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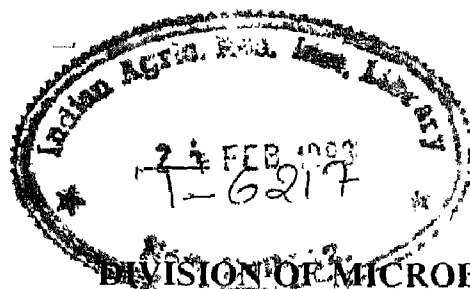
FLUORIDE TOLERANCE IN MICROALGAE AND ITS ECOLOGICAL IMPLICATIONS

MONICA BHATNAGAR

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DIVISION OF MICROBIOLOGY
INDIAN AGRICULTURAL RESEARCH INSTITUTE
NEW DELHI - 110 012

1997

FLUORIDE TOLERANCE IN MICROALGAE AND ITS ECOLOGICAL IMPLICATIONS

by

MONICA BHATNAGAR

A dissertation submitted to the Faculty of the Post Graduate School,
Indian Agricultural Research Institute, New Delhi,
in partial fulfilment of the requirements
for the degree of

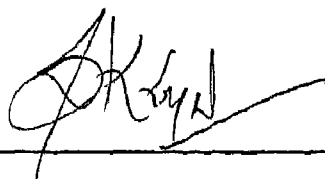
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(Chairman)



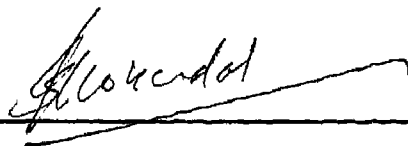
Dr. B.D. Kaushik

(Member)



Dr. K.R. Kaundal

(Member)



Dr. J.M.S. Mathur

(Member)



NATIONAL CENTRE FOR CONSERVATION & UTILIZATION OF BLUE GREEN ALGAE
Indian Agricultural Research Institute
New Delhi - 110 012 INDIA

Dr. S.K. Goyal
Principal Scientist and
Principal Investigator

26 June, 1997

CERTIFICATE

This is to certify that the thesis entitled **FLUORIDE TOLERANCE IN MICROALGAE AND ITS ECOLOGICAL IMPLICATIONS**, submitted to the Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology, embodies the result of *bona fide* research carried out by **Ms. Monica Bhatnagar** under my supervision and that no part of the thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help obtained during the course of study has been duly acknowledged by her.



(S.K. Goyal)

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Advisory Committee

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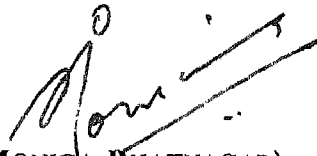
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INTRODUCTION

The ancient disease of gaddur, or gaddjax, was described in Icelandic literature 1000 years ago. Later known as dental fluorosis of cattle, the disease developed on domestic animals feeding on grass contaminated by ash deposited following volcanic eruptions. Fluorosis, an incurable crippling malady is caused by excessive intake of fluoride. In its innocuous form, it is symptomized by discoloured or stained teeth and joint pains. But in its severest form it leads to skeletal fluorosis causing deformities and finally permanent paralysis. The disease has become a major health concern today. Nearly 25 million Indians are affected by endemic fluorosis in 15 states of the country, while another estimated 25 million are at a risk.

The ultimate source of fluoride is the earth's crust where it is rated as the 13th most abundant element. Geological survey has revealed that the Indian subcontinent is heavily loaded with fluoride rich minerals like fluorite, topaz, apatite, rock phosphate, phosphatic nodules, and phosphorite. Fluoride from these sources leaches out and contaminates water and soil environment. Besides being naturally present, it enters the water supplies through effluents from several industrial units and sewage plants, run off from cultivated fields and by fluoridation of municipal water supplies. A number of industries use fluorides either as raw material or it appears as a by product in the effluent. Industries manufacturing aluminium, steel, phosphatic fertilizers, tooth paste, ceramics, bricks and automobiles are indicated in the process. Fluoride dust, fumes and F rich discharges from these establishments pollute the environment.

Fluoride is also the most phytotoxic of the common pollutants ranked fifth in

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importance after ozone, sulphur dioxide, oxidants other than ozone (peroxyacyl nitrates, nitrogen oxides) and pesticides. Susceptible species can be irreversibly damaged at atmospheric concentrations below 1ppb. Consistent exposure leads to bioaccumulation of fluoride in plants which along the food chain gets magnified to toxic levels in higher trophic forms.

Fluoride, the strongest known oxidising agent has multipronged effects on biological systems. Many of these effects as those on respiration, photosynthesis, pigment synthesis, protein synthesis and enzyme activity are potentially adverse to plant life.

Inspite of the voluminous information available on fluoride toxicity in higher plants, very few attempts have been made to study the response of microalgae to fluoride stress. Since microalgae constitute an important component of the biota, it was felt necessary to study the interaction between microalgae and fluoride. Thus the aim of the present study was to ascertain the limits of fluoride tolerance in microalgae, decipher the stress impacted physiology and examine if algae can scavenge fluoride from the ecosystem.

REVIEW OF LITERATURE

Sources of Fluorine

Soil

Water

Air

Fluorine and Man

Fluorine and Plants

Fluoride Uptake and Accumulation

Sensitivity and Symptoms

Physiological Responses

Membrane Systems

Photosynthesis

Algal Photosynthesis

Respiration

The Algal Perspective

Enzyme Systems

Metabolism

Fluoride Tolerance

Fluorine a member of the halogen family and symbolized as F, is a pale yellow gas discovered by Scheele in 1771 and isolated by Henri Moisson in 1886. Elemental fluorine with atomic number 9, exists as a diatomic molecule with a remarkably low dissociation energy of $36.8 \text{ kcal mol}^{-1}$. This makes it a highly reactive element. Being strongly electronegative and chemically active, it is always found in combined form. Binary fluorides have been demonstrated for all the available elements except He, Ne and Argon. The extremely electronegative behaviour of F is best illustrated by the volatile compounds it forms with nonmetals: the halogen fluorides as ClF, ClF₃, ClF₅, BrF, BrF₃, BrF₅, IF₃; the oxygen fluorides OF₂, O₂F₂ and O₄F₂ and the noble gas fluorides XeF₂, XeF₄, and KrF₂. Other non metals as N, S, and P also form volatile compounds with fluorine. The resulting fluorides are invariably more reactive than F itself. The fluorocarbons, particularly the fluorinated derivatives of CH₄ are widely used as refrigerating agents and propellants for aerosols. Polymers of fluorocarbons on the other hand possess marked chemical inertness and heat resistance. The most common product in use is PTFE (Polytetrafluoroethane, (CF₄)_n) sold under the trade name of Fluon or Teflon.

Fluorine is the strongest oxidising agent known. It displaces non-metallic elements from their compounds. It even displaces Cl from NaCl and O₂ from SiO₂ in glass and some ceramic materials. While in some ways F is a typical halogen, the difference between F and the next halogen Cl is so large that the properties of F containing compounds cannot be predicted by analogy with the compounds of Cl. Table 2.1 embodies some other important properties of F.

Table 2.1 Properties of Fluorine

Property	Value
Relative atomic mass	18.9984
Freezing Point	-219.61°C
Critical Pressure	55 atm
Density of gas at 0°C, 1 atm.	1.696 g l ⁻¹
Heat of Vaporization	1510 cal mol ⁻¹
Transition temperature (solid)	-227.61°C
Boiling point	-188.13°C
Critical temperature	-129.2°C
Density of liquid at boiling point	1.505 g ml ⁻¹
Dissociation energy	36.8 kcal mol ⁻¹
Heat of fusion	121.8 cal mol ⁻¹

Table 2.2 Status of Fluoride unsafe water bodies in India

State	Area	No. of Sources	No. of Samples	Range F(mgl ⁻¹)	No. of safe water sources	Reference
Gujarat	Amreli	55	-	0.8-11.0	-	RGNDWM
	Mehsana	127	139	1.58-9.9	-	RGNDWM
J & K	Doda	7	-	0.05-4.21	-	AIIMS
Maharashtra	Jalgaon	10	47	0.11-3.0	-	RGNDWM
	Bhandara	74	1200	1.5-10.2	-	RGNDWM
U.P.	Agra	-	-	0.2-3.2	-	Singh <i>et al.</i> , 1987
	Unnao	-	-	0.12-19.0	-	AIIMS
Karnataka	Dharwad	44	259	0.4-18.0	-	RGNDWM
	Gulberga	33	129	0.2-5.6	14	RGNDWM
	Raichur	147	488	0.4-8.5	137	RGNDWM
Haryana	Gurgaon	26	1440	0.17-24.17	10	RGNDWM
M.P.	Shivpuri	-	-	-	-	-
	and Jhabua	11	-	1.5-4.2	-	RGNDWM
Punjab	Sangarur	65	54	0.4-2.6	-	Benhi <i>et al.</i> , 1991
Rajasthan	Ajmer	-	503	0-16.2	37.0%	Gupta, 1991
	Banswara	-	229	0-3.2	89.5%	
	Baran	-	141	0-2.0	90.8%	
	Bhilwara	-	206	0-7.4	52.4%	
	Bundi	-	299	0-5.0	87.9%	
	Chittorgarh	-	479	0-6.6	91.4%	
	Dungarwara	-	150	0-6.2	73.3%	
	Jhalawar	-	103	0-1.2	100%	
	Kota	-	101	0-4.8	74.2%	
	Rajsamand	-	168	0-4.9	83.9%	
	Udaipur	-	270	0-5.9	85.6%	
	Nagaur	-	173	0-44.0	52%	Gupta., 1991
A.P.	Anantpuram	5	-	7.2-10.7	-	Sarlakunari <i>et al.</i> , 1993
	Nalgonda	-	410	0.4-20	-	Rajyalaxmi & Ramamohan Rao 1985

RGNDWM - Rajiv Gandhi National Drinking Water Mission Survey data ; AIIMS - Fluorosis Control Cell, AIIMS data (Susheela, 1993)

SOURCES OF FLUORINE

Fluorine, the 13th most abundant element (Paul and Huang, 1980) is roughly as plentiful as C, N or Cl. Its concentration in the earth's crust ranges from 20-1600 ppm. Both sedimentary and igneous rocks contain F. This fluorine is mostly insoluble, but a small amount enters the soil solution. Fluorspar (CaF_2) is the chief ore of fluoride. It also occurs in cryolite (Na_2AlF_6 or $3\text{NaF} \cdot \text{AlF}_3$), topaz ($\text{Al}_2\text{SiO}_4 (\text{OH}, \text{F}_2)$), phosphorite and fluorapatite. Rock phosphate ($\text{Ca}_5 \text{F}(\text{PO}_4)_3$) is also a plentiful source of F. The Indian subcontinent is rich in all these minerals.

SOIL

Fluoride levels in certain soils are known to be high (Weinstein, 1977). Background concentrations in the soil may reach 1%, although it is generally less than 0.05% in typical agricultural soils (Treshow and Anderson, 1989). Fluoride in soils generally arises as a result of their inherent mineral content. Artificially high levels can occur through contamination by polluted air (Agate *et al.*, 1949) through polluted irrigation water (Singh *et al.*, 1977; Benbi *et al.*, 1991), application of phosphatic fertilizers (Robinson and Edgington, 1946; Oelschlager, 1971), use of gypsum (contains upto 2-4% fluoride) amendments (Singh *et al.*, 1979) and use of inorganic or organic pesticides (Pattison, 1959; Metcalf, 1966). Deposition of fluoride in soil as a result of litter fall in a pine plantation has been reported by Amundson *et al.* (1990).

F sorption varies with the type and depth of soil (Saeki and Matsumoto, 1993). It increases with increasing depth. The upper organic matter rich horizon has less sorption capacity than the mineral soil (Amesen *et al.*, 1995). Heavy soils adsorb more

fluorine than the light soils (Shanwal *et al.*, 1989). Among the humus soils, mull soil has the highest sorption capacity followed by moder and mor soils (Wilke, 1987). Bower and Hatcher (1967) observed that alkaline soils adsorbed fluoride from soil water.

Plants absorb and accumulate F from the soil only when it contains a significant amount of soluble F (Singh *et al.*, 1979). This can be caused by industrial effluents which may contain substantial amount of soluble fluoride (Sidhu, 1979; Thompson *et al.*, 1979). Total F concentration in U.S. soils ranged from $<100 \mu\text{g g}^{-1}$ to over $1000 \mu\text{g g}^{-1}$ (NAS, 1971). Generally no relation exists between the fluoride content of soil and plants. Soil fluoride is normally taken up by plants only in small quantities. Absorption is greatest in acid soils in which fluorides are more soluble (Mac Intire, 1972; Cooke *et al.*, 1976; Fluhler *et al.*, 1981).

Accumulation of fluoride in soil indirectly affects the plant growth. Increased fluoride content of detritus and soil causes accumulation of organic matter in the surface of the soil (Rao and Pal, 1978). This may be because of the inhibition of microbial activity or regulation of enzymes responsible for fermentation of carbohydrate rich organic matter (Saha *et al.*, 1981). A significant negative correlation was observed between fluoride and activity of dehydrogenase, phosphatase, arylsulfatase (Wilke, 1987) and processes such as ammonification, nitrification (Wilke, 1987; Wensen and Adema, 1991) and denitrification (Becker and Ottow, 1985). However, mull soils were free from these toxic effects probably due to their higher adsorption capacity.

Further, fluorides accelerate release of phosphate from insoluble forms, which

may form Mg and Ca fluorophosphates. The reduced availability of Mg and Ca curtails the supply of ATP, inhibiting the fermentation process (Nickerson and Chung, 1952).

WATER

The most likely soluble forms of fluorine in natural waters are the free anion (F^-) and the undissociated hydrofluoric acid (HF) and its complexes with Al, Fe and B (Pitter, 1985). The complexed forms have either little or no biological activity (Janecek *et al.*, 1974). Holl (1979) have shown that fluorine compounds that do not dissociate to give free F ions were physiologically inactive.

Fluoride in water arises either by solubilization of rocks and minerals rich in fluoride or through effluents from industries, run off from cultivated fields and fluoridation of municipal water supplies (Rose and Marrier, 1977). Though the fluoride level in natural water bodies is below 0.1 ppm ($<5\mu M$), these activities can increase the concentration to toxic levels. Effluents from phosphate fertilizer factories and aluminium smelters have been shown to increase the F level in water bodies (Connell and Airey, 1982). According to one estimate in Canada, of the total 23,500 t of inorganic fluoride emitted into the Canadian environment, 13,500 t was released into water, of which 80% (11,000 t) was attributed to the phosphate fertilizer production alone.

The Water Quality Criteria set by the Bureau of Indian Standards has put 0.7 ppm as the desirable and 1.0 ppm as the upper limit of fluoride in drinking water. A number of water sources in India have been tested for their fluoride content by various workers (Table 2.2). The survey revealed that safe water sources coexisted with excess fluoride containing sources, within the same area village or location. Use of polluted

water for irrigation may lead to its accumulation beyond the critical limits in the soil. This may affect the quality of the crop because most of the applied fluorine was persistently retained in the upper soil surface (Specht and Mac Intire, 1961). Fluoride toxicity has been reported by Kaur and Duffus (1989) and Pillai (1984) in *Cyamopsis*, irrigated with fertilizer factory effluents containing $> 3000 \text{ mg l}^{-1} \text{ F}$. Mac Intire (1957) and Leone *et al.* (1948) suggested 10 ppm as the safe limit for irrigation water.

Fluoride is considered to be a major component of sea water and is present at a concentration of $1\text{-}3 \text{ mg kg}^{-1}$ (Wilson, 1975). The Water Quality Criteria, 1972 (USEPA, 1973) stipulated that F level, equal to or exceeding 1.5 mg l^{-1} is hazardous to the marine environment also. Fluoride loaded effluents discharged into the marine environment restrict the abundance and distribution of biota as has been observed by Pankhurst *et al.* (1980).

AIR

The problem of air borne fluoride in agriculture and forestry is of paramount importance. It has become a global concern because of growth of industries that use hydrofluoric acid and/or fluoride containing minerals and salts as raw material. Fluoride also arises as a byproduct or end product of many industrial processes. The industries that directly or indirectly use fluoride include aluminium, steel works, mining, enamel, pottery, glass, bricks, phosphatic fertilizers, welding, refrigeration, rust removal, oil refinery, plastics, pharmaceuticals, tooth paste, chemical industry and automobile industry. Fluoride from these industries is emitted as dust, fumes and in the effluent. The amount of fluoride released into the atmosphere may range from a few to several hundred pounds per day depending on the size of the industry and type of

fluoride containing raw material used. According to an estimate made in the United States (Waldbolt *et al.*, 1978), steel factories alone emit 40,100 t of inorganic fluoride per year into the environment. Extent of fluorides emitted by some of the major industries in the U.S. is given in Table 2.3. In most extreme cases, atmospheric fluoride concentrations have exceeded $100 \mu\text{g m}^{-3}$ but concentrations above $1 \mu\text{g m}^{-3}$ are now usual which is enough to be harmful (Treshow, 1971).

Airborne fluorides may be in gaseous or particulate form. Particulate fluorides settle on plant foliage where they add to the total leaf fluoride content. This is of utmost importance if forage plants or agricultural crops are involved. Several cases of extensive damage to forests and vegetation due to air borne fluoride have been reported (Mayrhofer, 1893; Ullmann, 1896; Haselhoff and Lindau, 1903; Adams *et al.*, 1952; Sidhu, 1979). Vegetation around an aluminium factory in Czechoslovakia was killed except for one exceptionally tolerant species *Conium maculatum* (Poison hemlock). Conifers were similarly killed in the vicinity of aluminium factories in the north west U.S. Typically needles of the more sensitive conifers such as Ponderosa pine (*Pinus ponderosa*) and Douglas fir (*Pseudotsuga menziesii*) became necrotic and the trees were killed over areas of few square miles (Treshow, 1980). Fluoride emission had devastated about 81 ha of Douglas fir trees in the vicinity of a phosphate reduction plant (Anderson, 1967).

FLUORIDE AND MAN

Fluoride has never been shown to be essential for plants, animals or humans and therefore when found to be a component of food, it should be considered as a contaminant rather than a nutrient (Foulkes, 1996a). Excess fluoride intake is known

Table 2.3 Estimated total inorganic fluoride emission from major industries in the United States.

S. No.	Source ^a	Emissions (t yr ⁻¹)
1.	Steel	40100
2.	Ceramics	21200
3.	Phosphate fertilizer & processing industries	18700
4.	Aluminium industries	16000
5.	Combustion of coal	16000
6.	Non ferrous metal foundries	4000
7.	Welding operations	2700

(After Waldbolt *et al.*, 1978)

Table 2.4 Fluoride content in agricultural crops and other food items.

Food Item	F (ppm)	Food Item	F (ppm)
Cereals		Leafy Vegetables	
Wheat	2.59-4.60	Amaranth	4.91-7.14
Rice	3.27-14.03	Mint	4.80
Bajra	1.72-2.23, 74.00*	Bathua	6.30
Maize	5.60	Lettuce	5.70
Pulses & Legumes		Spinach	0.77-4.14
Bengal Gram	3.84-6.20, 14.80*	Cabbage	1.28-3.30
Green Gram	2.34-4.84, 21.20*	Chowli	1.79-7.33
Red Gram	2.34-4.84, 52.80*	Other Vegetables	
Soybean	4.00	Cucumber	2.57-4.10
Roots & Tubers		Ladies Finger	2.20-4.00
Beet Root	4.20	Snake Gourd	2.16-3.44
Carrot	1.90-4.90	Brinjal	1.62-2.48
Potato	1.27-2.92	Tomato	1.00-3.40
Onion	1.00-3.70	French Beans	1.07-1.96
Sweet Potato	3.20	Spices & Condiments	
Fruits		Cardamom (Big Elaichi)	14.40
Apple	1.05-5.70	Red Pepper	8.07-10.70
Guava	0.24-5.10	Small Cardamom	8.30
Dates	4.50	Coriander Powder	
8.30			
Figs	4.20	Areca Nut	3.80-12.00
Mango	0.80-3.70	Betal Leaf	7.80-12.00
Banana	0.84-2.90	Tobacco	3.10-38.00
Grapes	0.84-1.74	Garlic	5.00
Nuts & Oil Seeds		Ginger	2.00
Almond	4.00	Turmeric	3.30
Cashewnut	4.10	Fenugreek Seeds	3.60
Coconut	4.40	Cloves	3.20
Mustard Seeds	5.70	Ajwain	2.90
Groundnut	5.10	Food from Animal Sources	
Beverages		Mutton	3.00-3.50
Tea (Dry leaves)	39.80-68.59	Beef	4.00-5.00
Tea Infusion (1 gm in 125 ml hot water)	18.13-56.19	Pork	3.00-4.50
		Fishes	1.00-6.50

(After Sengupta & Pal, 1971; Nanda, 1972; Lakdawala & Punecker, 1973; Chari et al., 1982 and Rajyalaxmi, 1982).

to cause fluorosis, an incurable, slow crippling malady, affecting young and old alike. It is symptomised by discoloured or stained teeth and rheumatism in its mild state manifesting into loss of teeth at an early age (dental fluorosis), deformed bones (skeletal fluorosis) and permanent paralysis. Fluoride toxicity is also known to decrease fertility rate (Freni, 1994; Narayana and Chinoy, 1994) and reduced IQ in infants (Waldbolt *et al.*, 1978).

Fluorosis appears to be fairly widespread in the world. Realizing the magnitude of the problem, the year 1995 was declared as the World Fluoride Year. Fluorosis has been known in India since the beginning of the current century. Today it is known to be prevalent in at least 150 districts of 15 states of India (Fig. 2.1).

It is believed that fluoride is required in minor concentrations during the early period of child growth when teeth and bones are being formed because fluorine protects against carious teeth and gives strength to the bones. So keeping this in view WHO (1984) and USEPA (1973) stipulated minimum and maximum levels of 0.7 and 1.0 ppm fluoride in drinking water. Fluoride deficiency has long been implicated in causing dental caries however a recent report from WHO (1992) shows that dental caries increase as the fluoride concentration increases towards the claimed optimum of 1 ppm (Ziegelbecker and Ziegelbecker, 1993). Further the phenomenon of 'paradoxical response' to fluoride in which high doses may paradoxically cause less damage than lower dose has also been reported by Foulkes (1996b).

Besides water, fluoride reaches human body through the air and food. Fluoride content of some of the agricultural crops and other food items included in our diet have been listed in Table 2.4. Sometimes interaction of F with other food ingredients may

Fig. 2.1 Endemic states for fluorosis in India.



have synergistic effect on its toxicity. Fluoride present in caffeine, tea, coffee and cola results in an increased plasma fluoride (Chan *et al.*, 1990).

FLUORIDE AND PLANTS

FLUORIDE UPTAKE AND ACCUMULATION

Plants absorb and accumulate fluoride from the atmosphere, soils or water. Root absorption is a significant route of entry into the plant, if the soil or water have significant amount of soluble fluoride. Uptake of fluoride by roots is generally regarded as a passive process (Venkateshwarlu *et al.*, 1965) and there is always a concentration gradient between the root and shoot. Plants like *Camellia* sp. however, were able to accumulate F in much higher quantity than the surroundings (Zimmerman *et al.*, 1957).

Air borne fluorides are the major sources contributing to their excessive accumulation in plants. Gaseous fluorides enter the plant chiefly through the stomata and enter the intercellular spaces. Fluorine is either directly absorbed by the cells in contact or it is transported through vascular tissues and redistributed to the specific sites of accumulation like leaves. Ares *et al.* (1980) studied some xerophytic plants exposed to F pollution in the vicinity of an aluminium smelter and showed that F accumulation was mainly by deposition or adsorption upon external leaf surface followed by a slow diffusion to the inner tissues, regulated by the water potential at the epidermis and cuticle. Absence of F accumulation in substomatic tissues lead to the conclusion that the uptake of gaseous fluoride through stomata may be relatively unimportant for these plants.

Once fluoride has passed through the cell wall it reacts initially with the

cytoplasmic membrane where a fraction of it may be retained. Inside the cells, upto 60% of F is found to be associated with the chloroplast (Chang and Thompson, 1966; Ledbetter *et al.*, 1960) and the rest enters the vacuoles. Continued accumulation of fluoride substantially increases its concentrations in the senescent leaves (Benedict *et al.*, 1964). Rapid growth dilutes F concentration. Accumulation and toxicity of fluoride depends on characteristics of the plant species, duration of exposure, concentration in the atmosphere and the chemical form of fluoride (McCune *et al.*, 1965; Pack *et al.*, 1959). *Dichapetalum*, *Thea*, *Gastrolobium*, *Camellia*, *Oxylobium*, *Acacia*, *Palicourea* and *Gossypium* sp. can accumulate as much as 4000 ppm concentrations of fluoride without any visible foliar injury (Jacobson *et al.*, 1966) and even in an unpolluted environment (Zimmerman *et al.*, 1957; Jacobson *et al.*, 1966).

Based on the appearance of foliar lesions it has been generalized that conifers are more sensitive species. Broad leaved species tend to be more tolerant (with exceptions, as some fruit trees are highly sensitive). Vegetables and field crops are highly resistant. Ornamental plants show variable sensitivity. Gladiolus and tulips are susceptible even to 1 ppb of atmospheric fluoride concentrations. The tolerant plants are able to accumulate higher concentrations of fluoride. Broad leaved species accumulate more F than conifers when they occur together. According to a case study by Pandey (1981), trees were more susceptible to F damage followed by shrubs, grasses and herbs.

The Italian rye grass (*Lolium multiflorum*) is known to accumulate 189 - 275 $\mu\text{gF g}^{-1}$ dry matter, when exposed to 1.1-1.35 $\mu\text{g HF m}^{-3}$ (Kostka, 1994). This grass is used as a standard for fluoride estimation. Considerable information is available on

the effect of fluoride on agriculturally important plants such as rice (Sun and Su, 1985; 1991), sunflower (Cooke *et al.*, 1978), soybean (Ghiasseddin *et al.*, 1981), spinach (Troiani *et al.*, 1994), parsley (Kabasakalis and Tsolaki, 1994), cabbage and cauliflower (Leone, 1980), maize (Kronberger and Halbwachs, 1978) and fruit trees such as apple (Reuter and Dassler, 1983) and grapes (Weinstein, 1984).

Fluoride content of vegetation is indicative of the degree of atmospheric contamination. Thompson *et al.* (1979) reported 44-281 ppm fluoride in vegetation around a phosphorus plant in New Foundland. Concentrations of fluoride in plant species colonizing fluorspar mine waste (that contain fluoride levels of the order 22-184mg g⁻¹ DW) varied between 0.05-5 mg F g⁻¹ DW (Cooke *et al.*, 1976; Johnson, 1976). However individual species from different sites showed consistent concentrations of fluoride (Cooke *et al.*, 1976) indicating interspecific variation in the uptake of soil fluoride (Cooke and Johnson, 1978). Kassabi and Assimi (1984) reported fluoride concentrations upto 4300 ppm in plants growing near a phosphate factory in Morocco.

The defluoridation potential of plants that can accumulate fluoride from water has also been studied for *Spirodela polyrrhiza* by Shirke and Chandra (1991) and for *Eicchornia crassipes* by Rao *et al.* (1973) under laboratory conditions. Substantial uptake was reported in *Spirodela* suggesting that this plant could be employed to remove fluoride from polluted waste waters. *Eicchornia crassipes* accumulated upto 75 mg fluoride per plant. Since this plant has a high threshold value, it can prove useful for treating industrial effluents containing very high levels of fluoride.

Very few studies have been reported on the fluoride uptake by algae. Amongst

the marine forms, red algae have been reported to accumulate higher levels of fluoride (Young and Langille, 1958). Kesava Rao and Indusekhar (1984) reported 11.35-20.04 mg kg⁻¹ F in red algae as compared to 4.78 - 17.82 and 3.02- 18.86 mg kg⁻¹ F in brown and green algae, respectively. This range of fluoride observed in algae was much higher than what occurs naturally in land plants (<10 mg kg⁻¹). Studies on the distribution of certain halogens in sea weeds (Kesava Rao and Indusekhar, 1989) showed that the halogen content in the sea weeds was in the order Cl > Br > I > F, where as in sea water the trend was Cl > Br > F > I. Their concentration factors were in the order I > F > Br > Cl. Iodine enters biological systems quicker than F but the latter's absorption was faster than Br and Cl. Chlorine was not accumulated by sea weeds but they contain substantial amounts of I, F and Br. It appeared that their uptake and accumulation was mutually independent. On the contrary Hemens and Warwick (1972) did not observe any significant bioaccumulation of halogens in marine algae.

Mac Arthur *et al.* (1983) reported fluoride uptake by *Synechococcus leopoliensis* (a fresh water cyanophyte). The five freshwater algal forms (*S. leopoliensis*, *Oscillatoria limnetica*, *Ankistrodesmus braunii*, *Scenedesmus quadricauda*, *Cyclotella meneghiniana* and *Stephanodiscus minutus*) studied by Hekman *et al.* (1984) showed variable response. No fluorine was detected in the green algae even after 50 h exposure to 50 ppm fluoride but was found to be associated in the cyanophytes and *C. meneghiniana*. Their F content increased with increasing F concentrations in the growth medium. *S. leopoliensis*, *O. limnetica* and *C. meneghiniana* accumulated 54 ±16, 71 ±17 and 116 + 18 ppm DW of F (control 30

+20, 26 +15, 36+16 ppm DW of F), when exposed to 2.6 mM F. Accumulation of fluoride appears to be regulated by the differential rates of F absorption and pH of the medium (Nichol *et al.*, 1987). Fluoride sensitive cells of *S. leopoliensis* showed an intracellular increase of 6.1 mM F over control on exposure to 2.6 mM fluorine at pH 6.26. However uptake decreased with increasing pH values. Fluoride resistant *S. leopoliensis* on the other hand showed no measurable increase in cell F. Another green alga *Chlorella pyrenoidosa* also showed no detectable increase on addition of 7.9 mM NaF even at a lower pH of 5.9.

SENSITIVITY AND SYMPTOMS

The exposed leaves show marginal leaf necrosis followed by light to dark brown necrotic lesions near or at the leaf tip, mottling and finally chlorosis. In extreme cases over 90 % of the affected leaves may become necrotic and over 90 % of the leaves of a tree may show injury (Treshow and Anderson, 1989). Badly injured leaves still remain on the tree (Brewer, 1960). Build up of intracellular fluoride concentration depends upon the external amount of fluoride and the symptoms appear only when a critical threshold has been reached. Given optimal conditions, sensitive plants may show susceptibility even at 1.0 ppb (Mc Cune, 1969; Treshow and Pack, 1970). When leaf injury is severe, growth is suppressed and the tree may ultimately die. In absence of necrosis and chlorosis, growth generally remains unaffected. It is also known to cause the well known suture red spot of peach, shrivel tip of cherry and similar symptoms of local necrosis on pears and apricots (Treshow, 1970) .

Severe fluoride toxicity leads to loss in productivity. Impairment of growth of conifers (Treshow *et al.*, 1967; Keller, 1977) and reduced fruiting in Valencia oranges

was observed when fluoride concentrations reached 200 ppm (Leonard and Graves, 1966). Yield of tomato fruits was substantially reduced when foliar fluoride content reach 1252 ppm (Pack, 1966). Development of pollen tube and receptivity has been shown to be impaired by Sulzback and Pack (1972). Seed and seedlings appear to be potentially more susceptible to fluorides. Holub and Navara (1966) found that 100 ppm F caused total inhibition of germination of *Pisum sativum* (pea) seeds. Purohit and Sharma (1985) reported germination inhibition of *Brassica campestris* (field cabbage) by only 2.5 ppm fluoride. According to Chang (1967), NaF inhibits germination of maize seeds by inhibiting phytase activity. Fluoride complexes with magnesium thereby making it unavailable for the activity of the enzyme.

Diverse effects of fluoride on the growth of microalgae have been reported. Most of these effects were apparent at millimolar and lower concentrations. Some freshwater microalgae have been shown to be sensitive to fluoride. Growth of *Chlorella pyrenoidosa* was reduced by 37% in the presence of 2 ppm (100 μ M) fluoride at pH 6.8 (Smith and Woodson, 1965) whereas 43 ppm (2.3 mM) F⁻ proved to be lethal to *Scenedesmus* sp. at an unstated pH (McKee and Wolf, 1963). Out of six fresh water planktonic microalgae, growth of cyanobacterium *Synechococcus leopoliensis* was only inhibited on addition of 2.6 mM NaF during mid logarithmic phase (Hekman *et al.*, 1984). The effect was however, transient showing resumption at reduced fluoride levels. Based on their experience and earlier observations, they suggested that F⁻ level found even in fluoride polluted waters should have minimal effects on algal populations.

Joy and Balakrishnan (1990) reported enhancement of growth in *Nitzschia palea*

and *Amphora coffeaformis* between 10-110 mg l⁻¹ of fluoride with significant stimulation at 70 mg l⁻¹. These authors together with Oliviera *et al.* (1978) suspected a nutritional role of F in algae.

PHYSIOLOGICAL RESPONSES TO FLUORIDE

The initial impact of a pollutant is at the molecular level. Specific enzymes and enzymatic reactions particularly involving photosynthesis and respiration are commonly the first to be affected. The physiological mechanisms vary with the pollutant involved.

Membrane Systems

Permeability and transport of fluoride across the cell membrane is the root cause of all the toxic effects of fluoride on the cell. Membranes could be affected either because of slow synthesis of membrane components or the membrane lipids may be hydrolysed and used as alternative sources of energy. Because lipids are major components of cell membrane, any change in the lipid or fatty acid composition leads to altered membrane structure and function. Changes in membrane permeability causing greater electrolyte leakage have been reported in *Pinus banksiana* and *Pinus strobus* seedlings (Zwiazek and Shay, 1988c). In *Pinus banksiana* lipid materials accumulated near plasmalemma, tonoplast, chloroplast and mitochondrial membranes during early stages of fluoride injury (Zwiazek and Shay, 1987).

Simola and Koskimies-Soininen (1980) observed an increase in the proportions of palmitic acid and decrease in linoleic acid in KF treated *Sphagnum fimbriatum* gametophytes and hypothesized that fluoride ions inhibit lengthening of the fatty acid chain. Fluoride fiddles with the Na pump disturbing the equilibrium between intracellular potassium and extracellular sodium (Ballantyne and Glover, 1981). Many

of the fluoride sensitive enzymes are membrane bound. Malic dehydrogenase has been shown to be sensitive to fluoride (Psenak *et al.*, 1977). Magnesium fluoride induced inhibition of plasmalemmal ATPase by preventing the binding of the normal substrate Mg ATP has been reported in *Beta vulgaris* at concentrations as low as 5 mM. (Giannini *et al.*, 1987b).

Wei (1972) reported that during fluoride fumigation of soybeans the first membrane to be disrupted was tonoplast. The tonoplast ATPase was subsequently shown to be inhibited by fluoride (Clowes and Juniper, 1968; Giannini *et al.*, 1987a). Fluoride at 10 mM inhibits the maintenance of the transmembrane pH gradient. The cause of this disruption is blockage of chloride antiport movement inwardly during proton extrusion. At high concentrations ATPase is directly inhibited. It was postulated that the blockage of chloride movement was because of competitive exclusion by bound fluoride. Once the tonoplast breaks down, phenols and organic substances are released into the cytoplasm which induce osmotic changes as well as exert toxic effects on it thereby hastening degeneration of other cell organelles.

A steady state model for F transport across the membrane and its subcellular partitioning was proposed by Miller *et al.* (1986). The basis of this model resides in the weak acid characteristics of fluoride in solution. Hydrogen fluoride is a weak acid (pK_a 3.45), and dissociates in solution into two forms HF and F^- . Their relative concentration depends on the pH of the immediate environment. This at a given pH can be predicted mathematically from the Handerson- Hasselbach equation.

$$pH = pK_a + \log [F^-] / [HF]$$

The two ionic species differ greatly in their abilities to pass through lipid

membranes. (Gutnecht and Walter, 1980). The charged ion has a permeability coefficient of 4.9×10^{-11} cm sec⁻¹, six times slower than the protonated ion that crosses a membrane at a rate of 1.4×10^{-4} cm sec⁻¹. In the cytoplasm, accumulated fluorides subsequently partition into chloroplasts, mitochondria and vacuoles, driven by transmembrane pH gradients and concentration differences. The transmembrane movement continues until the organelle function is disrupted. On a similar analogy, the partitioning would be expected to occur during the movement of dissolved fluoride with the transpirational stream through the apoplast enroute to the leaf margin. The consequence will be accumulation within the plant cells and subcellular compartments. The direction of movement would also be controlled by the magnitude of the pH drop across the membrane. Based upon the pH dependence, several reports (Whitford *et al.*, 1979; Armstrong and Singer, 1980) have suggested that F⁻ crosses membrane in the nonionic form HF. Effects of fluoride on mitochondrial and chloroplast membranes and the associated ATPase systems have also been studied (reviewed by Elsair and Khelfat, 1988; Pushnik and Miller, 1990; Miller, 1993).

Photosynthesis

Photosynthesis has long been recognized to be inhibited by fluoride even though the effect appears to be transient (reviewed by NAS, 1971; Amundson and Weinstein, 1980; Weinstein and Alscher-Herman, 1982) Table 2.5 summarises the effect of HF and NaF on photosynthesis of higher plants. Fluorine at 1-12 ppb level caused reduction in chlorophyll a and b content but the effect was transient unless the exposure was not prolonged (Weinstein, 1961). Evidence for fluoride interference with chlorophyll biosynthesis in higher plant leaves has been provided by McNulty

Table 2.5 Reported effects of fluoride (HF and NaF) on photosynthesis.

Genus or Species	$\mu\text{gHF.m}^{-3}$	Duration	Response	Reference
<i>Gladiolus</i>	0.8-8.0	7d	Total interruption in AP, with	Thomas & Hendricks, 1956
<i>Hordeum</i>	32	4-8h	recovery in few hours to days	
<i>Medicago</i>	200	4-8h		
Fruit trees	16-40	4-8h		
<i>Medicago</i>	32	2h	AP reduced during exposure,	Beimett & Hill, 1973
<i>Hordeum</i>	32	2h	with recovery after exposure	
<i>Vicia</i>	40	1 and 14d	AP reduced during exposure,	Horvath <i>et al.</i> , 1978
			with recovery after exposure	
<i>Lycopersicon</i>	1.4-5.2	4 weeks	No effect	Hill <i>et</i> <i>al.</i> , 1959
	0.9-11.2	3 weeks	No effect	
Fruit trees	2.1	183h	14% reduction in AP	Thomas, 1958
<i>Gladiolus</i>	3.1-5.2	30-205d	Reduction in AP	
<i>Gossypium</i>	13.6	138h	No effect	
<i>Citrus</i>	0.32-0.77	Growing season	No effect	Thompson <i>et</i> <i>al.</i> , 1967
<i>Gladiolus</i>	0.8	39d	No effect	Hill, 1969
	1.2	27d	3% reduction in AP	
<i>Fragaria</i>	2.3	63d	No effect	
	38	1d	50% reduction in AP	
<i>Lycopersicon</i>	5.1/12	17/21d	No effect	
<i>Prunus</i>	1.6	42d	No effect	
<i>Zea</i>	7.7	16d	No effect	
<i>Sorghum</i>	0.7	14d	No effect	
	2.2 then	12/2d	Reduced, with recovery after	McCune <i>et</i> <i>al.</i> , 1976
	1.7		exposure	
	3.5 then	7/7d	Reduced during $3.5\mu\text{g.m}^{-3}$	
	5+		exposure, then severely injured. Little recovery.	
<i>Pinus sylvestris</i> , <i>P. nigra</i> , <i>P. strobus</i> , <i>Larix decidua</i> , <i>L. leptolepis</i> , <i>Quercus</i> <i>borealis</i> , <i>Alnus incana</i> , <i>Pseudotsuga menziesii</i> , <i>Acer pseudoplatanus</i> , <i>Picea excelsa</i>	Ambient near source	Nov-Apr	Reduced AP of whole plant due to loss of foliage	Keller, 1977
<i>Liquidambar</i> sp. <i>Plantanus</i> <i>occidentalis</i> , <i>Acer rubrum</i> , <i>Liriodendron tulipifera</i> , <i>Oxydendrum arboreum</i> , <i>Pinus strobus</i> , <i>P. taeda</i> <i>P.</i> <i>echinata</i> , <i>Cornus florida</i>	1.9, 19 or 190ppm NaF	24h	AP reduced in older needles of <i>P. taeda</i> and <i>P. echinata</i> at 1.9ppm NaF. AP reduced in all species at 19 and 190ppm NaF	McLaughlin and Barnes, 1975
<i>Picea excelsa</i>	100ppm	Winter- spring	AP reduced in old foliage, new injured	Keller, 1980

and Newman (1961), Bogorod (1976) and Ivinskis and Murray (1984). Chloroplast disorganisation manifesting into chlorosis has also been reported in higher plants (Solberg and Adams, 1956; McNulty and Newman, 1961; Comeau and Le Blanc, 1972; Bligny *et al.*, 1973; Horvath *et al.*, 1978).

A varied photosynthetic response to fluoride has been observed. Exposure to high level of net F ($> 10 \mu\text{g m}^{-3}$) have been reported to reduce net photosynthesis in every species tested with the exception of cotton (Thomas and Hendricks, 1956; Hill, 1969; Bennett *et al.*, 1973). Prolonged exposure of sorghum to relatively low levels of HF resulted in reversible reduction in the rate of AP (Apparent Photosynthesis) (McCune *et al.*, 1976). The recovery process was often rapid but frequently incomplete (Thomas and Alther, 1966). Others have reported no effect on photosynthesis in the absence of any foliar injury (Hill, 1969; Thompson *et al.*, 1979). The more sensitive *Gladiolus* sp. however, showed reduction in AP under long term exposure (27 d) even at 1.4 ppb HF (Hill, 1969).

The inhibition of CO_2 uptake and photosynthesis may be due to the inhibition of enzymes or coenzymes with metal components involved in utilizing light energy (Spikes *et al.*, 1955). Ballantyne (1972) suggested that fluoride may complex magnesium which is essential for thylakoid functioning. Doley (1988) reported stimulation of apparent photosynthesis in young needles of *Pinus caribaea* var *bahamensis* and var *hondurensis* exposed to $1\text{--}2 \mu\text{gF m}^{-3}$.

Algal photosynthesis

Fluoride is a classical inhibitor of algal photosynthesis. Warburg *et al.* (1955, 1956 cited in Bishop and Gaffron, 1958) showed that fluoride entered cells of

Chlorella at pH 4.0 and inhibited photosynthesis by limiting CO₂ utilization. They obtained an immediate burst of CO₂ on addition of sodium fluoride to acid grown *Chlorella*. Bishop and Gaffron (1958) confirmed this and further showed that this CO₂ burst was a fluoride induced decarboxylation of an organic acid. The phenomenon however was not a generalised response to fluoride as several other algal forms studied as *Chlorella*, *Scenedesmus obliquus*, *Ankistrodesmus braunii* and *Chlamydomonas moewusii* did not show fluoride induced CO₂ gushes.

Vennesland and Turkington (1966) identified two loci of fluoride action - a primary locus in the light independent reaction of photosynthesis and the other locus was associated with the site of Hill reaction. The Hill reaction was less sensitive than photosynthetic O₂ release in *Anabaena variabilis* and *Plectonema boryanum*. Sensitivity increased with decrease in pH. The adverse effect of fluoride on Hill reaction was appreciably reduced at higher levels of CO₂ concentration showing optimum influence at 0.5 %.

Evidence for action of fluoride on the light independent reactions of photosynthesis was obtained for *Synechococcus leopoliensis* by Hekman *et al.* (1984), where photosynthetic O₂ release was inhibited by growth inhibitory F concentration. They observed that the retardation of growth was more drastic and proposed that inhibition was probably due to a loss of cell potassium. Loss of upto 90% of cell K within a few hours of exposure to 7.9 mM F was shown by Mac Arthur *et al.* (1983) and resumption of growth was preceded by reabsorption of K from the medium.

According to Nichol *et al.* (1987) fluoride toxicity is a pH related phenomenon. When growth and photosynthesis were measured at the same pH both showed similar

sensitivity to fluoride. They observed that O₂ evolution in light was completely inhibited in *Synechococcus leopoliensis* with in 3-5 min of addition of 3 mM NaF (57.7 ppm) at pH 6.6 but at pH 6.16, only 1.05 mM NaF brought about inhibition. Again the Hill reaction at this pH and F concentration was only marginally inhibited. They opined that photosynthesis was ATP limited in the presence of fluoride. Giannini *et al.* (1985) reported interaction of fluoride with the thylakoid F₀ F₁ - ATPase of chloroplasts. Fluoride stress has been shown to affect the thylakoid organisation within the chloroplasts of the dinoflagellate *Amphidinium carterii*. The envelope of the thylakoids squeezed away from the periphery of the chloroplast towards inside probably due to osmotic changes and/or molecular changes in its envelope membrane. In a similar effect on cyanobacterial thylakoids, fluoride may inhibit photophosphorylation. Alternatively the depletion of cellular ATP may be caused by a reduction in cytoplasmic pH. Thus the effect may be caused by the reduction of pH gradient across the thylakoids on which photophosphorylation depends and activation of the plasma membrane proton extruding ATPase, thus causing ATP consumption.

Such activation is well documented for weak acids (Sanders *et al.*, 1981; Marre *et al.*, 1982). A decrease in internal pH by as much as 0.4 units within 15 min of NaF treatment has also been reported. The consequent ATP depletion may affect a wide range of cellular functions like inhibition of phosphate uptake (Budd and Kerson, 1987) and loss of K⁺ ions (Mac Arthur *et al.*, 1983) also.

Respiration

One of the immediate manifestation of fluoride toxicity in plants is a change in respiration rates (Applegate and Adams, 1960; Miller and Miller, 1974). Stimulation

and inhibition depend on a number of factors such as plant species, concentration of fluoride, age of tissues, length of exposure, pH of culture medium and interactions between various mineral elements and fluoride (Miller, 1993).

Just as general plant susceptibility to fluoride varies with the species, so does the effect of fluoride on respiration (Table 2.6). Fluoride inhibition of respiration has been reported in a number of plant species including *Chenopodium*, *Glycine max*, *Oryza sativa*, *Triticum vulgare* and *Rubus hispidus*. Generally in higher plants, exposure to high or low concentrations of fluoride exhibits an initial stimulation followed by inhibition of respiration (Yu and Miller, 1967). Respiratory inhibition starts at the mitochondrial membrane with restriction of the movement of Na^+ and K^+ ions (Miller *et al.*, 1982) which in turn inhibit the activity of systems necessary for the process. Succinic, malic and NADH dehydrogenases are sensitive to fluoride. Succinic dehydrogenase in the presence of phosphate is the most susceptible enzyme (Lovelace and Miller, 1967a). Enzyme-membrane association further enhances the F action (Psenak *et al.*, 1977).

Miller and Miller (1974) studied soybean response to HF and found good correlation between tissue respiration, mitochondrial activity and mitochondrial ATPase activity. In F inhibited tissue, concomitant to reduction in respiration, the respiration rate and ATPase activity were also reduced. Converse was also true. One can therefore conclude that respiration was controlled by the relative ratio of ADP/ATP. In none of the cases however, the membrane integrity was lost (Pushnik and Miller, 1985). Mitochondria isolated from five day old etiolated corn seedlings when exposed to 30 mM NaF showed an increase in oxygen uptake accompanied with a decrease in ATP

Table 2.6 Reported response of respiration to fluoride treatment in higher plants.

Species	F conc.	Duration	Response	Reference
<i>Gladiolus</i> sp. <i>Lycopersicon</i> <i>esculentum</i> <i>Fragaria</i> sp. <i>Apium</i> <i>graveolens</i> <i>Prunus</i> sp. <i>Hordeum vulgare</i>	0.38-56 ppb HF	21-73 d	Stimulation of dark respiration in Gladioli at 0.38-1.8 ppb; no effect on other sp.	Hill <i>et al.</i> , 1959
<i>Phaseolus</i> <i>vulgaris</i> cv. Burpee	2.7 ppb HF	24 h growing season	Stimulation of respiration following 24h fumigation. On continuous exposure, inhibition seen during first 8 d after planting, followed by stimulation.	Applegate & Adams, 1960
<i>Lycopersicon</i> <i>esculentum</i> <i>Phaseolus</i> <i>vulgaris</i> cv. Tendergreen	1.6 ppb HF	8 d	Stimulation of dark respiration by 67 & 72% respectively.	Weinstein, 1961
<i>Chenopodium</i> <i>murale</i>	6 ppb HF	5-6 d	Stimulation of dark respiration and visible injury	Ross <i>et al.</i> , 1962
Soybean cv. Hawkeye	9-12 $\mu\text{g.m}^{-3}$ HF	4 d	Respiration stimulation in 2 d experiment and inhibition after 4 d of treatment.	Miller & Miller, 1974
Three <i>Pinus</i> sp. and six hardwoods	0.1-1.0 mol.m^{-3} NaF (1.9-190 ppm F)	24 h	Stimulation of dark respiration	McLaughlin & Barnes, 1975
<i>Triticum</i> <i>vulgare</i> cv. Zlatka	NaF soln. 5×10^{-3} M to 1×10^{-2} M	2 d	No effect	Holub, 1975
Rice cv. Milyang 23 & Jinheung	10 mg.m^{-3} HF	12 h day 12h night 24 h D/N	Decrease in respiration except in 12 h night treatment Increased respiration in all treatments	Kim <i>et al.</i> , 1979
<i>Gladiolus</i> <i>gandavensis</i> <i>Iris hollandica</i> <i>Crocus</i> <i>vernus</i>	--	8 h dark, 8h light	Respiration decreased Respiration increased	Suketa <i>et al.</i> , 1975
<i>Vicia faba</i> cv. Inovec	50 ppb HF	24 h	Stimulation of dark respiration, 2-6 h after fumigation.	Horvath <i>et al.</i> , 1978
<i>Pinus</i> <i>banksiana</i>	NaF Soln.	1 d	Decrease in respiratory rates & Respiratory Quotient	Zwiazek & Shay, 1988a

formation. Sub-mitochondrial preparations and trypsin solubilized ATPase also showed a 30% reduction. Therefore, it was concluded that fluoride did not inhibit the electron transfer process, but had a direct impact on the mitochondrial F_1 ATPase (oxidative phosphorylation takes place through the operation of cristae bound Mg requiring ATPases).

Oxygen uptake in plants is accelerated at lower concentrations of fluoride (Yu and Miller, 1967). McNulty and Lords (1960) and Ballantyne (1984) reported higher concentrations of phosphorylated nucleotides in pea plants that were HF fumigated. The disturbances in the energy metabolism were thus postulated to increase the amounts of phosphate acceptors (ADP) or phosphate donors (ATP) which in turn regulated the glycolytic or mitochondrial activities. Yang and Miller (1963a,b) and Miller (1993) proposed that elevated concentrations of ADP were responsible for deregulating carbohydrate metabolism and stimulated O_2 uptake. Regulation of respiration by relative ratio of ADP/ATP has also been suggested by Miller and Miller (1974). Dinitrophenol, a classical uncoupler of oxidative phosphorylation increased respiration in control tissue but had no effect on fluoride treated tissue (Yu and Miller 1967). It was further speculated that the membrane potential, a coordinating factor between oxidative phosphorylation and mitochondrial electron transport was reduced by 10% under fluoride stress (Giannini *et al.*, 1988). This reduction would reduce the electrochemical back pressure on the respiratory chain, allowing it to function more rapidly than under control conditions. Thus it was proposed that fluoride acts as a weak classical uncoupler similar to DNP and enters mitochondria by a non ionic diffusion gradient.

Increased O₂ consumption has been related to light exposure in *Gladiolus* leaves. According to Suketa *et al.* (1975) O₂ uptake by gladiolus leaves in light was increased by HF but in darkness HF slightly decreased oxygen uptake.

Polygonum and *Chenopodium* plants exhibited an increased use of pentose phosphate pathway over glycolysis. As enolase has been shown to be F sensitive (Miller, 1958) (perhaps because of the formation of a magnesium fluorophosphate complex) it is possible that an inhibition of glycolysis leads to an increased flow of carbon through the PPP. Subsequently activities of cytochrome oxidase, peroxidase, catalase and G6P dehydrogenase were shown to be enhanced in fluoride injured tissues. Fluoride may thus disrupt basic cellular energetics in some manner and increase O₂ consumption.

The algal perspective

At 1.05×10^{-3} M fluoride, oxygen consumption in *Chlorella* increased to more than 107 % and esterified phosphate increased to 153 %. But at 1.05×10^{-2} M although, there was no further increase in the rate of oxygen consumption, the esterified phosphate increased to 160 %. This increase in esterified phosphate indicated that fluoride affected multiple enzyme systems. This is in agreement with the mechanism of multiple enzyme inhibition resulting in a stimulation of the oxygen consumption (Reiners, 1947). The application of Reiners' theoretical treatment depends essentially on the increased esterified phosphates being ATP. Hacket (1959) indicated that the overall control of respiration depends upon the concentration of the acceptor (ADP) rather than the donor (ATP).

Of the three algal species studied by Hekman *et al.* (1984) only *Synechococcus*

leopoliensis showed a stimulation of respiration by a 5 h exposure to 2.6 mM fluoride, a growth inhibitory concentration. Its mechanism is not clear. Either 'wound respiration' (a secondary response to F induced physiological damage) or more directly, uncoupling of energy conservation from electron flow as proposed by Miller and Miller (1974) for F action on plants, may be involved. However, stimulation of respiration was inconsistent with the effect of fluoride on enolase activity. Growth inhibitory NaF treatments have been shown to decrease the ATP level in the cells of *S. leopoliensis* by 75 % and phosphate uptake was also abolished (Nichol *et al.*, 1987).

Fluoride adapted *Amphidinium carterii* showed a stronger evidence of changes in the mitochondrial matrix. Based on this observation Klut *et al.* (1981) suggested that fluoride competitively inhibits the matrix protein and associated enzymes, presumably those of the Krebs cycle (believed to be concentrated in the mitochondrial matrix) (Avers, 1976). They further proposed that it is possible that the fluoroacetate inhibition of endogenous respiration reported for another dinoflagellate (Hochachka and Teal, 1964) may be related to a fluoride effect if we consider that exogenously supplied F is metabolized to fluoroacetate within the cell as is known to happen in the case of fluoride resistant plants (Vickery and Vickery, 1975).

Enzyme Systems

Manifestation of fluoride toxicity is a result of interference with a number of enzyme systems, particularly those associated with photosynthesis and respiratory systems. A list of some of the important enzymes and systems affected by fluoride is presented in Table 2.7. The underlying mechanism of fluoride action may be in interfering with metal ion transport and inactivating metal ions at their sites of

Table 2.7 **Reported effects of fluoride on metabolic pathways and enzymes.**

Enzyme or system	Organism	Fluoride	Effect	Reference
Glycolysis				
Pyruvate kinase	<i>Phaseolus vulgaris</i>		3	McCune <i>et al.</i> , 1964
Phosphoglucumutase	Soybean	1mM	1	Yang & Miller, 1963a
UDP glucose fructose transglucosylase	Soybean	10mM	2	Yang & Miller, 1963b
Hexokinase	Yeast	-	2	Melchoir & Melchoir, 1956
G-6-P dehydrogenase	Soybean	-	3	Lee <i>et al.</i> , 1965
	Wheat seedlings	NaF moniodo acetate + NH_4^+	3	Olyunina <i>et al.</i> , 1988
Pentose Phosphate Shunt (PPP)				
	Plant leaves	-	3	Ross <i>et al.</i> , 1962
	Wheat seedlings	NaF moniodo acetate + NH_4^+	3	Olyunina <i>et al.</i> , 1988
	Higher plants	-	3	McCune & Weinstein, 1971
Enolase	Pea seeds extract	5mM	1	Miller, 1958
PEP carboxylase	Soybean	-	3	Yang & Miller, 1963b
Respiration				
Cytochrome oxidase	<i>Pellargonium zonale</i>	10mM	3	Poovaliah & Wiebe, 1971
Peroxidase	Plants	fumigation	3	Cao, 1989
	Jute seeds	20 mM, NaF	3	Sarkar & Mukherjee, 1981
	Soybean	10mM	2,3	Lee <i>et al.</i> , 1966
	<i>Pellargonium zonale</i>	10mM	2,3	Poovaliah & Wiebe, 1971
	Soil	NaF	3	Kremlenkova & Gaponyuk, 1984
	<i>Sesamum</i> , <i>Phaseolus</i> , <i>Vigna</i>	10^{-7} MF	3	Bora <i>et al.</i> , 1979
Catalase	Soybean	10^{-3} mM	3	Lee <i>et al.</i> , 1965
	Jute seed	20 mM, NaF	3	Sarkar & Mukherjee, 1981
Polyphenol oxidase	Soybean	10	2,4	Lee <i>et al.</i> , 1965
	Soil	NaF	3	Kremlenkova & Gaponyuk, 1984
	<i>Sesamum</i> , <i>Vigna</i> , <i>Phaseolus</i>	10^{-3} mM	3	Bora <i>et al.</i> , 1979
Succinic dehydrogenase	<i>Pellargonium zonale</i>	10mM	2,4	Lovelace & Miller, 1967a,b
ATPase	Soybean cv Hawkeye	9-12 μgHF	1,3	Miller & Miller, 1974
	Corn shoots	30mM	1,3	Pushnik & Miller, 1985
	Rice cv Milyang	10 mgm ^3HF	4	Kim <i>et al.</i> , 1979
	cv. Jinheung		3	
	<i>Pinus strobus</i> seedlings	0.4 $\mu\text{g m}^{-3}\text{HF}$	3	Rakowski <i>et al.</i> , 1995
Rhizobial peribacteroid membrane	<i>Rhizobium</i>		inhibits formation	Domigan <i>et al.</i> , 1988
Malic dehydrogenase	<i>Pellargonium zonale</i>	10mM	4	Lovelace & Miller, 1967b
	Soybean, Winter rape & Perennial ryegrass	-	4	Moeri, 1979
Dehydrogenases	Soil		4	Wilke <i>et al.</i> , 1987
	Soybean	15 ppb, 96 h F fumigation	4	Poovaliah, 1971
Amylase, α amylase, β amylase, Invertase	<i>Lasiurus sindicus</i>	NaF	2	Rockwood, 1919
			3	Choudhury & Bohra, 1988
Carbohydrate catabolism	<i>Azospirillum A. brasiliense</i> Sp 7 <i>A. lipoferum</i> Sp-59b	Fluoropyruvate	4 Differential growth inhibition	Martinez-Drets <i>et al.</i> , 1984
Cellulose synthesis	<i>Avena</i> sp.		2	Ordin & Skoe, 1963
Photosynthesis				
Hill reaction	Bean		2	Ballantyne, 1972
Chlorophyll biosynthesis	<i>Nicotiana tabacum</i>		2	Newman, 1962
			2	Wallis <i>et al.</i> , 1974
Chloroplast ATPase	Sugarbeet	30mM	2	Giannini <i>et al.</i> , 1988
	Soybean	10mM	2	Giannini <i>et al.</i> , 1985
Uroporphyrinogen decarboxylase	Tobacco		2	Chen & Miller, 1974

contd...

Table 2.7 contd...

Enzyme or system	Organism	Fluoride	Effect	Reference
Membrane				
Ultrastructure	Soybean leaf cells		1	Wei, 1972
Plasmamembrane	Sugarbeet	5mM	1	Giannini <i>et al.</i> , 1987a
ATPase	Maize		4	Facanha <i>et al.</i> , 1995
Tonoplast ATPase	Sugarbeet	30mM	1	Giannini <i>et al.</i> , 1987b
N Metabolism				
Nitrate reductase	<i>Sorghum bicolor</i>		2	Kadam <i>et al.</i> , 1980
	<i>Escherichia coli</i>	NaF Anaerobic	4	Aida <i>et al.</i> , 1986
		Aerobic	5	
	<i>Oryza sativa</i>	0-100mM	4 No effect <i>in vitro</i>	Srinivasan & Rama Rao, 1980
	<i>Pso. tetragonolobus</i> cv. Nigerian Local		4	Munjal & Desai, 1991
Nitrogenase	Lucerne cv Vernal	0.01-100 $\mu\text{g l}^{-1}$ ml ⁻¹ pH 2-6	5	Porter & Sheridan, 1981
	<i>Nostoc calcicola</i>	10 mM NaF pH 7.3	4 pH dependent	Rai <i>et al.</i> , 1980
	Alfalfa	0-100 $\mu\text{g F ml}^{-1}$ pH 2-6	5	Porter & Sheridan, 1981
Urease			2	Dixon <i>et al.</i> , 1980
Protein synthesis	Maize grain	PMSF	2	Kahnnikov & Tolokonnikov, 1971
Cysteine protease	Potato cv Spunta		5	Isola & Franzoni, 1993
Serine Protease	Pea	Ethyl benzene sulfoxyl fluoride	4	Bushnell <i>et al.</i> , 1993
Alanine amino transferase (GOT)	<i>Lasiurus indicus</i>	NaF	3	Choudhury & Bohra, 1988
Glutamate dehydrogenase	<i>L. multiflorum</i> & Pea		3	Jensch & Jager, 1983
Lipid Synthesis				
Lipid Synthesis	Higher Plants		2,4	Harwood & Stumpf, 1971
	<i>Sphagnum fimbriatum</i>		2,4	Simola & KoskimiesSoininen, 1980
Esterase	Pea and Bean		2,3	Mendoza <i>et al.</i> , 1969, Yee-Meiler, 1975
Others				
Superoxide dismutase	Plants China	fumigation	3	Cao, 1989
	<i>Poa pratensis</i>		2	Lorenz-Kubis & Morawieck, 1978
	Soybean & <i>Pelargonium</i>	15pph 96h F ⁺ fumigation	3	Poovaliah, 1971
	Lichen- <i>Cladonia rangiferina</i>		4 $\pm 50\%$	Lane & Puckett, 1979
Acid phosphatase	<i>Gladiolus</i> corms		4	Suketa <i>et al.</i> , 1974
	Plants		4	Zwiazek & Shay, 1988a,b
Succinic TTC reductase	<i>Nostoc calcicola</i>	NaF pH 7.3 (0.2 ⁺ mM)	pH dependent inhibition	Rai <i>et al.</i> , 1980
Alkaline phosphatase	Soil		4	Wilke, 1987
Alkaline organic phosphatase	Plant	10 mM NaF	4	Viola & Davies, 1991
Phosphoenol pyruvate phosphatase	<i>Brassica nigra</i>		4	Duff <i>et al.</i> , 1989
Inorganic pyrophosphatase	Chloroplast		2	El-Badry & Bassham, 1970
Acid pyrophosphatase	<i>Phaseolus</i> & <i>Vigna</i>		3	Bora <i>et al.</i> , 1979
Phosphopyruvate dehydrogenase	Pea mitochondria		4	Miernyk & Randall, 1987
phosphatase				
Aryl sulfatase	Soil		4	Wilke, 1987
Phytase	Pea seeds		2	Hauskrecht, 1972
Ascorbic acid oxidase	Jute Seeds	20 mM NaF	3	Sarkar & Mukherji, 1981
RNase	(3d) Jute Seedlings	20 mM NaF	Slight diseased	Sarkar & Mukherji, 1981

1- very sensitive in vitro; 2 - inhibition in vitro; 3 - enhanced in vivo; 4 - inhibited in vivo; 5 - normal activity

physiological activity (Treshow and Anderson, 1989). But it is equally likely that a number of enzymes are affected directly, thus inhibiting several reactions.

Enolase, the key enzyme of the glycolytic pathway is particularly sensitive to fluoride and was inhibited *in vitro* at concentrations as low as 10^{-4} M (Miller, 1958). He further demonstrated that Mg^{2+} was required as an activator of enolase and observed that fluoride inhibition was due to an interaction between magnesium, fluoride and phosphate (probably a magnesium fluorophosphate complex was formed). The constant k ($C_{Mg} \cdot C_{PO} \cdot C_F^2 \cdot \text{Residual activity} / \text{Inhibited activity}$) was found to be 7×10^{-12} . Increasing any one of the variables and holding the others constant increased the inhibition. Over 30% inhibition was obtained when the phosphate and magnesium concentrations were 1 mM and fluoride concentration was 0.5 mM in the reaction mixture. Enolase from the alga *Synechococcus leopoliensis* was also shown to be inactivated by fluoride (Hekman *et al.*, 1984).

Inhibition of enolase, implying glycolysis is accompanied by the channelling of carbohydrates through the pentose phosphate pathway (Weinstein and Alscher-Herman, 1982). An increase in the relative proportion of the pentose phosphate pathway over glycolysis in leaves has been reported (Ross *et al.*, 1962; McCune *et al.*, 1964; Weinstein and Alscher-Herman, 1982). Subsequently a stimulation of glucose-6-phosphate dehydrogenase, the first enzyme of the pentose phosphate shunt was observed by Malhotra and Khan (1984) and Miller (1993). Viola and Davies (1991) reported a transient stimulation of triose phosphate cycling increasing the 3-phosphoglycerate content. This was followed by increase in triose phosphate, fructose 1,6 bisphosphate and hexose phosphate in potato tubers on exposure to 10 mM NaF.

The shift in the metabolism led to an inhibition of starch synthesis, increase in sucrose synthesis and accumulation of pyrophosphate. It has been hypothesized that pp_i accumulates in the amyloplast due to the inhibition of alkaline inorganic pyrophosphatase by fluoride ion. An increase in sucrose synthesis along with a decrease in phosphoglucomutase activity has been reported by Pushnik and Miller (1990). Detailed studies revealed a requirement of Mg^{2+} or Mn^{2+} as a metal cofactor for enzyme activation. The enhanced inhibition of the enzyme on exposure to 1 mM fluoride and increasing substrate concentrations independent of the metal cofactor concentration suggested a complex interaction between this enzyme and fluoride.

Many enzymes of the respiratory system such as succinic, malic and NADH dehydrogenases are sensitive to fluoride, succinic dehydrogenase being the most sensitive in the presence of phosphate (Lovelace and Miller, 1967a,b). Oxidative enzymes as catalase, peroxidase, ascorbic acid oxidase, polyphenol oxidase and cytochrome oxidase are stimulated (Lee *et al.*, 1965).

Since fluoride disrupts membrane integrity, the membrane bound enzymes are the worst affected. The ATPase residing in the membrane systems are variously involved in different functions *viz*, form part of the transport system at the plasma membrane, carry out oxidative phosphorylation when present in the mitochondrial membrane and photophosphorylation when attached with the chloroplast membrane. Any alterations in any of these membranes leads to massive disturbances in the basic cellular energetics. Photophosphorylation was inhibited by 30% at 10 mM fluoride (Giannini *et al.*, 1985). The ion interacted with CF part of the enzyme and inactivated it (Giannini *et al.*, 1988). Similarly the inactivation of F_1 ATPase with fluoride

severely inhibits mitochondrial phosphorylation (Pushnik and Miller, 1990). Some authors have reported an initial stimulation of ATPase which has been attributed to fluoride behaving as a classic uncoupler similar to dinitrophenol. Clowes and Juniper (1968), Wei (1972) and Pushnik and Miller (1985) showed that fluoride inhibits the tonoplast ATPase by destruction of the transmembrane pH gradient due to blockage of chloride antiport movement inwardly during proton extrusion. At higher concentrations ATPase was directly inhibited.

Kinetic evidences indicate that plasma membrane linked Ca^{2+} ATPases and H^{+} ATPases are inhibited by the binding of fluoride to the MgF_2 complex (Coll and Murphy, 1992; Murphy and Coll, 1992; Facanha and de Meis, 1995).

Fluoride inhibited *in vivo* nitrate reduction by blocking the supply of pyruvate required for the citric acid cycle (Munjal and Desai, 1991).

Metabolism

Pretreatment of tobacco leaves with 5 mM NaF stimulated uptake of phosphate. However, a simultaneous treatment of both fluoride and phosphate inhibited P uptake (Penot and Diouris, 1969). In the presence of Ca, F stimulated P uptake in potato (Diouris and Penot, 1977). Calcium treated tissue contained twice as much fluoride as compared to treatment with F alone. According to Garrec and Chopin (1982) Ca migrates to the sites of fluoride accumulation and protects plants against fluoride injury. In polluted fir needles Garrec *et al.* (1978) reported an increase in Ca concentrations mainly as oxalate when F concentrations exceed $500 \mu\text{g g}^{-1}$.

Fluoride also tends to reduce the uptake of S, Cl, NO_3 (Penot and Diouris, 1969) Ca, Sr (Semendyaena and Zheronkina, 1988), Mg and Mn (Garrec *et al.*, 1978).

Leakage of intracellular K takes place in the presence of fluoride. An increase in proline content in some plants under fluoride stress has also been reported (Cao, 1989; Choudhary and Bohra, 1988).

A decrease in the level of magnesium and manganese on exposure to HF (Garrec *et al.*, 1977) limits the processes of transcription and translation (Pilet, 1970; Chang, 1968, 1970). A 25-30% decrease in the ribosomal content and protein synthesis occurred in the NaF treated roots. Suketa *et al.* (1975) also reported a decrease in the protein content of the leaves in *Gladiolus* on exposure to HF for 8 h. An increase in the soluble protein content in 3 day old jute seedlings has been shown on exposure to 20 mM NaF by Sarkar and Mukherji (1981). Troiani *et al.* (1994) reported no effect on crude protein content of irrigated spinach with water containing 16-20 ppm fluoride. Fluoride modifies the ratios of free nucleotides and the nucleotide ratio of RNA (Chang, 1968) and decrease the total ribosomal RNA. Pilet (1970) suggested that F affects RNA synthesis also. Factors affecting RNA metabolism have an indirect influence on the rate of protein synthesis. A substantial increase in ribonuclease activity has also been reported by Pilet (1970). Because ribonuclease activity *in vitro* is thought to be controlled by Ca, Mg and K ratios (Hanson, 1960), it is possible that fluoride exerted its action through a disruption of the Ca:Mg.

Changes in keto acids, organic acids, amino acids, amides, free sugars, DNA, RNA, phosphorus, starch and non starch polysaccharides have also been found in plants exposed to fluorides (NAS, 1971). Response of sugar metabolism to the fluoride treatment varied amongst different plants. In general, fluoride caused a decrease in the total sugar content of treated tissues. Viola and Davies (1991) reported an inhibition

of starch synthesis in potato tuber discs but it increased sucrose synthesis. A decrease in fructose, glucose, sucrose and raffinose in the fir (*Abies alba*) needles in response to increase in fluoride has been reported by Garrec *et al.* (1981). Zwiazek and Shay (1988a,b) and Simola (1977) have also reported a decrease in starch synthesis on fluoride exposure. In contrast, low concentrations of fluoride (10^{-7} M) have been reported to increase the total sugar and reducing sugar level in sesamum (Bora *et al.*, 1979).

Reported changes in sugars, polysaccharides and organic acid concentrations are necessarily associated with the activity of various enzymes. Inhibition of phosphoglucomutase may account for the increase in concentrations of non reducing sugars. This in turn could account for the decrease in starch concentrations (Koziol, 1984).

FLUORIDE TOLERANCE

In almost every ecosystem, a few species or at least a few individuals show compatibility with environmental stresses. Tolerance is usually considered to be element specific and an inherited characteristic (Baker, 1987). Plant species and genotypes exhibit a broad range of tolerance to fluoride injury (Vike *et al.*, 1995).

In *Plantago lanceolata* development of tolerance appeared to be at the cost of productivity (Horner and Bell, 1995). Populations of *Deschampsia cespitosa* and *Agrostis tenuis* growing in the effluent from fluorspar mine showed an increase in dry weight when irrigated with NaF solutions (Cooke and Johnson, 1978).

According to Bradshaw (1976), the mechanism of tolerance or resistance involves

- (a) Exclusion of the pollutant by modification of the cuticle, stomata and uptake mechanisms,
- (b) Complexing of the pollutant internally in a way to render it innocuous,
- (c) degradation of the pollutant to non-toxic byproducts, and
- (d) development of resistant enzymes which can function in elevated internal levels of the pollutant.

Synthesis of low molecular weight substances has been observed in plants accumulating elements to high concentrations. Some 30 plant species classified as fluoride accumulators have been shown to accumulate fluoroacetate (reviewed by Harper and O'Hagan, 1994). Concentrations of upto 0.23 mg fluoroacetate g⁻¹ (fresh wt) have been reported from the species *Dichapetalum cymosum* growing in low fluoride soils. The West African species *D. toxicarium* could even utilize the compound as fluoroacetyl CoA in the elaboration of fluorofatty acids (Peters *et al.*, 1960; Peters, 1972). In the activated form (fluoroacetyl CoA) it is highly toxic to animals because it leads to inhibition of citric acid cycle. Fluoroacetyl CoA competes with acetyl CoA for the enzyme citrate synthase where it reacts with oxaloacetate and is involved in the synthesis of the toxic 2R, 3R - fluorocitrate, instead of citrate. Fluorocitrate is also highly toxic to animals but plants can tolerate high concentrations without adverse effects probably as a result of an enzymatic adaptation. Meyer *et al.* (1992) showed that an enzyme fluoroacetyl CoA hydrolase in *D. cymosum* can exclusively hydrolyse fluoroacetyl CoA to fluoroacetate without hydrolysing to acetyl CoA. Fluorocitrate is therefore not formed.

Chara fragilis thalli as shown by X-ray crystallography can complex fluoride

into crystals of CaF_2 that increase in amounts with increasing F concentrations at the expense of CaCO_3 (Levey and Strauss, 1973).

Resistance to fluoride toxicity in microalgae has been correlated to the lack of fluoride uptake as in case of *Chlorella* and other chlorophytes studied by Hekman *et al.* (1984). According to Nichol *et al.* (1987) this may be due to formation in the cell membrane of a pathway for passive F^- anion movement. Such a passive permeation is unlikely for fluoride sensitive cells as supported by the data on the kinetics of ^{18}F exit from sensitive and resistant cells of *S. leopoliensis* (Nichol *et al.*, 1987). Fluoride resistance shown by the diatom *C. meneghiniana* which did accumulate fluoride (Hekman *et al.*, 1984) remains unexplained.

Klut *et al.* (1981) demonstrated slow but successful adaptation to inhibitory fluoride concentrations ($200 \mu\text{g ml}^{-1}$) in the dinoflagellate *Amphidinium carterii*. The adaptation was associated with major structural alterations. Amongst the three essential organelles namely chloroplast, mitochondria and nucleus, chloroplast appeared to be the most affected giving perforated appearance of the thylakoids. Appearance of microbodies in the cell cytoplasm that are otherwise absent or extremely rare might signify a key biochemical lesion caused by intracellular F accumulation.

MATERIAL AND METHODS

Survey of Fluoride in Water

Isolation of Algae and Cyanobacteria

Experimental Organisms

Maintenance and Growth

The Experiments

Fluoride Tolerance in Microalgae

pH and Fluoride Toxicity

Fluoride Uptake and Biosorption

Physiological Response of *Anabaena khannae*

The Analytical Techniques

Estimation of Pigments

Chlorophyll

Phycobilin

Carotenoids

Estimation of Proteins

Estimation of Carbohydrates

Estimation of $^{14}\text{CO}_2$ Uptake

Estimation of Dissolved Oxygen

Electrical Conductivity Measurement

Solute Leakage Determination

Estimation of Fluoride

Extracellular Fluoride

Fluoride in Algal Biomass

Acetylene Reduction Assay

SURVEY OF FLUORIDE IN WATER

Hydrogeology of an area, availability of fluorominerals and their solubility affect fluoride content in water. Solubility in turn is dependent on the pH of water, temperature and rainfall pattern of the area. Ajmer, a district in south-east Rajasthan is reported to be severely contaminated with fluoride (Gupta *et al.*, 1993). Thus fluoride content of some water bodies in and around Ajmer was explored.

ISOLATION OF ALGAE AND CYANOBACTERIA

Three sites viz. Arjunpura, Kesarpura and MDS University, were identified that contained comparatively higher concentration of fluoride. To know the effect of seasonal changes on F content of water, samples were collected at three month intervals from May '94 to May '95. The wells at the selected sites had adjoining concrete tanks, in which water pulled from the well was stored for animals. Samples of algae growing at the bottom and on the walls and the surface of water were collected from these tanks. After homogenisation 5 ml each was suspended in BG 11 medium (Stanier *et al.*, 1971) with or without nitrogen. The composition of BG 11 medium is given in Table 3.1.

Another set of BG 11 (+N or -N) supplemented with 20 $\mu\text{g ml}^{-1}$ fluoride as sodium fluoride was also inoculated. The algal flora of the fresh sample as well as that of the enriched culture (15 days old) was identified with the help of taxonomic keys (Smith, 1950; Desikachary, 1959).

EXPERIMENTAL ORGANISMS

From fluoride amended cultures eight microalgae were isolated by spread plating 0.1 ml of homogenised suspension on agar medium. Twelve algal and

Table 3.1 **Composition of BG 11 medium (Stanier *et al.*, 1971).**

Macroelements	g l ⁻¹	Trace element mix	g l ⁻¹
NaNO ₃	1.5	H ₃ BO ₃	2.86
K ₂ HPO ₄	0.04	MnCl ₂ ·4H ₂ O	1.81
MgSO ₄ ·7H ₂ O	0.075	ZnSO ₄ ·7H ₂ O	0.222
CaCl ₂ ·2H ₂ O	0.036	Na ₂ MoO ₄ ·2H ₂ O	0.39
Citric Acid	0.006	CuSO ₄ ·5H ₂ O	0.079
Ferric Ammonium Citrate	0.006	Ca(NO ₃) ₂ ·6H ₂ O	0.0494
EDTA	0.001		
Na ₂ CO ₃	0.02		
Trace elements mixture	1ml l ⁻¹		

cyanobacterial forms were examined for fluoride compatibility. Out of these, *Chlorella minutissima* ARC 2, a unicellular green alga isolated from domestic sewage, *Anabaena fertilissima* ARM 742, an alkalophilic diazotrophic cyanobacterium isolated from the soils of Hardoi district (U.P.), *Westiellopsis prolifica* ARM 365, a halotolerant diazotroph and *Anabaena iyengerii* ARM 375, an isolate from neutral rice field soils were obtained from the National Centre for Conservation and Utilization of Blue Green Algae, IARI, New Delhi. Rest of the microalgae were those isolated from fluoride rich samples collected from the study area.

MAINTENANCE AND GROWTH

'Borosil' make glassware after rinsing with chromic acid solution and repeated washing with tap water and finally with distilled water was heat sterilized in a hot air oven at 160°C for 2 h. Culture medium contained in suitable culture vessels, was sterilized in an autoclave at 15 lb inch⁻² pressure (121.6°C) for 15-20 minutes. Since fluoride is known to react with glass, sodium fluoride solutions were sterilized in autoclavable polycarbonate or polypropylene bottles. The experiments involving the use of fluoride, were conducted in autoclavable alkathene bags plugged with cotton plugs. These bags were secured in position on the culture racks by suspending them with clips from a cross wire. Cultures were shaken manually once a day.

The diazotrophic forms were maintained and grown in BG-11 medium without sodium nitrate (BG11-N) and non nitrogen fixing cyanobacteria and algae were grown in complete BG-11 medium. Subculturing was done at 15 d intervals. All subculturing and inoculation operations were carried out in laminar flow hood or inoculation room.

All cultures for maintenance (Mother cultures and stock cultures) and

experimentation were maintained and incubated at $28 \pm 1^\circ\text{C}$ temperature at 2000-2500 lux light intensity provided in 12/12 light/dark cycles in a fully automated growth room. Experiments were conducted in triplicates unless stated otherwise.

THE EXPERIMENTS

FLUORIDE TOLERANCE IN MICROALGAE

Growth (as chlorophyll content) response of seven cyanobacteria and five forms of green algae to graded concentrations of fluoride (0, 25, 50, 75, 100, 125, 150, 175 and $200\ \mu\text{g ml}^{-1}$) was measured after 15 d. This end point study formed the basis for the selection of *Anabaena khannae*, a diazotrophic cyanobacterium and the green alga *Chlorococcum humicola* for further investigations. In addition to stability at higher fluoride concentration, *C. humicola* possessed a comparatively larger size and had wide spread occurrence in fluoride infested area.

pH AND FLUORIDE TOXICITY

The combined effect of pH of the medium (pH 6, 7 and 8) and graded concentration of fluoride (0, 5, 25, 50, $100\ \mu\text{g ml}^{-1}$) on the growth of *A. khannae* and *C. humicola* was determined in terms of changes in pigment content and biomass production at the end of 15 d incubation period. Physiological effects on the processes of nitrogen fixation (ARA activity), photosynthesis and respiration (measured as ^{14}C uptake and oxygen evolution or consumption) were observed in short term experiments on 15 d old, homogenised cultures of *Anabaena* and *Chlorococcum*. The pH of the medium was adjusted using 0.25M MOPS for pH 6.0 and 0.25M HEPES for pH 7.0 and 8.0.

In the cultures of *Anabaena khannae* incubated at low pH and high fluoride

concentration, extensive leaching of phycobilins indicated disruption of cellular membranes. Hence the extent of membrane damage was measured as changes in the electrical conductivity of the medium over a period of 5 h. Leaching of solutes and phycobilins were quantified at the end of the incubation period.

FLUORIDE UPTAKE AND BIOSORPTION

Acid labile fluoride in fresh biomass and the available extracellular fluoride was measured in batch cultures of *A. khannae* and *C. humicola* after 15 days of incubation. For studies on biosorption, the two algae were first mass cultured under aseptic conditions. Biomass was collected from 20 d old cultures and dried in a hot air oven at 60°C. Separate 100 ml conical flasks, containing 10 ml distilled water supplemented with 0, 25, 50, 100, 200 and 500 $\mu\text{g ml}^{-1}$ sodium fluoride were added with 50 mg each of the dried algal biomass. Residual fluoride was estimated after 2 h of incubation on a rotary shaker.

PHYSIOLOGICAL RESPONSE OF *ANABAENA KHANNAE*

A time course study with *A. khannae* was conducted to determine various growth parameters as a function of fluoride concentration ranging from 0-100 $\mu\text{g ml}^{-1}$. Chlorophyll, phycobilin, carotene, dry weight, cellular protein and carbohydrates were quantified at 5 d intervals over a period of 25 days.

THE ANALYTICAL TECHNIQUES

ESTIMATION OF PIGMENTS

Chlorophyll

Total chlorophyll was estimated following the method of Mac Kinney (1941). Known amount of homogenised algal suspension (10 ml) was centrifuged at 5000 rpm

for 10 min. The supernatant was drained and the pellet suspended in 10 ml of 95% methanol. The tubes were covered with aluminium foil and kept on a water bath at 60°C for 30 min. to ensure complete extraction. The extract was cooled, volume made up to 10 ml with methanol and centrifuged. Absorbance of the supernatant was measured at 650 and 665 nm in a Beckman DU-64 spectrophotometer using 95% methanol as blank. Chlorophyll content was calculated using the following formula

$$\text{Total chlorophyll (mg ml}^{-1}\text{)} = \{2.55 \times 10^{-2} \times \text{O.D}_{650} + 0.4 \times 10^{-2} \times \text{O.D}_{665}\} \times \text{Dilution factor}$$

Phycobilin

A known volume of algal suspension (10 ml) was centrifuged at 5000 rpm for 10 min. The pellet was resuspended in 10 ml of 0.05 M phosphate buffer (pH 7.0) and subjected to repeated freezing and thawing for the release of phycobilins. The suspension was then centrifuged and c-phycocyanin (PC) c-phycoerythrin (PE) and Allophycocyanin (APC) were quantified in mg ml⁻¹ from the absorbance of the supernatant read at 562, 615 and 652 nm using the equations derived from the extinction coefficients of purified phycobiliproteins (Bennet and Bogorad, 1971).

$$\text{PC} = (\text{A}_{615} - 0.474 \times \text{A}_{652}) / 5.34$$

$$\text{APC} = (\text{A}_{652} - 0.208 \times \text{A}_{615}) / 5.09$$

$$\text{PE} = (\text{A}_{562} - 2.41 \times \text{PC} - 0.94 \times \text{APC}) / 9.62$$

Carotenoid

Carotenoids were extracted in 90% acetone (Jensen, 1978). An aliquot of 10 ml of homogenised algal suspension was centrifuged at 5000 rpm for 10 min and the pellet washed twice with distilled water. The pellet after adding 2-3 ml of 90% acetone was subjected to repeated freezing and thawing. Repeated extractions were done till

colourless acetone was obtained. Portions of supernatant containing pigment were pooled and final volume recorded. Absorbance of the extract was recorded at 450 nm using 90% acetone as blank. Concentration of carotenoids was calculated using the following formula

$$C=(D*V*F*10)/2500$$

where, D is the absorbance at 450 nm; V is the volume of the extract and f, the dilution factor (assuming that average extinction coefficient of pigment is 2500). The carotenoid concentration was expressed as mg ml⁻¹.

ESTIMATION OF PROTEINS

Total protein content was estimated following the method of Lowry *et al.* (1951).

Reagents

- a. Alkaline sodium carbonate solution : 10.0 g of sodium carbonate was dissolved in a little quantity of distilled water. After adding 2.0 g of sodium hydroxide to it, the volume was made up to 500 ml.
- b. Copper sulphate solution : 5.0 g of sodium potassium tartarate was dissolved in 100 ml distilled water.
- c. Sodium potassium tartarate solution : Ten gram sodium potassium tartarate was dissolved in 100 ml distilled water.
- d. Copper sulphate - sodium potassium tartarate solution :
One part each of reagent 'f' and reagent 'c' were mixed and diluted with eight parts of distilled water. The solution was freshly prepared and used.
- e. Alkaline reagent : Prepared by mixing 50 ml reagent 'a' and 1 ml of reagent

'd'. This was also prepared afresh before use.

f. Folin - Ciocalteu reagent : The reagent was a mixture of sodium tungstate ($\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$) and sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) in phosphoric and sulphuric acids. The reagent was diluted with distilled water to 1N strength and confirmed by titration against 1N sodium hydroxide to a phenolphthelin end point

g. Trichloroacetic acid (TCA) : 6% w/v

h. Standard protein solution : Bovine serum albumin (BSA) (1 mg ml^{-1}) stored in a freezer.

Ten ml of homogenized algal suspension was centrifuged and pellet washed with distilled water. Proteins were precipitated by suspending the pellet in 10 ml of 6% TCA for 1 min. TCA was removed by filtering the suspension through a sintered filter by suction and the filtrate discarded. Protein thus precipitated was dissolved in 4.5 ml of hot (55°C) reagent 'c' and allowed to stand for 3 min. After the reaction, the filtrate was collected in a test tube by suction and the volume made up to 5 ml by adding reagent 'e'. Then 0.5 ml of reagent 'f' was added, mixed rapidly and allowed to stand for 30 min at room temperature. Absorbance was read at 750 nm using a mixture of reagent 'e' and 'f' as blank. Total protein content was estimated from a standard curve prepared by using graded concentration (10-100 μg) of standard bovine serum albumin solution.

ESTIMATION OF CARBOHYDRATES

Total cellular carbohydrates were estimated following the method of Dubois *et al.* (1956).

A known volume of homogenized algal suspension was centrifuged at 5000 rpm

for 10 min. The resulting pellet was washed twice with distilled water. The pellet was suspended in 1 ml of distilled water and 1 ml of 5 % phenol was added to it. After thorough mixing, 5 ml of concentrated sulphuric acid was rapidly added. It was then allowed to stand at room temperature for 30 min. The absorbance was read at 490 nm in a Beckman DU-64 Spectrophotometer against distilled water blank. The amount of carbohydrates was calculated using a standard curve.

ESTIMATION OF $^{14}\text{CO}_2$ UPTAKE

The uptake of $\text{NaH}^{14}\text{CO}_3$ was estimated following the methods suggested by Kumar *et al.* (1982) and Larlandner (1989). Ten ml of homogenised culture suspension, pre-incubated in dark for 2 h, was supplemented with 100 μl of $\text{NaH}^{14}\text{CO}_3$ (specific radioactivity 0.003 $\mu\text{Ci}\mu\text{mol}^{-1}$). The algal samples were allowed to photosynthesise for 2 h at 2500 lux light intensity. Another set was kept in dark under same conditions. At the end of incubation period, algal activity was stopped by adding 100 μl of 37% formaldehyde solution. After centrifugation, the unused radio labelled bicarbonate was driven off from the pellet by adding 1 ml of concentrated acetic acid and drying it in an oven at 60°C. The pellet was then suspended in 10 ml of scintillation cocktail in a scintillation vial. Samples were counted in a Beckman LS-6000 SC Liquid Scintillation Counter. The rate of $^{14}\text{CO}_2$ uptake was expressed in DPM (disintegrations per minute) or μmol of $\text{Na H}^{14}\text{CO}_3$ fixed mg chl^{-1} .

ESTIMATION OF DISSOLVED OXYGEN

Oxygen evolution or consumption during photosynthesis or respiration was measured after the method proposed by Thomas (1988). The method involves use of a clear BOD bottle for photosynthesis and a dark bottle of known initial oxygen content

incubated for a suitable time in a natural or simulated environment.

A homogenised algal suspension was sparged with Argon for 30 minutes to reduce the initial oxygen level, filled in BOD bottles upto the brim and stoppered. Care was taken to avoid any bubbles in the bottle. Dark bottles were completely covered with foil. All the bottles were placed under water contained in a transparent plexiglass vessel. Light bottles were provided with 2500 lux light intensity. Initial bottle oxygen was recorded and final oxygen content was read after 2 h of incubation with DO analyser WTW Oxi-91. The contents of the bottles were filtered through predried and preweighed Whatman 41 filter papers to determine dry weight.

Results were expressed as Gross Photosynthesis (PG), Net Photosynthesis (PN) and Respiration (R) in $\text{mgC m}^{-3} \text{h}^{-1}$ based on the following equations

$$\text{PG} = 375.9(\text{LB} - \text{DB}) / (\text{N} \times \text{PQ})$$

$$\text{PN} = 375.9 (\text{LB} - \text{IB}) / (\text{N} \times \text{PQ})$$

$$\text{R} = 375.9 (\text{IB} - \text{DB}) \times \text{RQ} / \text{N}$$

Where IB, LB and DB are oxygen levels of initial, light, and dark bottles. N is the incubation time in h, PQ the Photosynthetic Quotient (1.2) and RQ the Respiratory Quotient (1.0). Factor 375.9 converts the results to a standard volume (1m^3). The results were further calculated on per mg chlorophyll and per mg dry weight basis.

ELECTRICAL CONDUCTIVITY MEASUREMENT

The electrical conductivity as a measure of membrane damage was determined using Systronics Conductivity Bridge 305. Potassium chloride solution 0.01N was used as a standard reference solution that has an electrical conductivity of $1411.8 \times 10^{-6} \text{ mhos cm}^{-1}$ at 25°C . The electrical conductivity of the solution at the temperature of

measurement (t) was calculated from the relation $EC_t = k/R_t$

where $k = EC_{25} \times R_{25}$. A temperature conversion factor (f_t) obtained from standard conversion table was used to convert conductivity values to 25°C. Thus

$$EC_{25} = EC_t \times f_t = kf_t / R_t$$

SOLUTE LEAKAGE DETERMINATION

Algal suspension was centrifuged at 10,000 rpm. The absorption spectrum of the supernatant was plotted in the UV range using Beckman DU 64 spectrophotometer. Absorbance for all treatments was read at a wavelength where the control showed maximum absorption (APHA, 1995). The results were expressed as the mean UV absorbance (cm^{-1}).

ESTIMATION OF FLUORIDE

Fluoride was estimated by the SPADNS spectrophotometric method (Bellack and Schoube, 1968), which is based on the reaction between fluoride and a zirconium dye lake.

Reagents

- a. Stock fluoride solution : Dissolved 22.01 mg anhydrous sodium fluoride, NaF in distilled water and diluted to 1000 ml; 1.00 ml = 100 μg F
- b. Standard fluoride solution : 100 ml of the stock fluoride solution was diluted to 1000 ml with distilled water; 1 ml = 10.0 μg F
- c. SPADNS solution : 958 mg SPADNS (Sodium 2- (parasulfophenylazo)-1,8-dihydroxy-3-(parasulfophenylazo)-2,7-naphthalene disulfonic acid trisodium salt) was dissolved in distilled water and diluted to 500 ml.
- d. Zirconyl acid reagent : Dissolved 133 mg zirconyl chloride octahydrate

($\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$) in about 25 ml distilled water and after adding 350 ml conc. HCl the volume was made up to 500 ml with distilled water.

e. Acid zirconyl - SPADNS reagent : Prepared by mixing equal volumes of SPADNS solution and zirconyl acid reagent.

f. Reference solution : 10 ml SPADNS solution was added to 100 ml distilled water. In a test tube 7 ml conc HCl was diluted and then added to the diluted SPADNS solution.

Extracellular Fluoride

A homogenised algal suspension was filtered. 50 ml filtrate or a portion diluted to 50 ml with distilled water was taken. 5 ml each of SPADNS solution and zirconyl acid reagent or 10 ml acid zirconyl - SPADNS reagent was added. After proper mixing absorbance was read at 570 nm setting the reference point (zero) of the spectrophotometer with solution 'f'.

The fluoride content was quantitated from a standard curve prepared in the range of 0-1.4 mgF l^{-1} by diluting appropriate quantities of the standard fluoride solution to 50 ml with distilled water.

Fluoride in Algal Biomass

The diffusion technique proposed by Hall (1960) was employed to extract fluoride from the algal biomass without prior ashing. Fresh algal biomass was harvested by centrifugation and washed once with distilled water. The pellet was taken in a 50 ml polypropylene tube with screw cap. A strip of whatman No. 51 filter paper impregnated with 50 μl of 3N potassium hydroxide (containing 1ml of 0.05% w/v ethanolic solution of phenolphthalein per 100ml KOH) was suspended in the tubes with

a thread. Carefully 2ml of 70% w/v cold perchloric acid was placed in the tube ensuring that none touched the inside of the neck and the tubes were immediately capped. After sealing the tubes with silicon grease they were placed in an oven at 60°C for 24 h. The filter papers were removed, the pale yellow area with red edges was cut off and extracted with 3 ml of water in a boiling water bath for 2 min. When cool, the samples were diluted to 50ml with double distilled water in a polypropylene volumetric flask and subjected to SPADNS spectrophotometric analysis.

Diffusion blanks containing 1 ml of water instead of test solution and diffusion standards in the range of 0-1.4 $\mu\text{g ml}^{-1}$, prepared by diluting appropriate quantity of stock NaF solution to 1ml were subjected to the same diffusion and determination technique as the sample.

ACETYLENE REDUCTION ASSAY

The nitrogenase activity was measured after Hardy *et al.* (1973). Five ml homogenised algal suspension was incubated in 15 ml vials and stoppered with subba seals. A 10% equivalent of the gas phase in the vials was replaced by an equivalent volume of acetylene using disposable syringes. The vials were incubated at 2500 lux illumination for 90 min at $28 \pm 1^\circ\text{C}$. The reaction was terminated by injecting 0.1 ml TCA (50%) and the gas phase assayed for ethylene in AIMIL-Nucon Gas Chromatograph 5700 with 4' long Porapak T column of 1/4" outer diameter and 3 mm internal diameter. Mesh range was 80-100. The column temperature was maintained at 80°C and the detector and injector were maintained at 110°C. Nitrogen was used as a carrier gas. Hydrogen and air were used to produce a flame to ionise the sample in the mobile nitrogen phase. The gas flow used was 35 ml min^{-1} . Activity as nmol C_2H_4

formed $\mu\text{g}^{-1} \text{ h}^{-1}$ and calculated as nmol C_2H_4 formed $\mu\text{g chlorophyll}^{-1} \text{ h}^{-1}$ using the formula

$$(E * P_{\text{sm}} * A_{\text{sm}} * V_{\text{a}} * 60) / (P_{\text{st}} * A_{\text{st}} * V_{\text{g}} * T * V_{\text{s}})$$

where E is nmol ethylene in the standard peak, P_{st} and P_{sm} are peak heights (cm) of standard and sample respectively, A_{st} and A_{sm} are attenuations for standard and sample, V_{a} the air space in the vial, V_{g} volume of gas sample injected, V_{s} sample volume and T is the incubation period (min).

Survey of Fluoride in Water

Seasonal Variation

Fluoride Concentration

Algal Distribution

Arjunpura

Kesarpura

University : Site 1 (Nursery)

University : Site 2

Fluoride Tolerance in Algae and Cyanobacteria

Response of Algae and Cyanobacteria to pH and Fluoride

Effect on Growth and Pigments

Anabaena khannae

Chlorophyll

Phycobilin

Carotenes

Pigment Ratios

Biomass

Chlorococcum humicola

Chlorophyll

Carotenes

Biomass

Membrane Damage

Electrical conductivity

Phycobilin Leaching

Absorbance in UV Region

Algal Activity Mediated pH Changes

Physiological Responses

Photosynthesis and Respiration

¹⁴C Uptake

Net Gas Exchange

Nitrogenase Activity

Influence of Fluoride and Incubation Period on

Growth and Cellular Constituents of *Anabaena khannae*

Biomass

Chlorophyll

Phycobilins

Carotenes

Proteins

Carbohydrates

pH and Fluoride Accumulation

Biosorption

SURVEY OF FLUORIDE IN WATER

A preliminary survey of various water bodies in and around Ajmer was made to identify the sites from which fluoride tolerant forms of algae and cyanobacteria could be obtained. The survey showed that 46% of the sources contained more than $1 \mu\text{gF ml}^{-1}$ (Table 4.1).

SEASONAL VARIATION

FLUORIDE CONCENTRATION

Kesarpura, Arjunpura and M.D.S. University campus were selected as the sites for seasonal studies on fluoride and algal distribution. The observations showed that samples had minimum fluoride concentration during winter (November) and maximum in summer (May) (Table 4.2).

ALGAL DISTRIBUTION

Arjunpura

Observations on algae appearing in enrichment cultures and natural populations showed the dominance of three filamentous cyanobacteria namely, *Lyngbya taylorii*, *Oscillatoria formosa* and *Plectonema notatum*, followed by *Navicula*, *Gloeocapsa aeruginosa* and *Chlorococcum humicola* at the beginning of the summer season during the month of May (Table 4.3). Towards the end of rainy season during August, *Oscillatoria obscura*, *O. subbrevis* and *Phormidium angustissimum* replaced the above algae. These were later followed by *Rhizoclonium*, *Stauroneis* and *Cymbella alpina*. The only organisms showing presence in both the seasons were *Chlorococcum humicola*, *Chlorella vulgaris* and *Navicula albeita* in lesser numbers than that in May.

Phormidium angustissimum continued its marked dominance into November, the beginning of winter. This, along with *Ulothrix* replaced all other algae. The

Table 4.1 Fluoride concentration of samples from different locations in Ajmer district.

Sampling Time	Site	Water Source	Fluoride ($\mu\text{g ml}^{-1}$)
Aug-Sept	Arjunpura	WW	3.30
	Budha Pushkar	Pond	0.12
	Hokra	HP	0.35
	Kesarpura	WW 1	3.50
		WW2	2.90
	Masuda	WW	2.00
		Pond	0.55
	MDSUniv., Ajmer	TW1	2.00
		TW2	2.50
	Revenue Board, Ajmer	HP	0.30
Oct-Nov	Shastri Nagar, Ajmer	HP	0.14
	Anasagar	Lake	1.15
	Arain	WW	0.30
	Chausala	HP	1.33
	Daanta	HP	0.26
	Jethana	WW	0.79
	Katsura	WW1	1.11
		WW2	0.30
	Kishangarh	WW	1.06
	MDS University	TW3	1.12
	Peer	HP	0.48
	Peesangan	WW1	1.48
		WW2	0.47
		WW3	0.52
		TW	0.46
	Salora	TW	1.80

WW: Well Water; HP: Hand Pump; TW: Tube Well

Table 4.2 Seasonal variation in fluoride content ($\mu\text{g ml}^{-1}$) of the samples at selected stations.

S.No.	Station	Aug	May	Aug	Nov	Feb
1.	Arjunpura	3.3	3.8	2.0	2.7	3.2
2.	Kesarpura	3.5	3.7	2.1	2.2	2.7
3.	M.D.S. University					
	Site 1	2.0	3.0	1.8	2.1	2.8
	Site 2	1.8	2.6	1.5	1.0	1.5

Table 4.3. Seasonal distribution of different algae in the samples collected from fluoride infested locations

S.No.	SITE	ARJUNPURA				KESARPURA				UNIV. SITE 1				UNIV. SITE 2			
	ORGANISM	M	A	N	F	M	A	N	P	M	A	N	F	M	A	N	F
CYANOPHYCEAE																	
1	<i>Anabaena</i> sp.	-E ^s	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	<i>A.khannae</i>	-	-	-	-	-	R	-	R	-	-	-	-	-	-	-	-
3	<i>Chroococcus dispersus</i>	-	-	-	-	-	D	C	R	-	-	-	-	-	R	-E	-
4	<i>C. giganteum</i>	-	-	-	-	-	-	-E	-	-	-	-	-	-	-	-	-
5	<i>C. minimus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-
6	<i>C. minor</i>	-	-	-	-	-	-	-	-	VC	-	-E	VC	C	D	-E	-
7	<i>Chroococcum gomontii</i>	-	-E ^s	-	-	-	-	-	-	-	-	-	-	-	C	-	-
10	<i>C. turgidus</i>	-	-E	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	<i>C. versicolor</i>	-	-E	-	-E	-	-	-	-	-	-	-	-	-	-	-	-
12	<i>Gloeocapsa</i> sp.	-	-	-	-	-	-	-	-E	-	-	-E	-E	-	-	-E	C
13	<i>G. aeruginosa</i>	VC	-	-E	R	-	VC	R	R	-	-	-	-	-	VC	-	-
14	<i>Hapalosiphon</i>	-	-E	-	-E	-	-	-	-	-	-	-	-	-	-	-	-
15	<i>Lyngbya borgerii</i>	-	-	-	-	D	R	R	R	-	-	-	-	-	-	-	-
16	<i>L. cryptovaginata</i>	-	-	-E	-	-	-	-	-	-	-	-	-	-	-	-	-
17	<i>L. taylorii</i>	D	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
18	<i>Merismopedia glauca</i>	-	-	-	-	-	-	-	-	-E	-	-	-E	-	C	-	-VC
19	<i>Myxosarcina burmensis</i>	R	-	-	R	C	D	R	R	R	-	-	-	VC	-	-	C
		E ^s			E ^s												
20	<i>Nostochopsis</i>	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	<i>Nostoc punctiforme</i>	CE ^s	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-
22	<i>N. sphaericum</i>	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	<i>Oscillatoria agardhii</i>	-	-E	-	-	-	-	-	-	-E	-	-	R	-	-	-	-
24	<i>O. formosa</i>	D	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-
25	<i>O. granulata</i>	-	-E	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	<i>O. limosa</i>	-	-	-	-	R	-	-	R	-	-	-	-	-	-	-	-
27	<i>O. obscura</i>	-	D	R	R	-	-E	-	-	-	-	-	-	C	-	-	R
28	<i>O. subbrevis</i>	-	D	R	R	-	-	-	-	-	-	-	-	-	-	-	-
29	<i>O. terebriformis</i>	-	-	-	-	-	-	-E	C	-	-	-	-	-	-	-	-
30	<i>Phormidium angustissimum</i>	-	D	D	C	-	-	-	-	-	R	VC	R	-	-	-	-
31	<i>P. inundatum</i>	-	-	-	-	C	D	R	R	-	-	-	-	-	-	-	-
32	<i>P. tenue</i>	-	-	-	-	-	-	-	-	-	-	-E	VC	C	-E	-	-D
33	<i>Plectonema nostocarium</i>	-	-	-	-	VC	-	-	VC	-	-	-	-	C	C	-E	-E
34	<i>P. notatum</i>	D	-	-E	C	-	-	-	-	VC	-	-	D	VC	-	-	R
35	<i>P. purpureum</i>	-	-	-	-	D	-	C	VC	-	-	-	-	-	-	-	-
36	<i>Spirulina</i>	-	-	-	-	-	-	C	R	-	-	-	-	-	VC	C	-
37	<i>Synechocystis</i>	-	-	-	-	-	-	-	-	VC	-	-	C	VC	-	-	-
38	<i>Synechococcus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-E ^s	-	-	RE ^s

Contd.

Table 4.3 Contd.

S.No.	SITE	ARJUNPURA				KESARPURA				UNIV. SITE 1				UNIV. SITE 2			
ORGANISM		M	A	N	F	M	A	N	F	M	A	N	F	M	A	N	F
CHLOROPHYCEAE																	
39	<i>Ankistrodesmus falcatus</i>	-	-	-	-	-	-	-	-	-	-	-E	C	-	-	-	-
40	<i>Chlamydomonas</i>	C	-	-E	-E	-	-	-E	-	R	-	-E	C	-	-	-	-
41	<i>Chlorella (small)</i>	C	-	-	-	-	-	-E	-	VC	-	C	VC	-	R	-	-
42	<i>C. vulgaris</i>	C	R	-E	-E	-	-	-	-	-	-	-	-	-	-	-	-
43	<i>Chlorococcum humicola</i>	VC	C	R	R	-	-	-E	-	-	RE	R	C	D	R	-	R
44	<i>Coelastrum</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-E	R
45	<i>Eudorina</i>	-	-	-	-	-	-	-E ^s	-	RE ^s	-	-	CE ^s	-	-	-	-
46	<i>Gonium</i>	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-
47	<i>Kirchineriella obesa</i>	CE ^s	-	-RE ^s	-	-	-	-	-	-	-	-	-	-	-	-	-
48	<i>Pandorina</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-
49	<i>Protococcus</i>	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	C
50	<i>Rhizoclonium</i>	-	VC	-	-	-	-	-	-	-	-	-	-	-	C	-	-
51	<i>Scenedesmus bijuga</i>	-	-	-	-	-	-	-	-	-	D	VC	D	C	C	-	-
52	<i>S. quadricauda</i>	-	-	-	-	-	-	-E	R	R	-	D	VC	C	R	-	R
53	<i>S. quadricauda longispina</i>	-	-	-	-	-	-	-	-	-	VC	R	R	-	-	-	-
54	<i>Spirogyra</i>	-	-	C	R	-	-	-	-	-	-	-	R	-	-	-	-
55	<i>Ulothrix</i>	-	-	D	VC	-	-	-	-	-	-	-	-	-	-	-	-
BACILLARIOPHYCEAE																	
56	<i>Achnanthes</i>	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57	<i>Amphora</i>	-	-	-	-	-	-	-	-	-	-	-	-	VC	C	-	R
58	<i>Caloneis</i>	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-
59	<i>Cymbella alpina</i>	-	VC	-	-	-	-	C	-	-	-	-	-	R	-	-	-
60	<i>Navicula</i>	VC	C	-	R	-	VC	VC	C	D	-	R	C	D	R	-	R
61	<i>Nitzscherella</i>	-	-	-	-	-	-	C	R	-	-	-	-	-	-	-	-
62	<i>Nitzschia</i>	-	-	VC	C	-	-	-	R	-E	-	-	-	VC	-	-	R
63	<i>Pinnularia</i>	-	C	R	R	-	-	R	C	-	-	-	-	-	-	-	-
64	<i>Stauroneis</i>	-	VC	-	-	-	-	VC	VC	-	-	-	-	R	-	-	-
65	<i>Synedra ulna</i>	-	R	C	R	-	C	D	D	-	-	-	-	-	-	-	-
DESMIDACEAE																	
66	<i>Cosmarium ocellatum</i>	C	-	-	R	-	-	-	-	-	-	-	R	-	-	-	-
67	<i>C. taxichondriforme</i>	-	-	-	-	-	-	VC	R	C	-	-	C	R	D	D	VC

-, not present; -E, not present in the fresh sample but observed on enrichment; E^s, observed on enrichment but was susceptible to 20 µg l⁻¹; D, Dominant; VC, Very Common; C, Common; R, Rare; M, May; A, August; N, November; F, February
 *Otherwise mentioned, all the forms were present in fresh samples and enrichment cultures amended with 20 µg l⁻¹.

representation of *Chlorococcum humicola* was further reduced. *Nitzschia* was found in considerable numbers, and *O. obscura*, *O. subbrevis* and *Pinnularia* made sporadic appearance. *Synedra ulna* increased in number over the August count. Towards the end of winter season during February, *L. taylorii*, *Nitzschia*, *O. formosa*, *Phormidium angustissimum* and *P. notatum* were found to be evenly distributed. *Ulothrix* however dominated the samples.

Kesarpura

At Kesarpura, the seasonal occurrence of different algal forms was almost similar to that observed at Arjunpura. *L. borgerii* and *Plectonema purpureum* were the dominant forms in May. The incidence of former was however, progressively reduced during rest of the three seasons but the latter was found commonly distributed during November and February.

In August, *Chroococcus dispersus*, *Myxosarcina burmensis* and *Phormidium innundatum* were the dominant ones, followed by *Navicula* and *Gloeocapsa aeruginosa*. *Myxosarcina burmensis* and *P. innundatum* were common in May and rare in November and February. *Navicula* remained very common in November and common in February. *C. dispersus* and *G. aeruginosa* also showed a continued presence.

In November, *Synedra ulna* was the dominant alga. It was common in August and regained the dominance in February. Very common presence could be observed of *Stauroneis*, *Navicula*, *Nitzscherella* and *Cosmarium taxichondriforme*. Distribution of *Stauroneis* remained unchanged in February but was reduced in the following seasons. Other than *Synedra* and *Stauroneis*, *Plectonema nostocorum* and *P. purpureum* were very common in February. T-6217

University: Site 1 (Nursery)

Navicula was the dominant alga in May followed by *Plectonema notatum*, *Chlorella vulgaris*, *Chroococcus minor* and *Synechocystis*. It was rare in November but became common in February. The winter season encouraged growth of *Synechocystis* and *Chlorella* with *Chroococcus minor* dominating the habitat.

There were only four forms in August viz. *Scenedesmus bijuga*, *S. quadricauda*, *Chlorococcum humicola* and *Phormidium angustissimum*. While the first alga was dominant and the second was common, the rest showed rare appearance. *S. quadricauda* was dominant in November and very common in February. Subsequently *S. bijuga* became the most abundant alga along with *Navicula*, *P. tenue*, *Chlorella vulgaris* and *C. minor*.

University: Site 2

Navicula and *Chlorococcum humicola* dominated site 2 in May followed by *Amphora*, *Myxosarcina burmensis*, *Nitzschia*, *Plectonema notatum* and *Synechocystis*.

During August, *Cosmarium taxichondriforme* and *Chroococcus minor* outgrew others while *Spirulina* and *Gloeocapsa aeruginosa* were very common. Only two forms *C. taxichondriforme* and *Spirulina* could be observed in samples collected during November. *Phormidium tenue* became dominant during February followed by *Merismopedia glauca* and *C. taxichondriforme*.

FLUORIDE TOLERANCE IN ALGAE AND CYANOBACTERIA

In order to know the incidence of fluoride tolerant algae and cyanobacteria, all the water samples were enriched in BG 11 medium unamended or amended with 20 $\mu\text{g ml}^{-1}$ fluoride added as NaF. All the forms observed in the unamended enrichment cultures were also observed growing in presence of F except *Anabaena*,

Nostoc punctiforme, *Eudorina* and *Synechococcus* sp. (Table 4.3), which may be sensitive to fluoride.

From the F amended enrichment cultures, eight unialgal forms representing four genera each of green algae and cyanobacteria were isolated (Table 4.4). These forms, together with another green alga *Chlorella minutissima* and three diazotrophic cyanobacteria *Anabaena fertilissima*, *A. iyengarii* and *Westiellopsis prolifica* (obtained from NCCUBGA) were selected for further studies.

Sensitivity to fluoride was tested by growing the selected algal forms in BG 11 medium supplemented with graded concentrations of fluoride (0-200 $\mu\text{g ml}^{-1}$). Cyanobacteria were found to be more sensitive to fluoride as compared to green algae which grew happily even at 200 $\mu\text{g ml}^{-1}$ (Tables 4.5a,b).

But for the increase in chlorophyll content of *Plectonema* and *A. fertilissima* at 25-75 $\mu\text{gF ml}^{-1}$, presence of fluoride progressively reduced chlorophyll synthesis at increasing F levels in all cyanobacteria. The growth of both these algae was significantly benefitted by the presence of F upto 50 $\mu\text{g ml}^{-1}$ beyond which it showed a decreasing trend (Fig.4.1). However, at 75 $\mu\text{gF ml}^{-1}$ it was still comparable to control. Majority of the tested cyanobacteria except *A. khannae* appeared to tolerate upto 50 $\mu\text{gF ml}^{-1}$, as their chlorophyll content at this level was still comparable to that of control. *A. khannae* proved to be the most sensitive cyanobacterium as even at 25 $\mu\text{gF ml}^{-1}$ level it showed 27% reduction in chlorophyll content. At 200 $\mu\text{gF ml}^{-1}$, except *Gloeocapsa aeruginosa* which showed only 34-39% reduction in chlorophyll content, the performance of all the other forms was drastically reduced ranging between 76-100% (Table 4.5a).

Green algae in contrast were not significantly affected by fluoride (Fig.4.2).

Table 4.4 Unialgal isolates obtained from enrichment cultures grown at 20 μgml^{-1} fluoride concentration .

S.No.	Organism	Month of Isolation	Source
1.	<i>Scenedesmus bijuga</i> var. <i>irregularis</i>	May	Univ. Site 1
2.	<i>Chlorococcum humicola</i>	May	Univ. Site1
3.	<i>Gloeocapsa aeruginosa</i>	August	Kesarpura
4.	<i>Selenastrum minutum</i>	November	Kesarpura
5.	<i>Anabaena khannae</i>	August	Kesarpura
6.	<i>Lyngbya cryptovaginata</i>	November	Arjunpura
7.	<i>Chlorella</i> sp.	November	Arjunpura
8.	<i>Plectonema</i> sp.	November	Arjunpura

Table 4.5a **Response of cyanobacteria ($\mu\text{g chlorophyll ml}^{-1}$) to graded concentrations of fluoride after 15 days incubation.**

Organism F($\mu\text{g ml}^{-1}$)	<i>L.cryptovaginata</i>	<i>Plectonema</i>	<i>G.aeruginosa</i>	<i>A.fertilissinma</i>	<i>A.iyengerii</i>	<i>W.prolifica</i>	<i>A.khanna</i>	Mean
Initial	0.024	0.037	0.054	0.023	0.049	0.040	0.035	
0	1.922	5.563	5.103	2.534	3.204	2.585	4.469	3.626
25	1.664	7.368	4.847	3.619	2.875	2.485	3.285	3.735
50	1.533	8.468	4.816	3.226	2.727	2.299	2.399	3.638
75	1.331	6.826	4.696	2.535	2.581	2.029	1.463	3.066
100	1.253	0.885	4.559	2.151	2.550	1.745	1.236	2.054
125	0.618	0.835	4.475	1.880	1.897	1.426	0.899	1.718
150	0.259	0.818	4.383	1.823	0.987	1.314	0.192	1.397
175	0.059	0.813	4.265	0.874	0.717	0.768	0.095	1.084
200	0.052	0.083	3.348	0.03	0.139	0.623	0.000	0.611
Mean	0.966	3.518	4.499	2.075	1.964	1.697	1.559	
CD _{5%}	0.355	1.451	1.233	0.913	0.508	0.487	0.455	
LC ₅₀	111.6	86.3	228.5*	167.7	134.4	147.9	53	

CD_{5%} : A (organism), 0.338; B (F), 0.305; AxB, 0.819

* Extrapolated

Table 4.5b **Response of green algae ($\mu\text{g chlorophyll ml}^{-1}$) to graded concentrations of fluoride after 15 days incubation.**

Organism / F ($\mu\text{g ml}^{-1}$)	<i>C. minutissima</i>	<i>S. bijuga</i>	<i>S. minutum</i>	<i>Chlorella</i> sp.	<i>C. humicola</i>	Mean
Initial	0.169	0.145	0.058	0.086	0.109	
0	18.650	7.458	14.062	14.671	12.896	13.548
25	16.976	7.812	16.074	13.570	12.431	13.373
50	15.905	7.102	15.018	14.636	11.220	12.776
75	15.374	7.148	14.612	13.652	11.489	12.455
100	14.247	7.677	13.197	13.124	12.830	12.215
125	14.556	7.242	14.289	13.294	12.809	12.438
150	15.117	7.573	14.254	13.904	12.958	12.761
175	12.761	7.508	11.617	13.783	12.917	11.717
200	13.251	7.309	9.356	12.145	12.520	10.916
Mean	15.204	7.425	13.609	13.642	12.452	
CD _{5%}	5.522	2.191	2.838	1.963	2.922	
LC50	275*	-	232*	375*	-	

CD 5% = A (organism), 1.301; B (fluoride), 1.404; AxB, 3.203

* Extrapolated

Fig.4.1 Response of cyanobacteria to graded concentrations of fluoride.

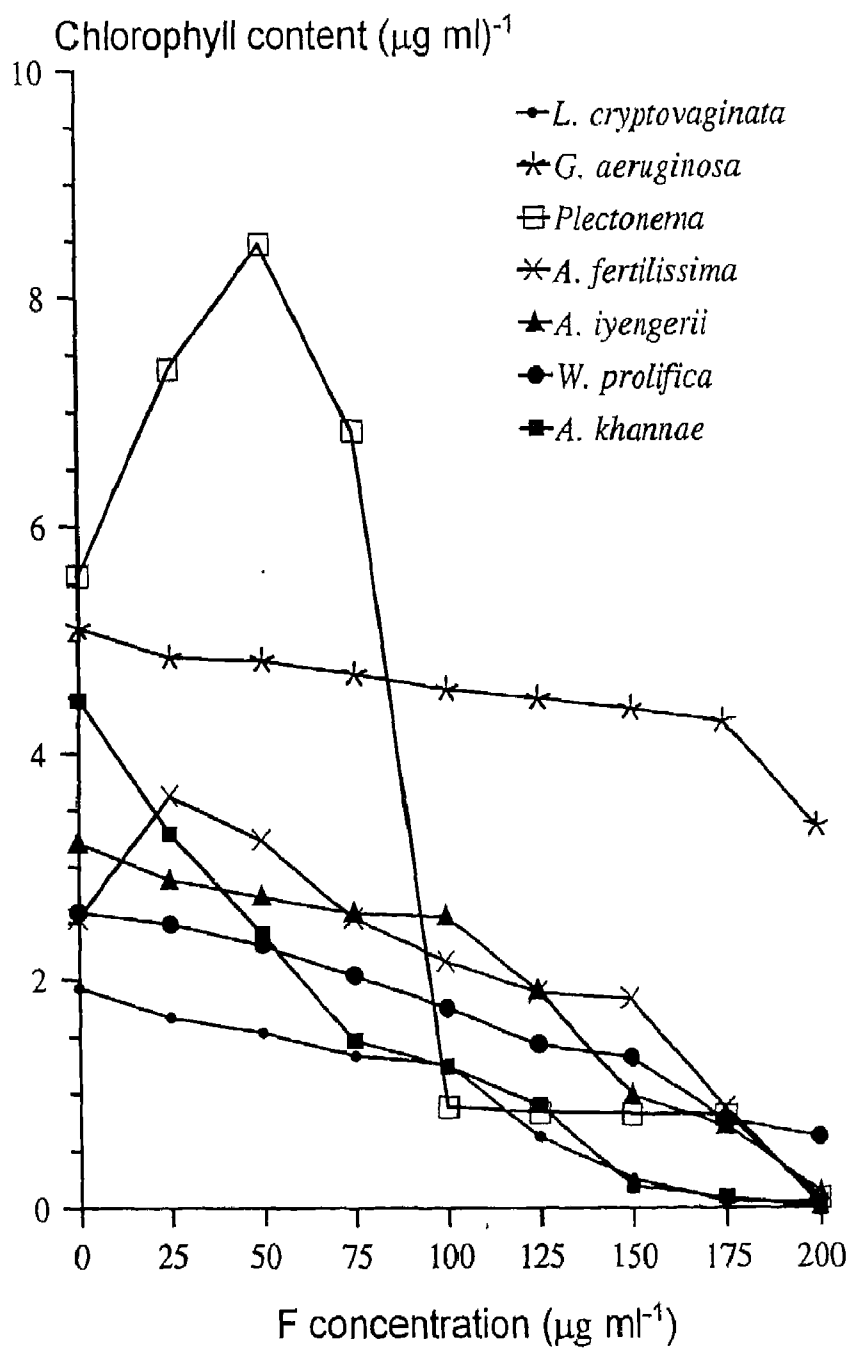
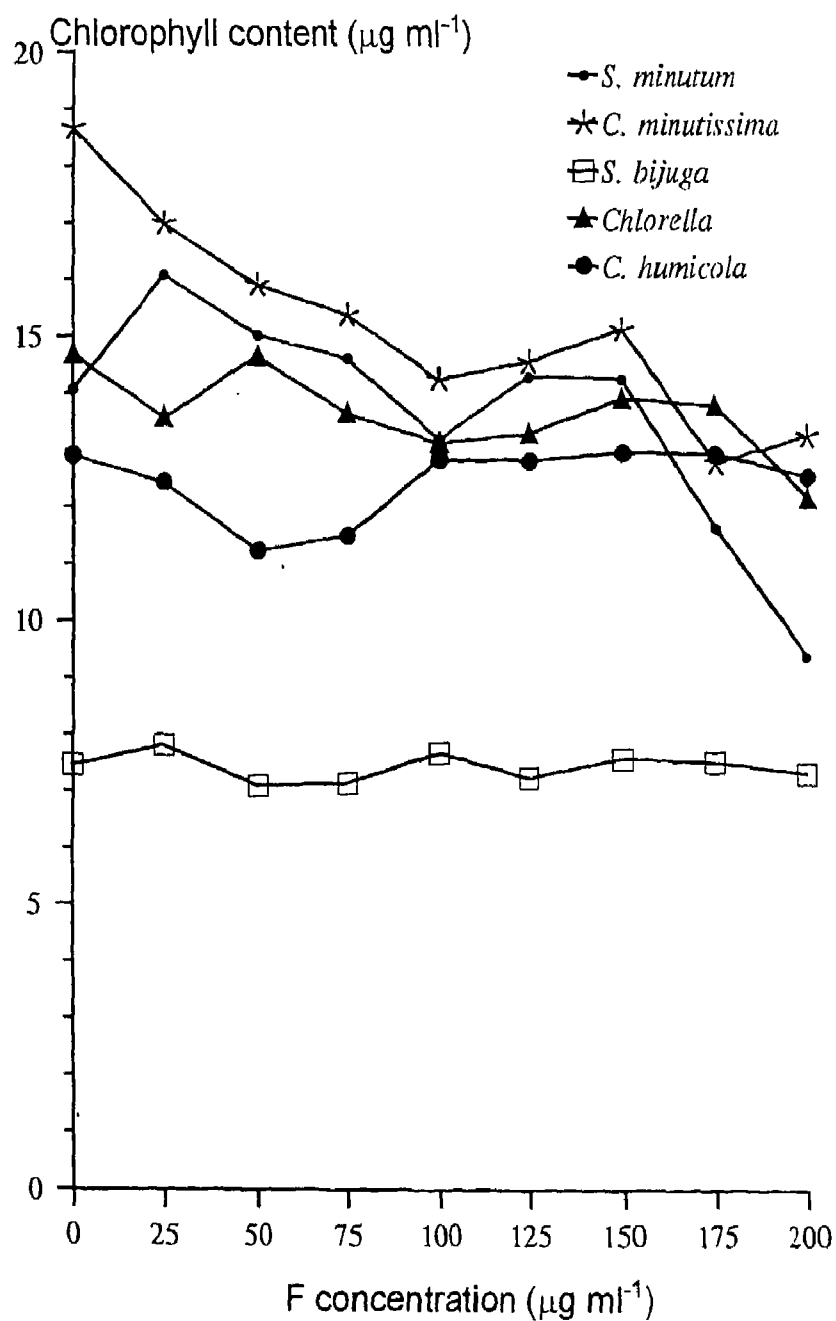


Fig. 4.2 **Response of green algae to graded concentrations of fluoride.**



Only a slight reduction in chlorophyll content was observed at concentrations above 150 $\mu\text{gF ml}^{-1}$ in *Selenastrum minutum* and *Chlorella minutissima*. *Scenedesmus bijuga* and *Chlorococcum humicola* showed uniform growth at all fluoride concentrations. *Chlorella* sp. was only partially affected at 200 $\mu\text{g ml}^{-1}$ fluoride treatment (Table 4.5b).

Estimates of EC_{50} (Table 4.5a,b) for the test algae and cyanobacteria showed that the sensitivity profile of these organisms was, *A. khannae* > *Plectonema* sp. > *L. cryptovaginata* > *A. iyengerii* > *W. prolifica* > *A. fertilissima* (all cyanobacteria) > *S. minutum* > *C. minutissima* > *Chlorella* sp. (all chlorophytes) while two green algae, namely *C. humicola* and *S. bijuga* appeared completely insensitive to fluoride.

RESPONSE OF ALGAE AND CYANOBACTERIA TO pH AND FLUORIDE

In general, cyanobacteria and green algae responded to fluoride in diagonally opposite fashion (Fig.4.1, 4.2). The former showed very high sensitivity while the latter appeared almost resistant to it. Since the pH of the medium regulates the availability of both cations and anions, further experiments were conducted to observe the interaction between fluoride and pH, as affecting growth of the most sensitive cyanobacterium *A. khannae* and the resistant chlorophyte *C. humicola*.

EFFECT ON GROWTH AND PIGMENTS

Anabaena khannae

Chlorophyll Content

In unamended medium, *A. khannae* showed optimum growth at pH 8.0 (Fig 4.3). Reduction in pH was unfavourable to the cyanobacterium expounded by a 53% decrease in chlorophyll content at pH 7.0 and 58% at pH 6.0. The effect of low pH became more perceptible in presence of F stress. At pH 6.0, even at low

concentration of 5 $\mu\text{gF ml}^{-1}$, the chlorophyll content was reduced by 33%. Further increase upto 25 $\mu\text{gF ml}^{-1}$, did not appreciably enhance the adverse effect but beyond this level the effect was drastic and the alga was completely bleached (Table 4.6a).

Contrastingly, at pH 7.0 the organism showed a 39% increase in chlorophyll content with 5 $\mu\text{g ml}^{-1}$ fluoride treatment. Although at 25 $\mu\text{gF ml}^{-1}$ the pigment content was marginally reduced, yet it was still higher than the control. Further increase in F drastically affected the pigment synthesis but the organism did not bleach as it happened at pH 6.0.

Changes in chlorophyll content per unit dry weight (DW) were statistically nonsignificant at pH 7.0 and 8.0 (Table 4.6a). At pH 6.0 significant decrease was visible at 50 and 100 $\mu\text{gF ml}^{-1}$.

Phycobilin Content

Phycobilins form a major component of the cells of *A. khannae* constituting upto 28% of the biomass. Ideal pH supporting optimum phycobilin synthesis in unamended medium appeared to be 8.0 (Fig. 4.4). Lowering the pH adversely affected the process. As seen in Table 4.6b, there was 52.4 and 63.3% decrease in total phycobilins at pH 7.0 and 6.0, respectively. Same trend was observed in presence of F at different pH levels. Higher pH appeared to have a protective effect on the cyanobacterium against F toxicity. The pace of reduction in phycobilin content per ml culture and per mg DW at lower pH was more drastic than at higher pH. At pH 6.0, the presence of 25 $\mu\text{gF ml}^{-1}$ reduced the phycobilin content by 54.5% as compared to only 28.34% at pH 8.0. This effect was enhanced at 50 $\mu\text{gF ml}^{-1}$ to 92.7 and 40.8% respectively.

Fig. 4.3 Effect of pH and graded concentrations of fluoride on chlorophyll content of *A. khannae* after 15d incubation.

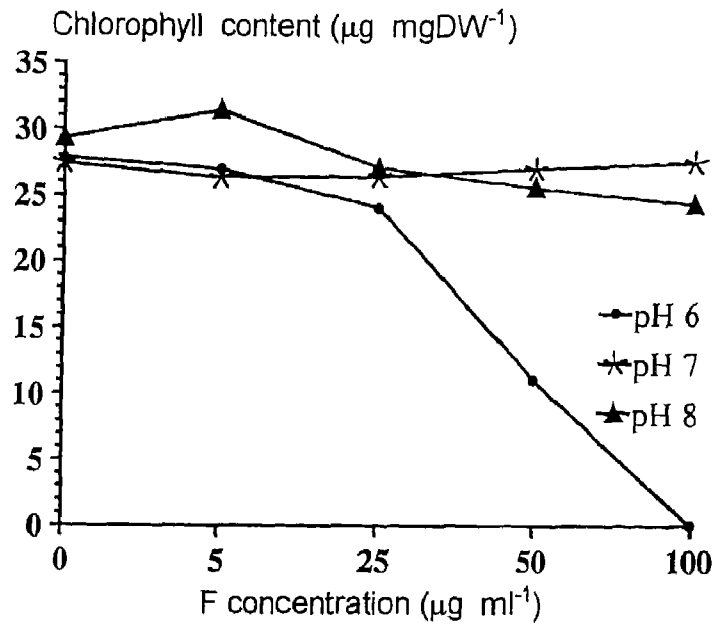


Fig. 4.4 Effect of pH and graded concentrations of fluoride on phycobilin content of *A. khannae* after 15d incubation.

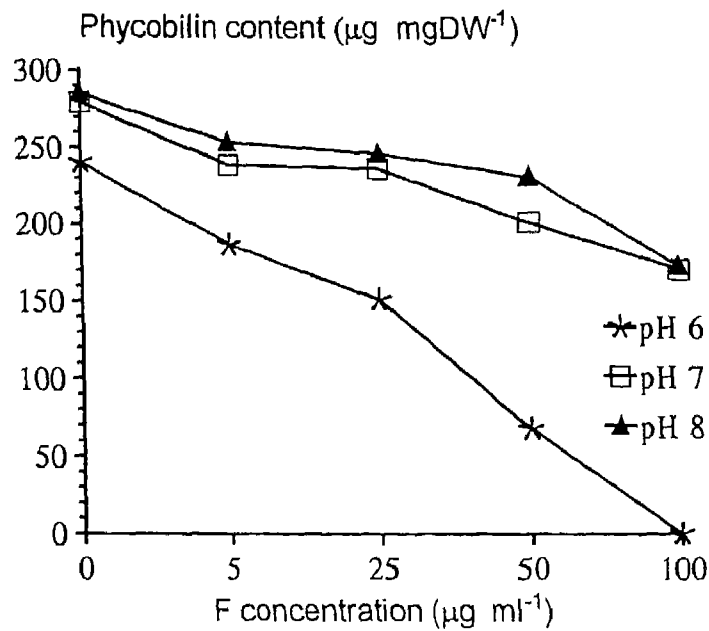


Table 4.6a Effect of graded concentrations of fluoride and pH on the chlorophyll content of *A. khannae*.

pH	6		7		8	
F($\mu\text{g ml}^{-1}$)	A	B	A	B	A	B
0	4.046	27.784	4.600	27.380	9.718	29.221
5	2.711	26.970	6.398	26.321	8.932	31.378
25	2.558	24.082	5.066	26.373	7.813	27.065
50	0.449	11.035	2.355	27.007	7.086	25.591
100	0.000	0.000	1.763	27.521	4.419	24.399

A: $\mu\text{g chlorophyll ml}^{-1}$

Factor CD_{5%}

pH 0.410

F 0.740

pH x F 1.312

B: $\mu\text{g chlorophyll mg DW}^{-1}$

Factor CD_{5%}

pH 12.261

F 5.505

pH x F 11.309

Table 4.6b Effect of graded concentrations of fluoride and pH on the phycobilin content of *A. khannae*.

pH	6		7		8	
F ($\mu\text{g ml}^{-1}$)	A	B	A	B	A	B
0	36.382	239.83	47.180	279.01	99.124	284.84
5	20.718	186.65	58.689	238.57	76.180	253.26
25	16.552	151.42	45.148	236.38	71.028	246.03
50	2.656	67.76	18.044	201.61	58.721	231.15
100	0.000	0.000	10.971	171.42	31.458	173.34

A: $\mu\text{g phycobilins ml}^{-1}$

Factor CD_{5%}

pH 4.600

F 7.617

pH x F 13.549

B: $\mu\text{g phycobilins mgDW}^{-1}$

Factor CD_{5%}

pH 46.56

F 46.84

pH x F 86.37

Carotenes

In medium without F, pH induced changes in total carotenes closely resembled the trend followed by chlorophyll and phycobilins (Fig. 4.5) with only 52 and 57% carotene synthesized at pH 6.0 and 7.0 respectively in comparison to that at pH 8.0.

Addition of fluoride to the medium significantly reduced the total carotene content per ml at all the pH values (Table 4.6c). Here also, high pH reduced the F toxicity. While at pH 6.0, increase in F concentration progressively reduced the carotene content, at pH 7.0 and 8.0, this effect was greatly mollified. However the concentration per unit DW showed statistically nonsignificant change at pH 7.0 and 8.0. At pH 6.0 the per unit DW carotene synthesis was reduced by 33-82% on addition of 5-50 $\mu\text{gF ml}^{-1}$.

Pigment Ratios

Phycobilins seemed to be more sensitive to fluoride at pH 6.0. Increase in fluoride concentration from 5-100 $\mu\text{g ml}^{-1}$ decreased the phycobilin:chlorophyll ratio from 8.5 at 5 $\mu\text{gF ml}^{-1}$ to 1.0 at 100 $\mu\text{g ml}^{-1}$ (Table 4.6d). Fluoride concentration dependent decline in the ratio was also observed at pH 7.0 and 8.0 but the effect was considerably subdued. It was statistically significant only at 100 $\mu\text{gF ml}^{-1}$ amendment. Variation in chlorophyll:carotene ratio was statistically nonsignificant in response to pH and fluoride interactions except at concentrations above 25 $\mu\text{gF ml}^{-1}$ at pH 6.0 where a conspicuous reduction was observed (Table 4.6e).

Biomass

pH and fluoride individually and in combination showed adverse effect not only on pigment content but also on biomass production. Even in absence of fluoride, biomass production was almost halved at pH 6.0 and 7.0 as compared to that at pH

Table 4.6c Effect of graded concentrations of fluoride and pH on carotene content of *A. khannae*.

pH	6		7		8	
F($\mu\text{g ml}^{-1}$)	A	B	A	B	A	B
0	8.70	0.058	9.65	0.058	16.90	0.051
5	5.87	0.054	15.40	0.063	14.70	0.053
25	4.30	0.040	10.70	0.057	12.90	0.052
50	1.53	0.040	5.11	0.058	12.90	0.049
100	0.00	0.000	3.50	0.054	8.95	0.049

A: $\times 10^{-3}$ μg carotene ml^{-1}

Factor	CD _{5%}
pH	0.0011
F	0.0011
pH x F	0.0034

B: μg carotene mg DW^{-1}

Factor	CD _{5%}
pH	0.0143
F	0.0096
pH x F	0.0186

Table 4.6d Effect of graded concentrations of fluoride and pH on phycobilin : chlorophyll ratio of *A. khannae*

pH/ F($\mu\text{g ml}^{-1}$)	0	5	25	50	100
6	9.374	8.478	6.906	6.191	1.000
7	10.293	9.393	9.178	7.977	6.240
8	10.209	8.541	9.096	8.429	6.983

CD_{5%} pH, 2.166; F, 2.050; pH x F, 3.804

Table 4.6e Effect of graded concentrations of fluoride and pH on chlorophyll : carotene ratio of *A. khannae*

pH/ F ($\mu\text{g ml}^{-1}$)	0	5	25	50	100
6	467.34	475.11	595.81	281.98	1.00
7	476.82	420.05	471.90	470.91	471.09
8	555.12	582.67	559.94	550.15	513.31

CD_{5%} pH, 129.43; F, 96.75; pH x F, 184.61

Fig. 4.5 Effect of pH and graded concentrations of fluoride on carotene content of *A. khannae* after 15d incubation.

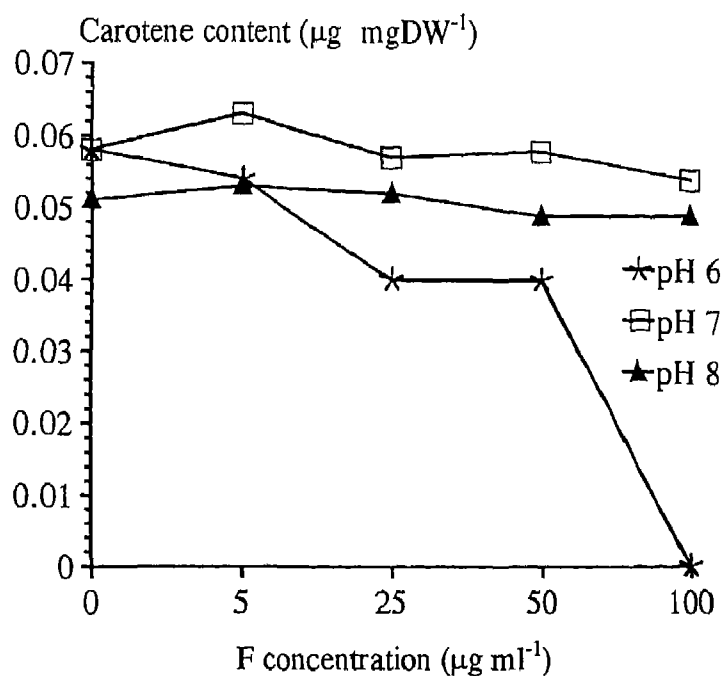
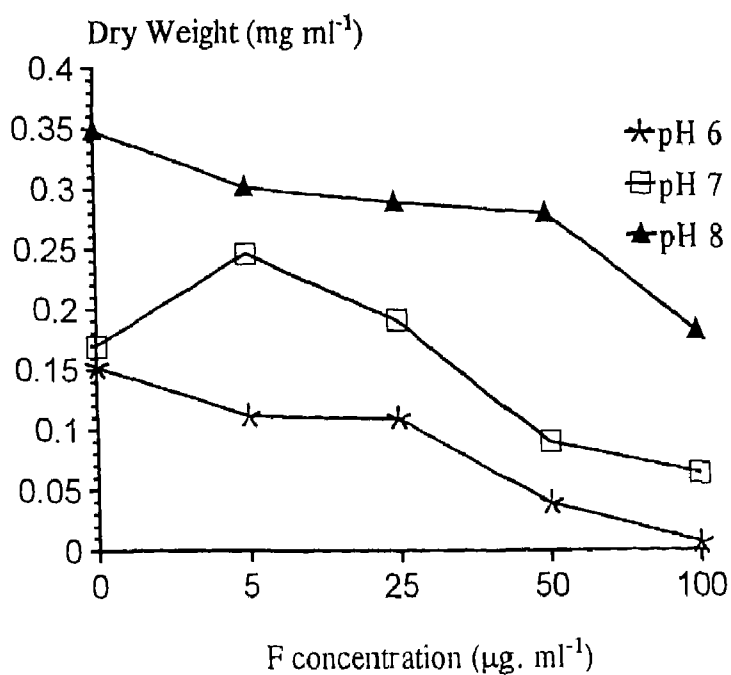


Fig. 4.6 Effect of pH and graded concentrations of fluoride on biomass production by *A. khannae* after 15d incubation.



8.0 (Table 4.6f). Presence of fluoride showed a synergistic effect but at every F level, protective effect of increasing pH was clearly discernible (Fig. 4.6). However at pH 7.0 more biomass was generated when the medium was supplemented with 5-25 $\mu\text{gF ml}^{-1}$ with maximum synthesis at 5 $\mu\text{gF ml}^{-1}$. Beyond this concentration, there was inhibition in biomass production.

Chlorococcum humicola

Though green algae are known to sustain better than cyanobacteria at low pH, *C. humicola* appeared to be an alkalophile like *A. khannae*. Growth in terms of pigment content as well as biomass production was severely affected at acidic as well as neutral pH.

Chlorophyll

In contrast to the adverse effect of fluoride on the growth of *A. khannae*, *Chlorococcum* showed no significant change in chlorophyll content even at increasing levels of F in the medium (Table 4.7a). In the absence of fluoride, chlorophyll synthesised per unit dry weight was significantly reduced at low pH. Interestingly, the effect was more severe at pH 7.0 than at 6.0 (Fig. 4.7). On dry weight basis, chlorophyll content at pH 6.0 was higher, probably as a result of decrease in biomass produced at this pH (Table 4.7c). At pH 7.0 the chlorophyll synthesis was reduced by 72% as compared to 86.5% reduction at pH 6.0, whereas the biomass was reduced only by 55.2% and 82.6% respectively,

Increasing levels of fluoride resulted in a gradual increase in the chlorophyll content per unit DW at pH 6.0 from 23.8 in control to 33.1 μg at 100 $\mu\text{gF ml}^{-1}$ added. Increase in pH to 7.0 or 8.0 although reduced the chlorophyll content, the values were not statistically significant. These observations show that although the

Table 4.6f **Effect of graded concentrations of fluoride and pH on biomass production (mg DW ml⁻¹) by *A. khannae*.**

pH/ F (μg ml ⁻¹)	0	5	25	50	100
6	0.152	0.111	0.109	0.390	0.006
7	0.169	0.246	0.191	0.090	0.064
8	0.348	0.301	0.289	0.280	0.182

CD_{5%} pH, 0.0657; F, 0.0364; pH x F, 0.0725

Table 4.7a **Effect of graded concentrations of fluoride and pH on the chlorophyll content of *C. humicola***

pH	6		7		8	
F(μg ml ⁻¹)	A	B	A	B	A	B
0	0.806	23.789	1.669	19.317	5.972	30.771
5	0.905	26.309	1.672	18.356	6.071	30.309
25	0.874	27.609	1.670	18.019	6.450	26.927
50	0.835	28.315	1.668	18.430	6.009	26.858
100	0.827	33.102	1.589	17.553	5.862	27.314

A: μg chlorophyll ml⁻¹

Factor	CD _{5%}
pH	0.208
F	0.217
pH x F	0.398

B: μg chlorophyll mg DW⁻¹

Factor	CD _{5%}
pH	1.431
F	2.615
pH x F	4.631

Fig. 4.7 Effect of pH and graded concentrations of fluoride on chlorophyll content of *C. humicola* after 15d incubation.

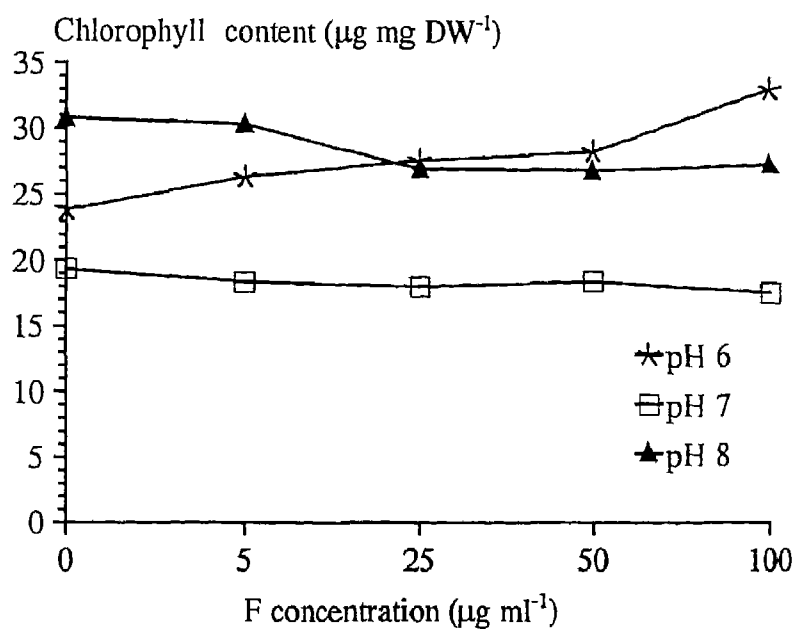
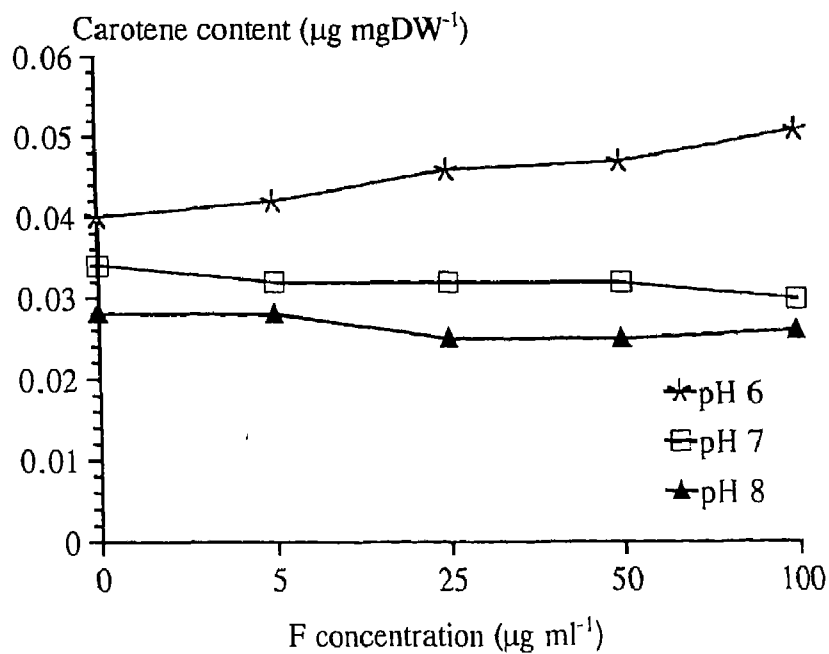


Fig. 4.8 Effect of pH and graded concentrations of fluoride on carotene content of *C. humicola* after 15d incubation.



total chlorophyll content in the fluoride exposed cultures was appreciably reduced, the chlorophyll synthesis per g biomass was significantly increased. This implies that even at 100 $\mu\text{gF ml}^{-1}$, the chlorophyll apparatus is not impaired in this alga.

Carotenes

A sharp and significant reduction in carotene synthesis was observed in *C. humicola* in response to decrease in pH in unstressed cultures (Fig. 4.8). However, per unit dry weight carotene synthesis was maximum at pH 6 followed by pH 7.0 and 8.0. Increasing concentrations of fluoride had no statistically significant effect on carotene biosynthesis at all the three pH levels (Table 4.7b).

Biomass

The alkalophilic nature of the alga *Chlorococcum humicola*, is further supported by the maximum biomass production at pH 8.0 (Fig. 4.9). The biomass produced at pH 8.0 was 82.5% higher than at pH 6.0 in the absence of fluoride.

Presence of fluoride in the medium, adversely affected biomass production at pH 6.0 with upto 27% reduction at 100 $\mu\text{gF ml}^{-1}$ (Table 4.7c). Maximum biomass production was observed at 25 $\mu\text{gF ml}^{-1}$ amendment at pH 7.0 and 8.0. The protection provided by higher pH against fluoride toxicity was evident from the observation that at pH 7.0 and 8.0 even at 100 $\mu\text{gF ml}^{-1}$, there was no appreciable reduction in the biomass production but at pH 6.0, the biomass was progressively reduced beyond 25 $\mu\text{gF ml}^{-1}$. On the other hand a minor stimulatory effect of fluoride at pH 7.0 and pH 8.0 can be discerned from the data embodied in Table 4.7c.

MEMBRANE DAMAGE

Cell membrane is the primary site of action for most of the ions. Such ionic interactions with the membrane might damage it irreversibly, causing leakage or even

Table 4.7b Effect of graded concentrations of fluoride and pH on the carotene content of *C. humicola*.

pH	6		7		8	
F($\mu\text{g ml}^{-1}$)	A	B	A	B	A	B
0	1.35	0.040	2.90	0.034	5.48	0.028
5	1.45	0.042	2.96	0.032	5.64	0.028
25	1.45	0.046	2.96	0.032	6.13	0.025
50	1.38	0.047	2.87	0.032	5.63	0.025
100	1.26	0.051	2.70	0.030	5.59	0.026

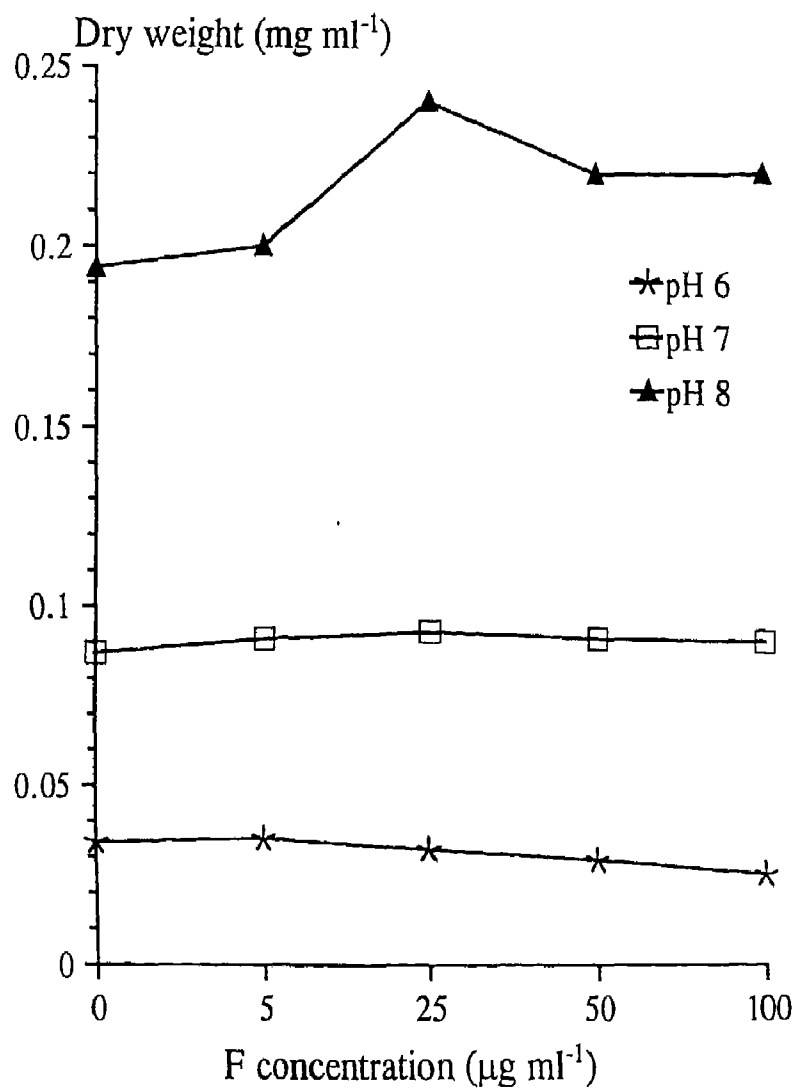
A: $\mu\text{g carotene ml}^{-1}$		B: $\mu\text{g carotene mg DW}^{-1}$	
Factor	CD _{5%}	Factor	CD _{5%}
pH	0.0010	pH	0.0019
F	0.0008	F	0.0071
pH x F	0.0015	pH x F	0.0124

Table 4.7c Effect of graded concentrations of fluoride and pH on the biomass production (mg DW ml^{-1}) by *C. humicola*.

pH/ F ($\mu\text{g ml}^{-1}$)	0	5	25	50	100
6	0.034	0.035	0.032	0.029	0.025
7	0.087	0.091	0.093	0.091	0.090
8	0.194	0.200	0.240	0.220	0.220

CD_{5%} pH, 0.007; F, 0.012; pH x F, 0.021

Fig. 4.9 Effect of pH and graded concentrations of fluoride on biomass production by *C. humicola*.



lysis of the cells. This in turn, may change the ionic strength of the medium and add organic solutes into it. Therefore, change in electrical conductivity (E.C.) and increase in the absorbance of the medium in UV region were studied to judge the effect of fluoride on the cell membrane of *A. khannae* and *C. humicola*.

Electrical conductivity

Illuminated cultures of *A. khannae* showed an initial drop in conductivity within 15 min. of incubation irrespective of the treatments (Table 4.8a). In the absence of fluoride increase in the incubation time registered a recovery at pH 6.0 and 7.0, showing appreciable increase over control upto 90 min., while at pH 8.0 the E.C. was significantly reduced. In all the cases however, the E.C. ultimately tended to become comparable to control at 300 min. stage.

Treatment with sodium fluoride at pH 6.0 induced a 12.38% increase in the E.C. at 5 $\mu\text{gF ml}^{-1}$ concentration in 5 h, which is 5.2% more than the maximal increase (253.8 $\mu\text{S cm}^{-1}$) observed for control. Increase in fluoride concentration pushed up the E.C. by 42% over control at 50 $\mu\text{gF ml}^{-1}$ after 5h, indicating massive membrane damage. Higher pH appeared to reduce the severity of damage as the E.C. at pH 6.0 was 12.38 and 42.36% more at concentrations 5 and 50 $\mu\text{gF ml}^{-1}$ as compared to only nil and 29.5% increase at pH 8.0.

C. humicola in contrast, did not exhibit any uniform trend or statistically significant changes in response to pH and fluoride (Table 4.8d).

Phycobilin leaching

The trend in excretion of water soluble pigments phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) also indicated that presence of fluoride damaged the cell membrane integrity, but the effect was mollified at higher

Table 4.8a. Effect of pH and fluoride on membrane permeability of *A. khannae*. I. Electrolyte leakage (E.C. in $\mu\text{S cm}^{-1}$)

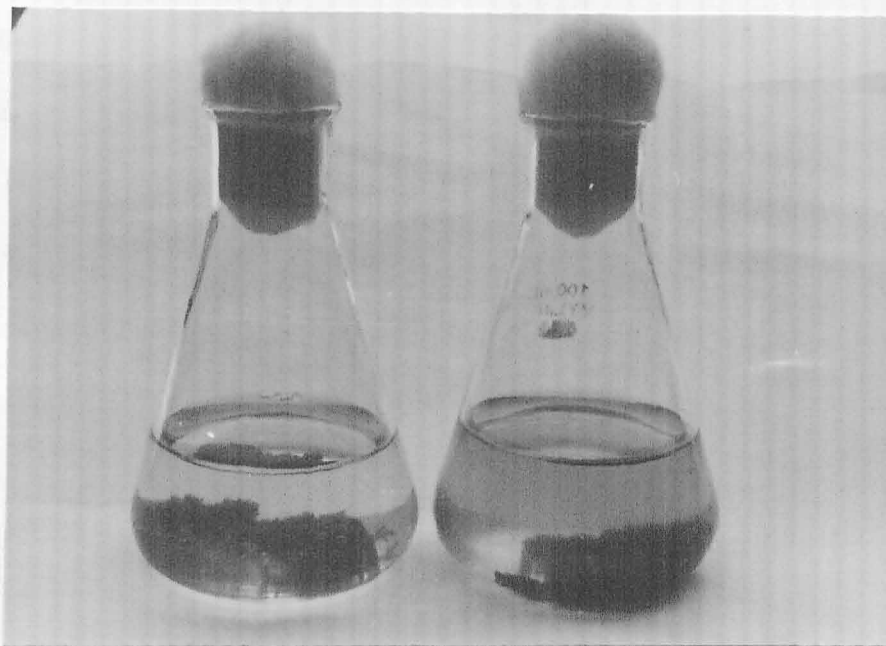
pH	6			7			8		
Time(min) / F ($\mu\text{g ml}^{-1}$)	0	5	50	0	5	50	0	5	50
0	236.80	256.00	442.40	235.2	265.60	463.20	241.60	268.00	467.20
15	222.17	249.75	427.04	220.46	245.90	442.46	240.50	251.29	464.43
30	240.83	263.33	448.30	236.67	260.11	470.00	240.83	274.17	465.00
60	242.50	269.17	470.00	241.67	270.00	473.33	242.50	277.50	468.33
90	253.80	280.74	487.60	256.41	272.69	490.21	235.10	292.90	479.78
150	249.11	282.90	557.94	249.11	275.00	504.51	235.75	272.69	514.43
180	248.33	284.48	612.95	247.54	279.87	557.93	238.11	269.02	559.80
300	241.24	287.68	629.78	248.47	283.79	583.12	237.92	264.83	605.09
CD _{5%} Two Factor (F x Time) ANOVA									
F	12.956			31.099			9.558		
Time	16.372			20.399			23.733		
F x Time	29.553			40.026			41.596		
CD _{5%} Three Factor ANOVA									
pH							7.987		
F							9.167		
Time							11.622		
pH x F							16.701		
pH x Time							20.129		
FxTime							20.928		

Table 4.8b Effect of pH and fluoride on membrane permeability of *A. khannae* II. Phycobilin leaching at 50 $\mu\text{g ml}^{-1}$ F

pH	6		7		8	
Time(h)/ Pigment	3	6	3	6	3	6
PC	1.438	3.034	0.482	0.689	0	0.369
APC	0.925	4.119	0.426	1.089	0	0.826
PE	1.845	2.307	0.466	0.355	0	0.244
Total	4.208	9.460	1.374	2.133	0	1.439

PC: $\mu\text{g phycocyanin ml}^{-1}$; APC: $\mu\text{g allo-phycocyanin ml}^{-1}$; PE: $\mu\text{g phycoerythrin ml}^{-1}$

Plate 1. Phycobilin leaching in *Anabaena khannae* on treatment with $50\mu\text{g F ml}^{-1}$ at pH 6.0 as compared to control.



pH (Fig 4.10). In *A. khannae*, increase in electrical conductivity of the medium was accompanied by a visible leaching of phycobiliproteins (Platel) into the medium. At 50 $\mu\text{gF ml}^{-1}$ and pH 6.0, 6.3% of the total phycobilins leached out in 3 h, which increased to 14.3% after 6 h. At pH 7.0 and 8.0, only 3.2% and 2.1% phycobilins appeared in the medium under similar conditions (Table 4.8b). Microscopic examination of these samples showed massive disruption of the cells in the medium supplemented with 50 μgF at pH 6.0 and 7.0, indicating damage to the cell wall also.

Absorbance in U.V. region

After filtering the algal biomass, the filtrate was scanned for absorbance in the UV region for analysing total dissolved organic compounds released by the alga. The scan of untreated cultures of *A. khannae* showed a peak at 210 nm wavelength. The absorbance response of the treated samples was compared with that of control to indicate the quantum of dissolved organic compounds as affected by fluoride stress.

In unstressed cultures, there was an inverse relationship between pH and organic compounds released by the alga into the medium (Fig. 4.11) showing a progressive reduction at increasing pH levels. Addition of 5 $\mu\text{gF ml}^{-1}$ increased this release by 1.75 times at pH 6.0 within 3 h of incubation (Table 4.8c). The rate was slowed down to 1.5 times of control at pH 7.0 while no change in the absorbance could be observed at pH 8.0. On extending the period of fluoride treatment to 6 h, a similar but more severe effect was observed.

Increase in the fluoride concentration resulted in enhanced absorption which was considerably reduced at higher pH levels. The release of organic matter by the alga in 6 h at 50 $\mu\text{g ml}^{-1}$ fluoride supplementation was approximately ten and two folds higher at pH 6.0 and pH 7.0 respectively as compared to that at pH 8.0.

Table 4.8c **Effect of pH and fluoride on membrane permeability of *A. khannae* III Absorbance of medium at 210 nm.**

pH	6		7		8	
Time(h)/ F ($\mu\text{g ml}^{-1}$)	3	6	3	6	3	6
0	0.084	0.134	0.105	0.030	0.000	0.017
5	0.147	0.384	0.022	0.046	0.000	0.031
50	0.413	0.796	0.084	0.150	0.046	0.082

absorbance in cm^{-1}

Table 4.8d **Effect of pH and fluoride on membrane permeability of *C.humicola*. Electrolyte leakage (E.C. in $\mu\text{S cm}^{-1}$)**

pH	6			7			8		
Time (min) / F ($\mu\text{g ml}^{-1}$)	0	5	50	0	5	50	0	5	50
0	2116	2134	2272	2112	2127	2275	2104	2118	2272
90	2071	2120	2246	2092	2118	2274	2089	2112	2270
180	2094	2100	2257	2096	2074	2298	2078	2116	2270
240	2096	2125	2256	2109	2145	2296	2132	2134	2281
300	2078	2120	2234	2103	2132	2292	2092	2125	2261

C.D_{5%} Two Factor (F x Time) ANOVA

Fluoride	44.9	73.2	37.3
Time	38.2	42.2	36.4
F x Time	74.5	90.6	69.3

CD_{5%} Three Factor ANOVA

pH	20.4
F	17.6
Time	21.6
pH x F	34.7
pH x Time	37.7
F x Time	37.7

Fig. 4.10 Effect of pH and fluoride on membrane damage of *A. khannae* II. Phycobilin leaching with $50 \mu\text{gF ml}^{-1}$.

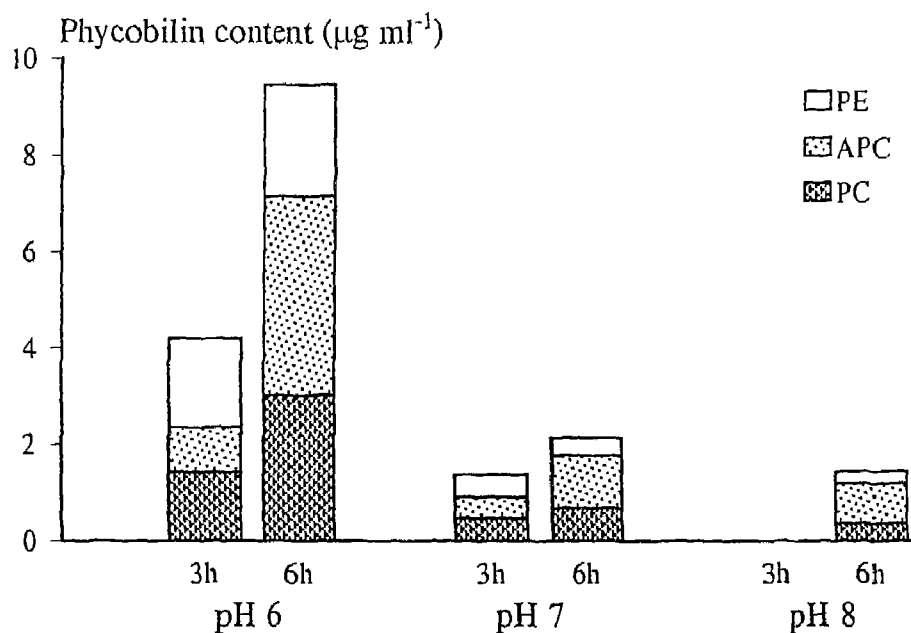
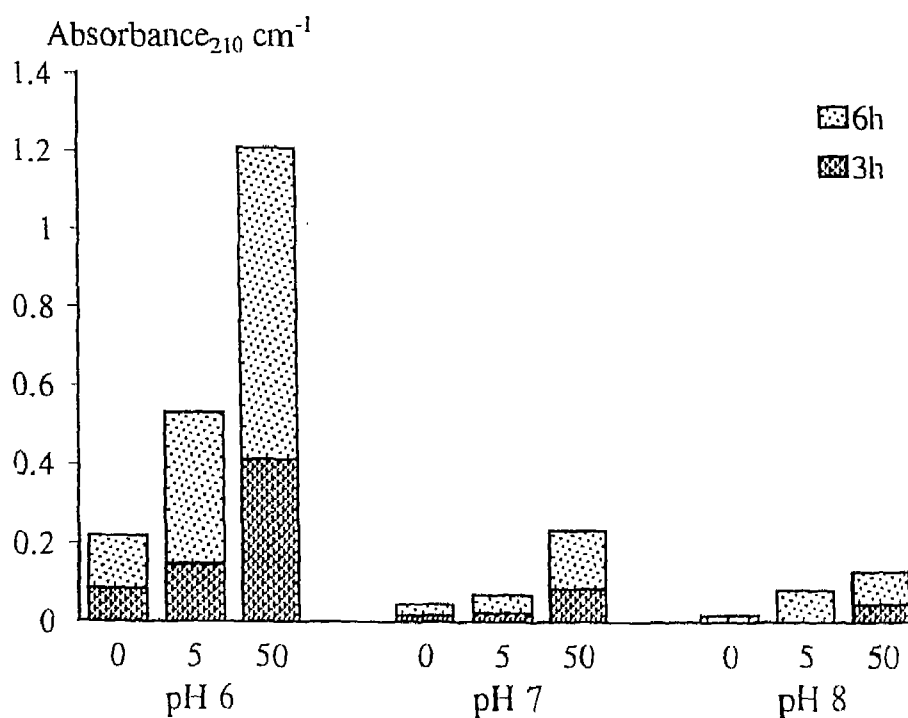


Fig.4.11 Effect of pH and fluoride on membrane permeability of *A. khannae* III. Absorbance of the medium.



ALGAL ACTIVITY MEDIATED pH CHANGES

Change in the initial and final pH of the medium was recorded for the E.C. experiment as the experiment was conducted with unbuffered medium. Since observations showed significant changes, a separate set of experiments under similar conditions was carried out to observe changes in pH of the medium as a result of algal activity.

Anabaena khannae growing in BG 11 medium in the absence of nitrogen, raised the pH of the medium to 6.8, 8.5 and 9.0 within 1.5 h of growth in media set at initial pH of 6.0, 7.0 and 8.0 respectively. The pH finally got stabilised at 6.7, 8.4 and 9.1 after 5 h (Table 4.9). Addition of fluoride to the medium at the rate of $5 \mu\text{g ml}^{-1}$ and $50 \mu\text{g ml}^{-1}$ did not induce much change except that at pH 6.0 with $50 \mu\text{g ml}^{-1}$, the rise in pH value was extended over a longer period of 3.5 h.

In presence of nitrate nitrogen the rise in pH was of the degree of 1 to 1.7 units increasing the pH from 6.0 to 7.4, 7.0 to 8.7 and 8.0 to 9.1. Addition of fluoride at pH 6.0 however reduced this quantum of increase to only 0.9 and 0.7 units at $5 \mu\text{g ml}^{-1}$ respectively. No significant response to fluoride could be observed at other pH values. These observations suggest that fluoride does not have a synergistic effect on the algae mediated change in the pH. One could observe comparatively lesser change in the pH of the medium under nitrogen fixing conditions than under nitrogen assimilation mode when the alga was utilizing nitrate nitrogen.

In contrast to *A. khannae*, non-nitrogen fixing green alga *Chlorococcum humicola*, drastically changed the pH when grown in the presence of nitrate as a source of nitrogen (Fig. 4.12). The pH initially set at 6.0, increased steadily to 9.8 in 3.5 h and stabilised thereafter. Whereas media set at pH 7.0 and 8.0 increased to

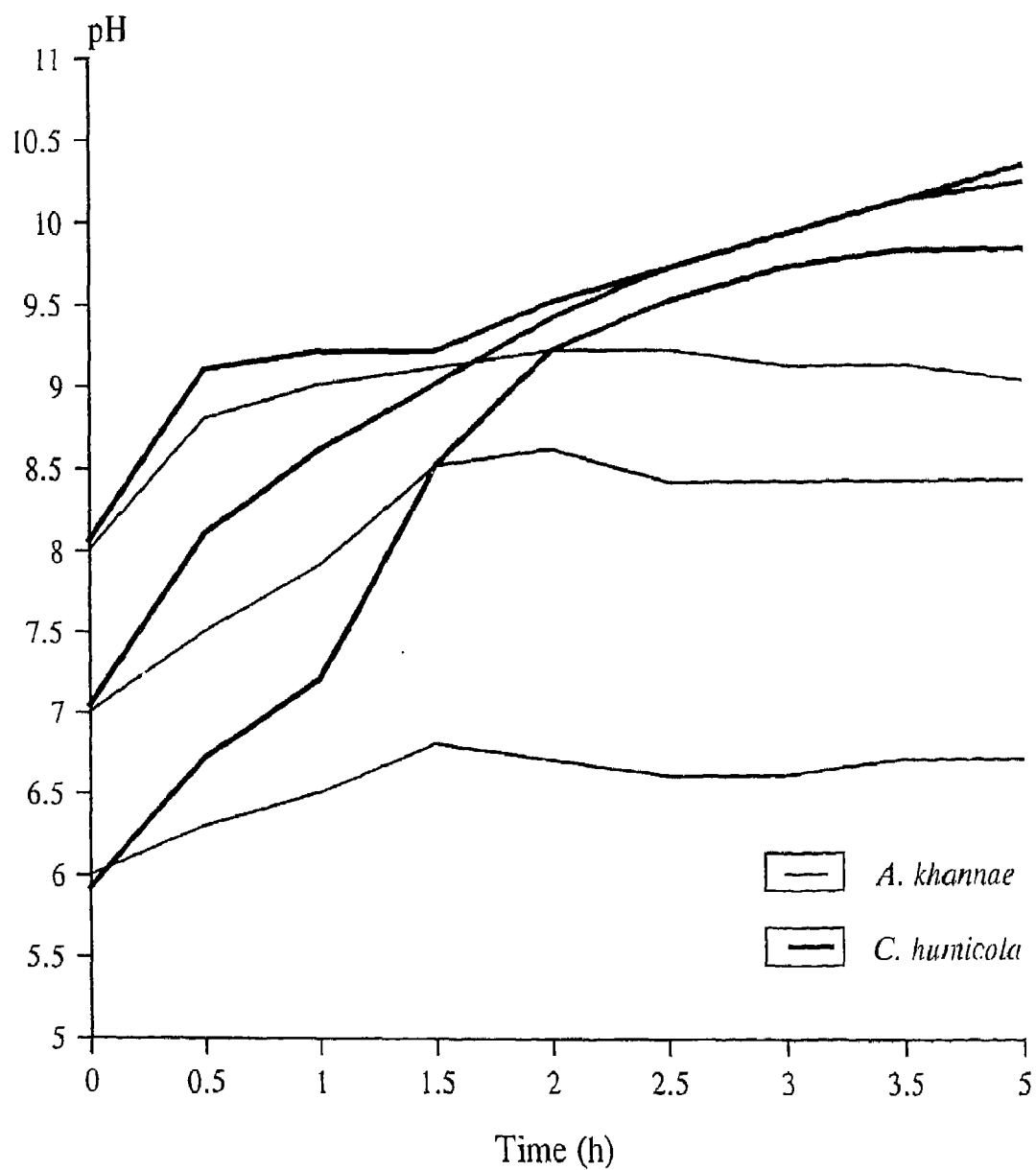
Table 4.9a Changes in pH of the medium during growth of *A. khannae* in the presence or absence of nitrate nitrogen.

pH	6			7			8		
Time (h) / F ($\mu\text{g ml}^{-1}$)	0	5	50	0	5	50	0	5	50
BG-N									
0	6.0	6.1	6.0	7.0	7.0	7.1	8.0	8.0	8.0
0.5	6.3	6.4	6.0	7.5	7.4	7.7	8.6	8.8	8.8
1.0	6.5	6.7	6.1	7.9	7.9	8.2	8.9	9.0	9.0
1.5	6.8	6.8	6.4	8.5	8.3	8.4	9.0	9.1	9.1
2.0	6.7	6.9	6.5	8.6	8.5	8.5	9.2	9.2	9.1
2.5	6.6	6.8	6.7	8.6	8.4	8.5	9.2	9.2	9.1
3.0	6.6	6.7	6.7	8.4	8.4	8.5	9.1	9.1	9.1
3.5	6.7	6.9	6.8	8.4	8.4	8.4	9.0	9.1	9.1
4.0	6.7	6.8	6.8	8.4	8.4	8.4	9.0	9.1	9.1
5.0	6.7	6.8	6.8	8.4	8.4	8.4	9.0	9.0	9.1
BG+N									
0	6.0	6.0	5.9	7.0	7.0	7.1	8.0	8.0	7.9
0.5	6.7	6.5	6.2	8.0	8.0	7.5	8.7	8.5	8.3
1.0	7.1	6.7	6.3	8.5	8.5	8.0	8.9	8.8	8.6
1.5	7.2	6.7	6.4	8.6	8.6	8.2	9.0	8.9	8.8
2.0	7.2	6.8	6.5	8.7	8.7	8.3	9.1	8.9	8.9
2.5	7.3	6.8	6.6	8.7	8.7	8.4	9.0	9.0	8.9
3.0	7.3	6.9	6.6	8.7	8.7	8.5	9.0	8.9	8.9
5.0	7.4	6.9	6.7	8.7	8.7	8.5	9.1	8.9	8.8

Table 4.9b Changes in the pH of the medium during growth of *C. humicola* in presence of nitrate nitrogen

pH	6			7			8		
F($\mu\text{g ml}^{-1}$) Time(h)	0	5	50	0	5	50	0	5	50
0	5.9	5.9	5.9	7.0	7.0	7.0	8.0	8.0	8.0
0.5	6.7	6.2	6.4	8.1	7.8	7.6	9.1	9.0	8.6
1.0	7.2	6.6	6.8	8.6	8.5	8.2	9.2	9.1	9.3
1.5	8.5	7.7	7.5	9.0	9.0	8.9	9.2	9.2	9.1
2.0	9.2	8.9	8.8	9.4	9.3	9.3	9.5	9.5	9.4
2.5	9.5	9.4	9.2	9.7	9.6	9.6	9.7	9.8	9.6
3.0	9.7	9.6	9.5	9.9	9.9	9.9	9.9	10.0	9.8
3.5	9.8	9.7	9.6	10.1	10.1	10.1	10.1	10.1	10.0
5.0	9.8	9.8	9.7	10.2	10.2	10.2	10.3	10.3	10.2

Fig. 4.12 Changes in pH of the medium due to microalgal activity.



10.2 in 5 h (Table 4.9b). This is rightly so because utilization of nitrate nitrogen increases the pH. Addition of fluoride initially reduced the rate of change in pH but subsequently at 5 h stage it became comparable to control.

PHYSIOLOGICAL RESPONSES

Fluoride ions exert diverse effects on physiological processes like photosynthesis, respiration, synthesis of pigments and proteins and on several enzymes, which adversely affect the biology of an organism. Number of reports are available on animal, plant and bacterial systems, but studies on algae and cyanobacteria are rare. Therefore, experiments were conducted to study the effect of pH and fluoride on photosynthesis, respiration and nitrogenase enzyme of the two test organisms.

Photosynthesis and Respiration

¹⁴C Uptake

Anabaena khannae utilized much higher amount of ¹⁴C in presence of light than in dark (Table 4.10a). The reduction in unstressed cells was as much as 90% and did not respond to change in the pH (Fig. 4.13). Almost the same trend was maintained in presence of 5 $\mu\text{gF ml}^{-1}$ where, the ¹⁴C uptake remained comparable to that of unstressed cells. At higher concentration of 50 $\mu\text{gF ml}^{-1}$, the carbon fixation was adversely affected at lower pH but at pH 8.0, the values were comparable to that of control. Even in dark, higher pH proved to be better than the lower pH for stressed cells.

C. humicola appeared to be better than *A. khannae* in utilizing ¹⁴C in presence and absence of fluoride (Fig. 4.13). The illuminated cultures of *C. humicola* fixed as much as 58.30 μmol as compared to only 25.17 μmol in *A. khannae* under unstressed

Fig. 4.13 Effect of pH and fluoride on ^{14}C uptake activity of *A. khannae* and *C. humicola*.

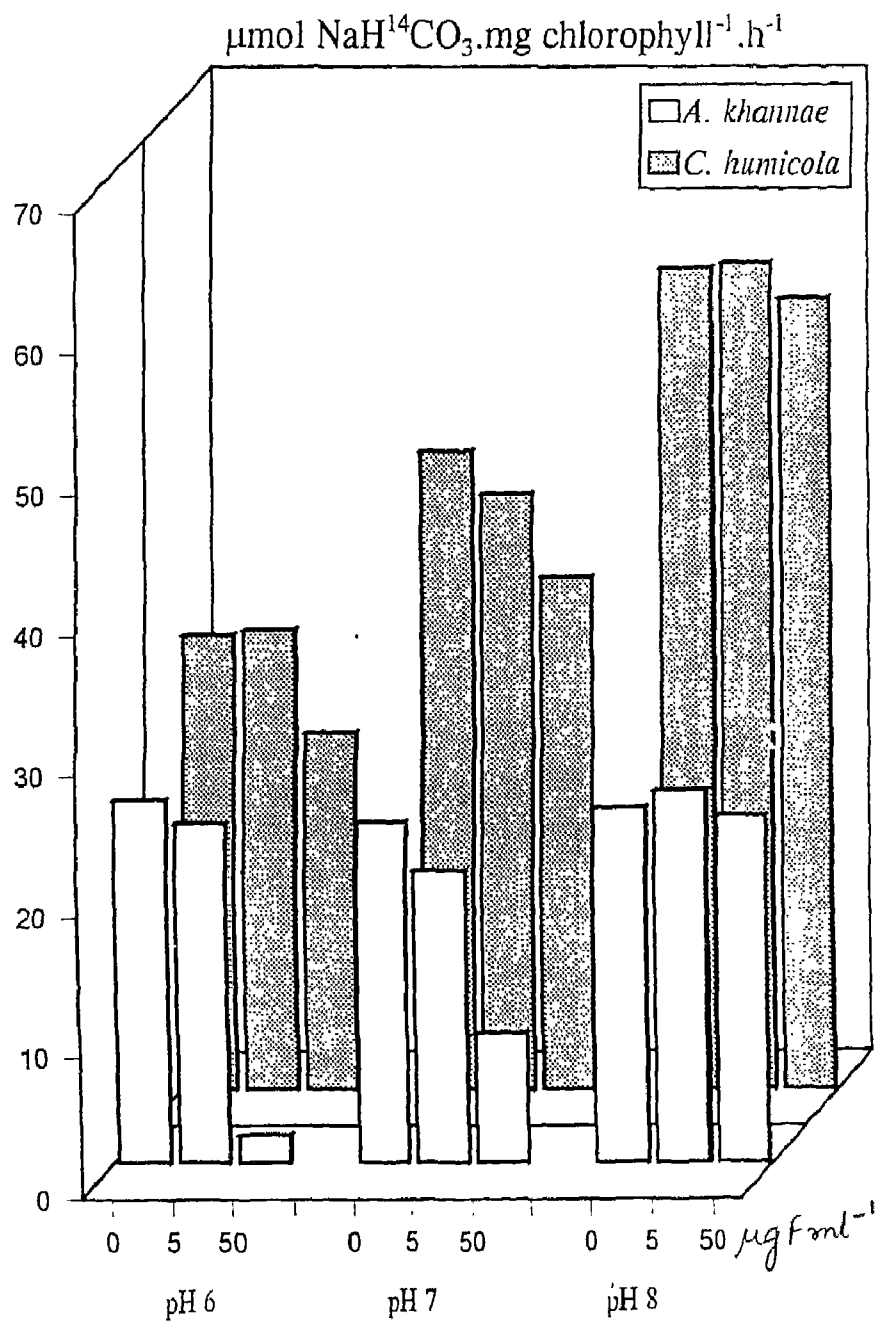


Table 4.10a Effect of pH and fluoride concentrations on ^{14}C uptake activity ($\mu\text{ mol NaH}^{14}\text{CO}_3\text{ mg chl}^{-1}\text{ h}^{-1}$) of *A. khannae*.

pH/F($\mu\text{g ml}^{-1}$)	LIGHT			DARK		
	6	7	8	6	7	8
0	25.77	24.19	25.17	2.13	2.21	2.29
5	24.09	20.75	26.42	1.58	2.58	3.03
50	1.95	9.14	24.60	1.43	1.99	3.51
CD _{5%}						
pH		11.50			0.414	
F		3.42			0.418	
pH x F		8.99			0.800	

Table 4.10b Effect of pH and fluoride concentrations on ^{14}C uptake activity ($\mu\text{ mol NaH}^{14}\text{CO}_3\text{ mg chl}^{-1}\text{ h}^{-1}$) of *C. humicola*.

pH/F($\mu\text{g ml}^{-1}$)	LIGHT			DARK		
	6	7	8	6	7	8
0	32.32	45.29	58.32	17.48	15.16	29.99
5	32.66	42.26	58.70	12.32	14.64	27.29
50	25.36	36.33	56.17	3.05	3.30	30.69
CD _{5%}						
pH		11.97			5.57	
F		10.54			1.94	
pH x F		20.69			4.89	

conditions (Table 4.10b). Even in dark, the performance was far superior than the cyanobacterium. Increase in the pH from 6.0 to 8.0, significantly enhanced the carbon utilization in illuminated and dark cultures showing 80 and 67% increase respectively.

The beneficial effect of increasing pH levels was obvious at both, the levels of fluoride and in presence and absence of light. In terms of total uptake, illuminated cultures proved to be the best in presence of 5 $\mu\text{gF ml}^{-1}$ at pH 8.0. Here again, at higher concentrations of 50 $\mu\text{gF ml}^{-1}$ the reduction was more at pH 6.0 (21%) than at pH 7.0 (19%) and 8.0 (3%). Surprisingly, in dark grown cultures, the protective effect of high pH against fluoride toxicity was substantial where, at 50 $\mu\text{gF ml}^{-1}$, the ^{14}C uptake was reduced by 82 and 78% over control at pH 6.0 and 7.0 and showed a gain of 3% at pH 8.0.

Net Gas Exchange

Net photosynthesis (PN) in *A. khannae*, measured in the absence of F as net oxygen exchange was maximum at pH 8.0 (213.6 mgC fixed. mg chl $^{-1}$ h $^{-1}$) (Table 4.11). This was gradually reduced to 193.47 and 87.73 at pH 7.0 and 6.0. Conversely, the process of respiration showed maximum value of 128.21 at pH 7.0 and lowering or increasing the pH reduced this activity to 110.96 and 96.64 respectively. These observations show that photosynthesis favourably responded to increase in pH, while pH optima for respiration was 7.0 (Fig. 4.14).

Fluoride at 5 $\mu\text{g ml}^{-1}$, marginally inhibited PN at all levels of pH but the respiratory activity remained almost unaffected. On the other hand at 50 $\mu\text{g ml}^{-1}$, PN was reduced by 9.8 and 46.27% at pH 8.0 and pH 7.0 and at pH 6.0, no PN could be detected.

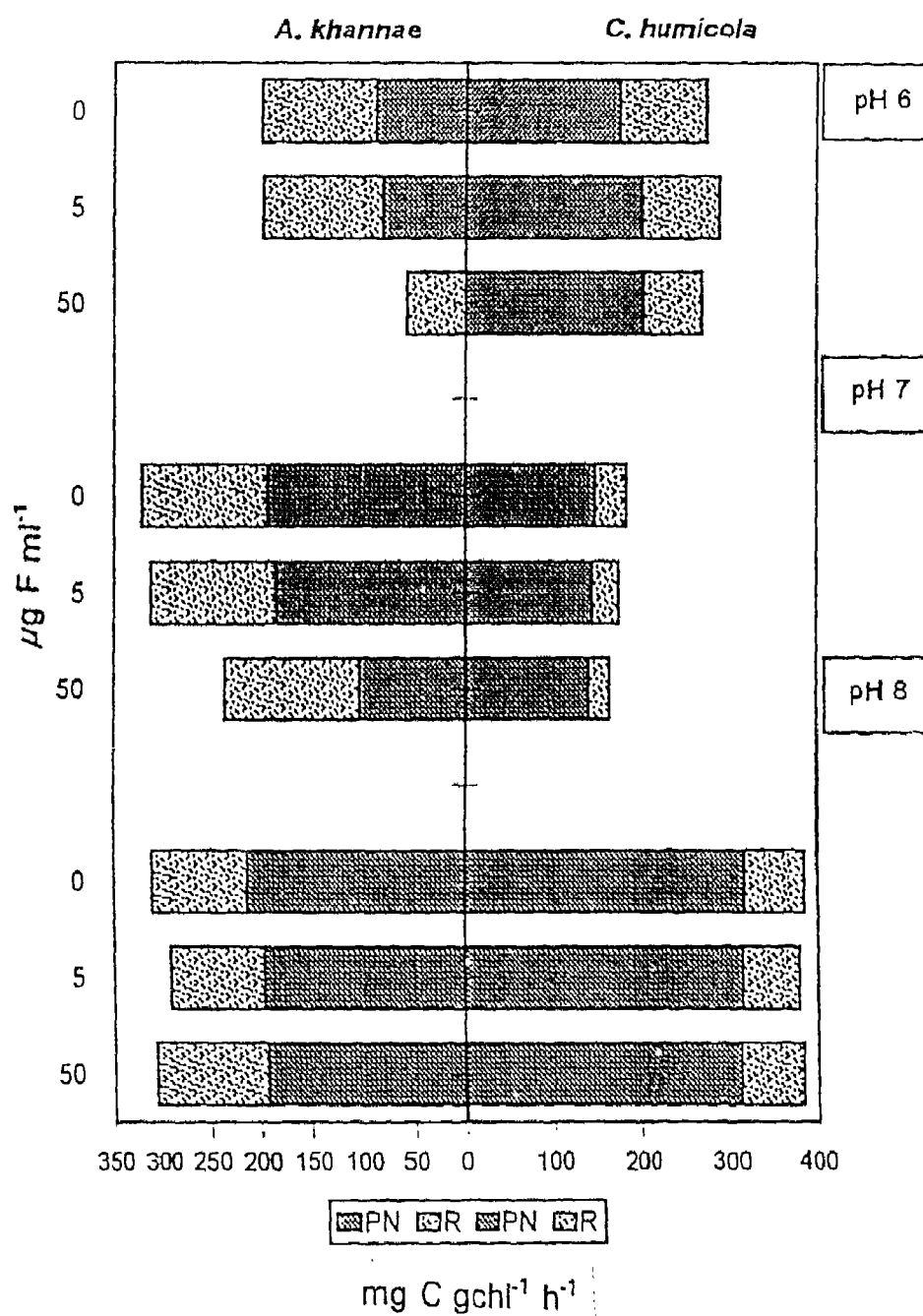
Table 4.11 **Effect of pH and fluoride concentration on photosynthesis and respiration of microalgae.**

pH	6			7			8		
F($\mu\text{g ml}^{-1}$)	0	5	50	0	5	50	0	5	50
<i>A. khannae</i>									
PG	180.19	177.82	0	300.31	282.99	213.69	294.13	289.12	287.13
PN	87.73	80.61	0	193.47	184.81	103.96	213.60	196.09	192.59
R	110.96	116.65	56.90	128.21	127.82	131.68	96.64	95.63	113.45
PN:R	0.79	0.69	0	1.51	1.45	0.79	2.21	2.05	1.70
<i>C. humicola</i>									
PG	256.79	273.07	256.79	175.54	167.99	151.00	384.44	379.47	372.49
PN	175.41	199.83	200.73	147.23	143.45	139.68	316.36	314.41	312.41
R	97.65	87.89	67.72	33.98	29.45	23.90	69.69	65.47	72.09
PN:R	1.80	2.27	2.96	4.33	4.87	5.84	4.54	4.80	4.33

PG: Gross Photosynthesis; PN: Net Photosynthesis; R: Respiration

* PG, PN, R values in $\text{mg C gchl}^{-1} \text{ h}^{-1}$

Fig. 4.14 Effect of pH and fluoride on photosynthesis and respiration in microalgae.



Despite the complete inhibition of the photosynthetic process at pH 6.0 by 50 $\mu\text{gF ml}^{-1}$, 51.3% respiratory activity was still observed. Respiration remained unaffected by fluoride at pH 7.0 and 8.0, however, PG decreased on addition of fluoride, more prominently at 50 $\mu\text{g ml}^{-1}$ F amendment. PN: R ratio decreased with increase in fluoride at all pH levels, implying that photosynthesis is more sensitive to fluoride than respiration (Table 4.11).

Productivity of *C. humicola* was maximum at pH 8.0 (384.4 mg) and the loss by way of respiration was only 69.69 mgC (Table 4.11). Reduction of pH to 7.0 negatively affected the processes, but at pH 6.0, appreciable recovery was observed in PN and R values. In terms of PN:R ratio, pH 7.0 and 8.0 were comparable but a marked reduction was observed at pH 6.0.

In contrast to *A. khannae* where the photosynthetic process (PN) was adversely affected by fluoride at all pH levels, *Chlorococcum* showed a stimulation with concomitant reduction in respiration at reducing pH levels. Addition of 50 $\mu\text{gF ml}^{-1}$ caused upto 14% increase in PN at pH 6.0 and decreased the respiratory activity by 30%. The gross productivity at pH 6.0, in presence of 5 $\mu\text{gF ml}^{-1}$ was increased by 6% and remained unchanged at 50 $\mu\text{gF ml}^{-1}$.

C. humicola also showed an increase in the PN:R ratio from 1.8 at pH 6.0 to 4.33 at pH 7.0 and 4.54 at pH 8.0. Presence of fluoride raised the ratio at pH 6.0 and 7.0, but no significant effect could be observed at pH 8.0. Thus in contrast to *A. khannae*, in the green alga *C. humicola* photosynthesis appears to be stimulated in the presence of fluoride.

Fluoride appears to have more severely affected the process of respiration as evident from the increasing PN:R values at the three pH levels. At pH 6.0 and 7.0,

while the PN values consistently increased, the R values showed a decreasing trend. This resulted in consistent increase in the PN:R values. This effect was more prominently visible at higher pH levels.

Nitrogenase Activity

Nitrogenase, the key enzyme involved in nitrogen fixation is present in diazotrophic cyanobacteria. Activity of nitrogenase is directly or indirectly regulated by the process of photosynthesis which forms the main source of energy, reductant and carbon skeletons used for tagging of ammonia. Since fluoride was observed to effectively regulate the photosynthetic activity of *A. khannae* an attempt was made to study how it affected the process of nitrogen fixation.

Effect of pH and fluoride on nitrogen fixation by *A. khannae* was determined by first suspending the cyanobacterium in fluoride free medium adjusted to pH 6.0, 7.0 and 8.0. After allowing an hour of acclimatization, the media were fortified with sodium fluoride, to attain a final fluoride concentration of 5 and 50 $\mu\text{g ml}^{-1}$.

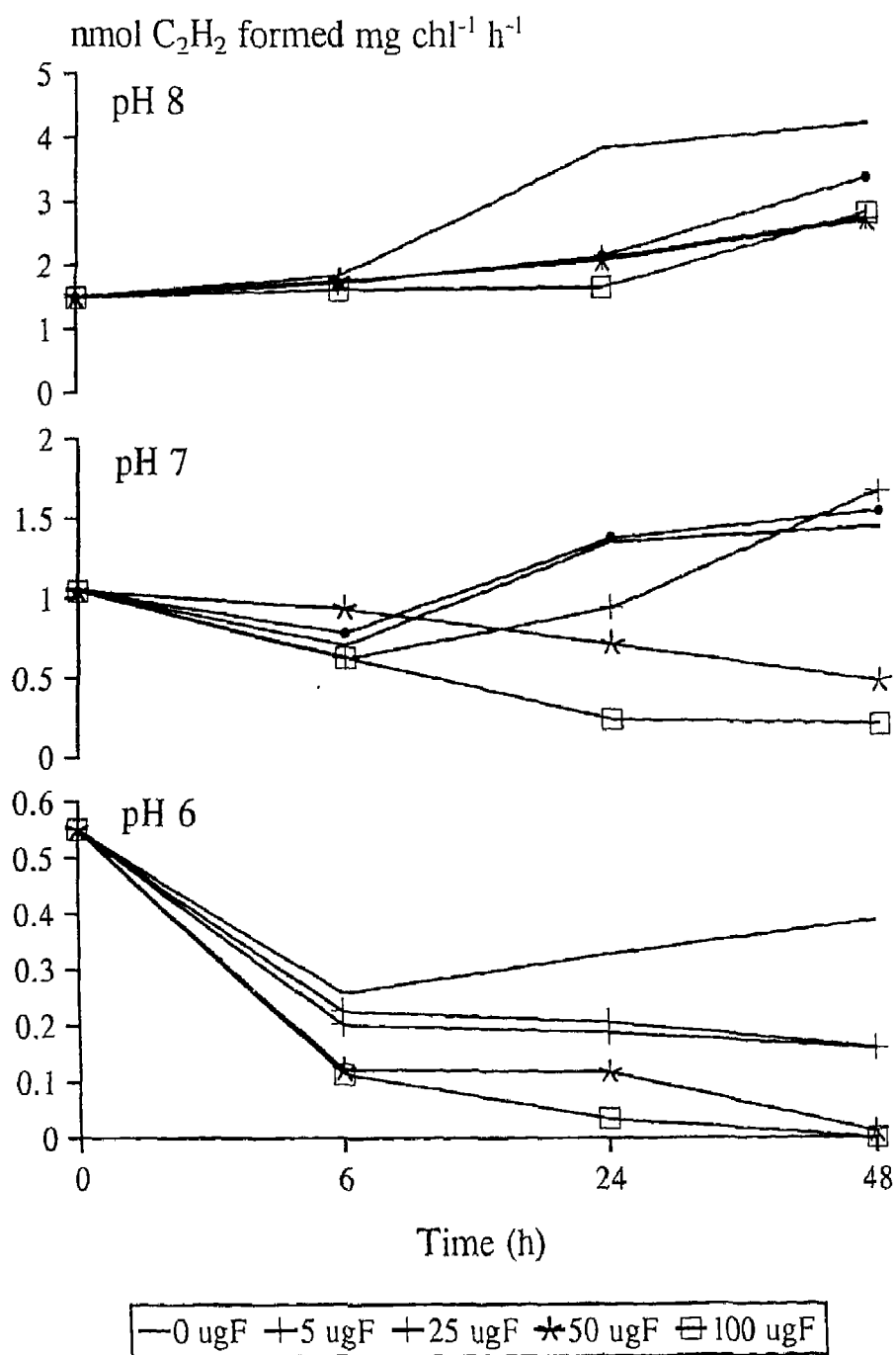
Effects of varying pH in presence of graded concentrations of fluoride on acetylene reduction activity by *A. khannae* are given in Table 4.12. Nitrogenase activity was optimum at pH 8.0 and lowering of pH reduced the activity by 30% for each unit decrease in pH from 8.0 to 6.0. Addition of fluoride synergistically enhanced the negative effect at low pH (Fig. 4.15). These observations further substantiated the alkalophilic nature of the alga.

Increasing presence of fluoride progressively reduced the ARA at all the time intervals and pH levels. Maximum susceptibility of the process was observed in presence of 50 $\mu\text{gF ml}^{-1}$ at pH 6.0 and at 100 $\mu\text{gF ml}^{-1}$, the process was completely annihilated. The protective role of higher pH was visible at every level of fluoride.

Table 4.12 Effect of pH and fluoride concentration on the nitrogenase activity (nmol C₂ H₂ formed mg chl⁻¹ h⁻¹) of *A. khannae*.

pH	6			7			8		
F(μgm ^l ⁻¹)/ Time(h)	6	24	48	6	24	48	6	24	48
0	0.257	0.327	0.387	0.701	1.346	1.441	1.828	3.842	4.241
5	0.225	0.206	0.161	0.775	1.372	1.541	1.713	2.151	3.380
25	0.201	0.188	0.159	0.617	0.932	1.671	1.720	2.112	2.754
50	0.121	0.117	0.011	0.928	0.705	0.472	1.750	2.072	2.713
100	0.114	0.034	0.000	0.622	0.234	0.205	1.606	1.652	2.837
Activity before addition of F	0.551			1.042			1.492		
CD _{5%} Two Factor (F x Time) ANOVA									
F	0.072			0.439			0.593		
Time	0.111			0.323			0.807		
F x Time	0.198			0.618			1.451		
CD _{5%} Three Factor ANOVA									
pH							0.462		
F							0.194		
pH x Time							0.273		
pH x F							0.455		
F x pH							0.618		
Time x F							0.473		

Fig. 4.15 Effect of pH and fluoride on nitrogenase activity of *A. khannae*



INFLUENCE OF GRADED CONCENTRATIONS OF FLUORIDE AND INCUBATION PERIOD ON GROWTH AND CELLULAR CONSTITUENTS OF *A. KHANNAE*

Earlier studies on fluoride-pH interaction showed remarkable synergistic effect on the growth of *A. khannae*, while the green alga *C. humicola* remained largely unaffected. Further, lower pH enhanced the toxic effect, which distinctly smothered at pH 8.0. It was pH 7.0 at which fluoride proved to be significantly deleterious yet, not lethal to *A. khannae*. Hence, a time scale study was conducted at this pH to determine the effect of different levels of F on growth, pigments and other cell constituents of this cyanobacterium.

BIOMASS

Anabaena khannae showed a time course dependent increase in biomass upto 25 d in the absence of fluoride (Table 4.13a). Low concentration of 5 $\mu\text{gF ml}^{-1}$ of the medium, stimulated biomass production by 19% over control at 15 d stage. However on longer incubation, growth stabilised and became comparable to control. Higher concentrations of fluoride showed a decrease in total biomass which became more severe with increasing levels of fluoride. After 25 days at 50 $\mu\text{gF ml}^{-1}$, the biomass was reduced by 36.3% which increased to 81.6% at 100 $\mu\text{gF ml}^{-1}$.

CHLOROPHYLL CONTENT

Chlorophyll content as affected by increasing fluoride stress presented a very interesting situation (Table 4.13b). While the chlorophyll per unit volume of the culture was drastically reduced at higher fluoride levels, the reduction was not that severe in terms of chlorophyll per unit biomass of the alga. This shows that the process of biomass production is more readily influenced by fluoride as may be seen

Table 4.13a Effect of graded concentrations of fluoride and incubation period on the biomass production (mg ml^{-1}) by *A. khannae*.

Days/ F($\mu\text{g ml}^{-1}$)	0	5	25	50	100
5	0.054	0.055	0.057	0.041	0.036
10	0.090	0.099	0.099	0.063	0.047
15	0.175	0.208	0.198	0.116	0.063
20	0.311	0.291	0.274	0.133	0.076
25	0.479	0.472	0.386	0.305	0.088

Initial value: 0.0033 mg ml CD_{F} : F, 0.036; Time, 0.026; F x Time, 0.060

Table 4.13b Effect of graded concentrations of fluoride and incubation period on the chlorophyll content of *A. khannae*.

Days/ F($\mu\text{g ml}^{-1}$)		0	5	25	50	100
5	A	0.64	0.48	0.46	0.45	0.40
	B	12.64	8.63	8.09	10.98	12.24
10	A	1.56	1.84	1.51	1.15	0.55
	B	21.77	18.48	15.18	15.45	12.20
15	A	3.47	4.55	3.70	1.87	0.79
	B	20.70	21.85	18.69	17.12	12.50
20	A	6.69	6.23	5.67	2.42	0.94
	B	21.66	21.69	21.17	18.20	13.51
25	A	7.87	8.34	7.63	6.28	1.02
	B	16.42	18.18	19.84	21.22	11.58

$\text{CD}_{5\%}$

Factor	A ($\mu\text{g chlorophyll ml}^{-1}$)	B ($\mu\text{g chlorophyll mg DW}^{-1}$)
F	0.42	4.78
Time	0.33	3.25
FxTime	0.77	7.73

Initial value: 0.0608 $\mu\text{g ml}^{-1}$

in Table 4.13a. Low concentrations of fluoride increased the chlorophyll content of the culture. The cyanobacterium performed better at every stage of observation in the presence of $5 \mu\text{gF ml}^{-1}$ registering a 6% increase in chlorophyll ml^{-1} over control at the end of 25d incubation. Raising the fluoride concentration to $25 \mu\text{g ml}^{-1}$ marginally reduced the quantum of increase making it comparable to control. At still higher concentrations of 50 and $100 \mu\text{gF ml}^{-1}$, the differential effect of fluoride on chlorophyll content per unit volume and per unit biomass became more obvious from 15 d stage onwards. Chlorophyll content per ml of culture was progressively reduced with increase in concentration of fluoride and incubation period. The pace of reduction however was gradual upto $50 \mu\text{gF ml}^{-1}$ but beyond this it was more rapid. After 25 d growth the reduction in chlorophyll content at $50 \mu\text{gF ml}^{-1}$ was merely 3%, which increased to 87% at $100 \mu\text{gF ml}^{-1}$.

Chlorophyll content per unit DW however showed a different trend (Table 4.13b). In the absence of fluoride, maximum amount of chlorophyll per unit biomass produced was reached within 10 days of continuous growth, thereby increasing proportionately to the biomass for the next 10 days. After 20 days of incubation it dropped, indicating an increase in biomass synthesis thereafter. Addition of fluoride upto $50 \mu\text{g ml}^{-1}$ of the medium showed no significant changes in chlorophyll mgDW^{-1} over control with time. Although chlorophyll synthesis was drastically reduced by $100 \mu\text{gF ml}^{-1}$, the ratio of chlorophyll to biomass was maintained all through the incubation period at around 12.

PHYCOBILIN CONTENT

Phycobilins form an important cell constituent in cyanobacteria. Constituting the major component of the accessory pigments, their content denotes the

physiological status of the cell. From the data presented in Table 4.13c , one could see a time dependent increase in phycobilin content of *A. khannae* in the absence of fluoride. This increase was proportionate to increase in biomass as reflected by the observations on the amount of phycobilins per ml culture and per mg dry weight of the alga.

The fluoride affected phycobilin content in a fashion similar to chlorophyll. At 5 $\mu\text{gF ml}^{-1}$ there was none or only marginal variation over control values. Further increase in fluoride concentration induced a gradual to steep reduction in phycobilin content. At 25 $\mu\text{gF ml}^{-1}$ the reduction was only 7% as compared to 41 and 89% at 50 and 100 $\mu\text{gF ml}^{-1}$ at the end of the experiment.

In terms of total phycobilins synthesised per unit biomass, presence of 5 $\mu\text{gF ml}^{-1}$ did not have any appreciable effect as compared to control. Further increase in the fluoride stress did reduce the phycobilin synthesis but the effect was much less than that observed on total phycobilin content of the culture. After 25 d incubation, notwithstanding the marginal increase at 25 $\mu\text{gF ml}^{-1}$ reduction in phycobilin synthesis was 5 and 37% at 50 and 100 $\mu\text{gF ml}^{-1}$, as compared to 41 and 89% reduction in the total phycobilin content at corresponding fluoride levels.

The comparable time dependent increase in the content and synthesis of phycobilins in absence and presence of 5 and 25 $\mu\text{gF ml}^{-1}$, show that presence of lower levels of fluoride in the medium does not appreciably affect the main light harvesting pigment system, which is reflected in terms of unaltered rate of photosynthesis.

CAROTENE CONTENT

Data embodied in Table 4.13d show no significant influence of fluoride on

Table 4.13c Effect of graded concentrations of fluoride and incubation period on the phycobilin content of *A. khannae*.

Days/ F($\mu\text{g ml}^{-1}$)		0	5	25	50	100
5	A	4.61	4.63	4.54	3.19	1.48
	B	86.02	83.77	79.26	79.09	38.81
10	A	16.14	17.18	14.48	7.95	2.25
	B	228.0	173.66	145.98	131.16	46.33
15	A	40.31	46.00	36.43	14.30	3.81
	B	236.84	221.54	184.03	136.45	60.22
20	A	71.19	67.55	55.14	21.64	8.06
	B	233.10	232.89	207.01	172.05	111.50
25	A	108.47	123.36	100.27	63.14	11.90
	B	225.81	266.24	257.40	213.81	140.73
CD _{5%}						
Factor		A($\mu\text{g phycobilins ml}^{-1}$)			B($\mu\text{g phycobilins mg DW}^{-1}$)	
F		4.59			44.60	
Time		7.24			35.23	
F x Time		16.36			81.43	
initial value: 0.758 $\mu\text{g ml}^{-1}$						

Table 4.13d Effect of graded concentrations of fluoride and incubation period on carotene content of *A. khannae*.

Days/ F($\mu\text{g ml}^{-1}$)		0	5	25	50	100
5	A	1.12	0.84	0.84	0.58	0.4
	B	0.0207	0.0152	0.0146	0.0142	0.0123
10	A	1.94	2.12	1.98	0.88	0.46
	B	0.0268	0.0213	0.0200	0.0148	0.0098
15	A	5.21	6.54	5.39	2.23	1.09
	B	0.0307	0.0314	0.0272	0.0187	0.0172
20	A	15.03	14.08	11.31	4.40	2.40
	B	0.0495	0.0488	0.0426	0.0341	0.0343
25	A	15.60	15.40	15.10	12.1	2.35
	B	0.0326	0.0336	0.0400	0.0410	0.0290
C.D. 5%:						
Factor		A ($\times 10^{-3} \mu\text{g carotene ml}^{-1}$)		B ($\mu\text{g carotene mg DW}^{-1}$)		
F		0.01		0.001		
Time		0.01		0.001		
F x Time		0.02		0.002		
initial value: $0.036 \times 10^{-3} \mu\text{g ml}^{-1}$						

carotene content upto 25 $\mu\text{g ml}^{-1}$ concentration. Addition of 50 $\mu\text{gF ml}^{-1}$ had a negative effect and at 100 $\mu\text{g ml}^{-1}$ concentration the organism synthesized only 15% of that produced in absence of fluoride in 25 days. The carotene synthesis measured in terms of $\mu\text{g carotene mgDW}^{-1}$ remained unaffected even at 50 $\mu\text{gF ml}^{-1}$ at 25 d stage. Although, the time course study showed a marginal reduction in carotene synthesis after 10 and 15 days in presence of 25 and 50 $\mu\text{gF ml}^{-1}$, the cyanobacterium recovered back from the effect at 25 d stage. The adverse effect of 100 $\mu\text{gF ml}^{-1}$ was visible from the fifth day onwards without any trace of recovery.

PROTEIN CONTENT

In unamended medium the cyanobacterium synthesized a maximum of 92.8 $\mu\text{g protein ml}^{-1}$ in 20 days, which was approximately 30% of the dry weight (Table 4.13e). After 25 d, the activity appeared to slow down and both $\mu\text{g protein ml}^{-1}$ and mg DW^{-1} were reduced from 92.8 and 298.4 at 20d to 73.5 and 153.5, respectively. Presence of fluoride resulted in significant increase in protein content of the culture at 5 $\mu\text{gF ml}^{-1}$. Although at 25 and 50 $\mu\text{gF ml}^{-1}$, the protein content was appreciably reduced but still it was significantly better than in control. The protein content per unit biomass consistently increased upto 100 $\mu\text{gF ml}^{-1}$. Interestingly, at this level, while total protein content of the culture was reduced by 62%, the protein content on dry weight basis increased by 107% over control. Production of 318.6 $\mu\text{g protein per unit dry weight}$ as against 153.5 μg synthesized in control when supplemented with 100 $\mu\text{gF ml}^{-1}$, which implies that the increase in protein is probably an adaptive response to fluoride.

CARBOHYDRATE CONTENT

In the absence of fluoride, the carbohydrate content of the medium consistently

Table 4.13e Effect of graded concentrations of fluoride and incubation period on the protein content of *A. khannae*.

Days/ F($\mu\text{g ml}^{-1}$)		0	5	25	50	100
5	A	9.3	7.7	8.6	6.3	5.3
	B	172.0	139.1	151.6	153.7	155.7
10	A	18.2	17.7	19.1	13.2	6.1
	B	204.5	179.2	192.1	168.6	134.3
15	A	40.8	45.6	40.5	27.4	14.9
	B	233.4	219.1	204.4	236.1	236.7
20	A	92.8	63.3	67.8	30.0	18.5
	B	298.4	204.6	247.4	255.6	262.6
25	A	73.5	92.6	80.6	83.4	27.3
	B	153.5	196.2	208.7	273.6	318.6
C.D. 5%:						
Factor		A($\mu\text{g protein ml}^{-1}$)			B($\mu\text{g protein mg DW}^{-1}$)	
Fluoride		8.4			52.1	
Time		6.4			31.5	
Fluoride x Time		14.9			77.8	
Initial value: $0.780 \mu\text{g ml}^{-1}$						

increased with the time of incubation except for a marginal decrease at 25 d stage (Table 4.13f). At lower level ($5 \mu\text{gF ml}^{-1}$) presence of fluoride benefitted carbohydrate production, but at higher concentrations the process was adversely affected. After 25 d, in presence of $5 \mu\text{gF ml}^{-1}$, the carbohydrate content was 21 % more than control as compared to 23 and 73 % reduction at 25 and $100 \mu\text{gF ml}^{-1}$.

Unlike the protein synthesis, the carbohydrate content per unit dry weight, consistently decreased with increase in the level of fluoride and incubation period. At 25 d stage, increase in fluoride concentration from 5 to $100 \mu\text{g ml}^{-1}$ resulted in 15 % decrease in carbohydrate synthesis.

These observations show that although the increasing fluoride levels competitively reduced the carbohydrate content, the effect on synthesis was of a lower magnitude.

pH AND FLUORIDE ACCUMULATION

A. khannae accumulated significantly high amounts of fluoride. The concentration of fluoride associated with the algal biomass increased with decrease in pH (Table 4.14). The cyanobacterium at $5 \mu\text{gF ml}^{-1}$ accumulated $0.4 \text{ mg F g DW}^{-1}$ from the medium adjusted to pH 6.0. This was reduced to 0.378 and 0.157 when the pH was raised to 7.0 and 8.0. In presence of $50 \mu\text{gF ml}^{-1}$ the accumulated fluoride increased by 12, 5 and 11 folds over $5 \mu\text{gF ml}^{-1}$ at pH 6.0, 7.0 and 8.0 respectively.

The green alga *C. humicola* accumulated comparatively less fluoride (Fig. 4.16). Although in this alga also, the process appeared to be influenced by the fluoride concentration in the medium, but the extent of accumulation was markedly reduced.

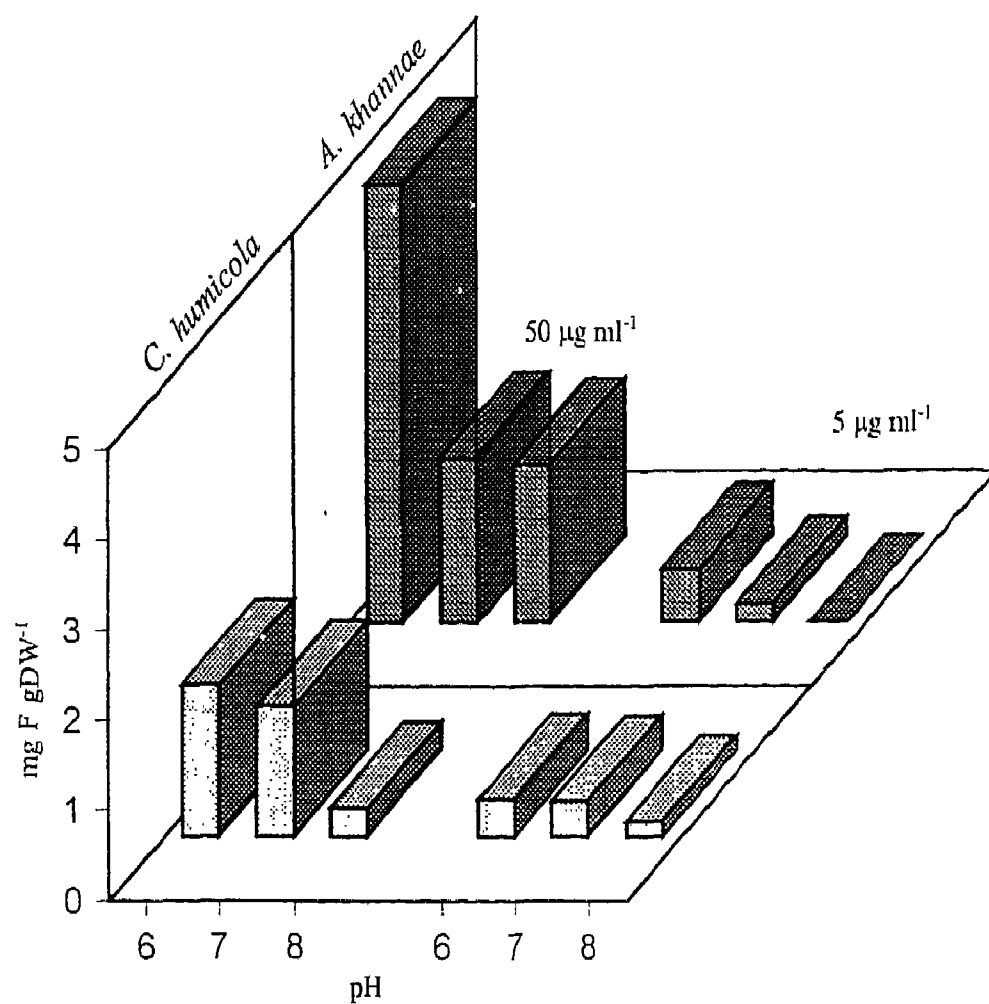
Table 4.13f Effect of graded concentrations of fluoride and incubation period on the carbohydrate content of *A. khannae*.

Days/ F($\mu\text{g ml}^{-1}$)		0	5	25	50	100
5	A	7.5	5.9	6.2	4.0	3.6
	B	138.1	107.4	108.0	97.9	99.0
10	A	18.2	16.5	15.0	7.7	4.0
	B	204.9	166.9	151.5	98.4	91.8
15	A	56.7	59.5	42.9	11.8	8.1
	B	324.0	286.3	216.6	101.3	128.7
20	A	93.0	99.6	51.1	25.5	21.0
	B	299.0	242.4	186.4	191.4	176.9
25	A	86.0	104.3	65.4	54.5	22.7
	B	279.6	220.9	207.5	191.5	187.5
C.D. 5%:						
Factor		A ($\mu\text{g carbohydrate ml}^{-1}$)			B($\mu\text{g carbohydrate mgDW}^{-1}$)	
F		7.16			25.12	
Time		4.93			27.58	
F x Time		11.68			62.87	
Initial value: $0.919 \mu\text{g ml}^{-1}$						

Table 4.14 Effect of pH on fluoride removal efficiency of *A.khannae* and *C. humicola*.

	Dry Weight (mg ml ⁻¹)		Fluoride removed (mg gDW ⁻¹)	
	F in medium (μg ml ⁻¹)			
	5	50	5	50
<i>Anabaena khannae</i>				
pH 6	0.045	0.018	0.402	4.833
pH 7	0.067	0.045	0.378	1.794
pH 8	0.087	0.077	0.157	1.727
<i>Chlorococcum humicola</i>				
pH 6	0.033	0.026	0.2	1.44
pH 7	0.066	0.062	0.58	1.68
pH8	0.182	0.218	0	0.31

Fig. 4.16 Effect of pH on fluoride accumulation by microalgae.



BIOSORPTION

Experiments on biosorption by *A. khannae* showed pH and F concentration dependent variations. Maximum sorption took place at 100 $\mu\text{gF ml}^{-1}$ at all pH values (Table 4.15). The amount removed ranged between 2.1-2.6 mgF gDW⁻¹. Although the observations show higher efficiency of fluoride removal at 100 $\mu\text{gF ml}^{-1}$ at pH 7.0, the differences with values at pH 6.0 and 8.0 were not so significant.

Biomass of *C. humicola* showed a concentration dependent increase in the amount of sorbed fluoride. Maximum biosorption was observed at pH 7.0. Upto 1.96 mg F was found to be sorbed at 100 $\mu\text{g ml}^{-1}$ fluoride treatment. Desorption in 1N HCl did not show any consistent results.

Table 4.15 **Effect of pH on fluoride biosorption in *A. khannae* and *C. humicola*.**

$\mu\text{g F ml}^{-1}$	<i>A. khannae</i>		<i>C. humicola</i>	
	Biosorbed*	Desorbed'	Biosorbed*	Desorbed'
pH 6				
25	0.978	0.051	0.587	0.148
50	1.34	0.067	1.294	0.152
100	2.132	0.126	1.868	0.247
pH 7				
25	1.252	0.046	0.309	0.067
50	1.882	0.017	1.711	0.048
100	2.619	0.124	1.960	0.111
pH 8				
25	1.303	0.002	0.152	0.033
50	1.769	0.139	0.878	0.089
100	2.24	0.173	1.586	0.124

* . ' Observations were made after 2h of biomass suspension in fluoride solution for sorption studies and after 12h of suspension of F containing biomass for desorption in 1N HCl

** values in mg F g^{-1}

Fluoride content in nature was never found to be beyond $3.8 \mu\text{g ml}^{-1}$. Enrichment of samples from selected sites in unamended and F ($20\mu\text{g ml}^{-1}$) amended medium showed similar patterns of algal distribution. Further studies on the extent of F tolerance with eight algal isolates viz., *Lyngbya cryptovaginata*, *Plectonema* sp., *Gloeocapsa aeruginosa*, *Anabaena khannae*, *Scenedesmus bijuga*, *Selenastrum minutum*, *Chlorella* sp., *Chlorococcum humicola* obtained from amended enrichment cultures and four forms obtained from the germplasm collection at NCCUBGA, *Anabaena fertilissima*, *A. iyengerii*, *Westiellopsis prolifica* and *Chlorella minutissima*, showed that tolerance to fluoride varied with species. When exposed to graded levels of fluoride, the cyanobacteria showed 50% lethality at concentrations below $200 \mu\text{gF ml}^{-1}$ except for *Gloeocapsa aeruginosa*. The threshold concentration affecting their growth was $50 \mu\text{gF ml}^{-1}$. However, chlorophytes remained unaffected even upto $150 \mu\text{gF ml}^{-1}$.

These experiments showed that the ambient fluoride concentrations observed in nature should have minimal effects on the autochthonous algae and one does not expect that presence of fluoride at that level will induce any degree of tolerance or resistance in the algae occurring at the test sites. Insensitivity of *Chlorella pyrenoidosa* to 7.9 mM F ($150 \mu\text{g ml}^{-1}$) has been reported by Nichol *et al.* (1987). Hekman *et al.* (1984) also found no significant effect of fluoride upto 2.6 mM ($50 \mu\text{g ml}^{-1}$) on the growth of algae and diatoms. Results of the present study found further support in their observation that at this level, the cyanobacterium *Synechococcus leopoliensis* showed highly reduced rate of growth. Most of the cyanobacteria studied so far have been found to be susceptible to fluoride (Vennesland and Turkington, 1966; Nichol *et al.*, 1987). Based on these reports it can be inferred that freshwater

phytoplankton vary in their sensitivity to the fluoride and that the greens are more stable than the blue greens under fluoride stress. The latter part of the statement gathers support in the response shown by the green algae and cyanobacteria obtained from the germplasm collection, which never had an exposure to fluoride.

The apparent stimulation of growth observed in *Plectonema* sp. and *A. fertilissima* at fluoride concentration of upto $50 \mu\text{g ml}^{-1}$ could be ascribed to hormoligosis, a phenomenon in which sub-harmful amounts of many stress agents may be helpful when presented to organisms in suboptimal environments (Luckey, 1959). A similar enhancement of growth in *Nitzschia palea* and *Amphora coffeaeformis* on exposure to $10\text{-}110 \mu\text{g ml}^{-1}$ fluoride has been reported by Joy and Balakrishnan (1990). However, these authors together with Oliviera *et al.* (1978) suspected a nutritional role of F in algae.

Fluoride toxicity was found to be pH dependent in *A. khannae*. Addition of fluoride at a low pH of 6.0 adversely affected pigment synthesis as well as biomass production. Evidence in favour of interference of fluoride with pigment synthesis has been provided by McNulty and Newman (1961) in higher plants and in the dinoflagellate *Amphidinium carterii* by Klut *et al.* (1981). Fluoride supplementation at higher pH although, reduced biomass production but chlorophyll and carotene synthesis showed no significant change. Increase in pH showed a protective effect against fluoride stress especially at lower F concentrations. Phycobilins were found to be ten times more sensitive to fluoride than chlorophyll. Hypersensitivity of phycobilins to fluoride followed by chlorophyll and carotenes is further supported by the data on pigment ratios.

The synergistic effect of fluoride and low pH in reducing algal growth has

been reported earlier by Nichol *et al.* (1987) for *Synechococcus leopoliensis* and *Oscillatoria limnetica* and on higher plants by Horner and Bell (1995). The observed dependence of fluoride toxicity on pH may be a consequence of the pH transformed chemical form of fluoride in solution. It has been hypothesised that F transport through biological membranes occurs primarily through non ionic diffusion of HF (Armstrong and Singer, 1980) that crosses artificial lipid bilayers five to seven times faster than the F⁻ ion (Gutknecht and Walter, 1980). Since HF is a relatively weak acid (pK_a 3.45; Weast, 1978), significant concentrations of the HF molecule exist at physiological pH and increase as pH falls. Thus it is the HF concentration and not the quantity of fluoride which governs toxicity. HF concentration can be calculated from the Handerson-Hasselbach equation:

$$\text{pH} = \text{pK}_a + \log (\text{F}^-/\text{HF})$$

Application of this equation to the data showed the threshold to be 0.742 μM HF, below which fluoride exerts no influence on growth of *A. khannae*. This assumption holds true for *A. khannae* grown at increasing levels of fluoride (upto 100 $\mu\text{g ml}^{-1}$) and pH 6.0 and 7.0. However, the adverse effect of high concentration of fluoride (50 and 100 $\mu\text{g ml}^{-1}$) on biomass production and phycobilin content at pH 8.0 can not be explained on the basis of this hypothesis.

In contrast to *A. khannae*, the green alga *C. humicola* showed no appreciable effect of F supplementation on biomass production at pH 6.0 and 7.0, whereas a significant stimulation was apparent at pH 8.0. Pigment synthesis varied in concurrence with the changes in biomass at pH 7.0 and 8.0. Tolerance of *Nitzschia palea* and *Amphora coffeaeformis* to comparatively high concentration of F at pH 7.6-8.2 has been reported by Joy and Balakrishnan (1990). They further suggested that

the stimulation of growth upto 90 mg l⁻¹ fluoride could be a result of F⁻ induced increase in cell multiplication. Fluoride at concentrations upto 7.9 mM and pH values down to 5.9 could not generate any growth response in *Chlorella pyrenoidosa* (Nichol *et al.*, 1987). This resistance was correlated to absence of fluoride uptake and the same was found for the chlorophytes studied by Hekman *et al.* (1984). According to these authors, sensitive cells are permeable only to HF while resistant cells are permeable to both F⁻ as well as HF.

Once fluoride has passed through the cell wall it reacts initially with the cytoplasmic membrane. Disruption of cellular membranes may manifest into toxicity as suggested by our results with *A. khannae*.

Leakage of electrolytes from the cells of *A. khannae* measured in terms of changes in the EC of the medium, increased within 5h of suspension in medium containing 50 µg F ml⁻¹ at pH 6.0 and 7.0. This, accompanied with a visible leaching of phycobilins and organic solutes, further supports our contention that the primary site of fluoride action is the cell membrane.

Membrane permeability is known to be altered under various environmental stresses and also when tissues are treated with inhibitors of respiration (Younis, 1969; Younis *et al.*, 1969; McCullough and Simon, 1973). Zwiazek and Shay (1987) reported alterations in membrane permeability causing greater electrolyte leakage in fluoride treated jackpine seedlings. Membranes could be affected either due to insufficient energy available for rapid synthesis of membrane components or because membrane lipids are hydrolysed and used as alternative sources of energy (McCullough and Simon, 1973; Zwiazek and Shay, 1988a,b). However heavy leaching of the electrolytes and phycobiliproteins observed in the present study along

with disruption of cells at higher F concentrations within a few hours of exposure indicates at a direct oxidative damage of the cell envelope.

The green alga *C. humicola* appeared to have more stable cell membrane as it did not exhibit any significant change in the electrical conductivity of the medium. One of the reasons for this may be the substantial increase in pH of the unbuffered medium due to its growth. The alga raises the pH of the medium from 6, 7 and 8.0 to 9.8, 10.2 and 10.3, respectively. The increase in pH might be because of the rapid utilization of nitrate nitrogen, involving an $\text{OH}^-/\text{NO}_3^-$ antiport system leading to a net efflux of OH^- (Deane-Drummond, 1990), aided with a rapid rate of photosynthesis. High pH has been shown to provide protection against fluoride toxicity. A change in the pH value of one unit leads to a ten fold decrease in HF concentration. At pH 10, the absolute concentration of HF is only $7.42 \times 10^{-4} \mu\text{M}$ at $50 \mu\text{g F ml}^{-1}$.

The alga appears to circumvent the fluoride toxicity by way of i) requirement of high threshold concentration of fluoride for induction of a response, and ii) creation of high pH microenvironment around the cells that protects the organism by reducing the availability of HF, the form in which fluoride is able to pass through the cell membrane. The minor amount of fluoride that is able to pass through the cell membrane in some way stimulates the metabolism thereby improving growth. Beneficial effects of lower levels of fluoride on growth and metabolism of plants have been reported earlier (Brewer, 1960; Treshow, 1970; NAS, 1971; Miller, 1993). These authors however, did not consider fluoride as an essential nutrient and the perceived benefits were related to a 'sparing action' of F on the requirement for the halogen chloride .

Inorganic carbon transport is carried out by an active C_4 transport system that

enables -algae to concentrate carbon from the external environment. When this transport process is fully induced, cells are able to utilize both CO_2 and HCO_3^- as substrates (Badger and Andrews, 1982; Miller *et al.*, 1990). ^{14}C uptake in illuminated cultures of *A. khannae* and *C. humicola* was inhibited by $50 \mu\text{g F ml}^{-1}$ at pH 6.0 and 7.0 but remained unaffected at pH 8.0. The inhibitory effect was more severe on *A. khannae* than *C. humicola*. C_i transport in algae is linked to photosynthetic electron transport and ATP generation (Badger, 1987). The observed inhibition might be the consequence of the fluoride sensitivity of membrane ATPases (Giannini *et al.*, 1987a,b) that are involved in active C_i uptake.

In *A. khannae*, loss of ^{14}C uptake activity was accompanied by a complete annihilation of the photosynthetic process in presence of fluoride at pH 6.0. According to Nichol *et al.* (1987) fluoride toxicity is a pH related phenomenon. They observed complete inhibition of photosynthetic O_2 evolution by NaF at pH 6.6 in *Synechococcus leopoliensis* whereas it was only slightly inhibited at pH 8.0 (Hekman *et al.*, 1984). Photosynthesis has been shown to be inhibited in *Chlorella* at acidic pH (Bishop and Gaffron, 1958). The process was reported to be unaffected in *Scenedesmus quadricauda* and *Ankistrodesmus braunii* by $50 \mu\text{g F ml}^{-1}$ concentration.

Vennesland and Turkington (1966) identified two sites of F action. A primary site in the light independent reactions and a secondary site associated with the Hill reaction, the latter being less sensitive than photosynthetic O_2 release. Giannini *et al.* (1985) showed that the site of F action in plants was the thylakoid ATPase. On a similar analogy fluoride might be inhibiting photophosphorylation occurring on algal thylakoids. Nichol *et al.* (1987) suggested that photosynthesis was ATP limited in the presence of fluoride, either as a result of reduction in cytoplasmic pH that decreases

the pH gradient across the thylakoids on which photophosphorylation occurs, or by activation of the plasma membrane proton extruding ATPase leading to ATP consumption.

Reduced carbon uptake on the other hand did not appear to limit the process of photosynthesis in *C. humicola* as PN exhibited no appreciable response to fluoride amendments. In the absence of fluoride at pH 6.0, the rate of photosynthesis showed a significant increase over pH 7.0 but still it was about 50% less than that at pH 8.0. Since the organism was grown in presence of nitrate nitrogen, at low pH, the nitrate uptake is expected to increase tremendously (Deane-Drummond, 1990). Utilization of nitrate is essentially a reductive process requiring continuous supply of NADH produced during respiration. This in turn is regulated by the continuous supply of respiratory substrates synthesised during photosynthesis. Perhaps the observed increase of both respiration and photosynthesis at pH 6.0 was to cope up with the increased demand of reducing power generated by the rapid uptake of nitrate nitrogen.

Alterations in oxygen consumption and CO₂ evolution of plants exposed to fluoride result from changes in glucose metabolism caused as a result of stimulation (Applegate and Adams, 1960) or inhibition (Hill *et al.*, 1959) of respiratory pathways.

The process of respiration in *A. khannae* was considerably tolerant to fluoride toxicity. Rather, a marginal improvement in the activity on F supplementation could be discerned. Inhibition observed with 50 $\mu\text{g ml}^{-1}$ fluoride amendment at pH 6.0 could be a consequence of complete annihilation of photosynthesis causing a scarcity of carbon sources. Stimulation of dark respiration by fluoride was earlier reported in

Chlorella (McNulty and Lords, 1960) and *Synechococcus leopoliensis* (Hekman *et al.*, 1984). Acceleration of O₂ uptake in plants at lower concentrations of fluoride is also known (Yu and Miller, 1967).

A shift in metabolism occurs from glycolysis to pentose phosphate pathway on exposure to fluoride. (Miller, 1958, 1993) This is caused by the inhibition of enolase, a key enzyme of glycolysis. Enolase has also been reported to be inhibited in algae by Hekman *et al.* (1984) on F treatment. However, there was no correlation between enolase inhibition and dark respiration. On the other hand they proposed 'wound respiration' (a secondary response to F induced physiological damage) as the root cause for the observed effects. Fluoride may disrupt basic cellular energetics and thus increase oxygen consumption by increasing ADP levels. Miller (1993) showed that fluoride acts as classical uncoupler of oxidative phosphorylation.

In contrast to *A. khannae*, respiration in the green alga *C. humicola* was inhibited on exposure to fluoride at pH 6.0 and 7.0, while at pH 8.0 the process remained unaffected. Protective effect of rise in pH on respiration has been demonstrated by Younis (1958). Since *Chlorococcum* exhibited no effect on its growth to upto 100 µg ml⁻¹ fluoride after 15d of incubation as observed in earlier experiments the observed inhibition was probably a transient one. Decrease in respiration may be attributed to inhibition of respiratory enzymes. Klut *et al.* (1981) have suggested competitive inhibition by F of enzymes of the Krebs cycle present in the mitochondrial matrix of *Amphidinium carterii*. Miller (1993) on the basis of earlier reports suggested mitochondrial F₁ ATPase as the site of F action. Fluoroacetate inhibition of endogenous respiration in a dinoflagellate has also been proposed. (Hochachka and Teal, 1964).

Thus both the organisms exhibited divergent response to fluoride in terms of photosynthesis and respiration. Inhibition of photosynthesis was accompanied by stimulation of respiration in *A. khannae* while the converse was true for *C. humicola*. The two processes appear to be controlled by a common determining factor ATP/ADP ratio which in turn depends upon the ATPase activity. ATPase activity indirectly regulates numerous enzymes and has been demonstrated to be differentially inhibited by fluoride. While increased ADP pools stimulate respiration, the photosynthetic process becomes ATP limited (Nichol *et al.*, 1987).

Stress on the processes of photosynthesis and respiration leads to alterations in a number of other functions. Diazotrophs fulfill their N requirement by fixing atmospheric nitrogen through the Mo requiring enzyme nitrogenase. Inhibition of nitrogen fixation by fluoride has been shown in plants by Sheridan (1979) and in *Nostoc calcicola* by Rai *et al.* (1980). Bahal and Talpasayi (1971) reported heterocyst inhibition in *Anabaena ambigua* under F stress. In the present study a marked inhibition of nitrogen fixation was observed in fluoride stressed *A. khannae*. The cyanobacterium showed a dose dependent decrease in nitrogenase activity at all the pH levels. The effect became more prominent after 48 h. The inhibition could be a result of direct involvement of fluoride with the enzyme or more logically due to alterations in photosynthesis and respiration that provide the necessary reductants and carbon skeletons for this energy intensive process.

The active uptake or concentration of elements by living microbial cells is termed bioaccumulation. Hekman *et al.* (1984) reported bioconcentration of fluoride in cyanobacteria and a diatom *Cyclotella meneghiniana* in presence of 50 $\mu\text{gF ml}^{-1}$ at an unstated pH. In present study, both *Anabaena* and *Chlorococcum* showed a

concentration dependent increase in F uptake, which was effectively subdued at high pH. The cyanobacterium accumulated comparatively more fluoride than the chlorophyte which may be because of the apparent differences in the cell wall organisation and the physiology of the two organisms. According to Gutknecht and Walter (1980), Whitford (1983) and Nichol *et al.* (1987), fluoride crosses the cell membrane almost exclusively as HF. If this be true, there should have been negligible accumulation of fluoride at pH 8.0. However the concentration of fluoride associated with the biomass of *A. khannae* at 50 $\mu\text{g F ml}^{-1}$ was comparable to that accumulated at pH 7.0. Though no significant effect could be observed on the processes of photosynthesis and respiration that involved short term exposures to fluoride, long term experiments on growth of *A. khannae* showed a reduction in overall growth of the organism with high F concentrations (50 & 100 $\mu\text{g ml}^{-1}$). This implies that on extended exposure, fluoride does enter the cell even at a high pH to inhibitory levels, albeit at a much slower rate.

Resistance to fluoride in *C. humicola* inspite of the observed accumulation may be attributed to the higher threshold concentration of fluoride for inducing any response as mentioned earlier. A complex cell wall and intracellular compartmentalization as compared to the primitive prokaryotic organisation of *A. khannae* might be advantageous to the green alga. That, F enters the cells of *C. humicola* at low pH and high fluoride concentrations can be derived by the fact that an appreciable response in relation to fluoride was exhibited by the organism in terms of photosynthesis and respiration at pH 6.0 and 7.0.

CONCLUSION

Studies on the seasonal variation in the distributional pattern of algae in the four fluoride infested water bodies showed that presence of fluoride should have minimal effects on algal populations. The distribution observed was more a reflection of climatic changes rather than variation in fluoride level. Response of isolated autochthonous algae and strains from the germplasm collection to graded fluoride concentrations further supported this observation. None of the algal forms tested showed any effect of fluoride upto $25 \mu\text{gF ml}^{-1}$ concentration. Chlorophytes were more comfortable with fluoride and remained unaffected even at $200 \mu\text{g ml}^{-1}$ as compared to the cyanobacteria that showed a threshold concentration of $25 \mu\text{g ml}^{-1}$.

Detailed studies with the widely distributed green alga *Chlorococcum humicola* and the cyanobacterium *Anabaena khannae*, showed that fluoride toxicity was pH and concentration dependent. The enhancement of toxicity at low pH and the mollifying effect of high pH can be correlated to the species of fluoride which is pH specific. At low pH, fluoride exists as HF, the form in which it can easily cross the cell membrane barrier and accumulate in the cell to toxic levels.

Comparatively more complex cell wall in *C. humicola* may impede the entry of fluoride into the cell specially at lower concentrations. Physical compartmentalization of various metabolic processes is expected to need higher intracellular fluoride concentrations. Stability of the green alga at higher fluoride concentrations is evidently the consequence of these phenomena. The resistance may be further enhanced by the rising pH of the medium due to progressive utilization of nitrate nitrogen by the green alga.

Fluoride has diverse effects on various physiological processes. While *C. humicola*

exhibited stimulation of photosynthesis, the respiratory process was inhibited. Converse was true for the cyanobacterium. It has been suggested that probably both the processes are regulated by the ATP/ADP levels. Increase in ADP pools stimulated respiration while at the same time photosynthesis becomes ATP limited. Inhibition of photosynthesis in *A. khannae* showed a good correlation with C_1 uptake which was limited probably as a consequence of membrane disruption. Alterations in photosynthesis and respiration also manifested into inhibition of nitrogenase activity.

Fluoride scavenging through algal biomass appears to be mainly a process of biosorption. In the blue green alga, the polysaccharidic sheath provided an ideal surface for sorption of fluoride, compensating the filamentous nature of the alga. In *C. humicola*, the microscopic unicellular nature provided substantially increased cell surface for adsorption of fluoride.

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