MICHIGAN DEPARTMENT OF CONSERVATION
Research and Development Report No. 7*

April 14, 1964

STUDIES ON IRON-BINDING ORGANIC COMPOUNDS FROM MICHIGAN WATERS[1]

By Fred Kent and F. F. Hooper

Introduction

Amines isolated from lake water have been shown to affect the growth rate of algae (Fogg and Westlake, 1955). An artificial chelating agent (HEDTA) was found to influence the iron cycle of a Michigan lake (Schelske, Hooper, and Haertl, 1962). The purpose of the present study is to explore the relationship between various naturally occurring organic compounds and the utilization of iron by algae. By testing the ability of organic extracts from natural waters to increase the growth rate of algae in the presence of iron, we hope to better understand (1) the kinds, amounts, and distributions of naturally occurring iron-binding compounds in Michigan waters and (2) the relative importance of these compounds as regulators of primary production.

---

* Institute for Fisheries Research Report No. 1686.

[1] Contribution from Dingell-Johnson Project F-27-R, Michigan.

Methods

Five-gallon water samples were filtered through a glass tube (400 mm x 60 mm) containing activated cocoanut charcoal (6-14 mesh). Organic compounds were eluted from the charcoal using first a mixture of 100 ml of petroleum ether and 100 ml of ethyl ether followed by 200 ml dichloromethane and finally 200 ml methanol. The solvents were evaporated, the residues were combined and brought to dryness. One ml of methanol was added to the dried residue to dissolve tars which might have prevented the solvent from penetrating the mass. After evaporating the methanol, 30 ml of ether was added and the ether insoluble fraction was removed. The ether solution was then extracted three times with water using 15 ml of water per 30 ml of ether solution. The water soluble portion was removed and evaporated to dryness. The ether layer was extracted with 1 N hydrochloric acid. This extract was made strongly basic with 25% sodium hydroxide in order to free the amines from their acid salts. After cooling, the basic solution was extracted several times with ether and the ether was evaporated to dryness. The resulting residue was the amine fraction.

A yellow organic acid fraction was obtained from the carbon filter residue by extraction with 80% ethanol and acidification with hydrochloric acid. This residue was then dried and extracted with ethyl acetate. The method used has been described by Shapiro (1957). He found yellow organic acid extracts to be hydroxy carboxylic acids which had a phenolic or enolic character.

Algae culture technique.--Chlamydomonas Reinhardi was grown in Beyernick's solution which has the following composition:

NH_4NO_3	- 0.5 gm	K_2HPO_4	- 6 ml M/15
K_2HPO_4	- 0.2 gm	KH_2PO_4	- 4 ml M/15
$MgSO_4 \cdot H_2O$	- 0.2 gm	Distilled water to bring	
$CaCl_2 \cdot H_2O$	- 0.1 gm	volume to 1 liter.	

Flasks containing 200 ml of the culture media were inoculated with 5 ml of a stock culture of Chlamydomonas. Cultures were illuminated continuously with a fluorescent bulb. A mechanical shaker was used to keep the cells in suspension. The extracts being tested (amines or organic acid) were added to test flasks along with iron (as ferric chloride). Control culture flasks contained either the iron or the extract but not both. Experimental and control flasks were incubated for 10 days at laboratory temperature (70 F). After incubation the transmittance of the cell suspension was measured in a spectrophotometer at a wave length setting of 710 mμ.

To determine if the amine fraction had an iron-binding capability the following procedure was carried out. The amine fraction was separated from the algae cultures containing iron (as ferric chloride) by passing the culture through a charcoal filter and eluting and separating the amine fractions as outlined above.

The amine residue was dissolved in 0.1 ml of petroleum ether and a 10 lambda sample was transferred to a piece of No. 1 Whatman filter paper. The sample was subjected to two-dimensional chromatography. The first phase was accomplished by using either of the following two solvent systems.

I.	n-butanol	- 120 ml	II.	ethanol 95%	- 180 ml
	glacial acetic acid	- 30 ml		water	- 10 ml
	water	- 50 ml		ammonia (0.8 N)	- 10 ml

The following solvent system was used for the second phase of the two-dimensional chromatogram: t-amyl alcohol - 30 ml; water - 20 ml; formic acid - 30 ml; ethyl acetate - 20 ml. After the first phase of chromatography a 0.2% solution of ninhydrin in acetone was used to detect the amine on the filter paper. An iron fraction separated from the amine fraction during the second phase of chromatography. Iron was identified by spraying the paper with a solution prepared by dissolving 10 mg of diphenylthiocarbazone in 50 ml of chloroform.

A similar procedure was used to demonstrate that the yellow organic acid extracts held iron. In this case the first phase of the two-dimensional chromatography was carried out with a solvent consisting of 25 ml of pyridine, 25 ml of t-amyl alcohol, 1 ml of diethylamine and 25 ml of distilled water.

Results

In a series of tests made on water samples collected from the Huron River and Whitmore Lake (Livingston and Washtenaw counties, Michigan) we used three sets of controls. The first control consisted of the algae culture media and the extracted amine but lacked iron; the second contained iron but lacked amine; and the third contained only culture solution. After 10 days of incubation the controls containing only iron or only amine did not differ significantly from one another in transmittance. These controls, however, showed a lower transmittance than controls containing only culture medium.

Amine extracts from both the Huron River and Whitmore Lake gave a transmittance after 10 days incubation that was significantly lower than any of the control series indicating that both waters contained amines which enhanced algal growth in the presence of iron (Table 1). Growth of cultures containing amines from the Huron River was significantly greater than growth of cultures containing amines from Whitmore Lake. Since these comparisons are based on unit volumes of samples collected in the field, not upon units of amine, it is not possible to decide whether this is a difference in potency of the two extracts or a difference in concentration of amine in the water. Since the Huron River collects water from a variety of drainages, some diversity in dissolved organic substance might be expected. It should also be pointed out that these extracts were added to culture solutions at concentration levels somewhat higher than are found in natural waters. Hence quantitative differences in the response would be expected when these compounds are present in the concentration range found in nature.

The possibility was evaluated that the amine fraction found in natural waters may be partially or totally bound and that it is released to perform chelation by the filtration and extraction procedures. To investigate this possibility ferric chloride (1%) was added to a 10-gallon water sample from the Huron River until a concentration of 0.05 ppm soluble iron was attained. The sample was then filtered through charcoal. The amine fraction was then separated and added to algae cultures. Even if the iron was added before filtration there was a significant increase in growth rate over the control containing only the amine ($t = 3.15$ for 10 d.f.) without a subsequent addition of iron (Table 2). It is clear that a second addition of iron after filtration further increased the growth rate and it is also apparent that there is a greater increase in growth when iron is added to the culture flask than when iron is added to the original water (Table 2). These results suggest that some unknown substance may have occupied the iron-binding sites on the amine. Filtration and purification of the amine may have removed some of these substances and made these sites available for additional iron binding. Although purification and isolation of the amine fractions enhances its ability to bind iron and thereby increases the growth rate of algae, it is clear from the above tests that the unmodified amine was also active.

The series of waters analyzed from the Upper Peninsula are representative of the numerous small, soft-water lakes within the Hiawatha National Forest in Alger, Delta, and Schoolcraft counties. The lakes studied have methyl orange alkalinities ranging between 3 and 5 ppm. Stoner Lake contains little coloring matter. Timijon, Irwin, and Starvation are colored with humic colloids. Waters and Ball (1957) and Waters (1956, 1957) have discussed limnological features of these lakes and have described attempts to increase their productivity by liming.

Extracts from all of these lakes except Stoner gave a statistically significant decrease in transmittance over that of controls (Table 3). Starvation had a far greater activity than any of these lakes and, in fact, had a much greater activity per unit water volume than any of the waters we have tested to date.

To determine whether or not the yellow coloring matter in these lakes had an iron-binding capacity, the yellow organic acid complexes of Starvation and Stoner lakes were isolated and tested in the same manner as amine extracts. A limited number of tests indicated that the acid extracts from both of these lakes produced growth rate increases in the presence of iron as did the amine fractions. The extract from Starvation gave a higher growth rate increase than the extract from Stoner. This is to be expected since the latter was only slightly colored.

Discussion

The observation that amines isolated from natural water will complex and bind nutrients tends to confirm earlier observations by Fogg and Westlake (1955) of complex formation by peptides isolated from Anabaena. These authors note peptide binding of copper, iron, zinc, and phosphorus. The evidence that naturally occurring amines and organic acids affect primary production through interaction with iron further emphasizes the role played by dissolved organic substances in lake metabolism. Since natural waters appear to differ, at least quantitatively, in these substances, it would seem desirable to study the dissolved organic substances of Michigan lakes and streams on a systematic basis.

Of particular interest are the results from the Upper Peninsula bog lakes. The extraordinary activity shown by the amine fraction isolated from Starvation Lake indicates that amines may be a source of diversity among small colored bog lakes otherwise considered quite similar. Amines may account for the differences noted between Starvation and the other bog lakes in this area. Starvation had phosphorus concentrations ranging from two- to eightfold greater than any of the four lakes studied by Waters (1956). Of the colored lakes treated with lime (Timijon and Starvation) only Starvation gave a well defined biological response (Waters, 1957). After liming this lake developed a bloom of *Microcystis* and a large population of *Dinobryon*. It seems appropriate therefore to suggest that some of the chemical differences between Starvation and the other lakes, as well as the differences in response to lime, might be accounted for by the mobilization of nutrients by amines carried in the lake water.

Acknowledgments

The authors wish to express their thanks to J. S. Marshall, formerly of the Department of Zoology, University of Michigan, who supplied the algae cultures, to James Merna of the Institute for Fisheries Research who assisted in collecting samples, and to J. T. McFadden and James R. Ryckman of the Institute for Fisheries Research who gave us statistical advice.

Literature cited

Fogg, G. E., and D. F. Westlake. 1955. The importance of extracellular products of algae in freshwater. Proc. Int. Assoc. of Limnol. 12: 219-232.

Schelske, Claire L., Frank F. Hooper, and E. J. Haertl. 1962. Responses of a marl lake to chelated iron and fertilizer. Ecol. 43(4): 646-653.

Shapiro, Joseph. 1957. Chemical and biological studies on the yellow organic acids of lake water. Limnol. and Oceanog. 2(3): 161-179.

Waters, Thomas F. 1956. Chemical and biological effects of lime application to bog lakes in northern Michigan. Ph.D. thesis, Michigan State University.

Waters, Thomas F. 1957. The effects of lime application to acid bog lakes in northern Michigan. Trans. Am. Fisheries Soc. (1956) 86: 329-344.

Waters, Thomas F., and Robert C. Ball. 1957. Lime application to a soft-water, unproductive lake in northern Michigan. J. Wildl. Mgmt. 27(4): 385-391.

INSTITUTE FOR FISHERIES RESEARCH

Fred Kent and F. F. Hooper

Report approved by W. R. Crowe

Typed by M. S. McClure

Table 1. --Transmittance of *Chlamydomonas* cultures containing amine extracts from Whitmore Lake and the Huron River (Livingston and Washtenaw counties, Michigan)

Water and extracts	Percentage transmittance after 10 days of incubation						
	Flasks						Mean, and 95% confidence interval[1]
	1	2	3	4	5	6	
Whitmore Lake							
Amine and Iron	76	72	69	75	73	70	72.5 ± 3.3
Amine only (control)	84	83	77	85	79	82	83.2 ± 3.3
Huron River							
Amine and Iron	62	68	60	66	63	65	64.0 ± 3.3
Amine only (control)	81	76	79	83	84	80	80.5 ± 3.3
Culture media only (controls)							
With Iron	80	78	84	77	82	76	79.5 ± 3.3
Without Iron	89	94	96	87	91	90	91.2 ± 3.3

[1] Confidence interval calculated from error term taken from analysis of variance of data given above and data of Table 3.

Table 2.--Effect of filtration and extraction procedures upon amine extracted from the Huron River

Treatment	Percentage transmittance of *Chlamydomonas* culture after incubation for 10 days with amine extract						
	Flasks						Mean and standard error
	1	2	3	4	5	6	
Iron added to water sample before filtration	78	72	81	74	79	76	77.0 ± 1.49
Iron added to culture	67	63	69	74	72	64	68.0 ± 1.49
Iron added to water sample before filtration and to culture	60	69	62	65	63	67	64.3 ± 1.49
Amine only (control)	82	86	88	81	78	83	83.0 ± 1.49

Table 3.--Transmittance of *Chlamydomonas* cultures containing amine extracts from Upper Peninsula lakes (Alger, Delta and Schoolcraft counties, Michigan)

Lake and extract	Percentage transmittance after 10 days incubation						
	Flasks						Mean, and 95% confidence interval[1]
	1	2	3	4	5	6	
Timijon (Amine + Iron)	66	69	64	72	70	68	68.2 ± 3.3
Control (Amine only)	79	85	82	76	84	80	81.0 ± 3.3
Stoner (Amine + Iron)	78	74	70	69	75	79	74.2 ± 3.3
Control (Amine only)	83	80	77	76	84	81	80.2 ± 3.3
Irwin (Amine + Iron)	78	71	72	68	70	73	72.0 ± 3.3
Control (Amine only)	88	80	84	79	82	80	82.2 ± 3.3
Starvation (Amine + Iron)	39	44	34	37	35	40	38.2 ± 3.3
Control (Amine only)	71	78	69	72	74	70	72.3 ± 3.3

[1] Confidence interval calculated from error term taken from analysis of variance of above data and data of Table 1.