Effects of Lowered Oxygen Tension on the Susceptibility of Daphnia Magna to Certain Inorganic Salts.

Edward Joseph Fairchild
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EFFECTS OF LOWERED OXYGEN TENSION
ON THE SUSCEPTIBILITY OF DAPHNIA MAGNA
TO CERTAIN INORGANIC SALTS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Zoology, Physiology and Entomology

by

Edward Joseph Fairchild, II
B. S., Louisiana State University, 1948
M. S., Louisiana State University, 1950
August, 1964
MANUSCRIPT THESSES

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ABSTRACT

Studies were conducted to determine effects of oxygen tensions lower than atmospheric upon the susceptibility of the microcrustacean, Daphnia magna Straus, when exposed to solutions of certain inorganic salts known to be present in various industrial effluents. This investigation is one of few to attempt a correlation between conditions of lowered oxygen concentrations and presence of toxic materials; these factors often occur simultaneously in natural waters which are polluted and each, in itself, can contribute to an unfavorable aquatic environment.

Culture methods are given in detail for several types of media employed and reproduction and longevity data are reported. Young daphnids of similar age groups cultured in standard media were used in conducting experiments.

Apparatus was devised whereby mixtures of air and nitrogen were obtained in desired ratio by measuring their partial pressures; the gas mixtures could be duplicated at any time provided changes in barometric pressure were accounted for. Tests with D. magna exposed to solutions of salts were conducted at varied oxygen tensions which were maintained throughout test periods by admitting gas mixtures under constant pressure, temperature (23 ± 1°C.) and rate of flow. Maximum deviation above and below mean oxygen concentration was usually less than 0.1 p.p.m. A critical evaluation of advantages and
disadvantages of apparatus and technique is presented.

Tests at different oxygen tensions were conducted with nine salts. Each experiment employed 10 daphnids in each of nine concentrations of salt in a series of dilutions in geometrical progression, and 10 controls in a standardized, laboratory prepared, dilution water. Experiments were conducted at dissolved oxygen levels of approximate 1.6, 3.0, 4.6, 6.6, and 9.0 p.p.m.; each salt, however, was not tested at all levels. Median toxicity thresholds were calculated for 25, 50, and 100 hours. Results are presented graphically and in tabular form.

The 25- and 50-hour thresholds (average) for D. magna tested with sodium chloride and ammonium sulfate at 4.3 p.p.m. dissolved oxygen showed no significant decrease from thresholds at atmospheric oxygen (approx. 6.5 p.p.m.), but the 100-hour thresholds did show slightly significant decreases. This oxygen level did not appreciably alter susceptibility of daphnids until the latter part of test periods.

Most thresholds for D. magna tested at 3.0 p.p.m. dissolved oxygen showed further decreases. Some 25-hour threshold drops were not significant. Compounds listed in order of greatest relative drop in threshold for 100 hours are: sodium carbonate, ammonium sulfate, ammonium chloride, sodium chloride, calcium chloride, and sodium chromate. The first two salts showed threshold drops greater than 50% while the remainder showed less. Tests at 1.5 p.p.m. oxygen showed still further lowering of thresholds. Compounds listed in order of greatest relative drop in 100-hour thresholds are: ammonium
sulfate, ammonium chloride, sodium carbonate, sodium sulfate, calcium chloride, sodium chloride, sodium bisulfate and sodium chromate. Threshold drops for the first five salts indicated decreases of 100% or more. The greatest drop was 148%. Thresholds for D. magna tested with sodium bisulfite at lowered oxygen tensions showed rises above thresholds at atmospheric oxygen. Tests at high oxygen, 9.0 p.p.m., showed similar results. These responses have been attributed to oxidation of sulfite to the less toxic sulfate. Tests with this salt are not considered conclusive.

Results indicate that D. magna in the presence of low oxygen is most susceptible to compounds which alter osmotic pressure and to sodium carbonate which imparts high pH. Results further indicate that daphnids are least affected by the "truly toxic" compound, sodium chromate, and by sodium bisulfate which imparts low pH. Modes of action of the salts are reviewed and discussed in relation to findings of this study. Analysis of data conclusively shows that D. magna tested under lowered oxygen tensions exhibited lower thresholds than when tested at atmospheric oxygen. This was indicated for all salts, with the exception of sodium bisulfite. The degree of threshold drop depended upon oxygen tension; decrease in oxygen progressively increased the susceptibility to the compound. It is concluded that toxicity to D. magna varies with oxygen tension, with low oxygen being synergistic.

Findings are in agreement with investigations of similar nature conducted upon fish, and give additional proof that presence or absence of toxic substances must be considered before final evaluation
of minimum concentrations of oxygen can be made. Similarly, effects of low oxygen must be taken into account prior to establishment of safe minimum concentrations of chemicals tolerated by aquatic organisms.
INTRODUCTION

This study concerns one small phase of the vast amount of research being conducted by academic groups, industries, federal agencies and state agencies in an effort to acquire information leading to an effective water pollution abatement program.

It is generally recognized that the conservation of natural resources is one of the most important problems at present before this country. Some of the most serious phases of this problem are connected with conservation of natural waters. One of our most important natural resources is water and all of us should be concerned about it. Most of us, however, have paid slight attention to our water resources because of their apparently limitless quantities. Nevertheless, as the populations of our cities have increased, and as industrial expansion has occurred, more of us have begun to realize that our water resources are not by any means limitless.

In many areas of our country the natural waters have become polluted—so much so in a few areas that they are rendered unsuitable for many uses. Therefore, a growing demand for some consistent reduction and control of the pollution of surface waters by sewage and industrial wastes has come about. Governmental and industrial officials are cognizant of the pressure being placed on them by organizations and individuals and are doing much to
alleviate the matter and meet these demands. Federal and state agencies have established various types of research divisions which are primarily concerned with problems of stream control and pollution abatement. For example, in Louisiana the Wild Life and Fisheries Commission has established research groups which concern themselves with the many problems of pollution correction. Besides state and government agencies, industries have established research groups which are concerned with the pollution problems of the industry they represent. The Louisiana Petroleum Refiner's Waste Control Council, under whose sponsorship this study has been conducted, is one such industrial organization.

Adequate control of industrial and domestic waste discharge into natural waterways demands that these materials be tolerated by the essential organisms found in the receiving waters. Although pollution correction has been a matter of concern for some time, there is a limited amount of literature pertaining to the effects of waste materials on organisms other than fish. Of course, the first step to be taken in resolving pollution problems was the determination of the direct effect of pollutants (or pure chemicals) on fish, inasmuch as they are the end products of aquatic production. It has been pointed out, however (Anderson, et al, 1948), that materials reduced to a level where they are not harmful to fish may, in the course of time, completely eliminate them by destroying fish foods.

Studies with microcrustacea have indicated that these organisms
are much more susceptible to toxic substances than are fish. (Anderson, et al, 1948; Fowler, 1950). They are widely distributed in natural waters and have been shown to be an important source of food for most fish during some stage of their life cycle (Pennak, 1953). It is conceivable, therefore, that slight pollutional effects which do not harm fish directly, might eventually eliminate them in an indirect way, i.e., by destroying aquatic invertebrates that serve in the fish food chain.

This study concerns the use of one species of microcrustacea. The organism, *Daphnia magna* Straus, has been used by various investigators in evaluating the effects of toxic substances (Naumann, 1938; Anderson, 1946, '48; Fowler, 1950; Freeman and Fowler, 1963; and Freeman, 1953). These studies have been of value in establishing minimum safe concentrations of toxic substances for *Daphnia* and for aquatic invertebrates in general. However, there are other factors besides toxicity of a material which probably should be considered before safe concentrations can be set. One of prime importance is the availability of dissolved oxygen.

Inadequate dissolved oxygen in natural waters can contribute to an unfavorable environment for aquatic life; in fact, from this standpoint, it may be regarded as a corollary pollutant. Low oxygen tensions in an aquatic environment can be brought about in several ways: biological oxidation, chemical oxidation, rise in water temperatures, and activity of chlorophyll-bearing plants. However, from pollution aspects the first two mentioned factors are probably
of the greatest significance. When pollutants, such as combinations of organic and inorganic wastes, are discharged into a natural body of water, biological and chemical oxidation begin immediately. If oxygen in the water is sufficient there will result stable final products with subsequent lowering of dissolved oxygen. However, if oxygen tension is already low it may be entirely depleted by the processes of oxidation. The action of microorganisms, by the process of anaerobic and aerobic decomposition, account for the greater part of the oxygen depletion, but oxygen demand due to the presence of compounds which unite chemically with oxygen also play an important part. It follows from this that oxygen levels may be reduced to such an extent that healthy aquatic life ordinarily associated with normal conditions cannot be sustained. Consequently, any toxic materials present in the wastes are exercising their effect upon the animal life which is already handicapped by low oxygen tensions. The question arises, then, just how much damage is done by depleting an oxygen supply even though such depletion is not below the minimum required for the absolute maintenance of life?

There is a paucity of information in the literature concerning the foregoing question. Southgate, et al, (1933) investigating the River Tees in England which received sewage and industrial effluents, found the water to contain substances poisonous to salmon and to be only partially saturated with dissolved oxygen. Subsequent tests indicated that the toxicity of the two main pollutants was increased with a decrease in oxygen. Townsend, et al, (1944) conducted
studies to determine the effect of pH on salmon when exposed to low oxygen tensions after finding that waste sulfite liquor brought about these conditions in a large estuary. It was found that the pH had a definite effect upon the ability of the fish to withstand low oxygen tensions. With the exception of these investigations, no other references can be cited.

It is the primary aim of this study, therefore, to determine the effects of lowered oxygen tension on the susceptibility of an aquatic invertebrate, *Daphnia magna*, when it is exposed to various toxic substances. Similarly, it can be stated that a corollary purpose has been to study the influential effect of the compounds tested upon the probable lethality of low concentrations of oxygen.
MATERIALS AND METHODS

Certain aspects of the materials and methods employed during the course of this study are extensively described and discussed since they are original with the writer. Considerable detail is desirable in order that it might prove beneficial to others who wish to conduct investigations of a similar nature.

The Test Organism

*Daphnia magna* is a member of the Order Cladocera, Class Crustacea. Cladocera are primarily fresh-water organisms, but there are several marine species. The group as a whole has representative forms which may be found in just about any type of water, excluding those which are grossly polluted. Each species or species group has an affinity for a specific habitat. Some are open water or limnetic forms; others collect in the vegetation at margins of lakes, rivers, ponds and pools. The significance of Cladocera in the aquatic food cycle as food for young and adult fish alike was first emphasized by Forbes in 1885, and since then by innumerable investigators. Various studies of the stomach contents of young fish show from one to 95 per cent Cladocera by volume, and very few studies show less than ten per cent (Pennak, 1953). Other groups of less importance which utilize Cladocera in the diet are *Hydra* and immature and mature insects which in turn are eaten by fish. As pointed out by Anderson, et al (1948), Freeman and Fowler (1953), and others, *D. magna* meets
certain requirements which must be taken into consideration in order to serve as a test organism. Besides being a source of fish food, *D. magna* is easily handled and cultured in great numbers in the laboratory, thus making it available at all times of the year. Furthermore, *D. magna* has been shown to be intermediate in sensitivity to chemicals, being less sensitive than certain other invertebrate organisms, but more sensitive than fish (Anderson, et al, 1948; Fowler, 1950; and Fairchild, 1953). For these reasons and others presented herein, *Daphnia magna* has been recognized as a good, representative experimental animal with which to conduct bioassay.

The method of culturing *Daphnia magna* used in these investigations is similar to that of Anderson (1948) as modified by Freeman and Fowler (1953). The original culture media of Anderson employed filtered lake water as the diluent whereas the latter used an "artificial water". This water, generally designated as "standard reference water" (hereafter SRW), was developed by Hart, Doudoroff and Greenbank (1945) and modified by Abegg (1948), Williams (1948) and Freeman (1949). It is a laboratory-prepared medium, free of organics and contains all the major ions in concentrations and proportions typical of a mean surface water of the United States. Besides being used as a culture medium water, it was employed as the dilution medium in all of the tests conducted in this study.

Culture medium was prepared by mixing five grams of air-dried horse manure with 25 grams of dried sandy muck in one liter of SRW.
The infusion was left undisturbed for two or three days and then strained through silk bolting cloth. The filtrate was then left an additional four to six days before being used. Young *D. magna* used in tests were cultured in four ounce wide mouth bottles. One mature female was placed in each of a series of bottles containing culture medium. After four or five days, two mg. of yeast was added every other day. The yeast was prepared by mixing two mg. of dried yeast per ml. of SRW. Daphnids were fed by adding one ml. of the resultant suspension to each bottle of culture medium. The young were removed every day to prevent depletion of the food supply and, when not being used in tests, were put into stock tanks.

Occasional outbreaks of "sewage fungus" (filamentous bacteria, genus *Sphaerotilus*) gave considerable difficulty. Control measures were employed but without much success. Attempts were made to use autoclaved media, but it proved unsatisfactory inasmuch as daphnids lived only a few days.

The SRW used in this study differs from that used by Freeman and Fowler (1953) in that it was made with single-distilled water (distilled in a Barnstead still) whereas double-distilled water (redistilled in a Pyrex glass still) was used by the above mentioned authors. Concerning this point, Freeman and Fowler say, "Daphnids will not live in single-distilled water, but are easily maintained in good condition in reference water made with double-distilled water." Most of the tests reported herein were conducted by the
use of single-distilled water. Some of the earlier tests employed
double-distilled water, but the majority did not. No statistically
significant differences in results were encountered when SRW made
from both types of water was used with the same chemicals. There­
fore, it is felt that some unaccountable factor must have influenced
Freeman and Fowler's findings.

As pointed out by various investigators (Abegg, 1948; Williams,
1948; and Freeman, 1953), the use of SRW as a dilution medium is well
justified in that it enhances the duplication of testing conditions.
It has been used successfully for experimental studies with inverte­
brates other than *Daphnia magna* (Bennett and Jenkins, 1950; Prince,
1953; and Weber, 1953), and with fish (Abegg, 1948; Williams, 1948;
and Freeman, 1953).

Banta (1921) and Anderson (1933; 1942) have pointed out that
the simplest criterion that might be used as an index of the general
metabolic condition of daphnids is their rate of reproduction.
Anderson (1948), employed filtered lake water media and found that
well fed *Daphnia magna* females produced approximately 20 young
every two or three days. On the other hand, Freeman and Fowler
(1953), using standard reference water media, point out that
their daphnids produced 30 young per brood each two and one-half
days, and Freeman (1953) obtained a reproduction rate of 20 young
per brood. The last mentioned investigators point out that no
difficulty was encountered with standard reference water. The
author, however, has not found SRW for culture medium water
completely satisfactory, inasmuch as the reproduction rate of
D. magna could not be consistently maintained as high as that
reported by Freeman and Fowler. During preliminary investigations
it was noted that the daphnid reproduction rate occasionally de-
clined to a noticeably low average, approximately 14 to 16 per
brood. In spite of care with which media were made, this repeatedly
occurred. It soon became apparent that some factor, other than
care in making media, was exerting its influence. It was thought
that perhaps some of the chemicals in the SRW stock solutions had
deteriorated or precipitated. Consequently all solutions were re-
made with the utmost care. Similarly, different soil types and a
carefully selected horse manure (free of fungus) were used since it
has been shown by numerous investigators (Anderson and Jenkins, 1942;
MacArthur and Baillie, 1929; Ingle, Wood, and Banta, 1937) that
striking reductions in numbers of young produced will occur if cul-
tural conditions become poor. These measures proved of no avail,
however, for the same low rate of reproduction was obtained. An
alternative explanation of these happenings was the possibility
that the daphnid stock had become genetically different, perhaps
through mutations. Banta (1933) has shown that this occurs oc-
casionally and further points out that genetic differences often
become apparent in reduced reproduction. This possibility, how-
ever, was immediately ruled out when it was found that daphnids,
after being placed in "natural water", immediately began to repro-
duce in great numbers. This water was introduced into the laboratory
by V. Lambou, a former junior research fellow in my laboratory, who had taken it from small dirt- and grass-bottom pools where it had collected as runoff. Therefore, it has been designated as "runoff water". Some of the daphnids, these being second generation progeny of females doing so poorly in SRW media, produced as many as 45 young per brood for three successive broods while being cultured in "runoff water". The average brood size for 15 daphnids was 26.0 and this number included the first broods which are usually small. It was later found that daphnids produced still larger broods in this type medium, some as many as 60 per brood for two successive broods. These data are presented in Tables II and III.

It became apparent from these observations that some material, beneficial to the well-being of the daphnids, was lacking or had gradually become depleted from the SRW media which had been employed. It is the considered opinion of the writer that the beneficial factor or factors, whether micro organisms, inorganic or organic chemicals, became "lost" to the daphnids. Apparently "it" was not being supplied in SRW media as in Anderson's filtered lake water medium and "runoff water". If it were supplied, the amount must have been insufficient for good reproduction. This assumption seems valid inasmuch as one cannot expect the observed response to "runoff water" if the daphnid stock had become genetically different. It follows that the missing ingredient could only be associated with the use of SRW as the culture medium water over a prolonged period of time. The original D. magna stock brought to the laboratory came from clones which had been...
cultured in lake water media and were obtained from Dr. B. G. Anderson in 1949 by Dr. I. Fowler. Subsequent culturing methods employed SRW media. Reproduction records (which the writer has in his possession) were good and in general agreed with what Anderson (1948) reported. Within the next one and one-half years the reproduction rate began to decline. Variations occurred at times in that individual daphnids produced above average broods, but on the whole the reproduction rate was below the considered norm. On the other hand, when cultured in "runoff water", daphnids reproduced young in numbers well above the average as set forth by Anderson.

Accordingly, with these observations in mind, several batches of media were prepared. One of these, designated as medium #1, was a mixture of SRW media and "runoff water", each comprising one-half of the total volume. A second medium, #2, consisted of the same materials and proportions save the "runoff water" was filtered through silk bolting cloth and fine Whatman filter paper. Medium #3 was the usual SRW medium which had been innoculated with a small amount of "runoff water", five ml. per 100 ml.

These media were used in an attempt to determine the beneficial effects of "runoff water" upon reproduction of daphnids in SRW media. Filtered "runoff" was used in medium #2 in order to determine whether the presence of microorganisms was a contributing factor. Admittedly, this type of filtering did not exclude the possibility of extremely minute forms, but it did keep back the larger organisms usually found in SRW media and in "runoff water". As previously mentioned, medium
had been autoclaved in an attempt to destroy "sewage fungus" and it was found that daphnids would not thrive in it. One cannot conclude from this, however, that daphnids did not do well due to a lack of microorganisms (which of course were killed) as a food source, inasmuch as they were fed yeast. Heat labile organics, some possibly being essential to daphnids, were also probably destroyed.

Medium #3, inoculated medium, was employed with the intention of gaining additional information concerning the possible influence of microorganisms. If these were an important factor, either by acting as a food source or by supplying essential metabolites, would their growth and multiplication bring about increased daphnid reproduction?

Each medium was set up in the usual manner, i.e., approximately 100 ml. in each of ten four-ounce bottles. A young female daphnid was placed in each bottle and fed the prescribed two mg. of yeast every other day. Each day the young were taken from the bottles and their numbers recorded. As shown in Table I, daphnids cultured in media #1 and #2 exhibited similar average reproduction rates, these being 21.8 and 22.7 young per brood respectively, or 8.7 and 9.1 young per day. However, medium #3 did not yield as high reproduction figures as did media #1 and #2. Actually it was about representative for SRW media as it had been used prior to these investigations. These data are not conclusive, but they do indicate that the beneficial material in "runoff water" is not essentially associated with microorganisms. It is seen that the unfiltered
medium did not induce a significant increase in reproduction as compared to filtered medium. Similarly, medium #3 (innoculated with unfiltered "runoff") did not bring about increased daphnid reproduction as would be expected if the microorganisms, which reproduced in great numbers, played an essential role. Therefore, it appears that the beneficial constituent present in "runoff water" is in the form of dissolved organics and/or inorganics. The author does not consider this phase conclusive. The experiments were not entirely controlled, i.e., known age groups were not used. The daphnids were selected at random and the ages were approximated. For this reason they are designated as "young females". In later experiments the daphnids were selected from stock of known history and ages. In addition the filter methods were inadequate.

Even though "runoff water" has certain characteristics which bring about good daphnid reproduction, it lacks other characteristics which are desirable. Culture media water must, of necessity, be available at any and all times. "Runoff water", of course, does not meet this requirement in that it is only available during and after periods of rainfall; the rain water runs through pastures and fields and collects in small pools. During periods of drought or scant rainfall these pools dry up. Secondly, "runoff" is not very desirable for a study of this type because of the lack of uniformity of the constituents, hence the possibility of unknown variables. SRW, on the other hand, is of known composition, thus easily duplicated from time to time. Admittedly, the soil and manure mixture
are of unknown character but even these can be repeatedly collected with some assurance of duplication.

Fortunately, it was found that daphnids, after having lived several generations in media of "runoff water", could be cultured in SRW media with good success. Therefore, it was the general practice of the writer to keep several large glass aquaria filled with "runoff" well stocked with daphnids. This supply furnished the stock from which were taken individuals to be cultured in SRW media, and these in turn were cultured to supply the test daphnids. It should be noted that the "runoff water" alone was not used in the preparation of stock media. In addition it contained about two grams (per litre of "runoff") of decayed leaves (mostly oak) and detritus which was collected from the bottom of shallow pools containing the water. This mixture was allowed to settle, usually requiring two to three days, before the introduction of stock daphnids. It should also be noted that occasionally the material taken from the pools contained green grasses and newly fallen leaves. It was found that these, as a result of decomposition and fermentation, brought about a marked lowering of the stock medium pH (5.8 to 6.4). Consequently, when this occurred the medium was filtered in order to exclude these materials. After standing several days the medium was ready for daphnids.

Following these procedures, it has been found that *D. magna* reproduces well, thus meeting the criterion of a good, general metabolic condition and assurance of "physiologically fit" test organisms.
Reproduction data, inasmuch as they have not been reported for daphnids cultured in these types of media, are presented herein. It has been the aim of the author, aside from the primary purpose of supplying test organisms, to obtain information concerning longevity relative to reproduction rates. Consequently, protocol has been made of several groups of daphnids cultured in SRW media and "runoff water" media. All of the daphnids, from which these data were obtained, were of the same age, i.e., less than 24 hours, at the time of introduction into culture media. Each daphnid was fed two mg. of yeast every second day. The young were taken out each day and their numbers recorded.

Table II gives reproduction and longevity records of six groups of daphnids, three being cultured in "runoff water" media and three in SRW media. It is seen from these data that daphnids cultured in "runoff" did considerably better than those in SRW media. However, the latter exhibited good yields, also. The difference is immediately seen by comparing the mean value of total numbers of young produced, 375.1 for "runoff" and 283.3 for SRW, with the average total days reproducing, 30.0 and 30.1 respectively. Using these figures, the average number of young produced per day was calculated, these being 11.6 and 9.3 respectively. Similarly, the average number of young produced every two and one half days was calculated as the mean brood size, these being 30.7 and 22.7 respectively. It will be noticed that daphnids cultured in SRW media exhibited greater longevity than did those in "runoff" media (48.6 days compared to 42.6), although those in the latter reproduced at a greater rate. This indicates
that the parent daphnids in "runoff" media were, metabolically, more active, i.e., they produced more young in a shorter life span. In association with this, it is noted in passing that the life span of D. magna is easily lengthened by controlled feeding of one mg. of yeast every third day or by semi-starvation for seven days before starting the feeding schedule. The author has recorded instances of daphnids living as long as 68 days at 23 ± 1°C. These findings are in accord with those of other investigations inasmuch as Ingle, Banta and Wood (1937) show that well fed daphnids grow more rapidly, reproduce in greater numbers, and have a higher heart beat frequency than those reared on limited food. Similarly, MacArthur and Brailly (1929 a) in their experiments on effects of temperature and longevity, found that D. magna lived 29.2 days at 28°C. and 44.7 days at 18°C. Anderson and Jenkins (1942) found still greater longevity and assume from this that their cultural conditions were more favorable. Animals used in the investigations reported herein lived longer than would be expected from their results. Therefore, it appears that the cultural conditions were more favorable than those used by these workers.

Table III is presented in order to show the sequence of increasing and decreasing brood sizes as obtained by daphnids cultured in the two types of media. The mean values shown for the last four broods (13 through 16) are somewhat misleading. A few females produced at a slow rate for the individual few broods but their last broods were unusually large. Furthermore, only a small percentage
of females (half or less than half of the original number of parents) produced as many as 13 broods. Therefore the mean size of the last few broods has been magnified to some extent.

The culturing technique employed to obtain the above data was more exacting than that used in supplying test animals. Most of the test daphnids were produced by females selected at random from stock media. Age and history of the parent was not requisite, therefore they were approximated.

It is believed that additional research of this nature might eventually lead to the development of a completely satisfactory culture method for daphnids, since, according to various investigators one is still to be found. The primary purpose of this investigation, however, did not concern itself with a detailed study of the nutritional aspects of culturing methods.

Gas Mixing and Dispensing Apparatus

A schematic diagram of the apparatus designed for mixing and dispensing gases is shown in Figure 1. Bottles B-1, B-2, B-3, and B-4 had a capacity of approximately 18 liters each. Gases were mixed in bottles B-3 and B-4 while B-1 and B-2 contained nitrogen saturated water. This was used to force the gas mixture from B-2 and B-4 into the test system. The apparatus was designed to operate in series, a gas mixture being dispensed from either the right half (B-1, B-3) or the left half (B-2, B-4) of the system. After depletion of gases in one side, the other side was used. However,
Table I

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of brood females</th>
<th>Av. total no. young produced</th>
<th>Av. total no. days reproducing</th>
<th>Av. No. young produced / day</th>
<th>Av. No. young / brood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium #1</td>
<td>10</td>
<td>870</td>
<td>31</td>
<td>9.7</td>
<td>21.6</td>
</tr>
<tr>
<td>Medium #2</td>
<td>10</td>
<td>834</td>
<td>88</td>
<td>9.1</td>
<td>98.7</td>
</tr>
<tr>
<td>Medium #3</td>
<td>10</td>
<td>197</td>
<td>33</td>
<td>6.0</td>
<td>14.9</td>
</tr>
</tbody>
</table>

Table I. Reproduction records of *Daphnia magna* cultured in three types of media at 23 ± 1°C.
<table>
<thead>
<tr>
<th>Media</th>
<th># of brood females</th>
<th>Av. total young produced</th>
<th>Av. total days reproducing</th>
<th>Av. total days lived</th>
<th>Av. # young produced/day</th>
<th>Av. # young/brood/2 1/2 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRW 15</td>
<td>316.6 (109 - 424)</td>
<td>32.7 (15 - 47)</td>
<td>52.7 (30 - 68)</td>
<td>9.7 (7.9 - 13.7)</td>
<td>24.1 (18.1 - 31.0)</td>
<td></td>
</tr>
<tr>
<td>SRW 20</td>
<td>298.5 (158 - 523)</td>
<td>32.5 (18 - 45)</td>
<td>47.3 (39 - 66)</td>
<td>9.1 (4.4 - 13.0)</td>
<td>22.6 (11.1 - 32.6)</td>
<td></td>
</tr>
<tr>
<td>SRW 20</td>
<td>235 (114 - 521)</td>
<td>25 (15 - 44)</td>
<td>45.8 (26 - 70)</td>
<td>9.2 (6.0 - 13.6)</td>
<td>21.5 (14.9 - 30.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>av. = 283.3</td>
<td>av. = 30.1</td>
<td>av. = 48.6</td>
<td>av. = 9.3</td>
<td>av. = 22.7</td>
<td></td>
</tr>
</tbody>
</table>

"Runoff" 20

| "Runoff" 20 | 312.2 (176 - 449) | 27.8 (18 - 39) | 38.7 (26 - 48) | 11.2 (9.1 - 12.7) | 27.9 (22.7 - 31.8) |

"Runoff" 17*

| "Runoff" 17* | 443.1 (149 - 610) | 31.3 (28 - 43) | 46.0 (33 - 55) | 13.7 (5.7 - 19.8) | 34.3 (14.3 - 49.5) |

"Runoff" 19**

| "Runoff" 19** | 370.1 (180 - 545) | 30.4 (26 - 35) | 43.1 (37 - 47) | 9.8 (7.0 - 15.5) | 29.9 (17.3 - 38.9) |
|               | av. = 375.1       | av. = 30.0     | av. = 42.6     | av. = 11.6       | av. = 30.7         |

* Three brood females died within three days.
** One brood female died second day.

Table II. Reproduction records (averages and extremes) of *Daphnia magna* cultured in SRW media and "Runoff" media at 23 ± 1°C.
Table III

<table>
<thead>
<tr>
<th>Brood #</th>
<th>Average no. young produced</th>
<th>No. of brood females</th>
<th>% of original brood females</th>
<th>Brood #</th>
<th>Average no. young produced</th>
<th>No. of brood females</th>
<th>% of original brood females</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.0</td>
<td>30</td>
<td>100</td>
<td>1</td>
<td>8.6</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>15.6</td>
<td>30</td>
<td>100</td>
<td>2</td>
<td>14.6</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>20.5</td>
<td>30</td>
<td>100</td>
<td>3</td>
<td>13.5</td>
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<td>100</td>
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<tr>
<td>4</td>
<td>30.6</td>
<td>30</td>
<td>100</td>
<td>4</td>
<td>18.1</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>38.8</td>
<td>30</td>
<td>100</td>
<td>5</td>
<td>29.0</td>
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<td>6</td>
<td>36.7</td>
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<td>6</td>
<td>34.3</td>
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<tr>
<td>7</td>
<td>33.4</td>
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<td>100</td>
<td>7</td>
<td>46.3</td>
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<tr>
<td>8</td>
<td>31.7</td>
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<td>93.3</td>
<td>8</td>
<td>42.8</td>
<td>29</td>
<td>96.6</td>
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<tr>
<td>9</td>
<td>29.9</td>
<td>27</td>
<td>90.0</td>
<td>9</td>
<td>41.5</td>
<td>28</td>
<td>93.3</td>
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<tr>
<td>10</td>
<td>21.0</td>
<td>26</td>
<td>83.3</td>
<td>10</td>
<td>36.1</td>
<td>26</td>
<td>86.6</td>
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<tr>
<td>11</td>
<td>23.1</td>
<td>23</td>
<td>76.6</td>
<td>11</td>
<td>36.8</td>
<td>24</td>
<td>80.0</td>
</tr>
<tr>
<td>12</td>
<td>26.5</td>
<td>19</td>
<td>63.3</td>
<td>12</td>
<td>37.7</td>
<td>19</td>
<td>63.6</td>
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<tr>
<td>13</td>
<td>22.3</td>
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<td>33.3</td>
<td>14</td>
<td>32.7</td>
<td>9</td>
<td>30.0</td>
</tr>
<tr>
<td>15</td>
<td>18.6</td>
<td>7</td>
<td>23.3</td>
<td>15</td>
<td>16.6</td>
<td>4</td>
<td>18.3</td>
</tr>
<tr>
<td>16</td>
<td>22.6</td>
<td>6</td>
<td>20.0</td>
<td>16</td>
<td>12.0</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td>17</td>
<td>16.5</td>
<td>2</td>
<td>6.6</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table III. Brood sizes produced by *Daphnia magna* cultured in SRW media and "Runoff" media at 23 ± 1°C.
gas mixtures could be dispensed from both halves at the same time.

In order to obtain known reproducible gas mixtures in bottles B-3 and B-4, all air was evacuated by means of a vacuum pump. This was accomplished by opening and closing certain stopcocks, depending upon whether B-3 or B-4 was to be evacuated. Each bottle was connected by pressure tubing and a series of stopcocks to the manometer (Fig. 1, M) and the vacuum pump. By adjusting stopcocks S-5 and S-6 (Fig. 1, S-5, S-6) air was removed from either B-3 or B-4. The air passed through a calcium chloride drying tube in order to keep moisture from the vacuum pump. Stopcock S-6 could be opened either to atmospheric air or to the evacuating system. This shunt to the outside of the system was sometimes used when evacuation was undesirable while the pump was in operation. Stopcock S-5 could be adjusted to allow evacuation of either gas mixing bottle, but not both at once. If B-3 was to be evacuated stopcock S-1 was opened, S-3 closed, and S-4 opened to the manometer hose. Similarly, pinchelamps P-3 and P-4 were tightly closed to keep water from leaving B-1 and B-2 and entering B-3 during evacuation. On the other hand, if B-4 was to be evacuated, the corresponding stopcocks (S-1', S-3', and S-4') and pinchelamps (P-3' and P-4') on the other half of the apparatus were adjusted.

Since a complete vacuum, which would assure removal of all air, was difficult to obtain, a water depth of approximately two inches was kept in each of bottles B-3 and B-4. In this manner
it was possible to remove air without having to obtain complete vacuum. This was accomplished by evacuating until a manometer reading indicated that the only pressure being exerted from inside B-3 or B-4 was that of the vapor pressure of the two inches of water. The vapor pressure of water at 23°C, is approximately 21 mm. of mercury. Similarly, at 22 and 24°C, it is 19.8 and 22.2 mm. Hg. respectively. Therefore, when the manometer showed a change of pressure approximately equivalent to the barometric pressure minus the vapor pressure of water, it was known that all air had been removed from the gas mixing chamber. For example, if the barometric pressure at time of mixing was 760 mm. Hg. and the temperature was 23°C., the calculation was:

\[760 - 21 = 739\]

A pressure equivalent of 739 mm. Hg. had been removed while 21 mm. Hg. pressure (due to the water) yet remained.

After evacuation of air was obtained, stopcocks S-1 (or S-1' if B-4 was evacuated), S-5 and S-6 were closed so that no air could enter the mixing bottles. Bottles B-3 and B-4, having been voided of atmospheric air, were then ready for introduction of air and pure nitrogen in known ratio. During preliminary experiments with the apparatus, gas mixtures were not made until the evacuated mixing bottle had been flushed several times with nitrogen. It was found in subsequent tests, however, that flushing with nitrogen more than once was apparently unnecessary; dissolved oxygen determinations in
the test system (Fig. 2) indicated no significant differences in
the two procedures.

Oxygen tensions were obtained by using mixtures of air and
nitrogen. The original intention had been to use oxygen with
nitrogen, but it was found in preliminary investigations that this
mixture was not very satisfactory for obtaining the lower limit
of dissolved oxygen (approximately 1.6 p.p.m.) because extremely
small amounts of oxygen had to be used. These were measured by
their partial pressures, and a slight variation in observed pressures
tended to magnify an increase or decrease of the oxygen-nitrogen
ratio. On the other hand, by using air-nitrogen mixtures, it was
possible to use a greater amount of air in proportion to nitrogen,
inasmuch as air is composed of oxygen and nitrogen in the relative
volume of twenty-one to seventy-nine per cent. Therefore, air-
nitrogen mixtures, particularly those used to produce the lower
limit of dissolved oxygen, were more easily measured and more
accurately reproduced. It is noted that preliminary use of
oxygen-nitrogen mixtures proved satisfactory in producing medium
oxygen concentrations (approximately 4.5 p.p.m.), but this type of
mixture was not used in subsequent testing since air-nitrogen was
used to obtain low oxygen levels. It is believed that air-nitrogen
mixtures, from the standpoint of simulating natural conditions,
probably served the purpose better since they contained some
carbon dioxide, thus giving a more normal complement.

Known reproducible mixtures of air and nitrogen were obtained
by measuring their partial pressures on manometer M (Fig. 1). The principle of this method is based on Dalton's law of partial pressures which states that the total pressure of a mixture of gases is the sum of the partial pressures of the several gases. Table IV shows the relative partial pressures and volumes of air-nitrogen mixtures used. The relative volume of air to nitrogen is not the only factor, however, which brought about a desired oxygen concentration in the test solutions. Other factors to be considered were the rate of flow of the mixtures through the test system and the hydrostatic pressure. These are discussed later.

Mixtures were obtained in bottles B-3 or B-4 by allowing the desired amount of each gas to enter the evacuated bottle through stopcocks S-2 or S-2' respectively. Each stopcock had a double inlet, one for air and the other for nitrogen, but only one gas could enter at a time. In this procedure air was always admitted first. As air entered the mixing bottle, the mercury column dropped, thus indicating the pressure exerted by the air. In this manner, any desired volume of air was obtained by measuring its pressure on the millimeter scale of the manometer. For example, it is seen in Table IV that a volume of air which exerted a partial pressure of 133 mm. Hg. was used (in mixture with nitrogen) to bring about the lower limit of dissolved oxygen.

The nitrogen partial pressures were obtained by allowing pure nitrogen to enter the second inlet of stopcock S-2 or S-2'. However, the procedure for obtaining nitrogen pressures was slightly
different. When the combined pressures of air and nitrogen in the
gas mixing bottle attained the equivalence of atmospheric pressure
on the outside of the bottle, it became necessary to force nitrogen
into the mixing chamber under pressure. This was easily done since
the nitrogen was highly compressed in a steel cylinder. Thus, it
was possible to adjust the final pressure in the mixing bottle to
a desired level. The final pressure was always adjusted so that
the total exerted was 860 mm. Hg. Since this was greater than
atmospheric, there was a pressure equivalent to 100 mm. Hg. ex­
erted from the inside of the gas mixing bottle. In order to keep
this pressure from forcing the stoppers out of the mixing bottle,
thus producing leakage, it was necessary to devise clamps where­
by the stoppers were tightly fitted. These are shown in Figure
1 as L and L'. It is seen in Table IV that a nitrogen partial
pressure of 706 mm. Hg. mixed with an air pressure of 133 mm. Hg.
produced an approximate oxygen tension of 1.5 p.p.m., providing
other factors of hydrostatic pressure and bubble count were met.
It follows from the law of partial pressure that:
\[
\text{vapor pressure of water} + \text{partial pressure of air} + \text{partial pressure of nitrogen} = \text{total pressure of mixture}
\]
Substituting the above values we have:
\[
21 + 133 + 706 = 860
\]

As previously mentioned, barometric pressure must be considered and
accounted for in the manometer readings during introduction of gases.
Once the desired gas mixture was obtained, it was ready to be forced into the test system. Gas mixtures were made up well in advance (at least 1 to 8 hours) of being used so that mixing of the air and nitrogen would occur. Mixing was facilitated by gently shaking the mixing bottle, thus producing some agitation of the two inches of water.

Gases were forced from the mixing bottle by opening pinch-clamps P-3 or P-3', thus allowing a gravitational flow of the nitrogen saturated water from bottles B-1 or B-2 into B-3 or B-4 respectively. By opening stopcock S-3 or S-3', the gas mixture was forced into the test system. It is noted, however, that the initial flow of gases into the test system could not be accomplished by simply opening stopcock S-3 or S-3'. As previously pointed out, there was, in the mixing bottle, a pressure of 100 mm. Hg. over and above atmospheric pressure. This pressure was great enough to keep water from flowing from bottles B-1 or B-2. Therefore, the gases exerting this excess pressure were used before opening pinch-clamps P-3 or P-3'. Sometimes the excess pressure was not passed through the test system. In such cases it was bled into the atmosphere (by means of the three way stopcock S-3, S-3') until the pressure was low enough to permit the flow of water.

During the course of a test period the gas mixtures were depleted and had to be remixed. Before a second gas mixture could be made it was necessary to remove the water which had been used to displace the gases from the mixing bottle. This was done by
use of a water pump (Fig. 1, WP) which forced the water from B-3 or B-4. These were emptied by opening pincholamps P-4, P-3 or P-4', P-2' respectively. Prior to emptying the bottles it was necessary to open stopcocks S-3 or S-3' in order to prevent a vacuum as the water left. The water outlets, designated as T-3 and T-3', were adjusted to a length that would permit withdrawal of all but two inches of water. Water entered bottles B-1, B-2 by way of inlets T-2, T-2'. These inlets extended to the bottom of the water bottles so that as little agitation as possible occurred as the water entered, thus keeping aeration to a minimum. Aeration, however, was not too troublesome, inasmuch as oxygen picked up by the water as it passed through the water pump was displaced with nitrogen. Compressed nitrogen was forced through a hose which branched at Y-1 and entered bottles B-1 or B-2 by way of dispersing stones D or D'. These diffusers assured rapid uptake of nitrogen and subsequent displacement of oxygen through outlets P-1 and P-1'. Oxygen content of the water could be determined by taking samples through serum bottle stoppers R and R'. During test periods, while the contents of B-1 or B-2 were being used to displace the gas mixtures in B-3 or B-4, nitrogen was bubbled through the water at a slow, steady rate.

Testing Apparatus

The 16 wide mouth flasks (250 ml.) shown in Figure 2 comprise the test system which was connected by means of rubber tubing with the gas mixing apparatus. The schematic diagram shows some detail, but for most flasks it merely indicates the directional flow of
<table>
<thead>
<tr>
<th></th>
<th>1.5</th>
<th>3.0</th>
<th>4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approximate Dissolved Oxygen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(p.p.m.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Vapor pressure of water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mm. Hg. at 23°C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial pressure of air</td>
<td>133</td>
<td>236</td>
<td>347</td>
</tr>
<tr>
<td>mm. Hg.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Partial pressure of nitrogen</td>
<td>706</td>
<td>603</td>
<td>494</td>
</tr>
<tr>
<td>mm. Hg.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% air of total pressure</td>
<td>15.46</td>
<td>27.44</td>
<td>40.34</td>
</tr>
<tr>
<td>% nitrogen of total pressure</td>
<td>82.09</td>
<td>70.12</td>
<td>57.55</td>
</tr>
</tbody>
</table>

Table IV. Relative partial pressures and volumes of air-nitrogen mixtures used to obtain oxygen tensions.
Figure 1

Schematic Diagram of Gas Mixing Apparatus

Legend

B-1, B-2 - Containers for nitrogen saturated water (18 l.)
B-3, B-4 - Containers for gas mixtures (18 l.)
D, D' - Gas dispersing stones
L, L' - Bottle stopper holding clamps
M - Mercury manometer
P, P' - Screw type pinch clamps
R, R' - Serum bottle rubber stoppers
S, S' - Stopcocks
T, T' - Glass tubing
#P - Water pump
Y - Glass "Y" tubes

- Dotted hoses represent pressure tubing
Figure 1. Schematic diagram of gas mixing apparatus
gases through the system. Figure 3 shows a detailed diagram of the first four flasks.

Each flask shown in Figures 2 and 3 had a double gas inlet (8 mm. O.D. tubing) and a single outlet which bifurcates. One inlet (#1 in Figs. 2 and 3) was short enough to allow any gas being forced through it to enter above the surface of liquids contained in the flasks. The other inlet (#2 in Figs. 2 and 3) connected with a gas dispersion tube with a fritted glass cylinder of coarse porosity. Gases forced through this inlet were dispersed into fine bubbles throughout the liquid contents of the flasks. Gas could be passed through either inlet by use of pinchelamps on the rubber tubing which connected one flask with the next. In addition, each flask had a glass rotating rod (Fig. 3, RR) whose proximal end was bent at a right angle in order to facilitate the suspension of a glass vial (Fig. 3, V). The rotating rod was sealed at both ends, the distal end having been flared and flattened (as a nail head) so that it fitted tightly into a length of rubber tubing which in turn fitted over a length of glass tubing. The glass tubing served as a casing for the smaller rotating rod and allowed the latter to be rotated freely. The distal end of the tubing which held the rotating rod was bent double and clamped so as to prevent a source of leakage. The rotating rod mechanism is shown in detail in flasks F-2, F-3, Figure 3.

It will be noticed by inspection of Figure 2 that the testing apparatus was divided into two units, each unit comprised one half
of the system. Gases entered the left unit, passed through flasks F-a, F-b, F-1, F-2, F-3, F-4, F-5 and F-c, and left by way of cylinder C-1. Gases entered flask F-d of the right unit and exited through cylinder C-1'. During preliminary investigations, prior to dividing the system into two units, gases were forced through all flasks at the same time. Consequently, an oxygen gradient was set-up in the system. In the case of pure nitrogen it was found that the first flasks (F-a, F-b) which received the gas were depleted of their dissolved oxygen much quicker than were the last flasks (F-f, F-2). At the end of four or five minutes of bubbling, flask F-a contained somewhat less oxygen than did flask F-e. Similarly, upon introduction of air-nitrogen mixtures, the contents of flasks F-a and F-b attained the desired oxygen from the mixture in about two minutes, whereas flasks F-f and F-e did not take up as much oxygen in the same period of time. Apparently these effects were produced because, in each instance, the first flasks (F-a, F-b) were receiving "pure" gases as they entered the system. On the other hand, the last flasks (F-e, F-f) were receiving, for a short time at least, gases which were "scrubbed" from the solution (by vigorous bubbling) in the preceding flasks. They received, so to speak, "unpure" or "diluted" gases. This problem of gradient build-up was partially overcome by dividing the system into the two separate units. Another measure employed to offset gradient build-up was that of introducing the gases through two flasks at a time, one in each of the two units. This procedure is discussed below in detail.
Prior to testing, each of flasks F-1 through F-9 contained 100 ml. of test solution and a five ml. vial of dilution water (SRW) which was suspended by means of a thread loop from the glass rotating red. The contents of the vial served as a holding medium for the young *D. magna* used in testing. The procedure employed in making dilution sequences and the handling of test animals is discussed later. Flask F-10 served as a control; it contained 100 ml. of dilution water (no chemical) and the vial plus daphnids. Each of flasks F-a, F-b, F-c, F-d, F-e and F-f contained 100 ml. of dilution water plus that in the vial. The last mentioned containers served as check points for dissolved oxygen. As shown in Figure 2, these flasks had openings through which were tightly fitted serum bottle stoppers. The stoppers facilitated oxygen determinations. It is noted that the author originally planned to use these stoppers in all of the test containers, but it was found that the rubber was toxic to *D. magna*. Apparently the toxicity occurred because of the relatively great mass of rubber in so little solution, inasmuch as rubber tubing in large volumes of liquid did not display toxic effects. An alternative explanation would be that the rubber from which the stoppers were made contained materials which were more toxic than are materials in rubber tubing. However, the use of stoppers in all of the flasks would not have proved feasible; it was later found that the compounds tested interfered with the chemical method used in determination of dissolved oxygen. In connection with this, it is further noted that flasks F-a, F-c and F-e were originally included in the test system
for the purpose of holding solutions of the chemical being tested in the same respective concentrations as contained in flasks F-1, F-5 and F-9. These flasks (F-a, F-c and F-e) were to be used as dissolved oxygen check points in conjunction with flasks F-b, F-d and F-f. It was hoped that this method would serve to determine any differences in oxygen concentration which might be caused by the chemical being tested. For example, it is known that a liquid which contains a solute, and especially an ionizable solute, will dissolve less gas than the pure liquid. Therefore, a cross-check for this type of effect would have been desirable; but as pointed out above, the materials tested interfered with the determination of oxygen. The aspects of this problem are discussed later.

Each of the flasks, subsequent to being readied for testing, was gently but firmly fitted to its respective stopper. Once this was done, the system was intact and ready for introduction of gases. In order to determine whether gases were leaking during their passage through the system, a small amount of water was blown from a wash-bottle into the trough formed where each stopper fitted into the mouth of the flask. Any escape of gas was easily found by the ensuing bubbling and was stopped by pressing the stopper more firmly into the flask. Since each flask, tube and solution contained atmospheric air and oxygen, it was necessary to deplete the system of these gases in order that a known desired oxygen level could be obtained. This was accomplished by forcing nitrogen, which is physiologically inert, through the system. This gas was bubbled
at a known, constant, pressure through the fritted glass dispersion tubes of two flasks, one in each unit of the system. For example, flasks F-a and F-d were depleted at the same time by regulating pinchclamps P-3 and P-3' (Fig. 2). Similarly, nitrogen was forced through F-b and F-6 after the oxygen was depleted to a desired level in the preceding flasks. It follows then, that the last pair of flasks which were depleted were F-e and F-f. Each flask received nitrogen for a specific period of time, depending upon the degree of oxygen depletion desired. This in turn depended upon the final oxygen level to be used during the test. Bubbling periods, for the oxygen levels used, are presented below. Each flask received the gases at a constant pressure which was regulated and controlled in three ways: (a) the force with which nitrogen entered was regulated by means of pinchclamps P-3, P-3', and at the nitrogen inlet; (b) pressures were observed at manometer M by opening either pinchclamp P-2 or P-2', depending upon whether pressure was to be measured in the left unit or right unit of the system; and (c) internal pressures were controlled by hydrostatic pressure. The latter was measured by the height of a water column in cylinders C-1 and C-1'. After the dissolved oxygen was lowered to a desired level (as determined by micro-dissolved oxygen technique described later), the system was ready to receive the gas mixture stored in the mixing bottles.

The gas mixture was forced into the flasks in the sequence used for the introduction of nitrogen, except that it was bubbled
through the contents for different periods of time. By adjusting pinchclamps P-3, P-3' and that on the gas inlet, the gas mixture, like nitrogen, was bubbled at constant pressure. Each flask received the air-nitrogen mixture for a specific period of time. The bubbling period are presented below. After the mixture had bubbled through the contents of each flask, the pinchclamp on the inlet hose was moved to the hose of the next flask to receive the gases. Thus, after removal of the pinchclamp, gases entered the flasks by way of the short inlet tubes which opened above the surface of the liquids. By the time the last two flasks, F-c and F-f, had the mixture passed through their contents, all of the preceding flasks were receiving the mixture through the short inlet tubes. Then, by adjusting pinchclamps P-3, P-3' and stopcocks S-1 and S-2, the rate of gas flow through the entire system was slowed to a rate of 9 - 12 bubbles per minute. This rate was determined by bubble count at the two exits, C-1 and C-1'. Subsequent to passing the air-nitrogen mixture through the liquids, but prior to the beginning of test time, a 15 to 15 minute waiting period elapsed in order that equilibrium was reached between the dissolved gases and those in the atmosphere inside the flasks. Apparently, oxygen was taken up in solution until it reached a maximum for that amount available in the gas mixture under the conditions of temperature, pressure, and volume of liquid used.

During standardization experiments the oxygen levels were checked before exposing the daphnids to test solutions. At the
beginning of test time the animals (contained in the vials) were exposed by turning the rotating rod with a quick twist. Thus, the vial dropped from the arm of the rod into the test solution. In case the vial did not drop the first time, it was easily forced off by turning the rod back and forth with quick successive movements. Vials in flasks F-a through F-f, which contained dilution water only, were thrown off in the same manner.

It is noted that dilution water contained in the vials had a different oxygen tension than did the liquid in the flasks, inasmuch as the vial contents did not have nitrogen passed through them. Apparently this did not appreciably effect the previously adjusted oxygen content of the flasks. Dissolved oxygen determinations (in preliminary experiments), a few minutes after the vial contents were spilled into the flask, showed no significant alteration of oxygen tensions. From this it appears that the excess oxygen from the vial of water was given up to the atmosphere within the flasks and swept from the system through exits C-1 and C-1'. It is assumed that the excess oxygen in solution exerted a partial pressure above that of the oxygen in the atmosphere within the container and had a tendency to escape from the dissolved to the atmospheric state at the liquid-air interface.

In summary, the procedure outlined below was followed for obtaining and maintaining the various oxygen tensions employed in this study.
I. For approximate 1.5 p.p.m. dissolved oxygen:

1. The water level was adjusted in cylinders C-1, C-1' to 7 cc. above the gas outlet.

2. Nitrogen was bubbled through each gas dispersing tube for six minutes. This decreased the dissolved oxygen from atmospheric to 0.6 - 0.8 p.p.m.

3. Nitrogen was bubbled through each flask at a manometer reading of 18 - 20 mm. Hg.

4. Air-nitrogen mixture (15.5 to 82.1%) was bubbled through each gas dispersing tube for 60 seconds at 18 - 20 mm. Hg. This increased the dissolved oxygen level to about 1.5 p.p.m.

5. The air-nitrogen mixture was allowed to pass through inlets above the surface of flask contents for 12 - 16 minutes at an adjusted flow rate of 9 - 12 bubbles per minute.

II. For approximate 3.0 p.p.m. dissolved oxygen:

1. The water level was adjusted in cylinders C-1, C-1' to 10 cc. above the gas outlet.

2. Nitrogen was bubbled through each gas dispersing tube for four minutes. This decreased the dissolved oxygen from atmospheric to about 1.9 - 2.1 p.p.m.

3. Nitrogen was bubbled through each flask at the same manometer reading as in I-3 above.

4. Air-nitrogen mixture (27.4 to 70.1%) was bubbled through each gas dispersing tube for two minutes at 18 - 20 mm. Hg. This increased the dissolved oxygen level to about 3.0 p.p.m.

5. The air-nitrogen mixture was allowed to pass through the test system as in I-5 above.

III. For approximate 4.5 p.p.m. dissolved oxygen:

1. The water level was adjusted in cylinder C-1, C-1' to 14 cc. above the gas outlet.
2. Nitrogen was bubbled through each gas dispersing tube for same length of time as in II-2 above.

3. Nitrogen was bubbled through each flask at same manometer reading as in I-3, II-3 above.

4. Air-nitrogen mixture (40.3 to 57.6 %) was bubbled through each gas dispersing tube for three to three and one-half minutes. Thus dissolved oxygen level was decreased to about 4.5 p.p.m.

5. The air-nitrogen mixture was allowed to pass through the test system as in I-5, II-5 above.

Dissolved Oxygen Determination

A number of modifications of the original technique of Winkler for the determination of dissolved oxygen have been used by various workers. The accuracy of both the original method and any of its modifications is necessarily limited by the accuracy of the sampling techniques employed. This is especially important when dealing with water of low oxygen tension, inasmuch as accurate analysis demands that the water sample be kept from contact with atmospheric air. Krogh (1935) described a syringe pipette which could be used in the estimation of the oxygen concentration of water; by the use of such a pipette, contamination of the water sample with atmospheric air was completely avoided. Van Dam (1933, '35) modified Krogh's syringe pipette, and later Fox and Wingfield (1938) modified Van Dam's pipette.
Figure 2
Schematic Diagram of Testing Apparatus

Legend

C-1, C-1' - Graduated cylinders (100 ml.)
D - Fritted glass dispersing tubes
F-1 - F-10 - Test containers (250 ml. wide mouth)
F-a - F-f - Oxygen checkpoint containers (250 ml. wide mouth)
M - Mercury manometer
P - Screw-type pinchclamps
RR - Glass rotating rod
RS - Serum bottle rubber stoppers
S-1, S-2 - Stopcocks
V - Glass vial (5 ml.)
Figure 2. Schematic diagram of testing apparatus
Figure 3

Schematic Diagram of Test Containers

Legend

D - Fritted glass dispersing tubes

F-1 - F-4 - Test containers (250 ml. wide mouth)

P-1 - Screw-type pinchclamps

RR - Glass rotating rod

V - Glass vial (5 ml.)

1 - Gas inlet (above surface of solution)

2 - Gas inlet (beneath surface of solution)
Figure 3. Schematic diagram of test containers
Two different types of syringe pipettes have been employed in this study, these being the Fox-Wingfield and the Krogh-Keyes. The Krogh-Keyes syringe pipette proved more satisfactory for the technique described herein, since it was made to use with a hypodermic needle whereas the Fox-Wingfield syringe was not. Therefore, in order to adapt the latter instrument to the needs of this study some modification in design was made. This consisted of fitting a short length of thick gum rubber tubing (approximately 2.5 mm. I.D.) to the nozzle of the syringe. To the other end of the tubing was fitted an adapter by which a hypodermic needle could be attached.

The Krogh-Keyes syringe pipette is shown in Figure 4. The Fox-Wingfield syringe is not shown since it is basically the same in design. The glass syringe (total capacity five ml.) is fixed and stabilized in a stainless steel frame. The head-screw (extreme right), which can be rotated, moves in a threaded sleeve which is attached to the frame. The end of the glass plunger of the syringe is kept in contact with the end of the head-screw shaft by means of rubber bands attached to the end of the plunger and the metal frame. Vernier adjustment of the head-screw may be set to any desired fraction for reproduction. This was accomplished by means of a locknut which could be secured in any position on the head-screw. For my work the locknuts were adjusted and then secured in place with

1Purchased from Messrs. Phillip Harris and Co., 144 Edmond Street, Birmingham, England.

several drops of Censo-Sealstix cement. This fixed position of the locknut assured a constant, previously calibrated (see below), pipette volume. It is noted that the needle was also sealed to the syringe by means of cement.

Each of the syringes pipettes was calibrated according to the method of Fox and Wingfield (1938). The pipette volume (barrel plus dead space in nozzle and needle) was determined chemically as follows. The head-screw was screwed down until the locknut was in contact with the sleeve and then the syringe was filled with a standardized solution (N/40 - M/240) of potassium iodate. This was delivered from the syringe into a titration vessel, after which the syringe was rinsed out twice with distilled water. The potassium iodate was standardized against sodium thiosulfate (N/40) which had been previously standardized against potassium bi-iodate (N/10). Then one ml. of one per cent potassium iodide solution and three drops of o-phosphoric acid were added to the titration vessel. The iodine liberated, which was quantitatively equivalent to the amount of potassium iodate originally present, was titrated against the standardized sodium thiosulfate (N/40), using starch as indicator. This titration, using macro quantities, was done with a 10 ml burette. The pipette volume was calculated from the results of the titration. In like manner, the volume of the dead space was determined, the dead space (needle plus nozzle) alone having been filled with potassium iodate solution. This solution was then drawn up into the barrel of the syringe together with some distilled water,
washed out, and the amount of iodate present was determined by titration with a micro burette. The fixed pipette volume was found by subtracting the volume of the dead space from the total determined volume for the syringe. Similarly, the volume corresponding to one turn of the head-screw was found by titration in the manner described above.

Calculations involved in the calibration of the pipettes are as follows:

I. Determination of the volume of the syringes (dead space and barrel).

Syringe #1 - (Fox-Wingfield) Filled with N/40 solution of potassium iodate.
Amount sodium thiosulfate (N/40) used in titration = 3.07 ml. (av.).
Therefore, vol. of syringe = ml. Na₂S₂O₃ used above x \frac{ml. KI03}{ml. Na₂S₂O₃}

vol. of syringe #1 = 3.07 x \frac{2.00}{3.94} = 1.5564 ml.

Syringe #2 - (Krogh-Keyes) Filled with N/40 solution of potassium iodate.
Amount of sodium thiosulfate (N/40) used in titration = 1.64 ml. (av.).
Therefore, volume of syringe #2 = 1.64 x \frac{10.00}{9.85} = 1.664 ml.

Syringe #3 - (Krogh-Keyes) Filled with N/40 solution of potassium iodate.
Amount of sodium thiosulfate used in titration = 1.65 ml. (av.).
Therefore, volume of syringe #3 = 1.65 x \frac{10.00}{9.87} = 1.68 ml.
II. Determination of the volume of the dead space (nozzle and needle).

Syringe #1 - Dead space filled with N/40 potassium iodate.
Amount of sodium thiosulfate (N/40) used in titration = 0.1368 ml. (av.).
Therefore, volume of dead space = 0.1368 x 10.00 = 0.1368 ml.
Therefore, pipette volume used = 1.6664 - 0.1368 = 1.5296 ml.

Syringe #2 - Dead space filled with N/40 potassium iodate.
Amount of sodium thiosulfate (N/60) used in titration = 0.1766 ml. (av.).
Therefore, volume of dead space = 0.1766 x 2.00 = 0.0883 ml.
Therefore, pipette volume used = 1.6664 - 0.0865 = 1.5799 ml.

Syringe #3 - Dead space filled with N/40 potassium iodate.
Amount sodium thiosulfate (N/60) used = 0.1740 ml. (av.).
Therefore, volume of dead space = 0.1740 x 2.00 = 0.0877 ml.
Therefore, pipette volume used = 1.6664 - 0.0857 = 1.5807 ml.

III. Determination of the volume of liquid introduced with one turn of the head-screw was done in a similar manner and found to be:

Syringe #1 = 0.035 ml. (av.)
Syringe #2 = 0.063 ml. (av.)
Syringe #3 = 0.062 ml. (av.)

It is noted in passing that all standard solutions were re-standardized periodically in order to assure accuracy of determinations.
The method employed in this study for determining dissolved oxygen is a modification of that used by Fox and Wingfield (1938). Table VII gives results obtained with the ordinary Winkler method compared to results obtained by the Fox-Wingfield method. It is seen that the two methods give closely comparable results at high oxygen concentration, but that at low oxygen concentrations the Fox-Wingfield method gives constantly lower results. This is pointed out by the authors as probably due to atmospheric contamination of the water sample in the ordinary Winkler method.

Inasmuch as the syringe pipettes described herein had greater dead spaces (due to the hypodermic needles) than did the syringe used by the above mentioned investigators, different concentrations of chemical reagents were used in analysis of water samples. In order to determine the oxygen content of a sample, the following procedure was used. The head-screw was screwed down until the lock-nut met the sleeve. The dead space was filled with aqueous manganous chloride solution. The stock MnCl₂ was made up 40 g. in 100 ml. of solution. The stock solutions were diluted in varying amounts, according to the calculated dead spaces of the syringes. After dilutions, each syringe dead space held 0.008 g. of MnCl₂. In order to fill the dead space with MnCl₂, the solution was drawn into the syringe, which was then turned so that the nozzle and needle pointed upwards. The plunger was raised, expelling any air bubbles, so that only the dead space remained filled with the solution. The outside of the needle was then washed with distilled water, and the sample
to be analyzed was drawn into the syringe by piercing the serum bottle stopper with the hypodermic needle. The plunger was withdrawn until its head made contact with the shaft of the head-screw, thus indicating that a known quantity of sample had been drawn into the syringe. Alkaline iodide solution was next drawn into the syringe by unscrewing the head-screw a specified number of turns.

A stock solution of alkaline iodide was made with 32 g. of NaOH and 10 g. of KI in 100 ml. of solution. This stock solution was diluted so that each syringe took in 0.014 g. NaOH and 0.004 g. KI. The quantity of this solution drawn in was made equal to about twice the volume of manganous chloride solution in the dead space. This was found to be given by five turns of the head-screw of syringe #1 and three turns for syringe #2 and #3. After the alkaline iodide was taken in, the syringe pipette was shaken until the precipitate of manganous hydroxide was evenly distributed. It was then put aside for three minutes in order to complete the absorption of oxygen by the precipitate. After further shaking of the syringe, o-phosphoric acid was drawn in by five turns of the head-screw of syringe #1, and three turns of the head-screw of #2 and #3. The instrument was again shaken until all of the precipitate had disappeared and iodine was liberated. The solution was then ejected into a micro titration vessel of approximately 10 ml. capacity and the syringe barrel was washed out with distilled water, the washing having been ejected into the vessel. The iodine was titrated against standard sodium thiosulfate solution.
After withdrawal of each water sample from the oxygen check point flasks, an equivalent amount of SRF of appropriate oxygen content was replaced in the same manner by which it was taken out. A flask of SRF fitted with a serum bottle stopper and a nitrogen inlet was used in order to obtain the sample replacement whenever needed. With this procedure it was possible to keep equal volumes of liquid in the check point flasks at all times.

Preliminary investigations employed a Rehberg micro burette of 100 cu. mm. (0.1 ml.) capacity for micro titrations. However, this did not prove very satisfactory in that the column of mercury, activated by a mercury plunger-type valve, made contact with the volumetric solution (sodium thiosulfate) and formed a sludge. Therefore, subsequent work employed a Linderstrom-Lang and Holter, air interface type micro burette. This burette was specially constructed for those standard solutions which might attack or be influenced by contact with mercury. The air interface is accomplished by sealing an open-top vertical glass tube into the horizontal portion of the burette. It assures a constant air space between the mercury and the sodium thiosulfate. The burette is made of glass tubing of small bore with a graduate scale from 0 cmm. on the top to 50 cmm. (0.05 ml.) on the bottom, divided into 0.2 cmm. (0.0002 ml.). A mercury plunger-type valve, controlled by a horizontal screw with a very fine thread, activates a column of mercury which in turn activates the air space in contact with the volumetric solution (sodium thiosulfate). The air space forces a discharge of minute volumes
of solution with utmost precision beneath the surface of the mate-
rial being titrated.

During titration of the iodine liberated in a dissolved oxygen determination, the titration vessel was held in a support so that the tip of the micro burette dipped just below the surface of the contents of the vessel. The solution was stirred throughout the titration with a glass rod bent so that its end formed a ring at right angles to the rod. The stirring rod was moved up and down by means of a thread over a series of pulleys and levers. Two drops of dilute starch solution were used as indicator. The colorless end point of the liquid in the titration was compared with distilled water containing the same amount of starch in a similar glass vessel.

Derivation of the formula (Fox and Wingfield) for calculating the oxygen content of a water sample is as follows:

One cc. of M solution sodium thiosulfate corresponds to 0.008 g. of oxygen.

Let = normality of the sodium thiosulfate used. Then 1 cc. of a solution of sodium thiosulfate of normality

\[ = 0.008 \times \text{h g. oxygen} \]

\[ = \frac{0.008 \times \text{h} \times 22.400}{32} \]

\[ = 5.6 \times \text{h cc. oxygen at N.T.P.} \]

But \( h = 2 \frac{1}{t} \), where \( t \) = normality of potassium iodate used in the standardization of the sodium thio-
sulfate solution and \( t \) = titre of the sodium thiosulfate (2 cc. of potassium iodate solution was used).
Therefore 1 cc. of h N solution of sodium thiosulfate

\[ \frac{5.6 \times 31}{t} : 11.8 \times \frac{1}{t} \] cc. oxygen at N.T.P.

Let the volume of the water sample be \( v \) ml. and the volume of sodium thiosulfate required in the estimation be \( n \) ml. Then the oxygen concentration of the water sample expressed in ml./l.

\[ \frac{1000 \times n \times 1 \times 11.2}{v \times t} \]

\[ \frac{11.200 \times n}{vt} \]

Now, substituting actual values in a numerical example we have:

\[ n = 0.1208 \text{ ml.} \quad v = 1.420 \text{ ml.} \]

\[ l = 0.0850 \quad t = 3.97 \]

To convert to p.p.m., multiply by 1.43 since one ml. of oxygen weighs 1.489 mg.

Therefore, \( 5.99 \times 1.43 = 8.87 \) p.p.m.

Dissolved oxygen determinations were made periodically during tests and recorded. During preliminary investigation the oxygen content of the flasks was determined regularly every few hours. However, when it became apparent that oxygen levels remained relatively constant with a standard amount of fluctuation for the various air-nitrogen mixtures used, the determinations were less frequently made, these being random spot checks. Typical examples as to the apparent degree of fluctuation during some of these tests are presented in Table VIII. The maximum deviation above and below the mean was usually less than 0.1 p.p.m. of dissolved oxygen and not greater than 0.16 p.p.m. The dissolved oxygen values listed in Tables X through XVIII represent mean values and were derived by averaging the determinations obtained during a test period.
Table V

<table>
<thead>
<tr>
<th>Analysis No.</th>
<th>Fox-Wingfield Method</th>
<th>Ordinary Winkler Method</th>
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<tr>
<td>1</td>
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<td>7.90</td>
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</tr>
<tr>
<td>12</td>
<td>0.95</td>
<td>1.03</td>
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</table>

Table V. Comparison of ordinary Winkler method with Fox-Wingfield method in the analyses of the oxygen content of fresh water at 10°C. (After Fox and Wingfield, 1938)
### Table VI

<table>
<thead>
<tr>
<th>Period in Hours</th>
<th>Average dissolved oxygen (p.p.m.)</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td></td>
<td>for air-nitrogen mixtures</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0 - 15</td>
<td></td>
<td>1.53</td>
<td>3.02</td>
<td>4.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.51 - 1.63)</td>
<td>(2.91 - 3.08)</td>
<td>(4.45 - 4.52)</td>
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<tr>
<td>15 - 25</td>
<td></td>
<td>1.52</td>
<td>2.99</td>
<td>4.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.48 - 1.58)</td>
<td>(2.89 - 3.08)</td>
<td>(4.52 - 4.60)</td>
</tr>
<tr>
<td>25 - 50</td>
<td></td>
<td>1.54</td>
<td>2.98</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.45 - 1.61)</td>
<td>(2.92 - 3.01)</td>
<td>(4.35 - 4.48)</td>
</tr>
<tr>
<td>50 - 75</td>
<td></td>
<td>1.56</td>
<td>3.04</td>
<td>4.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.51 - 1.60)</td>
<td>(2.93 - 3.10)</td>
<td>(4.38 - 4.48)</td>
</tr>
<tr>
<td>75 - 100</td>
<td></td>
<td>1.48</td>
<td>2.96</td>
<td>4.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.42 - 1.56)</td>
<td>(2.91 - 3.04)</td>
<td>(4.43 - 4.58)</td>
</tr>
</tbody>
</table>

|                      | Av. for total test period        | 1.53 | 2.99 | 4.48 |
|                      | Maximum deviation between extremes | 0.19 | 0.81 | 0.26 |
|                      | % maximum deviation              | 12.6 | 7.1  | 5.1 |

**Table VI.** Average and extremes of dissolved oxygen determinations during test periods with air-nitrogen mixtures. Each value represents averages of determinations taken at check points in test system.
Figure 4. Krogh-Keyes syringe pipette used in micro dissolved oxygen determinations.
Calculation and Statistical Analysis of Thresholds

The median toxicity threshold, as used here, is defined as that concentration of test material in which 50 per cent of the test organisms are immobilized in a given period of time. The 25-hour, 50-hour, and 100-hour threshold, therefore, is that concentration which immobilizes 50 per cent of the animals in 25, 50 and 100 hours, respectively. This value, which has been designated by others in various ways, such as median tolerance limit (TLM), fifty per cent lethal dose (LD 50), and median lethal dose (MLD), can be calculated in several ways. The method used to calculate thresholds is that of Anderson, et al, (1948).

The initial phase of the calculation is to determine the concentration, in terms of container number, in which half the animals were immobilized. The term "immobilized" is used in preference to "killed" or "dead" inasmuch as it is sometimes difficult to determine, by observing in glass, whether all life processes of a daphnid have ceased. In order to illustrate the method of calculation, the results of three observations are shown in Table IV. Observations other than the 25-, 50-, and 100-hour were made but are intermediate and need not be considered in calculating final thresholds.

In calculating the 25-hour threshold, it is seen that all of the animals were immobilized in container #1. In #2 two were alive, in #3 seven were alive, in #4 nine were alive, and in #5 all were alive.

Assume that the two animals in container #2 would have died
half-way between the concentrations in container #1 and #2. Call this concentration container #1.5, and multiply by 2 (number of daphnids alive in #2); this gives a product of 3.0.

Seven daphnids were alive in container #3 and two were alive in #2. Assume that these five died half-way between #2 and #3. Call this concentration container #2.5. Multiply by 5 (number of daphnids alive in #3 minus those alive in #2) which makes 12.5.

Nine daphnids were alive in #4 and seven were alive in #3. Assume two would have died at 3.5 and multiply by 2, the product of which is 7.0.

All ten daphnids were alive in #5. Since nine were alive in #4 assume that one died at 4.5. Multiply 4.5 by 1 and the product is 4.5.

Add the products (the sum of which is 27), and divide by 10 since that is the number of animals introduced into each test container. This gives the threshold concentration in terms of container number, in this case 2.7.

Summarizing we have:

<table>
<thead>
<tr>
<th>Test Container #</th>
<th>Number living daphnids = product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>3.5</td>
<td>2</td>
</tr>
<tr>
<td>4.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

27.0/10 = 2.7, the threshold concentration in terms of container number.
The following equation was used to convert the threshold concentration in terms of container number to the actual concentration:

\[ \log c_1 - (a-1) \log b = c_a \]

Where \( c_1 \) is the concentration in container #1, \( a \) is the threshold concentration in terms of container number, \( b \) is the dilution factor, and \( c_a \) is the concentration (median threshold) of the test material in #\( a \).

The concentration in container #1 was 12,000 p.p.m. Therefore, \( c_1 = 12,000 \) and the \( \log c_1 = 4.07918 \).

The threshold in terms of test container number was 2.7 (see preceding calculations). Therefore, \( a = 2.7 \).

The sequence of dilutions used in this test were in the powers of 1.33, therefore the dilution factor (\( b \)) was 1.33 and \( \log b = 0.12385 \).

Substituting the above values in the equation, we have:

\[ 4.07918 - (2.7-1) 0.12385 = \log c_a \]

\[ \log c_a = 3.86863 \]

\[ c_a = 7390 \text{ p.p.m.} \]

This \( c_a \) value, however, is not final inasmuch as the test solution was diluted with five ml. of SRW upon exposing the daphnids. In such cases the actual threshold concentration must be calculated. Since, in each container, there was 100 ml. of the original solution plus five ml. of SRW containing the daphnids, it is seen that

\[ 739000 / 106 = 7038 \text{ p.p.m.} \]

which is the actual median threshold concentration.
If we apply the same procedures to the 50- and 100-hour data we find that the threshold values are 6288 and 6195 p.p.m. After calculating for dilution we find that the values have dropped to 5932 and 4948 p.p.m. respectively.

However, some of the tests, i.e., many of those conducted in the four ounce bottles under atmospheric conditions, did not undergo the same degree of dilution. Some were diluted only by one half ml. of the yeast suspension plus one ml. of SRW used in pipetting daphnids into test solutions. Others were diluted by two and even three ml. of SRW when transferring daphnids. Therefore, the last calculation presented above varied to fit the occasion.

Since most of the experiments were carried out at least in duplicate, the threshold was calculated for each, and this value averaged.

Additional statistical treatment of threshold values is shown in Table V. These are median thresholds obtained for sodium chloride and all tests were carried out under atmospheric conditions, dissolved oxygen concentration approximately 6.4 p.p.m. This example is taken because sodium chloride was more extensively tested than any of the other compounds presented herein. It is seen for the 26-, 50-, and 100-hour thresholds, that the per cent range above the minimum threshold is 8.1, 7.2, and 6.6 % respectively. As pointed out and discussed later, these data indicate that the 26- and 50-hour thresholds are not as reliable as the 100-hour. However, the main point of interest is the per cent deviation from the mean.
As shown, the greatest percentage deviation from the mean at the 25-, 50-, and 100-hour level is 4.49, 3.55 and 3.33 % respectively. These data indicate that this series of tests were reproducible within 5, 3.6, and 3.3 % of the mean thresholds obtained for 25-, 50-, and 100-hour tests. This reproducibility, however, was not always obtained. Table X - XVIII give the per cent range of thresholds for all tests reported herein. Although values for per cent deviation from mean thresholds are not given, these can be estimated by comparing the range variations (per cent range above minimum threshold).

Chemicals Tested and Test Solutions

These studies have been limited to inorganic salts which are known to be present in various types of industrial effluents. Since the work was sponsored by certain petroleum refiners, one of the primary objectives was to test compounds which are of interest to them as well as to general biological research. Tests were necessarily restricted to non-volatile and inorganic compounds. Volatile materials would most certainly lose some of their toxic components during the vigorous bubbling of gases through them prior to testing. Similarly, compounds which are easily altered by chemical and biological oxidation were undesirable.

The compounds tested and reported here were: (1) ammonium chloride, (2) ammonium sulfate, (3) calcium chloride, (4) sodium carbonate, (5) sodium chloride, (6) sodium chromate, (7) sodium
Table VII

<table>
<thead>
<tr>
<th>Container</th>
<th>0</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
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</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
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</tr>
<tr>
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<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table VII. Number of motile *Daphnia magna* in test container at time indicated.
<table>
<thead>
<tr>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>Deviation from mean (p.p.m.)</th>
<th>% Deviation of extremes from mean</th>
<th>% Range above minimum threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>7212</td>
<td>171</td>
<td>3.23 - 4.49</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>(6988 - 7445)</td>
<td>(233 - 324)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>6111</td>
<td>146</td>
<td>3.38 - 3.55</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>(5894 - 6318)</td>
<td>(207 - 217)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>5098</td>
<td>104</td>
<td>3.10 - 3.33</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>(4940 - 6268)</td>
<td>(158 - 170)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table VIII. Threshold data for *Daphnia magna* calculated from six tests with Sodium chloride at atmospheric oxygen.
bisulfite, (8) sodium bisulfate, and (9) sodium sulfate.

The test solutions were made up in 100 ml. quantities by diluting the chemicals with standard reference water. Dilutions were made in geometrical progression in varying powers. For example, in some instances of low oxygen a preliminary experiment had to be carried out with dilutions in powers of 1.78. This large step dilution sequence covered a wide range of concentrations and showed the approximate threshold of toxicity. After having estimated the approximate range, the other tests were set up in another series of dilutions covering a narrower range of concentrations. These were usually in powers of 1.58, 1.41 or 1.33, depending upon the anticipated effects of lowered oxygen as indicated by the preliminary experiments. Table IX gives the various dilution sequences\(^1\) which were used. They were calculated for 100 ml. dilutions.

**Testing Procedure**

The procedure employed for conducting tests was as follows:

1. The test solutions were usually set up in duplicate and sometimes in triplicate, one series of solutions in flasks and one or two in four ounce bottles. The solutions were made in 100 ml. quantities, nine concentrations being made in a series of dilutions in geometrical progression. The tenth container of each test contained dilution water and served as a control. In addition to

\(^1\)From Dr. E. G. Anderson. Obtained by personal communication through Dr. I. Fowler.
Table IX

<table>
<thead>
<tr>
<th>Bottle Number</th>
<th>Log Dilution</th>
<th>Arith. Dilution</th>
<th>Bottle Number</th>
<th>Log Dilution</th>
<th>Arith. Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>1.0</td>
<td>1</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>1.78</td>
<td>2</td>
<td>0.2</td>
<td>1.68</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>3.16</td>
<td>3</td>
<td>0.4</td>
<td>2.51</td>
</tr>
<tr>
<td>4</td>
<td>0.75</td>
<td>6.82</td>
<td>4</td>
<td>0.6</td>
<td>3.98</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>10.0</td>
<td>5</td>
<td>0.8</td>
<td>6.31</td>
</tr>
<tr>
<td>6</td>
<td>1.25</td>
<td>17.78</td>
<td>6</td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>31.52</td>
<td>7</td>
<td>1.2</td>
<td>15.85</td>
</tr>
<tr>
<td>8</td>
<td>1.75</td>
<td>56.25</td>
<td>8</td>
<td>1.4</td>
<td>25.12</td>
</tr>
<tr>
<td>9</td>
<td>2.0</td>
<td>100.0</td>
<td>9</td>
<td>1.6</td>
<td>39.81</td>
</tr>
</tbody>
</table>

Starting volume = 228.49 mls. = 270.97 mls.

<table>
<thead>
<tr>
<th>Bottle Number</th>
<th>Log Dilution</th>
<th>Arith. Dilution</th>
<th>Bottle Number</th>
<th>Log Dilution</th>
<th>Arith. Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>1.0</td>
<td>1</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.15</td>
<td>1.41</td>
<td>2</td>
<td>0.125</td>
<td>1.33</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>2.0</td>
<td>3</td>
<td>0.25</td>
<td>1.78</td>
</tr>
<tr>
<td>4</td>
<td>0.45</td>
<td>2.82</td>
<td>4</td>
<td>0.375</td>
<td>2.37</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
<td>3.98</td>
<td>5</td>
<td>0.500</td>
<td>3.16</td>
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<tr>
<td>6</td>
<td>0.75</td>
<td>5.62</td>
<td>6</td>
<td>0.625</td>
<td>4.22</td>
</tr>
<tr>
<td>7</td>
<td>0.9</td>
<td>7.94</td>
<td>7</td>
<td>0.75</td>
<td>5.62</td>
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<tr>
<td>8</td>
<td>1.05</td>
<td>11.22</td>
<td>8</td>
<td>0.875</td>
<td>7.50</td>
</tr>
<tr>
<td>9</td>
<td>1.20</td>
<td>15.85</td>
<td>9</td>
<td>1.0</td>
<td>10.00</td>
</tr>
</tbody>
</table>

Starting volume = 342.42 ml. = 399.85 mls.

Table IX. Dilution sequences used in making 100 ml. test solutions. (After Anderson)
these, each of the six flasks which were to serve as oxygen check-
points contained dilution water.

2. The gas mixtures were then made up in ratio according
to the oxygen tensions to be used for the particular test.

3. After having completed the above steps, daphnids were
pipetted from the culture bottles into a bottle of dilution water
from which they were transferred to more dilution water. Ten
daphnids were placed in each of ten vials of reference water (approx-
imately 4.5 ml. per vial). In many instances these vials were also
used to introduce the test animal into the test solution contained
in the four ounce bottles. At other times the daphnids were pipetted
directly from SRW into the test solution. The former method is con-
sidered advantageous over the latter in that subsequent calculations
for dilution from the added SRW were more accurate. Similarly, the
beginning of test time was more specifically determined. Vials which
were used in the check-point flasks were filled with SRW but did not
contain daphnids. Ten to 12 hours prior to testing, all young
daphnids were removed from the culture bottles. Thus, animals re-
moved for tests were of the same age class.

4. One half ml. of yeast suspension containing 0.6 mg.
of yeast was added to each of the test containers which was to re-
ceive daphnids.

5. The vials were then suspended from the rotating rod
arms after which the flask stoppers were firmly seated in the flasks.

6. Each of the glass to rubber connecting points was
spotted with Cello-Seal before being joined. This material, a heavy stopcock grease, gave a gas-tight seal between glass and rubber tubing. Water was also used as a precautionary means in preventing leaks, as well as detecting leaks, by placing it in the trough formed at the junction of stopper with flask.

7. Nitrogen was then forced through the test system, according to the method previously described. Once the oxygen concentration had become depleted within a desired level, the air-nitrogen mixture was forced from the gas mixing apparatus into the test system for the stipulated time and pressure.

8. Micro dissolved oxygen determinations were made after adjusting the rate of gas flow.

9. After the approximate oxygen tension was obtained, the vials and contents were dropped into the test solutions by manipulation of the rotating rods, thus exposing the daphnids to the test solution. This was the beginning of test time. The paralleled tests under atmospheric oxygen were usually begun at about the same time as the test under lowered oxygen by dropping the vials of daphnids or by pipetting the daphnids into the test solutions made up in the four ounce bottles.

10. Periodic observations were made throughout the 100-hour test period, but those used in calculating the median toxicity thresholds were the 25-, 50-, and 100-hour. Observations consisted of recording the number of living daphnids in each test container.
11. *Periodic dissolved oxygen determinations were made.*

If the oxygen level either rose or declined, the gas flow rate was adjusted accordingly.
RESULTS

A total of 84 tests were conducted upon nine inorganic salts by use of *Daphnia magna*. The 25-, 50-, and 100-hour median toxicity thresholds were determined from each salt. Of the 84 tests, 39 were conducted at atmospheric oxygen tensions, 5 at approximately 9.0 p.p.m. oxygen, 4 at approximately 4.4 p.p.m. oxygen, 16 at approximately 3.0 p.p.m. oxygen, and 20 at approximately 1.6 p.p.m. oxygen. Six of the compounds tested were salts of sodium, two were salts of ammonium, while the other was a salt of calcium. All of these are known to be present in various types of industrial wastes. The results of these tests are presented in Tables X through XVIII and are shown graphically in Figures 5 through 13. Excluding those compounds tested but once, each of the thresholds and the oxygen concentrations are average values. The per cent range above the minimum threshold for each test has been calculated in order to show the degree of variation encountered. Similarly, the part per million change (drop or rise) in threshold, as influenced by varied oxygen content, is given for each test or series of tests. In order to show the relative effects of lowered oxygen on the susceptibility of *D. magna* to the chemicals, the latter values have been converted to per cent drop or rise.
Ammonium chloride

Nine tests were conducted with this salt. Four of these were at atmospheric oxygen, approximately 6.4 p.p.m. The average thresholds were 389.5, 274.9 and 246.6 p.p.m. at 25, 50 and 100 hours respectively. Two tests at an average oxygen tension of 3.16 p.p.m. showed drops in threshold concentration to 280.5, 233.0 and 174.3 p.p.m. respectively. The respective per cent drop in thresholds was 4.8, 17.9 and 41.5%. Three tests at an average oxygen tension of 1.48 p.p.m. showed further drops in threshold, these being 124.4, 111.8 and 99.7 p.p.m. The latter values show a decrease of 197.0, 145.8 and 147.1% from the 25-, 50-, and 100-hour thresholds obtained at atmospheric oxygen. The data obtained for the salt are presented in Table I; results are represented graphically in Figure 5.

Ammonium sulfate

Eight tests were conducted with this salt, three at atmospheric oxygen (approximately 6.8 p.p.m.), one at 4.61 p.p.m. (av.), two at 2.91 p.p.m. (av.), and two at 1.58 p.p.m. (av.). The 25-, 50-, and 100-hour thresholds at atmospheric oxygen were 415.9, 362.8 and 288.5 p.p.m. respectively. Similarly, the respective thresholds at 4.61 p.p.m. oxygen were 391.2, 356.1 and 155.4 p.p.m. The per cent differences between these threshold concentrations and those at atmospheric oxygen were slight, the drop being 6.3, 1.9 and 9.9% at 25, 50 and 100 hours respectively. Inasmuch as the differences were no greater than the range of variation in other tests
with this salt, the decreases cannot be considered as statistically significant. On the other hand, percentage decrease in thresholds for tests conducted at 2.91 and 1.68 p.p.m. of oxygen are significant. The 25-, 50-, and 100-hour thresholds for the former were 290.9, 264.7 and 155.4 p.p.m. and represented drops of 51.3, 61.8 and 85.8% respectively. The respective thresholds for 1.58 p.p.m. oxygen were 141.7, 126.5 and 116.2 p.p.m. of salt and showed decreases of 193.5, 186.7 and 148.3% respectively. Data for tests with ammonium sulfate are given in Tables X and XI and are represented graphically in Figure 6.

**Calcium chloride**

This salt, the only one of calcium, was employed in seven tests. Three were conducted at atmospheric oxygen (approximately 6.7 p.p.m.), while four were conducted at two lowered oxygen tensions. Two of the latter were at 3.16 p.p.m. (av.) oxygen and the other two at 1.54 p.p.m. (av.) oxygen. Calculated thresholds for the 25-, 50-, and 100-hour levels at atmospheric oxygen were 4626, 4454 and 3978 p.p.m. respectively. Thresholds for the same periods at 3.16 and 1.54 p.p.m. oxygen were 4365, 3553 and 2824 p.p.m., and 2820, 2459 and 2049 p.p.m. respectively. The per cent decrease in 25-, 50-, and 100-hour threshold values for the 3.16 and 1.54 p.p.m. lowered oxygen tensions were 6.0, 25.4 and 40.7%, and 94.0, 81.1 and 93.9% respectively. The drop in threshold for the 25-hour level at 3.16 p.p.m. oxygen was not significant, inasmuch as it was less than the per cent range of variation for other thresholds found for
this salt. Each of the other decreases in threshold values were significant. Data for tests with calcium chloride are given in Tables XI and XII and are shown graphically in Figure 7.

Sodium carbonate

This salt was used in nine different tests. However, results are reported for only eight because one of the preliminary tests at low oxygen was conducted with a range of concentrations which were too high. Four tests were at atmospheric oxygen, approximately 6.5 p.p.m., and two each at 3.1 p.p.m. (av.) and 1.53 p.p.m. (av.) oxygen. The 25-, 50-, and 100-hour thresholds (av.) at atmospheric oxygen were 614.7, 565.8 and 558.4 p.p.m. respectively. Similarly, the respective average thresholds at 3.1 p.p.m. oxygen were 387.5, 333.2 and 322.4 p.p.m. sodium carbonate. The per cent drop from those thresholds at atmospheric oxygen were 58.6, 69.8 and 71.3%. Tests at the 1.53 p.p.m. oxygen level showed still greater decrease in thresholds. These were 287.3, 280.2, and 266.6 p.p.m. for the respective 25-, 50-, and 100-hour periods. Percentage decreases for these values are 113.9, 101.9 and 107.2% respectively. These data show that relative decrease in thresholds for *D. magna* tested in sodium carbonate are approximately 50 and 100% lower at 3.1 and 1.6 p.p.m. oxygen respectively than thresholds determined at atmospheric oxygen. Sodium carbonate thresholds for *D. magna* were limited, in part at least, by the high pH of test solutions. The threshold concentrations showed pH values
which ranged from 9.5 to 9.8. Results of tests with this salt are presented in Tables XII and XIII and Figure 8.

**Sodium chloride**

Sodium chloride was the most extensively tested salt of those reported herein. Fourteen tests were conducted; six of these were at atmospheric oxygen (about 6.4 p.p.m.) while the remainder were at lowered oxygen tensions. The 25-, 50-, and 100-hour test periods gave average thresholds of 7212, 6111 and 5098 p.p.m. sodium chloride at atmospheric oxygen. Two tests at an average 4.44 p.p.m. oxygen gave a slight decrease in thresholds, these being 6676, 5709 and 4178 p.p.m. for the respective periods. The per cent drop in thresholds was 9.7, 7.0 and 22.0% respectively. The first two values are not significant in that they fall within the per cent range of variation, but the latter does have some significance. It would indicate that a 4.4 p.p.m. oxygen tension does have a slight effect on the susceptibility of *D. magna* when exposed to the salt for 100 hours. The 25-, 50-, and 100-hour average thresholds exhibited for three tests at an average 2.89 p.p.m. oxygen were 4766, 4105 and 3448 p.p.m. sodium chloride respectively. Similarly, thresholds for three tests at 1.48 p.p.m. oxygen were 4106, 3807 and 3170 p.p.m. of salt for the respective periods. These values show relative drops in thresholds for the 2.89 p.p.m. oxygen level to be 51.6, 48.9 and 47.9%. For the 1.48 p.p.m. oxygen level they were 75.6, 60.5 and 60.7% at the respective 25-, 50-, and 100-hour levels. These data show that
the lower oxygen tensions, around 3.0 and 1.5 p.p.m., affect the thresholds of *D. magna* to sodium chloride. They are lowered approximately 50 and 65% respectively from thresholds at atmospheric oxygen. Results of these tests are presented in Tables XIII and XIV and Figure 9.

**Sodium chromate**

Eight tests were conducted with this salt. Four tests were at atmospheric oxygen, approximately 6.6 p.p.m., and two each at average oxygen tensions of 3.17 and 1.56 p.p.m. The 25-, 50-, and 100-hour thresholds at atmospheric oxygen were 0.94, 0.71 and 0.61 p.p.m. respectively. Similarly, the respective average thresholds at 3.17 p.p.m. oxygen were 0.89, 0.605 and 0.41 p.p.m. sodium chromate. The per cent drop from those thresholds found at atmospheric oxygen were 5.6, 16.7 and 24.4% respectively. The first drop is not significant while the latter two are, to a small extent. Tests at 1.56 p.p.m. oxygen showed slightly greater decreases in threshold values. These were 29.2, 35.8 and 39.7% drops for the 25-, 50-, and 100-hour thresholds which were 0.73, 0.52 and 0.365 p.p.m. respectively. Sodium chromate was the most toxic salt to *D. magna* of those tested. It was found, however, that the relative decrease in thresholds brought about by lowered oxygen tension was considerably less than for other salts. This is shown by the percentage drops which did not exceed 25% at 3.0 p.p.m. oxygen level, or 40% at the 1.5 p.p.m. level. These results are given in Tables XIV and XV and shown graphically in Figure 10.
Sodium bisulfite

A total of 14 tests were conducted with this salt. Nine of these employed bisulfite which has been designated, for reasons discussed later, as "old". Five tests were conducted with "new" bisulfite. Four of the tests with "old" bisulfite were at atmospheric oxygen, approximately 6.5 p.p.m., two were at high oxygen tension which averaged 9.3 p.p.m., one was at 3.16 p.p.m. average oxygen, and two at 1.61 p.p.m. average oxygen. The 25-, 50-, and 100-hour toxicity thresholds at 6.5 p.p.m. gave averages of 125.6, 91.2 and 61.4 p.p.m. respectively. The per cent range in variation was high, these being 25.1, 20.0 and 14.7 % for the respective threshold groups. When D. magna was tested in this bisulfite at high oxygen (9.3 p.p.m.) the threshold values rose. For 25-, 50-, and 100-hour test periods they were 205.3, 146.9 and 106.7 p.p.m. respectively. These values show the per cent rise above the respective thresholds at atmospheric oxygen to be 63.5, 63.3 and 72.0 %. Similarly, tests at lowered oxygen tensions showed rises in thresholds from those at atmospheric oxygen. The 25-, 50-, and 100-hour thresholds at 3.16 p.p.m. oxygen were 158.3, 134.9 and 76.8 p.p.m. salt respectively, and at 1.61 p.p.m. oxygen they were 159.6, 125.1 and 70.3 p.p.m. salt. The per cent rise in thresholds above those at atmospheric were 21.3, 47.9 and 25.1 % respectively for the 3.16 p.p.m. oxygen level. At the 1.61 p.p.m. oxygen level the respective rises were 27.1, 37.2 and 14.5 %. Therefore, it is
seen that thresholds for *D. magna* to bisulfite were all higher than those found in tests at atmospheric oxygen.

The same trend was found for tests with "new" bisulfite. Threshold averages at 25, 50 and 100 hours for the atmospheric oxygen level were 99.1, 72.4 and 57.5 p.p.m. Here again the percent range in variation was high, this being 37.1, 14.5 and 14.4% respectively. It is noted that these threshold values are somewhat lower than those obtained with "old" bisulfite at atmospheric oxygen. Interpretation of this and other results with this salt are discussed later. Two other tests were conducted, these being at high oxygen (9.2 p.p.m.). The threshold for the respective periods were 127.1, 111.5 and 99.1 p.p.m. of "new" bisulfite. These values represent rises of 26.3, 54.1 and 72.3% respectively above thresholds at atmospheric oxygen. This is similar to findings with "old" bisulfite; 100-hour thresholds for both at high oxygen showed the same relative rise in threshold, 72.0 and 72.3%. The 50-hour thresholds were somewhat similar, 53 and 54%, but the 25-hour were not (53.5 and 28.3%). It is pointed out that the thresholds for "new" bisulfite are considerably lower than those for the "old". These aspects are discussed later. Sodium bisulfite exerts much of its toxic effect on *D. magna* by lowering the pH of test solution. This is also discussed at a later time. Data from bisulfite tests are presented in Tables XV and XVI. The results are shown graphically in Figure 11. The broken lines represent
threshold curves for the "old" bisulfite, while tests with the "new" are represented by solid lines (a), (b), and (c).

**Sodium bisulfate**

This salt was tested with the intention of gaining information concerning the results encountered with sodium bisulfite. Both give a low pH in solution. Seven tests were made; four at atmospheric oxygen, approximately 6.6 p.p.m., one at high oxygen tension (8.9 p.p.m.), and two at low oxygen (1.56 p.p.m.). The 25-, 50-, and 100-hour thresholds at atmospheric oxygen were 206.7, 177.4 and 153.4 p.p.m. respectively. At 8.9 p.p.m. oxygen the thresholds for the respective periods were 210.3, 183.4 and 145.3 p.p.m. of bisulfate. The 25- and 50-hour thresholds show a slight rise (1.7 and 3.4 %) above threshold for the same periods at atmospheric oxygen. The 100-hour threshold shows a slight drop (5.6 %). These differences, however, are not significant because they fall well within the per cent range of variation between thresholds. On the other hand, the 25-, 50-, and 100-hour thresholds found at 1.56 p.p.m. oxygen do show slightly significant decreases in threshold values. These were 159.2, 113.4 and 105.5 p.p.m. respectively and indicate relative drops of 29.8, 56.4 and 45.4 % from the thresholds at atmospheric oxygen. Results of tests with this salt are presented in Table XVII and in Figure 12.
Sodium sulfate

Eight tests were conducted with this salt. Four tests were at atmospheric oxygen, approximately 6.6 p.p.m., and two each at average oxygen tensions of 2.92 and 1.46 p.p.m. The 25-, 50-, and 100-hour thresholds at atmospheric oxygen were 6949, 5966 and 5514 p.p.m. respectively. Similarly, the respective average thresholds at 2.92 p.p.m. oxygen were 6490, 5056 and 4135 p.p.m. sodium sulfate. The percent drop from those thresholds found at atmospheric oxygen were 7.1, 18.0 and 33.4 % respectively. The 25-hour decrease (7.1 %) is not significant, while the 50- and 100-hour decreases (18.0 and 33.4 %) are, especially the latter.

Tests at 1.46 p.p.m. oxygen showed still greater lowering of threshold values. These were 14.0, 23.7 and 100.4 % drops for the 25-, 50-, and 100-hour threshold which were 6097, 4822 and 2752 p.p.m. respectively. These data indicate that the susceptibility of *D. magna* when exposed to sodium sulfate at lowered oxygen tension is, relatively speaking, progressively increased throughout a 100-hour test period. This is seen by comparison of threshold drops for the 25-, 50-, and 100-hour periods. In 25 hours the threshold drop at 2.92 p.p.m. oxygen was not significant, whereas at 1.46 p.p.m. oxygen it was. In 50 hours the drop at 2.92 p.p.m. oxygen was 18 %, whereas at 1.46 p.p.m. it dropped further to 24 %. In 100 hours the thresholds were lowered by 33 and 100 % for the respective oxygen tensions. Therefore, it is seen that the
100 % drop at 1.46 p.p.m. oxygen tripled that at 2.92 p.p.m. oxygen.

The data presented above are given in Tables XVII and XVIII and represented graphically in Figure 13.
<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests (100 daphnids / test)</th>
<th>Oxygen tension (p.p.m.)</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (p.p.m.)</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium chloride</td>
<td>4</td>
<td>25</td>
<td></td>
<td>369.5</td>
<td>4.9</td>
<td>(364.4 - 382.6)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td></td>
<td>274.2</td>
<td>9.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td>246.6</td>
<td>14.2</td>
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<tr>
<td></td>
<td>atm. approx. 6.4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>av. 3.18</td>
<td>25</td>
<td></td>
<td>250.5</td>
<td>9.8</td>
<td>109.0 (-)</td>
<td>41.8 (-)</td>
</tr>
<tr>
<td></td>
<td>av. 5.18</td>
<td>50</td>
<td></td>
<td>233.0</td>
<td>5.6</td>
<td>41.9 (-)</td>
<td>17.9 (-)</td>
</tr>
<tr>
<td></td>
<td>av. 1.48</td>
<td>100</td>
<td></td>
<td>174.3</td>
<td>6.1</td>
<td>72.3 (-)</td>
<td>41.5 (-)</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
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<td>atm. approx. 6.8</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>av. 5.6</td>
<td>25</td>
<td></td>
<td>415.9</td>
<td>2.9</td>
<td>(408.3 - 420.5)</td>
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<td></td>
<td></td>
<td>50</td>
<td></td>
<td>382.8</td>
<td></td>
<td>(356.1 - 366.8)</td>
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</tbody>
</table>

Table X. Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature 23 ± 1°C. (Ammonium chloride and Ammonium sulfate)
Table XI

<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests (100 daphnids / test)</th>
<th>Oxygen tension (p.p.m.)</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (−) or rise (+) from av. threshold at atmospheric O₂ (p.p.m.)</th>
<th>Drop (−) or rise (+) from av. threshold at atmospheric O₂ (%)</th>
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</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td></td>
<td></td>
<td>6.6</td>
<td>288.5</td>
<td>3.0</td>
<td>29.3 (−)</td>
<td>6.3 (−)</td>
</tr>
<tr>
<td>(contd.)</td>
<td></td>
<td></td>
<td>25</td>
<td>391.2</td>
<td></td>
<td>141.0 (−)</td>
<td>51.3 (−)</td>
</tr>
<tr>
<td></td>
<td>av. 4.61</td>
<td></td>
<td>50</td>
<td>356.1</td>
<td></td>
<td>138.6 (−)</td>
<td>61.8 (−)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>282.4</td>
<td></td>
<td>131.1 (−)</td>
<td>85.6 (−)</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>3</td>
<td>atmos. approx. 6.7</td>
<td>25</td>
<td>4626</td>
<td>5.0</td>
<td>274.2 (−)</td>
<td>193.5 (−)</td>
</tr>
</tbody>
</table>

Table XI. Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature 23 ± 1°C. (Ammonium sulfate and Calcium chloride)
<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests (100 daphnids / test)</th>
<th>Oxxygen tension (p.p.m.)</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (p.p.m.)</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (%)</th>
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</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>50</td>
<td>4454</td>
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<td>(4347 - 4567)</td>
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<td>261.0 (-)</td>
<td>6.0 (-)</td>
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<tr>
<td>(contd.)</td>
<td>6.7</td>
<td>3972</td>
<td></td>
<td>(3966 - 3981)</td>
<td>0.3</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>25</td>
<td>4365</td>
<td></td>
<td>(4166 - 4564)</td>
<td>9.6</td>
<td>1806.0 (-)</td>
<td>64.0 (-)</td>
</tr>
<tr>
<td>av. 3.16</td>
<td>50</td>
<td>3553</td>
<td></td>
<td>(3472 - 3634)</td>
<td>4.7</td>
<td>1995.0 (-)</td>
<td>81.1 (-)</td>
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<tr>
<td>5</td>
<td>100</td>
<td>2824</td>
<td></td>
<td>(2755 - 2893)</td>
<td>5.0</td>
<td>1923.0 (-)</td>
<td>93.9 (-)</td>
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<tr>
<td>2</td>
<td>25</td>
<td>2820</td>
<td></td>
<td>(2755 - 2884)</td>
<td>4.7</td>
<td>1806.0 (-)</td>
<td>64.0 (-)</td>
</tr>
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<td>av. 1.54</td>
<td>50</td>
<td>2459</td>
<td></td>
<td>(2403 - 2515)</td>
<td>6.4</td>
<td>1995.0 (-)</td>
<td>81.1 (-)</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>2049</td>
<td></td>
<td>(2002 - 2096)</td>
<td>7.4</td>
<td>1923.0 (-)</td>
<td>93.9 (-)</td>
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<tr>
<td>Sodium carbonate</td>
<td>4</td>
<td>25</td>
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<td>614.7</td>
<td>3.5</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>(606.1 - 627.6)</td>
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<td>Atmospheric approx. 6.6</td>
<td>50</td>
<td>565.8</td>
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<td>(556.8 - 572.4)</td>
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<tr>
<td></td>
<td></td>
<td>552.4</td>
<td></td>
<td>(546.8 - 556.8)</td>
<td>1.2</td>
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Table XII. Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature 23 ± 1°C. (Calcium chloride and Sodium carbonate)
### Table XIII

<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests (100 daphnids / test)</th>
<th>Oxygen tension (p.p.m.)</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from avg. threshold at atmospheric $O_2$ (p.p.m.)</th>
<th>Drop (-) or rise (+) from avg. threshold at atmospheric $O_2$ (%)</th>
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<tbody>
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<td>Sodium carbonate</td>
<td>2</td>
<td>3.10</td>
<td>25</td>
<td>367.5</td>
<td>10.1</td>
<td>227.2 (-)</td>
<td>58.6 (-)</td>
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<td>(contd.)</td>
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<td></td>
<td>50</td>
<td>333.2</td>
<td>6.7</td>
<td>232.6 (-)</td>
<td>69.8 (-)</td>
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<td></td>
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<td></td>
<td>100</td>
<td>322.4</td>
<td>3.7</td>
<td>230.0 (-)</td>
<td>71.3 (-)</td>
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<tr>
<td>Sodium chloride</td>
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<td>1.53</td>
<td>25</td>
<td>287.3</td>
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<td>327.4 (-)</td>
<td>113.9 (-)</td>
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<td></td>
<td>50</td>
<td>280.2</td>
<td>6.2</td>
<td>285.6 (-)</td>
<td>101.9 (-)</td>
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<td></td>
<td></td>
<td>100</td>
<td>266.6</td>
<td>4.9</td>
<td>285.8 (-)</td>
<td>107.2 (-)</td>
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**Table XIII.** Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature $23 \pm 1^\circ C$. (Sodium carbonate and Sodium chloride)
Table XIV

<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests (100 daphnids / test)</th>
<th>Oxygen tension (p.p.m.)</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (%)</th>
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</thead>
<tbody>
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<td>Sodium chloride</td>
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<td>4.44</td>
<td>100</td>
<td>4178</td>
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<td>920.0 (-)</td>
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<td>(3792 - 4564)</td>
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<td>22.0 (-)</td>
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<td>3</td>
<td>4.44</td>
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<td>50</td>
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<td>50</td>
<td>4105</td>
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<td>2006.0 (-)</td>
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<td>(3982 - 4347)</td>
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<td>3448</td>
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<td>(3311 - 3544)</td>
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<td>Sodium chromate</td>
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<td>25</td>
<td>4106</td>
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<td>3807</td>
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<td>100</td>
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<td>3170</td>
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<td>(3013 - 3306)</td>
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<td>(0.91 - 0.97)</td>
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<td>(0.91 - 0.97)</td>
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<td>0.71</td>
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<td>(0.68 - 0.74)</td>
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<td>(0.68 - 0.74)</td>
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<td>(0.49 - 0.53)</td>
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Table XIV. Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature 23 ± 1°C. (Sodium chloride and Sodium chromate)
Table XV

<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests (100 daphnids / test)</th>
<th>Oxygen tension (p.p.m.)</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (p.p.m.)</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chromate</td>
<td>50</td>
<td>0.306 (0.60 - 0.61)</td>
<td>3.17</td>
<td>1.7</td>
<td>0.10 (-)</td>
<td>16.7 (-)</td>
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<tr>
<td></td>
<td>100</td>
<td>0.41 (0.38 - 0.44)</td>
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<tr>
<td>Sodium chromate</td>
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<tr>
<td></td>
<td>25</td>
<td>0.73 (0.71 - 0.75)</td>
<td>5.6</td>
<td>5.8</td>
<td>0.21 (-)</td>
<td>29.2 (-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.52 (0.49 - 0.56)</td>
<td>12.2</td>
<td>0.19 (-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.365 (0.35 - 0.38)</td>
<td>8.6</td>
<td>0.15 (-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium bisulfite</td>
<td>25</td>
<td>126.6 (115.9 - 145.0)</td>
<td>25.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;old&quot;</td>
<td>50</td>
<td>91.2 (80.5 - 96.6)</td>
<td>20.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>61.4 (55.9 - 64.1)</td>
<td>14.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>205.3 (201.0 - 209.6)</td>
<td>4.3</td>
<td>79.7 (+)</td>
<td>63.5 (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>148.9 (145.5 - 152.3)</td>
<td>4.7</td>
<td>57.7 (+)</td>
<td>63.3 (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>109.7 (101.4 - 116.9)</td>
<td>14.3</td>
<td>47.3 (+)</td>
<td>72.0 (+)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table XV. Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature 23 ± 1°C. (Sodium chromate and Sodium bisulfite)
<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests</th>
<th>Oxygen tension (p.p.m.)</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric $O_2$ (p.p.m.)</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric $O_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bisulfite</td>
<td>1</td>
<td></td>
<td></td>
<td>25</td>
<td>162.3</td>
<td>26.7 (+)</td>
<td>21.3 (+)</td>
</tr>
<tr>
<td>&quot;old&quot;</td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>154.9</td>
<td>43.7 (+)</td>
<td>47.9 (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>78.8</td>
<td>15.4 (+)</td>
<td>25.1 (+)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>25</td>
<td>169.6</td>
<td>9.6</td>
<td>34.0 (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(152.3 - 166.9)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>50</td>
<td>125.1</td>
<td>8.8</td>
<td>33.9 (+)</td>
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<td></td>
<td></td>
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<td>(119.8 - 130.3)</td>
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<td>100</td>
<td>70.3</td>
<td>9.5</td>
<td>8.9 (+)</td>
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<td>(67.1 - 73.5)</td>
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<td>Sodium</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bisulfite</td>
<td>3</td>
<td></td>
<td></td>
<td>25</td>
<td>99.1</td>
<td>37.1</td>
<td></td>
</tr>
<tr>
<td>&quot;new&quot;</td>
<td></td>
<td></td>
<td></td>
<td>(84.5 - 11.9)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>72.4</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(57.1 - 65.8)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>57.5</td>
<td>14.4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(53.4 - 61.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>25</td>
<td>127.1</td>
<td>0.3</td>
<td>28.0 (+)</td>
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<td></td>
<td></td>
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<td>(126.9 - 127.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>111.6</td>
<td>7.9</td>
<td>39.2 (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(107.4 - 116.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>99.1</td>
<td>4.9</td>
<td>41.6 (+)</td>
</tr>
</tbody>
</table>

Table XVI. Threshold data for salts determined with \textit{Daphnia magna} at different oxygen tensions. Temperature $23 \pm 1^\circ$C. (Sodium bisulfite "old" and Sodium bisulfite "new")
<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests</th>
<th>Oxygen tension</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (p.p.m.)</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bisulfate</td>
<td>4</td>
<td></td>
<td>25</td>
<td>208.7</td>
<td>(200.3 - 219.4)</td>
<td>9.5</td>
<td>1.7 (♦)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>177.4</td>
<td>(174.7 - 182.7)</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>155.4</td>
<td>(148.4 - 159.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>av. 8.9</td>
<td></td>
<td>25</td>
<td>210.3</td>
<td></td>
<td>3.6 (+)</td>
<td>1.7 (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>183.4</td>
<td></td>
<td>6.0 (+)</td>
<td>3.4 (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>145.3</td>
<td></td>
<td>8.1 (-)</td>
<td>5.6 (-)</td>
</tr>
<tr>
<td>2</td>
<td>av. 1.55</td>
<td></td>
<td>25</td>
<td>159.2</td>
<td>(152.3 - 166.0)</td>
<td>9.0</td>
<td>47.5 (-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>113.4</td>
<td>(110.3 - 115.9)</td>
<td>4.6</td>
<td>64.0 (-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>105.5</td>
<td>(101.0 - 110.0)</td>
<td>8.9</td>
<td>47.9 (-)</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>4</td>
<td></td>
<td>25</td>
<td>6949</td>
<td>(6684 - 7080)</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>5966</td>
<td>(5764 - 6310)</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>5514</td>
<td>(5309 - 5624)</td>
<td>5.9</td>
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</tr>
</tbody>
</table>

Table XVII. Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature 23 ± 1°C. (Sodium bisulfate and Sodium sulfate)
Table XVIII

<table>
<thead>
<tr>
<th>Salt</th>
<th>No. of tests</th>
<th>Oxygen tension</th>
<th>Hours</th>
<th>Threshold values (p.p.m.)</th>
<th>% Range above minimum threshold</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (p.p.m.)</th>
<th>Drop (-) or rise (+) from av. threshold at atmospheric O₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium sulfate</td>
<td>2</td>
<td>25</td>
<td>6490</td>
<td>3.9</td>
<td>459.0 (-)</td>
<td>7.1 (-)</td>
<td></td>
</tr>
<tr>
<td>(contd.)</td>
<td></td>
<td>50</td>
<td>5056</td>
<td>0</td>
<td>210.0 (-)</td>
<td>18.0 (-)</td>
<td></td>
</tr>
<tr>
<td>av.</td>
<td></td>
<td>100</td>
<td>4136</td>
<td>8.4</td>
<td>1379.0 (-)</td>
<td>33.4 (-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4016 - 4264)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25</td>
<td>6097</td>
<td>6.9</td>
<td>852.0 (-)</td>
<td>14.0 (-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>4822</td>
<td>1.8</td>
<td>1144.0 (-)</td>
<td>23.7 (-)</td>
<td></td>
</tr>
<tr>
<td>av.</td>
<td></td>
<td>100</td>
<td>2762</td>
<td>5.0</td>
<td>2762.0 (-)</td>
<td>100.4 (-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2685 - 2819)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table XVIII. Threshold data for salts determined with *Daphnia magna* at different oxygen tensions. Temperature 23 ± 1°C. (Sodium sulfate)
Figure 5. Ammonium chloride thresholds for *Daphnia magna* when exposed to varied oxygen tensions at 23 ± 1°C.

Figure 6. Ammonium sulfate thresholds for *Daphnia magna* when exposed to varied oxygen tensions at 23 ± 1°C.
Figure 7. Calcium chloride thresholds for Daphnia magna when exposed to varied oxygen tensions at 23 ± 1°C.

Figure 8. Sodium carbonate thresholds for Daphnia magna when exposed to varied oxygen tensions at 23 ± 1°C.
Figure 9. Sodium chloride thresholds for *Daphnia magna* when exposed to varied oxygen tensions at 23 ± 1°C.

Figure 10. Sodium chromate thresholds for *Daphnia magna* when exposed to varied oxygen tensions at 23 ± 1°C.
Figure 11. Sodium bisulfite thresholds for *Daphnia magna* when exposed to varied oxygen tensions at 23 ± 1°C.

Figure 12. Sodium bisulfate thresholds for *Laphnia magna* when exposed to varied oxygen tensions at 23 ± 1°C.
Figure 13. Sodium sulfate thresholds for *Daphnia magna* when exposed to varied oxygen tensions at $23 \pm 1^\circ$C.
DISCUSSION

Apparatus and Technique

The primary purpose of this study, as previously mentioned, has been to determine the effects of different low oxygen tensions on the susceptibility of *Daphnia magna* when exposed to the salts tested and reported herein. An investigation of this nature demanded that special apparatus be devised whereby low dissolved oxygen tensions could be obtained and maintained with minimum fluctuation during test periods. Inasmuch as the literature revealed a scarcity of references concerning the type of apparatus needed, much of the technique employed has been original with the author. Therefore, another aim of this study has been to develop, to test for practicability, and to standardize apparatus and technique with which desired and reproducible dissolved oxygen tensions could be obtained and maintained. Several investigators have reported methods by which they were able to control dissolved oxygen tensions (Southgate et al, 1933; Townsend, et al, 1938; Lindroth, 1949; and Jones, 1952). These were adapted to tests with fish, however, and could only be used in testing one concentration of chemical at a time. They were also used mostly for short term experiments. Dr. J. N. Phillips¹ has devised an ingenious method

¹Laboratory of Algal Physiology, Univ. of Texas. Personal communication to the author.
whereby known gas mixtures can be obtained and maintained for long periods. Construction of the apparatus is rather costly, but would well serve, with some modification, the needs of a work of this nature.

It is noteworthy that apparatus used here had certain advantages over those of the first mentioned group of investigators. Gases of known composition in any desired ratio could be mixed with utmost precision. Similarly, since the gas mixing apparatus was designed to operate in series, mixtures could be reduplicated over long periods of time provided changes in temperature and barometric pressure were accounted for. It is further noted that the apparatus would also be suitable for mixing and testing other types of gases. The testing apparatus was advantageous in that several concentrations of chemicals could be tested within the same period of time and under the same given conditions. Thus, more test organisms were used within a single testing period, giving greater numbers from which threshold determinations were calculated. Also, the testing apparatus made possible the exact determination of the beginning of test time. This was accomplished by holding the test organisms in open vials which could be dropped into test solutions at the time desired. In spite of some fluctuation, dissolved oxygen levels were maintained over long periods of time. Fluctuation in levels could be controlled within a minimum standard range, but this may not have been less than that encountered by other workers, inasmuch as no mention is made by them concerning this aspect. In this work, the
maximum deviation above and below the mean oxygen tension was usually less than 0.1 p.p.m. Just how much of the determined fluctuation was actual is not known. Inherent experimental error might have accounted for some change. For example, it was found that introduction of minute air bubbles into the syringe pipette while taking samples would give as much as 0.3 to 0.5 p.p.m. difference in oxygen determinations. Therefore, care had to be exercised in order to eliminate this source of error. Also, some fluctuation might have been due to slight changes in temperature inasmuch as tests were conducted at 23 ± 1°C. Another probable advantage of the methods employed herein over the usual Winkler method was the use of syringe pipettes for micro dissolved oxygen determinations. Greater accuracy was obtained since samples were never in contact with atmospheric air. Similarly, syringe pipettes facilitated speed of determinations.

On the other hand, a critical evaluation of the methods employed herein do reveal some shortcomings and limitations. In describing the testing apparatus it was pointed out that oxygen check points were maintained at either end of the test system proper, and reasons have been given for the use of this procedure. Because of this, it was necessary to assume that oxygen concentrations determined at the bracketing check points were the same or closely approximated the conditions of oxygen tension in the test containers. This assumption seems valid, inasmuch as each of the flasks in the test system contained identical volumes of liquid which took up gases
under identical pressures and rates of flow, and at the same tempera-
ture. Each of these factors has an important role in governing the
solubilities of gases in liquids and each was controlled as closely
as possible. There is, however, the possibility of exception to the
assumption set forth above. It is known that an ionizable solute
in a liquid will cause less gas to be dissolved in that liquid than
would be the case if the liquid contained no solute (Bircher, 1940).
Therefore, it is possible that test flasks which contained solutions
in high concentrations, such as the first and second flasks of the
test system, might actually have had lower oxygen levels than indi-
cated by determinations at the check points. It would seem, how-
ever, that these influencing factors were of no great significance
in this study. The chemicals tested were of relatively low con-
centration and should not have appreciably affected the degree of
oxygen uptake into the solutions. The difference, at the most,
would probably be less than that encountered in apparent fluctuation
throughout the tests. A more thorough investigation of this subject
might be warranted in studies which employ much higher concentra-
tions of salts.

A notation has been made in connection with the above, that
determination of dissolved oxygen in the test solutions was not
possible, inasmuch as the salts tested interfered with the chemical
determination. In the last few years considerable literature has
been published concerning polarographic methods for the determina-
tion of dissolved oxygen (Moore, et al, 1948; Lingane, 1949).
Polarographic methodology has certain advantages over chemical methods (Winkler and modifications thereof) in that interfering substances do not alter the accuracy of determinations. Furthermore, it has been adapted to use with small volumes of liquid. Perhaps the use of such apparatus would shed greater light on the question of just how much difference in oxygen absorption is brought about by various compounds in solution. Polarographs are expensive, however, and may not be warranted for studies of this type.

There are certain limitations to the use of the testing apparatus as described herein. First, the chemicals (or effluents) tested are necessarily restricted to those which are non-volatile and also those which are not easily oxidized. Materials in these categories would, in all probability, lose some of their toxic properties because of the vigorous bubbling they would undergo while adjusting oxygen tensions. An example of alteration of toxicity which probably came about in this manner is that of sodium bisulfite. Results of experiments with this compound are discussed later. However, it is noted at this time that the sulfite radical was apparently oxidized, to some extent, to the sulfate. The latter is less toxic to *D. magna*, therefore threshold for what was thought to be bisulfite were actually too high. A second limitation to the use of the testing apparatus is the size of the bioassay organism. The apparatus was specially designed for use with *D. magna*. It was originally thought, however, that young fish (three to five mm. in length) would be used. Several experiments were conducted using
fish, but without success. From all appearances the fish, five in each of the vials, were asphyxiated because of oxygen depletion in the vial contents. Or perhaps death was caused from a high carbon dioxide build-up in the vial SRW. At any rate, most of the animals died prior to beginning of test time. Several modifications of this procedure were tried, but to no avail. Indications are, then, that test organisms larger than D. magna are not particularly suitable for use in the apparatus.

In parallel with the above critique, it could be argued, in view of the findings with young fish, that D. magna might also have been affected by low tensions of oxygen brought about by consumption from the vial contents. It is unlikely, however, that oxygen declined to critical levels before the beginning of test time. O'Connor (1948), using D. pulex and D. obtusata, found that the maximum oxygen consumption at 27°C, was 0.37 ml. per daphnid per hour or 3.7 ml. per 100 daphnids.

In view of the evaluation of the methods and techniques employed herein, it is felt that they could well be adapted, perhaps with certain modification, to wide usage in studies of a similar nature.

**Test Results**

Each of the compounds tested and reported here has been used by one or more investigators in determining toxicity thresholds for Daphnia magna in solutions of atmospheric oxygen tensions (Naumann, 1938; Anderson, 1946, '48; Fowler, 1950; and Freeman and Fowler,
1953). Tests at lowered oxygen tensions, however, have not been reported in the literature. Most of the thresholds determined at atmospheric oxygen in this study have been found to be higher than those reported by other workers. Fowler (1950), and Freeman and Fowler (1953), reported lower 100-hour thresholds for ammonium chloride, calcium chloride, sodium chloride, sodium chromate and sodium carbonate. They found slightly higher thresholds for sodium sulfate and sodium bisulfite. Anderson, et al, (1948), reported slightly lower 100-hour thresholds for ammonium sulfate. For comparison, however, the work of Freeman and Fowler is of greater significance, inasmuch as testing conditions were similar to those used in this study. There are several possibilities as to why thresholds differ, but from the standpoint of this study the differences are of no particular importance. The objective has been to determine relative differences in thresholds as influenced by lowered oxygen tensions.

Anderson (1948) pointed out that daphnids are much more sensitive to some substances at the time of molting than during the period between molts. He further noted that test periods should be long enough so that all individuals will have had time to molt. Daphnia magna adults molt once every three days or less at 25°C. As the temperature is lowered the duration of molting periods is greater. Young daphnids, under normal conditions, will molt at least two times during a 100-hour test period (Fowler, 1950). As a consequence of these characteristics, the 25- and 50-hour test
conducted under the usual testing procedure have not been considered to be too reliable from standpoints of reproducibility and determination of apparent toxicity. Several investigators have shown for different species of *Daphnia* that feeding brings about greater metabolic activity with consequent shorter duration between instars and faster growth than in the case of starved or semi-starved daphnids (MacArthur and Baillie, 1939; Ingle, et al, 1937; and Baylor, 1948). The testing procedure, as used in this study and previously set forth, has been to use 0.5 mg. of yeast (in suspension) per 10 daphnids in each of the test and control solutions. Feeding of daphnids at the beginning of tests hastened molting and growth. By use of this modification of the usual procedure, it was found that less variation in apparent thresholds occurred, thus resulting in good reproducibility of thresholds. Similarly, the 25- and 50-hour tests were of greater significance. This was the primary aim of feeding the daphnids while they were undergoing tests. It was felt that better comprehension and interpretation of the effects of low oxygen on the susceptibility of daphnids to the chemicals could be gained if 25- and 50-hour tests were used as well as the 100-hour test. This assumption proved to be valid.

Inspection of results show that the per cent variation in threshold values was less than 10% for the majority of tests. However, some of the tests showed variations of 14 to 16%, and in the case of sodium bisulfite as high as 25 to 37%. The average
per cent range for the 86-, 60-, and 100-hour thresholds of all compounds tested was 8.58, 7.07 and 8.02 % respectively. The average per cent range for compounds tested under atmospheric oxygen only was 10.80, 8.49 and 7.69 % respectively. Both groups of percentage values include the unusually high variation found for sodium bisulfite. These high variations have tended to magnify the values of the atmospheric oxygen tests, inasmuch as the total average per cent was calculated from fewer tests. Even so, it is seen that tests conducted at atmospheric oxygen show less variation for the 100-hour thresholds than for the 86- and 60-hour thresholds. This is the anticipated result in view of the fact that molting of daphnids occurred in the earlier phase of tests. On the other hand, averages for all tests, regardless of conditions of oxygen, show about the same degree of variation for 86-, 60-, and 100-hour periods. These data indicate that tests under lowered oxygen have given a greater relative variation in 100-hour thresholds and lower relative variations in 86- and 60-hour thresholds. This was a trend noticed in many of the tests conducted under lowered tensions. It is probably explained by the fact that fluctuation in oxygen level apparently occurred to some extent in all tests. A possible alternative explanation would be the acclimatization of the daphnids to lowered oxygen. It has been found that certain crustacea and fish are able to acclimatize, without apparent ill effects, to as little as 0.2 and 0.7 p.p.m. dissolved oxygen respectively (Van Horn, 1952). It would appear then, that D. magna might have acclimated to the
lowered oxygen, some more than others.

Young *D. magna* tested under lowered oxygen exhibited lower thresholds for each of the compounds (with the exception of sodium bisulfite) than when they were tested at atmospheric oxygen. The degree of threshold drop varied for each of the compounds and conditions of oxygen. Examination of results shows that the greatest relative drop in 100-hour threshold for *D. magna* occurred with the ammonium salts. These were 147.1 and 148.3% for ammonium chloride and ammonium sulfate respectively when tested at approximately 1.5 p.p.m. oxygen. Other compounds, listed in order of greatest relative drop for the same test period and oxygen, are sodium carbonate (107.2%), sodium sulfate (100.4%), calcium chloride (93.9%), sodium chloride (60.7%), sodium bisulfate (45.4%), sodium chromate (39.7%) and sodium bisulfite. With the exception of sodium sulfate, the above relationship was generally the same for 50-hour thresholds. The 25-hour thresholds showed roughly the same tendency as the 50-hour. The difference in threshold drop for sodium sulfate cannot be explained, inasmuch as it supposedly has the same mode of action as the chloride of sodium and calcium, i.e., by modifying the osmotic pressure. However, the data indicate that daphnids were not affected to any great extent during the first 50 hours. This was not the case with calcium and sodium chloride. The differences are immediately seen upon examination of Figures 3, 5 and 9. In passing, it is noted that sodium sulfate, unlike the chlorides, affects the water balance of higher organisms by the impermeability
of the membrane to the sulfate ion (Hawk, et al., 1947; and Abegg, 1950). On the other hand, membranes are much more permeable to chlorides. It is unlikely, however, that such differences in permeability could account for the above observed effects, since each of these salts produce the same end result, i.e., dehydration. Another interesting relationship exists between some of the other salts tested at the 1.6 p.p.m. oxygen level. It is seen that drops in 25-hour thresholds for ammonium chloride, ammonium sulfate and sodium carbonate are similar. Each showed a relatively greater percentage drop between 25-hour thresholds than between 100-hour thresholds. This would indicate that the low oxygen tension exerted just as great an effect during the first 25 hours as it did at the end of 100 hours. Other tests did not show this trend.

The usual effect of low oxygen upon the susceptibility of daphnids to the test solutions was one of gradation. That is to say, the relative lowering of thresholds increased with the duration of the test period.

Upon comparing the threshold data obtained at an approximate 1.5 p.p.m. oxygen concentration with those data obtained at the 3.0 p.p.m. level, it is noted that the relationship of greatest relative drops in threshold is altered. Sodium carbonate thresholds, relatively speaking, show the greatest decrease as influenced by lowered oxygen. Drops for each of these thresholds are at least 60% below thresholds at atmospheric oxygen. The only other compound which shows decrease of thresholds below 60% is ammonium sulfate. The
remainder show less than 50\% drop in threshold values at the 3.0 p.p.m. oxygen level. Therefore, these data indicate that daphnids tested at this oxygen level are most affected by sodium carbonate and ammonium sulfate. The other compounds, listed in order of greatest relative threshold drop are sodium chloride, ammonium chloride, calcium chloride, sodium sulfate, and sodium chromate. Sodium bisulfate was not tested at this oxygen level. This relationship did not exist for all 25-hour thresholds, but in general it is indicative of the trend.

Few tests were conducted at the 4.5 p.p.m. oxygen level since it was found in preliminary experiments that daphnids were apparently not seriously affected. Results of these tests with sodium chloride and ammonium sulfate showed slight drops in threshold values, but in most cases these were not statistically significant. However, it is noted that those threshold decreases which were apparently significant were at the 100-hour level. This would indicate that daphnids were not affected appreciably until the latter part of the test periods.

It is felt that results of tests conducted with sodium bisulfate and bisulfite deserve separate discussion, inasmuch as findings with the latter are confusing to say the least. Preliminary experiments with bisulfite indicated that *D. magna* was not at all hampered by lowered oxygen tensions in warding off effects of this salt. In fact, results show that thresholds rose above those at atmospheric oxygen. This would imply, then, that low oxygen was
antagonistic to the effects of sodium bisulfite on *D. magna*. Since this salt imparts a low pH, it appeared that daphnids were more resistant to a low pH when in a low oxygen environment than when in a high or atmospheric oxygen. However, in view of later work the above apparentances are unlikely. It was found in subsequent investigations that oxidation of bisulfite solutions by use of high oxygen content had actually brought about a chemical reaction whereby the sulfite radical (SO₃) was altered to sulfate (SO₄). Determinations were made with a Hellige turbidimeter according to the method of Sheen, et al, (1935). Taking into account the concentration of sulfates in SRW, it was found that of 125 p.p.m. sulfite originally started with, 70 p.p.m. was oxidized to sulfate in 90 hours when dissolved oxygen was kept at about 9.6 p.p.m. Similarly, 16 - 20 p.p.m. was oxidized to sulfate in about 12 hours. Therefore, it has been assumed that thresholds for *D. magna* at lowered oxygen tension are higher than thresholds determined at atmospheric oxygen because of chemical oxidation and ensuing loss of toxicity. Some oxidation of sulfite probably occurred due to the vigorous bubbling of gases as they were forced through test solutions. Also, since oxygen in gas mixtures was always present under slight pressure within the flasks, additional oxidation probably occurred. It is further assumed that less oxidation occurred in the open test bottles under atmospheric conditions than occurred in flasks in the test system. The question arises then: were the tests conducted in the open bottles actually at atmospheric oxygen? Probably not,
Inasmuch as some oxidation occurred in these solutions too, this factor could not be checked since dissolved oxygen determination could only be taken from the control bottles which contained SRW. Subsequent tests with sodium bisulfate showed that sulfate is less toxic than sulfite. Therefore, the more oxidation that occurred, the less toxic was the sulfite-sulfate mixture. In general, the threshold values, when plotted, show this trend (Fig. 7).

Another factor to consider in the transition of sulfite to sulfate is that of pH. In solutions of bisulfite a low pH is produced, the degree depends upon concentration. The pH of threshold concentrations was about 6.9. The lower pH limit tolerated by *D. magna* is about 6.0 (Anderson, 1946). When sulfite is converted to sulfate there results a greater hydrogen ion concentration from the formation of bisulfate. Therefore, the question comes up as to which component produces the greatest toxicity. Apparently the toxicity of the bisulfite plus its pH effect is slightly greater than that of bisulfate plus its slightly lower pH effect since subsequent tests with sodium bisulfate solutions showed higher thresholds than with bisulfite. Therefore, it is further assumed that the toxicity of bisulfite solutions tested was lessened by oxidation of some sulfite to sulfate. In connection with this, it is noted that pH effect of bisulfite apparently contributed most of the toxicity during early periods of testing. It was found that daphnids in bisulfite buffered with weak sodium hydroxide solution to pH 6.8 - 7.0 showed higher thresholds (809 p.p.m.) at the end of
25 hours. However, at 50 and 100 hours the thresholds had dropped to about the same level for unbuffered tests.

During turbidimetric determinations it was found that the stock sodium bisulfite which was used for testing had been partially oxidized in the bottle. This meant that tests had been conducted with impure bisulfite. Subsequent tests employed a new supply of bisulfite and, as previously pointed out, the thresholds for daphnids were slightly lower. For these reasons the two groups of tests with sodium bisulfite have been designated as "old" bisulfite and "new" bisulfite. In view of the uncertainty of the nature of the sodium bisulfite and conditions of testing, the author does not consider experiments with this compound conclusive.

As previously stated, tests with sodium bisulfate were conducted with the intention of gaining information which might help explain results encountered with bisulfite. Tests of high oxygen were run as a check against high oxygen tests with bisulfite. Results showed that thresholds were not appreciably changed from those found at atmospheric oxygen. It would seem that these results give further support to the belief that rises in thresholds found for bisulfite were due to oxidation of the sulfite to sulfate. Similarly, since both materials produced a low pH it was found expedient to test at lowered oxygen. Results showed that daphnids were more susceptible to low pH at lowered oxygen. The decrease in thresholds, however, was not great. The drop at a 1.56 p.p.m. oxygen tension was 45% for the 100-hour test. This would indicate that
Daphnids subjected to low oxygen are not as susceptible to low pH as they are to the high pH imparted by sodium carbonate. Generalizations as to all pH effects cannot be made, inasmuch as only two compounds (excluding sodium bisulfite) which show pH effects were tested.

The objective of this work has not been one of determining the modes of action of the chemicals used, inasmuch as this has been established by other investigators. However, it is expedient to consider these aspects so that lowered oxygen effects on the susceptibility of *D. magna* to the salts might be more clearly defined. Ellis (1936, '46) states that aquatic organisms are affected by compounds either through changes in pH or by the compounds being specifically toxic. Anderson (1946) notes that all salts are toxic when they are present in concentrations high enough to exert unfavorable osmotic pressure. Most investigators engaged in bioassay testing usually classify compounds into one of three groups: (1) those truly toxic, (2) those altering pH, and (3) those modifying the osmotic pressure. Most of the salts tested in this study fall into the last group. Sodium chromate is a "truly toxic" compound. Sodium bisulfite and bisulfate produce a low pH, whereas sodium carbonate imparts a high pH. High concentrations of the ammonium salts will give a lowered pH, but not in amounts used here. It is seen then, that *D. magna* is apparently most affected by low oxygen when subjected to the salts which altered osmotic pressure and to sodium carbonate which produced a high pH. Daphnids were least
affected by the "truly toxic" compound sodium chromate in combination with low oxygen. There is a paucity of literature pertaining to studies of a nature similar to this. Nothing has been found concerning the effects of lowered oxygen tensions on the susceptibility of invertebrates to chemicals. However, the findings reported here are in agreement with investigations conducted with fish. Southgate, et al, (1933) found that the toxicity of potassium cyanide and p-creosol to salmon was increased as the oxygen tension was lowered. Similarly, Townsend, et al, (1944) found that pH of water had a definite effect upon silver salmon to withstand low dissolved oxygen. These authors concluded that low dissolved oxygen concentrations accompanied by changes in hydrogen ion concentration are important factors contributing to mortality of fish in polluted areas. Neither of the writers presented their interpretations as to the underlying cause of such effects, but it is significant that their results are indicative of the trend found for the invertebrate, _D. magna_.

Fritsche (1916) made determinations of the freezing-point depression of the blood of _Daphnia magna_ and found that the osmotic concentration was consistently higher than that of the surrounding medium. According to Krogh (1939), _D. magna_ is a homeoosmotic organism and concentration differences across its membranes can be regulated to some extent. In the case of salts whose action is one of creating an imbalance in osmotic pressure, the main effect is a shift of water from the organism into the surrounding medium.
In order to offset ensuing dehydration the organism must attempt to arrive at a "steady state" between internal and external medium. This can be accomplished only by the steady expenditure of energy in special mechanisms adapted for this purpose. Similarly, *D. magna* can maintain isotonicity to a certain extent by absorbing some salts (Fritsche, 1915); this too requires expenditure of energy. Rosenfels (1935) has shown that uptake of bromide into *Elyodes* cells was brought to a standstill by lack of oxygen. It appears then that much of the observed effect of lowered oxygen tensions on the susceptibility of *D. magna* to the salts tested, particularly those which have their main action by altering osmotic pressure, is due to lowered energy potentials. Besides factors such as ion absorption mechanisms and osmoregulation, others of great importance which are intimately associated with energy potential are undoubtedly concerned. Such regulatory mechanisms as oxidation-reduction potentials and blood buffering systems probably lose, in part, their efficiency of normal function under the handicap of lowered oxygen. However, this is merely conjecture on the part of the author, inasmuch as no information was gathered in this study concerning these factors.

In considering the experimental data obtained from the foregoing tests, it is difficult to speculate on the application of such data to this or other organisms in their natural habitat. The knowledge of the actual lower limit of dissolved oxygen, for most aquatic organisms, is lacking. There is a great deal of literature pertaining to the subject, especially for fish, but much of it is mean-
ingless and cannot be correlated. The minimum level of oxygen, of course, varies with the species concerned, the age of the organism, prior acclimatization, activity, and with temperature. Moore (1942) conducted tests with several species of fish and found that the median fish will die at oxygen tensions of 3.1 p.p.m. in summer and 1.4 p.p.m. in winter. Ellis (1937) states that 3.0 p.p.m. oxygen is regarded as hazardous to fish life. In contrast, Lindroth (1949) reported that salmon parr lived at least five days at 2.2 p.p.m. of oxygen and in winter they tolerated 1.0 to 2.0 p.p.m. Mollusks show good resistance to low oxygen and will live for weeks at very low tensions (Mitchell, 1914). Acclimatized minnows and crayfish lived five days in water containing as little as 0.7 and 0.2 p.p.m. dissolved oxygen respectively when the temperature was held at 12°C. (Van Horn, 1952). Lindroth (1949) found the lower limit of oxygen for crayfish to be 1.4 p.p.m. at 16°C. The lower tolerance limit for _D. magna_ at 23 ± 1°C. is apparently around 1 p.p.m. dissolved oxygen. In preliminary experiments it was found that some daphnids died at oxygen concentrations near the 1 p.p.m. level, whereas mortality did not occur at approximately 1.5 p.p.m. in standard reference water.

In view of the foregoing it is seen that there are many factors which must be considered in estimating the minimum safe concentration of oxygen at which aquatic organisms will be unharmed under natural conditions. Whatever agreement is eventually reached, the findings of this study give additional proof that the presence
or absence of toxic substances is another important factor to be considered before a final evaluation as to the minimum safe concentration of oxygen can be made. Similarly, before safe minimum concentrations of chemicals can be established, the effects of low oxygen concentrations must be taken into account.
SUMMARY AND CONCLUSIONS

1. Apparatus was devised with which mixtures of air and nitrogen were obtained in desired ratio. Gas mixtures could be reduplicated over a 100-hour test period provided changes in barometric pressure were accounted for.

2. Testing apparatus was devised whereby tests with solutions of inorganic salts were conducted by use of Daphnia magna exposed to varied oxygen tensions. Oxygen levels were maintained throughout 100-hour test periods by admitting gas mixtures under known, constant pressures and rates of flow. The maximum deviation above and below the mean oxygen tension was usually less than 0.1 p.p.m.

3. Tests at different oxygen tensions were conducted with nine salts: ammonium chloride, ammonium sulfate, calcium chloride, sodium carbonate, sodium chloride, sodium chromate, sodium bisulfite, sodium bisulfate, and sodium sulfate. Each of these is known to be present in various types of industrial effluents. The 25-, 50-, and 100-hour median toxicity thresholds for Daphnia magna were calculated for each test.

4. Twenty-five and 50-hour thresholds for Daphnia magna tested with sodium chloride and ammonium sulfate at approximately 4.5 p.p.m. dissolved oxygen showed no significant decrease from those thresholds at atmospheric oxygen. One hundred-hour thresholds...
did show slightly significant decreases. It is concluded that this oxygen level does not appreciably alter the susceptibility of daphnids to these compounds until the latter part of test periods.

5. Most of the 25-, 50-, and 100-hour thresholds for *Daphnia magna* tested with seven of the salts at approximately 3.0 p.p.m. dissolved oxygen showed further decreases from those thresholds at atmospheric oxygen. Some of the 25-hour threshold drops were not significant. The compounds listed in order of greatest relative drop in thresholds for the 100-hour period are: sodium carbonate, ammonium sulfate, ammonium chloride, sodium chloride, calcium chloride and sodium chromate. The first two salts showed threshold drops greater than 50% while the remainder showed drops less than 50%.

6. The 25-, 50-, and 100-hour thresholds for *Daphnia magna* tested with eight of the salts at approximately 1.5 p.p.m. dissolved oxygen showed still further decrease from those thresholds at atmospheric oxygen. The compounds listed in order of greatest relative drop in 100-hour thresholds are ammonium sulfate, ammonium chloride, sodium carbonate, sodium sulfate, calcium chloride, sodium chloride, sodium bisulfate and sodium chromate. Threshold drops for the first five salts listed indicated decreases of 100% or more. The greatest drop was 148%.

7. Thresholds for *Daphnia magna* tested with sodium bisulfite at the lowered oxygen tensions showed rises above those thresholds
at atmospheric oxygen. Tests at high oxygen, approximately 9.0 p.p.m., showed similar results. These observed responses have been attributed to oxidation of sulfite to the less toxic sulfate. Tests with this salt are not considered conclusive inasmuch as the nature of the chemical and the testing conditions were unknown.

8. Experimental results indicate that *Daphnia magna* in the presence of lowered oxygen tension is most susceptible to those compounds which alter osmotic pressure and to sodium carbonate which imparts a high pH. Results further indicate that daphnids are least affected by the "truly toxic" compound, sodium chromate, and by sodium bisulfate which imparts a low pH.

9. Analysis of the data conclusively shows that *Daphnia magna* tested under lowered oxygen tensions exhibited lower thresholds than when tested at atmospheric oxygen. This was indicated for all salts tested, with the exception of sodium bisulfite. The degree of threshold drop depended upon the oxygen tension: decrease in oxygen progressively increased the susceptibility to the compound.

10. The toxicity of inorganic salts to *Daphnia magna* varies with oxygen tension, with low oxygen being synergistic.


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