THE USE OF *ULVA LACTUCA* L. AS AN INDICATOR ORGANISM FOR MARINE POLLUTION.

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

by

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November, 1975.

To my father and mother.

Photograph showing prolific growth of Ulva lactuca in Holes Bay - taken at low tide.

CONTENTS

SUMMARY

Prolific growth of *Ulva lactuca* in some marine environments into which sewage is discharged causes many problems. An understanding of the reasons for this growth is required so that it can be controlled or prevented. The thesis is concerned with tackling this problem and also investigating whether *Ulva lactuca* can be used as an indicator organism for pollution.

Prior to studies on growth of *Ulva* in polluted conditions, its pattern of life-history and mode of growth had to be elucidated. These findings led to the development of suitable laboratory culture facilities and development of a technique for studying growth by use of excised discs of thallus tissue. Laboratory culture was based upon the use of Erdschreiber medium and growth measured by percentage increase in area after two weeks in culture.

Laboratory nutrient bioassays using growth rate measurements were used to determine the effects of likely growth rate stimulating substances found in sewage, including three nitrogen forms, (nitrate-N, nitrite-N and ammonium-N), orthophosphate, acetate, adenine and kinetin, and sewage-contaminated mud itself. Only ammonium-nitrogen within the range 0.4 to 7.8 mg dm^{-3}, and sewage-contaminated mud, were found to significantly stimulate the growth rate of *Ulva* above that of Erdschreiber control medium.

Further growth rate assessments were made with samples of field seawater collected at points throughout two sewage-polluted harbours, Poole in Dorset with an *Ulva* problem, and Langstone in Hampshire at present without a problem. The results obtained were correlated with the concentrations of nutrients in the water samples, and with results of the earlier artificially enriched seawater experiments. Ammonium-

nitrogen was confirmed to be the only compound in field water which significantly stimulated growth of Ulva over that obtained with the control medium. The regions where prolific growth of Ulva occurred in Poole Harbour were the only areas which had field water with ammonium-nitrogen levels elevated to the range shown in laboratory studies to cause significantly higher growth rates of Ulva discs. Water from Langstone Harbour although sewage-polluted did not stimulate growth of Ulva discs and water analysis revealed ammonium-nitrogen levels below those determined as growth stimulatory.

The ability of Ulva to act as a test organism for a range of toxic substances including industrial/domestic sewage sludge, detergent, copper, zinc, lead, cadmium and mercury was studied. Graded growth responses were obtained for these components, and toxic levels of them were found to be similar to those for other macro-algae.

Ammonium-nitrogen was thus found to be the factor responsible for prolific growth of Ulva in the field. In order to prevent or minimise nuisance caused by enhanced Ulva growth it is recommended that the level of ammonium-nitrogen is kept below 0.3 mg dm^{-3} in seawater at all times.

Because of specificity of response to a simgle compound it is concluded that Ulva cannot be used as a general pollution indicator species, but its prolific growth in the field certainly indicates elevated ammonium-nitrogen levels.

CHAPTER 1.

Introduction

Since the beginning of this century there has been a widespread increase in domestic and industrial pollution of the sea, especially along coasts and estuaries where population and industry is concentrated. However, not until the last decade or two has there been growing concern to find the ways in which pollution affects marine life. This in turn necessitates finding means of detecting and evaluating the effect of pollution on organisms and biological communities. As a result biological indicators, organisms for identifying and/or quantifying environmental changes, have been sought.

In order to obtain a picture of the dispersion and variation of pollutants in a particular area, physical and chemical determinations are essential. Determination of physical and chemical conditions in sea water can indicate the ways in which pollutants are dispersed in the sea, but biological indicators are needed to determine the effects of the pollutants on the actual ecosystem. Thus biological indicators are often a better means of assessing pollution effects on aquatic life than physical and chemical measurements.

As indicated by Oglesby (1967) and Burrows (1971) there are two levels at which biological indicators can be utilized for measuring pollution effects. The first is at the community level. The assessment is achieved by observing changes in biomass, species diversity and number of individuals of a particular species within the community. Such monitoring work at the community level has been carried out in the field by North (1964) with Macrocystis communities on the Pacific coast of the United States of America. Copeland (1967), using model

systems, showed that such a study may be a sensitive method for assessing the degree to which the community is affected by pollution. However, as pointed out by Burrows (1971) and Butler, Andren, Bonde, Jernelov & Reish (1972) it is difficult to show whether these community changes are brought about by pollution or are a result of natural changes in the environment.

The second approach is at the species level. Individual species can be used to detect and evaluate pollution effects. Biological indicators at the species level have been used in a number of ways in relation to pollution studies. In freshwater environments the presence or absence of an indicator or a group of indicator species is often used to assess pollution. Here the absence, or presence, of an indicator organism means that the environment under investigation is already polluted. In marine and estuarine situations, where pollutants are generally present in a lower level due to dilution by a larger volume of sea water and tidal mixing, the abundance of indicator organisms is often employed to assess environmental changes as a result of pollution rather than using the absent/present criterion as in freshwater studies. The most useful indicator organisms are those which have the following characteristics:

(a) attachment so that movement away from the area of pollution is not possible,

(b) a graded tolerance to stress e.g. salinity and pollutants,

(c) a rapid response to changes in the environment,

(d) wide geographical and ecological distribution to enable comparisons to be made between different areas,

(e) simple morphology and uniformity of growth of the organism to provide ease of assessment of changes, e.g. in growth rate, and

(f) ease of handling in the field and in laboratory culture.

Clearly it is often impossible for one indicator organism to possess all of

these characteristics.

Though benthic algae possess several of the characteristics required of pollution indicators (Burrows, 1971a; Butler et al, 1972) little effort has been made to utilize them as such. The pioneer work in Liverpool using species of Laminaria has been one of the very few reported (Burrows & Pybus, 1970, 1971; Burrows, 1971a, b; Burrows & Sharples, 1972, 1973; Hopkins & Kain, 1971). Laminaria species are generally satisfactory for pollution indication (Burrows, 1971a), but they have one or two characters which reduce their overall usefulness. Firstly, they grow by use of intercalary meristems, and thus in order to assess growth, whole plants have to be cultured. Secondly, it takes 4-5 months to culture sporophytes to a suitable size for testing.

A few benthic algae are known as species frequently found associated with certain types of pollutant discharge as well as being widely distributed in apparently unspoilt environments. Ulva lactuca is one of these. As early as the beginning of this century, it was observed that this alga grew abundantly in domestic-sewage polluted and also "naturally" (drainage from decaying seaweeds) polluted areas (Letts & Richards, 1911; Cotton, 1911). Since then numerous other similar reports have been made (Nasr & Aleem, 1948; Bruce, 1953; Williams, 1960; Wilkinson, 1964; Sawyer, 1965; Hanks, 1966; Ehrhardt, 1968; Golubic, 1970; Bellan & Bellan -Santini, 1972; Ott, 1972), including the one reported here in Holes Bay, Poole Harbour in Dorset. Its prolific growth in sewage-polluted areas gives rise to considerable nuisance in that it can block power-station cooling-water filters, foul fishermen's nets, produce smell nuisance and blacken paint by the hydrogen sulphide evolved on its decomposition.

Despite the much reported phenomenon of excessive Ulva growth associated with sewage pollution, it is only recently that culture work has begun with the aim of assessing the feasibility of using this alga to indicate or measure pollution (Burrows, 1971a). Burrows concluded that it might be used as an efficient indicator for pollution but that further detailed work was necessary.

Ulva possesses many characteristics which make it convenient to use as a pollution indicator. It is euryhaline growing equally well in marine and estuarine environments. The alga has a very simple morphology which allows easy measurement of growth rate. Excised portions from the thallus continue to grow apparently normally, and it is easy to handle and culture in the laboratory. Hence it would seem to be excellent material for experimentation, and a promising organism for assessment of pollution effects.

The aim of this work is therefore two-fold - to test the feasibility of using Ulva lactuca as a biological indicator for marine and estuarine pollution, and to explain why it grows so well in sewage-polluted areas.

Before these objectives could be tackled it was necessary to investigate the alga's pattern of life-history and mode of growth , as no unequivocal information is available on either of these topics, for it is only with such knowledge that suitable growth conditions and method of growth assessment can be developed. Following this initial work it was envisaged that the main objectives could be tackled by use of a combination of nutrient enrichment cultures under controlled laboratory conditions and growth bioassays of field water coupled with chemical analysis of the water.

CHAPTER 2.

Taxonomy, Growth Habit and Life History of *Ulva lactuca*

2.1 Taxonomy and identification.

With a genus such as *Ulva* where there may still be some problems of species identification, and especially as no studies of phenotypic variation are available, it was necessary to be convinced that material collected for use could be identified with certainty as *Ulva lactuca*. Possible recommendations as to the use of *U. lactuca* as an indicator organism also clearly require such confidence.

Since the establishment of the genus *Ulva* by Linnaeus in 1753 there has been a major modification by Thuret (1854) who removed the monostromatic species. Thus he brought together within the genus *Ulva* species comprising distromatic thallii with two cell layers adhering to each other. At that time no difficulty was encountered in identifying the entity *U. lactuca*. Only in the last twenty years has the original species been split into 2-4 species on the basis of genetic experiments (Foyn, 1955) and on more critical morphological study (Hoek, 1964; Bliding, 1968).

In trying to identify the different species of *Ulva*, many phycologists (e.g. Hoek, 1964; Bliding, 1968) have made use of the following criteria:-

(a) external morphology of the thallus,

(b) the thickness of the thallus,

(c) arrangement of the vegetative cells,

(d) cell size,

(e) number of pyrenoids in each cell,

(f) the mode of reproduction and size and nature of swarmers,

(g) interfertility among sexually reproducing species,

(h) the mode of growth and morphology of the young germlings.

These criteria can be used to distinguish U. lactuca from the other three known species of Ulva present in the British Isles (Parke & Dixon, 1968), U. olivascens P. Dang., U. rigida (C.Ag.)Thur., and U. thuretii Foyn. Table I lists all the British Ulva species and the diagnostic characters by which they can be distinguished one from the other. The asterisks in the table indicate those features of the three other species that can best be used to distinguish them from U. lactuca. Even so, despite these apparent straightforward distinguishing features there can still be some uncertainties in identifying these species (Hoek & Donze ,1966) since of all the delimiting criteria mentioned above only the mode of reproduction and the interbreeding behaviour are almost constant for each species. The other criteria can be extremely variable.

2.2 Habitat of U. lactuca.

Ulva lactuca is a cosmopolitan alga . Its geographic distribution ranges from the tropics (John, 1972) to the arctic/sub-arctic (Kjellman, 1883; Blinova, 1968; Munda, 1970) and sub-antarctic (Knox, 1960).

On the shore, it usually grows in the [1]lower eulittoral zone and the sublittoral zone down to a depth of at least fifteen metres. It attaches by its holdfast to rocks, shells or other larger algae. [2]Ulva can also be found in rock pools in the higher eulittoral zone, especially in spring and summer (Knight & Parke, 1931). When detached plants form floating populations and continue to grow, particularly in sheltered areas, they can accumulate on mud-flats or other substrates, covering extensive areas (Salim, 1965; Donze, 1969). Ulva has been reported

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[1]Terminology for zonation follows Lewis (1964).

[2]The species nomenclature Ulva lactuca is now shortened to Ulva.

Table 1. A list of diagnostic features for identification of the British species of Ulva.

	U. lactuca	U. rigida	U. olivascens	U. thuretii
Margin of thallus	Smooth	tooth-like* microscopic protuberances	Smooth	Smooth
Thallus thickness (μm)	50-100	40-200	60-115	30-80
Arrangement of cells	mostly ordered	mostly ordered	unordered	mostly ordered
Number of pyrenoids in each cell	mostly 1	mostly 2 or more	mostly 1	mostly 1
Nature of swarmers and their average size (μm)	anisogamous gametes ♀ 7.6 x 4.1 ♂ 5.3 x 2.3 zoospores 10.9 x 6.4	anisogamous gametes ♀ 6.8 x 3.8 ♂ 5.9 x 2.9 zoospores 5.9 x 2.9	neutral quadriflagellate* and/or biflagellate swarmers 5.8-9.2 x 2.3-3.4	isogamous * gametes ♀ and ♂ 7.5 x 3.5

* Features best used to distinguish from U. lactuca

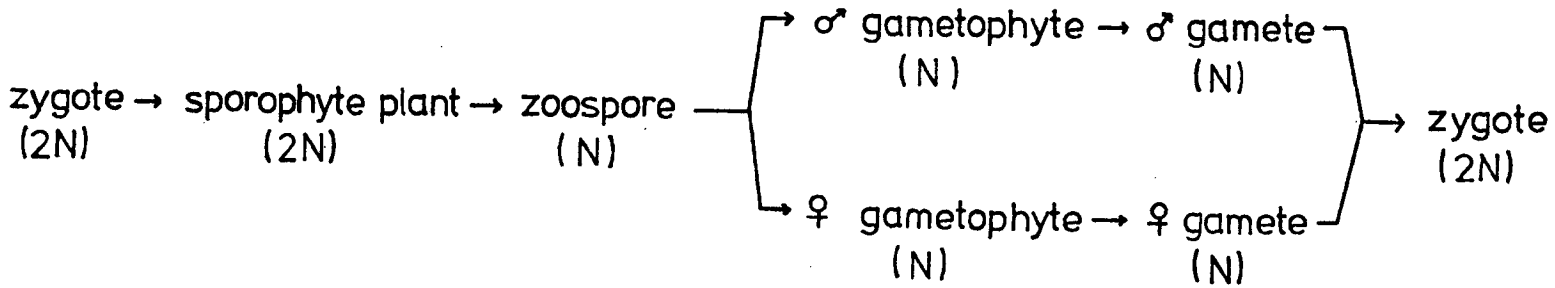
to grow "well" in areas where there is either freshwater run-off (Cotton, 1912; Knight & Parke, 1931) or "natural pollution", produced by the decay of marine animals and plants (Cotton, 1911); or where there is sewage contamination (Cotton, 1911; Letts & Richards, 1911; Salim, 1965; Sawyer, 1965).

2.3 Structure, reproduction and life-history.

Since the work of Schiller (1907) on the reproduction of *U. lactuca* there has been a lot of investigation concerned with its life-history and mode of reproduction. *Ulva* has a diplohaplontic life-history with isomorphic alternation of generations (fig. 1).

During the course of this work, observations were made on the reproduction and development of both the sporophyte and the gametophyte generations with the object of seeing whether there was any difference in the development of the two generations. This is important since if there is a difference, care must then be taken in selecting plants for experiments. Furthermore, because of the possibility that effects of pollution may be more acute at a particular developmental stage, it was essential to elucidate and confirm both inter-relationships of stages in and features of morphogenesis throughout its life-history. The structure of *Ulva lactuca* was therefore determined at stages throughout its life-history as was the temporal relationship between these stages.

All plant materials used for this investigation were obtained from one population on Hilbre Island off West Kirby in Cheshire. The cultures were illuminated by incandescent lights at an intensity of 4-4.6 klux and a day length of 15.5 hours. Temperature was kept at $12 \pm 1^{\circ}C$ and culture water continuously aerated with compressed air.



(N) = haploid

(2N) = diploid

Fig. 1. Life history of Ulva lactuca.

2.3.1 The sporophyte generation.

Following cytoplasmic fusion of the gametes the zygotes became negatively phototactic and swarmed towards the darker regions of the container. On settlement the zygote rounded-off and the flagella were absorbed or shed. The zygote was 5 µm in diameter when first measured (fig. 2, F), but after a few days had grown to 10-15 µm in diameter (fig. 2, G). Prior to the first cell division the zygote became pear-shaped (fig. 2, H).

At the first cell division the zygote elongated (fig. 2, H). One of the two cells (fig. 2, I) formed was the primary rhizoidal cell eventually giving rise to the holdfast; the other dividing to form the thallus. Subsequent transverse divisions of the thallus cell resulted in the formation of a uniseriate filament (fig. 2, J-L). The onset of division in the primary rhizoidal cell was usually delayed until five or even more of the thallus cells had been formed. This feature is said to be characteristic to the genus Ulva (Gayral, 1967). Longitudinal division of the thallus cells occurred (fig. 2, M) after the filament reached about the ten-celled stage. After 20 to 30 days, and as a result of longitudinal divisions, the filament became tubular (fig. 2, N). This condition remained until the plant reached about 0.5 cm in length (2-3 months) when the tube started to compress. The newly contiguous cell-walls apparently adhered and became bound together thus giving a distromatic thallus, but at this stage the plant still remained fairly narrow (fig. 3, A). The typical appearance developed after about six months (fig. 3 B), by which time the thallus measured some 6-18 cm in length and had a width of 2.5 to 7.5 cm. Such cultured Ulva plants seemed to have the same morphology as those collected from the field, indicating that this species could be reared normally in the laboratory.

Fig. 2. Detailed morphology of different stages in the life history of Ulva lactuca. A female gamete, B male gamete, C, D "sideways" and"head-on" fusion of gametes, E zoospores, F-N germination of zygote producing sporophyte plant.

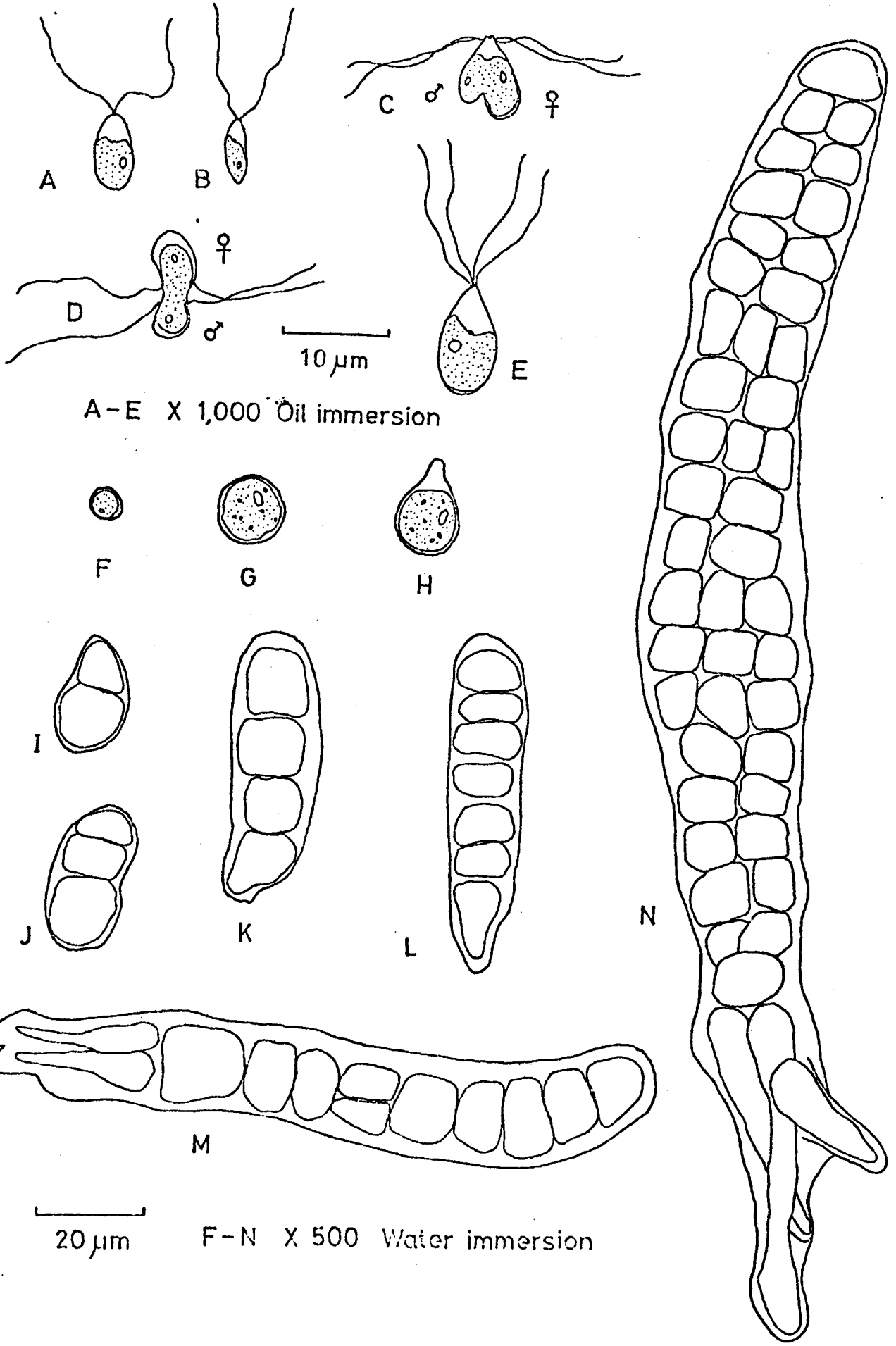
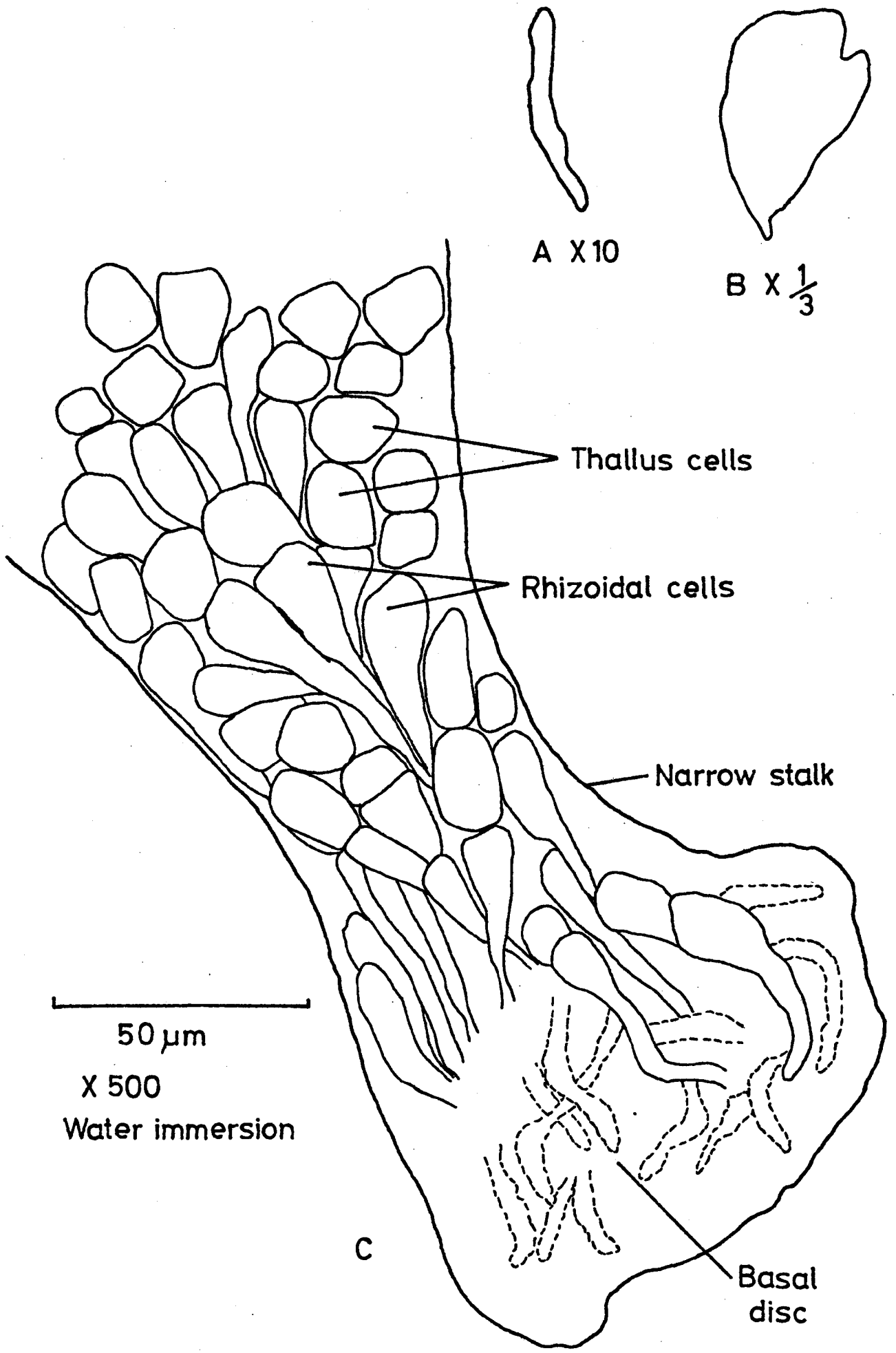

A
B
C
♂
♀
D
♀
♂
10 µm
E
A-E X 1,000 Oil immersion
F
G
H
I
J
K
L
M
N
20 µm
F-N X 500 Water immersion

Fig. 3. Ulva lactuca. A development of germling into distromatic thallus (2-3 months after germination), B mature plant (after 6 months growth), C detailed morphology of rhizoidal cells in and near basal region.


A X10
B X 1/3
Thallus cells
Rhizoidal cells
Narrow stalk
50 µm
X 500
Water immersion
C
Basal disc

The thallus cells contained one parietal cup-shaped chloroplast which possessed one and sometimes two pyrenoids. The chloroplast was usually situated adjacent to the cell wall so that when examined under the microscope the former could be clearly seen. One nucleus was present in each cell but was usually obscured by the chloroplast.

During the course of germling development, the primary rhizoidal cell also divided. The cells formed, differentiated, lost their chloroplasts and elongated to give rhizoidal strands at one end (fig. 3,C). Each cell also became multi-nucleate. As the plant matured the cells became interwoven and the strands adhered forming a disc-like base (fig. 3, C) which acted as a means of attachment to the substrate. A narrow stalk bridged the basal disc and the thallus.

As the sporophyte matured, dark green areas formed around the edge of the thallus. The cells within these future swarming areas divided repeatedly three or four times to form 8 or 16 quadriflagellate zoospores. Meiosis was presumed to have taken place during the process of zoospore formation.

2.3.2 The gametophyte generation.

When released the zoospores measured 10-12 μm by 5-6 μm (fig. 2, E) and were at first positively phototactic, but after a while, usually in a few hours or even less, they became negatively phototactic. Zoospores tended to be less active than both male and female gametes.

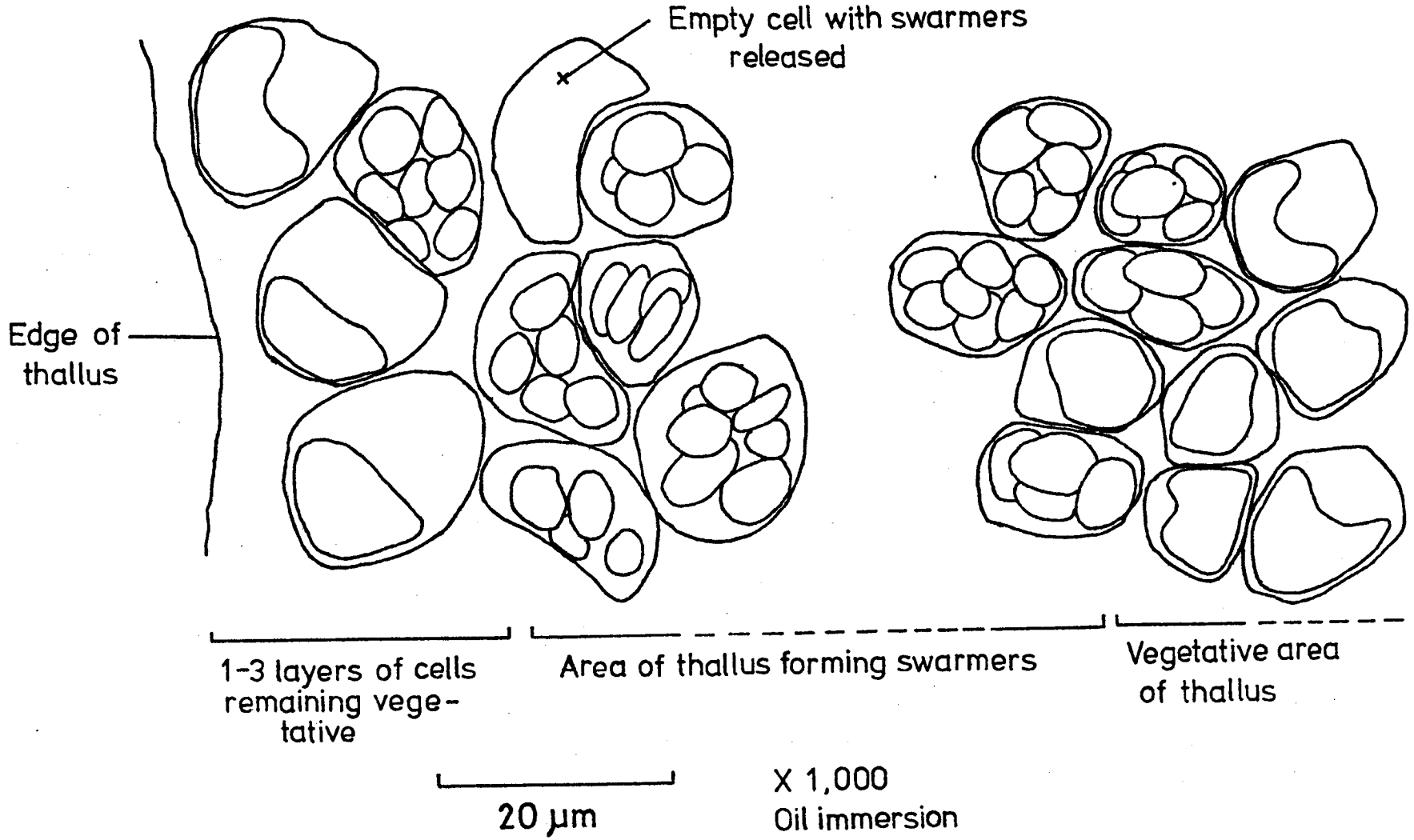
After settlement the zoospore which was now some 6 μm in diameter lost its flagella and subsequently increased to 12-15 μm diameter. Development of the zoospore was similar to that of the zygote, giving directly male or female gametophytic plants.

Both the male (+) and female (-) gametophytes appeared morphologically identical. As they matured the cells around the edges of the distromatic thallus produced swarmers. Each cell usually divided four or five times, resulting in 16 or 32 swarmers. The male and female plants could readily be distinguished by the colour of the swarming area. In male plants it was yellow whilst in female it was yellow-green. The development of swarmers occurred first near the edge of the thallus and then proceeded to about one third of the way from the centre (fig. 4). However, at the edge of the thallus, the outermost one to three layers of cells remained undivided and thus did not form swarmers. This feature has never been reported for Ulva lactuca, although Smith (1947) reported a similar observation for five other species of Ulva collected along the Pacific Coast of United States of America.

Ulva lactuca showed anisogamy; the male gametes looked yellowish while the female gametes appeared green (fig. 2, A-B). The newly swarmed male gametes were on average 5.3 x 2.3 µm, and the female gametes 7.6 x 4.1 µm. On one occasion Ulva material obtained from Osmington, Dorset, showed isogamy. This may have been the 'southern' species of Ulva identified and named as Ulva thuretii by Foyn in 1955. Hence the material was not used in culture experiments. Apart from being isogamous - U. lactuca is anisogamous - Foyn also found that cross-breeding U. thuretii with U. lactuca failed to produce germlings that grew further than the seven-cell stage. Dangeard (1965) reported the presence of both U. lactuca and U. thuretii on the south coast of England.

Before fusion, both types of gametes released from separate haploid plants showed positive phototaxis. They swarmed actively by means of their flagella. If no fusion occurred they remained active

Fig. 4. Surface of Ulva thallus showing vegetative and swarming areas.


Empty cell with swarmers released
Edge of thallus
1-3 layers of cells remaining vegetative
Area of thallus forming swarmers
Vegetative area of thallus
20 µm
X 1,000
Oil immersion

for at least 24 hours. When male and female gametes met they readily reacted and fusion took place. They fused either side-ways (fig. 2,C) or head-on (fig. 2, D).

The female gametes were sometimes observed to develop parthenogenetically in exactly the same way as the zygotes, but male gametes failed to do so although some workers have also reported parthenogenetic development of male gametes (Carter, 1926; Kapraun, 1970). The failure of the male gametes to develop may be due to chloroplast disintegration. Braten (1971, 1973) reported this disintegration after fusion in Ulva mutabilis.

Swarming could be induced by renewing the culture medium two to three days after collection of the plants from the field. The release of swarmers from a plant occurred almost immediately and simultaneously. Often within a minute or two most of the swarmers had escaped from the cells leaving the swarming part of the thallus colourless and devoid of any contents. The swarmers escaped through a perforation of the outer surface wall of each cell.

Since both the zygotes and zoospores apparently developed in the same way to maturity and no difference in growth rate was detected between the two, it was considered that both gametophyte and sporophyte plants could be used for future experiments.

CHAPTER 3

Materials and General Methods.

3.1 Materials

3.1.1. Sampling site and collection of *Ulva* material

Most *Ulva* material for culture and physiological work was obtained from the 'Little Eye' the smallest of the three islands comprising the Hilbre Islands at the mouth of the River Dee, about one mile off the coast of West Kirby, Wirral, Cheshire. The three islands form a chain lying in a north-westerly direction and roughly parallel with the river channel, with the Little Eye facing landwards. A system of reefs surrounds and connects the three islands, forming numerous rock-pools and shallow water channels. Geologically the islands are composed of soft Bunter sandstone. The Department of the Environment (1971) classified the whole of River Dee as a Class I (unpolluted) river, though there is a substantial amount of raw domestic sewage emptied daily from nearby residential areas into the river.

Ulva lactuca was collected, as required, from the same population in a small channel to the west of Little Eye. At every collection the size of *Ulva* plants and their abundance were recorded, as were the temperature and salinity of the water so that some idea of the range of temperature and salinity to which the crop of *Ulva* was subjected throughout the year would be determined. From more than fifty collections over a period of two and half years, useful physical and biological data of the sampling site emerged. For example, the temperature of the water varied from 3°C in January to a maximum of nearly 20°C in July, averaging 10.1°C over the year. Due to its estuarine nature, the salinity of the water varied from 20‰ to 31.2‰

at low water. But the range was most often between 23‰ and 30‰ with an average of 27.75‰. Ulva lactuca was the dominant species of macroalga occurring at the sampling site and all the year round a continuous supply was present. It was attached to rocks, shells or pebbles. U. lactuca was the only species of Ulva found at the site. In fact since the earliest records of the algal flora on Hilbre, this has been the only species of Ulva recorded (Gibson, 1889, 1891; Russell, 1969, 1971a,b, 1972, 1973). Periodic checks have confirmed this to be so. The size of the crop of Ulva varied with the period of swarming rather than with the season of the year. The crop built up to a maximum just before the plants swarmed, after which it dropped considerably and built up gradually again until the next period of swarming. Periodic swarming tended to occur in the few days around the spring tides occurring twice every month, however sporadic swarming was observed at other times.

Ulva plants were picked from all parts of the population and put into polyethylene bags which in turn were stored in a plastic bottle to prevent desiccation. The material was transported back to the laboratory immediately and then put into Erdschreiber medium. Time required for transportation was usually less than two hours. The freshly collected Ulva was allowed to acclimatise to the culture conditions in the laboratory for one or two days before use in experiments.

3.1.2. Chemicals

All chemicals used, unless otherwise stated, were of Analar grade.

3.2. General methods

3.2.1. Glassware cleaning

All new glassware used for culture work was treated with a 0.1% (W/V)

solution of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) for 48 hours in order to saturate phosphate absorbing sites on the inner surface before the usual cleaning procedure. This solution also served as a cleansing agent.

Glassware used for both culture and physiological work was cleansed as follows. It was initially scrubbed with a sponge before being soaked in 0.3% of laboratory detergent for at least 12 hours followed by further thorough scrubbing. It was then rinsed with hot tap water 20 times followed by ten rinses with deionised water. Drying took place in an oven at a temperature of 70-80°C. This procedure has been previously shown to be satisfactory for preparing glassware for algal culture in this laboratory (e.g. de Silva & Burrows, 1973).

3.2.2. Culture medium and culture conditions

Erdschreiber medium, an enriched sea water employed by Foyn (1934), was used to culture Ulva lactuca. The detailed composition of Erdschreiber medium (E-S medium) is given below and details of preparation are given in Appendix I. The[1] medium was made by mixing 25 cm^3 of soil extract and 5 cm^3 of salt solution containing sodium nitrate and disodium hydrogen orthophosphate dihydrate, and made up to 1 dm^3 using filtered seawater. Five cm^3 of salt solution diluted to 1 dm^3 gave 1.65 $mg.dm^{-3}$ nitrate-nitrogen (NO_3-N) and 0.348 $mg.dm^{-3}$ orthophosphate phosphorus (PO_4-P). Soil extract provides growth factors, micronutrients and some macronutrients for the growth of Ulva.

Sea water, obtained from the Irish Sea, was filtered through two layers of Whatman No.1 filter paper before use. Storage of sea water was in polyethylene carboys in an 8°C cold-room. All sea water was used within a week after filtering.

Cultures were set up using one of the following two regimes. It was unfortunate that different conditions had to be used but this was necessary because of changes in availability of growth facilities.

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[1] E-S medium contained less than 0.01 $mg.dm^{-3}$ NH$_4$-N.

(1) Cold-room culture - Ulva material was placed in glass bricks, with fitting lids, of 0.35 dm^3 capacity. Temperature was maintained at 8°C with continuous illumination by Atlas 80 W Northlight fluorescent tubes giving a light intensity of 1.5 Klux. The medium was changed twice a week.

(2) Tank-room culture - Glass tanks with 5 dm^{-3} or 2.5 dm^{-3} capacity were used. The temperature of the cultures was maintained at 12 ± 1°C. by circulating around the culture vessels a mass of thermostatically controlled water contained within a large metal tank measuring 1.44m x 1.44mX 0.55m. Overhead illumination was provided by a battery of 300 W incandescent lamps giving an intensity of 4 to 4.6 Klux on the surface of the culture medium. Day length of 15.5 hours was maintained by an automatic time switch. Aeration was by compressed air which was freed of suspended particles by passage through a flask of deionised water. The culture medium was changed once every five or six days. A detailed description of the determination of suitable culture conditions is given in section 4.2.

3.2.3. Sampling of Ulva discs and their measurement

Using the results of the study of the mode of growth of the Ulva thallus given in section 4.1.2 , standardized methods for sampling Ulva discs were devised which gave consistent and satisfactory results in the culture experiments. Discs were punched from the Ulva thallus by using a 11.8 mm internal diameter sharpened cork borer. Care was taken to avoid the marginal and basal holdfast regions because the former frequently produced swarmers and growth would then stop, while the latter had a considerably slower growth rate than the rest of the thallus. Discs thus obtained had quite uniform growth rates as

indicated by the relatively small standard errors of the means in the culture experiments. The discs were distributed randomly into the different treatments of an experiment. Discs of other sizes were tried, but small discs (5.8 mm diameter) tended to have a large error of measurement and large size discs (16.2 mm diameter) needed a large amount of plant material and hence their usage would have caused sampling difficulties.

Cut discs of Ulva continue to grow in culture and largely keep their original shape. Growth can easily be measured by recording the diameter of the discs when required. For measurement discs were laid flat between two thin glass plates and the diameters measured against a sheet of millimeter graduated graph paper. Two diameter measurements, one perpendicular to and through the mid-point of the other, were taken for each disc and the values averaged, this value being taken as the mean diameter of the disc. This value was then used to calculate area and percentage increase in area. The method provided a quick and accurate assay of growth. The results thus obtained, when compared with those obtained from an automatic area-meter (Type AAM-5, Hayashi Denko Co. Ltd., Tokyo), agreed to within 2.5%. Error of the area-meter was given as less than 1%. The area-meter was not routinely used because the manual method was found to be quicker.

3.2.4. Settling of swarmers

Ulva plants that showed signs of swarming were separated into individual glass bricks containing freshly made E-S medium. Such plants usually released swarmers two to five minutes after the transfer.

Zoospore cultures were set up by placing about 10 cm^3 of a concentrated zoospore suspension, appearing as greenish streaks in the culture medium, into a 100 cm^3 measuring cylinder containing

E-S medium. A uniform suspension of zoospores was obtained by repeated inversion of the measuring cylinder. This suspension was then poured into a glass tank containing E-S medium over sterilized glass slides. The zoospores settled on the slide surfaces overnight and were then transferred to the different treatments of an experiment.

Zygote cultures were set up by mixing approximately equal amounts of male and female gametes. The compatibility of the gametes was first tested by microscopic examination, to see whether the gametes fused immediately upon mixing. The rest of the settling procedure followed that for the zoospores.

Growth rates for both zoospore and zygote germlings were assessed by putting them into a petri dish of filtered sea water and counting the number of cells in each plant with a microscope fitted with a coated water-immersion lens. This prevented desiccation and overheating of the germlings during the process of counting. Plants up to the 40-celled stage could be counted accurately by this method.

3.2.5. Measurement of photosynthesis and respiration

Both photosynthesis and respiration of Ulva were measured using a Gilson differential respirometer with refrigeration facilities (Model RG 14). Photosynthesis was measured on the basis of oxygen production and respiration on oxygen consumption. When measuring photosynthesis, an integral battery of fourteen 53 W incandescent light bulbs provided a light intensity of 10 Klux at the level of the reaction flasks. When respiration was to be measured the lights were switched off and the flasks covered completely by black cloth. When so covered, light penetration into the flask was minimal being less than 10 lux. Temperature was kept constant at either $8^{\circ}C$ or $12^{\circ}C$.

Twenty cm^3 reaction flasks were used and 7.5 cm^3 of sea water medium added to each flask. Despite the differential operation of this respirometer it was found necessary, when rates of gaseous

exchange were low - i.e. during respiration measurements, to use an additional control flask containing sea water only.

In order to prevent photosynthesis being limited by CO_2 level during a three hour experiment it was found necessary to supplement for carbon dioxide consumed. For this purpose both of the following additions were tried:

(i) 0.25 cm^3 of sodium hydrogen carbonate at a concentration of 0.2 $g.dm^{-3}$,

(ii) 0.25 cm^3 of a mixture of 0.1 M sodium bicarbonate and 0.1 M sodium hydrogen carbonate at a ratio of 3:17, giving 0.22% of carbon dioxide.

Supplementation by (ii) was selected and subsequently used since it provided a more prolonged and controlled supply of CO_2 than (i), as indicated by a constant rate of photosynthesis throughout the test period. This supplement was added to the sea water medium rather than the centre well as the process of CO_2 diffusion from the centre well into the sea water was found to be too slow.

Initial experiments showed that five discs of Ulva of diameter 11.8 mm, giving a total of approximately 10 mg. dry weight, was a suitable amount of material for satisfactory photosynthetic and respiratory measurements. Few discs, e.g. 2 discs, led to too small a rate of gaseous exchange especially for respiration measurement, while more, e.g. 10 discs, gave reduced rates. The latter would seem to be caused by a tendency of the discs to adhere and overlap, this causing self shading and inadequate gas penetration, thus effectively reducing the surface of Ulva receiving full light intensity and free gaseous exchange.

Further experiments showed that there were no significant differences in either photosynthetic or respiratory rates between young and mature plants; nor were there any differences in such rates between different

regions of the thallus - marginal, mid and basal, - calculated on a unit area or dry weight basis.

3.2.6. Protein estimation

Total protein content of Ulva was estimated using the method devised by Lowry et al. (1951). Details of this method are given in Appendix II. Estimations were made on 10 mg portions of dried material, because often there was not enough time to carry out protein estimation on fresh material the same day an experiment was terminated. Check determinations showed no significant loss of protein during the drying procedure. The method of preparation of dried material is given in the dry weight determination section.

Normally Ulva contains from 6-12% of protein by dry weight (see section 5.3.2.).

3.2.7. Chlorophyll estimation

For chlorophyll extraction and quantitative estimation Arnon's method (1949) was adopted, details of which are given in Appendix III. The amount of Ulva material used for this was the same as in the case of protein estimation. Strain et al.(1971) reported that it was necessary to pretreat Ulva by immersing it in boiling water for a minute followed by cooling to room temperature, in order to obtain a complete extraction of chlorophyll. This was found not to be the case in this study as no difference was observed, with or without the additional boiling treatment, in the amount of chlorophyll extracted. Both fresh and dried Ulva were used for extraction for reasons given in 3.2.7. It was found that in the dried material there was a slight decrease in chlorophyll a in relation to the amount of chlorophyll b. The chlorophyll a:b ratio dropped from 2:1 for fresh material to 1.7:1 in dried. This was probably due to the more heat-labile nature of chlorophyll a than chlorophyll b. No adjustments were attempted for

this as most estimations were made with dried material and thus direct comparison could be made. Drying resulted in an approximately 10% loss of chlorophyll. Dried Ulva was found to contain between 6 to 10 µg chlorophyll mg^{-1} dry weight. Chlorophyll content was found to be uniform throughout the thallus.

3.2.8. Dry weight determination

Ulva was dried in the following manner. The Ulva surface was first cleaned thoroughly of bacteria and micro-algae, using a fine brush, and washed in deionised water before being placed in a container made from tin-foil. It was dried in an oven at 40-50°C overnight, for a minimum duration of 12 hours, needed to achieve constant weight, cooled to room temperature and weighed.

3.2.9. Cell size measurement

Cells of Ulva were drawn onto a piece of paper using a camera-lucida at X500 magnification. A scale was also drawn from a calibration slide with graduated 10 µm divisions. The magnified cell drawings were cut out and the area estimated by an automatic area-meter (Type AAM-5, Hayashi Denko Co. Ltd., Tokyo). The actual cell areas were then calculated from the drawn scale.

CHAPTER 4.

Development of Sampling and Culturing Techniques for *Ulva lactuca*

4.1 Development of sampling techniques

Before trying to establish a suitable way of sampling material from an *Ulva lactuca* plant for culture experiments, a knowledge of the pattern of its growth is necessary. Though it is a species frequently used for experiments it is very disturbing that very little is known about its mode of growth. Its growth is often described inadequately as just "diffuse" (Morris, 1968) or "cell division may occur anywhere within a thallus" (Smith, 1955). However, this does not rule out any gradients of growth within the thallus. The following experiments were therefore carried out to determine the detailed mode of growth of the *Ulva* thallus.

4.1.1. Methods

Two methods were used, one involving the use of whole, intact plants, while in the other, excised portions of the thallus were utilized.

(a) Intact plants.

Initially plants with a thallus length in excess of 5 cm were used but many of them swarmed freely after a few days in culture. Since the swarming area stopped growth, in terms of area increase, these plants could not be used to observe the pattern of thallus growth. However, a tracing of one of these plants before and after swarming is recorded in figure 5 and gives an indication of the position and spread of the swarming area.

Experience showed that small *Ulva* plants (thallus height 5 cm or less) rarely swarmed when brought into laboratory culture, and 5 such plants were thus selected to repeat the experiment. On each thallus

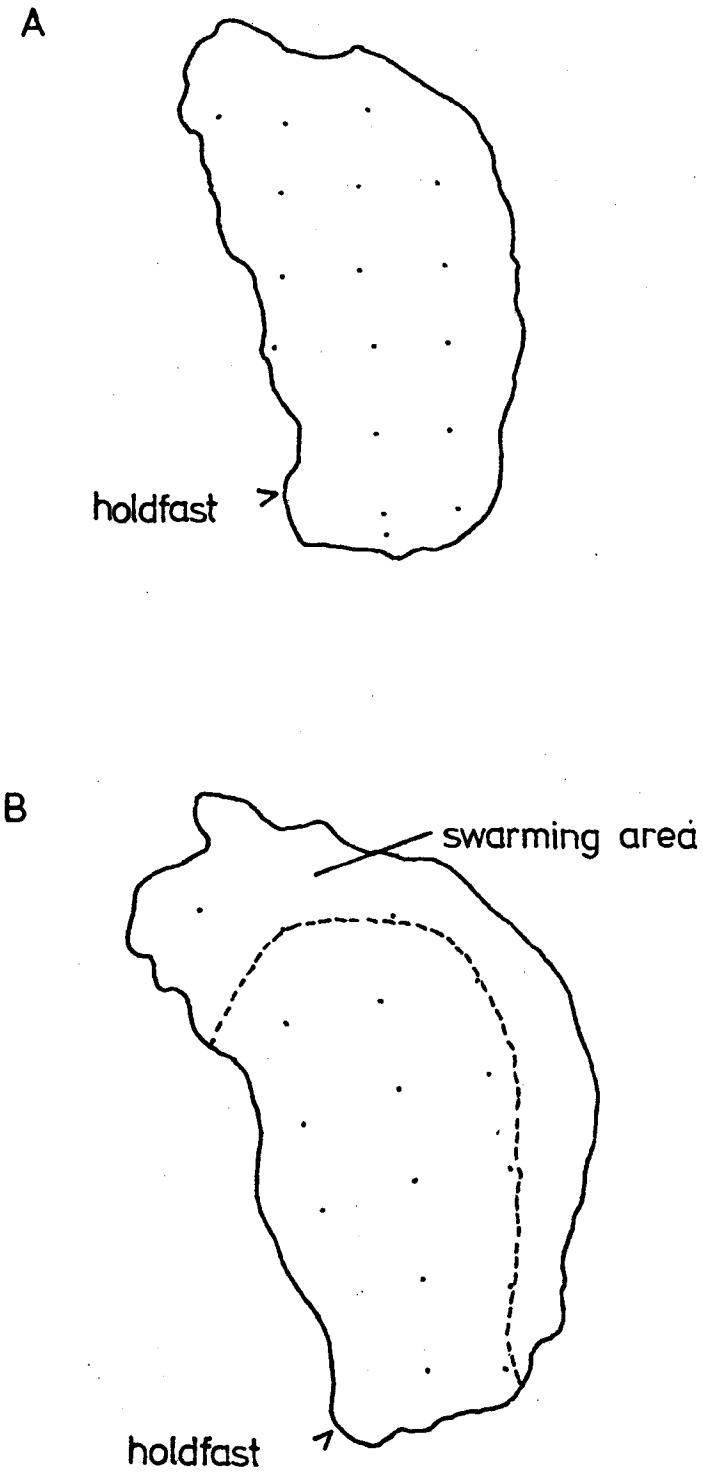


Fig. 5. Position and extent of swarming area.

holes were pierced, using a sharp stainless steel heat-sterilized needle, at regular intervals approximately 1 cm apart (as shown in figure 6A). The outline of the plant together with the positions of the pierced holes was traced onto paper. This tracing procedure was repeated five more times at intervals over the next thirty days. As a result the pattern of growth of the thallus was recorded. The plants were cultured in E-S medium, under incandescent light of 4.2 ± 0.2 Klux, at a temperature of 12 ± 1°C; a day length of 15.5 hours and with aeration by compressed air. The medium was changed every five days.

(b) Excised discs.

The thallus of Ulva was arbitrarily divided into three regions, namely, (1) basal region - area immediately around the holdfast, (2) marginal region and (3) mid region - area other than (1) and (2) (see figure 7). Forty discs of diameter 1.18 cm were cut, using a sharpened corkborer, from each of the three regions of the thallus. Discs from each of the regions were cultured separately in E-S medium under continuous illumination from North-light fluorescent tubes giving an intensity of 1.5 Klux. The diameter of the discs was measured after one week of growth and the percentage area increase calculated in each case.

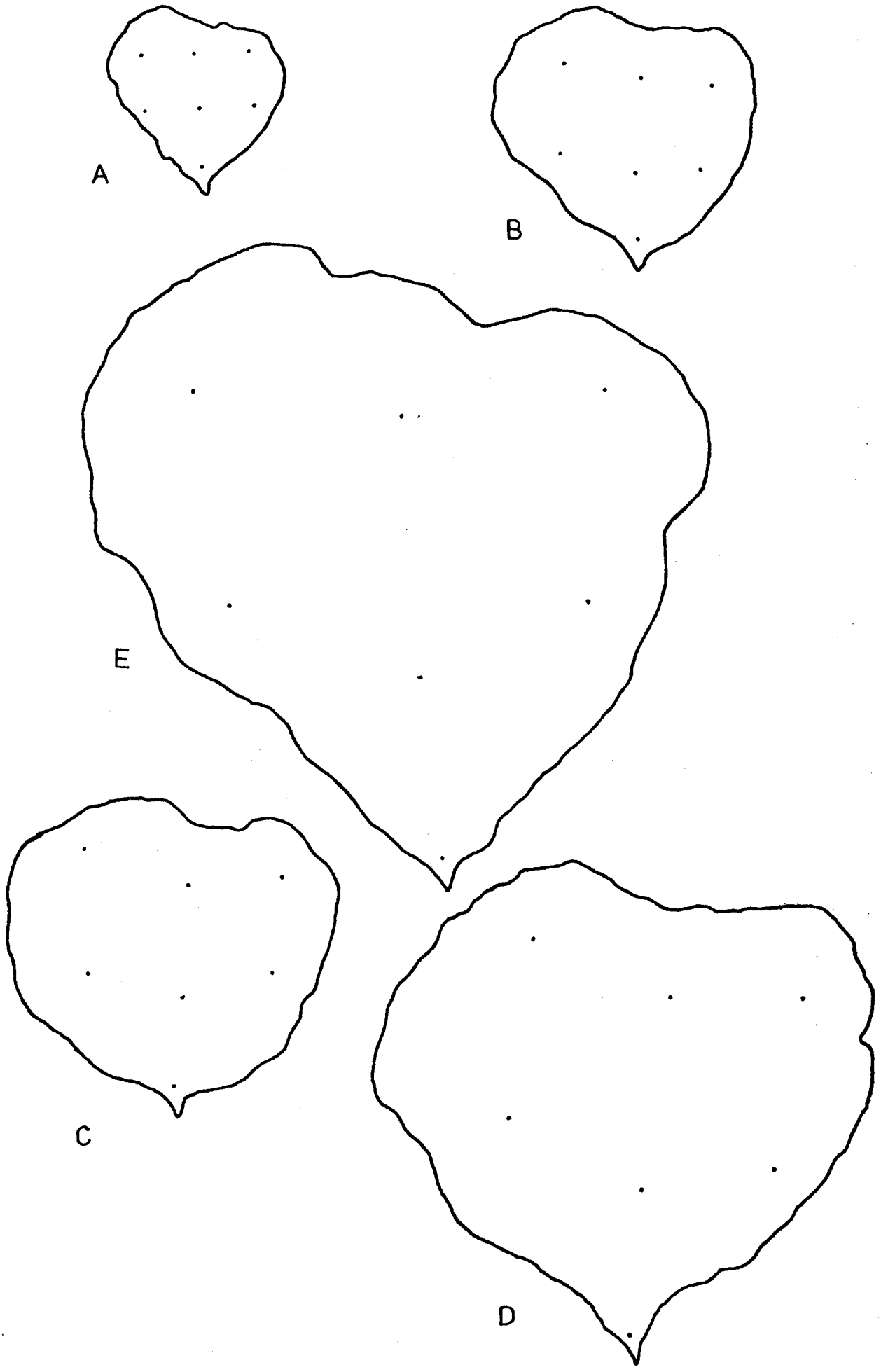
The experiment was repeated using discs obtained from regions (1) and (2) of the thallus with readings taken weekly over a period of three weeks. The culture conditions were as above.

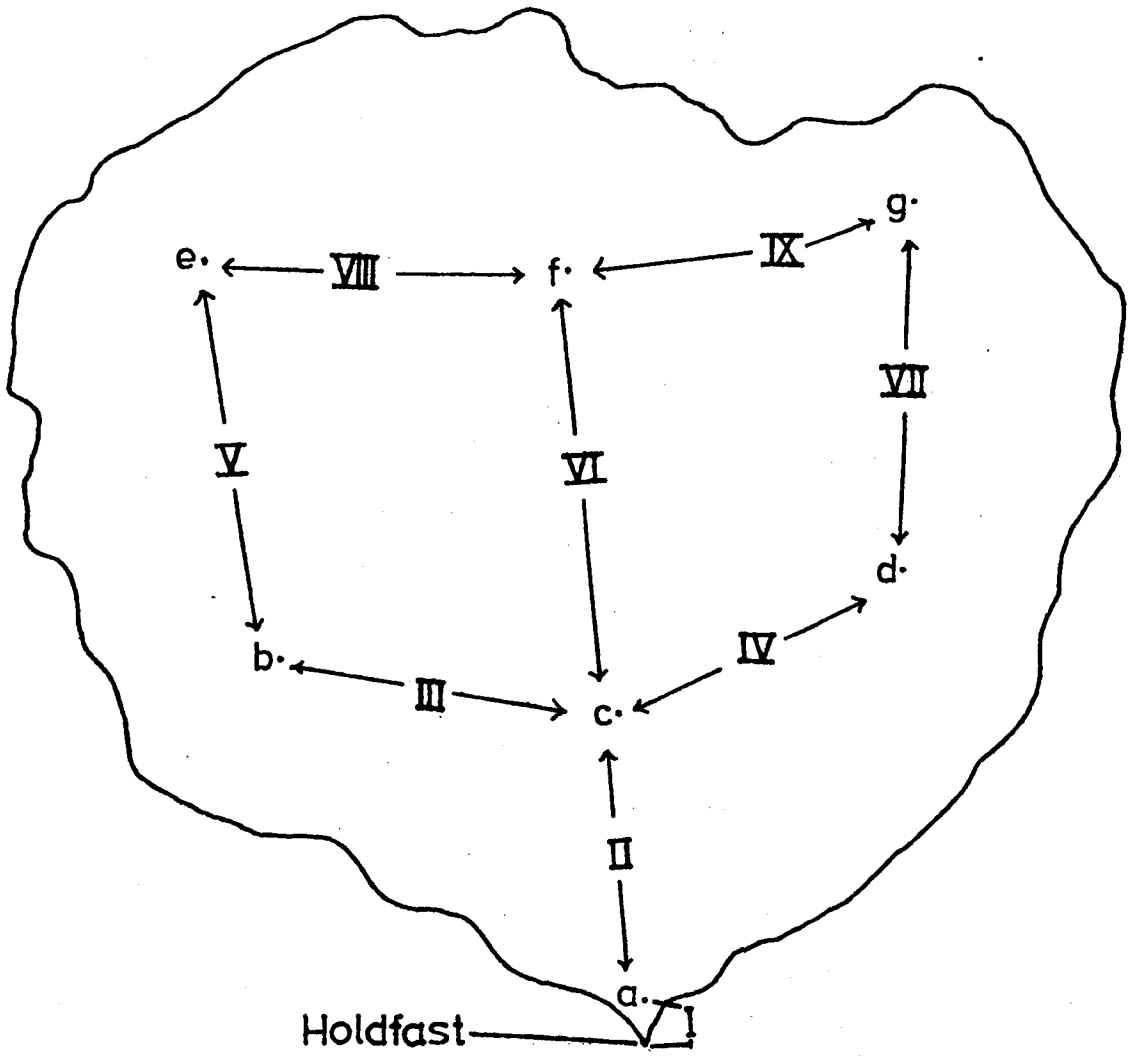
A third experiment was carried out over two weeks with discs from regions (1) and (3) under the same light and photoperiod regimes as in (a). Measurements were taken at the end of the culture period.

4.1.2. Results

(a) Intact plants

All the replicates showed similar results. The plant tracings in

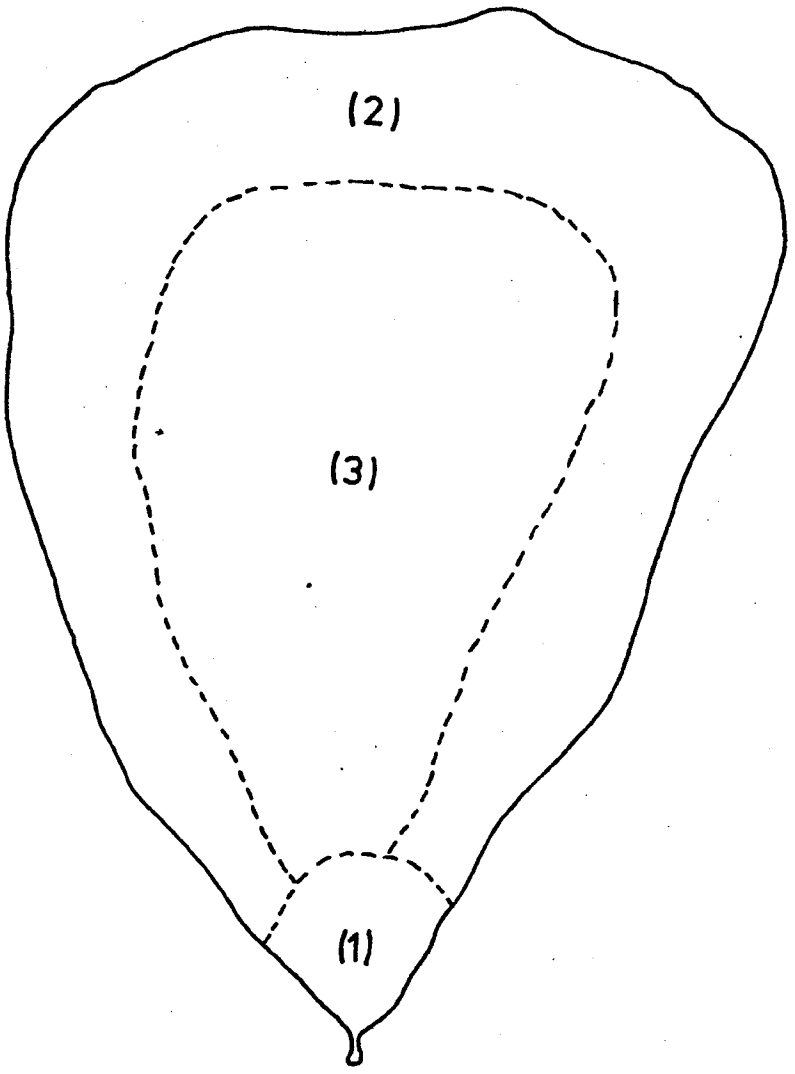

A
B
E
C
D



F.

Fig. 6. Tracings of an Ulva thallus (with pierced holes) over a period of 30 days growth. A Day 0, B Day 7, C Day 12, D Day 17, E Day 22, F Day 30.

Fig. 7. Diagram showing the three sampling regions of *Ulva* thallus - basal (1) mid (3) and marginal (2).


(2)
(3)
(1)

figures 6A to E show an example of growth of a plant with seven pierced holes (a to g) on its thallus. The distance between a and the tip of the basal disc, and those between a-c, b-c, c-d, b-e, c-f, d-g, e-f, f-g are listed I to IX respectively (see figure 6F). The respective percentage increases in distance between the holes are plotted against time in figure 8. The area of the whole plant in cm^2 and $\log_e$ of area are plotted against time in figure 9. From the results in figures 6 and 8, it is clear that at the holdfast (I), there was very little growth. A gradient of increase in growth rate was observed from the holdfast out towards the thallus margin. Thus for vertical distance increases- V, VI and VII have greater values (average 1.53 times) than II. For horizontal distance increases, VIII and IX showed faster growth (1.23 times) than III and IV. The graph in figure 9 shows growth of Ulva plant is exponential over the duration of the experiment. The growth of various segments, apart from I, also shows exponential trends (figure 8).

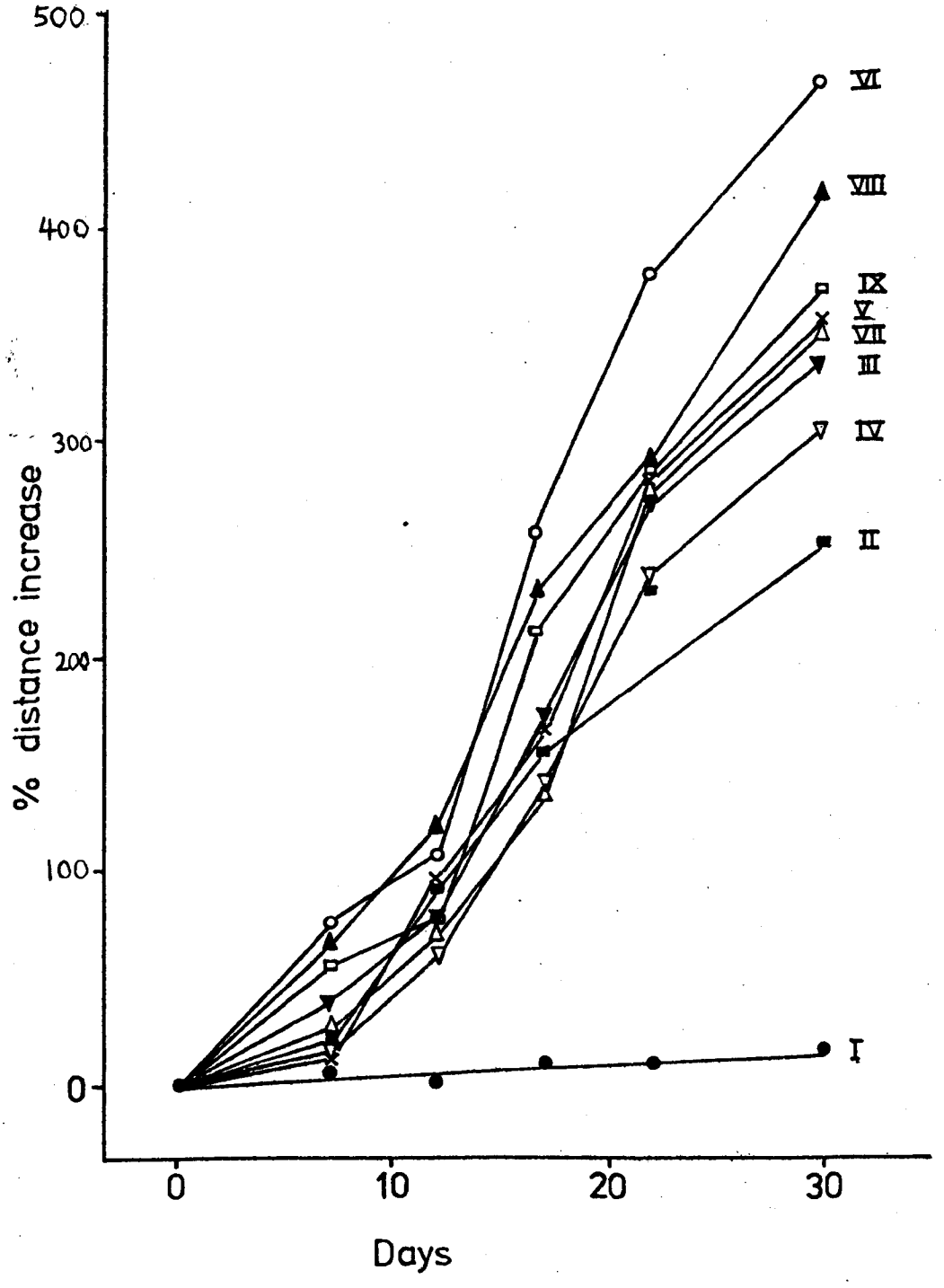
(b) Excised discs.

All three experiments gave very similar results as shown in figure 10. Discs obtained from both marginal and mid-regions of the thallus had a significantly greater growth rate than those from the basal/ holdfast region. There was a suggestion of a slightly higher growth rate in discs taken from the marginal region compared with those from the mid-region of the thallus, but the difference was not significant.

4.1.3. Discussion

Clearly every part of the Ulva thallus is capable of expansion and hence growth in terms of increase in area. Thus the growth of Ulva can be regarded as diffuse or intercalary rather than apical as can be the case for Enteromorpha (Burrows, 1959). Both the intact plant and excised disc experiments reflected this in that the plant grew in

Fig. 8. Mode of thallus growth experiment, showing percentage increase in distance between the holes pierced in the thallus, over a 30 day period.


500
400
300
200
100
0
% distance increase
0
10
20
30
Days
VI
VIII
IX
V
VII
III
IV
II
I

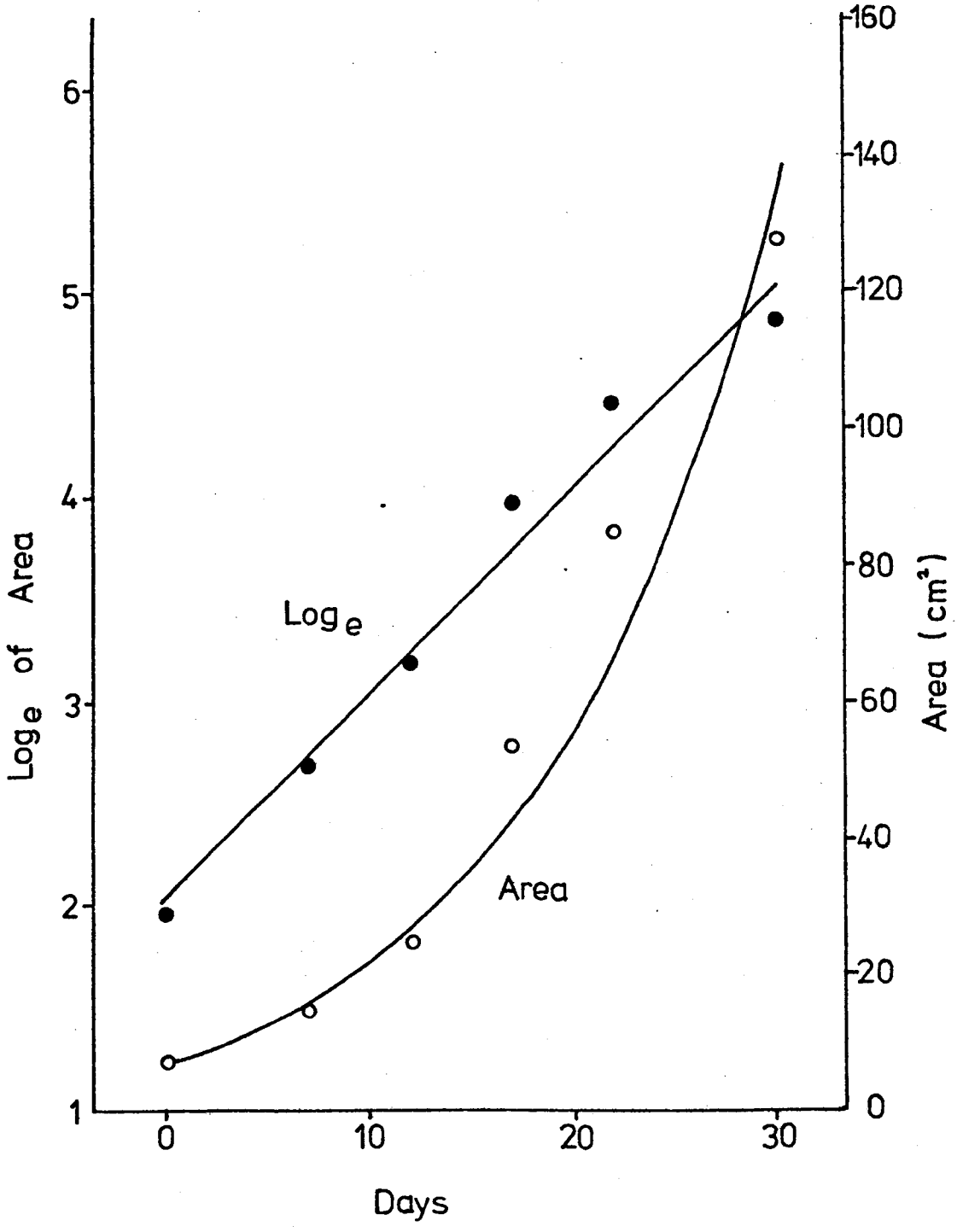


Fig. 9. Area increase of an Ulva thallus with time.

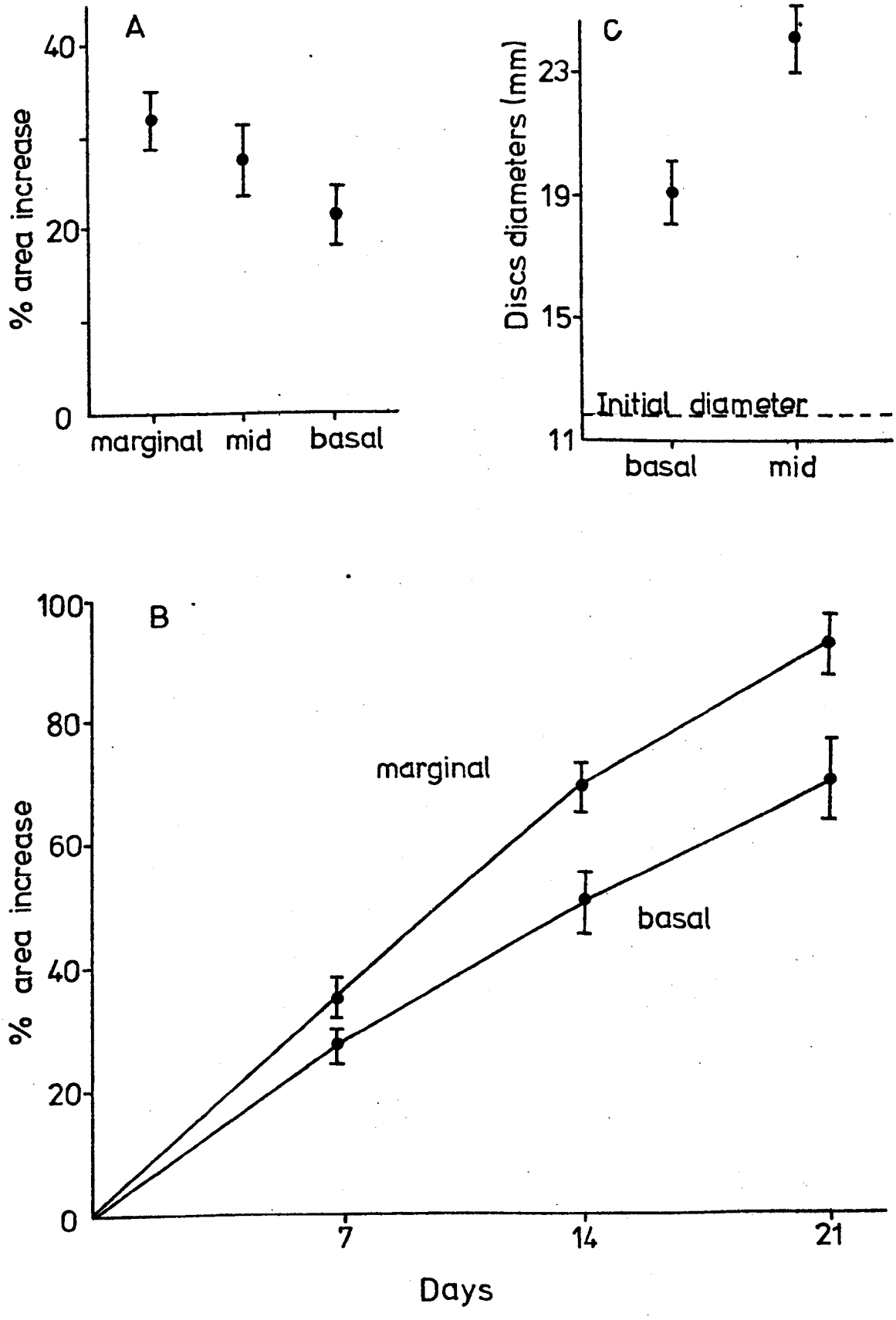
Fig. 10. Growth of excised Ulva discs obtained from the basal, mid and marginal regions of the thallus with time.

A, experiment using discs from all three regions, growth over 7 days.

B, experiment using discs from basal and marginal regions, and

C, experiment with discs from basal and mid regions, growth over 14 days.

Values given are means with twice standard error on each side.


A
40
20
0
% area increase
marginal
mid
basal
C
23
19
15
11
Discs diameters (mm)
Initial diameter
basal
mid
B
100
80
60
40
20
0
% area increase
marginal
basal
7
14
21
Days

every direction and discs grew symmetrically. The results also showed different growth rates in different parts of the thallus. A gradient of increasing growth rate was apparent from the holdfast towards the margin. This is clearly shown in figure 8 where the marginal region (V, VII - IX) and mid-region (III, IV, VI) had linear growth rates on average 1.46 times and 1.44 times that of the basal region (II). Growth at the holdfast was very slow throughout the experimental period. This growth pattern remained very similar throughout the growth period during which plant height and breadth increased from 3.5 cm and 3.2 cm to 13.0 cm and 13.9 cm respectively. As the plant attained a considerable size at the end of the culture experiment, one can assume that this pattern of growth applies to both small and large Ulva plants.

The fact that excised discs of Ulva showed growth results almost identical with those of the whole thallus, indicates that variation of growth rate within the thallus is at least partly genetically determined. The similarity in behaviour of isolated discs and intact thallus was supported by many observations which showed there was a much greater tendency for discs obtained from marginal regions to swarm than those obtained from near the holdfast. Thus discs retained the basic characteristics of the region of the intact plant from which they had been taken.

Other species of Ulva have been shown to exhibit similar growth patterns. Kale and Krishnamurthy (1964) using excised strips from different regions of Ulva rigida obtained similar growth behaviour. Lövile (1964), working with Ulva mutabilis, demonstrated a gradient of growth within the thallus of intact plants.

Despite the very similar morphology throughout the Ulva thallus the above has shown a definite pattern of variation in its growth. Some physiological differences are also apparent within the thallus.

Subbaramaiah (1967) found a gradient of ascorbic acid concentration in Ulva fasciata, it being highest in the basal region and lowest around the marginal region. How exactly growth and differentiation of Ulva is controlled is not known. There are too few facts to give one a clear picture. Indirect evidence suggests both growth regulators (Provasoli, 1958; Kale & Krishnamurthy, 1969) and genetic factors (Løvile, 1964) each play some role in this.

Pioneer work by Provasoli (1958) showed that under aseptic conditions, Ulva lactuca grows as atypical, short, filamentous germlings which do not develop into a foliaceous thallus. The normal flat blade could be obtained only by addition of a certain combination of adenine and kinetin to the aseptic growth medium.

By supplying 50 $\mu g\ dm^{-3}$ exogenous indole acetic acid (IAA) to pieces from different regions of Ulva rigida before further culture, Kale & Krishnamurthy (1969) detected stimulation of growth in all except the midbasal region where retardation of growth occurred. They then suggested the presence of auxin within the thallus and summarized its distribution pattern. They concluded that a supra-optimal amount of auxin occurred in the midbasal region, a near-optimal at marginal and apical regions, suboptimal at the basal region, while in the mid region the amount present was very low. Their suggestion needs to be treated with great caution since positive response of a plant towards an exogenous source of growth hormone does not by any means prove its presence within the plant under natural conditions. Much further work is required on the occurrence and function of growth factors in Ulva lactuca.

If indeed endogenous hormones are present within Ulva, it seems unlikely that they are manufactured in localized region(s) and then diffuse to the other parts. This is indicated by the fact that excised pieces of the plant from different regions continue to grow and retain

their different growth rates. However, this can be explained if synthesis occurs in all parts of the thallus or supply of hormones is usually obtained from outside as was postulated by Provasoli & Pintner (1964a, b, 1972). It is difficult to explain the differences in growth rates in different regions of the thallus. It may be due to the varying amounts of hormones synthesized within the thallus or to the difference in response to a given amount or type of hormone in the different regions of the thallus.

Whatever the actual mechanisms controlling growth and differentiation within the Ulva thallus, it would seem likely that both hormonal (endogenous and/or exogenous) and genetic factors each contribute significantly to the overall control.

Finally, as a result of the above investigation, it was concluded that the mid region of the thallus (refer to figure 7) was the most satisfactory area for sampling purposes. The basal region - area immediately around the holdfast - has too slow a growth rate to give adequate growth response. The marginal region, though having the fastest growth rate, frequently produced swarmers and growth would then cease and the cells disintegrate after the release of swarmers. Thus both the basal and marginal regions were avoided when sampling material from the Ulva thallus for experiments.

4.2. Development of culturing techniques

Before testing the effects of different pollutants on the growth of Ulva discs in culture, it was necessary to determine appropriate standard culture conditions in relation to:-

i) duration of culture required, to obtain a genuine growth rate measurement,

ii) salinity,

iii) light intensity, and

iv) temperature.

A knowledge of how environmental factors such as salinity, light intensity and temperature affect the growth of Ulva in the field also helps to give some insight into how it behaves under similar conditions in the laboratory. For example, it is known that Ulva grows well in areas with some freshwater run-off (refer to section 2.2) thus indicating that the plant can tolerate reduced salinities. Laboratory culture results on how these factors affect the growth of Ulva may in turn lead to some understanding of its growth potential and distribution in the field: why Ulva flourishes in some areas and not in others.

The following experiments were carried out to find how the growth of Ulva discs was affected by the duration of the experiment and by salinity, light intensity and temperature in culture.

4.2.1. Duration of culture.

4.2.1.1. Method.

Two experiments were carried out, one for a shorter culture period of 14 days, and the other for a period of 30 days.

In both experiments, 50 freshly cut Ulva discs were cultured in 2 dm^3 of E-S medium at a temperature of 12 $\pm$ 1°C and incandescent light of 4 Klux intensity. The daylength was 15.5 hours. The culture medium was changed every 5 days and was kept aerated with compressed air.

In the first experiment, disc diameter was measured every day for the first 12 days and then finally at day 14. Measurements were made between 16.00 and 17.00 hours every day except on day 10 when measurement was at 09.00 hours.

In the second culture experiment of 30 days, the diameter of the Ulva discs was measured every 6 days thus providing a total of five measurements.

4.2.1.2. Results.

Growth performance of Ulva discs expressed as percentage increase

in area is summarized in figure 11. In the 14 day culture experiment (fig. 11A) it is apparent that there was a lag phase of growth for the first 4 or 5 days followed by a period of 6-7 days when rate of growth with time was linear, with a suggestion of a reduction of growth rate in the final 2 days of culture.

Results of the 30 day culture (fig. 11B) showed a very similar rate of growth of discs over the first 6 to 12 days or so but then growth rate became exponential and remained so until the end of the culture period.

It is concluded that a culture period of between 14 and 21 days would be sufficient to give an amount of growth in Ulva (150 to 300 percentage increase in area) which is sufficient to allow accurate measurement and where only a small proportion of the culture time is the initial culture stabilization phase. Exceptionally experiments were continued for up to 24 days.

4.2.2 Salinity.

4.2.2.1. Method.

To assay any effect of salinity on growth rate, standard discs of Ulva were cultured for 21 days in seawater media diluted with appropriate amounts of deionised water giving the following salinities (g. dm^{-3} salt, or as usually written ‰): 33, 25, 16.5, 8.3 and 0. To each salinity sample was then added the standard amount of salt solution and soil extract as specified in E-S medium (refer to Appendix I). Fifty discs of Ulva were used in each medium and the culture conditions were as previously described (section 3.2.2) for the tank-room.

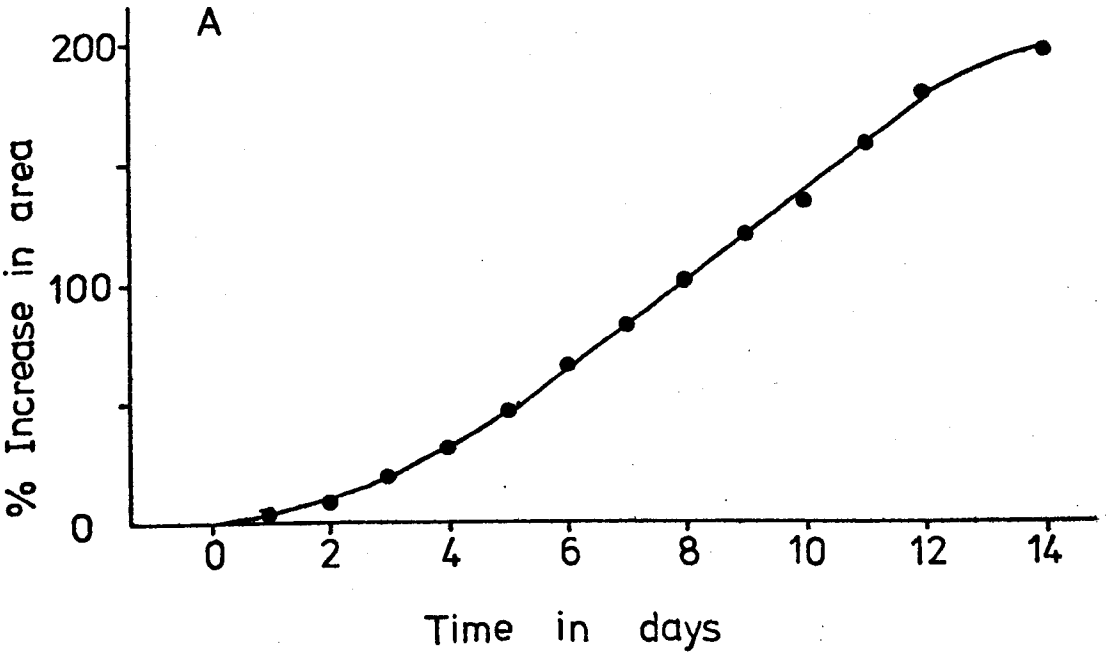
Another experiment was also completed to find out in more detail how Ulva was affected by low salinity. The salinities used were: 35 (as control), 10, 8, 6, 4, 2 and 0 ‰. The method of preparation of the waters was as above, with culture conditions as described for

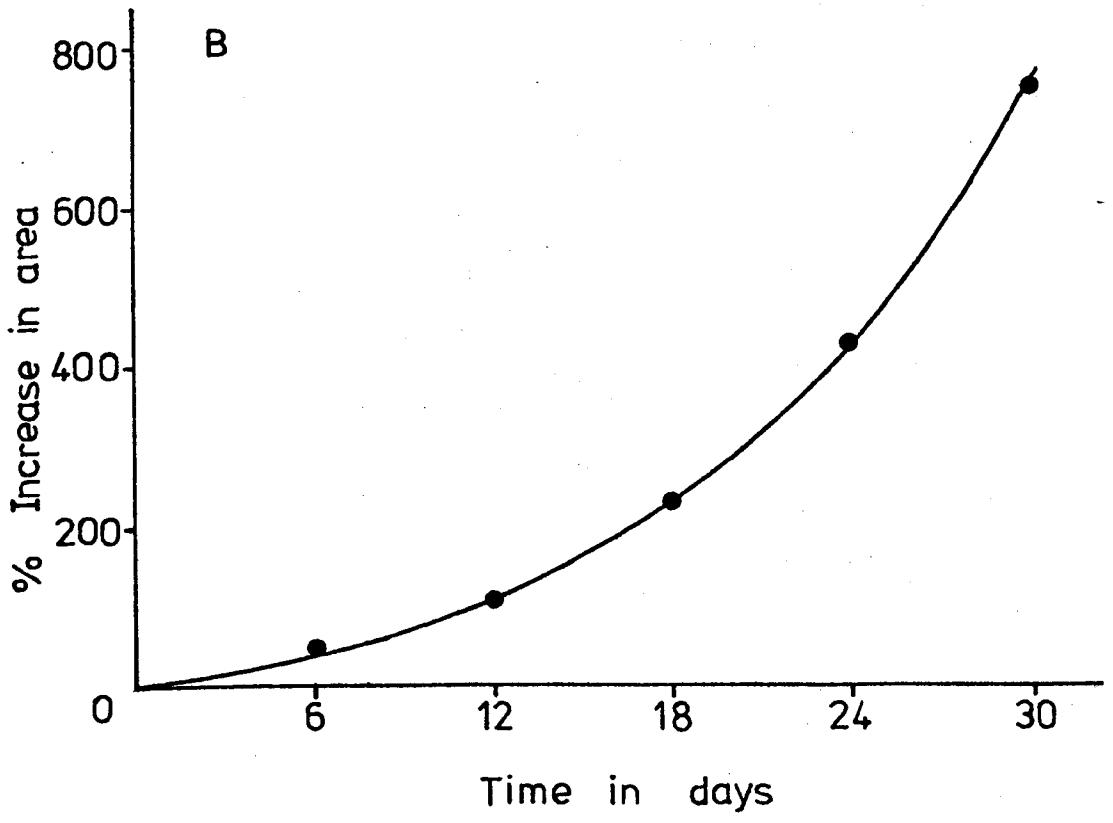
Fig. 11. Growth of Ulva discs with time.

A, culture of 14 days duration.

B, culture of 30 days duration.

Values given are means of 50 discs.


A
200
100
0
% Increase in area
0
2
4
6
8
10
12
14
Time in days


B
800
600
400
200
0
% Increase in area
6
12
18
24
30
Time in days

the cold room (section 3.2.2). The growth period was 24 days. In addition to growth measurement a cytological examination, by light microscopy, was made at the end of the culture period to see whether there was any change in appearance of the cells due to reduced salinity treatment.

Effects of different salinities on the photosynthetic and respiratory rates of Ulva discs were also assessed. The following range of salinities was employed: 34, 25, 10 and 4‰. Methods of measuring photosynthesis and respiration were as given in the general method section 3.2.5.

4.2.2.2. Results.

Results of the two culture experiments are given in figures 12A and 13. In the first culture experiment (fig. 12A) it was shown that growth rate of Ulva remained constant with seawater dilutions to half (16.5‰) normal salinity. Further dilution caused decrease in growth performance. At one quarter of normal salinity (8.3‰) the growth rate was only 52% of that of full strength seawater and practically no growth occurred in deionised water.

The second culture experiment (fig. 13) showed that at the end of the culture all the diluted seawater samples gave significantly lower and graded Ulva growth over the control (35‰). Cultures of salinities 10, 8 and 6‰ had similar growth responses but significantly higher growth rates than 4‰ which in turn showed significantly better growth performance than 2‰. As in the previous experiment (fig. 12A) very little growth occurred in deionised water. It is interesting to note that during the early part of the culture period (day 0 to 6) salinities 0 to 10‰ showed very similar growth inhibition in relation to the control. But a graded response was apparent from Day 12 onwards indicating graded inhibition effects may take a while to develop. Round, swollen cells of approximately 20 µm diameter were

Fig. 12. Effect of different salinities on Ulva discs.

A grown at the end of 21 days of culture, data plotted as twice standard errors on each side of mean.

B net photosynthetic -●-●- , and respiratory -o-o- rates , discs from plants grown in normal seawater, placed in and assayed at appropriate salinity before measurements commenced.

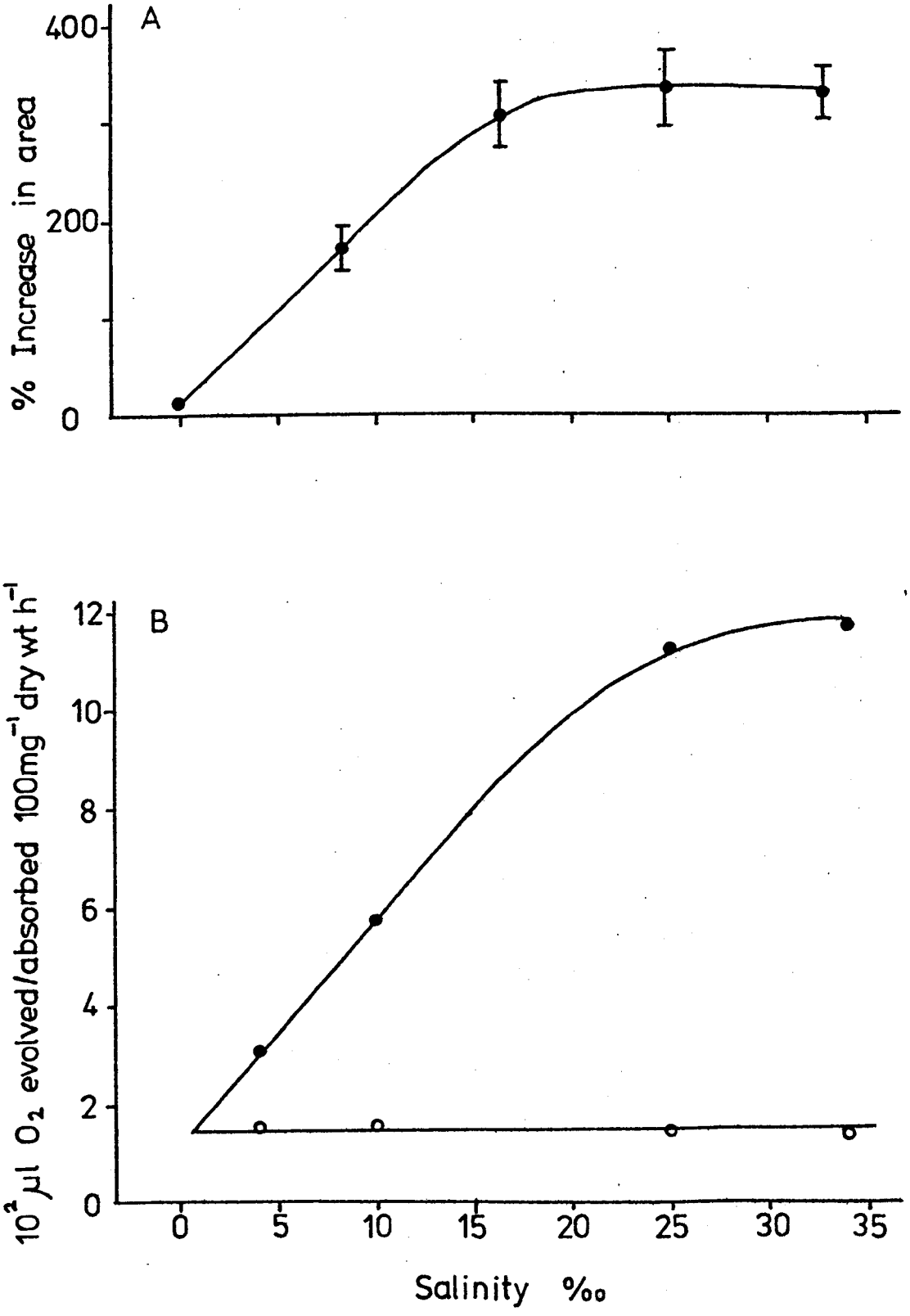
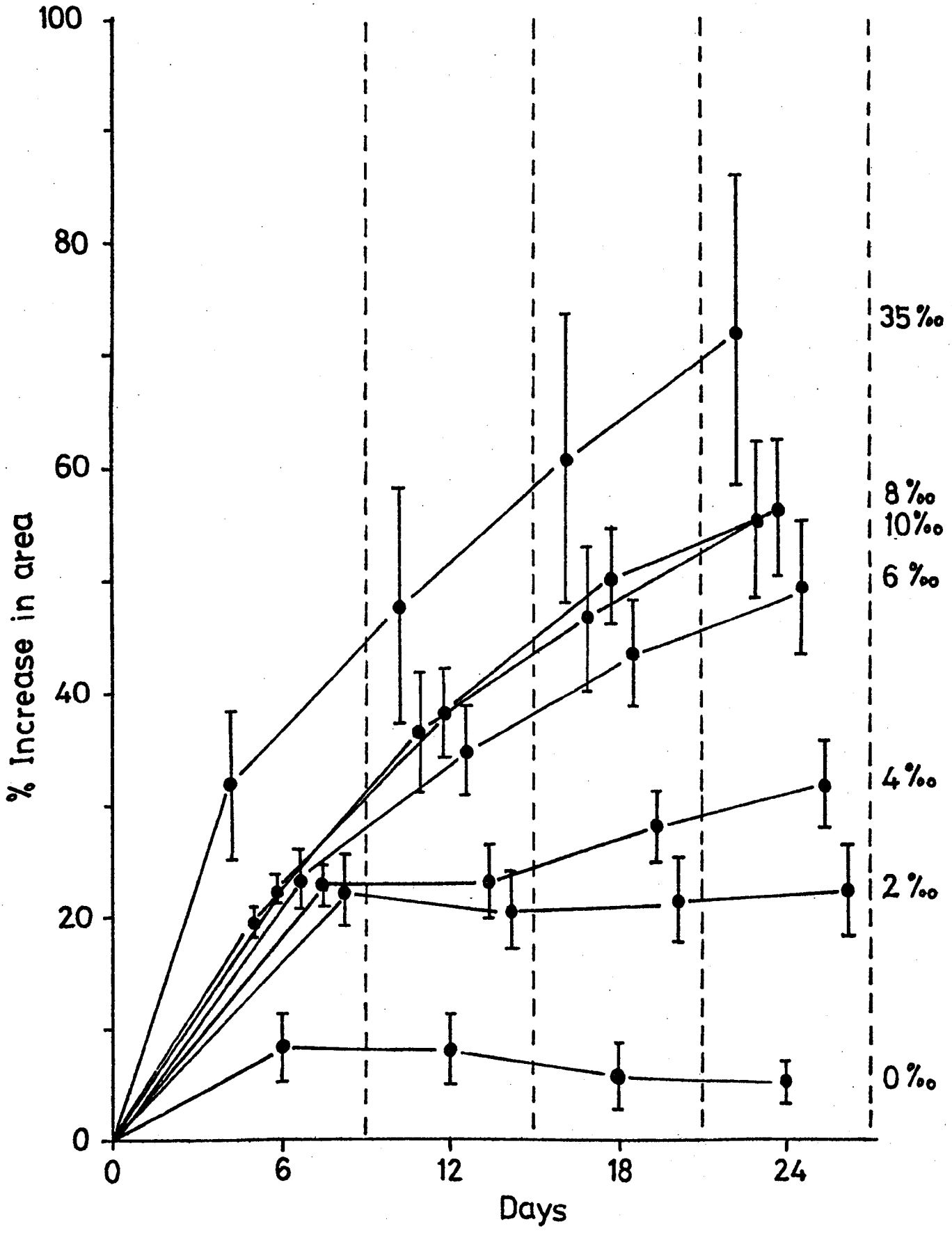

A
400
200
0
% Increase in area
B
12
10
8
6
4
2
0
10^2 µl O_2 evolved/absorbed 100mg^{-1} dry wt h^{-1}
0
5
10
15
20
25
30
35
Salinity ‰

Fig. 13. Percentage increase in area of Ulva discs over 24 days in media of low salinities. Values show mean and twice standard errors on each side of mean.


100
80
60
40
20
0
% Increase in area
0
6
12
18
24
Days
35‰
8‰
10‰
6‰
4‰
2‰
0‰

detected in the disc samples from reduced salinity cultures of 0, 2, 4, 6, 8 and 10‰, whereas under normal sea salinity Ulva cells measurements are very constant within the range 15-18 μm in length and 10-13 μm in width.

Similar graded responses to salinity were reflected in the photosynthetic measurements (fig. 12B). Photosynthetic rates of Ulva discs were virtually the same in salinities of 34 and 25‰. At 10‰ and at 4‰ photosynthetic rates decreased to 57% and 33% respectively of that of discs in full strength seawater. Dark respiratory rates were measured, but they were similar in all treatments (fig. 12B).

The above experiments showed that Ulva was remarkably tolerant of reduced salinities down to at least half that of normal full-strength seawater, but the growth photosynthetic and respiratory rates achieved with full salinity were never exceeded with reduced salinity. E-S medium, having a salinity of around 33‰, is thus suitable for the culture of Ulva.

4.2.3. Light intensity.

4.2.3.1. Method.

Ulva discs were cultured for 30 days using the following light intensities: 0.5, 1.0, 2.0, 3.0 and 4.0 Klux. These were obtained by covering the culture tanks and lids with a combination of the appropriate amount of black and white gauze and polyethylene sheets. All other culture conditions were as in the duration of culture experiments. Ulva disc diameters were measured every 6 days.

Photosynthesis of Ulva discs was also measured under 0, 2, 4, 6, 8 and 10 Klux of light at a temperature of 12°C.

4.2.3.2. Results.

The growth responses of Ulva discs under different light intensities are given in figures 14 and 15. Figure 14 shows percentage increase

in area of Ulva discs against time. From the graph it is clear that under limited light intensity (1 Klux or below) growth was linear with time up to the end of the culture period. At higher light intensity (2 Klux or above) growth was linear for the first 12 days or so but then became exponential. This growth pattern at high light intensity (2 Klux or above) confirms the earlier findings in the duration of culture experiment with a 4 Klux light level. Growth of Ulva discs increased linearly with light intensity as shown in figure 15. At 4 Klux there was still no sign of light saturation.

With light intensities of up to about 4 Klux net photosynthetic rate of Ulva discs increased linearly with light intensity (figure 16). The light saturation point at the tested temperature was around 8-10 Klux, but increase in rate between 6 and 10 Klux was only about 13%. Growth and net photosynthetic rate thus correlate very well. Four Klux, the highest intensity tested, and also the highest which could be provided in the available culture system,was used in all future culture work. Thus, because of faster growth at the higher light intensity, the culture period could be kept short and ensured that development of any response to environmental variables other than light was not light limited - a very important feature.

4.2.4. Temperature.

4.2.4.1. Method.

An experiment was designed to find out the effects of temperature on the growth of Ulva discs in culture. Discs of Ulva were placed in culture tanks situated in thermostatically controlled water baths at the following temperatures: 6, 12, 18, 24 and 30°C. All other culture conditions were as in the duration of culture experiment (section 4.2.1.). The experiment lasted for 14 days, at the end of which the diameter of the discs (50 per treatment) was measured and percentage increase in

Fig. 14. Culture of Ulva discs under different light intensities over a period of 30 days.

Values are means of 50 discs.

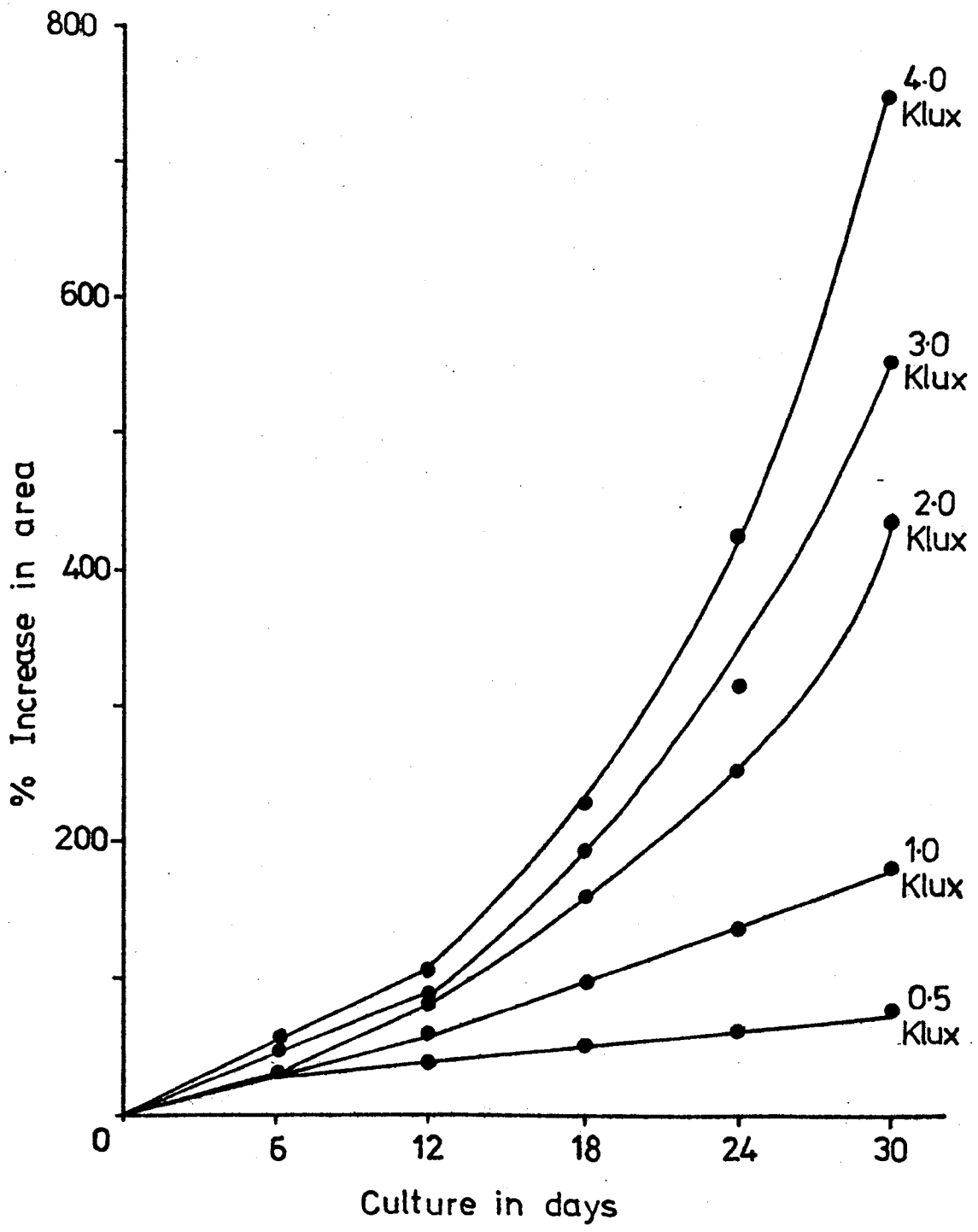
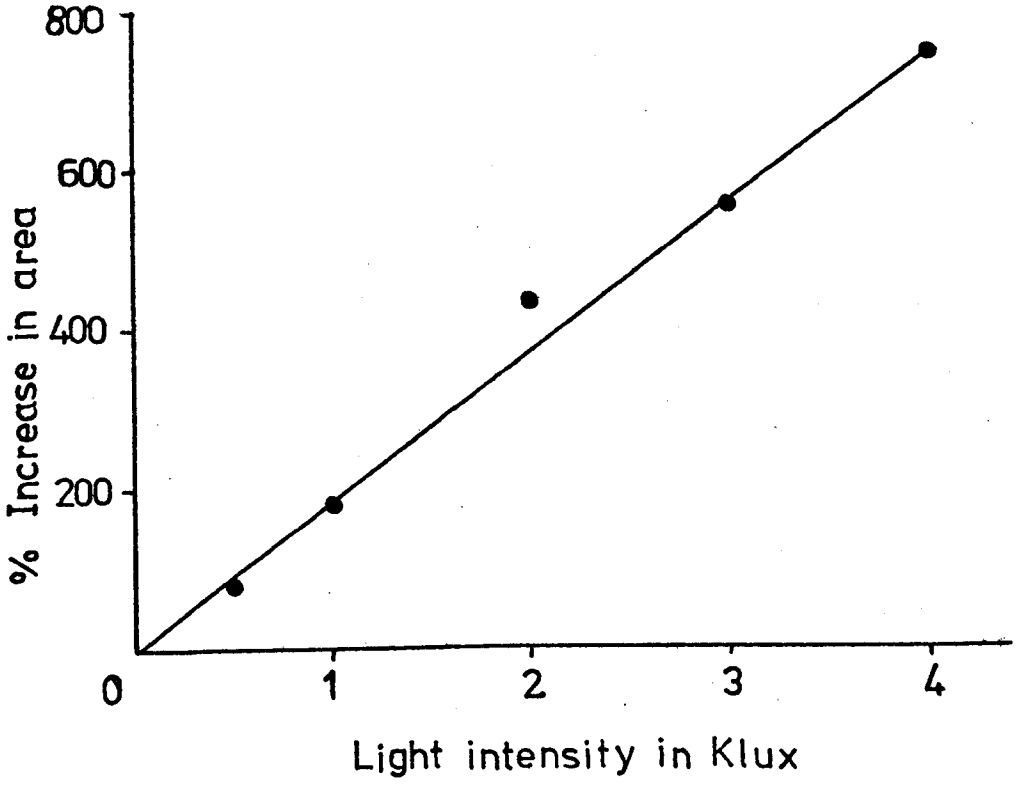
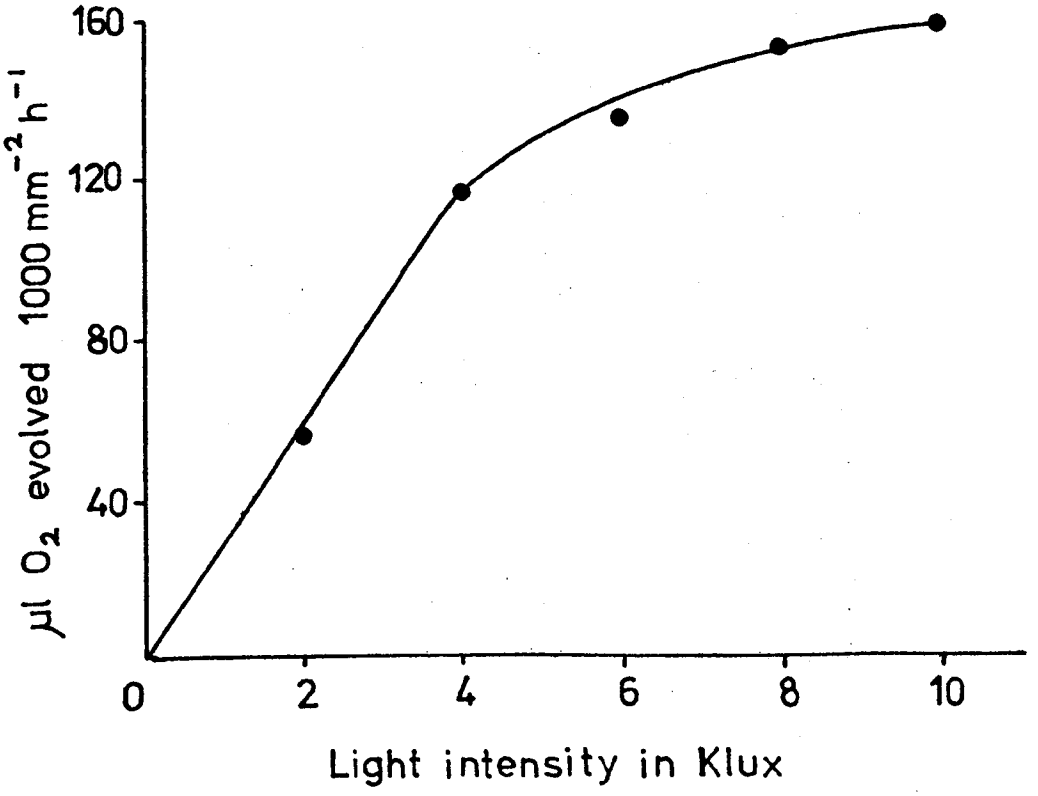

800
600
400
200
0
% Increase in area
4·0 Klux
3·0 Klux
2·0 Klux
1·0 Klux
0·5 Klux
6
12
18
24
30
Culture in days

Fig. 15. Ulva disc growth as a function of light intensity at the end of 30 days culture.
Values are means of 50 discs.

Fig. 16. The effect of light intensity on the photosynthetic rate of Ulva discs.
Values are means of triplicates.


800
600
400
200
0
% Increase in area
1
2
3
4
Light intensity in Klux


160
120
80
40
0
µl O2 evolved 1000 mm-2 h-1
2
4
6
8
10
Light intensity in Klux

area calculated.

Both photosynthetic and respiratory rates of Ulva were determined under the same temperature range using 10 Klux light intensity.

4.2.4.2. Results.

The results are summarized in figures 17 and 18. In culture, the growth rate of Ulva increased with temperature up to between 12 and 18°C where the optimum rate occurred. To determine a more exact optimum temperature under these culture conditions more observations between 12 and 18°C would be required. The growth rate decreased when the temperature was beyond 18°C and at 30°C the discs were dead half way through the culture period.

Measurements of net photosynthesis and respiration are given in figure 18. Net photosynthetic rate increased exponentially with temperature to around 24°C where the highest rate occurred. This was followed by a slight drop with further increase in temperature. Interestingly the respiration pattern was different in that the respiration rate increased linearly throughout the whole range of temperatures tested.

A temperature of 12°C was selected for future culture work. Higher temperatures up to approximately 18°C although increasing growth rate slightly also encourage the undesirable growth of blue-green algae in the cultures (see discussion below).

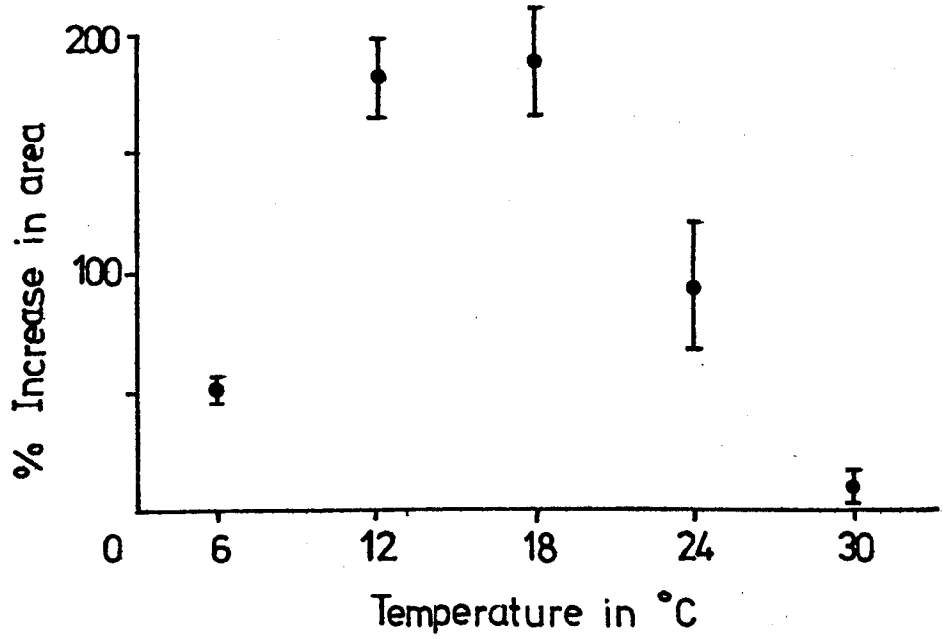
4.2.5. Discussion.

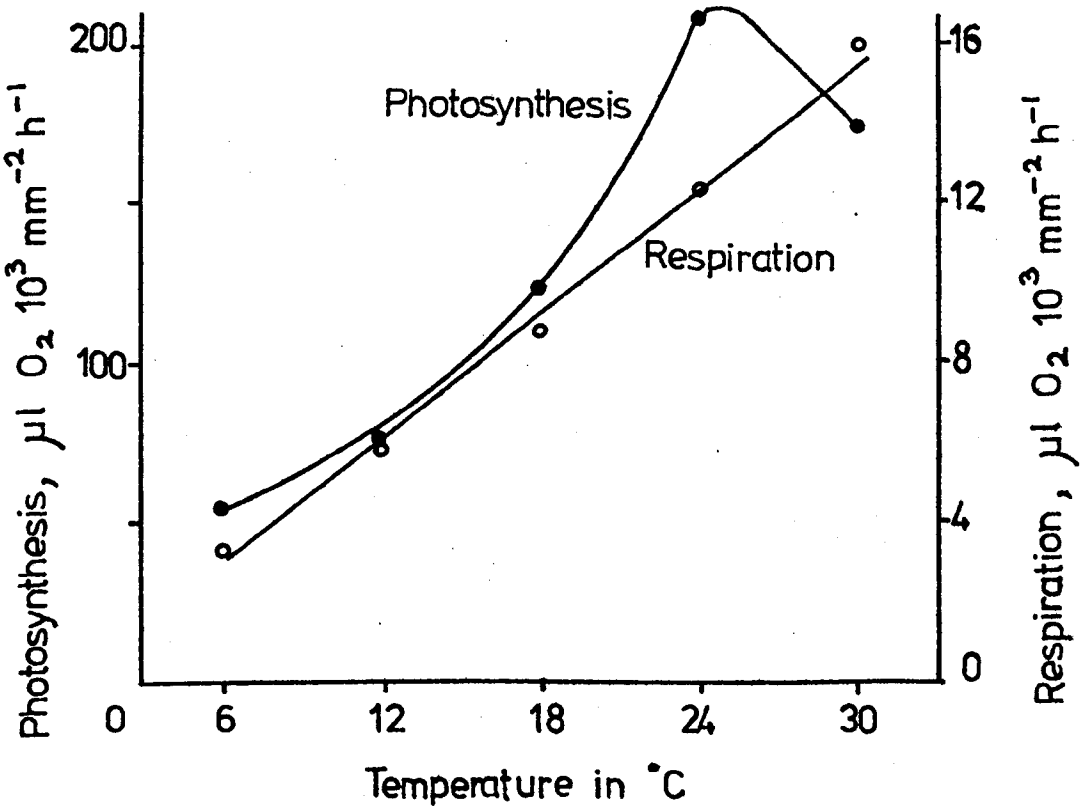
Growth of discs cut from Ulva plants was seen to be very similar to whole plants, both with respect to rate and time (compare fig. 11B with 9). This emphasises that cut portions of Ulva thallus behave in a very similar way to whole plants and thus supports the validity of using the more convenient discs. The suggested reduction of growth rate over the last two days of the first culture (figure 11A) might have

Fig. 17. Effect of temperature on the growth of Ulva discs over 14 days of culture. Values shown are mean with twice standard deviation either side of mean.

Fig. 18. Effects of temperature on the photosynthesis and respiration of Ulva over 2 hour period.

net photosynthesis —●—●— respiration —o—o—


% Increase in area
200
100
0
6
12
18
24
30
Temperature in °C


Photosynthesis
Respiration
Photosynthesis, µl O2 10³ mm⁻² h⁻¹
Respiration, µl O2 10³ mm⁻² h⁻¹
200
100
0
16
12
8
4
0
6
12
18
24
30
Temperature in °C

been caused by damage done to the discs as a result of frequent measurements throughout the experiment. There is indication that growth, in terms of area increase, in Ulva may take place largely during the photoperiod. Measurements at day 10 (fig. 11A) were made at 0900 hours instead of the usual 1600 - 1700 hours period. The values recorded were lower than would have been expected if growth rate was the same throughout a 24 hour period. It has been reported (Lövile, 1964; Kapraun, 1970; Braten & Lövile, 1968) that most cell division in Ulva occurred around midnight. If this is so then the process of cell expansion following cell division must take a considerable period of time to complete as area increase still occurs in the daytime. Whether this actually is the case could be tested by alternating the daily measuring time, (e.g. measuring at 0900 and 2100 hours on alternate days) to see whether the daily growth measurement recorded varies accordingly.

Experiments showed that U. lactuca was remarkably tolerant to reduction in salinity down to half (ca. 16.5‰) the strength of normal seawater. Photosynthetic measurements showed similar results (fig. 12) - i.e. insensitivity at medium dilution but much inhibition at low salt concentration. This agrees well with results obtained by Gesmer & Hammer (1960) who reported that photosynthesis in U. lactuca decreased by 77% when transferred from seawater to freshwater. The present result showed a drop of 67% in photosynthetic rate when seawater was diluted to 4‰. Kjeldsen (1967) and Ogata & Matsui (1965a, b) showed similar decreases in photosynthetic rates with seawater dilution in U. expansa and U. pertusa. Kjeldsen (1967), in agreement with this work (fig. 12), also found that photosynthesis was more sensitive than respiration to dilution. This was due he said to the fact that lowering the salinity (and pH) changed the equilibrium in the CO_2 supply system in seawater and consequently reduced the CO_2 supply for

photosynthesis. Respiration was less affected by the availability of CO_2 and hence it was less sensitive to dilution effects. Occasionally enhancement of photosynthesis at moderate dilution (down to around 20‰) has been reported (Kjeldsen, 1967; Legendre, 1921), but this was never observed in this investigation. Thus results obtained in this work, as well as those by other workers, lead to the conclusion that Ulva is tolerant to a fairly wide range of salinity - but grows best at maximum salinity. Marked inhibition of growth and photosynthesis occur however when the salt concentration falls to around 10‰. Such low concentrations cause visible changes in the plant, e.g. cells become swollen, chlorophyll levels fall or in severe cases death occurs, probably as a result of the osmotic shock and deprivation of carbon dioxide supply at low salinity. On many occasions it was found that low salinity caused an inhibition of swarming in U. lactuca. A similar observation was reported by Mohsen, Nasr & Metawalli (1972) for U. fasciata.

Observations in the field on the distribution of Ulva lactuca in relation to salinity variation agree well with the laboratory findings. In the River Mersey, U. lactuca was found growing as far up the river as Grassendale and Eastham Locks. Here salinity of water covering Ulva often falls to around 17‰. Many other workers have reported similar observations in other areas (e.g. Sudene, 1953; Wood & Palmatier, 1954; Gillham, 1957; Nelson-Smith, 1967). It must be remembered that in the field exposure to low salinity is often transient, so that strict comparisons between performance in the field and laboratory would have to take account of length of period of exposure to reduced salinity, e.g. 4 hours exposure per day to 8‰ water would not be likely to affect performance in the same way as a full 24 hours in the same water. Similarly one cannot equate "average" field salinity with fixed laboratory

salinity performance.

At low light intensities of 1.0 or 0.5 Klux it was shown (fig. 14) that Ulva growth was linear with time for at least 30 days. This was probably due to growth restriction under insufficient light intensity. With higher light intensities of 2 to 4 Klux, however, growth was exponential after an initial linear phase. This linear phase is probably due to adaptation of the Ulva discs to the growth conditions in the laboratory. A linear relationship between light intensity and Ulva growth rate occurred up to 4 Klux, the highest value tested (as shown in fig. 15). This was similarly reflected in the photosynthesis experiment (fig. 17). Here, net photosynthetic rate increased linearly with light intensity to around 4 Klux; beyond this, light saturation with respect to photosynthesis was soon approached. The rate of maximum photosynthesis was temperature dependent, and at 12°C light saturation occurred at around 8 to 10 Klux. Since photosynthetic saturation in Ulva occurs at light levels only a fraction of the intensity of full sunlight (value between approximately 40 and 80 Klux according to season), it means that providing CO_2 is not a limiting factor, the alga growing in shallow water in the field would photosynthesise most of the day at its maximum capacity. This may also partially explain why Ulva can grow very well in fairly turbid conditions such as in harbours and estuaries where light intensities incident on the plant will be low.

In the laboratory Ulva was not very tolerant of high temperature (fig. 17). This may have been due to competition with a contaminant filamentous blue-green alga growing on the surface of the Ulva discs in culture at 24°C and especially at 30°C. Slight contamination was also observed in the 18°C culture. There is probably more tolerance of higher temperatures in nature since Ulva is known to grow well in the summer in temperatures up to around 25°C. One point which should

not be overlooked is that in a field situation the temperature fluctuates, high temperatures are only maintained for a few hours at a time whereas in the culture experiment they were constant over a period of two weeks. While photosynthesis increased exponentially with rise in temperature (fig. 18), respiration increased only linearly. These results do not agree with those obtained by Kanwisher (1966) who reported for U. lactuca that oxygen utilization increased exponentially with temperature increase between 0 and 30°C. It should be stressed that respiration measured in this experiment, and in Kanwisher's work, was dark respiration. No data were obtained for respiration of Ulva in the light. Hardwick (personal communication) has showed evidence of Ulva having a very low photorespiration value and this may mean that the alga possesses the C4 - dicarboxylic acid pathway for photosynthesis and thus is highly efficient in its assimilation of CO_2 at low light intensities.

In view of the foregoing findings and available culture facilities the following conditions were adopted for future work:

i) duration of culture 14 to 21 days - giving a significant period of growth free from the adaptation to culture phase, and 150 - 300% increase in area,

ii) salinity 33‰ - which is optimal,

iii) light intensity 4 Klux - approaches the optimal for photosynthesis, and is the highest intensity readily maintained in culture tanks.

iv) temperature 12°C - approaches the optimal for growth and is below that producing possible troublesome growth of blue-green algae.

CHAPTER 5.

Growth of *Ulva lactuca* in nutrient enriched seawater

5.1 Introduction.

There has been a substantial amount of investigation into the effects of nutrients (e.g. nitrogen, phosphorus) on the growth of micro-algae e.g. O'Kelley, 1968; Morris, 1973. However very little is known about nutrient utilization in the macro-algae - including *Ulva*.

Before an attempt is made to use *Ulva* as an indicator for pollution it is necessary to have a comprehensive knowledge of how *Ulva* responds to major nutrients, or even to the different forms of the same nutrient. Thus for example, it is not only necessary to know how nitrogen as a nutrient affects the growth of *Ulva*, but also how *Ulva* responds to different sources of nitrogen (e.g. as nitrate, nitrite and ammonia).

Excessive *Ulva* growth in sewage-polluted areas has been reported previously on many occasions (e.g. Letts & Richards, 1911; Wilkinson, 1964). A detailed study into the effects of the nutrients on *Ulva* could help to explain this excessive growth response if the stimulation is caused by the nutrient fraction of the sewage. Hence with a knowledge of response to various nutrients in the laboratory it may be possible to relate these data to the growth and distribution of *Ulva* in polluted areas.

The effects of the following nutrients and growth substances on the growth rate of *Ulva* were examined: nitrogen, as nitrate, nitrite and ammonia, phosphorus as phosphate; acetate as a form of organic carbon; sewage contaminated mud; and the growth substances kinetin and adenine. Under aseptic conditions the addition of both kinetin and adenine were found (Provasoli, 1958) necessary in order to maintain normal growth of *Ulva*. All culture experiments, unless otherwise stated, were performed under tank-room culture conditions described in the general methods section 3.2.2.

5.2. Selection of control.

In every bioassay test a control is needed for comparative purposes. Earlier experiments using limited volume culture indicated that Ulva stopped growing after about a week in a medium of seawater collected from the Irish Sea without any nutrient enrichment. This would seem likely to be caused by the lack of nutrients and/or growth substances. To support a continual and active growth Erdschreiber medium (E-S medium) was the control medium selected for this work. The composition of E-S medium is given in section 3.2.2. This medium has been employed to culture macro-algae in this laboratory (Burrows, 1961) for many years and has given active growth and consistent results. It is important to bear in mind however that it is not a precisely defined medium. There are two components, seawater and soil extract, that vary in composition. Both of these components contain variable amounts of nitrate and phosphate. Analysis of the major nutrient fractions, nitrate and phosphate, in both seawater and soil extract have been made and it was found that together they contributed only a small proportion (NO_3-N .06 mg dm^{-3}, PO_4-P .03 mg dm^{-3}) of these particular nutrients. Since relatively much larger amounts of nitrate and phosphate (30 and 11 times respectively) are present in the salt solution fraction of E-S medium (when compared with the amounts present in seawater and soil extract) it would mean that effectively the composition of E-S medium is acceptably constant. This would seem to be so in practice since numerous experiments throughout the whole period of this work with E-S medium produced growth rates of discs which were not significantly different at 95% confidence limits. These consistent results give support to the use of E-S medium as a suitable control medium for the present work. Furthermore, when cultured in E-S medium in the laboratory Ulva had a mass to area ratio, chlorophyll and protein

content similar to plants collected fresh from the Hilbre Islands. With all these findings, it was concluded that E-S medium provides a suitable control medium for Ulva growth reference. This view was further supported by much additional circumstantial evidence at later stages in this work.

5.3. Nutrient bioassay tests.

5.3.1. Nitrate-nitrogen (NO_3-N).

5.3.1.1. Method.

The growth rate of Ulva in culture was tested in different concentrations of NO_3-N, as sodium nitrate, ranging from 0 to 38.9 mg dm^{-3} NO_3-N in 8 steps. Both soil extract and the standard amount of phosphate as specified in E-S medium were added to every culture. Thus the added nutrients in the test cultures consisted of:

(1) Soil extract (as specified in E-S medium),

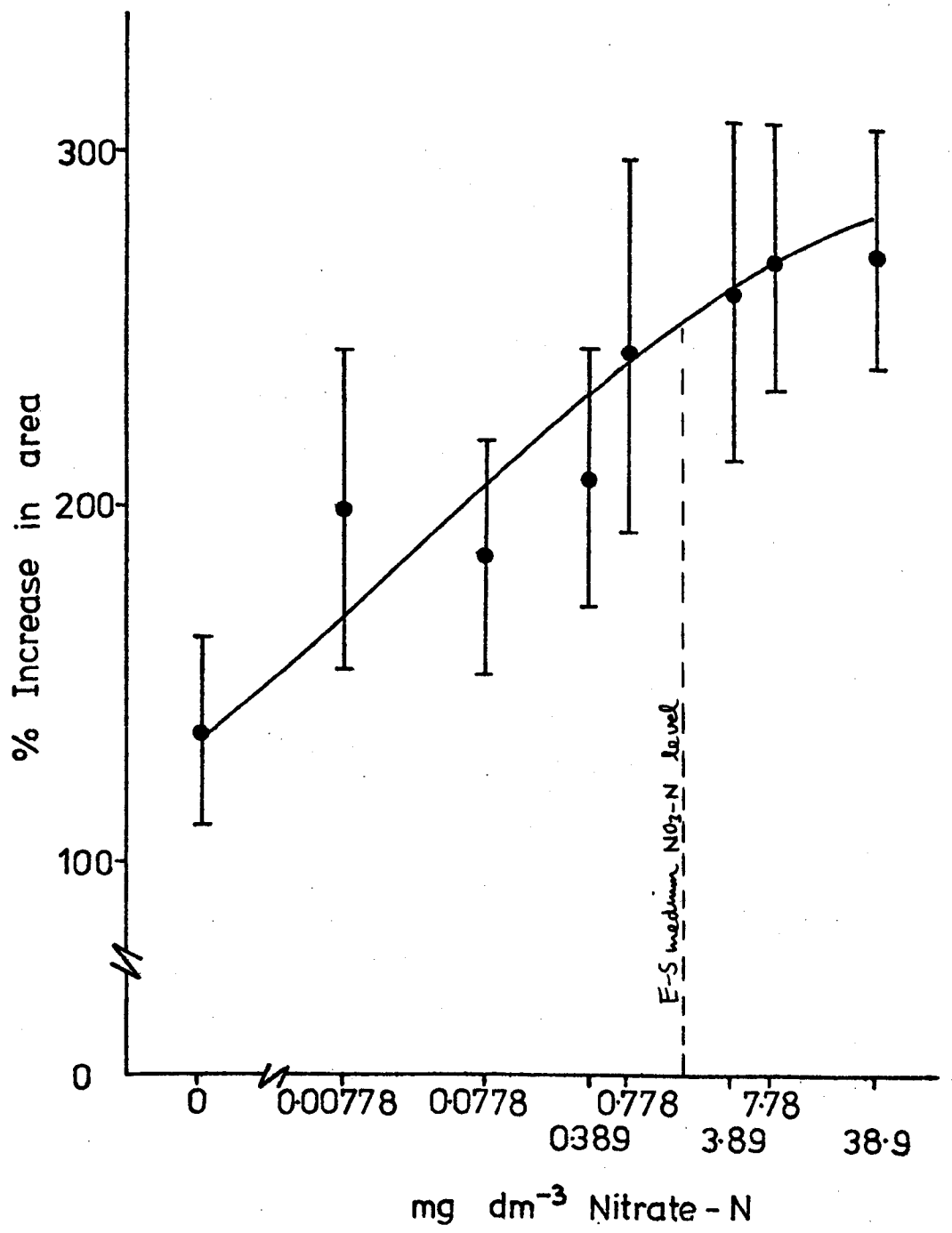
(2) Phosphate ("),

and (3) the appropriate amount of nitrate under the tank-room regime (see section 3.2.2). The culture period was 14 days. Chlorophyll and protein contents of the Ulva samples from each culture were determined using methods indicated in section 3.2.7 and 3.2.6 respectively. Triplicates containing 10 mg dried Ulva were used for each analysis, from each of the NO_3-N treatments.

5.3.1.2. Results.

The results of the culture tests are given in figure 19. These show an initial phase of sharply increasing growth rate of Ulva with increasing NO_3-N concentration from 0 mg dm^{-3} - 3.89 mg dm^{-3}, followed by a second phase where growth still increased with increasing concentration up to 38.9 mg dm^{-3}, (269% area increase) but much less markedly, suggesting a saturation level is reached around the higher NO_3-N level tested. E-S medium contains 1.7 mg dm^{-3} NO_3-N.

Fig. 19. Growth of Ulva discs after 14 days in different concentrations of NO_3, values plotted as mean percentage increase in area with twice standard errors on either side of the mean.


300
200
100
0
% Increase in area
E-S medium NO3-N level
0
0·00778
0·0778
0·778
7·78
0·389
3·89
38·9
mg dm-3 Nitrate - N

Chlorophyll analysis of the Ulva discs (fig. 20A) showed that cultures with low concentrations of nitrate, from 0 to 0.778 mg dm^{-3} NO_3-N, produced relatively low and very similar amounts of chlorophyll in Ulva of around 2 µg chlorophyll per 10 mg dry weight. There was a rapid increase in chlorophyll content from 2 to around 7.5 µg 10 mg^{-1} dry weight when the nitrate concentration in culture rose from 0.778 to 3.89 mg dm^{-3}-N. Further increase in NO_3-N produced no significant increase in chlorophyll level. Protein analysis (fig. 20B) revealed a trend in change of protein with NO_3-N level very similar to that of chlorophyll.

5.3.2. Ammonium-nitrogen (NH_4-N).

5.3.2.1. Method.

Two culture tests with a range of NH_4-N from 0 to 38.9 mg dm^{-3} NH_4-N were carried out. The first culture was under the cold-room culture conditions while the second was in the tank-room culture regime (refer to section 3.2.2). A third test employing a more limited range of NH_4-N (0.389 to 7.78 mg dm^{-3} NH_4-N) which was found to give significant stimulation of growth rate of Ulva was carried out after the completion of the first two cultures to determine more precisely the optimum NH_4-N level and the range giving growth stimulation. Ammonium chloride was the salt used for NH_4-N supply in culture. Apart from the appropriate amounts of NH_4-N, both soil and salt extracts were added to all cultures. The nutrient cultures thus consisted of:

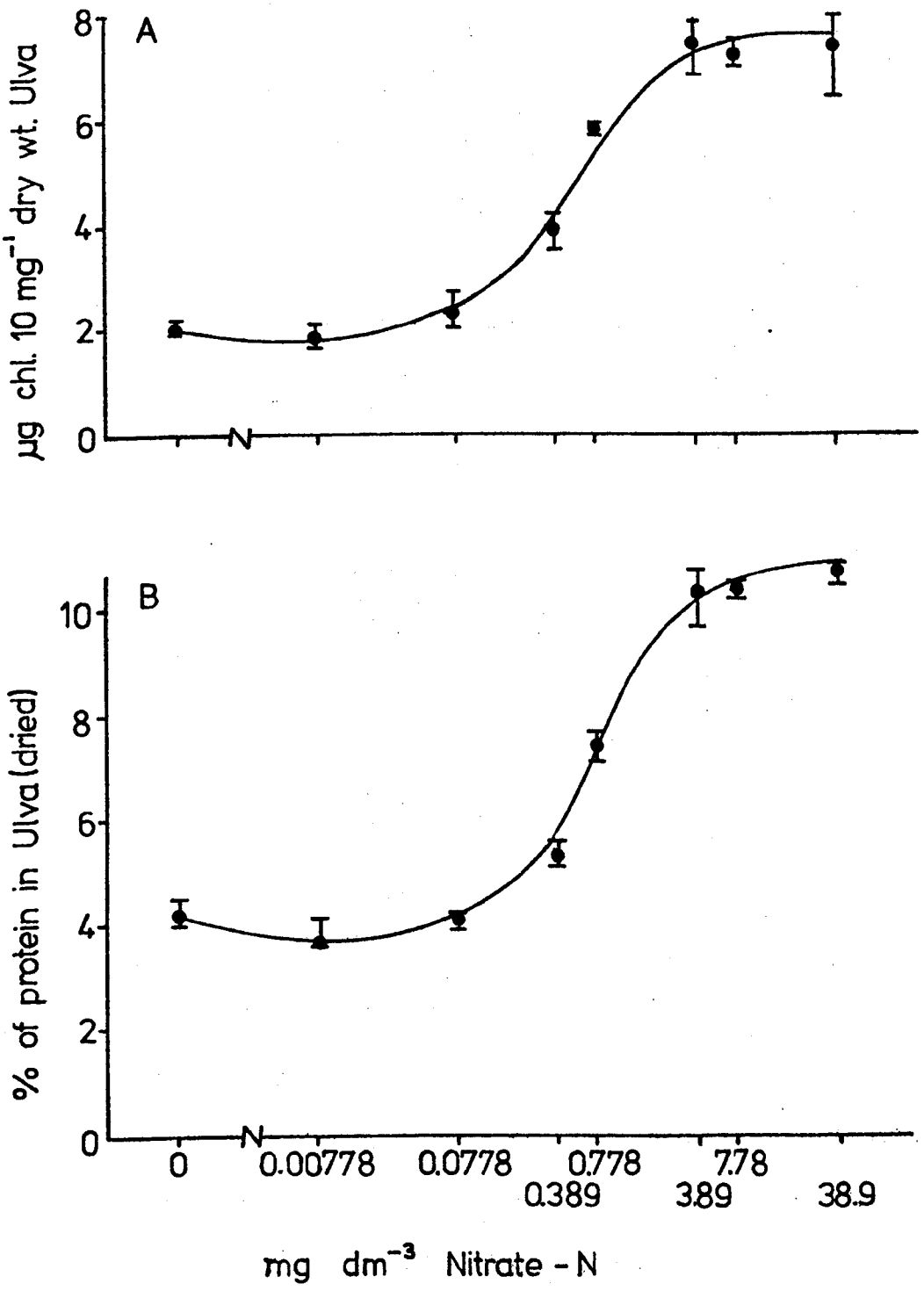
(1) Soil extract (amount as specified in E-S medium),

(2) Nitrate and phosphate ("), and

(3) the appropriate amounts of ammonium ions.

Chlorophyll and protein contents of the Ulva samples were analysed for the second culture series.

Fig. 20. Analysis of Ulva discs grown in various concentrations of NO_3-N, A chlorophyll content, B protein content. Mean value and range are shown.


A
µg chl. 10 mg⁻¹ dry wt. Ulva
8
6
4
2
0
N
B
% of protein in Ulva (dried)
10
8
6
4
2
0
N
0
0.00778
0.0778
0.778
0.389
7.78
3.89
38.9
mg dm⁻³ Nitrate - N

5.3.2.2. Results.

The three graphs in figure 21 show results of the culture tests for Ulva growth rate in ranges of added NH_4-N. In the first and second cultures where a wide range of NH_4-N was used, significant stimulation of growth in Ulva over the control of E-S medium (fig. 21A, B) occurred between 0.389 and 38.9 mg dm^{-3} NH_4-N and 0.389 and 7.78 mg dm^{-3} NH_4-N respectively. This difference in the range of stimulation in the two cultures might have been due partly to the short duration (7 days) of the first culture and partly to the different culture conditions. Greatest growth stimulation in both cases, however, was found to be at 3.89 mg dm^{-3} NH_4-N where growth rate was 57% more than the control in the first culture whilst in the second it was 82% more. At 38.9 mg dm^{-3} NH_4N the growth was 91% and 33% that of the control in the first and second cultures respectively. This reduction of growth was presumably due to a toxic effect of high concentrations of ammonium ions. In the third culture a narrow range of NH_4-N concentration was used with more values around the area of stimulation to define the limits of significant stimulation. From 0.389 to 3.89 mg dm^{-3} NH_4-N there was a linear increase in growth rate of Ulva over the control (fig. 21C). At 5.835 mg dm^{-3} NH_4-N the growth rate was not significantly lower than at 3.89 mg dm^{-3}, but beyond 5.835 growth stimulation was reduced and at 7.78 mg dm^{-3} NH_4-N was similar to that of the control.

The chlorophyll content of Ulva increased steadily (fig. 22A) from the control with 4.85 µg chlorophyll per 10 mg dry Ulva, to 8.48 µg chlorophyll per 10 mg dry Ulva of discs grown in 3.89 mg dm^{-3} NH_4-N . This was followed by a drop, similar to that in growth rate in the second culture (fig. 21B), in the amount of chlorophyll per unit dry weight present. The percentage of protein in Ulva (on a dry weight basis) increased gradually from 8.1% in the control to 10.5% in tissue

Fig. 21. Growth of Ulva discs after 14 days in various concentrations of NH_4-N, plotted as mean percentage increase in area with twice standard error on each side of the mean. A, wide range of NH_4-N - cold room culture, B, wide range of NH_4-N - tank room culture, C, narrow range of NH_4-N - tank room culture.

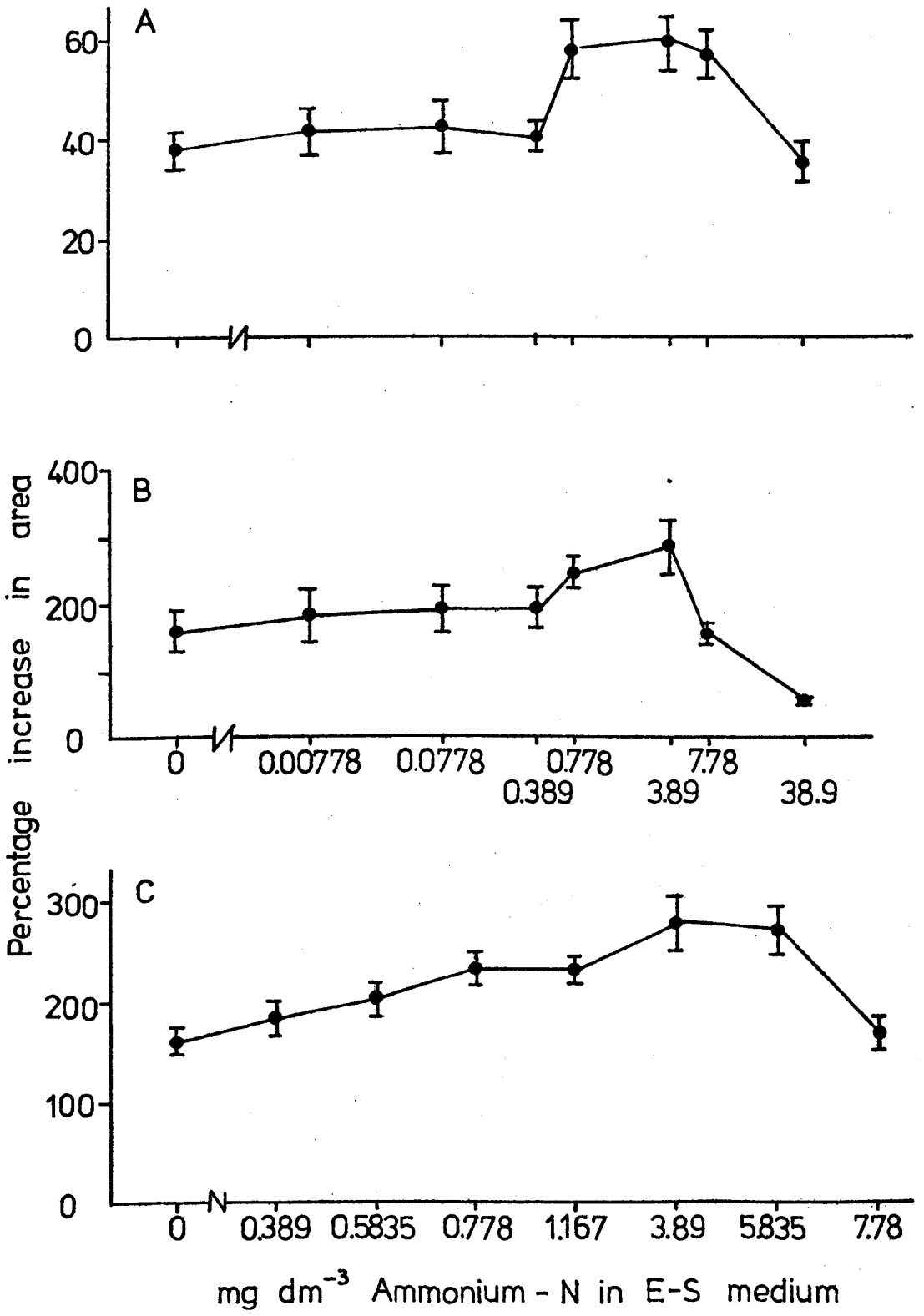
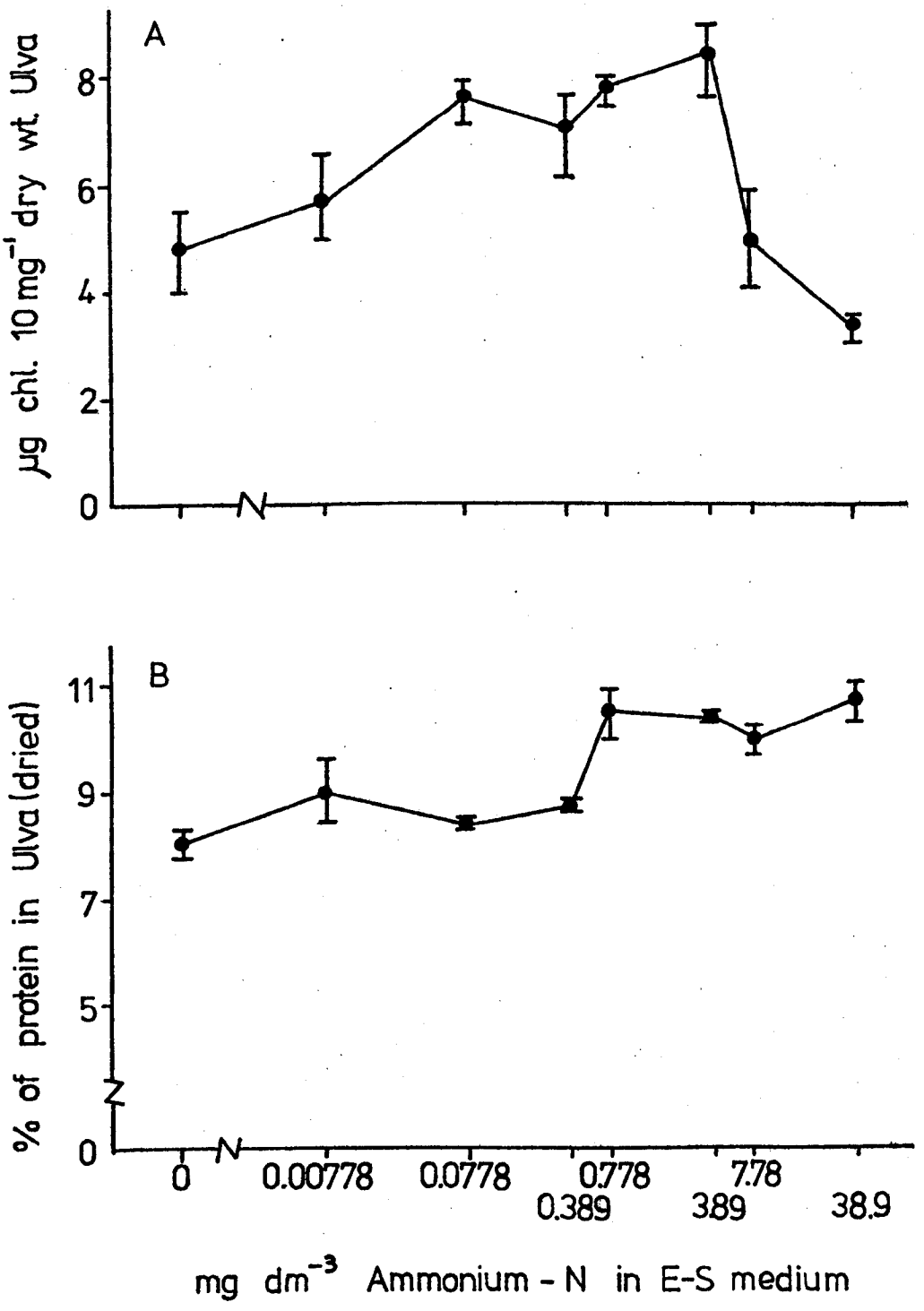

A
60
40
20
0
B
400
200
0
Percentage increase in area
0
0.00778
0.0778
0.778
7.78
0.389
3.89
38.9
C
300
200
100
0
N
0
0.389
0.5835
0.778
1.167
3.89
5.835
7.78
mg dm-3 Ammonium - N in E-S medium

Fig. 22. Analysis of Ulva discs cultured in various concentrations of NH_4-N. A, chlorophyll content, B, protein content. Mean value and range are shown.


A
µg chl. 10 mg⁻¹ dry wt. Ulva
0
2
4
6
8
B
% of protein in Ulva (dried)
0
5
7
9
11
0
0.00778
0.0778
0.778
0.389
7.78
389
38.9
mg dm⁻³ Ammonium - N in E-S medium

from the 0.778 mg dm^{-3} NH_4-N culture, but unlike the situation with growth and chlorophyll further increase in NH_4-N did not result in any reduction of protein, its level remained high to the highest concentration of NH_4-N (fig. 22B) tested. Thus even in the presence of toxic amounts of ammonium ions so far as growth is concerned, the protein content of Ulva remained maximal.

5.3.3. Nitrate-Ammonium-ion mixture.

5.3.3.1. Introduction.

Field water will rarely if ever contain only one nitrogen form, and in nearly all cases the 2 major forms are nitrate and ammonium nitrogen. To test the relative effectiveness of nitrate and ammonium ions as nitrogen sources where the total amount of nitrogen is constant, cultures of a mixture of the two forms of nitrogen were used.

5.3.3.2. Method.

Five cultures were set up with the individual concentrations of nitrate, ammonium ions or a mixture of them as given in the table below:

Table II: The nitrate-ammonium ion combinations used in the experiment.

Culture Number	NO_3-N (mg dm^{-3})	NH_4-N (mg dm^{-3})
1	3.89	0
2	2.92	0.97
3	1.945	1.945
4	0.97	2.92
5	0	3.89

All cultures thus contained a total of 3.89 mg of added nitrogen per dm^{-3} of seawater medium. Soil extract and phosphate were added in each case. Thus the cultures contained nutrient enrichments as:

(1) soil extract (amount as specified in E-S medium),

(2) phosphate (") and

(3) the appropriate amounts of nitrate and/or ammonium ions.

The Ulva discs harvested at the end of the culture period of 14 days (in tank-room conditions, see section 3.2.2), were analysed for growth and chlorophyll and protein contents.

5.3.3.3. Results.

Data from the culture experiment (fig. 23) showed that the growth rate of Ulva was significantly less with nitrogen in the form of nitrate than in all of the mixture treatments of NO_3-N + NH_4-N and when the form of nitrogen was solely ammonium. It is important to stress that the amount of nitrogen present in all treatments was the same. This shows clearly that nitrogen supplied as its ammonium form stimulated the growth of Ulva more than NO_3-N. A mixture of the two nitrogen forms also provided a significantly greater growth rate than NO_3-N used alone. These results support those found in NH_4-N culture experiments (fig. 21A, B and C) in that NH_4-N has a greater potential to stimulate the growth of Ulva than NO_3-N.

A similar pattern of results was obtained from the chlorophyll and protein analysis. Chlorophyll content of Ulva growing in NO_3-N alone averaged 5.10 µg per 10 mg dry weight (fig. 24A) whilst that of tissue from NO_3-N + NH_4-N cultures was between 6.91 and 8.58 µg, per 10 mg dry weight. As for protein content (fig. 24B) the values (on a dry weight basis) were 10.0% for NO_3-N treatment and between 10.4 and 10.9% for NO_3-N + NH_4-N mixtures and for NH_4-N added alone.

5.3.4. Nitrite-nitrogen (NO_2-N).

5.3.4.1. Method.

Sodium nitrite ranging in concentration from 0-38.9 mg dm^{-3} NO_2-N in 8 steps was added together with soil extract and salts solution to Ulva disc cultures. Thus the nutrient enrichment consisted of:

Fig. 23. Growth of Ulva discs after 14 days in different mixtures of NO_3-N and NH_4-N, values plotted as mean percentage increase in area with twice standard error on either side of the mean.

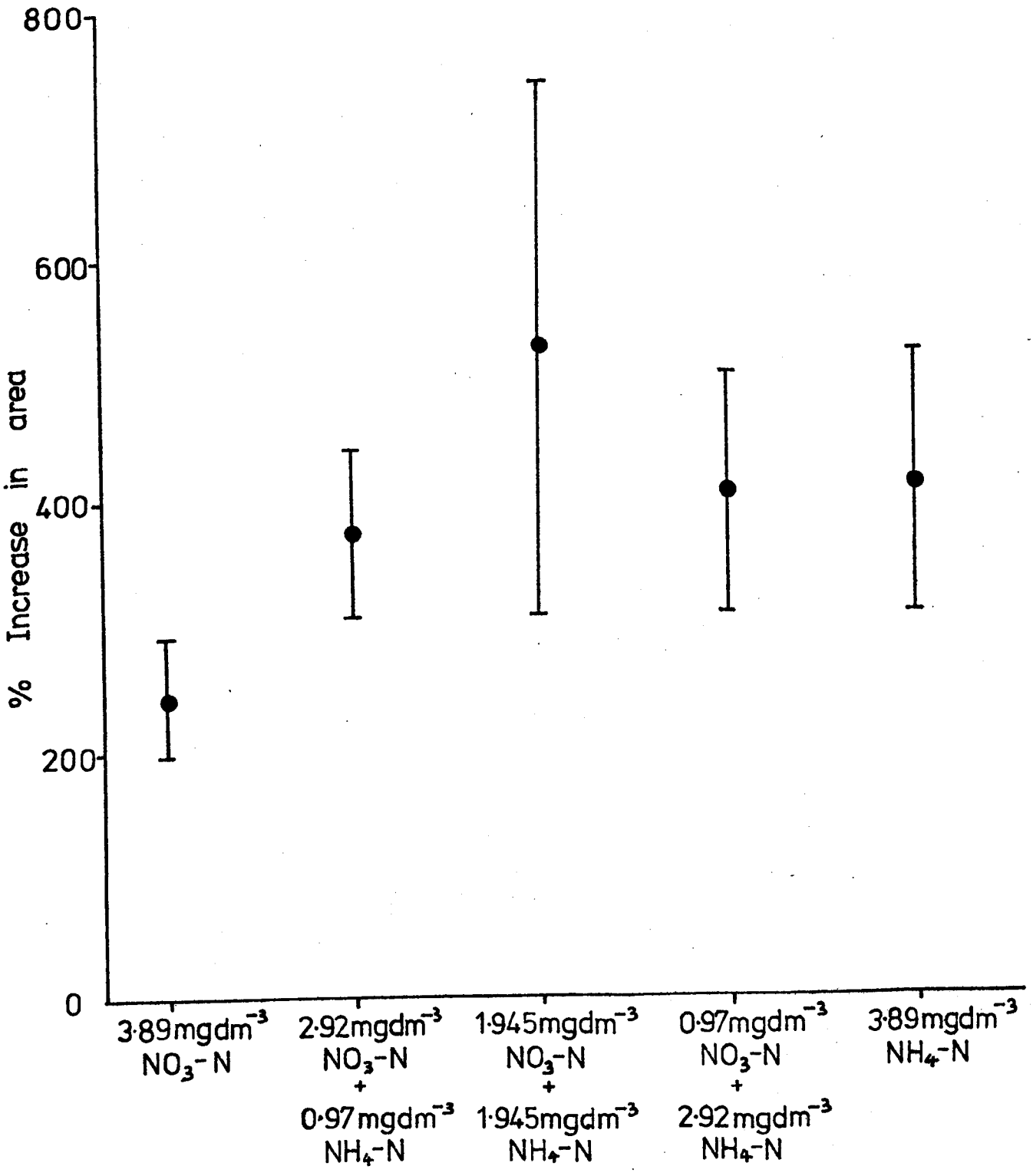
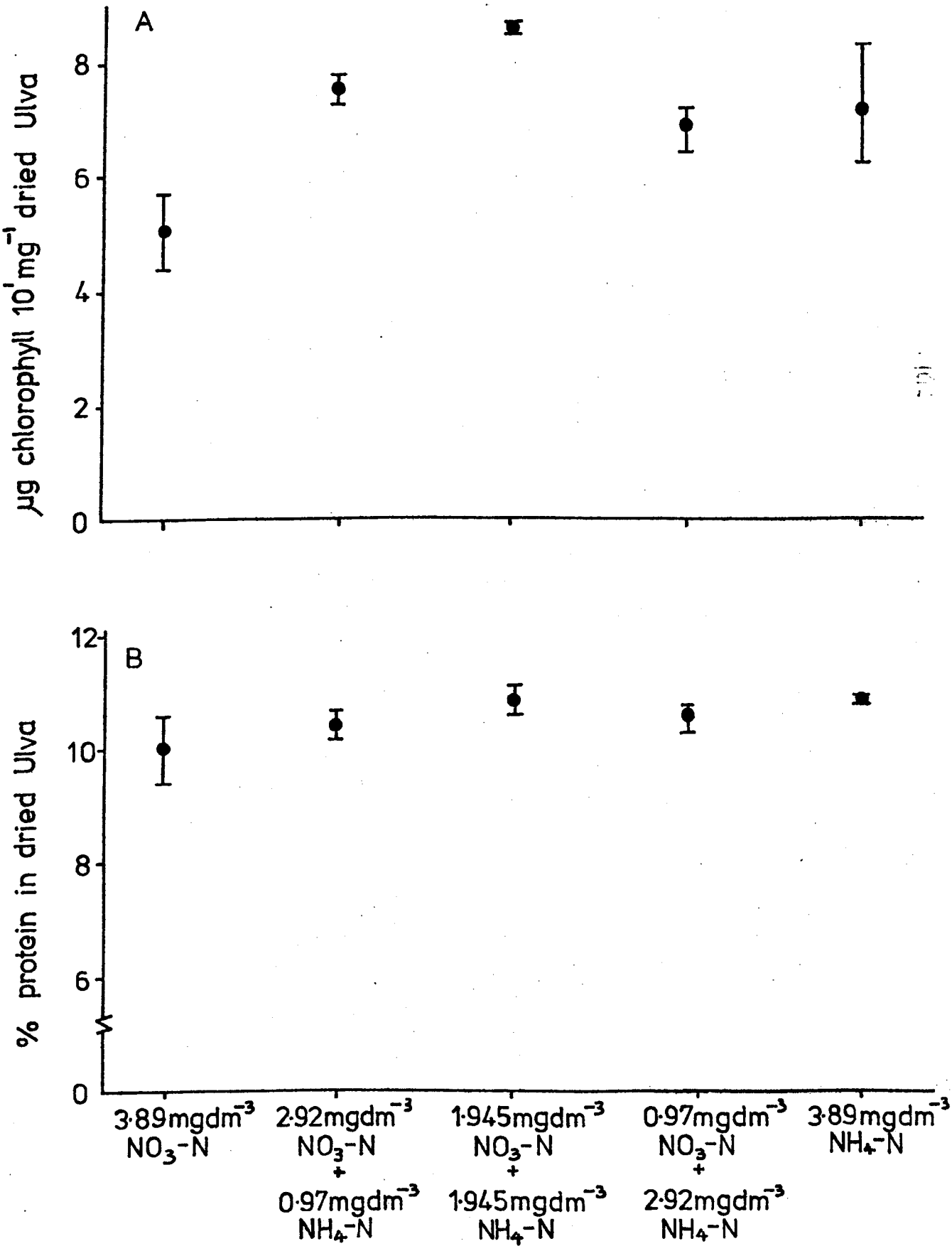

800
600
400
200
0
% Increase in area
3·89mgdm⁻³ NO₃-N
2·92mgdm⁻³ NO₃-N + 0·97mgdm⁻³ NH₄-N
1·945mgdm⁻³ NO₃-N + 1·945mgdm⁻³ NH₄-N
0·97mgdm⁻³ NO₃-N + 2·92mgdm⁻³ NH₄-N
3·89mgdm⁻³ NH₄-N

Fig. 24. Analysis of Ulva discs cultured in mixtures of NO_3-N and NH_4-N, A, chlorophyll content, B, protein content. Mean value and range are shown.


A
µg chlorophyll 10^{1}mg^{-1} dried Ulva
0
2
4
6
8
B
% protein in dried Ulva
0
6
8
10
12
3·89mgdm^{-3} NO$_3$-N
2·92mgdm^{-3} NO$_3$-N + 0·97mgdm^{-3} NH$_4$-N
1·945mgdm^{-3} NO$_3$-N + 1·945mgdm^{-3} NH$_4$-N
0·97mgdm^{-3} NO$_3$-N + 2·92mgdm^{-3} NH$_4$-N
3·89mgdm^{-3} NH$_4$-N

(1) soil extract (amount as specified in E-S medium),

(2) nitrate + phosphate (") and

(3) the appropriate amount of nitrite.

5.3.4.2. Results.

Culture results (fig. 25) indicated that the rate of growth of Ulva decreased with increasing amount of nitrite throughout the whole range of concentration used. At a concentration of 38.9 mg dm^{-3} NO_2-N, growth rate of Ulva was only 28% of that of the control.

5.3.5. Phosphate-phosphorus (PO_4-P).

5.3.5.1. Method.

The effects of different concentrations of PO_4-P, added as disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), on the growth of Ulva in culture were tested. The range of concentration employed was 0 - 50 mg dm^{-3} PO_4-P. Soil extract and nitrate were added to every treatment. Thus the culture medium consisted of the following additions:

(1) soil extract (amount as specified in E-S medium),

(2) nitrate ("), and

(3) the appropriate amount of phosphate.

5.3.5.2. Results.

No significant difference in the area increase was detected with Ulva when cultured in media with between 0 and 10 mg dm^{-3} PO_4-P (fig. 26). However, at the highest concentration tested - 50 mg dm^{-3} - there was a very marked reduction of growth rate in Ulva, it being less than 2% of the usual rate. This reduction in growth rate could have been partly caused by the formation of crystalline "sheets" of phosphate covering the surface of the discs and hence reducing the amount of light available for photosynthetic assimilation.

Fig. 25. Growth of Ulva discs after 14 days in various concentrations of NO_2-N, values plotted as mean percentage increase in area with twice standard error on either side of mean.

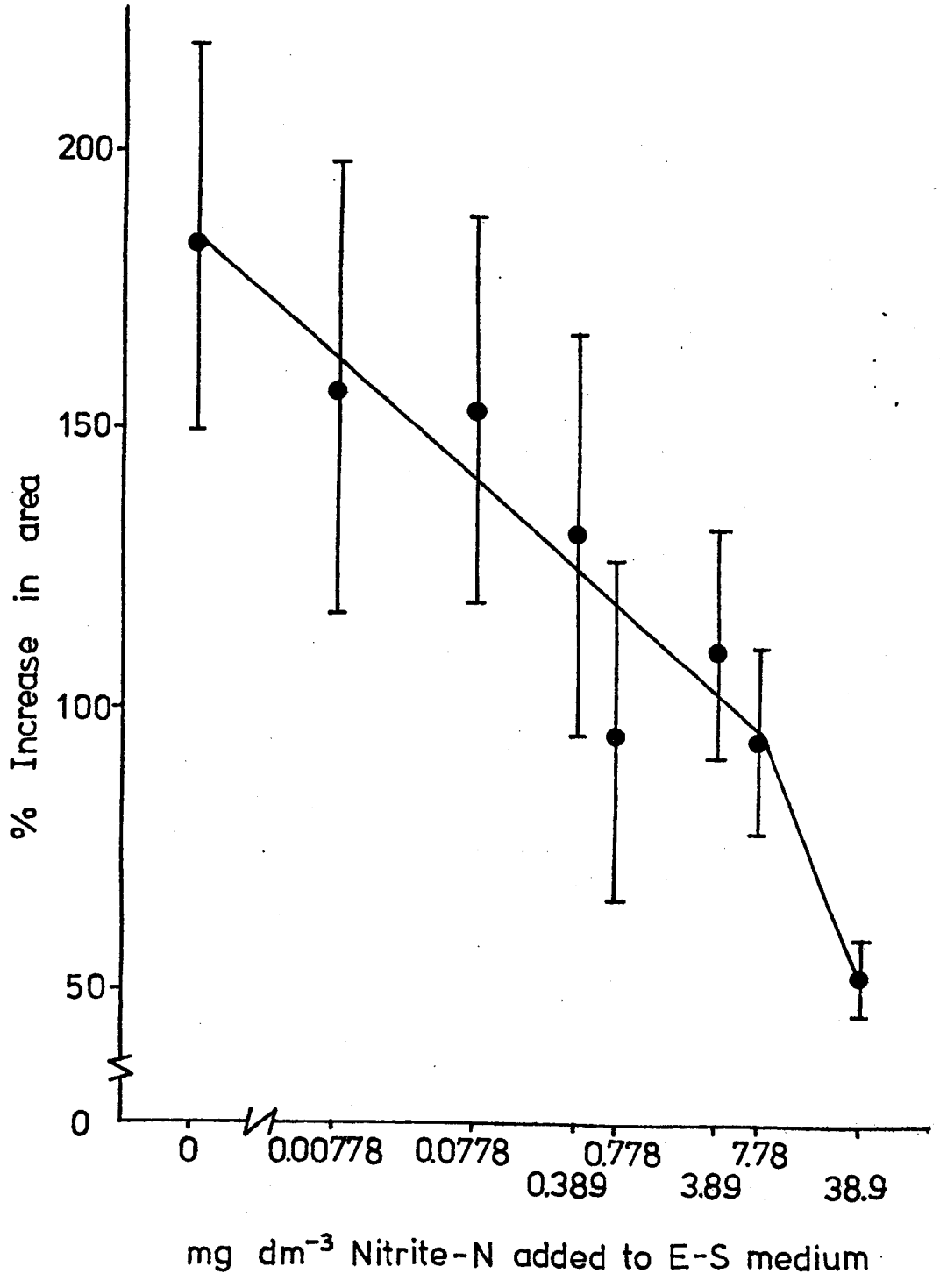
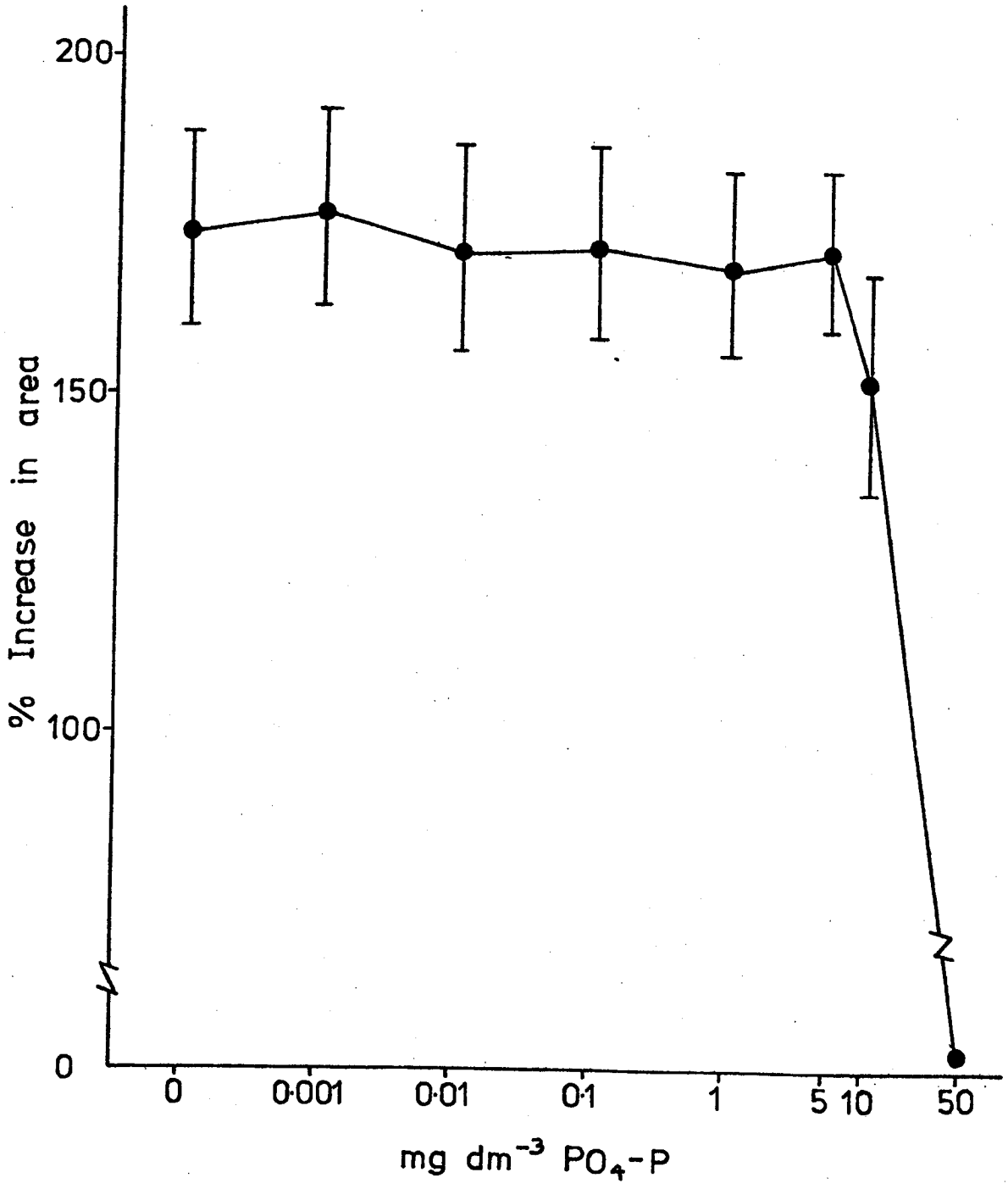

200
150
100
50
0
% Increase in area
0
0.00778
0.0778
0.778
7.78
0.389
3.89
38.9
mg dm⁻³ Nitrite-N added to E-S medium

Fig. 26. Growth of Ulva discs after 14 days in different concentrations of PO_4-P values plotted as mean percentage increase in area with twice standard error on either side of the mean.


200
150
100
0
% Increase in area
0
0·001
0·01
0·1
1
5
10
50
mg dm⁻³ PO₄-P

5.3.6. Phosphate - Ammonium mixture.

5.3.6.1. Introduction.

Waite and Gregory (1972) reported an apparent synergistic interaction of phosphate and ammonium ions on the photosynthetic ^{14}C incorporation into Ulva lactuca. Such enhancement of photosynthesis might be expected to be reflected in faster growth. To compare the above findings under the present conditions, Ulva disc growth tests were carried out with a range of phosphate level in the presence of optimal NH_4-N.

5.3.6.2. Method.

Ulva discs were cultured in a range of phosphate concentration (0 - 10 mg dm^{-3} PO_4-P) and a fixed amount of 3.89 mg dm^{-3} NH_4-N (from ammonium chloride). The usual amount of soil extract was also added. The test cultures consisted of the following substances added to seawater:

(1) soil extract (amount as specified in E-S medium),

(2) nitrate ("),

(3) ammonium-nitrogen at a concentration of 3.89 mg dm^{-3}, and

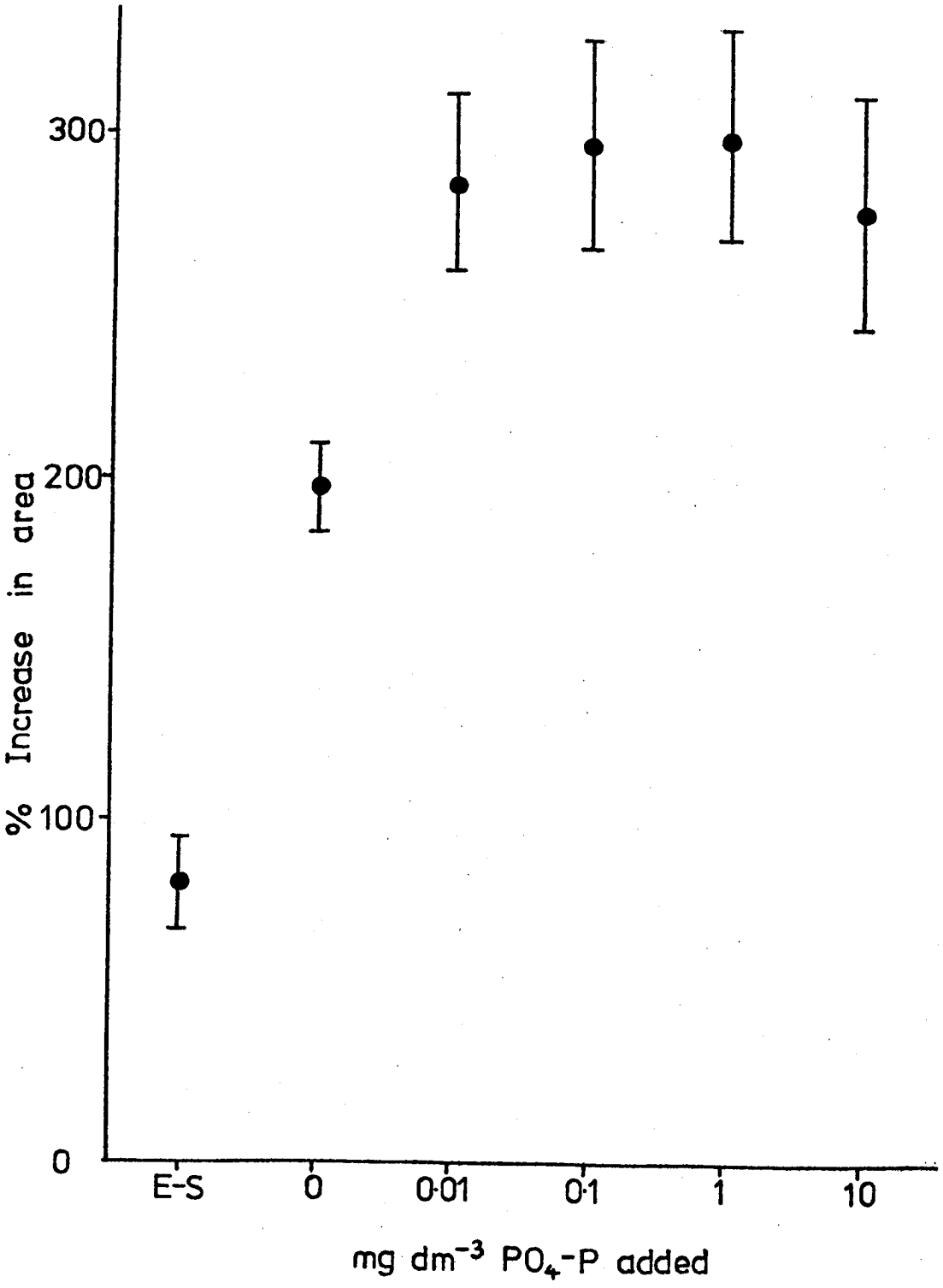
(4) the appropriate amount of phosphate.

A control of E-S medium was also set up.

5.3.6.3. Results.

All treatments with the addition of NH_4-N enhanced significantly the rate of growth of Ulva - two and a half to nearly four times - over that of the control of E-S medium (fig. 27). This confirmed previous results of the NH_4-N cultures (section 5.3.2.2.). It also showed that only a relatively small amount of phosphate, 0.01 mg dm^{-3} PO_4-P or less was required to give the optimal growth rate of Ulva. Thus the percentage increase in area of Ulva was very similar, and not significantly different, with any discs cultured in concentrations of PO_4-P ranging from 0.01 to 10 mg dm^{-3}. With no PO_4-P added however,

Fig. 27. Growth of Ulva discs after 14 days in different concentrations of PO_4-P plus fixed amounts of NO_3, NH_4 and soil extract. Values plotted as mean percentage increase in area with twice standard error on either side of mean.


300
200
100
0
% Increase in area
E-S
0
0·01
0·1
1
10
mg dm⁻³ PO₄-P added

the growth rate of Ulva was significantly lower, but even so it surpassed that of the control.

5.3.7. Sewage-contaminated mud.

5.3.7.1. Method.

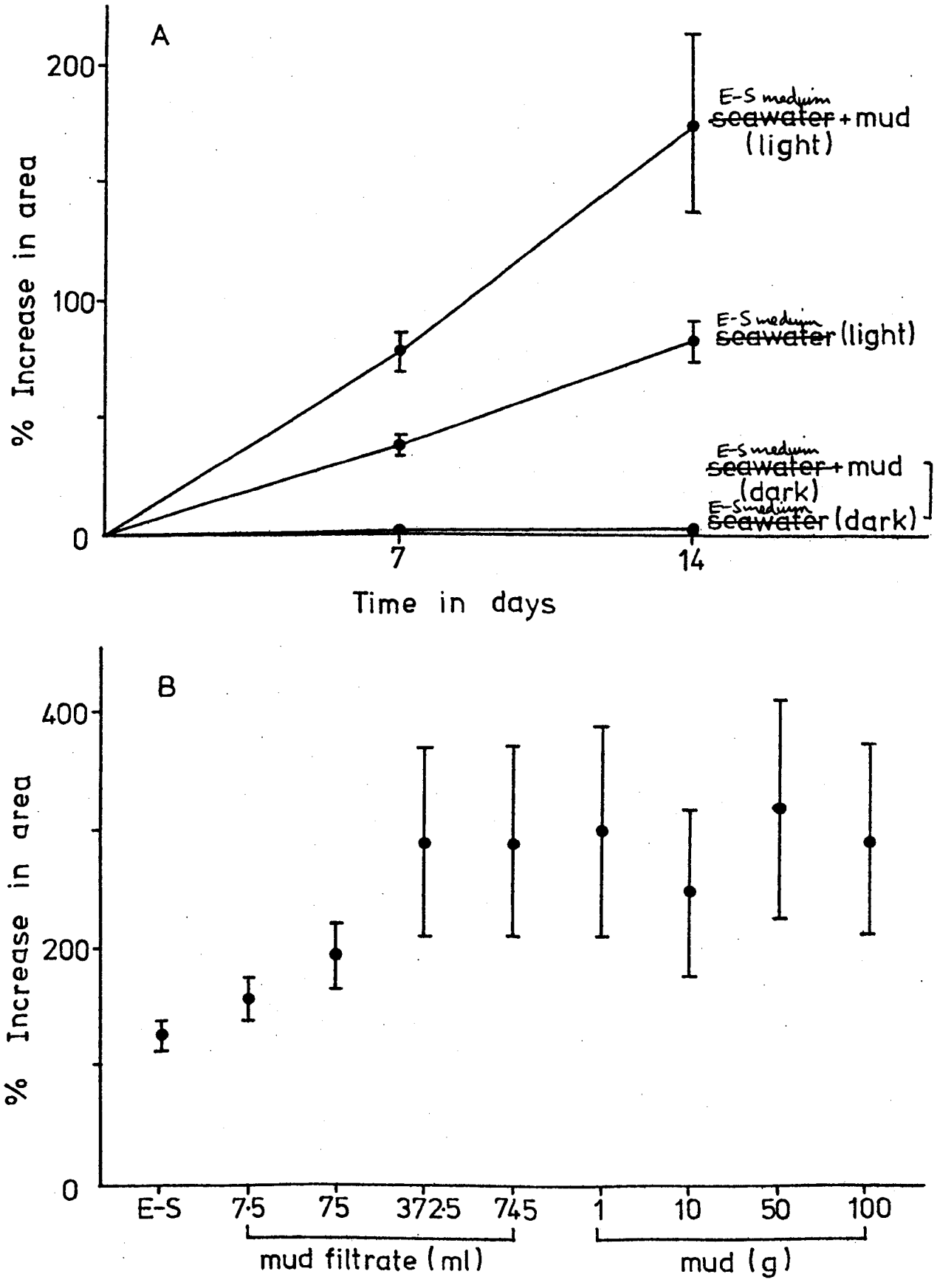
The effect of sewage-contaminated mud collected from Poole Harbour, Dorset, on the growth rate of Ulva was tested under light and dark conditions against a control of E-S medium. Fifty grams of mud were added to 5 dm^{-3} of E-S medium, and the suspension allowed to settle overnight before freshly cut Ulva discs were added. All treatments were maintained under cold-room culture conditions. Disc diameter was measured after 7 and 14 days of culture. Measurement of Ulva discs in the dark treatments took place in a dark room under a dim green safe-light.

A further experiment was carried out with Ulva discs to find whether direct contact of Ulva with mud was necessary to induce the growth effects. Different amounts of mud (1, 10, 50 and 100 g) or mud-filtrate (7.5, 75, 372.5 and 745 cm^3) were added to tanks containing E-S medium, giving a total of 8 cultures plus the E-S control. Twenty-four hours were allowed to elapse before Ulva discs were added to the different treatments. Mud-filtrate was prepared by placing 161 g of Poole Harbour mud in 1.2 dm^{-3} of seawater, which was then kept in the dark at 10°C for three days. The seawater was filtered under vacuum through two layers of Whatman No. 1 filter paper. E-S medium, mud and freshly prepared mud-filtrate were changed every five days. When not in use, mud for the above two experiments was stored at -18°C.

5.3.7.2. Results.

The data plotted in figure 28A show clearly that sewage-contaminated mud induced significant stimulation of growth of Ulva over the control in the light, but in both treatments, (plus mud and control), Ulva discs failed to grow in the dark. Similarly, mud-filtrate (and again

Fig. 28. A Growth of Ulva discs after 7 and 14 days in ~~seawater~~ E-S medium + sewage contaminated mud and ± light. B Growth of Ulva discs after 14 days incubation in ~~seawater~~ E-S medium with various amounts of added mud-filtrate or mud. Values given are mean percentage increase in area with twice standard error on either side of mean.


A
200
100
0
% Increase in area
E-S medium
seawater + mud
(light)
E-S medium
seawater (light)
E-S medium
seawater + mud
(dark)
E-S medium
seawater (dark)
7
14
Time in days
B
400
200
0
% Increase in area
E-S
7·5
75
372·5
745
1
10
50
100
mud filtrate (ml)
mud (g)

sewage contaminated mud) enhanced growth of Ulva (fig. 28B). Thus is indicated the soluble nature of at least part of the constituent(s) that stimulated growth. When a sufficient volume of mud-filtrate was present (372.5 cm^3 or more) the degree of stimulation induced equalled that in the presence of mud itself. A small volume of mud-filtrate gave less significant stimulation than the mud treatments and this was due probably to a quantitative difference rather than a qualitative one, i.e. the stimulation induced by both mud and mud-filtrate was caused by the same substance(s). This would be because when mud was in direct contact with the growth medium, a continuous supply of the stimulant(s) was possible from the former while in the case of mud-filtrate no replenishment was possible once the stimulant(s) has been used up.

5.3.8. Acetate-carbon ($CH_3.COO-C$).

5.3.8.1. Method.

Acetate is a common source of organic carbon (Hunter & Heukelekian, 1965) in sewage and because it can form an alternative carbon source for photosynthesis in some algae its effect on the growth rate of Ulva was tested. A range of concentrations from 0 - 20 mg dm^{-3} CH_3COO - C was employed using sodium acetate ($CH_3.COO\ Na.3H_2O$) as a source. Ulva discs were cultured for a period of 23 days under tank-room conditions and the diameter attained at the end of the culture measured.

5.3.8.2. Results.

There was no significant difference between growth of the control discs and those in any acetate-C concentration tested (fig. 29).

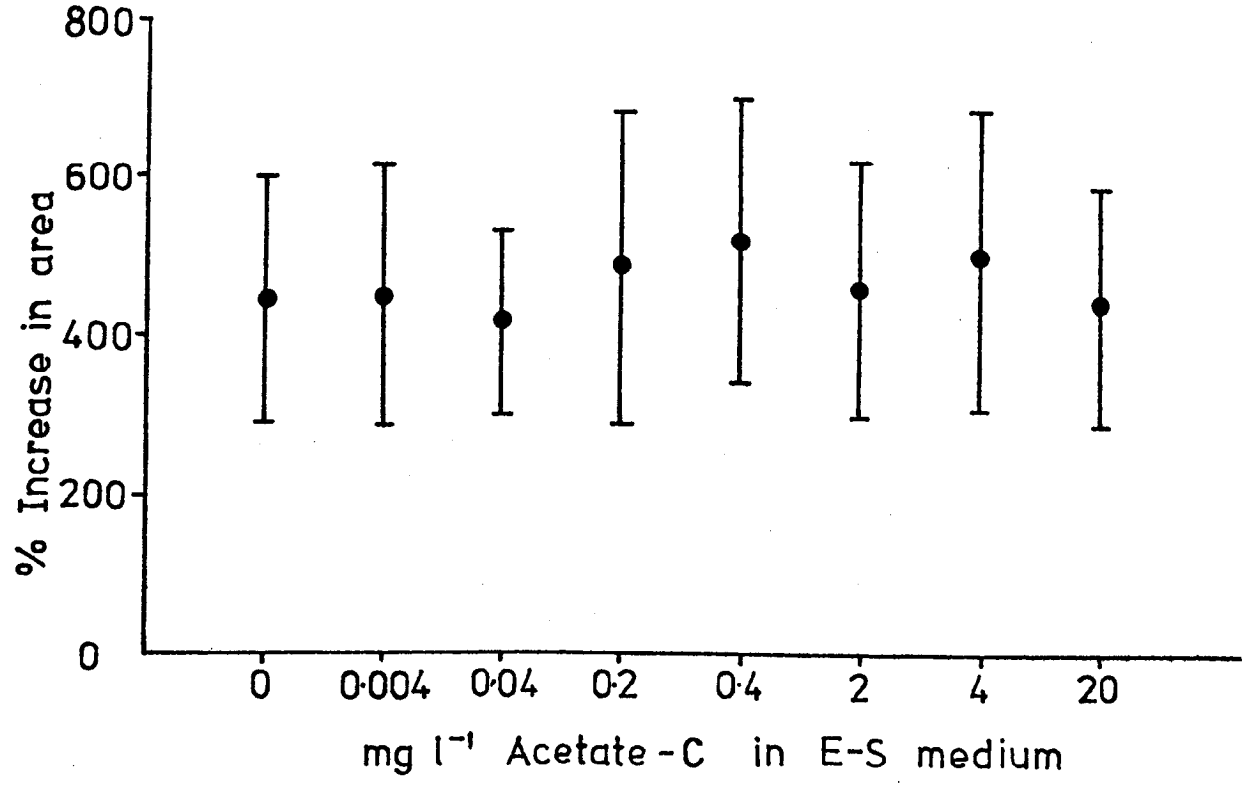
5.3.9. Adenine and kinetin.

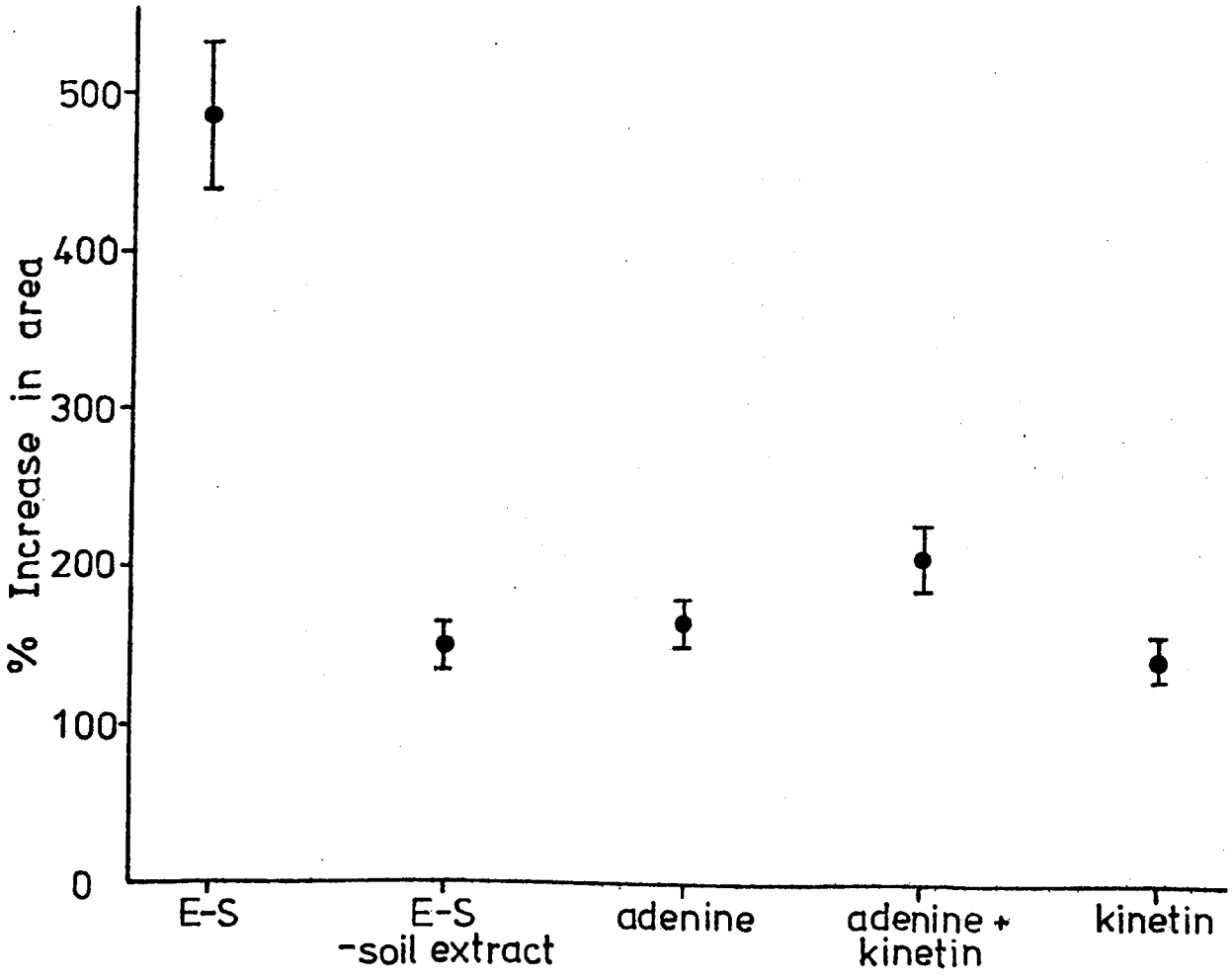
5.3.9.1. Method.

Both adenine and kinetin are claimed to act as growth control factors (Provasoli, 1958) in Ulva under aseptic conditions. Whether these chemicals are important to Ulva under normal growth conditions

Fig. 29. Growth of Ulva discs after 23 days in various concentrations of added acetate, values plotted as mean percentage increase in area with twice standard error on either side of mean.

Fig. 30. Growth of Ulva discs after 16 days with and without the addition of "growth factors", values plotted as mean percentage increase in area with twice standard error on either side of the mean.


800
600
400
200
0
% Increase in area
0
0·004
0·04
0·2
0·4
2
4
20
mg l⁻¹ Acetate-C in E-S medium


500
400
300
200
100
0
% Increase in area
E-S
E-S
-soil extract
adenine
adenine +
kinetin
kinetin

is unknown. The following experiment was designed to find if addition of these compounds affected the growth rate of Ulva or could replace the soil extract (added specifically for its content of biological growth factors in other cultures) in E-S medium. Five different treatments were set up as follows: (1) E-S medium, (2) seawater with nitrate and phosphate added, (3) seawater with adenine at 10 mg dm^{-3}, nitrate and phosphate added, (4) seawater with kinetin at 0.33 mg dm^{-3}, nitrate and phosphate added, (5) seawater with 10 mg dm^{-3} of adenine, 0.33 mg dm^{-3} of kinetin, nitrate and phosphate added. Soil extract was only added in treatment 1. Ulva discs were cultured for 16 days in these treatments under tank-room conditions. Diameters were measured at the end of the culture period.

5.3.9.2. Results.

It was found that neither adenine nor kinetin could replace the role of soil extract in E-S medium (see figure 30). Growth rates of Ulva in E-S medium without soil extract, or adenine and kinetin were similar, but all were significantly lower than that in E-S medium. A combination of adenine and kinetin, did, however, provide a significantly faster growth rate of Ulva than E-S medium without soil extract. It is interesting to note that the difference between E-S medium and E-S medium without soil extract, for the growth rate of Ulva discs, was significant (the former gave 3.2 times more growth than the latter), hence indicating the importance of the soil extract component(s) in E-S medium.

5.4. Discussion.

Nitrate and ammonium ions are the most important sources of nitrogen present in the aquatic environment for growth of algae (Round, 1965). Confirmation that E-S contains sufficient nitrogen for maximum growth comes from a comparison of nitrogen level and growth in the

NO_3-N supplement experiments. Here it was shown that optimal Ulva growth rate was reached with 0.778 mg dm^{-3} NO_3-N (fig. 19). As the minimum amount of nitrate present in E-S medium is 1.65 mg dm^{-3} NO_3-N it thus provides a more than sufficient amount of nitrogen for the growth of Ulva in culture. Further discussion of the possible role of NO_3-N in promoting Ulva growth in polluted harbours and estuaries is delayed until after chapter 6.

A significant amount (mg dm^{-3} levels) of NH_4-N is often present in coastal waters - especially where there is domestic sewage contamination. Organic nitrogen from sewage is converted through the action of bacteria, into NH_4-N which often is a usable source of nitrogen. The effects of NH_4-N on algae are varied. Thus at a concentration of 8.4 mg dm^{-3} nitrogen, Paasche (1971) found that ammonia was better as a source of nitrogen for the growth of Dunaliella tertiolecta than nitrate, while Burrows & Sharples (1972) reported significant inhibition in growth, by NH_4-N at 0.0778 mg dm^{-3}, for both Phaeocystis pouchetii and Skeletonema costatum . The present work shows clearly that the ammonium-ion is a better source of nitrogen than nitrate for Ulva disc growth (fig. 21A, B, C; fig. 23). Stimulation of Ulva growth rate over that of the control, (with its main source of nitrogen as NO_3-N) occurred between 0.389 and 7.78 mg dm^{-3} NH_4-N. With a constant flow culture system, Waite & Gregory (1969a,b) found that NH_4-N stimulated Ulva growth at concentrations between 0.5 and 0.9 mg dm^{-3} with maximal growth rate at 0.7 mg dm^{-3}. This stimulatory range is appreciably less than the one found here. This may be attributable to the difference in culture technique since a constant level of NH_4-N was available in the former case while this was not strictly so in the latter. Furthermore it is conceivable that the Ulva used in the different experiments was of a different physiological ecotype. The "blank" seawater control Waite & Gregory

used contained 0.4 mg dm^{-3} NH_4-N which was already found stimulatory in this work. The preference for NH_4-N over NO_4-N by Ulva may be due to the large amount of energy required for nitrate reduction prior to the incorporation of nitrogen from NO_3 into amino acids, whereas ammonia is directly incorporated into amino acid synthesis by the enzymic action of glutamic dehydrogenase (Jacobi, 1957) and much less energy is needed. Thus energetically NH_4-N is a more favourable nitrogen source. However, ammonium ions are toxic at high concentration, a possible reason being pH-induced damage to the tissue caused by accumulation of ammonia in the cytoplasm due to the inability of the plant to provide sufficient carbon-skeletons (via respiration) with which it can be combined in amino acid synthesis, and hence detoxified. Hence a balance must exist between ease of assimilation and toxicity. If the concentration is low enough to favour the former, stimulation of growth occurs while for too high a concentration the reverse results. This was clearly shown by the results of the first and second culture experiments (fig. 19A, B).

Nitrite was not a satisfactory source of nitrogen, and at high concentrations it was toxic to Ulva (fig. 25). Morris (1974) said that its toxicity at high concentration makes nitrite less suitable as a source of nitrogen than ammonia or nitrate in culture. In the sea, only a minute amount of nitrite is normally present, less than 0.1 to 50 µg NO_2-N dm^{-3} (Riley & Chester, 1971), in a transitional state, it being either reduced to ammonia or oxidized to nitrate by bacterial action. It clearly could not be responsible for stimulating Ulva growth.

Only a small amount of phosphate was required to provide maximal - in relation to phosphate - growth of Ulva (fig. 26). Analysis shows that a total amount of 0.01 mg dm^{-3} PO_4-P was present in seawater and

soil extract, and this amount is sufficient such that phosphate is not a limiting nutrient. With an optimal amount of NH_4-N present, at 3.89 mg dm^{-3}, the growth rate of Ulva was not affected by the amount of phosphorus added (fig. 27). Similar results were obtained by Waite & Mitchell (1972). They measured photosynthetic ^{14}C incorporation as a growth parameter of Ulva and found that little difference in "productivity" occurred with PO_4-P concentrations ranging from 0.04 mg dm^{-3} to 0.8 mg dm^{-3} when 0.6 mg dm^{-3} of NH_4-N, the level which produced their maximum growth stimulation, was present.

The stimulatory effect of sewage-contaminated mud from Poole Harbour on the growth rate of Ulva is shown in figures 28A and B. Burrows (1971) using mud collected from Dublin Harbour obtained similar results. Exactly how mud causes this effect is not known, but it is very likely to be by the provision of nutrients. Muds, especially those contaminated with sewage, contain appreciable amounts of organic substances which, as a result of bacterial action, are converted into inorganic forms and thus easily taken up and assimilated by Ulva. Thus organic nitrogen is degraded to ammonia and the latter clearly stimulates the growth rate of Ulva. As this process of organic decay takes place gradually this would provide a constant supply of nutrients for the plant. Further work on this topic, including analysis of the solution in contact with the mud for components such as ammonium ions and nitrate and phosphate should be rewarding.

Apart from carbon dioxide, carbon may be available to algae in an organic form such as acetate (Eppley, Gee & Saltman, 1963). Organic carbon is especially abundant in sewage. Hunter & Heukelekian (1965) analysed domestic sewage and found that acetic acid was one of its many sources. Burrows (1971) gave evidence of ^{14}C from labelled acetate being assimilated by Ulva and of growth stimulation by acetate. The

labelled ^{14}C-acetate might not necessarily have been assimilated directly, but bacterial action could have released $^{14}CO_2$ from it which was subsequently assimilated by the alga. The present culture work did not reveal any stimulatory effect of acetate on the growth rate of Ulva (fig. 29). Using a much higher concentration of acetate (1 to 2 g dm^{-3}) Mohsen, Nasr & Metwalli (1972) found that the dry weight of cultured Ulva fasciata doubled compared with the control. However, it is almost inconceivable that such a high concentration ever exists even under highly polluted conditions.

Work by Provasoli (1958) showed that Ulva required the addition of adenine and kinetin for normal development under aseptic conditions of culture. This, however, would not apply under natural conditions where most probably sufficient growth substances synthesized by bacteria and subsequently released are present in seawater. The importance of soil extract in culture as a source of such micronutrients/ hormones was clearly shown (fig. 30), but it is very unlikely that they contribute to stimulated growth in polluted conditions even though higher than normal concentrations will be present from organic breakdown.

In conclusion, ~~Ulva lactuca grows better on inorganic nutrients than organic ones. Phosphate, nitrate, ammonia and dissolved carbon dioxide were the main forms of nutrients assimilated.~~ Ammonia acted as a better source of nitrogen than nitrate for growth and it was postulated that stimulatory growth of Ulva in sewage contaminated areas could be caused by the presence of appreciable amounts of ammonia in those regions. The culture work showed that Ulva was tolerant to the application of a wide range of concentrations of different substances and the response of Ulva to these substances was both sensitive and reproducible, and thus so far supports the proposition that Ulva might be used as an indicator organism for pollution.

CHAPTER 6.

Bioassay of water quality in Poole Harbour, Dorset and Langstone Harbour, Hampshire.

6.1 Introduction

Previous laboratory culture experiments with Ulva showed a close correlation between growth and the amount of certain nutrients present. Because of this correlation it is possible that Ulva might be used as a test organism for pollutants. Furthermore, data collected in the laboratory on the general behaviour of Ulva under different environmental conditions strengthened the possibility of using this plant to identify and evaluate environmental changes. An attempt was thus made to assess water quality at polluted sites, using the growth rate of Ulva in water samples collected from the field as the diagnostic test feature.

Poole Harbour in Dorset was chosen as the main sampling area from which seawater was collected. There were several reasons for this choice. Firstly, parts of Poole Harbour (Holes Bay and Lytchett Bay) are known to have an abundant Ulva growth, while this is not so for other areas within the harbour. Secondly, there have been some hydrographic surveys of the area and thus information on the nature and flow of tidal waters within the harbour is available. Thirdly, for the last few years (and still continuing) regular water sampling and chemical analysis of the harbour water have been carried out by Poole Corporation. Samples have been analysed for salinity, percentage saturation with dissolved oxygen, turbidity, phosphate, oxidized nitrogen and ammonia. Information on the general nutrient level of water within the harbour was thus available. Fourthly, from the analyses available it seems that at least some parts of the harbour have high nutrient levels and thus should provide suitable conditions for assessing the alga's ability to act as a pollution indicator. It was hoped that

this study might also lead to an explanation of why Ulva grows so well in polluted areas and thus enable suggestions for its control or elimination to be put forward.

Water collections were also made from Langstone Harbour, Hampshire. No excessive Ulva growth occurs here at present though the harbour is eutrophicated by discharged sewage. This location thus acts as a comparison with the situation in Poole Harbour and should help to assess the effectiveness of Ulva as an indicator organism. Data are also available on the tidal characteristics, biological activity and nutrient levels in and around Langstone Harbour (Dunn, 1972).

6.2 Description of sampling areas

General hydrographical information on Poole and Langstone Harbours is given by Green (1940, 1952) and Dunn (1972) respectively.

Poole Harbour is part of the drowned valley of the ancient Frome-Solent river in Dorset. The present geographical setting is illustrated in figure 31. The harbour is enclosed and opens to the sea only through a narrow entrance to the east at Sandbanks. Two main rivers, the Puddle and the Frome, drain into the harbour. Two inlets, Holes Bay and Lychett Bay, lie to the north of the harbour. The main sewage outflow for the town of Poole empties into the upper part of Holes Bay.

Due to the small tidal range within the harbour, around 0.35 m at neap tides and 2 m at spring tides, water currents are slow with speeds of only 1.0 to 1.6 m sec^{-1}. The tidal regime at Poole is of some interest as it shows a double high water, especially marked at neap tides. This feature is shown clearly on the tide curve recorded at Poole Quay (figure 33). At a given moment the state of tide varies with location within the harbour. Thus first high water occurs initially at Pilot's Pier (refer to figure 31 for geographic location), half an hour later at Poole Bridge and three-quarters of an hour later at

Fig. 31. Map of Poole Harbour, Dorset.

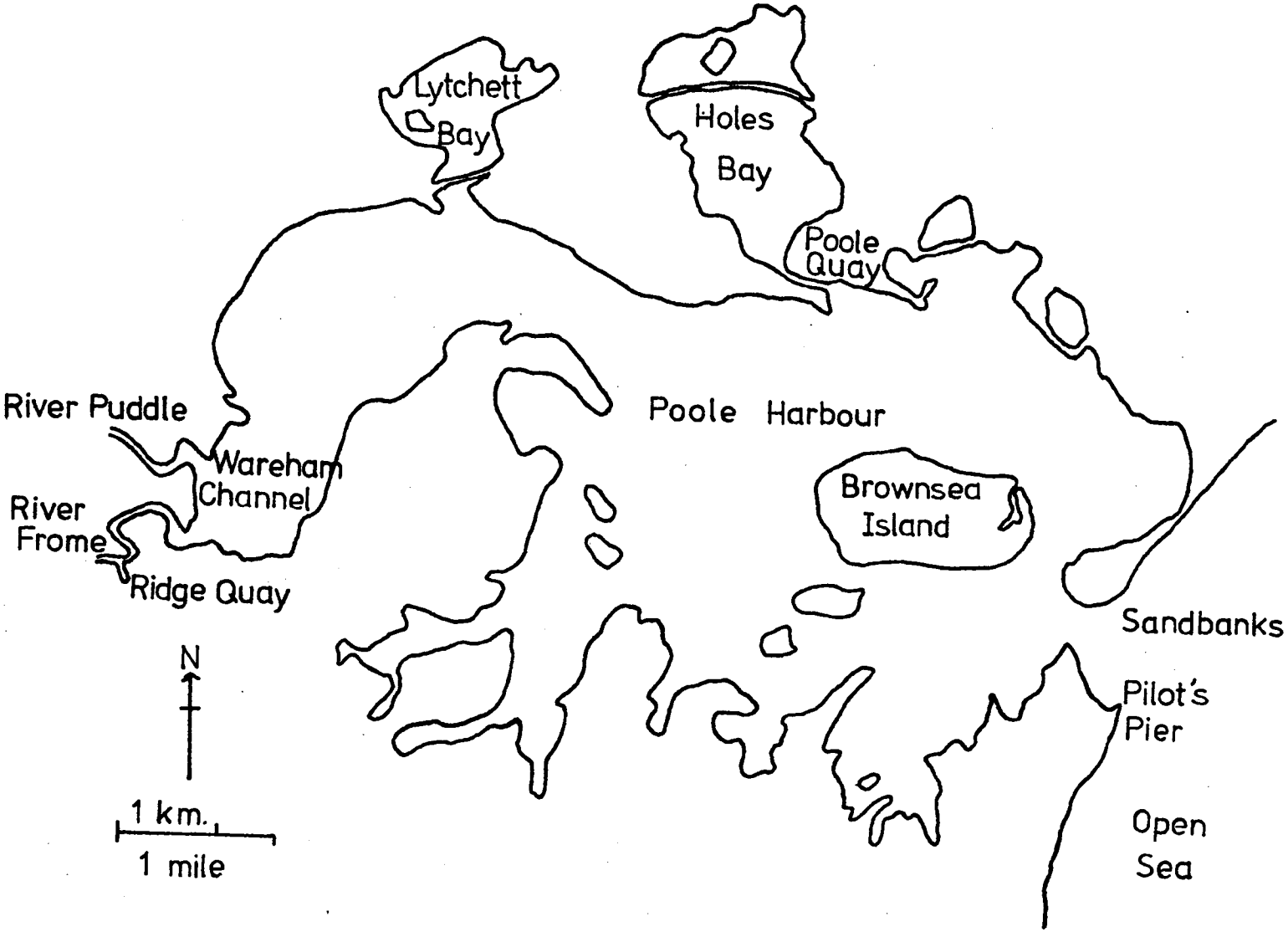

Lytchett Bay
Holes Bay
Poole Quay
River Puddle
Wareham Channel
River Frome
Ridge Quay
Poole Harbour
Brownsea Island
Sandbanks
Pilot's Pier
Open Sea
N
1 km.
1 mile

Fig. 32. Map of Langstone Harbour, Hampshire.

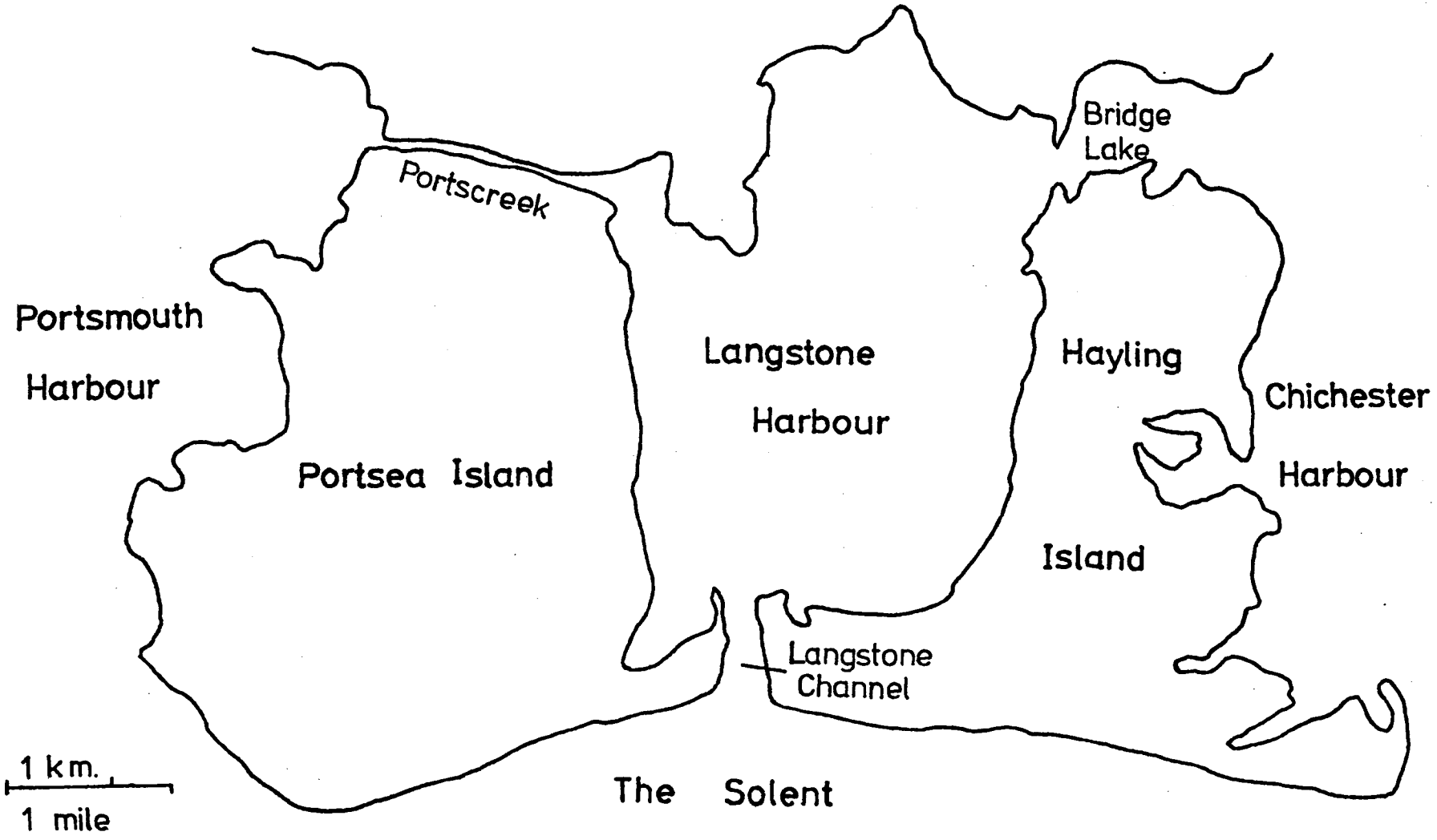
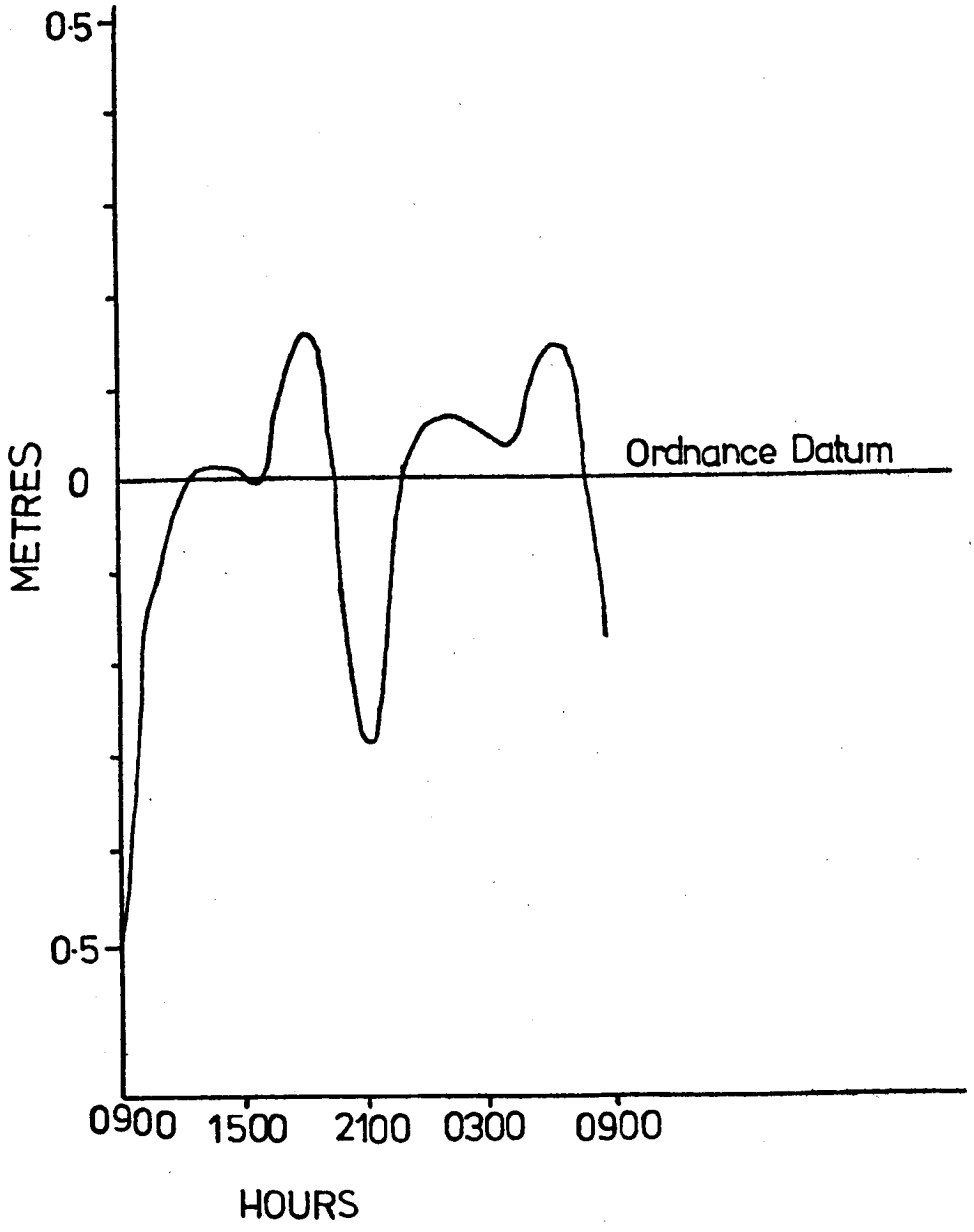

Portscreek
Bridge
Lake
Portsmouth
Harbour
Portsea Island
Langstone
Harbour
Hayling
Island
Chichester
Harbour
Langstone
Channel
1 km.
1 mile
The Solent

Fig. 33. Tide curve (neap tide) recorded off Poole Quay on 9th and 10th of April, 1973.


0·5
METRES
0
Ordnance Datum
0·5
0900
1500
2100
0300
0900
HOURS

Ridge Quay. Second high water timings shows similar features. A greater time-lag occurs for low water. At Poole Bridge low water is 40 minutes later than at Pilots' Pier whilst at Ridge Quay the lag is about one and three-quarter hours. This time lag on both high and low water in the harbour enabled water sampling to be carried out at the same state of the tide in different harbour positions. Automatic tide gauges are installed at different areas (Pilots' Pier, Poole Bridge and the Power Station) within the harbour.

Langstone Harbour, the other sampling area, lies between Portsea Island and Hayling Island in Hampshire (fig. 32). It is connected by two channels, Portscreek and Bridge lake, to Portsmouth Harbour and Chichester Harbour respectively. Langstone Harbour is connected with the Solent by the Langstone Channel to the south.

The tidal range is greater than that of Poole Harbour, with a range of 4.7 m at spring tides and 1.5 m at neap tides (Houghton, 1959). Water currents in the harbour can reach a speed of 2.5 m sec^{-1} (Hydraulic Research Station, 1970). Though a great portion of the tidal water passes through the Langstone Channel, some of it goes through Portscreek and Bridge Lake into or from the two surrounding harbours. A few small streams empty into the harbour but the small volume of freshwater introduced has little effect on the salinity of the harbour water which averaged 33.6‰.

Sewage effluents enter the harbour at two locations, one at Budd's Farm to the north-east and the other at Fort Cumberland at the mouth of Langstone Harbour (refer to fig. 35).

6.3 Preliminary sampling runs

Two initial sampling runs were made in Poole Harbour. These were to test whether Ulva showed any differential growth response when cultured in water collected at points spread throughout the harbour.

One would expect the water quality at a particular sample point to vary at high and low tide; thus two sampling runs were attempted at different states of tide. The first run was made during a neap high tide on the 9th of April, 1973, and the second on a neap low tide on the 11th of May in the same year. Collections were from 7 sampling sites (stations 1 to 7) the positions of which are given in figure 34A. It was assumed that water quality would change through the Harbour in relation both to the distance from the source of pollution and source(s) of fresh water. Most sampling points were therefore sited so as to relate to the likely path of movement of sewage from the discharge point towards the open sea. Also the sampling positions were such that sampling time could be kept to a minimum. This was necessary so as to enable water sampling to be carried out at the same state of the tide in different harbour positions. Originally it was intended to obtain a sample from within Lytchett Bay, since it was known that sewage was emptied into the Bay, but this site was abandoned as it was not accessible by the boats used. The sampling was carried out from motorised boats.

6.3.1 Sampling procedures

Seawater samples for culture work were collected, with either a pump or a plastic bucket, from a depth of 0.2-0.5 m. They were stored in heavy polyethylene carboys of 10 or 25 dm^{-3} capacity. Water temperature, pH and salinity were measured at the time of collection. The time of each sample collection was recorded and checked against the tide curve to ensure that collection had been made at the correct state of the tide. The water samples were taken back to the University of Liverpool immediately and were frozen at -18°C the same day.

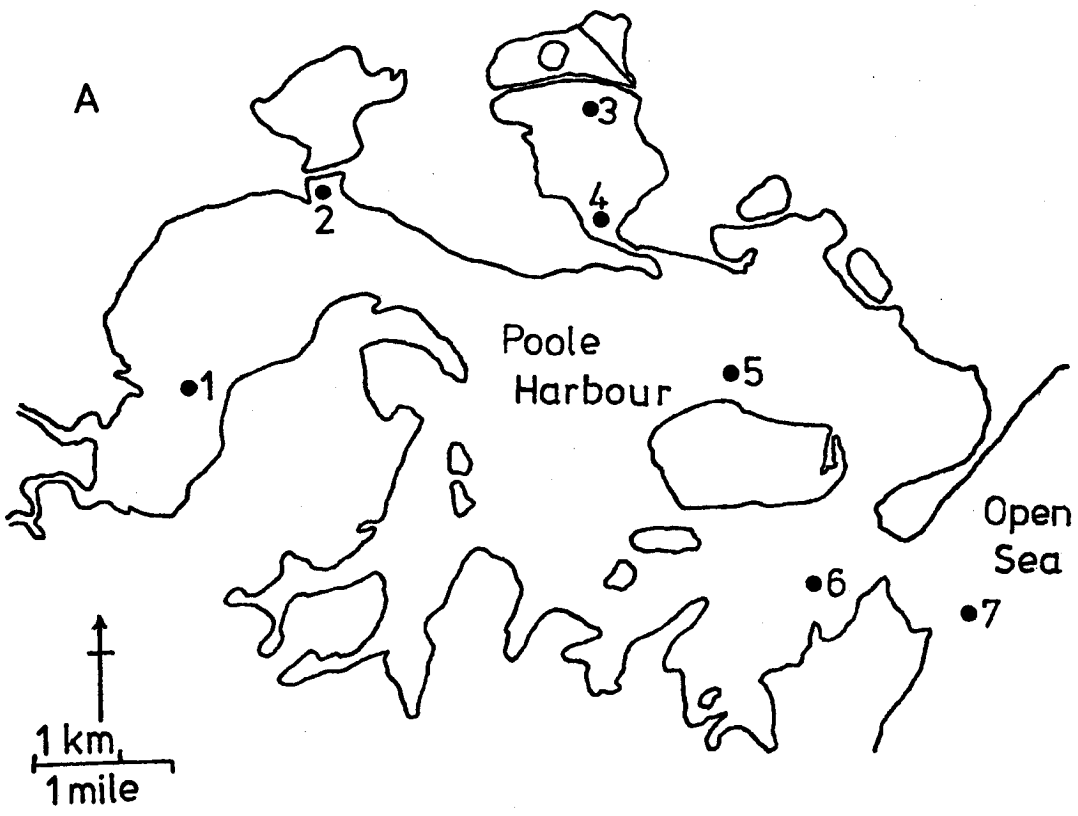
6.3.2 Laboratory experimental method

Cultures of Ulva discs were set up using the collected seawater samples the day after their collection. Tank-room culture conditions

Fig. 34. Sampling sites in Poole Harbour.

A, Sites in Poole Harbour, main section,

B, sites in Holes Bay.


A
1
2
3
4
5
6
7
Poole
Harbour
Open
Sea
1 km
1 mile

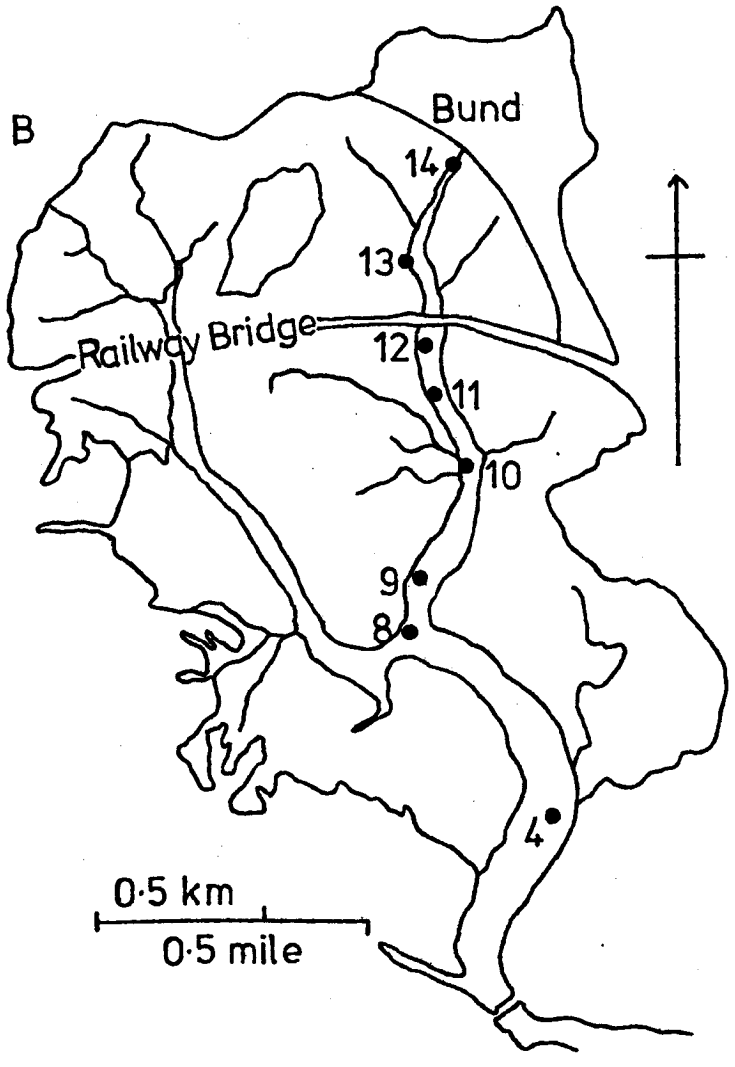
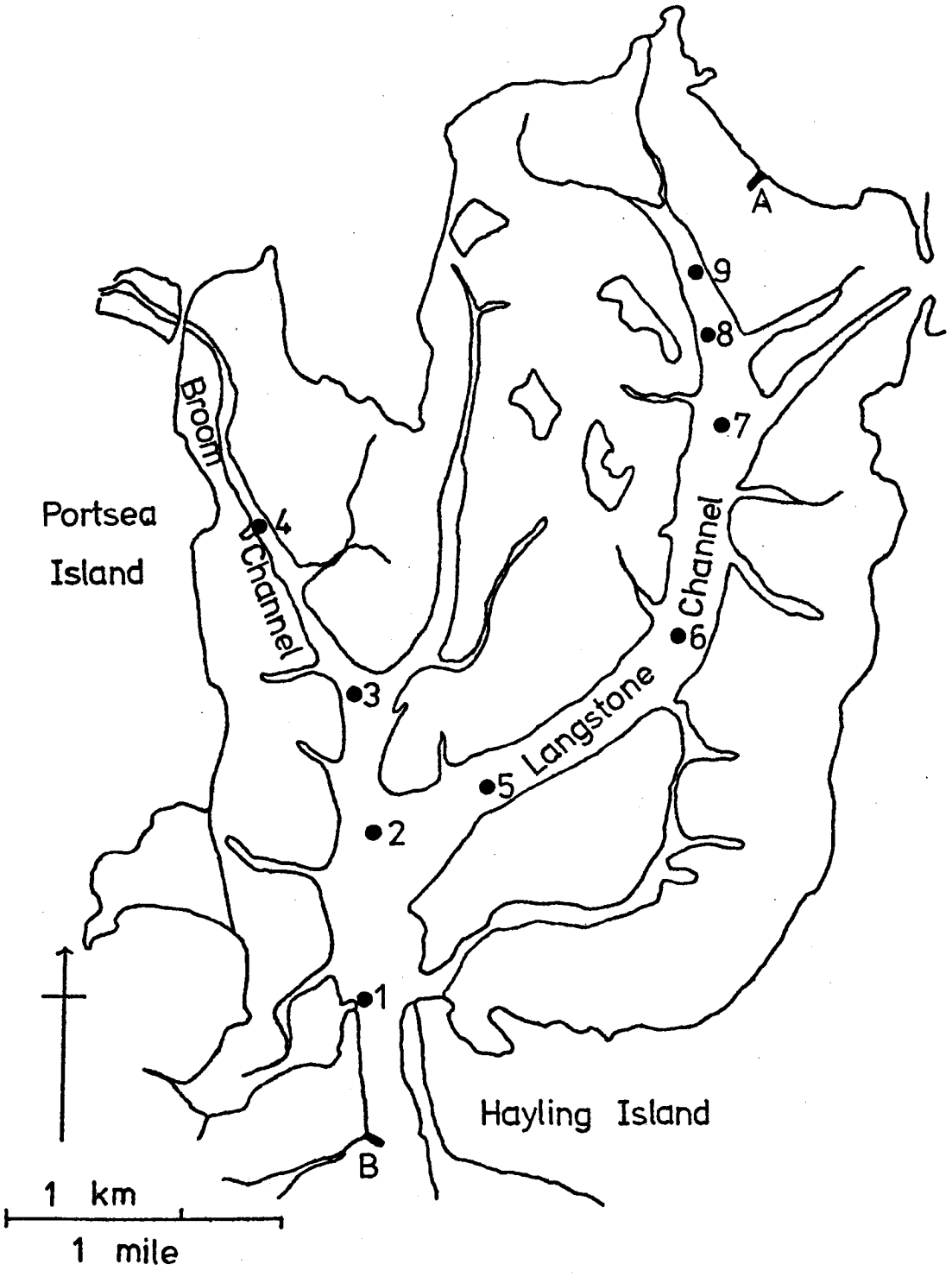

B
Bund
14
13
Railway Bridge
12
11
10
9
8
4
0·5 km
0·5 mile

Fig. 35. Sampling sites in Langstone Harbour.

A Budd's Farm)
) Sewage effluents
B Fort Cumberland)


A
9
8
7
6
5
4
3
2
1
B
Broom Channel
Portsea Island
Channel
Langstone
Hayling Island
1 km
1 mile

were used (see section 3.2.2). Irish seawater (ISW) was used as the control medium. Disc growth and chlorophyll and protein contents were estimated as before.

6.3.3 Results

Temperature, salinity and pH of the water samples at the time of collection are given in Table III. Growth performance of Ulva discs together with their chlorophyll and protein contents at the end of each culture period are summarized in figures 36 and 37.

Table III. Temperature, salinity and pH measurements of water samples collected on 9.4.73 (Neap high tide) and 11.5.73 (Neap low tide) from Poole Harbour.

Station No.		Temperature (°C)	Salinity (‰)	pH
Neap high	1	8.0	22.1	7.95
tide - April	2	7.1	19.5	7.92
collection.	3	8.0	23.4	8.01
	4	7.8	26.0	7.97
	5	8.3	26.0	7.97
	6	8.4	29.9	8.01
	7	8.5	32.5	8.13
Neap low	1	13.8	18.9	7.93
tide - May	2	14.2	18.5	7.87
collection.	3	15.3	22.8	7.93
	4	13.8	23.8	7.86
	5	12.8	25.7	7.82
	6	12.7	26.9	7.87
	7	12.8	31.9	7.93

It is interesting to note that in the April collection, water temperatures at and near the sea (station 5, 6 and 7) were higher than those within Holes Bay and near the Wareham Channel (stations 1 - 4). This situation was reversed in the May collection. This was due probably

to the shallow nature of the harbour and the direct influence of fresh-water run-off at the landward stations, where water cools down and warms up more quickly than in the deeper waters near to or in the open sea. The salinity at Wareham Channel (station 1) averaged 20.5‰ and was considerably lower than that at station 7 which averaged 32.2‰. There was a gradient of increase in salinity from Wareham Channel towards the sea.

Culture results (fig. 36 & 37) showed the highest Ulva growth in the seawater sample obtained from station 3 within Holes Bay. Seawater samples collected in or near the Wareham Channel (station 1 and 2) supported better Ulva growth than water obtained from the seaward side of the harbour (station 5 and 6) and at the sea (station 7). Sewage effluents are present in Holes Bay and in the Wareham Channel (the latter discharged from the town of Wareham). Water in these areas would be expected to have higher nutrient levels due to sewage contamination. This seemed to be reflected by the greater growth rate of Ulva cultured in water samples from these areas. Thus there was a good indication that Ulva showed a positive response to the water quality within the harbour in relation to sewage effluents. The control ISW gave the least growth; this was to be expected as off-shore waters generally have much lower nutrient levels than in-shore waters. The chlorophyll and protein contents of the discs both follow the same pattern of content with sampling position (cf. fig. 36B and C, fig. 37B and C). Growth and level of these important physiological components are also - as might be expected - positively correlated.

6.4 Monitoring water quality using Ulva

The preliminary studies have thus indicated a likely correlation between the growth of Ulva and water quality. The growth rate in water samples decreasing with distance from the source of sewage pollution in Poole Harbour, both of the sampling runs showing the greatest Ulva

Fig. 36. Effects of seawater samples collected from different stations in Poole Harbour at neap high tide (9.4.73) on Ulva disc growth and composition, parameters determined at the end of 14 days incubation in seawater samples.

A Percentage increase in area of Ulva discs. Values given are mean and twice standard error on either side of mean.

B Chlorophyll content of discs.) Values are mean
C Protein content of discs.) and range.

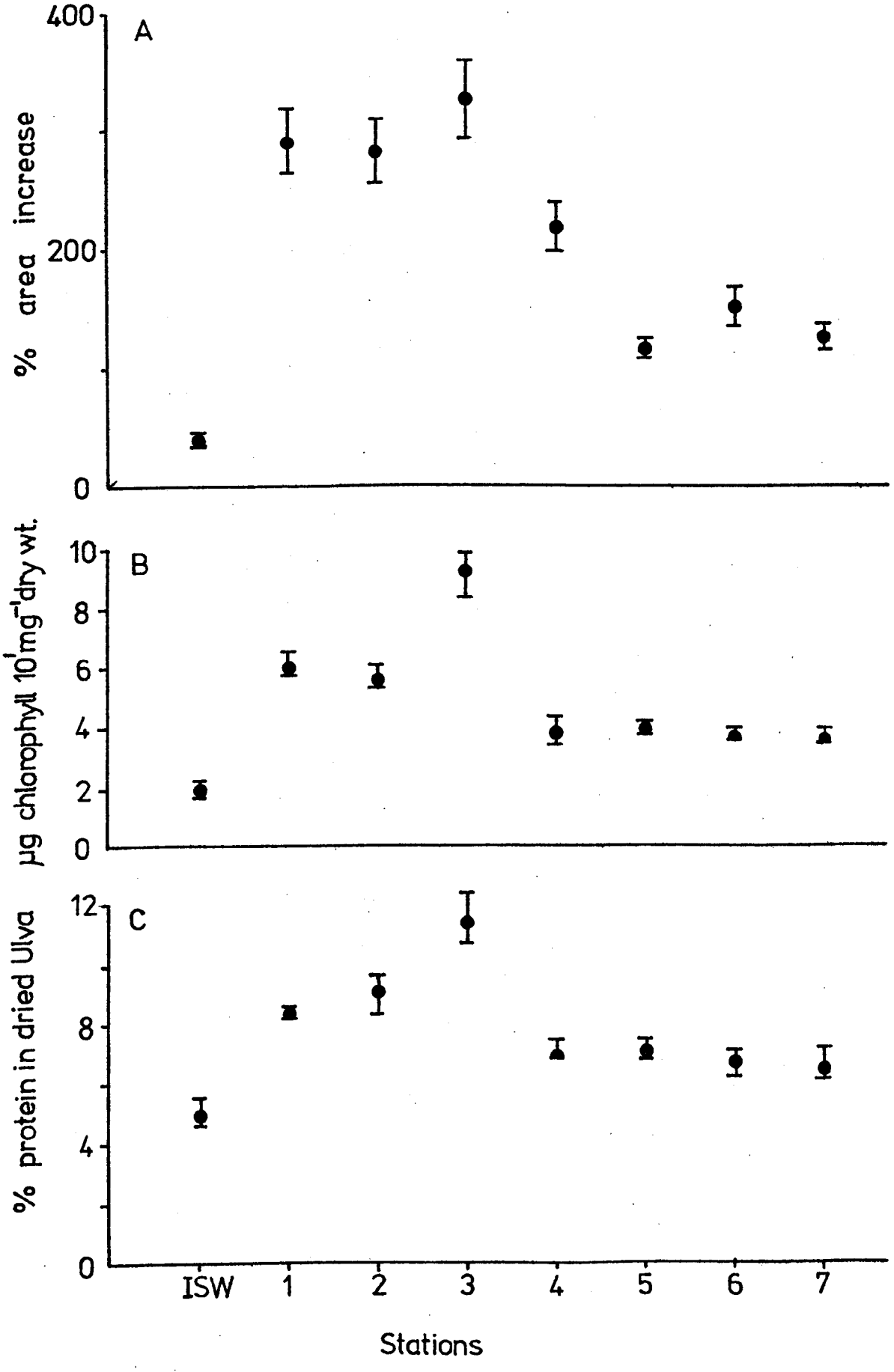
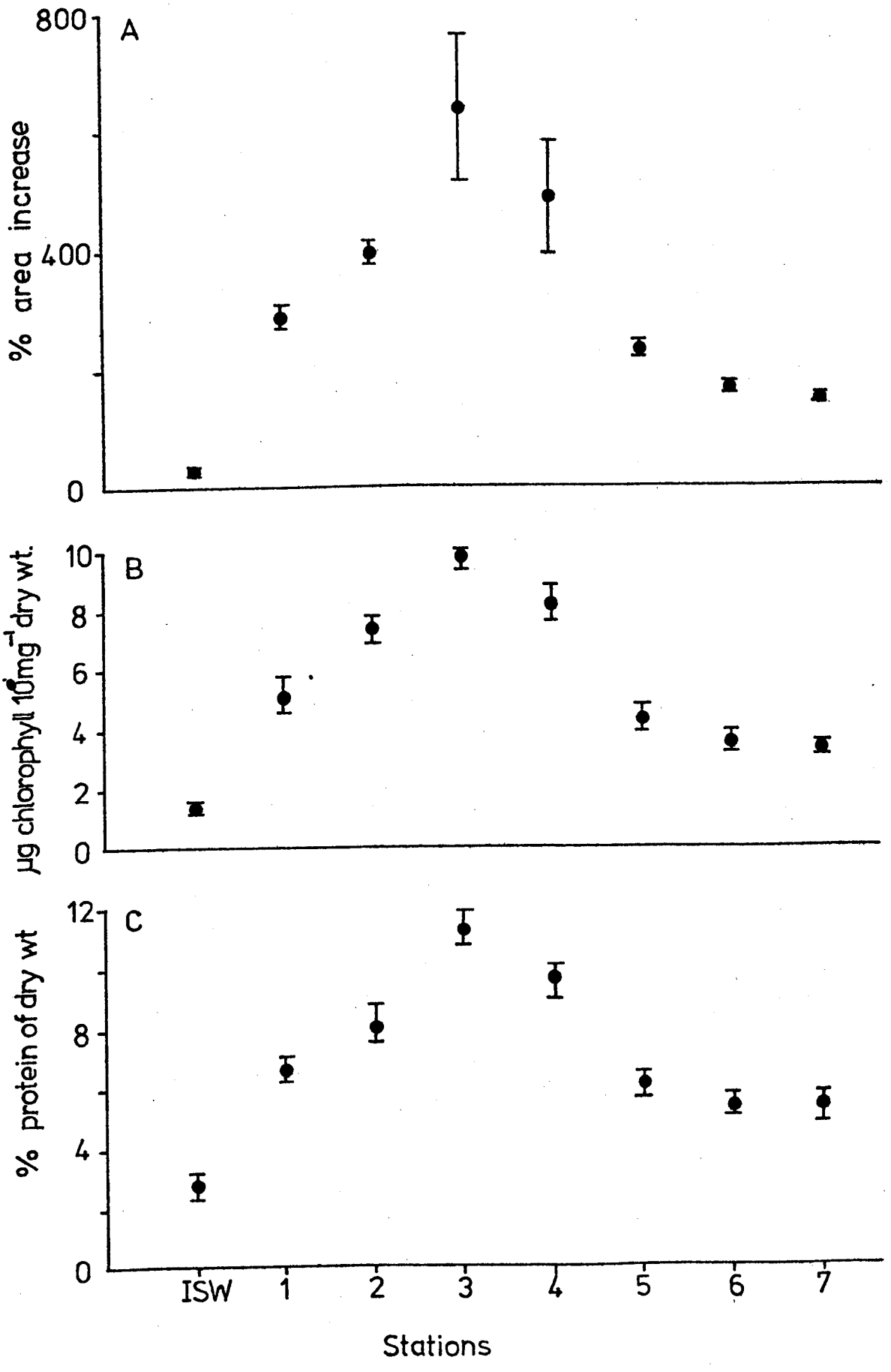

A
400
200
0
% area increase
B
10
8
6
4
2
0
µg chlorophyll 10¹mg⁻¹dry wt.
C
12
8
4
0
% protein in dried Ulva
ISW
1
2
3
4
5
6
7
Stations

Fig. 37. Effects of seawater samples collected from different stations in Poole Harbour at neap low tide (11.5.73) on Ulva disc growth and composition, parameters determined at the end of 14 days incubation in seawater samples.

A Percentage increase in area of Ulva discs.
Values given are mean and twice standard error on either side of mean.

B Chlorophyll content of discs.) Values are mean
C Protein content of discs.) and range.


A
800
400
0
% area increase
B
10
8
6
4
2
0
µg chlorophyll 10mg⁻¹ dry wt.
C
12
8
4
0
% protein of dry wt
ISW
1
2
3
4
5
6
7
Stations

growth in water obtained from within Holes Bay and near the sewage outflow, when compared with water samples obtained elsewhere in the Harbour and with the control ISW. Furthermore, a prolific amount of Ulva is found within Holes Bay and not in other areas within the Harbour, thus strengthening the possibility of a real correlation between prolific Ulva growth and sewage contamination.

To investigate the problem in more detail a further series of sampling sites was established solely within Holes Bay at increasing distances from the sewage effluent source. As amount of sewage contamination will be inversely proportional to the distance from the source of effluent, due to dilution by seawater, it should be possible, from observing the performance of Ulva growth in water samples obtained from these sites, to rigorously test the ability of the plant to reflect water quality. In conjunction with this second series of growth bioassays, quantitative chemical analysis of particular nutrients in the seawater samples was carried out. Components analysed were: NO_3-N, NO_2-N, NH_4-N and PO_4-P. This thus enables nutrient levels of water samples to be directly compared with the growth performance of Ulva. It was in this second series of combined growth bioassays and nutrient analyses that the other harbour, Langstone Harbour in Hampshire, which is polluted but at present without an Ulva problem, was used. It was considered a particularly useful site in that whilst being polluted and yet not apparently promoting Ulva growth it should really test the feasibility of using Ulva as either a general or specific pollution indicator, specific that is to one component of the polluting sewage mixture.

6.4.1 Sampling sites

The sampling sites in Poole Harbour are given in figures 34A and B. Seawater samples were collected from stations 4-6 and 8-14 thus forming a series of sites from near the sewage effluent (station 14) to the seaward end of the Harbour (station 6). Collections were made on two

separate occasions. The first was made at low spring tide on the 14th September, 1973 and the second at high spring tide on the 12th November the same year. These two dates were chosen since one would expect there would be at particular sampling sites maximum contamination and effect of sewage effluent at low spring tide and minimum contamination and effect during the high spring tide. Figure 35 gives the position of the sampling sites in Langstone Harbour. Only one collection was possible here, on the 18th of January, 1974 during a low neap tide. As shown on the map (fig. 35) sewage effluents are discharged near stations 1 and 9.

6.4.2 Sampling procedures and seawater analysis

Seawater sampling procedures were very similar to those for the preliminary runs as detailed in section 6.3.1. Seawater samples were also collected into 1 dm^3 polyethylene bottles for analysis of salinity, turbidity and PO_4-P, NH_4-N, NO_3-N and NO_2-N concentration. The analyses were carried out by Mr. Barry Silk of the Poole Purification Works. Technical Staff in the Marine Station of Portsmouth Polytechnic also helped in the analysis of the nutrient content of seawater samples collected in Langstone Harbour, but duplicate analyses of some of the Langstone Harbour samples were made by Mr. Barry Silk to ensure accuracy and comparability of analyses. Seawater samples for disc culture were taken back to the University of Liverpool immediately and were frozen at -18°C the same day.

Any effect of cold-storage on the nutrient levels in the seawater was also investigated. This was necessary because the culture experiments lasted for 14 days and the culture medium was renewed every 5 days. For this check, an additional water sample was taken at station 11 in Poole Harbour and was frozen at -18°C in Poole on the 14th September, 1973. The levels of NH_4-N, NO_3-N and NO_2-N in sub-samples from this water were analysed at the Poole Purification Works at intervals over a period of 14 days.

6.4.3 Laboratory experimental method

The method for the culture of Ulva and the subsequent measurement of its growth and protein and chlorophyll contents were as given in section 6.3.2. In addition, the sizes of Ulva cells in each treatment were measured using the method described in section 3.2.9. Three replicates, each having a minimum of 30 cells, were measured in samples from each culture. By comparing the sizes of Ulva cells among the treatments it was possible to see whether the increase in size of Ulva discs was a result of cell division or cell enlargement or a combination of both; see also for comparison section 4.2.2.2.

6.4.4 Results

6.4.4.1 Seawater analysis

Values obtained from chemical analysis of the seawater samples are summarized in tables IV, V, VI and VII.

Lower values of pH, percentage saturation of dissolved oxygen and chlorinity were found within Holes Bay than in the main body of Poole Harbour. Nutrient concentrations, however, were found to be about ten times higher, or even more, within Holes Bay than in the main Harbour (refer to table IV and V). NH_4-N was found to be the major nitrogen-form present inside Holes Bay. In Langstone Harbour the levels of NO_3 and NO_2 were high and similar to those found in Holes Bay (cf. table VI and IV, V), whilst the levels of phosphate and ammonia were low and similar to those in the main part of Poole Harbour, i.e. excluding Holes Bay. Thus when compared with Holes Bay, Langstone Harbour has less phosphate and ammonia but a similar amount of nitrate. Unlike the situation in the Poole Harbour complex the nutrient levels were fairly uniform throughout Langstone Harbour.

The effect of storage at -18°C on nutrient levels of seawater samples is shown in table VII. There are certainly changes in amounts of the components over the 14 days period and although trends of change are apparent there is more variation in levels than might have

Table IV. Analysis of seawater samples collected from Poole Harbour at low spring tide on September 14th, 1973.

Station no.	6	5	4	8	9	10	11	12	13	14
Temp. ^{o}C	17	16	16	15	15	15	14.5	15	15	N.D.
pH	7.91	7.95	7.80	7.70	7.65	7.64	7.63	7.52	7.46	7.29
% saturation of dissolved O_2	104	102	79	66	60	60	74	76	77	N.D.
Chlorinity ($g.dm^{-3}$)	16.8	13.6	14.8	16.2	13.6	14.2	10.0	9.6	8.6	7.6
Turbidity (R.T.U.)	16.5	33.0	14.0	14.0	22.0	25.0	36.5	49.0	58.0	15.5
PO_4-P	.046	.060	.44	.56	.93	.70	1.45	1.80	2.06	2.19
NH_4-N	.065	.128	1.33	4.28	3.04	3.38	5.61	6.95	8.13	9.18
NO_2-N	N.D.	.002	.035	.053	.074	.0219	.027	.0198	.0219	.033
NO_3-N	.002	.008	.073	.222	.115	.2031	.311	.4052	.2131	.127

Nutrient values are given as $mg.dm^{-3}$

N.D. = not determined

R.T.U. = Relative Turbidity Units

Table V. Analysis of seawater samples collected from Poole Harbour at high spring tide on November 12,1973.

Station no.	6	5	4	8	9	10	11	12	13	14
Temp. ^{o}C	11.7	11.2	10.2	10.0	9.9	9.5	9.5	8.5	9.0	12.0
pH	8.05	8.00	7.95	7.95	7.90	7.90	7.85	7.80	7.70	7.30
% saturation of dissolved O_2	102	104	96	96	94	83	83	78	80	76
Chlorinity ($g.dm^{-3}$)	19.5	19.3	18.6	18.2	17.9	17.7	17.4	16.4	16.8	3.6
Turbidity (R.T.U.)	6.4	8.4	10.4	10.4	11.1	7.8	8.6	11.1	8.6	22.5
PO_4-P	.02	.07	.05	.09	.07	.10	.08	.20	.13	5.58
NH_4-N	.04	.17	.15	.22	.35	.37	.33	1.12	.66	21.4
NO_2-N	.004	.007	.011	.015	.017	.015	.018	.048	.030	.044
NO_3-N	.04	.10	.07	.09	.18	.26	.29	.33	.18	.29

Nutrient values are given as $mg.dm^{-3}$

Table VI. Analysis of seawater samples collected from Langstone Harbour at low neap tide on January 18,1974.

Station No.	1	2	3	4	5	6	7	8	9
PO_4-P	.058	.067	.038	.038	.083	.077	.061	.070	.058
NH_4-N	.137	.165	.127	.140	.175	.182	.171	.162	.161
NO_2-N	.017	.018	.014	.014	.020	.020	.018	.018	.017
NO_3-N	.262	.314	.232	.248	.346	.340	.314	.284	.244

Values given as $mg.dm^{-3}$

Table VII. Changes in nitrogen level in the time over a period of 14 days. Samples collected at station 11 from Poole Harbour on September 14, 1973, and stored at -18°C.

Day of Storage	NH_4-N	NO_3-N	NO_2-N
0	5.61	.311	.027
3	5.61	.311	.027
6	6.38	.172	.044
7	7.31	.175	N.D.
10	7.35	.187	.077
14	6.06	.199	.044

N.D. = none detected.
Values given as $mg\,dm^{-3}$.

been expected. The trend of change in NH_4-N is up - by approximately 10% after 14 days, but up some 30% at day 10. With nitrate the trend of change is more uniformly down - some 30% down at day 14, but 40% down at day 10. For nitrite the trend is up, but with values at day 7 (more detectable) and at day 10 rather excessively up, difficult to explain. Even so the contribution of nitrite to total nitrogen is very small. It is likely that part of the NO_3 was converted to NO_2 and then to NH_3. It would however be unwise to place too much emphasis on the detailed patterns of change without further replicated analyses. Wide as they are it will be seen later that changes of such magnitude do not affect the validity of conclusions to be drawn from the culture results.

6.4.4.2 Laboratory culture experiments with field water samples.

Growth performance of Ulva discs in seawater samples collected from Poole and Langstone Harbours, together with the chlorophyll and protein contents of the discs at the end of each culture period are summarized in figures 38 to 40. Figure 41 gives the mean area of Ulva disc cells at the end of each culture experiment.

The growth of Ulva in the ISW in all three collections was less than in any of the seawater samples from either Harbour apart from station 14 of the spring high tide Poole Collection, indicating a degree of eutrophication throughout the Harbour. Erdschreiber medium gave consistent results throughout and can therefore be used with confidence as a control. Culture data from the spring low tide collection (fig. 38A) show a wider range of Ulva growth rate than found with the spring high tide collection (fig. 39A). This would seem to be correlated with the generally higher nutrient levels and wider range among the sampling stations of the former (refer to table IV and V). This would be expected from the minimum dilution effects of the low spring tide on the sewage effluent flowing out of the Bund (station 14). At the

Fig. 38. Effects of seawater samples collected from different stations in Poole Harbour at spring low tide (14.9.73) on Ulva disc growth and composition, parameters determined at the end of 14 days incubation in seawater samples.

A Percentage increase in area of Ulva discs. Values given are mean and twice standard error on either side of mean.

B Chlorophyll content of discs.) Values are mean
C Protein content of discs.) and range.

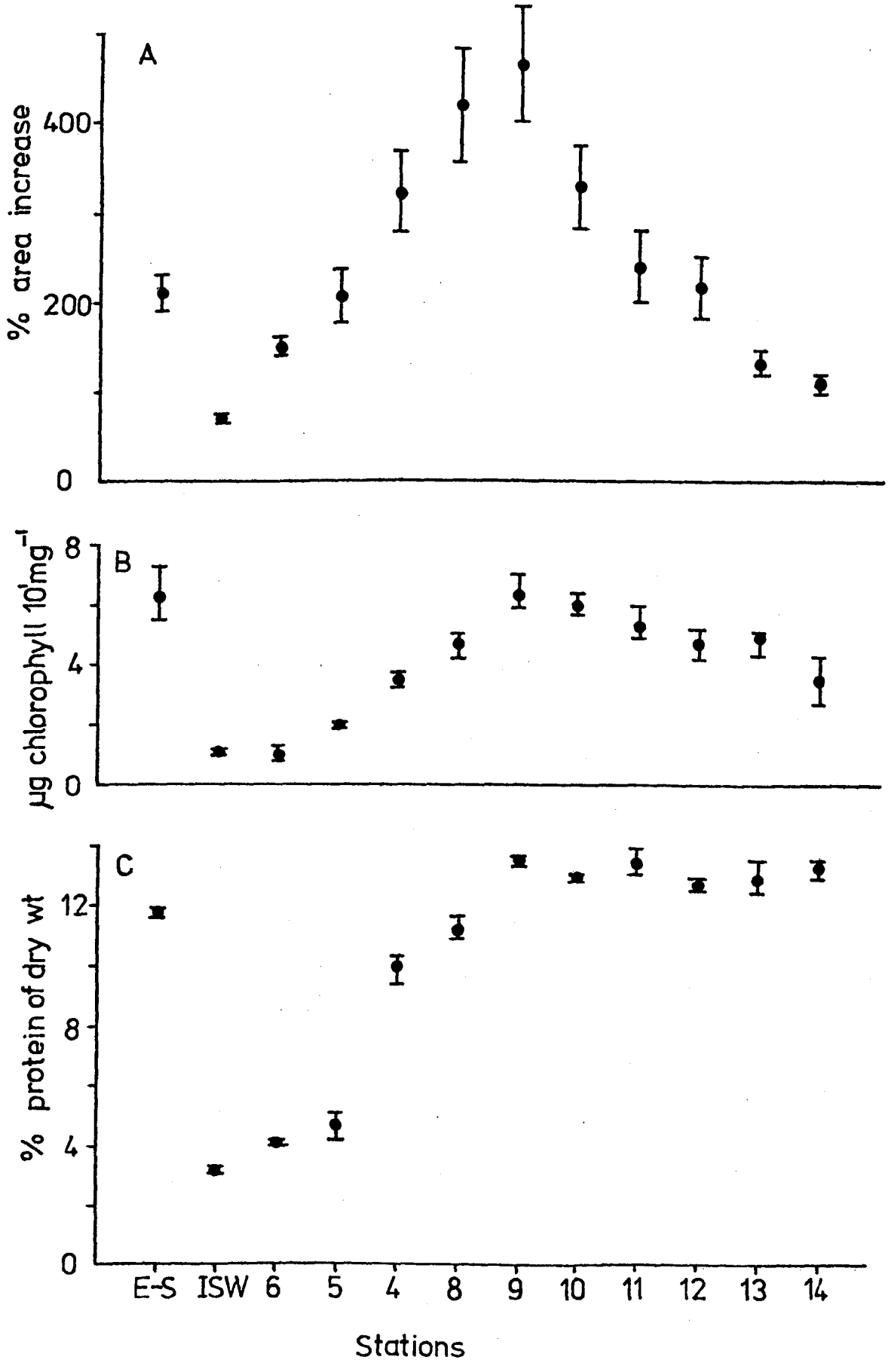

A
% area increase
400
200
0
B
µg chlorophyll 10'mg⁻¹
8
4
0
C
% protein of dry wt
12
8
4
0
E-S
ISW
6
5
4
8
9
10
11
12
13
14
Stations

Fig. 39. Effects of seawater samples collected from different stations in Poole Harbour at spring high tide (12.11.73) on Ulva disc growth and composition, parameters determined at the end of 14 days incubation in seawater samples.

A Percentage increase in area of Ulva discs. Values given are mean and twice standard error on either side of mean.

B Chlorophyll content of discs.) Values are mean
C Protein content of discs.) and range.

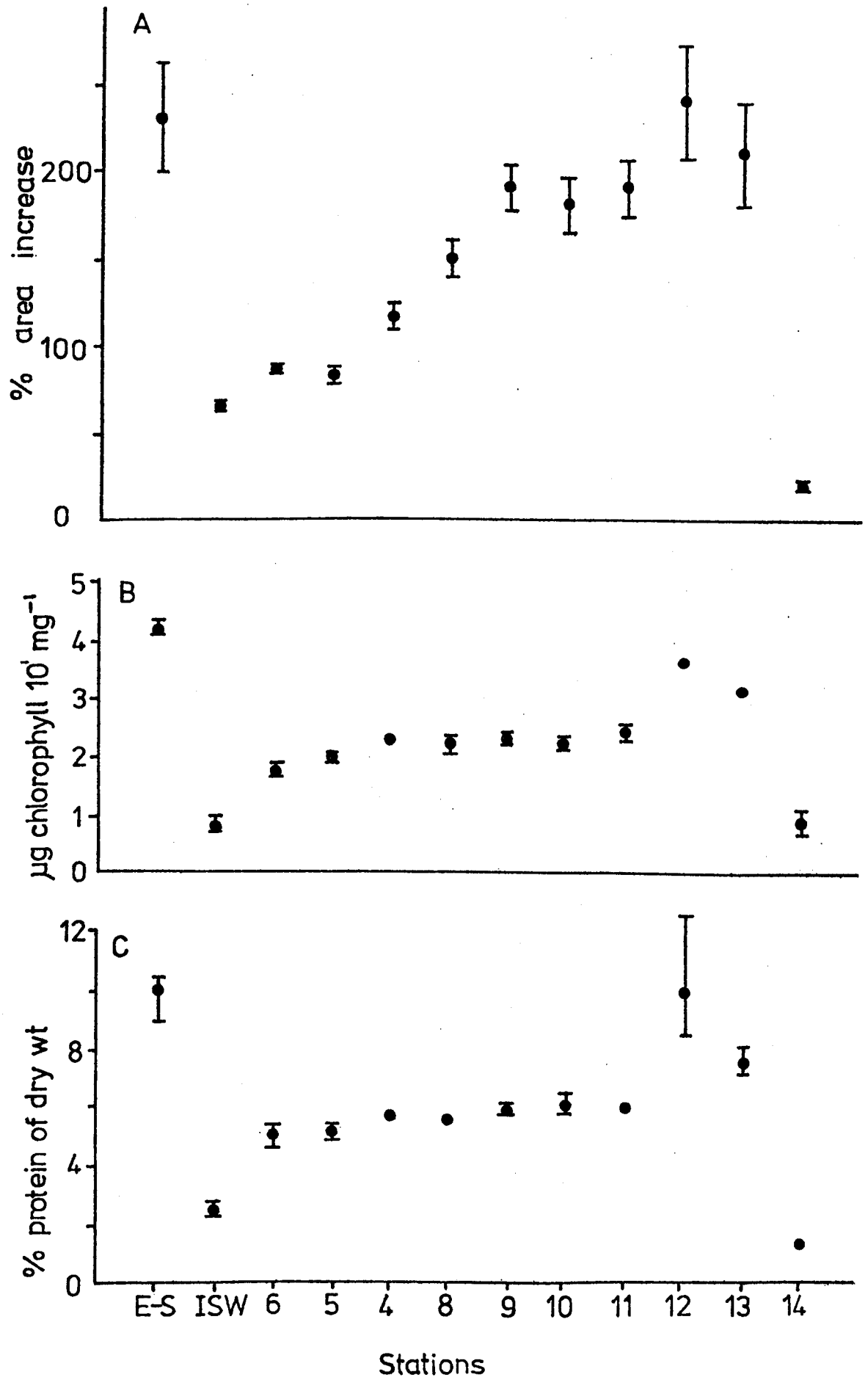
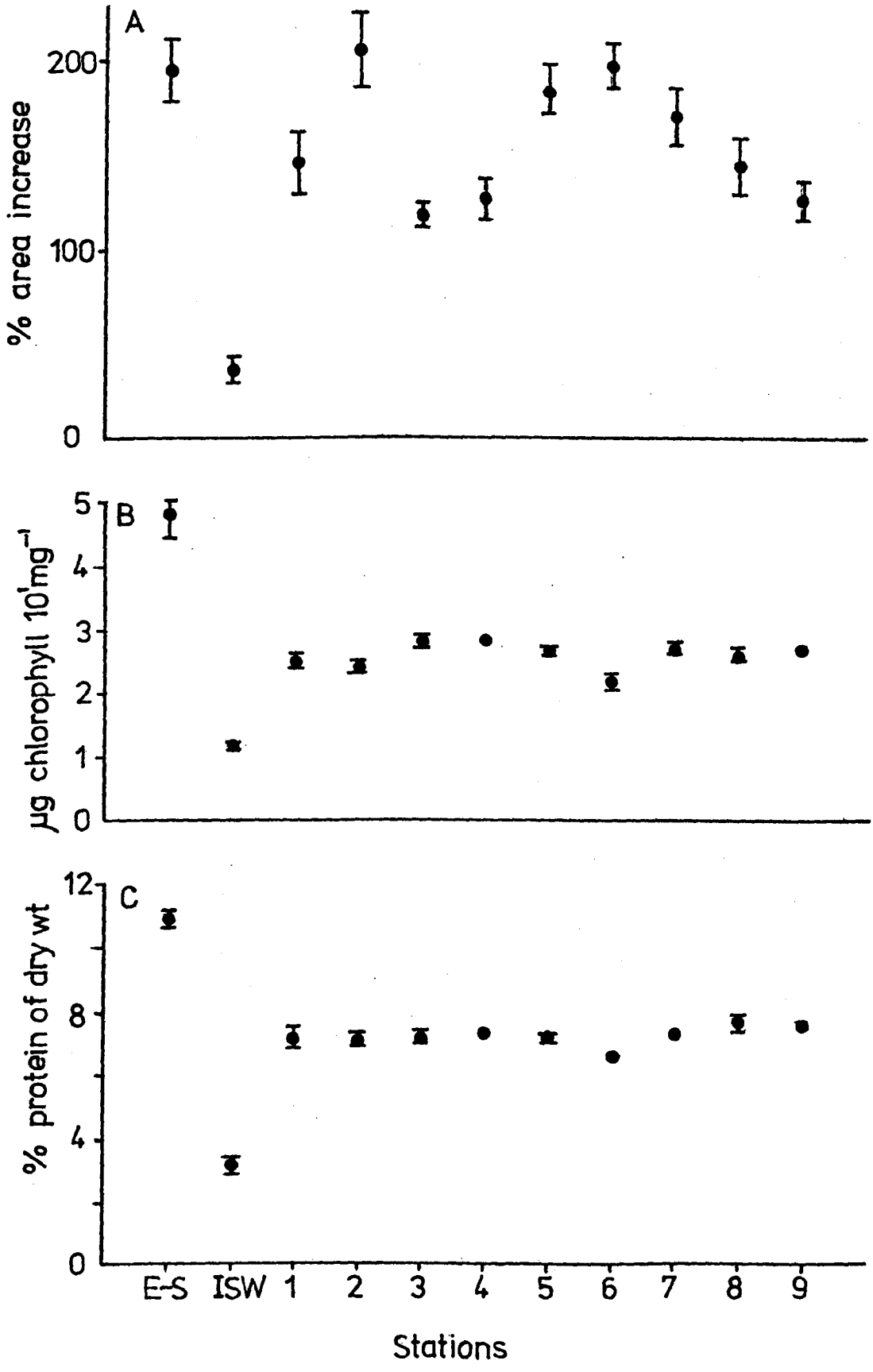

A
% area increase
200
100
0
B
µg chlorophyll 10' mg⁻¹
5
4
3
2
1
0
C
% protein of dry wt
12
8
4
0
E-S
ISW
6
5
4
8
9
10
11
12
13
14
Stations

Fig. 40. Effects of seawater samples collected from different stations in Langstone Harbour at neap low tide (18.1.74) on Ulva disc growth and composition, parameters determined at the end of 14 days incubation in seawater samples.

A Percentage increase in area of Ulva discs. Values given are mean and twice standard error on either side of mean.

B Chlorophyll content of discs.) Values are mean
C Protein content of discs.) and range.


A
% area increase
200
100
0
B
µg chlorophyll 10'mg⁻¹
5
4
3
2
1
0
C
% protein of dry wt
12
8
4
0
E-S
ISW
1
2
3
4
5
6
7
8
9
Stations

spring high tide, maximum dilution effects had occurred as a result of the influx of seawater into Holes Bay. Thus the concentration of nutrients within Holes Bay was up to ten times higher in spring low tide water than in spring high tide water at any one station. With the spring low tide sampling, culture water from stations 4-10 gave significantly greater growth of Ulva than did E-S medium, whereas for the spring high tide culture, none of the water samples induced more growth than the E-S medium. With all Langstone Harbour water samples growth produced was lower than E-S control medium.

The relative changes of growth rate and chlorophyll and protein content throughout the full series of field-water bioassays are certainly complex. In general chlorophyll and protein content of discs varied less than growth in relation to nutrient levels. This is clearly seen when the nutrient level is high as e.g. in samples from stations 9 to 14 in Poole Harbour collection at low spring tide (fig. 38). Here although Ulva growth rate dropped steadily, and with significant differences between all adjacent stations except 11 and 12, from stations 9 to 14, both chlorophyll and especially protein content remained high and very similar (because of limited replication of chlorophyll and protein analysis no significance tests were made). Similarly in the Langstone Harbour situation although there is much significant growth variation between stations, variation in chlorophyll and protein content is again very low; variation from mean values is approximately only 8% for protein, 16% for chlorophyll, but as high as 50% for growth. Comparison of growth, chlorophyll and protein content in the E-S control discs with those from field-water cultures is also interesting. Where nutrient level is low - especially so for NH_4-N - chlorophyll and protein levels remain very similar but when the water induces a growth increase of approximately 200% or more i.e. from around the E-S growth response, chlorophyll and protein levels do change very markedly and whilst in

proportion to growth is more in absolute terms e.g. from fig. 38 comparing stations 5 and 9 - growth increase is approximately 125% whilst chlorophyll and protein each increase by over 300%. Hence a change in water conditions sufficient to increase growth rate by 200% or more is needed to cause a change (increase) in chlorophyll and protein. Above this point increased growth stimulation is outpaced by increase in chlorophyll and protein, but then finally when nutrient/pollution level is high enough to reduce growth significantly below its maximum, chlorophyll and especially protein levels still remain at around the maximum values.

From these results it seems that neither chlorophyll nor protein data could be used with as much sensitivity to indicate water quality. The explanation of the different responses would however seem as difficult as the situation is complex without further experimentation.

Ulva mean cell area varied between 110-190 μm^2 in discs from all the three collection cultures (fig. 41), but the size of cells produced within each culture collection had less variation (less than 40 μm^2 difference between the lowest and the highest). The only exception where cell size was significantly different from all other cultures occurred in station 14 of the Poole culture which gave significantly larger cell size values than some of the other stations and seems likely to be due to the lower salinity of these water samples. The similarity in cell size between the cultures of an experiment means that any differences in the size of Ulva discs were not due to difference in cell size but rather to a difference in cell division rate. There is in fact a suggestion that when disc area increase is high, cell size is marginally lower, although with present sampling not significantly so.

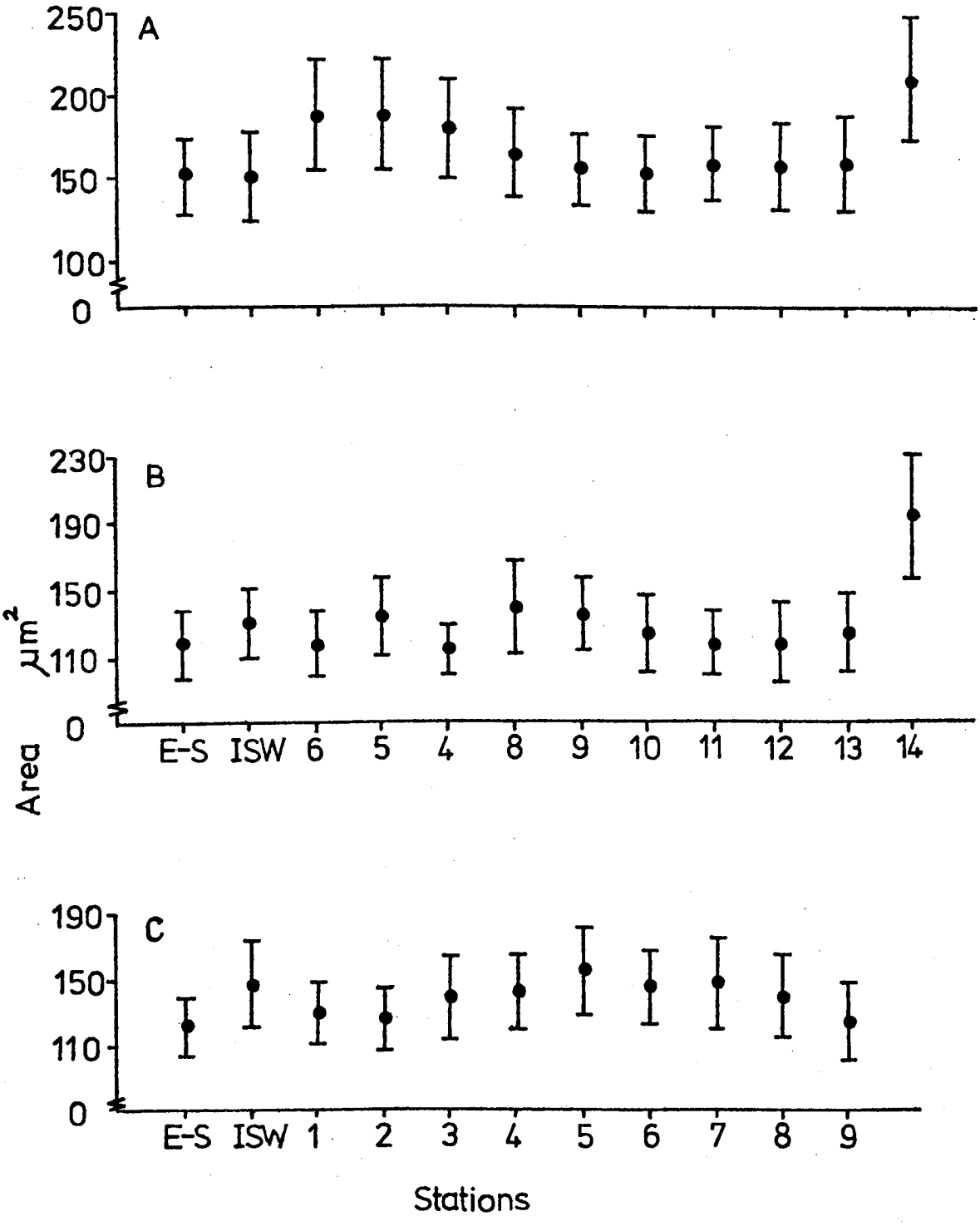
Comparison between the nutrient concentration and form, and growth data shows a clear correlation between growth rate and concentration

Fig. 41. Size of Ulva cells in discs cultured in different seawater samples from stations in Poole Harbour.

Water collected on:

A Spring low tide (14.9.73), B Spring high tide (12.11.73), and C Samples collected in Langstone Harbour, neap-low tide (18.1.74).

All values are mean with twice standard error on either side of mean.


A
250
200
150
100
0
B
230
190
150
110
0
Area µm²
E-S ISW 6 5 4 8 9 10 11 12 13 14
C
190
150
110
0
E-S ISW 1 2 3 4 5 6 7 8 9
Stations

of nutrients. To assess the effects of different concentrations of each nutrient and its relative importance on the growth of Ulva, the logarithmic concentration of each nutrient determined in all samples is plotted against percentage area increase of Ulva discs (fig. 42). The coefficient of regression (b) was calculated in each case and the fitted regression line drawn. The 'student's' t-distribution (t) for sample size smaller than 30; or for sample size larger than 30 the standard deviation (d), was also calculated. The level of significance of the regression line from a zero regression was found in each case.

Both the plotted lines of regression of NH_4-N (fig. 42A) and PO_4-P (fig. 42C) were similar, suggesting that there may be some relationship between the two. Initially the growth of Ulva increased with increase in concentration of both nutrients, but a drop in growth rate followed as the nutrient concentrations exceeded a certain level. It is important, and most interesting, to note that this stimulation and toxic range of concentrations coincided with that found in the NH_4-N bioassay experiment (refer to section 5.3.2.2, fig. 21). The NO_3-N (fig. 42B) results, however, indicated that there was no significant relationship between the NO_3-N concentration and the growth of Ulva.

The similarity in the results for NH_4-N and PO_4-P analyses means, either that both of these nutrients affect Ulva growth in a similar manner or that only one nutrient has an effect but that the other shows a "false correlation" with growth as a result of a similar dilution process and a positive correlation between NH_4-N and PO_4-P concentration in all samples. To assess the relative importance of NH_4-N and PO_4-P concentrations on the growth of Ulva, laboratory culture data obtained for the nutrient enrichment laboratory test (section 5.3.2 and 5.3.5) were plotted as in figure 42. Figures 43A and B are plots of NH_4-N and

Fig. 42. Regression analysis between percentage area in crease of Ulva discs and nutrient concentration of seawater samples collected from Poole and Langstone Harbours.

Ulva growth against A Log NH_4-N

B Log NO_3-N and

C Log PO_4-P

b = coefficient of regression, t = students' t-distribution, s = standard deviation.

(for explanation see text)

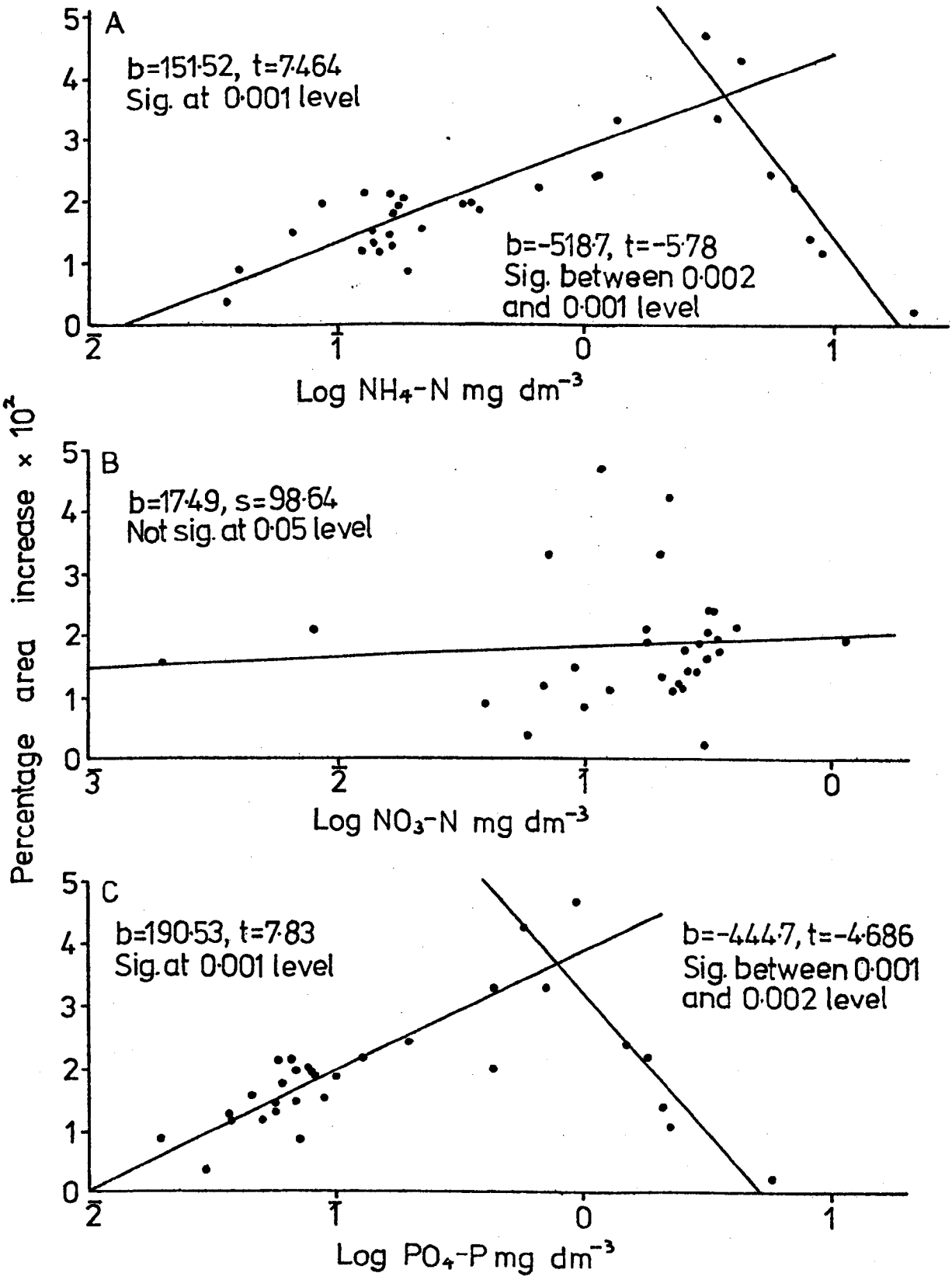
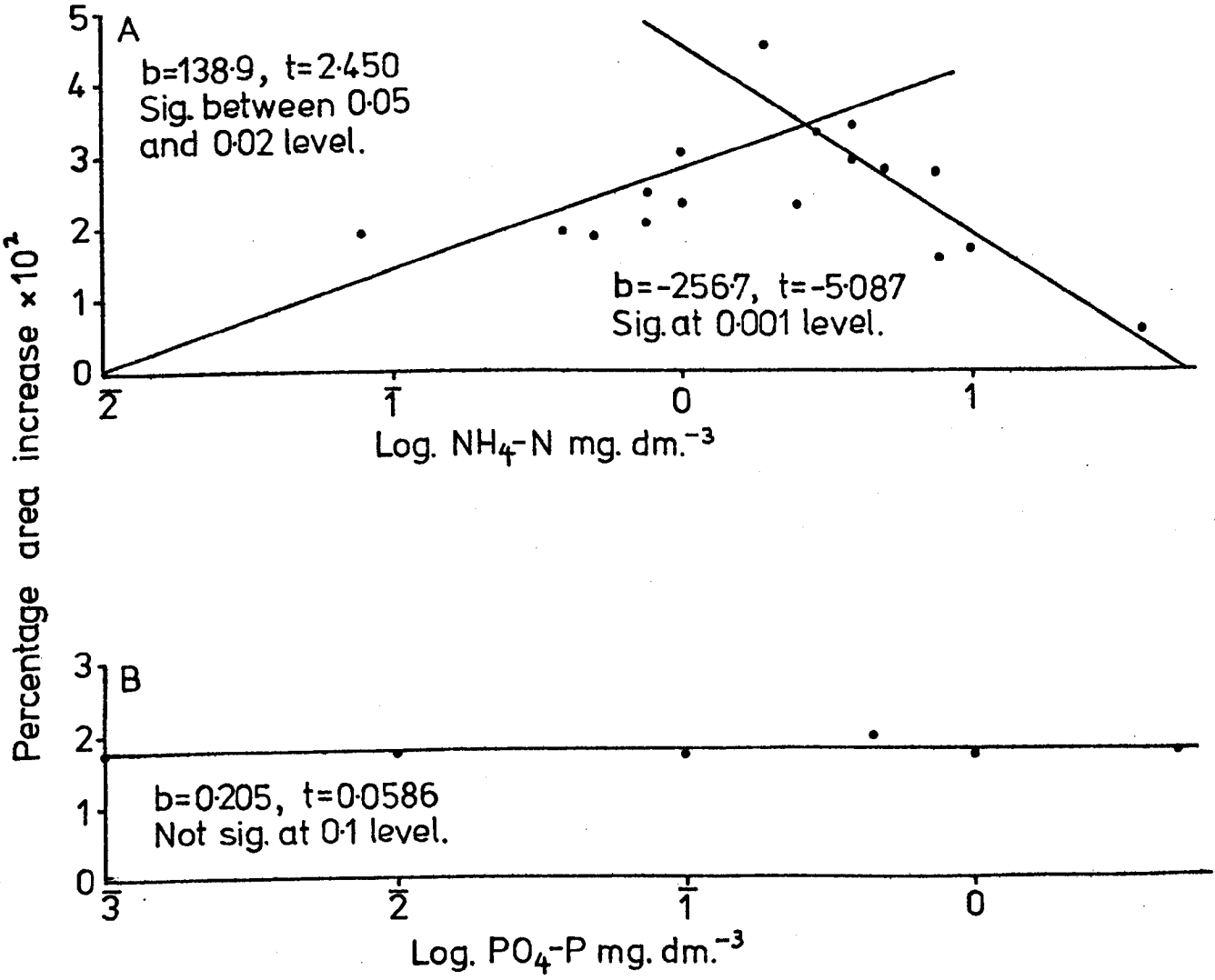

A
b=151·52, t=7·464
Sig. at 0·001 level
b=-518·7, t=-5·78
Sig. between 0·002
and 0·001 level
Log NH4-N mg dm-3
B
b=17·49, s=98·64
Not sig. at 0·05 level
Log NO3-N mg dm-3
C
b=190·53, t=7·83
Sig. at 0·001 level
b=-444·7, t=-4·686
Sig. between 0·001
and 0·002 level
Log PO4-P mg dm-3
Percentage area increase × 10²

Fig. 43. Regression analysis between percentage area increase of Ulva discs and components of laboratory nutrient cultures.

Ulva growth against A Log NH_4-N

B Log PO_4-P

b = coefficient of regression, t = 'students' t-distribution. (for explanation see text)


A
b=138·9, t=2·450
Sig. between 0·05
and 0·02 level.
b=-256·7, t=-5·087
Sig. at 0·001 level.
Percentage area increase ×10²
Log. NH4-N mg. dm.⁻³
B
b=0·205, t=0·0586
Not sig. at 0·1 level.
Log. PO4-P mg. dm.⁻³

PO_4-P logarithmic concentrations respectively against percentage area increase of Ulva. Comparing figure 43A with 42A, it is obvious that there is a close relationship between the two and in fact the slopes of the positive regression lines are not significantly different from each other at the 0.1 level. In both cases it is clear that NH_4-N directly influenced the growth of Ulva. It is of interest to note that the negative regression line in figure 43A is noticeably (significantly different between 0.02 and 0.01 level) less steep than that of the corresponding one in figure 42A. This is probably because in the latter, apart from the inhibitory effect of higher levels of ammonia, other toxic substances from the sewage effluents may also have inhibited Ulva growth, thus intensifying the toxic effect. In the case of PO_4-P (compare figure 43B with 42B), however, the patterns with enriched culture and field water bioassay were entirely different. In fact figure 43B showed that over the concentration range tested which covers the full range of levels found in the field, PO_4-P did not have any significant influence on the rate of Ulva growth. Thus this confirms that there is only an "apparent" correlation between the field - PO_4-P concentration and the growth of Ulva and that this is due to the positive correlation between PO_4 and NH_4 concentrations present in the water samples. This positive correlation in concentration of PO_4 and NH_4 can easily be shown by plotting the concentration of PO_4-P against that of NH_4-N (fig. 44B). This correlation was significant at the 0.001 level. No significant correlation was found between NO_3-N and NH_4-N levels (fig. 44A). Figure 44A also shows that a large drop in NH_4-N concentration was matched proportionally by a small drop in NO_3-N. This may have been the result of conversion of NH_4-N into NO_3-N under oxidizing conditions. Ammonia may also be favourably taken up by Ulva and bacteria.

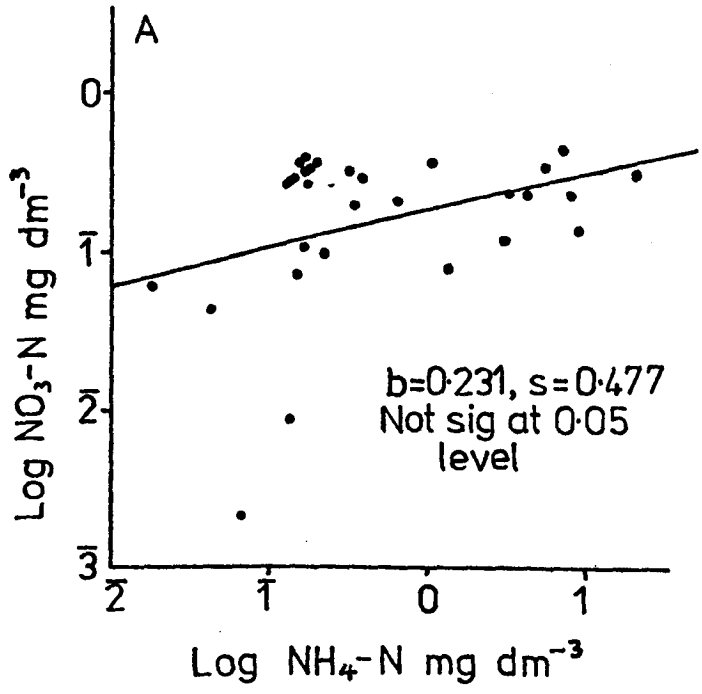
Fig. 44. Regression analysis on concentrations of different nutrients present in seawater samples collected from Poole and Langstone Harbour.

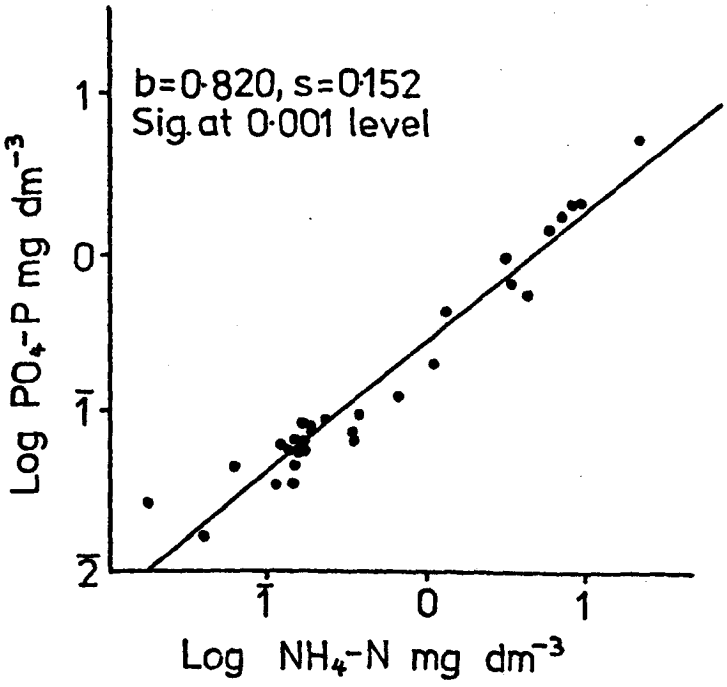
A Log NO_3-N against Log NH_4-N,

B Log PO_4-P against Log NH_4-N.

b = coefficient of regression, s = standard deviation.

(for explanation see text)


A
0
1̄
2̄
3̄
Log NO_3-N mg dm^{-3}
b=0·231, s=0·477
Not sig at 0·05
level
2̄
1̄
0
1
Log NH_4-N mg dm^{-3}


b=0·820, s=0·152
Sig. at 0·001 level
1
0
1̄
2̄
Log PO_4-P mg dm^{-3}
1̄
0
1
Log NH_4-N mg dm^{-3}

The culture results indicate that when compared with E-S medium, the range of stimulation of Ulva growth by NH_4-N was from just over 1 mg dm^{-3} to 8 mg dm^{-3} with an optimal stimulatory concentration at 3 mg dm^{-3}. Concentrations of over 8 mg dm^{-3} brought about the onset of inhibition of Ulva growth. These experimental results fit in with the actual field distribution of Ulva in Holes Bay. All water samples collected from the field, with the exception of that from station 14 collected at high water of spring tide gave a greater growth rate for Ulva than those of the ISW control samples. This shows the sensitivity of Ulva towards nutrient levels in the field samples as reflected by its corresponding growth response.

In addition to the effects of ammonia on growth, other interesting concentration-dependent developmental effects were consistently found. For example, swarming of Ulva was completely inhibited when the NH_4-N concentration was 10 mg dm^{-3} or above. Ammonia concentrations in the growth rate stimulating range also tended to suppress swarming in Ulva. This evidence supports field observations. In Holes Bay, where a relatively high amount of ammonia is present, and Ulva growth is prolific, no swarming of Ulva material was ever observed in the field during the period of study. Further investigation of the relationship between specific nutrient level and swarming should prove worthwhile.

6.5. Discussion.

The discussion in this section is restricted to interpretation of the water bioassay and chemical analysis data, its relationship to the nutrient enrichment culture work and a comparison of the growth responses in water from Poole and Langstone harbours, both eutrophicated with sewage. A more detailed discussion of these results in relation to the very small amount of relevant published work is withheld until the final discussion in Chapter 8.

By comparing the nutrient concentrations present in the water

samples collected from the harbours and their effect on the growth of Ulva there is a strong indication that ammonia is the nutrient which causes the prolific growth of Ulva. The field studies showed that significantly more Ulva growth occurred in water samples with a range of NH_4-N between 1.33 and 4.28 mg dm^{-3} than in E-S medium. With NH_4-N concentration from 5.61 to 6.95 mg dm^{-3} growth rate was similar to that in E-S control. But at 8.13 mg dm^{-3} NH_4-N or above, a significantly lower Ulva growth rate was found than with the E-S control. This range of NH_4-N concentration giving significantly greater Ulva growth over the E-S control fell within the limits observed in the NH_4-N laboratory bioassay test (section 5.3.2.2.) where the range of stimulation was found to lie between 0.389 and 5.835 mg dm^{-3} NH_4-N. The water samples, collected from the field, that gave a significant stimulation of Ulva growth over the E-S control contained NH_4-N concentrations within the stimulatory range found in the laboratory culture experiments. This further supports the thesis that the level of NH_4-N in the water is the causal factor of the prolific growth of Ulva in Holes Bay. It was interesting to note that the range of NH_4-N concentration causing stimulation in the field samples was narrower than that observed in controlled laboratory NH_4-N-added cultures. This was probably due to the presence in the field samples of other additional substances which can also influence the growth of Ulva. This would apply especially to water samples collected near the sewage outfall where there may be high concentrations of toxic substances e.g. detergents.

As already shown, it is clear that both NO_3 and PO_4 have less effect on the growth rate of Ulva than NH_3. These results are again supported by those found in the laboratory bioassay cultures.

Two sampling runs were taken in Poole Harbour one at spring low and one at spring high tide. This means that maximum pollution/nutrient

levels could be expected in the former and minimum in the latter. This difference led to further interesting and important observations. Firstly, growth performance of Ulva in water samples from low spring tide was generally much higher than that in spring high tide samples. A maximum value of Ulva growth, of 450% increase in area over the period of two weeks, was found in the spring low tide collection (fig. 38), this compares with only a 200% increase in area of discs cultured in water from the spring high tide collection over the same growth period (fig. 39). Secondly, a difference in the collections was found in the distance from the sewage outflow point to the sampling station which gave maximum Ulva growth. In the spring low tide collection, maximum Ulva growth occurred in station 9 water which is around 1310 m from the sewage outflow, yet in the spring high tide collection it was water from station 12, only some 590 m from the sewage outflow point which promoted maximum growth. Since the two collections were timed to achieve maximum and minimum nutrient levels within the Harbour the area in which maximum Ulva growth occurs should lie between these two stations, i.e. from just north of Junction Channel (station 9) to the Railway Bridge (station 12) within Holes Bay (refer to fig. 34 for geographic location). Interestingly the mean NH_4-N concentrations in the year 1973, observed by Mr. Silk of the Poole Purification Works, at Stations 9 and 12 were 0.7 and 3.4 mg dm^{-3} respectively. Furthermore the greatest Ulva growth has been and still is, observed to lie between these two stations in Holes Bay. Thus again this gives a further very strong indication that it is NH_4-N level which controls the growth of Ulva within Holes Bay.

Apart from the positive response to NH_4-N, the growth rate of Ulva discs also indicated the particular level of this nutrient in the water samples. A graded growth response in relation to the NH_4-N

nutrient status was observed in Ulva growth with water samples from the harbour when compared with the ISW control. For example, this was shown in water from stations 5 and 6 in the low spring tide run (fig. 38) and stations 6-13 (fig. 39) in the high spring tide collection. Thus Ulva is suitable, not only for monitoring stimulatory growth due to a limited range of NH_3 concentration but it can also give a clear indication of a very wide NH_4-N nutrient range in the water samples.

Of the two harbours under the present investigation only Holes Bay in Poole Harbour has an extensive growth of Ulva. This is of great significance in evaluating the ability of Ulva to act as a pollution indicator, since both harbours are known to be sewage polluted. The following is an attempt, using results obtained from the present work together with information published by others (e.g. Green, 1940; Green, Ovington & Madginck, 1957; Dunn, 1972), to make a detailed comparison of the two harbour situations.

In Poole Harbour the major source of nutrient input is treated domestic/industrial sewage emptying into the higher part of Holes Bay near station 14 (refer to fig. 35B) at the Bund. Construction of the Bund was completed in 1973. In 1971 the dry weather flow had already reached 5.46×10^4 m^3 day^{-1} (Sawyer, 1971). As the only outlet to the main harbour is at the opposite end of the Bay this means that the effluent flows through and mixes with seawater along its entire length. This in turn has two consequences. Firstly, there exists a concentration gradient of nutrients, high in the upper part of Holes Bay at the Bund and reducing towards Poole Quay at the other end. Secondly, on the whole, the level of nutrients within the Bay is high. Nutrient analysis of samples from within the Bay showed that this was so. The high level of nutrients, in particular ammonia, results in the abundant growth of Ulva. The average level of ammonia in most parts of Holes Bay being within the range found to cause significant growth stimulation

over that occurring with the E-S control medium.

On an ebbtide the contents of Holes Bay empty into the main harbour and are much diluted. Thus the level of nutrients in Poole Harbour proper is of an order of magnitude lower than that of Holes Bay. This drop in nutrient levels appears to prevent abundant Ulva growth.

As indicated earlier (section 6.2) there are two main sources of sewage effluent in Langstone Harbour. Budd's Farm, situated in the north-east of the harbour (fig. 35), discharges a dry weather flow of 3.41×10^4 m^3 day^{-1}. At Fort Cumberland (fig. 35) a dry weather flow of 6.82×10^4 m^3 day^{-1} empties into the mouth of Langstone Harbour. As effluents are discharged at both ends of the harbour this would be expected to lead to a fairly uniform distribution of nutrients within the harbour and in fact no definite gradient of nutrients was detected from one end of the harbour to the other. Nutrient analysis of samples taken within the harbour showed both NO_3 and PO_4 were present in the same order of concentration as in Holes Bay, and were somewhat higher that those in Poole Harbour. Ammonia, however, was about ten times lower than that of Holes Bay. Furthermore, the level of NH_3 present in Langstone Harbour was below the range found to give stimulation of Ulva growth in nutrient bioassay and Poole Harbour culture experiments. Thus it would seem that the main reason why Ulva does not grow excessively in Langstone Harbour is because of the low level of NH_3. Laboratory culture experiments using Ulva in seawater samples collected from Langstone Harbour confirmed this as no stimulation in growth was found.

CHAPTER 7

Ulva lactuca as a test organism - Toxicity Tests

Much work has been done on the effects of chemicals, mainly toxic in nature, on fish, invertebrates (e.g. molluscs) and micro-algae (Battelles Columbus Laboratories, 1971). Many of the chemicals tested are basic components of domestic and industrial sewage sludge. The nature of any potentially toxic substances depends mainly on the types of wastes entering the sewage system. Heavy metals and detergents are two common types of toxic substance present in sewage sludge that affect aquatic life. As *Ulva* grows in areas contaminated with sewage (e.g. Cotton, 1910; Ehrhardt, 1968), it is often subjected to heavy metals and detergents. A knowledge of how these substances affect the growth of *Ulva* is essential for an understanding of its distribution in sewage-contaminated harbours and estuaries and for determining how useful *Ulva* might be as a test organism for toxic chemicals. At the present time no data are available on the effects of heavy metals and detergents on the growth of *Ulva*. Experiments were therefore carried out to determine the effects of: sewage sludge; detergent (as Blusyl) and the heavy metals copper, mercury, zinc, lead and cadmium on *Ulva* growth. All experiments were performed under regime (ii) conditions as described in section 3.2.2. and growth measurements were made at the end of 14 days of culture.

7.1. Sewage sludge.

7.1.1. Method.

Sludge used in this experiment was supplied by the Davyhulme Sewage Works in December, 1972. The sludge which was a mixture of domestic and industrial origin, was stored at 2°C immediately it was received from the sewage works, and until used.

For culture work E-S medium was used as basic culture medium and a number of treatments set up with varying amounts of sludge added. The dilutions used were 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1.0%, 5.0% and 10.0% v/v based on wet sludge mixed with E-S medium. A control of just E-S medium was also included. The range of sludge dilutions used was similar to ~~these~~ that used by Burrows and Sharples (1972), for tests with other marine algae.

7.1.2. Results.

The average composition of the Davyhulme sludge during the week of collection, determined by a sewage work analyst, is given in table VIII.

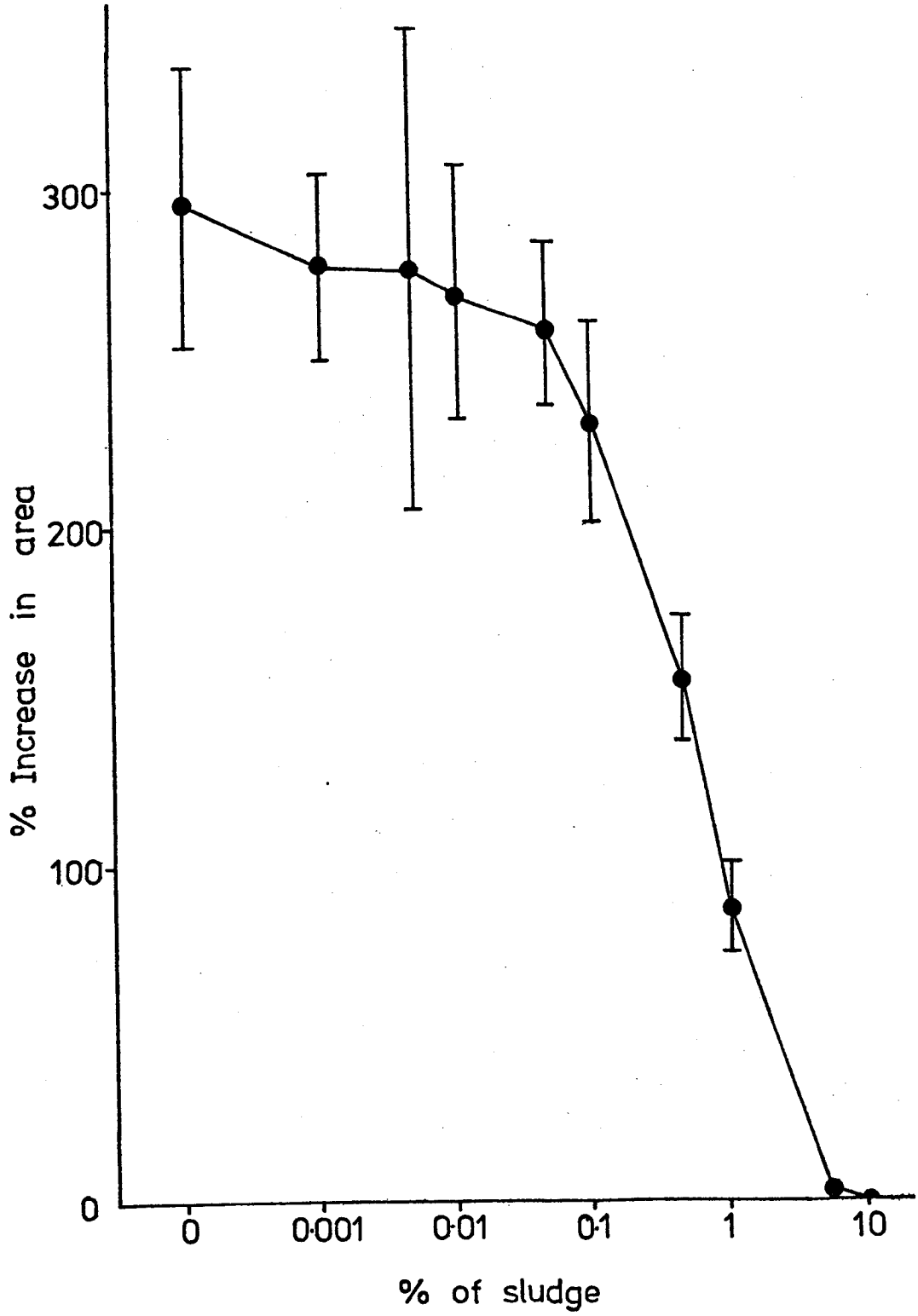
The culture results showed no significant differences in the growth of Ulva from the control up to 0.1% sludge concentration, after which there followed a rapid drop (fig. 45) in growth with increasing sludge concentration. Virtually no growth occurred in 5% and 10% sludge. At 0.1% sludge concentration the rate of Ulva growth was just significantly lower than that of the control.

Table VIII. Composition of Davyhulme sewage sludge.

Component	On dry wt. base	On wet wt. base
Dry wt.	-	1.74%
Mineral	31%	0.54%
Organic	69%	1.20%
Total P	1.58%	0.027%
Total N	2.20%	0.038%
Pb	431 ppm	7.50 ppm
Cd	35 ppm	0.61 ppm
Hg	25 ppm	0.44 ppm
Cu	1781 ppm	30.99 ppm
Zn	1868 ppm	32.50 ppm
Detergent	0.70%	0.012%

Fig. 45. Growth of Ulva discs after 14 days culture in various concentrations of sewage sludge added to E-S medium.

Values show mean and twice standard error either side of mean.


300
200
100
0
% Increase in area
0
0·001
0·01
0·1
1
10
% of sludge

7.2 Anionic detergent (Blusyl).

7.2.1. Method.

A laboratory anionic detergent, Blusyl, was used for the culture tests. The sample of detergent used had an undiluted strength of 165,000 mg dm^{-3} (16.5% w/w) Manoxol O.T. units (sodium dicotyl sulphosuccinate). The use of Manoxol O.T. as an ionic detergent reference standard was recommended by Longwell & Maniece (1955) as it is readily available in a high state of purity. It measures the detergent action and enables different detergents to be compared.

The following additions of detergent to E-S medium were prepared for the toxicity tests: 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.5 and 11.0 mg.dm^{-3} Manoxol O.T. units. Again the range of dilution used is within that used by Burrows and Sharples (1972).

7.2.2. Results.

A summary of the results is given in figure 46. Similar growth performances to the E-S control occurred with concentrations of up to 0.1 mg dm^{-3} detergent (Manoxol O.T.). This was followed by a sudden and significant reduction in growth obtained with detergent concentrations above 0.1 mg dm^{-3}, and at 5.5 mg dm^{-3} detergent, very little growth at all was observed. At 0.5 and 1.0 mg dm^{-3} growth was some 60% of the control.

7.3. Heavy metals.

7.3.1. Method.

Similar culture tests were carried out with each of copper, zinc, mercury, lead and cadmium. The range of concentrations tested and the salts in which the metals were supplied is given in table IX. The metals were added to E-S culture medium; the concentration ranges used being relative to the levels found at sea and under heavily polluted conditions, and similar to those used by Burrows and Sharples (1972).

Fig. 46. The effect of detergent (Blusyl) on the growth of *Ulva* discs in E-S medium. Growth over a period of 14 days.

Values show mean and twice standard error either side of mean.

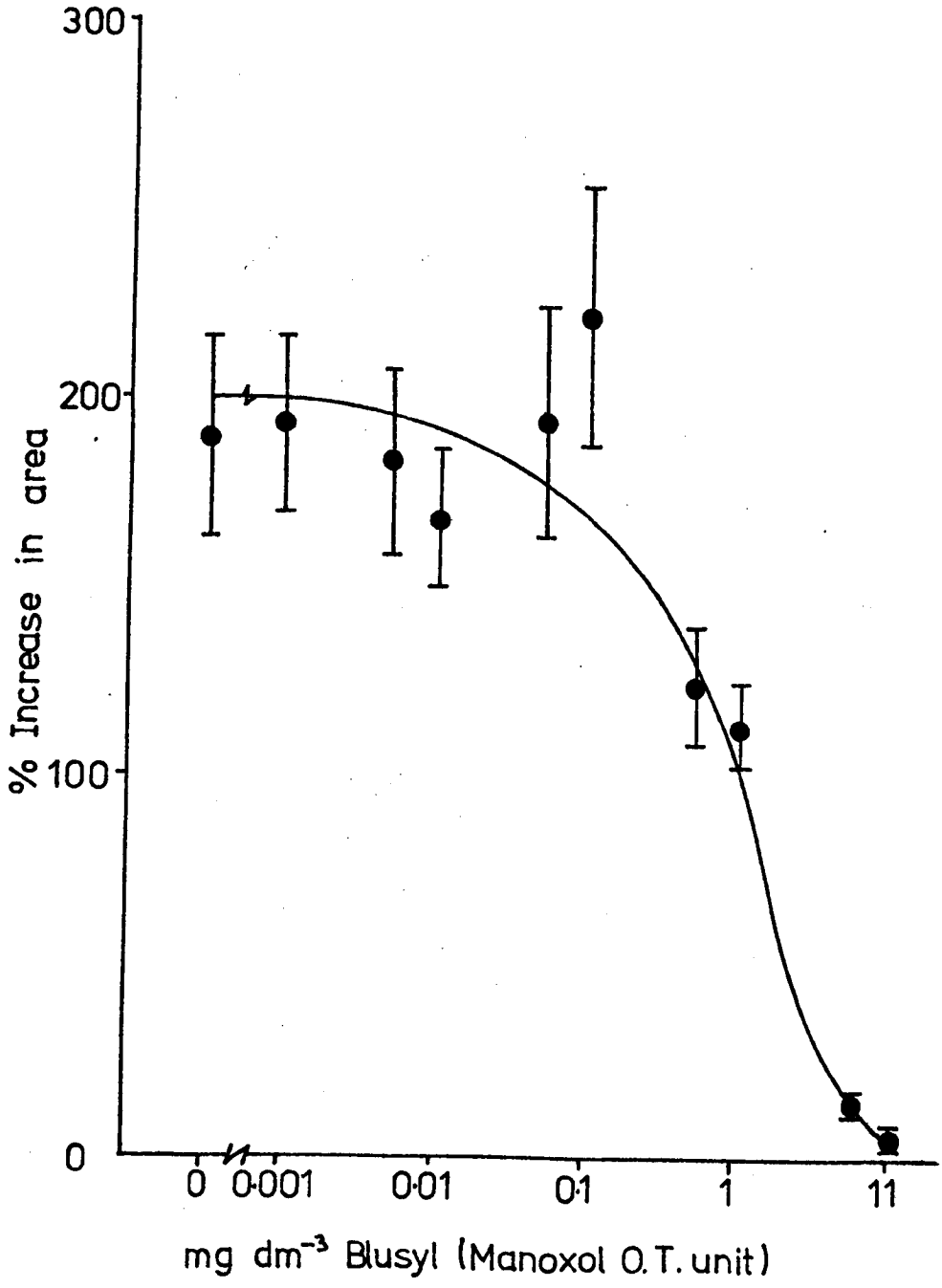

300
200
100
0
% Increase in area
0
0·001
0·01
0·1
1
11
mg dm⁻³ Blusyl (Manoxol O.T. unit)

Table IX. Concentration ranges of metal ion used and source in Ulva culture.

	Cu	Zn	Hg	Pb	Cd
Concentration range mg dm^{-3}	0.01 - 1.0	0.001 - 5.0	0.001 - 1.0	0.01 - 10.0	0.01 - 10.0
Form of salt used	$CuCl_2.2H_2O$	$ZnSO_4.7H_2O$	$HgCl_2$	$Pb(NO_3)_2$	$(CH_3.COO)_2$ $Cd.2H_2O$

7.3.2. Results.

The effects of heavy metals on the growth of Ulva are given in figures 47 to 51 and summarized in table X. All the metals tested seem to affect Ulva in a similar pattern with concentration. At low concentration no detectable effect occurred. This was followed by

Table X. Summary of the effect of heavy metals on the growth of Ulva.

Note: All values given as mg dm^{-3}

	Cu	Zn	Hg	Pb	Cd
Average concentration found at sea.(a,b)	0.003	0.01 - 0.005	0.00003- 0.00005	0.00003	0.00005- 0.00011
Minimum concentration showing significant inhibition over E-S control.	0.05	0.5	0.1	5.0	0.1
Lethal concentration.	0.2	5.0	0.5	10.0	5.0
Concentration present in Davyhulme sludge.	30.99	32.5	0.44	5.5	0.81
Concentration present in which inhibition occurred in sewage sludge culture.	0.03	0.03	0.00044	0.0055	0.00081
Concentration in which death occurred in sewage sludge culture.	1.5	1.5	0.022	0.28	0.041

a, Riley & Chester, 1971; b, Goldberg, 1965; c, Death occurred when Ulva discs stopped growth and lost their chlorophyll contents.

Fig. 47. Growth of Ulva discs after 14 days in various concentrations of copper added to E-S medium. Values show mean and twice standard error either side of mean.

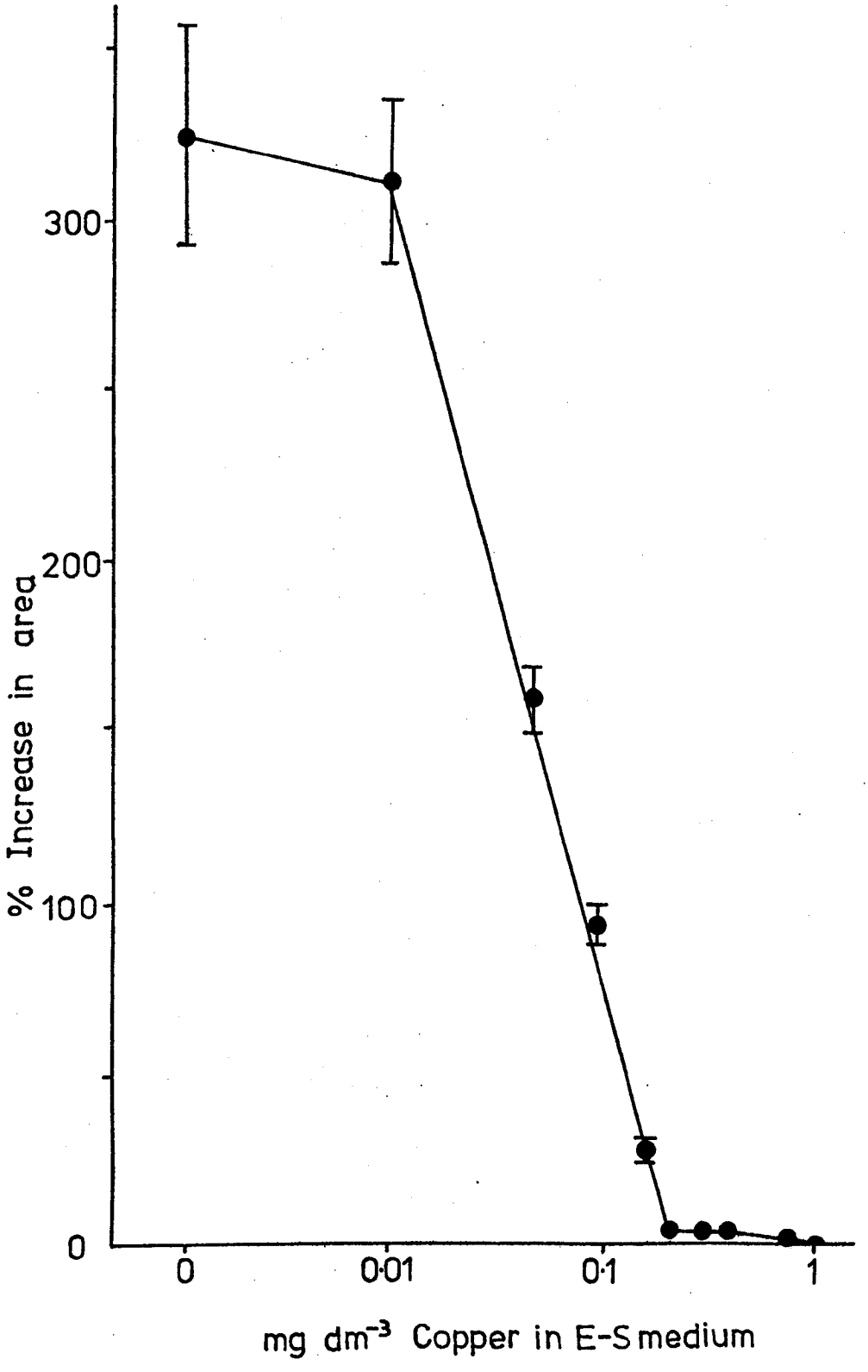

300
200
100
0
% Increase in area
0
0·01
0·1
1
mg dm⁻³ Copper in E-S medium

Fig. 48. Growth of Ulva discs after 14 days in various concentrations of zinc added to E-S medium. Values show mean and twice standard error either side of mean.

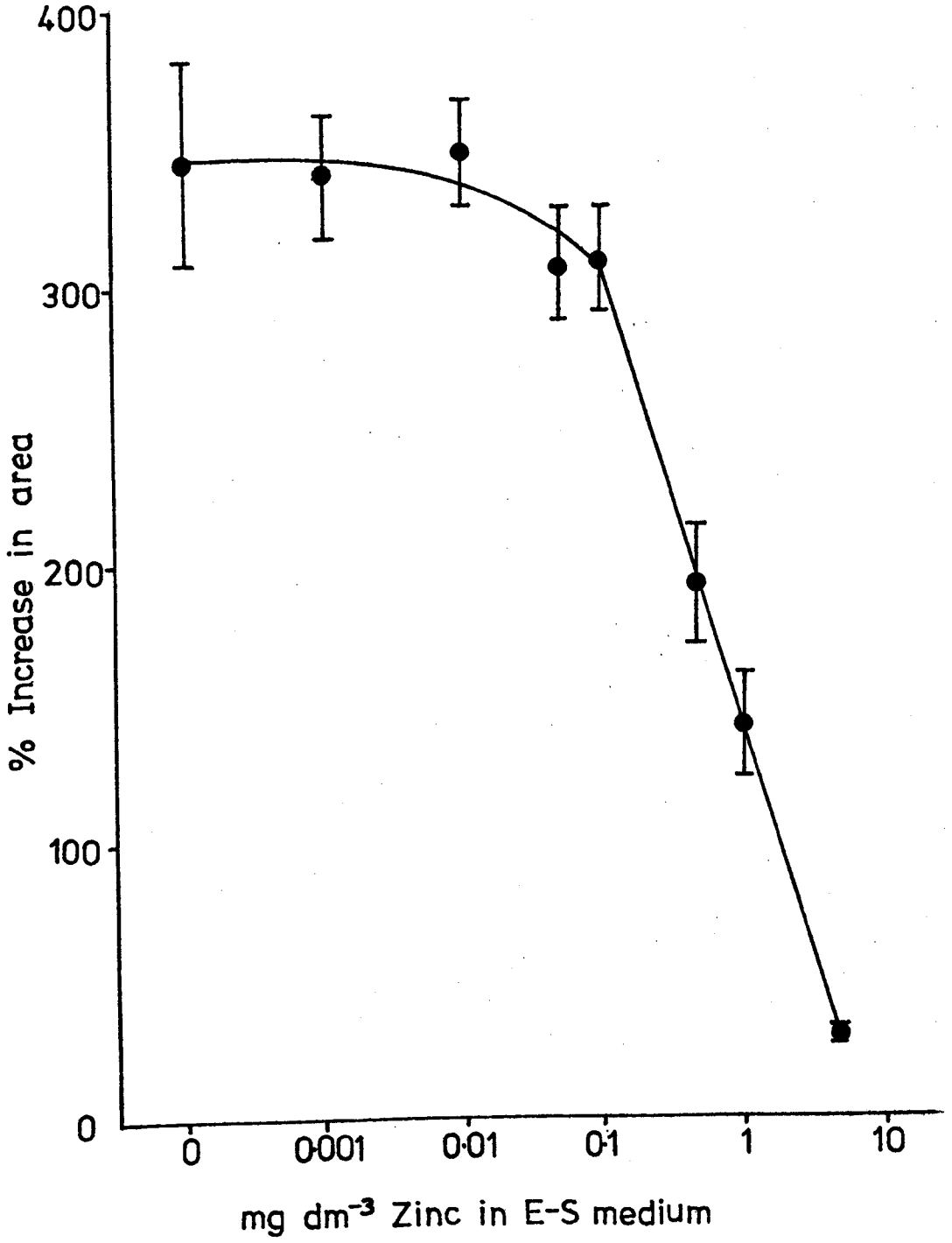
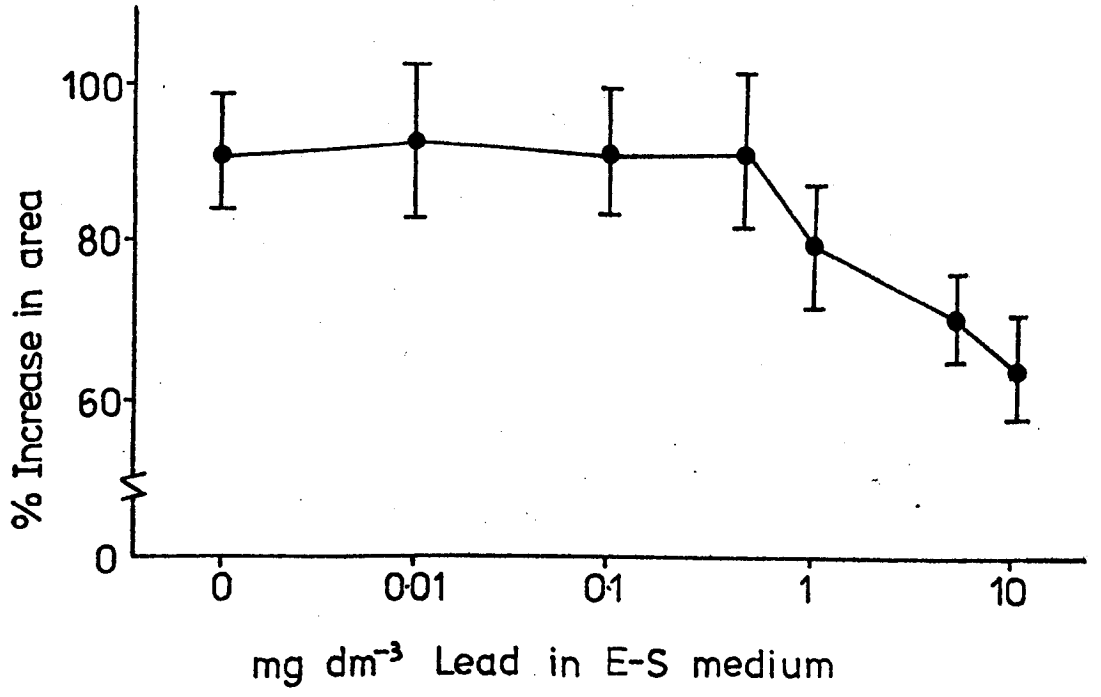

400
300
200
100
0
% Increase in area
0
0·001
0·01
0·1
1
10
mg dm⁻³ Zinc in E-S medium

Fig. 49. Growth of Ulva discs after 14 days in various concentrations of lead added to E-S medium. Values show mean and twice standard error either side of mean.

Fig. 50. Growth of Ulva discs after 14 days in various concentrations of cadmium added to E-S medium. Values show mean and twice standard error either side of mean.


100
80
60
0
% Increase in area
0
0·01
0·1
1
10
mg dm⁻³ Lead in E-S medium

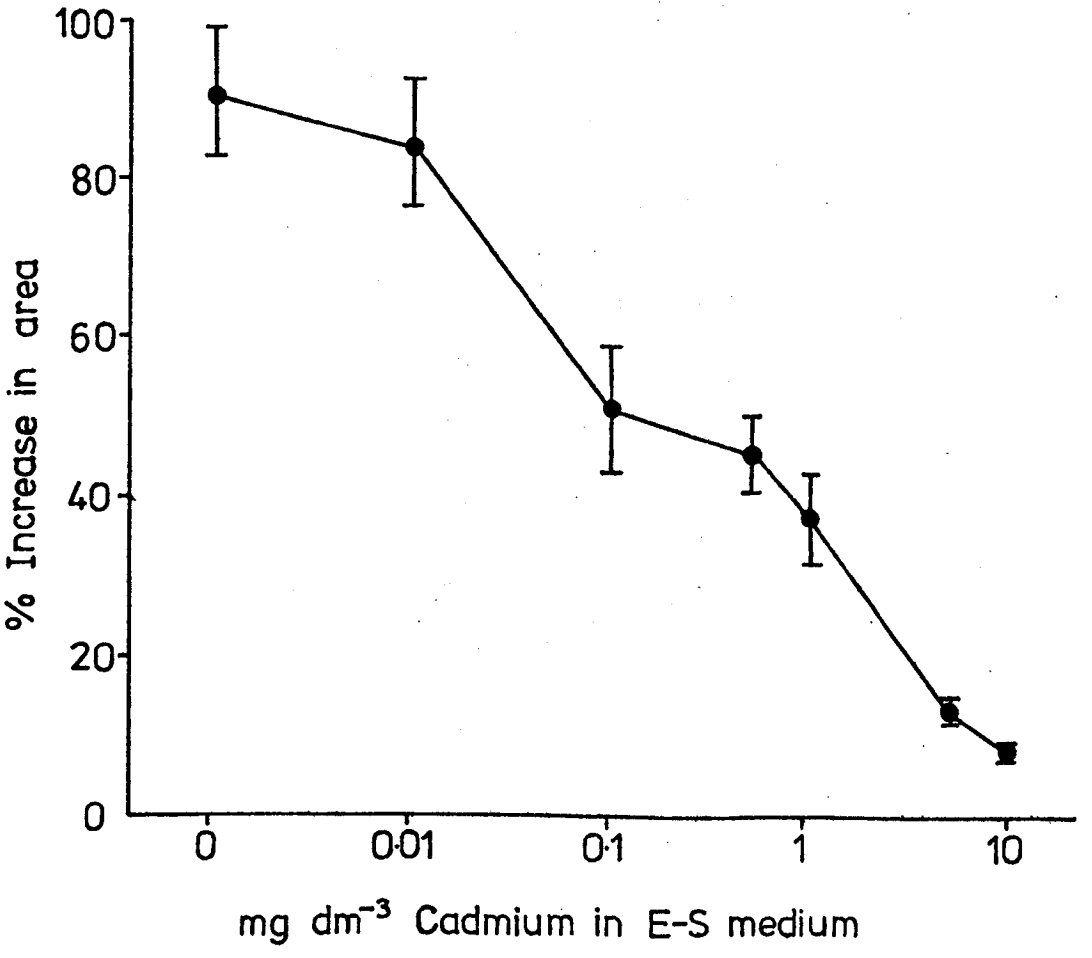
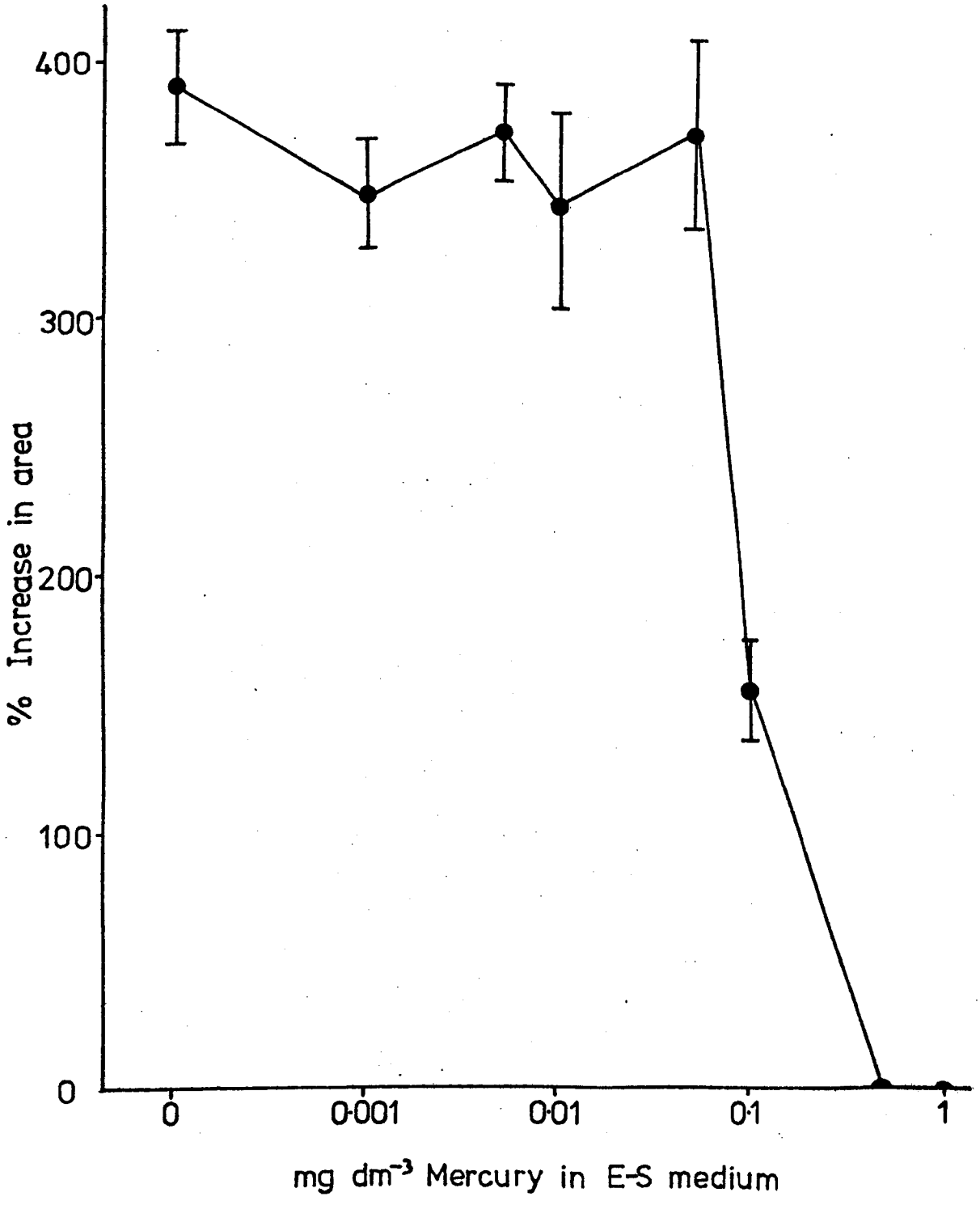

100
80
60
40
20
0
% Increase in area
0
0·01
0·1
1
10
mg dm⁻³ Cadmium in E-S medium

Fig. 51. Growth of Ulva discs after 14 days in various concentrations of mercury added to E-S medium. Values show mean and twice standard error either side of mean.


400
300
200
100
0
% Increase in area
0
0·001
0·01
0·1
1
mg dm⁻³ Mercury in E-S medium

a fairly sharp decrease in the growth of Ulva at the sub-lethal concentration until death occurred at a point in the concentration range tested as indicated in the table above and in figures 47 to 51. Results showed that the order of decreasing toxicity of the metals tested at sub-lethal concentration was Cu > Hg, Cd > Zn > Pb; and that of lethal concentration being Cu > Hg > Cd > Zn > Pb. Thus copper was the most toxic metal of the five while lead was the least.

The last two horizontal columns in table X show the amount of metals present in the sewage sludge cultures (section 7.1) where growth inhibition and lethal effects on Ulva of sludge occurred. This of course does not necessarily imply that the concentrations listed here were actually causing growth inhibitions and/or lethal effects on Ulva. They merely serve as references to which comparison can be made with the growth performance figures obtained in these metal toxicity test cultures. Comparison of these figures, between horizontal columns 1, 2 and 5, 6 respectively, indicates that, of the metals present in the sewage sludge, only copper and possibly zinc were likely to have caused inhibition of the growth of Ulva. Mercury, lead and cadmium were each present in too small an amount in the sludge to have induced any harmful effect on Ulva. Further work to find out if the effect of heavy metals was additive (in culture addition work), would make extrapolation to the likely effect of total heavy metal content in sewage much easier.

7.4. Discussion.

Since the sludge sample collected from Davyhulme Works was of combined domestic and industrial origin and its relative proportions uncontrolled, the number and concentration of potentially biotoxic substances could be appreciably different from other sample collections, depending on the quantities and frequencies of the discharge into the

Works at the time of collection. An example of the variation in two samples was provided by Burrows & Sharples (1972). Moreover, the figures of sewage analysis were averages of different estimates taken over a week. Hence the composition of the particular sample of sludge used may differ somewhat from the figures provided. Furthermore, the sample of sludge used in this work had yet a different detailed composition from the samples used by Burrows and Sharples (1972). Interpretation and comparison of the results obtained in this work with others can thus at best only be somewhat speculative.

Inhibition of growth of Ulva with a high percentage of sewage was probably due to a combination of three main factors, i) heavy metals, ii) detergents, iii) reduced light penetration. Culture tests on Davyhulme sludge suggested that a 2×10^{3} dilution would be required to eliminate inhibition of growth in Ulva. None of the dilutions tested has any stimulatory effect on the growth of Ulva. This agrees with the findings of Burrows & Sharples (1972) when they used Laminaria saccharina, Skeletonema costatum and Phaeocystis pouchetii as test organisms.

Detergents, as most of them contain polyphosphates, can act as a potential source of phosphorus and thus can lead to eutrophication of the environment. At high concentration, however, they are toxic to living organisms since they will act on the cell membrane, disturbing organisation of the lipid component with destruction of the controlled permeability. The lethal concentration of the anionic detergent Blusyl for Ulva was found to be approximately 5.5 mg dm^{-3} Manoxol O.T. units. This concentration was also the minimum to induce death of Skeletonema costatum and Phaeocystis pouchetii (Burrows & Sharples, 1972). They also found that death occurred in Laminaria saccharina when Blusyl reached 1.0 mg dm^{-3} Manoxol O.T. unit. Working on young sporophytes of Laminaria hyperborea, Hopkin & Kain (1972) found that

Blusyl was toxic at 1.65 mg dm^{-3} Manoxol O.T. units. Thus it appears that *Laminaria* species are more sensitive to Blusyl than *Ulva*. However, the variation in lethal concentrations of Blusyl for the algae tested is narrow and within the same order of magnitude. There were also some differences between the algae in relation to the minimum concentration giving significant inhibition over the control in culture. The order of decreasing sensitivity was *Phaeocystis pouchetii* > *Skeletonema costatum* > *Laminaria saccharina*, *L. hyperborea*, *Ulva lactuca*.

It is interesting to note that in the sewage sludge culture, the lethal and sub-lethal concentrations of sludge for *Ulva* contained 6.1 mg dm^{-3} and 0.61 mg dm^{-3} Manoxol O.T. units of detergent respectively. These concentrations corresponded closely with those found in the Blusyl culture experiment.

Copper and zinc, when present in minute concentrations, parts per billion level, stimulate algal growth (Walker, 1953; 1954) and thus act as micronutrients. Mercury, lead and cadmium lack this stimulatory effect. All metals when present in high enough concentrations, however, inhibit growth. Toxic threshold concentrations for *Ulva* were determined in the present work. It must be emphasised that toxic thresholds can vary with the form of the metal present. Thus it has been found that inorganic mercury was more toxic than dimethyl mercury to *Phaeodactylum tricornutum* when used in the same concentration (Hannan & Patonillet, 1972). The same authors also found that growth inhibition by a toxicant varied inversely with the concentration of nutrients available. This interaction means that toxicity would be better defined in the context of a given nutrient level. Many workers have found that a mixture of metals is more toxic to algae than when the individual metal is applied alone, total concentrations the same (e.g. Burrows & Sharples, 1973; Younge & Lisk, 1972). But sometimes the

reverse is true as Young & Lisk showed (1972) that a mixture of copper and silver was less toxic to Anacystis nidulans than was copper added alone, amounts of metals equal in each case. The carbonate, hydrogen ion and organic content of the water can each contribute to simple competitive inactivation of ions, effectively reducing the amount of metals available to the algae. Burrows & Sharples (1972) growing Skeletonema costatum in concentrations of copper in i) E-S medium containing soil extract which has chelating properties, and ii) von Stosch medium, without added EDTA as a chelating agent, found that the former medium lowered the toxicity of copper by the chelating effect of the soil extract present. Morris & Russell (1973) working on Ectocarpus siliculosus reported similar results and pointed out that in the marine environment, much of the copper present will be rendered non-toxic by complex formation with organic material. Thus care must be taken in trying to predict from laboratory toxicity-threshold results likely in field reaction of organisms to particular levels of polluting components. Apart from toxic effects of metals, decrease in the growth of Ulva might also be due to the reduction of light intensity reaching the discs caused by the suspended particles present.

In conclusion, Ulva responded to toxic substances in a sensitive way and could be effectively used as a test organism. Toxicity tests, carried out under controlled conditions in the laboratory, help to interpret and evaluate conditions in the field, though great care must be taken in extrapolation.

CHAPTER VIII. Final Discussion

It is well known that prolific growth of *Ulva* occurs in many marine sewage contaminated areas and causes considerable social and economic nuisance. The aim of this project was to find out why such growth occurs and to determine whether *Ulva* can be used as an indicator to monitor its own environment and perhaps also serve as a general biological indicator of pollution in a marine environment.

The validity of the conclusions reached in relation to the field problem, i.e. that the ammonia content of sewage polluted sea water is responsible for the extensive growth of *Ulva*, and that the alga can be used as an indicator for its own environment, depends on the validity of a number of assumptions made in the course of the work. Firstly, that the discs cut from the *Ulva* thallus and used in growth rate measurement reflected the growth rate behaviour of the plant as a whole. The second assumption was that Erdschreiber medium provided a suitable control medium for experiments designed to test whether or not growth rate was stimulated or inhibited by the chemical composition of the seawater. Thirdly, that the results of the laboratory experiments can be extrapolated to interpret the field situation.

The validity of using *Ulva* discs for growth rate measurement was supported by results obtained in section 4.1.2 which showed clearly that both intact plants and excised discs have very similar growth rates. In fact torn pieces of *Ulva* have the ability to continue growth and behave "like separate plants" (Cotton, 1911). This capacity almost certainly increases the ability of the plant to propagate and survive in the field, especially in polluted environments.

Whilst non-nutrient-enriched seawater can be used for germination of sporelings and for isolation of micro-algae (McLachlan, 1973), it is not suitable for algal culture in a limited volume of water. Nor do

algae grow well in a completely artificially defined medium as indicated by the work of McLachlan (1973). If algal culture is required for an extended period and continuous flow culture facilities are not available, as is the case here, enrichment of the seawater medium is necessary. Since the introduction by Foyn (1934) of E-S medium for the culture of Cladophora, it has subsequently proved very suitable for culture work generally (e.g. Provasoli, McLaughlin & Droop, 1957; Burrows, 1961). Because of the possible difference in source of the seawater (here all water for E-S medium was collected from the same position, as far as is navigationally possible, in the Irish Sea midway between Liverpool and Douglas, Isle of Man) and the presence of the soil extract (prepared at various times throughout the work) in the medium, E-S is not an exactly defined medium. Its use as a control medium was however vindicated by the fact that it gave a very consistent growth rate performance for Ulva throughout three years work (refer to section 6.4.4.2). In fact there was no significant difference between the mean growth rate in the different experiments. Also incidentally this latter point indicates that material collected throughout the year and over three separate years was very consistent, allowing direct comparison between experiments widely separated in time. The observed growth rate of Ulva in this work, around 200% increase in area in 14 days, is also very similar to the 170% increase in 14 days obtained by Waite & Gregory (1969a,b) using continuous flow culture; further evidence of the adequacy of the culture technique used throughout this work. From the nutrient bioassay experiments it was found that in order to obtain a healthy growth of Ulva in culture, a minimum of about 0.78 mg dm^{-3} NO_3-N and 0.01 mg dm^{-3} PO_4-P was required. Thus, for the growth of Ulva, E-S medium contains about twice the necessary amount of NO_3-N whilst the amount of PO_4-P present is excessive, (but not deleteriously so) some 35 times more than

the amount required. The phosphate level could safely be reduced for similar future culture work. In conclusion therefore the culture technique adopted was considered very adequate for the type of work carried out.

In trying to find out what causes the prolific growth of Ulva in sewage contaminated areas, experiments were conducted under controlled laboratory conditions to assess the effect of likely nutrient components of sewage effluent on growth rate of the alga. Field samples of seawater were then collected and tested for growth rate effects on Ulva discs, and growth was then related to the concentrations of the nutrients in the water samples. The results obtained with the field water samples and those from the controlled nutrient enrichment test samples were also correlated. As discussed previously (section 6.5) overwhelming evidence indicated that it was NH_4-N which was responsible for the prolific growth of Ulva in Holes Bay. It is important to note that the collected water samples giving significantly higher growth rates of discs than the E-S medium were all from within Holes Bay. Analysis of the nutrient levels in water samples regularly collected in different parts of Holes Bay and analysed by the Poole Purification Board showed that the whole area of the Bay north of Station 8 has an average NH_4-N concentration of 0.7 mg dm^{-3} or above. It is estimated that this area occupies over 80% of the total area of Holes Bay. Hence it is clear that a large proportion of water in the Bay has an NH_4-N level above that which gives enhanced growth of Ulva in laboratory cultures with seawater with increased NH_4-N levels. The fact that over much of Holes Bay, especially north of station 8, prolific growth of the alga does occur, would confirm the predictions from laboratory work of the NH_4-N level required for growth stimulation of the alga in the field.

Phosphate appeared to have a similar effect to ammonia on Ulva growth rate - significant positive correlation between growth rate and PO_4-P concentration - but this was shown to be due to a significant positive correlation between the concentrations of these two components in field water samples (refer to section 6.4.4.2). This possible misinterpretation of the role of phosphate concentration in the growth rate stimulation of Ulva, which would have been deduced from growth rate determinations and analysis of field water only, emphasises the need for the dual approach to the problem i.e. the use of and correlation between the former and experiments involving controlled additions of individual likely growth stimulating compounds to unpolluted seawater. Phosphate is essential for normal growth of Ulva but the level required is much less than the amount present in eutrophicated situations such as in Holes Bay and Langstone Harbour. Both the present work and that by Waite & Mitchell (1972) showed that when NH_4-N is in adequate supply, a low concentration of PO_4-P, at 0.01 to 0.05 mg dm^{-3} level is sufficient to achieve maximal growth rate of Ulva.

It now seems profitable to consider the nutrient status of the waters in other areas reported to have similar prolific growth of Ulva and compare them with the present case. The estuary of the Avon and Heathcote Rivers in Christchurch, New Zealand, is one which has a similar Ulva growth problem to that in Holes Bay, Poole Harbour. Bruce (1953) in her report gave some chemical data for the water within the Avon and Heathcote estuary and found a correlation between the abundance of Ulva and the increased "nutrient level" of the estuary due to increase in sewage effluent input. An area of dense Ulva growth was found in the vicinity of sewage effluents. The NH_4-N level in these dense growth areas was as high as 0.5 to 0.8 mg dm^{-3} but Bruce did not specifically correlate growth stimulation with NH_4-N level.

Boston Harbour in the United States of America also had a similar Ulva problem. Here the area of densest Ulva growth was adjacent to the town of Winthrop where the alga densely covered an area of about 101 ha of tidal mud flats. Sawyer (1965) collected and analysed water samples from within the harbour on four occasions in May and the NH_4-N concentration in the Winthrop area was found to be from 0.06 to 0.28 mg dm^{-3}. As all four collections were made within $\pm$ 2 hours of high tide, it is almost certain that during lower tide states, the NH_4-N level would go much higher than this range. Letts & Richards (1911) investigated the prolific growth of Ulva in Belfast Lough and found that the NH_4-N level within the estuary was generally high. In locations where abundant Ulva was growing the NH_4-N level averaged from 0.2 to 0.36 mg dm^{-3}. Thus evidence from other Ulva-affected areas also points to the general high nutrient level, and especially NH_4-N, as the cause of such prolific growth though the latter correlation was not commented on by the authors concerned.

Growth of Ulva was also found to be stimulated by contact with sewage-contaminated-mud (refer to section 5.3.7). The decaying organic mud will be likely to release ammonia which may then be absorbed and subsequently stimulate Ulva growth; this being possible if Ulva is lying on top of the mud. Here the general ammonia level of the surrounding water may or may not reach that required for prolific growth of Ulva and yet local high concentrations resulting in such growth might occur. Hence great care must be taken in sampling and analysing field water in such conditions. Further study on the effects of nutrients on the growth of Ulva which would eliminate the need for laboratory culture experiments could be attempted by putting pieces of the alga, suitably anchored, into different locations in the field and observing

their growth rates. The nutrient levels of the water in these areas could be monitored at the same time and correlation could then be made with the growth rates obtained. Such an approach is, however, not free from problems e.g. the alga could be damaged and torn off by wave action (Letts & Richards, 1911) thus making accurate growth measurements difficult if not impossible. The thallus may also be grazed by animals such as Hydrobia ulvae, as was observed to happen in the field in Holes Bay.

Apart from nutrients, suitable substrates for anchorage of Ulva thallus such as pebbles and shells (Cotton, 1911) have also been reported to encourage its abundant growth. In Holes Bay, however, there are few pebbles for anchorage but the majority of Ulva plants float freely in the almost still water as the tide comes in, and then lie on the sewage-contaminated mud flats when the tide ebbs.

In the foregoing discussion it has been established that the method of sampling and culturing Ulva was acceptable and the conclusion made that high levels of ammonia in the sewage-polluted waters in Holes Bay, Poole Harbour are responsible for the extensive growth of Ulva found, even though a certain amount of circumstantial evidence was included in the reasoning. It is now possible to evaluate the use of Ulva lactuca as an indicator organism for marine pollution.

The characteristics required of an efficient indicator organism have already been referred to earlier (see page 2) and included features such as, cosmopolitan nature of the species; inability to move away from the source of pollution; simple morphology leading to ease of assessment of growth rate; ease of handling for experimentation and finally rapid and graded response to the effects of pollutants. It is thus by examination of the extent to which Ulva meets such requirements, that an assessment of its efficiency as a biological indicator for pollution can be made. The following is an attempt at

such an assessment.

Firstly Ulva adapts to and grows in a wide range of environmental conditions and has been reported growing throughout the world (e.g. Lewis, 1964). Secondly it is usually attached to substrate but when in quantity may lie on mud or float in water yet tending to remain in the same lateral position in the absence of strong currents, as is the case in Holes Bay. Ulva has a very simple morphology, and growth rate measurement using detached discs has been shown to be very straightforward. Once a suitable method of culture of the alga had been determined, both with respect to medium and length of culture period, Ulva was very easily handled in the laboratory. When compared with Laminaria saccharina as regards to the relative ease of handling in culture, Ulva is superior in two ways. Firstly, as the growth rate of Laminaria plants collected from the field varied widely sporophytes had to be specially grown for up to 5 months in the laboratory before experiments could be started. Secondly due to its larger size, Laminaria requires larger, and more containers and seawater medium than Ulva. Compared with microalgae (phytoplankton) Ulva is also considered better because the former require complex isolation procedures to establish pure cultures before experiments can begin. Ulva has no such requirement. Thus from the above discussion it can be seen that Ulva adequately fulfilsthe requirements of the first four listed characteristics required of a pollution indicator species.

As far as the final and most important characteristic is concerned, that of a graded response to pollutants, Ulva growth rate showed such a response only to NH_4-N and inhibiting substances such as heavy metals and detergent. It did not show a graded response to common nutrients such as NO_3-N and PO_4-P except when the concentration

present was below the requirement for normal growth and a level which is usually present in unpolluted seawater. From the responses obtained it is clear that the growth rate of Ulva can only be used as an indicator for NH_4-N concentration, and even then only if inhibitors are not present in significant amounts to affect the issue. Thus Ulva, at best, can only be used to monitor its own environment, in that if Ulva grows prolificly in a location, as in Holes Bay and other reported areas (e.g. Letts & Richards, 1911; Bruce, 1953; Sawyer, 1965), a high level of NH_4-N would certainly be present in that area. The inability of Ulva to indicate eutrophication in general was clearly shown in the case of Langstone Harbour where high levels of PO_4-P and NO_3-N occurred, but the alga failed to respond to either of these.

Problems posed by prolific Ulva growth have been discussed earlier. (page 3). It is thought that by introducing proper corrective measures, present problems caused by excessive Ulva growth could either be prevented from intensifying or could even be overcome completely. Certainly in the light of the present findings it is clear that provided action is taken to control sewage discharge into any new location troublesome growth of Ulva could be avoided. The following are some suggestions for minimising or avoiding Ulva trouble.

The most obvious step that could be taken to avoid Ulva nuisance is to cut down the amount of NH_4-N present in the effluent. Results obtained and estimated from this work, as well as those obtained by others (Waite & Gregory, 1969a,b; Waite & Mitchell, 1972), indicate that it would be necessary for the NH_4-N level to be kept below 0.3 mg dm^{-3} at all times in the water into which the sewage was discharged, and therefore the level in the sewage would have to be adjusted accordingly. In order to control the level of NH_4-N , several methods can be employed.

Firstly, the level of NH_4-N in sewage effluent (or any other NH_4-N source) can be lowered by oxidation and hence formation of nitrate which does not affect Ulva growth in the same way. Nevertheless if this method was chosen care would be needed to make sure the nitrate level was not increased excessively so as to cause rapid Ulva growth, and checks would be required to ensure no other environmental problem arose. Secondly, the sewage effluent could be diverted to an area where rapid dilution would reduce the level of NH_4-N to below the recommended growth restricting level of 0.3 mg dm^{-3}. At the moment in Holes Bay the limited tidal effect is so inefficient in diluting NH_4-N that in most areas of the Bay the average concentration of NH_4-N is 0.7 mg dm^{-3} or above. Thirdly, Ulva growth could be restrained by the administration of growth inhibition or destruction chemicals such as algicides and herbicides. Needless to say such chemicals would have to be administered repeatedly to achieve the desired effect. Chemical control has been tried in Belfast Lough (Letts & Richards, 1911) and Boston Harbour (Sawyer, 1965) but achieved only very limited success due to the rapid dilution of the chemicals by the incoming tides. Furthermore administration of large quantities of such chemicals is undesirable as further serious upset of the environment may occur. Of the three recommendations made, the first two would seem to be best for eliminating the Ulva problem, and not creating others.

To conclude, prolific growth of Ulva is caused by increased NH_4-N levels in seawater as a result of sewage discharge, growth rate stimulation being caused by NH_4-N levels between 0.4 and 7.8 mg dm^{-3}; it can only be used as an indicator species in its own environment and for NH_4-N eutrophication, and is thus unsuitable as a general pollution indicator. To reduce or prevent prolific Ulva growth NH_4-N must be kept below 0.3 mg dm^{-3} at all times.

In a modern society and with increasing population it is inevitable that increasing waste disposal will be required; however it is necessary to establish safe levels for such disposals. This can be done by using a range of indicator organisms to assess safe levels and by employing the type of approach used in this work.

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Appendix I

Composition of Erdschreiber culture medium:

To 1 dm^3 of filtered seawater are added:-

25 cm^3 soil extract

5 cm^3 salt solution

Soil extract preparation.

100 gms. of garden soil (not recently fertilized) added to 1 dm^3 deionised water, suspension autoclaved at 15 lbs. $in.^{-2}$ pressure for 40 minutes; filtered when cool and filtrate re-autoclaved at same pressure for 20 minutes.

Salt solution preparation.

To 1 dm^3 of deionised water are added:

2.0 gms. sodium nitrate

0.4 gm. disodium hydrogen orthophosphate-dihydrate ($Na_2HPO_4.2H_2O$).

Appendix II

Protein estimation by Lowry-Folin method:

Materials

5% Trichloroacetic acid solution (TCA)

Folin-Ciocalteu's reagent

2% Sodium carbonate solution

0.05% Sodium, potassium tartarate in 0.025% Copper sulphate solution.

When making Na, K tartarate and copper sulphate solution each salt was separately dissolved, then solutions combined and made up to 100 cm^3. Two cm^3 of this solution was then added to 98 cm^3 of the 2% sodium carbonate solution. This final solution being designated solution A.

Method

10 mg. dry weight of Ulva were ground in cold 5% TCA with a glass mortar and pestle. Homogenate centrifuged and supernatant discarded, pellet resuspended in more 5% TCA and recentrifuged. Washing with TCA of pellet repeated 3 more times. Protein was solubilized in 5 cm^3 of 1 M NaOH overnight at 25°C. Suspension then centrifuged and supernatant decanted and retained. Pellet was washed with 2.5 cm^3 of 1 M NaOH and suspension recentrifuged, supernatant again decanted and retained. Two supernatants were pooled and protein solution made up to standard volume with 1 M NaOH.

Fresh Folin-Ciocalteu reagent was diluted 1:3 with deionised water before use in assay.

For the protein assay 0.5 cm^3 of protein solution was added to 4 cm^3 of freshly prepared solution A, solutions were well mixed and allowed to stand at room temperature for 10 minutes. 0.5 cm^3 of diluted Folin-

Ciocalteu reagent was then added to each tube. Tubes were shaken and allowed to stand for 45 minutes at room temperature and then the optical density at 750 nm estimated. A calibration graph for protein was prepared with bovine serum albumin assayed as above, and used for conversion of optical density values to protein.

Appendix III

Chlorophyll extraction and estimation (Arnon, 1949).

Portions of 10 mg of dried Ulva tissue were homogenised in a few drops of 85% acetone by a glass mortar and pestle. The homogenate was centrifuged at 3,000 r.p.m. for 5 minutes. The supernatant was decanted and kept, and the pellet resuspended in a small quantity of 85% acetone and the suspension recentrifuged as above. The second supernatant was decanted and added to the first and the resultant chlorophyll solution made up to standard volume with 85% acetone. The optical density of the solution was determined at 645 and 663 nm.

Total chlorophyll was then computed from the following formula:-

$$\text{Total chlorophyll} = \text{chlorophyll a} + \text{chlorophyll b}$$

$$= (0.0202 \times O.D._{645} + 0.00802 \times O.D._{663}) mg.cm^{-3}$$

The extraction and estimation procedure took less than one hour and the loss of chlorophyll during that time was estimated to be less than 5%.

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