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TOXIC WATERBLOOMS OF BLUE-GREEN ALGAE[1]

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ABSTRACT

UNIALGAL CULTURES of several species and strains of blue-green algae, including those most suspected of causing animal deaths, have been grown and found to vary greatly in toxicity. At least four toxic factors have been recognized. One produces fast deaths and is algal in origin. The others produce slow deaths and are bacterial in origin. The fast-death factor (FDF) is an endotoxin that so far has been encountered only with strains of *Microcystis aeruginosa* Kütz. *emend.* Elenkin. Its production is genetically and physiologically controlled. An FDF-producing strain of *M. aeruginosa* has been cultured on a large scale and the cells shown to be toxic when administered orally to sheep, calves, and smaller animals. FDF isolated from these cells has been identified as a quite stable cyclic polypeptide having an intraperitoneal LD_{50} for white mice of 0.47 mg. per kg. body weight. The slow-death factors may also contribute to the toxicity of waterblooms. It is concluded that a complex of interdependent variables determines the degree and kind of toxicity that a waterbloom can develop.

INTRODUCTION

For the past 80 years blue-green algae have been suspected of causing sickness or death to mammals, waterfowl and fish, and allergy, dermatitis or gastro-intestinal disorders in man. There have been several important investigations and the subject has been reviewed by Fitch *et al.* (6), Olson (23), Vinberg (36), Ingram and Prescott (14), Schwimmer and Schwimmer (25), and Grant and Hughes (briefly) (10). In this account, the emphasis will be mainly on animal sickness and deaths, since these are most frequent in occurrence and therefore of greatest importance. The first case was described in 1878 by Francis (7) in Australia. He reported that a waterbloom of *Nodularia spumigera* was responsible for the deaths of sheep, horses, pigs, and dogs. Since then, there have been

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†This paper is based upon a lecture presented before Section 3, Phycology, Ninth International Botanical Congress, Montreal, Que., August 19–29, 1959. It represents the results of a study carried out over a five-year period with various collaborators, as follows: E. F. L. J. Anet, C. T. Bishop, R. E. Harris, E. O. Hughes, M. McBride, B. Simpson, and A. Zehnder, presently or formerly of these laboratories, and H. Konst and P. D. McKercher, Animal Diseases Research Institute, Canada Department of Agriculture, Hull, Que.

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many reports of toxic waterblooms of blue-green algae from different parts the world, such as the U.S.A., Canada, Israel, South Africa, and the U.S.S. There have been at least four published accounts of outbreaks of algal poisoni in Canada during the last 25 years (12, 21, 22, 31), and losses of valuable liv stock, waterfowl, and pets are continually being reported.

With few exceptions, the investigations of toxic algae in Canada and elsewhe have depended upon collections of waterblooms — as and when they ha occurred. Attempts have been made at laboratory culture of the algae suspecte of toxicity. These have either failed (30, 37) or the yields of algae or of tox substances have been low (8, 21, 23) and the approach has, therefore, bee abandoned. The unpredictable occurrence and variable persistence of wate blooms have been a serious handicap to investigators. An additional handicap ha been the fact that the toxicity of a particular bloom sometimes varies widel during a period of a few days (5, 23). It sometimes increases during the earl stages of decomposition (10), but usually disappears in the advanced stage (6, 32, 33). Depending on the state of algal decomposition, the toxic factor o factors may or may not be present in the water surrounding the algal cell (6, 17, 20). There are records of fast deaths (1 to 2 hours or less) and of slo deaths (several hours to 1 or 2 days) following the ingestion of waterbloom (14, 23), but little or no attention seems to have been paid to these difference in survival times. Death may be preceded by pallor, convulsions, dyspnea paralysis of the hind limbs, foamy tears, or other symptoms (14, 23). Live damage is frequently reported, and there is occasional mention of inflammation o the mucous membranes of stomach and intestine. The toxic factor has been reported by various workers to be dialyzable, alcohol-soluble, fairly stable to heat and acid, unstable to alkali. It may be an alkaloid, but is not botulinal toxin, cyanide, cyanogenetic glucoside, arsenic, strychnine, phycocyanin, or an infectious agent (2, 14, 17, 23).

Five species of blue-green algae have been reported toxic (6, 10): *Microcystis aeruginosa* Kütz. *emend.* Elenkin [including M. *toxica* Stephens (30)], *Anabaena flos-aquae* (Lyngb.) DéBred., *Aphanizomenon flos-aquae* (L.) Ralfs, *Coelosphaerium Kützingianum* Nägeli, and *Gloeotrichia echinulata* (J. E. Smith) Richter (Figure 1). Previous studies have not answered two very important questions: 1. Is it the algae, or the bacteria associated with the algae, that produce the toxic factor? 2. Is more than one toxic factor involved? In the work to be described we have endeavoured to answer these questions, at least in part, by using the laboratory culture approach. We have isolated and grown unialgal cultures of a number of different species or strains of blue-green algae (9, 13) and have tested them for toxicity. We have isolated and identified one of the principal toxins (2), and have studied its effects on large and small animals (15). We have also examined the toxicity of the bacterial associates of a toxic strain of blue-green alga (27).

Experiments and Results

Toxic Microcystis

We began by isolating a unialgal strain of *Microcystis aeruginosa*, called NRC-1, from a bloom in which this species was a minor constituent. We then

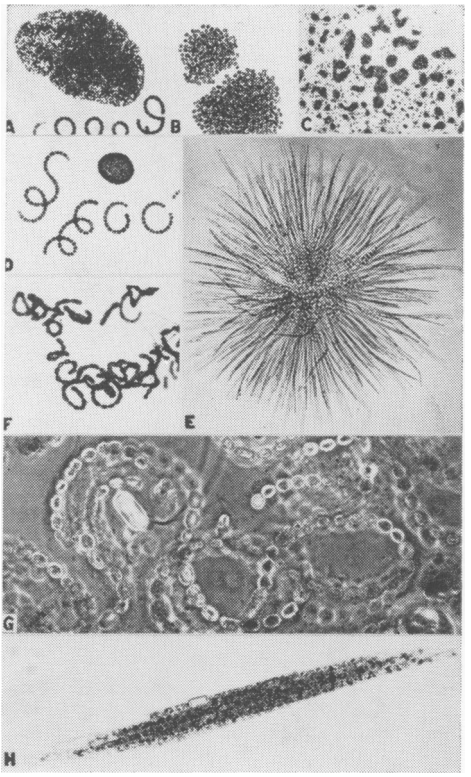

FIGURE 1. Species of blue-green algae most suspected of being toxic. A. *Microcystis aeruginosa* (top) and *Anabaena spiroides* (bottom—not particularly suspect), x 100. B. *Microcystis aeruginosa*, x 200. C. *Microcystis aeruginosa* with pronounced gelatinous sheath, x 40. D. *Coelosphaerium Kützingianum* (top) and *Anabaena spiroides* (bottom), x 100. E. *Gloeotrichia echinulata*, x 125. F. *Anabaena flos-aquae*, x 110. G. *Anabaena flos-aquae*, x 400. H. *Aphinizomenon flos-aquae*, x 400.

improved the culture conditions until we obtained maximal yields of better than 1 g. of dried cells per liter per week (13). Various bacteriological media, plus microscopic examination were used to test for contaminants. To test for toxicity, lyophilized cells or extracts were generally administered intraperitoneally or orally to white mice. The results were expressed in terms of the minimal lethal dose (MLD or LD_{100}) in mg. per kg. body weight.

M. aeruginosa NRC-1 proved to be toxic. It produced two distinct factors (13). One of these factors caused fast deaths (1 to 2 hours) preceded by pallor, convulsions, and prostration. It appeared to be an algal endotoxin which was released by incubation, freezing, or sonic disintegration. The other factor caused slow deaths (4 to 48 hours) at high dosages, preceded by piloerection, dyspnea, and lethargy. It appeared to be bacterial in origin. The slow-death factor, or SDF, could be detected only when the fast-death factor, or FDF, did not mask its presence.

The effects of culture conditions on the production of FDF by *M. aeruginosa* NRC-1 were investigated (11). One of the more interesting observations was that the best yields of algae occurred at 28 to 32.5°C. but the maximum produc-

tion of FDF occurred at 25°C. At 32.5°C. little or no FDF was produced. There was an initial lag in toxin production at all temperatures. At 25°C. the amount of FDF in the cell fraction reached a maximum in about 4 days, thereafter declining sharply. A small amount was detected in the culture filtrate, but the large loss from the cells could not be accounted for.

TABLE I

TOXICITY OF DISINTEGRATED LYOPHILIZED CELLS FROM CULTURES OF BLUE-GREEN AND GREEN ALGAE INJECTED INTRAPERITONEALLY INTO WHITE MICE. DOSAGE RANGE 20–640 MG. PER KG. BODY WEIGHT UNLESS OTHERWISE STATED.

Algae	Cultures Tested	Cultures Producing: Fast Deaths	Slow Deaths	No Deaths
*Anabaena**	3	—	—	3
Aphanizomenon	1	—	1	—
Coelosphaerium	1	—	1	—
Gloeotrichia	1	—	—	1
*Microcystis***	19	8	6	5
*Anacystis***	11	—	6	5
Nodularia	1	—	—	1
*Scenedesmus**	1	—	—	1
*Chlorella**	5	—	2	3

*Only fresh cells of one species of *Anabaena*, of *Scenedesmus*, and of two species of *Chlorella* were tested at doses of 80 to 120 mg. per kg. body wt. [in collaboration with Chaput and Grant (3)].

**Eleven cultures of *Microcystis* and six cultures of *Anacystis* were also tested at 1280 mg. per kg. body weight.

Toxicity of other blue-green algae

On the basis of the experience and results obtained with *M. aeruginosa* NRC-1, we isolated, grew and tested the toxicity of 43 species or strains representing seven genera of blue-green and two genera of green algae. As a rule, we measured growth and tested toxicity of both cells and media at intervals for 5 days. A few cultures grew very slowly and these had to be tested over a period of weeks. With few exceptions, the cells were disintegrated and lyophilized (to insure release of any FDF) and tested at dosages ranging up to 640 mg. per kg. or higher (Table I). Most of the culture filtrates were also concentrated, extracted and tested at the highest possible dosages. FDF was detected only in the cell homogenates (or culture filtrates) of strains of *Microcystis*. Moreover, only 8 out of 19 strains of *Microcystis* produced it. Of the remaining strains 6 produced slow deaths, and 5 produced no deaths. The other species or strains that were tested produced either no deaths or slow deaths of the SDF-type. This provided a further indication that SDF was bacterial in origin. Chaput and Grant (3), meanwhile, grew and tested both fresh and incubated (37°C.) cells of 17 species of blue-greens (including some of the same cultures tested by us). They found that six of these (one being another strain of *Microcystis*) produced deaths in mice injected intraperitoneally at dosages of 36 to greater than 200 mg. per kg. body weight. In every case, incubation was necessary to produce significant toxicity and the toxin was slow-acting.

Origin of FDF

A determined effort was made to obtain an uncontaminated culture of *M. aeruginosa* NRC-1 in an attempt to clearly establish the origin of FDF (27). Treatments with ultra-violet radiation, and with a variety of antiseptics, antibiotics, and bacteriostatic agents given singly, in combination, or in succession, enabled us to reduce the numbers of bacteria and to eliminate some but not all of them. Our conclusion that FDF originates in the algal cells is based on the following evidence: 1. An almost completely lysed culture was separated by differential centrifugation into an algae-rich fraction which contained FDF and a bacteria-rich fraction which contained only SDF. 2. A similar experiment with 40 litres of actively growing culture produced similar results. 3. Five out of six recognized bacterial contaminants were isolated and grown in sufficient amounts to be tested in the same way as the alga, and none of these produced any detectable FDF. 4. Colony isolates were made from three different FDF-producing blooms in which *M. aeruginosa* predominated. Both FDF- and SDF-producing strains of *M. aeruginosa* were obtained from each bloom, even though isolates from any one bloom would probably have had a common bacterial flora. 5. A non-toxic strain of *M. aeruginosa* was deliberately contaminated with the six bacterial contaminants from *M. aeruginosa* NRC-1 but these failed to induce the production of FDF. 6. Finally, some 30 single-cell isolations were made from *M. aeruginosa* NRC-1. From these we succeeded in growing two clones, one of which produced FDF, while the other produced SDF. This last discovery not only helped to establish the algal origin of FDF, but it also established that only certain genetic strains produce it. The selective growth of non-toxic clones may be a possible explanation of the observation mentioned earlier that no FDF was produced by genetically heterogeneous *M. aeruginosa* NRC-1 when grown at 32.5°C. (11). It may also be one of the reasons why a bloom can persist, seemingly unchanged, and yet lose its toxicity (5, 23).

Origin of SDF

While we were mostly concerned with the origin of FDF, we paid some attention to the origin of SDF too. Four of the six bacterial contaminants of *M. aeruginosa* NRC-1 (Gram-negative cocci, diplococci, coccobacilli, or bacilli) produced slow deaths when injected intraperitoneally into mice at 600 to 640 mg. per kg. (27). A pronounced inflammation of the intestine and peritoneal lining was noted in some cases. Following incubation, a different type of fast death was observed with one of these four contaminants when injected intraperitoneally, but no effects were observed when the four were administered orally at 1600 mg. per kg. Recently, Thomson *et al.* (34, 35) described a Gram-negative bacterium isolated from *Microcystis* and a Gram-positive *Bacillus* isolated from *Anacystis* which produce slow deaths when injected intraperitoneally into mice at low dosage (20 mg. per kg.). The two organisms produce different toxins which also appear to be different from SDF. The toxin from the Gram-negative organism is described as a heat-stable, acid-stable, acetone-precipitable neurotoxin, while the toxin from the *Bacillus* sp. is described as heat-labile with antigenic properties. In a survey of bacteria from 25 algal collections, Thomson found that the majority

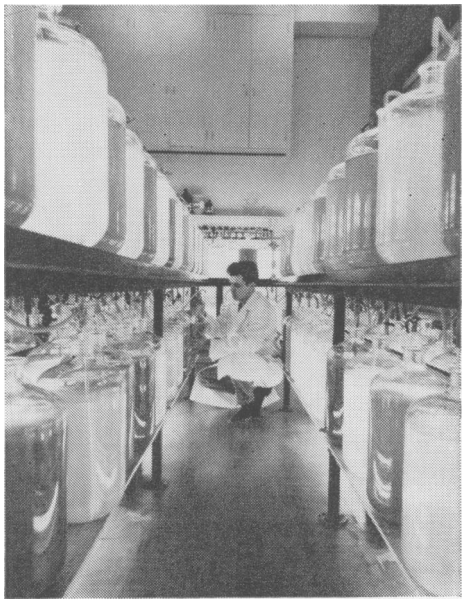

FIGURE 2. Large-scale culture of *Microcystis aeruginosa* NRC-1. Aerated cultures in 9-liter bottles illuminated by fluorescent lamps situated between the rows of bottles.

of the isolates were Gram-negative rods with a variety of biochemical reactions. Twenty-two out of twenty-six such isolates produced the heat-stable toxin while only two isolates produced the heat-labile one. Thomson, unfortunately, did not report on the effects of oral administration, nor did he report any autopsies. While our observations indicated that SDF was bacterial in origin, we were inclined to regard it as being of more significance in the laboratory than in the field since there were no deaths when the bacteria isolated from one strain of algae were administered orally. Thomson's finding of other slow-death factors produced by bacteria which are effective in low dosage when injected intraperitoneally indicates that other factors do occur, and some of these may very well contribute to the toxicity of waterblooms. This view is reinforced by our observation that symptoms of inflammation, such as have been recorded in a number of field cases, seem to be caused by bacteria rather than by algae. At the very least, it seems fair to suggest that the presence of large numbers of bacteria in a waterbloom will be an additional complication which must be considered in ascertaining the cause of any toxicity.

Identity of FDF

For the isolation and identification of FDF (2) we had, first of all, to develop large-scale culture facilities or an "algal factory" (Figure 2) from which we could harvest a sufficient amount of cells of *M. aeruginosa* NRC-1 to do some meaningful chemistry. At peak production we had about 300 subcultures of various ages growing in 9-liter bottles and were harvesting between 1 and 2 kg. of lyophilized cells per month. Previous work had suggested that FDF was an alkaloid (17, 26) but we were unable to confirm this. For a long time, we had no idea of the class of compound we were dealing with. Countercurrent distribution studies indicated that it existed both in a salt form (soluble) and as a free acid

(sparingly soluble). Electrophoresis on paper under acid conditions indicated that it was one of a mixture of polypeptides. By paper electrophoresis under mildly alkaline conditions in the presence of borate buffer we were able to resolve the mixture into five distinct polypeptides, only one of which was toxic. Upon hydrolysis, all five polypeptides were found to possess five amino acids in common. This explains why their separation was so difficult. FDF was found to contain seven amino acids in the following proportions: 1 aspartic, 2 glutamic, 1 *D*-serine, 1 valine, 1 ornithine, 2 alanine, and 2 leucine. It was totally resistant to attack by a variety of proteases. The dinitrofluorobenzene test for terminal amino groups was negative. Hence, we concluded that the molecule is cyclic. Because it dialyzes slowly, the molecular weight is probably of the order of 1300 to 2600. It contains at least one D-amino acid, which was detected by the use of D-amino acid oxidase. The biological activity of the molecule may be the consequence of any or all three of the following properties: being cyclic, having a D-amino acid, or containing ornithine. The weakly acidic character is accounted for by the presence of aspartic and glutamic acids. The LD_{50} (intraperitoneal) of purified FDF for white mice proved to be 0.47 mg. per kg. body weight. This is large in comparison with an MLD (intraperitoneal, mouse) of less than 5×10^{-3} μg. per kg. for certain botulinal toxins (16). The purified toxin appears to have no antibiotic activity.

FDF toxic to large animals

The yield from the "algal factory" was sufficiently great to allow us to conduct some toxicity tests with lyophilized *M. aeruginosa* NRC-1 cells on large animals (15). When administered orally to fasted animals, the alga proved toxic to mice, guinea pigs, rabbits, chickens, sheep, and calves, but domestic ducks were completely resistant. The largest animals survived 9 to 18 hr. Convulsions were not always observed, but the autopsy findings in all animals were quite constant. A pronounced hyperemia of the liver, indicative of congestion and hemorrhage, was the most characteristic symptom. The symptoms and autopsy findings agreed quite well with those reported for *Microcystis* blooms by Wheeler *et al.* (37), Mason and Wheeler (19) and Ashworth and Mason (1) in the U.S.A.; Steyn (32, 33), Louw (17), and Smit (29) in South Africa; and Shelubsky (26) in Israel. Hence we believe that *Microcystis* FDF is at least one of the important waterbloom toxins. The fact that domestic ducks are resistant to FDF by the oral route is particularly interesting since it eliminates it as a cause of duck sickness.

Toxicity of several waterblooms

In the course of this work, we had an opportunity to test the toxicity of 11 different waterblooms by intraperitoneal injection of mice (Table II). Seven of the collections came from eastern Ontario or neighbouring Quebec, two from Manitoba, and two from Saskatchewan. With the exception of *Lyngbya Birgei*, the dominant species in these blooms were all ones suspected of toxicity. Only two types of deaths were observed—fast and slow—accompanied by the same symptoms as those produced by FDF and SDF from cultures of *M. aeruginosa* NRC-1 (13). FDF was detected only in blooms in which *Microcystis aeruginosa*

TABLE II

TOXICITY OF LYOPHILIZED CELLS FROM 11 WATERBLOOMS OF BLUE-GREEN ALGAE INJECTED INTRAPERITONEALLY INTO WHITE MICE. DOSAGE RANGE 40 TO 640 MG. PER KG. BODY WEIGHT

Dominant Species	Blooms Tested	Blooms Producing: Fast Deaths	Slow Deaths
Anabaena flos-aquae	3	—	3
Aphanizomenon flos-aquae	1	—	1
Lyngbya Birgei	1	—	1
Microcystis aeruginosa	6	3	3

was dominant, while SDF was detected in some of these and in all the other blooms. This is consistent with the conclusion that SDF is bacterial in origin. Two of the *Microcystis* blooms were tested in connection with a severe outbreak of algal poisoning which occurred in Saskatchewan in the summer of 1959. Both samples had unusually large numbers of bacteria and the minimal lethal dosages for FDF and SDF were approximately the same. The Saskatchewan authorities reported that a number of the dead animals which were brought to them had a pronounced enteritis. This, as mentioned previously, seems to be a symptom produced by bacterial factors. The toxicity of waterblooms may thus be caused sometimes by the algae, sometimes by the bacteria associated with the algae, and sometimes by a combination of both.

DISCUSSION

There are a number of observations reported in the literature on toxic waterblooms which are difficult to explain satisfactorily on the basis of our present knowledge. For example, large animals (such as a 1,000-lb. cow) are reported to have died within 30 to 60 minutes of consuming a concentrated suspension of blue-green algae (21, 22, 31). In our experiments, large animals fed toxic *Microcystis* cells survived nine hours or more. It is probable that we administered little more than a minimum lethal dose. Therefore, it is conceivable that we might have shortened the survival time by increasing the dosage. We have found, however, that overdosing mice with FDF shortens the survival time, but the decrease is generally small. This rather suggests that conditions can sometimes exist which promote more rapid penetration of FDF or that another factor is responsible or involved. Another example is a report from India (28), where blooms are of frequent and widespread occurrence, that many experiments with different types have failed to disclose any that are poisonous or fatal. This is very surprising in view of the widespread occurrence of toxic blooms throughout the rest of the world. A possible explanation for this is that higher prevailing growth temperatures have selectively favoured the development of non-toxic strains. The poisoning of fish by waterblooms is generally believed to be caused by oxygen depletion or by toxic agents such as amines released during decay (18, 24). There is a report of carp having died when injected intraperitoneally with a *Microcystis* bloom (26) that was not decomposing and appears to have contained FDF. This suggests that fish may be susceptible to other factors besides anoxia and amines.

There is obviously a need for continued, detailed study of blue-green algal

waterblooms—both directly from collections and also from laboratory cultures. This is made all the more necessary by the fact that waterblooms are frequently a nuisance and a potential hazard to public health (14, 23). While humans are unlikely, except under duress, to consume sufficient amounts of waterbloom or its products at any one time to cause death from FDF, they may very well consume enough (perhaps over a period of time) to make them ill. In some cases, allergies may be produced (4); in other cases it may be the associated bacteria rather than the algae that will be the direct cause of different kinds of distress.

Conclusions

Based on the evidence which is presently available, we can summarize our knowledge of toxic waterblooms as follows:

1. At least one species of blue-green algae, *Microcystis aeruginosa*, can, on occasion, be acutely poisonous to animals if ingested in fairly large quantities in a condition suitable for the release of a fast-death-producing peptide. Only certain genetic strains of this alga are toxic [*cf* (30)].

2. The existence of species (or strains) of algae other than *Microcystis* which are capable of causing fast deaths (possibly by a toxin which is similar to if not identical with FDF) is indicated by case histories but so far has not been clearly established.

3. There are indications that the bacterial associates of blue-green algae can be poisonous. Some species, or their products, appear to be harmless if ingested, but others may not be. From the symptoms which have been reported in connection with some poisonous blooms, the toxicity may have been caused primarily by bacteria.

4. The variable toxicity of waterblooms seems to depend upon such factors as the species and strains of algae which are dominant, the types and number of bacterial associates, the conditions of growth, collection, and decomposition, the degree of animal starvation and susceptibility, and the amounts consumed.

Résumé

On a fait croître séparément plusieurs espèces et plusieurs souches d'algues bleu-vert comprenant en particulier celles que l'on soupçonne le plus d'être mortelles pour des animaux et on a constaté de grandes variations de toxicité selon les espèces et les souches. Au moins quatre facteurs toxiques ont été reconnus. L'un provoque une mort rapide et il est d'origine algueuse. Les autres provoquent une mort lente et ils sont d'origine bactérienne. Le facteur de mort rapide est une endotoxine rencontrée jusqu'à présent seulement dans des souches de *Microcystis aeruginosa* Kütz. *emend.* Elenkin. Sa production est contrôlée génétiquement et physiologiquement. Une souche de *M. aeruginosa* qui produit le facteur de mort rapide a été cultivée sur une grande échelle et on a constaté que les cellules en étaient toxiques lorsqu'elles étaient administrées par voie buccale à des moutons, à des veaux et à de plus petits animaux. Le facteur de mort rapide extrait de ces cellules a été identifié comme étant un polypeptide cyclique très stable ayant un LD_{50} intrapéritonéal pour souris blanches de 0.47

mg. par kg. de poids du corps. Les facteurs de mort lente peuvent également contribuer à la toxicité des fleurs d'eau des algues bleu-vert. La conclusion est qu'un ensemble de variables interdépendantes détermine le degré et le genre de toxicité à laquelle une fleur d'eau peut donner lieu.

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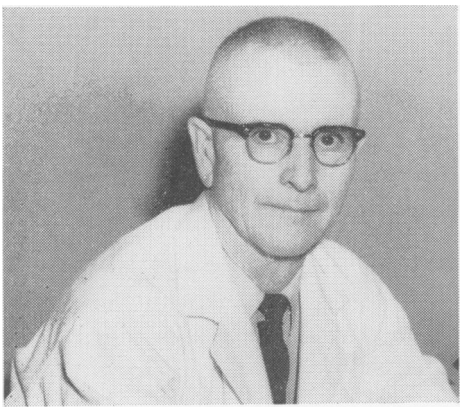

Dr. W. J. Turnbull, Western Representative on the Board of Directors of the C.V.M.A.

Dr. Bill Turnbull hails from Manitoba. He graduated from O.V.C. in 1925 and went into private practice for two years. He then joined the Health of Animals Branch and served in many parts of Canada. From 1941–1943 he was in the armed services and then returned to H. of A. In 1945, he joined the Saskatoon city health department and has been there ever since, except for a year's leave of absence in 1950–51 to take the D.V.P.H. course at the School of Hygiene, University of Toronto.

Since 1954, Dr. Turnbull has been veterinary representative on the Senate of the University of Saskatchewan. He was Vice-President of the Saskatchewan Veterinary Medical Association in 1956 and President in 1957 and 1958. He is now Secretary-Treasurer and Registrar.