the tank bottoms regularly. Hence, the vast majority of the systems were microbially contaminated. A typical biomass was found in some storage tanks, accompanied by an aqueous phase of low pH (3–5). Poorly maintained storage systems were characterised by frequent need to change filters, both in the storage system and in the vehicles, and reduced engine efficiency. Increased replacement of fuel nozzles was also noted, a problem linked by other workers to microbial

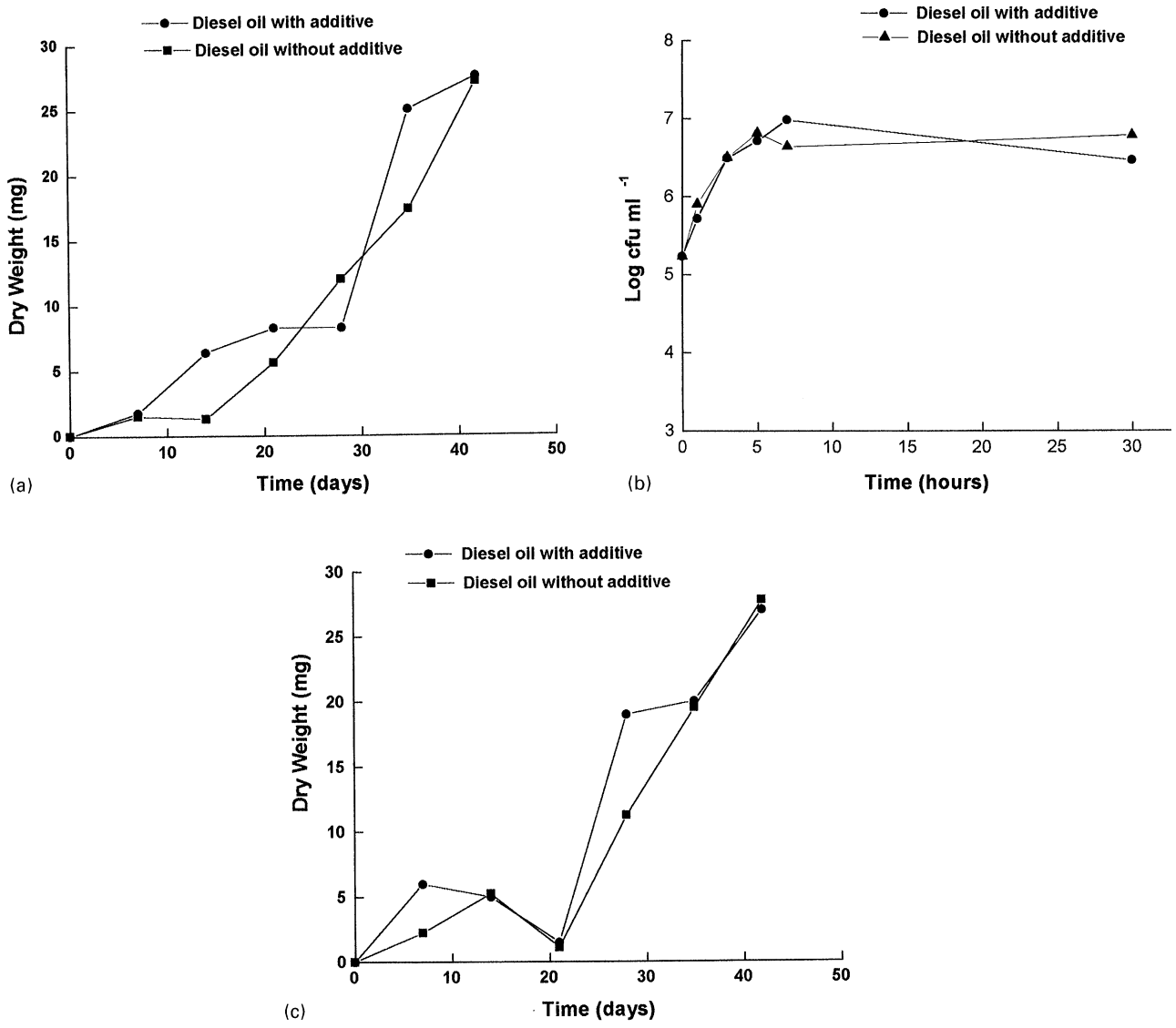

Fig. 1. Growth of fungi in diesel/water systems with and without additive package. (a) *Hormoconis resinae*, (b) *Candida silvicola*, (c) mixture of *Hormoconis resinae*, *Aspergillus niger*, *Aspergillus fumigatus* and *Paecilomyces variotii*. Mean of five replicates.

contamination (Haggatt and Morchat, 1992). In some garages, water was drained daily, while in others 3–6 months could elapse between draining. Several of the tanks in use were already corroded, as could be detected by the presence of rust particles in the drainage water, once again indicating an increased level of suspended solids. It was not possible to show a statistical correlation between the level of microbial contamination and premature failure of engine parts, but there was a relationship between the latter and the use of the additive. However, as seen, this additive did not promote microbial growth in laboratory studies.

Many of the companies visited commented on the fact that vehicle maintenance problems had decreased after underground storage tanks were replaced by aerial systems. One company in Rio Grande do Sul had lost considerable quantities of fuel because of corrosion of in-ground storage tanks (and had thereby caused unnoticed pollution of the surrounding area). When the tanks were replaced by an aerial system with storage tanks inclined at 30° to the horizontal to facilitate water drainage and removal, problems were reduced.

3.3. Ability of biocide to control microbial contamination

During storage of the diesel at room temperature over a bottom of Bushnell–Haas mineral salts, the following stages were noted visually:

Stage 1: Increased turbidity of the aqueous phase, seen at the same time as bacteria were detected by plating.

Stage 2: Formation of a dark, membrane-like layer at the oil/water interface.

Stage 3: Darkening of the oil itself.

Table 2
Dry weight of interfacial biomass in the model diesel/water systems after storage at room temperature for 400 days

Biocide content (ppm)	Biomass in diesel without additive[a] (mg)	Biomass in diesel with additive[a] (mg)	pH of aqueous phase (mean of the two diesels)
0 (control)	24.6	23.1	3.5
0.1	38.2	35.4	5.1
1.0	4.6	3.7	5.85
10.0	3.3	5.9	6.15

[a]Mean of five replicates.

The last stage is due to oxidation of the oil, which, according to Reddy (1988), is accompanied by the formation of a soluble gum. This was delayed in the presence of the additive, which contains an anti-oxidant, but Stages 1 and 2 were observed at the same time in all samples without biocide or with 0.01 ppm biocide. They became turbid after only 2 days of incubation. Biocide at 0.1 ppm was able to maintain the model systems clean for up to 30 days for both types of diesel, while no growth was seen throughout 400 days in the presence of 1 or 10 ppm biocide, showing its efficacy as a control agent.

The first microorganisms detected in the aqueous phase were aerobic bacteria (day 2), including *Micrococcus* and oxidative Gram negative rods, followed by yeasts and filamentous fungi from day 9. Bacteria and yeasts are normally the first colonisers of fuel/water systems and may condition the environment for the growth of filamentous fungi by the production of low molecular weight fatty acids, reducing the pH to that appropriate for fungal growth (Bruce, 1982). In our untreated systems, after 145 days the numbers of viable bacteria decreased considerably. This could have been due to increased acidity, the pH value falling to 3.5 during storage (Table 2). Towards the end of the storage period, at 400 days, SRB were detected. Neihof and May (1983) state that SRB are rarely detected in ship fuel storage tanks with a high fungal and yeast population, but our results show that these anaerobic bacteria may be present in low numbers and remain undetected by traditional techniques until the biomass becomes sufficiently large to provide suitable conditions of anaerobiosis for their growth. Inhibiting the formation of the interfacial biomass at the oil/water interface will thus considerably reduce the risk of SRB-associated corrosion in the system, by avoiding the production of this anaerobic niche.

The fungi most frequently detected in the non-biocide-treated systems were *H. resinae*, *A. fumigatus*, *P. variotii*, a *Penicillium* sp., and the yeasts, *Rhodotorula glutinis* and *Candida silvicola*. The latter was frequently found associated with *H. resinae*. The total biomass after 400 days of storage is shown in Table 2 and confirms the lack of effect of the additive on microbial growth and the efficacy of biocide at 1 and 10 ppm. It is interesting to note that the biocide at 0.1 ppm caused increased biomass formation. Although insufficient replicates were performed to carry out statistical analysis, it seems clear that sub-inhibitory

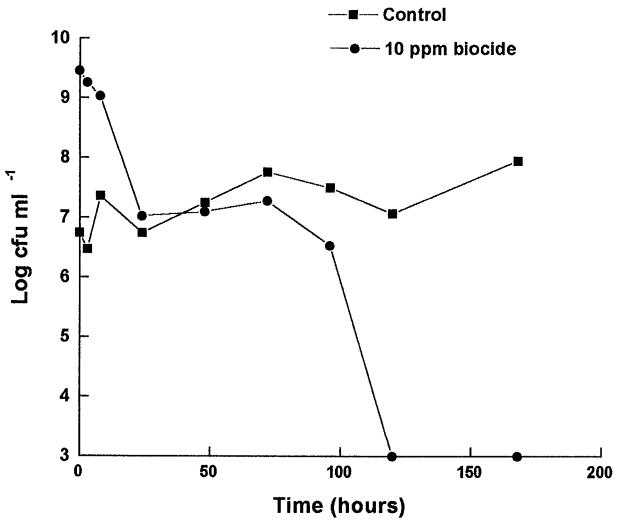

Fig. 2. Effect of addition of 10 ppm isothiazolone biocide on total viable aerobic bacteria and yeast numbers in a diesel/water system previously incubated for 145 days without biocide. Mean of two replicates.

concentrations of the isothiazolone mixture can increase microbial growth, possibly acting as a nutrient source. This has been reported previously for algal populations in the presence of isothiazolone biocide (Ortega-Calvo et al., 1991) and is an important caveat for the biocide user.

The addition of 10 ppm biocide to a highly contaminated system (a control flask after 145 days storage) resulted in a 2-log reduction in the total aerobic bacterial and yeast population within 24 h (Fig. 2). This was accompanied by a visible decrease in turbidity in the aqueous phase. No viable filamentous fungi were detected at this time, indicating that their numbers were < 5 ml^{-1}. From 24 to 49 h, the population was a mixture of aerobic bacteria and the yeast *C. silvicola*, but thereafter the yeast became dominant and was the only organism growing on the plates, indicating that the aerobic bacterial population had decreased to below detection limit (10^3 cfu ml^{-1}). This emphasises the need for an effective anti-yeast biocide in diesel/water systems, but indicates that microbial contamination in these systems can be controlled by the use of chemical agents.

Currently, biocides are not used by the bus companies visited in our survey, but the Brazilian petroleum company,

Petrobrás, has carried out tests on lorries and diesel storage tanks to show that considerable reductions in microbiological problems can be achieved by the use of isothiazolones (Rodrigues et al., 1996). Biocides are employed in other countries to control microbial problems in fuels and this practice should be considered in Brazil, especially in view of the poor maintenance carried out in many oil handling systems.

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