



## Assessment and Control of Biomass Growth in Fuel Storage Tanks at the KNPC Phase II: Evaluation of the Effectiveness of Biocides in Controlling the Microorganisms Causing Problems in Oil Products Tanks

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### Abstract

Microbiologically induced corrosion (MIC) can lead to several deteriorating outcomes in the oil industry. The growth of microorganisms (bacteria) in fuel storage tanks results in costly maintenance measures as well as alteration in oil product specifications, which lead to issues such as corrosion of steel and fiberglass reinforced plastic tanks, tank linings, elastomeric seals and hoses, low points in the piping, leak detectors, turbine pump components, filters, valves, etc.

The objectives of the project were to screen different biocides on controlling MIC in storage tanks, to identify the optimum dosage of biocide needed to control microbial growth in each petroleum product, and to analyze the fuel quality after treatment with biocides. Minimum inhibitory concentrations of seven biocides were estimated and three biocides were selected for detailed study (Acticide CMG, Kathon FP 1.5 Biocide, and Predator 6000). Predator 8000 was found to be similar to Acticide CMG, Kathon FP 1.5 Biocide in chemical composition, and MBC, so it was not included in the coupon weight loss study. Predator 8000 was found to be similar to Acticide CMG, Kathon FP 1.5 Biocide in chemical composition, and MBC, so it was not included in the coupon weight loss study. These biocides are equally effective in the prevention of microbial growth; did not change weight of mild steel coupons (custom made circular coupons of an average 2.45 cm diameter and 0.35 cm thickness, which were made from the same material as of the fuel storage tank were used to check whether the biocide have a corrosive effect); and did not affect the quality of fuels. But under the electron microscopy study, it was proved that Predator 6000 treated coupons had lesser degree of corrosion when compared to that of Acticide CMG and Kathon FP 1.5 Biocide treated coupons. So Predator 6000 was selected as the best biocide.

**Keywords:** Bacteria, Electron Microscopy, Predator 6000

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### Introduction

Biocide is defined as a chemical substance or composition used to kill microorganisms and/ pest organisms considered to be undesirable (Sondossi, 2010). Biocides, which are oxidizing or nonoxidizing, are used widely to control microbial induced corrosion (MIC). Chlorine, bromine, and ozone are typical oxidizing biocides, while nonoxidizing

biocides include formaldehyde, glutaraldehyde, isothiazolones, and quaternary ammonia compounds (Videla and Herrera, 2005). Oxidizing biocides are effective only in freshwater because they are inactivated by organic matter. Nonoxidizing biocides are more effective as they can control bacteria, fungi and algae and are pH independent. Combinations of oxidizing and nonoxidizing biocides are also used. Laboratory studies proved that isothiazolone mixture and quaternary ammonium compounds are more effective than glutaraldehyde and formaldehyde releasing agents (Allsopp et al., 2004). But toxicity of biocides leads to environmental concerns.

Biocides act by interacting with external cellular structures (e.g. Ethylenediaminetetraacetic acid, glutaraldehyde, phenols etc.), cell membranes (ethanol, surfactants, i-propanol, etc.), and cytoplasm (alkylating agents, proflavine, aldehydes, arylmethan, and acridine dyes). Requirements of good biocides include high biocidal activity and wide spectrum of action; long-term action; good solubility in water and hydrocarbons; effective activity at low concentration without fuel deterioration; chemical and thermal stability; corrosion-inertness with regard to the equipment used; ecological safety; compatibility with components of borehole solutions without changing their physical and chemical properties; not causing damage of catalysts at oil refining factories; made from cheap and accessible raw materials; and cost effectiveness (Yemashova et al., 2007; Rossmoore et al. 1988; Gaylarde et al. 1999; Neale, 2003).

4-(2-nitrobutyl) morpholine and 4, 4'-(2-ethyl-2-nitrotrimethylene)-di-

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morpholine Blend: These morpholine derivatives come under C- hydroxymethyl compounds (Fig. 1). Monomethylol nitropropane and dimethylol intermediates react with morpholine to form the end products (Paulus, 2005). This mixture is highly soluble in nonpolar solvents. A concentration between 500 and 1000 ppm is active against microbial deterioration.

The blend is active against bacteria and fungus. Even though they are produced from formaldehyde, morpholine, and nitropropane, they do not exert their antimicrobial effect through the release of formaldehyde (Zielinski and Chilson, 2009).

### Materials and Methods

The present study is aimed at monitoring and controlling bacterial growth and activity in the fuel storage tanks in Kuwait. The biocide, Predator 6000 (4-(nitrobutyl)morpholine (60-100%) and N,N'-methylenebismorpholine (4.99-9.99%)) (Innospec Limited, UK) was imported for the evaluation of its inhibitory effect on bacteria causing corrosion in fuel storage tanks. A group of four microbial strains (*Bacillus* sp., *Brachy bacterium* sp., *Kocuria* sp., and *Staphylococcus* sp.), which are most active, the principal groups isolated from different Kuwait storage fuel tanks (Mataqi et al., 2012), and are able to cause pitting, local spotting (start of corrosion), and biofilm formation were used as the microbial source. In fuel storage tanks, all bacterial species coexist, and to reproduce this situation, a mix culture (prepared by mixing the four selected bacterial cultures) was also used. The inhibitory effects of the biocide were investigated by using the agar well-diffusion method.

### Screening of the Biocide by Agar Well Diffusion Method and Estimation of Minimum Inhibitory Concentration

Nine concentrations from the biocide ranging from 100 ppm to 32,000 ppm were tested. Overnight, nutrient broth cultures of the four bacterial strains and the mix cultures were spread uniformly on to nutrient agar plates. Biocide (150 µl) was placed in wells within the nutrient agar plates. Control plates (without biocide) for each bacterial strain and mix culture were also maintained. Triplicate plates were kept for each concentration. The inhibitory effect was measured by the size of the inhibitory zone formed around the well. Zone diameters were measured after incubating at 37°C for 24 h. Minimum inhibitory concentration was determined as the antilog of the zero intercept of a linear regression of the squared size of these inhibition zone radii (in cm) plotted against the logarithm of the biocide concentration (Bonev et al., 2008).

### Estimation of Minimum Bactericidal Concentration

Minimum bactericidal concentration (MBC) is the minimal amount of antibiotic that results in a ≥99.9% decrease in the initial inoculum within 24h in a standard test and MBCs were estimated according to Suarez et al. (2003) with modifications. Pure broth cultures of *Bacillus* sp., *Brachy bacterium* sp., *Kocuria* sp., and *Staphylococcus* sp. were grown overnight and absorbance was measured at 600 nm and diluted to 2 x 10<sup>6</sup> cells/ml. A mix culture with 2 x 10<sup>6</sup> cells/ml of each bacterial strain was also prepared. Stock solutions of the biocide were prepared and different dilutions were constituted. A volume of 500 µl culture was dispensed into each micro centrifuge tube, and biocide dilutions were added to get a final concentration of 500 to 8000 ppm. Duplicates were maintained. Positive controls of all cultures without biocides and negative controls (nutrient broth alone) were also maintained. All of the tubes were maintained at 37 °C for 24 h. From each tube, an aliquot of 200 µl was cultured on nutrient agar plates and incubated at 37 °C. Bacterial colonies were counted.

### Evaluation of the Selected Biocide by Coupon Weight Loss Method

The biocide (Predator 6000) was further evaluated by coupon weight loss method in five fuels (ATK, Diesel, Kerosene, Mo-Gas, and Ultra) to determine the effect of the biocide on bacteria and mild steel coupons. Custom made mild steel circular coupons of an average 2.45 cm diameter and 0.35 cm thickness and 13 g weight were grinded, smoothed and polished prior to use. Coupons were cleaned with distilled water, and then with acetone. These coupons were then ground with silicon carbide papers (SiC) (Struers, Denmark) with a series of grit sizes; 220, 320, 500, 1000, 1200, 2500, and 4000. Distilled water was used in between and the coupons were grinded for 60 to 180s using each SiC paper placed on the turn table. They were ground to a smooth and scratch free finish.

The coupons were further polished on a polishing device (Struers, Denmark). Polycrystalline diamond suspension (30 ml) (MetaD Supreme, Buehler, USA) ranging in sizes 15, 9, 6, 3, and 1µm was applied on a disc with nap surface (MD-NAP-T, Struers, Denmark) and each coupon was polished for 60 to 180 s. After polishing the coupons were wrapped in tissue paper and stored in a desiccator until use.

### Coupon Weight Loss Experiment

The coupons were defatted in 95% alcohol for 15 min, air dried, and weighed. The coupons, flame sterilized with absolute alcohol, were incubated in each of the five fuels (50 ml) with a mix culture (2 x 10<sup>6</sup> cells/ml of each bacterial strain) and the respective bactericidal concentration of the biocide for 15 d in glass flasks, with occasional mixing. A total volume of 300 µl mix culture in nutrient broth was added to each flask. Biocide control (w/o mix culture, with 300 µl nutrient broth) for the biocide and mix culture control (w/o biocide) and fuel control (w/o biocide, w/o mix culture, with 300 µl nutrient broth) were maintained. The experiment was done in duplicates. Post incubation, from each flask, a volume of 200 µl was plated on nutrient agar to check the inhibition of bacterial growth. The coupons were rinsed with distilled water, incubated with 1N HCl for 30s to remove the corrosion products, wiped with paper towel, and air dried. To determine the percent weight loss, coupons were weighed (Mataqi et al., 2012) on semi-micro balance (CPA225D; Sartorius, Germany) and calculated as follows:

$$\% \text{ Weight loss} = (\text{Weight of coupon before incubation} - \text{Weight of coupon after incubation})$$

$$(\text{Weight of coupon before incubation}) \times 100$$

### Scanning Electron Microscopy of Coupons

These coupons were further analyzed for corrosion by electron microscopy (Analytical Scanning Electron Microscope; JSM-6010LA; Jeol).

### Quality Analysis of Fuels after Treatment with the Biocide

A site was prepared at the Kuwait Institute for Scientific Research campus and small model tanks (5 l) were installed in the field (Fig. 2) for the storage of fuel samples (ATK, Diesel, Kerosene, Mo-Gas, and Ultra) received from the oil sectors in Kuwait. These are small-scale tanks made from the same material (mild steel) used in Kuwait oil storage tanks, and these tanks are with outlets for easy sampling.

Product quality analysis of ATK, Diesel, Kerosene, Mo-Gas, and Ultra after the addition of biocide was conducted at the Petroleum Research Center (PRC), Ahmadi. The facility is equipped to conduct all of the required analyses according to ASTM and IP standard testing procedures. The Predator 6000, at its bactericidal concentration (2000 ppm) was added to 500 ml fuel and was incubated at room temperature for 15 d with occasional mixing. Controls (without the addition of the biocide) were also maintained.

Density, American Petroleum Institute (API) gravity, color, water content, atmospheric distillation, and smoke point of fuels were analyzed and compared with the controls. Bacterial presence in fuels after treatment with the biocide was tested by culturing 200 µl in nutrient agar

Parameter	Reference Method	Equipment
Density	IP190 (Energy Institute, 2005)	Density meter (Anton Paar GmbH, Austria)
API gravity	IP200 (Energy Institute, 2008)	Density meter (Anton Paar GmbH, Austria)
Color	D6045 (ASTM, 2012)	Spectrophotometric colorimeter (PFXi-195; Lovibond® Colour Measurement; Tintometer® Group, UK)
Water content	D6304 (ASTM, 2016)	Karl Fischer Titrator (71000 Aquamax Coulometric Karl Fischer Titrator; G.R. Scientific Limited, UK)
Atmospheric distillation	D86 (ASTM, 2016)	Automated distillation analyzer (OptiDist; PAC, USA)
Smoke point	D1322 (ASTM, 2015)	Smoke point analyzer (Smoke point-SP 10; Ad Systems, France)

**Table 1:** Reference Method and Equipment Used for the Parameters Studied

### Data Analysis

Inhibitory zone data was analyzed by linear regression. Data of the coupon weight loss experiment was analyzed by one-way Analysis of Variance (ANOVA) and data of the product quality experiments were analyzed by Anova: 2 factor.  $p < 0.05$  was considered significant and the post hoc analysis was performed by Tukey HSD test.

### Results

The biocide was screened for its inhibitory effect by agar well diffusion technique and was further studied to find MBC to weight loss experiment. The effect of biocide on mild steel coupons and different fuels were studied. Results of initial screening, MBC, effects of biocide on mild steel coupons, and product quality analysis are presented in the following sections.

#### Minimum Inhibitory Concentrations of the Biocide.

The biocide (Predator 6000) was evaluated for the inhibitory effect on four bacterial strains and the mix culture.

In control plates without biocide, complete bacterial growth was observed. Inhibition zones were absent in lower concentrations, and diameter of the zones increased with increasing concentrations. Fig. 3 shows linear regression of the squared inhibition zone radii ( $x^2$ ) against the logarithm of the concentration of the predator 6000.

MIC of the biocide for each bacterial culture and mix culture, which are the antilogs of the zero intercepts of the regression lines, is presented in Fig. 3. Predator 6000 was most effective against *Brachybacterium* sp. than the other bacterial strains (Table 2).

#### Coupon Weight Loss Experiment

In Diesel, Kerosene, and Mo-Gas, the biocide did not differ significantly ( $P > 0.05$ ) with regard to coupon weight loss (Table 3). Coupons treated with the biocide (w/o mix culture) did not differ significantly when compared to coupons in fuel controls (w/o biocide, w/o mix culture). No bacterial growth was detected in the biocide-treated fuels. Predator 6000 was completely soluble in all the fuels.

Biocide	MIC for <i>Brachybacterium</i> sp.	MIC for <i>Bacillus</i> sp. (ppm) 1308.93	MIC for <i>Staphylococcus</i> sp.	MIC for <i>Kocuria</i> sp.	MIC for *Mix culture
Predator 6000	559.88	1308.93	2970.61	838.67	1927.10

**Table 2:** Minimum Inhibitory Concentration of the Biocide for *Brachybacterium* sp., *Bacillus* sp., *Staphylococcus* sp., *Kocuria* sp. and Mix Culture

\* Mix culture: Equal proportions of the four bacterial strains *Brachybacterium* sp., *Bacillus* sp., *Staphylococcus* sp., and *Kocuria* sp.

Fuels	Predator 6000 + Mix Culture	Predator 6000 w/o Mix Culture	w/o Biocide w/o Mix Culture	w/o Biocide with Mix Culture
ATK	0.08 ± 0.017b	0.13 ± 0.02ab	0.25 ± 0.0066ab	0.16 ± 0.0027ab
Diesel	0.0025 ± 0.00025c	0.01 ± 0.002bc	0.2 ± 0.024a	0.16 ± 0.015ab
Kerosene	0.04 ± 0.016	0.041 ± 0.015	0.16 ± 0.0063	0.14 ± 0.073
Mo-Gas	0.27 ± 0.12	0.15 ± 0.15	0.31 ± 0.0086	0.24 ± 0.11
Ultra	0.07 ± 0.012bc	0.027 ± 0.00034c	0.27 ± 0.042a	0.29 ± 0.06a

**Table 3:** Percent Weight Loss of Mild Steel Coupons Treated with Mix Culture and the Biocide in Different Fuels Values are means ± standard deviation,  $n = 2$  per treatment group. Means in a row without a common superscript letter differ ( $P < 0.05$ ) as analyzed by one-way ANOVA and the TUKEY test.

### Scanning Electron Microscopy of Coupons

Pits corruptions were not observed on coupons after a 15d treatment with the biocide and mix culture, in ATK, Diesel, Kerosene, Mo-Gas and Ultra (Fig. 4).

### Quality of Fuels Treated with Biocide

ATK, Diesel, Kerosene, Mo-Gas, and Ultra were treated with the biocide Predator 6000 for 15 d and the details of fuel quality are given subsequently.

### Density, API gravity, Color, and Smoke Point of Biocide Treated Fuels

Densities of the fuels treated with Predator 6000 (2000 ppm) did not show any significant difference ( $P > 0.05$ ) from the respective controls

(Fig. 5). API gravities (Fig. 6) and color (data not shown) and smoke points (Fig. 7) of ATK, Diesel, kerosene, Mo-Gas, and Ultra also did not change ( $P > 0.05$ ) after treatment with the biocide.

### Water Content of Biocide Treated Fuels

Water content of all the five fuels increased ( $P < 0.05$ ) after the addition of the biocide (Table 4). In ATK, water content increased from 22 ppm to 43 ppm from control after treatment with the biocide. In diesel and Mo 95, the lowest increase in water content was in Predator 6000 treatment, whereas kerosene had similar increase in water content after treatment with Predator 6000 (27 ppm). When compared to the control, water content in ultra-increased by 210 ppm, after treatment with Predator 6000 (Table 4).

Fuels	Control (ppm)	Predator 6000 (ppm)
ATK	32.97	75.82
Diesel	48.94	67.09
Kerosene	33.48	60.35
Mo 95	286.08	355.81
Ultra	170.17	380.47

**Table 4:** Water Content of ATK, Diesel, Kerosene, Mo-Gas, and Ultra after Treatment with the Biocide

### Atmospheric Distillation of Biocide Treated Fuels

Atmospheric distillation results of ATK (Table 5), diesel (Table 6), kerosene (Table 7), Mo-Gas (Table 8), and Ultra (Table 9) are given in this section. Atmospheric distillation temperatures of the fuels did not change considerably from the controls. In ATK, maximum difference was in 95% distillation of Predator 6000 treatment, which showed a decrease in 4.4 °C. Initial boiling Point (IBP) and final boiling point (FBP) did not change considerably (Table 5). 95% distillation of diesel

showed an increase of 12.9 °C in Predator 6000 treatment (Table 6). IBP decreased by 1.2 °C in kerosene after treatment with Predator 6000 (Table 7). In Mo-Gas, 95% distillation temperature increased in Predator 6000 by 5.8 °C treatment (Table 8). IBP of Ultra decreased by 0.5 °C after treatment with Predator 6000 (Table 9), whereas FBP increased by 2.6 °C after treatment with Predator 6000. No microbial growth was detected in any of the fuels after treatment with biocide, and a few colonies were detected in the controls w/o the biocide.

Atmospheric Distillation	Control (°C)	Predator 6000 (°C)
IBP*	154.1	153.4
5%	163.5	163.5
10%	166.0	166.4
15%	168.6	168.4
20%	171.5	171.1
30%	175.3	175.3
40%	180.8	180.5
50%	186.9	186.5
60%	194.0	193.7
70%	202.4	201.9
80%	213.0	212.1
85%	220.0	218.6
90%	229.2	227.2
95%	246.0	241.6
FBP*	262.5	261.8

**Table 5:** Atmospheric Distillation Results of ATK Treated with Predator 6000

\*IBP – Initial boiling point; FBP – Final boiling point

Atmospheric Distillation	Control (°C)	Predator 6000 (°C)
IBP*	209.3	206.6
5%	238.3	236.8
10%	247.4	245.7
15%	254.3	252.1
20%	259.0	258.1
30%	269.7	269.5
40%	280.2	280.2
50%	289.6	290.3
60%	299.4	301.1
70%	310.4	313.2
80%	322.6	327.3
85%	329.3	335.7
90%	337.6	346.1
95%	347.8	360.7
FBP*	374.6	374.5

**Table 6:** Atmospheric Distillation Results of Diesel Treated with Predator 6000

Atmospheric Distillation	Control (°C)	Predator 6000 (°C)
IBP	155.6	154.4
5%	169.7	170.2
10%	174.8	175.0
15%	178.3	178.4
20%	181.2	181.3
30%	186.6	186.8
40%	191.8	191.7
50%	197.3	197.2
60%	203.4	203.3
70%	210.5	210.6
80%	219.1	219.1
85%	224.2	224.3
90%	230.6	230.5
95%	239.1	239.2
FBP	254.9	253.5

**Table 7:** Atmospheric Distillation Results of Kerosene Treated with Predator 6000  
\*IBP – Initial boiling point; FBP – Final boiling point

Atmospheric Distillation	Control (°C)	Predator 6000 (°C)
IBP	41.5	40.7
5%	61	60.9
10%	67.1	67.2
15%	71.9	72.1
20%	76.6	76.9
30%	86.4	87.5
40%	98.6	99.5
50%	109.8	109.7
60%	120.9	121.9
70%	133.1	134.1
80%	145.9	147.1
85%	153.2	154.9
90%	162.4	165.1
95%	178.5	184.3
FBP	188.6	184.3

**Table 8:** Atmospheric Distillation Results of Mo-Gas 95 Treated with Predator 6000  
\*IBP – Initial boiling point; FBP – Final boiling point

Atmospheric Distillation	Control (°C)	Predator 6000 (°C)
IBP	37.7	37.2
5%	58.2	56.3
10%	66.3	64.0
15%	72.5	70.2
20%	78.5	76.0
30%	90.7	87.7
40%	104.4	101.1
50%	114.5	111.4
60%	125.4	122.8
70%	135.8	132.9
80%	146.5	143.9
85%	151.3	149.4
90%	160.8	157.3
95%	179.7	170.4
FBP	186.5	189.1

**Table 9:** Atmospheric Distillation Results of Ultra Treated with Predator 6000  
\*IBP – Initial boiling point; FBP – Final boiling point

## Discussion

Microbiologically-induced corrosion is a major problem in the fuel storage tanks used in the oil industry. Microbiological corrosion is induced by bacteria, fungi, and algae (Lane, 2005; Little et al., 1997). For prevention and control of microbiologically-induced corrosion, the system needs to be clean (Lane, 2005; Videla and Herrera, 2005; Videla, 2002), which is not always practical (Videla and Herrera, 2005). Application of biocides can keep corrosion under control. Biocides can be either oxidizing or nonoxidizing, of which nonoxidizing are more effective. Nonoxidizing biocides are usually formaldehyde, glutaraldehyde, isothiazolones, and quaternary ammonia compounds (Videla and Herrera, 2005). The Predator 6000 is a mixture of 4-(nitrobutyl) morpholine (60-100%) and N,N'-methylenebismorpholine (4.99-9.99%). Even though it releases small amounts of formaldehyde, this will not contribute to substantial antimicrobial effect (Zielinski and Chilson, 2009). Oil-soluble corrosion inhibitors such as Predator 6000 may form a persistent monolayer film adsorbed at the metal solution interface, which prevents corrosion (Rajasekar et al., 2007).

The MIC of an antibacterial agent for a given organism is the lowest concentration of the agent required to inhibit the growth of an inoculum of the bacterium in a standard test. The MBC is the minimal concentration of antibiotic that kills the inoculum and can be determined by sub-culturing to agar media without antibiotics. MBC is the minimal amount of antibiotic that results in a  $\geq 99.9\%$  decrease in the initial inoculum within 24 h in a standard test (French, 2006). MBC of the Predator 6000 is 2000 ppm. MIC for Predator 6000 ranged from 560 to 2971 ppm of the product for pure cultures.

The coupon weight loss experiment demonstrated that corrosion in Predator 6000 coupons was very less when compared to the controls. This biocide was completely soluble in all the fuels tested. This result is in contrast to the view that isothiazolone mixture and quaternary ammonium compounds are more effective than glutaraldehyde and formaldehyde releasing agents (Yemashova et al., 2007; Allsopp et al., 2004).

Quality of the all the five fuels (ATK, Diesel, Kerosene, Mo-Gas, and Ultra) did not change in terms of density, API, color (data not shown), and smoke point after treatment with the biocide (Predator 6000). But water content of all the fuels increased considerably when treated with each of the biocide. Use of biocide in powder form may solve this problem. Atmospheric distillation temperatures of the fuels increased or decreased slightly in some cases but not much as to affect the performance of the fuels. So to conclude; inhibitory effects of the biocide was screened against a mix culture of *Bacillus* sp., *Brachybacillus* sp., *Kocuria* sp., and *Staphylococcus* sp. by agar well diffusion technique, and MICs were estimated. The biocide, Predator 6000, was further studied to find its effect on the quality of fuels (ATK, Diesel, Kerosene, Mo-Gas, and Ultra) and on the mild steel coupons. Quality of the all the five fuels did not change in terms of density, API, color, smoke point, and atmospheric distillation, after treatment with Predator 6000. Water content of the fuels increased after treatment with the biocide.

Coupon weight loss study proved that the biocide did not change the weight of the coupons significantly. But coupons in fuel controls (w/o biocide, w/o mix culture, with 300  $\mu$ l nutrient broth) were corroded. Interestingly, Predator 6000 showed very less degree of corrosion when compared to fuel controls. So Predator 6000 can be considered as a very effective biocide against the bacteria in the fuel storage tank in Kuwait.

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