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(54) Title: A RAPID METHOD FOR THE ASSESSMENT OF THE INHIBITION AND KILL OF ANAEROBIC BACTERIA BY TOXIC COMPOUNDS

(57) Abstract

A rapid method for determining the minimum inhibitory concentration of biocides or biocidal agents for use in anaerobically contaminated aqueous systems is disclosed. This rapid technique provides for an opportunity to determine the minimum amount of antimicrobial agents, or biocides either singularly or combination, to control anaerobic growth in aqueous streams contaminated by microorganisms. The test involves the use of pH indicator dye systems which respond to the environmental changes caused by the production of byproducts of carbohydrate metabolism by anaerobes.

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# A RAPID METHOD FOR THE ASSESSMENT OF THE INHIBITION AND KILL OF ANAEROBIC BACTERIA BY TOXIC COMPOUNDS

## Field of the Invention

A rapid method for determining the minimum inhibitory concentration of biocides or biocidal agents for use in anaerobically contaminated aqueous systems is disclosed. This rapid technique provides for an opportunity to determine the minimum amount of antimicrobial agents, or biocides either singularly or combination, to control anaerobic growth in aqueous streams contaminated by microorganisms. The test involves the use of pH indicator dye systems which respond to the environmental changes caused by the production of byproducts of carbohydrate metabolism by anaerobes.

The technique provides an answer within a time period of from about 30 minutes to about 8-10 hours as opposed to normal testing procedure that can require up to 1-2 weeks. The test procedures use multiple columns of multiple sample wells on a microtitration plate and techniques for transferring prescribed aliquots of contaminated aqueous systems, nutrients, pH indicator dyes, and incremental and serially diluted concentrations of anti-microbial agents.

## Background of the Invention

In many areas of industrial water use, biocides must be used to control bacterial growth to avoid, prevent or control microbiological accumulation of bacterial deposits and accumulation of the byproducts derived therefrom. The presence of these bacterial and other microbiological organisms often interfere with water processing or manufacturing of various products where these various industrial waters are being used. Industrial waters may include various waters such as those circulating in once through cooling water systems, waters recirculating in a closed loop cooling water system with or without a blow down feature, waters used and collected for processing prior to their use

in industrial processes, waters being discharged as effluent waters both before or after treatment to meet specified water quality standards, process waters used in the manufacture of various products, for example of paper or textile products, and other process waters such as those waters used in recovery of crude oil or in the processing of hydrocarbon chemicals.

In each of the uses of industrial waters, biocides have been and are being used presently to reduce, prevent, and control the presence of microorganisms and detrimental bacterial and similar growing organisms. Not only do these growths cause fouling, but their presence may also lead to corrosion of metal surfaces on which these microbiological colonies are deposited.

In the use of various biocides, a process is involved which includes the addition of a biocide into the industrial water being treated in such strength so as to control the growth of these microbiological organisms. By controlling the growth we mean to include not only total elimination of biological growth and elimination of the microorganism, but also static control of microorganism population so that extremes in growth population are controlled though not all organisms are killed. In the process of testing and determining the efficiency and type of biocide to be used, often procedures are required which last for from 24 to about 48 hours, and sometimes for 1 week and up to 2 weeks or more to determine the efficacious use of a particular biocide and particularly to determine the amount of that biocide which is necessary to reach static control or total elimination of the particular microorganisms being treated in the particular industrial water environment in which these organisms occur.

These long test periods are not only time consuming but wasteful of resources and the tests are expensive in regard to the determination of the proper biocide and its strength and concentration of optimal use to

maintain microbiological control of a particular contaminated industrial source.

Microbiocides are added to aqueous systems in a variety of industrial and recreational applications. Some of these applications include the addition of microbiocides to control the growth of algae, bacteria, fungi and protozoa in industrial cooling water systems, recreational water systems such as pools and spas, the addition of microbiocides to control bacteria in the manufacture of paper, the use of microbiocides to control bacterial growth during the processing of raw sugar, and the like. The invention may also find utility in the application of biocides used to control invertebrates including but not limited to zebra mussels, blue mussels, and Asiatic clams in industrial and municipal water intake systems and industrial cooling water systems. While particularly applicable to aqueous systems, the invention may also find utility in non-aqueous systems. As used herein, the terms "microbiocide" and "biocide" are used interchangeably and are meant to include chemicals used to control "pests" as defined under the Federal Insecticides Fungicide and Rodenticide Act (FIFRA) in both aqueous and non-aqueous fluid systems.

Current methods for the determination of the amount of microbiocide present in a fluid system tend to be time consuming measurements of the amount of bacterial growth (an indirect method) in the system or wet-chemical analysis of samples for active microbiocide (a direct method). The indirect method includes culturing a sample taken from the fluid system to determine bacterial growth. If bacterial growth is present, more microbiocide is generally fed into the system until a culture shows a steady or decreasing amount of microbiological growth. Wet chemical analysis methods are time consuming, labor intensive and subject to great error when conducted in the field rather than a well equipped laboratory. Until the time of making this invention there has existed no

convenient field method to rapidly and accurately determine how much biocide an anaerobe-contaminated system requires by an indirect method.

The use of tracer materials to monitor the effect of treatment chemicals such as corrosion and scale inhibitors in industrial water systems is well-known. Hoots U. S. 4,783,314 discloses the use of inert transition metal tracer materials for monitoring the concentration of corrosion and scale inhibitors using fluorometry. Hoots et al U. S. 4,966,711 and U. S. 5,041,386 teaches the use of inert fluorescent additives which are added in direct proportion to the amount of a corrosion and/or scale inhibitor to monitor the concentration of a corrosion and/or scale inhibitor in a given industrial water system. U. S. 4,992,380, 5,006,311, and 5,132,096 disclose methods and equipment to monitor fluorescent tracers used in industrial water treatment applications.

U. S. 5,128,419 and 5,171,450 disclose water soluble polymers having fluorescent moieties that are used to monitor their concentration in industrial water treatment applications. Japanese Patent No. 55003668 (1980) discloses an atomic adsorption spectroscopy method for monitoring biocide concentrations by adding and measuring lithium salt materials to indirectly determine the concentration of microbiocides. This method requires the separate addition of tracer material and requires the use of atomic adsorption spectroscopy to obtain results. Atomic adsorption spectroscopy is relatively expensive compared to fluorometry and has the additional disadvantage that atomic adsorption spectroscopy is not readily adaptable to field use due to the complex equipment involved, as well as open flame and flammable gas supplies.

U. S. 4,242,602 discloses an ultraviolet spectroscopy technique to monitor multiple water treatment components. The method involves the use of expensive analytical equipment along with computer hardware having specially written software. In addition, equipment must be calibrated on a site specific basis and recalibration may be necessary if

conditions in the water change. European Patent Application 466303 discloses a method involving the addition of a substance to treated water and how it reacts with the microbiocide. The substance reacting with the microbiocide is continuously measured and the concentration of the microbiocide is determined by loss of the substance. The method is cumbersome, requires special equipment, and two separate chemical feeds.

Biocides and microbiocides are used to control or eliminate bacterial growth in a number of different industrial aqueous media. Often, one biocide or microbiocide is insufficient to control all bacterial growth in the aqueous medium being treated. The presence of bacterial or other microbiological organisms interferes with the processing of industrial waters being treated and may lead to corrosion and other problems with equipment that is in contact with these contaminated waters.

U.S. Pat. No. 5,206,151, the disclosure of which is incorporated herein by reference, discloses a method to rapidly determine the effectiveness of biocides and microbiocides in various industrial waters by using a rapid screening technique which takes multiple samples of a contaminated aqueous medium containing microbiological organisms; adds thereto an indicator dye, preferably an oxidation-reduction indicator dye capable of reacting with dehydrogenase enzymes produced by the microbiological organisms; and then adds a nutrient accelerator to the treated solutions containing this oxidation-reduction indicator dye. The treated samples are contained on a titration plate with a serially diluted amount of an antimicrobial agent, and the titration plate is incubated at temperatures equivalent to the operational temperatures of the industrial aqueous systems in which the microbiological organisms exist. This method causes growth and accelerates microbiological organism activity which produces an increased concentration of reducing enzymes which

react with the oxidation-reduction indicator dye to cause a change in color. The change in color is then noted.

By comparing the first change in colors in the dyes relative to untreated columns, it is possible to determine the minimum inhibitor concentration (M.I.C.) of antimicrobial agents which inhibit the growth of the microbiological organisms contained in the contaminated aqueous system.

In the reduced environment necessary to support anaerobic bacteria the redox indicator dyes such as resazurin dye would change color in response to the reduced environment. Therefore, color change could not indicate whether or not anaerobes were present, as the environment would lead to a falsely positive result.

The '151 reference describes a method which may be utilized to test water that is not highly contaminated with anaerobes and therefore does not have a pre-existing low (negative) ORP. ORP is oxidation-reduction potential, as measured in mV. The method disclosed in col. 6, lines 52 - 57 and col. 6, lines 67 - col. 7 line 4 may be utilized to evaluate a primarily facultative population. Such facultative populations primarily respire aerobically in the test water, yet under anaerobic conditions may utilize anaerobic respiration/fermentation. For this reason, a test which effectively uses a redox dye to monitor facultative bacteria under mild anaerobic conditions, will not give an accurate result when anaerobic population are to be monitored under moderate to strong anaerobic conditions. As moderate to strong anaerobic conditions will have an effect on the redox dye giving a false reading. By contrast, the method disclosed herein is to be used when anaerobes (obligate and facultative) are the predominant populations and wherein ORPs are too low to detect changes in metabolic activity by resazurin.

U.S. Patent No. 5,374,536 discloses a product selection test for rapid determination of the presence of synergistic blends of the biocide or

the presence of biocide blends in contaminated waters. The method uses a reduction oxidation dye system, supplied nutrients, admixtures of one or more biocides or blends thereof and incubation times and temperatures providing for a variation of color changes of the dye system.

Color change to detect microbiological fouling of felts is disclosed in U.S. Patent No. 5,413,680. Iodonitrotetrazolium is utilized as the indicator therein.

An Autoreader for rapid MIC testing is disclosed in the Journal of Clinical Microbiology, Jan. 1988, p. 1-7. However, this method is inconvenient for field use, as it requires costly equipment which is not readily transportable. Moreover, the technique described utilizes an artificial broth for growth, not a native fluid. This broth, with a high protein content, would preclude use in testing with two of the most commonly used biocides in the paper industry, glutaraldehyde and isothiazolin, since they are inactivated by proteins. Furthermore, this test only works with pure cultures of organisms. Since a mixed population is normally present in paper mills or cooling tower fluids to be tested, this technique would not be useful for the purposes intended herein.

Luminescence is utilized to count the number of living microorganisms of test solutions in U.S. Patent No. 5,627,042.

PCT Application WO 92/19764 describes pH sensitive absorbency based dyes which are utilized to detect microbial growth in collected bodily fluids. This reference describes how the presence or absence of microbial growth may be detected. The method disclosed herein determines the effectiveness of microbiocides on anaerobes.

The subject invention solves many of the problems detailed above by providing an easy to use, accurate, continuous indirect method for the determination of microbiocide concentration needed to control microbial growth in fluid systems, particularly industrial water systems.

It is therefore an object of this invention to provide for a quick method to screen various and multiple microbiocides and/or toxicants useful in treating industrial waters to provide microbiological control and especially in treating those industrial waters used in the process of papermaking.

It further is an object of this invention to provide for a quick visual screening procedure to screen and test toxicants and biocides useful in control of microorganism growth and provide for control or elimination of microorganism growth in industrial waters, particularly in the process of manufacturing paper.

It is further an object of this invention to use in the visual procedure, certain pH indicators (often called pH indicator dyes) which change color in the visual spectrum such that a determination of the minimal inhibitory concentration (or MIC) of a particular antimicrobial agent in an aqueous system contaminated by microbiological organisms can be made.

It is an object of this invention to provide a quick method for screening various and multiple microbiocides and/or toxicants useful in treating industrial waters in the field, rather than in the laboratory environment.

It is a further object of this invention to provide for an easily handled microtitration plate containing multiple sample cells, (or sample wells) each cell being capable of holding a determined volume or aliquot of the contaminated aqueous system. To this aliquot of aqueous sample is then added a known and prescribed amount of a pH indicator dye capable of reacting to environmental changes due to carbohydrate metabolites formed by increased metabolic rates of microbiological systems. A chemical reaction between the carbohydrate metabolites formed at increased metabolic rates and the pH indicator dye compounds then produces a change in the color of this indicator dye which is a measure of

the increased microbiological metabolism caused by adding nutrients to the aqueous aliquot contained in the samples wells of the microtitration plates. These multiple aliquots then are tested (visually observed) for color changes. The object of the invention also includes procedures designed to determine, through a series of controlled dilutions giving various concentrations of antimicrobial agents to determine the minimal inhibitory concentrations of a particular antimicrobial agent or biocide or any combination of these antimicrobial agents or biocides in a particular contaminated aqueous system, for example, a certain paper furnish, or waters used in the manufacture of paper.

## Summary of the Invention

A rapid method for determining the minimum inhibitory concentration of biocides or biocidal agents for use in anaerobically contaminated aqueous systems is disclosed. This rapid technique provides for an opportunity to determine the minimum amount of antimicrobial agents, or biocides either singularly or combination, to control anaerobic growth in aqueous streams contaminated by microorganisms. The test involves the use of pH indicator dye systems which respond to the environmental changes caused by the production of byproducts of carbohydrate metabolism by anaerobes. The technique provides an answer within a time period of from about 30 minutes to about 8-10 hours as opposed to normal testing procedure that can require up to 1-2 weeks. The test procedures uses multiple columns of multiple sample wells on a microtitration plate and techniques for transferring prescribed aliquots of contaminated aqueous systems, nutrients, pH indicator dyes, and incremental and serially diluted concentrations of anti-microbial agents.

## Description of the Invention

The method is a means of determining which biocides/antimicrobial agents are optimal for use in paper machine fluids to control the growth of

microorganisms. The uncontrolled growth of microorganisms can cause unsightly deposits that can cause paper breaks or holes that result in costly downtime. When testing for optimal agents, it is important to test against the native population in the population's environment, and in the actual fluids of the dynamic system in which the population exists. Both of these factors will influence the efficacy of the biocide/antimicrobial agent. Because the antimicrobial agents are very costly and can only be applied to selected areas, selection of optimally active biocides for a system is extremely important. The testing should be conducted at a mill site. This may be several thousand miles from a full service laboratory designed for testing paper machine fluids. During shipment, the mixed population of microbes can change over time, or the fluids themselves may change chemically. These factors could both influence the outcome of the testing and result in a poor match of biocides.

**THE BIOCIDES**

The term biocide is clearly defined in the art as any type of antimicrobial agent that is capable of producing kill in biological systems. The term biocide is also used in the art to refer to antimicrobial agents (a subset of biocides) that are inhibitory, and are agents that prevent biological growth or biological systems from actively metabolizing. The antimicrobial agent is defined as containing one or a blend of one or more biocides. In general, an antimicrobial agent is microcidal or microstatic, although microcidal can also refer to biocidal, meaning "cidal" (killing) against biologic systems or microbiocidal, meaning "cidal" against microbial systems, a subgroup of biological systems. These terms are used interchangeably in industry and are understood to be interchangeable by one familiar with the art.

Illustrative biocides are listed in Table I, but this invention is not limited to only those biocides listed. The method should work with any biocide.

**TABLE 1**
**BIOCIDE**

| Biocide | Actives | Typical Use |
|---|---|---|
| A | 3,5 Dimethyl-tetrahydro-2H-1,3,5-thiadiazine-2-thione | Preservative for starch and clay |
| B | Methylene bisthiocyanate | Slimicide |
| C | 1-Alkyl($C_{16}$-$C_{18}$)amino-3-aminopropane acetate & Bis(trichloromethyl) sulfone | Slimicide |
| D | 5-Chloro-2-methyl-4-isothiazolin-3-one & 2-Methyl-4-isothiazolin-3-one | Slimicide Preservative |
| E | Alkyl dimethylbenzyl ammonium chloride & Dialkyl methyl benzyl ammonium chloride | Slimicide |
| F | 2,2-Dibromo-3-nitrilo-propionamide (DBNPA) | Slimicide |
| G | 2-(Thiocyanomethylthio)-benzothiazole & Bis(trichloromethyl) sulfone | Slimicide |
| H | Sodium dimethyldithiocarbamate & Disodium ethylene bis-dithiocarbamate | Slimicide |
| I | Glutaraldehyde(1,5 pentanediol) | Slimicide |
| J | 1-(3-Chloroallyl)-3,5,7-triazoniaadamatane chloride | Preservative |
| K | N-4-Dihydroxy-alpha oxobenzene chloride | Slimicide |
| L | Sodium hypochlorite | |
| M | 4,5-dichloro-1,2-dithio-3-one | |
| N | Decythioethylamine | |

THE NUTRIENTS

The nutrients named are simply foods, which are defined in the art as anything nutritious. Nutritious is further defined as providing nutrition. Nutrition is further defined as the process by which an organism takes in and assimilates food.

The term nutrient broth is a term of art obtained, for example, from the DIFCO manual and specified by the American Public Health

Association in "Standard Methods for the Examination of Water and Waste Water". Nutrient broth, a single example of a nutrient, may be obtained from either commercial supplier, such as DIFCO or BBL and is normally the same formula as required by "Standard Methods", above. The formula and specifications are tightly controlled by public health and medical industries so the term nutrient broth is not a generic term but is understood to mean a mixture of dehydrated powders containing a ratio of three parts beef extract to five parts peptone as normally used. Nutrient broth is a nutrient that is a food which accelerates activity and growth of microbiological organisms. Nutrient broth is a specific type of nutrient.

Glucose and diary half and half pasteurized creamer are also nutrients. Glucose is also known as dextrose and is a simple crystal sugar. Dairy half and half, according to the Code of Federal Regulations, Chapter 21, Section 131.180 is a food mix of milk and cream containing between 10.5% and 18% milk fat. Ultra pasteurized is a term defined by the Pasteurized Milk Ordinance Code as milk fluids treated at a time and temperature minimum of 280° F for two seconds. The addition of nutrients as defined permits and is designed to accelerate growth, which produces carbohydrate metabolites which react with the dyes, unless the growth is terminated or is made static by the addition of the antimicrobial agents being added.

The function of the added nutrients is to increase microbial metabolism in order to allow the microorganisms, or by-products produced by the organisms, to react with the pH dye. This reaction is indicated by a color change in the fluid. The purpose of the nutrient accelerator in the method described herein is to increase microbial metabolism..

Almost any nutrient or food can be called a growth medium or an agar, if agar-agar is added, to make a material for isolation or enumeration of various microorganisms. The materials found in the accelerator are commonly used in agar growth media (except for the cream). Almost any

food source can serve as a nutrient in a growth medium. Some of these include V-8 juice and corn meal, commonly used in environmental microbiological agars.

The concentrations of nutrients used in a nutrient accelerator are different than those used in a growth medium. Again, the purpose of the nutrients is different. In the accelerator, one attempts to increase microbial metabolism, and at the same time not to grossly change the conditions of the commercial fluid. The design of the accelerator solution takes into account that proteins, used at the concentrations found in agars, can interfere with the activity of certain biocide and may deactivate them. The accelerator solution was designed to minimize this problem while still promoting rapid microbial metabolism.

One of the benefits of the instant invention is that there are no requirements for sterilization, therefore there are no provisions set forth for sterilization. Sterilization need not be done so there is no requirement for sterilization before or after the addition of nutrients. Moreover, the sterilization procedures that are normally attendant with the laboratory procedure are neither necessary nor can they be achieved in the field.

Sterility/sterilization of the nutrients, solutions, materials, etc. is not required in the method outlined herein. Since the population in the commercial water is being measured in a short time period (1-8 hours), it is the native population of the water that will provide the microbial activity observed in the method. This short time period is one of the big advantages of the method over standard biocide selection techniques.

Typically, the population in a test sample of commercial water will contain 500,000-10,000,000 colony forming units (cfu) bacteria per mL. If the population is lower than 500,000 cfu/mL the contents of the test wells will not react within the eight hour time period of the method and, more importantly, the test will not provide a proper challenge to the biocides. It is very easy to kill low numbers of bacteria with almost any

commercial antimicrobial agent. The challenge comes when trying to control the population when there are millions of organisms per mL of fluid. This challenge has been met by in the method disclosed herein.

Although it is not necessary to perform the test under sterile conditions, it is necessary to use clean, non-spoiled materials. Any materials that show signs of contamination must not be used in the test.

The nutrients which may be added to the aliquot samples treated with the pH indicator dyes described above include any nutrient in solution or suspension with water or any base nutrient mix which includes glucose, sucrose, fructose, beef extract, peptone, (the combination of beef extract and peptone is often called "nutrient broth") tryptone, milk, half and half cream, yeast extract, or any mixture of the above. The specific nutrients to be preferred in the use of our rapid method to determine the minimum inhibitory concentration (MIC) of a particular antimicrobial agent is a mixture of glucose, nutrient broth and half and half cream in the volume ratio of approximately 1 to 8 to 10. However these nutrients may be blended in any reasonable ratios one to the other and successfully accelerate microbiological organism activity which in turn accelerates the metabolism rates and formation of carbohydrate metabolites which may in turn react with the pH indicator dyes of this invention. By adding the nutrients to the solutions directly on the microtitration plate, microbial activity or microbial respiration is increased and metabolism rates or metabolic processes of the microorganism increases to provide the results above. Therefore the interaction of these metabolites with the pH indicator dyes provides for monitoring cell viability via the organisms cellular electron transfer system.

## MICROTITRATION PLATES

The microtitration plates used are preferably clear plastic plates which contain multiple columns of depressions, called sample wells and multiple wells in each column. Most preferably the microtitration plate

contains at least two columns of at least three samples wells in each column. Preferably the microtitration plate contains from about 6 to about 12 columns, each column containing from about 6 to 12 sample wells. However, these plates may also be, for example, white ceramic plates, or any other plate-like construction of proper background to provide easy reading or visualization of color changes. These plates may be obtained from Co-star Corporation, Cambridge, Mass.

Into an upper series of sample wells running transversely across the multiple columns of the microtitrations plate are added equal volumes of aliquots from the aqueous system being tested. To these same series of sample wells are added a prescribed concentration of pH indicator dye, nutrient and antimicrobial agent. The antimicrobial agent is added at a concentration ranging from about 0.1 parts per million in concentration up to about 5,000 parts per million. Then by using multiple pipettes, serially diluted amounts of antimicrobial having concentrations of antimicrobial agents ranging from about 0.1 parts per million to about 5,000 parts per million are added to each of the sample wells in each of the columns thereby achieving sample wells containing the biologically contaminated test waters and a serially diluted amount of antimicrobial agent in each of the sample wells in each column. Either before or after the antimicrobial agent a known amount of a pH indicator dye is added to each sample well and a known amount of a nutrient media is also added thereto. This obtains a series, in each column, of sample wells containing a serially diluted sequence of equal aliquots of the contaminated aqueous system to which ever decreasing amounts of antimicrobial agent has been added. This series of decreasing amounts of antimicrobial agent, in the presence of nutrients and a pH indicator dye then measures microbiological or metabolic activity reflecting increased activity of the organisms cellular electron transfer system. Each column tests a different biocide against

the microbiological organisms contained in the aliquot sample of nutrient treated media.

This titration plate containing separate columns of multiple sample wells, which sample wells in turn contain serially decreasing concentrations of antimicrobial agent in equal aliquots of microorganism and equal amounts of nutrient and pH indicator dye is then incubated at temperatures which may be essentially equivalent to the temperature of the contaminated aqueous system from which the microbiological organisms contaminated samples were originally obtained. Essentially equivalent temperatures is meant to include actual environment temperature plus or minus about 10°-15° C. These incubation temperatures are at least 20° C, but normally do not exceed about 90° C. This incubation is for a period of time sufficient to develop the change in the indicator dye color through the reaction of the indicator dye with the environmental changes caused by the production of the byproducts of carbohydrate metabolism produced by nutrient accelerated activity in the test media. The sample plates must be incubated anaerobically. The Anaero Pack System from Mitsubishi Gas Chemical Company which can be purchased through Remel may be employed for field use.

This incubator time period may be as little as approximately 30 minutes and may be as long as from 8-10 hours. This testing time, preferably about 4 to about 8 hours, is considerably shorter in time and considerably more sensitive to the presence of minimum inhibitory concentrations of antimicrobial agents than previous tests for antimicrobial activity. After an appropriate incubating time of between approximately 30 minutes and 8-10 hours, the titration plate is removed and visually examined to determine color changes which have occurred in each of the sample wells in each of the columns of the titration plate. Plates are read when the untreated wells turn the appropriate color, depending upon which dye was utilized. The minimum inhibitory concentration of

antimicrobial agent contained in each column is determined at that level of concentration where the first color change is observed going from a lower to a higher concentration of antimicrobial agent in that column. Of course, each column contains a separate antimicrobial agent (or biocide) or separate mixture thereof.

The process may be repeated using different initial concentrations of antimicrobial agent until repeated experiments confirm the minimum inhibitor concentration which will control and maintain control of microbiological organisms in the aqueous media being treated with the antimicrobial agent.

Our method of determining minimum inhibitory concentration of antimicrobial agents is particularly found useful where the contaminated aqueous systems being tested are stock or furnish waters obtained from various locations in a pulp and paper manufacturing mill. The nutrients are preferably chosen from nutrient broth, glucose, dairy half and half ultra pasteurized creamer, and mixtures thereof, which mixture may also include the other nutrients described above. The microtitration plate contains at least three, preferably four columns of at least four and preferably at least eight sample wells each. The microtitration plate are normally made of glass, ceramic, but are preferably clear plastic or any other media which may tolerate the incubation temperatures and time periods and which provide a reasonable background on which colors can be observed and interpreted. The treated titration plate is normally incubated anaerobically at temperatures ranging from about 30° C. to about 50° C. for a period of time ranging from about 30 minutes to about 4 to 6 hours.

In addition, the incubation may be completed in an inert gas atmosphere such as that atmosphere provided by nitrogen, helium, argon, and carbon dioxide among others. When the incubation is performed under inert gas atmosphere additional results will be obtained which will

give the operator information in regard to the response of organisms to biocides under anaerobic conditions contaminated aqueous systems.

The invention is a method of determining the minimum inhibitory concentration of an antimicrobial agent in an anaerobe contaminated aqueous system comprising the steps of:

a) obtaining a sample of said contaminated aqueous system;

b) adding a pH indicator dye to said sample which reacts with environmental changes caused by carbohydrate metabolites of said anaerobes;

c) adding nutrient to said sample to form a dye treated nutrient aqueous system;

d) obtaining aliquots of the dye treated nutrient aqueous system;

e) performing multiple serial dilutions of an antimicrobial agent to be tested and forming mixtures of said aliquots of the dye treated nutrient aqueous system with each of the said serial dilutions;

f) incubating said mixtures anaerobically at a temperature essentially equivalent to the temperature of the contaminated aqueous system for a period of time sufficient to develop the change in the dye color by the reaction of the dye with said environmental changes caused by carbohydrate metabolites; and

g) determining the minimum inhibitory concentration of antimicrobial agent that inhibits anaerobes contained in said contaminated aqueous system by observing a change in color.

Furthermore, the anaerobe contaminated aqueous systems may be selected from the group consisting of pulp and paper processing waters, paper mill furnish waters, paper mill white waters, brown stock waters, paper mill effluent waters, open recirculating cooling waters, closed recirculating cooling waters, boiler feed waters, sugar mill processing

waters, chemical process streams, fermentation streams food processing waters, and petroleum and refinery processing and effluent waters.

Additionally, the nutrient may be selected from the group consisting of half and half dairy creamer, yeast extract, glucose, sucrose, fructose, glycerol, beef extract, peptone, tryptone, milk and mixtures thereof.

The serial dilutions may be performed so that the final concentrations of said antimicrobial agents in the serial dilutions comprise concentrations ranging from about 0.1 parts per million to about 10,000 parts per million, on the basis of the total volume of the dye treated, nutrient treated aqueous system.

The pH indicator dye may be selected from the group consisting of bromocresol purple, bromocresol green, bromothymol blue, chlorophenol red, methylene blue chloride, methyl red and phenol red.

The serial dilutions of said dye treated nutrient aqueous system may be incubated on a micro titration plate containing at least four columns of at least eight wells each, and the incubation is performed at a temperature within the range of from about 25° C. to about 60° C. and for a time period within the range of from about 60 minutes to about 24 hours.

Moreover, during incubation an atmosphere containing predominantly an inert gas is maintained over the treated titration plate.

The inert gas may be selected from the group consisting of $CO_2$ and $N_2$.

For the practice of this invention, step b) is delayed from 30 minutes to 20 hours.

To provide further information about our method for determining minimum inhibitor concentration of antimicrobial agents in contaminated aqueous systems, the following examples are given.

**EXAMPLES**

Approximately 100 ml. of filtered paper furnish either from stock furnish, tissue furnish, or other furnish was obtained.

These filtered solutions, which are filtered through a coarse filter, such as through tissue paper or a filter having a mesh size of about US Std Sieve Series No. 90, then have approximately 5 ml. of a nutrient solution, and 1 mL of pH indicator dye solution added thereto. The nutrient solution contains 0.4 dry powder from a BBL or Difco nutrient broth and 1 g dextrose in 100 mL water. To this nutrient solution is added 0.5 ml of ultra pasteurized half and half creamer which may be obtained at a local grocery store. The pH indicator dye solution contains 0.15 g dye in 100 mL of 0.01% NaOH. Approximately 150 microliters of this treated filtered water furnish solution is then pipetted into each of the microtitration sample wells using a multi-channel micropipette, such as supplied by Flow Laboratories as their Titertek ® and in as many columns as there are biocide preparations to be tested. Titertek is a registered Trademark of Flow Laboratories

One mL of a 1% solution of biocide was added to 4 mL of nutrient-dye-furnish containing sample to form a result 2,000 ppm solution of biocide in the sample.

These commercial or test biocidal agents can include, but are not necessarily limited to, polyamines, isothiazolins, organosulfurs, quaternary amines, organobromines, carbamates, methylene bis thiocyanate, or combinations thereof, or any other additional biocidal agents known.

Immediately after forming this 2000 ppm solution, 150 microliters of this treatment solution are removed and placed in a first micro sample well of the first test column of the microtitration plate. One column is left untreated by biocide. The solution formed in the microtitration sample well is then drawn up into the pipette used to transfer the biocide solution 2 to 3 times and placed back into the same sample well, thereby mixing the solutions. After mixing, 150 microliter of the mixture is removed from this first sample well and dispensed into the next lower sample well in the same column. Again the mixing procedure occurs and a final removal of

150 microliter of this second sample well solution is removed by using the micro pipette, This second aliquot in this series is placed in a third sample well (in the same column) on the microtitration plate. This procedure is repeated until all of the sample wells in a single column contains serially diluted concentrations of antimicrobial agent (biocide) added thereto and mixed therewith. The last 150 microliter aliquot taken from the last of the sample wells in the column is discarded.

This procedure is repeated in each column for all biocides to be tested. One column of sample wells is left untreated to act as a blank, that is, a test column containing test solution, nutrient, pH dye, but without any addition of biocidal or antimicrobial agents.

The microtitration plates containing multiple columns of multiple sample wells are then incubated. The temperature for performance of the technique is preferably those temperatures occurring with the process stream being tested, plus or minus from about 10°-15° C. These temperatures usually range from about 20° C. to about 80° C. and are usually from about 25°-30° C. to approximately 50°-60° C. Since the microtitration plate is to be incubated anaerobically in a carbon dioxide environment, i.e., the plate is placed in an anaerobic environment such as provided, for example, by an Anaero Pack System, available from Mitsubishi Gas Chemical Company, before incubation. This commercially available system provides for conversion of oxygen in the air over the sample to carbon dioxide and $H_2O$ thereby providing for a $CO_2$ atmosphere.

Following the above procedures, the concentration gradient, i.e., the serially diluted concentration of antimicrobial agents, will range from about 1000 parts per million in the first high concentration sample well to approximately 8 parts per million (or below) in the lowest concentration sample well. In the presence of dyes, the wells which do not contain sufficient antimicrobial agent change to a second color. Those that

contain sufficient antimicrobial agent remain the initial color. The test is read when all of the control wells (untreated) are the color of the dyes endpoint — yellow or clear. By comparing the concentrations in the serially diluted sample wells to those of the untreated wells, the MIC for each antimicrobial agent can be determined visually at the sample well where the lowest concentration of antimicrobial agent which controls growth of microbiological organisms in the sample tested occurs.

The bacteria which have been found to react to this test and which bacteria are commonly found in paper mills, cooling towers, among others, and have been found to include *Clostridia, Sulfate-Reducing Bacteria and Klebsiella*. By using an appropriate choice of initial concentration of antimicrobial agents or biocide, it is possible to screen these biocides at concentration ranges from approximately 10,000 parts per million down to and including concentration below one part per million.

The following examples are presented to describe preferred embodiments and utilities of the invention and are not meant to limit the invention unless otherwise stated in the claims appended hereto.

**Example 1**

To perform the experiment, filtered white water obtained from a pulp and paper mill was adjusted to pH 6.5 with either NaOH or HCl. A 100 mL aliquot of the pH adjusted furnish was placed into a Whirlpak® bag along with 5 mL of hydrated MiniTox™ (available from Nalco Chemical Corporation of Naperville, Illinois, containing 0.4 g nutrient broth, 1.0 g dextrose, 100 mL $H_2O$) nutrient solution, 1 mL of 0.15% chlorophenol red, and 0.5 mL of half and half creamer. The solution was mixed thoroughly until the color is evenly dispersed. This solution will be referred to as AnnaTox treated furnish hereafter.

Add 150 $\mu$L of the AnnaTox treated furnish into each well of a microtitre tray. Then, 4 mL of the AnnaTox-treated furnish was placed into 10 mL beakers (one beaker for each biocide being screened). Next, 1

mL of 1% (based on weight) biocide solution was added into each beaker and mixed. Immediately, 150 μL was withdrawn and a serial dilution down the column was begun . At the end of the column, the 150 μL was discarded. This procedure was repeated for all of the biocides of interest. One row of wells was not treated with biocide, to act as the control.

The tray was next sealed with transparent tape to prevent evaporation and incubated anaerobically until the control wells turn yellow, with no agitation at 37°C. Periodically the tray was checked for color change from red to yellow. The chlorophenol red is completely red at pH 6.8 and completely yellow at pH 5.1. A reduction in pH is achieved by the fermentative bi-products of the anaerobic bacterial populations present in the sample. If there is no biocide, color change may occur in from 1 to 24 hours, depending upon the type of anaerobic population in the sample. If the biocide was effective in either inhibiting or killing these bacteria, a color change will not be observed. Minimum inhibitory concentrations are read once all of the control wells have turned yellow. The MIC is read as the concentration in which the well below has turned yellow. This indicates that at that particular point, not enough biocide was added to have an effect. Thus a qualitative determination may be made as to effectiveness of a biocide treatment.

To ensure that the color change was due to actual pH change and not due to other external influences, an experiment was set-up using the AnnaTox method; and, the pH was measured after the MIC was read. The results showed that on average the MIC reading corresponded to a significant drop in pH between the concentrations. See Table IX for results.

To determine if the pH change was due to microbial growth and not trapped $CO_2$ or other influences, the furnish was autoclaved for 30 minutes at 121°C prior to running the assay. Instead of using transparent tape to seal the wells from evaporation, a couple drops of paraffin wax

was used in each well. The test was run side by side with unautoclaved furnish. Paraffin was also used to seal the wells. The MIC's showed normal variation as far as biocide efficacy is concerned in the unautoclaved sample. The assay that was run with the sterilized furnish did not show a color change within a 21 hour time period. The test was terminated at that point.

This test procedure may also be utilized to quantitatively determine how many microorganisms are viable after the biocide has been added. Aliquots would be taken from each sample well, and standard plating methods would be utilized to determine the bacterial counts.

It is important to note that the test is run under anaerobic conditions. The microbial growth that is referred to includes obligate anaerobes and facultative anaerobes. Obligate anaerobes are those microorgansims which cannot utilize oxygen and facultative anaerobes are those microorganisms that can grow with or without oxygen..

This procedure can be run using any of the following pH indicators:

Table II

| Trade Name | Chemical Name | Supplier |
|---|---|---|
| Bromocresol Purple | 5,5-dibromo-o-cresolsulfonphthalein | Baker |
| Bromocresol Green | 4,4-(2,2-dioxido-3H-1,2-benzoxathiol-3-ylidene) bis [2,6-dibromo-3-methyl-mono sodium salt] | EM Science |
| Bromothymol Blue | 3,3-dibromothymolsulfonphthalein | Baker |
| Chlorophenol Red | 4,4-(1,1-dioxido-3H-2,1-benzoxathiol-3-ylidene)bis[2-chloro-9Cl] | Acros |
| Methylene Blue Chloride | Phenothiazin-5ium,3,7-bis(dimethylamino)-chloride (9Cl) | EM Science |
| Methyl Red | (o-[[p-(dimethyl amino)phenyl]azo]benzoic acid | Baker |
| Phenol Red | Phenolsulfonphthalein, sodium salt | Baker |

The benefits of using different indicators is that the pH of the sample being tested won't have to be drastically adjusted, but rather may be chosen in view of the inherent pH of the particular system in question to be tested.

The experimental procedure described above was utilized to determine MIC's in Table III for a stock obtained from a Southeastern pulp and paper mill.

TABLE III

| Biocide | MIC (ppm) |
|---|---|
| A[1] | 1000 |
| B[2] | 8 |
| C[3] | 8 |
| D[4] | 8 |
| E[5] | 32 |
| F[6] | 8 |
| G[7] | 16 |

1 = Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione, available from Buckman

2 = Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate, available from Alco Chemical

3 = 1,3 pentandial (glutaraldehyde), available from Union Carbide

4 = 1,3 pentanedial and N-alkyl dimethyl benzyl ammonium chloride, available from Union Carbide

5 = 1-alkyl ($C_8$-$C_{18}$)3-amino-3-aminopropane monoacetate and bis(trichloromethyl) sulfone, available from Akzo

6 = 5-chloro-2-methyl-4-isothiazolin-3-one and methyl-4-isothiazolin-3-one, available from Rohm & Haas

7 = N-alkyl dimethyl benzyl-ammonium chloride and N-dialkyl methyl benzene ammonium chloride, available from Mason

**Example 2**

The results of Table IV were obtained utilizing the procedure described in Example I by testing a stock from a different Southeastern pulp and paper mill.

TABLE IV

| Biocide | MIC (ppm) |
|---|---|
| A[1] | 1000 |
| B[2] | 8 |
| C[3] | 32 |
| D[4] | 64 |
| E[5] | 125 |
| F[6] | 32 |
| G[7] | 125 |

1 = Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione, available from Buckman

2 = Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate, available from Alco Chemical

3 = 1,3 pentandial (glutaraldehyde), available from Union Carbide

4 = 1,3 pentanedial and N-alkyl dimethyl benzyl ammonium chloride, available from Union Carbide

5 = 1-alkyl ($C_8$-$C_{18}$)3-amino-3-aminopropane monoacetate and bis(trichloromethyl) sulfone, available from Akzo

6 = 5-chloro-2-methyl-4-isothiazolin-3-one and methyl-4-isothiazolin-3-one, available from Rohm & Haas

7 = N-alkyl dimethylbenzylammonium chloride and N-dialkylmethylbenzene ammonium chloride, available from Mason

**Example 3**

The results of Table V were obtained by testing a different stock from another southeastern pulp and paper mill, utilizing the experimental procedure described in Example 1.

TABLE V

| Biocide | MIC (ppm) |
|---|---|
| A[1] | 1000 |
| B[2] | 8 |
| C[3] | 8 |
| D[4] | 8 |
| H[8] | 16 |
| E[5] | 8 |
| F[6] | 8 |
| G[7] | 8 |

1 = Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione, available from Buckman

2 = Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate, available from Alco Chemical

3 = 1,3 pentandial (glutaraldehyde), available from Union Carbide

4 = 1,3 pentanedial and N-alkyl dimethyl benzyl ammonium chloride, available from Union Carbide

5 = 1-alkyl ($C_8$-$C_{18}$)3-amino-3-aminopropane monoacetate and bis(trichloromethyl) sulfone, available from Akzo

6 = 5-chloro-2-methyl-4-isothiazolin-3-one and methyl-4-isothiazolin-3-one, available from Rohm & Haas

7 = N-alkyldimethylbenzylammonium chloride and N-dialkyl methyl benzene ammonium chloride, available from Mason

8 = 2-bromo-2-nitropropane-1,3-diol (bronopol), available from Angus

**Example 4**

The results of Table VI were obtained by utilizing the procedure described in Example 1 for testing yet another Southeastern pulp and paper mill stock.

TABLE VI

| Biocide | MIC (ppm) |
|---|---|
| A[1] | 1000 |
| B[2] | 16 |
| C[3] | 8 |
| D[4] | 8 |
| H[8] | 32 |
| F[5] | 32 |
| G[6] | 32 |

1 = Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione, available from Buckman

2 = Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate, available from Alco Chemical

3 = 1,3 pentandial (glutaraldehyde), available from Union Carbide

4 = 1,3 pentanedial and N-alkyl dimethyl benzyl ammonium chloride, available from Union Carbide

5 = 5-chloro-2-methyl-4-isothiazolin-3-one and methyl-4-isothiazolin-3-one, available from Akzo

6 = N-alkyl dimethyl benzyl-ammonium chloride and N-dialkyl methyl benzene ammonium chloride, available from Rohm & Haas

8 = 2-bromo-2-nitropropane-1,3-diol (bronopol), available from Angus

**Example 5**

The procedure of Example 1 was utilized to obtain the results of Table VII, obtained by testing still another Southeastern pulp and paper mill stock.

TABLE VII

| Biocide | MIC (ppm) |
|---|---|
| $B^2$ | 64 |
| $C^3$ | 32 |
| $H^8$ | 32 |
| $E^5$ | 64 |
| $G^7$ | 64 |

2 = Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate, available from Alco Chemical

3 = 1,3 pentandial (glutaraldehyde), available from Union Carbide

5 = 1-alkyl ($C_8$-$C_{18}$)3-amino-3-aminopropane monoacetate and bis(trichloromethyl) sulfone, available from Akzo

7 = N-alkyl dimethyl benzyl-ammonium chloride and N-dialkyl methyl benzene ammonium chloride, available from Mason

8 = 2-bromo-2-nitropropane-1,3-diol (bronopol), available from Angus

**Example 6**

The procedure described in Example 1 was utilized to obtain the results of Table VIII, testing still another Southeastern pulp and paper mill stock.

TABLE VIII

| Biocide | MIC (ppm) |
|---|---|
| A[1] | 1000 |
| B[2] | 16 |
| I[9] | 250 |
| J[10] | 250 |
| G[7] | 32 |

1 = Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione, available from Buckman

2 = Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate, available from Alco Chemical

7 = N-alkyldimethylbenzyl ammonium chloride and N-dialkylmethylbenzene ammonium chloride, available from Mason

9 = methylene bis (thiocyanate), available from Rohm and Haas

10 = 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane, available from Dow Chemical Co. of Midland, Michigan

**Example 7**

Table IX illustrates that a pH change occurs as biocide is added to the system at various concentrations. In each case, the break point pH is indicated with bold print. For the case wherein no biocide is added (H), no significant pH change is evident in the system. It is this pH change which the indicator dyes follow in the method of this invention.

TABLE IX

| | pH change with addition of biocide | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| concentration (ppm) | A[1] | B[2] | C[3] | D[4] | E[5] | F[6] | G[7] | H[8] |
| 1000 | 4.66 | 6.95 | 6.34 | 6.59 | 7.08 | 6.87 | 6.87 | 5.19 |
| 500 | 4.71 | 6.89 | 6.70 | 6.70 | 6.82 | 6.94 | 6.92 | 4.76 |
| 250 | 5.20 | 7.01 | 6.76 | 6.80 | 6.82 | 6.99 | 6.85 | 4.72 |
| 125 | 4.67 | 7.21 | 6.80 | 6.80 | 6.93 | 6.95 | 6.85 | 4.92 |
| 64 | 4.86 | 6.97 | 6.70 | 6.70 | 7.06 | 5.61 | 6.12 | -- |
| 32 | 4.79 | 5.95 | 6.61 | 6.60 | ***6.50*** | ***4.87*** | ***5.79*** | -- |
| 16 | 4.67 | ***5.91*** | 5.76 | 5.33 | 4.56 | 4.88 | 4.62 | -- |
| 8 | 4.87 | 4.48 | ***5.64*** | ***5.52*** | 4.47 | 4.82 | 4.83 | -- |
| Initial pH | 7.21 | 6.95 | 6.95 | 6.89 | 7.20 | 7.16 | 6.81 | 6.97 |

1 = Tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione, available from Buckman

2 = Sodium dimethyl dithiocarbamate and disodium ethylene bisdithiocarbamate, available from Alco Chemical

3 = 1,3 pentandial (glutaraldehyde), available from Union Carbide

4 = N-alkyldimethylbenzyl ammonium chloride and N-dialkylmethylbenzene ammonium chloride, available from Mason

5 = 2-bromo-2-nitropropane-1,3-diol (bronopol), available from Angus

6 = 5-chloro-2-methyl-4-isothiazolin-3-one and methyl-4-isothiazolin-3-one, available from Rohm & Haas

7 = a combination of N-alkyl dimethyl benzyl-ammonium chloride and N-dialkyl methyl benzene ammonium chloride, available from Mason and 1,3 pentandial (glutaraldehyde), available from Union Carbide

8 = control, no biocide added

Changes can be made in the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims:

## Claims

1. A method of determining the minimum inhibitory concentration of an antimicrobial agent in an anaerobe contaminated aqueous system comprising the steps of:

a) obtaining a sample of said contaminated aqueous system;

b) adding a pH indicator dye to said sample which reacts with environmental changes caused by carbohydrate metabolites of said anaerobes;

c) adding nutrient to said sample to form a dye treated nutrient aqueous system;

d) obtaining aliquots of the dye treated nutrient aqueous system;

e) performing multiple serial dilutions of an antimicrobial agent to be tested and forming mixtures of said aliquots of the dye treated nutrient aqueous system with each of the said serial dilutions;

f) incubating said mixtures anaerobically at a temperature essentially equivalent to the temperature of the contaminated aqueous system for a period of time sufficient to develop the change in the dye color by the reaction of the dye with said environmental changes caused by carbohydrate metabolites; and

g) determining the minimum inhibitory concentration of antimicrobial agent that inhibits anaerobes contained in said contaminated aqueous system by observing a change in color.

2. The method of claim 1 wherein said anaerobe contaminated aqueous systems are selected from the group consisting of pulp and paper processing waters, paper mill furnish waters, paper mill white waters, brown stock waters, paper mill effluent waters, open recirculating cooling waters, closed recirculating cooling waters, boiler feed waters, sugar mill processing

waters, chemical process streams, fermentation streams food processing waters, and petroleum and refinery processing and effluent waters.

3. The method of claim 1 wherein said nutrient is selected from the group consisting of half and half dairy creamer, yeast extract, glucose, sucrose, fructose, glycerol, beef extract, peptone, tryptone, milk and mixtures thereof.

4. The method of claim 1 wherein said serial dilutions are performed so that the final concentrations of said antimicrobial agents in the serial dilutions comprise concentrations ranging from about 0.1 parts per million to about 10,000 parts per million, on the basis of the total volume of the dye treated, nutrient treated aqueous system.

5. The method of claim 1 wherein said pH indicator dye is selected from the group consisting of bromocresol purple, bromocresol green, bromothymol blue, chlorophenol red, methylene blue chloride, methyl red and phenol red.

6. The method of claim 1 wherein said serial dilutions of said dye treated nutrient aqueous system are incubated on a micro titration plate containing at least four columns of at least eight wells each, and the incubation is performed at a temperature within the range of from about 25° C. to about 60° C. and for a time period within the range of from about 60 minutes to about 24 hours.

7. The method of claim 6 wherein during incubation an atmosphere containing predominantly an inert gas is maintained over the treated titration plate.

8. The method of claim 7 wherein the inert gas is selected from the group consisting of $CO_2$ and $N_2$.

9. The method of claim 1 wherein step b) is delayed from 30 minutes to 20 hours.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/25864

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12Q 1/18, 1/22
US CL : 435/32, 34
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/32, 34, 287.4, 288.4; 436/163

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, WPIDS
search terms: antimicrob?, inhib? or mic, aqueous or water, enzyme, nalco

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 5,374,536 A (ROBERTSON, L.) 20 December 1994, col. 1, lines 30-54; col. 2, lines 29-33; col. 3, lines 48-52; col. 6, lines 32-36; col. 9, lines 31-42. | 1-9 |
| Y | US 5,252,484 A (MATNER et al) 12 October 1993, col. 16, lines 24-43. | 1-9 |
| Y | US 5,206,151 A (ROBERTSON, L.) 27 April 1993, col. 5, lines 37-38; col. 9, lines 4-7; col. 11, lines 50-59; col. 12, lines 49-56 and lines 65-66. | 1, 4, 6-9 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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07 JANUARY 1999

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