IV. DISCUSSION

DISCUSSION

Eversince Harder and Witsch (1942) of Germany and Spoehr (1951) of the United States of America suggested the possibility of utilizing the fast growing unicellular algae for food or feed, algal cultures have assumed an industrial dimension (Burlew,1953). The potentiality of algae as source of food, feed, fodder and manure has been further established by the extensive researches carried out during the past few decades. The economic utilization of algae necessitates the development of techniques for axenic culturing of these organisms on a large scale. Though many media have been devised for culturing different algae, in the opinion of Venkataraman (1960a) no single medium can be said as the best one. From the studies with different algal species Gopinathan (1986) also arrived at the same conclusion. Since the nutritional requirements of algae vary with species, the successful and long term culturing of any algal species demands a thorough

understanding of its nutritional requirements which can be studied only under controlled laboratory conditions using unialgal cultures.

In the present investigation, an attempt has been made to assess the role of some essential elements like phosphorus (P), calcium (Ca) and magnesium (Mg) (macronutrients) and iron (Fe), manganese (Mn) and zinc (Zn) (micronutrients) on the growth and metabolism of three algal species *viz.* *Chlorococcum humicola*, (Naeg.) Rabenhorst, *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim. The study also puts an endeavour to evaluate the optimum concentrations of above mentioned nutrients that would provide maximum output of these economically important algal species under culture conditions. Various parameters observed include cell concentration, cellular contents of photosynthetic pigments, carbohydrate, protein and productivity.

The experiments were carried out using pure cultures of three algal species raised in Ward and Parish medium. The cells made deficient of a particular element were treated with varying concentrations of the concerned element and the results were compared with that of control raised in the absence of the above nutrient.

PHOSPHORUS

Phosphorus is one of the major nutrient elements required for normal growth of algae (Myers, 1951; Ketchum, 1954; Krauss, 1958; Provasoli, 1958). According to Adolf Kuhl (1962), the growth of a wide variety of algae in their natural environment as well as in the laboratory conditions depends on the amount of available phosphorus. The importance of phosphorus on the growth of any organism lies in the fact that the energy rich compound adenosine triphosphate (ATP) and numerous other phosphorylated compounds are involved in practically every synthetic action of the cells.

Fogg (1971) reviewed the relationship between algae and phosphorus and proposed that many species could absorb orthophosphate from solutions containing less than 1 ppm P and when P-deficient most species were capable of producing powerful surface or extracellular phosphatases which enable them to obtain phosphate from a great variety of inorganic and organic phosphorus compounds including synthetic detergents. The release of alkaline phosphatase under salinity stress by a cyanobacterium *Anabaena doliolum* was reported by Abraham (2001). These discoveries confirm the extreme dependence of algae on phosphorus for adequate growth and cellular activities.

The present study too establishes the importance of phosphorus on the growth of three studied phytoplankton species as they showed an arrested

growth throughout the test procedure in P-deficit medium. A similar observation was made by Jordan and Bender (1973) in their *in situ* enrichment experiment where they found an elimination of all growth responses of phytoplankton communities of a natural lake due to the omission of phosphate from the treatment mixture. Gernot Falkner *et al.* (1984) reported the cessation of algal growth and an increase in the membrane permeability for phosphate when blue-green algae in a lake were suffered from phosphate deficiency.

Addition of phosphorus to the medium induced growth responses in the test organisms and they attained maximum cell density at the respective optimum concentrations which was found to be 0.24 ppm for all the three species. Pitcairn and Hawkes (1973) studied growth of *Cladophora* in synthetic media containing levels of phosphorus from 1 to 7 mg/l and reported the lack of significant growth with an increase of phosphorus above 1 mg/l, and a significant reduction below 1 mg P/l. Ivor and David (1985) proposed that the cellular requirements of phosphorus in *Selenastrum minutum* increased more rapidly than those for nitrogen with the increase in growth rate. The explorations of Wang *et al.* (1995) on the effects of nitrogen, phosphorus, vitamins and trace metals on the growth of red tide organism *Prorocentrum micans* have shown that phosphorus more than any other nutrient limts the growth of *P. micans.*

Higher phytoplankton density and productivity in various aquatic ecosystem by the increased levels of phosphorus were reported by Condit

(1972), Halmann (1972), Gloosechenko and Alvis (1973), Gopinathan *et al.* (1984), Axier *et al.* (1991), Peeters *et al.* (1993), Foellimi (1994), Gemza (1995), Connors *et al.* (1996) and Wu chonghua *et al.* (1998). But this increase in the phytoplankton population with the addition of phosphorus cannot be regarded as a universal phenomenon as the works of some investigators failed to produce any positive effect with the increased levels of phosphorus (Grambast - Fessard and Guerlesquin, 1991; Bachmann *et al.*, 1995; and Levine *et al.*, 1997).

Chu (1942) investigated the requirement of phosphorus by various planktonic algae. He observed a good growth of planktonic algae in nutrient solutions with 0.1 to 2.0 ppm P, limited growth in 0.05 ppm P and noticed an inhibitory effect when the concentration of phosphorus exceeded 20 ppm. When the test organisms were exposed to different levels of phosphorus *viz.* 0.06 ppm, 0.12 ppm, 0.24 ppm, 0.48 ppm and 0.96 ppm, the growth response in terms of cell density was maximum in 0.24 ppm and a retarding effect was noticed in the lower and higher levels of phosphorus. According to Rodhe (1948), freshwater algae can be grouped into three categories as low, medium and high on the basis of their phosphorus tolerance ranges falling below, around or above 20 μg/litre. In this regard, it is possible to include the three species under study in the category 'high' as they showed tolerance range above 20 μg/litre.

Healey (1973) from his studies on P-deficiency in *Anabaena* proposed the characteristics of P-deficiency as loss of heterocysts, a decline in

chlorophyll *a*, protein, RNA and cellular phosphorus and an increase in carbohydrate/dry wt. In the experiments conducted it was observed that chlorophyll *a* content/cell was higher in P-deficient cells on 5th day; but a decline in chlorophyll *a*/cell was noticed in the later stages. A similar result was shown by P-deficient cells of *Ankistrodesmus braunii* (Pirson *et al.*, 1952) and P-deficit thalli of *Hydrodictyon reticulatum* (Neeb, 1952). An increased production of chlorophyll *a* in the earlier stages of P-deficiency may be an adaptation of the organism to thrive in the adverse conditions.

Studies indicate the inhibitory effect of P-deficiency on the production of accessory pigments. P-deficit control cells of *Chlorococcum humicola*, (Naeg.) Rabenhorst showed highly retarded values for chlorophyll *b* and carotenoid in all the days of estimation, while the presence of chlorophyll *c* remained undetected throughout the test procedure in control cells. The cells of *Chlorella ellipsoidea*, Gerneck, starved of phosphorus possessed detectable level of accessory pigments only on 5th day. Thereafter, its presence could not be ascertained till the end of experiment. *Scenedesmus bijuga*, (Turp.) Lagerheim too unveiled the least contents of these pigments when grown in P-free medium.

Kwang - Guk An *et al.* (2003) report variations in the chlorophyll content with changing concentrations of phosphorus in an artificial lentic system. The works of Ammini Joseph (1983) on *Isochrysis* and *Tetraselmis* and that of Sathi (1992) on some phytoflagellates have shown that in higher

concentrations of nitrate and phosphate, the chlorophyll content was more reduced than in the optimum and lower concentrations of these macronutrients. However, the results of the investigation undertaken indicate much lessened values of photosynthetic pigments in the cells exposed to lower levels of phosphorus. This difference may be due to the varying degrees of tolerance exhibited by different algal species towards phosphate.

The specific participation of phosphorylated compounds and phosphorylation reactions in photosynthesis was clearly indicated by many investigations that have employed algae (Strehler, 1952; Brown and Frenkel, 1953; Arnon, 1957, 1960; Kandler, 1960). In view of the close dependence of photosynthesis on phosphate, Pirson (1955) suggests the possibility of photosynthesis and in turn other metabolic reactions being affected by P-deficiency. Pirson *et al.* (1952) observed an inhibition of photosynthetic oxygen production in P-defective cells which was at least partly reversed by the addition of phosphate. Joseph (1988) proposes that the varying concentrations of nitrate and phosphate influence the productivity in culture system. In the present study, significantly high productivity values were obtained in optimum and slightly higher levels of phosphate compared to very low and very high concentrations and this tallies with the earlier observations of Sathi (1992).

With regard to biochemical compounds, a slight initial increase in the carbohydrate and decrease in protein contents were noticed in the three

test orgaisms under P-deficiency. This is in accordance with the view of Healey (1973). In the opinion of Adolf Kuhl (1962) the interference of P-deficiency with nitrogen metabolism may be the reason for the accumulation of starch. The decrease in protein content may be an outcome of the failure of the formation of ATP due to inadequate phosphorus supply (Hewitt, 1951). The excess amount of phosphorus in the medium beyond the optimum level led to a decrease in the contents of these metabolites suggesting the adverse effect of higher levels of this macronutrient on the algal metabolism.

The evaluation of various growth parameters of three test organisms *viz. Chlorococcum humicola,* (Naeg.) Rabenhorst, *Chlorella ellipsoidea,* Gerneck and *Scenedesmus bijuga,* (Turp.) Lagerheim confirms the indispensable role of phosphorus on the metabolism of these organisms. The observations also suggest that beyond a particular level, this macronutrient can become toxic to algal growth and metabolism.

CALCIUM

Calcium ions undoubtedly play an important part in the maintenance of cytoplasmic membrane and wall structures (Fritsch, 1935, 1945; Blinks, 1951; Round, 1973). The physiological effects of calcium on the state of protoplasm are probably best reflected by its action in decreasing cell permeability and antagonising the action of K^+ in this respect (Steward, 1963). At enzymatic level calcium has been shown to act as an activator for certain enzymes as arginine kinase, adenosine triphosphate, adenyl kinase *etc*. Several phospholipases from a number of different organisms including higher plants are known to be activated by Ca ions (Steward, 1963). In addition, calcium plays a stabilizing role in maintaining the proper configuration of amylase molecule which in turn provides them the resistance to proteolytic degradation. Apart from these various functions of calcium in different organisms, the available literature shows that the absolute requirement of calcium for the growth of algae is a disputed one. Calcium was found to be unnecessary for *Coccochloris peniocystis* (Gerloff *et al.*, 1950b) and *Anacystis montana* (Venkataraman, 1962d). However, there is the possibility that these organisms might require only traces of calcium and calcium impurities present in nutrients used might have satisfied their requirements (Venkataraman, 1969).

According to O'Kelley (1968), all algae have a calcium requirement at least in the absence of strontium, though demonstrating it is difficult in

some cases. Early studies of Molisch (1895) and Pringsheim (1926) showed that different algae require different quantities of calcium. In the opinion of Chu (1949) minimum requirement for calcium depends on the medium and algal species used. O'kelley and Herndon (1961) are of the view that the magnitude of calcium requirement is related to the synthesis and amount of pectic substances in the cell wall and most of the algal species respond to calcium in macronutrient levels.

There is much controversial account regarding the requirement of calcium by *Chlorella*. Early workers like Hopkins and Wann (1925), Trelease and Selsam (1939), Scott (1943) and Myers (1944) were of the opinion that calcium is not essential for the growth of *Chlorella*. However, Noack *et al.* (1940) and Manual (1944) reported the requirement of calcium by *Chlorella* for its proper growth. Stegmann (1940) who investigated the role of calcium in the metabolism of *Chlorella* revealed that this element may be considered as a macronutrient or micronutrient depending on the metabolic function involved as some metabolic processes required this element in relatively high concentrations while others in very small amounts. It has also been suggested that strontium can substitute for the small calcium requirements of *Chlorella* (Round, 1973).

From the results of the present investigation it can be commented that calcium is essential for the growth of *Chlorococcum humicola*, (Naeg.) Rabenhorst, *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim. Of different concentrations of calcium tried *viz.*, 0.4 ppm,

0.8 ppm, 1.6 ppm, 3.2 ppm and 6.4 ppm, 1.6 ppm Ca was found to be optimum for *Chlorococcum humicola*, (Naeg.) Rabenhorst, while for *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim optimum concentration of calcium was found to be 0.8 ppm. Beyond and below these optimum levels a retarding effect was noticed in all the parameters.

Ca-deficit cells of *Chlorococcum humicola*, (Naeg.) Rabenhorst exhibited the least cell number throughout the test procedure and they showed exponential growth only for 17 days. In lower concentration of calcium applied *ie.* in 0.4 ppm, log phase of growth prolonged to 19^{th} day, while in all other concentrations, the alga exhibited a continuous enhancement in growth rate till 21^{st} day. The contents of photosynthetic pigments, carbohydrate and protein too possessed minimum values in Ca-deficient control. All these observations indicate that calcium is an absolute requirement for the adequate growth and metabolism of *Chlorococcum humicola*, (Naeg.) Rabenhorst.

For *Chlorella ellipsoidea*, Gerneck higher concentration of calcium applied *ie.* 6.4 ppm was found to be more detrimental to cell division than its deficiency as least cell number was always obtained in the sample treated with 6.4 ppm Ca. In control as well as in concentrations above the optimum levels, growth was continuous only upto 17^{th} day followed by a decline in accompanying days. Throughout the experimentation, chlorophyll *b* and *c* revealed least contents in 6.4 ppm Ca. Chlorophyll *a* and carotenoid showed

minimum values in 6.4 ppm Ca in later and earlier stages of growth respectively. Not much difference in productivity was exhibited by the cells grown in Ca-deficient medium and those supplied with 6.4 ppm Ca. Biochemical compounds like carbohydrate and protein always possessed least values in Ca-free medium. It may be suggested that higher concentration of calcium is more deleterious to the growth of *Chlorella ellipsoidea*, Gerneck than its deficiency and the response of the species to higher Ca-levels varies with the metabolic function involved.

Deficiency as well as the excess amount of calcium in the medium resulted in an adverse effect on *Scenedesmus bijuga,* (Turp.) Lagerheim, the degree of which varied with the age of the culture and the metabolic process involved. 3-4 celled colonies of *S. bijuga*, (Turp.) Lagerheim in contrast to normal two celled colonies were observed in Ca-deficient control. The failure in rapid cell multiplication and the separation of daughter cells formed may be the reason for the formation of multicelled colonies of *Scenedesmus bijuga*, (Turp.) Lagerheim under Ca-deficiency. Similar formation of multicelled colonies of *Gonium pectorale* at higher levels of calcium that prevent cell multiplication was reported by Groves and Kostir (1961).

Variations in the growth of *Selenastrum westii* with varying concentrations of calcium was reported by Natarajan (1960). Rahimian (1972) studied the effect of calcium on the growth and morphogenesis of *Chlorella, Golenkinia* and *Scenedesmus* and recorded an increased cell number, greater

primary production and higher starch content in the presence of calcium and a significant decrease in these parameters when calcium was substituted by strontium or barium.

In the present study too, a decreased cell number, lesser primary production and a decline in the pigment, carbohydrate and protein contents were revealed by Ca-deficient cells of test organisms. Reduced enzymatic activities due to Ca-deficiency may be the reason for the reduced growth responses of the algae in Ca-free medium. Studies also show that beyond optimum levels, the macronutrient calcium exerts toxic effect on the microalgae and it resulted in their reduced growth responses in the medium with higher concentrations of calcium.

MAGNESIUM

Due to the strategic position magnesium occupies in the photosynthetic apparatus, the algal species have an absolute requirement for this element (Venkataraman, 1969). Magnesium is included under the major element or ions in any culture medium along with potassium, sulphate and phosphate. Minimum requirement of this element appears to be greater than that of calcium. This nutrient is always found in excess of normal requirements in natural water bodies, hence is not considered as a limiting factor for the growth of planktonic species.

Some of the earlier literature available suggest the varied requirements of this element by different algal species. Thus, when as little as 0.1 mg/l Mg results in optimum growth of *Ankistrodesmus falcatus* (Rodhe, 1948), the minimum concentration of this element that produces good growth in *Staurastrum paradoxum* is about 4 mg/l (Chu, 1949). For *Anacystis montana*, Venkataraman (1962 d) proposes a concentration of 0.125 ppm Mg for its maximum growth. According to Natarajan (1960), a concentration of 1.25 ppm Mg is needed for *Selenastrum westii* for abundant growth.

In the present study, when the test organisms were exposed to a range of magnesium viz., 0.365 ppm, 0.73 ppm, 1.46 ppm, 2.9 ppm and 5.8 ppm, *Chlorococcum humicola*, (Naeg.) Rabenhorst and *Scenedesmus bijuga*, (Turp.) Lagerheim showed maximum growth in 2.9 ppm Mg, while the optimum concentration of magnesium for *Chlorella ellipsoidea*, Gerneck was found to be 1.46 ppm. It was also seen that this optimum concentration

of magnesium if supplied as a combination of both $MgCl_2$ and $MgSO_4$ induced more growth than when it was provided in the form of $MgCl_2$ or $MgSO_4$ alone. It may be because sulphur in magnesium sulphate ($MgSO_4$) is stimulating the algal growth as it was the only source of sulphur in the medium.

Importance of sulphur on the algal growth was established by the studies of Hase *et al.* (1957). They observed an arrested development and cell division in S-deficient cells. They also noticed an active assimilation of sulphur in the ripening phase of algal cultures thus substantiating the indispensable role of this element in the process of cell division.

It has been advocated that in Mg-free medium young photo-synthetically active cells produce lesser number of daughter cells which were unable to grow normally (Hase *et al.*, 1957). According to Tamiya (1960), Mg-deficiency did not affect the growth and division of cells although there was a slight time lag in cell division and the resultant daughter cells were highly etiolated and were incapable of further division. An interrupted cell division was recognised in *Chlorella* and *Ankistrodesmus* due to Mg-deficiency (Round, 1973). A similar suppression of growth and cell multiplication was observed in Mg-deficient cells of three algal species studied and they showed the least cell number in Mg-free medium throughout the experiment.

Being a constituent of the chlorophyll molecule magnesium is obviously concerned in photosynthesis. It is the only metal contained in

chlorophyll and comprises 2.7% of the molecule. Whether or not magnesium of chlorophyll serves as an active site in the photosynthetic process is still undetermined (Alvin Nason and William D. Mc clory, 1963). Stimulatory action of magnesium on the Hill-reaction was observed by Susor and Krogmann (1964) in cell-free preparations of *Anabaena variables*. Hill and Whittingham (1955) reported a significant depression in photosynthetic rate in *Chlorella* deprived of magnesium and it is in accordance with the current observations. The microalgae under study revealed the minimum rate of carbon production in Mg-free medium. The addition of this macronutrient to the medium provoked C-production which attained its maximum level in respective optimum concentration and a further increase in the Mg-concentration resulted in an adverse effect on the productivity of these organisms.

Decreased chlorophyll content in Mg-defective cells confirms the influence of this element in the chlorophyll synthesis. Hase *et al.* (1957) detected etiolation in the cells of *Chlorella ellipsoidea* when nitrogen or magnesium was eliminated from the medium. Similar etiolation as well as reduction in the size of cells were shown by the experimental algae in Mg-deficient control samples. Quantitative estimation of chlorophylls and carotenoid revealed reduced values in the cells wanting magnesium, thus confirming the necessity of this element in the synthesis of photosynthetic pigments. The reduced rate of C-fixation may be an outcome of the reduced chlorophyll content.

Early disturbance of nitrogen metabolism, a temporary accumulation of non-digestible carbohydrate (Pirson and Badour, 1961), a high content of labile phosphate (Badour, 1961), inhibition of RNA and protein synthesis (Galling, 1963), increase in the acid soluble nitrogen compounds, hypoxanthine and uracil (O'kelley, 1968) were put forward as the symptoms of Mg-deficiency. Magnesium plays a predominant role in promoting the formation of the enzyme - substrate complex and the resultant intermediate of the reaction. The disrupted biochemical reactions together with the reduced photosynthetic activity may cause decreased carbohydrate content in the cells wanting magnesium.

Magnesium is necessary for the integrity of ribosomes. According to Bonner (1965), deficiency of this element affects the aggregation of ribosomal units to form functional particle. This may interfere with the protein synthesis. Reduced protein content obtained in control cells devoid of magnesium thus agrees with the previous observations.

There is not much recent literature regarding the influence of magnesium on algal growth. The studies conducted highlight that magnesium is an absolute requirement for the algal species *Chlorococcum humicola*, (Naeg.) Rabenhorst, *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim. However, above optimum levels, this macronutrient appeared to be harmful to the organisms as various parameters observed *viz.*, cell number, productivity, the contents of photosynthetic pigments, carbohydrate and protein showed a declining trend with higher levels of magnesium.

IRON

Iron has long been considered as an essential element to algae. Most of the algal species require this nutrient only in microquantities, hence it is included under micronutrients or trace elements. The earlier works of Myers (1947), Goldberg (1952), Venkataraman and Dutta (1959), Natarajan (1960), Venkataraman (1960 b, 1962 d) have substantiated the importance of iron for the growth of algae. In all natural waters iron exists in ionic form and its amount is found to be less in alkaline water.

The possible role of iron as an ecological factor for algal growth was discussed by earlier workers like Uspenski (1927) and Gran (1933). According to Uspenski (1927), the distribution of plants like *Spirogyra rivularis*, *Cladophora fracta* and *Drepanocladus fluitans* depends on the concentration of active iron. Gran (1933) suggested that periodic rises in iron concentration of inshore waters along with nutrients and humic compounds washed from the shore caused the fluctuations in the population density of phytoplankton in the Gulf of Maine. Recent researches by Banse (1991), Bizsel *et al.* (1997) and Paerl (1997) have shown that the reactive iron is an important factor for the phytoplankton bloom and its distribution in coastal water.

Wiessner (1962) suggests that the optimum amount of iron for algal growth depends on the species investigated and to some extent on the composition of the nutrient solution. The experiments of Knauss and Porter

(1954) have shown that the absorption of iron and other metallic cations by *Chlorella pyrenoidosa* is directly proportional to the concentration available in the nutrient solution. The amount of iron and certain other elements are found to be generally higher in brown algae (Pillai, 1956) and it is assumed that the cations may be partly bound by the alginic acid in the walls (Wasserman, 1948). Hudson and Morel (1989) have noticed adsorbed and precipitated form of iron on the cell surface of marine phytoplankton *Pleurochrysis carterae* and *Thalassiosira weissflogii* in addition to the metabolically active and stored forms of this element.

In the present investigation the three test algae, *Chlorococcum humicola*, (Naeg.) Rabenhorst, *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim were exposed to a range of iron supplied in the form of $FeCl_3$ as 0.0075 ppm, 0.015 ppm, 0.03 ppm, 0.06 ppm and 0.12 ppm. Concentrations ranging from 0.0075 ppm to 0.015 ppm were found to be favourable for the growth of *Chlorococcum humicola*, (Naeg.) Rabenhorst whereas, the optimum concentration of iron for *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim was found to be 0.015 ppm. A harmful effect was noticed in all the three algal species when the amount of iron exceeded the optimum levels.

Gerloff and Skoog (1957 a) have reported the critical levels of iron for the maximum growth of *Microcystis aeruginosa* to be 100 ppm when supplied as EDTA chelates. This large requirement may be explained by the fact that iron forms complexes with the chelating agent EDTA and such

bound form of iron is relatively unavailable to algae. To compensate for such biological unavailability additional quantities of elements must be added to the culture medium (Wiessner, 1962).

Bryan and Bogorad (1967) observed that the growth and chlorophyll production in wild type *Chlorella vulgaris* and in a mutant C-10 were affected by the concentration of iron in the medium. They also noticed the influencing role of iron levels on the protochlorophyll *a* to chlorophyll ratio in mutant C-10. Elimination of iron from the medium adversely affected the algal growth as cell number, productivity and the contents of pigments, carbohydrate and protein of all the algal species under investigation furnished lesser values in Fe-deficient medium. Reduction in cell volume and etiolation were also observed in the cells deprived of iron.

For *Chlorococcum humicola,* (Naeg.) Rabenhorst, the highest concentration tried (0.12 ppm) was found to be more detrimental to algal growth than its deficiency. The cells treated with 0.12 ppm Fe furnished minimum values for number of cells, pigment content and productivity throughout the test procedure. During the early stages of growth, Fe-deficit cells of *Chlorococcum humicola* (Naeg.) Rabenhorst revealed least contents of carbohydrate and protein. As the cultures became aged, the deleterious effect of excess iron surpassed that of Fe-deficiency and from 13^{th} day onwards, minimum carbohydrate and protein contents were possessed by the cells exposed to 0.12 ppm Fe.

Scarcity of iron exerted more growth inhibition in *Chlorella ellipsoidea*, Gerneck than its higher concentrations. For *Scenedesmus bijuga,* (Turp.) Lagerheim, during the early stages of growth, cell division and productivity were greatly suppressed by the excess amount of iron in the medium; where as with the ageing of culture, control cells deprived of Fe possessed least values for these parameters. The contents of pigments, carbohydrate and protein were always minimum in Fe-deficit cells of *Scenedesmus bijuga,* (Turp.) Lagerheim.

The above observations suggest that algal species vary in their tolerance to iron. *Chlorococcum humicola*, (Naeg.) Rabenhorst appeared to be more susceptible to higher concentration of iron than *Chlorella ellipsoidea,* Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim.

Iron is a constituent of many enzymes like catalase and peroxidase and of ferredoxin, cytochromes and certain other porphyrins; hence its deficiency will obviously lead to retarded growth. Iron containing acids of unknown structure considered to be associated with co-enzyme A, has been isolated from *Chlorella vulgaris*, *Scenedesmus quadricaudata* and *Dunaliella viridis* (Boichenko and Zarin, 1965). Iron has also been found to be essential for the hydrogenase development (Yanagi and Sasa, 1966). Fogg (1952) and Venkataraman (1961) showed that the extent of secretion of extracellular products, an invariable concomitant of growth of blue-green algae, increased in older cultures of *Anabaena cylindrica* and *Nostoc* respectively by deficiencies in mineral nutrients such as iron.

A major fraction of iron in the cell is found to be associated with chloroplast. Decreased synthesis of protein in the chloroplast (Pirson *et al.*, 1952) and reduced incorporation of magnesium into the porphyrin molecule (Granick, 1951, 1955) were suggested as the probable reasons for the reduced chlorophyll synthesis in the chloroplast due to iron scarcity.

Reduced rate of photosynthesis due to decreased chlorophyll may account for the retarded algal growth in Fe-deficient medium (Kennedy, 1940; Pirson *et al.*, 1952). Duysens (1954) and Chance and Sager (1957) are of the view that since cytochromes are iron porphyrins, the non-availability of iron may intervene with their formation, thereby reducing the photosynthetic process.

Lin *et al.* (1994) observed a close correlation between the concentration of dissolvable iron and chlorophyll *a* content of red tide diatoms especially *Skeletonema costatum*. They also noticed that addition of iron promoted the rate of cell division in these organisms. A similar result was obtained by Wang *et al.* (1995) and Chow *et al.* (1998) in their explorations on red tide organism *Prorocentrum micans* and *Chlorella ellipsoidea*. It has been found that the addition of iron to the test cultures provoked algal growth and all parameters studied like cell number, productivity, carbohydrate, protein and pigment content unveiled higher values in the presence of iron than its absence. The current results thus tally with the earlier observations.

MANGANESE

Small amounts of manganese are present in all natural waters. Owing to its role in nitrogen metabolism it is probably a requirement for all algae. The seasonal variation in manganese concentration in surface waters along with the different requirements by various algae may be playing a regulatory role in the species composition of the phytoplankton in the aquatic systems. According to Knauer (1996), manganese acts as a growth limiting factor for sensitive algal species in natural water bodies.

Manganese has been established as a necessary nutrient for the growth of various algae such as *Chlorella* (Walker, 1953, 1954; Pirson and Bergmann, 1955; Reisner and Thompson, 1956a, b; Eyster *et al.*, 1956, 1958a, b; Brown *et al.*, 1958), *Ankistrodesmus* (Pirson *et al.*, 1952), diatoms (Von stosch, 1942; Spencer, 1957) and some algal flagellates (Harvey, 1947; Provasoli and Pintner, 1953).

It has been suggested that photosynthesis and growth can be stimulated by the addition of manganese to algal culture solutions (Round, 1973). Normal growth of *Euglena gracilis* (Constantopoulos, 1970) and *Dunaliella tertiolecta* (Noro, 1978) was found to be strongly dependent on manganese. Noro (1978) observed a reduced growth rate in *Dunaliella tertiolecta* when the concentration of manganese was less than 0.1 ppm. Brand *et al.* (1983) report that in marine phytoplankton reproductive rates

are limited by manganese ion activity of 10^{-10} M and 10^{-11} M. The addition of as little as 10^{-9} M $MnCl_2$ was found to be stimulatory to the growth of *Chaetoceros socialis*, where as in a natural community of phytoplankton maximum stimulation of growth was observed in 10^{-6} M to 10^{-7} M Mn (Sunda *et al.*, 1981). Srisudha (1989) has described that concentrations of manganese greater than 0.05 ppm enhanced the growth rate in *Isochrysis galbana* and *Synechocystis salina*.

In the present investigation, of the series of Mn-concentrations applied *viz.* 0.0175 ppm, 0.035 ppm, 0.07 ppm, 0.14 ppm and 0.28 ppm, 0.07 ppm Mn was found to be more favourable for the growth of *Chlorococcum humicola,* (Naeg.) Rabenhorst, while a range of 0.035 ppm to 0.07 ppm Mn was found to be stimulatory to *Chlorella ellipsoidea,* Gerneck and *Scenedesmus bijuga,* (Turp.) Lagerheim. The capacity of *Scenedesmus* to store manganese in their cells and utilise it during favourable conditions has been observed by Jahnke and Soulen (1978).

The study undertaken clearly reveals the absolute requirement of this micronutrient for the growth of three experimental algae as they showed reduced values for all the parameters when grown in Mn-deficit medium. Addition of manganese to the medium stimulated growth responses in these algae which reached to the extreme level in the respective optimum concentration and its further addition appeared to be harmful to these organisms. When compared to *Chlorococcum humicola,* (Naeg.) Rabenhorst and *Chlorella ellipsoidea*, Gerneck, *Scenedemus bijuga*, (Turp.) Lagerheim

was found to be more adversely affected by 0.28 ppm Mn as this alga showed the least cell number in this concentration throughout the experiment. About 32% reduction in cell number as compared to control was registered by *Scenedesmus bijuga*, (Turp.) Lagerheim exposed to 0.28ppm on 21st day. Therefore, the tolerance limit of microalgae to this trace element appears to be varying with the species.

Higher concentrations of manganese are found to be toxic to various algal species. A depression in growth rate and 50% reduction in cell volume have been noticed in *Selenastrum capricornutum* and *Chlorella stigmatophora* in 31 mg/l and 50 mg/l Mn respectively (Christensen *et al.*, 1979). Knauer (1996) and Angadi *et al.* (1996) too observed toxic effect of high concentrations of manganese on freshwater phytoplankton and these results are found to be in accordance with that of present investigations. The studies conducted by Wiessner (1962) and O' Kelley (1974) had substantiated the role of manganese in the reactions of some enzymes in the Kreb's cycle and other metabolic processes. The toxicity of higher levels of manganese may be due to the enhanced respiratory activities of algae under these concentrations. It can also be possible that the interaction of various factors in a complex manner produce toxic effects. It has also been recognised that manganese can act as a protective agent against heavy metals like copper, cadmium and zinc (Stauber and Florence, 1985; Sunda and Huntsman, 1996). Srisudha (1989) reported an inverse relationship between pH and Mn-toxicity as the toxicity showed a declining trend with the increase in pH in *Isochrysis galbana* and *Synechocystis salina*.

Influence of manganese on the rate of photosynthesis was clearly revealed by the earlier works of Pirson *et al.* (1952), Arnon (1954), Kessler (1955), Kessler *et al.* (1957) and Pirson (1958a). They recorded a decreased rate of photosynthesis under Mn-deficiency both in weak light and light saturation. Brown (1954) and Eyster *et al.* (1956, 1958 b) found complete suppression of the Hill-reaction in the absence of manganese. Teichler - Zalden (1969) emphasised the necessity of manganese to carry out photosynthetic reactions involving photosystem II in *Anacystis* and *Chlamydomonas.*

From the explorations with *Ankistrodesmus braunii*, Kessler (1955) and Kessler *et al.* (1957) pointed out that manganese may affect the oxygen production mechanism in photosynthesis. The impact of manganese on the oxygen evolving system of photosynthesis was extensively studied by Vernon (1962) and Cheniae and Martin (1968, 1969). No inhibitory effect of Mn-deficiency upon the initial simultaneous production of hydrogen and oxygen was observed in *Chlorella vulgaris* illuminated in anaerobic conditions (Spruit, 1958). According to him, it is only during steady state photosynthesis that oxygen evolution requires manganese. The decreased rate of photosynthesis in three algal cultures grown in Mn-defective medium confirms the importance of manganese in the photosynthetic process.

Wiessner (1962) was of the opinion that chlorosis can never be considered as a symptom of Mn-deficiency. Bergmann (1955) even suggested an increase in the chlorophyll content per dry weight of *Chlorella vulgaris*

and *Chlorella pyrenoidosa* respectively due to inadequate supply of manganese. However, Kessler *et al.* (1957) reported a much less chlorophyll content in Mn-deficient three days old culture of *Chlorella* than in similarly treated non-deficient culture. He even postulated a manganese : chlorophyll ratio of 1 : 600 in *Chlorella* grown in a medium containing manganese just sufficient for maximum photosynthesis and Hill-reaction. Eyster *et al.* (1958 b) proposed that in the absence of manganese, chlorophyll became more sensitive to destruction by light, hence chlorosis in the cells devoid of manganese may be considered as a secondary symptom of the deficiency. O'Kelley (1968) suggests Mn-deficiency as the cause for the chlorotic appearance of some algal cells. Decreased chlorophyll content in Mn-deficit cells of *Isochrysis galbana* and *Synechocystis salina* was noticed by Srisudha (1989). The least pigment content in the control cells of the three experimental algae is in accordance wth the previous observations.

Manganese acts as the activator of many enzymes like oxidases, peroxidases, dehydrogenases and decarboxylases which are involved in most of the biochemical reactions of the cell. It may be speculated that reduced rate of biochemical reactions resulted in lowered contents of metabolites like carbohydrate and protein in Mn-defective cells.

ZINC

Earliest report on the requirement of zinc for algal growth was that of Eilers (1926) in *Stichococcus bacillaris*. Investigations conducted thereafter established the necessity of this element for the growth of many algal species though only in microquantities (Stegmann, 1940; Provasoli and Pintner, 1953; Walker, 1953, 1954) and much work has been done on its metabolism in *Euglena* (O'Kelley, 1974). Under optimal conditions, the growth rate of *Euglena* was shown to be a linear function of the zinc content of cells (Price and Quigley, 1966).

According to Provasoli and Pintner (1953), several algae show a requirement of zinc in concentrations above 10^{-1}–10^{-2} mg/l. Walker (1954) suggested a requirement of 4.5 mg/l Zn for the growth of *Chlorella* species. Zinc is known to form stable complexes with DNA and RNA thus maintaining their stability. Decreased RNA content and an increased DNA and amino acids in photosynthesizing Zn-deficient cells were observed by Wacker (1962). Zinc also plays a vital role in maintaining the integrity of ribosomes. Praske and Plocke (1971) noticed that under conditions of Zn-deficiency ribosomes were disappeared in *Euglena* but reappeared when zinc was added.

Highly reduced growth responses of the test algae *viz.* *Chlorococcum humicola*, (Naeg.) Rabenhorst, *Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim in the control raised in Zn-deficient medium confirm the necessity of this element for the adequate

growth of these organisms. Stegmann (1940) detected a decrease in chlorophyll formation and photosynthetic activity in cultures deficient in zinc. The addition of zinc resulted in a steep rise in chlorophyll production followed by the recovery of photosynthesis. Reduced photosynthetic activity due to Zn-deficiency was further proved by the explorations of Warburg and Luttagens (1946) who observed a subsided rate of photosynthesis by the addition of O-phenantholine which forms specific complexes with zinc. The occurrence of this metal in carbonic anhydrase (Keilin and Mann, 1940) suggests that this element may participate in photosynthesis at the level of CO_2 fixation (Wiessner, 1962). Walker (1954) demonstrated a relative decrease in dry weight at lower concentrations of zinc.

Addition of zinc to the test cultures resulted in an increase in cell number and C-fixation, higher production of pigment, carbohydrate and protein. It was also found that the requirement of zinc by the three algal species varied with the age of the culture. 0.04 ppm to 0.06 ppm Zn was found to be stimulatory to the growth of *Chlorococcum humicola*, (Naeg.) Rabenhorst. The optimum range for *Chlorella ellipsoidea,* Gerneck was found to be 0.04 ppm to 0.08 ppm and that for *Scenedesmus bijuga,* (Turp.) Lagerheim was 0.06 ppm to 0.08 ppm Zn. Increase in growth responses by the addition of zinc in *Scenedesmus bijuga* was reported by Kanakavalli Susarala (1987). Srisudha (1989) suggests an optimum range of 0.01 ppm to 0.05 ppm zinc for *Isochrysis galbana*, Parke and 0.02 ppm to 0.05 ppm Zn for *Synechocystis salina,* Wislouch for the enhanced cell number, C-production and pigment content in these organisms.

Jensen *et al.* (1974) proposed that though zinc is an essential element, it becomes more toxic at higher levels and different species vary widely in their sensitivity to zinc toxicity. Retardation of growth in studied algal species exposed to zinc concentrations above the optimum level substantiate the toxic action of this trace element. The reports of previous investigations also point to a depression in cell division in various algal species at toxic levels of zinc which vary with species (Rachlin and Farran, 1974; Rosko and Rachlin, 1975; Rachlin *et al.*, 1982, 1983; Srisudha, 1989; Srivastava *et al.*, 1991; Thongra - ar and Matsuda, 1994). According to De Filippis *et al.* (1981), the ability of metals to inhibit NADP-oxidoreductase and the resultant lowering of cells' NADPH manifests itself in the declined growth rate.

A linear relationship between metal taken up and external metal concentration was observed by Bryan (1969), Canterford *et al.* (1978) and Srisudha (1989). It was suggested that the initial uptake of heavy metals was related to simple ion exchange where positively charged metal ions displace cations present on the cell surface (Davies, 1978). The amount of heavy metals bound to the cell surface at equillibrium is determined by the affinities of cations for the binding site and the free ion concentration in solution. Jensen *et al.* (1982) suggest that once inside the cell, zinc ions initially become compartmentalized into the cells' polyphosphate bodies and other organelles in an attempt to reduce the toxic burden of the metal, if it exceeds 0.1 ppm.

A retarded primary production at concentrations above the optimum level was shown by the experimental algae. In the opinion of De Filippis and Ziegler (1993), heavy metals including zinc decrease the activities of four enzymes involved in the fixation of CO_2 which lead to the decrease in the photosynthetic rate in lag phase, but with the ageing of the cultures some of their lost activities were found to be recovered. In the experiments conducted, *Chlorococcum humicola*, (Naeg.) Rabenhorst alone showed an increase in the rate of primary production with a rise in zinc concentration with ageing, while other two species *viz. Chlorella ellipsoidea*, Gerneck and *Scenedesmus bijuga*, (Turp.) Lagerheim failed to exhibit this phenomenon. This may be due to varying degrees of tolerance by different algae to the same concentration of trace metal.

Davies (1973) comments that increased growth responses at exalted levels of zinc with prolongation of exposure period may be explained as the exudation of waste products from the senescent cultures which complex the medium, will reduce the amount of metal taken up and will also decrease the rate of incorporation into plant cells. Both these processes provide a degree of protection against the toxic effect of the metal. The difference in the metal complexing capacities of the exudates in the log and senescent phases was confirmed by Fisher and Fabris (1982) suggesting a difference in the exudate composition.

Davies and Sleep (1979a, b) found that even too low concentrations of zinc as 0.01 ppm to 0.015 ppm can cause detectable inhibition of C-fixation *ie.* less than 90% of control values. Toxic effect of

higher concentrations of zinc on the photosynthetic reduction of phytoplankton was further supported by the explorations of Srisudha (1989) and Bhattacharyya *et al.* (2000).

However, investigations carried out on *Asterionella japonica* by Fisher *et al.* (1981) have shown that C-fixation rates and chlorophyll production increased with dosage of zinc and exceeded even that of control in higher levels of zinc. Van Assche and Clijsters (1990) and Katiyar and Katiyar (2000) too observed enhanced rate of C-fixation at elevated levels of zinc. This enhanced productivity was explained as the role of zinc in carbonic anhydrase activity and was speculated as a mechanism of self protection in order to overcome pollution (Katiyar and Katiyar, 2000). An accelerated production of chlorophyll *a* content with increased levels of zinc was demonstrated by Andrade *et al.* (1994) and Katiyar and Katiyar (2000).

However, in the present investigation it was found that high concentrations of zinc exerted a retarding effect on the pigment content thus contradicting the above observations. Disruptions of thylakoidal membrane within the chloroplast and the enlargement of inter-thylakoidal space due to exposure to elevated metal concentrations were reported by Wang *et al.* (1995). Such damages to the intrastructure of the chloroplast may account for the reduced chlorophyll content of algal species at high metal concentrations.

Subsided values for carbohydrate and protein content in test organisms exposed to elevated levels of zinc indicate the toxic action of this

metal on the algal metabolism. Cellular damage due to high zinc concentrations as explained by Voloshko and Gavrilova (1994) and Saygideger (1998) may be suggested as the possible reasons for the reduced content of metabolites. From the study undertaken, it can be deduced that the trace element zinc which has a positive effect as micronutrient at low concentrations becomes toxic at higher levels.

The investigation conducted elucidates that among the three macronutrients, phosphorus, calcium and magnesium, the element magnesium is needed in relatively large quantities for the adequate growth of test algae. Their requirement for the macronutrients was in the order Mg>Ca>P. Though phosphorus was needed only in very small quantities, its absence resulted in almost complete suppression of the growth in these organisms, thus confirming its role as major growth limiting nutrient. Among the micronutrients, iron, manganese and zinc, the least requirement by the algae was noted for iron. Above the optimum levels, each of these essential elements was found to be toxic to algal growth.