1975

Nutritional Studies On Cladophora Glomerata, With The Identification Of Silicon And Thiamine As Required Nutrients

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NUTRITIONAL STUDIES ON *CLADOPHORA GLOMERATA*, WITH THE IDENTIFICATION OF SILICON AND THIAMINE AS REQUIRED NUTRIENTS

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Submitted in partial fulfillment

of the requirement for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

September, 1975

ABSTRACT

One of the signs of the eutrophication of certain aquatic ecosystems is the massive overproduction of Cladophora glomerata. The manipulation of nutrient concentrations has been suggested as the best method of preventing this overproduction. Since the nutritional requirements of this alga are not well defined, research into its nutritional requirements was initiated.

Initially, quantitative methods for the assessment of growth of unialgal cultures were developed to replace qualitative procedures which had been employed previously. A standard inorganic medium was selected and the optimum pH, 8.5, was established. Requirements for calcium, sulphur and nitrogen were investigated and optimal concentrations of approximately 20 mg/l S and 45 mg/l N were established. Growth

enhancement continued with increasing calcium concentrations up to 25.6 mg/l, the highest concentration tested.

Soil water extract (SWE) has been used for decades to supplement certain algal culture media, including those for C. glomerata, but its active components have been essentially unknown. Analysis of SWE showed that an organic heat-labile component was significant, and when thiamine was substituted for SWE, it provided 80% of the stimulation induced by SWE. Subsequent investigations of the requirement for thiamine by C. glomerata demonstrated that 1 µg/l, provided either by SWE or as pure vitamin, stimulated satisfactory growth, while 10 µg/l is the apparent optimum concentration.

Further analysis of SWE showed that SWE ash enhanced the growth of the alga when added to media already supplemented with thiamine. This suggested the importance of inorganic components of SWE and subsequent chemical analyses of SWE showed the presence of high concentrations of calcium (100 mg/l) and silicon (8 mg/l). The calcium, provided by SWE, promoted growth, as expected from earlier experiments. Silicon gave slight growth enhancement at 1 mg/l and more enhancement at 5 mg/l. More silicon could be extracted from the alga grown with added silicon and the cell

walls from the algae grown at 5 mg/l Si showed a distinct electron-dense outer layer which was absent in cell walls from algae grown in media with no added silicon. Germanium, known to be a specific inhibitor of silicic acid metabolism, is inhibitory at moderate concentrations only to those plants that require silicon. Germanium dioxide at 25 mg/l and 75 mg/l inhibited the growth of C. glomerata. Cross wall formation was disrupted by the addition of 25 mg/l GeO_2 and the cell walls became extremely thickened and cell division stopped at 75 mg/l GeO_2.

The optimum pH for unialgal cultures of C. glomerata is 8.5, and the requirement for calcium is high, thus confirming the alga's preference for calcareous, alkaline environments in nature.

The most interesting results came from the investigation into the active components of SWE. Two previously unknown nutritional requirements, thiamine and silicon, were identified. Thiamine may be a very significant nutrient in determining the spread and overproduction of this alga in nature. Silicon has not been previously reported as a required nutrient for any member of the Chlorophyta.

ACKNOWLEDGEMENTS

I wish to sincerely thank my supervisor, Dr. D. A. McLarty for his willingness to explore new areas and for his many conversations that gave me constant encouragement. I also thank my advisors, Dr. W. E. McKeen and Dr. R. B. van Huystee for their valuable suggestions and for critically reading the thesis. Dr. J. A. Traquair prepared the material for the electron microscope with the assistance of Mr. R. Smith. This is an important part of the work and I greatly appreciate their assistance. Other members of the department that provided special assistance were Mr. P. E. Dabinett, who helped with the light microscope, Mrs. S. Legeza who provided the cultures of Phycomyces blakesleeanus, and especially Mrs. V. Zvagulis who assisted me in innumerable ways. Valued moral support was provided by Dr. D. Wilson, and by the graduate students and staff of the department who freely gave both assistance and friendship.

Special thanks to my wife, Chi-Wing, who accepted this work as a rival with inspiring equanimity and at its completion prepared the manuscript.

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CHAPTER 1: GENERAL REVIEW

1.1 Introduction

Cladophora glomerata is a member of the division Chlorophyta and the order Cladopherales. Unusual characteristics, including multinucleate cells, variable thallus structure and an ill-defined life cycle, make it difficult to determine its affinities with other members of this division. The taxonomic problems of this order, having been extensively reviewed in the monograph of Van den Hoek (1963), will be dealt with only briefly in this thesis.

Although the taxonomic problems associated with this group of algae may be difficult, questions pertaining to its physiology and ecology are perhaps even more complex. The review of Whitton (1970) and the lengthy introduction provided by Jackson and Lin (1968) supply much of the background information in these areas, and although they will be referred to often, their discussions will not be repeated here.

The massive overgrowth of Cladophora in certain aquatic ecosystems has made research into the physiology and ecology of this alga essential. Once there is a basic understanding in these areas, water management practices can be designed to control or

prevent these overgrowths.

The nutritional requirements of Cladophora are not well understood. Certainly, to obtain effective control of a problem alga in a large body of water, the best approach is to limit the input of all nutrients, or at least to lower the level of one nutrient below the concentration required for good growth of the alga. In the Laurentian Great Lakes, there are basically two types of problem algae, Cladophora at the shores and blue-greens in the open water. A nutrient reduction program has been implemented in the lakes, with phosphorus removal planned for sewage treatment plants on both the Canadian and American shores of lakes Ontario and Erie.

Cladophora certainly requires a rich phosphorus supply, but there is no guarantee that phosphorus removal from sewage influents will reduce the growth of C. glomerata in these lakes. A more complete understanding of the nutritional requirements of this alga would give a rational basis for a reduction in nutrient loading aimed at control of Cladophora. If a requirement for a vitamin or an unusual element were uncovered, this might be the key to the control of problem growths. For these reasons, the nutritional requirements of C. glomerata, the most common species in

this area were investigated, with emphasis on the identification of previously unknown nutritional requirements.

1.2 The Natural Environment of *Cladophora*

1.2.1 Physical Requirements

A stable substrate is a necessity for growth of *C. glomerata*. Calcareous rocks are its favourite substrate, but other rough surfaced rocks, pilings and concrete erosion control barriers are also colonized. The most prominent growths in Lake Ontario occur where the bedrock shelves out gradually from shore.

Vigorous water movement has also been shown to be necessary for this alga. In streams, it commonly occurs in riffles and in lakes, wave washed shores are colonized, and laboratory experiments have shown that shaken cultures are more productive than still ones (Jackson and Lin 1968).

Moderate temperatures produce the best growth. Active growth in the field has been reported from between 5° C to 25° C. Laboratory tests have shown that 25° C (Zuraw 1969) and approximately 20° C (Bellis 1966) are near the optimum temperature.

Light intensity requirements for this alga are very high. Whitton (1967) found that the growth

rate increased with increasing light intensity up to 7500 lx, the highest value tested.

There is no known light periodicity for C. glomerata but Storr and Sweeny (1971) showed that the alga grows much better with 14 hour light periods than with 12 hour periods.

1.2.2 Chemical Requirements

C. glomerata requires a pH of between 7 and 9.5 for good growth in natural waters. Bellis (1968) found that the optimum pH for cultures of this alga was between 7.3 and 9.0. Another important characteristic of any natural environment that supports C. glomerata is a high water-hardness, indicating a high concentration of calcium. Laboratory studies have shown healthy growth of the alga at calcium levels that cause precipitation in the medium (Bellis 1968).

The distribution of the alga in the Great Lakes suggests that high nutrient concentrations are required for good growth of the alga. In lakes Ontario, Erie and Michigan the average nutrient level is high and C. glomerata occupies most of the suitable substrates. There are, however, larger growths associated with major suppliers of nutrients, such as a sewage treatment outfall or a river. In lakes Huron and Superior the nu-

trient level is generally low and C. glomerata only occurs where there is local input of additional nutrients. Of all the possible nutrients involved, phosphorus has been suggested as the one that may limit the growth of both C. glomerata and the other problem algae in the Great Lakes. There is little proof that phosphorus is the limiting factor in these areas, although it certainly is in many aquatic ecosystems. It is interesting that this genus has responded positively to the addition of nutrients to aquatic ecosystems by man and that it now dominates other genera that might occupy the same habitats.

1.3 The Control of Cladophora

Algicides and the harvesting of the standing crop could be used to control C. glomerata if the problem were very localized. However, the only way that the problem can be attacked in a large water system is to limit nutrients that are required for the growth of this alga. Liebig's Law of the Minimum states that if one nutrient can be reduced below the level required for optimum growth, growth will be reduced. Before attempts are made to reduce any nutrient, consideration must be given to the cycling of that nutrient in nature and to the nutritional requirements of the organisms

which the nutrient reduction is designed to inhibit. Phosphorus is often limiting in aquatic ecosystems and is relatively easy to remove from sewage effluents so it seems the most logical element to reduce. However, a great deal of phosphorus has been added to lakes Michigan, Erie and Ontario and some of this has been stored in the sediments. The potential for release of phosphorus from the sediments is still under discussion as plans go on for the removal of phosphorus from sewage. C. glomerata, of course, requires phosphorus but it appears that a considerable reduction in the phosphorus levels of the lakes would be required to produce a significant decrease in growth.

1.4 Important Studies on the Nutrition of Cladophora

1.4.1 Field Studies on Phosphorus and Cladophora

In his review of the literature on Cladophora, Whitton (1970) quoted seven field studies in which excessive growths of the alga were correlated with high levels of phosphorus. One of these studies was conducted on Lake Huron by Neil and Owen (1964). They applied four nutrient additions to areas of the lake that did not support Cladophora and seeded each area with algae from Lake Ontario. The addition of sheep manure produced the fastest response and a heavy growth of the

alga was produced. Phosphorus addition also produced a heavy growth of the alga while nitrogen added alone did not produce as much growth as the phosphorus addition. Even nitrogen added along with phosphorus did not improve on the addition of phosphorus alone.

Another study on the Great Lakes surveyed in Whitton's review was that of Herbst (1969). The observation was made in this study that Cladophora growths occur in the lakes that have a high phosphorus concentration (Erie, Ontario and Michigan), and not in those with lower concentrations (Huron and Superior), and it was recorded that local sources of phosphorus, especially cities, cause serious problem conditions. Herbst recommended that tertiary treatment, for the removal of phosphorus, be added to sewage facilities to reduce the problem growths of Cladophora.

A more recent study in England, (Pitcairn and Hawkes 1973), showed that the mean annual dry weight of Cladophora in seven rivers was significantly correlated with the mean annual concentration of phosphorus but not significantly correlated with the mean annual concentration of nitrogen. Rivers with less than 1 mg/l P tended to support only modest growth of Cladophora.

1.4.2 Laboratory Data Suggesting the Importance of Phosphorus

Few laboratory studies have been done on Cladophora due to the difficulty of freeing it from contamination and to the slow growth it usually exhibits in defined culture media. Pitcairn and Hawkes (1973), using partially clean cultures in a medium supplemented with natural water, found that 1 mg/l P was needed for optimum growth in culture. They also found that river water, taken from below a sewage outfall which was high in phosphorus, supported better growth of Cladophora in culture than water taken from above the outfall, which was low in phosphorus. This difference in growth could be abolished by adding phosphorus to the river water from above the outfall.

Jackson and Lin (1968) noted that Cladophora increased its rate of photosynthesis in culture when phosphorus was increased from 0.00 to 0.003 mg/l. They reported that the optimum concentration of phosphorus of between 0.16 and 1.6 mg/l while the preferred phosphate to nitrate ratio was between 1:5 and 1:20, high compared to a more common ratio of 1:30 for some green algae.

Fitzgerald (1970) showed that, according to his data on extractable phosphorus, Cladophora specimens

taken from the Madison Lakes were often phosphorus limited.

1.4.3 The Importance of Other Nutrients

The information in the preceding section suggests that Cladophora is growth limited by phosphorus in some environments. Mason (1965) studying five farm ponds near Ithaca, New York, found that high productivity of C. glomerata was directly associated with high nitrate and high biological oxygen demand and not with phosphate concentration. Bellis (1968) found that calcium was a requirement of the species that could not be replaced by magnesium. His cultures needed at least 6.4 mg/l Ca and 0.7 mg/l Mg for good growth.

The effect of carbon dioxide on cultures of Cladophora has given ambiguous results. Wood (1968) found that the rate of photosynthesis doubled when the initial carbon dioxide content was increased from 1587 to 1916 umoles/l while Zuraw (1969) reported that bubbling 2% CO_2 enriched air through cultures inhibited growth even though the medium was buffered with tris which kept the pH high.

1.4.4 Importance of Vitamins

The literature contains no reference to the

vitamin requirements of C. glomerata but there are many indications that such requirements exist. The closest thing to a description of a vitamin requirement was the work of Thomas (1963). He showed that the addition of certain bacteria to bacteria-free cultures stimulated the growth of the alga slightly and natural lake water, supplemented with nitrogen and phosphorus enhanced the growth considerably. The active bacterial product was water soluble, diffusible through agar and heat labile at 80° C. The active component in the lake water was also heat labile at 80° C. He tried to replace this component by the addition of vitamin B_{12}, thiocarbamide and the hormones 2-4-D and IAA. None of these compounds enhanced the growth of the alga, but the properties of the active component strongly suggest the importance of vitamins.

The other evidence that suggests a vitamin requirement is the constant need of an undefined addition to culture media. Whitton (1967) and Pitcairn and Hawkes (1973) added natural water to their cultures while Bellis (1968) and Van den Hoek (1963) used soil extracts in their cultures. The taxonomy of this genus by Van den Hoek is the accepted guide, and many of his conclusions were based on culture studies. To obtain healthy unialgal cultures with natural morphology, soil

extract or pond water had to be added to the culture medium.

Zuray (1969) obtained some stimulation of growth with 0.5% soil extract in his cultures but obtained no enhancement with the addition of a vitamin mixture that included B_{12} and thiamine (B_1). This result suggests that the active components of soil extract are not vitamins despite other evidence that suggests the importance of vitamins. One of the principle objectives of the research for this thesis was to establish the nature of the active components of soil extract.

1.5 The Determination of Silicon Requirements by the Use of Germanium.

Germanium is the next higher analogue of silicon in Group IV A of the periodic table of elements. Germanium acts as a competitive inhibitor of silicon metabolism and at moderate concentrations is only inhibitory to those plants that require silicon (Lewin 1966, Werner 1967a). A number of higher plants and all diatoms tested were inhibited by germanium at concentrations between 15 and 75 mg/l. A range of fungi, bacteria and algae from the divisions Chlorophyceae, Rhodophyceae and Cyanophyceae were not inhibited even by much higher concentrations (Werner 1967a). One non-silicon con-

taining alga was not inhibited by 400 mg/l germanium (Lewin 1966). The inhibitory effect of germanium can be reduced by adding a high concentration of silicon (Lewin 1966).

CHAPTER 2: MATERIALS AND METHODS

2.1 Sources of Unialgal Cultures of *Cladophora*

2.1.1 The Isolation of Medway Creek *C. glomerata*

Algae from Medway Creek were used in most of the experiments and unless specific reference is made to the source of the alga, this is the culture used. This isolate was collected and cleared of contamination by Mrs. W. Zvagulis. The technique used to obtain unialgal cultures consisted of plating out vigourously washed filament tips on agar, allowing some growth to occur and then removing the new tips and transferring these to algal culture media in tubes. The tips that grew and were unialgal were then transferred to flasks and large cultures were obtained.

This culture was identified as *C. glomerata* by Bellis (1966) using the criteria of Van den Hoek (1963).

2.1.2 Lake Ontario *C. glomerata*

This isolate was collected at Coronation Park, Oakville, Ontario (Lake Ontario) by Dr. D. A. McLarty. It was isolated as described above.

2.1.3 European Cladophora

The Indiana Culture Collection of Algae provided C. glomerata cultures LB 1484, LB 1486 and LB 1488 and C. fracta culture LB 1487. All were collected in Europe and identified by Van den Hoek (1963).

2.2 Selection of Soil and the Preparation of Soil Water Extract (SWE)

The soil was selected according to the principles set out by Pringsheim (1950), the originator of the soil water culture technique. The site of soil collection was in a well cultivated flower bed that had not been recently fertilized. The soil, a grey garden loam with medium humus and clay content, was found to be very suitable for the culture of C. glomerata. The site of soil collection was within the watershed of the Medway Creek, from which the C. glomerata had been isolated and since the algal and the soil sample were taken within a few hundred yards of each other, the components of soil water extract were probably all found in the natural environment of the alga. The other soil sample which was used to make SWE 2, was collected outside of the watershed of the Medway Creek. It also was a grey garden loam but it had a higher sand content and less clay than the original soil sample. In preliminary

tests SWE 2 was less effective than SWE in stimulating the growth of C. glomerata.

Both SWE and SWE 2 were prepared by autoclaving 1 liter of distilled water and 1 kg. of soil for 1 hour. When partially cool, this slurry was filtered through a Whatman #1 filter paper in a Buchner funnel. The filtrate was frozen for storage and thawed only immediately before use.

2.3 Preparation of the Basic Algal Culture Medium

Tris buffered inorganic medium (TBIM) (Smith and Weidman 1964), was used as the basic inorganic medium. The concentration of nutrients in this medium is shown in Table 2.3-1, and the procedure for its preparation is given in Table 2.3-2. Distilled, deionized water was used to make the medium and the pH was adjusted to 8.3 - 8.4, using 0.1 N HCl.

2.4 Maintenance of Stock Cultures

Stock cultures of each Cladophora were maintained in 2800 ml Fernback flasks containing 900 ml of TBIM and 100 ml (10%) of SWE. The initial pH of this medium was adjusted with 0.1 N HCl to 8.4. Subcultures were made each month using 1 - 2 grams fresh weight of the alga, to inoculate each liter of fresh medium. The cultures were maintained on a large reciprocating shake

Table 2.3-1 Composition of TBIM

	Concentration mg/l
K	99.6
Na	46.0
P	31.0
N	28.1
Ca	4.0
Mg	7.3
Cl	7.6
S	11.3
B	2.0
Fe	1.0
Zn	2.0
Mn	0.4
Mo	0.5
Cu	0.4
Co	0.1
TRIS	606.0 (5000 uM)
EDTA	50.0

Table 2.3-2

Procedure for the Preparation of TBIM

Major Nutrients

KNO_3	20 ml of 0.1M soln
Na_2HPO_4	10 ml of 0.1M soln
$MgSO_4 \cdot 7H_2O$	3 ml of 0.1M soln
$CaCl_2 \cdot 2H_2O$	1 ml of 0.1M soln
Tris*	25 ml of 0.2M soln

Add each of the above to 800 - 900 ml of deionized water and add 1 ml of each of the following micro-nutrient solutions. Make up 1 liter.

Micro Nutrients

I EDTA 50 g
KOH 31 g per liter deionized water

II H_3BO_3 11.42 g per liter deionized water

III $FeSO_4 \cdot 7H_2O$ 4.98 g per liter deionized water

IV

$ZnSO_4 \cdot 7H_2O$	8.82 g	
$MnCl_2 \cdot 4H_2O$	1.44 g	
$Mo\ O_3$	0.71 g	per liter acidified water
$CuSO_4 \cdot 5H_2O$	1.57 g	
$Co(NO_3)_2 \cdot 6H_2O$	0.49g	

* Tris (hydroxymethyl) aminomethane

table in an algal culture room at a temperature of 14° C. Cool white fluorescent tubes and incandescent bulbs provided a light intensity of approximately 5400 lux, at the level of the cultures, for 14 hours each day. Each stock culture, in a period of about 4 weeks, produced 15 to 20 grams of healthy material which was used as inocula for experimental purposes.

2.5 Initial Attempts to Quantify the Growth of *Cladophora*

Most of the experimental work done on *C. glomerata* has depended on some qualitative means of assessing growth. The only methods that have given quantitative results are oxygen production data such as that gathered by Jackson and Lin (1968) and the volume data gathered by Storr and Sweeny (1971). Pitcairn and Hawkes (1973) had some success in gathering quantitative dry weight data but they had considerable difficulty in standardizing their inocula.

For the nutrient experiments described in this thesis, growth over time was the most desirable measurement, therefore methods were devised that would yield either quantitative dry weight or chlorophyll data. The first of these methods was to allow *C. glomerata* filaments to attach to asbestos plates and then to culture the attached algae in a large shake flask.

This method failed because of poor attachment to the plates and contamination by other organisms.

The second method attempted was to obtain the fresh weight of an aliquot of Cladophora before and after or possibly during an experiment. The aliquot was weighed in a pre-weighed screw-cap vial containing a few ml. of sterile water. This method was very cumbersome and time consuming, and had all the problems that are normally associated with taking fresh weights. The most serious of these problems is the inclusion of free water with the algal aliquot. This method was used for the initial experiments that were designed to test the effect of various organics on the growth of the alga.

2.6 Two Successful Methods of Experimenting with Cladophora

2.6.1 Large Flasks Experiments

These experiments were done using 2800 ml Fernbach flasks containing 1 liter of medium. The flasks were randomly arranged on the same shake table that held the stock cultures. An inoculum of 1.0 or 1.5 grams fresh weight was used for each flask. These inocula were obtained by dissecting a blot-dried sample, from a stock culture, into small pieces and then the

required inoculum was weighed using a top loading balance. After about 20 days of incubation, under the same conditions as described for the maintenance of stock cultures, the algal growth was assessed using dry weight determinations. This method was very effective and gave reproducible results. The variability of inocula for this method is shown in Table 2.6 a.

Another advantage of this method is that large growths can be accommodated in the flasks so that differences between treatments may be clearly recognised. The main disadvantage of this method is that it requires a large space for very few flasks and consequently, the number of treatments and the number of replications is severely limited. However, this method has yielded the most reliable results and is the recommended method for further investigations.

2.6.2 Small Flask Experiments

To test the effect of various levels of additives, it was desirable to use more treatments than were possible in the procedure described above. 250 ml flasks with 50 ml of experimental medium were used for these experiments. The experimental design for these experiments was either 5 replicates of 7 treatments or 7 replicates of 5 treatments, randomly arranged on a

Table 2.6 Variability of Inocula

a). Dry weight of 4 inocula with a fresh weight of 1.0 gram

28.9 mg
27.4
29.2
28.4
28.5 $\bar{x}$
0.4 SE

b). Dry weight of 5 inocula obtained by using the unmodified small flask method

5.1 gm
3.5
5.8
6.3
3.1
4.8 $\bar{x}$
0.6 SE

c). Dry weight of 5 inocula obtained using the modified small flask method

5.8 mg
5.2
5.5
6.3
5.9
5.7 $\bar{x}$
0.2 SE

rotary shake table operated at 100 rpm.

Two methods were used to obtain equal quantities of C. glomerata for each of the experimental flasks. The first of these consisted of dissecting the algal mat from a stock culture into squares about 1 cm to a side. The alga was given 24 hours recovery time in the original spent medium and then was blended in 300 ml of spent medium for 30 seconds in a sterile Waring blender. This treatment produced filaments ranging up to 20 cells in length, which were collected by straining through sterile cheese cloth. The filament fragments were then suspended in the quantity of medium that was to be common to all of the experimental flasks. The substance under study was added to each flask in various amounts, followed by the addition of equal amounts of the algal suspension and the common components of the media. At the end of the incubation period in the algal culture room, dry weights or chlorophyll data were obtained.

This method yielded some good results and the original inocula per flask were fairly equal (Table 2.6 b).

The main problem with this method was handling the large volume of medium that contained the algal suspension. This suspension had a volume of about 1.5

liter and from it, 35 equal aliquots of about 45 ml had to be taken. Since some of the filament fragments were still quite large, a dispensing syringe could not be used. Equal volumes of the suspension were obtained by pouring portions of it into a beaker and then measuring volumes with a graduated cylinder.

To streamline this method, the following modifications were employed. After collecting the filament fragments on the cheese cloth, the excess medium was pressed out and seven subsamples, each of 1 gram fresh weight, were obtained. Each of these was transferred to one of seven beakers containing 250 ml of experimental medium, covering a range of concentrations of the supplements to be tested. Five aliquots of 50 ml each were then obtained from each of these suspensions. Equal quantities of algae in each aliquot was assured, to within reasonable limits, by constant and forceful agitation of the suspensions of algae. These final 50 ml samples were incubated in 250 ml flasks for 20 days under the same conditions described for the maintenance of stock cultures. Dry weights or chlorophyll analyses were obtained at the end of each experiment.

This modified small flask method was an improvement since no more than 250 ml of algal suspension

was handled at one time. The other main advantage was that each treatment in every experiment was given a similar inoculum when this method was used. The original inoculum per flask was fairly equal in this method (Table 2.6 c).

2.6.3 The Determination of the Growth Curve of C. glomerata

Time course studies demanded a somewhat different experimental design. The procedure outlined in Section 2.6.2 was followed with the exception that 70 flasks were prepared instead of the normal 35.

To half of these (35 flasks), 5 ml of sterile filtered thiamine HCl (10 mg/l) was added, thus providing a concentration of 1 mg/l thiamine when diluted by the addition of 45 ml of the TBIM algal medium. To each of the other series of 35 flasks, 5 ml of sterile filtered SWE were added, to produce a concentration of 10% SWE when diluted to 50 ml by the addition of the TBIM algal mixture. The 70 flasks were randomly distributed on two rotary shake tables and incubated as described in Section 2.6.2.

Each week, for seven weeks, five flasks from each of the two series (+ thiamine and + SWE) were analysed, three being used for dry weight determin-

ations and two for chlorophyll a determinations.

2.7 Chlorophyll Analysis

Chlorophyll analysis was used in place of or in support of dry weight measurements in determining the growth curve of the alga and for analysing experiments where an additive could become deposited on the alga, artificially increasing the dry weight.

The extraction of chlorophyll was done according to Bain (1969) and the absorbance of the resulting extract was measured at 665 nm on a Spectronic 20 equipped with a "red" tube and filter. Calculation of the amount of chlorophyll was done according to the formula: $C_a = 14.3 \times A_{665}$ (Standard Methods 1971)

C_a = mg/l chlorophyll a in the extract.

This calculation assumes a light path of 1 cm which is only approximate for the round curvette of the Spectronic 20. To convert this value to the amount of chlorophyll in the culture medium, the following formula was used:

$X_a = 14.3 \times 1/5 \times A_{665}$ (small flasks)

$X_a = 14.3 \times 1/20 \times A_{665}$ (large flasks)

X_a = mg/l chlorophyll a in the medium.

The conversion factor of 1/5 is employed because in the small flask experiments, the chlorophyll a

from 50 ml of culture was concentrated in 10 ml of extract. For large flask experiments the conversion factor is 1/20 because the chlorophyll a from 1000 ml of culture was concentrated in 50 ml of extract.

2.8 Methods for the Analysis of Soil

2.8.1 Ashing Procedures

500 ml of SWE was concentrated to 25 ml by boiling in an open beaker. This concentrate was transferred to a crucible, dried at 100° C overnight and ashed at 650° C for 10 hours. This ash was redissolved in 500 ml of acidified glass distilled water and used in place of SWE to determine whether the active components of SWE resided in its ash.

2.8.2 Differential Heating to Determine the Heat Stability of the Active Component of SWE

Three 500 ml aliquots of one sample of SWE were sterilized by three different methods. One aliquot was filtered, first through a glass fiber filter and then through a sterile 0.45 µm membrane filter, another was steamed for 20 minutes and a third was autoclaved for 15 minutes at 15 psi.

2.8.3 The Determination of Thiamine in Soil

The Phycomyces blakesleeanus thiamine bioassay as employed by Burkholder and McVeigh (1940) was used to test the level of thiamine in the various samples of SWE. The SWE was sterilized by filtration through a 0.45 µm membrane filter as were the thiamine control solutions.

2.8.4 The Chemical Analysis of SWE

Analyses were done for calcium, nitrate, phosphate (total and soluble), silicon, sulphate and alkalinity. Alkalinity was determined according to Standard Methods (1971) while the other tests were done on a Hach DR-EL-2 spectrophotometer. Most of the Hach tests were modified from Standard Methods (1971) and they gave a reasonable estimate of the chemical content of SWE. A brief outline of each method used is given in Table 2.8.4-1.

2.9 Bacterial Examination of the Culture Medium

The bacterial population in the algal culture medium was examined because of the possible mediating effect the bacteria could have on the response of the alga to thiamine.

Table 2.8.4-1

Procedures for the Chemical Analysis of SWE

Analysis for:	Test Applied:
Alkalinity (as $CaCO_3$)	Total alkalinity determined, using titration with 0.02N H_2SO_4 and brom cresol green-methyl red as an indicator. (Standard Methods 1971)
Nitrate	Cadmium reduction method (Hach Manual 1973)
Phosphate (total and soluble)	Ascorbic acid method, digestion with hot, dilute sulphuric acid for total phosphate. (Hach Manual 1973) (Standard Methods 1971)
Silicon	Heteropoly blue method (Hach Manual 1973) (Standard Methods 1971)
Sulphate	Turbidimetric method (Hach Manual 1973) (Standard Methods 1971)
Calcium (hardness as $CaCO_3$)	Titration method (Hach Manual 1973) (Standard Methods 1971)

2.9.1 Isolation of the Bacteria

A sample medium from a culture 20 days old was diluted to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. 0.5 ml of each dilution was used to inoculate 9 cm petri plates containing TBIM agar supplemented with 10% (v/v) SWE and 0.1% (w/v) peptone. Each type of bacteria observed was examined after gram staining.

2.9.2 Determination of Thiamine Requirements for the Isolated Bacteria

Both types of bacteria were streaked on plates of TBIM agar with or without the addition of 20 µg/l thiamine. Five plates of each bacterium were prepared with thiamine and another five plates of each were prepared without thiamine. These plates were incubated in the algal culture room in the same conditions of light and temperature as the stock cultures of C. glomerata. Observations of the growth of these bacteria were made in five days.

2.10 The Use of Antibiotics to Reduce the Bacterial Population in the Cladophora Cultures

2.10.1 Screening for Suitable Antibiotics

Chloramphenicol, erythromycin and tetracycline were tested using 125 ml flasks with 50 ml of me-

dium under conditions which were the same as for the maintenance of stock cultures. The actual concentrations and combinations of antibiotics screened are shown in Table 2.10.1-1. Two small tufts of washed C. glomerata were added to each treatment flask both as an inoculation of bacteria and to check on the viability of the alga in the treatment. After 24 hours, one of each of the surviving C. glomerata tufts was transferred to fresh medium while the other remained in the treatment flask.

The presence of bacteria was checked after 24 hours' in a semi-quantitative way. Tubes containing 25 ml TBIM + 10% SWE + 1% w/v peptone were prepared and inoculated with 1 ml of medium from each of the treatment flasks. After 4 days of incubation, 5 ml of this medium was transferred to a Spectronic 20 cuvette and the percent transmission was measured at 450 nm. The condition of the C. glomerata in all flasks was noted.

2.10.2 Application of Antibiotics to Reduce Bacteria Under Experimental Conditions

Twelve 125 ml flasks, containing 50 ml of sterile TBIM each, were inoculated with 1 gm fresh weight of C. glomerata. Six of these flasks contained

Table 2.10.1-1

The Concentrations and Combinations of Antibiotics Screened

Concentration in mg/l

Flask #	Chloramphenicol	Erythromycin	Tetracycline
1	20	–	–
2	–	20	–
3	–	–	20
4	20	20	–
5	–	20	20
6	20	–	20
7	20	20	20
8	40	–	–
9	–	40	–
10	–	–	40
11	40	40	–
12	–	40	40
13	40	–	40
14	80	–	–
15	–	80	–
16	–	–	80
17	100	–	–
18	–	100	–
19	–	–	100
20	–	–	–

100 mg/l chloramphenicol and 50 mg/l erythromycin and the other six were controls containing TBIM alone. These flasks were placed on a rotary shake table in the dark in the algal culture room. After 18 hours the entire content of each flask was emptied into one of twelve Fernback flasks containing 950 ml of experimental medium. The final concentrations were 5 mg/l chloramphenicol and 2.5 mg/l erythromycin.

2.10.3 Procedure for Bacterial Plate Counts

The medium used was TBIM + 10% v/v SWE + 0.1% w/v peptone solidified with 2% w/v agar. The bacterial suspension in the algal culture medium was diluted to 10^{-4} of the original concentration and 0.5 ml of this final dilution was spread over the surface of the plate. After 5 days the bacterial colonies were counted.

2.11 The Extraction of Silicon from Cladophora and its Determination

Silicon determinations were made by the Hach Chemical Co. method (Hach Manual 1973), which is modified from the Heteropoly Blue procedure of Standard Methods (1971). The test gave reproducible results and the accuracy was estimated at $\pm$ 0.1 mg/l Si, over the effective range of the test, 0.1 mg/l to 2.0 mg/l Si.

Silicon was extracted from the alga by inserting each sample of dried alga on its filter paper into 500 ml Nalgene bottles each with 100 ml of deionized distilled water and 400 mg of $NaHCO_3$. The bottles were loosely capped and autoclaved for one hour. After cooling 4.8 ml of concentrated H_2SO_4 was added to each bottle and the silicon test was done immediately. The digestion with $NaHCO_3$ should both extract the silicon and render it into a form which would react in the test applied (Standard Methods 1971).

CHAPTER 3: RESULTS

3.1 The Optimum pH and an Examination of the Inorganic Nutritional Requirements of *C. glomerata*

3.1.1 The Determination of the Optimum Initial pH for Cultures of *C. glomerata*

Using the method outlined in Section 2.6.2, seven treatments of pH 6.5 to 9.5, at 0.5 pH unit intervals were investigated. The highest and lowest of these initial pH values would be expected to be somewhat unstable since tris, used as the buffering system in all samples, is effective only within the pH 7.0 to pH 9.0 range. The object of this experiment was to determine simply the initial pH which provided the greatest increase in dry mass. Figure 3.1.1-1 shows that pH 8.5 was the treatment closest to the optimum initial pH for cultures of *C. glomerata*.

The error bars (SE) shown in this figure are quite long, showing that variation within the treatments was large. This variation indicated that the procedure was too insensitive to test a narrower range of pH values.

3.1.2 Calcium Requirements of *C. glomerata*

The calcium requirements of *C. glomerata* were examined over the range of 0 to approximately 26 mg/l.

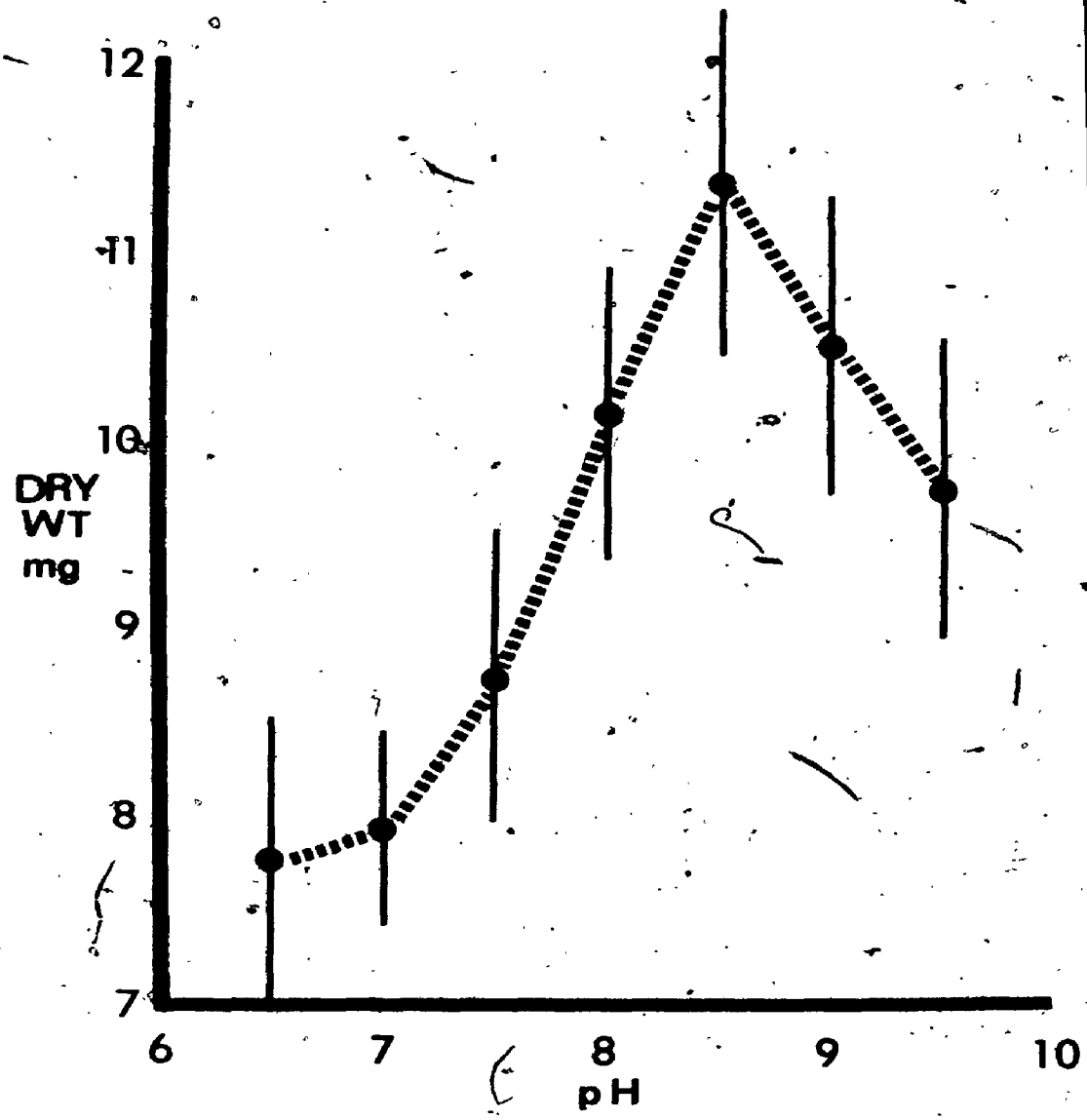


Figure 3.1.1-1 The growth of C. glomera at pH values of between 6.5 to 9.5.
ANOVA: F < 2 N.S. (.05)

At the highest concentrations, calcium was beginning to cause precipitation in the medium. The method described in Section 2.6.2 was used for both of the experiments described here. Figure 3.1.2-1 shows the response of C. glomerata over the entire range of calcium concentrations tested. Growth increased with increased calcium concentration even up to 25.6 mg/l, the highest concentration tested. In the three lowest concentrations the alga was somewhat bleached and deterioration had begun. The anomolous result shown for the third lowest concentration (1.6 mg/l) may have been due to an oversized inoculum. The very large increase in dry weight at the highest concentration was due partly to the adherance of the precipitate, although the alga was very healthy at this concentration.

To examine in more detail the effect of calcium at low concentrations, the results of another experiment are shown in Figure 3.1.2-2. These results confirm the observation that the dry weight of C. glomerata increases with increasing calcium concentration. The addition of 0.32 mg/l Ca did not significantly improve the growth but 0.64 mg/l Ca brought the level of growth to a plateau that was not significantly improved by the addition of 1.28 or 2.56 mg/l Ca. The two highest concentrations tested (5.12 and 10.24 mg/l) show the same increase in growth with increasing concentra-

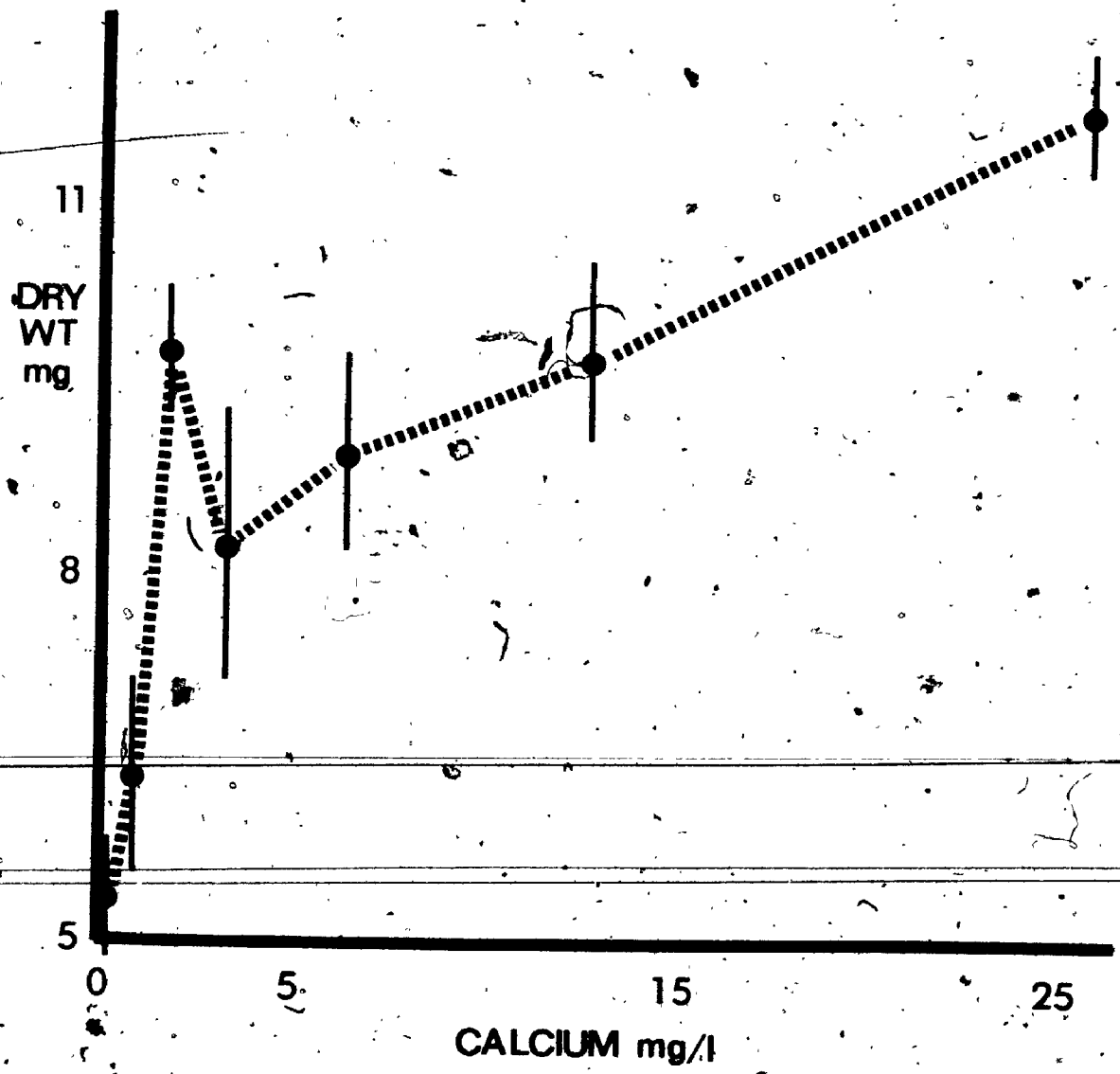


Figure 3.1.2-1 The response of C. glomerata to Ca between 0.0 and 25.6 mg/l.

ANOVA: $F > 3$ *(.05)

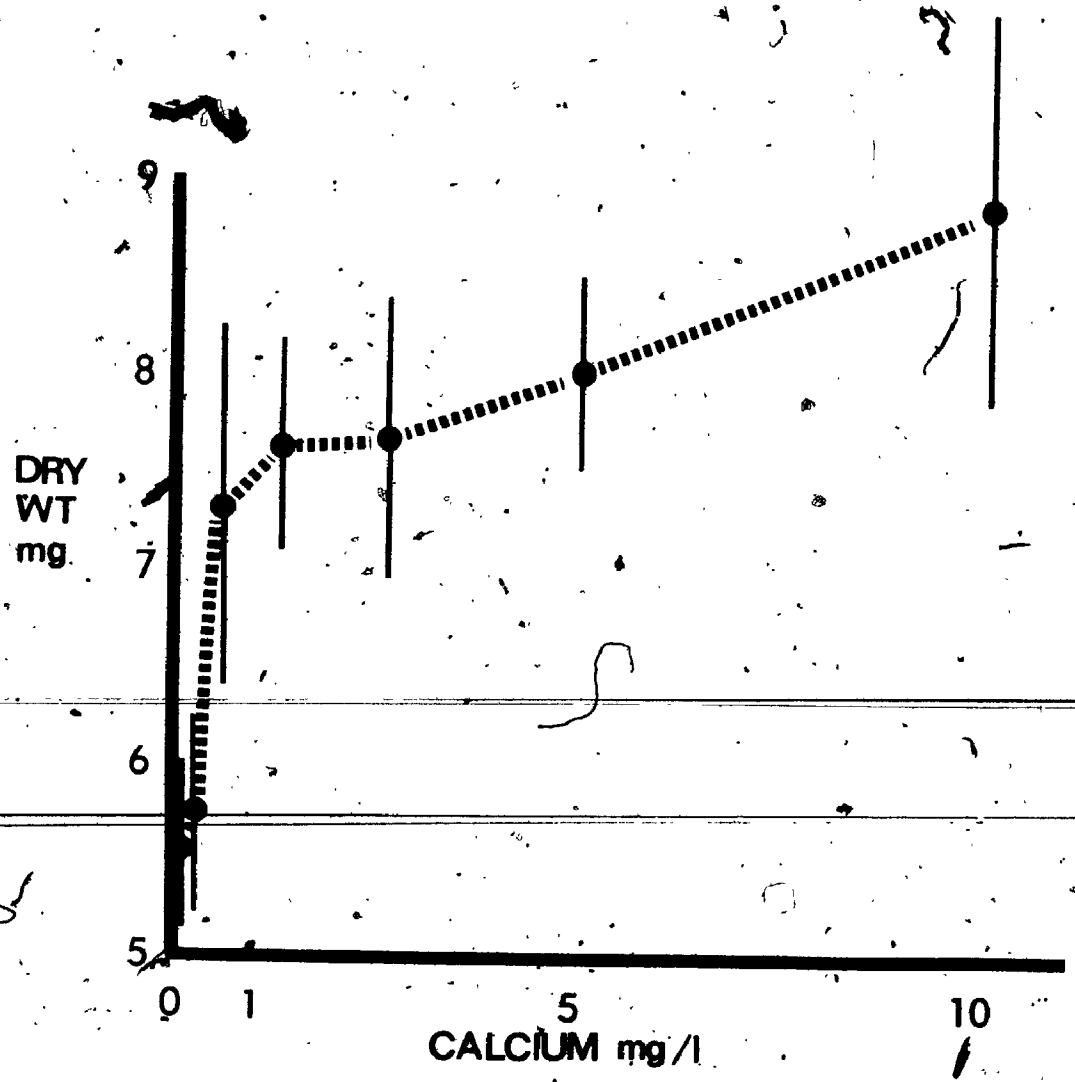


Figure 3.1.2-2 The response of C. glomerata to Ca between 0.0 and 10.24 mg/l.

ANOVA: $F > 3$ *(.05)

tion shown in Figure 3.1.2-1. Only the algae exposed to these two highest concentrations were healthy in appearance.

3.1.3 Sulphur Requirements of C. glomerata

The requirement of C. glomerata for sulphur was examinedusing the methods outlined in Section 2.6.2. A preliminary experiment examining sulphur concentrations in the range of 20 to 1810 mg/l showed that sulphur was inhibitory beyond 100 mg/l. Figure 3.1.3-1 shows the results of an experiment over the range of 0 to 96.2 mg/l S. At 96.2 mg/l S the highest concentrations tested, the expected inhibition was observed. The dry weight increased with increasing concentrations of sulphur up to 19.4 mg/l with a very small additional increase at 48.1 mg/l. In other experiments the minimum requirement for good growth seemed to be lower, sometimes as low as 5 mg/l as shown in Figure 3.1.3-2. The minimum requirement for sulphur appears to be at least 5 mg/l although 20 mg/l appeared to produce a significant improvement of growth over lower concentrations in some experiments. 100 mg/l S or more is inhibitory to C. glomerata in culture.

3.1.4 Nitrogen Requirements of C. glomerata

Once again, using the methods outlined in

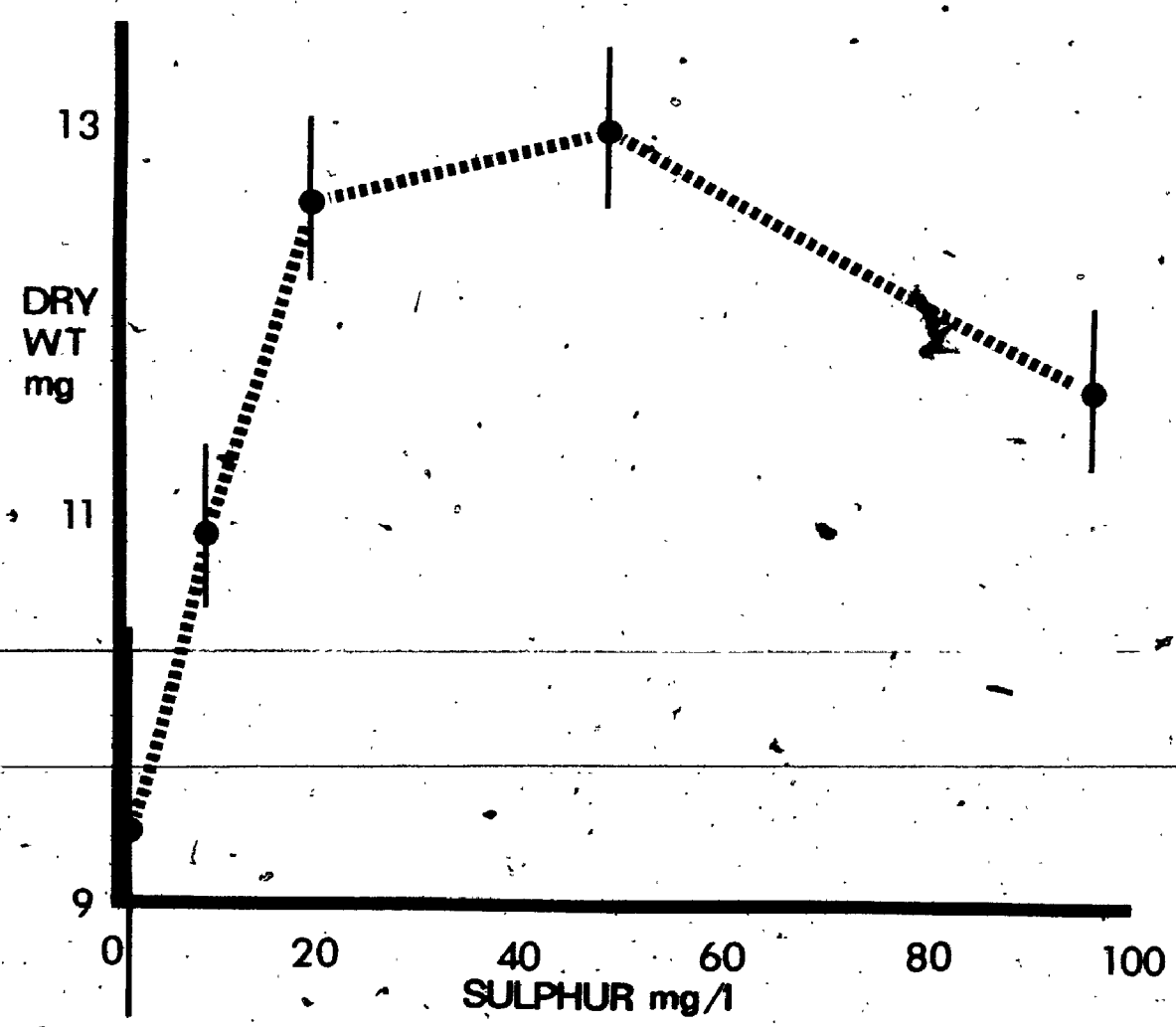


Figure 3.1.3-1 The response of C. glomerata to S between 0.0 and 96.2 mg/l.

ANOVA: $F < 2$ N.S. (.05)

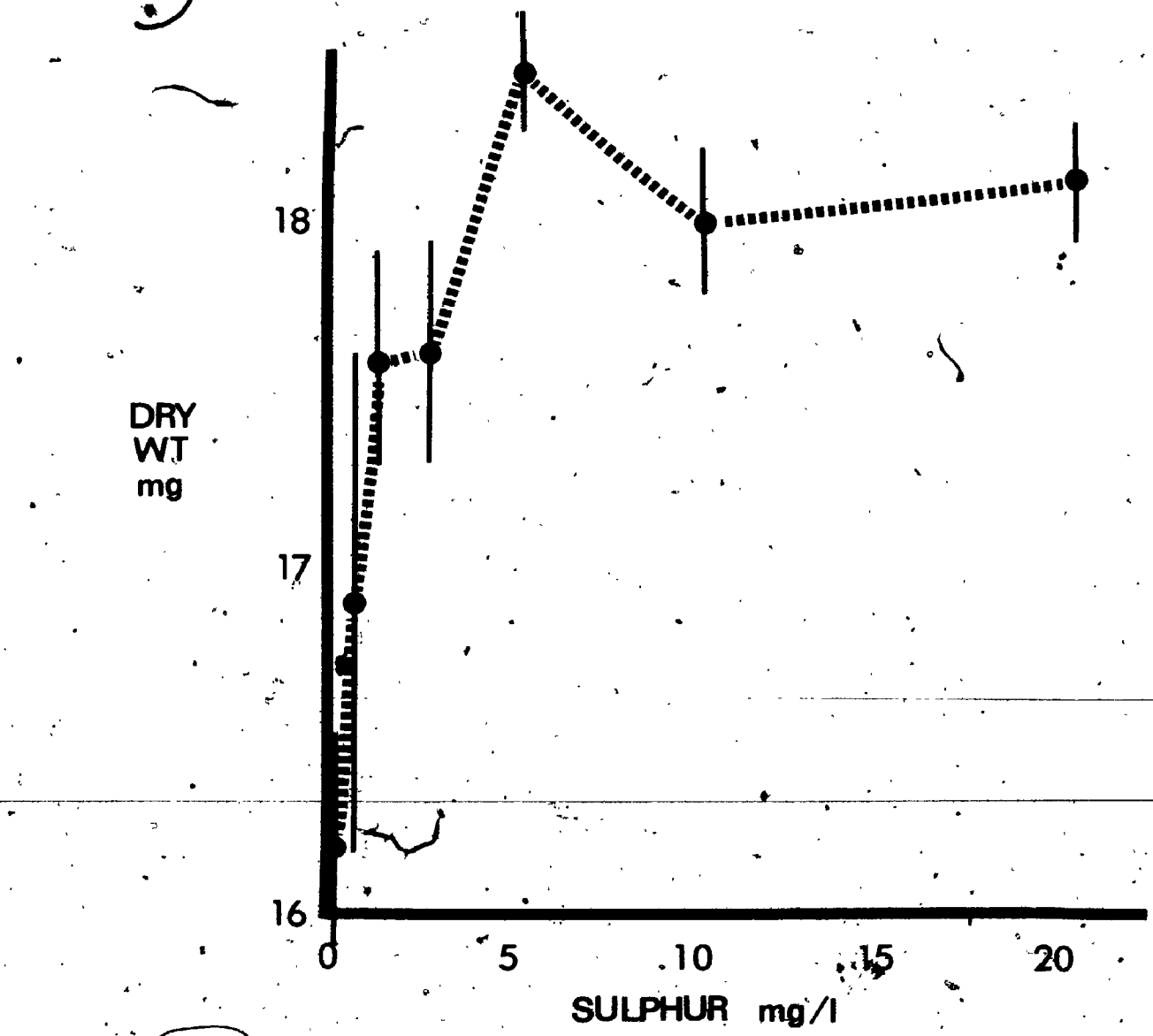


Figure 3.1.3-2 The response of C. glomerata to S between 0.0 and 21 mg/l.

ANOVA: $F > 3$ *(.05)

Section 2.6.2, The influence of nitrogen was examined. Concentrations of nitrogen from 0.05 mg/l to 89.65 mg/l were investigated and the results are shown in Figure 3:1.4-1. Almost as much growth was promoted by 5.65 mg/l N as by any other treatment although there is a small improvement at 11.25 mg/l and again at 44.85 mg/l. One complicating factor of analysing the reaction to nitrogen is that the buffer tris in the TBIM provides 40 mg/l N. To check on the possible influence of tris, one treatment in the experiment described above had nitrogen added as tris instead of as nitrate. This treatment produced better growth than any of the other treatments, the dry weight produced by the addition of double tris was 13.14 mg compared to 12.04 mg produced by the addition of 44.85 mg/l N as nitrate. To determine if this enhancement of growth by tris was reproducible, another experiment was set up using the same method used in the previous experiment except that tris was used to supply nitrogen at levels from 0 to 179.2 mg/l. Table 3.1.4-1 shows the influence on the growth of the C. glomerata as well as the effect on the pH of these various concentrations of tris. Concentrations of tris at 0.8 mM and below, each produced an average dry weight of just over 11 mg, while the treatments of 1.6 mM to 12.8 mM each produced a dry weight of just under 13 mg. The effect of the various concentrations of tris on

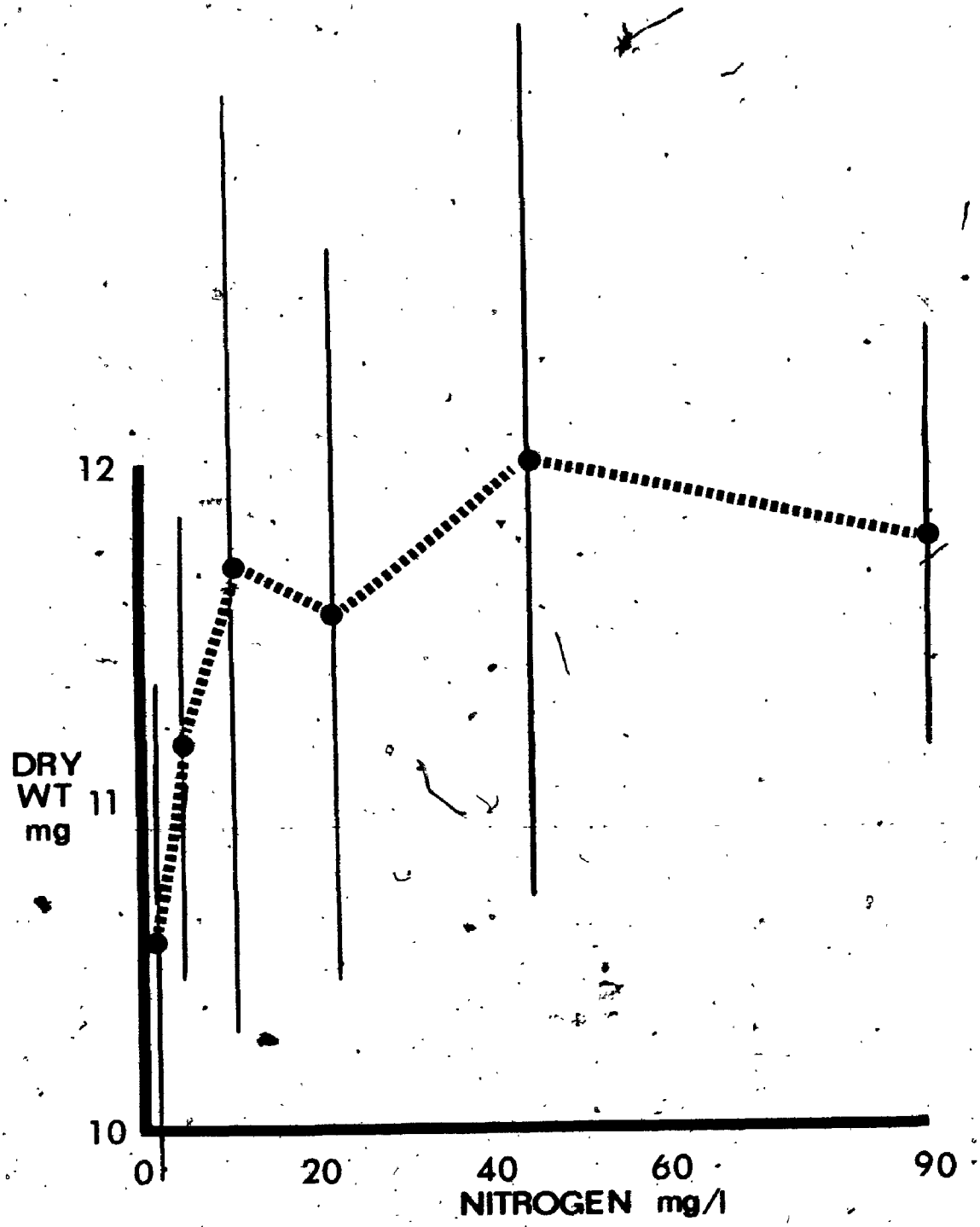


Figure 3.1.4-1 The response of C. glomerata to N between 0.05 and 89.65 mg/l.

ANOVA: $F < 2$ N.S. (.05)

Table 3.1.4-1

The Influence of Various Levels of Tris Buffer on the pH of the Medium and on the Growth of *C. glomerata*.

Conc. of tris (mM)	Conc. of N (mg/l)	Initial pH adjusted to 8.5	Final pH	Dry wt. of *C. glomerata* (mg ± SE)
0.0	0.0	7.2	7.8	11.6 ± 0.6
0.4	5.6	8.0	7.9	11.0 ± 0.8
0.8	11.2	8.6	8.0	11.3 ± 0.7
1.6	22.4	8.8	8.0	13.0 ± 0.5
3.2	44.8	9.3	8.1	11.6 ± 0.8
6.4	89.6	9.5	8.1	12.5 ± 0.3
12.8	179.2	9.8	8.2	12.9 ± 0.5

ANOVA: $F < 2$ N.S. (.05)

Table 3.1.4-2

The influence of Various Levels of Nitrogen on the Growth of C. glomerata

Concentration of N (mg/l)	Dry Wt. of C. glomerata (mg ± SE)
0.05	33.7 ± 3.0
11.25	56.8 ± 4.4
44.85	64.0 ± 12.0
179.25	56.2 ± 6.1

ANOVA: $F < 4.0$ N.S. (.05)

the final pH was not dramatic but there was a small increase in pH with increasing tris.

Another experiment testing the effect of a range of nitrogen concentrations, using the somewhat more reliable large flask method listed in Section 2.6.1, was set up. The results of this experiment are shown in Table 3.1.4-2. Once again 44.85 mg/l N was the treatment that produced the greatest dry weight. There is an increase in dry weight of almost 70% from the control, (with 0.05 mg/l N), to the 11.25 mg/l treatment and a further increase at the 44.85 mg/l treatment for a total increase of 100% over the control. The highest level of nitrogen tested was 179.25 mg/l. At this concentration suboptimal growth occurred.

3.2 The Response of C. glomerata to SWE

SWE was used as a supplement to maintain the stock cultures, but was always omitted from experiments since its contribution of nutrients would be unknown. However, some preliminary experiments established that some unknown components of SWE were significant in the nutrition of C. glomerata. Table 3.2-1 shows that SWE + calcium + sulphur produced five times the growth observed in the control cultures using TBIM alone while the addition of either calcium or sulphur alone produced only a small improvement in growth.

Table 3.2-1

The Relative Influence of Calcium, Sulphur and SWE on the Growth of *C. glomerata*

Treatment	Dry Wt.(mg ± SE)
TBIM + S (12.8 mg/l)	120.7 ± 11.6
TBIM + Ca (16 mg/l)	141.8 ± 13.2
TBIM + S (12.8 mg/l) + Ca (16 mg/l) + SWE (10% v/v)	495.9 ± 8.9
TBIM alone	95.9 ± 5.7

ANOVA: $F > 8$ ** (.01)

In an experiment, set up to determine the optimum concentration of SWE, the lowest concentrations tested, 0.5% and 1%, produced a marked improvement in growth (Figure 3.2-1). The best growth occurred at 20% SWE, the highest concentration tested, but 10% SWE yielded almost as much fresh weight.

The growth stimulation effected by the two different SWEs described in Section 2.2 were then tested. The results of these experiments are shown in Figures 3.2-2 and 3.2-3. SWE produced the same pattern of stimulation observed in Figure 3.2-1, with 90% of its stimulation at the 10% level and only a small additional stimulation at 20%. SWE 2, in a similar experiment produced less than 40% of its stimulation at the 10% level with a dramatic stimulation observed at 20%.

3.3 The Analysis of SWE

3.3.1 The Thiamine Content of SWE as Determined by *Phycomyces blakesleeanus* Thiamine Bioassays

The *Phycomyces* thiamine bioassay, a very sensitive assay for thiamine (Burkholder and McVeigh, 1940), was used to determine the level of thiamine in extracts made from the two samples of soil previously described in Section 2.2. The bioassays (Table 3.3.1-1) show that SWE (the more extensively used extract) contained 10 µg/l thiamine, while the second extract, SWE 2, con-

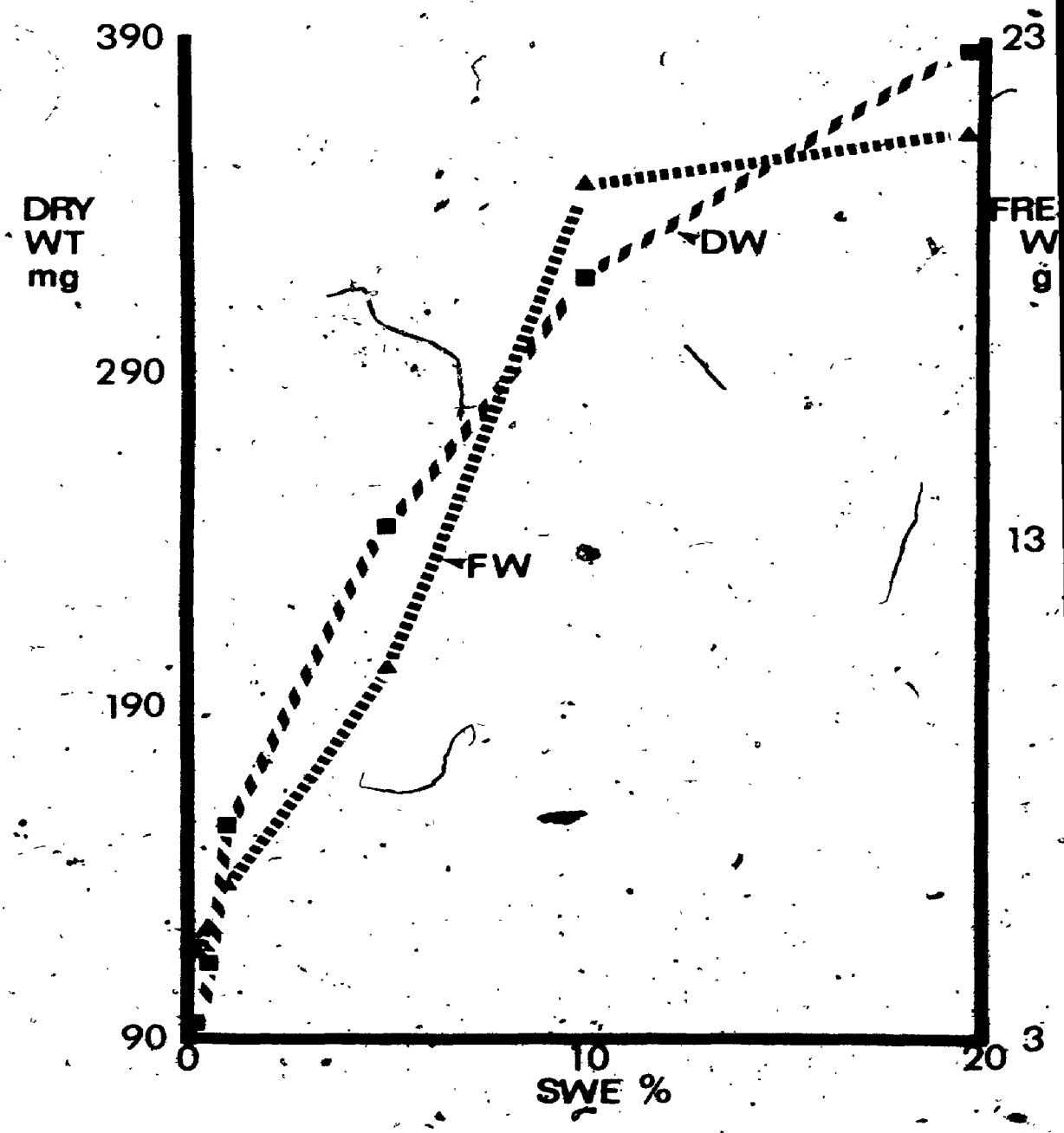


Figure 3.2-1 The results of a non-replica experiment showing the response of C. glomera to concentrations between 0 and 20% SWE.

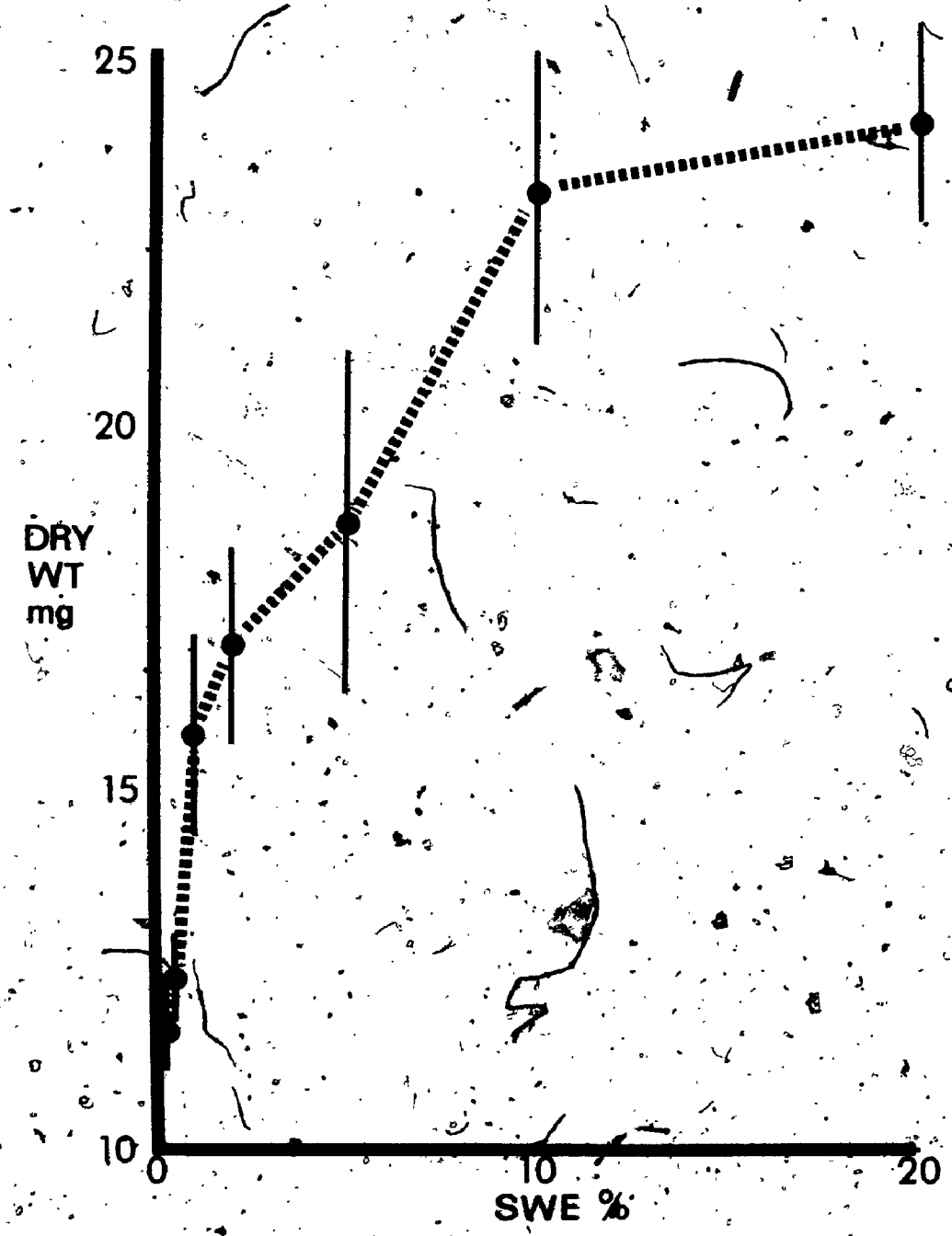


Figure 3.2-2 The response of C. glomerata to concentrations between 0 and 20% SWE.

ANOVA: $F > 3$ * (.05)

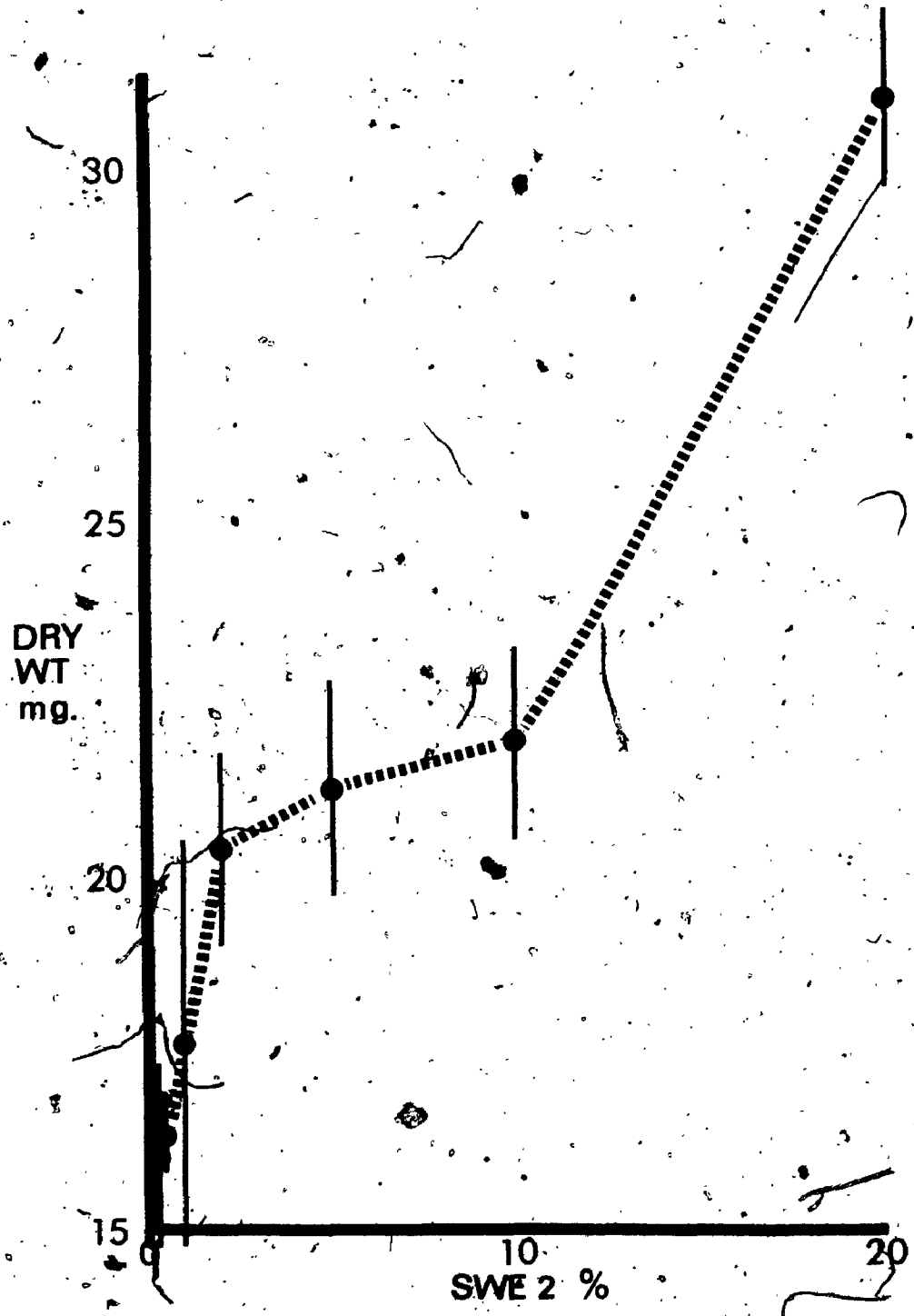


Figure 3.2-3 The response of C. glomerata to concentrations between 0 and 20% SWE 2.

ANOVA: $F > 3$ * (.05)

Table 3.3.1-1

Result of the Phycomyces Thiamine Bioassay and Calculation of the Level of Thiamine (in 100% of the sample) in Two Samples of SWE

Treatment	Dry Wt. of Phycomyces (mg ± SE)	Thiamine concentration
Controls		
0.0 µg/l thiamine	9.0 ± 0.6	
0.5 µg/l thiamine	19.5 ± 0.6	
1.0 µg/l thiamine	25.2 ± 3.2	
2.5 µg/l thiamine	39.4 ± 0.6	
Soil samples		
SWE (5%)	19.1 ± 0.5	10 µg/l
SWE 2 (10%)	15.0 ± 1.4	3 µg/l

tained only 3 µg/l.

3.3.2 The General Nature of the Active Components of SWE as Determined by Ashing and Differential Heating

Using the methods described in Section 2.6.2, two experiments were performed to determine the general nature of the active components in SWE. The first experiment was designed to test whether the active components of SWE resided in its ash and the second, examined what effect the three methods of sterilizing SWE (described in Section 2.8.2) had on its activity. The solution of SWE ash, prepared as described in Section 2.8.1, at a concentration of 10%, with 90% TBIM, produced 111.9 ± 7.6 mg dry weight which was almost identical to the dry weight of 112.6 ± 5.8 mg produced in the control of TBIM alone. The SWE that had not been ashed, produced the typical stimulation of growth and 393.0 ± 5.6 mg dry weight were produced. These results are shown in Table 3.3.2-1 along with the results of the differential heating experiment. This second experiment shows that SWE, after any of the three methods of sterilization, was capable of considerable stimulation of growth of C. glomerata. The control treatment of TBIM alone yielded only 62.7 ± 3.1 mg dry weight while the least effective of the SWE treatments was the autoclaved SWE which yielded 232.1 ± 6.0 mg dry weight.

Table 3.3.2-1

Growth of C. glomerata on Media Supplemented by SWE or Ashed SWE

Treatment	Dry Wt.(mg ± SE)
TBIM alone	112.6 ± 5.75
TBIM + 10% SWE	393.0 ± 5.6
TBIM + 10% SWE ashed	111.9 ± 7.6

Growth of C. glomerata on Media Supplemented by SWE Which Had Been Exposed to Three Levels of Heating During Sterilization

Treatment	Dry Wt.(mg ± SE)
TBIM alone	62.7 ± 3.1
TBIM + 10% SWE autoclaved	232.1 ± 6.0
TBIM + 10% SWE steamed	269.2 ± 14.3
TBIM + 10% SWE sterile filtered	316.8 ± 15.9

ANOVA: $F > 8$ ** (.01) [both expts]

However there was a considerable improvement in growth when less heat was applied during sterilization. The steamed SWE yielded 269.2 ± 14.3 mg dry weight and the sterile filtered SWE produced 316.8 ± 15.9 mg dry weight.

3.3.3 The Partial Chemical Analysis of SWE

Using the methods described in Section 2.8.4, analyses were done for calcium, nitrate, phosphate (total and soluble), silicon, sulphate and alkalinity.

Calcium was determined by establishing the calcium hardness. One sample (3 replicates) of SWE was found to have 300 mg/l hardness as $CaCO_3$, which gave a calculated calcium concentration of approximately 120 mg/l. 210 mg/l calcium hardness, as $CaCO_3$, was found in another sample (2 replicates) which gave a calculated calcium concentration of approximately 82 mg/l. This is a very high concentration of calcium and even a 10% solution of SWE would provide about 10 mg/l Ca which would be a considerable addition since TBIM contains only 4 mg/l Ca (Table 2.3-1).

The nitrate, phosphate and sulphate concentration all proved to be unspectacular as shown in Table 3.3.3. None of these could be expected to cause a significant increase in growth since TBIM contained much more of each of these elements. The alkalinity at 60 mg/l as $CaCO_3$ was approximately as expected with

Table 3.3.3

Inorganic Components of SWE

Component	Measured as	Concentration (mg/l)
Ca	$CaCO_3$	Sample 1 120.0 [Ca]
		Sample 2 82.0 [Ca]
N	NO_3	0.28 [N]
P	soluble PO_4	0.43 [P]
	total PO_4	0.53 [P]
Si	SiO_2	7.55 [Si]
S	SO_4	0.67 [S]
Bicarbonate alkalinity	$CaCO_3$	60.0

the pH of the SWE at 7.6. Silicon, however, was present at a high concentration. 10% SWE would provide almost 1 mg/l Si. TBIM provided no silicon to the alga except that leached from the glassware.

3.4 The Determination of the Active Factors in SWE

Thiamine was examined as one of the possible active components in SWE since the initial analyses of SWE, described in Section 3.3.2, suggest that a somewhat heat labile organic component is involved. Thiamine was also implicated since it is known to be required by a number of algae (Lewin 1961), is water soluble, and was found to be present in SWE (Section 3.3.1).

The relative effectiveness of thiamine and SWE in enhancing the growth of C. glomerata was investigated using both the standard method outlined in Section 2.6.1 and by a comparative growth curve study. The growth curve study was set up as described in Section 2.6.3. The effectiveness of calcium and silicon, found in SWE, were also investigated.

3.4.1 The Relative Effectiveness of Thiamine and SWE

Table 3.4.1-1 shows that TBIM supplemented with 1 mg/l thiamine produced 80% of the stimulation observed when TBIM is supplemented with 10% SWE. When both 1 mg/l thiamine and 10% SWE were used to supplement TBIM, great-

Table 3.4.1-1

Growth of *C. glomerata* on Media Supplemented by SWE or Thiamine, Alone or in Combination

Treatment	Dry Wt. (mg ± SE)
TBIM alone	34.5 ± 3.1
TBIM + thiamine (1 mg/l)	256.6 ± 5.3
TBIM + SWE (10%)	305.6 ± 4.9
TBIM + thiamine (1 mg/l). + SWE (10%)	334.0 ± 13.0

ANOVA: $F > 8$ ** (.01)

Table 3.4.1-2

Growth of Four Cladophora Isolates from the Indiana Culture Collection on Media Supplemented by SWE or Thiamine.

	C. fracta	C. glomerata		
	LB 1487	LB 1484	LB 1486	LB 1488
Treatment	Dry Wt. (mg)	Dry Wt. (mg)	Dry Wt. (mg)	Dry Wt. (mg)
TBIM	47.1	92.9	95.8	125.4
TBIM + thiamine (10 ug/l)	271.0	234.6	237.3	248.3
TBIM + SWE (10%)	312.1	324.4	279.1	311.1

er enhancement of growth was observed than that resulting from the addition of SWE alone. In addition, Table 3.4.1-2 shows the growth response of four other Cladophora isolates to media supplemented with thiamine or SWE.

3.4.2 Growth Curve Studies with TBIM Supplemented with SWE or Thiamine

Using the method outlined in Section 2.6.3, the growth of C. glomerata over time was investigated. The basic medium TBIM was supplemented either by 10% SWE or 1 mg/l thiamine. Growth of each treatment was assessed weekly by both dry weight and chlorophyll a determinations.

The chlorophyll a content of the two series of cultures over the six week period is shown in Figure 3.4.2-1. During the first week there was little increase but over the second and third week, chlorophyll a content rose rapidly in both treatments. There was no net production of chlorophyll a during the fourth week, and later the chlorophyll a content declined. The pattern of accumulation of chlorophyll a and the later decline was very similar for both treatments with the biggest differences between treatments being the constantly larger chlorophyll content induced by the addition of SWE.

Figure 3.4.2-1 The chlorophyll a content of C. glomerata cultures measured over six weeks. The dotted line shows the results from cultures supplemented with 10% SWE and the solid line shows the results from cultures supplemented with 1 mg/l thiamine.

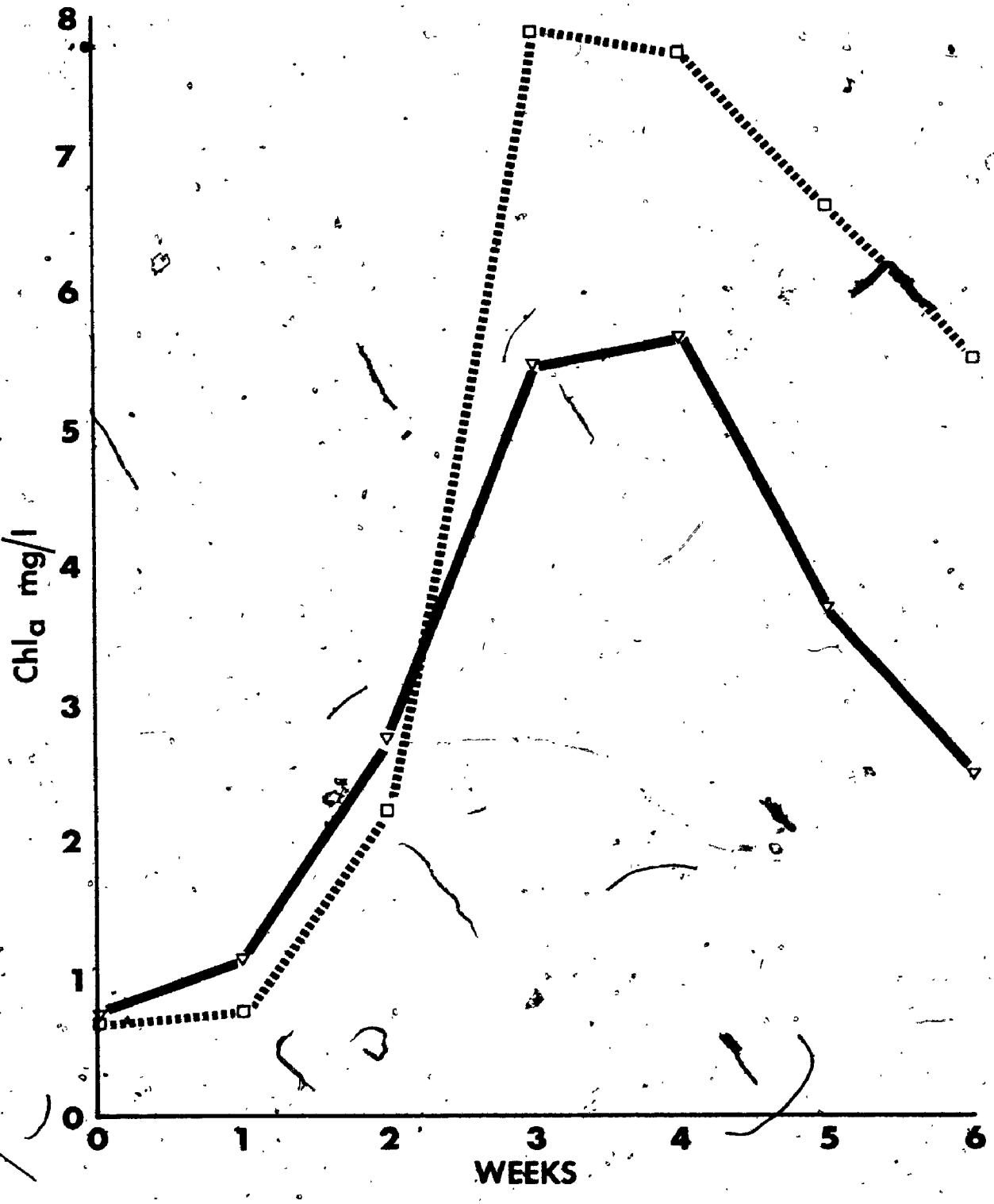
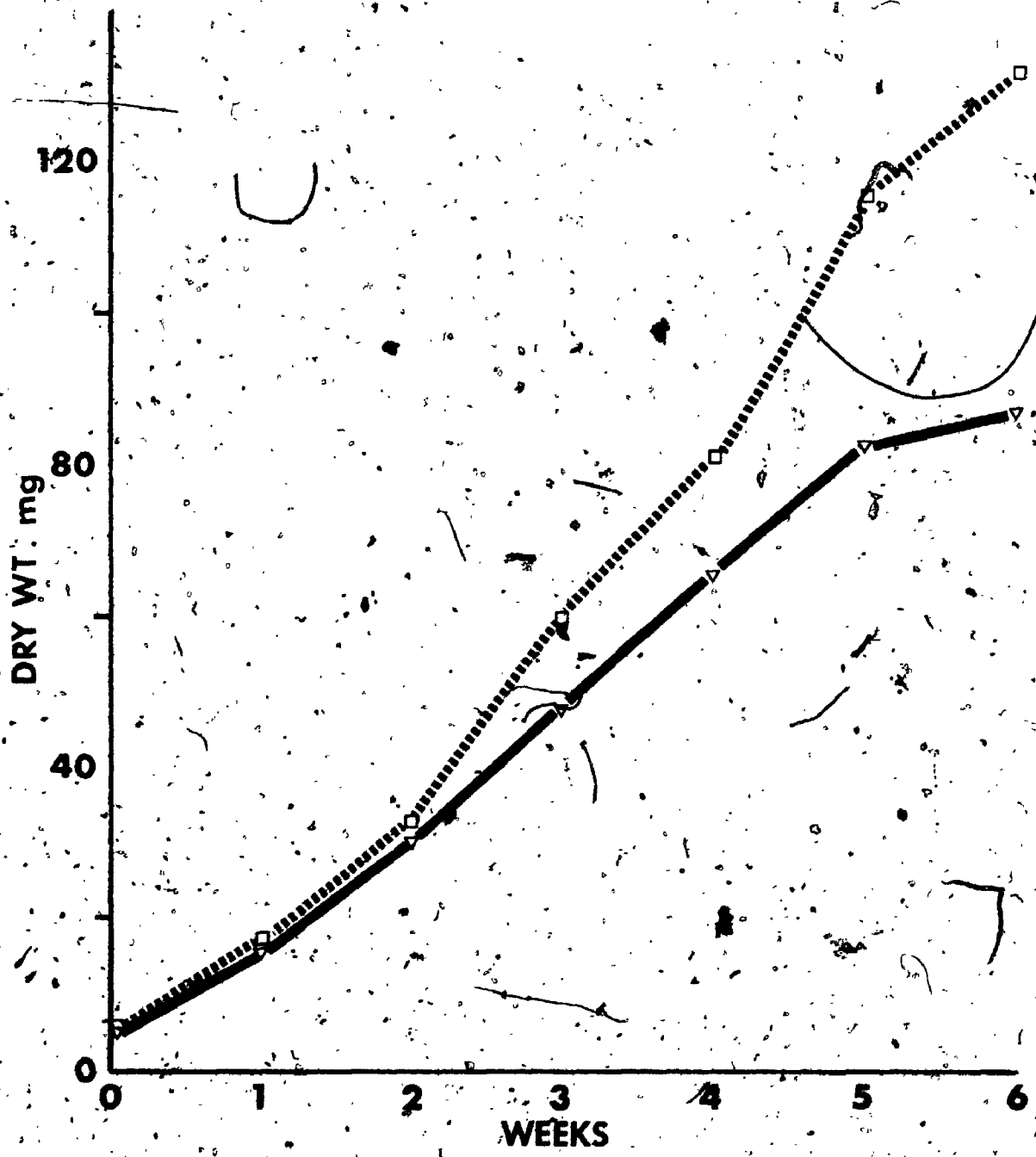

8
7
6
5
4
3
2
1
0
Chl a mg/l
0
1
2
3
4
5
6
WEEKS

Figure 3.4.2-2 The dry weight of C. glomerata cultures measured over six weeks. The dotted line shows the results from cultures supplemented with 10% SWE and the solid line shows the results from cultures supplemented with 1 mg/l thiamine.


120
80
40
0
DRY WT. mg
0
1
2
3
4
5
6
WEEKS

The results of the dry weight measurements, which were taken simultaneously with the chlorophyll a measurements, are shown in Figure 3.4.2-2. These graphs show an essentially steady increase in dry weight over the first five week period, and a slight decrease in rate of increase of dry weight in the sixth week. The dry weight is a measure of accumulated biomass and so it cannot decline until decomposition begins. The pattern of increase of dry weight was similar for both treatments. The only important difference between treatments, again, was that SWE produced a greater stimulation of growth than did the addition of thiamine.

3.4.3 The Determination of the Optimum Level of Thiamine

The results of three experiments, carried out according to the methods outlined in Section 2.6.2, are illustrated in Figure 3.4.3-1 and 3.4.3-2. Figure 3.4.3-1 shows the results of a preliminary experiment which investigated the range of thiamine from 0 to 625 µg/l. The lowest concentration tested (0.2 µg/l) resulted in a dramatic enhancement of growth. Further additions of 1 µg/l and 5 µg/l resulted in further enhancement but additions of 25 µg/l to 625 µg/l produced uniform growth at a level only slightly below that pro-

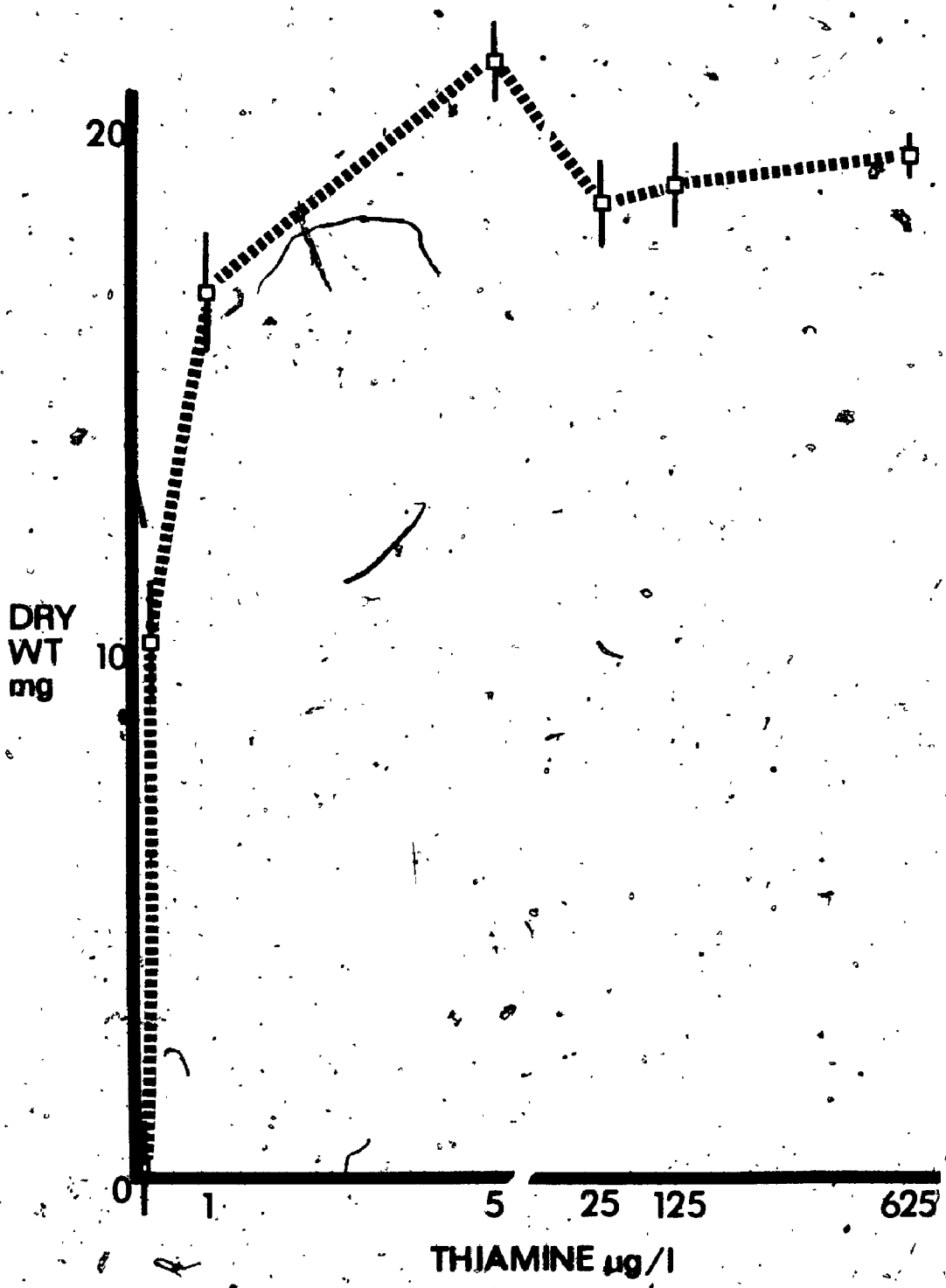


Figure 3.4.3-1 The influence of thiamine at concentrations of 0 to 625 µg/l on the growth of C. glomerata.

ANOVA: $F > 4.0$ ** (.01)

duced at 5 µg/l.

Further experimentation was carried out to find the optimum concentration which apparently falls somewhere between 5 and 25 µg/l thiamine. Figure 3.4.3-2 shows the results of two experiments covering a range of thiamine concentrations of 0 - 20 µg/l and 0 - 15 µg/l. The lowest concentration examined in the experiment that produced Graph 1 was 0.1 µg/l and even at this concentration a marked increase in growth was observed. Both Graphs 1 and 2 show 10 µg/l as the optimum concentration of thiamine for the enhancement of the growth of C. glomerata. Graph 1 shows a marked improvement of growth at 10 µg/l compared to 5 µg/l and only a slight reduction in enhancement at 20 µg/l. Graph 2 shows only a slight improvement in growth at 10 µg/l compared with 6 µg/l but a marked decrease in growth at 15 µg/l. This difference in the pattern of enhancement of growth is due, apparently, to minor vagrancies in the quality and quantity of the inoculum for each treatment.

In all three of these experiments, 1 µg/l thiamine is sufficient to provide most of the enhancement of growth which was observed.

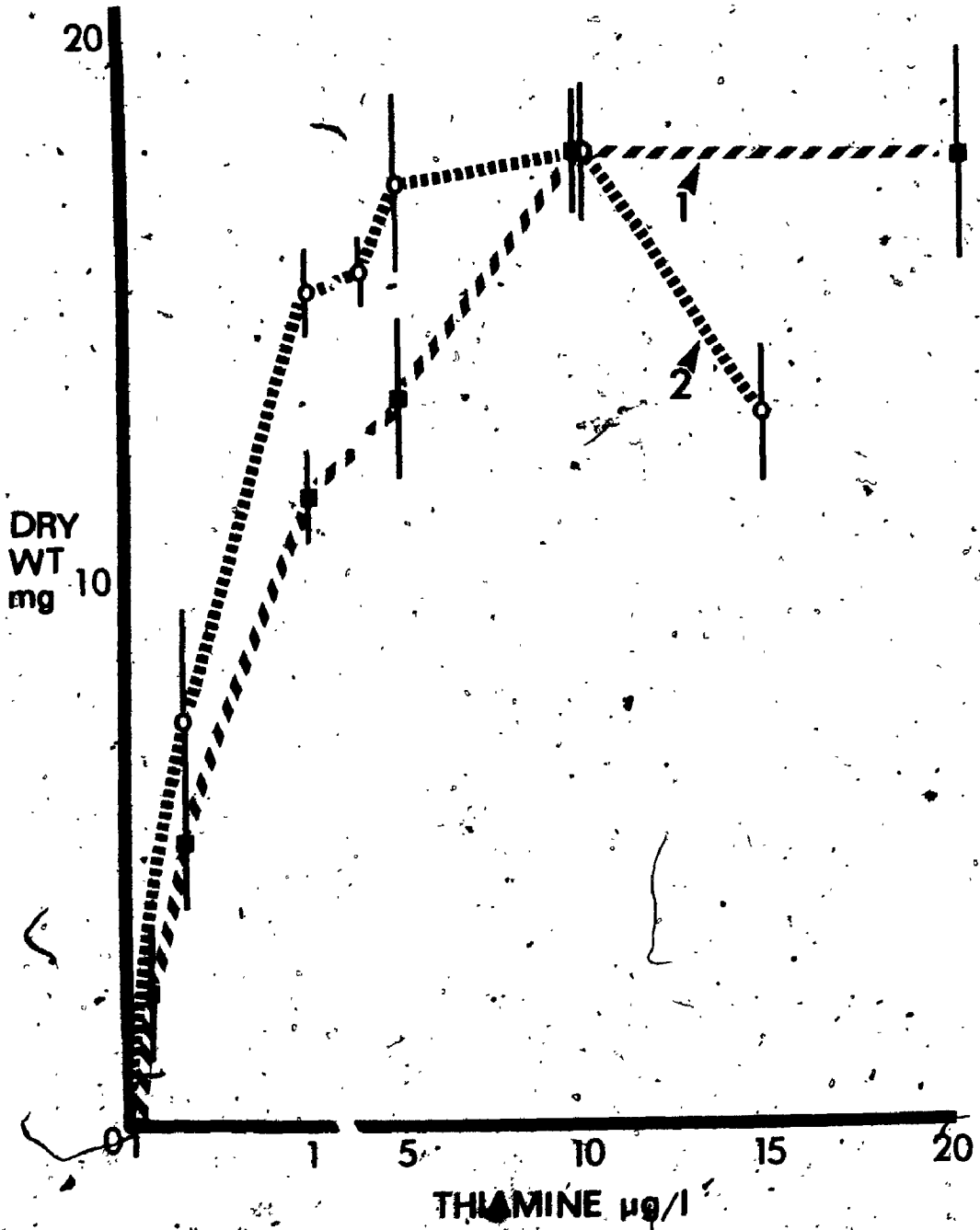


Figure 3.4.3-2 The influence of thiamine at concentrations below 20 µg/l on the growth of C. glomerata.

ANOVA: $F > 4:0$ ** (.01) [both expts]

3.4.4 The Effectiveness of the Ash of SWE when Added with Thiamine

The addition of SWE consistantly produces 15 to 25 % more growth than the addition of thiamine while SWE ash alone was shown to be inactive (Table 3.3.2-1). However, SWE ash might still contain compounds that could enhance growth if applied in the presence of thiamine. The results of an initial experiment, using SWE ash prepared as described in Section 2.8.1, are shown in Table 3.4.4-1. The addition of SWE produced approximately 84 mg more dry weight than the addition of thiamine alone but the addition of SWE produced only 24 mg more dry weight than the addition of SWE ash along with thiamine.

Further investigations of this additional enhancement of growth by SWE ash were carried out using the methods described in Section 2.6.2. Two similar experiments were conducted on the various combinations of SWE, SWE ash and thiamine. For one experiment, a normal stock culture, maintained on TBIM + 10% SWE, was used as the source of inocula while, for the other experiment, a special stock culture maintained on TBIM + 10 µg/l thiamine was used for the inocula. The results of these experiments showed similar responses to most treatments, however those cultures maintained on thiamine rich media showed less response to the addition of thia-

Table 3.4.4-1

The Influence of SWE Ash, when Added to TBIM Supplemented with Thiamine, on the Growth of *C. glomerata*

Treatment	Dry Wt. (mg ± SE)
TBIM + thiamine (10 µg/l)	234.6 ± 11.6
TBIM + thiamine (10 µg/l) + 10% SWE (ashed)	283.9 ± 5.7
TBIM + thiamine (10 µg/l) + 10% SWE	318.3 ± 5.8

ANOVA: $F > 8$ ** (.01)

Table 3.4.4-2

The Interactions of SWE, SWE Ash and Thiamine on the Growth Enhancement of *C. glomerata*

Treatment	Inocula maintained on TBIM + 10% SWE Dry Wt.(mg ± SE)	Inocula maintained on TBIM + thiamine Dry Wt.(mg ± SE)
TBIM	25.0 ± 1.6	26.1 ± 0.9
TBIM + SWE (10%)	35.8 ± 1.6	35.1 ± 1.7
TBIM + SWE (10%) + thiamine (10 µg/l)	37.5 ± 1.7	38.0 ± 2.2
TBIM + SWE (10%) + SWE ash (10%)	38.8 ± 1.2	36.5 ± 1.5
TBIM + SWE ash (10%)	24.5 ± 2.7	21.3 ± 2.5
TBIM + SWE ash (10%) + thiamine (10 µg/l)	39.9 ± 2.5	29.2 ± 2.2
TBIM + thiamine (10 µg/l)	35.1 ± 1.3	25.1 ± 1.4

ANOVA: $F < 2$ N.S. (.05) [both expts]

mine (Table 3.4.4-2). In both experiments the two lowest dry weights were produced by the treatments of TBIM alone and by TBIM fortified only by SWE ash.

Considering the acouracy of the experimental method, all of the other treatments in the first experiment gave approximately equal enhancement of growth. The second experiment showed almost equal growth for the three treatments with SWE but all of the other treatments, including the addition of thiamine, produced less enhancement of growth. The addition of thiamine and SWE ash gave a modest improvement over the control of TBIM alone. One constant feature in the two sets of results is that the enhancement of growth due to the addition of SWE alone is not as great as that due to the further addition of either thiamine or SWE ash.

3.4.5 The Significance of the Calcium Contribution from SWE

SWE was found to have a concentration of calcium of approximately 100 mg/l (Section 3.3.2). TBIM, on the other hand, provides only 4.0 mg/l Ca. Earlier experiments (Section 3.1.2) showed that calcium continues to enhance growth even at 25 mg/l. A 10% addition of SWE to TBIM would provide about 10 mg/l Ca to the culture medium and should induce a significant enhancement of growth. An experiment was set up, following

Table 3.4.5-1

The Significance of the Calcium Content of SWE on the Enhancement of Growth of *C. glomerata*

Treatment	Dry Wt. (mg ± SE)
TBIM + thiamine (10 µg/l)	223.1 ± 6.3
TBIM + thiamine (10 µg/l) + calcium (16 mg/l)	242.2 ± 8.8
TBIM + thiamine (10 µg/l) + 10% SWE	278.7 ± 5.0

ANOVA: $F > 8$ ** (.01)

the methods outlined in Section 2.6.1, to test the significance of calcium and the results are shown in Table 3.4.5-1. There is a clear improvement in growth with the addition of calcium at 16 mg/l which is only a slightly higher concentration of calcium than that provided by an addition of 10% SWE.

3.4.6 The Significance of the Silicon Component of SWE

The addition of calcium alone was not as effective as the addition of SWE ash, so some other inorganic component was implicated. Phosphorus, nitrogen and sulphur, if indeed they remained in the ash, were at lower concentrations in SWE than in TBIM so they are not likely involved. However, silicon would remain in the ash and a 10% solution of SWE would provide 0.8 mg/l since there is 7.6 mg/l in undiluted SWE (Table 3.3.2-1) while TBIM provides no ~~sili~~con. Table 3.4.6-1 shows the results of an initial experiment, designed to determine the effect of silicon. This experiment was conducted using the method outlined in Section 2.6.1, except that two treatments instead of four were used. The results show only a very small increase in growth with the addition of silicon.

Since this initial experiment gave an indication that silicon was having an effect, the effect of a range of higher concentrations of silicon was inves-

Table 3.4.6-1

The Influence of Silicon at the Levels Found in SWE on The Growth of *C. glomerata*

Treatment	Dry Wt.(mg ± SE)
TBIM + thiamine (10 µg/l) + calcium (16 mg/l)	199.3 ± 3.3
TBIM + thiamine (10 µg/l) + calcium (16 mg/l) + silicon (1.0 mg/l)	205.6 ± 4.7

ANOVA: $F < 5$ N.S. (.05)

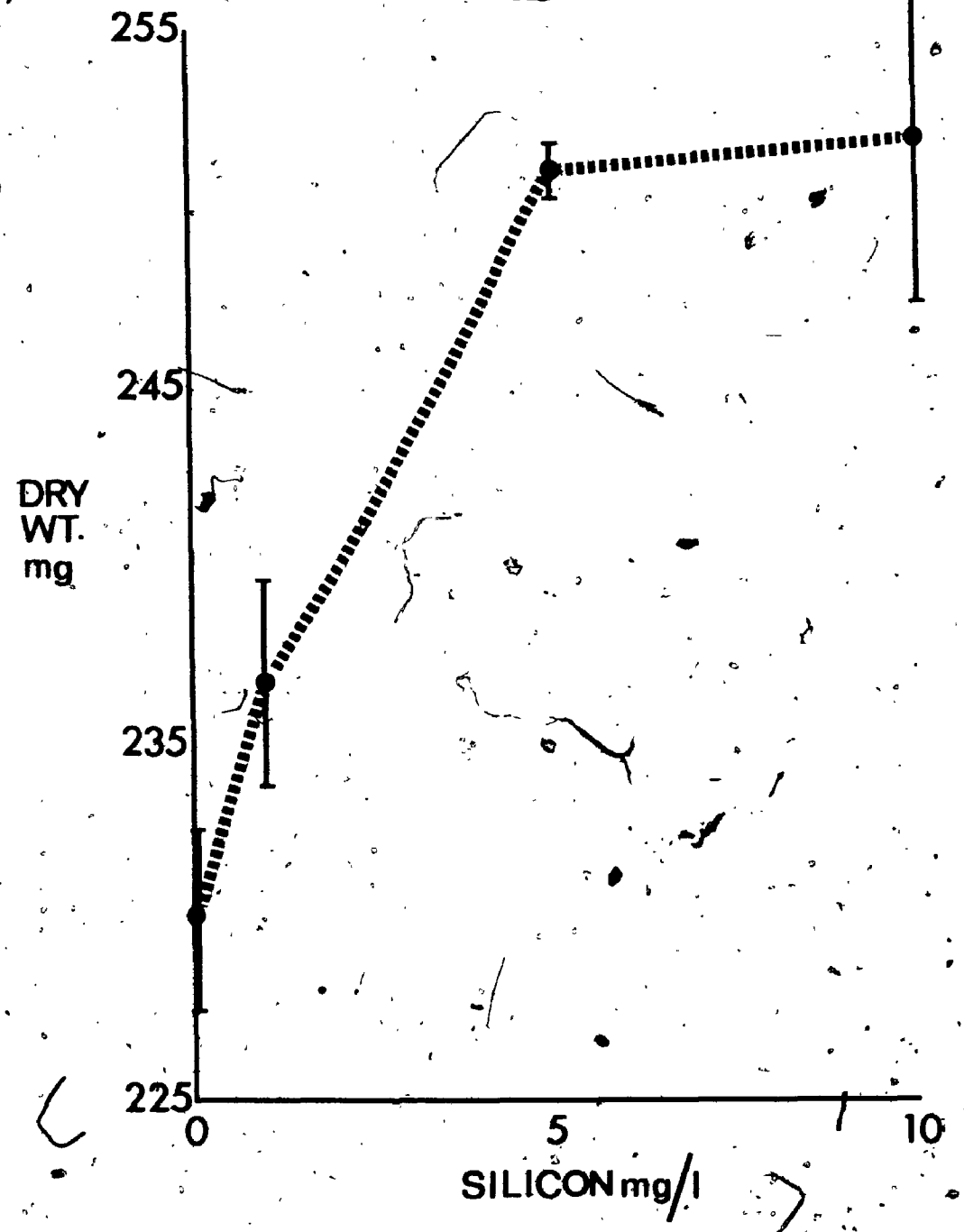


Figure 3.4.6-1. The influence of silicon at concentrations between 0 and 10 mg/l on the growth of C. glomerata.

ANOVA: F > 8 ** (.01)

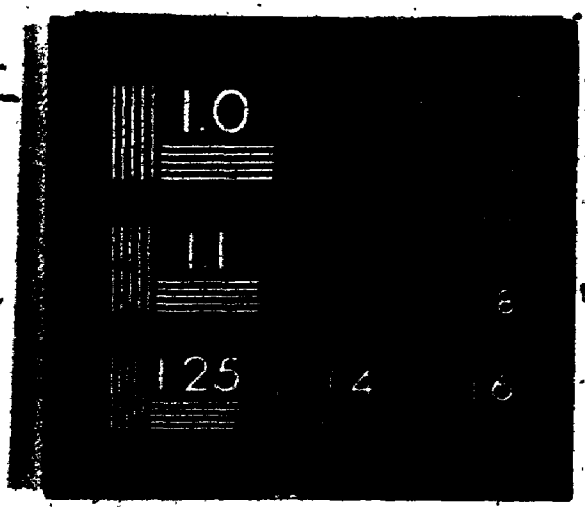
Table 3.4.6-2

Analyses of Silicon in the Spent Media an
in the Alga from the Experiment Shown in Figure 3.

Treatment	Si in spent media	Extractable Si in algae	g
TBIM + Ca(16mg/l) + thiamine(10µg/l)	0.27 mg/l	0.22 mg	
TBIM + Ca(16mg/l) + thiamine(10µg/l) + Si(1mg/l)	1.0 mg/l	0.38 mg	
TBIM + Ca(16mg/l) + thiamine(10µg/l) + Si(5mg/l)	4.8 mg/l	0.52 mg	
TBIM + Ca(16mg/l) + thiamine(10µg/l) + Si(10mg/l)	9.9 mg/l	0.53 mg	

2 2

OF/DE



tigated using the methods outlined in Section 2.6.1. Figure 3.4.6-1 shows that, at 1 mg/l, a small increase, comparable to that seen in the previous experiment, was obtained. A considerable enhancement in growth was induced by the addition of 5 mg/l Si, but no further enhancement was observed by the addition of 10 mg/l.

At the conclusion of this experiment, both the spent media and the harvested algae were analysed for silicon, using the methods outlined in Section 2.11, the results of these analyses are shown in Table 3.4.6-2. The treatment with no added silicon contained 0.27 mg/l Si in the medium and 0.22 mg Si in the alga harvested from 1 liter. This silicon must have been derived from the glass vessels. Only a very minor removal of silicon from the medium was detected by the method used, but quite large differences in silicon content of the alga were found which correlated with the observed growth enhancement.

3.4.7 The Partial Replacement of the Activity of SWE by Thiamine, Silicon and Calcium

The effectiveness of additions of SWE, SWE ash solution and a combination of calcium and silicon were compared using the experimental procedure outlined in Section 2.6.1. Thiamine at a concentration of 10 µg/l was added to each treatment to ensure that the

Table 3.4.7-1

A Comparison of the Enhancement of the Growth of C. glomerata by SWE, SWE Ash or a Combination of Calcium and Silicon.

Treatment	Dry Wt.(mg ± SE)
TBIM + thiamine (10 µg/l) + SWE (10%)	225.6 ± 4.2
TBIM + thiamine (10 µg/l) + SWE ash soltn (10%)	202.5 ± 5.8
TBIM + thiamine (10 µg/l) + Si (5 mg/l) + Ca (16 mg/l)	209.6 ± 5.9

ANOVA: $F > 5$ *(.05)

thiamine content of SWE was not a limiting factor in the growth enhancement. The addition of calcium and silicon effectively duplicated the influence of the SWE ash solution but still did not quite equal the stimulation provided by SWE itself (Table 3.4.7-1).

3.4.8 The Influence of Germanium

Since GeO_2, at concentrations below 75 mg/l, is toxic only to plants that require silicon (Werner 1967 a), the effect on C. glomerata of 75 mg/l GeO_2 and lower concentrations was investigated. Figure 3.4.8-1 shows that 75 mg/l GeO_2 severely inhibited growth, 25 mg/l gave moderate inhibition and 5 mg/l did not result in a decrease in dry weight. The algae in both the 25 mg/l and the 5 mg/l treatments appeared very healthy and actually appeared greener than the control although cultures exposed to 25 mg/l produced a much smaller mass. Microscopic examination showed that the cells in all of the germanium treatments were somewhat enlarged and the chloroplasts were more complete than in the control treatment. Cross wall formation was severely inhibited in the 25 mg/l treatment (Plate 3.4.8-1 C and D). The enlargement of the cells and the bulging of the cell walls is illustrated clearly in Plate 3.4.8-1 (B and D). A comparison of this Plate with Plate 3.4.8-2, showing cells from the control treatment,

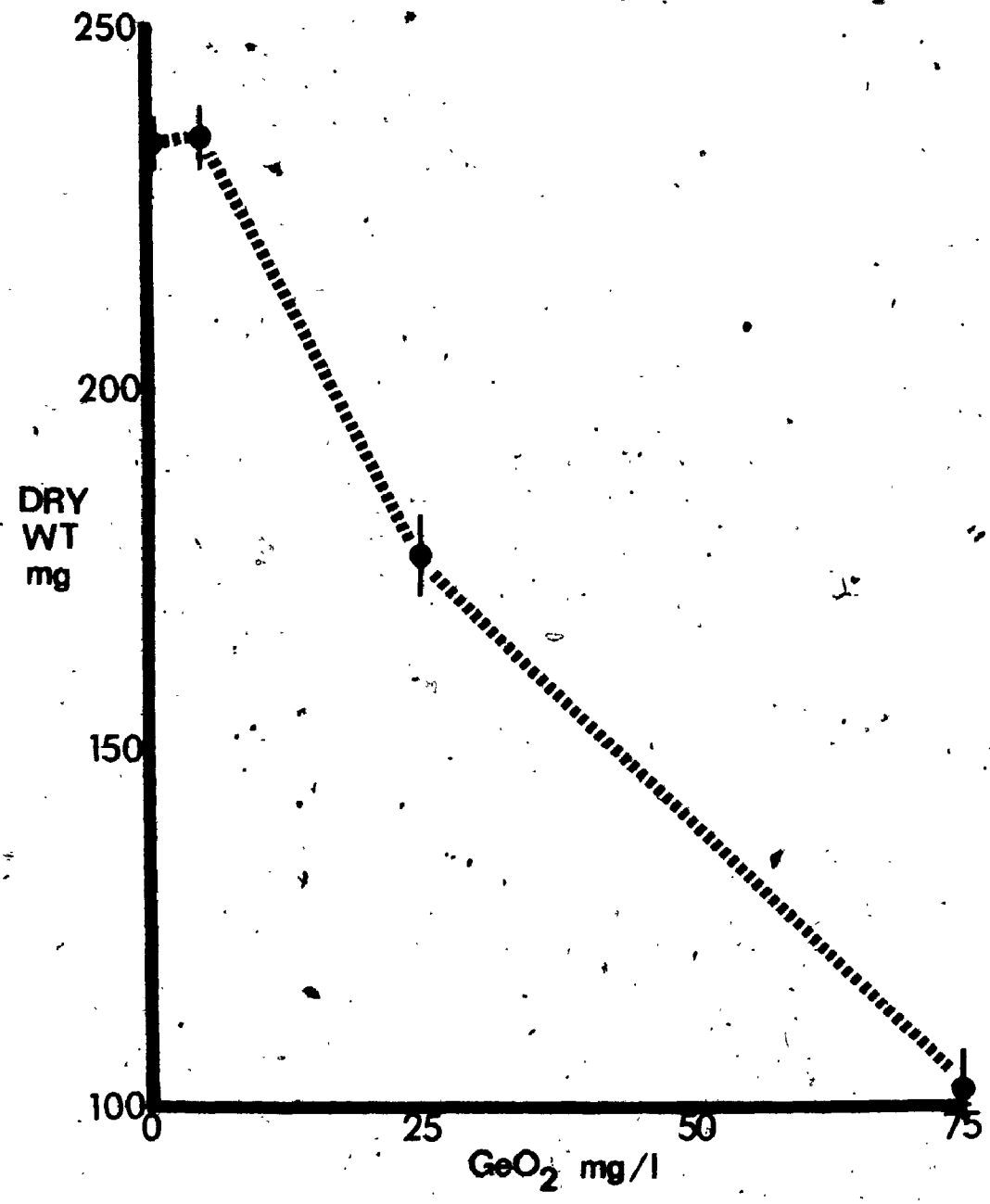


Figure 3.4.8-1 The influence of GeO_2 at concentrations between 0 and 75 mg/l on the growth of C. glomerata.

ANOVA: $F > 8$ ** (.01)

Plate 3.4.8-1 Light micrographs of living material of C. glomerata incubated in medium with 25 mg/l GeO_2. Scale = 30 µm.

A - Part of two cells with an incomplete cross wall, showing continuous cytoplasm, a complete chloroplast and the deposition of an undefined substance inside the cell wall.

B - A cell illustrating bulging walls and an abnormal cross wall.

C - A length of filament with numerous incomplete cross walls and the deposition of some substance on the inside of the walls.

D - Part of two cells showing incomplete cross walls and bulging cell walls.

Plate 3.4.8-2 Light micrographs of living material of C. glomerata incubated in medium with no added GeO_2. Scale = 30 µm. These filaments show the normal condition of this alga in culture.

A - Part of two cells with a normal cross wall.

B, C - Cells with disperse chloroplasts, cytoplasmic strands and normal cell walls.

D - Low magnification view showing the length of cells and condition of the chloroplast.

Plate 3.4.8-3 Light micrographs of living material of C. glomerata incubated in medium with 75 mg/l GeO_2. Scale = 30 μm.

A - A high magnification view of a highly abnormal cross wall showing deposition of large amounts of material on either side of the cross wall.

B, C - The extreme thickening of the cell wall is shown.

D - Some of the distorted cell shapes common in this treatment are shown.

clearly shows the difference in cell size. Plate 3.4.8-2 also shows normal cross walls and the typical dispersed chloroplasts which are normally found in cultured C. glomerata. Plate 3.4.8-3 illustrates algae from the 75 mg/l GeO_2 treatment in which the striking feature is the massive thickening of the inner layer of the cell wall. Some attempts at cell division were evident in these cells but the appearance of the cells and the reduction of the final size of the culture suggested that most of these cells had not divided but merely enlarged and thickened their walls.

In another experiment, 20 mg/l GeO_2 was supplied to one set of cultures while 20 mg/l GeO_2 and 20 mg/l Si were supplied to another. The treatment of germanium alone, produced the typical disruption of cross walls, while the addition of the silicon slightly reduced the occurrence of incomplete cross walls but did not increase the dry weight produced by the inhibited cells.

3.4.9 Observations of the Cell Wall with the Electron Microscope

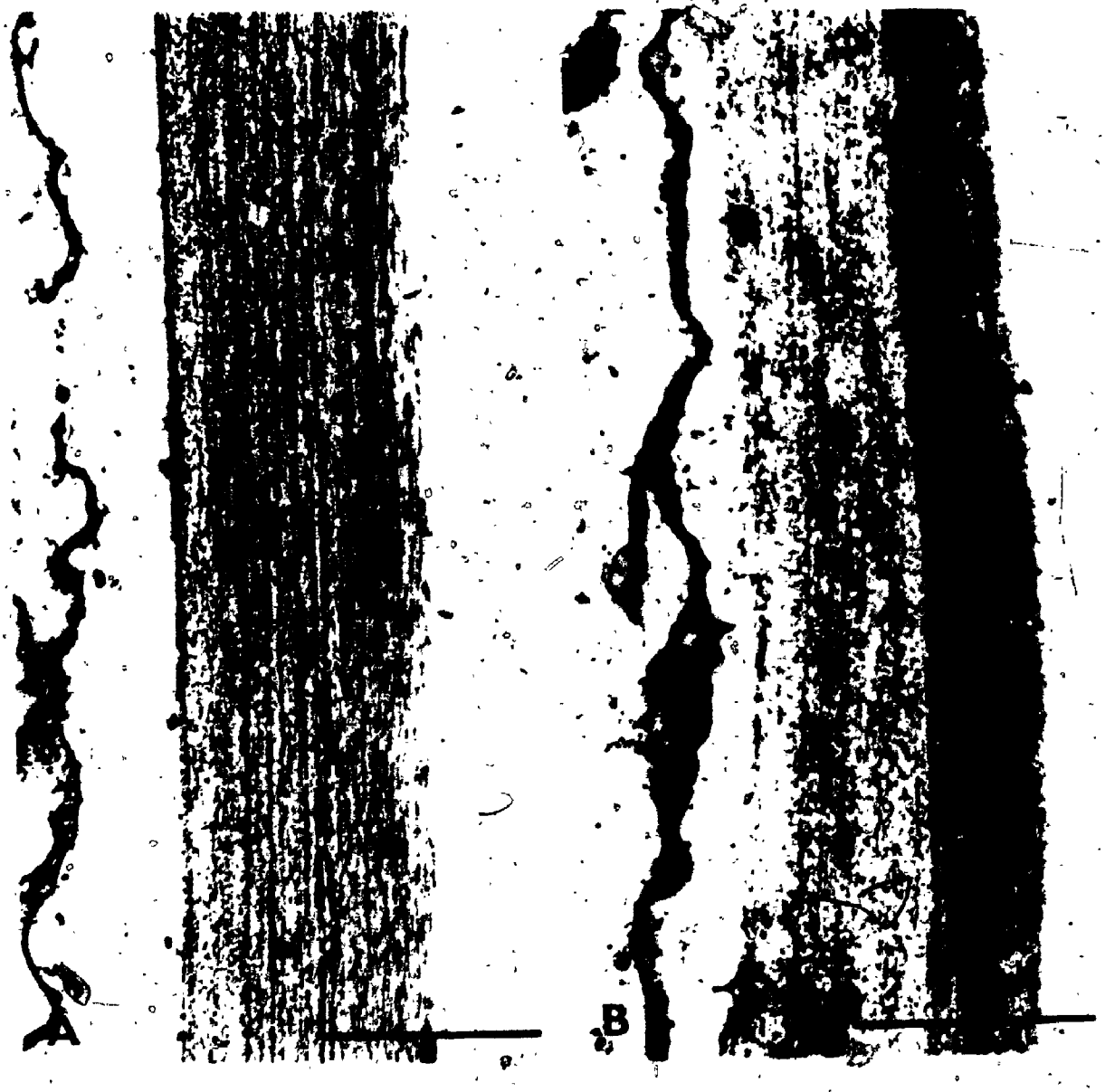
To locate silicon in the cell, a sample of C. glomerata grown with added silicon and another control sample were prepared for the electron microscope, using the methods outlined by Franke et. al. (1969). The

Plate 3.4.9-1 Electron micrographs of lateral cell walls of C. glomerata. Scale = 1 μm.

A - No added silicon.

B - 5 mg/l silicon.

Note the electron-dense outer band characteristic of cell walls from silicon enriched cultures.


A
B

outer layer of the cell wall, which closely resembled the proteinaceous cuticle of C. rupestris described by Hanic and Craigie (1969), was found to be considerably more electron-dense in cells that had access to supplementary silicon (5 mg/l Si treatment shown in Figure 3.4.6-1) than in cells growing in media with no added silicon (Plate 3.4.9-1).

3.5 The Influence of Additional Carbon, Both Organic and Inorganic on the Growth of C. glomerata

In preliminary studies, soluble starch was found to give large increases in dry weight consistantly, while other carbon additions, peptone, yeast extract, dextrose, sucrose and sodium bicarbonate, generally caused a decrease in dry weight compared to the control which contained no additional carbon.

Subsequent experiments using the method outlined in Section 2.6.1, showed once again that soluble starch gives a substantial increase in dry weight. However, 250 mg of soluble starch were added to each flask and the increase in dry weight was approximately 250 mg per flask. The increase in dry weight seemed more due to the adherance of the soluble starch to the filaments than to real growth. Another experiment, using the modified method in Section 2.6.2, tested the effect of soluble starch and $NaHCO_3$, using chlorophyll analyses, as

Table 3.5-1

The Apparent Influence of Soluble Starch (SS) on the Growth of *C. glomerata*

Treatment	Dry Wt. (mg ± SE)
TBIM + SWE 10% (v/v)	263.6 ± 8.2
TBIM + SS (250 mg/l) + Ca (16 mg/l)	466.5 ± 2.1
TBIM + SS (250 mg/l) + thiamine (10 µg/l) + Ca (16 mg/l)	519.3 ± 7.6
TBIM + thiamine (10 µg/l) + Ca (16 mg/l)	254.7 ± 3.5

ANOVA: $F > 8$ * (.01)

Table 3.5-2

The Influence of Soluble Starch (SS) and $NaHCO_3$ on the Chlorophyll Content of C. glomerata

Treatment	mg Chl/l ± SE
TBIM + 10% SWE	0.104 ± 0.011
TBIM + SS (250 mg/l) + Ca (16 mg/l)	0.029 ± 0.003
TBIM + SS (250 mg/l) + Ca (16 mg/l) + thiamine (10 µg/l)	0.024 ± 0.002
TBIM + $NaHCO_3$ (250 mg/l) + Ca (16 mg/l) + thiamine (10 µg/l)	0.020 ± 0.004
TBIM + $NaHCO_3$ (250 mg/l) + Ca (16 mg/l)	0.011 ± 0.001
TBIM + Ca (16 mg/l) + thiamine (10 µg/l)	0.095 ± 0.015
TBIM alone	0.095 ± 0.005

ANOVA: $F > 4$ * (.01)

described in Section 2.7, rather than dry weight determinations, assuming that adherance of the starch to the algal filaments would not be measured.

These results (Table 3.5-2), clearly show that the dramatic increase in dry weight was not due to growth but merely to adherance of the soluble starch on the filaments. The chlorophyll content of C. glomerata is actually reduced by the addition of soluble starch or $NaHCO_3$.

3.6 The Influence of Bacteria on the Response of C. glomerata to Thiamine

3.6.1 The Effectiveness of Antibiotics in Reducing the Bacterial Population of C. glomerata Cultures

Following the method described in Section 2.10.1 the antibiotics, chloramphenicol, erythromycin and tetracycline were screened as potentially useful agents to remove bacteria from the C. glomerata cultures. Although many of the algal inocula died during these treatments, Table 3.6.1-1 shows the degree of bacterial contamination of the eight surviving algal inocula indicated by the turbidity of the peptone tubes.

All these flasks showed some bacterial contamination but chloramphenicol, particularly at 40 and 80 mg/l, was quite effective at reducing the bacterial population. After one week, the algal inocula that had not been transferred out of the antibiotic mixtures were examined to determine whether any concentration of anti-

Table 3.6.1-1

The Effectiveness of the Antibiotic Treatments that were not Lethal to C. glomerata, in Reducing the Bacterial Population

Flask #	Antibiotic treatment	% Transmission* of peptone tube after 4 days incubation
1	Chloramphenicol (20 mg/l)	91
2	Erythromycin (20 mg/l)	70
8	Chloramphenicol (40 mg/l)	77
9	Erythromycin (40 mg/l)	74
11	Chloramphenicol (40 mg/l) and Erythromycin (40 mg/l)	97
14	Chloramphenicol (80 mg/l)	97
15	Erythromycin (80 mg/l)	92
20	None	24

* measured at 450 nm in a Spectronic 20

biotic could be maintained in the cultures without inhibiting growth. Only Flask #1, containing 20 mg/l chloramphenicol, and Flask #20, with no antibiotic, were healthy and growing. Bacterial checks on these flasks, following the method described above, showed 49% transmission for Flask #1 and 19% transmission for Flask #20.

3.6.2 The Effect, on the Response of C. glomerata to Thiamine, of Reducing the Bacterial Population by the Use of Antibiotics

Inocula of C. glomerata were prepared as described in Section 2.10.2. Three of the six antibiotic-containing flasks and three of the six control flasks were used to inoculate six experimental flasks which contained 950 ml of TBIM with 10 µg/l thiamine. The other three antibiotic containing flasks and three control flasks were used to inoculate six experimental flasks containing 950 ml of TBIM only. The experiment was then conducted as described in Section 2.6.1. After three weeks the algae were harvested and bacterial plate counts, as described in Section 2.10.3, were done. The results (Table 3.6.2-1) show that the antibiotic treatment was effective in reducing the bacterial population from approximately 1×10^8 bacteria/ml to 1×10^7 bacteria/ml. These bacteria numbers are very high but

Table 3.6.2-1

The Effectiveness of the Antibiotic Treatment for Reducing Bacteria and the Effect of this on the Response of *C. glomerata* to Thiamine.

Treatment	Dry Wt. (mg ± SE)	Bacteria / ml ± SE
Antibiotic treated inocula, 10 µg/l thiamine	204.0 ± 1.7	(1.1 ± 0.29) x 10^7
Antibiotic treated inocula, no thiamine	102.9 ± 9.2	(8.3 ± 0.22) x 10^6
Control inocula, 10 µg/l thiamine	218.7 ± 1.1	(1.2 ± 0.30) x 10^8
Control inocula, no thiamine	78.7 ± 9.0	(1.2 ± 0.08) x 10^8

ANOVA: F > 4 * (.05) [both Dry Wt and Bacteria / ml]

they represent counts from three week old cultures when there is considerable substrate for them to utilize.

The reduction in the number of bacteria had no effect on the enhancement of the growth of C. glomerata by thiamine which seems to indicate that the bacteria are not mediating this enhancement.

The bacteria on these plates were then examined and only two dominant colony types were observed. One was pale yellow and the other was white and both were composed of gram negative rods.

In order to determine the thiamine requirements of these bacteria, five plates of TBIM agar plus 1% dextrose were inoculated with one type of bacteria and another five plates were inoculated with the other type. Similarly, five plates of TBIM agar plus 1% dextrose and 20 µg/l thiamine were inoculated with each bacterium. The bacteria were observed to grow equally well on all plates showing that the bacteria do not require thiamine and providing additional evidence that the bacteria are not mediating the response of C. glomerata to thiamine.

3.7 Summary of Results

3.7.1 The pH Optimum and Inorganic Requirements Not Related to SWE

The results of these experiments are somewhat difficult to interpret because of the small growth of the alga and the large variations within treatments. However, some consistant results were obtained and these will be summarized here.

pH

The best growth of C. glomerata was observed when the initial pH of the medium was between 8.0 and 9.0. The apparent optimum initial pH was 8.5 (Figure 3.1.1-1).

Calcium

Calcium provided increased enhancement of growth with increasing concentrations up to 25.6 mg/l, the highest concentration tested (Figure 3.1.2-1).

The minimum concentration of calcium for survival seemed to be approximately 1 mg/l (Figure 3.1.2-2) and the alga had a healthy appearance only when growing in media with a concentration of calcium in excess of 5 mg/l.

Sulphur

Sulphur concentrations approaching or in excess of 100 mg/l are inhibitory to C. glomerata. 20 mg/l S seems to be required for good growth and 1 mg/l S

is the apparent minimum requirement for survival (Figure 3.1.3-1).

Nitrogen

Nitrogen requirements proved difficult to establish. An optimum of 45.0 mg/l N was found in one experiment (Figure 3.1.4-1) and this was confirmed in another experiment using a more reliable method (Table 3.1.4-1).

3.7.2 The Importance of Organic Compounds as Carbon Sources and the Effect of the Addition of Bicarbonate.

Soluble Starch

Soluble starch at first appeared to give a stimulation of growth (Table 3.5-1) but this was later found to be merely adherance of starch particles to the algal filaments (Table 3.5-2).

Other Organics

All other organics tested increased the bacterial growth in the media but resulted in an inhibition of the growth of C. glomerata.

Bicarbonate

The addition of bicarbonate caused the pH to shift upwards and inhibited the growth of the alga.

3.7.3 The Significant Components of SWE

Three components of SWE were found to be sig-

nificant in the enhancement of the growth of C. glomerata. These components were thiamine, calcium and silicon. Together they made up more than 90% of the effectiveness of SWE (Table 3.4.7-1). The outline below shows the main steps in the analysis of SWE which revealed these nutritional requirements.

Steps in the Analysis of SWE

a. SWE was ashed; the ash was not effective in enhancing growth (Table 3.3.2-1).

b. SWE was heated; heat decreased the activity of SWE (Table 3.3.2-1).

c. Thiamine when tested in place of SWE replaced 80% of the effectiveness of SWE (Table 3.4.1-1).

d. The Phycomyces bioassay for thiamine was done on SWE and SWE2. The extracts contained 10 µg/l and 3 µg/l thiamine respectively.

e. The growth enhancement effected by SWE and SWE2 at concentrations from 0 to 20% was investigated. The different patterns of stimulation shown in Figure 3.2-2 and 3.2-3 are correlated with the amount of thiamine provided by the extract. 10% SWE, providing 1 µg/l thiamine, produced good growth of the alga.

f. The optimum level of thiamine was then determined. 10 µg/l thiamine was the apparent optimum but 1 µg/l was sufficient to produce good growth of the alga (Figures 3.4.3-1 and 3.4.3-2).

g. The possible mediating effect of bacteria on the observed thiamine response was discounted by experiments that showed that reducing the bacterial population did not affect the thiamine response and that the bacteria did not require thiamine for their growth.

h. The effectiveness of SWE ash was tested again, in media already supplemented with thiamine. A considerable improvement in growth was observed with the addition of the ash (Table 3.4.4-1), suggesting that much of the activity of SWE that was not replaced by thiamine is due to inorganic components.

i. SWE was then analysed chemically (Table 3.3.2-1). It was found that SWE, in contrast to TBIM, the standard growth medium, provided less of all the nutrients tested except calcium and silicon. 10% SWE would provide 10 mg/l Ca and 0.8 mg/l Si, while TBIM provides only 4 mg/l Ca and no silicon.

j. The response to additional calcium was expected to be positive since earlier experiments had shown increased growth with increasing calcium concentrations. The calcium provided by SWE proved to be significant in the growth of C. glomerata. The addition of 16 mg/l Ca (approximately the amount provided by 10% SWE) to TBIM supplemented with 10 µg/l thiamine provided an additional 10% growth enhancement (Table 3.4.5-1).

k. Silicon at 1 mg/l, a concentration slightly higher than that provided by 10% SWE, gave only a marginal improvement of growth when added to TBIM with 10 µg/l thiamine and 16 mg/l Ca (Table 3.4.6-1). Higher concentrations of silicon proved stimulatory, with 5 mg/l Si promoting a considerable enhancement of growth (Figure 3.4.6-1).

l. GeO_2 inhibited growth at concentrations of 25 mg/l or more. 75 mg/l stopped cell division but cell enlargement and extreme cell wall thickening occurred. 25 mg/l GeO_2 disrupted cross wall formation. The disruptive effect of GeO_2 on the formation of cross walls was partially overcome by the simultaneous addition of an equal amount of silicon.

m. The ultrastructure of the wall revealed an outer layer or cuticle which was electron-dense only in those cells from media supplemented with silicon.

CHAPTER 4: DISCUSSION

4.1 The pH Optimum and Requirements for Sulphur, Calcium and Nitrogen

4.1.1 pH

All species of Cladophora normally thrive at relatively high pH (Whitton 1970), although one record of Cladophora at a pH below 7.0 was noted for a creek contaminated by acid mine waste (Bennett 1969, quoted by Whitton 1970).

Bellis (1966) in his laboratory investigation found a pH optimum of near 8.2 for C. glomerata, and Mason (1965) noted that the best growth of C. glomerata, in the farm ponds he studied, was in a pond with a stable pH over 8.0. The pH optimum, of near 8.5, determined in investigations for this thesis, agrees well with the results of previous investigations.

pH values of between 8.0 and 8.5 were maintained in the experiments described, by the use of tris buffer. The application of increasing concentrations of tris did improve growth of the alga, but the effect seemed to be due to an increase of the final pH with more tris, rather than because of a utilization of tris by the alga. Bellis (1966) found that tris maintained the pH to within 0.2 pH units of the initial pH even after 15 days of incubation. Since tris can maintain

a stable pH in the range of pH 7.0 to 9.0 and is non-utilizable, it is a very satisfactory buffer system for the culture of C. glomerata.

The importance of a high pH, in natural waters, as a determining factor for the presence of this genus, appears to be well established. Limestone or some other carbonate source is normally required to maintain these alkaline conditions since the carbonate-bicarbonate alkalinity system is very important in determining the pH in natural waters. Lakes Huron, Michigan, Erie and Ontario all have limestone bedrock for part of their bottom, which predisposes them to growths of Cladophora. Another interesting feature of a pH optimum this high is that almost all of the inorganic carbon is in the form of HCO_3^- at pH 8.5, which suggests that Cladophora can use this form of carbon directly as a carbon source for photosynthesis.

Whitford (1960), felt that the best single indicator of the type of algal flora is pH, since pH is high in eutrophic habitats, where nutrients and bicarbonate are generally abundant, while pH is low in oligotrophic habitats where the concentration of mineral nutrients is low. Certainly a high pH, with its concomitant conditions, is essential for satisfactory growth of C. glomerata.

4.1.2 Calcium

The enhancement of the growth of C. glomerata by increasing concentrations of calcium was expected from previous work done in this laboratory by Bellis (1966). He found that 1.2 mg/l Ca was required for survival, 6.4 mg/l Ca was required for growth and no inhibition occurred even at concentrations of calcium over 60 mg/l. Figure 3.1.2-1 and 3.1.2-2 show results almost identical to these. 1 mg/l Ca was required for survival, 5 mg/l Ca was required to produce healthy looking algae and 25.6 mg/l, the highest concentration tested, produced the best growth. This high concentration of calcium caused the pH to rise, which could also be beneficial for the growth of this alga.

The need by Cladophora for high water hardness and high concentrations of calcium has long been recognised. Liebmann (1951, quoted by Fjerdingstad 1965) concluded that Cladophora has a distinct preference for hard, calcium rich water, and Lundh (1951, quoted by Fjerdingstad 1965) did physiological work that indicated that the occurence of Cladophora is dependent on high concentrations of bicarbonate and calcium. More recently Blum (1960) reiterated these concepts. A typical natural environment where Cladophora can be found would contain in excess of 50 mg/l Ca (Fjerdingstad 1965).

Part of the requirement of a high water hard-

ness may be due to a direct need for high dissolved solids. Fjerdingstad (1965) quoted six reports of freshwater Cladophora from saline inland localities in Europe and many marine species of Cladophora are very similar to freshwater forms. Round (1965) suggested that C. glomerata is basically a marine species and, as such, naturally thrives only in water with high dissolved solids. Zuraw (1969) found that the addition of artificial seawater was beneficial for his unialgal cultures of Lake Michigan C. glomerata. It seems clear that part of the requirement for a high water hardness and high calcium is an expression of a preference for high dissolved solids, but the alga's actual requirement for calcium appears to be real. The classical view of the role of calcium in the nutrition of algae was expressed by Pringsheim (1949), who stated that calcium is not a required nutrient for most algae. However, Bellis (1966), after his studies on the calcium-magnesium ratio requirements of C. glomerata, concluded that calcium is a required nutrient for the alga and that magnesium could not replace this requirement. The observations reported in this thesis lend support to Bellis' contention.

4.1.3 Sulphur

Sulphur was found to enhance the growth of C.

glomerata up to concentrations of approximately 20 mg/l. Concentrations of 90 mg/l or more were inhibitory. If this growth response of the alga to high levels of sulphur holds true in natural situations, sulphur could be important in the creation of the Cladophora problem in the Laurentian Great Lakes.

Sulphur concentrations have doubled in Lake Ontario and Lake Erie over the last sixty years to current levels in excess of 8 mg/l S (Beeton 1961). The main sources of this sulphate are sulphonates, sulphur dioxide and industrial waste. Sulphonates are a major component of all modern household detergents. Attention has been paid to phosphates, the other major detergent component, but to date the possible role of the sulphonates in adding nutrients to aquatic ecosystems has been ignored. Sulphur dioxide, among the most serious of the air pollutants, is formed mainly by the burning of fossil fuels containing sulphur. The residence time of sulphur dioxide in the atmosphere is of the same order as that of water vapour (Liss 1970), therefore much of the sulphur dioxide generated by the industries around the Great Lakes would be contributed to the lakes. Direct industrial contribution of sulphur is also very significant. The International Joint Commission (1969) reported that twenty-one industries contributed 29 million kilograms of sulphate per year

to Lake Ontario and the St. Laurence River.

The enhancement of the growth of C. glomerata in culture, by high concentrations of sulphur, coupled with the increasing levels of sulphur in the Great Lakes could be interpreted as evidence that sulphur is significant in causing the Cladophora problem. However, all nutrients are increasing in the lakes and C. glomerata, in culture and in nature, probably prefers a high concentration of each nutrient. A proposal that can be made from these observations, is that sulphur should not be overlooked when the problem of eutrophication is approached. This view has been expressed before (Kappe and Kappe 1971), but to date virtually all the attention is given to phosphorus.

4.1.4 Nitrogen

Nitrogen and phosphorus are the two nutrients that are most often thought responsible for excessive algal productivity. In his exhaustive study of limiting nutrients on Lake Erie, Lange (1970) found that nitrogen was limiting in approximately two thirds of the water samples, while phosphorus was limiting in about one third. However, there is little evidence that growths of Cladophora are ever limited by nitrogen in nature. Whitton (1970), in his thorough review of the literature on Cladophora, found virtually no instances

where the lack of nitrogen had been indicated as the major factor limiting the growth of the alga. Pitcairn and Hawkes (1973) found a high correlation between growths of C. glomerata and concentrations of phosphorus, but a low correlation between growth of the alga and concentrations of nitrogen. Mason (1965), however, found that in the farm ponds he studied, there was direct association of the growth of C. glomerata, with high nitrogen and high biochemical oxygen demand but not with temperature, pH or phosphorus.

Investigations reported in this thesis, indicated that nitrogen was stimulatory up to a concentration of 44.9 mg/l which is about ten times the concentration of nitrogen found in the lower Great Lakes (International Joint Commission 1969). There is some direct field evidence that C. glomerata in Lake Ontario is sometimes nitrogen limited. During International Field Year on the Great Lakes, studies were done on populations of C. glomerata to determine if they were nitrogen or phosphorus limited. Hopkins and Carney (1972, quoted by Neil 1974) found that all samples of algae tested had ammonia N absorption rates of greater than 15 mg/10 mg algae x hour, which indicated that the algae were nitrogen limited. Alkaline phosphatase activity levels were all low and extractable phosphorus levels were all greater than 0.08 mg P/100 mg algae,

indicating that the algae contained surplus phosphorus. Neil (1974) concluded that "the role of nitrogen in limiting Cladophora growths needs to be investigated." Certainly the results of these field studies, along with the demonstrated need for very high concentrations of nitrogen in culture indicates that similar to sulphur, nitrogen should no longer be overlooked.

4.2 The Significance of Organic Compounds and Bicarbonate as Sources of Carbon

The theory has been put forward that carbon dioxide, generated from organic waste by bacteria, is important in the creation of algal blooms (Kuentzel 1969). Blue-green algal blooms particularly, may be controlled by the need for a supply of carbon in addition to that provided by the bicarbonate-carbonate alkalinity system. Pearsall (1932) stated "the abundance of blue-green algae is correlated with high concentrations of organic matter in the water, these algae being able to increase at very low concentrations of inorganic nutrients." Jernelov (quoted by Kuentzel 1969) found that in the Swedish lakes he studied, there were tremendous increases in the numbers of bacteria accompanying every algal bloom. This evidence of the importance of organic substances in the support of massive growths of blue-green algae suggested that large growths

of C. glomerata also would need carbon from this source. Mason (1965) found that high productivity of C. glomerata in the ponds he studied was directly associated with high biochemical oxygen demand and C. glomerata is generally considered to be an alga that thrives in the presence of some organic pollution (Fjerdingstad 1965).

Laboratory experiments on the stimulation of Cladophora productivity by certain organics at first appeared promising, with large increases in dry weights observed with the addition of starch. This later proved to be due to the accumulation of starch on the walls of the alga since chlorophyll measurements showed no increase when starch was added to the medium. These low chlorophyll measurements could have been caused by a suppression of chlorophyll synthesis after the uptake of carbohydrate (Edelman and Hanson 1972) but this phenomenon has been reported only with the addition of sucrose and not with glucose or other carbohydrates. It seems highly unlikely that this mechanism would be significant here, after the addition of starch. Other organics added to the medium supported a massive increase in bacteria which increased the turbidity and inhibited the growth of the alga.

Bicarbonate addition caused the pH of the medium to shift to over 9.5. This meant that some of the

bicarbonate was changed to carbonate which is known to be toxic to algae (King 1970). The addition of carbon, in either an organic or inorganic form, was not stimulatory to the growth of C. glomerata, indicating that carbon is not a limiting factor for the growth of this alga in shaken culture at pH 8.2 to 8.5.

4.3 The Identification of Nutritional Requirements of C. glomerata by the Analysis of SWE

Two nutrients required by C. glomerata were identified in this study. Thiamine was found first and proven to be the most significant component of SWE (Moore and McLarty, 1975). An inorganic, required nutrient element, silicon, was also identified. This is the first time a requirement for silicon has been shown for a member of the Chlorophyta. Both thiamine and silicon are provided by SWE. Calcium, another component of SWE was proven to be significant in the growth enhancement of C. glomerata.

4.3.1 Thiamine

Thiamine was tested as a possible replacement for SWE, after results of initial experiments showed that ashing SWE destroyed an active component and that the amount of stimulation by SWE was inversely proportional to the degree of heating during sterilzation. These

experiments suggested that an active component in SWE was a heat labile organic substance. The effectiveness of thiamine was investigated since it is a heat labile organic substance, water soluble and known to be present in most soils. Kononova (1966) reported that the soils he analysed contained 2.9 to 19.3 ug/kg thiamine. Since SWE is made by extracting 1 kg of soil with 1 liter of water, concentrations of thiamine in the 3 to 20 ug/l range were anticipated. Moreover, thiamine is required by forty species of algae (Lewin 1961), although this requirement has not been suggested for any alga closely related to C. glomerata.

Thiamine was found to replace 80% of the growth enhancement due to SWE. Thiamine, when added to a medium already supplemented with SWE produced a small additional enhancement of growth. These results indicated that thiamine was the most significant active component in SWE and, since supplementation by thiamine in addition to SWE further improved growth, SWE must supply a suboptimal concentration of thiamine.

The Phycomyces blakesleeanus thiamine bioassay, a sentitive assay for thiamine (Burkholder and McVeigh 1940), was used to determine the concentration of thiamine in SWE and SWE 2. SWE was found to contain 10 ug/l thiamine while SWE 2 contained 3 ug/l thiamine. The relative effectiveness of these two SWEs

were then investigated. SWE produced much better growth than SWE 2 at concentrations of from 1 to 10% with significant enhancement observed with 1% SWE and with 2% SWE 2. At 10% SWE, which provided 1 ug/l thiamine to the media, growth of the C. glomerata was near optimal while 10% SWE 2 provided much less enhancement of growth than 20%. These results showed that the growth enhancement by the SWEs tested is controlled by their thiamine content. As expected 10% SWE provided a somewhat suboptimal concentration of thiamine but the 1 ug/l thiamine it did provide was sufficient to produce good growth of the alga. Growth curve studies were then done with cultures supplemented either by SWE or by thiamine. The pattern of growth of the alga was the same in both treatments. Chlorophyll increased slowly for the first week then rapidly for the next two weeks, levelling off in the fourth week and declining thereafter. Dry weight determinations showed a relatively steady increase in both treatments for the first five weeks with a decrease in the rate of increase in the last week sampled. The thiamine treatment uniformally produced approximately 80% of the growth enhancement effected by SWE. The chlorophyll data showed clearly that the exponential phase of growth ended after three weeks, thus the termination of other experiments after three weeks incubation was justified. The dry weight

data did not show the normal growth curve since all the accumulated biomass is measured each time and a decrease in dry weight, due to decomposition would not occur until well after growth had stopped.

The effect on the growth of C. glomerata of a range of concentrations of thiamine was investigated. In an initial experiment, it was shown that optimal growth occurred at a concentration of between 1 and 25 ug/l with no further enhancement at very high concentrations. Further experiments established 10 ug/l as the optimum concentration although 1 ug/l consistantly was sufficient to provide most of the growth enhancement. 10% SWE also provided 1 ug/l thiamine and this also was sufficient to stimulate good growth.

These results demonstrate that C. glomerata requires thiamine for satisfactory growth in culture and that an important component in SWE for the stimulation of this alga is thiamine.

Four isolates of Cladophora obtained from the Indiana culture collection and an isolate from Lake Ontario were all found to require thiamine. Only the response of the Indiana isolates are presented in this thesis. One of the Indiana isolates, C. fracta, did particularly poorly in media lacking added thiamine and the thiamine supplemented medium produced 87% of the growth of the SWE supplemented medium.

Thiamine requirements appear to be widespread in C. glomerata and in closely related species. An earlier investigation that described the culture of Lake Michigan C. glomerata reported stimulation with soil extract or seawater but no stimulation with a vitamin mixture that included thiamine (Zuraw 1969). Excluding such possibilities as an inorganic deficiency in the medium or very low vitamin activity, this work suggests that not all isolates of C. glomerata require thiamine.

The bacteria that are associated with these unialgal cultures of C. glomerata could be influencing the observed responses of the alga to SWE and thiamine. One possibility is that a portion of the bacterial population responds to the addition of thiamine and produces another substance that enhances the growth of the alga. The best way to determine if the enhancement by thiamine is indirect would be to test the response of axenic cultures of the alga. Stein (1973) reported several researchers who have had success in obtaining bacteria free cultures of algae by the use of antibiotics but a number of attempts with various methods did not yield axenic cultures of C. glomerata. However, the bacterial populations could be decreased by antibiotic applications. Investigations using cultures with reduced bacterial populations showed that they

responded to thiamine in the same way as cultures with normal bacterial populations. Another indirect approach to this problem was to isolate the bacteria and determine whether they had a requirement for thiamine. When this was done the two dominant colony types were found to grow equally well on TBIM-dextrose agar with or without thiamine.

The bacteria in the cultures do not require thiamine and a reduction in the bacterial population did not affect the response of the alga to thiamine and so the enhancement of the growth of C. glomerata is likely a direct effect of thiamine.

Since C. glomerata represents a serious problem in many of our aquatic ecosystems, the possible significance of this thiamine requirement on the growth of the alga in nature must be considered. Natural water contains thiamine, one study showed concentrations of 0.2 ug/l and 0.03 ug/l in unfiltered and filtered pond water respectively (Hutchinson 1934), and another study reported 0.03 ug/l in oligotrophic Lake Tahoe and 0.4 ug/l in a eutrophic lake (Carlucci 1972). Both of these reports allude to the possible influence of thiamine on the algal populations of the lakes investigated. Sewage and soil runoff are thought to be the most important sources of thiamine to natural waters, although it has been reported that aquatic bacteria (Hagedorn 1969) and

phytoplankton (Carlucci 1970) can produce it in situ. The thiamine content of the Laurentian Great Lakes is unknown but it certainly must be increasing with the increased populations of humans and livestock in the watershed. The significance of this thiamine addition has not been proven but evidence presented here strongly suggests that increasing the level of thiamine from a presumably very low level would encourage the growth of C. glomerata if all other nutrients were there in adequate amounts.

4.3.2. Calcium

The requirement of C. glomerata for high concentrations of calcium was discussed in Section 4.1.3. The conclusion was reached that calcium is an essential element for this alga and that at least 5 mg/l Ca was required to produce healthy filaments, and growth enhancement continued up to concentrations over 25 mg/l. TBIM provided only 4 mg/l Ca and so additional calcium would be expected to enhance growth. SWE was found to contain approximately 100 mg/l Ca and so TBIM supplemented with 10% SWE would provide 14 mg/l Ca.

TBIM supplemented with thiamine and SWE ash yielded more dry weight than that produced when just thiamine was used as a supplement and together they yielded 89% of the growth produced by the addition of

thiamine and non-ashed SWE (Table 3.4.4-1). Similarly TBIM supplemented with thiamine and calcium yielded more dry weight than that produced when just thiamine was used as a supplement and together they yielded 87% of the growth produced by the addition of thiamine and non-ashed SWE (Table 3.4.5-1). These results confirm the importance of calcium in the nutrition of C. glomerata and show that calcium is an important inorganic component of SWE.

4.3.3 Silicon

The review, 'Silicon and Plant Growth' (Lewin and Reimann 1969) recognised silicon as an essential nutrient for diatoms, some other members of the Chrysophyta and a few higher plants, but no member of the Chlorophyta has been shown to require silicon. The evidence presented in this thesis indicates that silicon is a component of the cell wall of C. glomerata. SWE was found to contain 8 mg/l Si, therefore TBIM supplemented with 10% SWE would provide approximately 1 mg/l Si. This concentration of silicon consistantly promoted increased growth of the alga and higher concentrations, 5 and 10 mg/l, further increased growth (Figure 3.4.6-1). Silicon was extracted from the algae grown at these various concentrations and the amount of extractable silicon in the algae increased in pro-

portion with the amount of silicon supplied (Table 3.4.6-2). Natural populations of C. glomerata have been analysed for silicon and high levels, 1.35% of the ash (Kishler 1967) and 6.5% of the ash (OWRC 1964 unpublished) were found. This silicon was attributed to epiphytic diatoms. This promotion of growth by silicon and the increase in extractable silicon as the supply of silicon increased, suggests that silicon is required by C. glomerata.

Lewin (1966) and Werner (1967a) described a critical method of determining whether an organism requires silicon. Germanium, the next higher analogue of silicon in Group IVA of the periodic table is a specific inhibitor of silicic acid metabolism and only organisms that require silicon are inhibited by GeO_2 at concentrations below 75 mg/l (Werner 1967a). Consequently, germanium is very toxic at low concentrations to some higher plants and diatoms, but not toxic even at higher concentrations to algae from other divisions, bacteria, fungi and mammals (Werner 1967a). The severe inhibitory effects of GeO_2 at concentrations of 75 mg/l and 25 mg/l are shown in Figure 3.4.8-1 and Plates 3.4.8-1, -2 and -3. The inhibition of cross wall formation clearly shown in Plate 3.4.8-1 C may be due to an interruption of protein synthesis. Hanic and Craigie (1969) showed that both the outermost layer of the cell wall or cuticle and the cross wall disks were proteinaceous in C.

rupestris and Werner (1967) showed that in the diatom Cyclotella cryptica cell division and protein synthesis were interrupted by GeO_2. The combination of these two pieces of work with the clear observation of disruption of cross wall formation strongly suggests that protein synthesis is important in the formation of cross walls in C. glomerata and that silicon plays a part, since germanium is a specific inhibitor of silicic acid metabolism.

The other portion of the cell wall that is proteinaceous in some species of Cladophora, is the cuticle (Hanic and Craigie 1969). This appeared to be the layer where the silicon was deposited in the cell wall. Plate 3.4.9-1 clearly shows an outer band which was electron-dense in cells taken from silicon rich medium and not electron-dense in cells from medium with no added silicon. This electron-dense band was interpreted as a proteinaceous cuticle, rich in associated silicon. The presence of silicon in close association with other cell wall components has been described in several studies on higher plants (Engel 1953, Jones et al. 1963 and Lewin and Reimann 1969).

The inhibition of C. glomerata by concentrations of GeO_2, which would be toxic only to plants that require silicon, is good evidence that this alga has a requirement for silicon. Supportive evidence is the

growth promotion by silicon, the increase in extractable silicon and the electron-dense cuticle which appears only in the presence of silicon.

The presence of silicon in C. glomerata is also suggested by several observations that can be made in its natural environment. The rough texture and strength of the filaments could be explained by a siliceous cuticle especially since the earlier explanation of an outer layer of chitin (Wurdack 1923) has been discounted (Hanic and Craigie 1969). The large numbers of epiphytic diatoms that are attracted to Cladophora might also be explained by the presence of available silicon.

The ecological importance of this requirement for silicon for the growth of C. glomerata in the Great Lakes is difficult to assess. Silicon is very plentiful in the lithosphere but it is sparsely soluble in water. Silicon depletion has been forecast for Lake Michigan (Schelske and Stoermer 1971) due to massive increases in the diatom populations. Large growths of C. glomerata in the lake may also be contributing to this silicon depletion. In lakes where the dominant diatom population has been replaced by blue-green or green algae which do not require silicon, there would be more silicon available for C. glomerata and this could encourage excess productivity.

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