

**Ecophysiological Studies on some Algae and Bacteria
of Waste Stabilization Ponds**

by

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Thesis submitted in accordance with requirements of the
University of Liverpool for the Degree of Doctor in Philosophy

November, 1984

I dedicate this thesis to Dr C. G. SETTLE for his
endless patience, encouragement and support.

Field research on Waste Stabilization Ponds (W.S.P.) was carried out at EXTRABES (Station for the Biological Treatment of Sanitary Sewage) in the city of Campina Grande ($7^{\circ} 13' 11''$ S, $35^{\circ} 52' 31''$ W) Paraiba State, NE Brazil.

Two W.S.P. Systems were studied, both receiving raw municipal sewage: System I, a five-pond series comprising an anaerobic pond (A_1) receiving raw sewage, followed by a facultative pond (F_1) and three maturation ponds (M_1 , M_2 and M_3). System II comprised four-independently loaded facultative ponds (F_2 , F_3 , F_4 and F_5), each receiving raw sewage.

Algological research started in January 1978 and three sets of experiments were carried out at different BOD_5 surface loadings.

Samples of the pond effluent (at 5 cm below the surface) were obtained at 08.00h weekly and analysed for chlorophyll a concentration, algal genera identification and counting. Samples of the pond water column (collected at 5 different points of the pond) were also taken and analysed for the same parameters.

Algal genera present were related to the degree of treatment achieved. Flagellate genera, Euglena, Chlamydomonas, Phacus and Pyrobotrys, predominated in facultative ponds, whereas non-motile Scenedesmus, Chlorella and diatoms dominated in maturation ponds.

Algal densities (measured as chlorophyll a concentration) were greater between 170 and 230 $kg\ BOD_5\ ha^{-1}\ d^{-1}$. Ponds with loadings approaching 500 $kg\ BOD_5\ ha^{-1}\ d^{-1}$ supported little algal growth and ceased to function as W.S.P.'s.

Diurnal studies on the effluent of a maturation pond M_3 and a facultative pond F_5 were carried out to determine how the algal population might influence other effluent parameters. Greater fluctuation in the algal population and in the associated BOD_5 and SS, occurred in the effluent from F_5 than in M_3 .

Changes in effluent colour, from bright green to colourless was evident (particularly in facultative ponds), during effluent sampling. This colour variation suggested a diurnal movement and stratification of the algal population. In the facultative pond F_5 (where flagellate algae predominated) stratification during day light hours was more pronounced than in the maturation pond M_3 .

Algal primary productivity was measured by the oxygen light and dark bottle technique in pond F_1 , fed by effluent from an anaerobic pond, the maturation pond M_3 , the final pond of a five pond series and two facultative ponds receiving raw sewage (F_2 and F_4) at different BOD_5 surface loadings. Oxygen production decreased in all the ponds studied with increasing BOD_5 surface loadings. Gross primary production was reduced nearly to zero at BOD_5 surface loadings around 400 $kg\ BOD_5\ ha^{-1}\ d^{-1}$.

The laboratory studies show that selection may occur in the Escherichia coli population as a result of the different pond environments i.e. raw sewage, facultative and maturation ponds. This selection process makes it imperative that when experimental work is carried out on the bacterial die-off using E. coli the organism must have been isolated from the appropriate pond rather than from raw sewage.

The pH rather than high concentrations of ammonia, is the major factor reducing the growth of the bacterial population.

Of the two algal genera studied, Euglena appears to be more sensitive to ammonia than Chlorella, particularly at pH values above 7.0.

This thesis demonstrates the importance of algae as components of the W.S.P. system. Pond design must incorporate features which will provide optimum conditions for algal growth.

Acknowledgements

I would like to thank

- my supervisor, Dr H. W. Pearson for his assistance during the writing of this thesis;

- Prof. D. H. Jennings, for the use of the facilities of the Botany Department;

- my colleagues, Dr A. M. L. Jawad, Dr G. Malin, Dr W. D. F. Jardin, Dr C. G. Settle, Mrs C. L. Paiva (M.Sc.) and Miss R. C. R. Machado (M.Sc.) for their help, criticism and encouragement;

- all the staff of the Botany Department that so kindly helped me with all my requests;

- Mrs D. Lewis and Miss J. Farrell for typing this thesis;

- Mr Adielson Ferreira (M.Sc.) and Mr Jose Gomes from the Civil Engineering Department of the Federal University of Paraiba, Campina Grande, Brazil, for giving me the opportunity to do this Ph.D.

- Dr S. A. Silva, Chief of EXTRABES, where part of the work for this thesis was carried out;

- all the staff and colleagues of EXTRABES, especially members of the Algology Section, for their assistance in collecting the fieldwork data.

- and finally the British Council and the University of Paraiba for their financial support.

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CHAPTER 1

INTRODUCTION

The increasing world population, and the drift of people towards the cities and towns, have brought with it the problems associated with the disposal and treatment of wastes.

In industrialized countries, where the great majority of the population is connected to a sewage system, wastewater treatment is carried out in conventional treatment plants comprising secondary treatment by activated sludge systems or trickling filters and anaerobic digestion of sludge. This plant has high maintenance costs and energy demands, and also needs careful operation by trained and qualified personnel. These factors frequently militate against the widespread use of such systems in many developing countries. In addition, "conventional" treatment systems often do not provide any significant degree of pathogen removal, so important in developing countries where endemic pathogen levels in the population are high and medical facilities less than adequate.

Waste disposal is a very acute problem in developing countries where only 32% of the population may have adequate excreta and sewage disposal systems. It is in these countries that low cost and efficient methods of wastewater treatment are urgently needed.

It has long been accepted that tropical and subtropical climates provide an ideal environment for the use of Waste Stabilization Ponds (W.S.P.) as a method of waste water treatment (Ludwig et al. 1951). The advantages of using W.S.P. over conventional systems can be considerable and include their low cost of construction, maintenance and operation and the high percentage removal of pathogens and organic matter (Cadwell, 1946; Gloyna and Herman, 1956; Oswald et al., 1957; Herman, 1962; Gloyna and Espino, 1969). Furthermore, in arid and semi-arid regions of the world, provided that suitable precautions are taken, the effluent of a W.S.P. can be reclaimed to produce water for domestic use (Shillinglaw and Piertese, 1977) and for crop irrigation.

Although the disposal of human waste by a water borne sewerage system is the best from a public health point of view, its use, especially in rural areas, is often impractical, because of the high cost of construction. In these cases the use of other types of non-water borne excreta disposal are preferred, and include night soil collection, pit latrine, pour flush toilets and septic tanks. The choice of any method to a community in particular will depend on a careful integration of economical, social, climatological and design factors.

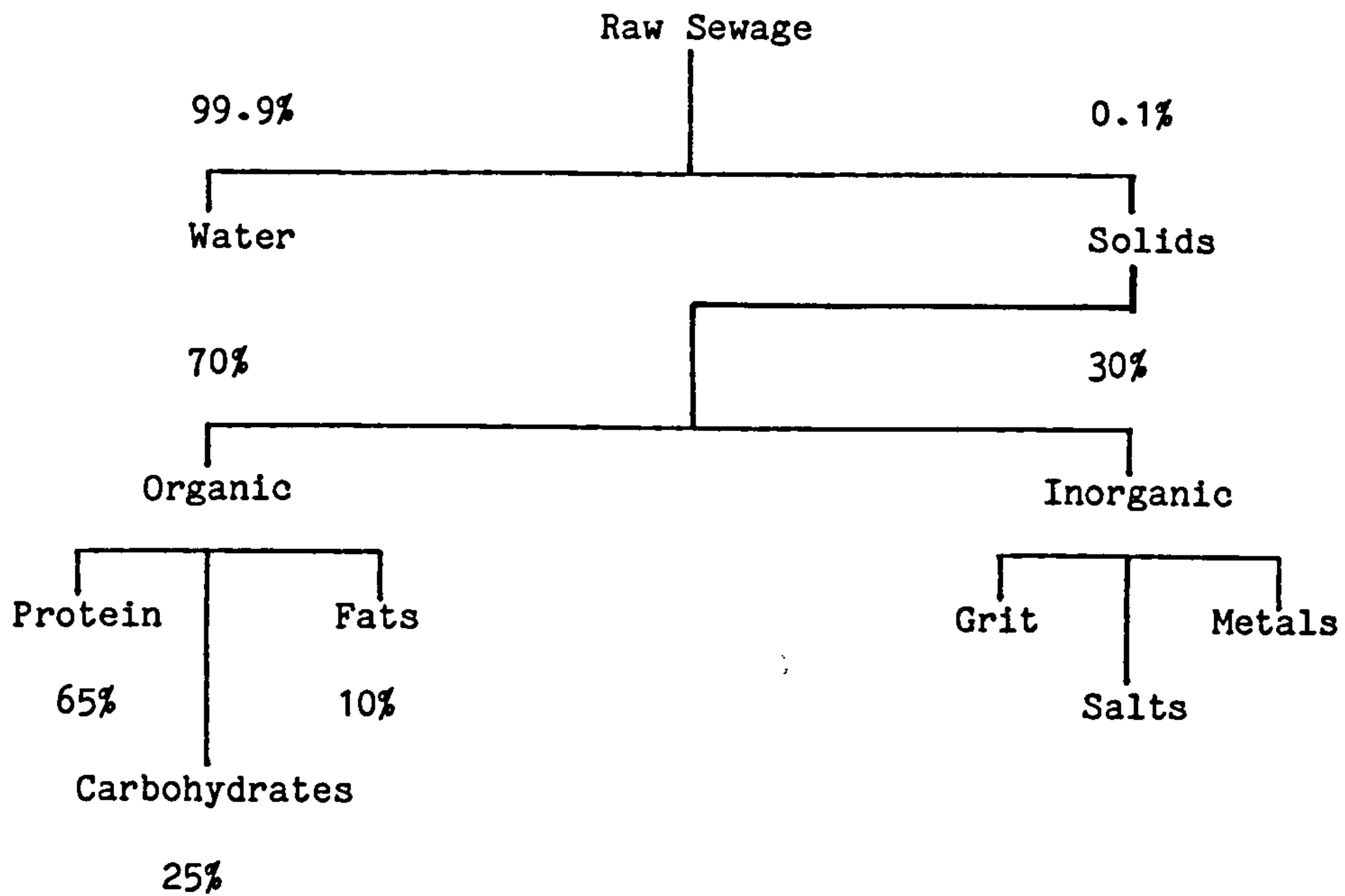
Ponds have been used for centuries to store and treat animal and household wastes _____ However, it was only in 1924

that the potential of oxidation ponds for sewage treatment was
accidentally discovered.^(Gray, 1940) Nowadays in the U.S.A. ponds are used
throughout the country, even in Alaska (1/3 of the domestic and
industrial waste water in the U.S.A. is treated by W.S.P.).
Successful experiences have also been reported from all over the
world, in the U.S.A. (Parker, 1962), Brazil (Silva, 1982), Israel
(Watson, 1962), South Africa (Shaw et al., 1962).

Wastewater or sewage can be purely domestic or contain indus-
trial discharges, agricultural run-off and urban storm drainage.
Domestic sewage, in particular, comprises human excreta and
sullage and, when fresh, is a grey turbid water with an inoffen-
sive odour. Human excreta comprises mainly faeces and urine
while sullage is the wastewater resulting from personal washing,
laundry, food preparation and the cleaning of kitchen utensils
(Mara, 1976). The composition of a typical domestic sewage is
shown in Diagram 1.1.

The chemical composition of sewage is complex and therefore
special parameters are used to characterize it. The principal
parameter is the oxygen demand, i.e. the amount of O_2 required to
oxidize the organic matter present in the sewage. This oxygen
demand of a waste water can be expressed in terms of COD and
BOD₅.

Diagram 1.1. - Composition of Sewage (Tebbutt, 1970)



COD measures the oxygen necessary to totally oxidize (chemically) the organic matter present in the waste into carbon dioxide and water.

BOD is usually expressed on a 5 day 20°C basis. Details of the methods for the measurements are found in APHA (Standard Methods, 1980).

Measurements of BOD₅ and COD are used to judge the sewage strength of a community. The higher the concentration of organic waste present the stronger the sewage is considered to be. Sewage strength is governed largely by the the water consumption of the community, thus in U.S.A. the water consumption is high, and

the sewage is weak ($BOD_5 = 200$ to 300 mg l^{-1}) whereas in tropical countries the sewage is strong ($BOD_5 = 400$ to 700 mg l^{-1}) as the water consumption is relatively low (Mara, 1976).

Sewage contains a large number of commensal bacteria originally from the human gut. The types of bacteria species commonly found in human faeces are listed in Table 1.1., which also shows that their numbers vary with community type and diet.

Some of the bacteria excreted are pathogens which give rise to disease (Table 1.2) although these virulent types are not normally found in the intestine of healthy people.

Human faeces can contain different types of excreted protozoal and helminth pathogens (Table 1.3) which also represent a health risk and have to be removed from the environment.

In warm developing countries sewage is frequently discharged without treatment into surface waters i.e. rivers, lakes, oceans and estuaries and the same water is utilized for drinking, swimming, bathing and washing. These poor sanitary conditions in addition to the lack of personal hygiene are very common. Under such circumstances the diseases caused by pathogenic bacteria present in the faeces easily spread causing considerable losses in human life, especially among young children.

Table 1.1 Bacterial microflora of human faeces by national diet
(from Feachen et al., 1983)

		Number of bacteria in faeces (mean log ₁₀ per gram)						
National diet	Country	Enterobacteria ^{a,b}	Enterococci ^b	Lactobacilli	Clostridia	Bacteroides	Bifidobacteria	Eubacteria
Largely carbohydrate	Guatemala	8.7	7.9	9.0	9.3	10.3	9.4	ND
	Hong Kong	7.0	5.8	6.1	4.7	9.8	9.1	8.5
	India	7.9	7.3	7.6	5.7	9.2	9.6	9.5
	Japan	9.4	8.1	7.4	5.6	9.4	9.7	9.6
	Nigeria	8.3	8.0	ND	5.9	7.3	10.0	ND
	Sudan	6.7	7.7	6.4	4.9	7.8	8.5	ND
	Uganda	8.0	7.0	7.2	5.1	8.2	9.4	9.3
Mixed Western	Denmark	7.0	6.8	6.4	6.3	9.8	9.9	9.3
	England	7.9	5.8	6.5	5.7	9.8	9.9	9.3
	Finland	7.0	7.8	8.0	6.2	9.7	9.7	9.5
	Scotland	7.6	5.3	7.7	5.6	9.8	9.9	9.3
	United States	7.4	5.9	6.5	5.4	9.7	9.9	9.3

ND. No data

Sources: England, India, Japan, Scotland, United States, Uganda (Drasar 1974); Denmark, Finland (International Agency for Research on Cancer 1977); Hong Kong (Crowther and others 1976; Nigeria, Sudan (Draser, personal communication); Guatemala (Mata, Carrillo and Villatoro 1969).

a. This group mainly contains Escherichia coli.

b. These two groups are the most commonly used faecal indicator bacteria.

Table 1.2 Bacterial pathogens excreted in faeces
(from Feachen et al., 1983)

Bacterium	Disease
<u>Campylobacter fetus</u> ssp. <u>jejuni</u>	Diarrhoea
Pathogenic <u>Escherichia coli</u> ^a	Diarrhoea
<u>Salmonella</u>	
<u>S. typhi</u>	Typhoid fever
<u>S. paratyphi</u>	Paratyphoid fever
Other salmonellae	Food poisoning and other salmonellosis
<u>Shigella</u> spp.	Bacillary dysentery
<u>Vibrio</u>	
<u>V. cholerae</u>	Cholera
Other vibrios	Diarrhoea
<u>Yersinia enterocolitica</u>	Diarrhoea and septicemia

a. includes enterotoxigenic, enteroinvasive, and enteropathogenic E. coli.

Therefore from the public health point of view an appropriate system of waste collection, treatment and disposal in any communal area, large or small, is necessary to prevent the spread of disease.

Table 1.3 Protozoal pathogens excreted in faeces (from Feachen et al., 1983)

Protozoon	Disease
<u>Balantidium coli</u>	Diarrh ^o ea, dysentery and colonic ulceration
<u>Entamoeba histolytica</u>	Colonic ulceration, am ^e bic dysentery, and liver abscess
<u>Giardia lamblia</u>	Diarrh ^o ea and malabsorption

Helminthic pathogens excreted in faeces

Helminth	Common name	Disease
<u>Ancylostoma duodenale</u>	Hookworm	Hookworm
<u>Ascaris lumbricoides</u>	Round worm	Ascariasis
<u>Clonorchis sinensis</u>	Chinese liver fluke	Clonorchiasis
<u>Diphyllobothrium latum</u>	Fish tapeworm	Diphyllobothriasis
<u>Enterobius vermicularis</u>	Pinworm	Enterobiasis
<u>Fasciola hepatica</u>	Sheep liver fluke	Fascioliasis
<u>Fasciolopsis buski</u>	Giant intestinal fluke	Fasciolopsiasis
<u>Gastrodiscoides hominis</u>	n.a.	Gastrodiscoidiasis
<u>Heterophyes heterophyes</u>	n.a.	Heterophyiasis
<u>Hymenolepis nana</u>	n.a.	Hymenolepiasis
<u>Metagonimus yokogawai</u>	n.a.	Metagonimiasis
<u>Necator americanis</u>	Hookworm	Hookworm
<u>Opisthorchis felinus</u>	Cat liver fluke	Opisthorchiasis
<u>O. viverrini</u>	n.a.	"
<u>Paragonimus westermani</u>	Lung fluke	Paragonimiasis
<u>Schistosoma haematobium</u>	Schistosome	Schistosomiasis; bilharziasis
<u>S. japonicum</u>	"	"
<u>S. mansoni</u>	"	"
<u>Strongyloides stercoralis</u>	Threadworm	Strongyloidiasis
<u>Taenia saginata</u>	Beef tapeworm	Taeniasis
<u>T. solium</u>	Pork tapeworm	Taeniasis
<u>Trichuris trichiura</u>	Whipworm	Trichuriasis

n.a. Not applicable

The effectiveness of any method of sewage treatment in reducing the number of pathogenic bacteria has to be measured in some way. This has been currently done by monitoring the numbers of a chosen faecal indicator organism.

A faecal indicator is a diagnostic organism, selected from the various bacteria found in human excreta, to indicate environmental faecal pollution and the possible presence of pathogens.

Many bacteria have been proposed as faecal indicators. The selection and use of Faecal coliforms (FC) and Faecal streptococci (FS) as indicators of warm blooded animal pollution arises from the fact that they fulfil almost all the requirements for an ideal bacterial indicator (Feachen et al., 1983). These include the fact that there are simple and relatively reliable laboratory procedures available for their identification and enumeration (Klock, 1971). FC are facultative anaerobes, gram negative, non-spore forming rods that ferment lactose with acid and gas production at 44°C within 24-48 hours. The most common FC, E. coli, can also produce indole from typtophan at that temperature. The FS group includes organisms mainly associated with animals but some species occur both in man and animals. Additionally some strains appear to be ubiquitous in both polluted and unpolluted environments. Aside from the problem of non-faecal strains, the FS have major advantages over the FC as indicators

these include their simple enumeration by the membrane filtration technique at 37°C (temperature readily obtained in any small laboratory) and the fact that they survive longer than FC and are less likely to show regrowth after chlorination (Feachen et al., 1983).

Although the presence of faecal indicator organisms is assumed to show the possible pathogen presence it does not show the level of the pathogen population or the pathogen type (virus, protozoa, cysts and helminth eggs).

The efficiency of W.S.P. in removing pathogen bacteria, helminths and protozoa has been extensively reported (Davis and Gloyna, 1972; Marais, 1974; Mara et al., 1983). Among the causes leading to FC and pathogen die-off in ponds are the exhaustion of their internal food resources after a period of time and lack of successful competition with other organisms in the pond, (Klock, 1971). Thus retention time in the W.S.P. system is an important factor. Factors such as temperature (Geldreich et al., 1964), U.V. radiation (Moeller and Calkins, 1980) and algal concentration are also important.

Reduction of E. coli (99.9%) and S. faecalis (99.9%) with variation in temperature and retention time were reported by Parker (1962). Efficiencies in FC removal higher than that (99.99993%) have been reported by Mara et al. (1983) with a retention time of 28.5 days.

The removal of organic matter (expressed as BOD₅) is achieved in W.S.P. ponds through two processes, viz. anaerobic and aerobic digestion. According to the relative dominance of each of these two processes, W.S.P. can be classified into anaerobic, facultative or aerobic (maturation) ponds.

Anaerobic ponds are heavily loaded ponds where anaerobic digestion of organic matter is carried out by acid forming and methanogenic bacteria. The former group convert organic compounds into organic acids, mainly acetic acid which is converted to methane, carbon dioxide and water by the latter group. The success of an anaerobic pond depends on the balance between these two types of bacteria, on temperatures remaining above 15°C and a pH between 6.8-7.4. A well designed anaerobic pond with a retention time of 24 h can remove at least 50% of the BOD₅ (Mara, 1977). Therefore the incorporation of an anaerobic pond as an initial treatment unit can reduce land requirements.

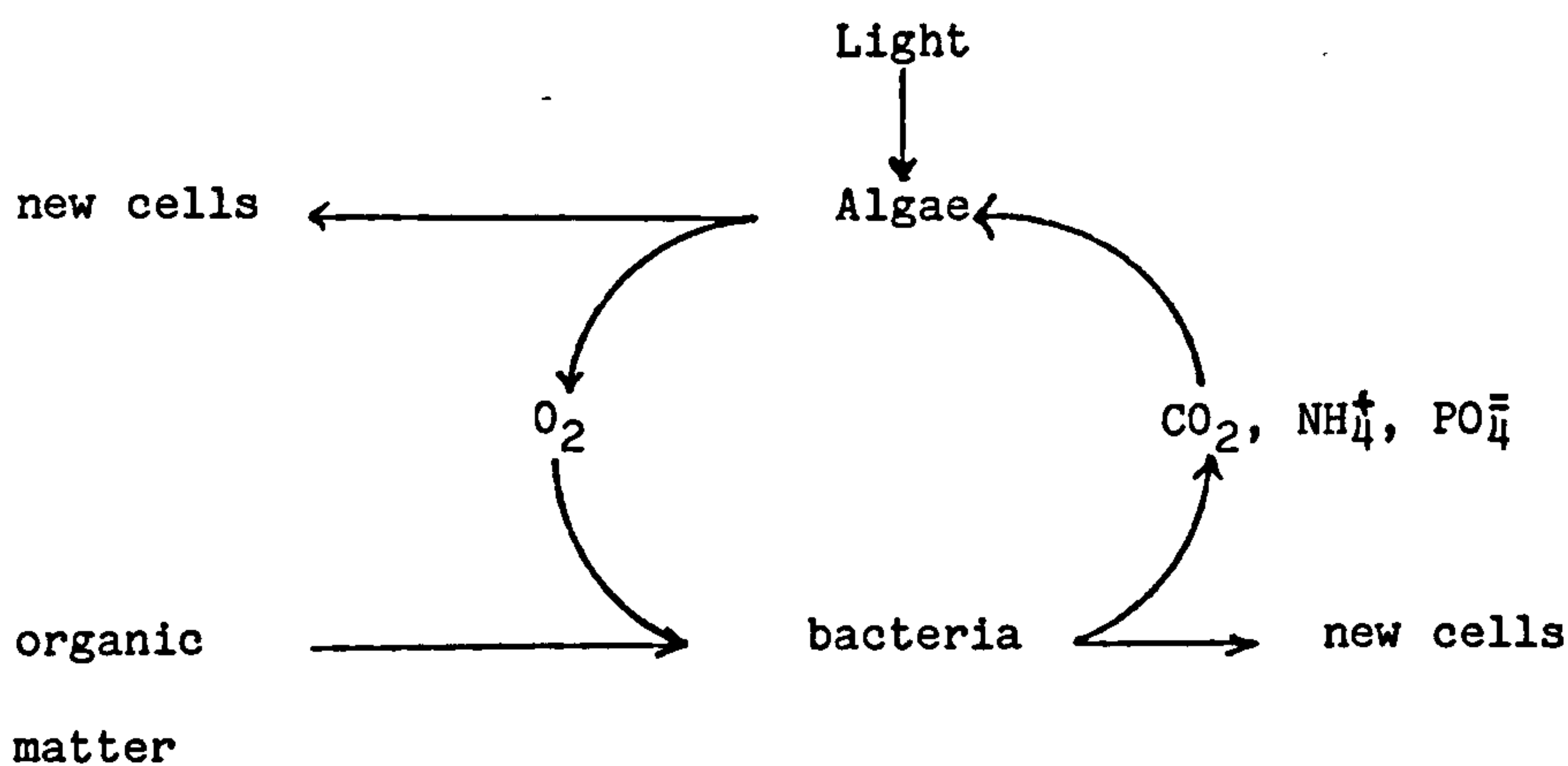
Facultative ponds are those ponds where aerobic conditions prevail towards the water surface and anoxic conditions exist towards the bottom.

Raw sewage or effluent from an anaerobic pond, septic tank or conventional treatment can be further treated by facultative ponds.

In a facultative pond bacterial oxidation processes convert

much of the organic material to carbon dioxide, ammonia and phosphate (Ludwig et al., 1951). Pseudomonas, Flavobacterium and Alcaligenes are some of the bacteria genera involved in these oxidation processes. The presence of these nutrients creates an environment suitable for the development of algae and these, through photosynthetic activity produce more oxygen. This oxygen is available for further bacterial oxidation of the organic material. The complementary activity between algae and bacteria has been extensively reported (Oswald et al., 1953; Neel and Hopkins, 1956; Towne & Davis 1957; Patil et al., 1975) and is illustrated in Diagram 1.2.

Diagram 1.2 The complementary activity of algae and bacteria in W.S.P.



There is some evidence that sewage pond algae can also utilize and thus degrade the organic matter (Oswald et al., 1953; Pipes and Gotaas, 1960).

W.S.P. oxygen supply depends upon the photosynthetic activity of the algae with only a small amount of oxygen coming from re-aeration through the water surface air interphase. Dissolved oxygen concentration in a facultative pond fluctuates with the photosynthetic activity of the algae with highest daily concentrations being attained in the late afternoon (Neel and Hopkins, 1956; Patil et al., 1975). Algae photosynthesis can sometimes remove carbon dioxide more rapidly than it is replaced by bacterial activity, causing an increase in the pond's pH. Values as high as pH 10.5 have been reported (Cadwell, 1946).

Pond water temperature approaches that of the air (Neel and Hopkins, 1956) and shows a daily pattern similar to solar radiation (Bush et al., 1961). On hot days, thermal stratification in the pond water column can occur and this inhibits mixing between the upper and lower layers of water because of their differences in density (Stahl and May, 1967). Mixing is important because it minimizes short-circuiting, ensures a reasonable vertical distribution of organic matter (BOD), oxygen and some algae, (especially the non-motile types which might otherwise settle on the sludge layer at the bottom).

Sludge is formed, at the bottom of a facultative pond, by the settling of the entering solids, but these will undergo anaerobic digestion if temperature and pH are adequate. The removal of

sludge from a facultative pond is therefore rarely required, only perhaps once every 10 years or so.

A depth of 1.5 m for a facultative pond is generally accepted as a compromise between the development of anaerobic conditions at greater depth and the danger of emergent vegetation and mosquito breeding at shallower depths. In arid regions where evaporation is high, water losses can be minimized by increasing pond depth up to 20 m (Mara, 1976).

Maturation ponds or aerobic ponds are employed either as the final treatment process in the sequence anaerobic-facultative-aerobic or as ponds to "polish" the effluent from conventional treatment plants. Maturation ponds are included in a W.S.P. system mainly to destroy pathogenic organisms and viruses and do not further reduce the BOD₅ to any great extent. The cysts and ova of intestinal parasites are removed from the sewage by settling in the bottom of the pond where they eventually die (Mara, 1976).

In maturation ponds the presence of the algae (Fogg, 1962), the greater number of algal species present (Toms, et al., 1975), and the high pH from algal photosynthesis (Parhad and Rao, 1974) together with other physical factors, (e.g. wind action, Marais, 1974) and climatological factors (e.g. ultraviolet light, Moeller and Calkins, 1980) are suggested to have an effect on the bacterial die-off rate.

A better quality effluent (both in terms of BOD and pathogen content) is produced by using a series of small ponds rather than a single pond of the same overall area (Gloyna and Herman, 1956; Marais, 1966). It is also known that maximum bacterial removal by ponds in series is achieved when the retention time of each pond is the same (Marais, 1974). It might be that ponds in series produce a better mixing pattern and reduce the phenomenon of short-circuiting (Parker, 1962).

In tropical countries the acceptable pond layout is either a facultative pond receiving raw sewage, followed by 2 or 3 maturation ponds or a system comprising an anaerobic, facultative and 2 or 3 maturation ponds

High Rate Algal Ponds (H.R.A.P.) or high-rate Waste Stabilization ponds are designed for the rapid conversion of organic wastes into algal biomass. They have received increasing attention as means of treating sewage (Gloyna, 1971) and producing utilisable algal biomass (Oswald, et al., 1953; Golueke et al., 1967; Shelef et al., 1980).

Since these ponds are very shallow, with depths varying from 0.2 to 0.6 m, the photic zone can be extended almost to the pond bottom; consequently high algae yield is achieved. In order to reduce the BOD₅ in the receiving water, the algae have to be removed from the effluent. The high cost of algal removal

methods and the need for skilled personnel for their operation and maintenance are a disadvantage for H.R.A.P. use, particularly in developing countries. In addition, the effluent produced has not an adequate bacteriological standard since they function at very short retention times (1 to 5 days).

The types of algae found in W.S.P. vary considerably but usually algae present in sewage belong to four Phyla; Cyanobacteria (e.g. Oscillatoria), Chlorophyta (Chlamydomonas, Chlorogonium, Chlorella), Euglenophyta (Euglena and Phacus) and Bacillariophyta (Navicula and Nitzschia).

A dense algae population in W.S.P. is indicative of healthy conditions in the pond (Parker, 1962). Apart from producing oxygen, algae are also important in the reduction of soluble salts especially ammonia and orthophosphate in the effluent (Rasche, 1970; Shillinglaw and Piertese, 1977).

Bush et al. (1961) give a detailed account of the various soluble inorganic salts removed from sewage during algal growth. Removal of more than 90% of the influent ammonia under normal summer conditions has been reported by van der Post and Engelbrecht (1973) and this efficiency was reduced by a decline in algal population.

Although ammonia can be efficiently utilized by alga ; concentrations over 2.0 mM at pH 8.0 or higher can inhibit algal

growth and photosynthesis (Abeliovich and Azov, 1976).

The phosphates are removed by precipitation, as hydroxyapatite, at high pH values which develop as a result of photosynthesis. The precipitation of phosphate starts at about pH 8.2 and for every increase of one unit of pH above that the concentration of phosphate remaining in the water decreases by a factor of 10 (Ellis, 1983).

Although vital for the treatment process, excessive algal growth in W.S.P. often causes problems when large numbers of algae are present in the effluent. These algae can contribute significantly to the BOD₅ which, if not diluted properly before discharge, can increase the oxygen demand of the receiving water body (Gloyna and Herman, 1956).

Tropical countries provide the ideal climatic conditions for the utilization of W.S.P. Intense solar radiation results in high pond water temperature, and periods of intense photosynthetic activity provide an accumulation of dissolved oxygen during the day for the use of the microorganisms during the night.

The overall advantages and disadvantages of various sewage treatment systems are presented in Table 1.4.

It is noticeable from Table 1.4 that W.S.P. either including or excluding anaerobic treatment are undoubtedly the best method

Table 1.4: Advantages and Disadvantages of Various Sewage Treatment Systems (from Arthur, 1982).

	Activated Sludge Plant	Trickling Filter	Extended Aeration Plant	Oxidation Ditch	Aerated Lagoon System	Waste Stabilization Pond System (including anaerobic units)	Waste Stabilization Pond System (excluding anaerobic units)
<u>Criteria</u>							
BOD ₅ Removal	**	**	**	***	***	***	***
FC Removal	*	*	**	**	***	***	***
SS Removal	***	***	***	***	**	**	**
Helminth Removal	**	*	*	**	**	***	***
Virus Removal	**	*	**	**	***	***	***
Ancillary Use Possibilities	*	*	*	*	***	***	***
Effluent Reuse Possibilities	*/a	*/a	**	**	***	***	***
Simple and Cheap Construction	*	*	*	*	*	***	***
Simple Operation	*	**	*	**	*	***	***
Land Requirement	***	***	***	***	**	**	*
Maintenance Costs	*	**	*	*	*	***	***
Energy Demand	*	**	*	*	*	***	***
Minimization of sludge for removal	**/b	**/b	**/b	*	**	***	***

a/ The effluents from activated sludge, and trickling filter frequently have high ammonia levels (> 5mg/l) and faecal bacterial concentrations, and are usually not suitable for irrigation or fish farming without tertiary treatment.

b/ Assumes provision of sludge digesters.
Key: *** good; ** fair; * poor.

of sewage treatment in tropical countries. Compared to the other sewage treatment systems, W.S.P. land requirements are high. However, in many developing countries, especially in the tropics, this is rarely a disadvantage since sufficient land is normally available at low cost (Mara, 1976).

1.1 Introduction to the Current Work

The biology of Waste Stabilization ponds is a large and complex topic and one that will need considerable further research and investigation if pond design is to be improved and rationalised on the basis of biological activity rather than on empirical engineering equations.

This thesis wishes to contribute to such a design improvement, and to indicate how pond performance might be efficiently monitored. The investigation can be divided into two interrelating sections. Firstly, the ecophysiological studies which have been made on the algal population and the Faecal Coliform (FC) bacteria present in the experimental pond complex at the Federal University of Paraiba at Campina Grande north-east of Brazil.

The field studies have concentrated on trying to determine the relationship between organic loading (and other related physico-chemical parameters) and algal speciation, activity and primary productivity. Some attempt has also been made to relate the

dynamics of the algal population to treatment efficiency and effluent quality in the various Waste Stabilization pond types.

Limited field studies have also been made on the diurnal activity of F.C. indicator bacteria with a view to aiding our understanding of the factors controlling their die-off rate.

Secondly, a series of laboratory experiments have been performed in an attempt to understand in more detail:

i) The nutritional and physical factors which may be important in affecting the growth of different algal species in Waste Stabilization Ponds;

ii) Algal/algal interactions which may affect species balance;

iii) The significance of ammonia toxicity in controlling algal activity and also that of E. coli isolates from sewage ponds.

CHAPTER 2

THE BASIC MATERIALS AND METHODS USED IN THE FIELD STUDIES

2.1 Research Station and the experimental pond system

The field study data included in this thesis were collected at EXTRABES (Estacao Experimental de Tratamentos Biologicos de Esgotos Sanitarios - Experimental Station for the Biological Treatment of Sanitary Sewage), which is part of the Federal University of Paraiba.

The station is located in the city of Campina Grande (latitude 7° 13' 11" south, longitude 35° 52' 31" west) Paraiba State in the north east of Brazil and occupies the premises of an old sewage treatment station of the city which ceased to operate in 1960 as it was unable to treat the increased volume of sewage and it was uneconomical to upgrade the plant. The sewage treatment plant and part of the site were donated by the State Water and Sewerage Company of Paraiba CAGEPA to the Federal University of Paraiba who initiated a research programme on waste stabilization ponds. The experimental pond systems started operating in March 1977. Silva (1982) describes in detail all the conversions that were made.

Since 1977, three basic systems have functioned at EXTRABES: System I, a five-pond series comprising an anaerobic pond receiving raw sewage (A1) followed by a facultative pond (F1) and

three maturation ponds (M1, M2 and M3); System II, comprising four independently loaded facultative ponds (F2, F3, F4 and F5), each one receiving raw sewage; System III, three anaerobic ponds (A2, A3 and A4); A2 and A3 were in series, running into a facultative pond F6 and A4 was independently loaded, running into a facultative pond F7. The pond dimensions for all the three systems are given in Table 2.1

All the three systems received raw municipal sewage. Effluents from all three systems were ultimately discharged back into the interceptor sewer running alongside the station.

Algological research on the facultative and maturation ponds was started in January 1978. As algae were not present in the anaerobic ponds, these ponds were not included in the algology research programme.

Data on total chlorophyll and species identification were collected for the facultative ponds F6 and F7 of System III. As both ponds had problems with infiltration through the bottom which made the results difficult to analyse - they are not included in this thesis.

The ponds of Systems I and II were studied in more detail. Three sets of experiments were carried out, where ponds were loaded with different amounts of BOD (Tables 2.2 and 2.3). Intrapond circulation was studied in ponds of System II during

Table 2.1 Physical characteristics of the ponds (from Silva, 1982)

Pond		Dimensions (m)	Area (m ²)	Volume (m ³)
S Y S T E M I	A ₁	10.00 x 3.35 x 1.25	34	42
	F ₁	10.00 x 3.35 x 1.00	34	34
	M ₁	10.00 x 3.35 x 1.00	34	34
	M ₂	10.00 x 3.35 x 1.00	34	34
	M ₃	10.70 x 3.35 x 1.00	36	36
S Y S T E M II	F ₂	25.70 x 7.50 x 1.25	193	241
	F ₃	26.40 x 7.40 x 1.25	195	244
	F ₄	25.70 x 7.40 x 1.25	190	238
	F ₅	25.70 x 7.30 x 1.25	188	235
	S Y S T E M III	A ₂	9.80 x 1.23 x 1.75	12
A ₃		5.00 x 1.23 x 1.65	6	10
A ₄		14.90 x 1.27 x 1.5	19	33

Tab 2.2 Experimental details for System I ponds.

Experiment Number	Pond	Raw Sewage flow rate ($m^3 d^{-1}$)	Mean Hydraulic retention time (days)	Calculated BOD ₅ Loadings		Actual BOD Loading	
				Areal ($kg BOD_5 ha^{-1} d^{-1}$)	Volumetric ($g BOD_5 m^3 d^{-1}$)	Areal ($kg BOD_5 na^{-1} d^{-1}$)	Volumetric ($g BOD_5 m^3 d^{-1}$)
1 Jan 77 to May 79	A ₁	6.17	6.8	544	45	-	35
	F ₁	6.17	5.5	-	-	116	-
	M ₁	6.17	5.5	-	-	83	-
	M ₂	6.17	5.5	-	-	46	-
	M ₃	6.17	5.8	-	-	35	-
2 June 79 to Nov 80	A ₁	21.24	2.0	1,874	150	-	118
	F ₁	21.24	1.6	-	-	375	-
	M ₁	21.24	1.6	-	-	338	-
	M ₂	21.24	1.6	-	-	262	-
	M ₃	21.24	1.7	-	-	190	-
3 Jan 81 to Dec 81	A ₁	10.56	4.0	932	75	-	73
	F ₁	10.56	3.2	-	-	290	-
	M ₁	10.56	3.2	-	-	245	-
	M ₂	10.56	3.2	-	-	156	-
	M ₃	10.56	3.4	-	-	109	-

**Assuming the BOD₅ of the Raw Sewage influent to be 300 mg l⁻¹. Table Modified after Silva (1982).

Tab. 2.3 Experimental details for System II ponds.

Experiment Number	Pond	Raw Sewage flow rate ($\text{m}^3 \text{d}^{-1}$)	Mean Hydraulic retention time (days)	Calculated BOD_5 Loadings **		Actual BOD_5 Loading	
				Areal ($\text{kg BOD}_5 \text{ha}^{-1} \text{d}^{-1}$)	Volumetric ($\text{g BOD}_5 \text{m}^3 \text{d}^{-1}$)	Areal ($\text{kg BOD}_5 \text{ha}^{-1} \text{d}^{-1}$)	Volumetric ($\text{g BOD}_5 \text{m}^3 \text{d}^{-1}$)
1 Jan 77 to May 79	F ₂	20.33	11.8	300	25	258	25
	F ₃	20.33	12.0	300	25	255	25
	F ₄	25.03	9.5	400	32	322	32
	F ₅	12.43	18.9	200	16	162	16
2 June 79 to Nov 80	F ₂	32.16	7.5	500	40	388	40
	F ₃	39.00	6.3	600	48	464	48
	F ₄	34.82	6.8	550	44	425	44
	F ₅	31.32	7.5	500	40	378	40
3 Jan 81 to Nov 81	F ₂	32.16	7.5	500	40	482	40
	F ₃	39.00	6.3	600	48	577	48
	F ₄	34.82	6.8	550	44	529	44
	F ₅	31.32	7.5	500	40	482	40

**Assuming the BOD_5 of the Raw Sewage influent to be 300 mg l^{-1} .
Table modified after Silva, (1982).

Experiment 3 using small submersible pumps.

The area or surface BOD loading of the facultative and maturation ponds (λ_s) were calculated from the formula described by Mara (1975), i.e.:

$$\lambda_s = \frac{10 L_i Q}{A}$$

where L_i = BOD₅ (mg l⁻¹)
 Q = flow (m³ d⁻¹)
 A = mid-depth area (ha)

λ_s (kg BOD ha⁻¹ d⁻¹)

Volumetric loading (λ_v) rather than surface loading rates are usually applied to anaerobic ponds (see Mara, 1976). They were calculated from the equation.

$$\lambda_v = \frac{L_i Q}{AD}$$

where L_i = BOD₅ (mg l⁻¹)
 Q = flow (m³ d⁻¹)
 AD = mid-depth area (m²)

λ_v (g BOD m⁻³ d⁻¹)

2.2 ALGOLOGICAL ANALYSES

2.2.1 Pond Water Sample Collection Methods

2.2.1.1 In-pond sampling

A perspex tube (2m long and 5cm in diameter) was used to obtain samples from the pond water throughout all the experiments. This tube had, at one end, a special remote closing device allowing it to be introduced open into the pond and removed closed. By using

this procedure, a sample representative of the whole water column of the ponds (except for the pond sludge) could be obtained.

Complete water column samples were collected from 5 different points in the pond. These samples were then mixed together in a bucket and composite sub-samples removed for subsequent analysis. This method was used in all the ponds.

2.2.1.2 Effluent sampling

Samples of the effluent leaving each pond were collected directly by bucket. The contents of these were also stirred before the sub-samples were removed.

Composite and effluent samples were collected weekly and analysed for chlorophyll a, phaeophytin, algal genera identification and algal cell counts. In the case of the effluent samples, the above measurements were made on portions of the same sample that was also being analysed for physico-chemical and bacteriological parameters.

2.2.2 Chlorophyll analysis

The algal biomass present in the pond (composite sample) or the pond effluent was determined by measurements of chlorophyll a and phaeophytin. A known volume of sample, between 5 and 50 ml, was filtered through a 25 mm diameter Whatman GFC pad in a Millipore Filtration System. A 2.5 ml aliquot of a 1% $MgCO_3$ suspension (in

water) was filtered through the GFC pad before the actual sample, to increase algal retention on the filter pad and to avoid a drop in the pH affecting the chlorophyll sample during the subsequent extraction with solvent. After filtration, the GFC pad was transferred to a graduated Pyrex glass centrifuge tube and 7 ml of cold 90% (v/v) acetone added to extract the pigments. This was done in the dark at 4°C for 16 hours by placing the tubes in a refrigerator. Preliminary studies had shown that minimal degradation of chlorophyll a occurred over the period under the described conditions. The tubes containing the pads were then centrifuged in a small bench centrifuge at 2500 RPM for 2 minutes to obtain a clear supernatant pigment extract. This supernatant was transferred into small tubes which were placed in the dark in an ice-box for the short period prior to spectrophotometric analysis. The optical density of each sample was measured at 663 nm and 750 nm in a Pye-Unicam spectrophotometer SP6-500 using 1 cm path length glass cuvette and blank of 90% (v/v) acetone. The same sample was then acidified with a drop of 1 M HCl, mixed thoroughly and measured again at 663 nm and 750 nm. The concentrations of chlorophyll a and phaeophytin were calculated using the equation of Talling and Driver(1963) with the recommended extinction coefficients of 89 and 56 for chlorophyll a and phaeophytin respectively (Golterman, 1978).

Thus:

$$1 \text{ Total Pigment } (\mu\text{g l}^{-1}) = \text{ODo} \times \frac{1000}{k_{\text{Chl a}}} \times \frac{\text{extract volume (ml)}}{\text{filtrate volume (l)}}$$

$$2 \text{ chlorophyll a } (\mu\text{g l}^{-1}) = 2.43 (\text{ODo}-\text{ODa}) \times \frac{1000}{k_{\text{Chl a}}} \times \frac{\text{extract volume (ml)}}{\text{filtrate volume (l)}}$$

$$3 \text{ phaeophytin } (\mu\text{g l}^{-1}) = \text{ODo} [2.43(\text{ODo}-\text{ODa})] \times \frac{1000}{k_{\text{phaeo}}} \times \frac{\text{extract volume (ml)}}{\text{filtrate volume (l)}}$$

where:

ODo = absorbance at 663 nm less absorbance at 750 nm before acidification

ODa = " " " " after acidification

k = extraction coefficient

2.2.3 Algal counts

Sub-samples from the composite and effluent samples were poured into separating funnels and 1 ml of 2% formaldehyde solution added. The contents were shaken and left to sediment to the bottom, *there being no buoyant algal present.* The basal 10 ml of liquid in the separating funnel, which contained the settled algae, was drawn off. Algal numbers were estimated using an Improved Neubauer Haemocytometer chamber. Counts for each algal genus were converted into cells per litre.

Qualitative estimates of the different algal genera present in the composite and effluent samples was also made by simple microscopic examination. The presence of each genus for each pond was recorded.

2.2.4 Bacteriological analyses

Viabile counts of faecal coliforms (FC) and faecal streptococci (FS) were made using standard membrane filtration methods.

Faecal and strepto coliforms were grown on m-FC Broth (Difco) and m-Enterococcus Agar (Difco) respectively. Full details of the methodology are given in the ^{APAA} (Standard Methods, 1975) and Report no. 71 on Public Health and Medical Subjects (1969).

2.3 PHYSICO-CHEMICAL ANALYSES

2.3.1 In-Pond measurements

The following parameters were determined in pond water during the experimental work:-

Dissolved Oxygen: Measurements of the dissolved oxygen concentration in the pond water column were made using a YSI model 54 ABP Dissolved Oxygen Meter with a YSI model 53-39 Dissolved Oxygen Electrode in conjunction with a YSI model 57-91 stirrer. The electrode plus stirrer were lowered carefully into the pond and readings were taken at 10 cm intervals throughout the depth of the pond.

pH: Measurements were made using a Radiometer model 29 pH meter with combined glass electrode. *Subsurface samples were brought to the surface before measurement.*

Light intensity: Measurements of irradiant energy in langleys were taken using a Gunn-Bellany Solar-Radiometer

(Baird and Tatlock, U.K.). Under water light measurements, in *Photosynthetically Active Radiation (PAR)* quanta $\mu\text{s}^{-1} \text{cm}^{-2}$, were made utilizing a Biospherical Digital Scalar Irradiance Meter (model QSP/170) with a sensor (model QSP/200).

Temperature: In situ measurements of the water measurements of the water column were made using a YSI model 47 telethermometer linked to a YSI model 401 thermistor probe spaced vertically 10 cm apart on an aluminium bar which was carefully lowered into the pond so that it was possible to take simultaneous temperature measurements from 5 cm above the pond surface down to the bottom of the pond. For convenience, in situ pond temperatures and also temperatures of the laboratory samples were sometimes taken using the built-in thermistor on the YSI 54A oxygen meter.

2.3.2 Effluent measurements

The following analyses were *routinely* determined on pond effluent samples and occasionally on other samples:

Biological oxygen demand (BOD_5) in $\text{mg O}_2 \text{ l}^{-1}$ changes in oxygen concentrations in the samples were measured using a Dissolved Oxygen Meter ^{YSI} model 54A and a self stirring YSI model 57-20 BOD_5 bottle probe.

Chemical oxygen demand (COD) in $\text{mg O}_2 \text{ l}^{-1}$ was measured by the Dicromate Reflux method.

pH was measured using a Radiometer model 29 pH meter with

combined glass electrode.

Nitrate nitrogen (mg l^{-1}) was measured by the Chromotropic Acid Method.

Ammoniacal nitrogen (mg l^{-1}) was measured with an Orion model 407-A Specific Ion Meter with an Orion Ammonia Electrode Model 95-10.

Chloride (Cl^-) (mg l^{-1}) was measured with an Orion model 407-A Specific Ion Meter with an Orion Chloride Electrode Model 96-10.

Total Phosphorus (Total P) (mg l^{-1}) was measured by the Persulphate Digestion Method.

Soluble Phosphorus (*Reactive the inorganic fraction* Soluble P) (mg l^{-1}) was measured by the Ascorbic Acid Method.

Suspended Solids (SS) (mg l^{-1}) were measured using 9 cm diameter Whatman GFC filters.

Sulphide ($\text{S}^=$) (mg l^{-1}) was measured by the Methylene Blue Method.

Total Alkalinity (CaCO_3) (mg l^{-1}) was measured by titration of a pre-selected pH.

Conductivity ($\mu\text{S cm}^{-1}$) at 25°C was measured using a YSI model 33 SCT Conductivity Meter fitted with an YSI model 3300 probe.

Precise details of all these methods can be found in ^{APHA} Standard Methods for the Examination of Water and Wastewater, 1975) 13th Edition.

The physico-chemical and bacteriological data mentioned in this thesis were analysed by other members of the EXTRABES team. This does not apply to the in situ physico-chemical measurements for dissolved oxygen, sulphide, pH, light intensity and temperatures taken during the special algological experiments described in Chapters 5, 6 and 7.

CHAPTER 3

ALGAL SPECIATION AND POPULATION STUDIES

Variation in algal biomass and speciation in response to changes in physico-chemical and biological factors are essential features of an aquatic environment.

The predominance of different algal species in response to the changing ecological factors in waste stabilization ponds has been recognised for some time (Cadwell, 1946; Neel and Hopkins, 1956; Fitzgerald and Rohlich, 1958).

The sewage characteristics, changes in light intensity and temperature are subject to seasonal variation and also vary with latitude. These variations have to be taken into consideration when comparing sewage pond systems from different localities.

Palmer (1969) collated numerous data from the literature on algae tolerant of high organic pollution and concluded from this that organic wastes tended to influence the speciation of the algal flora more than other factors in the aquatic environment, such as water hardness, light intensity, pH, dissolved oxygen, rate of flow, size of the water body, temperature and other types of pollutant.

In phytoplankton, species reported as most common in water bodies receiving domestic sewage vary in different parts of the world. Diatoms, pigmented flagellates, green algae and blue

green algae are all well represented among the pollutant tolerant genera. In experimental maturation pond systems in South Africa, a species of Chlorella was dominant in the mid-winter while Euglena sanguinea was reported to be dominant during mid-summer. Algae such as Chlamydomonas, Micractinium, Anabaena and Cryptomonas were also present and dominated the algal flora at times between mid-winter and mid-summer. The annual mean water temperature was above 20°C with seasonal minima and maxima of 13°C and 29.9°C (Shillinglaw and Piertese, 1977).

Patil and co-workers (1975) studied the succession of phytoplankton in a sewage stabilization pond in India with an average BOD₅ surface loading of 336.3 kg BOD₅ ha⁻¹ d⁻¹; they found interesting patterns of algal succession.

Among the Chlorophytes, the flagellate forms, e.g. Pyrobotrys gracilis, Chlorogonium elongatum, Chlamydomonas sphagnicola, predominated in the early period (mid-February to mid-March). However, Chlorella vulgaris and Ankistrodesmus falcatus were found in greater numbers only during the later period (early April). Two genera of euglenoids, Euglena and Phacus, constituted the major portion of the pond algal community during mid-March/early April. Each appeared to have its own peak period. The temperature of the pond water ranged from 25 to 34°C and showed minimum and maximum values during the morning and afternoon hours, respectively.

Raschke (1970) studying the algal composition of sewage ponds in Iowa (U.S.A.) where pond temperature ranged from 0.8°C to 29.9°C, identified 18 genera comprising 29 species of algae. Throughout the year green algae and flagellate species dominated the plankton whilst diatoms and blue-green algae dominated the benthic flora. The flagellates that were dominant in the winter and early spring were members of the Volvocales, Chlamydomonas, Chlorogonium, Eudorina and Euglenophyta, Euglena. In contrast, Chlorococcales, Ankistrodesmus, Chlorella, Coelastrum, Micractinium, Crucigenia, Oocystis and Scenedesmus were dominant throughout the summer and autumn.

The distribution of phytoplankton in a double-cell sewage pond at Hallan, Nebraska (U.S.A.) receiving an estimated BOD loading of 41 kg BOD₅ ha⁻¹ d⁻¹ was studied during the summer and autumn of 1965 (de Noyelles, 1967). Sixteen and eighteen algae species were recorded in the first and second ponds. The algae recorded in these ponds included Chlamydomonas, Chlorella, Euglena, Scenedesmus and Oscillatoria with Ankistrodesmus falcatus v. acicularis being the dominant species.

In this chapter the distribution of algal genera present in EXTRABES sewage ponds was investigated. This was done with a view to not merely producing a qualitative list of species but to linking the presence of different algal genera to the degree of treatment achieved in the various ponds in the system.

3.1 Algal speciation

The frequency of the different algal genera present in the ponds of System I and II was determined from presence and absence data recorded from 58 weekly samplings during the period January 1978 to March 1979, i.e. during Experiment 1. Details of the sampling technique, concentration of the samples and fixation have been described previously (see Chapter 2). The algal genera and their frequencies in System I and System II are shown in Fig. 3.1a and b respectively.

In System I the motile flagellate genera Euglena, Phacus, Pyrobotrys, Chlamydomonas, Eudorina, Pandorina and Chlorogonium were present in all the ponds (except A1) during the whole 58 weeks period (Fig. 3.1a). As purification of the sewage progresses through the system, from F1 to M3, the frequency of the flagellate genera tended to be reduced, except for Euglena whose frequency was sustained and Chlorogonium which actually increased. The non-motile alga Chlorella was also present in all the ponds of System I whereas the other non-motile algae such as Micractinium, Scenedesmus, Ankistrodesmus and Cyclotella were absent from F1 but appeared with increasing frequency from M1 to M3. The types of algae present in System I not only varied from pond to pond but also the total number of genera present increased from thirteen in F1 to eighteen in M3.

In System II, (Fig. 3.1b) with BOD surface loadings varying

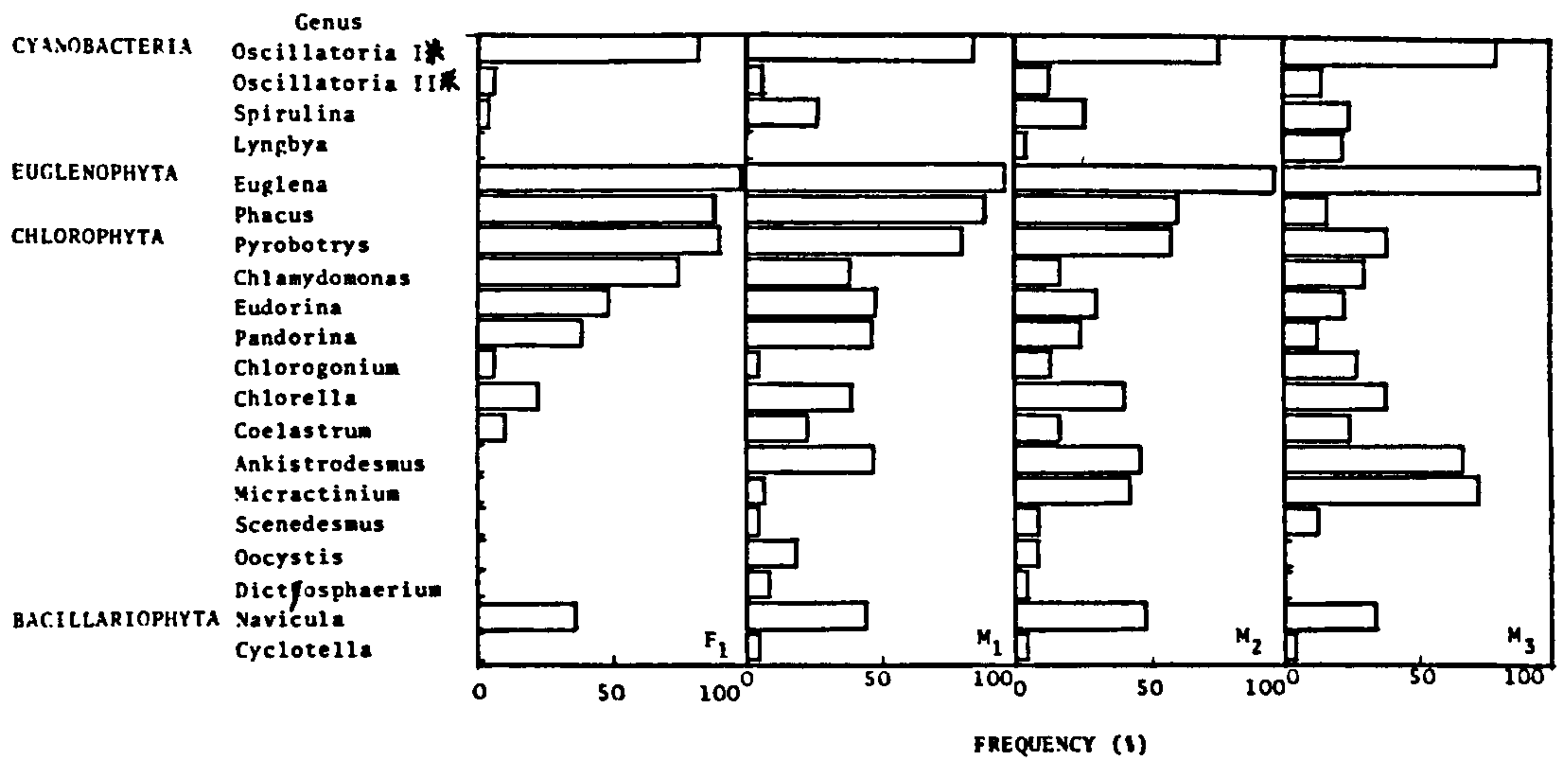


Fig. 3.1(a) Algal genera present in a facultative pond (F₁) and Maturation ponds (M₁ and M₂ and M₃) of System I. Frequency was determined from 58 weekly samples from each stabilization pond.

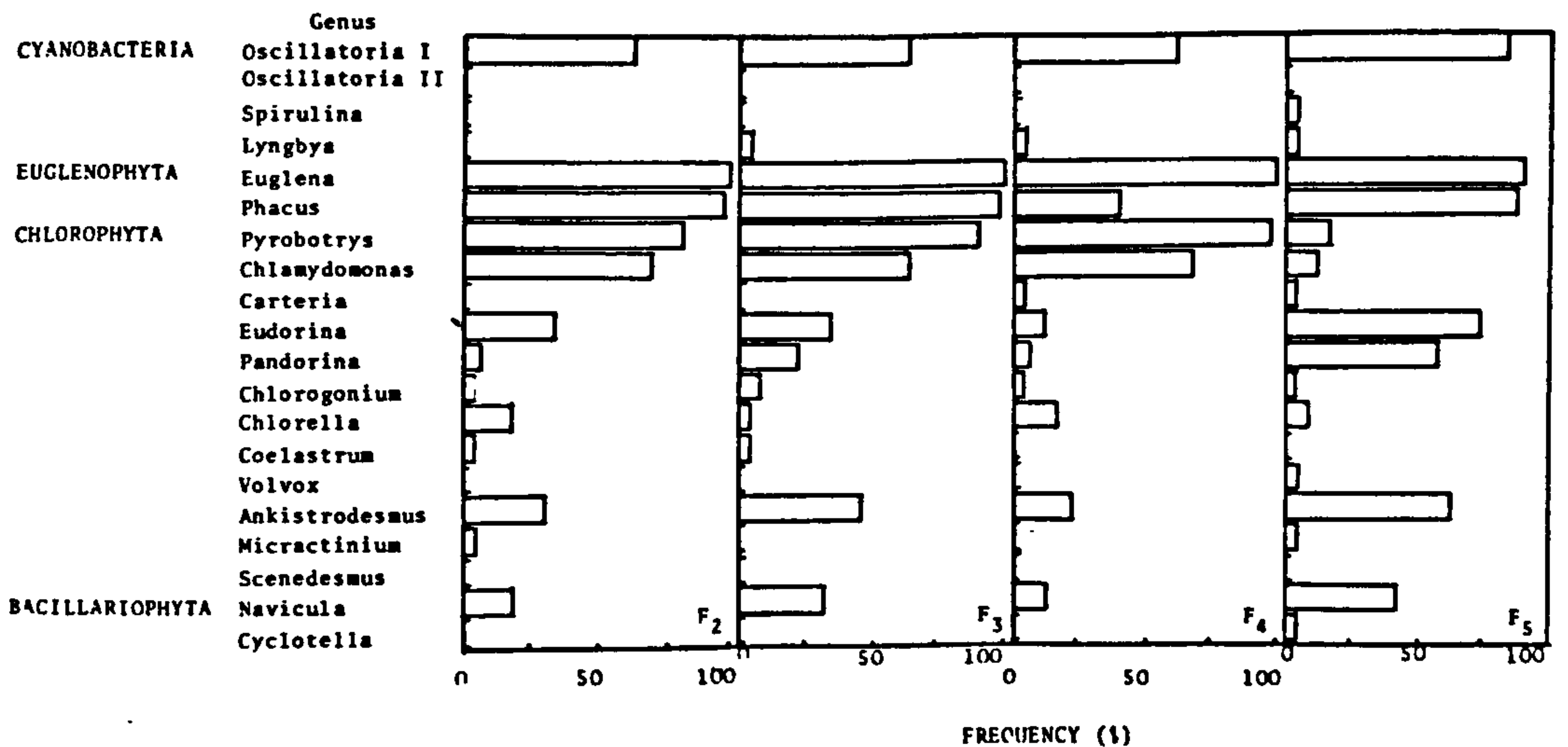


Fig. 3.1(b) Algal genera present in the Facultative ponds (F₂, F₃, F₄ and F₅) on System II. Frequency was determined from 58 weekly samples from each stabilization

* Oscillatoria I - The mean trichome diameter₁ (μm) = 10
 * Oscillatoria II - The mean trichome diameter₁ (μm) = 7

between 150 to 300 kg BOD₅ ha⁻¹ d⁻¹ depending on the pond, Euglena was the most frequent algae in all four ponds during the period examined. The genus Phacus had a high frequency in all the ponds except F₄ (which had the highest loading). Pyrobotrys and Chlamydomonas were also frequent in the ponds, except in F₅, which had the lowest loading of the four ponds. In contrast, the colonial flagellates Eudorina and Pandorina were more frequent in F₅ than in the other ponds. Navicula, the most frequent of the diatom species, occurred with approximately the same frequency as the non-motile algae Ankistrodesmus.

The facultative ponds of System II had an average of 13 genera present except for F₅ which had 17.

Although the frequency of the different genera present in the ponds of Systems I and II were not examined in detail for Experiments 2 and 3, the additional presence and absence data collected for the algal genera in these systems from time to time gave some indications of algal diversity.

Diversity data for ponds of Systems I and II based on the presence and absence of the different algae genera during Experiments 1, 2 and 3 are shown in Tables 3.1 and 3.2. A genus was scored present in a pond if it appeared in any sample taken during the experimental period. Despite the data being treated in a somewhat simplistic manner, some interesting patterns emerge.

TABLE 3.1 Presence and absence data for algal genera in System I during Experiments 1, 2 and 3, together with the mean values for the physico-chemical parameters measured during the course of these experiments (Table 3.1.1)

Experiment	1	1	1	3	1	3	2	3	2	3	2	2	2
Pond	M3	M2	M1	M3	F1	M2	M3	M1	M2	F1	M1	M1	F1
Retention time (days)	5.8	5.5	5.5	3.4	5.5	3.2	1.7	3.2	1.6	1.6	1.6	1.6	3.2
Area (m ²)	36	34	34	36	34	34	36	34	34	34	34	34	34
BOD surface load (λs)	35	46	83	109	116	156	190	245	262	290	338	375	375
Euglena	+	+	+	+	+	+	+	+	+	+	+	+	+
Chlamydomonas	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyrobotrys	+	+	+	+	+	+	+	+	+	+	+	+	+
Eudorina	+	+	+	+	+	+	+	+	+	-	+	+	-
Pandorina	+	+	+	+	+	+	+	+	+	+	+	+	-
Scenedesmus	+	+	+	+	-	+	+	+	+	-	+	+	-
Phacus	+	+	+	+	+	+	-	-	-	-	-	-	-
Dictyosphaerium	+	+	+	-	-	-	-	-	-	-	-	-	-
Oocystis	+	+	+	-	-	-	-	-	-	-	-	-	-
Cyclotella	+	+	-	-	-	-	-	-	-	-	-	-	-
Chlorogonium	+	+	+	-	+	-	+	-	+	+	+	+	-
Ankistrodesmus	+	+	+	-	-	-	+	-	-	-	-	-	-
Chlorella	+	+	+	-	+	-	+	-	+	+	+	+	-
Micractinium	+	+	+	+	-	-	+	-	+	-	-	-	-
Carteria	-	-	+	+	-	+	+	+	+	+	+	+	+
Coelastrum	+	+	+	-	+	-	+	-	-	-	-	+	-
Navicula	+	+	+	+	+	+	+	+	-	+	-	-	+

TABLE 3.1.1

BOD ₅ (mg l ⁻¹)	17	19	25	35	45	37	26	49	32	53	41	78
pH	8.38	8.10	7.99	8.21	7.69	8.08	7.99	7.97	7.91	7.75	7.84	7.78
NH ₃ -N (mg l ⁻¹)	8.4	14.1	19.6	22.1	26.6	24.6	29.1	27.1	30.3	32.3	31.0	29.6
NO ₃ -N (mg l ⁻¹)	1.41	0.98	0.44	0.31	0.24	0.30	0.17	0.32	0.17	0.19	0.17	0.34
SS (mg l ⁻¹)	45	43	61	72	74	66	51	78	49	53	50	69
Total P (mg l ⁻¹)	3.05	3.54	3.74	4.25	4.16	4.29	4.28	4.58	4.38	4.35	4.50	4.49
Soluble P (mg l ⁻¹)	2.42	3.22	3.46	2.94	3.72	3.08	3.26	3.17	3.29	3.21	3.31	3.04
Chlorophylla (µg l ⁻¹)	423	266	479	365	1122	272	142	323	132	33	84	134
Sulphide (S ²⁻) (mg l ⁻¹)	-	-	-	1.82	-	2.60	4.10	4.87	6.07	8.40	7.30	7.52

TABLE 3.2 Presence and absence data for algal genera in System II during Experiments 1, 2 and 3, together with the mean values for the physico-chemical parameters measured during the course of these experiments (Table 3.2.1)

Experiment	1	1	1	1	2	2	2	2	2	3	3	3	3
Pond	F5	F3	F2	F4	F5	F2	F4	F3	F2	F5	F3	F4	F3
Retention time (days)	18.9	12	11.8	9.4	7.5	7.5	6.8	6.3	7.5	7.5	6.3	6.8	6.3
Area (m ²)	188	195	193	190	188	193	190	195	193	188	193	190	190
BOD surface load (λ s)	162	255	258	322	387	388	425	464	482	482	482	529	577
Euglena	+	+	+	+	+	+	+	+	+	+	+	+	+
Chlamydononas	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyrobotrys	+	+	+	+	+	+	+	+	+	+	+	+	+
Chlorogonium	+	+	+	+	+	+	+	+	+	+	+	+	+
Chlorella	+	+	+	+	+	+	+	+	+	+	+	+	+
Ankistrodesmus	+	+	+	+	+	+	+	+	+	+	+	+	+
Coelastrum	+	+	+	+	+	+	+	+	+	+	+	+	+
Phacus	+	+	+	+	+	+	+	+	+	+	+	+	+
Dictyosphaerium	+	+	+	+	+	+	+	+	+	+	+	+	+
Volvox	+	+	+	+	+	+	+	+	+	+	+	+	+
Eudorina	+	+	+	+	+	+	+	+	+	+	+	+	+
Pandorina	+	+	+	+	+	+	+	+	+	+	+	+	+
Microactinium	+	+	+	+	+	+	+	+	+	+	+	+	+
Carteria	+	+	+	+	+	+	+	+	+	+	+	+	+
Oocyst-is	-	+	-	+	-	+	+	-	-	-	-	-	-
Navicula	+	+	+	-	+	+	+	+	+	+	+	+	+
BOD ₅ (mg l ⁻¹)	40	51	54	57	61	60	63	61	75	77	75	75	76
pH	7.83	7.66	7.66	7.60	7.95	7.92	7.94	7.92	7.77	7.80	7.77	7.76	7.77
NH ₃ -N (mg l ⁻¹)	17.1	19.5	20.6	21.5	25.4	25.8	26.4	27.5	26.4	26.2	26.6	26.6	26.6
NO ₃ -N (mg l ⁻¹)	0.33	0.29	0.31	0.32	0.17	0.17	0.17	0.17	0.34	0.36	0.33	0.33	0.33
SS (mg l ⁻¹)	96	91	90	85	83	84	86	78	85	80	81	77	77
Soluble P (mg l ⁻¹)	3.04	3.39	3.41	3.42	2.98	2.94	3.00	3.08	3.05	2.95	2.93	2.93	2.91
Total P (mg l ⁻¹)	3.58	3.92	3.98	3.86	4.23	4.26	4.39	4.44	4.53	4.47	4.58	4.58	4.43
Chlorophylla (μ g l ⁻¹)	1572	1273	1325	1168	486	469	443	357	198	166	199	137	137
Sulphide (S ²⁻) (mg l ⁻¹)	-	-	-	-	6.25	5.83	6.35	6.28	6.68	6.70	6.76	7.05	7.05

Comparing the two systems together the flagellates Euglena, Chlamydomonas and Pyrobotrys were ubiquitous to all the ponds, i.e. were present up to surface loadings of $577 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$. In contrast, certain other species only occurred in ponds with low surface loadings, for example Cyclotella was found at surface loadings up to $46 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$, Oocystis at surface loadings up to $83 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$. However, although other species showed patterns of intermediate tolerance to pond loadings, the patterns were not always clear cut. For instance Dictyosphaerium was not found at loads above $162 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$ in System II but did not appear at loads above $83 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$ in System I. Similarly, Phacus did not appear in any pond at loads above $322 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$ but was only present in ponds of System I with loads below $56 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$.

On the basis of its frequency in the ponds of System I one might have predicted that Scenedesmus should occur in System II ponds below a loading of 338 or possibly $262 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$ but it does not. Eudorina and Pandorina genera showed a clear pattern of distribution with loading through the ponds of System I but this pattern did not hold for System II where the presence or absence was erratic with both genera appearing at loads as high as $529 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$.

Similarly, Chorella, Chlorogonium, Ankistrodesmus and Coelastrum were erratic in System I but gave a clear pattern of

occurrence in System II. The distribution of Micractinium, Navicula and Carteria, however, were erratic in both Systems and could not be correlated with BOD₅ surface load at all.

3.2 Discussion

According to Patil et al. (1975) waste stabilization ponds represent a transitional stage between waste water and clean water. The availability of energy sources and physico-chemical changes associated with sewage treatment influence the population dynamics resulting in a marked succession of organisms during the process of purification.

In Systems I and II ponds, Euglena, Phacus, Pyrobotrys, Chlamydomonas, Eudorina, Pandorina and Chlorogonium were observed throughout the experimental period. The presence of these algae agrees with the comparative rating of algae tolerating organic pollution produced by Palmer (1969). In this rating the genus Euglena tops the list of his 60 genera most tolerant to pollution which also includes the other flagellates mentioned here.

The successful growth of these genera in sewage could be attributable to several different factors. Munawar (1970) related the abundance of the Euglenophyta in sewage ponds to high average concentrations of oxidizable organic matter (15.16 ppm) and high average concentrations of free carbon dioxide.

Alternatively, Provasoli (1958) and Provasoli and Pintner

(1960) have suggested that the distribution of Euglena which utilises ammonia as its sole source of inorganic nitrogen may be more dependent upon high levels of ammonia in the sewage than it is on carbon sources.

The sequential decrease in frequency of the other flagellates through System I, i.e. other than Euglena and Chlorogonium could be related either to the decrease in organic matter (organic carbon) from F1 to M3 or the decrease in ammonia concentration (Table 3.1.1). It was not possible at the time to determine which of these factors was the most important in controlling the decrease in flagellate algae through the system.

The decrease in the frequency of flagellates in System I was accompanied by a concomitant increase in the numbers and frequency of the non-motile genera Micractinium, Scenedesmus and Ankistrodesmus. Their presence coincided with a reduction in suspended solids and organic matter content in the water. In System I, there was an inverse relationship between the number of genera present and the organic loading of the ponds (Table 3.1.1).

The changes in the dominance and the increase in the algal genera from facultative to maturation ponds are analogous to the changes which occur as *any water system becomes less eutrophic.* In both cases the decrease in nutrient levels leads to an increase in algal diversity.

The sorts of changes in the patterns of frequency of algae present in System II ponds were in general similar to that of System I. However, whilst a correlation exists between the species and the degree of organic loading, ammonia concentration or suspended solids, this may not simply represent a direct response to these parameters. Changes in organic loading may coincide with other changes in water quality such as solubilisation of toxic substances or compounds which specifically aid or inhibit the growth of a particular algae.

Chlamydomonas has been reported as the only alga to grow successfully in ponds under conditions of high loading, approximately $336 \text{ kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$ and to survive the resulting anaerobic conditions (Cadwell, 1946; Neel and Hopkins, 1956). In these Brazilian ponds it was clear that other algae, such as Euglena, Phacus, and Pyrobotrys were just as capable as Chlamydomonas of surviving high loads and periods of anaerobiosis.

The Cyanobacteria present in the ponds of Systems I and II appeared not to be true components of the phytoplankton population but as epilithic species growing on the pond walls in a band close to the water surface or on floating pieces of the scum. Thus their occasional appearance in the pond water samples was because they had become detached from the walls, or the sample contained some scum.

It was interesting to note that during Experiment 1 the pattern of results for algal speciation and dominance in ponds F2 and F3 which had very similar loads, were the same. This suggests that the differences found in the algal population of the other ponds are differences related to variation in the in-pond parameters associated with loadings (Table 3.2.1).

Facultative ponds with similar BOD₅ surface loadings but from different Systems, e.g. F1 from System I (receiving settled sewage from an anaerobic pond) and F5 from System II (receiving raw sewage) showed only limited differences. They contained the same algal species except that F5 contained additionally, Ankistrodesmus, as a frequent component of the flora and occasionally Dictyosphaerium, Volvox and Micractinium. Insufficient data were collected, however, to determine whether the relative proportions of these algae remained the same.

It is interesting to note that the effluent produced by these two ponds was different in certain aspects notably in parameters such as ammonia and suspended solids (Tables 3.1.1 and 3.2.1). Furthermore, differences in certain ponds' characteristics, such as surface area, retention time and type of sewage have to be taken into consideration when making this kind of comparison. Intrapond circulation had no effect on the algal speciation and effluent quality when ponds F2 and F5 were under the same BOD₅ surface loadings during Experiment 3.

According to Patil et al. (1975) the predominance of various algae species is dependent on the loading factors and the physical design of the sewage ponds. Moreover, the degree of pretreatment and mixing influences the speciation in the ponds.

The experimental systems at EXTRABES have enabled the investigation of these points. The results obtained support to some extent the findings of Patil et al. However, changes in BOD₅ loading also involve changes in retention time at least when the origin of sewage is the same and so it is difficult to resolve any difference that the two factors might cause.

Since increasing the surface loading of the ponds reduced the number of genera present and the flagellates Euglena, Chlamydomonas and Pyrobotrys were the only algae to survive at high loadings, identification of the algal species present and the degree of diversification give a clear indication of the loading of a system and more important, the degree of treatment achieved. As yet, numerical values cannot be related to speciation but such a modelling approach should be possible in the long term with more detailed manipulation of the data available from EXTRABES and other sources. This sort of information would be of considerable value when attempting to make an immediate on-site estimation of the performance of ponds where the loadings are unknown or where a system does not appear to be functioning as designed.

3.3 Species Balance in Algal Populations

Although frequency data are a useful indicator of algal speciation and occurrence, they give no indication of the relative proportions and numbers of the algal types present. On the other hand, quantitative determinations of algal population by direct count methods may lead to confusing estimates of the biomass present if results are expressed simply as numbers per ml or litre. This is because of the wide range in cell and colony sizes between species within an algal population. Thus, if the phytoplankton population comprises different algae, simple counts may distort the values for the relative biomass contribution made by the various species. Some compensation may be attempted by, for example, transforming numerical records into volume (Paasche, 1960; Lund, 1964; Bellinger, 1968; Bellinger, 1974).

In this section of the work, algal counts for each species were made to allow fluctuations in the abundance of a particular species to be determined in the various ponds of System I. These data were also transformed into volume estimates so that the relative abundance of the various algal species comprising the population could be compared.

3.3.1 Methods

Geometric formulae were applied to the mean measurements of the linear dimensions of various algal species to obtain an estimate of cell volume. The cell dimensions of Euglena, Chlamydomonas, Pyrobotrys and Chlorella species present in pond M1 (System I) were measured and formulae chosen which seemed to best fit the geometry of the cells of a particular species. These data are presented in Table 3.3.

The most difficult species to fit a geometric formula to was Euglena because it continuously changed its cell shape, however, a cylinder seemed the closest shape to adopt. Since the cells tapered, it was decided to take a corrected length value which compensated for the shape not fitting a simple cylinder formula (Table 3.3).

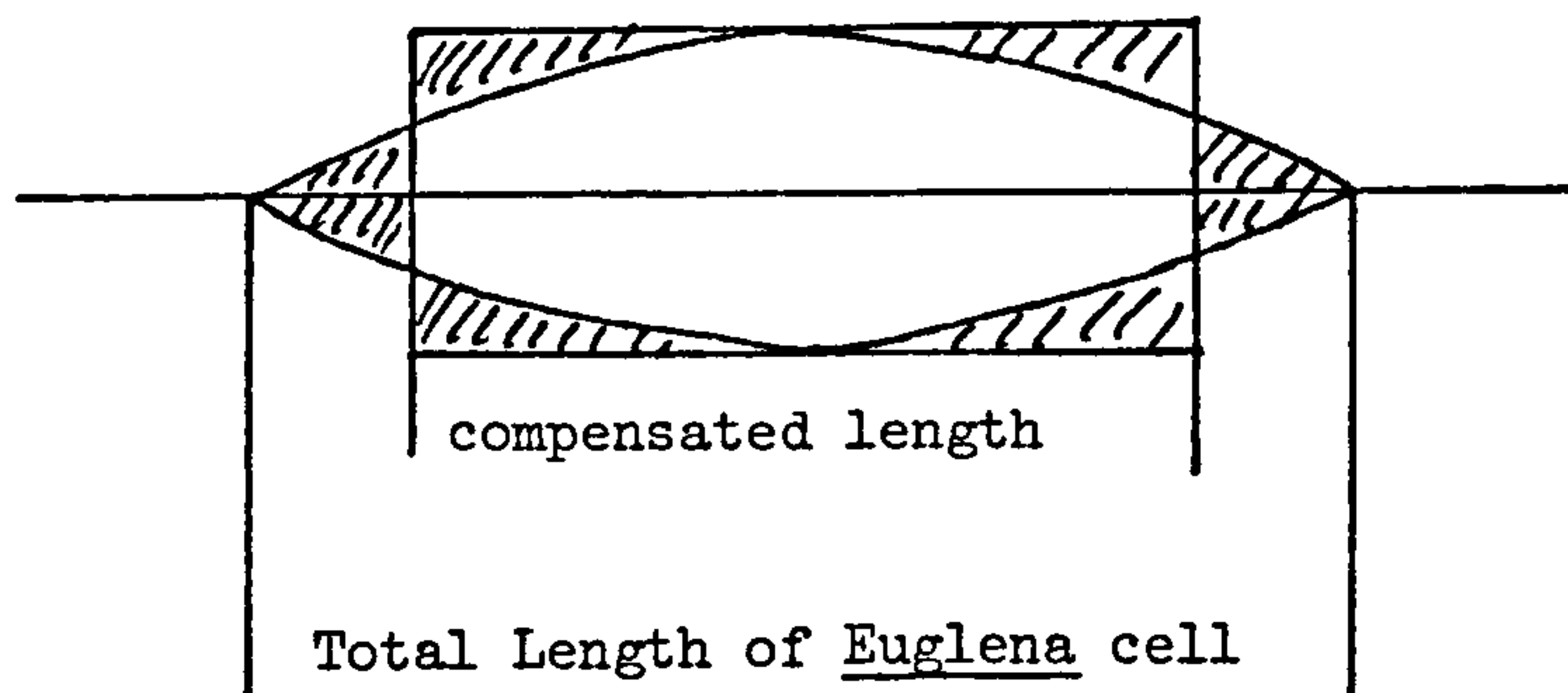


TABLE 3.3 Mean cell dimensions and mean cell volumes for Euglena, Pyrobotrys, Chlamydomonas and Chlorella calculated from 100 cells of each genus

Alga	Shape	Geometric formula	Mean cell dimensions		
			Length (μm)	Diameter (μm)	Volume (μm^3)
<u>Euglena</u>	cylinder (approx)	$v = \pi r^2 h$	135 103 (1)	11	6,172
<u>Pyrobotrys</u>	elipsoid about c	$v = 4/3 \pi abc$	9.7 (c)	6.0 (a and b)	185 3,000 (colony)
<u>Chlamydomonas</u>	elipsoid about c	$v = 4/3 \pi abc$	8.5 (c)	6.0 (a and b)	160
<u>Chlorella</u>	sphere	$v = 4/3 \pi r^3$	-	6.0	115

(1) compensated length

When counting the algae no distinction had been made between a rounded smaller and a larger more elongate form of Euglena because it was thought they were different stages of growth of the same species. When volume calculations for Euglena were made, therefore, these two sizes of Euglena were taken into consideration. The mean volume for the long form of Euglena was $9788 \mu\text{m}^3$ and for the smaller rounder form, $2557 \mu\text{m}^3$. Some microscopic observation of samples taken over several weeks, but subsequent to the main counting programme, showed that the ratio of the larger Euglena to the smaller one was approximately 1:1. Thus, the mean volume from combining both forms was calculated as follows:

Volume of the long form (μm^3)	=	9788
" " short form (μm^3)	=	2557

The mean volume = $\frac{9788 + 2557}{2} = 6,172 \mu\text{m}^3$

When the volume of the colonial flagellate alga Pyrobotrys was estimated, it was noticed that the numbers of individual cells in a Pyrobotrys colony was either 8, 16 or 32 cells. Microscopic examinations of several samples were made and the average cell number per Pyrobotrys colony was estimated to be 16. The

mean cell dimensions were calculated from at least 100 cells or colonies of each alga and are presented in Table 3.3.

3.3.2 Results

The variation in cell number with time of four algal genera, namely Euglena, Pyrobotrys, Chlamydomonas and Chlorella in the final four ponds of System I is shown in Fig. 3.2. Not all algal genera present in the different ponds were considered, only the most frequent ones. It can be seen from these results that there were no seasonal variations in number for any of the four algae in any of the ponds studied from September 1978 to May 1979.

In the facultative pond F1, of the four genera studied, Euglena and Pyrobotrys numbers fluctuated less dramatically than those of Chlamydomonas which on several occasions fell from relatively high cell numbers to very low. Chlorella appeared only during the last 2 weeks in October 1978 in a small number. In M1 in contrast, Chlorella was present in very high cells numbers except for a short period in which it almost disappeared, i.e. during a week in December 1978. Pyrobotrys more than Chlamydomonas exhibited a wax or wane pattern throughout the period examined. Euglena cell numbers did not fluctuate much, except towards April and May 1979.

In M2 and M3 the numbers of cells of the flagellate genera were much reduced. In fact, Pyrobotrys was absent from these two

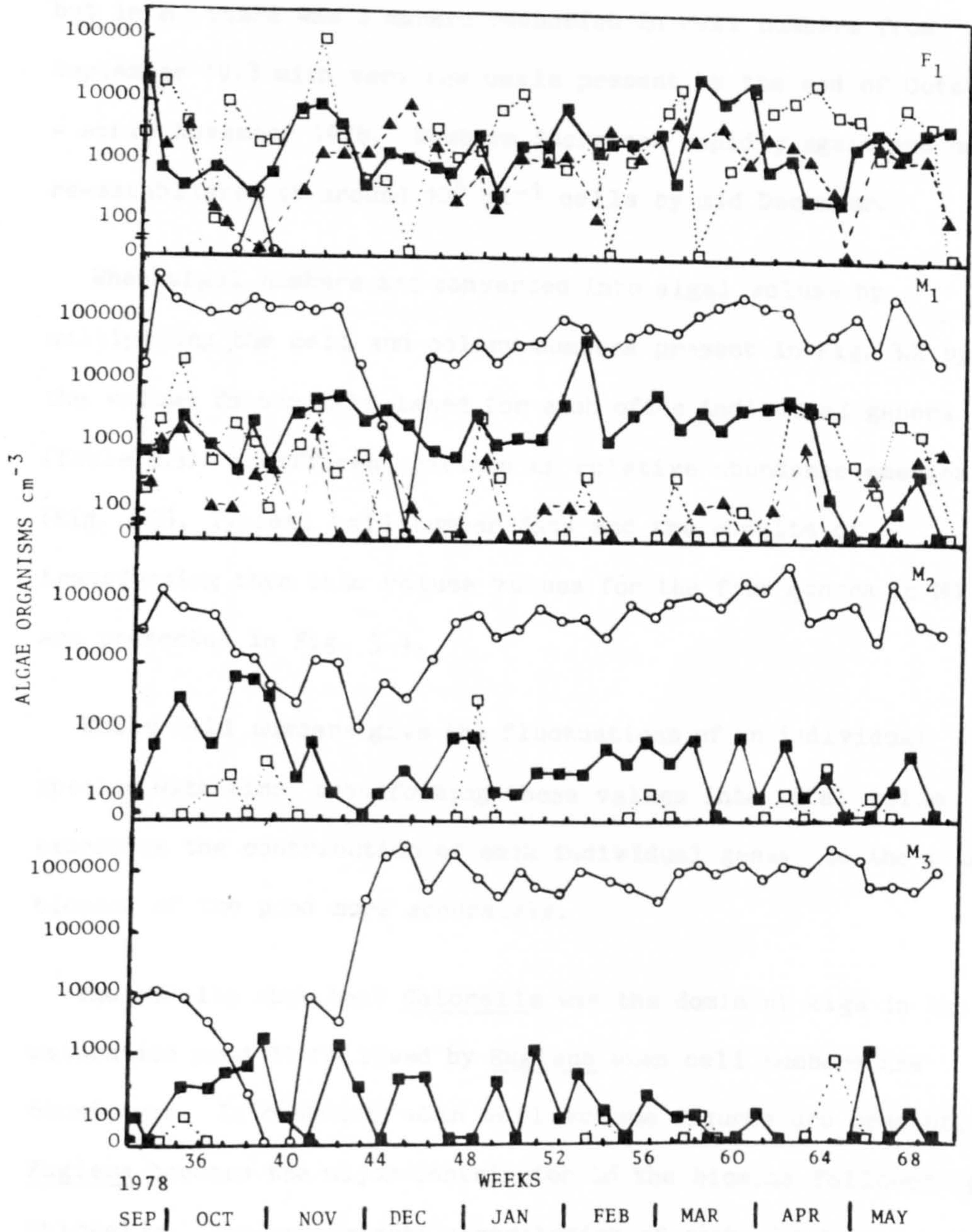


Fig. 3.2 Variation in cell number of Euglena (■), Chlorella (○), Chlamydomonas (□) and numbers of colonies of Pyrobotrys (▲) present in the final four ponds of System I.

ponds whereas Euglena numbers were reduced. The presence of Chlamydomonas was variable and the numbers of cells small. The cell numbers of Chlorella were high in both the M2 and M3 ponds but in M3 there was a marked reduction in cell numbers from September 1978 with very few cells present by the end of October - early November 1978. Numbers increased rapidly again and had re-established at around 10^6 ml⁻¹ cells by mid December.

When algal numbers are converted into algal volume by multiplying the cell and colony numbers present in Fig. 3.2 by the volume factor calculated for each of the individual genera (Table 3.3), a different pattern of relative abundance emerges (Fig. 3.3). Typical cell number data and the results of transforming them into volume values for the four genera in M1 are presented in Fig. 3.4.

While cell numbers give the fluctuations of an individual species with time, transforming these values into total volume expresses the contribution of each individual genus to the total biomass of the pond more accurately.

The results show that Chlorella was the dominant alga in the maturation pond M1 followed by Euglena when cell numbers are considered. In contrast, when cell volume figures are present, Euglena becomes the major contributor to the biomass followed by Chlorella. The approximate contribution of each algal genus expressed as a percentage of the total biomass in the different

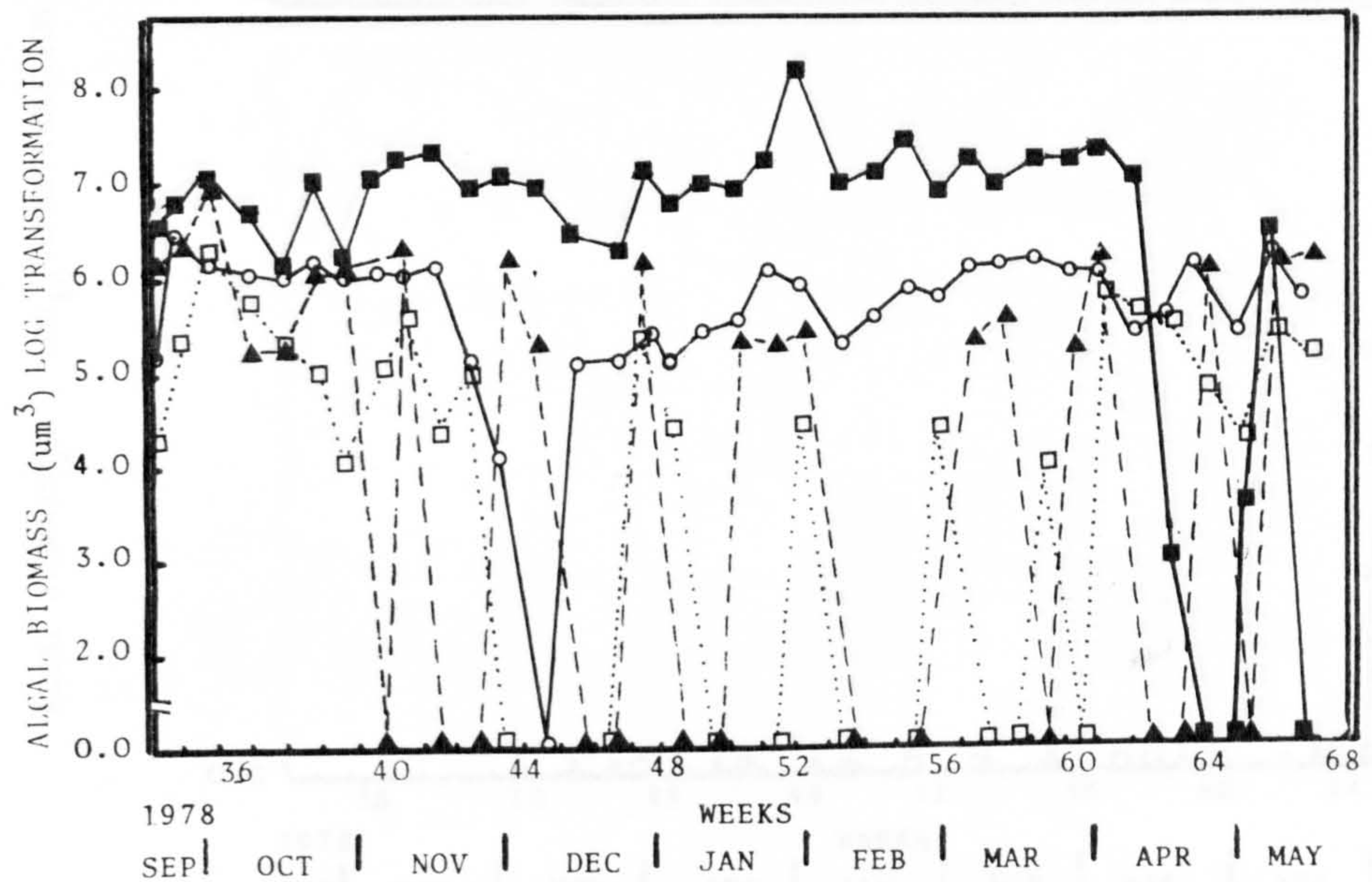


Fig. 3.3 Variation in algal biomass (volume) in the Maturation pond (M1). The data was obtained by multiplying the cell and colony numbers from Fig. 3.2 by a volume factor calculated for each of the individual species: Euglena (■), Chlorella (○), Chlamydomonas (□) and Pyrobotrys (▲).

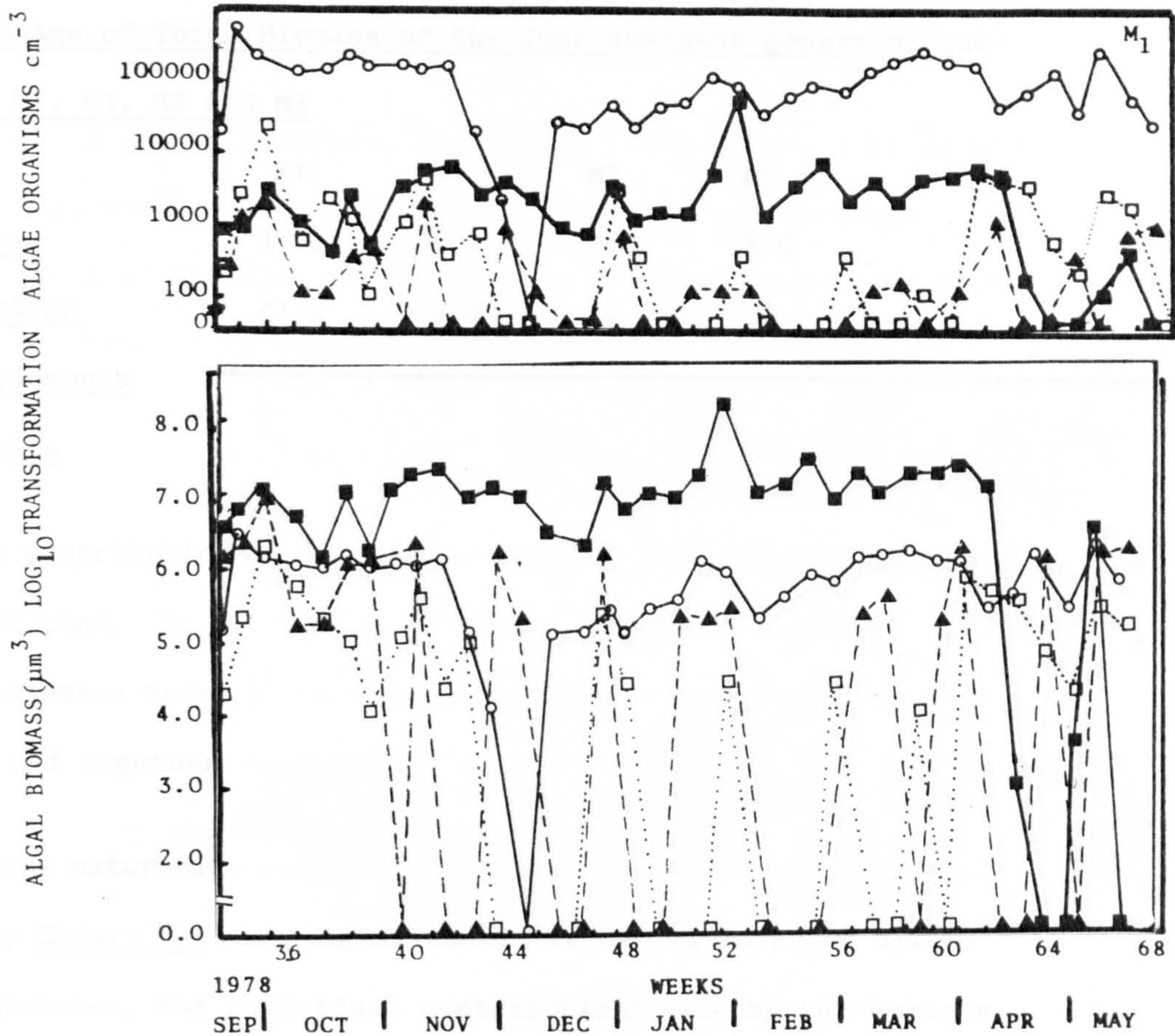


Fig. 3.4 A comparison between data from mean cell number (from Fig. 3.2) with data from mean cell volume (from Fig. 3.3) for the four algal genera present in the Maturation pond M1: Euglena (■), Chlorella (○), Chlamydomonas (□) and Pyrobotrys (▲).

ponds, is presented in Table 3.4.

TABLE 3.4

The approximate individual contribution of each genus as percentage of Total Biomass of the four dominant genera of the ponds F1, M1, M2 and M3

	F1	M1	M2	M3
<u>Euglena</u>	34.5	26.0	22.0	13.0
<u>Pyrobotrys</u>	29.0	9.0	0	0
<u>Chlamydomonas</u>	36.0	17.0	4.0	4.0
<u>Chlorella</u>	0.5	51.0	74.0	83.0

The contribution to the biomass made by each alga varied from pond to pond. In the facultative pond F1 the contribution to the total biomass made by the three flagellate genera was almost equal and together represented 99.5% of the total.

In the maturation pond the contribution to the total biomass made by Chlorella rose significantly (from 51% in M1 to 83% in M3). However, the individual contributions made by the various flagellates did not remain equal and Euglena made the greatest biomass contribution of these species, some 13%, which represented 76% of the total flagellate contribution in M3.

3.4 Discussion

The fluctuations in cell number shown by the different algal genera during the experimental period did not appear to be either

coincidental or sequential in the different ponds. These fluctuations could not be related to obvious variations in climate parameters such as air temperature, rainfall, evaporation or incident solar radiation (Table 3.5).

It would seem, therefore, that these fluctuations must in some way relate to the variations in the 'in-pond' environment. Unfortunately, the mean values for the physico-chemical data of the pond effluent throughout the experimental period (Tables 3.1.1. and 3.2.1) do not provide any clues as to the causes of these changes in algal biomass. This is probably because these data are only mean values based on single point analyses each day and are thus a poor indicator of diurnal and short term fluctuations in these parameters. Furthermore, they take no account of biotic factors such as algal grazers or specific pathogens. Elucidation of such a complex situation would clearly require a more sophisticated approach than was present in this study.

Apart from the conversion of algal number to volume to give a better indication of relative biomass, surface area to volume has been used by Bellinger (1974). This is considered to be an indicator of the potential rate of growth of particular algae. The higher this ratio is, the greater are the photosynthetic efficiency and nutrient absorption rates of the algae (Bellinger, 1974). Cells with such high rates might be expected to exhibit a more rapid population increase.

Table 3.5 Meteorological data for the period September 1978 to May 1979

Year	Mean air temperature	Rainfall total	Evaporation total (mm)	Solar radiation (langleys)	Sunshine hours (h)
September/1978	21.1	80.7	146.1	563	185.0
October	22.2	5.1	180.6	668	249.0
November	22.9	15.7	204.8	681	243.8
December	23.1	31.4	190.9	641	225.4
January	23.9	29.6	184.2	642	241.2
February	24.0	12.4	168.5	683	189.4
March	24.2	35.2	210.3	672	217.0
April	23.9	77.6	191.8	645	212.1
May/1979	22.2	144.3	147.8	491	133.0

Paasche (1960) studying the relationship between photosynthetic rate and standing crop in terms of numbers, volume and cell surface area showed that these three measurements gave different impressions of the importance of the various algal species, also total cell surface correlated best with the rate of photosynthesis ^(correlation coefficient $r = 0.74$) as compared with the cell volume (0.62) and cell number (0.45). In this study on mixed populations where algae vary in size, cell numbers gave little indication of the relative abundance of each species and their contribution to the total biomass.

Fig. 3.4 shows well how the apparent dominance of an algal species can be changed by the type of data manipulation used. Since volume data seemed to give a clear pattern of relative abundance, further transformation of the data to cells surface area was not attempted.

Transformations of cell number data have been recognised by researchers in fresh water ecosystems but have received little attention by workers on sewage pond systems. This makes the analysis of species dominance for such work difficult to interpret and to compare. If treatment is to be in any way related to the dominance of particular algal species then some quantitative analysis of speciation is necessary.

This is explained by the quantitative data on the relative abundance of certain genera present in Table 3.4 which suggest that the dominance of Pyrobotrys and Chlamydomonas and, to a lesser extent, Euglena, is indicative of facultative pond conditions, whereas dominance by Chlorella would suggest a high degree of treatment. Changes in species dominance may also provide useful information on variation in effluent quality when the loads of specific ponds are changed. Of course, speciation may be a factor of latitude and climate and so the actual flora might be different in Brazil compared to the United States. Thus a list of comparable species likely to occur in different latitudes with different degrees of treatment would be useful.

CHAPTER 4

ALGAL BIOMASS

4.1 Introduction

The size of the phytoplankton population (algal biomass) can be made by a variety of methods and includes direct methods in which the fresh or dry weight of the organism present in a volume of sample is determined. The measurement of dry weight is most commonly used. However, in environments such as sewage ponds this method has several disadvantages. The high content of suspended solids and microorganisms such as bacteria and protozoa can complicate accurate measurements of algal mass, since it is difficult to separate the weight of the algae from those of the non-algal components.

Many different indirect estimations of phytoplankton biomass have been attempted. These have included measurements of particulate organic carbon, nitrogen, turbidity, protein, DNA, ATP, cell counts, cell volume and chlorophyll. Of these, microscopic counts and chlorophyll measurements are most often used.

All the indirect methods of biomass measurement require calibration if absolute mass is to be determined. In practice, certain indirect measurements are accepted without absolute

correction. This is particularly true for chlorophyll determinations where the relative and absolute comparison between populations are clearly expressed, for example in $\mu\text{g chl a l}^{-1}$. Conversion factors to change chlorophyll concentration into dry weight are available. For example, assuming that chlorophyll constitutes, on average, 1.5% of the dry weight of organic matter (ash-free weight), estimations of dry weight can be made by multiplying the chlorophyll value by a factor of 67 (APHA, 1980). These values, however, are approximations and dry weight to chlorophyll relationships will depend on the metabolic state and the growing condition of the alga. The problems associated with the estimation of cell counts using a microscope and their subsequent transformation into cell volume have already been discussed in Chapter 3.

The use of chlorophyll as an estimate of algal biomass has become widespread in phytoplankton studies, largely because of the speed, simplicity and reproducibility associated with its measurement (Rai, 1980). As chlorophyll a is the only primary photosynthetic pigment in phytoplankton, the others (b, c, d) being accessory, the determination of chlorophyll a alone is sufficient for most routine monitoring (Nusch, 1980). It has been shown that correction for the chlorophyll a degradation products (phaeophytin and phaeophorbide) by a second absorption reading after acidification is more important than correcting for other chlorophylls. These degradation products may frequently

constitute 16 to 60% (or more) of the chlorophyll a content in both sea water (Jensen & Sakshaug, 1973) and freshwater (Glooschenko et al. 1972; Riemann, 1978). An unacidified/acidified (U/A) ratio of 1.7 (or slightly higher) is indicative of an extract containing 100% chlorophyll. In contrast, if the extract contains nothing but phaeophytin, the U/A ratio will be 1.0. The U/A ratio is used here as an indicator of the physiological state of the algal population with high ratios suggesting a potential for efficient photosynthetic activity and a healthy algae population whereas ratios approaching 1.0 would indicate a moribund one.

In this chapter algal biomass was studied in the ponds of Systems I and II. It was hoped that such measurements would also provide an opportunity to investigate the relationship between the size of the algal population and pond BOD₅ surface loading. Samples of the pond water and effluent were collected and analysed for chlorophyll a concentration.

The samples were obtained at the same time as those for physico-chemical analyses of the effluent, i.e. at 08.00h. This time represented the start of the working day and also allowed sufficient time for all the determinations to be completed and thus avoided unacceptable storage of samples. Also at this time, on the basis of measurements of temperature and pH made throughout the depth of the water column, the ponds were

apparently physico-chemically homogeneous. The extraction procedure and equations used to calculate the chlorophyll a concentrations were described in detail in Chapter 2.

4.2 Results

4.2.1 Chlorophyll measurements on Systems I and II Ponds

The results obtained from the chlorophyll a analyses of samples taken directly from the pond water column and pond effluent of Systems I and II are shown in Figs. 4.1 and 4.2. All the ponds comprising System II showed continuous fluctuations in chlorophyll a concentrations with time. The rapidity and amplitude of these fluctuations were greater in samples of the effluent than in water column samples from the same pond and the various peaks and troughs in the chlorophyll a concentrations did not necessarily coincide in the two sets of data. The weekly chlorophyll a means for effluent samples of each period were usually higher than the values calculated for the corresponding pond water column sample.

Ponds of System I similarly showed fluctuations in chlorophyll a concentrations with time in both pond water column and effluent samples. These fluctuations appeared to be more frequent in the facultative pond F1 than in the following three maturation ponds.

In F1, fluctuations in chlorophyll a concentration were

Fig. 4.1 Results of chlorophyll a analyses for samples taken from the water column (● - continuous line) and effluent (○ - broken line) of ponds from System I (F1, M1, M2, M3) during Experiments 1 and 2.

$\mu\text{g CHLOROPHYLL A l}^{-1}$

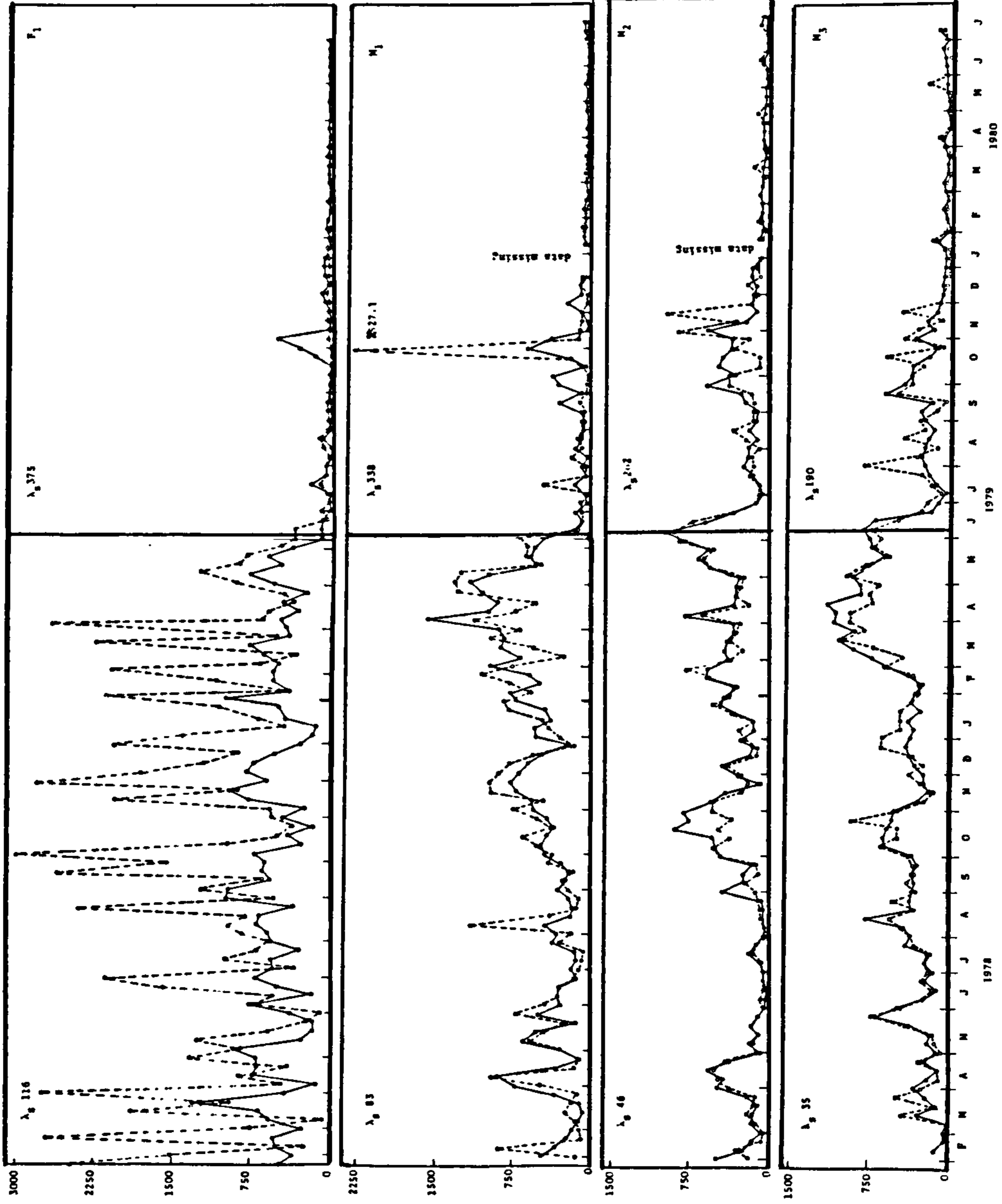
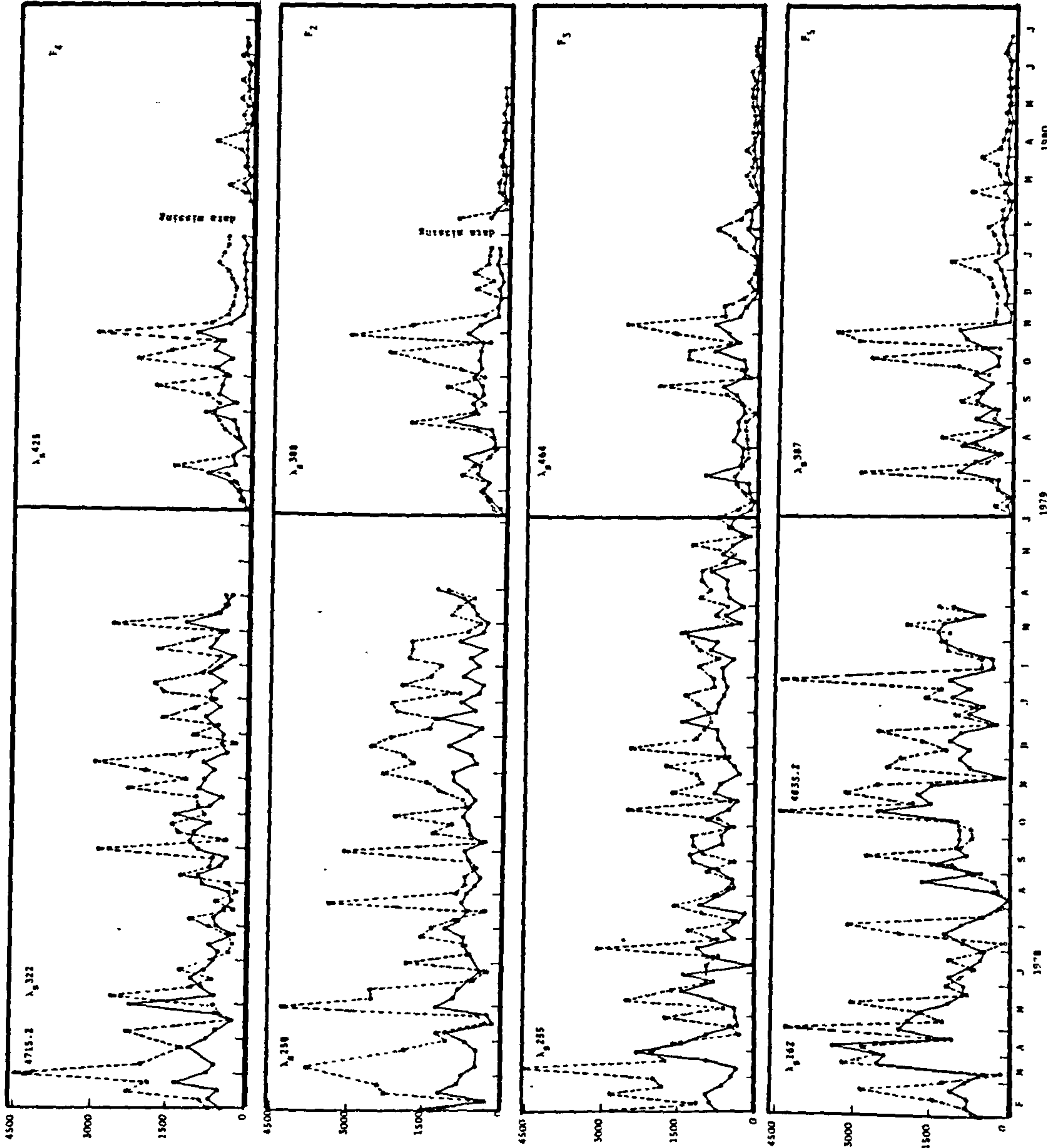


Fig. 4.2 Results for chlorophyll a analyses for samples taken from the water column (● - continuous line) and effluent (○ - broken line) of ponds from System II (F2; F3, F4, F5) during Experiments 1 and 2.

µg CHLOROPHYLL A l⁻¹



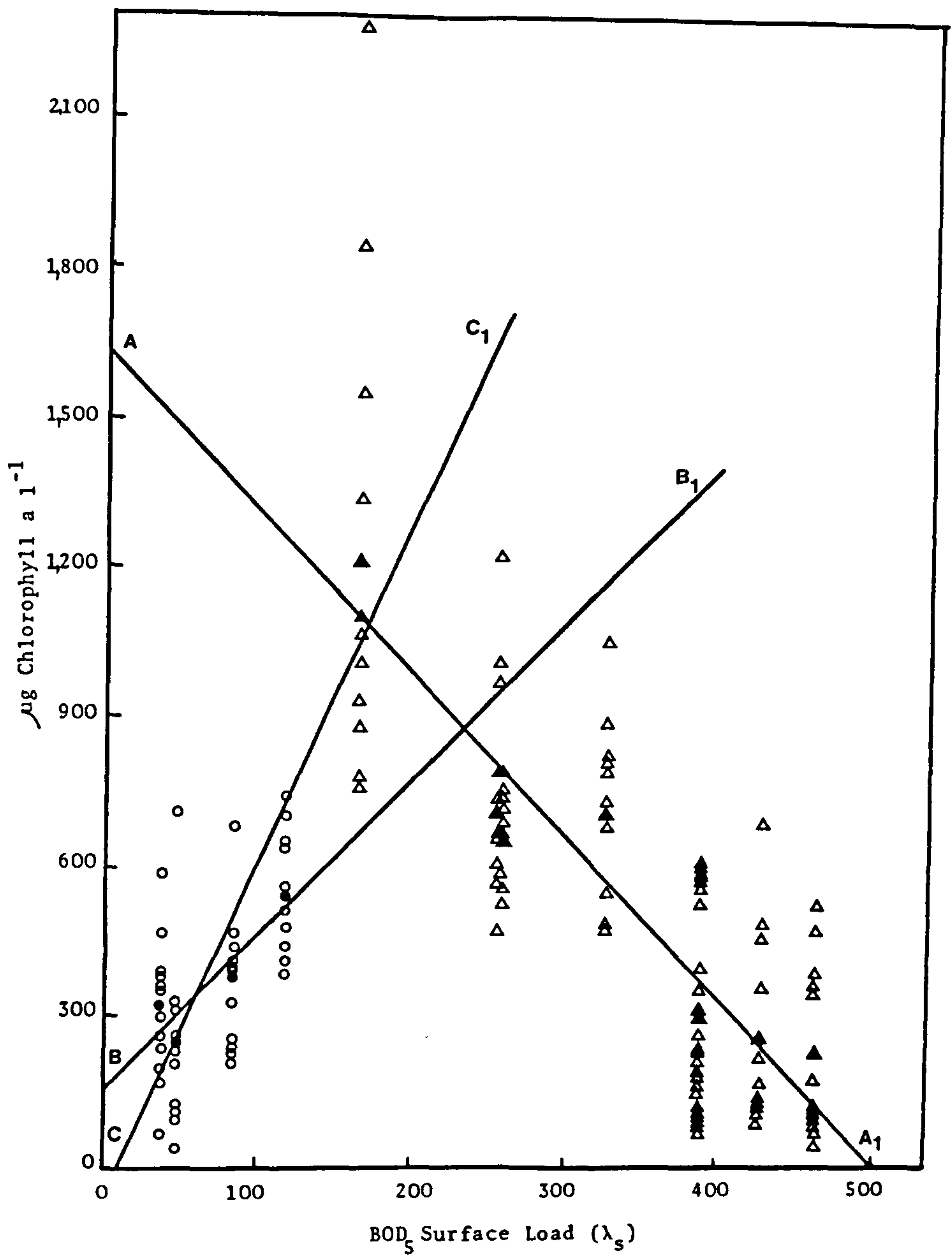
greater in the effluent samples than in the water column samples and thus similar to the facultative ponds of System II.

As sewage treatment progresses through the ponds from F1 to M3, the chlorophyll a values obtained for the water column and the effluent samples coincided more closely both in concentration and in the way they fluctuated with time.

The increase in BOD₅ surface loading on the ponds of both systems during Experiment 2 led to a reduction in the algal standing crop in each pond compared with the values obtained during Experiment 1. This reduction in chlorophyll a concentration in System I ponds was greater than might have been predicted. The F1 pond had become anoxic and a thick algal scum formed on ponds M1 and M2, which unfortunately was not removed on a regular basis by the pond operators. Additionally, floating macrophytes were grown on M3 during at least the latter period of Experiment 2.

The monthly means for chlorophyll a concentration obtained for the pond water column samples were regressed against the pond BOD₅ surface loading for the ponds of System I and II (Fig. 4.3).

These regression lines showed two trends depending on which System was being considered. A negative correlation between chlorophyll a concentration and BOD₅ surface loading was obtained for System II (Table 4.1) where data for Experiments 1 and 2 were



	n	r	confidence limit (%)	regression equation
System I	48	0.56	99.9	$y = 152.1 + 3.16x$
System II	96	-0.79	99.9	$y = 1630.8 - 3.23x$
Ponds below loadings of $200 \text{ BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$	60	0.76	99.9	$y = -66.3 + 6.83x$

combined (regression line A - A₁).

In contrast, a positive correlation (regression line B - B₁) was obtained for ponds of System I during Experiment 1 (Table 4.1). However, it should be mentioned that a negative correlation (not shown in Figure 4.3) was obtained with the data from System I ponds during Experiment 2.

Some monthly means for the chlorophyll a to phaeophytin ratio (U/A) on samples from the ponds of Systems I and II for Experiments 1 and 2 are shown in Table 4.2. The ratios were usually close to 1.7 (or higher) in all the ponds. At no time did these ratios approach 1.0. Even when the BOD₅ surface loading on the ponds was increased substantially (during Experiment 2) the ratios remained high. Thus the increase in organic loading on the pond has altered the biomass concentration and speciation but did not seem to cause any severe deterioration in the physiological state of the algal population.

During Experiment 2, pond F5 was studied to ascertain whether or not a gradation in chlorophyll a concentration occurred from the influent to the effluent. The results of three studies are shown in Fig. 4.4.

There was no significant alteration in the chlorophyll a concentration from the influent to the effluent. Regression analysis of the chlorophyll a concentration with distance from

Table 4.2 Chlorophyll a : phaeophytin ratio (U/A) on samples from ponds of Systems I and II during Experiments 1 and 2

Experiment 1

Pond	M3	M2	M1	F1	F5	F3	F2	F4
λ_s	35	46	83	116	162	255	258	322
<u>1978</u>								
February	1.37	1.53	1.52	1.55	1.66	1.65	1.61	1.72
March	1.54	1.42	1.40	1.66	1.60	1.57	1.54	1.63
April	1.59	1.40	1.33	1.60	1.54	1.39	1.56	1.99
May	1.60	1.36	1.41	1.41	1.55	1.60	1.52	1.62
June	1.44	1.27	1.37	1.46	1.42	1.54	1.52	1.44
July	1.47	1.30	1.42	1.47	1.45	1.55	1.52	1.45
August	1.63	1.31	1.59	1.25	1.62	1.76	1.75	1.62
September	1.78	1.60	1.64	1.77	1.69	1.69	1.75	1.69
October	1.71	1.70	1.65	1.94	1.72	1.85	1.91	1.77
November	1.52	1.46	1.53	1.75	1.57	1.62	1.92	1.73
December	1.56	1.43	1.46	1.64	1.60	1.66	1.76	1.72
<u>1979</u>								
January	1.61	1.57	1.53	2.23	1.63	1.77	1.74	1.70

Experiment 2

Pond	M3	M2	M1	F1	F5	F2	F4	F3
λ_s	190	262	338	375	387	388	425	464
<u>1979</u>								
June	1.80	1.96	1.89	1.84	2.09	1.57	1.58	1.60
July	3.14	1.28	1.90	1.80	1.65	1.65	1.57	1.64
August	2.22	1.64	1.80	1.96	1.54	1.74	1.50	1.70
September	1.60	1.70	1.71	1.39	1.66	1.66	1.62	1.65
October	1.55	1.61	1.58	1.44	1.59	1.65	1.63	1.54
November	1.91	1.50	1.58	1.32	1.70	1.75	1.89	1.83
December	1.70	1.71	1.83	1.73	1.86	1.84	1.86	1.56
<u>1980</u>								
January	1.58	1.54	1.59	1.54	2.12	1.91	1.93	1.78
February	1.70	1.76	1.70	1.68	1.85	1.96	1.86	1.80
March	2.00	1.93	1.89	1.96	1.80	1.68	1.89	1.76
April	1.69	1.44	1.42	1.22	1.72	1.75	1.68	1.73
May	1.51	1.55	1.42	1.42	1.70	1.65	1.75	1.70

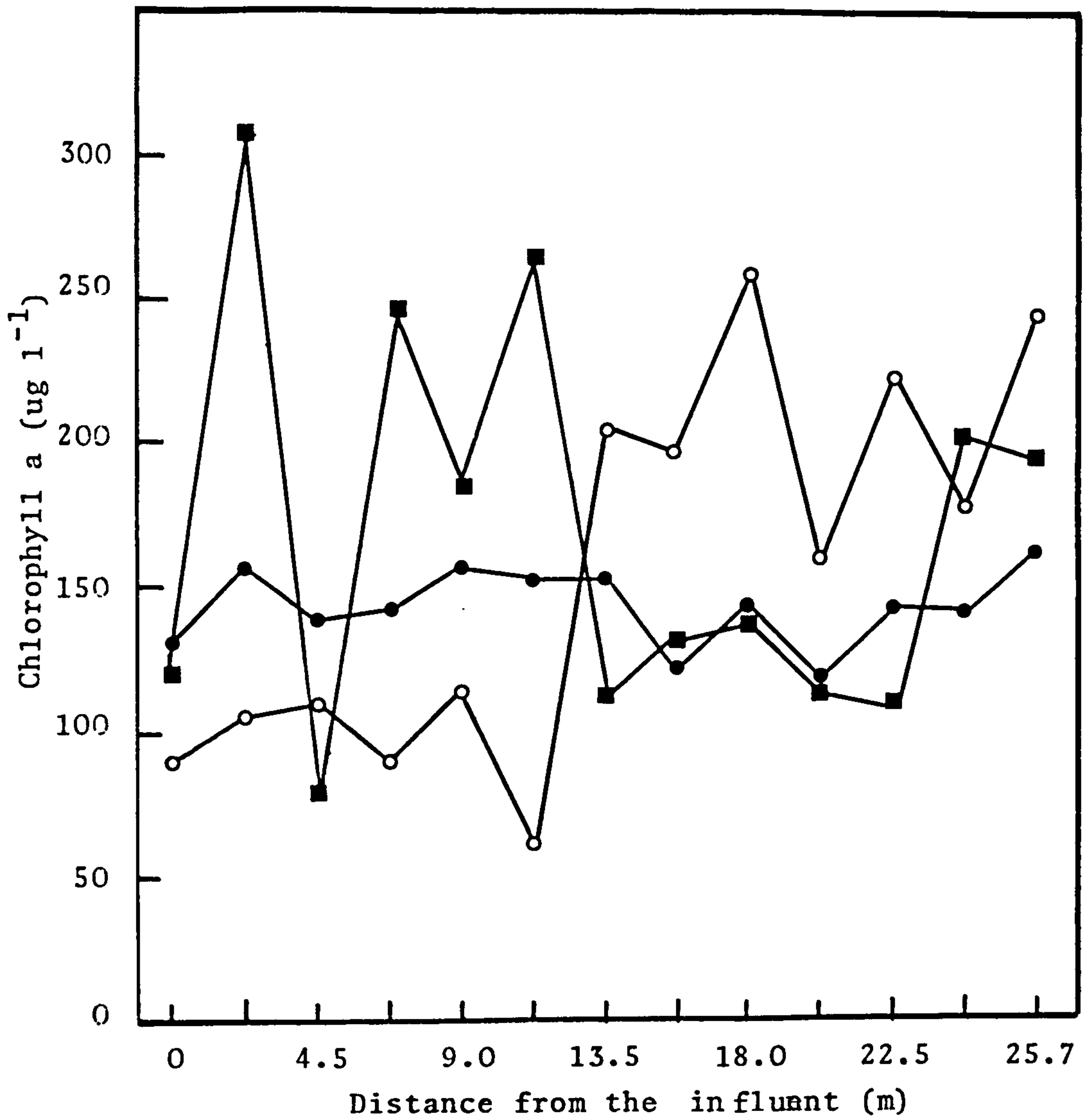


Fig. 4.4 Total water column chlorophyll a concentrations of samples at distances along the length of the pond F₅ (from the influant to the effluent) on three occasions: ○ 22.11.79; ■ 28.11.79 and ● 13.12.79.

the influent point showed a very low correlation ($n = 36$, $r = 0.14$) since the chlorophyll a concentrations were frequently 'patchy' throughout the length of the pond.

During Experiment 1 it was observed that during certain periods the GFC filter pads remained green, after the 16 hour extraction period in 90% (v/v) acetone indicating incomplete pigment extraction. This was only true of the GFC pads which had been used to filter algal samples from maturation ponds.

Microscopic examination of these samples showed a high cell concentration of the alga Chlorella. An attempt was made to improve the chlorophyll extraction by changing the solvent used and the method of pigment extraction. Boiling the filter pads for 2 minutes in a known volume of 90% (v/v) methanol was employed. As in acetone, spectrophotometric readings were carried out in samples stored in a refrigerator for 16 hours. No differences in the absorption readings were found between refrigerated samples and samples measured after boiling. The absorbance readings in these cases were converted into μg chlorophyll a l^{-1} using the recommended extinction coefficient of 77 (see Golterman, 1971).

In M3, chlorophyll a extraction using 90% (v/v) methanol was carried out in the period between 20.02.79 to 19.06.79 whereas in M1 and M2 from 07.05.79 to 11.06.79. It is possible that changing the solvent extraction method may distort the pattern of

biomass production and may be responsible for increasing the apparent yield. Scrutiny of the data in Figure 4.1 does show that biomass apparently increases significantly in M3 during the period of methanol extraction. However, no such increases occurred in M1 and M2 and errors produced due to the changes in solvent were probably less than would have occurred if poor extraction in acetone had been continued.

4.2.2 Algal contribution to COD

Laboratory experiments were done to determine the COD contribution made by various algae present in pond effluents.

Four different algae were used: Euglena, Pyrobotrys, Osillatoria and Chlorella. The latter two algae were isolated from a maturation pond and cultivated in the laboratory, whereas Euglena and Pyrobotrys were obtained as virtual monoculture directly from the effluent of different facultative ponds. The algae were centrifuged down, washed several times and resuspended in distilled water before suitable dilutions were made.

Chlorophyll a concentration and COD estimations were made on the appropriate cell dilutions of the four algal species.

Regression analysis between these parameters for each alga is shown in Table 4.3.

Positive correlation was obtained for each alga separately

(Fig. 4.5a) and when all the algal data were combined together regardless of species (Fig. 4.5b).

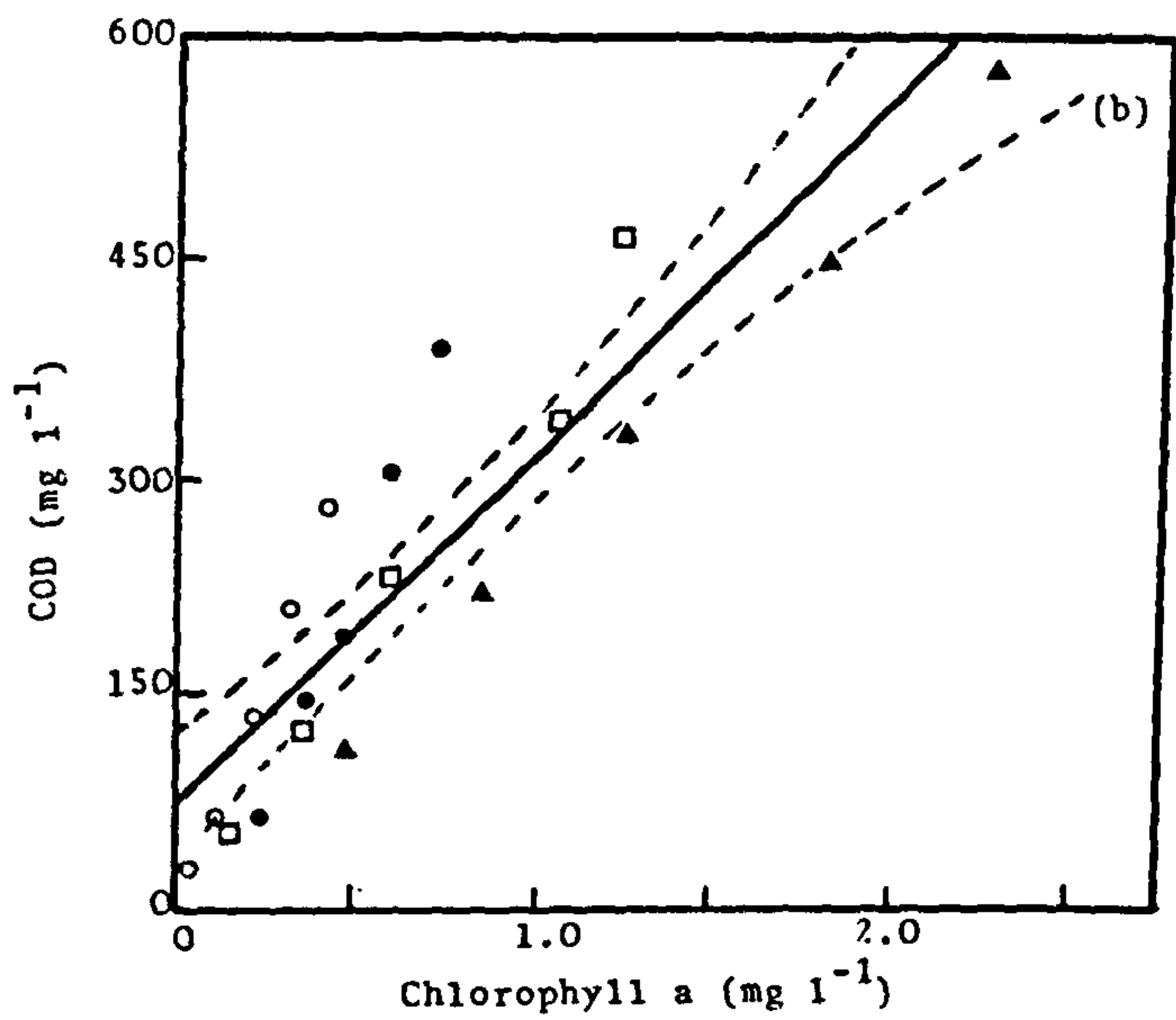
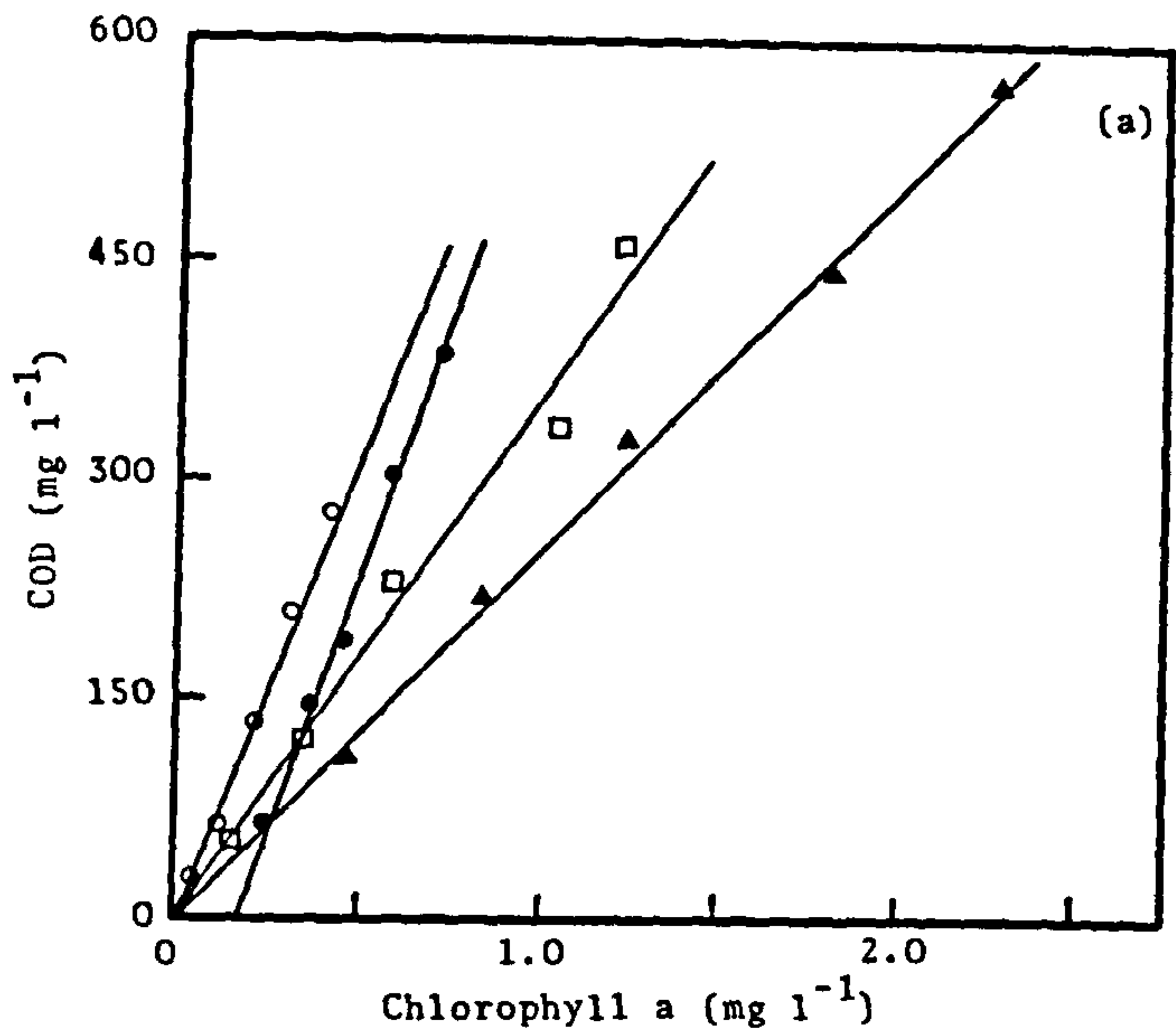
These regression data show that different algal species contribute different amounts of COD for the same unit of chlorophyll a concentration. Therefore the COD produced by an effluent with Chlorella as the predominant alga would be higher (some 2.5 times higher) than an effluent with Euglena, provided that the biomass (chlorophyll a concentrations) were the same.

It would be better to relate COD contribution to dry weight since it is possible that alga concentration of chlorophyll a may vary with environmental conditions. However, precise estimation of dry weight in a sewage pond environment is difficult to obtain because of the presence of non-algal suspended solids. After all that, measurements of chlorophyll a would be acceptable.

The different gradient obtained from the regression line for the different algae may be at least partly due to the fact that two of the species were grown in laboratory cultures. The regression line with the 95% confidence belt (Fig. 4.5b) obtained by manipulating all algal data together suggests that algal biomass concentration represented by 1 mg chlorophyll a l^{-1} is going to produce a COD contribution of nearly 300 mg l^{-1} .

- Fig. 4.5 (a) Regression lines between chlorophyll a and COD for 4 algal types: ○ Chlorella; ● Pyrobotrys; □ Oscillatoria and ▲ Euglena.
- (b) Regression line between chlorophyll a and COD using the combined algal data from Fig. 4.5(a) with the confidence belt set of 95%.

Tab. 4.3 Details for the regression analyses between chlorophyll a and COD for four algal types and the combined algal data shown in Fig. 4.5: n is the number of samples in the regression; r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line, where $y = \text{COD (mg l}^{-1}\text{)}$ and $x = \text{chlorophyll a (mg l}^{-1}\text{)}$.



	n	r	confidence limit (%)	regression equation
<u>Euglena</u>	5	0.999	99.9	$y = -3.43 + 257.98x$
<u>Pyrobotrys</u>	5	0.994	99.9	$y = -116.52 + 690.66x$
<u>Oscillatoria</u>	5	0.987	99 - 99.9	$y = 1.34 + 361.06x$
<u>Chlorella</u>	5	0.998	99.9	$y = -4.98 + 654.53x$
Combine algal data	20	0.904	99.9	$y = 74.36 + 240.10x$

4.3 Discussion

Fluctuations with time of the algal biomass present in sewage ponds have been reported by many authors. According to Shillinglaw and Piertese (1977) the 'wax and wane' pattern of algae in the effluent of South African ponds was due to changes in environmental conditions. Whereas van der Post and Torien (1974) observed that a decline in algal biomass only occurred during the warmest month of the year, severe reductions in algal population in sewage ponds as a result of heavy grazing by rotifers and cladocerans has also been reported (de Noyelles, 1967; Raschke, 1970).

The fluctuations in the algal biomass observed in the EXTRABES ponds during Experiment 1 did not appear to be related to either changes in the environmental conditions or to zooplankton grazing.

In the north east of Brazil air temperatures (and thus water temperatures) and light intensities undergo little variation throughout the year (see Table 3.5). Consequently no seasonal variation of the algal biomass would be expected nor in fact was found. The fluctuations that were observed could not be correlated with zooplankton activity in the ponds or with any short term changes in BOD₅ surface loadings caused by changes in sewage strength. The causes of these large and sudden variations are as yet unresolved.

The movement of the algae through the water column and their stratification into distinct zones were almost certainly responsible for the large fluctuations in chlorophyll a concentration recorded in the effluent samples and the discrepancies between effluent chlorophyll a values and those of the water column samples taken at the same time. Algal stratification also seemed more pronounced in the facultative ponds than the maturation ponds and when the algal band was near to the effluent take-off level it caused the effluent to become dark green. This algal movement has implications for the effluent quality and it is dealt with in more detail in Chapter 5.

The negative correlation between pond chlorophyll a and mean BOD₅ surface loading from System II ponds (line A-A₁, Fig. 4.3) emphasizes the reduction in algal biomass caused by increasing the loadings. This would also imply that maximum algal biomass would occur in clean water. This is unlikely since in such a situation algal growth would be impaired by nutrient limitation. This idea is supported by the positive gradient of the regression line B - B₁ for System I ponds which generally had the lowest surface loadings. The intercept of the regression line of System II (A - A₁) with the regression line of System I (B - B₁) in Fig. 4.3 gives some indication where this point of nutrient limitation might affect algal growth in sewage ponds. This intercept occurs at a pond loading of 230 kg BOD₅ ha⁻¹ d⁻¹.

Included in Fig. 4.3 is a third regression line ($C - C_1$) obtained using data from all the ponds with BOD_5 surface loading below $200 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$. In this case the intercept with $A - A_1$ occurs at $170 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$.

Therefore in north east of Brazil, BOD_5 surface loadings of between 170 and $230 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$ probably represents the range in which a maximum algal growth can be expected to occur with a fall off in biomass concentration at loadings above or below this range.

Ponds with loads approaching $500 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$ are, under the environmental conditions experienced in north east of Brazil, unlikely to support algal growth and will cease to function as Waste Stabilization Ponds

If the growth and harvesting of algae is required as part of an integrated sewage treatment plant and algal biomass production, system pond loads should remain within the range of $170-230 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$. In contrast if treatment of high quantities of sewage is the aim, much higher BOD_5 surface loadings can be used. Provided that retention time is adequate, a reasonable effluent quality will still be produced.

Based on the results obtained from Fig. 4.3 comparable values for chlorophyll a concentration would be expected for F1, in Experiment 2 and F4 in Experiment 1 since they had similar BOD_5

surface loadings (375 and 322 kg BOD₅ ha⁻¹ d⁻¹ respectively). This was not so since the mean value for F1 was 57.84 µg chlorophyll a l⁻¹ and 712.7 µg chlorophyll a l⁻¹ for F4. The unexpectedly low values of chlorophyll a concentration in F1 may be the result of other factors than simply BOD₅ surface loading. The probable cause for the very low chlorophyll a concentration in F1 was the much shorter retention time of 1.6 days as against 9.5 days of F4. A retention time of 1.6 days would be near to the algal doubling time and the algae would be washed out with the effluent.

The algal biomass results presented here would predict that for the climatic conditions prevailing in the north east of Brazil, Paraíba State in particular, Waste Stabilization Ponds with BOD₅ surface loading of up to 300 kg BOD₅ ha⁻¹ d⁻¹ are quite acceptable, whereas ponds with BOD₅ surface loadings of above 400 kg BOD₅ ha⁻¹ d⁻¹ whilst functioning acceptably (Silva, 1982) will almost certainly malfunction if the loading is inadvertently increased by a relatively small amount.

Chapter 5

DIURNAL STUDIES ON POND EFFLUENT QUALITY

5.1 Introduction

The performance and efficiency of any sewage treatment plant is judged on the quality of the effluent it produces. In addition, the sewage treatment plant should be free from objectionable odours, especially when located near human habitation.

Waste Stabilization pond (W.S.P.) performance is traditionally measured in terms of BOD₅, SS and FC removal. Values for BOD₅ and SS of a pond effluent depend to a large extent on the algal concentration and therefore these two parameters may not necessarily be good measures of the degree to which the sewage has been treated. However, in developing countries, where the effluent is to be re-used for irrigation or discharged into rivers and lakes which are used by local people for drinking, ablutions and recreation, effluent criteria should also emphasise pathogen standards. Various standards are recommended depending on how the effluents are to be utilised i.e. for irrigation of different types of crops or for discharge into receiving streams (Arthur 1982).

Evaluation of the effluent quality of sewage ponds is usually made at best from data obtained from weekly, bi-weekly or monthly samplings. Very little data is available on the diurnal

variation in physico-chemical, bacteriological and algological parameters.

If pond performance is to be gauged on the basis of occasional grab samples then the sample must at least be representative of the mean diurnal effluent quality. Thus the timing of sample collection may be critical.

In this section, effluents from a facultative and a maturation pond were monitored at regular intervals throughout 24 h periods. Special emphasis was given to how the algal population might influence the other effluent parameters during these experiments.

5.2 Method

Effluent samples from the facultative pond F4 and the maturation pond M3 were taken every two hours during a period of 24 h (from 08.00h to 08.00h next day). These studies were carried out on two different occasions: F4 in 20-21/09/78 and M3 in 24-25/10/78. The samples were analysed immediately for pH, suspended solids, BOD₅, COD, nitrate, ammonia total and soluble phosphorus, chlorophyll a and F. coliform. The method details have been previously described in Chapter 2. "In situ" measurements of dissolved oxygen and temperature were also recorded 5 cm below the pond surface (i.e. at the effluent take-off depth).

5.3 Results

The results obtained for these diurnal studies in F4 and M3 are presented in Figures 5.1 and 5.2 respectively.

The chlorophyll a concentration of the F4 effluent *ranged* between 10,617.8 and 63.4 μg , chlorophyll a l^{-1} with the maximum and minimum values being recorded at 12.00h and 24.00h respectively.

The highest concentrations of chlorophyll a were discharged in the effluent during day time with very few or no algae in the effluent at night. In contrast, the chlorophyll a concentration in M3 effluent varied much less, but again maximum values (1,137.1 μg l^{-1}) occurred during day time. At night the chlorophyll a concentration remained uniform around 500.0 μg l^{-1} .

"In situ" dissolved oxygen (D.O.) measurements in F4 at the effluent take-off depth showed a period of supersaturation between 10.00h and 18.00h and complete anaerobiosis during the night. In contrast, D.O. measurements in M3 showed that at 5cm depth, the pond was aerobic during the whole 24h period with supersaturation occurring between 12.00h and 16.00h.

These periods of oxygen supersaturation occurred during the day light hours when presumably algal photosynthesis was occurring but did not necessarily coincide with the maximum algal concentration at the effluent take-off level.

Fig. 5.1 Variations in F_4 of:

- a) Dissolved oxygen (\square), pH (\bullet), water temperature (\blacktriangle);
- b) Ammonia (\blacksquare), Total Phosphorus (\blacklozenge), Soluble Phosphorus (\diamond);
- c) Suspended solids (\circ), BOD₅ (\bullet);
- d) Chlorophyll a (\triangle), F. coliform (\blacktriangle)

Dissolved oxygen was measured "in situ" 5 cm below the surface i.e. at effluent take-off point.

Dotted lines between points indicate that the data is underestimated due either to instrument insensitivity or in the case of BOD₅, the dilution range was insufficient.

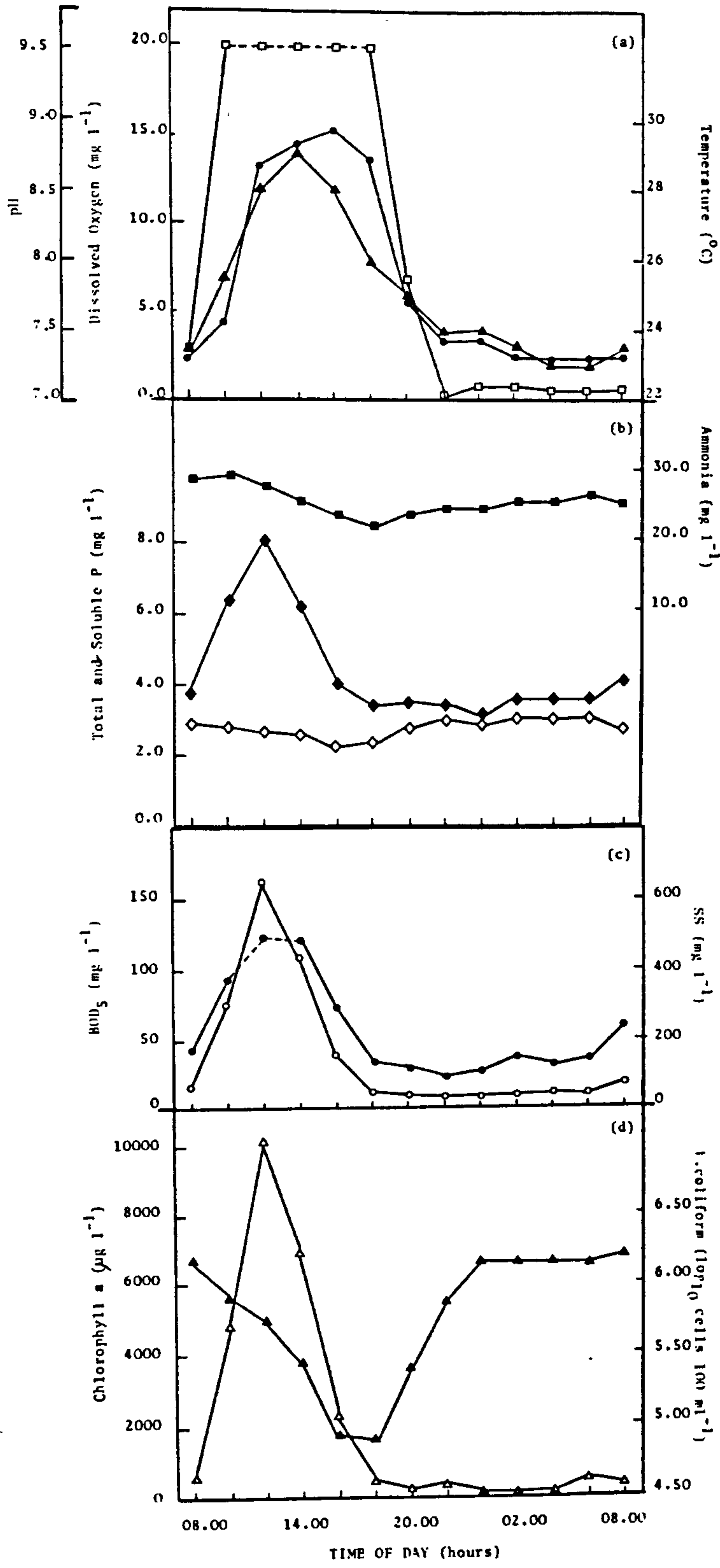
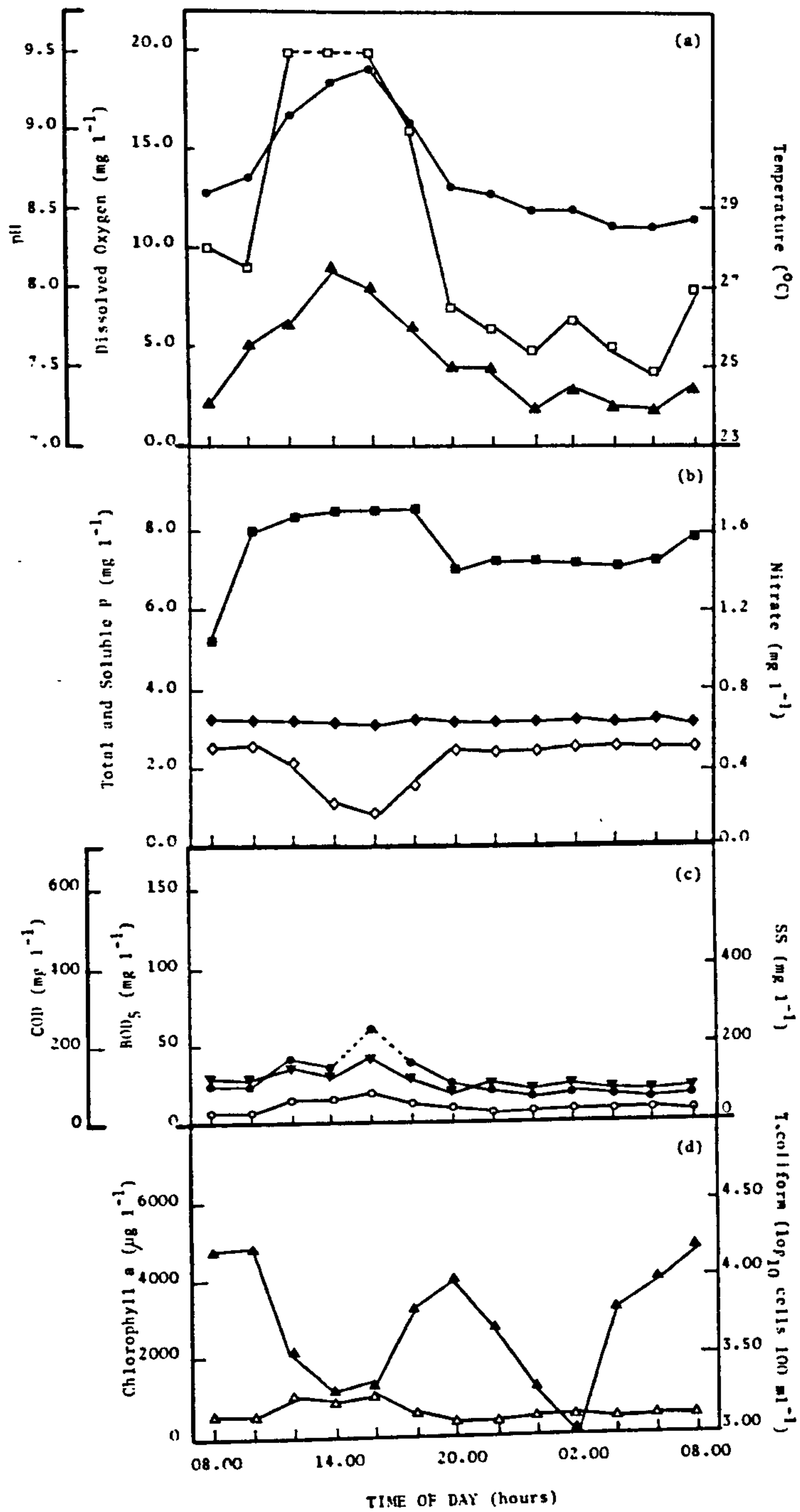


Fig. 5.2 Variations in *M3* of:

- a) Dissolved oxygen (\square), pH (\bullet), water temperature (\blacktriangle)
- b) *Nitrate* (\blacksquare), Total Phosphorus (\blacklozenge), Soluble Phosphorus (\diamond);
- c) Suspended solids (\circ), BOD₅ (\bullet), COD (\blacktriangledown);
- d) Chlorophyll a (\triangle), T. coliform (\blacktriangle)

Dissolved oxygen was measured "in situ" 5 cm below the surface i.e. at effluent take-off point.

Dotted lines between points indicate that the data is underestimated due either to instrument insensitivity or in the case of BOD₅, the dilution range was insufficient.



In the effluent of F4, the F. coliform counts varied by up to an order of magnitude during day time. Their low numbers coincided with high dissolved oxygen and frequently with high algal concentrations during daylight.

Total coliform counts were measured in the effluent of M3 by the MPN technique. This was done because it was felt that the low F. coliform counts would make it difficult to accurately measure variation in bacteria concentration with time. The results showed that T. coliform (like the results for F. coliform in F4) decreased during the day.

In both pond effluents the bacterial numbers increased again at night and by 08.00h the next morning, their numbers had returned to those found at the beginning of the diurnal study.

The clear pattern of recovery in numbers of F. coliform measured in effluent samples of F4 during the night was less distinct for T. coliform in M3. However, the fluctuations in M3 effluent samples might be related to the inherent inaccuracies in the MPN method and not represent a different behaviour pattern of the bacteria.

The routine monitoring of nitrate in F4 effluent had established that extremely low concentrations were present and so only ammonia was measured. In fact little variation in ammonia occurred during the experimental period. In contrast, in M3

similar routine monitoring had shown that it was the ammonia concentration that was low and so only nitrate was measured. Little variation in nitrate concentration was detectable over the experimental period.

In the effluents of both ponds the concentrations of soluble phosphorus were similar and decreased as the pH of the water increased during the day.

Microscopic examination of the effluent during these diurnal studies showed the predominance of flagellate algae (viz. Euglena, Pyrobotrys, Phacus and Chlamydomonas) in the effluent of F4, whereas non-motile algae (Chlorella, Micractinium) were the commonest algae in the effluent of M3.

Fluctuations in the parameters measured, and thus in effluent quality, were greater in F4 than M3.

In both pond effluents, good positive correlations were obtained for chlorophyll a against changes in SS, BOD₅, D.O. and pH (Table 5.1). Good positive correlation was also obtained for chlorophyll a against Total Phosphorus in F4 effluent.

In contrast, negative correlations between coliform bacteria and changes in pH and D.O. were obtained in F4 but were not as convincing in M3 (Table 5.1).

Table 5.1 Correlation coefficient (r) and confidence limit (%) for regression analyses between various effluent parameters measured bihourly over a period of 24h in F4 and M3.

The number of samples (n) was 13.

Correlated Parameters	POND			
	F4		M3	
	r	Confidence limit (%)	r	Confidence limit (%)
chl a x SS	0.99	99.9	0.94	99.9
chl a x BOD ₅	0.95	99.9	0.90	99.9
chl a x COD	-	-	0.94	99.9
chl a x D.O.	0.70	99 - 99.9	0.93	99.9
chl a x T. Phosp	0.98	99.9	0.16	< 80
chl a x pH	0.61	95 - 99	0.90	99.9
pH x D.O.	0.88	99.9	0.96	99.9
pH x log ₁₀ F. coliform number	-0.80	99.9	pH x log ₁₀ T. coliform	-0.39 80 - 90
D.O. x log ₁₀ F. coliform number	-0.75	99 - 99.9	D.O. x log ₁₀ T. coliform	-0.31 < 80
chl a x log ₁₀ F. coliform number	-0.16	< 80	chl a x log ₁₀ T. coliform	-0.36 < 80

5.4 Discussion

These results show that considerable variation occurred in certain of the effluent parameters measured over 24h and consequently in the quality of effluent during this period. The amplitude of these variations depended upon the type of pond.

In facultative pond (F4) the high algae concentrations in the effluent correlated positively with the observed increases in SS and BOD₅ (Table 5.1), a fact that had also been noted by earlier workers, including Cadwell (1946). It is therefore possible that at some stage during the day the effluent of a W.S.P., particularly a facultative one, may contain nearly as much organic material as the sewage entering it because of the high density of algae. However, the solids in the incoming sewage represent a public health hazard, because of the associated pathogens; in contrast the algae in the effluent are not recognised to be associated with pathogens which should have been killed during the retention in the pond. Furthermore a high algae concentration in the effluent frequently coincides with high levels of dissolved oxygen which may minimize the immediate oxygen demand in the receiving water bodies. This high algal concentration will represent an immediate oxygen demand at night due to respiration, and no photosynthetic activity, unless it has been diluted out in the receiving water.

Significant correlation was obtained between F. coliform numbers and both dissolved oxygen concentration and pH in the effluent of F4 and between T. coliform at these parameters for M3 (Table 5.1). The negative correlation between algal biomass (chlorophyll a concentrations) and coliform numbers however were less significant for both ponds. This suggests that it may not be the concentration of algae per se which is important but their rate of metabolic activity. Thus although high pH and high concentrations of dissolved oxygen coincide with low coliform numbers in the effluent, and could be the direct cause, they also indicate periods of rapid algal photosynthesis and thus high metabolic activity. It could be that during such periods effective concentrations of antibacterial compounds are also released by the algae. Furthermore antibacterial activity could be the result of the synergistic effect or combinations of several parameters.

On the basis of these data another factor that requires investigation is whether or not the change in bacterial numbers that occurred with time in the effluent of the same pond are the result of death or migration through the water column away from unfavourable conditions.

The reasons for the observed reductions in F coliform numbers in W.S.P. effluents has received considerable attention. Several hypotheses have been put forward to account for these reductions.

Pratt (1944) isolated from laboratory cultures of Chlorella a substance called chlorellin, which possesses antibacterial properties most effective against gram-positive bacteria. Cadwell (1946) suggested that the efficiency of W.S.P. in removing coliform bacteria was based upon the liberation of antibacterial substances by algae, presumably compounds like chlorellin.

The algae might also be affecting the bacteria in an indirect way (Cadwell, 1946; Oswald et al., 1957). Nutrient depletion especially in maturation ponds has been proposed as a factor responsible for reducing the bacterial population in W.S.P. (Smallhorst, 1953). The high oxygen levels found in pond effluents during the day and the rise in the pH of the water to 9.0 to 9.5 have also been suggested.

For instance, field observations by Parhad and Rao (1974) noted a reduction in E. coli numbers whenever the pond pH increased as a result of algal photosynthesis. Their laboratory studies confirmed their field observations. A rise in pH was responsible for the reduction of bacteria and E. coli could not grow in waste water when the pH was greater than 9.2.

The combination of ammonia concentration and pH measured in the effluent of F4 over the 24 h period (Fig. 5.1) were near to those considered toxic to algal growth and photosynthesis by

Abeliovich and Azov (1976) i.e. concentrations greater than 2.0 mM and pH values above 8.0.

Therefore the inherent ammonia concentration could be a key factor limiting the BOD₅ surface loading capacity of facultative ponds in these latitudes even when the sewage is predominantly of a domestic rather than industrial nature.

The diurnal measurements also showed that temporary increases in pH (rather than ammonia concentrations) may cause periods of inhibition of algal photosynthesis during the day which would not be predicted on the basis of 08.00 h measurements of pH. Such periods of inhibition however may or may not be reversible or be prolonged enough to alter overall pond performance considerably by affecting algal growth rates or "wash-out" of the algal population.

What does need to be clarified in the context of these observations, and those of Abeliovich and Azov (1976), is whether pond algal populations routinely subjected to high concentrations of ammonia at high pH increase their tolerance to ammonia above the suggested value of 2.0 mM when the pH exceeds 8.0. It should be noted that values are based on laboratory experiments on only a few species.

In a facultative pond in particular, high concentrations of algae in the effluent correlated positively with increases in the

amount of Total Phosphorus (Table 5.1). The algae can also indirectly affect the concentration of the soluble phosphorus present in the effluent as a result of photosynthetic activity. This raises the pH above 9.0 causing precipitation of soluble phosphorus.

Grab effluent samples may not truly represent the mean effluent quality of a pond since considerable fluctuations on certain parameters were recorded (Table 5.2). However the 08.00h sample, which represented the routine sampling hour in EXTRABES, did not greatly deviate from the 24th mean value (at least for this experiment) and thus gives reasonable representation of effluent quality.

Great care should therefore be taken in the interpretation of grab sample data when comparing ponds of the same locality, if samples were collected at different times.

The importance of the algae in determining the fluctuations in effluent parameters have been clearly shown here. Furthermore the removal of algae from the effluent prior to discharge could bring about a significant reduction in SS, BOD₅, COD and Total Phosphorus concentrations. It is possible that this might be feasible at least in facultative ponds by simply ensuring that the effluent is not taken from the algal rich zone. To this end a more detailed study of algal stratification is essential.

CHAPTER 6

MICROBIAL STRATIFICATION IN PONDS

6.1 Introduction

Algal stratifications in sewage oxidation ponds have been studied by several authors. Munawar & Zafar (1967) studying a bloom of Eudorina elegans in India during April 1963, showed some interesting patterns of vertical distribution of these algae. There was a movement from the bottom to the upper strata at noon, when bright sunshine prevailed. They concluded that the migration of Eudorina elegans was controlled by temperature and light intensity at different times of the day. They could not explain why the alga was not present in any of the observed strata of the pond water at dusk and suggested that it might be present in the silt at the bottom of the pond.

Hartley and Weiss (1970) studying the vertical migration of algae in a tertiary oxidation pond in North Carolina (U.S.A.) found similar results. Euglena rostifera was the dominant algae in these ponds and was able to move vertically and select an appropriate light intensity during daylight hours. Euglena rostifera moved into the sludge between 18.00h and 20.00h, remaining there through the night. *Their* laboratory studies showed that this algae could survive and grow in complete darkness for 2 weeks, indicating that Euglena rostifera is able to live chemosynthetically in the sludge at night in the oxidation ponds.

Vertical migration was independent of temperature in the range 20°C - 29°C.

Stratification of the algae in certain of the EXTRABES sewage ponds was first noticed during the collection of pond water column samples with the perspex tube, where a narrow green algal band varying in its depth in the water column could be observed. It was also observed that during the day the colour of the effluent could change from bright green to colourless. This suggested that not only did the algae stratify but that they also showed diurnal vertical movement in the water column.

This vertical migration of the phytoplankton present in EXTRABES waste stabilization ponds was examined in some detail. *Studies* were carried out soon after the diurnal studies on the pond effluent described in Chapter 5.

6.2 Materials and methods

Preliminary stratification studies in the facultative pond F4 and the maturation pond M3 were carried out using the perspex column fitted along its length with sampling posts. This method was soon abandoned because of the almost immediate destruction of the stratified algal layer, as the algae sank rapidly through the column of water in the tube while it was being removed from the pond.

An alternative method for sampling at different depths was employed. It consisted of a small manual peristaltic pump fitted with a teflon tube. This tube was approximately 80 cm long and was attached to two horizontal parallel plates of 10 cm in diameter set 5 cm apart as shown in the diagram. These plates had the advantage of restricting the vertical sample direction. The advantage of the peristaltic pump system of collection was that as it caused little disturbance of the vertical water layer, large volumes could be collected and the samples could be accurately obtained from a known 5 cm zone anywhere in the water column. Also there were no problems with the algae cells sinking during sampling or disturbance of algal stratification associated with the use of the perspex column.

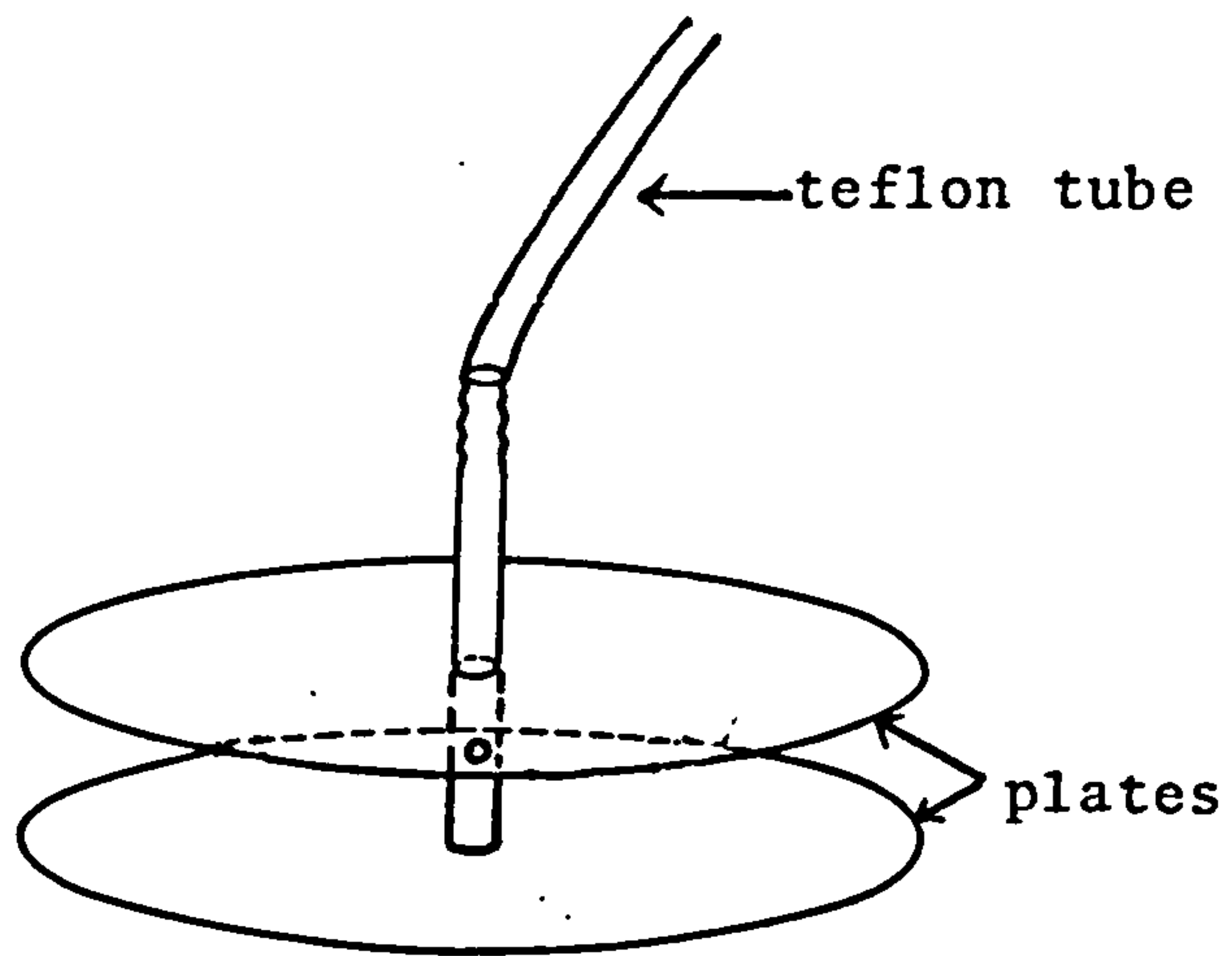


Diagram of the Peristaltic Pump

Sampling procedure using the peristaltic pump: the teflon tube (marked at 5 cm intervals) and the collector, were submerged carefully in the pond water and samples at the first depth were taken. Then the collector was slowly lowered to the next depth, but before taking the next sample pond water from the previous depth had to be removed. This was done by pumping pond water from the new depth until water from the previous sample was totally displaced. This procedure was followed for all depths sampled and during all the experiments. Samples for chlorophyll a analysis were collected from the water column during the daylight period of 12 hours (from 05.00h to 17.00h).

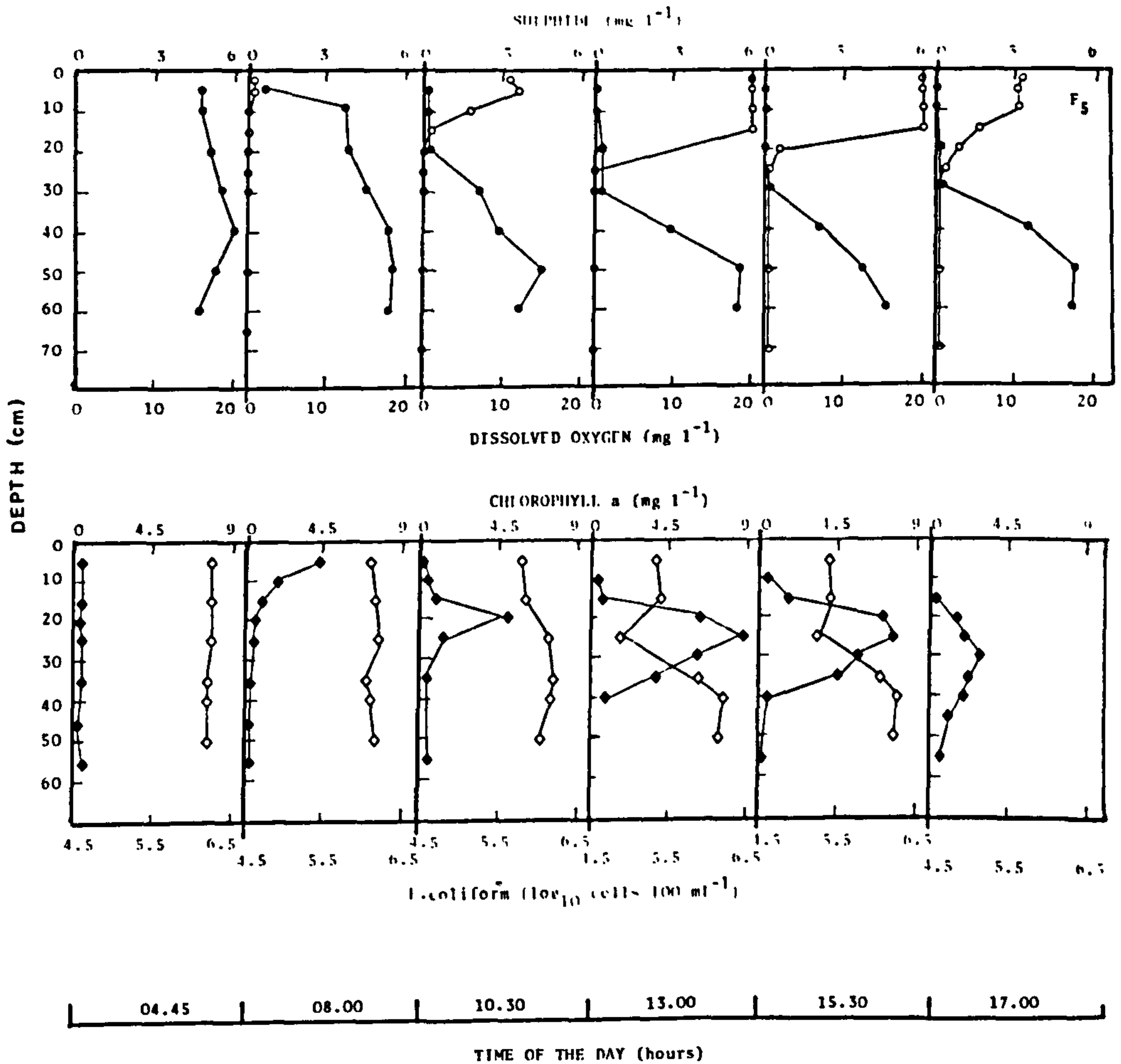
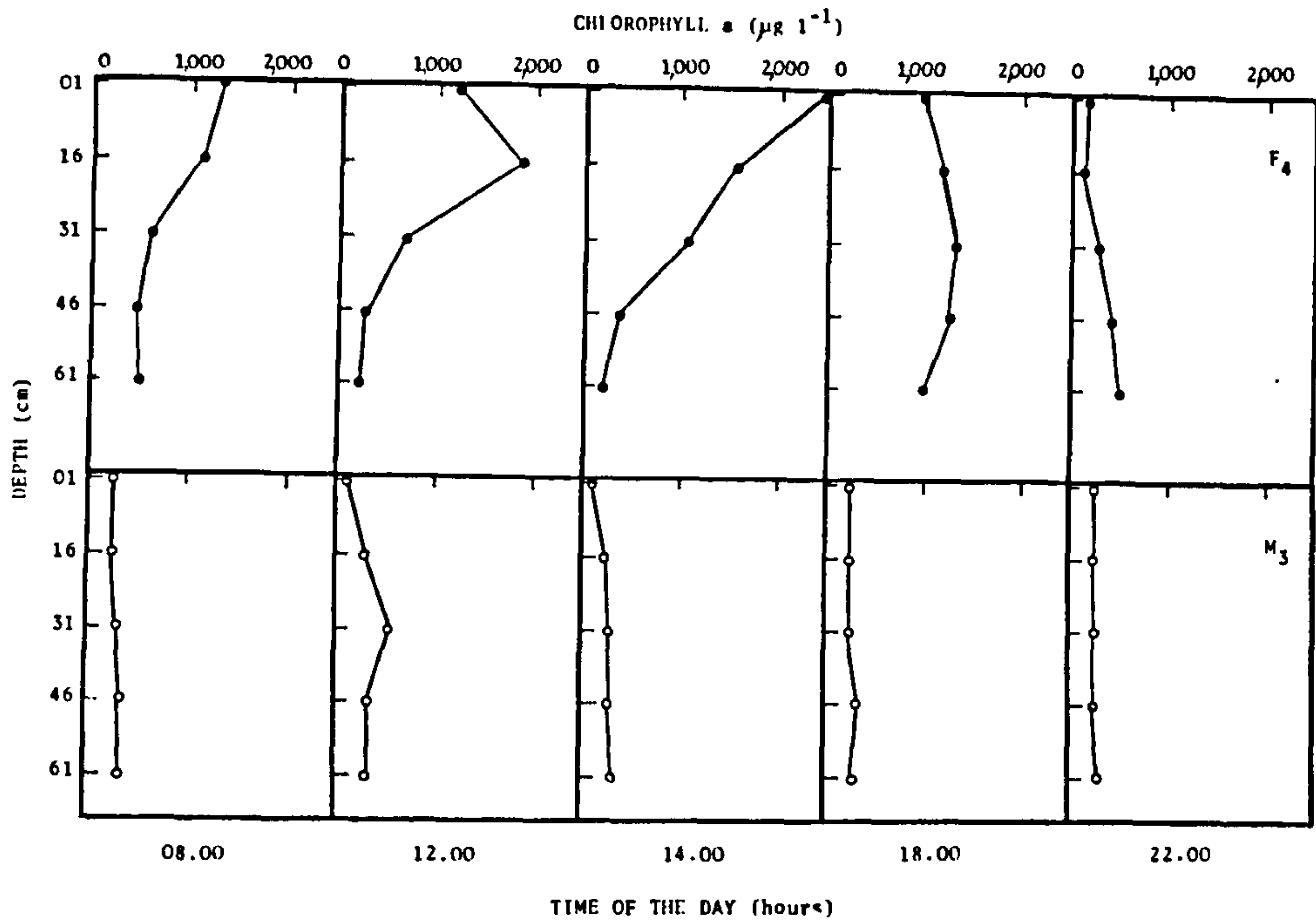
Logistically it was not possible to analyse replicated samples from every 5 cm zone of the water column for chlorophyll a. The chlorophyll a was therefore only extracted from the samples in the depth sequence which showed any indication of the presence of algae (either visually or by microscopic analysis). When FC numbers and sulphide concentrations were also to be estimated, these were determined in the same sample as the chlorophyll a estimations.

6.3 Results

Results for chlorophyll a concentrations at different depths in the facultative pond F4 and the maturation pond M3 using the perspex tube technique are shown in Fig. 6.1. In the facultative pond the algae showed evidence of stratification during daylight

Fig. 6.1 Chlorophyll a results obtained at different times and depths in the Facultative pond F₄ (●) and the Maturation pond M₃ (○) using the perspex tube technique.

Fig. 6.2 Results of chlorophyll a (◆), FC numbers (◇), sulphide (●) and "in situ" measurements of dissolved oxygen (○) obtained at different times of the day and depths in the Facultative pond F₅ using the peristaltic pump.



F. coliform (\log_{10} cells (ml ml^{-1}))

hours and the position of the zone of maximum algal concentration changed with time. Maximum stratification occurred between 12.00h and 14.00h, and the pond had almost completely destratified by 18.00h. In contrast, little or no algal stratification was apparent in the maturation pond.

In the more detailed studies on F5 using the peristaltic pump, the number of FC, chlorophyll a and sulphide concentrations were all determined from the same water sample. Dissolved oxygen profiles were measured in situ and typical data obtained are presented in Fig. 6.2.

The chlorophyll a analysis of samples taken at dawn (04.45h) showed very low concentrations throughout the water column. Three hours later at 08.00h, the algae had moved towards the surface and the highest values of chlorophyll a were found at the effluent take-off level (i.e. 5 cm below the surface). At subsequent sampling times (10.30h, 13.00h and 15.30h) the algae-rich zone moved down from the surface depths between 15 and 40 cm, with maximum concentrations of chlorophyll a occurring within the 20-25 cm zone. At no stage during the daylight did the algae reach a depth of more than 50 cm. At dusk (17.30h) the chlorophyll a values showed that the algal stratification was breaking down.

The FC numbers also showed a pattern of stratification with numbers showing a \log_{10} reduction in the oxygen and algae-rich

upper water layers during the day but showing a higher and more homogeneous distribution in the early hours.

The highest sulphide concentration was recorded in the pre-dawn sample (04.45h) and was between 5 and 6 mg⁻¹ throughout the water column. This concentration was reduced in the upper layers as the oxygen increased. Dissolved oxygen varied with time of day but was only ever detectable in the top 30-35 cm of the pond depth.

Microscopic examinations of samples from the different depths showed the predominance of flagellate algae, e.g. Euglena, Pyrobotrys, Chlamydomonas, Phacus in F4 and F5 compared to the non-motile algae species, Chlorella and Micractinium in M3.

6.4 Discussion

The results of these studies have shown that algal stratification was distinctly more evident in facultative ponds than in maturation ponds. It also explains the results of the studies on diurnal variation in effluent quality previously described for a facultative and maturation pond (see Chapter 5). Movement of the algae in the water column and their subsequent stratification seems to occur in response to external factors.

The absence of stratification at dawn (Fig. 6.2) and the gradual destruction at dusk of the stratification which had formed during the day supports the idea that these flagellate

algae move in response to light.

Low light intensities have been shown to induce positive phototactic responses in several flagellate algae (Bendix, 1960), including species predominant in facultative ponds. Nultsch & Häeder (1979) have shown that algae will also respond negatively to high light intensities.

At dawn, light intensities were probably still too low to induce positive algae phototaxis and this could explain the uniform distribution of algae within the water column. Between dawn and 08.00h, the increasing light intensity is probably the factor controlling the algal movement towards the surface and as surface light intensities can reach values of 400×10^5 quanta $s^{-1} cm^{-2}$ their subsequent movement downwards in the water column is away from the high surface intensities. Unfortunately, no instrumentation was available at the time of these studies to enable accurate light measurements to be made at the surface or in the water column.

Hartley and Weiss (1970), studying the vertical distribution of Euglena, found that this alga would migrate through the water column in a pond to avoid light intensities in excess of 75 cft^{-2} .

Given the observed homogeneous concentration of chlorophyll a through the top 60 cm at night and assuming that this

concentration is the same throughout the entire depth of the water column, then less algae seem present in the water column at night than during the period of intense stratification during the day. This finding lends support to the idea that flagellate algae move down into the sludge or sediment during darkness and rise into the water mass during the day (Round, 1973; Hartley & Weiss, 1970). Unfortunately, the sludge was not analysed for algae at night in this experiment, and previous studies showed only that no algae were present during the day.

The possible direct and indirect action of the algae in causing a reduction in FC counts in the effluent take-off zone in the pond have already been discussed (see Chapter 5). The results presented here confirm that FC numbers can be reduced at least one \log_{10} reduction in the richly oxygenated and algae rich upper zones of the pond.

The low FC counts coincided with the highest algae concentrations lending support to the early findings by Cadwell (1946) that the efficiency of waste stabilization ponds in removing coliform bacteria is a function of algal activity. Whether this is due to the algae increasing the pH, the oxygen tension or producing antibacterial substances cannot be determined from these experiments.

Concentrations of hydrogen sulphide in the water column of this particular pond (F5) were below the range of 6.5-8.15 mg l^{-1} of $\text{S}^{=}$

thought to cause the complete disappearance of algae in sewage lagoons (Parker, 1962; Gloyna & Espino, 1969).

The algal stratification in facultative ponds is a daily phenomenon and can cause high concentrations of algae to appear in the effluent at times during the day. This increases the values of the BOD₅, COD and Suspended Solids, etc. The occurrence of these high concentrations of algae, particularly in facultative ponds, could be prevented by either setting the effluent take-off level below 45 cm or using a more sophisticated variable effluent 'take-off' system such as is used for avoiding algal blooms in drinking water reservoirs. A lower 'take-off' depth would increase the FC numbers in the effluent from one pond (by approximately an order of magnitude) but in a series of ponds where a facultative pond is followed by one or more maturation ponds, this would not be significant since the pathogen die-off occurs predominantly in the maturation ponds. The role of a facultative pond is primarily to reduce BOD₅ and COD and such an effluent removal procedure would enhance this role.

The chlorophyll a gradients measured in M3 (Fig. 6.1) are relatively uniform and of a lower concentration than in the facultative pond F4. Therefore, a lower level 'take-off'

procedure would seem of little benefit in reducing BOD₅.

The final effluent from a pond series, ending in a maturation pond, should probably be removed close to the surface where FC are likely to be at their lowest concentration.

CHAPTER 7
ALGAL PRODUCTIVITY

7.1 Introduction

Primary productivity of an ecological system, community or any part of it, is defined as the rate at which radiant energy is stored by photosynthetic activity of producer organisms (green plants) in the form of organic substances which can be used as food material.

Most measurements of primary productivity have been based on some indirect quantity, such as the amount of raw material used (CO_2) or the amount of by-product released (O_2). The ideal way to measure productivity would be to measure the energy flow through the system but this has so far proved difficult to do (Odum, 1971).

Since there is a definite equivalence between oxygen produced and carbon fixed, oxygen evolution has been extensively used for determining productivity.

Among the methods for measuring oxygen production in aquatic environments, the "light and dark" bottles technique pioneered by Gaarder and Gra ν (1927), has long been a standard procedure for estimating primary productivity in both marine and fresh water environments.

The changes in oxygen concentration occurring in the light bottles is the resultant of the oxygen produced by chlorophyll containing organisms and the consumption of oxygen in respiration by both photosynthetic and non-photosynthetic organisms. The actual amount of oxygen produced in the light bottle is the measure of net oxygen production. In light bottles containing high concentrations of phytoplankton, the water may become over saturated with oxygen causing bubbles containing oxygen to form inside. This may cause errors in the final oxygen determination and possibly also lead to inhibition of photosynthesis (Hepher, 1962).

The respiratory demand of microorganisms present in the sample (e.g. algae, bacteria and protozoa) is obtained by measuring oxygen depletion occurring in the dark bottle.

In short term experiments, in which no great changes occur in numbers and kind of organisms in the sample, the amount of oxygen consumed in the dark bottle may be added to the amount produced by the light bottle to give an estimate of gross primary production. In doing this, the assumption is made that respiration by either plants or bacteria remains the same in the light and dark. Brown (1953) studied respiration of Chlorella whose oxygen supply was enriched with $^{17}\text{O}_2$ and by following the disappearance of $^{17}\text{O}_2$ from the air in equilibrium with the culture he could measure respiration independently of photosynthesis. He found

that the heavy isotope of oxygen was used up at the same rate in the dark (at least for a number of hours) as in the light, indicating that respiration should be the same in both light and dark during experiments of short duration.

The duration of the incubation time may vary according to the aquatic system studied from 24 h in oligotrophic lakes to half and hour or less in eutrophic waters. In productive waters an exposure of even one hour may lead to an oxygen supersaturation and raised pH in water samples incubated near the surface (Soeder and Talling, 1971). When very short exposure periods are used the above problems do not arise but the time occupied in manipulation can become a significant source of error.

Estimates of production per day have been calculated from long exposure (24 h or dawn to sunset), from half-day exposure (dawn to mid-day or mid-day to sunset) multiplied by 2 or from the summation of a sequence of short exposures (3-4 h) (Vollenweider and Nauwerk, 1961). Where pronounced diurnal changes occur in phytoplankton density and/or its activity, the summation of short exposure has an advantage over the other methods. Although the method of Steemann Nielsen (1952) based on the uptake of radioactive carbon (^{14}C) is more widely used than the "light and dark" method in measuring primary production, this method (providing incubation is short) measures gross photosynthesis. Therefore the older method of Gaarder and Gra λ (1927) is the only field

technique for the direct measurement of both gross and net production and has the added appeal of simplicity of equipment and procedure.

The vast majority of primary productivity studies deal with oligotrophic environments (lakes, oceans) and little is known about primary productivity in eutrophic ^{waters} especially those comprising sewage lagoons.

In this section, the primary productivity was studied in four different sewage ponds by estimating the oxygen production by using the light and dark technique. These include a facultative pond F₁ fed by effluent from an anaerobic pond, a maturation pond M₃ the final pond in a five pond series and two facultative ponds (F₂ and F₄) at different BOD₅ surface loadings and receiving raw sewage.

In terms of sewage treatment, measurements of primary productivity provides information on the amount of oxygen readily available for bacterial respiration at a particular time. The amount of photosynthetic oxygen production in ponds with different BOD₅ surface loading can also be evaluated with a view to determining their relative efficiencies.

7.2 Methods

Two litre samples of pond water from five different depths (i.e. 0 to 12, 12 to 24, etc.) between 0 to 60 cm below the surface were collected with a peristaltic pump described in Chapter 6, at 09.45 h. The necessary care was taken to minimise sample aeration and exposure to direct sunlight during collection and distribution.

For each depth, size 300 ml BOD flasks were filled with pond water, as following: the first two for measurements of initial oxygen and chlorophyll a concentration, the third and fourth to act as light bottles and the fifth and sixth, as dark bottles. The dark bottles were covered with aluminium foil to exclude light.

At 10.00 h, the two sets of paired light and dark bottles were suspended vertically in the pond at 12 cm depth intervals at the same depth from which the pond water sample had been originally collected. The necks of the uppermost bottles were just underneath the water surface and care was taken to ensure that there was no mutual shading by the bottles.

These experiments were carried out under all kinds of weather conditions and always at the same time of day and on the same day of the week.

The bottles were removed after 30 minutes incubation in the

pond and brought immediately to the laboratory where pH temperature, dissolved oxygen were measured and chlorophyll a concentration determined according to the methods described in Chapter 2.

In situ measurements of temperature and dissolved oxygen of the whole water column were taken at the beginning and end of the production study.

7.3 Results

The primary production data has been expressed on an area basis ($\text{g O}_2 \text{ m}^{-2} \text{ h}^{-1}$) to allow for ease of comparison between the ponds and for the sake of any subsequent comparison with other published work.

Variation in gross and net primary production for ponds of System I (F1 and M3 from Feb. 1978 to Oct. 1979) and System II (F2 and F4 from Feb. 1978 to May 1980) are shown in Fig. 7.1 and 7.2 respectively.

The results show that values for gross and net primary production fluctuated from month to month and that these fluctuations were more pronounced in the facultative ponds than the maturation pond.

The net oxygen production was generally a greater proportion of the total production in the maturation pond M3 compared to F1 (the facultative pond in the same series) and to the independ-

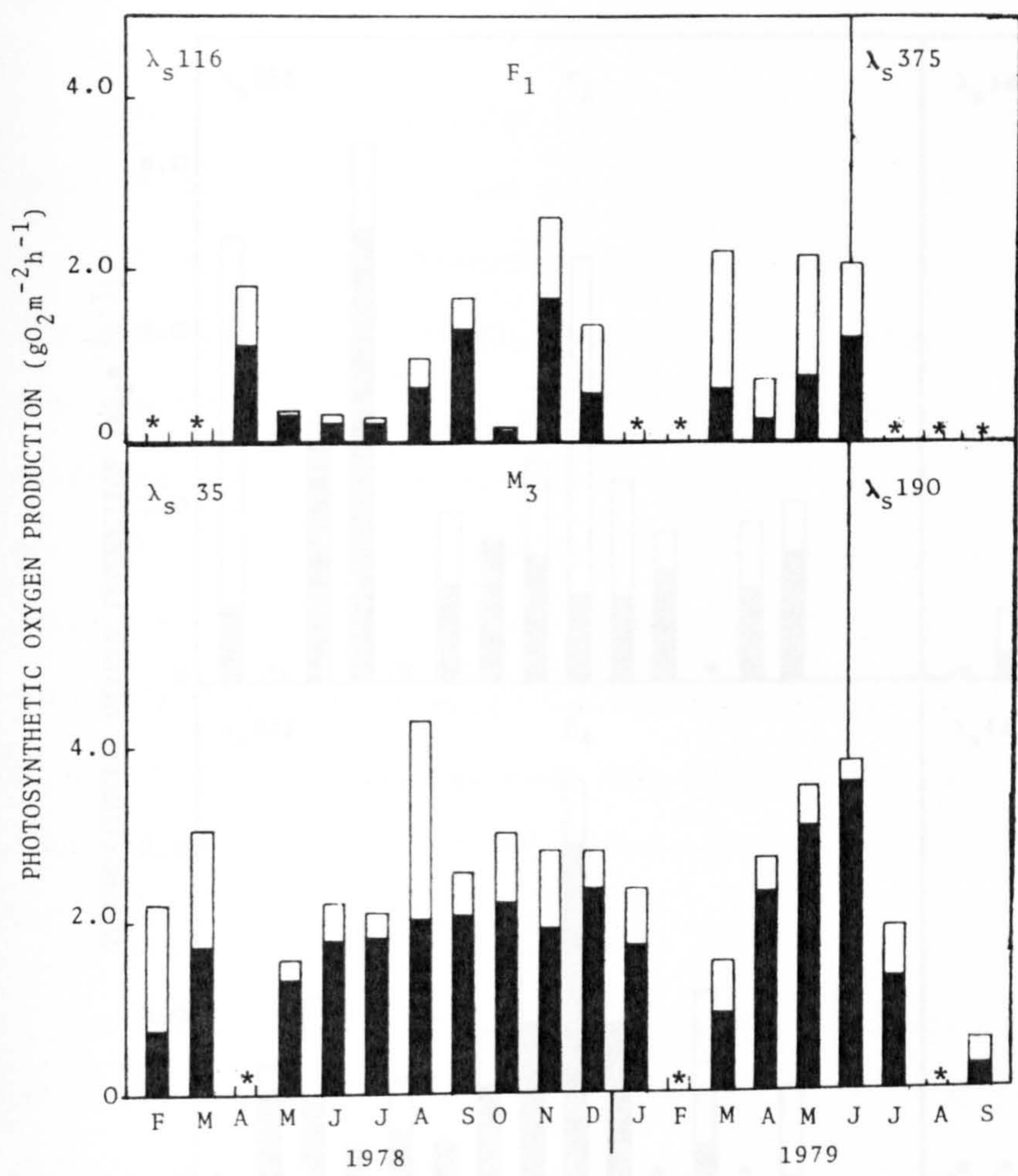


Fig. 7.1 Variation in gross primary production in F1 and M3 of System I during Experiment 1 and 2. The solid parts of the histograms represent the net primary production.

In some production experiments (*) the net production values were considered to be inaccurate due to some technical fault and therefore were not included.

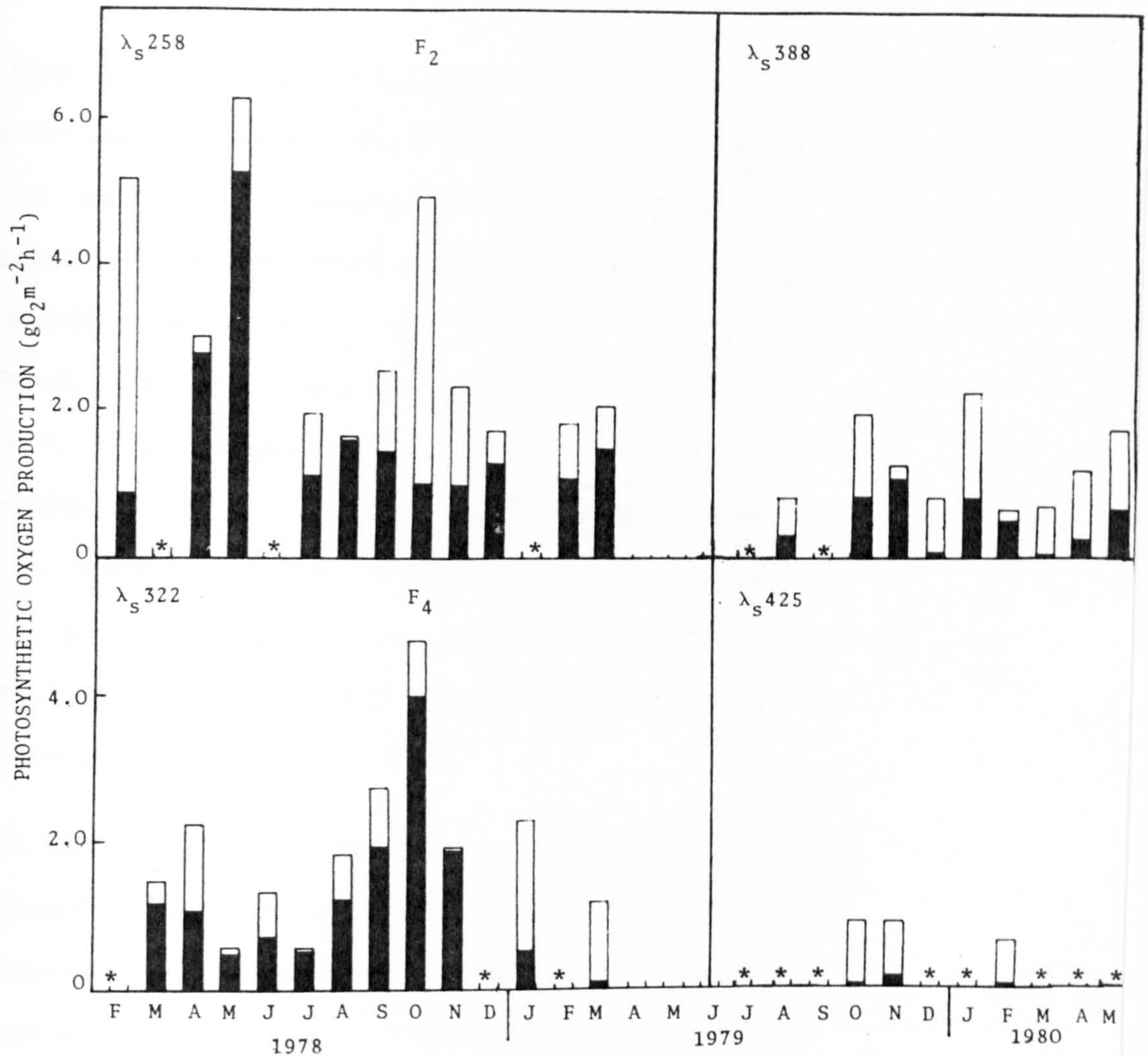


Fig. 7.2 Variation in gross primary production in F₂ and F₄ of System II during Experiment 1 and 2. The solids parts of the histograms represent the net primary production.

In some production experiments (*) the net production values were considered to be inaccurate due to some technical fault and therefore were not included. The ponds were emptied during April and May 1979 (for dispersion studies) and therefore no production studies were made

ently loaded facultative ponds F2 and F4 (Fig. 7.1 and 7.2).

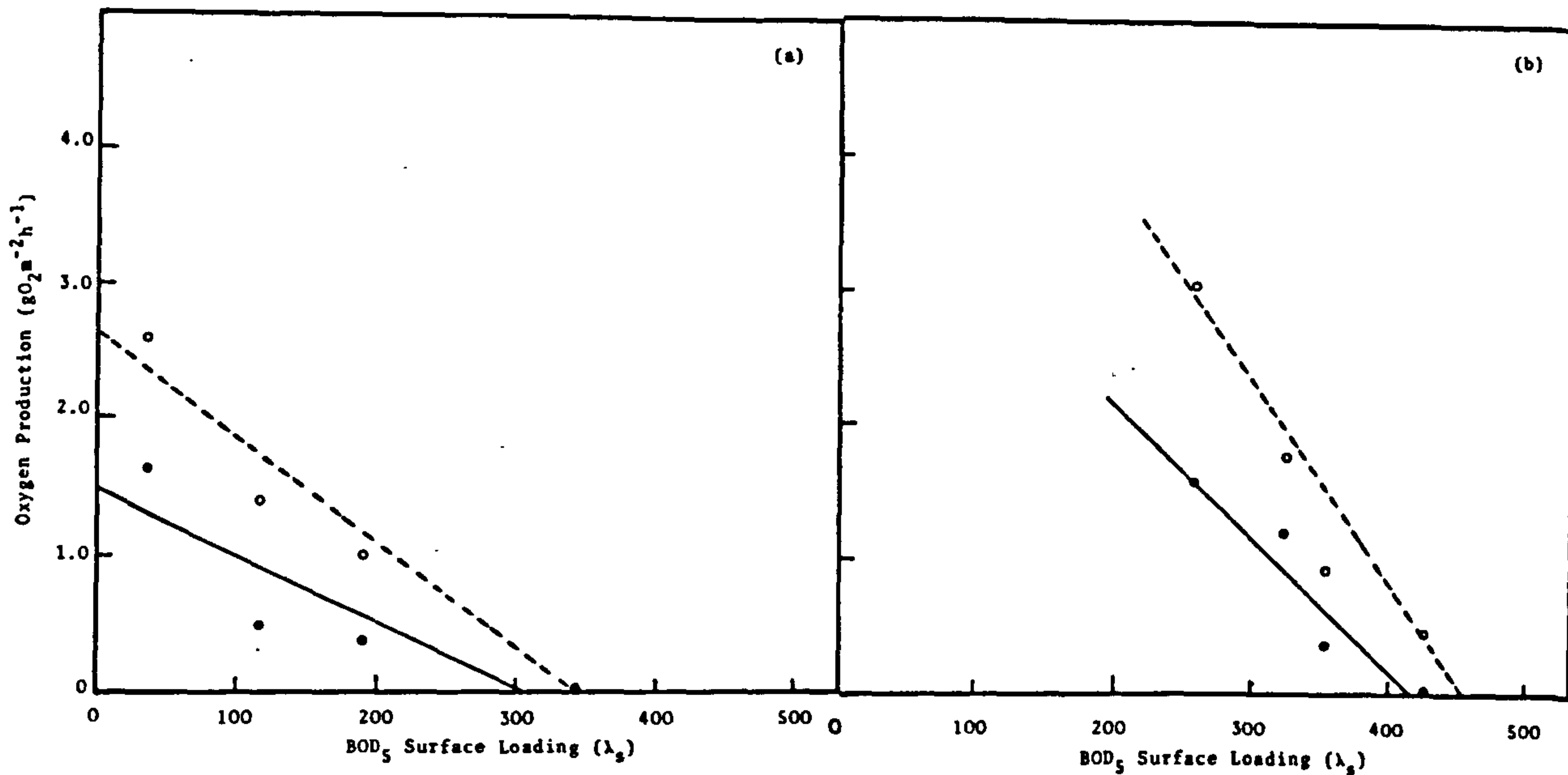
These monthly figures for pond primary production represent data for one day a month and are based on values obtained between 10.00 h and 10.30 h. Therefore it is not possible to obtain legitimate values for monthly and annual production. Furthermore the monthly values cannot be used to make comparison between different ponds since measurements were not taken simultaneously. However, taking the data for each pond as a whole, some comparison can be made between primary production and pond surface loading. The regression analyses for both net and gross primary production against mean BOD₅ surface loading for ponds of System I and System II showed primary production decreasing with increasing loading (Fig. 7.3).

The variations in chlorophyll a concentration and gross and net primary production with depth in certain ponds of System I and System II are shown in Fig. 7.4 and 7.5. The depth of maximum primary production did not always coincide with the zone of maximum chlorophyll a concentration. In the maturation pond net and gross primary production were usually measurable at depths up to 60 cm except when the load was increased to such an extent that M3 became facultative.

Although there was a temperature gradient in the pond, measurements did not change much compared to the pond mid-depth

Fig. 7.3 Regression lines for monthly gross (dotted line) and a net (continuous line) primary production values against mean BOD₅ surface loading for ponds of System I (a) and System II (b) using data from Experiments 1 and 2. For simplicity only the mean gross (○) and net (●) values for each set of data are included. See Table 7.1 for statistical details.

Table 7.1 Details for the regression analyses between monthly values of gross and net primary production and BOD₅ surface loading shown in Fig. 7.3 (a) and (b): n is the number of samples, r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line where y = gross or net primary production ($\text{g O}_2 \text{ m}^{-2} \text{ h}^{-1}$) and x = BOD₅ surface loading ($\text{kg BOD}_5 \text{ ha}^{-1} \text{ d}^{-1}$).



System I (Experiment 1 and 2)

	n	r	confidence limit (%)	regression equation
Gross Production x BOD_5 surface loading	42	-0.666	99.9	$y = 2.621 - 0.007x$
Net Production x BOD_5 surface loading	42	-0.570	99.9	$y = 1.508 - 0.005x$

System II (Experiment 1 and 2)

	n	r	confidence limit (%)	regression equation
Gross Production x BOD_5 surface loading	49	-0.673	99.9	$y = 6.801 - 0.015x$
Net Production x BOD_5 surface loading	49	-0.592	99.9	$y = 4.146 - 0.010x$

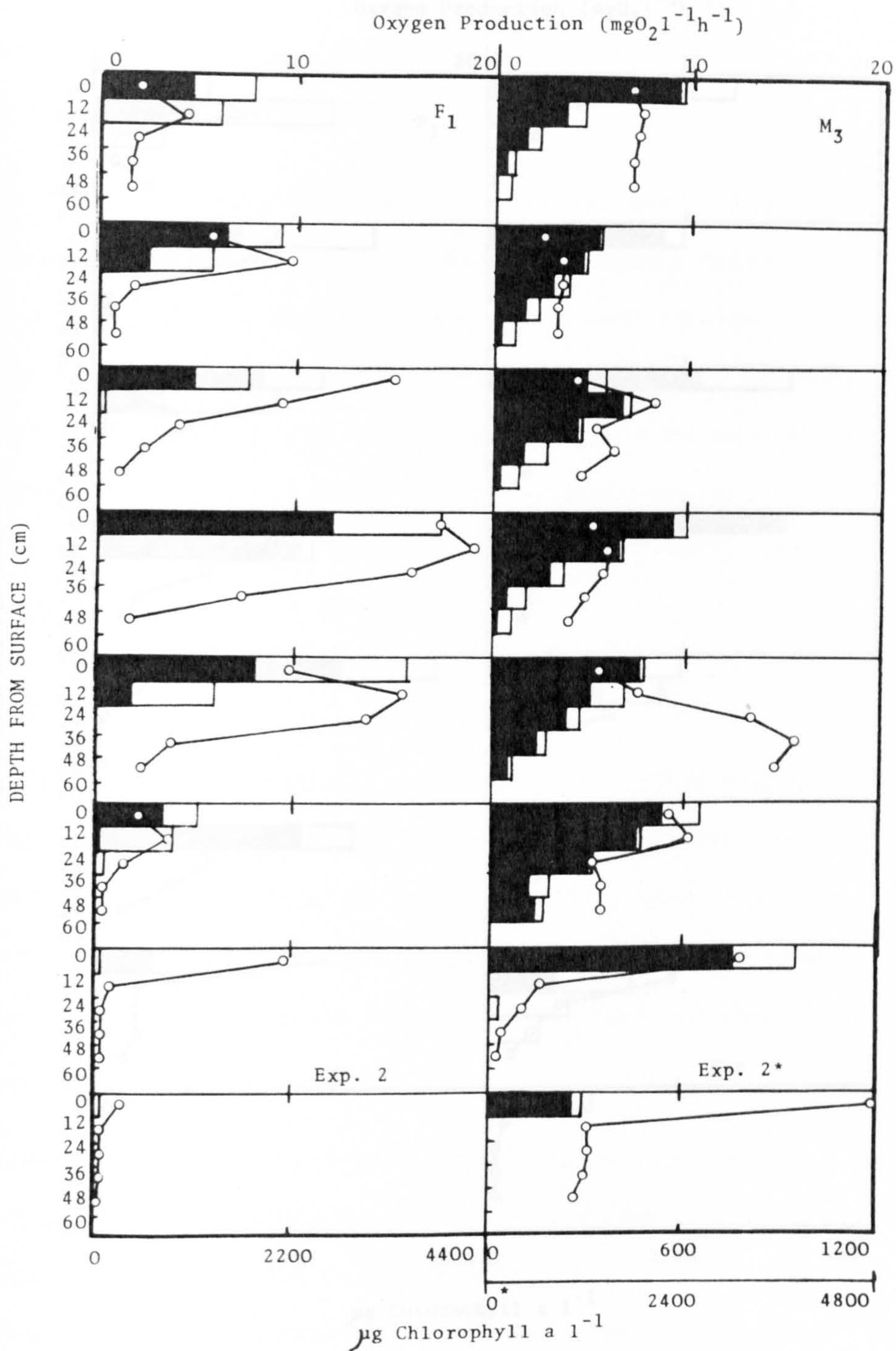


Fig. 7.4 Variation with depth in gross (full histograms) and net primary production (black area of the histograms) for a facultative pond F1 and a maturation pond M3 during Experiment 1 and 2. Variations in chlorophyll a concentrations with depth a is also shown (o).

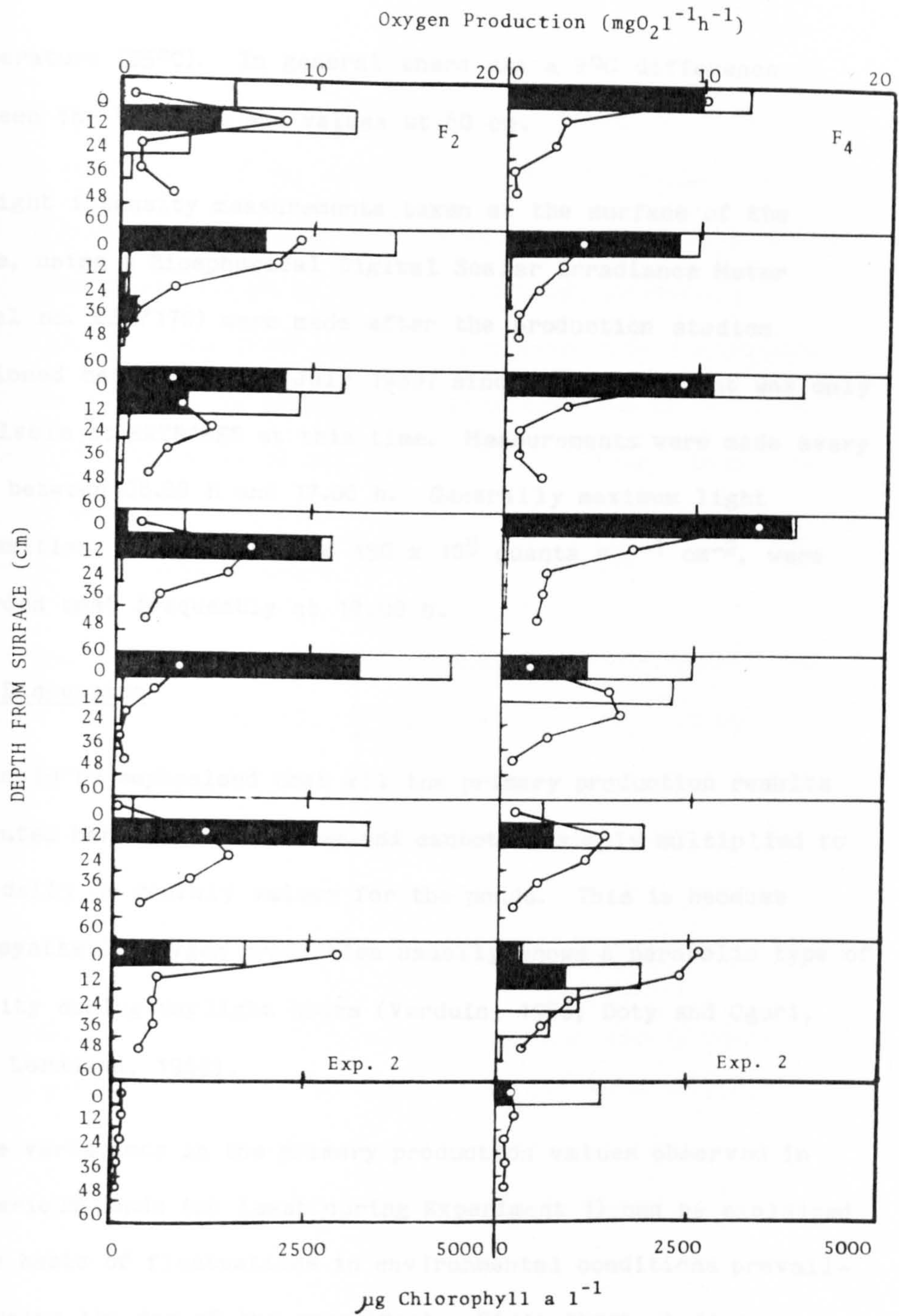


Fig. 7.5 Variations in depth in gross (full histograms) and net primary production (black area of the histograms) for facultative ponds F2 and F4 during Experiments 1 and 2. Variations in chlorophyll a concentrations with depth is also shown (o)

temperature (25°C). In general there was a 2°C difference between the top 5 cm and values at 60 cm.

Light intensity measurements taken at the surface of the ponds, using a Biospherical Digital Scalar Irradiance Meter (model no. QSP/170) were made after the production studies mentioned here (Feb. and July 1980) since this equipment was only available at EXTRABES at this time. Measurements were made every hour between 08.00 h and 17.00 h. Generally maximum light intensities, between 370 and 450×10^5 quanta $s^{-1} cm^{-2}$, were recorded most frequently at 10.00 h.

7.4 Discussion

It should be emphasised that all the primary production results presented here 10-11 h values and cannot be simply multiplied to give daily or monthly values for the ponds. This is because photosynthetic oxygen production usually shows a parabolic type of activity during daylight hours (Verduin, 1956; Doty and Oguri, 1957; Lorenzen, 1963).

The variations in the primary production values observed in the various ponds (at least during Experiment 1) can be explained on the basis of fluctuations in environmental conditions prevailing during the day of the experiment. Since these studies were carried out under all kinds of climatic conditions, i.e. rain, cloud cover, to a sunny day (or any combination of these) these

monthly results will give little direct information on the primary production of the different ponds. To obtain such data primary production would have to be studied in situ in all the ponds simultaneously or by a parallel primary production laboratory bioassay under controlled reproducible conditions. The first alternative is totally impractical due to the high numbers of samples to be analysed in a short time. Therefore the bioassay seems to be the only feasible alternative for obtaining relative data between ponds.

In System I during Experiment 2 oxygen production would seem to be directly attributable to the increasing BOD₅ surface loading rather than environmental conditions being more severe in F1 than M3. These results might be expected since the effects of increasing the load on the system would be significantly reduced by the time it reached M3, the final pond in the series.

Generally, increasing BOD₅ surface loadings has decreased primary production in all the ponds studied. This is showed by the regression lines (Fig. 7.3) and the negative correlation coefficients (Table 7.1) for ponds of both systems during Experiment 1 and 2.

These regression lines (Fig. 7.3) show that gross primary production is reduced near to zero at values of BOD₅ surface loading of around 400 kg BOD₅ ha⁻¹ d⁻¹ or more.

Although measurable algal population was present at these high BOD₅ surface loadings (see Chapter 4), the gross production was reduced significantly and could compromise seriously the algae-bacteria symbiosis and overall pond performance (Fig. 7e). If a reasonable supply of oxygen is needed, at least during the day, such high BOD₅ surface^{loadings} should be avoided since ponds could easily become totally anaerobic and cease to function as a facultative waste stabilization pond.

Results of primary production with depth showed differences exist between a facultative and a maturation pond. Net oxygen production is a smaller percentage of the gross production in facultative ponds compared to a maturation pond, emphasising the greater oxygen demand of the former type of pond.

Values for the chlorophyll a concentration at depths down to 60 cm confirm that as in previous experiments algal stratification occurs in the pond water column and it is more evident in facultative ponds.

The maximum chlorophyll a concentration did not always coincide with the depth of maximum oxygen production (photosynthesis) suggesting that self-shading effects by the algae are occurring.

The zone of maximum production at least for 10-11 h values for facultative and maturation ponds is usually between 10-20 cm

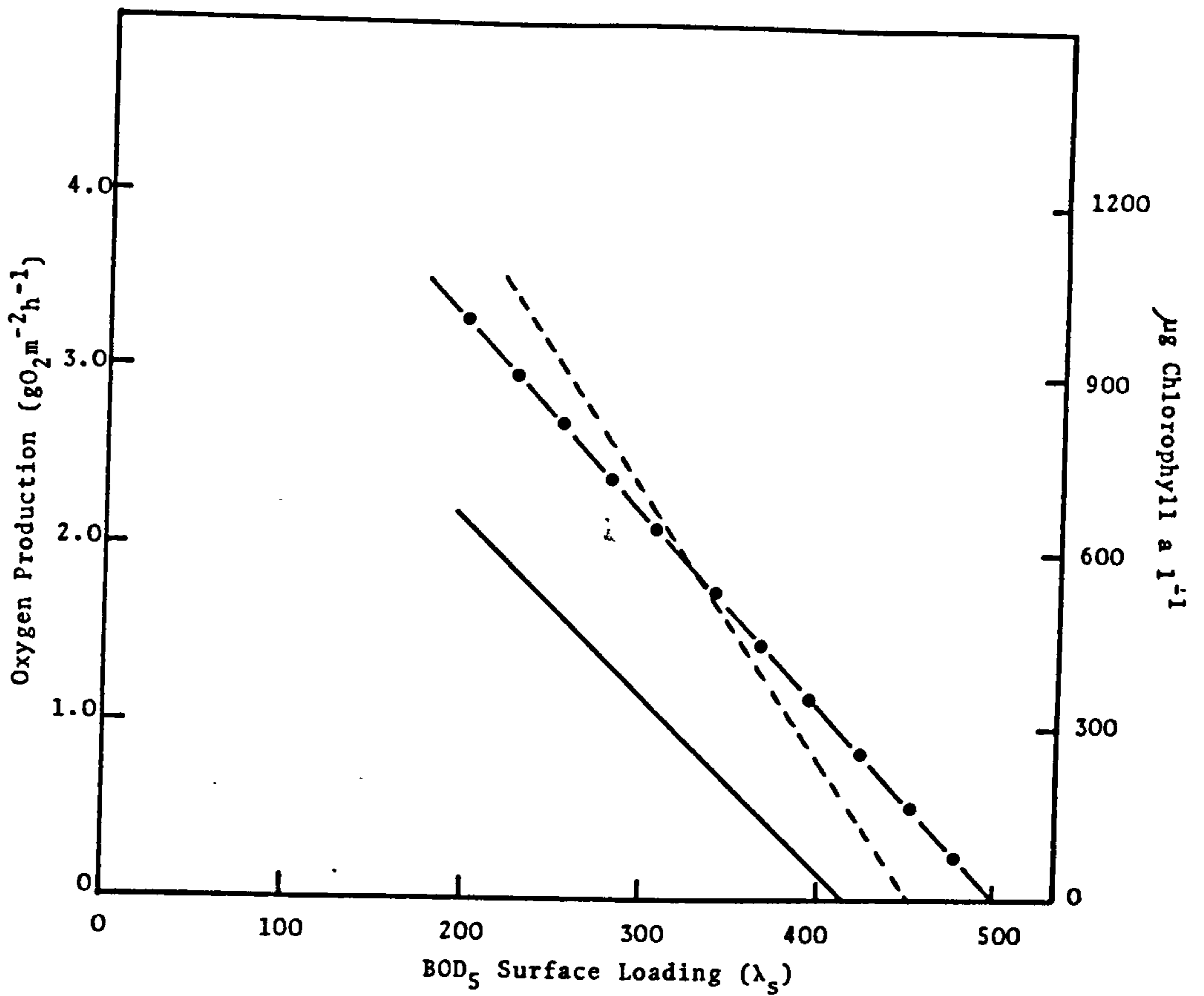


Fig. 7.6 Regression lines for gross production (dotted line), net production (continuous line) from Fig. 7.3 (b) and chlorophyll a concentration from (— • —) Fig. 4.3 against BOD₅ surface loading λ_s .

below the surface. One might suggest therefore that the algae present in these ponds are not inhibited by the light intensities measured in the north east of Brazil.

CHAPTER 8

BASIC METHODOLOGY OF LABORATORY STUDIES

8.1 Algal Purification

Unialgal cultures of Oscillatoria, Chlorella and Navicula were isolated from ponds of Systems I and II. The main method used to obtain such isolates of Chlorella and Navicula was to centrifuge the alga -containing pond sample at low speed, wash the pellet of algae in sterile distilled water, then resuspend it and repeat the process up to five times. The final resuspended sample was then used as a source of inoculum for the preparation of algal streak plates. The medium for these plates comprised agar dissolved in sterilised, filtered water from the appropriate pond. Colonies from these plates were used as sources of inoculum for new plates of liquid culture and the process being repeated until unialgal cultures were obtained. In the case of Oscillatoria, which was motile, the outermost radial filaments from the colonies, which moved free of contaminants and debris were picked off and placed into liquid culture medium or restreaked onto plates.

The unialgal cultures were cultivated under continuous fluorescent light at 25°C in static liquid culture using Modified Allen's Medium (Allen, 1968) or on plates where the liquid medium was solidified with 1.0% (w/v) Technical Agar No. 3 (Oxoid).

Filtered sewage was used to make up the medium instead of distilled water throughout the period that the algae were cultivated at EXTRABES. Algae were subcultured every month.

The flagellate algal species, Chlamydomonas, Euglena, Phacus and Pyrobotrys were not successfully isolated even in unialgal cultures. This was due to the intense growth of associated bacteria which 'swamped' the algae culture. Unfortunately, no suitable antibiotics were available which would suppress bacterial growth without affecting the algae.

In the Botany Department of the University of Liverpool the bacteria contaminating the unialgal cultures of Chlorella were isolated. A ten-fold dilution series (between 10^{-1} to 10^{-6}) was prepared using 1/4 strength Ringers Solution (Oxoid). A 1 ml aliquot was transferred from a particular dilution tube to a sterile petri dish. Six plates were prepared for each dilution of the series. Then 20 ml of molten (45°C) Nutrient Agar 1.2% w/v (Oxoid) was poured into each dish which was swirled to mix the inoculum and agar evenly. The plates were allowed to cool and solidify and then triplicate plates were incubated upside down at 28°C in the algal growth room or at 37°C in an incubator. Bacterial colonies appeared within 2-3 days in the growth room and within 24h in the incubator.

After incubation, single bacteria colonies were transferred

from the dilution plate into Nutrient Broth (Oxoid) and incubated overnight at the temperatures in which they had been previously grown. Triplicate bacterial lawns were prepared from each of the pure liquid cultures by pipetting 2 ml of bacterial culture into a petri dish and adding 20 ml of molten Nutrient Agar 1.2% w/v (Oxoid). When the mixture was solid and dry an Oxoid Multodisc (Oxoid) was placed on the plates to determine which antibiotic these bacterial contaminants of algal cultures were sensitive to. The Oxoid Multodisc used had the following antibiotics impregnated into each individual disc: Erythromycin (50 µg), Sulphafurazole (500 µg), Novobiocin (5 µg), Oleandomycin (10 µg), Penicillin (5 µg), Chloramphenicol (50 µg), Streptomycin (25 µg), Tetracyclin (50 µg) and Chloramphenicol (50 µg). ^{The last three} were the antibiotics efficient against the bacteria contaminating the Chlorella cultures.

The subsequent purification of the Chlorella cultures was performed using methods similar to those described by Droop (1967) except that the Chlorella was exposed to the three antibiotics separately instead of a mixture and the concentration employed was lower than the amounts recommended by Droop, i.e. 12.5, 25, 50, 100, 200 and 400 µg ml⁻¹. The lower concentrations of antibiotics were used so as to avoid chloroplast bleaching after 24 hours exposure (Jones et al. 1973). After exposure, Chlorella was samples were washed free of the antibiotic by repeated washing and centrifugation.

The algae were subcultured into antibiotic-free media using either Allen's Medium or Nutrient Broth (Oxoid). The use of Nutrient Broth was employed because there is no guarantee that the original medium that supported bacterial cultures will be suitable for axenic growth (Droop, 1967).

After three weeks, cultures were tested for sterility by streaking a loopful of liquid algae culture onto Nutrient Agar plates and incubating them at either 28°C or 37°C for a week. The Chlorella cultures which had been exposed to 100 µg ml⁻¹ of Streptomycin were axenic. No detectable microscopic changes had occurred in the antibiotic-treated algae when compared with the bacterised cultures and they grew well in both Allen's Medium and in Nutrient Broth (Oxoid).

The growth and purification of flagellate *genera* such as Euglena, Chlamydomonas and Pyrobotrys, common in sewage ponds, was not successful under laboratory conditions. It was therefore necessary to use laboratory cultures obtained from The Cambridge Culture of Algae and Protozoa (CCAP-36, Storey's Way, Cambridge CD3 ODT, U.K.). Some of these laboratory isolates required further purification using the techniques just described. The genera chosen were the same as those identified as being present in the ponds of System I and II of EXTRABES. When possible the specific strains chosen had been originally isolated from habi-

tats comparable to waste stabilization ponds. The following species were obtained:

Chlorella sp. - strain 211/28 H01972-UL 196 RI 200674 factory effluent - Malaya.

Chlorella vulgaris f. tertia F. et N strain 211/8K Sorokin et Myers, T x 7.11.05 high temperature strain, Texas, USA.

Euglena gracilis - Klebs - strain 1224/5Z Pringsheim 1950.

8.1.1 Algal Growth Methods

Biomass measurements of algae in culture were determined either by estimating Chlorophyll a concentrations after extraction in 90% methanol (see Chapter 2) or by measuring the optical density of the culture at 540 nm and converting the absorption readings for each alga into cell numbers per ml by means of a calibration curve. ^{See Appendix 1} In the microbial interaction experiments involving algae and bacteria, numbers of algal cells were counted using a calibrated Improved Neubauer Haemocytometer.

8.2 Bacterial purification and identification

FC bacteria were isolated from effluent samples of ponds from Systems I and II. They were isolated by the Microbiology Section of EXTRABES using the Millipore Membrane Filtration Technique (APHA, 1975). Two isolates from each pond effluent were selected and maintained on slopes of Nutrient Agar (Oxoid).

In this project the components of the heterogeneous FC population were identified using the Reaction Chart of Ewing (1970). However, to do this individual isolates from the mixed population of FC had to be prepared as follows.

The FC of each pond effluent was grown in suitable enriched medium. Fifty ml aliquots of Nutrient Broth (Oxoid) were poured into 100 ml conical flasks, sealed with sterile stoppers and covered with aluminium foil and then autoclaved for 15 min. at 15 lb in⁻².

A loopful of FC was inoculated in the Nutrient Broth and the static cultures were incubated at 37°C for 24 hours. After the incubation period, a ten-fold dilution series was produced using sterile 1/4 strength Ringers Solution Tablets (Oxoid). A sub sample of each dilution from each sample was transferred to petri dishes containing sterile Nutrient Agar (Oxoid) and spread using a glass rod. Sterile techniques were used throughout the identification procedure. Plates of the different dilutions were incubated upside down in a 37°C incubator for 24 hours. Plates containing visible colonies were chosen and from each pond type 10 colonies were picked off and tested for the biochemical characters of Ewing Reaction Chart (Table 8.1). All incubations were carried out at 37°C for 24 hours.

The precise methodology for each diagnostic test was taken from Bergey's Manual of Determinative Bacteriology (Buchanan &

TABLE 8.1 DIAGNOSTIC TABLE FOR IDENTIFICATION ENTEROBACTERIACEAE *

	Proteus vulgaris	Proteus mirabilis	Salmonella	Escherichia	Enterobacter aerogenes	Serratia	Erwinia
Ferment dextrose	+	+	+	+	+	+	+
Gas from dextrose	+	+	+	+	+	+	- (weak)
Lysine decarboxylase	-	-	+	+ or -	+	+	-
Ornithine decarboxylase	-	+	+	+ or -	+	+	-
H ₂ S	+	+	+	-	-	-	-
Indole	+	-	-	+	-	-	-
Fement lactose	-	-	-	+	+	-	-
Ferment dulcitol	-	-	+	+ or -	-	-	-
Phenylalanine demaminase	+	+	-	-	-	-	+ or -
NH ₃ from urea	+	+	-	-	-	+ or -	-
Citrate utilization	- or +	- or +	-	-	+	+	+
Nitrate reduction	+	+	+	+	+	+	-
Gelatin liquifaction	+	+	+ or -	-	- slow	+	+

* Simplified from Ewing, 1970 - Differentiation of Enterobacteriaceae by biochemical reaction, Center for Disease

Gibbons, 1974). After incubation time, the diagnostic test tubes were compared with a reaction chart. The results indicated that the postulated mixed population of FC from the different ponds was composed of one single species of the Enterobacteriaceae, E. coli.

The results were not unexpected, probably because since E. coli is the major component of the FC population and in suitable medium can easily outgrow the other species. It would seem that this had occurred during the isolation procedures carried out at EXTRABES.

8.2.2 Bacteria Growth Methods

Estimate of E. coli biomass was determined spectrophotometrically. A sub-sample of approximately 2 ml was placed in a 1 cm path length glass cuvette and absorbance measured at 590 nm (Adler, 1973) using a Pye Unicam SP8-100 UV/VIS Spectrophotometer. These spectrophotometric readings were converted into cell numbers per ml by using a calibration curve previously constructed by counting bacterial numbers in a series of dilutions of a known absorbance. ^{See appendix 1} Bacterial numbers were counted using a Coulter Counter (Coulter Electronics Ltd., Luton, U.K.). A known volume of a cell suspension (i.e. between 5-50 µl) was mixed with a fixed volume (20 ml) of Isoton (Azide free, Balanced, Electrolyte solution) a saline solution especially

designed for the Coulter Counter. A glass tube with an orifice diameter of 30 μm was used.

When the Coulter Counter could not be used, e.g. in algae/bacteria interaction, because different orifice size tubes had to be used for the algae and bacteria, the bacteria enumeration was made by plate counts on Nutrient Agar (Oxoid) after preparation of an appropriate dilution series using 1/4 strength Ringers Solution (Oxoid). The plates were counted after 24h incubation at 37°C. This method of enumeration is often termed viable count; in contrast to the electronic counting it measures only those cells that are capable of growth on the plating medium used.

CHAPTER 9

LABORATORY STUDIES ON SOME FACTORS REGULATING ALGAL AND FAECAL COLIFORM GROWTH

9.1 General Introduction

When microbes are transferred from the field to the laboratory for detailed studies on their physiology and metabolism, a question that frequently arises is whether as purified laboratory cultures they remain sufficiently similar in their characteristics to the naturally occurring population. It is almost certain that bacteria undergo changes of ecological significance when removed from their habitat and are grown in the laboratory. The magnitude of this alteration can be minimized in laboratory stock strains by attempting to simulate the natural habitat as far as possible.

In this Chapter the experimental work was divided into three sections: 1) algal growth studies in different media and under different laboratory conditions and also includes studies on algal-algal interaction; 2) bacterial growth in different media and their sensitivity to oxygen and antibiotics; 3) the growth of mixed populations of algae and bacteria.

9.2 Algae

9.2.1 Introduction

W.S.P. support large numbers of algae which mainly belong to the Euglenophyta and Chlorophyta (Neil and Hopkins, 1956, de Noyelles, 1957; Palmer 1969). Members of both these phyla grow in the dark chemosynthetically in the presence of organic compounds or photosynthetically or both.

Euglena rostifera will survive and grow in complete darkness (Oswald et al., 1953, Hartley & Weiss, 1970) and this adaptation is reversible (Cramer & Myres, 1952). Dark growth in Euglena can persist for long periods, up to 14 days, as reported by Hartley and Weiss (1970).

According to Pipes and Gotaas (1960) Chlorella when reproducing rapidly do utilize some of the organic compounds present in the sewage. There is also evidence for slow rates of organic carbon assimilation by Chlorella in the dark (Bush et al., 1961). The early evidence of the direct use of carbon sources by Chlorella was given by Oswald et al. (1953). They observed that a fair degree of sewage treatment was obtained even though bacterial growth was inhibited.

The ability of certain algae to both photosynthesise and utilize organic carbon in the dark is certainly an advantage especially in environments such as W.S.P. where light penetration

is restricted to the surface, and where water currents will move these algae (especially the non-motile species) into depths where light intensities are very low or non-existent.

In this section algal species commonly found in W.S.P. were grown in liquid media to assess their growth characteristics in media of different compositions and in different light regimes.

Heterotrophic growth of mono and mixed cultures of Euglena gracilis and Chlorella sp. (a pond isolate) were also investigated with a view to understanding aspects of algal speciation. By varying the ratios of these two algae (volume:volume) an attempt was made to investigate whether either of the algal species could obtain a competitive advantage over the other.

9.2.2 Materials and Methods

Axenic cultures of Chlorella sp. (isolated from a maturation pond) Chlorella vulgaris f. tertia (N strain 211/8K Sorokin and Myres) and Euglena gracilis Klebs (strain 1224/52 Pringsheim) were used. The Chlorella vulgaris and Euglena gracilis came from the Cambridge Collection of Algae and Protozoa (CCAP).

The algae were grown in either Allen's, (1968) or Cramer and Myrnes (1952) media. Unless otherwise stated 5 gl^{-1} of glucose was added to the medium. Nutrient Broth was added to Allen's Medium as recommended by Oxoid.

The algae were grown in 100 ml conical flasks with 50 ml sterile culture medium. They were placed in a Gallenkamp Rotary Shaker at 80 rev min⁻¹.

The temperatures of the growth rooms varied from 28 ± 2°C (continuous light) to 25 ± 1°C (12 h light/ 12 h dark).

The illumination of both continuous light and light/ dark rooms was provided by cool white fluorescent lamps giving an incident radiation of 66 ± 1 μE m⁻² S⁻¹. When growing in total darkness the culture flasks were wrapped in aluminium foil and placed at the same temperature as those under continuous light.

The cultures used as inocula had previously been grown under the same experimental conditions and inocula consisted of algae in the late exponential phase.

Algal growth was measured by Optical density (see Chapter 8) and converted to cell numbers by means of a calibration curve.

In the mixed algal growth experiments, axenic cultures of Chlorella sp. (isolated from a maturation pond) and Euglena gracilis were used.

Algae were grown in Cramer and Myers (1952) ^{medium} with 10 mM of PIPES (Sigma Chemical Co., St Louis, Mo. U.S.A., PKa = 6.8 at 25°C) supplemented with 25 mM of glucose. Growth took place in continuous light and in 12 h light/12h dark regimes. In both

growth rooms light intensities were approximately $60 \pm \mu\text{E m}^{-2}$
 s^{-1} and temperatures were $25.5 \pm 0.5^\circ\text{C}$.

Algae were grown on a stationary bench in 100 ml flasks containing 50 ml of Medium. Flasks were gently shaken once a day. Counts using an Improved Neubauer Haemocytometer were made every two days over a period of 18 days.

One ml of culture was aseptically withdrawn and the pH recorded. Samples were fixed with 2 drops of 2% formaldehyde and immediately counted.

In each light regime, monocultures and mixed cultures with initial ratios of 40 Chlorella sp. to 1 Euglena gracilis (V/V) and 80 Chlorella sp. to 1 Euglena gracilis(V/V) were set up. The volume of inoculum used in each treatment was pre-determined as follows: replicate counts from the stock cultures of Euglena gracilis and Chlorella sp. were made and then different volumes of stock of Euglena gracilis and Chlorella were added together to 50 ml of Distilled Water and further counts were made until the appropriate volumes of stock would give the desired ratios (V/V).

Maximum growth rates were calculated according to Fogg (1965).

9.2.3 Results

9.2.3.1 - General growth Chlorella sp, Chlorella vulgaris and Euglena gracilis

The growth under continuous light of Chlorella sp., Chlorella vulgaris and Euglena gracilis in Cramer and Myers (1952) Medium, Allen's Medium (1968) and Allen's Medium supplemented with Nutrient Broth (Oxoid) is shown in Fig. 9.2.1.

These results show that Chlorella vulgaris, grew better in the three different media compared to Chlorella sp. (pond isolate). For these two algae, the highest growth was obtained in Allen's Medium supplemented with Nutrient Broth, followed by Allen's Medium and Cramer and Myers ^{medium} Euglena gracilis, unable to grow in Allen's Medium, grew better when this medium was supplemented with Nutrient Broth, although it did not grow as well as the Chlorella species.

Although Cramer and Myers is considered a medium for Euglena gracilis it did not promote good growth for this alga under continuous light. Therefore the light regime and the algal requirement for an organic carbon source was investigated. The results for the growth of Chlorella sp., Chlorella vulgaris and Euglena gracilis under two different light regimes (continuous light and light/dark) in Cramer and Myers, with and without glucose, are shown in Fig. 9.2.2.

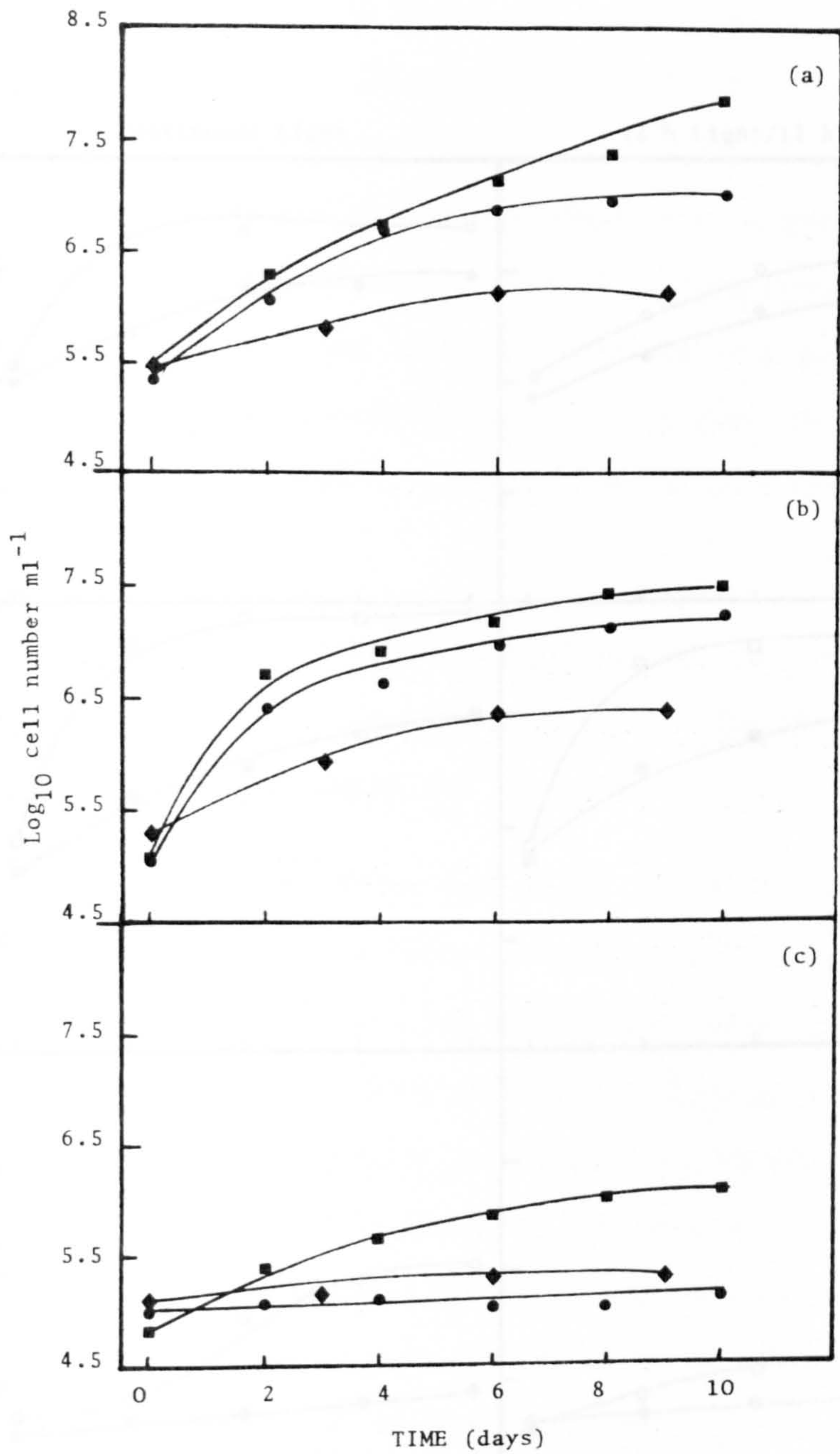


Figure 9.2.1 Growth of *Chlorella* sp. (a), *Chlorella vulgaris* (b) and *Euglena gracilis* (c) under continuous light in Allen's Medium (●), Cramer and Myers (◆) and Allen's Medium (■) supplemented by Nutrient Broth.

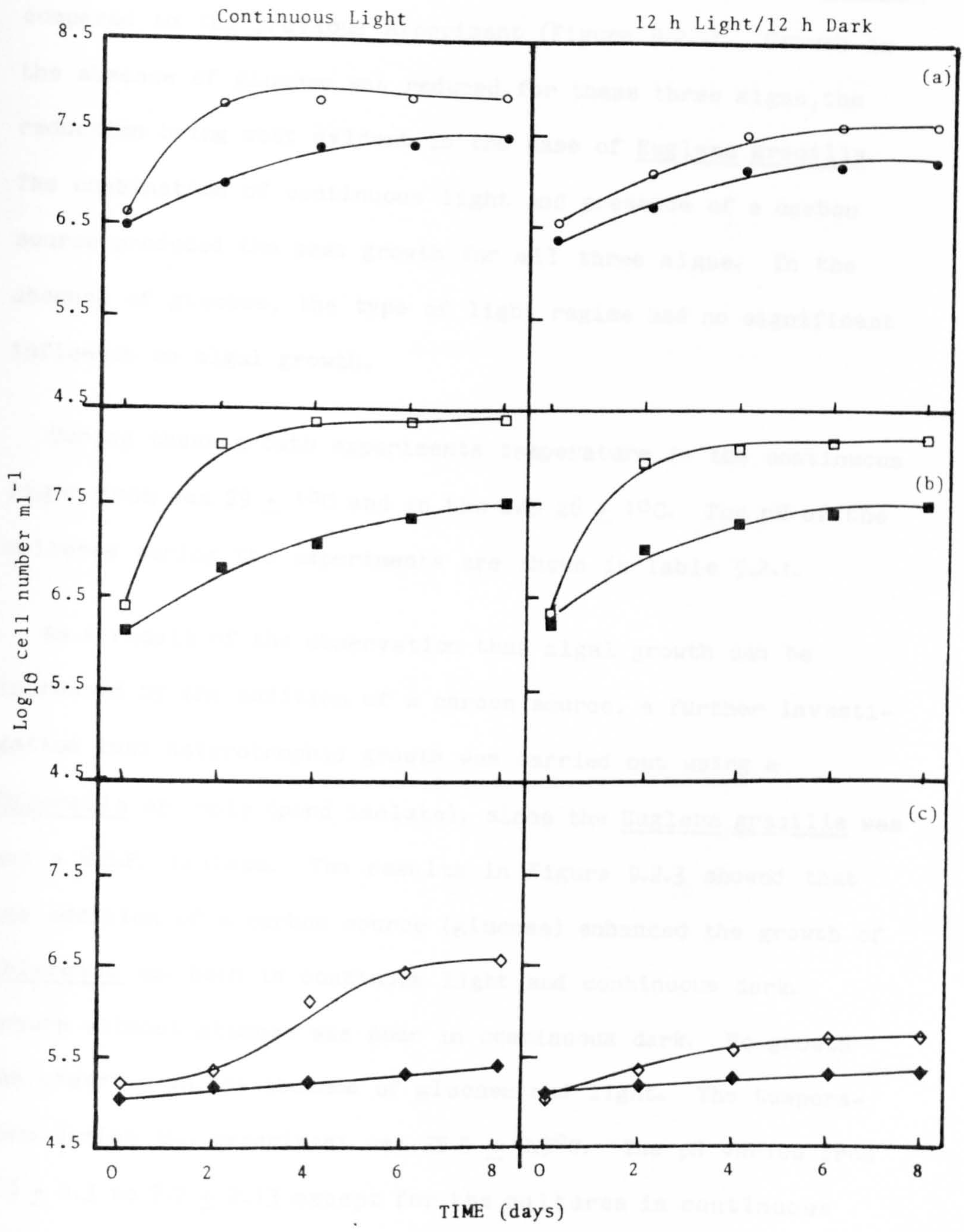


Figure 9.2.2 Growth of *Chlorella* sp. (a), *Chlorella vulgaris* (b) and *Euglena gracilis* (c) under continuous light and 12 h light/12 h dark in Cramer and Myers Medium. Open symbols indicated growth in the presence of glucose whereas closed symbols growth in its absence.

The addition of glucose to Cramer and Myers greatly improved growth of Euglena gracilis, Chlorella sp. and Chlorella vulgaris compared to the previous experiment (Figure 9.2.1). Growth in the absence of glucose was reduced for these three algae, the reduction being most evident in the case of Euglena gracilis. The combination of continuous light and presence of a carbon source produced the best growth for all three algae. In the absence of glucose, the type of light regime had no significant influence on algal growth.

During these growth experiments temperature in the continuous light room was $29 \pm 1^{\circ}\text{C}$ and in the L/D $26 \pm 1^{\circ}\text{C}$. The pH of the cultures during the experiments are shown in Table 9.2.1.

As a result of the observation that algal growth can be increased by the addition of a carbon source, a further investigation into heterotrophic growth was carried out using a Chlorella sp. only (pond isolate), since the Euglena gracilis was not a W.S.P. isolate. The results in Figure 9.2.3 showed that the addition of a carbon source (glucose) enhanced the growth of Chlorella sp. both in continuous light and continuous dark. Growth without glucose was poor in continuous dark. No growth was observed in the absence of glucose and light. The temperature during the experiment was $25.5 \pm 0.5^{\circ}\text{C}$. The pH varied from 7.3 ± 0.3 to 7.7 ± 0.13 except for the cultures in continuous light without glucose where the pH reached 9.7 ± 0.9 .

Table 9.2.1 The pH values for the growth in (a) continuous light and (b) light/dark of Chlorella sp., Chlorella vulgaris and Euglena gracilis in Cramer and Myers (1952) in the presence or absence of glucose.

a) Continuous Light		
	+ Glucose	- Glucose
<u>Chlorella</u> sp.	8.4 ± 0.4	8.0 ± 0.5
<u>Chlorella vulgaris</u>	8.6 ± 0.5	8.5 ± 0.3
<u>Euglena gracilis</u>	8.5 ± 0.5	7.9 ± 0.6
b) Light and Dark		
	+ Glucose	- Glucose
<u>Chlorella</u> sp.	7.8 ± 0.4	7.7 ± 0.3
<u>Chlorella vulgaris</u>	7.9 ± 0.6	7.7 ± 0.6
<u>Euglena gracilis</u>	8.0 ± 0.5	7.6 ± 0.5

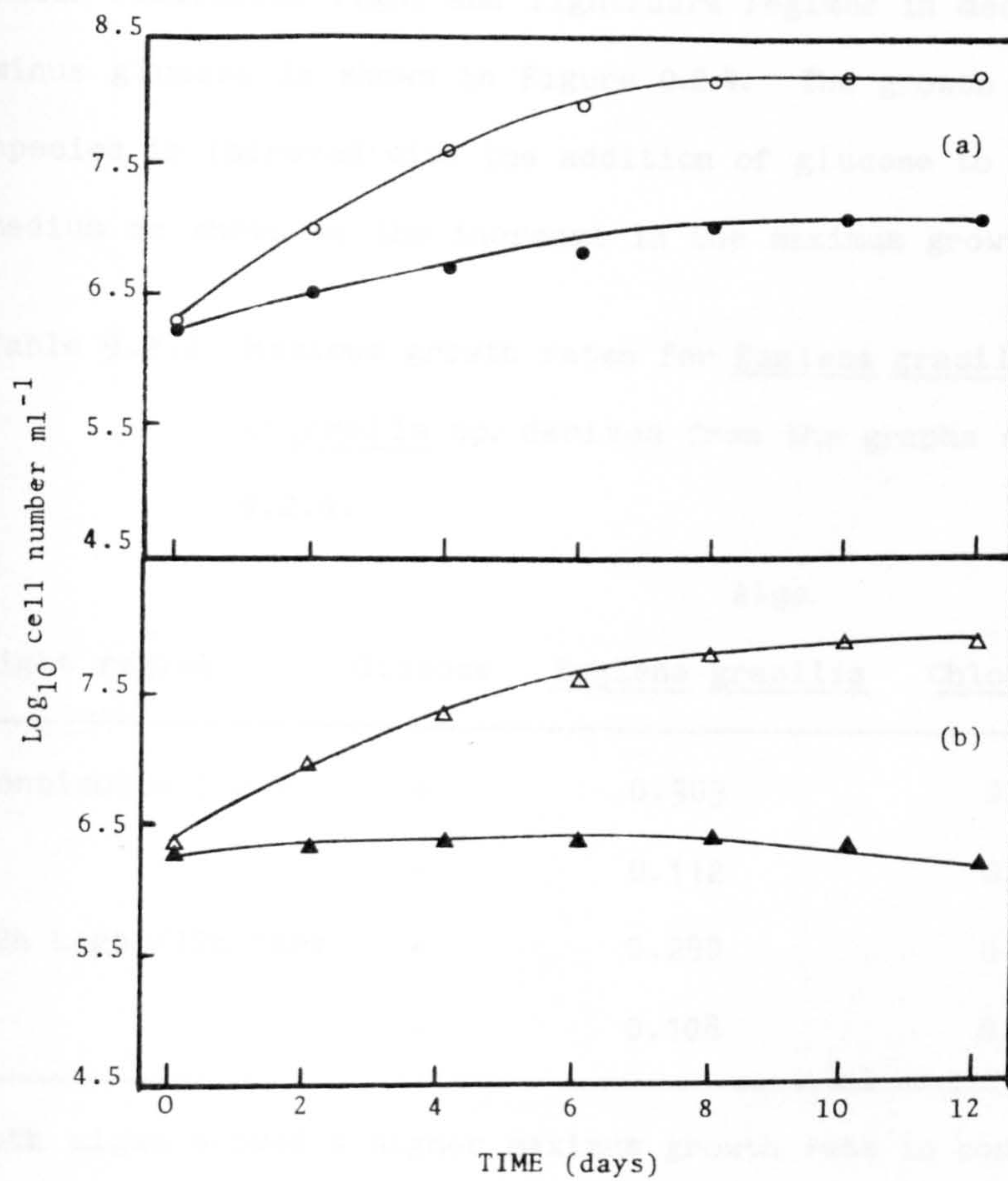


Figure 9.2.3 Growth of Chlorella sp. under continuous light (a) and continuous dark (b). Open symbols indicate growth in the presence of glucose whereas closed symbols growth in its absence.

9.2.3.2 - Mixed algal growth

The growth of Euglena gracilis and Chlorella sp. in monoculture under continuous light and light/dark regimes in media plus and minus glucose is shown in Figure 9.2.4. The growth of both algae species is improved with the addition of glucose to the culture medium as shown by the increase in the maximum growth rates.

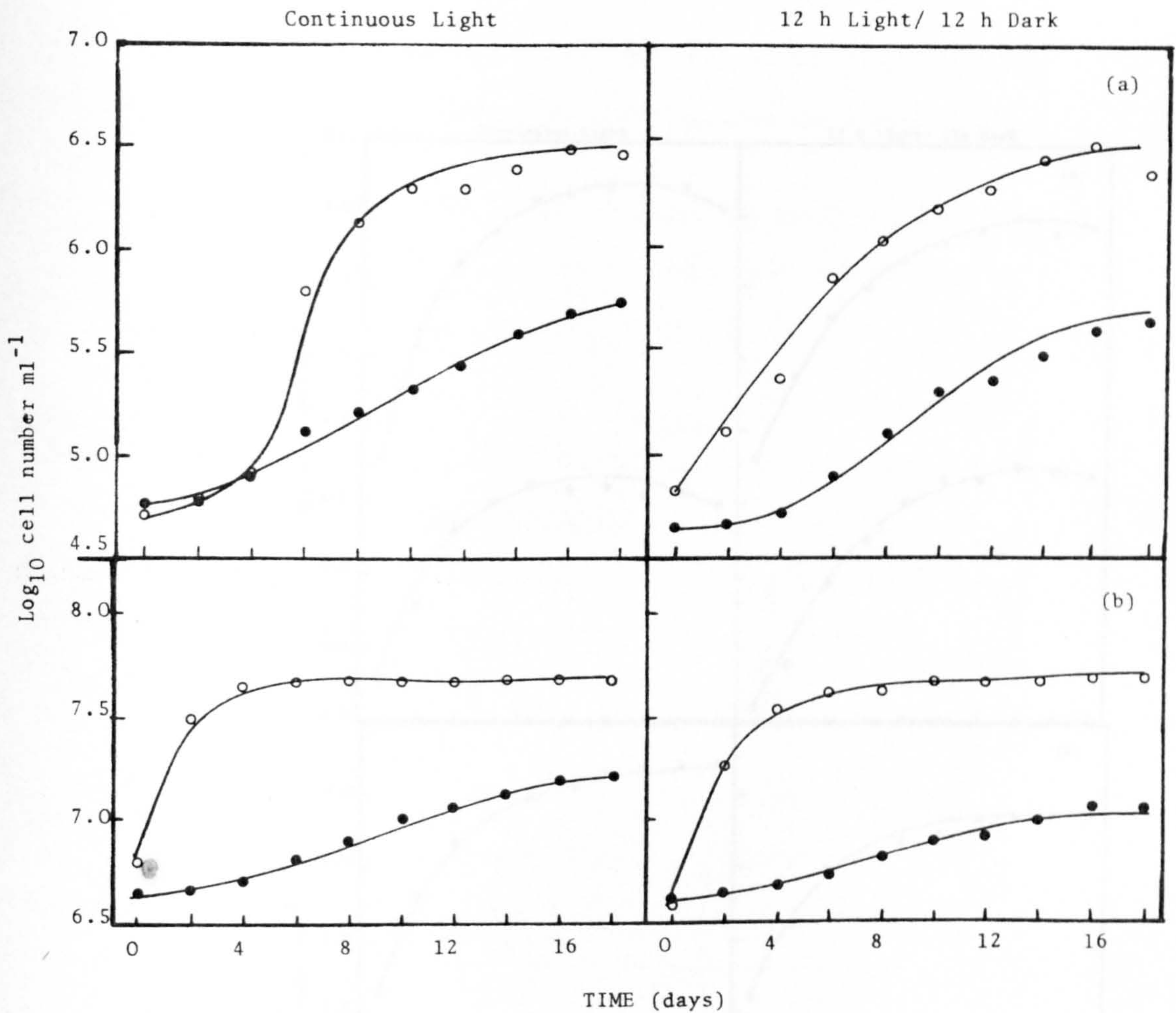
Table 9.2.2 Maximum growth rates for Euglena gracilis and Chlorella sp. derived from the graphs shown in Fig. 9.2.4.

Light regime	Glucose	Alga	
		<u>Euglena gracilis</u>	<u>Chlorella</u> sp.
Continuous light	+	0.303	0.350
	-	0.112	0.053
12h Light/12h Dark	+	0.250	0.340
	-	0.108	0.037

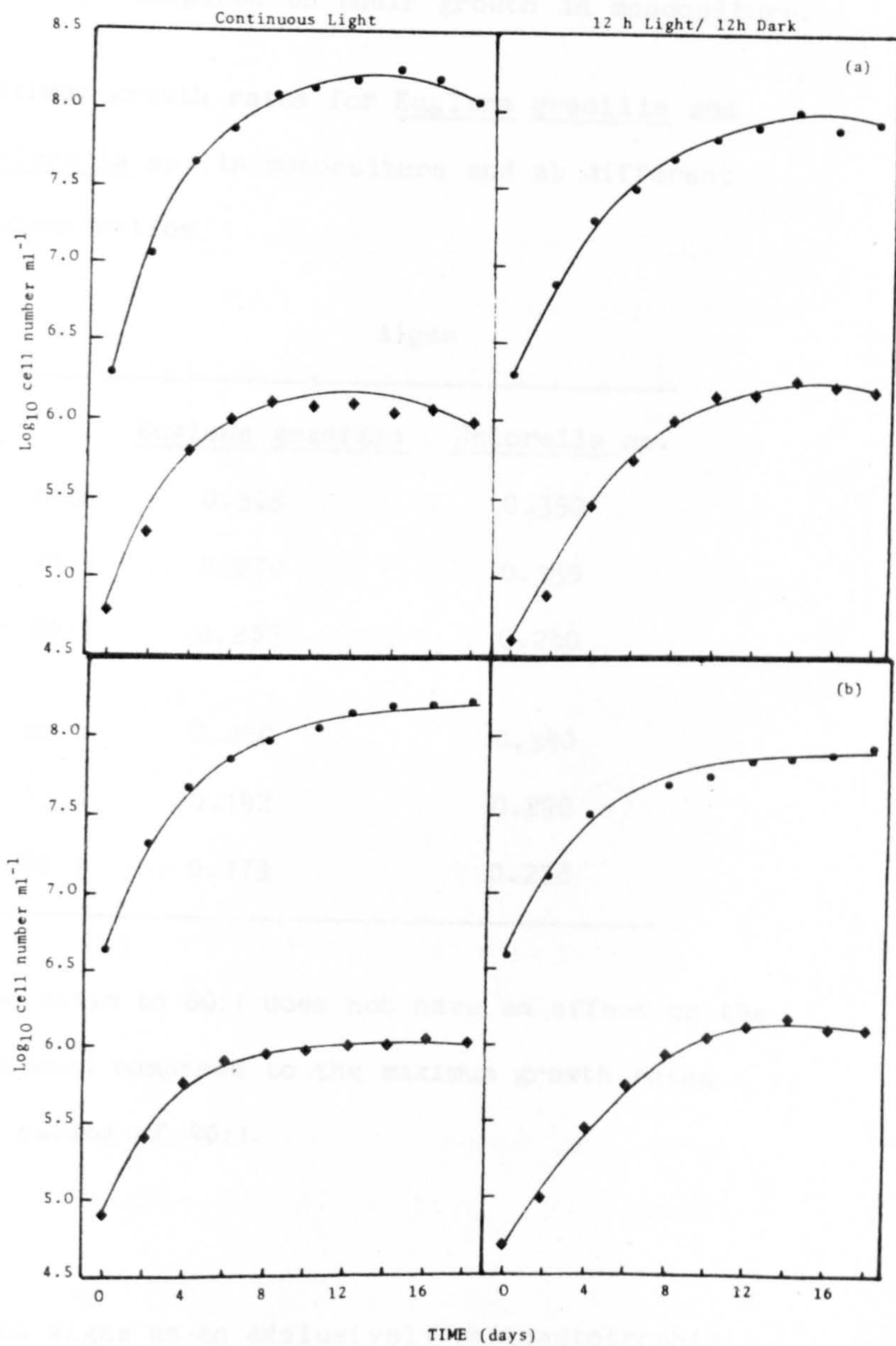
Both algae showed a higher maximum growth rate in continuous light whether or not glucose was present.

As the maximum growth rates for both species were greater in the presence of glucose. Light regimes remained the same as in the previous experiments.

The growth of Chlorella sp. together with Euglena gracilis at different volume ratios under continuous light and 12h light/12h



9.2.4 The growth of *Euglena gracilis* (a) and *Chlorella* sp. (b) in monoculture under continuous light and 12h light/12h dark regime. Open symbols represent growth in the presence of glucose whereas closed symbols, growth in its absence.



9.2.5 The growth of *Euglena gracilis* (◆) and *Chlorella* sp. (●)

in mixed culture under continuous light and 12h light/12h dark regimes in ratios of (a) 40: *Chlorella* to 1 *Euglena* and (b) 80 *Chlorella* to 1 *Euglena* (volume:volume).

dark is shown in Figure 9.2.5.

In both continuous light and light/dark the algae have the lowest maximum growth rates at the volume ratios of 80 Chlorella: 1 Euglena (Table 9.23) compared to their growth in monoculture.

Table 9.2.3 - Maximum growth rates for Euglena gracilis and Chlorella sp. in monoculture and at different volume ratios

Light Regime	Algae		
		<u>Euglena gracilis</u>	<u>Chlorella</u> sp.
Continuous Light	mono	0.303	0.350
	40:1	0.270	0.335
	80:1	0.253	0.260
12h Light/ 12H Dark	mono	0.250	0.340
	40:1	0.192	0.290
	80:1	0.173	0.228

Doubling the volume ratio to 80:1 does not have an effect on the maximum growth rate when compared to the maximum growth rates obtained at volume ratios of 40:1.

9.2.4 DISCUSSION

The concept of the algae as an exclusively photoautotrophic group has to be modified since there are many references in the

literature showing that utilization of organic compounds is a wide spread phenomenon. The substrates which support heterotrophic growth serve as both carbon and energy sources (Danforth, 1962).

In the case of Chlorella, organic compounds e.g. glucose, will be partially oxidized to release energy which in turn is necessary to convert the remaining part of the compound into cell material (Kuhl and Lorenzen, 1964). Carbon from external glucose is incorporated by Chlorella not only into polysaccharides but also to a lesser extent into lipids and, provided that ^{oxygen} is present glucose is also partly converted into lipids in the dark (Paschinger, 1969). Shihira and Krauss (1965) in a very detailed study on the taxonomy and physiology of Chlorella showed that growth of most of the species studied was stimulated by glucose both in light and dark.

The results presented here show that the algae show varying degrees of dependence on external carbon sources. Euglena gracilis shows considerable need for an external carbon source whereas Chlorella growth occurs in media with or without a carbon source. This difference in nutritional requirement will determine the success of these algae in any particular environment.

In environments where a high content of organic matter is expected (e.g. facultative) ponds, Euglena will be a successful

organism (Oswald et al., 1953). In contrast in low loaded ponds (e.g. maturation) a reduced number of Euglena would be expected (see Chapter 3).

In the case of Chlorella, although present in both pond types, (see Chapter 3) this alga has a competitive advantage over Euglena in maturation ponds since it is able to grow photoautotrophically as well as heterotrophically. Therefore, Chlorella will be ubiquitous as its nutritional requirements are less stringent than those of Euglena.

Since the mixed growth experiments using Euglena and Chlorella were not carried out under nutrient limitation conditions, these two algae were not in direct competition and therefore did not exclude each other as a result of the depletion of a common carbon source. More experimentation in this field of mixed populations is required especially under nutrient limitation.

9.3 Oxygen and antibiotic sensitivity of E. coli pond isolates

9.3.1 Introduction

Although sewage treatment by W.S.P. is known to reduce faecal coliform (FC) by up to 99.99993% (Mara et al., 1983) the factors promoting "die-off" are far from clearly understood (see introduction). Many of the bacterial "die-off" studies are currently done on E. coli strains isolated from Raw sewage

(Parhad and Rao, 1974; Davis and Gloyna, 1972; Marais, 1974) and the results obtained in this way might be misrepresentative since changes in the balance and behaviour of the bacterial populations can occur during sewage treatment.

One of the parameters which requires more detailed investigation is that of the effects of oxygen on bacterial growth. Since there was an inverse relationship between FC numbers and supersaturating oxygen concentrations in the pond (see Chapter 5), plus the fact that E. coli as a facultative anaerobe might be expected to be sensitive to high concentrations of dissolved oxygen, the relationship between F.C. survival and oxygen concentrations was further investigated.

The F.C. bacteria, particularly E. coli strains are known to transfer drug resistance (R factor) (Richmond, 1972) to other bacteria and are thus contributors to the increasing resistance to antibiotics found among disease causing bacteria. Garbow et al. (1974) reviewed the subject, suggesting that current determinations of FC with an acquired R factor should be part of sewage pond effluent criteria. The complete method for determination of FC with R factor is described by Sturtevant and Feary (1969).

Due to the ready availability and apparent high utilisation of antibiotics in Brazilian towns such as Campina Grande, the possibility that FC showing drug resistance might be present in

effluent from EXTRABES ponds was investigated.

The laboratory studies are on FC isolates from raw sewage and W.S.P. effluents include the selection of a growth medium, preliminary studies on the effects of oxygen on bacterial growth and bacterial sensitivity to different antibiotics.

9.3.2 Material and Methods

E. coli strains used here were isolated from raw sewage, and the effluent of various ponds at EXTRABES including F1 and M3.

The bacteria were grown in either Nutrient Broth (Oxoid) or in a defined basic Mineral Medium (Williams et al., 1983) supplemented with 10 gl^{-1} of glucose and 10 mM of ammonia. The bacteria were grown at $30 \pm 3^\circ\text{C}$ in 100 ml flasks containing 50 ml of medium and placed in a Gallenkamp Orbital incubator at 80 rev min^{-1} . The appropriate bacterial inocula for the experiments were prepared by growing them overnight in the same medium as was to be used in the experiments.

When the defined Mineral Medium was used, 0.2 M Phosphate Buffer was also added to prevent a drop pH during growth. Bacterial growth was followed turbidometrically as described in Chapter 8.

Unless otherwise stated the initial pH of the Mineral Medium

was adjusted before autoclaving to 7.0 and the Nutrient Broth to 7.4.

The effects of different partial pressures of oxygen on the growth of FC (from Raw Sewage) were studied in Nutrient Broth. Special conical flasks each fitted with a side arm made from a nephelometer tube were sealed with gas tight rubber stoppers to prevent gas exchange with the atmosphere. This arrangement allowed nephelometer readings to be taken throughout the experimental period without opening the flasks.

One ml of inoculum was placed in all flasks, each flask containing 100 ml of sterile Nutrient Broth. The flasks were sealed and N₂ bubbled through them for 5 minutes, using hypodermic needles and a gas manifold to remove any air present in the flasks. Different volumes of oxygen were introduced into the sealed flasks by means of a syringe (having just removed an equal volume of nitrogen from the flasks again by syringe). This was done to give a final series of flasks with oxygen concentrations as follows: 5, 10, 20, 40, 60, 80 and 100% at a total gas pressure of 1 atm. Nephelometer readings were taken at regular intervals (usually every hour).

The effects of different antibiotics on E. coli isolates from several ponds of System I and II were tested using Multodiscs (Oxoid). This test consists of applying the paper

discs impregnated with different antibiotics at known concentrations to lawns of the various E. coli isolates. The discs used were impregnated with the following antibiotics ($\mu\text{g ml}^{-1}$): Penicillin (5), Erythromycin (50), Novobiocin (5), Oleandomycin (10), Sulphafurazole (500), Chloramphenicol (50), Streptomycin (25) ^{Tetracycline (50)}. The lawns were prepared by mixing 1 ml of E. coli inoculum in Nutrient Broth with 20 ml of molten Nutrient Agar. When the lawn was solid, the Oxoid Multodisc was carefully placed on the surface of the lawn in the centre. Plates in triplicate for each bacterial isolate were incubated at 37°C for 24h. After the incubation period the bacteria were considered sensitive (+) to a particular antibiotic if a clear zone (greater than 1 mm) had formed around the antibiotic disc (indicating that no growth had occurred). In contrast, the bacteria would be considered resistant (-) to an antibiotic if bacterial growth had occurred up to and under the impregnated disc.

9.3.3 Results

9.3.3.1 Growth of FC in defined and undefined media.

Growing the bacteria in Nutrient Broth (in defined medium) was an attempt to simulate the high nutritional conditions in raw sewage. Bacteria were also grown in a defined mineral medium to simulate conditions in a maturation pond where the total organic loading is reduced.

The results for the growth of FC strains isolated from raw sewage and from the effluents of F1 and M3 in the mineral media supplemented with glucose and in Nutrient Broth are presented in Figure 9.3.1 Growth rates in Nutrient Broth were similar for Raw Sewage, F1 and M3 isolates and no significant changes in pH occurred. In contrast when the same isolates were grown in Defined Mineral Medium differences between the isolates could be detected (Figure 9.3.1). The raw sewage isolate grew more slowly than the other isolates and had long lagphase. Cell counts ($\approx 3 \times 10^9 \text{ml}^{-1}$) at the end of the experimental period were however similar for all the isolates. The pH of the medium decreased to 5.0 towards the end of the experimental period probably due to accumulation of end products of glucose fermentation and as a result of ammonia utilization.

9.3.3.2 Effects of different concentrations of Oxygen on FC growth

Since Nutrient Broth promoted good growth of all the isolates, this medium was chosen for the investigation of bacterial growth under different O_2 regimes.

The growth of the raw sewage FC isolate over a period of 8 hours under different percentages of Oxygen is shown in Figure 9.3.2. FC growth increased with increasing oxygen concentration. However, no clear distinctions between growth at 60, 80 and 100%

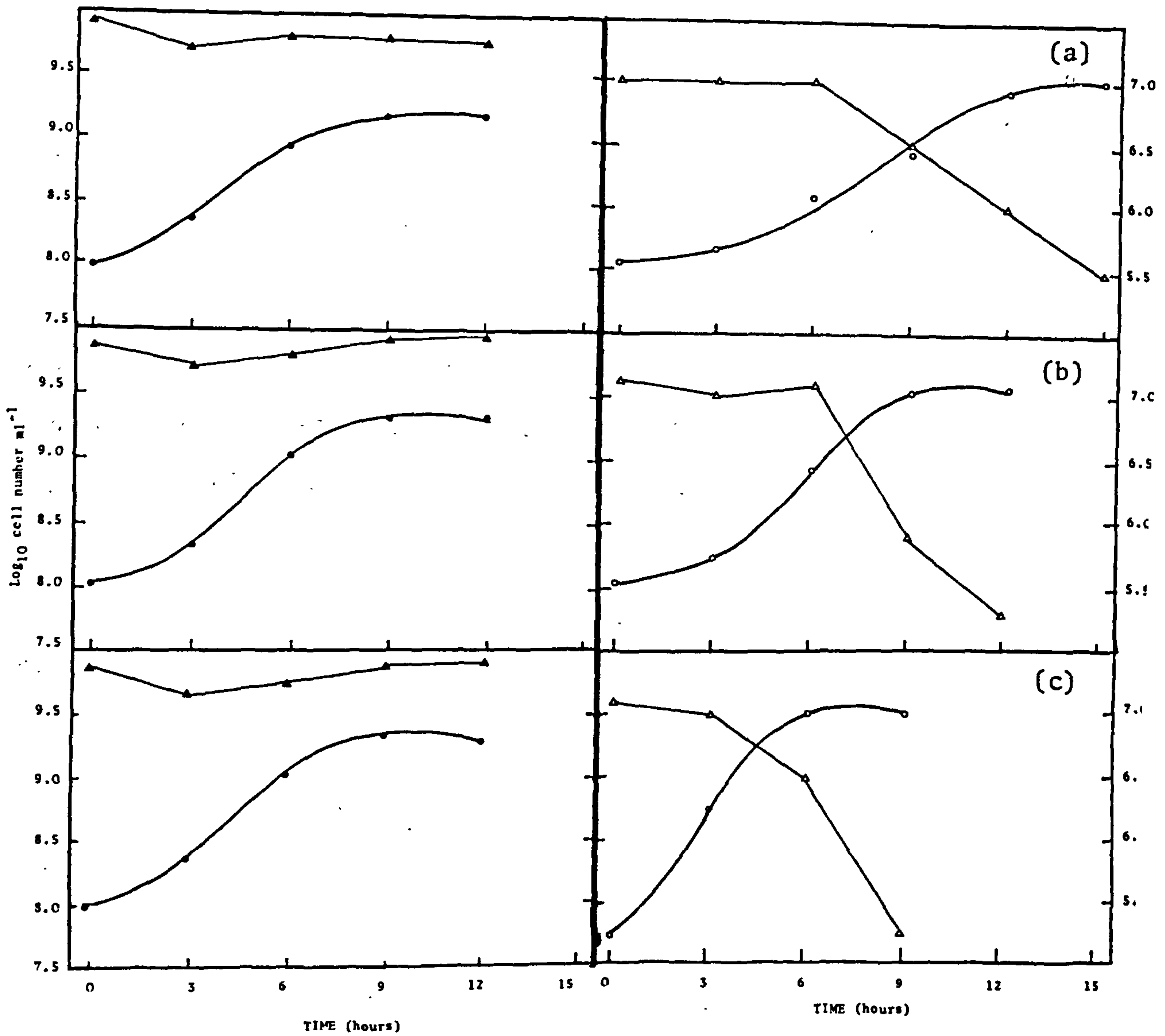


Figure 9.3.1 Growth ^{of FC} in Nutrient Broth (\bullet) Defined Mineral Medium plus glucose (\circ) of FC isolates from raw sewage ^(a) F1(b) and M3 (c). The variation in pH occurring during growth is also shown (\blacktriangle , \triangle).

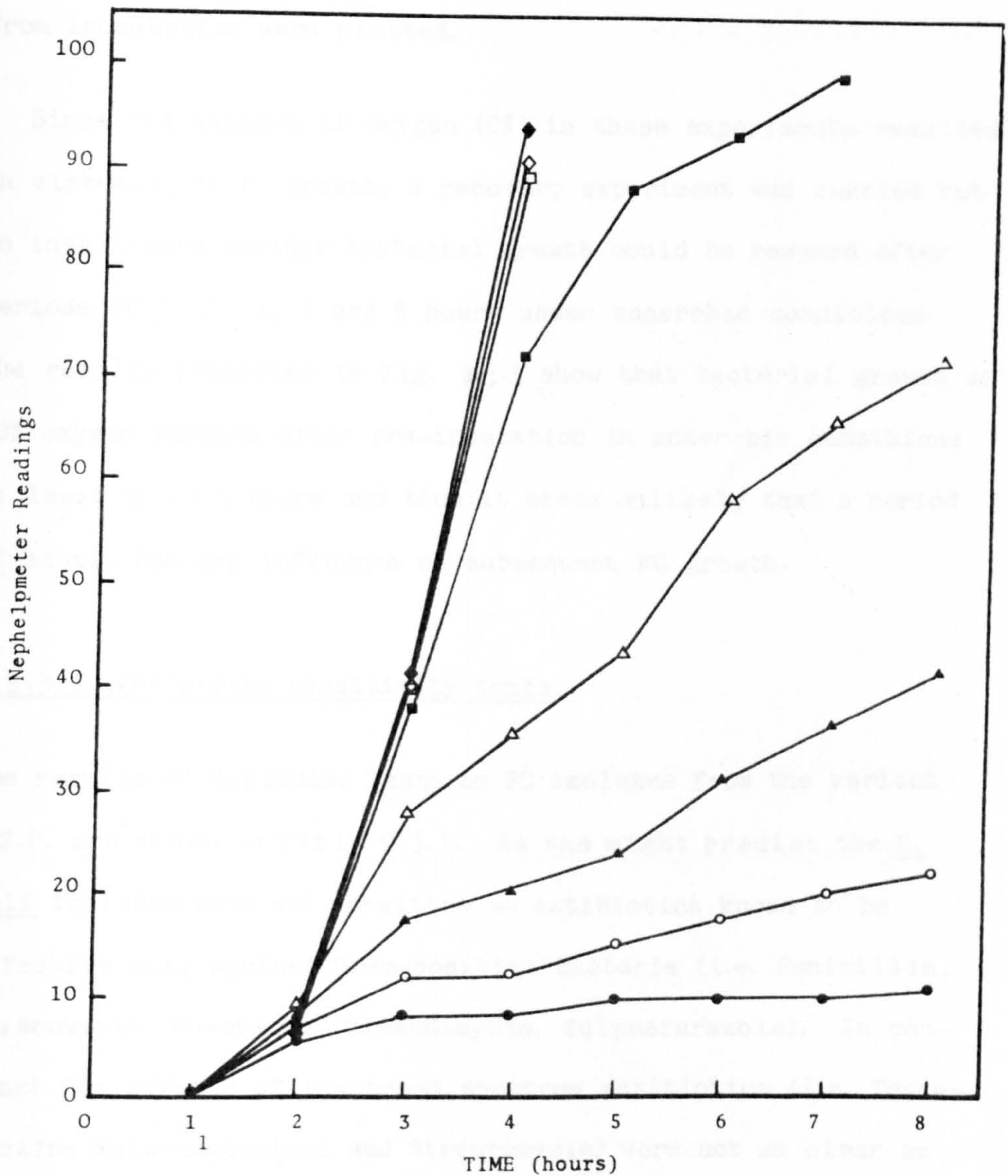


Figure 9.3.2 Growth in Nutrient Broth of an FC isolated from raw sewage under different percentages of oxygen:
 0% (●); 5% (○), 10% (▲), 20% (△),
 40% (■), 60% (□), 80% (◇) and 100% (◆).

were measurable 5 hours after inoculation as at these high oxygen concentrations the turbidity of the cultures was so great that they gave readings which exceeded the range of the nephelometer scale. Therefore in this case only readings taken after 4 hours from inoculation were plotted.

Since the absence of oxygen (0%) in these experiments resulted in virtually no FC growth, a recovery experiment was carried out to investigate whether bacterial growth could be resumed after periods of 1, 2, 3, 4 and 5 hours under anaerobic conditions. The results presented in Fig. 9.3.3 show that bacterial growth at 20% oxygen resumes after pre-incubation in anaerobic conditions at least up to 5 hours and thus it seems unlikely that a period of anoxia has any influence on subsequent FC growth.

9.3.3.3 Antibiotic sensitivity tests

The results of Multidisc Tests on FC isolates from the various W.S.P. are shown in Table 9.3.1. As one might predict the E. coli isolates were not sensitive to antibiotics known to be effective only against Gram-positive bacteria (i.e. Penicillin, Erythromycin, Novobiocin, Oleandomycin, Sulphafurazole). In contrast the effects of the broad spectrum antibiotics (i.e. Tetracycline, Chloramphenicol and Streptomycin) were not as clear as might be expected. All the E. coli isolates were sensitive to 25 $\mu\text{g ml}^{-1}$ of Streptomycin except for the isolates from the

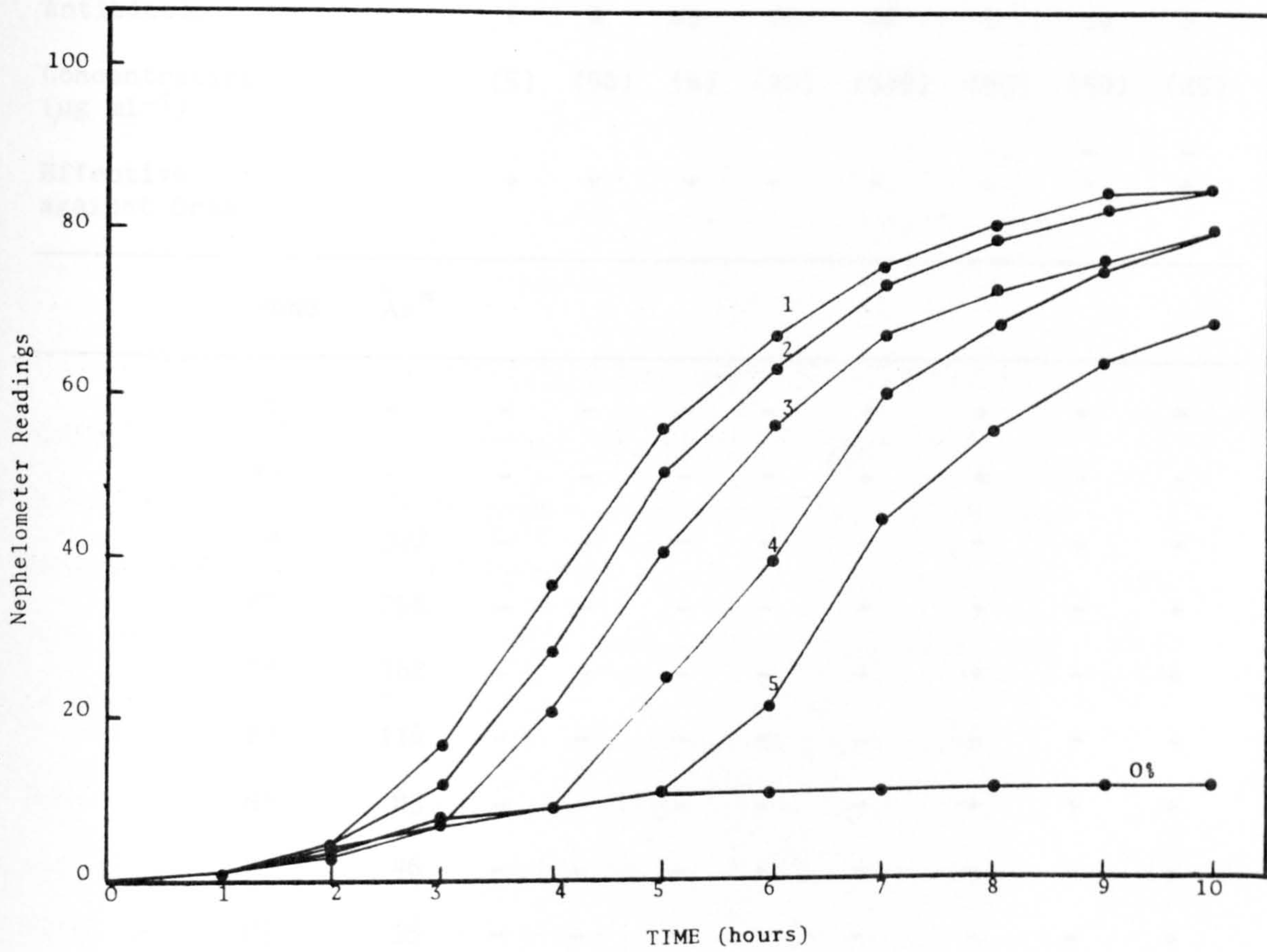


Figure 9.3.3 Growth in Nutrient Broth of an FC isolated from raw sewage after 1, 2, 3, 4 and 5 hours under anaerobic conditions.

Table 9.3.1. Antibiotic sensitivity test for E. coli isolates from raw sewage and effluents from the ponds of System I and II.

Antibiotic		P	E	NB	OL	SF	C	Te	S
Concentration ($\mu\text{g ml}^{-1}$)		(5)	(50)	(5)	(10)	(500)	(50)	(50)	(25)
Effective against Gram		+	+	+	+	+	-	-	-
	Pond	λ_s^*							
	RS	-	-	-	-	-	+	+	+
	A1	-	-	-	-	-	+	-	-
	F4	322	-	-	-	-	+	+	+
	F2	258	-	-	-	-	+	-	+
	F5	162	-	-	-	-	+	-	+
	F1	116	-	-	-	-	-	-	+
	M1	83	-	-	-	-	+	+	+
	M2	46	-	-	-	-	-	-	+
	M3	35	-	-	-	-	-	-	+

Sensitivity was recorded as + whereas - indicates resistance. The antibiotics employed were: Penicillin (P), Erythromycin (E), Novobiocin (NB), Oleandomycin (DL), Sulphafurazole (SF), Chloramphenicol (C), Tetracyclin (Te) and Streptomycin (S).

* BOD_5 surface loading.

anaerobic pond A1. The antibiotic Tetracycline ($50 \mu\text{g ml}^{-1}$) was not effective against the isolates from F2, F5 and M3.

Isolates from F1, M2 and M3 were not affected by $50 \mu\text{g ml}^{-1}$ of Chloramphenicol.

9.3.4 Discussion

The growth of FC in different media seems to vary depending on which pond the bacteria were isolated from.

An undefined medium such as Nutrient Broth promoted uniform growth and no differences between the three isolates were observed. In contrast the growth of these isolates in a Mineral Medium supplemented with glucose was different, especially for the isolate from raw sewage, which had a longer lag phase.

Abeliovich and Weisman (1978) have shown that raw sewage contains either low concentrations of glucose or none at all (depending on the method employed for its determination). Based on their findings and the fact that raw sewage contains on average 65% of protein (Tebbutt, 1970) it would seem reasonable to assume that bacteria isolated from raw sewage would grow well in a proteinaceous medium, such as Nutrient Broth.

As the sewage treatment progresses from pond to pond, reducing the BOD_5 , the substrates available for bacterial growth may also change resulting in a consequent selection of different

bacterial types. Therefore the reduction in nutritional status occurring from raw sewage to M3 could account for the slow growth of the isolate from raw sewage in mineral medium. This isolate would need a high nutritional status to grow well and in a lower nutritional status would need a longer lag phase to synthesise the appropriate enzymes (e.g. for glucose utilization). This would not be the case of isolates ~~for~~ F1 and M3 which would already possess the appropriate enzymes for glucose utilization, and growth occurs without a lag phase.

Faecal coliforms under aerobic conditions utilize a wide range of organic compounds as substrates for respiration and in conventional complex bacteriological media such as nutrient broth, the nitrogenous constituents available for respiration are amino acids and peptides.

The rapid increase in growth with increasing percentage of O_2 measured here agrees with the similar findings of Towers and et al. (1965) who found that the rate of growth of E. coli increased as the O_2 tension increased towards 1 atm and was only inhibited by oxygen at the higher pressures of 2-3 atm (i.e. hyperbaric oxygenation).

Although the FC showed low growth at 0% O_2 they were able to grow as soon as oxygen was reintroduced into the medium.

The preliminary tests on the FC isolates sensitivity to diff-

erent antibiotics showed that the EXTRABES W.S.P. effluents contained bacteria resistant to antibiotics such as Chloramphenicol, Tetracycline and Streptomycin and that FC isolates from different ponds showed variable sensitivity.

Since E. coli with the transferable drugs resistance factor (R) could be present in the effluent of various ponds, the possibility of the R factor transferring to pathogenic bacteria (e.g. Salmonella and Shigella) in the pond systems warrants further careful study.

Therefore in places where antibiotics are sold without any restrictions (e.g. northeast of Brazil) the suggestion made by Garbow^{etal (1974)} that the determination of the FC populations with an acquired R factor should be incorporated as criteria of pond effluent evaluation, should be considered.

9.4 Algae-Bacteria

9.4.1 Introduction

The complimentary activity of algae and heterotrophic bacteria in W.S.P. has been described earlier (see Chapter 1). This form of sewage treatment is particularly efficient at reducing the number pathogenic organisms which is usually measured indirectly by estimating the numbers of the faecal indicators. The FC numbers, and presumably the pathogens, decrease especially after passing

through a maturation pond.

Many reasons have been put forward to explain the causes of these reductions; from bacteriophages, predators, high pH values, nutrient deficiencies, algal competition, temperature, to wind action and UV radiation.

Laboratory studies have shown that Chlorella cultures produce an antibacterial agent (Pratt & Fong, 1940; Pratt, 1942, 1944, 1948; Pratt et al., 1945) called Chlorellin. Oswald et al., 1953, found that bacterial numbers in raw sewage containing Chlorella were lower than the bacterial numbers found when the algae was absent or when Euglena gracilis was used instead of Chlorella.

Although the evidence for algal antibacterial activity was obtained from laboratory experiments, its relevance in the field cannot be ignored. Laboratory experiments are important tools which help us to understand parts of the complex interactions between many variables which occur simultaneously in natural aquatic environments.

In this section, Chlorella and E. coli both isolated from a maturation pond were grown together under controlled laboratory conditions to investigate how variables such as pH and carbon source might influence these mixed populations.

9.4.2 Materials and Methods

Axenic algal cultures of a laboratory strain of Chlorella vulgaris and a Chlorella sp. (isolated from the maturation pond M3) were used in conjunction with an E. coli isolated from the effluent of M3.

The experiments on algal-bacterial interactions were divided into two sections: in the first, Chlorella vulgaris was grown in Allen's Medium (1968) with and without 5 g l⁻¹ of glucose. E. coli was added to the glucose plus algal culture on the 4th day. Bacterial growth was measured every 8 hours during day 4 and 5 and thereafter every two days. Algal growth was measured every two days by chlorophyll a determinations (see Chapter 8). During the period between days 4 and 5 the chlorophyll a measurements in the cultures of plus and minus bacteria were taken every 16h. The experiment was conducted over a 14 day period. In the second section, Chlorella sp. (pond isolate) and E. coli were grown in Allen's Medium with 5 mM of NH₄⁺ and buffered at pH's 7.2, 8.0 and 9.0 with 0.2 M HCl-TRIS buffer.

Algal growth was followed by determining increase in cell number. Algal and bacterial growth measurements were taken every two days during the first 8 days and a final measurement was taken on the 16th day.

In both sections, bacterial growth was measured by the plate

count technique using Nutrient Agar (Oxoid) as the growth medium. Plates were incubated at 37°C for 24 hours. Two hundred and fifty ml conical flasks containing 100 ml of Allen's Medium were used and placed on a Gallenkamp Orbital Shaker at 80 rev. min⁻¹ under continuous light intensity of $60 \pm 1 \mu\text{E m}^{-2} \text{ s}^{-1}$ at $28 \pm 2^\circ\text{C}$.

9.4.3 Results

The results for the growth of Chlorella vulgaris in the presence and absence of glucose is shown in Figure 9.4 (a). In the presence of glucose, growth was considerably greater than in the medium without glucose. In the cultures without glucose, variations in pH during the experimental period (i.e. from an initial pH of 7.8 to a final pH of 10.45) could account for the poor growth, in this unbuffered medium.

Although the addition of E. coli to a glucose plus algal culture (Figure 9.4.1(b)) slightly reduced the chlorophyll a content over the experimental period, the growth pattern for the cultures with and without bacteria were very similar. Algal growth however in the cultures with bacteria is reduced after the 6th day.

The growth of E. coli when mixed with Chlorella vulgaris increases between day 4 and 5 but thereafter decreases rapidly reaching a constant value by day 12 (Figure 9.4.1(c)).

The regression analyses of bacterial numbers against the pH of

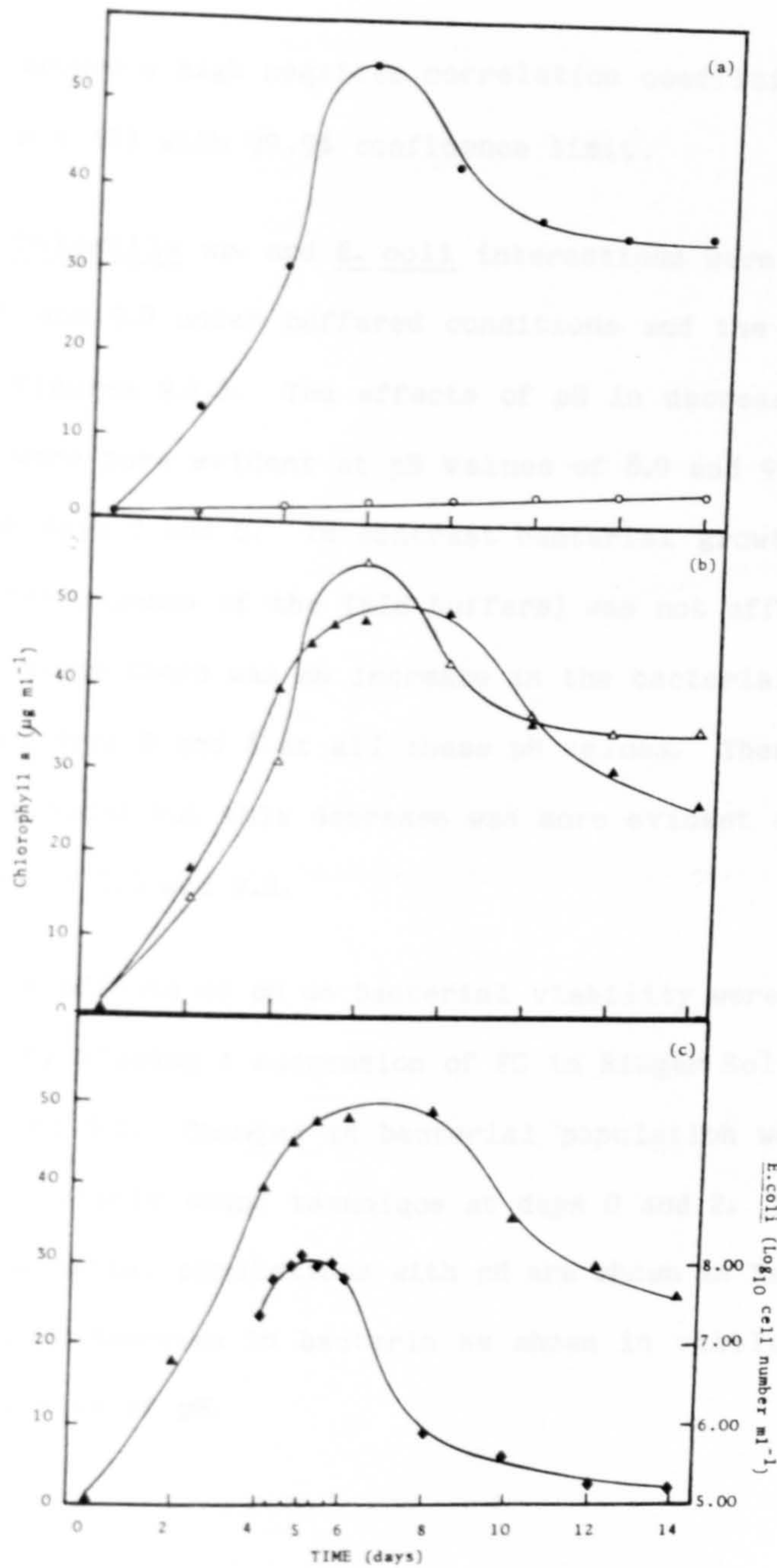


Figure 9.4.1 The growth in Allen's Medium of:

- (a) *Chlorella vulgaris* in the presence (●) and in the absence (○) of glucose;
- (b) *Chlorella vulgaris* in glucose, in the presence (▲) and absence of (Δ) of *E. coli*.
- (c) *Chlorella vulgaris* (▲) mixed with *E. coli* (◆).

the cultures showed a high negative correlation coefficient ($r = - 0.84$, $n = 11$) with 99.9% confidence limit.

Therefore Chlorella sp. and E. coli interactions were studied at pH 7.2, 8.0 and 9.0 under buffered conditions and the results are shown in Figures 9.4.2. The effects of pH in decreasing algal growth were more evident at pH values of 8.0 and 9.0 in the period between days 0 and 6. In contrast bacterial growth, (probably at the expense of the TRIS buffers) was not affected by the pH values since there was an increase in the bacterial numbers between days 0 and 6 at all these pH values. Thereafter their numbers reduced but this decrease was more evident at the high pH values of 8.0 and 9.0.

Therefore the effects of pH on bacterial viability were reinvestigated by placing a suspension of FC in Ringer Solution at pH 7.0, 8.0 and 9.0. Changes in bacterial population were estimated by the viable count technique at days 0 and 2. The variations in bacterial populations with pH are shown in Table 9.4.1. There was a decrease in bacteria as shown in viable counts with increase of pH.

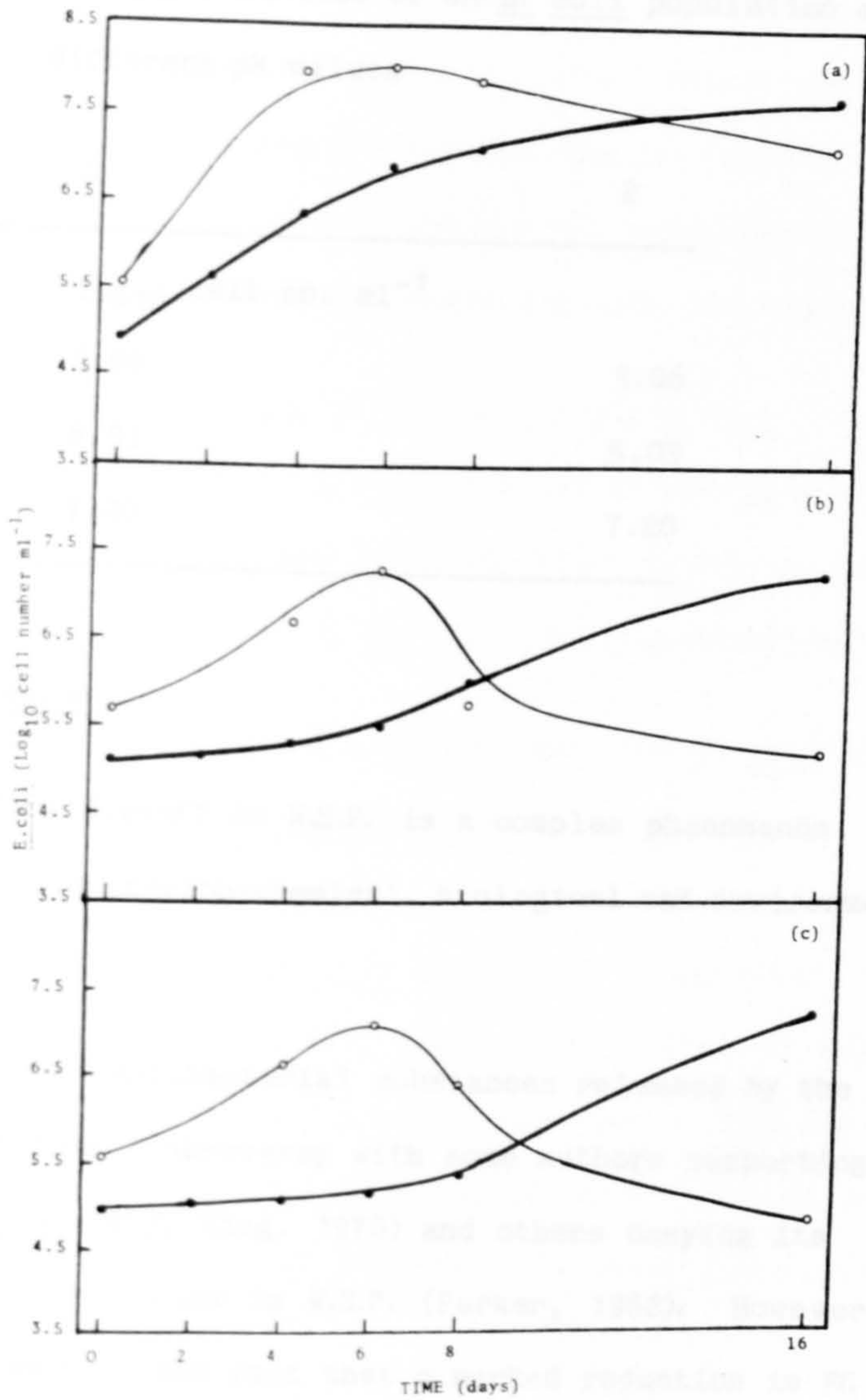


Figure 9.4.2 The growth in Allen's Medium of *Chlorella* sp.
 (●) mixed with *E. coli* (○) at pH 7.2 (a), 8.0
 (b) and 9.0 (c).

Table 9.4.1 Changes with time of an E. coli population at different pH values

Day	0	2
pH	log ₁₀ cell no. ml ⁻¹	
9.0	6.89	3.06
8.0	6.93	5.03
7.0	7.00	7.20

9.4.4 Discussion

Faecal coliform die-off in W.S.P. is a complex phenomenon involving various physico-chemical, biological and environmental conditions.

The presence of antibacterial substances released by the algae is a matter of some controversy with some authors supporting this idea (Oswald et al, 1953; King, 1970) and others denying its importance as a mechanism in W.S.P. (Parker, 1962). However, there is agreement on the fact that a marked reduction in FC occurs when algal growth is intense (Neel & Hopkins, 1956). It has been pointed out that one of the requirements for the excretion of antibacterial compounds by the algae is high pH (Pratt and Fong, 1940) which increases its solubility and possibly toxicity (Proctor, 1957).

Many authors have investigated the direct and indirect effects of pond algae on the removal of FC bacteria. Parhad and Rao (1974) found that algal growth increased the pH from 7.5 to more than 10.0 and that increasing pH caused the reduction of E.coli when the bacteria are grown in association with the algae.

Since CO₂ is an important growth factor for E. coli, its unavailability due to rapid utilization by the photosynthesising algae has also been suggested as an important factor in determining the removal of E. coli in natural environments (Gray 1975).

Although high pH values certainly reduced the E. coli numbers when in the presence of Chlorella the bacterial population did not disappear completely. According to Davis and Gloyna (1972) the small reduction of the E. coli population in unialgal Chlorella cultures indicates that axenic algal cultures contribute little to the die-off of enteric bacteria. A more rapid die-off was found when a single species of enteric bacterium was grown in a heterogeneous algal population (a situation closer to the natural environment).

The reduction but not the complete di-sappearance of E. coli when in the presence of Chlorella agrees with the findings of Vela and Guerra (1966). Their laboratory studies using mixed cultures of Chlorella pyre noidosa (TX 71105) and various bacteria found that bacteria can grow in cultures of Chlorella by utilising products excreted by the algae and an algal/bacterial

population equilibrium resulted.

The nutritional status of the medium (e.g. Ringer Solution) combined with a high pH can cause a reduction in the FC population.

Therefore, in maturation ponds, FC die-off is certainly the result of a combination of factors with nutritional status and high pH being only two of them.

CHAPTER 10

AMMONIA TOXICITY STUDIES

10.1 Introduction

The toxicity of ammonia to zooplankton and fish is a universal phenomenon that has been widely described (Shilo and Shilo, 1953; Warren, 1962; Natarajan, 1970).

This toxicity of ammonia to aquatic organisms has been attributed to the un-ionized form (NH_3) (Wuhrmann *et al.*, 1947; Wurhmann and Woker, 1948), while the ionized form (NH_4^+) is considered non-toxic (Tabata, 1962).

In aqueous solution ammonia is present as both NH_3 and the ammonium ion NH_4^+ , and the equilibrium between them can be represented by the equation:



Ammonia is regarded as toxic to fresh water organisms (Wurkmann and Woker, 1948; Downing and Merkem, 1955) because it is uncharged and lipid soluble, whereas the permeability of membranes to charged and hydrated ammonium ions is relatively low (Jacobs, 1940; Milne *et al.*, 1958).

The concentration of ammonia (NH_3) is dependent on a number of factors, in addition to the total concentration (i.e. $\text{NH}_3 + \text{NH}_4^+$). Most important among these are pH and temperature: the concen-

tration of ammonia increases with increasing pH and with increasing temperature (Emerson et al, 1975). It has been calculated that a 10°C rise in temperature will double the amount of NH_3 present in the ammonia solution (Ruffier et al, 1981).

The relative amounts of NH_3 and NH_4^+ determined by the pH is illustrated by Fig. 10.1. In acid solution, virtually all the ammonia is converted to ammonium ion whereas at pH 9.2, half of the ammonia will be in the form of the ammonium ion.

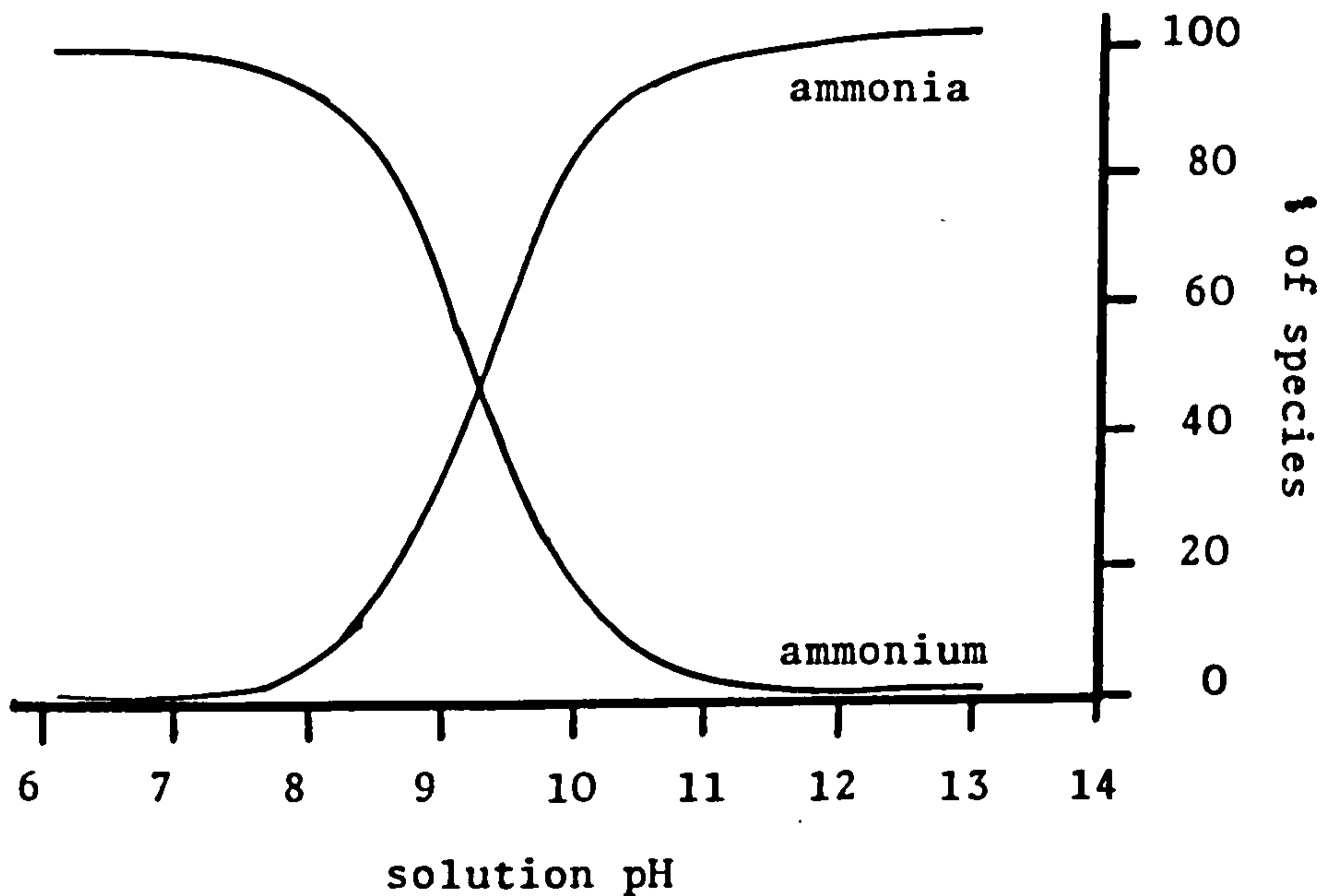


Fig. 10.1 Fraction of ammonia and ammonium ion as a function of pH ^{at 25°C} (taken from Instruction Manual ammonia electrode model 95.10 - Orion Research Incorporated, Ma., U.S.A.)

Ammonia is a common constituent of municipal waste water. It is released following the hydrolysis of urea and the biological degradation of amino acids and other nitrogenous organic matter. Concentrations of ammonia in raw municipal waste water usually range from 9 - 30 mg l⁻¹ of ammonia nitrogen (Ruffier et al., 1981) but can be higher (Abeliovich and Azov, 1976).

Abeliovich and Azov (1976) working on the toxicity of ammonia to algae in sewage ponds found that concentrations over 2.0 mM above pH 8.0 inhibited photosynthesis and the growth of Scenedesmus obliquus, a dominant species in high rate ponds.

This investigation is primarily concerned with ammonia toxicity to axenic cultures of Euglena gracilis and Chlorella vulgaris and also a Chlorella species isolated from a waste stabilization ponds at EXTRABES. Unfortunately it had not been possible to isolate and purify the Euglena species common in the Brazilian ponds. In addition the effects of ammonia on F. coliform strains (E. coli) isolated from EXTRABES ponds were also investigated.

10.2 Materials and Methods

10.2.1 Algae

The three axenic algal species used were Euglena gracilis Klebs *f. tertia F. et N* strain 1224/5z Pringsheim 1950; Chlorella vulgaris strain 211/8K *et Myers, TX 7.11.05* Sorokin (repurified from culture obtained from the Cambridge

Collection of Algae and Protozoa (C.C.A.P.), Cambridge, England, and a Chlorella species presumed to be Chlorella vulgaris, isolated from the EXTRABES maturation period (M3).

The algological experimentation was divided into 2 sections. In the first, the algae were grown in Cramer and Myers (1952) Medium (Chapter 9) buffered at pH 6.8, with 10 mM of PIPES (Sigma Chemical Co., St Louis, Mo., U.S.A.. pKa 6.8 at 25°C) at concentrations of ammonia varying from 5 to 40 mM. In the second, the algae were grown in Cramer and Myers (1952) Medium at concentration of 10 mM of ammonia and the pH was varied from 7.6 to 9.0 using 50 mM of BICINE buffer (Sigma Chemical Co., St Louis, Mo., U.S.A. pKa 8.3 at 25°C). The pH of the Medium was adjusted with 3mM NaOH. These experiments were carried out simultaneously in either continuous light or a 12h light/12h dark cycle.

The light intensities varied from $60 \pm 8 \mu\text{E m}^{-2} \text{ s}^{-1}$ in the continuous light growth room to $50 \pm 1.5 \mu\text{E m}^{-2} \text{ s}^{-1}$ in the light and dark growth room.

The temperatures of the growth rooms were maintained at $25 \pm 1^\circ\text{C}$.

The cultures used as inocula had previously been grown under the same experimental conditions. The inocula containing approximately 1×10^6 cells ml^{-1} for Chlorella and 5×10^6 cells ml^{-1} for Euglena gracilis were introduced aseptically into the

appropriate experimental flask.

Algal growth was estimated by optical density measurements at 540 nm as described previously in Chapter 8.

Ammonia concentrations were determined by the Indophenol Method described by Scheiner (1976).

The pH was measured using an Electronic Instrument Ltd. 7020 pH meter.

The effects of nitrate on algal growth were also investigated in the experiments by including cultures growing in nitrate at a nitrogen concentration equivalent to those of the ammonia series. This could not be done for Euglena since it does not grow in nitrate (Cramer and Myers, 1952).

10.2.2 Bacteria

F. coliform (E. coli) strains were isolated from effluent samples of various facultative and maturation ponds at EXTRABES.

The effects on the growth of E. coli at concentrations of ammonia varying from 5 to 40 mM at pH values between 7.0 and 9.0 were investigated.

The bacteria were grown in Mineral Medium buffered to the appropriate pH using 0.2 M Phosphate Buffer (Chapter 9) and supplemented with 10 g l⁻¹ of glucose. Different amounts of

$(\text{NH}_4)_2\text{SO}_4$ were added to give a final concentration of 5, 10, 20 and 40 mM ammonia - N. Analar grade chemicals were used throughout.

The glucose and $(\text{NH}_4)_2\text{SO}_4$ were ether-sterilized.

The Mineral Medium which had been previously autoclaved (15 psi 15 min.) was mixed with the glucose and $(\text{NH}_4)_2\text{SO}_4$ and 50 ml aliquots were aseptically distributed in sterile 100 ml conical flasks.

A 1 ml aliquot, taken from a bacterial culture, which had been grown overnight in Mineral Medium, was used as inoculum in each of these experiments.

Growth was measured every two hours as described in Chapter 8 and where necessary the pH was adjusted by the addition of sterile 3 M NaOH.

The flasks were incubated in the dark at $33.8 \pm 0.9^\circ\text{C}$ in a Gallenkamp Orbital Incubator shaking at 100 rev min^{-1} .

10.3 Results

10.3.1 Algae

The growth of Euglena gracilis, Chlorella sp. and Chlorella vulgaris at initial ammonia concentration of 5, 10, 20 and 40 mM (at pH 6.8) was followed during an eight day period. Replicates

of each concentration were grown either in continuous light or in a 12h light/12h dark cycle (shown in Fig. 10.2). These data show that at pH 6.8 the ammonia concentration used did not inhibit the growth of these three algae in either continuous light or in the light/dark regime. Furthermore, at pH 6.8 a concentration of 5 mM was sufficient to ensure that nitrogen was not limiting during the 8 day period.

Table 10.2 shows for each algae the changes in pH, ammonia concentrations and volatilization occurring with time for each of the ammonia concentrations in the two light regimes.

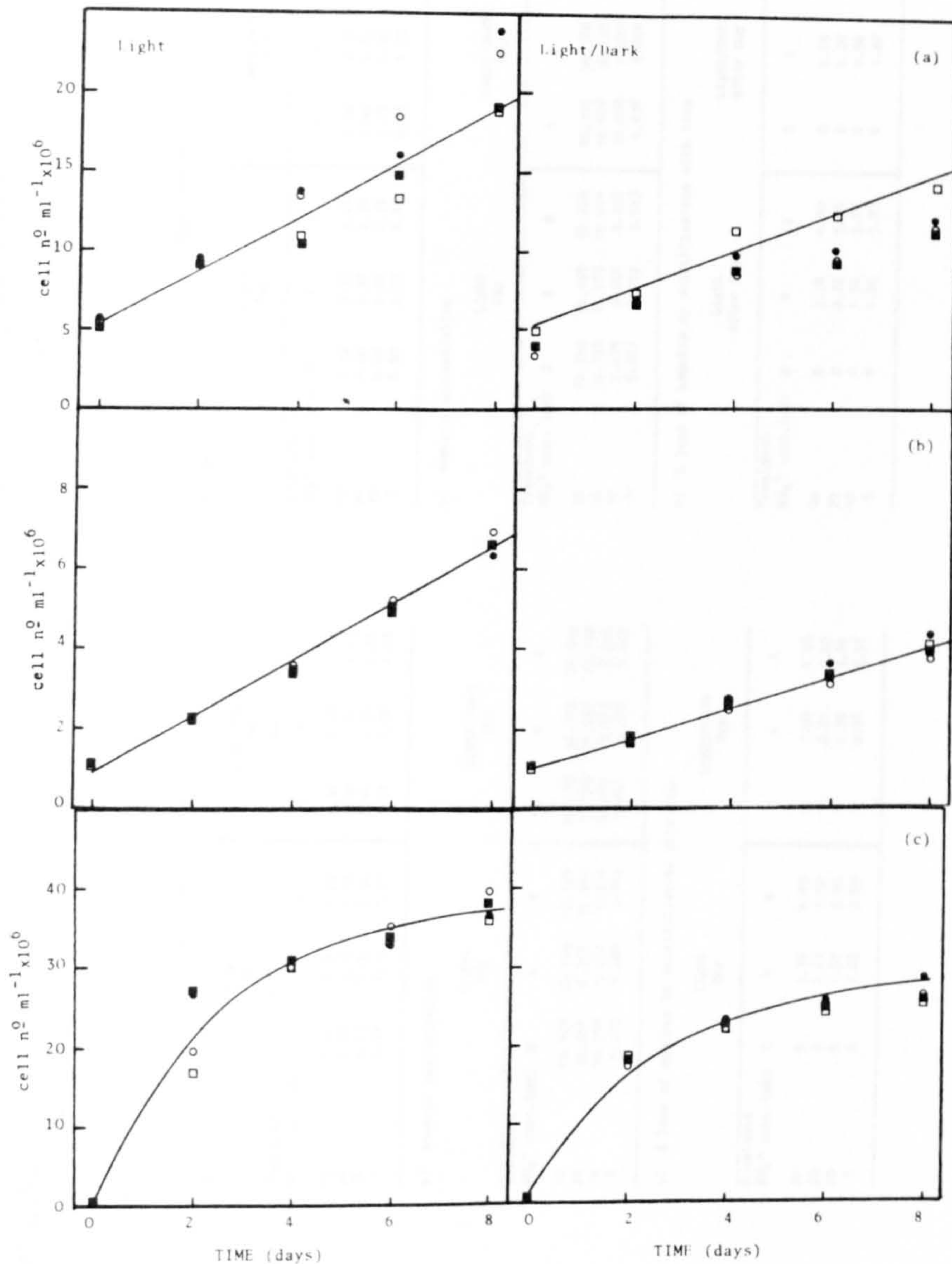
In both light regimes the changes in the ammonia concentration and pH were more pronounced in the Chlorella vulgaris culture which had the highest final cell numbers per ml (Fig. 10.2) than for the other two species.

Although under the light/dark regime (Fig. 10.2) growth was generally reduced no differences in response to ammonia concentration were evident when compared to the results for growth in continuous light.

The growth of Chlorella sp. and Chlorella vulgaris on nitrate at nitrogen concentrations equivalent to those of the ammonia series are shown in Fig. 10.3. Both algae showed similar growth patterns and rates of growth on nitrate as they did on ammonia. The changes in pH associated with algal growth in both light

Fig. 10.2 Growth of (a) Euglena gracilis, (b) Chlorella sp. and (c) Chlorella vulgaris during an eight day period at initial ammonia concentration of 5 (●), 10 (○), 20 (■) and 40 (□) mM at pH 6.8. All values are the mean of triplicate determinations. Standard deviations did not exceed 10% unless otherwise stated. All lines drawn are regression curves except in the case of Chlorella vulgaris (c). See statistical details in Table 10.1.

Table 10.1 Regression data for the cell number $\text{ml}^{-1} \times 10^6$ versus time curves shown in Fig. 10.2: n is the number of samples, r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line where y = cell number $\text{ml}^{-1} \times 10^6$ and x = time (days).



	LIGHT				LIGHT/DARK			
	n	r	Confidence limit %	Regression Equation	n	r	Confidence limit %	Regression Equation
<u>Euglena</u>	20	0.963	99.9	$y = 5.15 + 1.89x$	20	0.932	99.9	$y = 4.77 + 1.00x$
<u>Chlorella sp.</u>	20	0.990	99.9	$y = 0.94 + 0.69x$	20	0.990	99.9	$y = 1.07 + 0.38x$

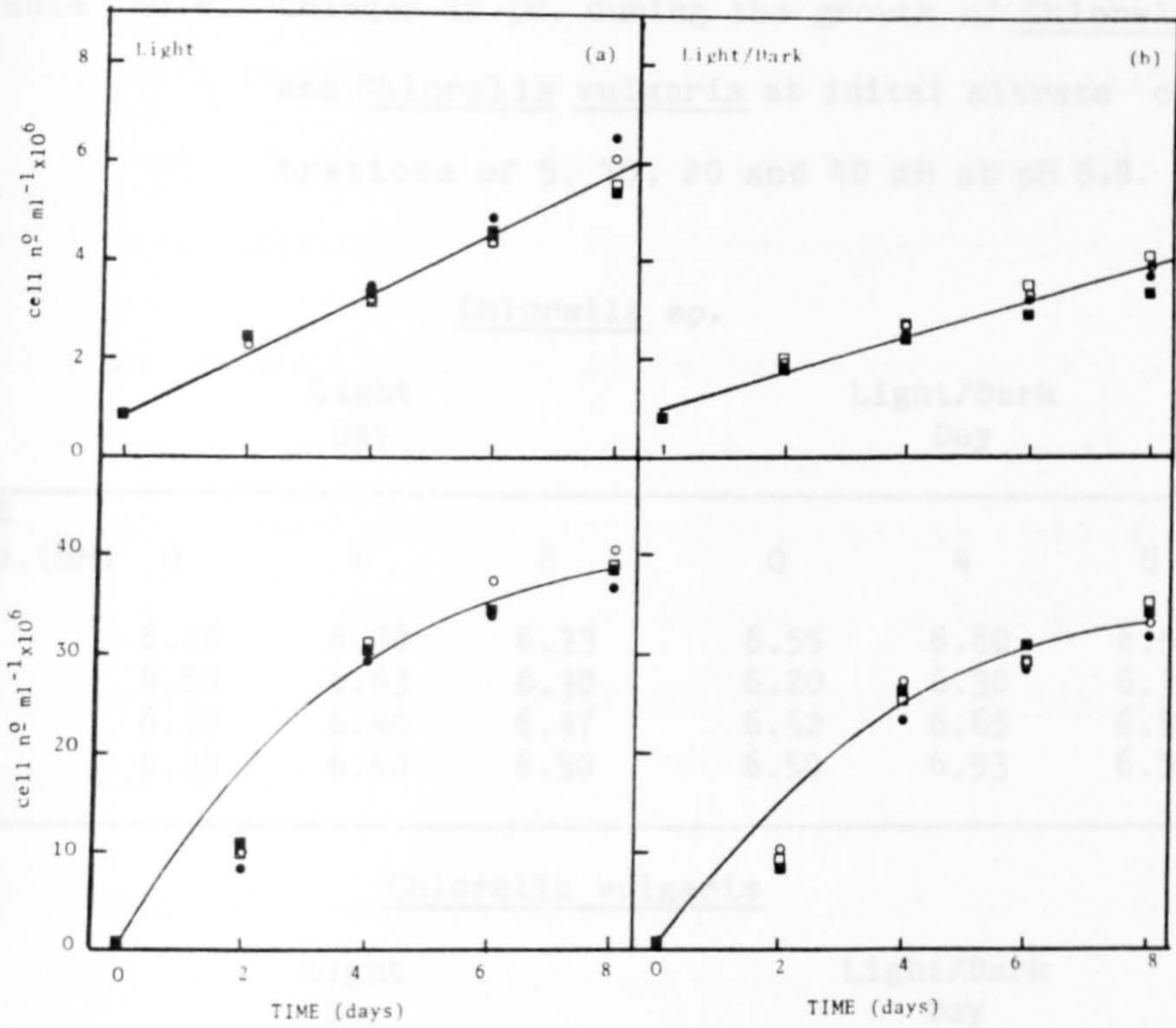
Table 10.2 Changes in pH, ammonia concentrations and loss of ammonia by volatilization during the growth of Euglena gracilis, Chlorella vulgaris and Chlorella sp. at initial ammonia concentrations of 5, 10, 20 and 40 mM at pH 6.8.

<u>Euglena gracilis</u>				<u>Chlorella vulgaris</u>				<u>Chlorella sp.</u>			
a) pH		Light Day		Light Day		Light/Dark Day		Light Day		Light/Dark Day	
Treatment	NH ₄ ⁺ conc.(mM)	0	8	0	8	0	8	0	8	0	8
40	6.70	6.66	6.63	6.53	6.53	6.42	7.13	6.42	7.18	6.20	6.33
20	6.70	6.66	6.66	6.53	7.15	6.30	7.13	6.30	7.10	6.50	6.30
10	6.60	6.50	6.43	6.60	7.08	6.60	7.35	6.60	7.38	6.26	6.47
5	6.60	6.60	6.63	6.50	7.13	6.58	7.33	6.58	7.28	6.33	6.50
b) Ammonia concentration											
Treatment	NH ₄ ⁺ conc.(mM)	0	8	0	8	0	8	0	8	0	8
40	40.059	35.909	35.828	40.158	37.865	40.148	26.000	40.148	36.782	40.150	32.288
20	19.494	12.194	12.177	19.794	13.196	19.745	15.750	19.745	24.993	19.454	12.032
10	10.045	9.016	8.967	10.150	9.549	10.149	8.615	10.149	9.113	10.018	9.553
5	5.094	4.147	4.099	5.040	4.350	5.025	4.208	5.025	4.725	5.034	3.996
c) % loss of ammonia by volatilization with time											
Treatment	NH ₄ ⁺ conc.(mM)	0	8	0	8	0	8	0	8	0	8
40	0	3.38	4.50	0	1.76	0	4.48	0	1.88	0	4.90
20	0	0.05	3.98	0	0.05	0	3.69	0	0.21	0	3.79
10	0	1.83	3.90	0	3.34	0	3.49	0	2.98	0	3.50
5	0	2.22	3.79	0	0.72	0	3.57	0	2.06	0	3.50

Fig. 10.3 Growth of Chlorella sp. (a) and Chlorella vulgaris (b) during an eight day period at initial nitrate concentration of 5 (●), 10 (○), 20 (■) and 40 (□) mM at pH 6.8. All values are the mean of triplicate determinations. Standard deviations did not exceed 10% unless otherwise stated. All lines drawn are regression curves. See statistical details in Table 10.3.

Table 10.3 Regression data for the cell number $\text{ml}^{-1} \times 10^6$ versus time curves shown in Fig. 10.3: n is the number of samples, r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line where y = cell number $\text{ml}^{-1} \times 10^6$ and x = time (days).

regions were again greater for *Chlorella vulgaris* than for *Chlorella* sp. (Table 10.4).



	LIGHT				LIGHT/DARK			
	n	r	Confidence limit %	Regression Equation	n	r	Confidence limit %	Regression Equation
<i>Chlorella</i> sp.	20	0.990	99.9	$y = 0.94 + 0.61x$	20	0.980	99.9	$y = 1.01 + 0.36x$

Chlorella sp. (laboratory culture) under both light regimes, at initial nitrate and ammonia concentrations of 13 mM and pH values of either 7.5 or 8.3 or 9.0 are shown in Figures 10.3 and 10.4.

regimes were again greater for Chlorella vulgaris than for Chlorella sp. (Table 10.4).

Table 10.4 Changes in pH, during the growth of Chlorella sp. and Chlorella vulgaris at initial nitrate concentrations of 5, 10, 20 and 40 mM at pH 6.8.

Chlorella sp.

Treatment	NO ₃ ⁻ conc.(mM)	Light Day			Light/Dark Day		
		0	4	8	0	4	8
40	6.20	6.38	6.33	6.55	6.60	6.57	
20	6.50	6.63	6.30	6.20	6.30	6.30	
10	6.30	6.40	6.47	6.52	6.65	6.57	
5	6.33	6.60	6.50	6.50	6.53	6.50	

Chlorella vulgaris

Treatment	NO ₃ ⁻ conc.(mM)	Light Day			Light/Dark Day		
		0	4	8	0	4	8
40	6.66	8.61	8.97	6.27	8.73	8.57	
20	6.23	8.73	8.75	6.15	7.85	7.92	
10	6.38	9.90	10.07	6.20	8.87	8.87	
5	6.15	8.00	8.37	6.22	8.20	8.17	

The growth of Chlorella sp. (pond isolate) and Chlorella vulgaris (i.e. the laboratory culture) under both light regimes, at initial nitrate and ammonia concentrations of 10 mM and pH values of either 7.6 or 8.3 or 9.0 are shown in Figures 10.4 and

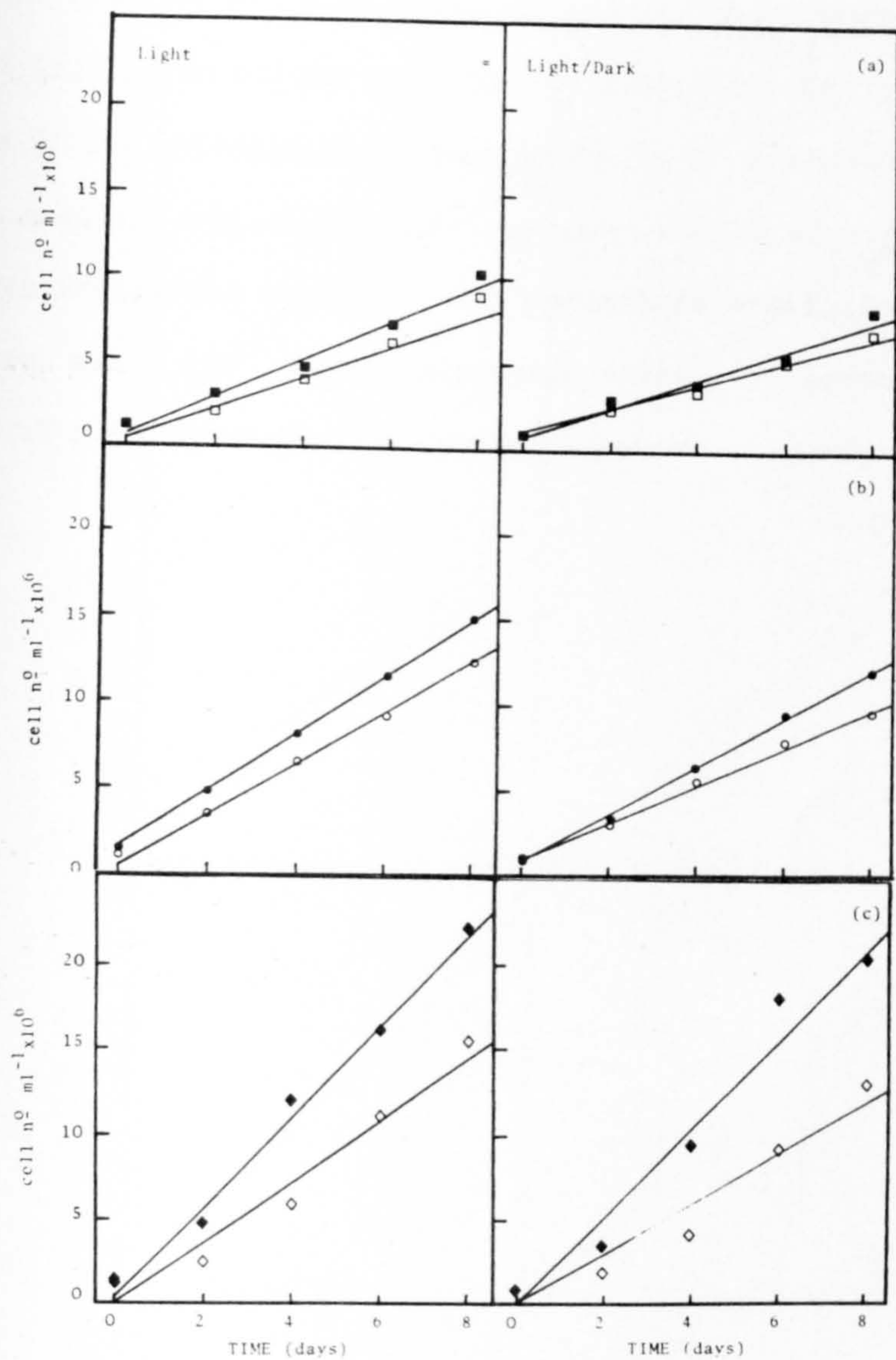
10.5. These results show that a concentration of 10 mM of ammonia did not inhibit the growth of these two algae at any pH. In fact the growth of Chlorella sp. and Chlorella vulgaris both improved with increasing pH as the increasing positive gradients show (Tables 10.5 and 10.6). However, growth was generally reduced under light/dark compared to continuous light regime.

Although no inhibition with increasing pH was observed (over the experimental period) the two Chlorella species did show differences in their preference for nitrate as the nitrogen source at elevated pH's. No major changes in pH were observed during the experimental period, except for Chlorella vulgaris at pH 9.0 (Table 10.7).

The growth data for Euglena gracilis in 10 mM ammonia at pH's of 7.6, 8.3 and 9.0 respectively are shown in Fig. 10.6. The previous results for the growth of Euglena gracilis at pH 6.8 at 10 mM were also included for comparison. In contrast to the results for the Chlorella species, growth of Euglena gracilis was inhibited at pH's of 8.3 and 9.0 under both continuous light and in a 12h light/12h dark regime. No major variations in pH occurred during the growth of Euglena gracilis (Table 10.9).

Fig. 10.4 Growth of Chlorella sp. at pH 7.6 (a), 8.3 (b) and 9.0 (c) during an eight day period at initial concentration of 10 mM of ammonia (open symbols) and 10 mM of nitrate (close symbols). All values are the mean of triplicate determinations. Standard deviations did not exceed 10% unless otherwise stated. All lines drawn are regression curves. See statistical details in Table 10.5.

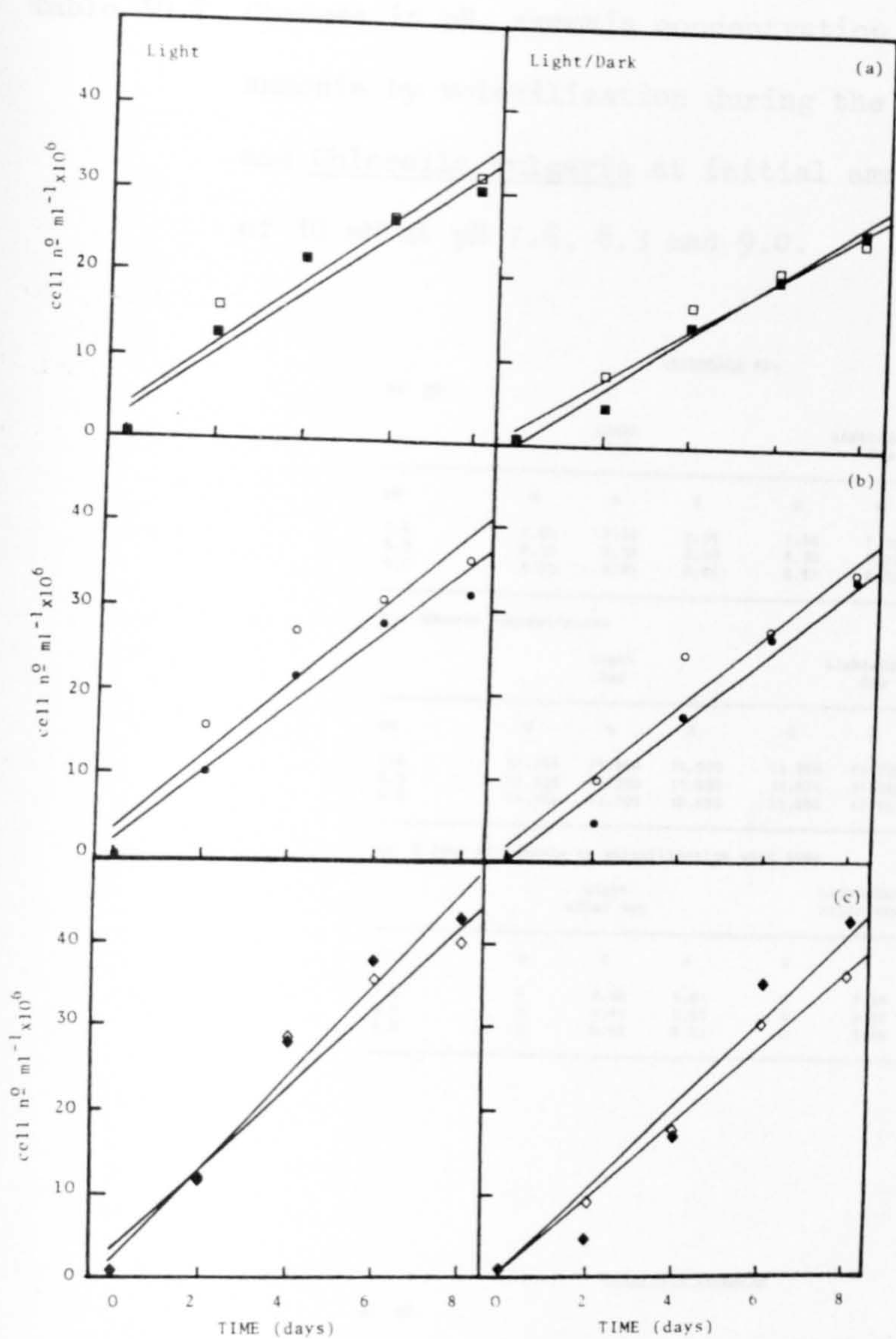
Table 10.5 Regression data for the cell number $\text{ml}^{-1} \times 10^6$ versus time curves shown in Fig. 10.4; n is the number of samples, r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line where y = cell number $\text{ml}^{-1} \times 10^6$ and x = time (days).



pH	N Source	LIGHT				LIGHT/DARK			
		n	r	Confidence limit %	Regression Equation	n	r	Confidence limit %	Regression Equation
7.6	NH ₄ ⁺	5	0.982	99-99.9	y = 0.68+0.95x	5	0.997	99.9	y = 1.0 +0.69x
	NO ₃ ⁻	5	0.991	99.9	y = 0.72+1.13x	5	0.986	99-99.9	y = 0.84+0.84x
8.3	NH ₄ ⁺	5	0.997	99.9	y = 0.93+1.43x	5	0.997	99.9	y = 1.20+1.06x
	NO ₃ ⁻	5	0.999	99.9	y = 1.49+1.67x	5	0.999	99.9	y = 0.97+1.39x
9.0	NH ₄ ⁺	5	0.980	99-99.9	y = -0.134+1.84x	5	0.970	99-99.9	y = -0.21+1.53
	NO ₃ ⁻	5	0.991	99.9	y = 0.67+2.64x	5	0.980	99-99.9	y = -0.03+2.64x

Fig. 10.5 Growth of Chlorella vulgaris at at pH 7.6 (a), 8.3 (b) and 9.0 (c) during an eight day period at initial concentration of 10 mM of ammonia (open symbols) and 10 mM of nitrate (close symbols). All values are the mean of triplicate determinations. Standard deviations did not exceed 10% unless otherwise stated. All lines drawn are regression curves. See statistical details in Table 10.6.

Table 10.6 Regression data for the cell number $\text{ml}^{-1} \times 10^6$ versus time curves shown in Fig. 10.5; n is the number of samples, r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line where y = cell number $\text{ml}^{-1} \times 10^6$ and x = time (days).



pH	N Source	LIGHT				LIGHT/DARK			
		n	r	Confidence limit %	Regression Equation	n	r	Confidence limit %	Regression Equation
7.6	NH_4^+	5	0.960	99-99.9	$y = 4.66+3.73x$	5	0.980	99-99.9	$y = 2.09+3.09x$
	NO_3^-	5	0.967	99-99.9	$y = 36.5+3.70x$	5	0.992	99.9	$y = -0.42+3.43x$
8.3	NH_4^+	5	0.960	99-99.9	$y = 4.73+4.35x$	5	0.980	99-99.9	$y = 2.09+4.34x$
	NO_3^-	5	0.983	99-99.9	$y = 2.25+4.13x$	5	0.991	99.9	$y = -0.712+4.37x$
9.0	NH_4^+	5	0.980	99.9	$y = 2.92+5.08x$	5	0.994	99.9	$y = 0.06+4.66x$
	NO_3^-	5	0.985	99.9	$y = 1.81+5.60x$	5	0.981	99-99.9	$y = -3.00+5.70x$

Table 10.7 Changes in pH, ammonia concentration and loss of ammonia by volatilization during the growth of Chlorella sp. and Chlorella vulgaris at initial ammonia concentration of 10 mM at pH 7.6, 8.3 and 9.0.

Chlorella sp.

a) pH

pH	Light Day			Light/Dark Day		
	0	4	8	0	4	8
7.6	7.60	7.60	7.55	7.60	7.55	7.58
8.3	8.30	8.32	8.28	8.30	8.27	8.32
9.0	8.83	8.85	8.85	8.87	8.82	8.88

b) Ammonia concentration

pH	Light Day			Light/Dark Day		
	0	4	8	0	4	8
7.6	11.766	11.692	11.590	11.866	11.735	11.123
8.3	11.428	11.280	11.089	11.676	11.662	11.258
9.0	11.766	11.709	10.690	11.666	11.951	10.755

c) % loss of ammonia by volatilization with time

pH	Light After day			Light/Dark After day		
	0	4	8	0	4	8
7.6	0	2.66	5.51	0	4.24	5.00
8.3	0	1.41	5.01	0	2.87	5.07
9.0	0	8.49	8.53	0	3.85	8.88

Chlorella vulgaris

a) pH

pH	Light Day			Light/Dark Day		
	0	4	8	0	4	8
7.6	7.53	7.75	7.70	7.75	7.75	7.70
8.3	8.23	8.42	8.40	8.28	8.40	8.33
9.0	8.80	9.10	9.10	8.85	8.87	9.02

b) Ammonia concentration

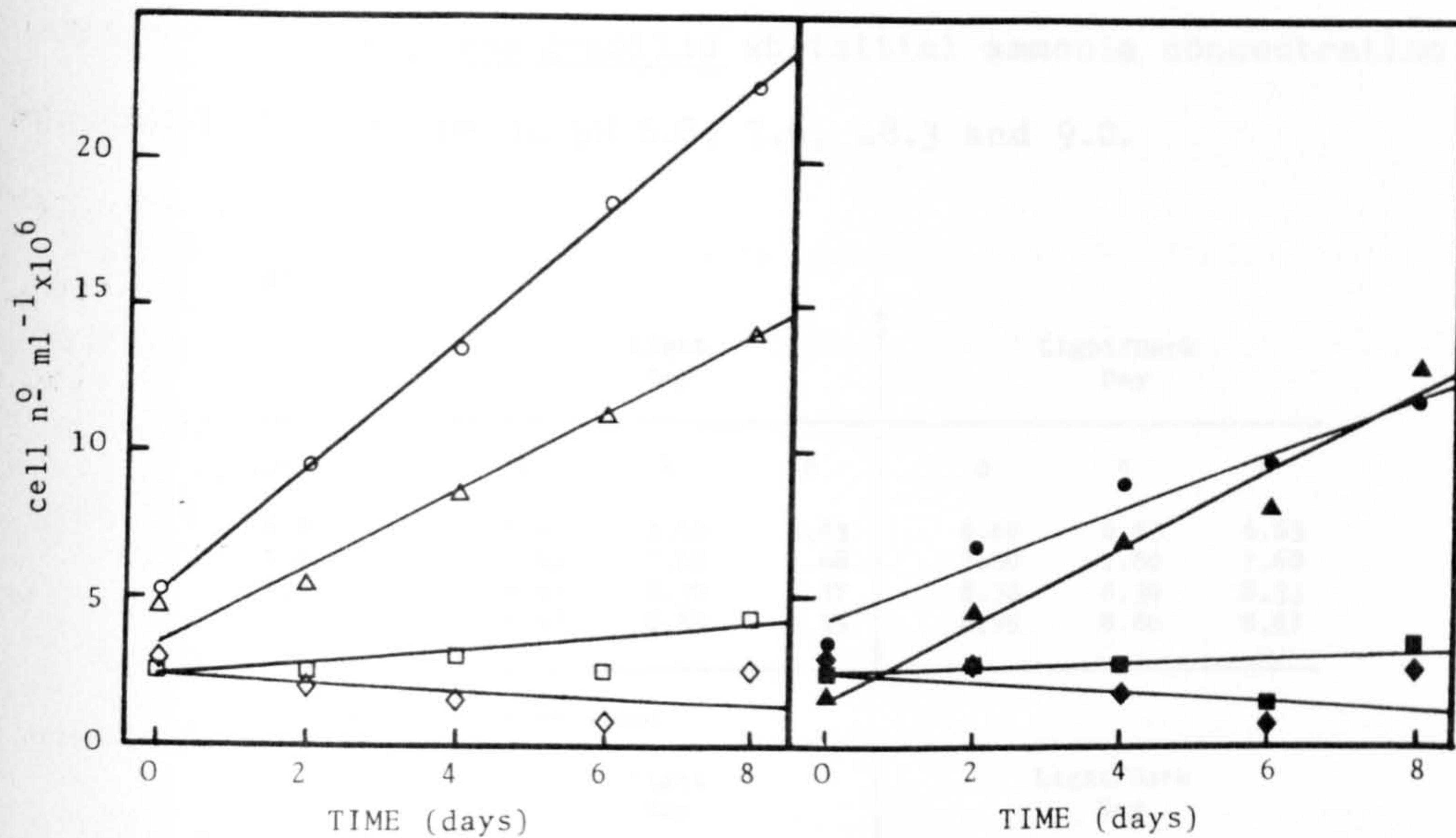
pH	Light Day			Light/Dark Day		
	0	4	8	0	4	8
7.6	11.670	11.121	10.256	11.666	10.671	10.250
8.3	11.666	11.393	10.197	11.863	11.648	10.908
9.0	11.766	10.367	9.090	11.701	11.102	9.817

c) % loss of ammonia by volatilisation with time

pH	Light After Day			Light/Dark After Day		
	0	4	8	0	4	8
7.6	0	2.02	5.00	0	3.78	5.24
8.3	0	1.44	5.07	0	2.54	5.50
9.0	0	8.01	8.02	0	4.50	8.81

Fig. 10.6 Growth of Euglena gracilis at pH 6.8 (○ ●), 7.6 (△ ▲), 8.3 (□ ■) and 9.0 (◇ ◆), during an eight day period at initial ammonia concentration of 10 mM. Open symbols indicate growth under continuous light whereas close symbols, under 12h light/12h dark. All values are means for triplicate determination and standard deviation did not exceed 10% unless otherwise stated. All lines drawn are regression curves. See statistical details in Table 10.8.

Table 10.8 Regression data for the cell number $\text{ml}^{-1} \times 10^6$ versus time curves shown in Fig. 10.6; n is the number of samples, r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line where y = cell number $\text{ml}^{-1} \times 10^6$ and x = time (days).



pH	N source	LIGHT				LIGHT/DARK			
		n	r	Confidence limit %	Regression Equation	n	r	Confidence limit %	Regression Equation
6.8	NH ₄ ⁺	5	0.996	99.9	y = 5.16+1.89x	5	0.979	99-99.9	y = 4.77+1.00x
7.6	NH ₄ ⁺	5	0.976	99-99.9	y = 3.61+1.22x	5	0.982	99-99.9	y = 1.59+1.30x
8.3	NH ₄ ⁺	5	0.728	80-90	y = 2.39+0.16x	5	0.161	< 80	y = 2.49-0.04x
9.0	NH ₄ ⁺	5	-0.456	<80	y = 2.43-0.12x	5	-0.530	< 80	y = 2.79-0.15x

Table 10.9 Change in pH, ammonia concentration and loss of ammonia by volatilization during the growth of Euglena gracilis at initial ammonia concentration of 10 mM at pH 6.8, 7.6, 8.3 and 9.0.

a) pH

pH	Light Day			Light/Dark Day		
	0	4	8	0	4	8
6.8	6.60	6.50	6.43	6.60	6.53	6.63
7.6	7.60	7.60	7.68	7.60	7.60	7.60
8.3	8.33	8.30	8.37	8.30	8.30	8.33
9.0	8.97	8.83	8.85	8.95	8.80	8.87

b) Ammonia concentration

pH	Light Day			Light/Dark Day		
	0	4	8	0	4	8
6.8	10.045	9.016	8.967	10.150	9.549	9.002
7.6	11.676	11.509	11.016	11.676	11.582	11.396
8.3	11.428	11.186	11.016	11.666	11.460	11.202
9.0	11.701	11.232	11.070	11.700	11.038	10.795

c) % loss of ammonia by volatilization with time

pH	Light After day			Light/Dark After day		
	0	4	8	0	4	8
6.8	0	1.83	3.90	0	3.34	3.72
7.6	0	1.38	4.50	0	3.32	5.48
8.3	0	1.44	5.10	0	2.22	5.93
9.0	0	7.50	7.52	0	5.15	8.75

10.4.2 Bacteria

The growth of E. coli strains isolated from raw sewage and various ponds of Systems I and II were investigated in Mineral Medium supplemented with glucose in various combinations of

ammonia and pH. Data for the bacteria originally isolated from raw sewage and ponds F1 and M3 of System I are presented in Figure 10.7 and for the bacteria isolated from F2, F4, F5 of System II in Figure 10.8.

At pH's 7.0 and 8.0 none of the ammonia concentrations had a toxic effect on bacterial growth except for the E. coli strain isolated from raw sewage. This strain did not show any visible growth during the 10h period.

Although the E. coli strain isolated from F1 grew at all combinations of pH and ammonia concentration, the data suggest a slight inhibition at pH 9.0 when the ammonia concentration was initially 40 mM.

While comparable results to those observed for the F1 isolate were obtained for the M3 isolate at pH's 7.0 and 8.0, this strain was more sensitive to 40 mM ammonia at pH 9.0.

The results for the isolates from the independently loaded facultative ponds (F2, F4, F5) of System II are shown in Figure 10.8. These isolates grew equally well at ammonia concentrations ranging from 5 to 40 mM at pH's 7.0 and 8.0. However, growth was reduced at all ammonia concentrations at pH 9.0.

The isolate of F5 was the most affected especially at high ammonia concentrations.

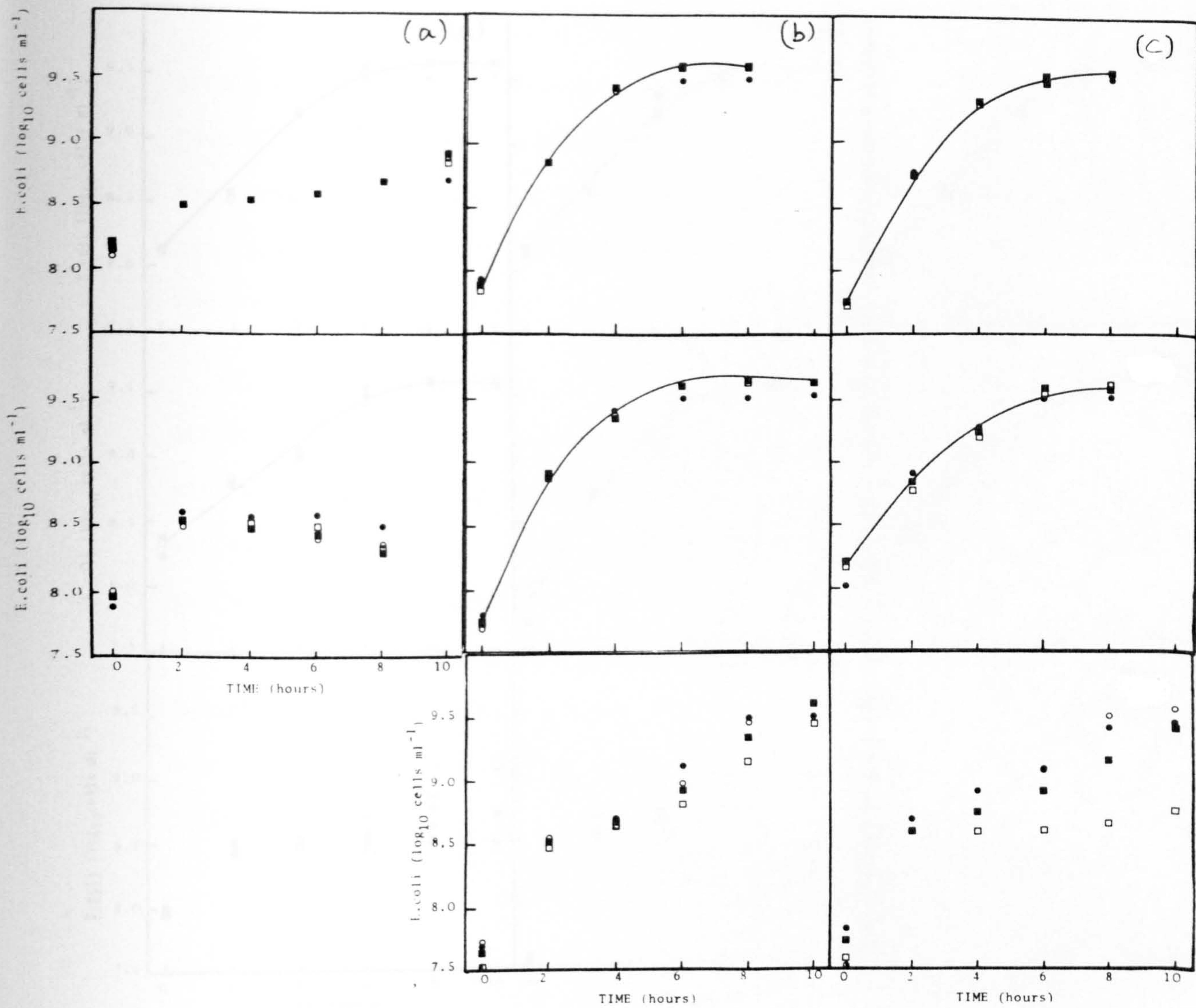


Fig. 10.7 Growth in Mineral Medium of *E. coli* isolates from (a) Raw Sewage, (b) F1 and (c) M3 at different pH's at initial ammonia concentrations of 40 (\square), 20 (\blacksquare), 10 (\circ) and 5 (\bullet) mM.

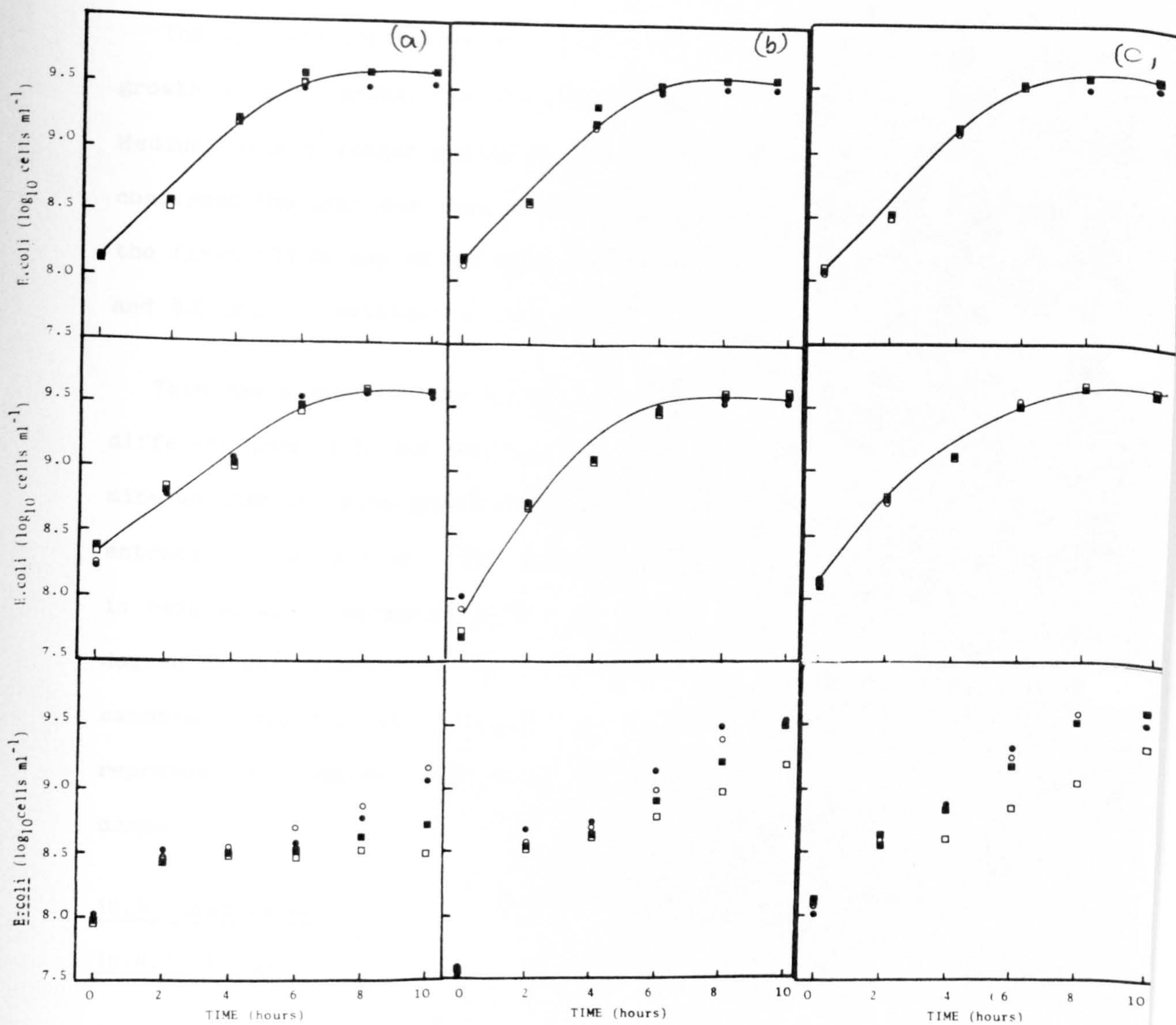


Fig. 10.8 Growth in Mineral Medium of *E. coli* isolates from (a) F5, (b) F2 and (c) F4 at different pH's at initial ammonia concentrations of 40 (□), 20 (■), 10 (○) and 5 (●) mM.

The apparent inhibitory effect of 5 mM of ammonia on the growth of a raw sewage isolate was re-investigated (in Mineral Medium) over a longer period of time (Fig. 10.9). These data confirmed the previous result that growth did not occur during the first 10h at any pH and subsequent growth was poor at pH 7.0 and 8.0 and non existent at pH 9.0 even after 24h incubation.

This raw sewage isolate was grown in nutrient broth (Oxoid) at different pH's (7.0, 8.0 and 9.0) without added ammonia to determine whether its slow growth was the result of the ammonia concentration or simply pH. The data (Fig. 10.10) show that growth is reduced with increasing pH but not inhibited to the same degree as was observed in the Mineral Medium in the presence of ammonia. Thus the results from Figs. 10.7 and 10.9 would seem to represent the combined effects of pH, ammonia and possibly media composition.

10.4 Discussion

10.4.1 Algae

The inhibitory effects of ammonia on algal growth and photosynthesis have been extensively reported (Avron and Shavit, 1965; Crofts, 1966; Natarajan, 1970; Abeliovich and Azov, 1976; Thomas et al., 1980) though little is known about how actual transport into the cells occurs (Raven, 1980). However, when absorbed at a slow rate, high concentrations of ammonia can be utilized and tolerated by most living organisms because of their highly

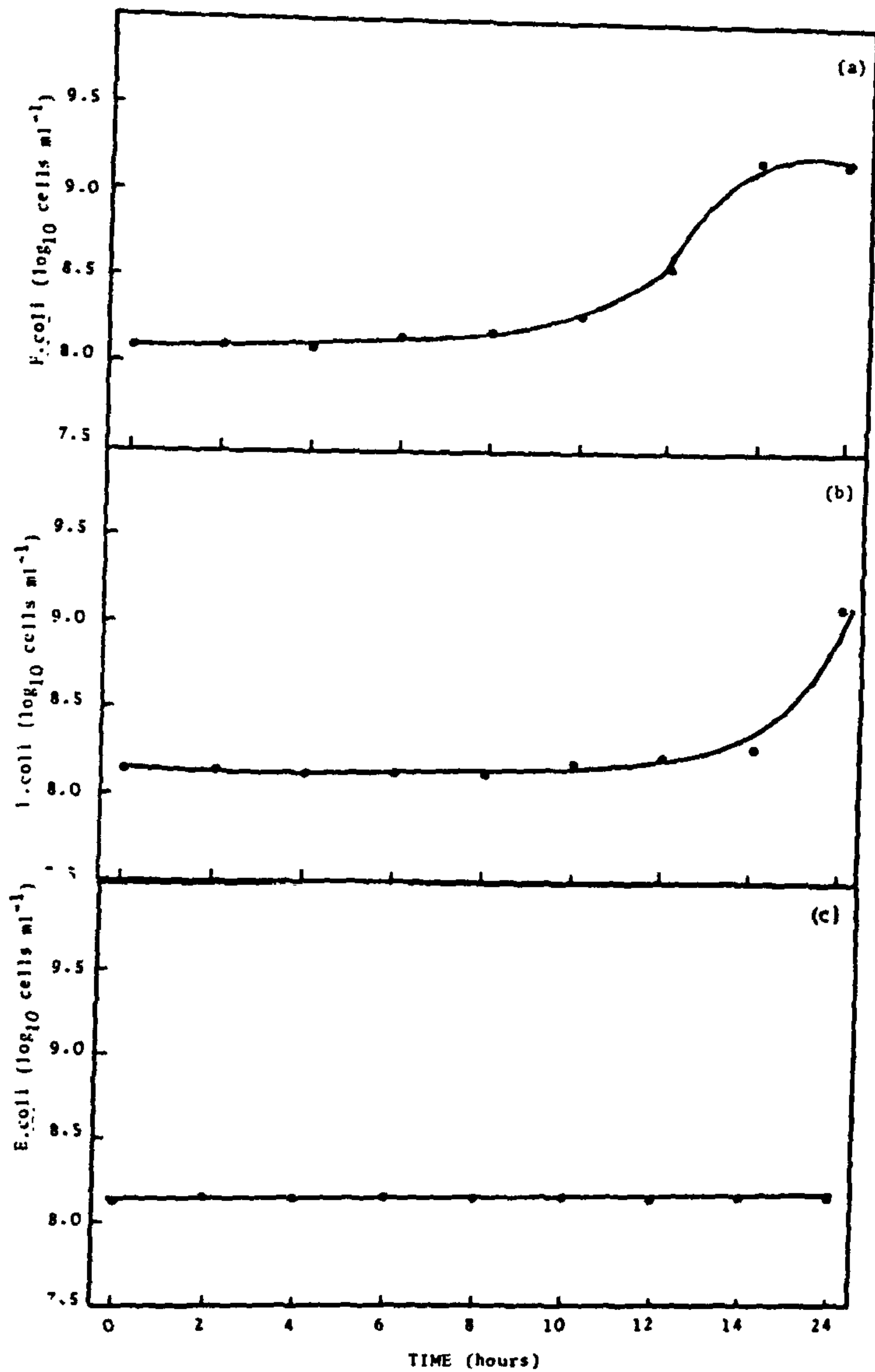
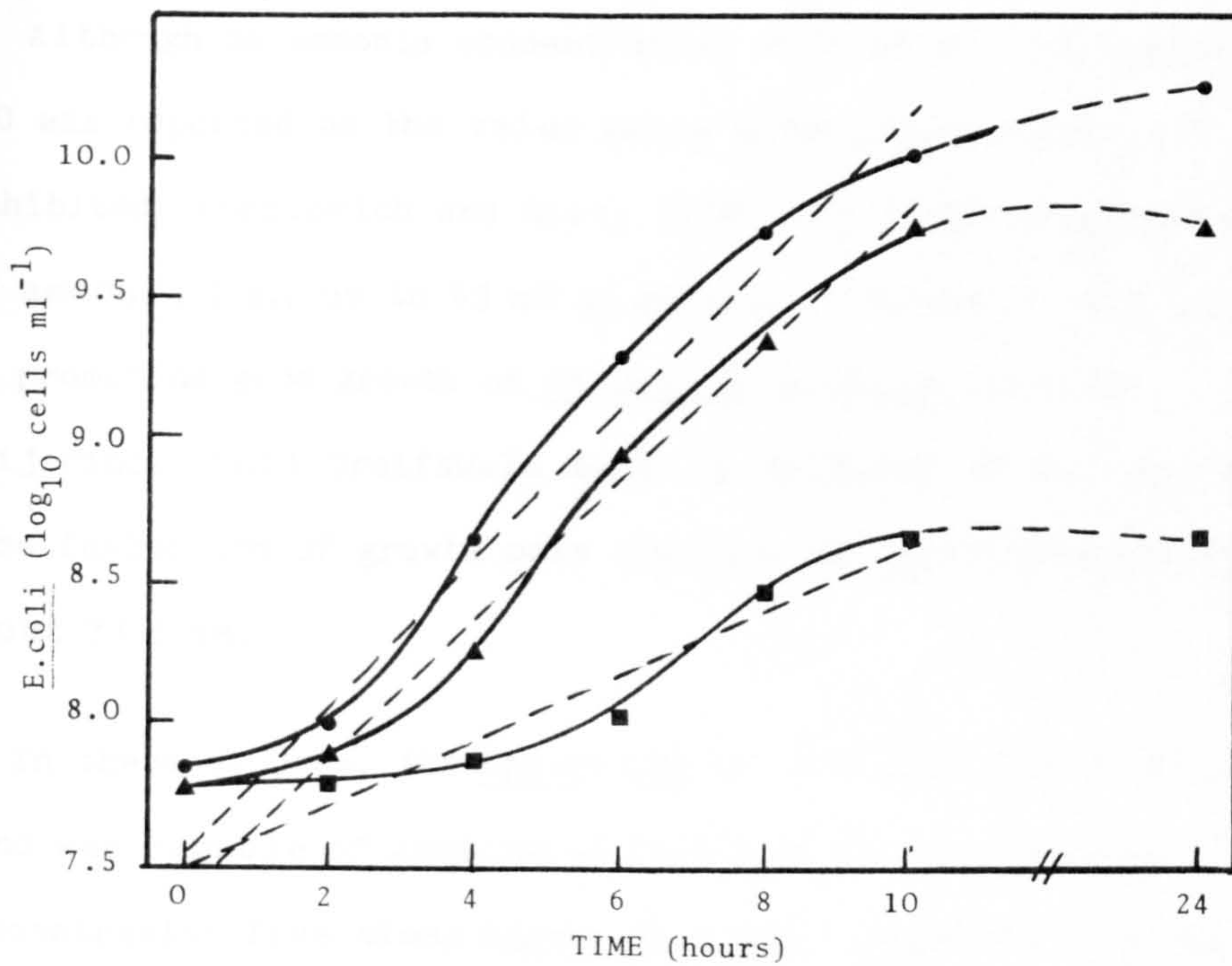


Fig. 10.9 Growth in Mineral Medium of *E. coli* isolated from Raw Sewage at (a) pH 7.0 (b) pH 8.0 and (c) pH 9.0 at initial ammonia concentration of 5 mM. Dotted line represents growth between 14h and 24h after inoculation.

Fig. 10.10 Growth of a Raw Sewage isolate of E. coli on Nutrient Broth at pH 7.0 (●), 8.0 (▲) and 9.0 (■). Dotted lines represents growth between 10h and 24h after inoculation.

Table 10.10 Regression data for the \log_{10} cell no. ml^{-1} versus time curves shown in Fig. 10.10. n is the number of samples, r is the correlation coefficient and $y = a + bx$ is the equation describing the regression line where $y = \log_{10}$ cell no. ml^{-1} and $x = \text{time (hours)}$.



pH	n	r	confidence limit (%)	regression equation
7.0	5	0.986	99-99.9	$y = 7.58 + 0.26x$
8.0	5	0.995	99.9	$y = 7.38 + 0.24x$
9.0	5	0.960	99-99.9	$y = 7.48 + 0.11x$

efficient detoxifying mechanisms through which urea, glutamine, and asparagine are formed (Warren, 1962).

Although an ammonia concentration of 2 mM at a pH higher than 8.0 was reported as the value above which algal growth was inhibited (Abeliovich and Azov, 1976) much higher concentrations of ammonia i.e., up to 43 mM at pH's up to 8.0 have been reported as promoting good growth of Chlorella vulgaris (strain Beijerinck, Coll Greifswald A-23) by Matusiak (1976). In this case inhibition of growth only occurred at concentrations of about 71.5 mM.

In these studies, the Chlorella sp. isolated from a maturation pond was capable of growing at pH 8.0 in 10 mM of ammonia i.e. a concentration five times higher than that suggested by Abeliovich and Azov as inhibitory to algal growth. Furthermore, Chlorella vulgaris (f. tertia F. et N strain 211/8K Sorokin et Myers T x 7.11.0) the laboratory strain was not sensitive to 10 mM even at pH 9.0. In contrast to the Chlorella species, Euglena gracilis was more sensitive to ammonia particularly at pH's above 7.0.

These findings support the suggestion made by Matusiak et al. (1977) that Chlorella species could be successfully used in W.S.P. to treat wastes with high concentrations of ammonia without the system collapsing due to ammonia toxicity.

Although at low pH (6.8 and 7.0) Chlorella vulgaris and

Chlorella sp. showed no preference between ammonia and nitrate at high pH i.e. 9.0, the Chlorella species isolated from W.S.P. showed some preference for nitrate.

The high concentrations of ammonia used in these laboratory studies and those reported by Matusiak are unlikely to occur in W.S.P. receiving domestic sewage. However, BOD₅ loading rates above those mentioned in Chapter 3 for F4 could bring ammonia concentrations up to levels considered toxic by Abeliovich and Azov (1976). It would seem therefore that tolerance to ammonia varies with algal species and strains and may also depend on the physiological state of the algae and the conditions under which they have previously been growing.

Given these findings and the apparent differences in ammonia toxicity levels reported it is likely that unless other factors are interacting, the algal population developing in a newly established pond will be appropriate for the prevailing ammonia concentration.

However, in existing waste stabilization ponds where it is intended to increase the load, and when such action may lead to a significant increase in ammonia concentrations, then "in situ" tests to evaluate the sensitivity of the algal population to ammonia should be made. Furthermore such tests should be done at a time when pH is predicted to be at its highest and before any

loading increase is started. Even if the existing algal population is likely to be sensitive to the predicted "new" ammonia concentration, if the increase is gradual it is probable that the algal population may alter its species balance to accommodate such changes without destabilisation of the system. It is clear that a series of ponds *is* less likely to be affected during such changes than a system comprising a single facultative pond.

Given the importance of flagellate algal species in facultative ponds and the apparent sensitivity of Euglena to ammonia, tests for ammonia toxicity to other flagellate algal species e.g. Pyrobotrys, Chlamydomonas and Phacus would provide valuable additional information as would toxicity studies on algae under heterotrophic growth conditions.

10.4.2 Bacteria

The growth data obtained for the different E. coli isolates show that not all F. coliforms respond similarly to the various combinations of pH and ammonia concentrations. This series emphasises the dangers of using information from work on a single isolate to predict the behaviour (and particularly the rate of "die-off") of the F. coliform population as a whole.

The increased sensitivity of the raw sewage isolate when compared with E. coli strains isolated from the various W.S.P. also cautions against the not infrequent use of raw sewage

isolates in the studies designed to establish the factors determining F. coliform "die-off" in facultative and maturation ponds (Parhad and Rao, 1974).

The variations in sensitivity to pH and ammonia between different isolates also suggest that the strains or species balance within the F. coliform population alters depending on the conditions prevailing in the various ponds. Although only single isolates from the ponds were available nevertheless the method of isolation almost certainly ensured that the particular isolate was the predominant one. If this variation in the balance of strains within the population is true then regrowth of certain E. coli strains within the pond system must occur.

Comparing growth of the raw sewage isolate at different pH's in Mineral Medium plus ammonia with its growth in nutrient broth shows that pH alone is a factor determining the survival of at least part of the F. coliform population, a factor also shown by Parhad and Rao (1974) working on E. coli isolated from waste water. The poor growth in Mineral Medium compared to nutrient broth particularly at the higher pH's used also emphasises the interaction between nutritional status and parameters such as pH in determining growth and thus presumably "die-off".

In systems where pH fluctuates diurnally then the length of the exposure time to high pH may affect recovery and thus the maintenance of the F coliform population. This has been investi-

gated here but requires more attention. It may also be that combinations of high pH, low nutrient status plus the presence of algal products and/or light may have a synergistic effect and enhance the rate of "die-off" of E. coli strains.

However, on the basis of these data it must be emphasised that several E. coli strains do survive and grow at pH's as high as 9.0 and the pH in the EXTRABES ponds rarely exceed this value for any prolonged period.

Finally, it would seem that F. coliform are unlikely to be as sensitive as the algae population to high concentrations of ammonia. However, this would not be particularly significant in terms of BOD₅ removal since F. coliform are not considered particularly important in this context (Mara, 1976) and one cannot extrapolate these results to the heterotrophic bacteria populations of the ponds as a whole.

CHAPTER 11

GENERAL DISCUSSION

The use of W.S.P. is becoming an increasingly popular method of sewage treatment, both in warm temperate climates (e.g. Mediterranean France) and tropical climates. There is therefore an urgent need to obtain more data on the performance of these ponds to further improve and refine the engineering design.

Measurements of pond performance and function have always been centered on BOD₅ reduction and pathogen removal and little consideration has been given by engineers to the relationship between the algae, bacteria and other microbial populations present in the pond and how their activities might affect pond design and operation (Parker, 1962).

A more complete understanding of the complex mechanisms occurring in W.S.P. and the way these affect the design of such systems requires information on biological and associated physico-chemical data to be collected in a rational and standardised way.

Very few systems are being monitored in a systematic way to obtain biological data and in those that are, the methods and timing of sampling are poorly described (Shillingdaw & Piertese, 1977). A literature survey has also shown that in most cases,

critical information on factors such as depth of effluent take-off and time of sampling are not mentioned. The data presented in this thesis (Chapters 5 and 6) have shown how important time and depth of effluent take-off can be in the evaluation of the quality of pond effluent.

Measurements of biological parameters, in particular the numbers and types of algal species present (as well as physico-chemical parameters) require a detailed programme of sampling to be carried out. To obtain an overall estimation of pond performance one has to sample the pond water body, and not just the effluent, since effluent sampling gives only the end product of pond function. The pond water body can be monitored by taking samples of the water column, e.g. by using a perspex column (see Chapter 4), and the effluent, by either taking a grab sample or composite sample. Composite effluent samples (e.g. daily, or weekly) are not appropriate for biological analyses and labile compounds such as ammonia and sulphide. Storage of such samples over a period of time may lead to changes in concentration of ammonia and sulphide as a result of absorption by living organisms and volatilization. The size of some populations e.g. of the algae may alter during storage, they may die and decompose, or if conditions are favourable they may actually increase in numbers.

Due to algal stratification in the water column, as a result

of changes in light intensity (Chapter 5), the algal biomass of the effluent will vary depending on the time of day at which it is sampled. Therefore any information on effluent sampling and analysis would require also that the precise time of the sampling be stated. In contrast, the use of the perspex column to sample the water body will help to overcome this difficulty, since the algae will be present in the water column no matter at what depth they occur below the surface of the pond. Therefore sampling the water body using a column can take place at any time of the day at least during daylight hours.

Probably
certain algae *genus*: such as Euglena actually enter the sediments at night and this may make representative sampling difficult. This tube type of sampling would also allow the determination of the mean pond algal population, by multiplying the chlorophyll a concentration of the water column by the total volume of the pond.

In small ponds, like those at EXTRABES, sampling the water column presents few difficulties as the whole surface of the ponds is accessible. In much larger ponds (e.g. 2-3 ha) a systematic sampling procedure (using a limnological approach), covering the whole area of the ponds, must be undertaken to obtain a representative sample.

The determination of the algal species present and their relative numbers can be used as an indication of pond type and

will relate, at least approximately, to the BOD₅ surface loading. In general flagellate genera (e.g. Euglena, Chlamydomonas, Phacus and Pyrobotrys) predominate in facultative ponds. The non-motile green algae (Scenedesmus and Chlorella) and diatoms are more dominant in maturation ponds. The numbers of species also increase with purification. The presence of one or two *genera* in a pond is indicative of a highly loaded facultative pond (e.g. 350 BOD₅ ha⁻¹d⁻¹) whereas as many as 15 species may be present in the final maturation pond of a series (e.g. 35 kg BOD₅ ha⁻¹d⁻¹). Changes in algal speciation i.e. from high numbers of non-motile species towards fewer numbers of flagellates would signal an increase in loading and a change in effluent quality. Therefore identification of algal species, or at least algal genera, in conjunction with the algal biomass, is indicative of the status and performance of a pond.

Measurements of these two parameters will therefore allow a rapid, relatively simple and cheap assessment of pond performance unlike BOD₅ measurements (which take 5 days) or FC estimations.

Sudden increases in the BOD₅ loading of ponds will increase the concentrations of substances such as ammonia and sulphide (Chapter 3), which are known to be toxic to the algae (Gloyne & Espino, 1969; Abeliovich & Azov, 1976), and the overall pond performance could be compromised. If the increase is too rapid the algae will have no time to adapt and will be washed-out

(Abeliovich and Azov 1976), but a gradual increase in BOD₅ surface loading would allow either existing algae to adapt or allow the selection of an algal population more tolerant to the new levels of ammonia and H₂S. This would almost certainly lead to a change in speciation or at least the species balance.

Measurements of species diversity and algal biomass allow rapid recognition of the build up of adverse conditions within the pond (e.g. increasing BOD₅ surface loading). Typical symptoms of impending breakdown would also be: odour problems, scum formation and the development of a purple colouration due to the presence of sulphur bacteria. In these cases, the inflow of sewage could be stopped in time to avoid total collapse of the system.

It is known that the presence of algae increases the BOD₅ and SS of pond effluent (Cadwell, 1946; Gloyna & Herman, 1956; Fitzgerald & Rohlich, 1958; Meron et al., 1965) and this may cause eutrophication problems in the receiving water body.

Algae can be removed directly from the effluent by micro-straining or chemical flocculation but these procedures are expensive and require well trained personnel, and therefore are frequently not suitable for developing countries. A more appropriate method of algal removal would be the growth of macrophytes in maturation ponds which will shade out the algae (and thus also reduce the BOD₅ and SS) and strip nutrients,

mainly nitrogen and phosphorus, from the water.

The choice of the type of macrophyte is important to ensure that mosquito breeding is not encouraged. The harvesting of macrophytes is relatively simple (easier than algae) and the biomass produced can be used as green fertilizer, animal feed or conversion into biogas.

It is claimed that algae can be economically harvested from High Rate Algal Ponds. This type of sewage treatment would only be acceptable if linked to the harvesting and sale of the algal products, otherwise an effluent with high BOD and SS will be produced by the retained algae. These algae can be used in animal feed, for the extraction of high grade materials

(Goldman, 1979). The efficient production and harvesting of algal protein from a H.R.A.P. requires the development and maintenance of a unialgal population for prolonged periods of time. Azov et al. (1980) discussed the problems of producing unialgal populations in H.R.A.P. and has developed a matrix in which parameters such as retention time, pH, temperature and organic matter can be manipulated to predict the dominant species which will occur in a given set of conditions.

Algae from the effluent of a W.S.P. can be used for irrigation especially in arid and semi-arid regions. Once in the soil the algae improve the water holding capacity, texture and

the organic content (by adding humus and colloids). They would also probably act as slow release fertilizers, liberating nitrogen and phosphorus as they are decomposed by microbes in the soil. They would also help to stabilise the soil surface by releasing mucilage and extracellular material which helps to aggregate soil particles.

A programme of irrigation using W.S.P. effluent should include assessment of the following:

1. the microbiological features of the effluent in order for it to be safe for the crops, agricultural workers and consumers (Arthur, 1982);
2. the chemical composition of the effluent especially the Na, Ca^{2+} and Mg^{2+} ; (see Watson, 1962);
3. the soil type to be irrigated;
4. the type of crop to be irrigated (Arthur, 1982);
5. the type of irrigation method (e.g. spray, drip irrigation).

The chemical composition of the soil is needed for the calculation of the Sodium Absorption Ratio (SAR) which is a key factor in affecting soil fertility. Sodium ions tend to get adsorbed into soil particles, displacing Mg^{2+} and Ca^{2+} . Unless there is some degree of drainage through the soil, evaporation will concentrate these added ions, causing the build up of a saline soil. Soil salinity restricts the types of crops which can be grown.

Algae grown in ponds containing high concentrations of heavy metals (e.g. in those treating industrial effluents) may become tolerant of them, allowing accumulation of the metals within the cells, in the cell walls or external mucilage. The presence of high concentrations of heavy metals would disqualify the algae from further utilization as animal feed supplements and it may prevent the effluent from being used for irrigation as the metal ion could accumulate in the soil.

It is also possible that algae may release or produce allelopathic (extracellular and phenolic) compounds, which may inhibit germination and growth of higher plants.

Algae in W.S.P., especially the maturation type, can be successfully used as a food source for herbivorous fish and by doing so the productivity of the system can be increased. Carp and Tilapia are the types of fish most commonly cultivated but polyculture, where several fish species are growing in the same pond, is widely practised. In this case the fish population is composed of either herbivorous fish, which eat either different species or different sizes of algae, or a combination of herbivorous and detritus fish feeders.

The health aspects of growing fish in W.S.P. have to be considered but some simple precautions could be applied to ensure pathogen control: 1) fish should reside in clean water for several

weeks before harvesting; 2) good hygiene standards at all stages of fish handling and processing; 3) discouraging the consumption of uncooked fish.

This thesis has shown that chlorophyll a concentrations can be correlated to BOD₅ loadings (see Chapter 4) as well as O₂ production (see Chapter 7). Using the regression lines developed from these analyses it is possible to predict at which BOD₅ loadings maximum chlorophyll a concentrations can be expected. The biological results here can be used to corroborate physico-chemical analysis data.

The linear relationship between chlorophyll a concentration and the COD contributions has shown that an algal concentration equivalent to 1 mg l⁻¹ of chlorophyll a will contribute some 300 mg l⁻¹ of COD. Once the COD:BOD ratio for the algae has been verified and given that algae represent 70% of the BOD of an unfiltered effluent, then chlorophyll a concentrations from an effluent can be used to give close approximations to the effluent BOD and COD. It would not be an accurate measurement but it would certainly indicate trends of BOD and COD in the effluent.

Although direct measurements of biomass by means of dry weight are not recommended (due to the high concentration of SS other than algae) biomass can be determined indirectly by chlorophyll a and cell counts. If algal counts are going to be used as a measure of biomass, they must be converted into volume

estimates for each algal genus present.

Experimentation in Chapter 10 indicated that the characteristics of the FC population are not constant. FC from differently loaded ponds have different tolerances to pH and ammonia, presumably as a result of selection in different pond environments. These findings have considerable implications for the "die-off" studies which are usually performed on bacteria isolated from raw sewage. Die-off studies should therefore only be performed on bacteria isolated from the particular pond under investigation.

Measurements of algal biomass and species diversity can provide a more rapid estimation of pond performance than the traditional measurements of pond effluent. Effluent quality evaluation can be inaccurate or misleading because it depends greatly on the time of day the sample was collected. An improvement in the effluent quality could be made (from an engineers point of view) by incorporating a system whereby the effluent could be taken off at variable depths. This could ensure minimum algal numbers. The production of algae-free effluents would make use of the stratification that occurs in the pond during the day by drawing the effluent from algae-free zones. An algal free effluent would have a lower BOD, COD, SS but the number of FC would not necessarily be reduced (see Chapter 5). Consequently the algal free effluent should serve only as the input to a

series of maturation ponds, where the die-off of FC usually occurs.

This thesis has shown that many aspects of pond biology have to be further investigated if biological parameters are going to help to improve engineering design.

One of these aspects is the complex interaction occurring between algae and bacteria. The most intensively studied interactions have been those associated with FC die-off, but there are other algal/bacterial interactions which are biologically important for the pond performance, i.e. the interactions between the non-pathogenic heterotrophic bacteria and algae. This type of study has seldom been carried out in full-scale ponds and laboratory studies always impose some artificial constraints upon the organism.

A full scale monitoring programme of a functioning pond would be necessary to provide more complete information on the interactions within the pond, and the interactions between the pond and the environment.

SUMMARY

1) Field research on Waste Stabilization Ponds (W.S.P.) was carried out at EXTRABES (Station for the Biological Treatment of Sanitary Sewage) in the city of Campina Grande ($7^{\circ} 13' 11''$ S, $35^{\circ} 52' 31''$ W) Paraiba State, NE Brazil.

2) Two W.S.P. Systems were studied, both receiving raw municipal sewage: System I, a five-pond series comprising an anaerobic pond (A_1) receiving raw sewage, followed by a facultative pond (F_1) and three maturation ponds (M_1 , M_2 and M_3). System II comprised four-independently loaded facultative ponds (F_2 , F_3 , F_4 and F_5), each receiving raw sewage.

3) The frequency data of the different algal genera present in ponds of System I and System II was determined using presence and absence data recorded from 58 weekly sampling (i.e. during Experiment 1). In System I and II ponds, Euglena, Phacus, Pyrobotrys, Chlamydomonas, Eudorina, Pandorina and Chlorogonium were always present.

In System I (BOD_5 surface loadings ranging from $116 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$ in the facultative pond F_1 to $35 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$ in the maturation pond M_3) there was a sequential decrease in frequency of flagellates genera i.e. other than Euglena and Chlorogonium. This decrease was accompanied by a concomitant increase in the number and frequency of the non-motile genera i.e. Micractinium, Ankistrodesmus and Cyclotella.

In System II there was an inverse relationship between the number of genera present and the organic loading of the ponds. In System II (BOD_5 surface loadings varying between 150 to $300 \text{ kg } BOD_5 \text{ ha}^{-1} \text{ d}^{-1}$), Euglena was the most frequent alga. The genus Phacus had high frequency in all the ponds except in F_4 (with the highest loading). In contrast, Pyrobotrys and Chlamydomonas were also frequent in all the ponds, except in F_5 (with the lowest loading). Colonial flagellate Eudorina and Pandorina were more frequent in F_5 .

Facultative ponds with similar BOD₅ surface loadings but from different Systems, eg. F₁ from System I (receiving settled sewage from an anaerobic pond) and F₅ from System II (receiving raw sewage) contained the same algal genera except that F₅ contained additionally Ankistrodesmus and occasionally Dictyosphaerium, Volvox and Micractinium.

Diversity data from ponds of System I and II (based on the presence and absence of algae genera) during Experiments 1, 2 and 3 showed that Euglena, Chlamydomonas and Pyrobotrys were present up to BOD₅ surface loadings of 577 kg BOD₅ ha⁻¹d⁻¹.

4) Quantitative determinations of algal populations by direct count should be converted into algal volume due to the wide range in cell and volume size between different genera. In doing so, the relative abundance of the various algal genera comprising an algal population can be compared.

In the maturation pond M₁, Chlorella was the dominant alga followed by Euglena, when cell number was considered. In contrast, by converting cell number into cell volume, Euglena becomes the major contributor to the biomass, followed by Chlorella.

5) All ponds of System I and II showed fluctuations in chlorophyll a concentrations with time in both water column and effluent samples. In System II the rapidity and amplitude of these fluctuations were greater in samples of the effluent than in the water column samples. In System I, these fluctuations in chlorophyll a concentrations were more frequent in the facultative pond F₁ than in the following three maturation ponds.

The increase in BOD₅ surface loading on the ponds of both Systems led to a reduction in algal density. The regression analyses between monthly means for chlorophyll a concentrations of the pond water column samples and the BOD₅ surface loading showed that algal density was greater at

BOD₅ surface loading of between 170 and 230 kg BOD₅ ha⁻¹d⁻¹ and there was a fall in algal biomass at loadings above or below this range.

For the environmental conditions in the NE of Brazil, ponds with BOD₅ surface loadings approaching 500 kg BOD₅ ha⁻¹d⁻¹ supported little growth and cease to function as a W.S.P.

6) Diurnal studies on the effluent quality of F₄ and M₃ showed that considerable variation on certain effluent parameters occurred and the amplitude of these variations depended on the type of pond. The chlorophyll a concentrations in the effluent were higher in F₄ than in M₃ with maximum values occurring during day time. In the effluent of the facultative pond F₄, high algae concentrations correlated positively with BOD₅, SS and Total Phosphorus. However, the high algae concentrations frequently coincides with high levels of D.O. which may minimize the immediate oxygen demand of the receiving water bodies.

During day time, there was a decrease in Faecal and Total coliforms with the increase of D.O. and pH. Although considerable fluctuations occurred on certain effluent parameters, the mean 24 h value did not greatly deviate from the 08.00 h value, which is the routine sampling hour in EXTRABES.

7) Algal stratification in the water column was distinctly more evident in the facultative pond F₅ than in the maturation pond M₃. In F₅, algal stratification occurred between 10.30 h and 15.30 h with maximum chlorophyll a concentrations occurring at depth of 20 - 25 cm. Dissolved oxygen was only measured at depth of 30 - 35 cm below the surface. The Faecal coliforms also stratified and were reduced in number in the oxygen and algae rich upper layers. Maximum sulphide concentrations (between 5 and 6 mg l⁻¹) was recorded at dawn (04.45 h) and was reduced in the upper layers as the oxygen concentrations increased.

8) The gross and net primary productivity, in the different ponds, fluctuated from month to month and these fluctuations were more pronounced in the facultative ponds than in the maturation ponds. The depth of the maximum production did not always coincide with the maximum chlorophyll a concentrations. In the maturation pond, net and gross primary production was measurable at depth up to 60 cm whereas in facultative ponds it did not exceed depth of 24 cm below the surface. The zone of maximum primary production (for 10 - 11 h) for both maturation and facultative ponds is between 10 - 20 cm below the surface.

9) Chlorella sp., an alga isolated from the effluent of the maturation pond M₃, was able to grow in continuous dark in the presence of glucose.

10) Faecal coliforms strains isolated from raw sewage, from the effluent of F₁ and M₃ showed different growth curves according to the medium in which they were growing. In Nutrient Broth, growth rates were similar whereas in Mineral Medium, the raw sewage grew more slowly than the other two isolates and had a log lag phase.

11) In Nutrient Broth, the growth of a raw sewage Faecal coliform increased with the increase of oxygen partial pressures. Growth at 0% oxygen was low but as soon as oxygen (20%) was reintroduced in the medium, growth resumes (even after pre-incubation in anaerobic conditions of at least up to 5 h).

12) Antibiotic Sensitivity Tests (using Oxoid Multodisc) on Faecal coliforms isolates from the various W.S.P. showed that all the Escherichia coli were sensitive to 25 $\mu\text{g ml}^{-1}$ of Streptomycin except for the isolate from the anaerobic pond A₁. Tetracycline (50 $\mu\text{g ml}^{-1}$) was not effective against the isolates of F₂, F₅ and M₃ and isolates from F₁, M₂ and M₃ were not affected by 50 $\mu\text{g ml}^{-1}$ of Chloramphenicol.

13) At pH values of 9.0 and in the presence of Chlorella sp., Escherichia coli numbers were reduced but the bacterial population did not disappear completely.

14) Growth of Escherichia coli isolated from F₁ and M₃ (System I) and F₂, F₄ and F₅ (System II) was not affected by initial ammonia concentrations of 5, 10, 20 and 40 mM and pH 7.0 and 8.0. At these ammonia concentrations and pH values only the raw sewage isolate did not show any visible growth. In medium without ammonia, growth of the raw sewage isolate decreased with increasing pH.

15) The growth of Euglena gracilis, Chlorella sp. and Chlorella vulgaris at initial ammonia concentrations of 5, 10, 20 and 40 mM and at pH 6.8, was not inhibited. Growth of Chlorella sp. and Chlorella vulgaris at pH 6.8 at equivalent nitrate concentrations as those for ammonia showed a growth pattern similar to that found for ammonia. Initial concentrations of nitrate and ammonia of 10 mM and pH values of 7.6, 8.3 or 9.0 did not inhibit the growth of either Chlorella sp. or Chlorella vulgaris. The growth of Euglena gracilis in 10 mM of ammonia was inhibited at pH's of 8.3 and 9.0.

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APPENDIX 1 - Calibration Curves

1.1 - Calibration Curves for Algae

Chlorella sp

Chlorella vulgaris

Euglena gracilis

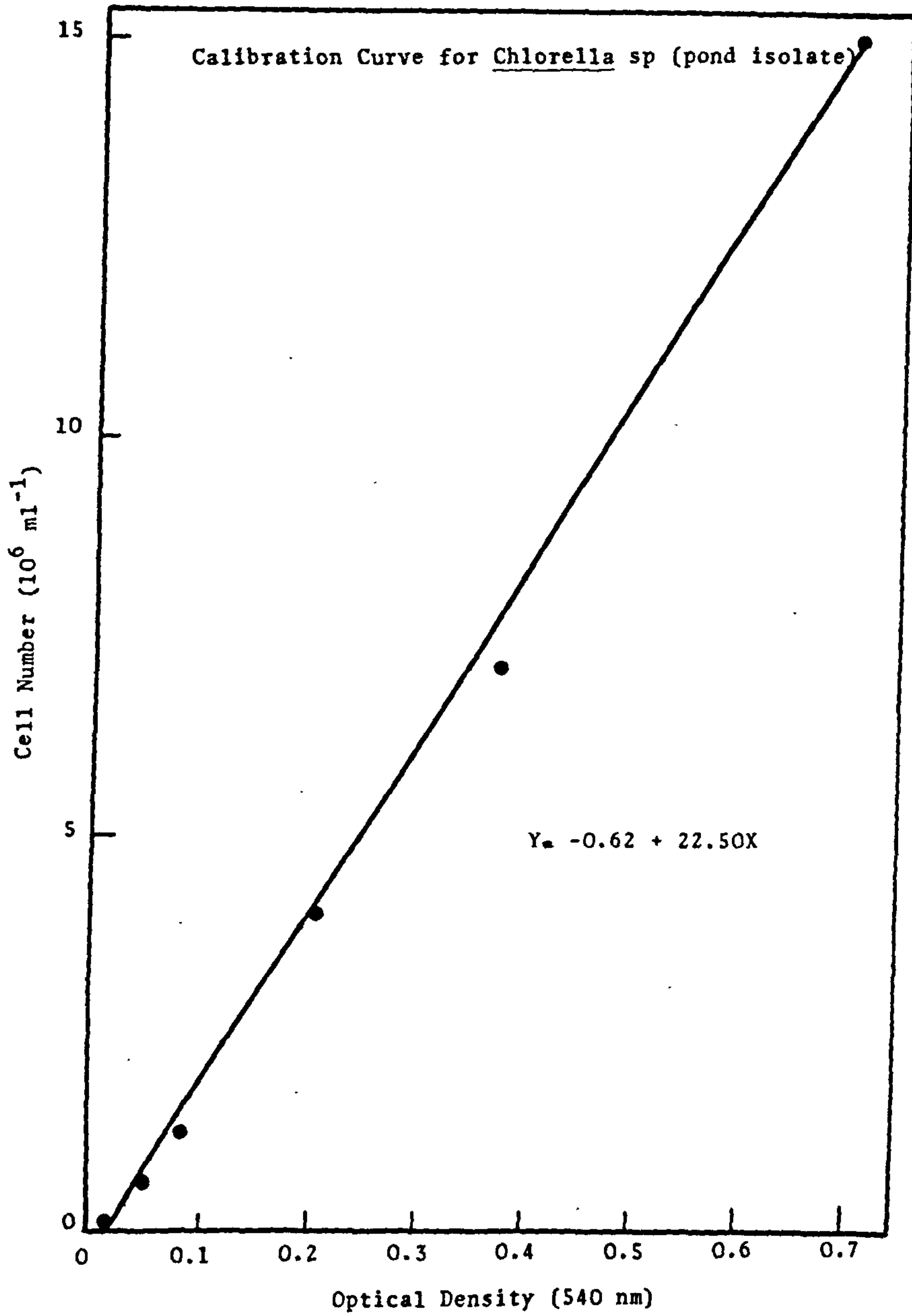
1.2 - Calibration Curves for Bacteria

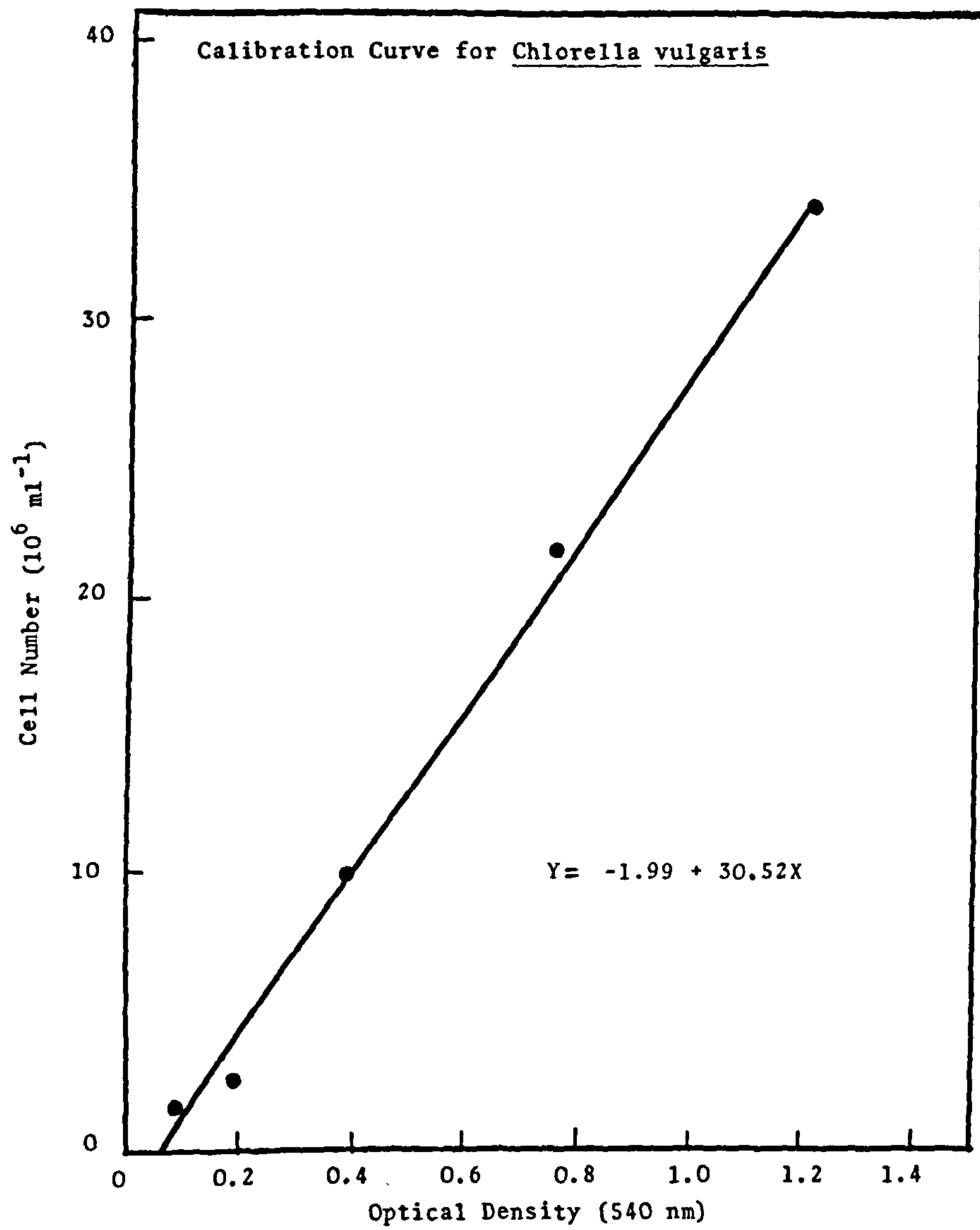
E.coli isolate from Raw Sewage in Defined Mineral Medium and Nutrient Broth (Oxoid).

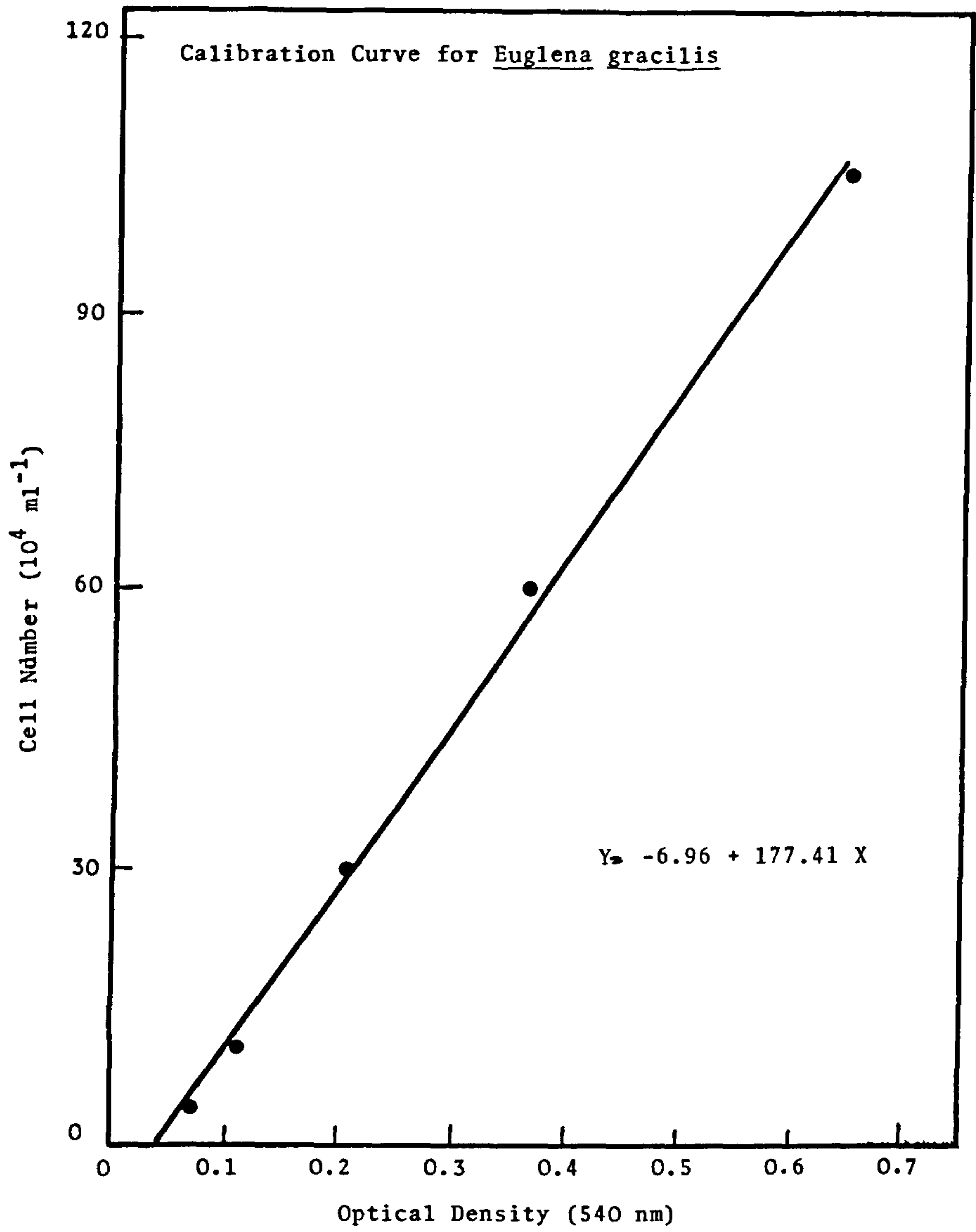
E.coli isolate from F₁ in Defined Mineral Medium and Nutrient Broth (Oxoid).

E.coli isolate from M₃ in Defined Mineral Medium and Nutrient Broth (Oxoid).

1.3 - Calibration Curve for Ammonia (Scheiner, 1976).







CALIBRATION CURVES FOR BACTERIA

