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A STUDY OF WATER-SOLUBLE INHIBITORY COMPOUNDS (ALGICIDES) PRODUCED BY FRESH-WATER ALGAE

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TABLE OF CONTENTS

LIST OF TABLES

Antibiotic Production by The Green Alga, *Pandorina Morum*

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ABSTRACT

A complex system of growth inhibitors was observed in the green algae (Volvocaceae). Inhibitors were found in the culture filtrates of some genera which limit their own growth (auto-inhibitors) while others in the family produce substances which check the growth of other genera (heteroinhibitors). These inhibitors were destroyed by autoclaving. It was decided that Pandorina morum, which produced the strongest inhibitor and Volvox tertius, the most sensitive to the inhibitor would make an excellent model system for a study of the chemical and physical properties of these naturally occurring algicides. The algicide could be removed from actively growing cultures about the 12th day after inoculation and maximum inhibition was recorded for the next 18 days. The substance could be diluted several times with the retention of at least partial activity. The inhibitor was relatively stable to high temperatures, moved slowly through a dialysis membrane and possessed anti-bacterial properties in that it inhibited the growth of Staphylococcus aureus. The material was relatively stable when exposed to acid, although exposure to a pH of 2.0 for 30 minutes did destroy most of the activity. The substance was soluble in benzene and chloroform. All attempts to degrade or destroy the inhibitor with the common proteolytic enzymes (trypsin, chymotrypsin and

pronase) proved unsuccessful, suggesting the substance is not proteinaceous in nature. In experiments with G-25 and G-50 Sephadex, the inhibitor was retained on the column, indicating a molecular weight of less than 5000. The Clark type oxygen electrode revealed that the inhibitor greatly reduced photosynthetic rates in Volvox. A 65% reduction in the rate of photosynthesis was observed after several minutes exposure to medium in which Pandorina morum had been growing. Respiration rates were apparently unaffected.

DESCRIPTORS:

algal toxins, aquatic algae, algal poisoning, aquatic weed control, Chlorophyta, nuisance algae, algal control, freshwater algae, scum, water pollution sources.

INTRODUCTION

The presence of algicides in culture filtrates of algae have been demonstrated by several workers (Harris, 1970; Von Denffer, 1948; Jörgensen, 1956; Mast, 1938; Levring, 1945; Pratt, 1942; Rice, 1954; Lefevre, 1952). During a recent physiological study, the author observed that Platydorina caudata Kofoid, a colonial green flagellate, produces a heat labile, extra-cellular substance which inhibits or checks its own growth (Harris, 1970).

In the present study several genera were examined from the same family (Volvocaceae) to determine if algicides are of common occurrence in this group of green algae. The production of naturally occurring substances which inhibit or check an organisms's own growth (autoinhibitors) as well as substances which inhibit the growth of other organisms (heteroinhibitors) were considered.

The manner in which these inhibitors stop or retard growth, their chemical nature and the effects of these substances upon other aquatic organisms, such as bacteria, fungi, protozoans, etc., are largely unknown. It is hoped that this and future studies on these naturally occurring algicides will shed some light on a method to control the indescriminate growth of algae in all water supplies. The biological controls are preferable to the chemical means in that the former have no adverse effect

on the environment. However, no biological control exists at the present and when we are forced to control or check nusiance algal growth, we must resort to the application of some chemical treatment.

MATERIALS AND METHODS

The strains employed for this study, with the exception of Platydorina caudata (Kan-3H and Kan-1E), were obtained from the Culture Collection of Algae at Indiana University. The Platydorina strains were obtained by the author as plankton from a natural population collected in 1965 in Neosho County, Kansas. The Indiana strains used in this study were Volvox globator (LB 106), Volvox tertius (LB 132), Pandorina charkowiensis (840), Pandorina morum (18), Eudorina Californica (LB 809), Eudorina cylindrica (1196), Eudorina elegans (1207), Eudorina illinoisensis (808), Gonium pectorale (197), Volvulina pringsheimii (LB 1020) and Volvulina steinii (LB 1524).

Cultures were maintained axenically at 20°C. Light was supplied by banks of cool white fluorescent tubes giving an intensity of 250-300 ft-c. with a regime of 8 hr darkness and 16 hr light. The cultures used for the survey of the inhibitory substances were grown in 500 ml of volvox medium (Provasoli and Pintner, 1959) in 200 ml Erlenmeyer flasks.

The survey for the presence of these inhibitors was performed in the following manner: (a) the cells were removed from the growth medium after 21 days growth by filtration through No. 1 filter paper. (b) 10 ml of the cell free medium was pipetted into several 18X150 mm culture tubes. (c) half the

tubes were autoclaved while the contents of the remaining tubes were filtered through a Millipore HA membrane (pore size 0.45u). (d) the tubes were inoculated with all test genera (5 - 10 colonies) in all possible combinations and incubated 14 days. (e) the tubes were checked for growth every 24 hr by optical density readings at 400 mu or in some cases checked by visual observations. (f) since inhibition was not present in the autoclaved tubes the amount of growth in the autoclaved culture filtrates was adopted as the standard. Earlier experiments demonstrated that fresh medium and autoclaved culture filtrates yielded approximately the same growth.

The following computations were made to determine the percentage of inhibitory activity of the test medium:

$$100 - \left(\frac{\text{Optical density, millipore filtered}}{\text{Optical density, autoclaved}} \right) \times 100 = \% \text{ Activity}$$

All reactions in which oxygen evolution was determined were carried out at 20°C in a water-jacketed, closed-system, reaction chamber constructed of lucite. The Clark electrode was employed to measure the rate of oxygen consumption and evolution in this closed chamber of 1.1 ml fluid volume. The flat surfaces of the horse-shoe-shaped chamber served

as the window for the light. The electrode was calibrated for oxygen evolution by calculating the solubility coefficient for oxygen in water. Light was obtained from tungsten lamps filtered through heat absorbing filters to exclude light in excess of 800mu. Respiration was measured as oxygen consumption and photosynthesis was measured as oxygen evolution.

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RESULTS

It was observed during the initial phases of this study that a rather complex system of growth inhibitors (algicides) are present in the family Volvocaceae. Medium in which Pandorina morum had grown was especially toxic and inhibited growth of all other genera examined, with the exception of Eudorina californica. Culture filtrates from Volvulina pringsheimii and Eudorina californica were inhibitory to most of the members of the family. Some autoinhibition was observed in the genus Volvulina. The genera surveyed and the patterns of inhibition are summarized in Table 1. In Table 2, the genera are ranked in the order of strongest inhibitor production. Based on the results summarized in Tables 1 and 2, it was decided that Pandorina morum, which produced the strongest inhibitor, and Volvox tertius, the most sensitive to the inhibitor would make an excellent model system for a study of the chemical and physical properties of these naturally occurring algicides. Except where specifically stated, the results obtained in the remainder of the study were derived from this model system.

To study the kinetics of the inhibitory substance in a growing culture of Pandorina morum, 20 ml of the culture medium were aseptically withdrawn at 48 hr intervals and assayed for activity. The results are shown in Table 3. Inhibition was

TABLE I

INHIBITION OF GROWTH IN SEVERAL VOLVOCACEAN GENERA BY CULTURE FILTRATES FROM MEMBERS OF THE SAME FAMILY

	Inoculum (test organism)										
Culture filtrate from	E. cylindrica	Pd. charkowiensis	G. pectorale	P. morum	V. globator	E. illinoinensis	V. pringsheimii	V. tertius	E. elegans	E. californica	P. caudata
Eudorina cyclindrica	0	+		+	+	+	+	+			
Pandorina charkowiensis		0		+			+		+		
Gonium pectorale		+	0		+		+	+			+
Pandorina morum	+	+	+	0	+	+	+	+	+		+
Volvox globator		+		+	0	+	+		+		
Eudorina illinoinensis	+	+		+	+	0		+	+		
Volvulina pringsheimii		+	+	+	+		A	+	+		+
Volvox tertius						+	+	0			
Eudorina elegans		+	+	+	+		+		0		
Eudorina californica	+	+		+	+		+	+	+	0	
Platydorina caudata		+			+		+	+			A

\+ = At least 20% inhibition of growth

A = Autoinhibition

0 = No autoinhibition

TABLE 2

RANKING OF INHIBITORS FOUND IN THE CULTURE FILTRATES OF SEVERAL MEMBERS OF VOLVOCACEAE

Organism	Inhibitor Production
Pandorina morum	+++
Volvulina pringsheimii	+++
Eudorina cylindrica	+++
Eudorina illinoinensis	+++
Pandorina charkowiensis	++
Eudorina californica	++
Platydorina caudata	++
Volvox tertius	++
Gonium pectorale	++
Eudorina elegans	+
Volvox globator	+

+++ = Excellent inhibitor production

++ = Fair inhibitor production

+ = Poor inhibitor production

TABLE 3

APPEARANCE OF THE INHIBITOR IN MEDIA PRODUCED BY *PANDORINA MORUM*

Days after inoculation	Growth		
	millipored	autoclaved	Controls Fresh medium
4	+++	+++	+++
6	+++	+++	+++
8	+++	+++	+++
10	+++	+++	+++
12	0	+++	+++
14	0	+++	+++
16	0	0	+++
18	0	0	+++
20	0	+	+++
22	0	+	+++
24	0	+	+++
26	0	+++	+++
28	+	+++	+++

+++ = Excellent growth

++ = Fair growth

+ = Poor growth

0 = No growth

observed about the 12th day after inoculation. By the 30th day after inoculation, activity had decreased and growth could be observed in the assay tubes. It was also observed that the material was partially destroyed by heat as recorded by growth in the autoclaved conditioned medium.

To determine the effects of the inhibitor on other members of the family, conditioned medium was harvested, divided into 10 ml samples and inoculated with several colonies. The results are summarized in Table 4. It was observed that medium in which Pandorina morum had grown inhibited most members of the group. Best inhibition was observed on Volvox tertius and Volvulina pringsheimii.

To ascertain to what extent dilution of the conditioned medium was possible with the retention of at least partial inhibitory activity, conditioned medium from 21 day cultures was harvested and diluted to various concentrations with fresh volvox medium. It was observed that dilutions varied with different batches of conditioned medium. These variations were attributed to such factors as strain differences and the time at which the medium was harvested. In general, however, the medium could be diluted 15 - 20 times with the retention of at least partial activity.

The inhibitor was relatively stable when exposed to high temperatures, i.e. 30 minutes at 100° C only slightly affected

TABLE 4

EFFECT OF THE INHIBITOR PRODUCED BY PANDORINA MORUM ON SEVERAL GENERA OF THE VOLVOCACEAE

Genus	Growth		
	Controls Fresh medium	Autoclaved extract	Millipored extract
Gonium pectorale	0.95	0.93	0.38
Pandorina morum	0.51	0.47	0.55
Pandorina charkowiensis	0.34	0.33	0.10
Volvulina pringsheimii	0.31	0.38	0.06
Eudorina cylindrica	0.79	0.85	0.44
Eudorina elegans	0.43	0.38	0.21
Eudorina illinoinensis	0.35	0.41	0.43
Volvox globator	0.10	0.10	0.04
Volvox tertius	0.06	0.05	0.01

Growth was recorded by measuring Optical Density at 400 mμ

the activity of the inhibitor. However, it was observed that heat inactivation of the substance was proportional to the concentration of the inhibitor. Material concentrated several times was more stable than that which had not been concentrated.

To gain some insight about the molecular size of the inhibitor and to examine the possibility that cessation of growth might be caused by depletion of some important component of the medium, 10 ml samples of conditioned medium were dialyzed against fresh volvox medium for periods varying from 24 to 48 hrs. The medium was changed every 12 hrs (approximately 400 ml per change). The dialysis was carried out at 4°C utilizing a magnetic stirrer to aid diffusion. The results are summarized in Table 5.

To examine the possibility of anti-bacterial properties, small wells (0.9 cm in diameter) were cut in Soy Broth agar plates. These plates were streaked with one of several bacteria, such as Escherichia coli, Streptococcus faecalis and Staphylococcus aureus. The plates were incubated at room temperature (20 - 22° C) while the wells were kept moist with conditioned medium. Inhibition was recorded as a circular zone around the well which showed no bacterial growth. A zone of inhibition was observed with Staphylococcus aureus.

TABLE 5

DIALYSIS OF THE INHIBITOR PRODUCED BY PANDORINA MORUM

Dialyzed		Non-dialyzed	
millipored	autoclaved	millipored	autoclaved
+	+++	0	+

+++ = Excellent growth

++ = Fair growth

+ = Poor growth

0 = No growth

The stability of the inhibitory substance in various concentrations of acid was examined as follows: 10 ml samples from a single batch of conditioned medium with an original pH of 8.0 were placed in each of several tubes and titrated with 1N HCL to pH values ranging from 2.0 to 7.0. The samples were kept at the various pH values for 30 min and then titrated back to pH 7.0 with 1N NaOH. The samples were then millipored, inoculated with bacteria-free colonies and incubated under standard culture conditions. After 14 days of incubation growth was assayed. The results are summarized in Table 6.

Several organic solvents and their effects upon the activity of the toxin were examined. In this experiment a 1 : 1 mixture of conditioned medium plus the solvent in question was prepared. These were mixed in a separatory funnel, shaken and allowed to stand at toom temperature for several minutes. The conditioned medium was separated from the solvent and assayed for activity. The results are summarized in Table 7.

To get some idea as to whether or not this inhibition of other members of the group occurs in mixed culture the following experiment was performed. A flask with 2 liters

TABLE 6

STABILITY OF THE SUBSTANCE AFTER 30 MIN EXPOSURE TO ACID pH

pH (for 30 minutes)	Growth	
	millipored	autoclaved
7.0	0	0
6.0	0	0
5.0	0	0
4.0	0	+
3.0	0	+
2.0	++	++

+++ = Excellent growth

++ = Fair growth

\+ = Poor growth

0 = No growth

TABLE 7

EFFECT OF SEVERAL ORGANIC SOLVENTS ON THE ACTIVITY OF THE TOXIN PRODUCED BY PANDORINA

Solvent	Growth	
	millipored medium	Autoclaved medium
Petroleum ether	0.0	0.0
Benzene	0.7	0.7
Chloroform	0.6	0.7
Carbon tetrachloride	0.0	0.01

Growth estimates were by measuring Optical Density at 400 mμ

of medium was inoculated with colonies representing several members of the family Volvocaceae. The genera are listed in Table 4. Relative percentages of each genus was recorded every 48 hrs over a 21 day period. At the end of the period Pandorina morum represented between 45 - 55% of the total colonies remaining while Platydorina caudata represented 25 - 30%, Eudorina 15 - 20% and the others about 2 - 5%. It is perhaps siginficant to mention that Volvox colonies had completely disappeared after 72 hrs in the mixed culture.

To gain some insight concerning the possibility that the toxin was protein in nature, several proteolytic enzymes and their effects upon the toxin were examined. These enzymes were trypsin, chymotrypsin and pronase. In determining the effect of these enzymes on the level of toxicity, the appropriate dry weight of enzyme was added to 10 ml of conditioned medium and allowed to incubate at room temperature for several hrs. At the end of the incubation period the enzymes were inactivated by heating and the conditioned medium was assayed for activity. No reduction in the level of activity was observed with any enzyme examined.

It was shown that the inhibitor moved slowly through a dialysis membrane; therefore a more critical examination of the molecular size was undertaken using Sephadex gel filtration. All Sephadex gels used in this investigation were packed in standard Pharmacia Company non-jacketed columns (K25/45). Before packing the columns, the gels were allowed to equilibrate overnight in volvox medium. The void volumes were calibrated using a dilute solution of India ink as recommended by the manufacturer. All filtrations were carried out at room temperature. Data and calibrations for all columns used are given in Table 8.

According to the theory of Sephadex filtrations, completely excluded molecules should begin to appear in the effluent after a volume equal to the void volume. Non-excluded molecules should appear after an additional volume equal to the internal volume. Table 9 shows that for the G-25 Sephadex, excluded molecules should begin to appear in the 65 - 70 fraction. The non-excluded molecule should appear in the 140 - 150 ml fraction. The results for G-25 Sephadex are summarized in Table 9. Here we can see activity was

TABLE 8

CALIBRATIONS FOR SEPHADEX COLUMNS

Gel	Mol Wt range of excluded molecule	Bed Volume (ml)	Wt dry Gel (g)	Void Volume (ml)	Internal Volume (ml)
G-25	5000	205	40	66	100
G-50	10000	205	25	56	125

TABLE 9

ACTIVITY OF FRACTIONS COLLECTED IN G-25 SEPHADEX GEL

Fraction collected (including void vol)	Growth		
	millipored	autoclaved	controls (fresh medium)
61 - 70 ml	+++	+++	+++
71 - 80	+++	+++	+++
81 - 90	+++	+++	+++
91 - 100	+++	+++	+++
101 - 110	+++	+++	+++
111 - 120	+++	+++	+++
121 - 130	+++	+++	+++
131 - 140	+	++	+++
141 - 150	0	+	+++
151 - 160	+++	+++	+++
161 - 170	+++	+++	+++
171 - 180	+++	+++	+++
181 - 190	+++	+++	+++
191 - 200	+++	+++	+++

+++ = Excellent growth

++ = Fair growth

+ = Poor growth

0 = No growth

between ml 131 - 150. In G-50 activity was observed between ml 181 - 190.

Some time ago, it was shown that Chlorellin, a substance produced by Chlorella, reduced respiration rates in the genus Chlorella (Swanson, 1943). It was therefore decided that an examination of the inhibitor on the rates of both photosynthesis and respiration would be beneficial.

Actively growing cultures of Volvox globator were harvested after 14 days growth under standard conditions. The colonies were removed from the growth medium by filtering through Whatmans number 1 filter paper and resuspended in a 1 : 1 solution composed of fresh medium and culture filtrate from Pandorina morum. The colonies were then placed in the closed chamber of the oxygen electrode and exposed to total darkness for 5 min to measure oxygen consumption (respiration). This dark period was followed by a 5 min exposure to light in order to measure the rate of oxygen evolution (photosynthesis). Measurements were taken at 4 hr intervals over a 13 hr period. The results are summarized in Table 10. It was observed that 1 hr exposure of Volvox to culture filtrates of Pandorina resulted in a 65% reduction in the rate of oxygen evolution. This reduction increased to 91% after 12 hrs exposure to the inhibitor. Respiration as measured by oxygen consumption was unaffected.

As mentioned earlier several reports of inhibitor production

TABLE 10

EFFECT OF CULTURE FILTRATES FROM PANDORINA MORUM ON OXYGEN EVOLUTION AND OXYGEN CONSUMPTION IN VOLVOX

Hrs after exposure to inhibitor	Hour 1	Hour 4	Hour 8	Hour 12
CONTROLS (FRESH MEDIUM)				
O_2 Evolution [1]	0.018	0.020	0.019	0.020
O_2 Consumption [1]	0.004	0.004	0.005	0.004
AFTER EXPOSURE TO CULTURE FILTRATES				
% inhibition of O_2 evolution	65	88	84	91
% inhibition of O_2 consumption	4	-3	-3	-2

[1] umoles O_2/ug chlorophyll/hr

by the algae have appeared in the literature. However, these have consisted primarily of records of occurrence or studies on physical properties. The work reported in this study is the first firm evidence of a possible mode of action of an algal inhibitor and it appears to be a specific inhibitor of photosynthesis. Work is preceeding to determine the exact site of inhibition within the photosynthesis system.

DISCUSSION

The inhibitor, in an actively growing culture of Pandorina morum, appeared on the 12th day after inoculation and for the next 14 days, inhibition was observed. However, on the 28th day after inoculation, natural degradation of the inhibitor had reduced activity to the point where growth once again was observed in the assay tubes.

The inhibitory substances produced by Pandorina morum inhibited growth of all members of the Volvocaceae (with the exception of Eudorina illinoisensis). Best inhibition was observed with Volvox tertius and Volvulina pringsheimii.

The inhibitory substance could be diluted 15 - 20 times with the retention of at least partial activity. A major problem and thus far observed in every known toxin producing algae, is the apparent lack of a correlation between the level of toxicity and the density of organisms in the medium (Collier, 1958; Gorham, 1960; Shilo, 1953). In some cases, high levels of growth resulted in a high level of toxicity.

The substance was relatively stable to heat. Autoclaving greatly reduced the level of activity but did not completely destroy it. The degree of heat lability was apparently proportional to the concentration of the inhibitor. This differs from the inhibitor produced by Platydorina caudata (Harris, 1970) which was destroyed by only 40 minutes exposure at 60° C.

To get some idea concerning the molecular size of the substance, samples of conditioned media were dialyzed against fresh medium for periods ranging from 24 to 48 hrs. The substance moved slowly through the membrane and a gradual loss of activity could be observed. This also differs from the inhibitor produced by Platydorina in that it did not move through the membrane.

The substance was also examined for anti-bacterial activities and proved to inhibit the growth of Staphylococcus aureus.

It was observed that the inhibitor was relatively stable when exposed to acid conditions. However, exposure of the substance to a pH of 2.0 for 30 minutes did destroy most of the activity. This is similar to the acid response of the autoinhibitory substance produced by Platydorina in that it was also degraded after 30 minutes exposure to a pH of 2.0.

Several organic solvents were surveyed in order to find suitable solvents to aid in future purification procedures. The substance was soluble in both benzene and chloroform.

The inhibitor produced by Pandorina morum stopped or greatly reduced the growth of most members of the Volvocaceae (colonial green flagellates). Best inhibition was observed on Volvox tertius and Volvulina pringsheimii. The only genus in the entire family completely unaffected by the substance

was Eudorina Illinoinensis. In a mixed culture experiment in which all members of the family were inoculated into a large carboy, Pandorina morum quickly became the dominant organism, making up some 55% of the total at the end of a 21 day growth period. Based on the effect of the inhibitor on most members of the family, these results in the mixed culture experiment were certainly not unexpected.

All attempts to degrade or destroy the inhibitor with the common proteolytic enzymes proved to be unsuccessful. This suggests that the substance is not proteinaceous in structure.

Sephadex was used rather extensively to get some idea of the molecular size of the inhibitor. In all experiments with G-25 and G-50, the inhibitor was retained on the column. This indicates a molecular weight of less than 5000. Work is presently in progress to determine more accurately molecular weight using the procedure of mass spectrophotometric analysis.

The Clark type oxygen electrode was used to determine the effects of the inhibitor on respiration and photosynthesis. It was readily apparent that photosynthetic rates were severely affected by the inhibitor. After several minutes exposure to the substance a 65% reduction in activity was observed. However, the rates of respiration were in general unaffected

or at best reduced only slightly.

The substance produced by Pandorina differs markedly from the autoinhibitor produced by Platydorina in that the former is non-specific and stops growth of most algae. Thus far, results suggest that the inhibitor is not proteinaceous and is less than 5000 molecular weight. This differs markedly from the inhibitor produced by Platydorina, a member of the same family.

It is hoped that this and future studies on the toxin (algicide) produced in this system will shed light on a method to control the indescriminate growth of algae in all water supplies, including those used by man. Studies are continuing to determine the chemical structure of the substance and just how these toxins operate biochemically in checking the growth of algae. Hopefully we can eventually synthesize the material in the laboratory.

LITERATURE

Collier, A. Some biochemical aspects of red tides and related oceanographic problems. Limnol. Oceanogr. 3 (1958): 33-39.

Von Dennffer, D. Uber einen Wachstum-Hemmstoff in alternden Diatomeenkulturen. Biol. Zentralbl. 67(1948): 7-13.

Gorham, P. R. Toxic waterblooms of blue-green algae. Can. Vet. J. 1(1960): 235-245.

Harris, D. O. An autoinhibitory substance produced by Platydorina caudata Kofoid. Plant Physiology. 45 (1970): 210-214.

Jørgensen, E. G. Growth inhibiting substances formed by algae. Physiol. Plant. 9(1956): 712-726.

Lefèvre, M., and Jakob, H. Sur quelques proprietes des substances actives tirèes des cultures d'algues d'eau douce. C. R. Acad. Sci. 229(1949): 234-236.

Levring, T. Some culture experiments with marine plankton diatoms. Med. Oceanogr. Inst. Göteborg. 3(1945): 12.

Mast, S. O., and Pace, D. M. The effects of substances produced by Chilomonas paramecium on the rate of reproduction. Physiol. Zool. 11(1938): 359-382.

Pratt, R. Studies on Chlorella vulgaris. V. Some of the properties of the growth inhibitors formed by Chlorella cells. Amer. J. Bot. 29(1942): 142-148.

Provasoli, L., and Pinter, T. J. Artificial media for fresh-water algae; problems and suggestions. In: Tryon, C. A., and R. T. Hartman, Ed., The Ecology of Algae. Spec. Publ. No. 2, Pymatuning Laboratory of Field Biology, University of Pittsburg (1959): 84-96.

Rice, T. R. Biotic influences affecting population growth of plankton algae. Fish. Bull. U. S. 54(1954): 227-245.

Shilo, M., and Aschner, M. Factors governing the toxicity of cultures containing the phytoflagellate Prymnesium parvum Carter. J. Gen. Microbiol. 8 (1953): 333-343.

Swanson, C. A. The effect of culture filtrates on respiration in Chlorella vulgaris. Amer. J. Bot. 30 (1943): 8-11.