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INDICATOR COMPOSITION FOR MICROORGANISMS AND TEST METHOD EMBODYING SAME

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This invention relates to an indicator composition for viable microorganisms and to a test method embodying said composition. More particularly, the invention relates to a composition and procedure suitable for field testing for the presence or absence of viable microorganisms in hydrocarbon fuels and/or water bottoms associated therewith.

In recent years, the adverse effects of microorganisms on hydrocarbon fuels, especially distillate fuels (e.g., jet fuel, diesel fuel, kerosene, home heating oil, etc.) have received wide recognition. As a consequence, a substantial need has developed for materials and procedures to identify and combat this problem. The present invention is addressed to satisfying the need for a simple, rapid, substantially foolproof field test for the presence or absence of viable microorganisms in hydrocarbon fuels and/or water bottoms associated therewith.

In accordance with the present invention, a sample of fuel and/or water bottoms is contacted with an indicator composition comprising an aqueous solution of nutrient salts, a normally liquid, preferably olefin-free material selected from the group consisting of hydrocarbons and mixtures of hydrocarbons, and a compound selected from the group consisting of the triphenyl monotetrazolium chlorides.

Heretofore the procedure for determining the presence or absence of microorganisms in hydrocarbon fuels and/or water bottoms involved the cumbersome, time consuming, and expensive steps of collecting a sample of fuel and/or water bottoms, forwarding it to a laboratory where the sample was assayed for viable microorganisms by conventional pour-plate techniques, and then transmitting the results from the laboratory back to the field.

By contrast, the procedure of the present invention can be completed in the field where the results are readily available within a matter of as little as 12 hours. This is made possible by the use of a triphenyl monotetrazolium chloride, taking advantage of the known phenomenon by which these colorless compounds are reducible to a colored formazan. What is surprising is that the necessary reduction and concomitant color formation can be accomplished as a result of the metabolism of a small quantity of microorganisms and within a very short period of time, generally from about 12 to 72 hours.

In addition to the triphenyl monotetrazolium chloride, the indicator composition contains an aqueous solution of nutrient salts. These salts are necessary to the survival of the microorganisms and should be present, at minimum, in an amount which will sustain the microorganisms for at least a period of time required to effect a reduction of the triphenyl monotetrazolium chloride to a formazan; usually from about 12 to about 72 hours. Generally, the combination of salts provided by the Bushnell-Haas medium (J. Bacteriology, vol. 41, p. 653 (1941)) is quite adequate for this purpose. It is sometimes desirable, however, to enrich this combination of nutrient salts with other ingredients to accelerate the metabolic activity of the microorganisms, and thus speed up the reduction of the

triphenyl monotetrazolium chloride. To this end amounts of ammonium nitrate and/or ferric chloride beyond those called for in the Bushnell-Haas formula may be utilized. Glycerine, lactates, citrates, succinates, sodium bicarbonate, manganous chloride, and sodium molybdate may also be used as enriching ingredients.

Other standard nutrient salt solutions may also be employed, including those disclosed by Davis, Chase, and Raymond (Applied Microbiology, vol. 4, pp. 310–315 (1956)); Webley and DeKock (Biochemical J., vol. 51, pp. 371–375 (1952)); Stone and Fenske (J. Bacteriology, vol. 44, pp. 169–178 (1942)); and Leadbetter and Foster (Archiv fur Mikrobiologie, vol. 35, page 92 (1960)).

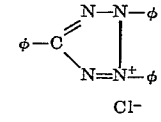
Another ingredient of the indicator composition is a generally liquid, preferably olefin-free hydrocarbon or a mixture of such hydrocarbons. Olefins are preferably omitted since they tend to interfere with the reduction of the tetrazolium salt, thus slowing the rate of color formation. Since the hydrocarbon ingredient is utilized by the microorganisms as an energy source, it is sometimes desirable to use a hydrocarbon or a mixture of hydrocarbons corresponding to those present in the fuel in which the microorganisms have been living. This obviates any problems the microorganisms might have in adapting to a new environment and is a practice which can be conveniently followed where the fuel is relatively free of olefins. In the case of home heating oil, however, where the olefin content may be substantial, it is preferable to use an olefin-free hydrocarbon as the energy source ingredient of the indicator composition.

In general, however, any hydrocarbon which can be readily ingested by the microorganisms is useful as an energy source, including a variety of refinery streams and products such as water white distillate, Stoddard solvent, jet fuel, and kerosene, or more broadly a distillate fuel (i.e., a fuel at least 30% of which boils above 400° F. and not more than 15% of which boils below 250° F.). Pure hydrocarbons and desirably the olefin-free grade of iso-octane, nonane, n-dodecane, tetradecane, n-hexadecane, and decahydronaphthalene, as well as mixtures of these, may also be used. Enough hydrocarbon to support life for 12 to 72 hours should be used. Generally, 0.1% to 1% by volume based on the aqueous solution of nutrient salts is adequate as a lower limit. More can be used if desired.

It has also been found, where a pure hydrocarbon is used as the energy source ingredient, that acceleration of color formation can be realized by the addition of minor amounts of the corresponding alcohol or aldehyde to the indicator composition. For instance, where n-dodecane is used as the energy source ingredient, the addition of as little as 2% by volume of n-dodecanol or, of dodecanal, accelerates color formation by a factor of 5 when compared with the same formulation containing no dodecanal or n-dodecanol.

While the invention contemplates the use of any known triphenyl monotetrazolium chloride, only two are commercially available and are therefore of particular interest. These two are:

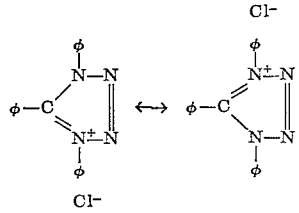
2,3,5 triphenyl tetrazolium 2H chloride (known commercially as TTC) which is believed to have the formula



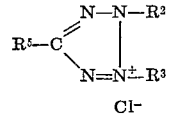
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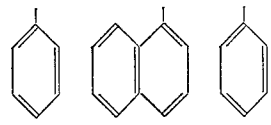
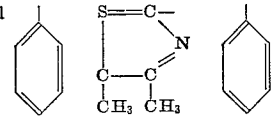
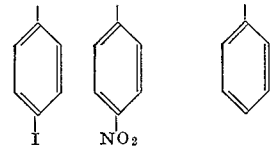
1,4,5 triphenyl tetrazolium chloride (known commer-

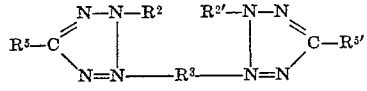
cially as Tetrazolium Red) which is believed to have the formula

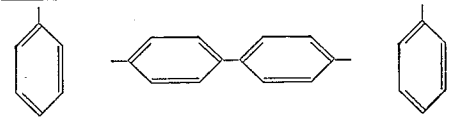
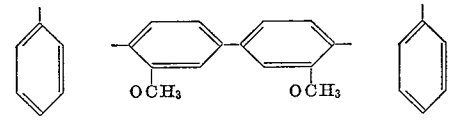
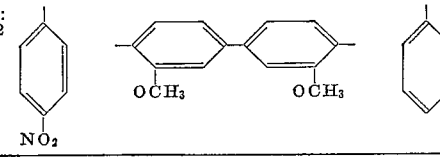


The usefulness of tetrazolium compounds for purposes of this invention is quite selective to the triphenyl monotetrazolium species. For reasons to be detailed presently, the compounds tabulated below were found inoperative:



	R^2	R^3	R^5
Tetrazolium Violet: (2,5 diphenyl 3a naphthyl 2H tetrazolium chloride).			
Thiazolyl Blue (MTT): 3(4,5 dimethyl thiazolyl 2) 2,5 diphenyl 2H tetrazolium bromide.			
Iodonitro Tetrazolium Violet (INT): 2 (p Iodophenyl)-3-(p-Nitrophenyl) 5 phenyl 2H tetrazolium chloride.			



	$R^{2'}$, $R^{2'}$	R^3	$R^{5'}$, $R^{5'}$
Neo-Tetrazolium (NT): (3,3' (4,4' biphenylene)-bis [2,5 diphenyl 2H tetrazolium chloride].			
Tetrazolium Blue (BT): (3,3' (3,3' dimethoxy 4,4' biphenylene)-bis [2,5 diphenyl 2H terrazolium chloride].			
p-Nitro Blue Tetrazolium Chloride (p-Nitro BT): (3,3' (3,3' dimethoxy 4,4' biphenylene)bis [2 (p-nitro phenyl) 5 (phenyl) 2H tetrazolium chloride].			

As another aspect of the invention, the indicator composition can be used to test the effectiveness of a biocide to sterilize a source of fuel or water bottoms known to be contaminated with microorganisms. If the microorganisms are killed before they have an opportunity to reduce the triphenyl tetrazolium chloride, no color formation will take place. In the case of a slow-acting biocide, it is possible that some reduction and color formation will take place before the microorganisms expire, but if the biocide is effective at all, the color should be faint when compared with the color formed in a biocide-free indicator composition.

It follows that in this test a biocide-free indicator composition should be run alongside the biocide-treated indicator composition. This will permit confirmation of the presence of microorganisms in the fuel or water bottom sample and possibly give some information about the speed with which the biocide kills.

The indicator composition is conveniently handled in serum bottles, though the usefulness of other containers will be readily apparent to those skilled in the art.

Serum bottles are provided with a split rubber stopper adapted to receive the needle portion of a syringe-needle unit, and a crimpable sealing ring adapted to secure the rubber stopper to the serum bottle. After being partially filled with indicator composition, the rubber stopper is inserted and crimped in place with the sealing ring, forming an air-tight package.

The sample of fuel and/or water to be tested can be drawn up into a syringe-needle unit and a measured amount of material can then be injected into the indicator composition via the split rubber stopper of the serum bottle.

To insure that the test sample of fuel and/or water is the only source of viable microorganisms introduced into the test procedure, it is important that the serum bottle and its parts, the unused syringe-needle unit, and the indicator composition itself, all be sterile.

With the exception of the triphenyl tetrazolium chloride, the indicator composition ingredients can be autoclaved together in the serum bottle. At sterilizing temperatures the triphenyl tetrazolium chlorides are reduced in the presence of hydrocarbons and so must be separately autoclaved, and when cool, added to the other indicator composition ingredients in the serum bottle.

Preparation of indicator compositions

Example I(a)

2500 ml. of Bushnell-Haas nutrient salt solution were prepared by adding to the following salts in the amounts indicated, enough distilled water to make up to 2500 ml.:

$MgSO_4$	grams	0.5
KH_2PO_4	do	2.5
K_2HPO_4	do	2.5
$CaCl_2.2H_2O$	do	0.05
NH_4NO_3	do	2.5
$FeCl_3$ (sat. soln)	drops	5

25 ml. of this nutrient salt solution were added to each of a series of 100 ml. serum bottles followed by the addition to each bottle of 50 ml. of a commercial jet fuel (Turbine Fuel A).

The split rubber stoppers were inserted loosely in each bottle and the sealing rings were put on but not crimped.

The assembled bottles were then autoclaved for 20 minutes at 121° C.

In a separate container 0.25 gram of 2,3,5 triphenyl tetrazolium 2H chloride (Triphenyl Tetrazolium Chloride TTC) was admixed with 20 ml. of distilled water, and the mixture was autoclaved for 20 minutes at 121° C.

When the serum bottles and the tetrazolium salt solution were cool enough to handle, 0.1 ml. of the latter was added to the former observing strict sterile techniques. The split rubber stoppers were then firmly inserted in the serum bottles, and the sealing rings were crimped in place. The bottles of colorless indicator composition were then ready for use.

Example I(b)

The procedure of Example I(a) was repeated with the following change in ingredients.

Instead of Turbine Fuel A as the hydrocarbon energy source, a mixture of 90 vol. percent n-dodecane and 10% n-hexadecane (olefin-free grades) was used.

Example II(a)

The procedure of Example I(a) was repeated with the following changes in ingredients.

1. An enriched nutrient salt solution was used which, in addition to the Bushnell-Haas recipe, contained the following:

$NaHCO_3$	grams	0.25
$Na_2MoO_4 \cdot 2H_2O$	do	0.0025
$MnCl_2 \cdot 2H_2O$	do	0.0025
$FeCl_3$ (sat. soln)	drops	5
Glycerine	grams	25
Sodium lactate syrup (60%)	ml.	10.3

2. A mixture of 90 vol. percent n-dodecane and 10% n-hexadecane (olefin-free grades) was substituted for Turbine Fuel A as the hydrocarbon energy source.

The results were a series of bottles containing a colorless indicator composition.

Example II(b)

The procedure of Example II(a) was repeated except that 1 ml. of a commercial biocide (a mixture of dioxaborinanes) was added to each serum bottle prior to autoclaving.

The results were a series of bottles containing a colorless biocide-containing indicator composition.

Example III

The procedure of Example II(a) was repeated except that 1,4,5 triphenyl tetrazolium chloride (Tetrazolium Red) was used instead of Triphenyl Tetrazolium Chloride (TTC).

The results were a series of bottles containing a colorless indicator composition.

Examples IV–IX

Attempts to repeat the procedure of Example II(a), using the following tetrazolium salts, proved unsuccessful because autoclaving to sterilize the salts resulted in their decomposition into colored dyes:

IV Tetrazolium Violet
V Thiazolyl Blue
VI Iodonitro Tetrazolium Violet
VII Neo-Tetrazolium
VIII Tetrazolium Blue
IX P-Nitro Blue Tetrazolium Chloride

To circumvent this problem, solutions of each of these salts were sterilized by ultrafiltration and then combined with the other ingredients per Example II(a). This resulted in a series of bottles of colorless (except for Examples V and IX which were yellow) indicator compositions.

Example X

A pair of serum bottles containing indicator compositions prepared in accordance with Examples I–IX were utilized in the following experiment.

One bottle ("A") from each pair served as a control and into the other ("B") was injected, via presterilized syringe-needle units, with 1 ml. of a "conditioned inoculum" of viable microorganisms. This inoculum is a water suspension of mixed cultures of bacteria and fungi collected from a number of petroleum stock storage facilities held in intimate contact with commercial turbine fuel for 30 days or more.

When plated, using standard dilution and pour-plate techniques, the number of viable organisms per ml. of inoculum was determined to be 2800.

All of the bottles, controls included, were shaken and then placed in a dark room at room temperature. The color of the solutions was checked at 12-hour intervals over a period of 48 hours, with the following results:

	Bottle	Hours			
		12	24	48	72
Example:					
I(a)	A	0	0	0	0
	B	0	0	1	2
II(a)	A	0	0	0	0
	B	2	4	4	4
I(b)	A	0	0	0	0
	B	1	1	4	4
II(b)	A	0	0	0	0
	B	0	0	0	0
III	A	0	0	0	0
	B	2	3	4	4
IV	A	0	0	0	0
	B	0	0	0	0
V	A	0	0	0	0
	B	0	0	0	0
VI	A	0	0	0	0
	B	0	0	0	0
VII	A	0	0	0	0
	B	0	0	0	0
VIII	A	0	0	0	0
	B	0	0	0	0
IX	A	0	0	0	0
	B	0	0	0	0

NOTE.—0=No color change; 1=Hint of pink; 2=Slightly pink; 3=pink 4=Red.

Recalling that the A and B bottles of each example differed only in that the latter contained viable microorganisms, it will be noted that none of the A bottles changed color during the 72-hour storage period.

Comparing the B bottles of various examples, the following information obtains:

Examples

I(a)–I(b).—Color formation appeared sooner and became more intense where an olefin-free hydrocarbon ingredient was used.

I(b)–II(a).—Color formation became more intense, earlier, where an enriched nutrient salt solution was used.

II(a)–II(b).—The use of a commercial biocide extinguished microbiological life and prevented color formation.

II(a)–III.—2,3,5 triphenyl tetrazolium 2H chloride and 1,4,5 triphenyl tetrazolium chloride are essentially equivalent for purposes of the invention.

IV–IX.—The tetrazolium salts used in these examples were inoperative for purposes of the invention since they did not undergo a color change.

FIELD TEST USE OF INDICATOR COMPOSITIONS

The following tests utilized indicator compositions prepared in accordance with Examples II(a) and II(b).

Example XI

The main inboard fuel tanks (Nos. 2 and 3) of a Convair 880 jet airplane were defueled in accordance with standard practices for the equipment. This left about ½ to 3 inches of fuel in the bottom of the tank, the depth varying with the contour of the tank.

The needle of a presterilized syringe-needle unit was dipped into this remaining fuel in tank No. 2 and a 2 ml. sample was drawn into syringe. 1 ml. of the fuel was then injected into a serum bottle containing indicator composition prepared in accordance with Examples II(a) and II(b). The two bottles were shaken and then stored in the dark at ambient temperatures.

Using fresh serum bottles, the procedure was repeated with a sample of fuel from the bottom of tank No. 3.

A sample of fuel from each of tank Nos. 2 and 3 was plated using standard pour-plate techniques. The number of viable organisms per ml. of inoculum in each case was reported as "TNTC" (too numerous to count).

The four bottles were held in storage for five days, and the following observations were made on a daily basis:

Days Storage	Tank No. 2		Tank No. 3	
	Indicator II(a)	Composition II(b)	Indicator II(a)	Composition II(b)
1	Pink	Colorless	Pink	Colorless.
2	do	do	do	Do.
3	do	do	do	Do.
4	do	do	do	Do.
5	do	do	do	Do.

These data show the fuel samples from both tanks were contaminated with microorganisms, as evidenced by the pour-plate results and confirmed by the pink coloration of the samples of biocide-free indicator composition. Also shown is that the biocide used was effective in killing the microorganism present in the fuel samples, as evidenced by the colorless appearance of the biocide-containing samples of indicator composition.

Example XII

During a four-month period following the test described in Example XI, fuel tank No. 3 of the Convair 880 was serviced with fuel containing the recommended dosage of the same commercial biocide used in preparing the indicator compositions of Example II(b). Fuel tank No. 2 was serviced with the same fuel but without the biocide.

At the end of the four-month period, tank Nos. 2 and 3 were again defueled, fuel samples taken and serum bottles inoculated, all as described in Example XI. After 72 hours' storage these observations of the serum bottles were made:

Tank No. 2		Tank No. 3	
Indicator II(a)	Composition II(b)	Indicator II(a)	Composition II(b)
Pink	Colorless	Colorless	Colorless.

These data confirm that the samples of biocide-treated fuel from tank No. 3 were sterile. Also shown is that the samples' biocide-free fuel from tank No. 2, while unsterile, could be sterilized by the biocide used in treating the fuel in tank No. 3.

Example XIII

2 ml. samples of fuel were withdrawn into presterilized syringe-needle units from the fuel tanks of each of two in-service railroad diesel locomotives. Half of each fuel sample was then injected into a serum bottle containing indicator composition prepared in accordance with Examples II(a) and II(b). The four bottles were then shaken and stored in the dark at ambient temperatures.

A visual inspection of the bottles was made after 24 hours' storage, with the following observations:

Locomotive No. 2639		Locomotive No. 2575	
Indicator II(a)	Composition II(b)	Indicator II(a)	Composition II(b)
Pink	Colorless	Pink	Colorless.

These data show that both fuels' samples were contaminated with microorganisms and that the presence of the biocide in the indicator composition sterilized the fuel samples.

While the indicator composition of this invention is particularly useful in determining the presence or absence of viable microorganisms associated with distillate fuels, it may also be used to test other hydrocarbon media such as gasoline, cutting oil, soluble oil, Stoddard solvent, hydraulic oil, lubricants, and the like.

I claim:

1. A sterile composition suitable for field testing for the presence or absence of viable microorganisms in hydrocarbon fuels and water-bottoms associated therewith, comprising an aqueous solution of nutrient salts, a water soluble lactate, a normally liquid substantially olefin-free hydrocarbon and a triphenyl tetrazolium chloride indicator.

2. The composition of claim 1 in which said triphenyl tetrazolium chloride is 2,3,5 triphenyl tetrazolium 2H chloride.

3. The composition of claim 1 in which said triphenyl tetrazolium chloride is 1,4,5 triphenyl tetrazolium chloride.

4. The composition of claim 2 in which said olefin-free material consists of a mixture of 90 vol. percent n-dodecane and 10 vol. percent n-hexadecane.

5. A sterile composition suitable for field testing for the presence or absence of viable microorganisms in hydrocarbon fuels and water bottoms associated therewith, comprising an aqueous solution contain per thousand ml. thereof:

$MgSO_4$	grams	0.2
KH_2PO_4	do	1.0
K_2HPO_4	do	1.0
$CaCl_2 \cdot 2H_2O$	do	0.02
$NaHCO_3$	do	0.10
$Na_2MoO_4 \cdot 2H_2O$	do	0.001
$MnCl_2 \cdot 2H_2O$	do	0.001
NH_4NO_3	do	1.0
$FeCl_3$ (sat. soln)	drops	4
Glycerine	grams	10
Sodium lactate syrup (60%)	ml	4.1

a mixture of 90 vol. percent n-dodecane and 10 vol. percent n-hexadecane (olefin-free grades) and 2,3,5 triphenyl tetrazolium 2H chloride.

6. A method of testing a substantially olefin-free hydrocarbon fuel and/or water-bottoms associated therewith for the presence or absence of viable microorganisms which comprises:

(a) forming a mixture of said hydrocarbon fuel and/or water-bottoms with the composition of claim 4,

(b) storing said mixture at ambient temperature for a period of from about 12 to about 72 hours, and

(c) observing any color transformation.

7. A method of testing a sample of hydrocarbon fuel and/or water bottoms associated therewith for the presence or absence of the viable microorganisms which comprises admixing said sample and the composition of claim 5, storing the mixture at ambient temperatures for a period of from about 12 to about 72 hours and observing any color change which takes place.

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U.S. Cl. X.R.

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