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BIOCATALYTIC OXIDATION

OF

NITRILOTRIACETATE

BY

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A THESIS

PRESENTED IN PARTIAL FULFILLMENT OF

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B. ABSTRACT

The specific scope of this work is to study the biological and chemical effects of a single genetic strain of bacteria, Bacillus subtilis #9524, on the speific destruction to innocuous products of sodium nitrilotriacetate (NTA) and sodium tripolyphosphate (STPP). Insofar as the same strain of bacteria was used each time, under limited variability and sterile conditions, the side effects of multiple bacterial strains and their inter-relationships among themselves and the organic substrates are eliminated. This has allowed for excellent replication of results.

This is a generalized technique for the evaluation of the biodegradability of a compound. Whereas tests with activated sewage sludge may show results of oxygen consumption, it may be due to multiple variable reasons associated with unknowns for the breakdown of the compound in question.

Tests also need to be extensive, in that while tests with lower levels of concentration (1-5ppm) may prove to be somewhat effective, higher concentration levels (10-20ppm) of the compound may prove to possess toxic effects on the bacterial strain in use, due to physical factors of altered solution surface tension and lack of isotonicity.

As a result of this study it was found that while there was a minute amount of oxygen consumption at lower levels of NTA, it was not enough to justify saying that NTA was biodegradable. Further experimentation at higher levels of concentration, while having slightly higher levels of oxygen consumption, also showed a trend of increasing toxicity, where an increase in the concentration of the NTA resulted in a lowering of the oxygen demand. Tests on the STPP proved that there was no breakdown, because there was relatively no oxygen consumption, but a known re-entry into the life biochemical processes of the very useful phosphate ion.

APPROVAL OF THESIS

BIOCATALYTIC OXIDATION

OF

NITRILOTRIACETATE

SAUL HAMMERMAN

FOR

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NEWARK COLLEGE OF ENGINEERING

BY

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I. INTRODUCTION

It is a disturbing thought that man is doing significantly little to save his own planet from becoming a "dead world". Earth one day may not be able to support its inhabitants. This is happening in spite of space travel, lunar exploration, and other great scientific accomplishments. As is the case with several species of animals (the dodo bird, the bald eagle, and the whale), man's abuse of his environment has brought about the partial or total destruction of what once seemed to be unlimited resources - such as water bodies; air; and the land from which we reap our foods, extract its many riches, and on which many other animals try to co-exist with us on the Earth. This abuse, negligence, and lack of foresight has caused serious health hazards.[1]

While a handful of people have warned Earth's inhabitants about the consequences of technological advances without consideration of all possible results, few people really listen. Yet, as the industrialized world progressed and thoughtful humans began looking around us to see where we were heading, some serious people did begin to talk of controls to help save our environment. They were concerned about the poisoning of the air we breathe, the water we drink, and the soil from which we obtain our food.

Regulations, ordinances, and laws have been promulgated to control the amounts of gases and solid particles that can be emitted by car engines and smoke stacks; waste liquids that can be piped into rivers; and the types and limits of usable insecticides. Unfortunately, in regard to the legislation that has been passed, very little of it is truly possible of effective enforcement.

In the field of interest of this report are the entries of pollutants and nutrients into our waterways to cause harmful results. Today the sewer and waste lines of industrial plants run into rivers, lakes, and other waterways - many of which are also important as water sources of large downstream cities. In earlier times, waste disposal was merely a matter of opening a valve to the sewer lines which ran to a convenient stream or lake. Times have actually changed the situation very little. There are still many industries that have no other effective ways to handle this problem. One case in point is the Lake Superior area where one asbestos plant is pumping its waste in large quantities into the waters of the lake. Medical tests have shown that asbestos fibers carried by the lake's waters, also used by cities for drinking purposes, have been found in the lung tissues of the people who have been drinking the water. Tests have also shown that workers at asbestos plants run great risks of devel-

oping lung cancer from ingesting air-borne asbestos fibers.

A large area of present concern has been the use of laundry detergents. When these agents were first introduced during the early 20th century, they consisted almost entirely of surfactants, (propylene tetramer benzene or PT benzene, dodecyl beta alanine, dodecane sulphonic acid, lauryl trimethylammonium chloride, etc.)[2]. These are still the basic ingredient of commercial detergents, phosphate or non-phosphate, and are complex synthetic chemicals, with ability to reduce surface tension in their action as "wetting agents".

Surfactants in commercial detergent preparations, are the ingredients chiefly responsible for actual soil removal. However, they clean cotton and some other fabrics rather poorly unless combined with chemicals known as "builders", which create conditions in the wash water that enable the surfactants to work efficiently. These "builders" control alkalinity, put soils in suspension, prevent dirt from redepositing in cloth, and emulsify oils and grease. This is absolutely necessary for machine washing. Some types of these "builders" are; trisodium phosphate, tetrasodium pyrophosphate, tetrapotassium pyrophosphate, sodium hexa metaphosphate, etc.

In 1947, these "builders" were added to detergents

by the Proctor & Gamble Company, when they introduced "Tide" detergent on the market. This breakthrough proved to be one of the great advances in soap business history. With phosphates added, detergents cleaned noticeably better than soap, and were cheaper too. Also because these agents left little residue, it made possible the advent of popular household automatic washing machines.

Recently, with the publicized outcry against phosphates; reverberations of the detergent controversy have reached a high pitch. Housewives, in their ignorance, are at a loss as to how to launder without either guilt or fear.[3] In recently sold detergents, the surfactants made up to 15 to 20 percent of the typical heavy duty formula; while in most detergents, phosphates in one form or another could be found to account for 35 to 55 percent by weight.

The cited danger behind the use of phosphates, is that some frantic ecologic experts consider the phosphates the major reason that our rivers and lakes are eutrophying. Eutrophication is an overfertilization with nutrients. What makes phosphates so controversial is their role as a chemically essential nutrient. Phosphate is an ion that all organisms need. Among these organisms are aquatic weeds and one-celled algae-microscopic plants that live in water.[4] When phosphates with other essential nutrients enter waterways, the water is enriched, and a eutrophic

stage is reached. Algae grow well in such an environment, and the critics of phosphate containing detergents therefore charge that the phosphates are responsible for most of the excessive growth of algae in U.S. waterways today.[5]

The problem is that while in limited amounts, algae are benefical, creating oxygen through photosynthesis and forming a link in the aquatic food chain; in excess, however, algae can become a danger to human survival. When the algae die, they can produce obnoxious odors and wash slime ashore. Furthermore, while live algae give off oxygen, dead algae - as any organic waste in water - create additional biochemical oxygen demand (BOD), by a process in which oxygen is consumed as part of the chemical breakdown of the algae. This in turn produces anerobic bacterial activity. The problem of decomposing organic matter is that it can destroy a lake's ability to supply sufficient oxygen for desirable varities of fish.

Critics point to many bodies of water as "dead", among these are Douglas Lake in Michigan, Lake Mendota in Wisconsin, Lake Washington in Washington, and Lake Erie.[6] The phosphate industry points to the latter, one of the most frequently mourned, as producing as much fish as all the other Great Lakes combined; yielding in recent years the greatest amounts in its history. This is not to be taken that the lake is not polluted. Soap industry experts feel,

however, that the change from phosphate detergents would not help.

In lakes, ponds, and other slow-moving waters which provide a good environment for excessive algal growth, the critical factor as to whether or not algal growth will become a danger to fish depends on the availability of many nutrients other than phosphates. Eutrophication is common in nature, for nutrients enter the water from sources as varied as agricultural-field-runoff-rainfall and the decomposition of dead fish. Wisconsin's Green Bay, for example, was blanketed with algae when white men first glimpsed it. Man's activities can greatly accelerate the process when he puts nutrients into lakes and rivers through domestic sewages, industrial wastes, and the runoff of agricultural fertilizer following rain.

Some ecologists feel that phosphorus is only one of about twenty different essential nutrients that algal growth depends on, admittedly the phosphorus in the form of phosphate compounds is one of the most important. They feel that if the phosphorus content is reduced by a large amount it could be made to be the limiting factor. They argue that when the limiting factor is depleted, algal growth will stop. Ecologists say that as much as 70 percent of incoming phosphorus comes from municipal sewage plants; and of this phosphorus as much as 70 percent may

come from phosphate detergents. These experts, therefore, feel that by changing detergent formulas to eliminate most or close to all the phosphorus in detergents, they may solve the problem of excessive algal growth.

On this point some opposing industry experts state that even if more than 99 percent of the phosphates were removed there would still be enough in solution to fill the phosphorus requirements of the algae. They state that while as much as 10,000 parts per billion of phosphorus can be found in sewage, all that the algae require is less than 10 ppb, or 0.01 mg per liter, and that all the rest is excess that is not used in algal growth. These experts state that the only way to help clean up waterways would be with better sewage treatment plants. These plants could chemically remove most nutrients not just phosphates. However, as of today this is a fiscally improbable solution, unfortunately.

The detergent issue came to a critical head during the late 1960's when there arose an organized hysterical clamor against phosphates. The outcries grew loud, and politicians started to listen. Legislation banning phosphates was publicly debated. In several cities laws banning or limiting the amounts of phosphates in detergents were passed. Pressure from vocal groups became so strong that Congress felt that it should act on the publicized issue.

Congressional committees went into action to hear about the faults of the phosphates and the dangers they posed. Critics of phosphates referred to tests showing that other substances could be substituted for the phosphates and still clean clothes. Among the possible polyphosphate replacements mentioned were citrates, gluconates, tartarates, silicates, and ethylene diamine tetraacetate (EDTA).

The detergent industry came back with voluminous data and performance result tests to refute these claims. The industry scientists stated that some of the substances could clean clothes, however, they would be in such high concentration that they would not be biodegradable; or that they would be dangerous to humans. Their tests also showed that in lower-biodegradable concentration, the substitutes proved ineffective in getting desirable results. Industry experts felt that all phosphates could not be removed because no substitute could safely perform the spectrum of functions that the polyphosphates had proven capable of over a 25-year period. They therefore argued that only a limited level of substitution was possible. A criteria for polyphosphate replacement was set with the following conditions set:[7]

1. It should be economically competitive with polyphosphates.
2. It must be capable of fulfilling the phosphate

functions in the detergent box.

3. It must have no serious pollution potential of its own.

There was one suggested phosphate substitute which seemed as if experts of the soap industry should not ignore, and that was a compound known as sodium nitrilotriacetate (NTA). This compound was first produced in Germany during the 1930's. For many years it was considered too expensive for general use. Then during the late 1950's, Dr. Singer, now of Hampshire Chemicals, working on his own, developed a more economical way of getting similar and highly purified chelating agents. While working as a chelate consultant, he heard of a Du Pont announcement of selling liquid hydrogen cyanide (HCN) for 20 cents or less a pound. Small concerns had been unable to get HCN so cheaply. He than began working on EDTA and other chelating agents. Before this work, sodium cyanide, formaldehyde, and an amide, gave an 80 to 85 percent pure compound. Now using HCN, Dr. Singer was able to get 99.5 percent purity. He found that by substituting ammonia for the ethylenediamine he would obtain NTA, by the following process:[8]

1. $6CH_2O + 4NH_3 \longrightarrow (CH_2)_6N_4 + 6H_2O$
2. $(CH_2)_6N_4 + 6CH_2O + 12HCN \longrightarrow 4N(CH_2CN)_3 + 6H_2O$
3. $4N(CH_2CN)_3 + 12\ NaOH + 12H_2O \longrightarrow 4N(CH_2COONa)_3 + 12NH_3$

4. $4N(CH_2COONa)_3 + 6H_2SO_4 \longrightarrow 4N(CH_2COOH)_3 + 6NaSO_4$

However, while he was able to demonstrate the cleaning power of the chelate, he could not get industry to use it because it was still far more expensive than the phosphates. In the early 1960's Standard Oil (Ohio) started to have huge amounts of HCN as a by-product at one of its plants, they offered the HCN at so low a price that it became economically possible to produce NTA in vast amounts. It then seemed that NTA conformed to the first two points of the criteria, and ecologists produced evidence that it met the third one also.

When it looked as if Congress was going to proceed to ban the use of phosphates in 1970, industry decided to act on its own and replace some of the phosphates with NTA, or up to 25 percent of the total weight. Construction of plants to produce NTA and HCN were begun. Millions of pounds of NTA were produced and billions were ordered. Then tragedy struck. In late December 1970, the U.S. Department of Health, Education, and Welfare banned the use of NTA because of fears it could cause birth defects. Laboratory tests with animals had shown that a combination of NTA and two heavy metals in waste water could cause a high incidence of fetal injuries.[9] This was later disproven by further tests presented by industry, as the giant

detergent companies tried to legalize the use of NTA, so as to be able to recover some of their vast losses in investment. However, the government continued the ban because of the need of more research to determine the carcinogenic and mutagenic hazards of the chemical.[10] Tests are continuing in this field in many areas, including those to determine if NTA would be biodegradable.

Environmentalists have been able to get a total ban of phosphates in some cities. In most of the country the phosphate content has been maximized at 35 percent or less of the heavy-duty laundry detergent formula, or 8.7 percent or less of elementary phosphorus. Many phosphate-free detergents, none produced by the three major detergent companies - until the middle of 1973, appeared on the market. Most of these have had to be removed from the market and reformulized because they were found to be hazardous to the health of the users - especially to children who get into all sorts of cleaning materials. Here again phosphates have proven to be better than any other known substitute. Phosphates have, over the last 25 years, shown themselves to be unusually safe as well as effective compounds. As already stated the phosphates enable the detergents to clean with less alkalinity, less even than soap; and although federal health agencies received approximately 4,000 reports of accidental ingestion of cleaning products

by young children, no deaths or even serious injuries are known to have been caused by phosphate laundry detergents in a generation of use.[11] In fact Americans consume several million pounds of phosphates in beer and soft drinks alone each year. As phosphates are one of the essential inorganic ions that are found in the human body, any small ingestion would not normally cause dangerous results. This is not the case with one substitute after another that has been tried since the dispute over phosphates began nearly a decade ago. In one case a fifteen-month-old girl died after inhaling a quantity of Arm & Hammer non-phosphate detergent.[12] This example is but one of about half a dozen deaths, and hundreds of children have been taken ill and needed hospitalization. Here also is one of the other dangers discovered in the short time these substitutes have been on the market. Many people developed rashes and infections from detergents which contained enzymes. In another case, it was discovered that washing-soda detergents could inactivate flameproof finishes on clothing made mainly to protect young children.[13]

Today, almost a decade after the whole furor began, the ecologists are still arguing for change, but have shown no really effective substitute to be available. The products some consumers are told to use do not perform a job people can be completely satisfied with but must settle for because of the anti-phosphate fears that have been

spread by environmentalists. Industry points to the using of NTA in Canada and Sweden, where the Health Boards of these nations have given NTA a "clean bill of health". Many times, however, human physiologic changes involved, if there are any, may not come out for a decade or more. No one could assure that any substitute would not create more problems than it would solve.

II. EXPERIMENTAL BACKGROUND

Bascillus subtilis belongs to family XIII of bacteria, the Bacillaceae, all of which are spore-forming saprophytes of soil. B. subtilis themselves are found to be widespread in soil, hay and straw, and decomposing organic matter.[14] They are mesophilic, motile, facultatively aerobic rods. B. subtilis themselves, measure 0.8 to 1.2 by 2.0 to 8.0 microns in size, possess from 6 to 8 flagella, and form a film on media in which they are grown. The older, fiber-forming cultures, which have resulted of a long period of time at a controlled temperature, contain suitable enzyme systems for use in particular organic matter. (When too great a film starts to form the seed solution should be remade because the film will clog the pipettes.) Colonies of Bacillus subtilis are found to be gram-positive and usually comprised of long chains of the bacteria. The zig-zag shape of the chain is due to their laying parallel, with individual rods meeting at obtuse angles. Spore formation occur in great numbers, appearing in the center of the rod. A membrane or pellicle begins to form around the spores as vegetative cells shrink.

Spores of Bacillus subtilis germinate equatorially, that is, a rod grows out of it at a right angle to the diameter. The optimum temperature for growth is 28 to 40°C, the minimum being 10°C and the maximum about 60°C.[15]

Therefore, the temperature range of this study, $20 \pm 1^{\circ}C$, is well within the limits of $10^{\circ}C$ to $60^{\circ}C$, although not within the optimum range for the Bacillus subtilis.

An excellant source of information on bacteria, their activity, and their properties, is John R. Porter's "Bacterial Chemistry and Physiology". This is a collection of information obtained by hundreds of investigators over a period of years.

While Bacillus subtilis are not disease producing organisms, it may be of interest to note other ways of inactivating or destroying them beside heat denaturation. The text describes the B. subtilis as one of the most difficult of bacteria to destroy, whether by chemical or physical action. The action of X-rays on agar alters the agar in such a way as to render the medium unsuitable for the growth of the B. subtilis. Submission to intense pressure (6,000 atms or more) for many hours (14) also destroys the organism. The Bacillus subtilis may also be destroyed by vigorous agitation for hours in a mechanical shaker with finely divided abrasives, such as glass beads, Pyrex chips, or boron carbide. The agitation causes a denaturation in the cellular proteins, transforming the colloidal state of their protoplasm or causing their cellular disintegration.

Studies on surface tension have shown it to be an important factor in the growth of a micro-organism. This is especially true for the Bacillus subtilis variety used since they grow in a surface pellicle of the liquid and find a considerable different environment from that in the bulk of the liquid.

The phenomenon of adsorption also plays a very important role in many systems. Adsorption is a surface affair and since it is at or on the surface that chemical reactions take place, adsorption is in many instances the precursor, if not the determiner, of these reactions. Because bacteria offer many surfaces, adsorption must be a very important factor in initiating surface chemical reactions and control other cellular activities.

It would be good to look at the type of reactions that occur here and in the systems of the waterways and sewage plants. Experiments have shown that in biological waste treatment systems, the micro-organisms oxidize the organic matter to form carbon dioxide and water to obtain energy, which converts some of the organic matter to cellular protoplasm. Eventually the organic matter in the waste waters is either completely oxidized or partially changed in form. The manner in which this process occurs is through the use of highly complex enzyme systems in the bacteria. Enzymes are a class of protein capable

of causing chemical changes without undergoing any chemical change themselves; i.e. catalytic action. The reaction is substrate $\xrightarrow{\text{enzyme}}$ products. While produced by living cells, enzymes act independently of such cells. Also the enzymes' activity is not inhibited by their removal from the cell that produced them. Well known enzymes include the ptyalin of the salivary glands, pepsin from the stomach glands, and trypsin of the pancreas. Bacteria produce enzymes that are similar to those produced by organs of higher life forms.[16]

A protein is a functional unit which is made up of an arrangement of various amino acids. Some twenty amino acids are known to be the building blocks of the proteins. The differences among proteins is the result of different number, sequence, and arrangement of the amino acids. Therefore, there is an infinite number of proteins belonging to different animal and plant species; however, a given organism has only a certain number. Enzymes represent large protein (molecular weight of 5×10^3 to 1×10^6 g/g-atom or more, and in the thousands of amino acids) combinations and permutations of varied spatial chemical configurarion.

As already indicated the enzymes are not destroyed during their chemical reactions; however, their activity may be inhibited by the products formed. An enzyme's ac-

tion is specific and each enzyme causes its own particular type of chemical change and that kind only on its specific substrate. Enzymes are effected by temperature and pH, each working in its limited range. They are delicate structures which can be destroyed or inhibited by change in their pH and temperature. While heat destroys them (heat denaturation), freezing only retards their action.

The biochemical reactions occuring in bacterial cells are complicated and interrelated. The cells are producing various types of enzymes. One large area of difference is between the ectoenzymes and endoenzymes. While the latter are liberated by the disintegration of the cell that produces them, the former are freed within the cell and diffuse through the plasma membrane into surrounding media. Bacterial enzymes may also be classified by the type of substrate it acts on. Carbohydrases act on carbohydrates, while those that cause biological oxidation are referred to as oxidases. There are adaptive enzymes which are free by organisms as a response to a substrate in the medium culture. The primary purpose of bacterial enzymes is to break complex food materials down into simple materials fit for assimilation by the bacteria.[17]

The specificity of certain bacteria for a particular class or several classes of organic compounds can be explained by the enzyme systems they use to metabolize

food. If, however, another food source is the only one available, the enzyme systems must be capable of modification so to be able to use the compound present. To achieve this, actual mutation of the organism may be necessary. Therefore, in nature it is found that mutation (by lower life forms) is the rule, not the exception. The only differences between organisms is the time necessary (generations) for the mutation to occur. The generation time of Aerobacter aerogenes is 17.2-17.4 minutes, while that of Rhizobiun japonicum, in a medium of mannitol-mineral yeast extract or dextrose, is 6 to 8 hours, an extraordinary long time for bacterium. The Bacillus subtilis has a generation time of 26 to 32 minutes.[18] It can then be seen that growth may proceed rapidly or it may require a considerable period of time.[19] This information is necessary for a thorough understanding of the complex processes that are going on in the treatment of wastes. In an actual situation where waste materials require treatment, biological oxidation is usually the route by which the waste is to be broken down, where the action of countless different species of bacteria will be useful to affect this treatment. In most cases biological digestion is the best means of breakdown because the greater the variety of microbial species present in the system, the greater the number of types of compounds which they can effectively metabolize.

However, as the number of compounds are reduced so should the number of bacterial enzymes present. At the far end of this projection is when one compound or a few select compounds are to be examined for specific reasons. When waste sewage sludge is used in tests on the above type solutions contradictory results can be obtained. This results because sewage from different areas contain different bacteria and the certain bacteria or combination of bacteria needed to breakdown a specific compound, while present in one sewage may be confined to that area alone for certain limiting reasons. This would make replication of results almost impossible. This is the case with NTA. While R.D. Swisher et al[20] obtained results of NTA breakdown, John McKenna[21] working on the effect of NTA on linear alkyl benzene sulfate (LAS), showed that NTA resulted in a lower breakdown of the surfactant. Therefore, not only did the NTA not breakdown, but in reaction with LAS prevented its breakdown possibly by inactivation of the enzymes. This test by McKenna also showed another area of contradictory results. While his results showed STPP as accelerating the breakdown of the surfactant, tests by G.W. Malaney and W.D. Sheets[22] showed that STPP had no effect on the surfactant they used. The use of a single strain of bacteria specifies to the tester the results that were found in the past using a specific organic compound. Indeed, excellent replications have been

obtained in the past using Bacillus subtilis #9524.

While we are examining the use of bacteria in the digestion and metabolism of food in respect to waste treatment, it should be remembered that this is not their prime function. As has been mentioned, metabolism of foods by bacterial enzymes usually result in the production of new compounds. Thus, single strain systems are used in the synthesizing of many organic chemicals. In fact, large scale bio-organic synthesizing has proven to be profitable, especially when compared with the cost of chemical synthesis. This is especially true in the area of vitamins.

A common example (given in most courses on biochemistry) is the case of fermentation. In this process carbohydrates are broken down by specific enzymes. The reaction can be shown as:

$$\underset{\text{glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{ethanol}}{2C_2H_5OH} + \underset{\text{carbon dioxide}}{2CO_2}$$

The more complex enzyme system can be found in Appendix I. By the changing or omission or addition of a single enzyme or more, a different compound is obtained. This can be demonstrated by showing other compounds derived by the enzymic breakdown of glucose:

$$C_6H_{12}O_6 \longrightarrow 2CH_3\text{-}CHOH\text{-}COOH$$
lactic acid

$$C_6H_{12}O_6 + O_2 \longrightarrow 2CH_3COOH + 2HCOOH$$
acetate formic acid

Here again it can be seen that one's use of specific enzymes is important. The products obtained in the fermentation process depend on the particular carbohydrates and the bacteria used since different bacteria exhibit different enzyme capabilities.

As mentioned previously a great deal of use of strain-specific systems is in the drug synthesising industry. Different strains of Bacillus subtilis are used variously in production of Adenylic acid, Aterumin, Bacillin, Bactiracin, Guanosine, Inosinic acid, etc. Table I[23] is illustrative of some compounds obtainable by use of strain-specific bacterial systems. The particular bacteria of this report, Bacillus subtilis is important in the assay of Penicillin and Streptothricin. This strain of bacteria is also used in the synthesis of many amino acids, as: Leucine, Aspartic acid, Tyrosine, and some others.[24]

Before describing my experiment it would be well to look at the way industry and government handles the problem of the phosphates in America's waterways, and the ideas of improving them. Since the time of NTA's general

Class	Compound	Organism
Alcohol	Ethanol	Saccharomyces cerevisiae
Alcohol	Beer	Saccharomyces sp.
Alcohol	Glycerol	Bacillus lichenformis
Antibiotic	Penicillin	Penicillum notatum
Antibiotic	Bacillin	Bacillus subtilis
Antibiotic	B_{12}	Bacillus megaterium
Antibiotic	Biotin	Sporobolomyces pararoseus
Antibiotic	Riboflavin	Bacillus megaterium
Acid	Acetic Acid	Lactobacillus plantarum
Acid	Butyric Acid	Butyrivibrio fibrisolvens
Acid	Fumaric Acid	Rhizopus arrhizus
Carbohydrate	Sorbose	Acetobacter suboxydans
Carbohydrate	Glucose	Trichoderma viride
Steroid	Ergosterol	Penicilium puberuium

Table I. Typical Synthetic Uses of Microbes

use in detergents was short lived, there never were any valid ideas of mass control ever put forth. However, the ideas on phosphate are more plentiful. It would be beneficial then to look at means proposed to rid the lakes and rivers of the world of the pollution that phosphates are supposed to have caused.

Today's conventional means remove about one-fifth of the phosphorus found in sewage. This is done primarily thru a settling process where the sewage goes through aeration and subsequent solid precipitation. A number of systems have been proposed in which the amount of phosphorus removed would be increased. One such process developed by Dorr-Oliver is referred to as the phosphate extraction process (PEP), which is a two-stage chemical-biological process.[25] In the PEP, lime is added to the primary settling tank, raising the pH to between 9.5 and 10.0. This results in an increase of suspended solids removed from 60 percent to 85 percent, and an increase of the BOD from 30 percent to about 75 percent. The amount of phosphate content is reduced by more than 90 percent. The balance is almost completely removed in the secondary aeration stage via a micro-organism's metabolic process. A tertiary stage (purely chemical) wherein any phosphate present in the effluent of the secondary stage is precipated, has proven to be economically unappealing.

In tests conducted by the Federal Water Pollution Control Administration, aluminum sulfate ($Al_2(SO_4)_3 \cdot 24H_2O$) was added prior to the aeration and cut the phosphate content by more than 95 percent. While the above represents a chemical approach to the problem, the FWPCA has also been conducting a study of a purely biological process. However, while this process works well in theory or on a small scale, problems arise in trying to use this process on a large scale. This process requires optimized conditions of aeration time and rate, suspended solids concentrations, and solid-liquid seperation time. These conditions are extremely difficult to maintain and only about 25 percent removal is obtained.

These processes hold out hope for the future that the problem of excessive phosphates in our waters may be alleviated. However, it would mean a commitment on the part of the federal government and industry to provide the money so that these type waste treatment plants could be built all over the country wherever it has been determined that waterways in those areas were endangered.

III. EXPERIMENTAL DETAILS

A. Preparation of Distilled Water Stock

Distilled water was obtained from the college chemical stock room in a large (20 liter) bottle, which had been previously cleaned with detergents, and thoroughly rinsed. The bottle was kept covered with a cotton plug. This water should be distilled from a block tin still, contain less than 0.01 mg/l copper, and be free of chlorine, chloramines, caustic alkalinity, organic materials, and acids.[26]

B. Preparation of Solutions Used in Testing

All compounds used were reagent-quality chemicals, ie., ACS grade or A.R. (analyzed reagent) grade; all were used without further purification. The reagents for the azide modification of the iodometric method for dissolved oxygen are made up as follows:[27]

1. Manganese Sulfate Solution

Dissolve 364 grams of $MnSO_4 \cdot H_2O$ in distilled water, filter, and dilute to 1 liter. The manganese sulfate solution should liberate not more than a trace of iodine when added to an acidified solution of potassium iodide.

2. Alkali-Iodide-Azide Reagent

Dissolve 500 grams of sodium hydroxide (NaOH)

and 135 grams of sodium iodide (NaI) in distilled water and dilute to 1 liter. To this solution add 10 grams sodium azide (NaN_3) dissolved in 40 mls distilled water. This reagent should not give a color with starch solution when diluted and acidified.

3. Sulfuric Acid, Concentrated

The strength of this acid is about 36 N. Hence 1 ml is equivalent to about 3 mls of the alkali-iodide-azide reagent.

4. Starch Solution

Prepare an emulsion of 5-6 grams soluble starch in a beaker with a small quantity of distilled water. Pour this emulsion into a liter of boiling water, allow to boil for a few minutes, and let settle overnight. Use the clear supernate. Test with free iodine for blue color. Remake frequently.

5. Sodium Thiosulfate Stock Solution, 0.10N

Dissolve 24.82 grams $Na_2S_2O_3 \cdot 5H_2O$ in boiling water and dilute to 1 liter. Preserve by adding 1 gram of NaOH per liter.

6. Standard Sodium Thiosulfate, 0.025N

Prepare by diluting 250 mls sodium thiosulfate

stock solution to 1 liter. Standard sodium thiosulfate solution, exactly 0.025N, is equivalent to 0.20 mg DO per 1.0 ml.

7. Potassium Permanganate Solution

Dissolve 6.3 grams of $KMnO_4$ in distilled water and dilute to 1 liter.

8. Potassium Oxalate Solution

Dissolve 2 grams $K_2C_2O_4 \cdot H_2O$ in 100 mls distilled water; 1 ml of this solution should be sufficient for the reduction of about 1.1 ml of the permanganate solution.

C. Procedure for Seeding Solution With Bacteria

Usually seeded dilution water should be used the same day it is made; however, in the ease of Bacillus subtilis, which can be stored at low temperatures in a refrigerator for as long as six months to a year, this is not necessary. B. subtilis are so stable that they can be used without error at high temperatures, as high as 55 to 60°C. The suspension was prepared as follows: Bacillus subtilis #9524, supplied by the National Type Culture Collection, comes in freeze-dried sealed standard glass vials. The vial is flamed, broken, and emptied into sterilized, distilled water. In this work 100 mls of water was used for the contents of the vial.

D. Preparation of Stock Solutions

Into a flask containing 1 liter of distilled water 0.1 gram of NTA was added resulting in a concentration of 100mg/l. The solution of NTA was then sterilized at 15 psig and 121°C for 45 minutes, capped with sterile cotton plugs. A similar procedure was followed for sodium tripolyphosphate (STPP).

E. Preparation of Culture Media

A broth for the cultivation of the Bacillus subtilis #9524 was made of the following nutrients:[28]

(A) Heart Infusion Broth (Difco B38) .. 20.0g
(B) Glycerol (U.S.P.) 60.0g
(C) Glucose (U.S.P.) 15.0g

This was added to 1 liter of distilled water. The broth was then autoclaved for 45 minutes at 15 psig and 121°C, using a sterile cotton plugged bottle. The broth was then allowed to cool to room temperature and placed in 30 test tubes which had been heated dry and sterilized at 200°C for 45 minutes and then allowed to cool. One milliliter seed bacterial suspension was added to each test tube (containing 9 mls of nutrient) and these were incubated for one week at 20°C before use.

F. Preparation of Other Requirements for Procedure

1. Sterilized water solutions were allowed to

equilibrated to 20°C and absorb oxygen for a week, through sterile cotton plugs covered with brown paper dust caps.

2. All pipettes and BOD bottles were sterilized in an oven at 450°F for 45 minutes. Glassware was previously washed and thoroughly rinsed with copious amounts of distilled water. Bottles, flasks, beakers, etc., were sealed with sterile cotton plugs and capped with a piece of brown paper covering to prevent entry of any foreign material. The BOD bottles, during sterilization, also had a piece of cord at the side of the plug to prevent too tight a seal, so as to be easier to open. The pipettes were sterilized in special tubular metal containers obtained for that purpose.

3. A hundred-ml pipette, used to fill the BOD bottles with sterilized H_2O, was sterilized at 450°F for 45 minutes, however, since it was too large for the metal container, it had to be wrapped in brown paperand sealed at both ends.

G. Loading Procedure

The BOD bottles were loaded in the following manner:

1. Sixteen sterile BOD bottles were arranged to be filled with 300 mls sterilized-oxygen saturated water, 1 ml bacterial suspension, and varying amounts of NTA or STPP.

2. The amounts of NTA or STPP added were such as to give concentrations of 0, 1/4, 1/2, 1, 1 1/2, 2, 4ppm. etc.

3. A set of bottles containing only sterile water and a set of NTA (STPP) at 1 ppm but with no bacteria were also set aside for testing.

IV. TESTING METHODS EMPOLYED

The methods used for carrying out the experimental work were essentially the same as those used in previous work by M. I. Marblestone and V. A. De Bari. These methods were originally taken from "Standard Methods", a well established reference for water treatment and evaluation procedures. Any modifications used were necessary so that work could proceed, and do not, to any extent, represent a serious deviation from accepted evaluation of water practices. Single strain bacterial inoculum and complete media sterility were necessary for replication.

A. The method used here was modified from the standard dilution method outlined in previous experiments so as to reduce the chances of losing the sterility needed to obtain meaningful results. All techniques utilized sterility as a modification of the BOD techniques based on the Rideal-Stewart-Winkler DO test.

1. The loading of the BOD bottles was previously described.

2. The BOD bottles were incubated for two set periods of time. The first was for a period of five days and another for a period of twelve days, all at approximately 20°C. This was done so as to evaluate the DO contents of the bottles

at two important stages in the BOD process. At the five-day stage the amount of oxygen consumed by the reaction should represent 68 percent of the bottles' DO content; and at the twelve-day stage it should represent 99 percent of that content.[30]

3. Determine the DO content of the incubated samples and blanks, using the azide modification of the iodometric method.

4. Calculate the BOD for the samples as follows:

$$\text{mg/l BOD} = \frac{D_1 - D_2}{P}$$

where: D_1 = DO of dilution water before incubation

D_2 = DO of dilution sample after incubation

P = fraction of sample used/total amount in bottle

B. The dissolved oxygen in the samples is determined by the azide modification of the Rideal-Stewart-Winkler iodometric method[31] and is outlined below:

1. After incubation, the sample, collected originally in a 300-ml sterile, standard DO bottle, has exactly 0.7 ml concentrated H_2SO_4 added,

well below the surface, followed by 1 ml $KMnO_4$. Stopper and mix by inversion. It is essential to add no more than 0.7 ml H_2SO_4 as the first step of pretreatment. For this reason, it is best that the acid be added with an 1-ml pipette, graduated to 0.1 ml. The amount of permanganate added should be sufficient to obtain a violet color which persists for 20 minutes. If the permanganate tinge is destroyed in a shorter time, add additional $KMnO_4$, but avoid large excesses. The acid and permanganate are added to destroy any living substance that may still be present after the incubation period.

2. Remove the permanganate color completely by adding 0.5-1.0 ml $K_2C_2O_4 \cdot H_2O$. Mix well and allow to stand in the dark (the reaction takes place more readily in the dark). Excess oxalate causes low results; add only an amount of oxalate which completely decolorizes the $KMnO_4$ without having an excess of more than 0.5 ml. Decolorization should occur in 2 to 10 minutes. If it is impossible to decolorize the sample without adding a large excess of oxalate, the result will be of little value.

3. Once a clear color is obtained, add 1 ml manganous sulfate solution followed by 3 mls alkali-iodide-azide reagent well below the surface of the liquid; stopper with care to exclude air bubbles and mix by inverting the bottle several times. After the precipitate settles, leaving a clear supernatent above the basic manganic oxide and/or manganous hydroxide floc mixtures, shake again. When settling has produced a clear supernant, carefully remove the stopper and immediately add 1 ml concentrated H_2SO_4 by gently allowing the acid to run down the neck of the bottle, restopper, and mix by gentle inversion until dissolution is complete. The iodine should be uniformly distributed throughout the bottle before decanting (here a 100-ml pipette was used) the amount needed for titration. Wait for 5 minutes before decanting. One hundred mls samples were used so that at least two titrations could be carried out with each 300-ml bottle.

4. Titrate with 0.025N sodium thiosulfate to a pale straw color. Add 1 ml of freshly prepared starch solution and continue the titration to the first disapperance of the blue color.

5. Calculation

Because 1 ml 0.025N sodium thiosulfate titrant is equivalent to 0.2 mg DO, each ml of sodium thiosulfate titrant, for a 100-ml sample, used is equivalent to 2 mg/l DO.

1 ml 0.025N thiosulfate =
0.025 equiv/l x 8 mg/equiv O_2 = 0.2 mg/l O_2

For a 100-ml sample, 1 ml 0.025N $Na_2S_2O_3$ =
0.2 mg/l x 1000ml/100 ml = 2 mg/l DO
1 mg/l DO x 1 g/1000 mg x 1 l/1000 ml =
1 g/1,000,000 = 1 ppm

C. Discussion of Test

The reactions involved in the various steps of the modified Winkler method are represented by the following equations:[32]

Manganous sulfate reacts with the potassium hydroxide in the alkaline potassium iodide mixture to produce a white flocculent precipitate of manganous (Mn^{+2}) hydroxide:

$$MnSO_4 + 2KOH \longrightarrow Mn(OH)_2 + K_2SO_4$$

If a white precipitate is obtained, there was no dissolved oxygen in the sample and there is no need to proceed further. A brown precipitate shows that

oxygen was present and reacted with the manganous hydroxide. The brown precipitate is manganic (Mn^{+4}) basic oxide:

$$2Mn(OH)_2 + O_2 \longrightarrow 2MnO(OH)_2$$

Upon addition of the acid, this precipitate is dissolved forming manganic sulfate:

$$MnO(OH)_2 + 2H_2SO_4 \longrightarrow Mn(SO_4)_2 + 3H_2O$$

There is an immediate reaction between this compound and the potassium iodide previously added, liberating iodine and resulting in the typical iodine coloration of the water:

$$Mn(SO_4)_2 + 2KI \longrightarrow MnSO_4 + K_2SO_4 + I_2$$

The quantity of iodine liberated by these reactions is chemically equivalent to the quantity of oxygen present in the sample. The quantity of iodine is determined by titrating a portion of the solution with a standard solution of sodium thiosulfate:

$$2Na_2S_2O_3 + I_2 \longrightarrow Na_2S_4O_6 + 2NaI$$

The thiosulfate is made of such a strength that 1 ml = 0.2 mg oxygen. Such a solution would have a normality obtained as follows:

1. 0.2 mg/ml = 0.2 g/l
2. Equivalent weight of oxygen = 8.0 g/equivalent
3. 0.2/8.0 = 0.025N

V. RESULTS AND INTERPRETATION

The data obtained from the titration of the solutions of NTA in the BOD bottles is given in Tables II and III. The data was plotted in Figures I and II.

While some of the oxygen was used up, it appears that some sort of inhibition occurs which prevents the breakdown of the NTA. No breakdown of STPP occurred since the titrations showed its DO content was similar to that of pure, sterilized water. This is to be expected since phosphate ion is involved in interchangeable phosphorylations of carbohydrates - high energy bond systems - in the metabolic cycles in life.

From the results that were obtained a curve of the type in Figure III can be derived. It can be divided into three sections. As one proceeds in section I, along the curve, one sees a steady rise. This rise corresponds to a portion of the curve where increased concentration of organic matter provides an increasing amount of food for the organisms. At the boundary of section I and section II, an optimum occurs. This corresponds to the optimum concentration of the substrate, for the bacteria under study, at the particular temperature at which the study is being made. Proceeding into section II, the curve falls off sharply. Along this portion of the curve

Conc.(ppm)	D.O. (100-ml sample)	BOD(mg/l)
H_2O (0 days)	9.10	-
H_2O	9.10	-
0	9.06	0.12
1/4	8.86	0.72
1/2	8.80	0.90
1.0	8.72	1.14
1.5	8.66	1.32
2.0	8.64	1.38
4.0	8.32	2.34
5.0	8.16	2.82
6.0	8.09	3.03
8.0	8.13	2.91
10.0	8.24	2.58
15.0	8.28	2.46
20.0	8.36	2.22

Table II. BOD Values For NTA After Five Days

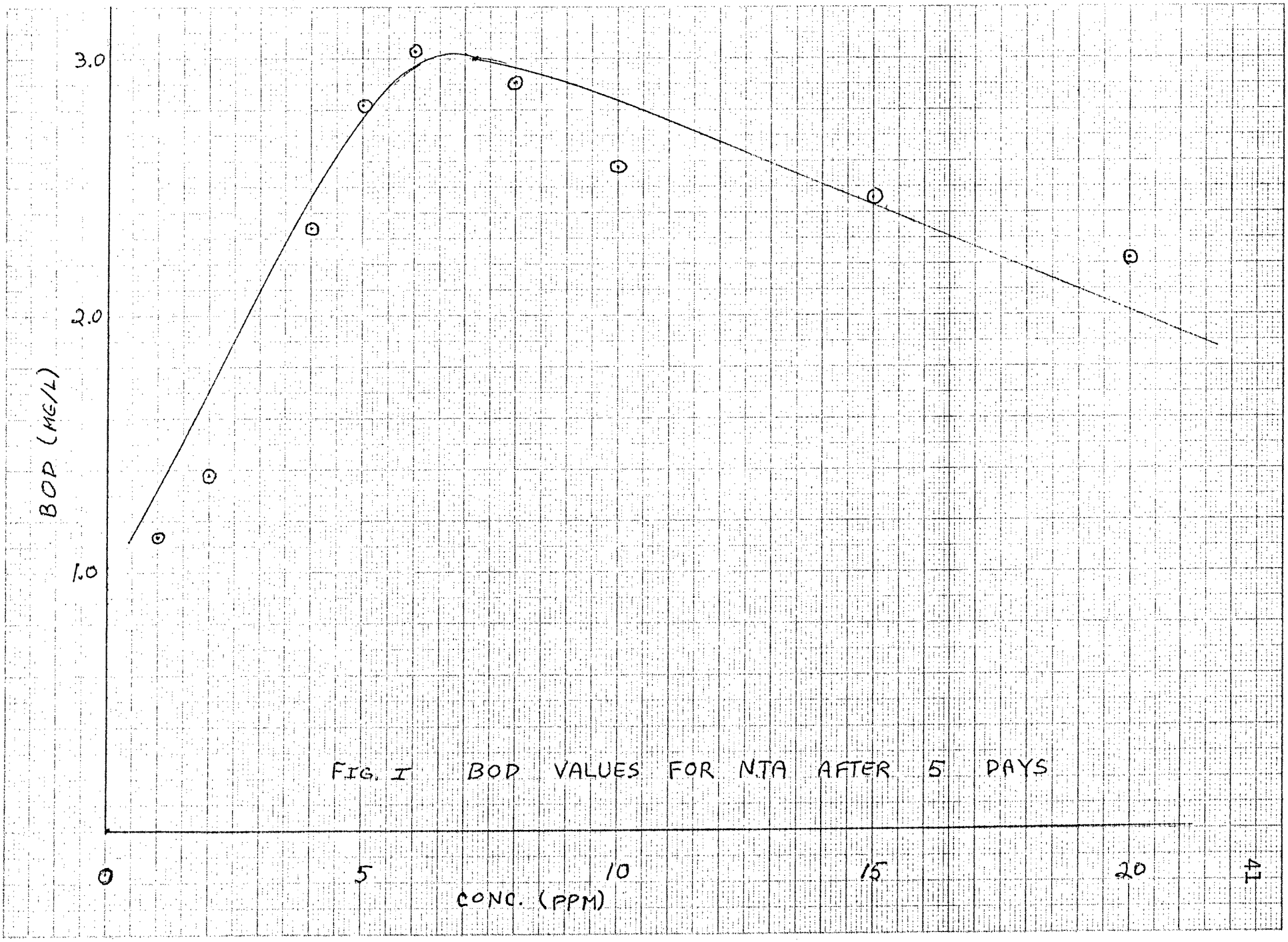

FIG. I BOD VALUES FOR NTA AFTER 5 DAYS

Conc.(ppm)	D.O. (100-ml sample)	BOD(mg/l)
H_2O	9.06	0.12
0	9.00	0.30
1/4	8.80	0.90
1/2	8.72	1.14
1.0	8.56	1.62
1.5	8.50	1.80
2.0	8.42	2.04
4.0	8.24	2.58
5.0	8.00	3.30
6.0	7.95	3.45
8.0	7.98	3.36
10.0	8.18	2.76
15.0	8.21	2.67
20.0	8.30	2.40

Table III. BOD Values For NTA After Twelve Days

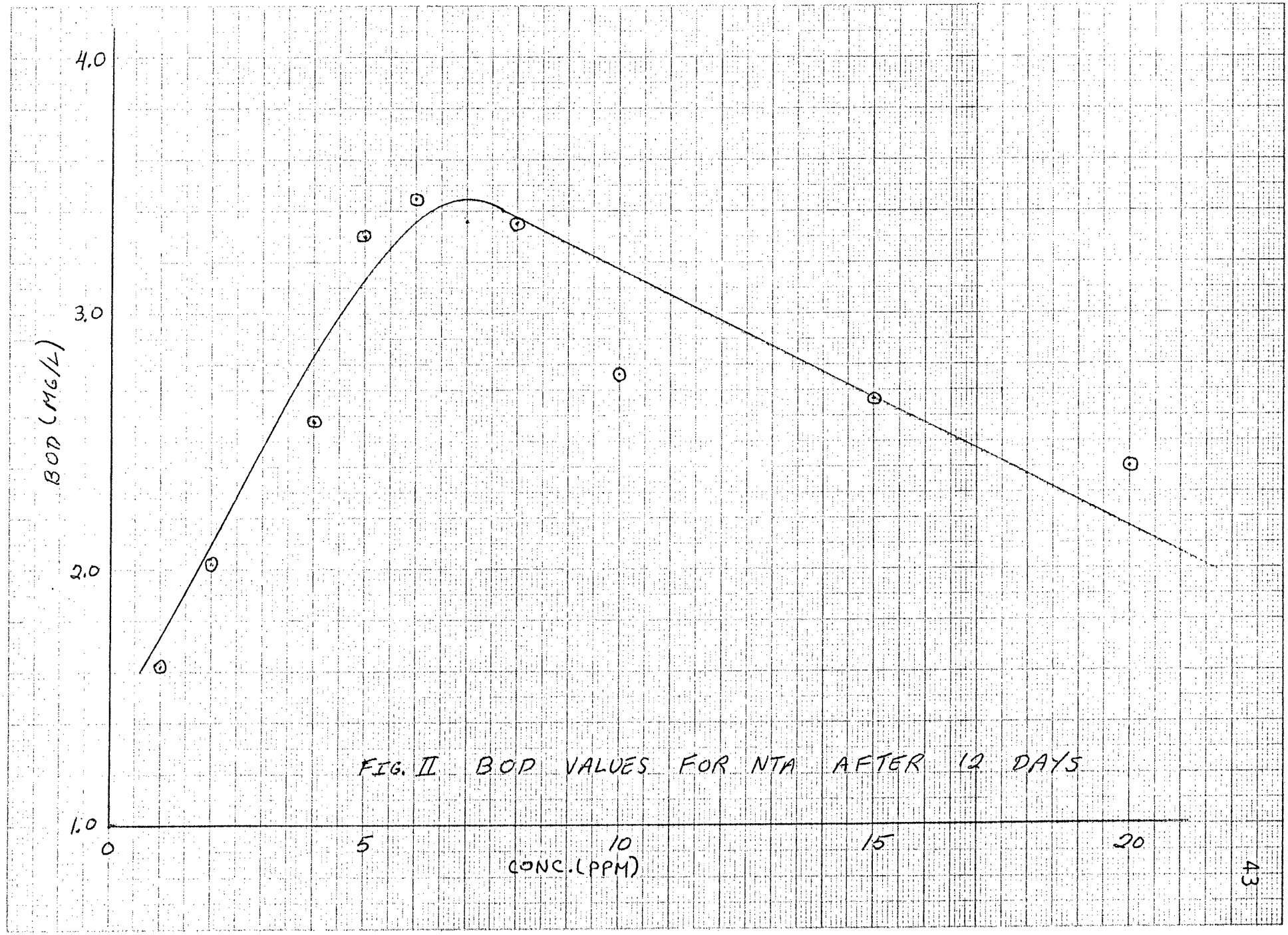

FIG. II BOD VALUES FOR NTA AFTER 12 DAYS

FIG III. EFFECT OF CONC. ON BOD EXERTION

the concentrations are steadily increasing in toxicity, and the utilization of substrate for food by the bacteria is rapidly falling off. The boundary of sections II and III illustrates the minumum of BOD exertion, at which point utilization of substrate by the bacteria virtually ceases; and this continues to be the case on into section III. The displacement from the level portion of the curve from zero BOD exertion is probably due to slow chemical oxidation or residual enzymatic action.

On examination of Figure I and II it would appear that there was oxygen consumption at a zero-ppm concentration level of NTA. This small amount of deviation from the curve is due to small amounts of evaporation from the 300-ml BOD bottle (especially 12-day incubation) and small changes in temperature in incubation which where beyond the control of this study. Extremely small amounts of oxygen are present, even dissolved, in such refactory materials as solids - i.e., glass, metals, etc.

Conc.(ppm)	D.O. (100-ml sample)	BOD(mg/l)
1/4	9.02	0.24
1/2	8.97	0.39
1.0	8.95	0.45
1.5	8.93	0.51
2.0	8.98	0.36

Table IV. BOD Values For STPP After Five Days

Conc.(ppm)	D.O. (100-ml sample)	BOD(mg/l)
1/4	9.00	0.30
1/2	8.95	0.45
1.0	8.93	0.51
1.5	8.90	0.60
2.0	8.95	0.45

Table V. BOD Values For STPP
After Thirteen Days

CONCLUSIONS AND RECOMMENDATIONS

From the results obtained in this study it can be seen that NTA will not breakdown easily. This could pose many problems if the NTA was to be used in extensive amounts. While a minute and inconclusive amount of breakdown occurred, this was at too low a concentration to be meaningful. Even if the lowest possible amount of NTA was present in commercial detergents, in place of the phosphates, multiplied by the millions of households that would use these detergents, NTA could cause catastropic results to humans. We are not sure of the chemical derivatives of NTA. We are alarmed at the threats of genetic damages and carcinogenesis.

We, as of today, do not know the derivatives of the NTA molecule if it were to be broken down. Also important along these lines are how these derivatives arrived at and what are the possible intermediate products that could enter into reactions with other intermediates, derivatives, or other compounds in its vicinity, involving life itself.

There is also the potential danger from NTA itself. Not all of the NTA (or any compound) in an effluent would be completely broken down in today's typical sewage treatment plant. Some of this NTA will inevitably find

its way into ground water supply without being treated at all. Therefore, today we face a number of possible dangers that we have no answer to.

A great amount of research will still have to be done to answer these questions and others posed in this paper. The unknown nature of its intermediates and the possibility that they cause carcinogenic and mutagenic hazards will have to be resolved. Therefore, to scream for the change from phosphates to NTA, or any other substitute, without knowing of the possible dangers that would be incurred in such a switch, is foolhardy.

In the end it does not matter what "builder" is used (NTA, STPP, etc.) if there is no effective way of preventing the entry of this chemical or its derivatives from entering our waterways before they are completely broken down into the simple compounds (CO_2, H_2O, etc.) or into simple ions or elements (PO_4^{-3}, NO_3^-, etc.) that can be usefully and safely reused. This calls for a commitment from everyone - industry, government, and the consumer - to see that waste water and sewage treatment plants are constructed that can handle the job required of them.

APPENDIX I

1. Glucose + ATP $\xrightarrow{\text{Glucokinase}}$ Glucose-6-phosphate
2. G-6-P $\xrightarrow[\text{Isomerase}]{\text{Phosphohexose}}$ Fructose-6-phosphate
3. F-6-P $\xrightarrow[\text{Kinase}]{\text{Phosphofructo-}}$ Fructose-1,6-Di-P
4. F-1,6-Di-P $\xrightarrow{\text{Aldolase}}$ Dihydroxyacetone-P + Glyceraldehyde-3-P
5. Dihydroxyacetone-P $\xrightarrow[\text{Isomerase}]{\text{Phosphotriose}}$ Glyceraldehyde-3-P
6. Glycer-3-P $\xrightarrow[\text{3-P Dehydrogenase}]{\text{Glyceraldehyde}}$ 1,3-Diphosphoglycerate
7. 1,3-DiP-Glycerate $\xrightarrow[\text{Kinase}]{\text{Phosphoglycerate}}$ 3-Phospho-glycerate
8. 3-P-Glycerate $\xrightarrow[\text{Mutase}]{\text{Phosphoglycerate}}$ 2-P-Glycerate
9. 2-P-Glycerate $\xrightarrow{\text{Enolase}}$ Phosphoenolpyruvate
10. P-enolpyruvate $\xrightarrow[\text{Kinase}]{\text{Pyruvate}}$ (Enol)-Pyruvate
11. (Enol)-Pyruvate $\longrightarrow$ (Keto)-Pyruvate

The above reaction converting Glucose ——— Pyruvate, is referred to as the Embden-Meyerhof Pathway.

12A. Pyrovate $\xrightarrow[\text{Dehydrogenase}]{\text{Lactate}}$ Lactate (anaerobic state)

12B. Pyruvate $\xrightarrow[H^+]{DPNH}$ Ethanol

FOOTNOTES

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