# The Quest for Characteristics Discriminating between *Phaeocystis* Strains

A thesis submitted in partial fullfillment of the MSc degree in Environmental Biology at the Rijksuniversiteit Groningen.

Hester Bosma

March 1996

## ACKNOWLEDGEMENTS

The practical work for this study has been performed in several labs. Therefore I would like to thank the people of the Microbial Ecology group in the Microbiology department and the Macro Algae and Micro Algae groups in the Marine Biology department. I thank Stella Boele-Bos for helping me with the RAPD-studies and Wytze Stam and Janine Olsen for the supervision of this part of the study. I would like to thank Ingmar Janse for helping me find my way in the lab and in the methanolysis procedure. Special thanks to Marion van Rijssel for the pleasant supervision.

## ABSTRACT

The aim of this project was to describe clonal similarities or dissimilarities of cultured *Phaeocystis* strains, in an attempt to clarify taxonomic confusion and methodological problems. The main obstacle in doing so was the change of composition of the sea water used during the course of the experiment which meant that obtained results could not be repeated. Three series of batch cultures were performed with five *P. globosa*-type strains originating from different sites in the North Sea. The strains were investigated on as many parameters as possible by performing culture-based and genetic studies. Autofluorescence of acetone extracted samples showed to be a good biomass estimation of colony forming *Phaeocystis*. Colony morphology had previously been used as a criterium for *Phaeocystis* taxonomy. In this study the shape and size of colonies varied daily and in every series of batch cultures. The strains could be distinguished visually during handling of the strains. It was demonstrated that percentage of cells in colonies was not a reliable criterium but the method was succesfully used. Cell sizes overlapped but have potential to discriminate between strains when used in combination with other taxonomic parameters. The ranges of the cell sizes fell into the ranges indicated in the literature. Growth rates of the strains differed for all the series of batch cultures of the same strain so it has not been used as a taxonomic parameter. Pigment analysis did not show high variation between the strains although pigments have previously been applied as chemotaxonomical markers for *Phaeocystis* taxonomy by several authors. This study indicates that on the level of strain discrimination pigment analysis is not sensitive enough. Sugar composition studies revealed that sugar patterns of strains were variable. Total sugar analysis showed an increase in amount of total sugars when nutrients were limiting. The filter method tested for the measurements of total sugar per cell was succesful. RAPD analysis allowed the strains to be grouped in two clusters corresponding with their geographical origin.

The main conclusion emerged from this study is that inter-population variation is high in *P. globosa* strains. Cell size and sugar fingerprints have potential to discriminate between strains and the RAPD data revealed clear differences between the strains in accordance with their geographical origin.

## ABBREVIATIONS

| | |
|---|---|
| Fuc | Fucoxanthin |
| Bfuc | Buta-fucoxanthin |
| Cfuc | *Cis*-Fucoxanthin |
| Neo | Neoxanthin |
| Diadi | Diadinoxanthin |
| Diato | Diatoxanthin |
| Peri | Peridin |
| Hfuc | 19'-Hexanoyloxyfucoxanthin |
| Viol | Violaxanthin |
| ßCaro | ß-Carotene |
| Chl *a* | Chlorophyll *a* |
| ara | arabinose |
| rha | rhamnose |
| xyl | xylose |
| glcA | glucuronic acid |
| man | mannose |
| gal | galactose |
| glu | glucose |
| galA | galacturonic acid |

**CONTENTS**

# 1. INTRODUCTION

***Phaeocystis.*** *Phaeocystis* is a bloom-forming alga recognised both as a nuisance alga and as an ecologically important member of the phytoplankton (Davidson and Marchant, 1992). In spring *Phaeocystis* regularly forms dense blooms in very contrasting nutrient rich areas of the world ocean (Lancelot *et al*, 1994). During the collapse of *Phaeocystis*-blooms, dissolved organic compounds are released and form massive foam deposits that accumulate on beaches (Eberlein *et al*, 1985).

*Phaeocystis* belongs to the class of the Prymnesiophytes which incorporates single celled algae smaller than $\pm$ 10 $\mu$m, bearing two flagella and a haptonema as well as organic scales on the outer side of the cell wall (Van den Hoek, 1978). The first description of the *Phaeocystis* life cycle was done by detailed microscopic work (Kornmann, 1955). This morphological study conducted on a cultured strain isolated from the North Sea gave evidence of a high complexity of the cycle, characterised by the alternance between different free living cells and mucilaginous colonies of non-motile coccoid cells. Colonies were shown to widely vary in shape and size, reaching several mm or up to 2 cm (own observations) at the stationary phase of their growth. Among the free living cells Kornmann (1955) distinguished three different types of flagellated cells, varying in size from 3 to 9 $\mu$m in diameter. Since Kormann (1955) numerous morphological studies were conducted to determine the factors controlling the transition from one form into another but no new information could be deduced from these studies (Rousseau *et al*, 1994). There is still a lot of confusion on the different life forms, since they can be difficult to distinguish using conventional observation techniques. An additional difficulty stems from the considerable taxonomic confusion and uncertainties about identity of *Phaeocystis* species or strains.

**Taxonomy.** Since the turn of the century nine species of *Phaeocystis* have been described. Sournia (1988) reviewed the diagnostic features of *Phaeocystis* and recognised two of the nine species as valid: *Phaeocystis scrobiculata* and *P. pouchetii*. Other studies (e.g. Jahnke and Baumann, 1987) found this to be an over-simplification. Recently, Baumann *et al* (1994) suggested that four *Phaeocystis* species exist, these being: *P. globosa, P. scrobiculata, P. pouchetii* and one undefined Antarctic species. These species are distinguished by colony and single cell morphology and by temperature tolerance.

The species status of three colony-forming species was supported by Medlin *et al* (1994): *P. globosa, P. pouchetii* and *P. antarctica*. The authors based separation of the species on morphological, physiological and genetic grounds. Both *P. pouchetii* and *P. antarctica* are cold-water species, *P. globosa* originates from temperate waters. According to Medlin (pers. comm.) there is evidence to divide *P. globosa* into sub-species.

The taxonomy of 16 *Phaeocystis*-strains was investigated by Vaulot *et al* (1994). The authors proposed a clustering of the strains into six groups based on genome size, pigment analysis, morphological differences and geographical origins. The clusters coincided with geographical origin. One cluster corresponded with *P. antarctica*, all the other clusters, despite their differences could be included in *P. globosa* according to the present state of taxonomy. It was suggested that the North European cluster should be considered as the true *P. globosa* and that the other clusters are probably yet-undescribed species or subspecies (Vaulot *et al*, 1994).

Some of the main criteria for *Phaeocystis* taxonomy have not been fully reliable. Pigment composition was emphasised as a very useful tool when discriminating between strains (Vaulot *et al*, 1994). Previously, Gieskes and Kraay (1986) and Buma *et al* (1991) indicated that *P. globosa* and the Antarctic strain could be distinguished on the basis of their pigment characteristics. The pigments characteristic for Prymnesiophytes were fucoxanthin and 19'-hexanoyloxyfucoxanthin.

A reliable criterium to establish distinction of *Phaeocystis* species is 18S sequence data (Medlin *et al,* 1994). Information on genetic variation could clarify relationships which remain uncertain and that were based on other techniques. The principal advantage of molecular analysis is that nucleic acids are affected to a lesser extent by external environmental conditions when compared to culture-based studies.

**Colony matrix.** *Phaeocystis* colonies consist of cells embedded in a mucilaginous matrix. Important physiological and ecological properties of the *P. globosa* colonies are to be related to the nature of the mucilaginous matrix (Lancelot and Rousseau, 1994). The polysaccharides of the matrix provide an energy reservoir for the cells dividing within it (Lancelot and Mathot, 1985; Veldhuis *et al*, 1985). This extracellular mechanism enables *Phaeocystis* colonies to continue growing through the dark without the need to accumulate large energy reserve inside the cells. This colonial reservoir thus gives a selective advantage to colonial cells over free-living cells (Lancelot and Rousseau, 1994).

The composition of *Phaeocystis* mucus is largely unknown. Guillard and Hellebust (1971) found that mucus of two *Phaeocystis* strains consisted of oligo- and polysaccharides of heterogenous composition. Painter (1983), following analysis of an impure bloom, showed that the mucus might be a very complex soluble proteoglycan. Recent findings indicate that a polysaccharide composed of 90% of glucose units characterises the *P. globosa* strain mucus gel (Lancelot and Rousseau, 1994). The *P. pouchetii*-like gel comprised a heteropolysaccharide containing some amino acid residues due to its significant nitrogen content (Verity *et al*, 1988 and 1991). Van Boekel (1992) showed evidence that the colony matrix is formed through gelling of carboxylated and sulfated polysaccharidic chains promoted by salt (calcium and magnesium) bridges. This may suggest that the

chemical structure of the polysaccharide matrix is species- or strain-specific. Janse *et al* (1996) suggest that variability in sugar fingerprints may reflect the presence of different strains.

**Object of this study:** The aim of this project was to describe the clonal similarities and dissimilarities of cultured *Phaeocystis* strains available at the University of Groningen. Due to the lack of information regarding inter- and intra-population variation there is confusion regarding *Phaeocystis* taxonomy. Physiological characteristics analysed clearly overlap between species and clones. This means that generalizations concerning physiological and biochemical responses of the alga should be exercised with care if the source of the strain is unknown (Baumann *et al*, 1994). In this study it is attempted to clarify some problems around inter-population variation. Strains were investigated on as many parameters as possible by performing 1) culture-based studies: biomass, growth rate, size of algal cells, percentage cells in colonies, pigments and biomass of interacting bacteria; and 2) molecular genetics: Random Amplification of Polymorphic DNA (RAPDs). Sugar-based studies were performed by investigating the amount of total sugar, and by using a methanolysis procedure to analyse sugar components.

## 2. MATERIAL AND METHODS

**Cultures**. The research was carried out with colony-forming *Phaeocystis*-strains originating from different sites (Table 1). The major part of the project was carried out with five strains (the top five in Table 1). These strains formed spherical colonies with cells arranged homogeneously within the gelatinous matrix (globosa-type, Jahnke and Baumann, 1987). All the cultures were grown in enriched seawater medium as described in Janse *et al* (1996). All cultures were incubated at 10°C (except Phaeo ant. which was incubated at 5°C) in 400 ml serum bottles on a roller device (2 rpm). A 14:10 L:D cycle was employed using a light intensity between 70 and 100 $\mu E/m^2/s$. Stocks of the strains were kept at 5°C and stirred twice on a weekly basis.

During the log- and exponential phase the cultures were sampled daily at approximately the same times to eliminate cell cycle effects. The frequency of measurements decreased during the stationairy phase and stopped when biomass declined.

Table 1: Names and origin of used cultures.

| Name | Species | Isolation date | Origin |
|---|---|---|---|
| Louis | *P. globosa* | 1992 (L.Peperzak) | North Sea |
| Plymouth | *P. globosa* | 1952 (M. Parke) | Channel |
| BCZ | *P. globosa* | 1993 (V. Rousseau) | Belgium |
| Phaeo ax | *P. globosa* | 1990 (W. van Boekel) | Marsdiep |
| Ingmar | *P. globosa* | 1994 (I. Janse) | Marsdiep |
| Ph'91 | *P. globosa* | 1991 (L. Peperzak) | North Sea |
| Phaeo ant | *P. antarctica* | 1988 (A. Buma) | Weddel Sea |

**Biomass.** A conventional method used for the daily biomass estimation of colony-forming *Phaeocystis* was the Utermöhl sedimentation method (Utermöhl, 1958). Samples were fixed in an acid Lugol solution (App. 1) which caused the colonies to dissociate. Following sedimentation, cell counts could be performed using an inverted microscope. This method was shown to be laborious and therefore daily biomass increase was measured using the method of Peeters and Peperzak (1990) as modified by Van Rijssel (pers. comm.). This method is based upon the autofluorescence of samples and acetone extracted samples.

Samples were centrifuged for 20 min at 715 *x g* to obtain a dense pellet. The supernatant was carefully removed until 0.5 ml remained at the bottom of the tube. Acetone

was then added until the volume was again 5 ml, thereby creating a 90% acetone solution for the pigment extraction. The pellet was resuspended by shaking and vortexing and the samples were put in an ultrasonic shake bath for 10 min. Centrifugation was repeated for 20 min and the supernatant was carefully removed into a clean quartz cuvette. The fluorescence was measured with a spectrometer Perkin-Elmer LS-5B with the following parameters: excitation, 435 nm; emission, 670 nm; and the band pass on 20 for excitation 0. Plots of cell counts versus fluorescence of extracted samples was linear with r=0.99 (App. 2, non-colony-forming *Phaeocystis*, with permission of P.J. van Hall).

**Bacterial counts.** Counts from xenic cultures and the axenic Phaeo ax strain for bacterial contamination was performed as described below with Hoechst dye using fluorescence microscopy (Paul, 1982):

- 0.2 $\mu$ pore size polycarbonate filter (Ankersmidt Breda)
- Irgalan black (0.2 g in 2% acetic acid)
- Hoechst dye nr 33258.

Samples were diluted 3 times by adding 2 ml artificial seawater and filtered over the black stained polycarbonate filters. A Zeiss microscope connected to a UV lamp (467259-9901) was used for the counting. The formula used to convert to the amount of bacteria per liter is the average of bacteria per square x 3.27 x $10^9$. Due to the high conversion factor this method has a high threshold value.

**Proportion of cells in colonies.** The percentage of cells in colonies was estimated by means of gentle filtration on to a 20 $\mu$m mesh size nylon filter. Only single cells and a few colonies with 2 or 4 cells were able to pass this filter. Counts showed that 95% of the single cells were present in the filtrate (Veldhuis and Admiraal, 1987). The fluorescence of both filtrate and culture was measured and the percentage of cells per colony was calculated using (1 - filtrate/culture) x 100.

**Cell size.** The size of algal cells was determined by fixing the cells in an acid Lugol solution. Diameter measurements were performed using an Olympus T041 inverted microscope at 150x magnification.

**Pigments.** For the analysis of pigments cultures were harvested during the exponential and stationairy phases of growth. Cultures were filtered without vacuum on to GF/C Whatman filters. Since culture densities varied, different volumes of the cultures were filtered. Filters were immediately stored at - 80°C after filtration until required for analysis. Prior to analysis filters were grinded and pigments extracted in 5 ml (90%) acetone. Analysis and identification of pigments using HPLC is described in detail by Villerius *et al* (1996).

**Total sugars.** Total sugar were analysed using the phenol-sulphuric-acid method (Liu *et al*, 1973). A known amount of culture was filtered over GF/C Whatman filters

(pre-ashed for 2 h at 180°C) without vacuum. The volume of sample to be filtered depended on the age and density of cultures. Filters were cut into small pieces, transferred into tubes (washed twice) and frozen until required. Reagents used were 1.8 ml distilled water, 0.2 ml 50% phenol and 4 ml concentrated sulfuric acid. Phenol and sulfuric acid were added to the distilled water whilst vortexing between the two steps. The mixture was incubated at room temperature for 30 min. Filters were then centrifuged for 10 min at 715 *x g*, the resulting supernatant transferred to clean Eppendorf tubes and recentrifuged for 20 min at 15,000 *x g* in an Eppendorf centrifuge. Total sugars were determined by absorbance at 485 nm.

**Qualitative sugar analysis**. Cultures were harvested during the exponential phase (autofluorescence approximately 200) and 200 ml was filtered without vacuum onto a 10 μm mesh size nylon filter. Biomass of the filtrate and the non-filtrated culture was measured using fluorescence and the samples were frozen to break the cell walls. The samples were then thawed in a water bath (37°C), shaken well and centrifuged for 20 min at 25,000 *x g* and 4°C. Total sugar content of the supernatant was then measured. After methanolysis a minimum of 200 nmol total sugars was needed to be able to detect peaks with the gas chromatograph. For this reason the samples needed to be concentrated using a Milipore mini-ultra filter with a pore size of 1 kDalton. In order to avoid bacterial contamination samples were maintained at approximately 4°C.

The final stage of the purification was the removal of salt from samples by dialysis according to the method described by Sambrook *et al* (1989). The dialysis tubes were made by Visking, cat nr. 256K02, pore size 24 Å. The samples were put in the cold room for approximately 3 days with the demineralised water being replaced twice daily.

The methanolysis step was based on that described by Kamerling and Vliegenthart (1988) with the modification that the concentration of MeOH/HCl was doubled from 1M to 2M. Due to the absence of N-acetylsugars, the N-reacetylation step was omitted.

In order to examine the impact of different nutrient levels on the sugar content the BCZ strain was incubated in five media which varied in the concentration of $PO_4$/$NO_3$/$NH_4$ (Table 2). The media correspond with the description in Janse *et al* (1996) except for the addition of these nutrients. The cultures were harvested during exponential phase.

Table 2: Nutrient concentration in media for BCZ-experiment.

| Medium | Concentration $PO_4/NO_3/NH_4$ (ml/400ml) |
|---|---|
| F0 | - |
| F40 | 0.2 |
| F20 | 0.4 |
| F10 | 0.8 |
| F5 | 1.6 |

**Extraction of DNA.** All cultures were harvested during the exponential phase by centrifugation at 2190 *x g* for 15 min. Extraction of DNA was performed after a method of H. Klerk (unpublished) using a CTAB (hexadecyltrimethylammonium bromide) procedure. DNA concentrations were calculated by measuring the ultraviolet (UV) absorbance of the DNA in solution at 260 nm (Sambrook *et al*, 1989). Average yields were 2-11 $\mu$g of high molecular DNA x $g^{-1}$ wet weight.

**DNA amplifications.** Amplifications were performed with 25 $\mu$l reactions which were prepared using 10 ng DNA (10 $\mu$l), 2.5 $\mu$l 10 x Buffer (SpaeroQ, Leiden, The Netherlands), 1.25 $\mu$l 10 x dNTP, 0.015 $\mu$l Supertaq (SpaeroQ, Leiden, The Netherlands), 0.65 $\mu$l of a 10-mer primer (Operon Technologies, Alameda, CA, Kit A, C and D, Table 3; 25 ng/$\mu$l). The reaction mixtures were overlaid with one drop of mineral oil and centrifuged briefly. PCR amplifications were performed in a Perkin Elmer Cetus thermocycler with an initial denaturation step of 94°C for 2 minutes followed by 45 cycles of 1 minute at 94°C, 2 minutes at 36°C, and 2 minutes at 72°C. A ramp time of 108 s (=1° per 3 s) was used between 36°C annealing and 72°C extension. Duplicate reactions were run to check for reproducibility of the RAPD reactions. After amplification 10 $\mu$l of each reaction was mixed with Orange-G loading dye, separated by electrophoresis on 1.5% TAE agarose gels and stained with EtBr according to standard methods (Sambrook *et al*, 1989). Bands were visualised by UV fluorescence and photographed using Polaroid 665 film.

**Distance analysis.** Distances were analysed using the DISTAN program package developed by Klerk *et al* (1996).

Table 3: Nucleotide sequence of primers used.

| Code of primer | Sequence of primer 5' to 3' |
|---|---|
| A-01 | CAGGCCCTTC |
| A-03 | AGTCAGCCAC |
| A-05 | AGGGGTCTTG |
| A-07 | GAAACGGGTG |
| A-16 | AGCCAGCGAA |
| A-17 | GACCGCTTGT |
| C-02 | GTGAGGCGTC |
| C-06 | GAACGGACTC |
| C-07 | GTCCCGACGA |
| C-08 | TGGACCGGTG |
| C-09 | CTCACCGTCC |
| C-10 | TGTCTGGGTG |
| C-11 | AAAGCTGCGG |
| C-12 | TGTCATCCCC |
| C-15 | GACGGATCAG |
| C-16 | CACACTCCAG |
| C-17 | TTCCCCCCAG |
| C-18 | TGAGTGGGTG |
| C-19 | GTTGCCAGCC |
| C-20 | ACTTCGCCAC |
| D-04 | TCTGGTGAGG |
| D-09 | CTCTGGAGAC |
| D-11 | AGCGCCATTG |
| D-13 | GGGGTGACGA |

## 3. RESULTS

**Batch cultures.** Five strains (see Table 1) were used for the main part of the research and three series of batch cultures were performed. The first series of batch cultures was carried out with four strains (see Table 4). Strain Phaeo ax started to form colonies only after the first series had finished and it was then included in the research. The Plymouth strain stopped growing during the second series of batch cultures but recovered weeks later. Data pertaining to this strain is therefore missing for this series.

**Seawater.** During the research three series of batch cultures were performed each with different seawater compositions due to evaporation of the water in the reservoir and leakage of nutrients out of the filter system. The nutrient concentration was higher during the second series of batch culture (pers. comm. J. Stefels and L. Villerius) although details on the nutrient concentrations were not available. The third series of batch cultures was performed with fresh seawater.

Table 4: List of strain characters deduced from the series of batch cultures.

| | Series of batch cultures | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 |
| Strain | | $\mu$ ($d^{-1}$) | | Peak fluorescence | | | Size ($\mu$m) |
| BCZ | 0.97 | 1.18 | 1.28 | 511 | 439 | 257 | 4.44 $\pm$ 0.80 |
| Ingmar | 0.88 | 0.91 | 0.92 | 492 | 336 | 434 | 3.96 $\pm$ 0.97 |
| Louis | 0.86 | 0.81 | 1.24 | 485 | 159 | 284 | 5.05 $\pm$ 0.92 |
| Phaeo ax | - | 1.05 | 1.20 | - | 550 | 338 | 3.92 $\pm$ 0.63 |
| Plymouth | 0.84 | - | 0.38 | 356 | - | - | 5.31 $\pm$ 0.94 |

**Biomass.** During the first series of batch cultures both Utermöhl sedimentation and fluorescence of extracted samples were performed to estimate daily biomass. The relationship between the two parameters is shown in Fig. 1. The graphs for strains Ingmar and Louis were linear with $r=0.97$ and $r=0.99$ respectively. Growth curves from the first series of batch cultures based on fluorescence are shown in Fig. 2 (see also Figs 4a and 4b for respectively the second and third series). The highest biomass measurements for each strain in every series of batch cultures with fluorescence is shown in Table 4. All strains behaved differently to the change in nutrient levels in the series of batch cultures.

**Cell size.** The size of cells varied between 3.9 and 5.3 $\mu$m which is in the range suggested by Vaulot *et al* (1994).

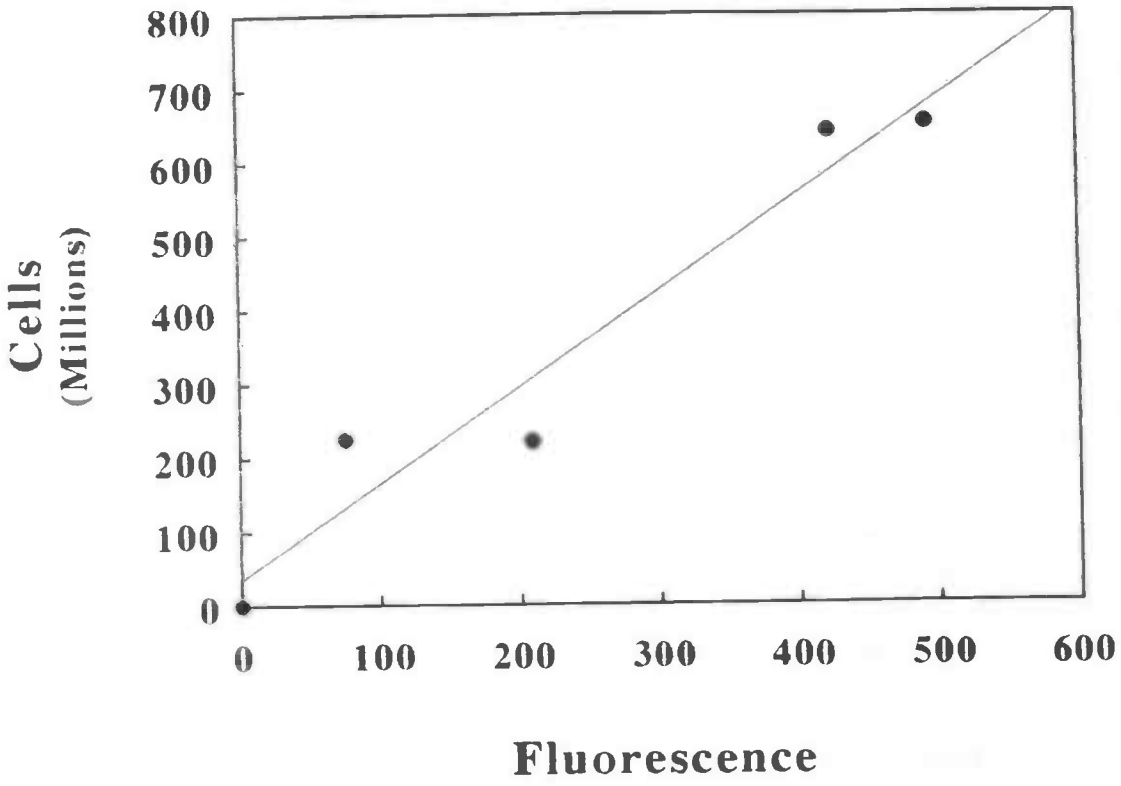
**Percentage cells in colonies**. The amount of cells per colony varied daily and per batch culture as presented in Fig. 3. Percentage in colonies of two series of batch cultures are shown.

**Observation during culturing.** During culturing of the strains it became apparent that the strains behaved differently and therefore could be distinguished visually. Colonies from the Plymouth strain were round or oval and pale in comparison with the other strains. The colonies looked rather weak instead of the firmness observed on colonies formed in the other strains. The Louis and Ingmar strain formed similar colonies which were smaller and round. Both strains could be rather green in batch culture due to the fact that they did not always form a great deal of colonies. The Phaeo ax strain always looked green regardless the proportion of cells in colonies, the colonies were round or oval and approximately the same size as colonies from the Plymouth strain. The colonies of the BCZ strain appeared in different forms, from round to tube-like. BCZ in batch culture always looked clear between the colonies because the vast majority of cells appeared to be in colony form. In addition to colony form and appearance the Plymouth strain was distinct in that it could not be kept in stock at 5°C, only at 10°C.

**Bacterial biomass.** Bacteria in the cultures were only counted during the first series of batch cultures. Biomass established during start of the stationary phase and the growth rate are shown in Table 5. Strain Louis maintained the highest biomass of bacteria in that phase.

Table 5: Biomass and growth rate of bacteria.

| Culture | $\mu$ ($d^{-1}$) | Biomass (cells/l) |
|---|---|---|
| BCZ | 0.98 | $4.2 \times 10^{10}$ |
| Ingmar | 0.79 | $2.5 \times 10^{10}$ |
| Louis | 0.87 | $9.8 \times 10^{10}$ |
| Plymouth | 0.85 | $2.1 \times 10^{10}$ |





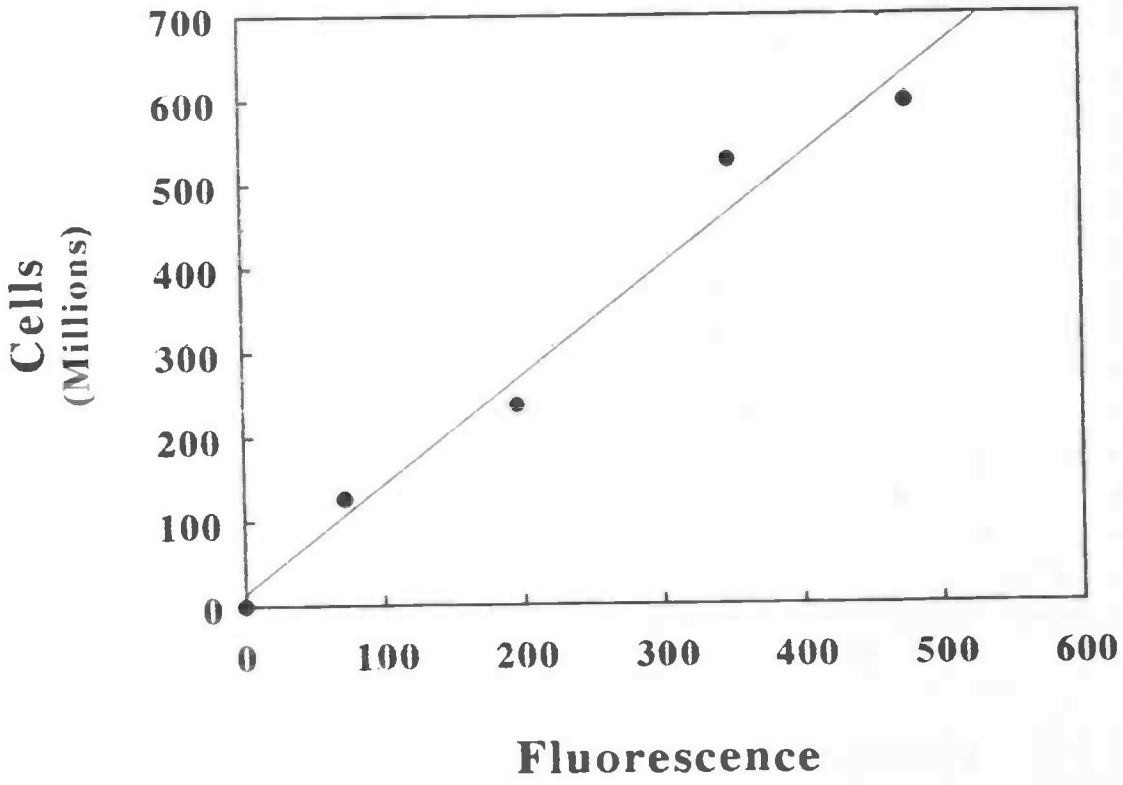




Fig. 1: Relationship between cell counts and fluorescence of extracted samples for 2 strains.

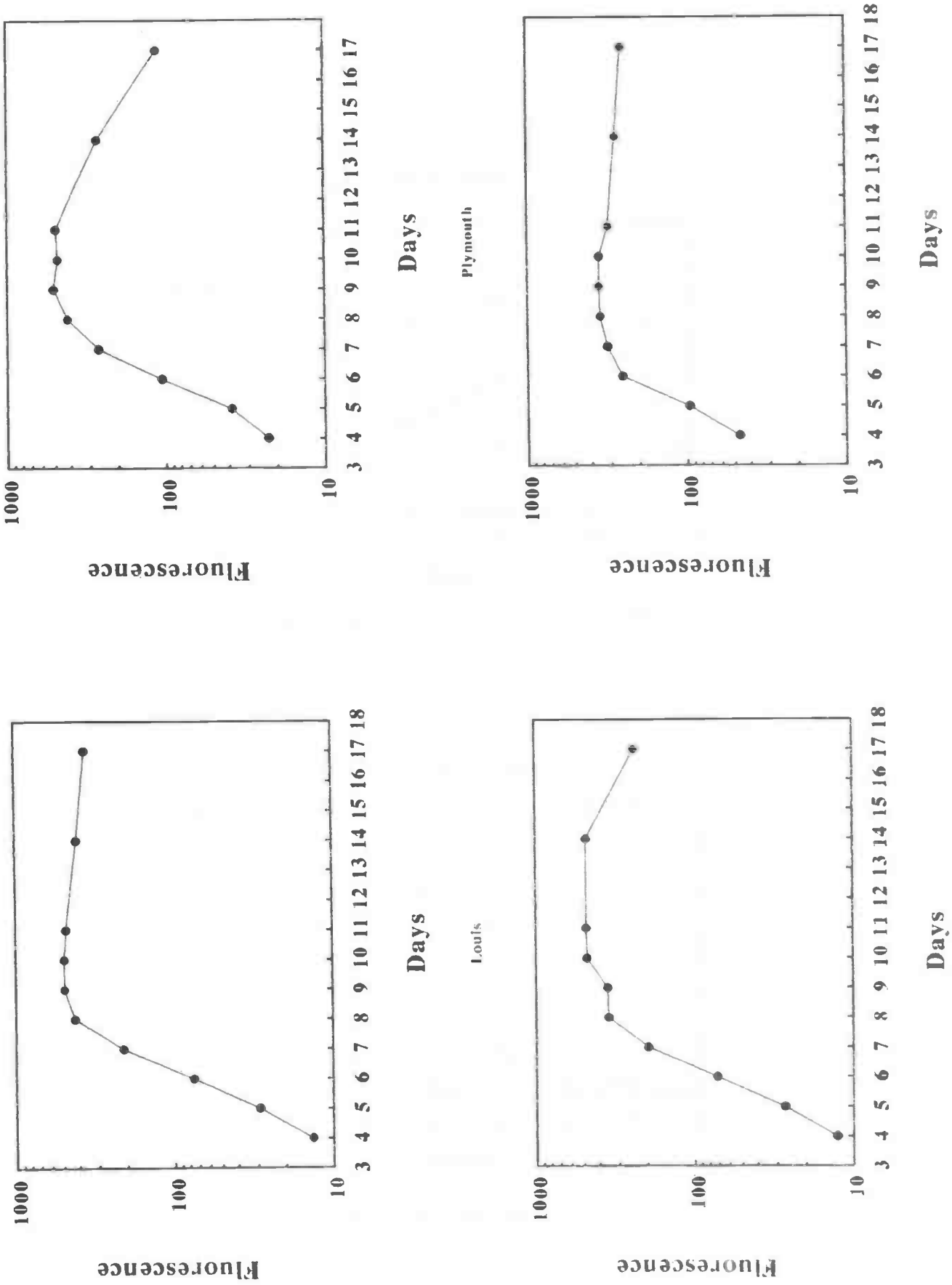
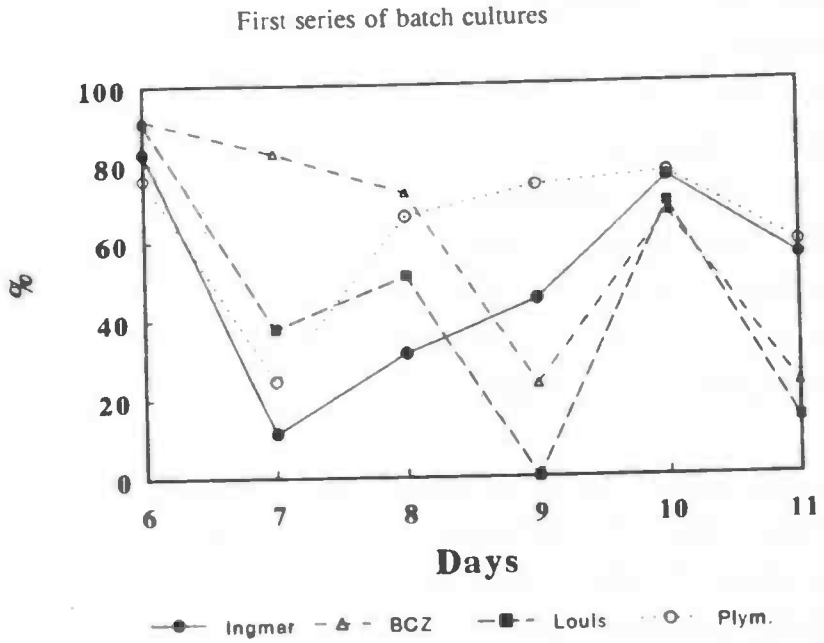



Fig. 2: Growth curves of the first series of batch culture.



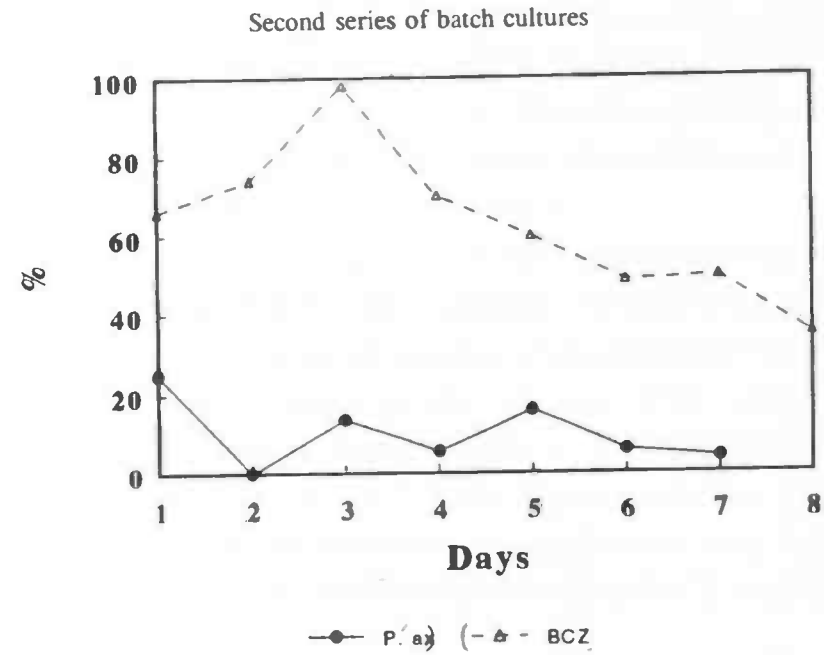




Fig. 3: Percentage of cells in colonies of two series of batch cultures.

**Total sugars.** During the second and third series of batch cultures total sugars were measured. To be able to relate total sugar amount to biomass the total sugar data are presented together with the corresponding growth curves (Fig. 4). The graphs illustrate that during the exponential phase the slopes of biomass and total sugars were virtually the same. In the stationary phase biomass declined first, sugars amounts decreased later. In the third series of batch cultures more sugars were produced when there was less biomass. The standard deviation of the total sugar measurements ranged between 0-65%.

**Pigments**. Analysis of pigments was performed during the first series of batch cultures on day 5 and day 9. Results are expressed as percentage of total carotenoids and ratio to chlorophyll *a* and presented in Table 6. All strains contained similar major accessory pigments which were Chl $C_1$, fucoxanthin, 19'-hexanoyloxyfucoxanthin, 19'-butanoyloxyfucoxanthin and diadinoxanthin. Strain Ingmar in stationairy phase (Is) lacked chlorophyll *a*. The pigments varied little between strains.

**Qualitative sugar analysis.** Throughout all the series of batch cultures the strains were analysed qualitatively for various sugars (Fig. 5). Glucose has been excluded from Fig. 5a since it was highly variable (Janse *et al*, 1996). The results are presented as percentage of total sugars minus glucose. The sugar pattern as demonstrated in Fig. 5a did not show variation between strains. The main sugar was found to be mannose. The Plymouth strain (sample P1) demonstrated a slightly different pattern with rhamnose as the main sugar and Phaeo ax produced more galactose than mannose in the third series of batch cultures. No glucuronic acids (i.e. galA and glcA) were present in the Plymouth strain despite the fact that this strain formed most colonies (Fig. 3). The glucose pattern for all sampled cultures is shown in Fig. 5b. Glucose is by far the major sugar in the *Phaeocystis* cultures. The percentage of glucose altered between the series of batch cultures. Results for filtered samples are not shown.

Fig. 6 presents the total sugar amount related to biomass in cultures incubated in the different media for the BCZ-nutrient-experiment. In media with no additional nutrients (F0) or with low concentrations of nutrients added (F40) more sugars were produced by the cells. Biomass and percentage of cells in colonies of the cultures were measured at the day of harvesting and showed variation (Table 7). The sugar pattern for the different nutrients levels is presented in Fig. 7. There appears to be little variation in sugars between samples. Mannose and arabinose are most abundant. Fig. 8 shows variation of glucose in the nutrient levels. No nutrient effect is related to glucose amount. Results for filtered samples are not shown.

Table 6: Pigments in strains in exponential (e) and stationairy (s) phase, the capital letters B, I, L and P stand for strains BCZ, Ingmar, Louis and Plymouth.

| | Fuc | ßfuc | Neo | Diadi | Diato | Peri | Hfuc | Viol | Cfuc | ßCaro |
|---|---|---|---|---|---|---|---|---|---|---|
| Be | 94.1 | 0 | 0 | 5.38 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ie | 93.8 | 0 | 0.41 | 4.44 | 0 | 0 | 0 | 0 | 0 | 0.74 |
| Le | 94 | 0 | 0.32 | 4.51 | 0.09 | 0 | 0 | 0 | 0 | 0.18 |
| Pe | 91.9 | 0 | 0 | 8.12 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bs | 91.9 | 0.09 | 0.32 | 4.31 | 1.59 | 0 | 0.3 | 0 | 1.25 | 0.2 |
| Is | 95.4 | 0.08 | 0.18 | 2.03 | 0 | 0.42 | 0.28 | 0.14 | 1.44 | 0 |
| Ls | 96.4 | 0.01 | 0.15 | 1.88 | 0.27 | 0 | 0.09 | 0 | 0 | 0.05 |
| Ps | 93.8 | 0.1 | 0.37 | 3.32 | 0.45 | 0 | 0.45 | 0 | 0 | 0.07 |

Ratio to Chl *a*

| | Chl $C_3$ | Chl $C_{1+2}$ | Carotenoids |
|---|---|---|---|
| Be | 0.122 | 0.477 | 1.817 |
| Ie | 0.216 | 0.603 | 4.149 |
| Le | 0.515 | 0 | 7.845 |
| Pe | 0.362 | 1.326 | 2.861 |
| Bs | 0.377 | 0.705 | 4.473 |
| Is | - | - | - |
| Ls | 0.504 | 0 | 8.132 |
| Ps | 0.525 | 1.209 | 7.040 |

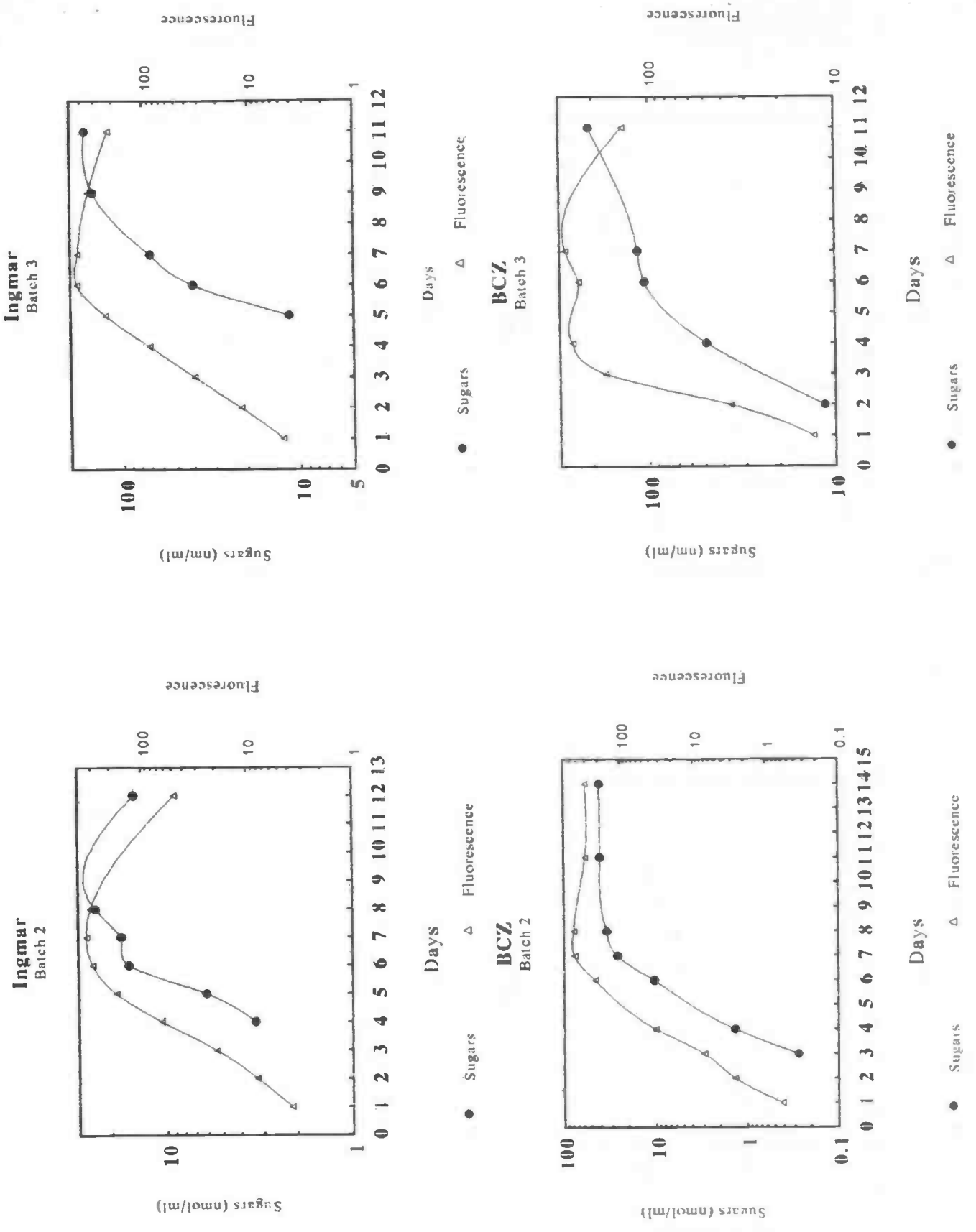
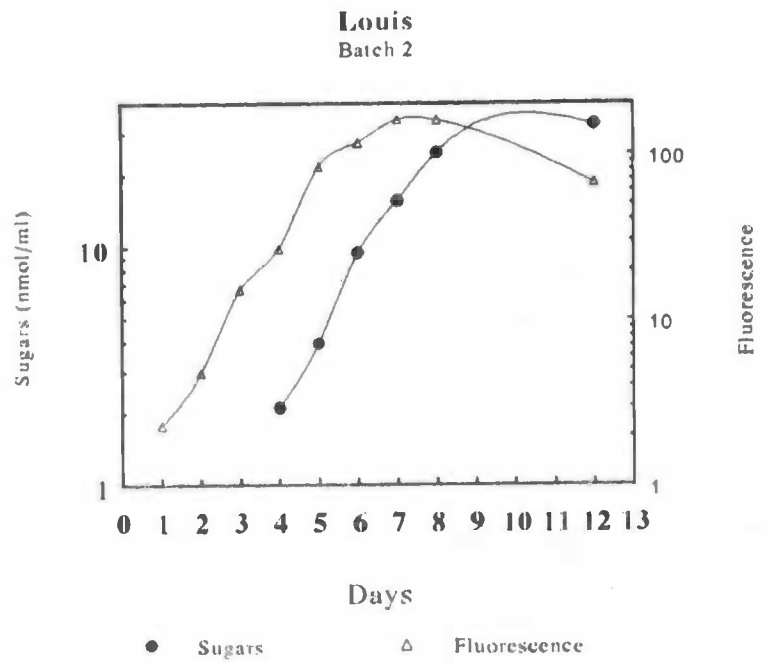
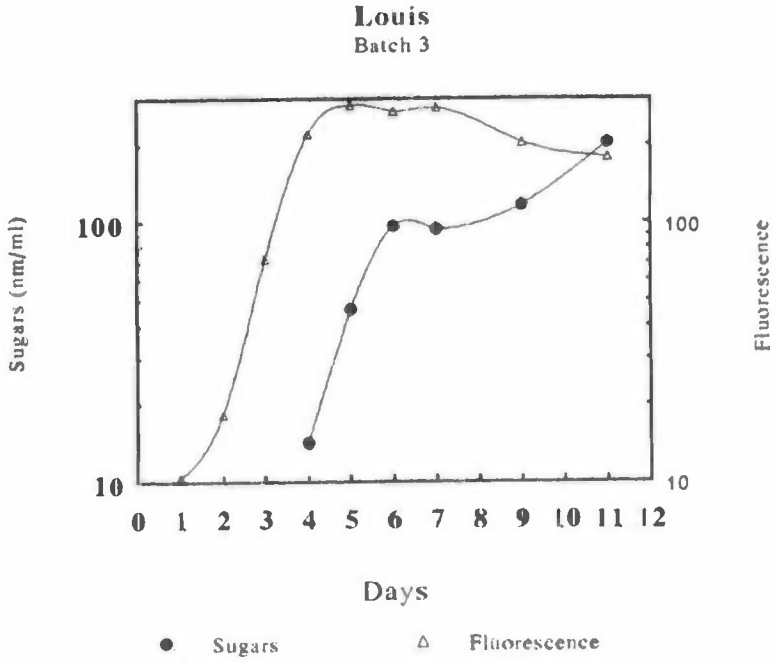
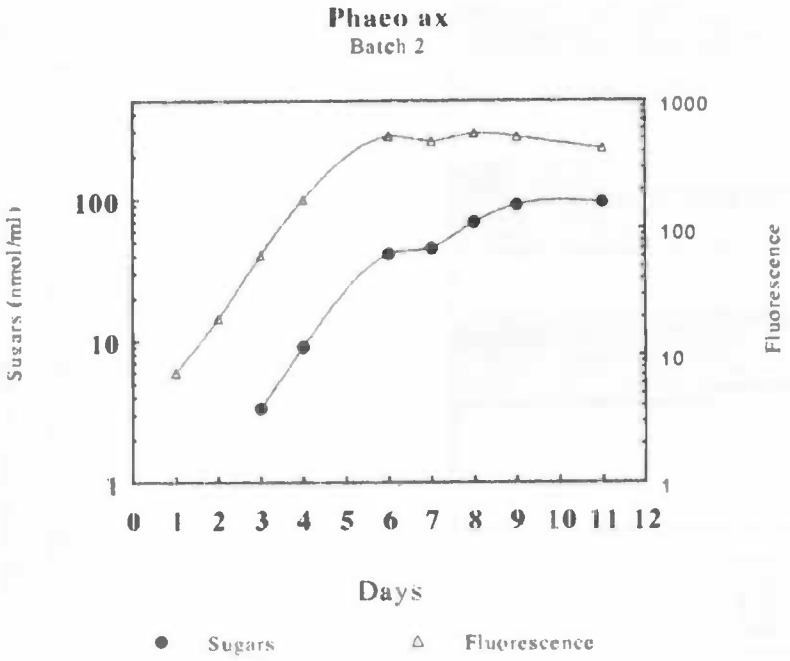



Fig. 4a: Total sugars and growth curves of two series of batch cultures.



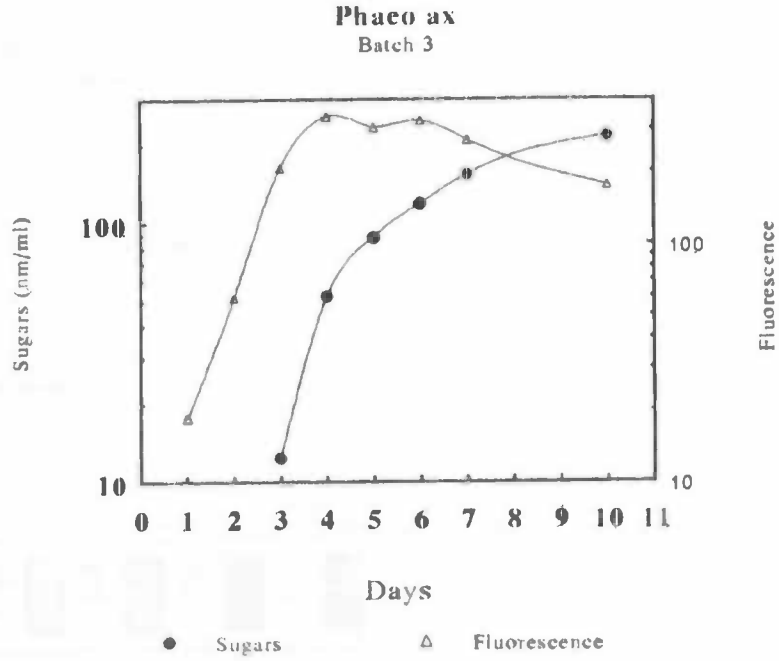




Fig. 4b: Total sugars and growth curves of two series of batch cultures.

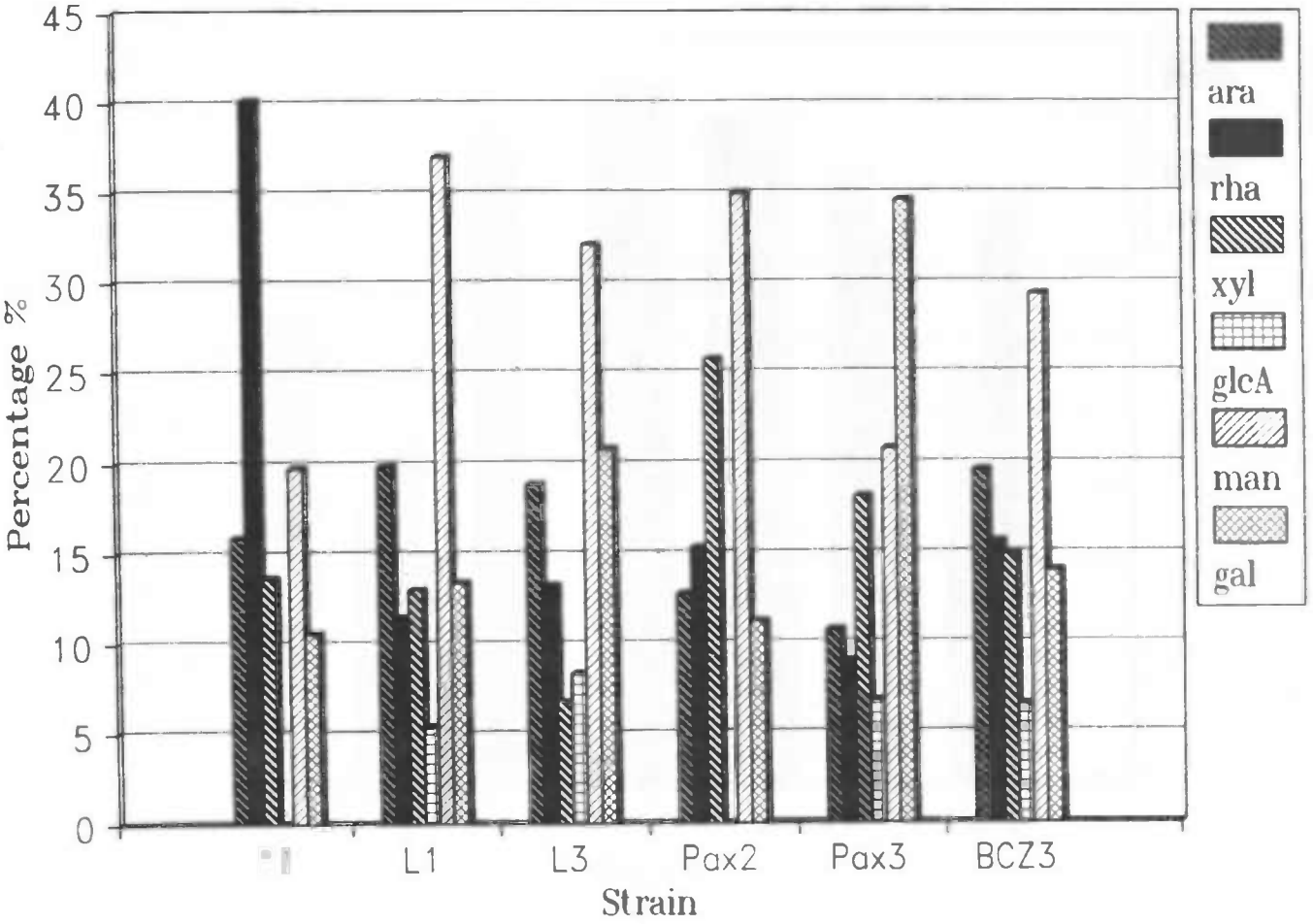



Fig. 5a: Sugar patterns. The letters P, L, Pax and BCZ stand for strains Plymouth, Louis, Phaeo ax and BCZ. The numbers indicate the series of batch cultures during which samples were taken.

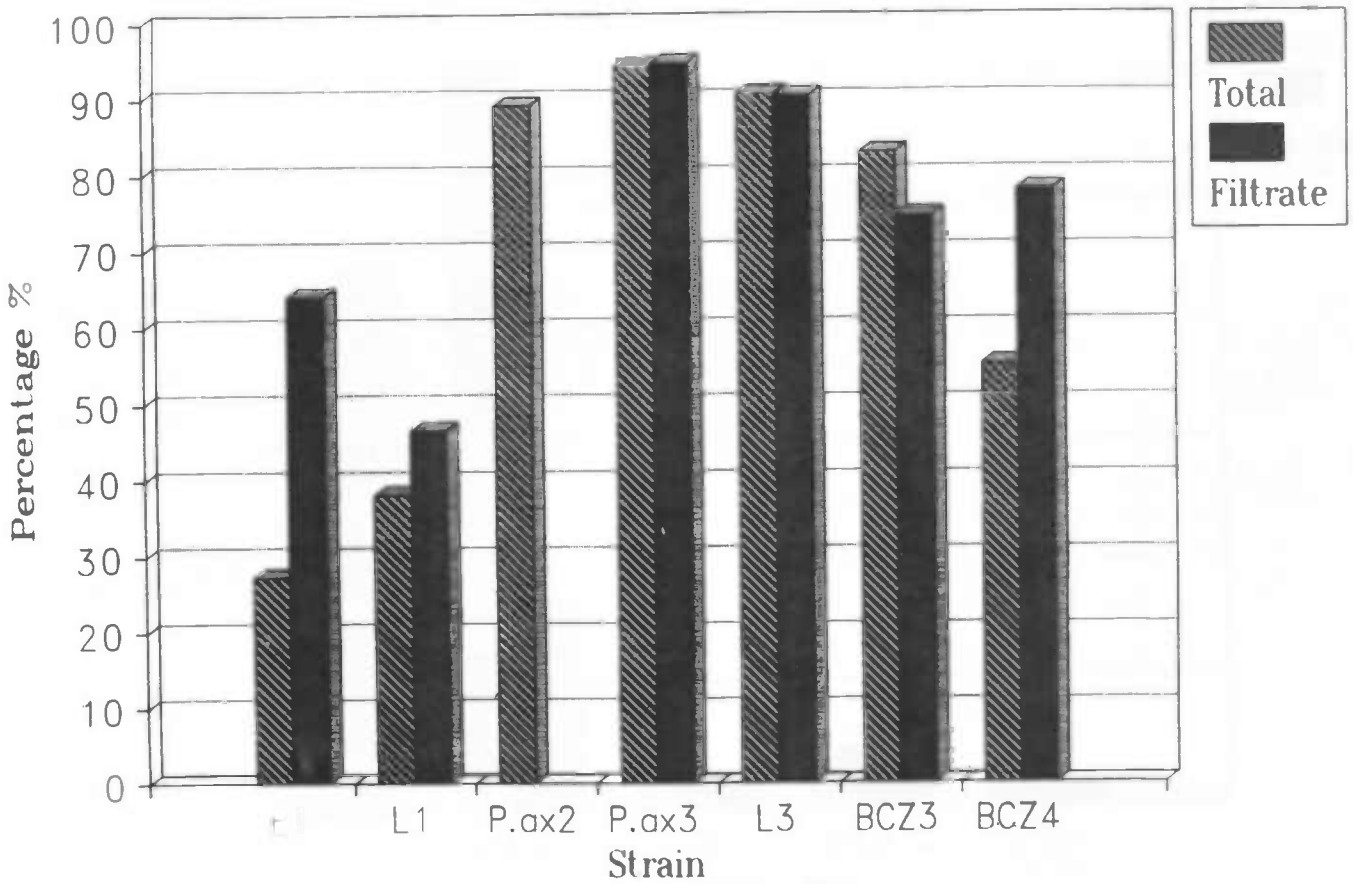



Fig. 5b: Percentage glucose of all sugars in strains during different series of batch cultures.

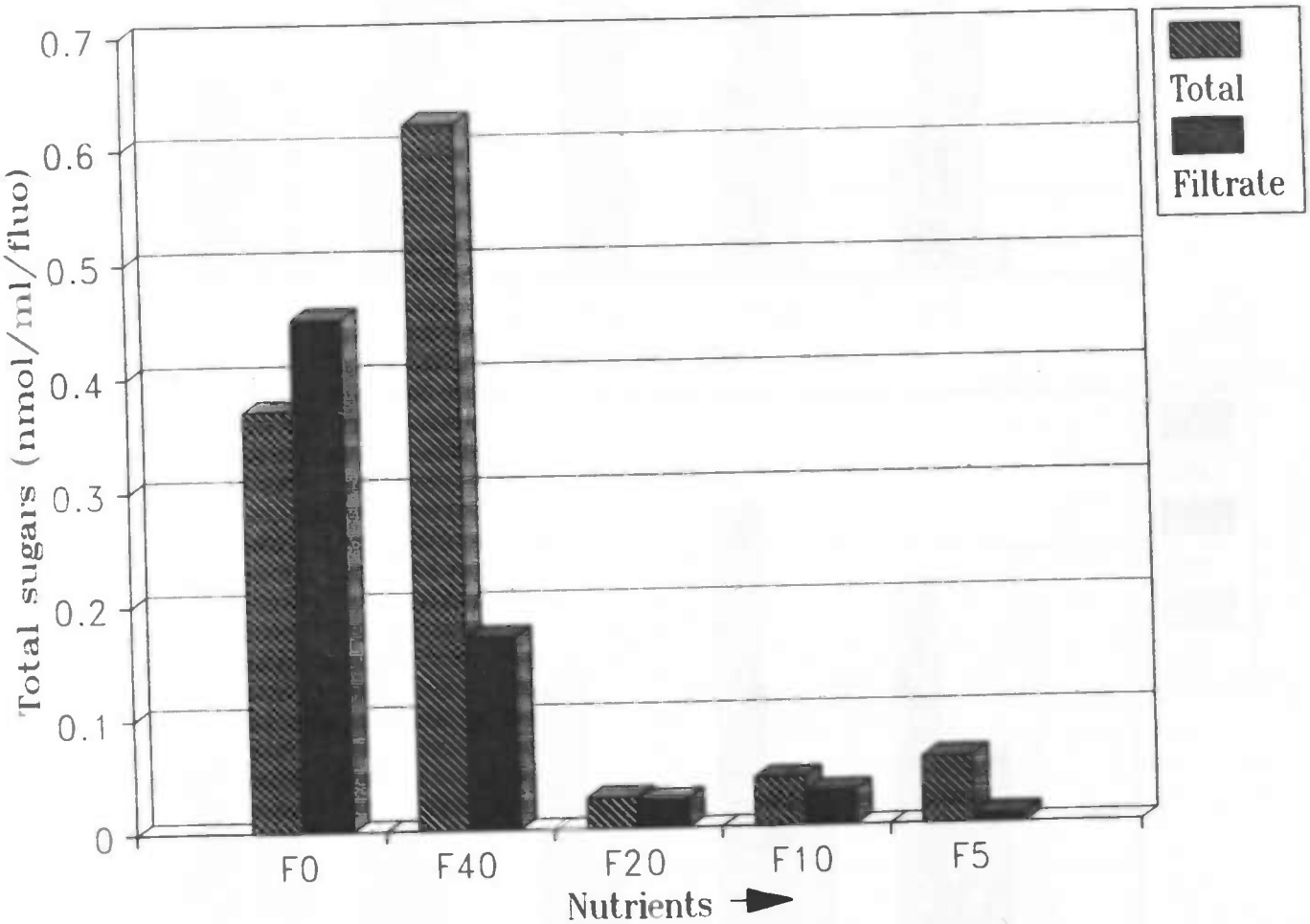



Fig. 6: Total sugars in samples from the BCZ-nutrient-experiment.

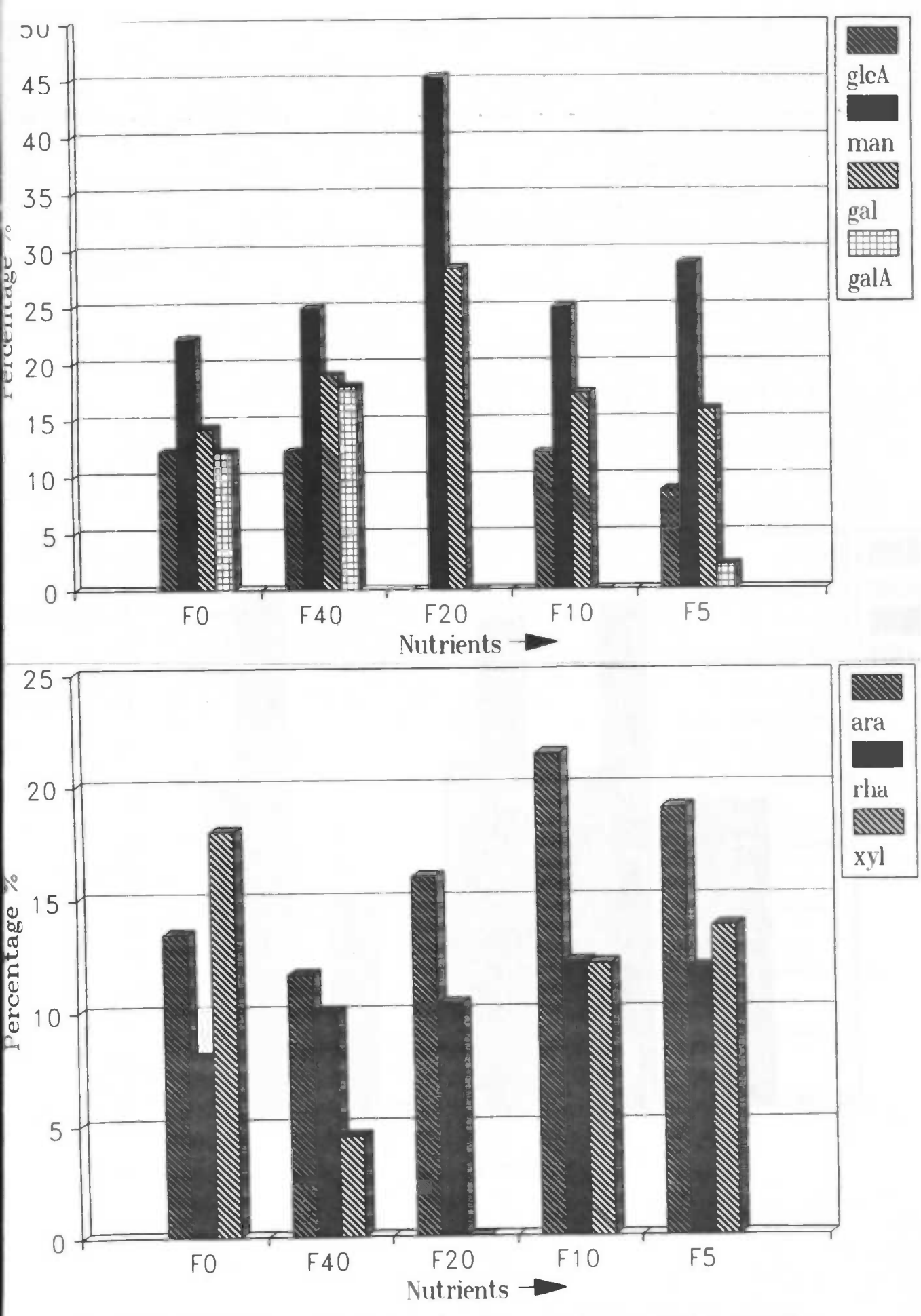



Fig. 7: Sugar pattern BCZ-nutrient-experiment.

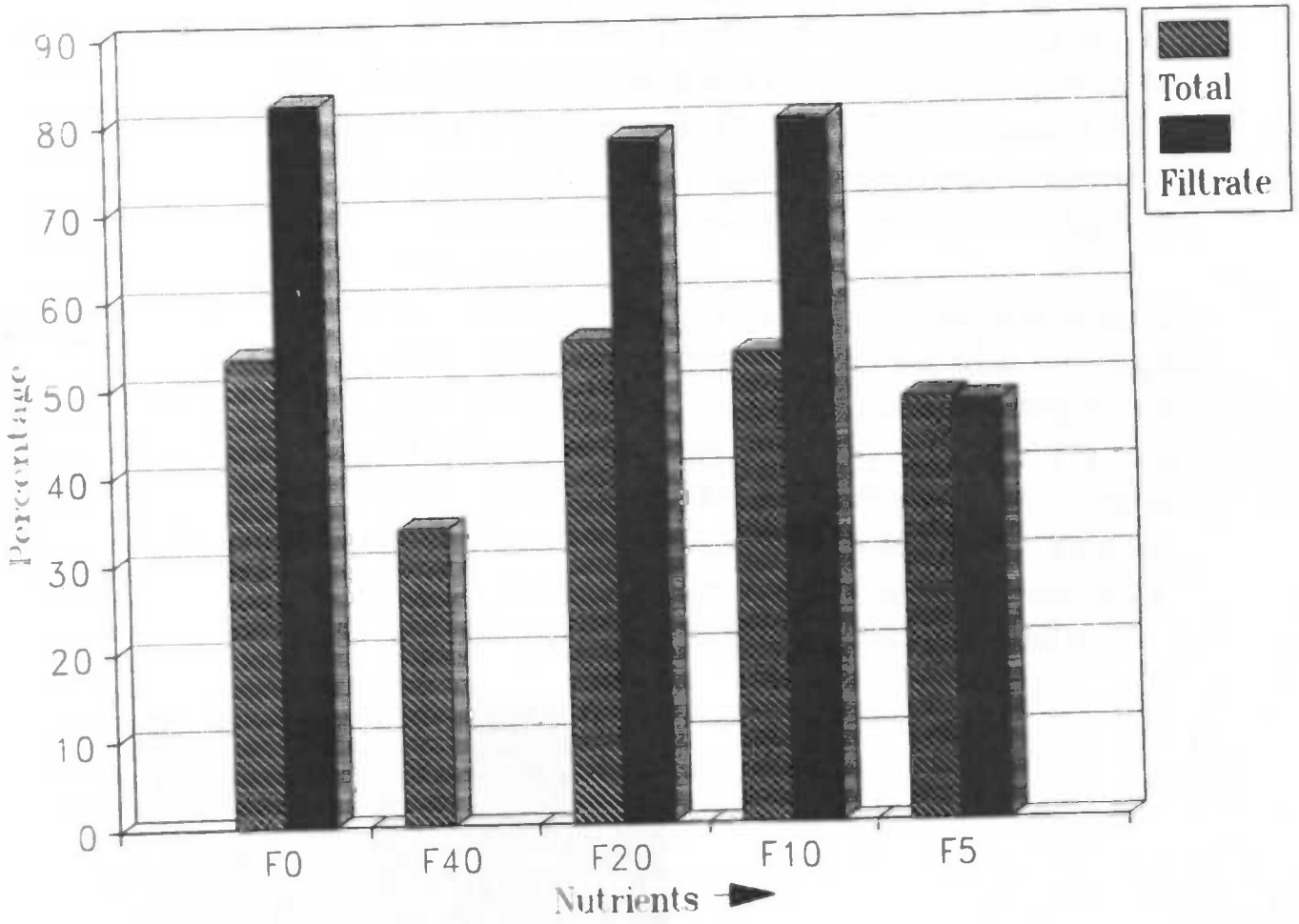



Fig. 8: Percentage glucose of all sugars in samples BCZ-nutrient-experiment.

Table 7: Batch culture data of BCZ-nutrient-experiment.

| Sample | Sampling day | Biomass in fluorescence units | Percentage of cells in colonies |
|---|---|---|---|
| F0 | 10 | 9.0 | 71 |
| F40 | 6 | 113 | 42 |
| F20 | 4 | 226 | 60 |
| F10 | 4 | 267 | 63 |
| F5 | 6 | 340 | 50 |

**RAPD data.** Scoring of the bands generated by RAPD amplification of 23 primers was performed twice in an attempt to eleminate the effect of overscoring. The RAPD amplification generated 151 and 171 scoreable bands. Only reproducible bands were scored except when just one of the duplicates showed a RAPD pattern. Strain Ph'91 was excluded from the RAPD data. Primers of series A did not produce any bands at all. Amplification with some primers gave a highly polymorphic RAPD pattern, whereas other primers amplified more conserved genomic sequences or very few sequences (Fig. 9) as was the case with strains Louis and BCZ.

**Distance analysis.** RAPD data were analysed using distance analysis by neighbour joining, resulting in the unrooted tree shown in Fig. 10. Bootstrap analysis was replicated 100 times and in 95 cases two clusters could be distinguished (bootstrap values are indicated in Fig. 10). Cluster South consists of *P. globosa* strains Louis, BCZ and Plymouth originating from the South of the North Sea and the Channel. Cluster North incorporates *P. globosa* strains Ingmar and Phaeo ax which originated from Marsdiep. *P. antarctica* strain Phaeo ant was used as an outgroup, it joined the tree in the North cluster. When parsimony analysis was used to analyse the RAPD data, the same two clusters were formed (data not shown).

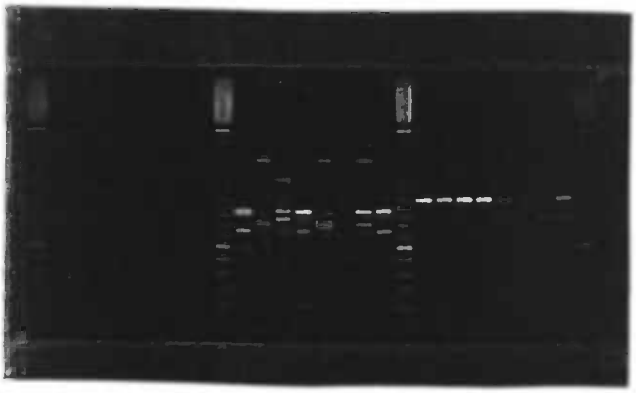


Fig. 9: RAPD pattern.

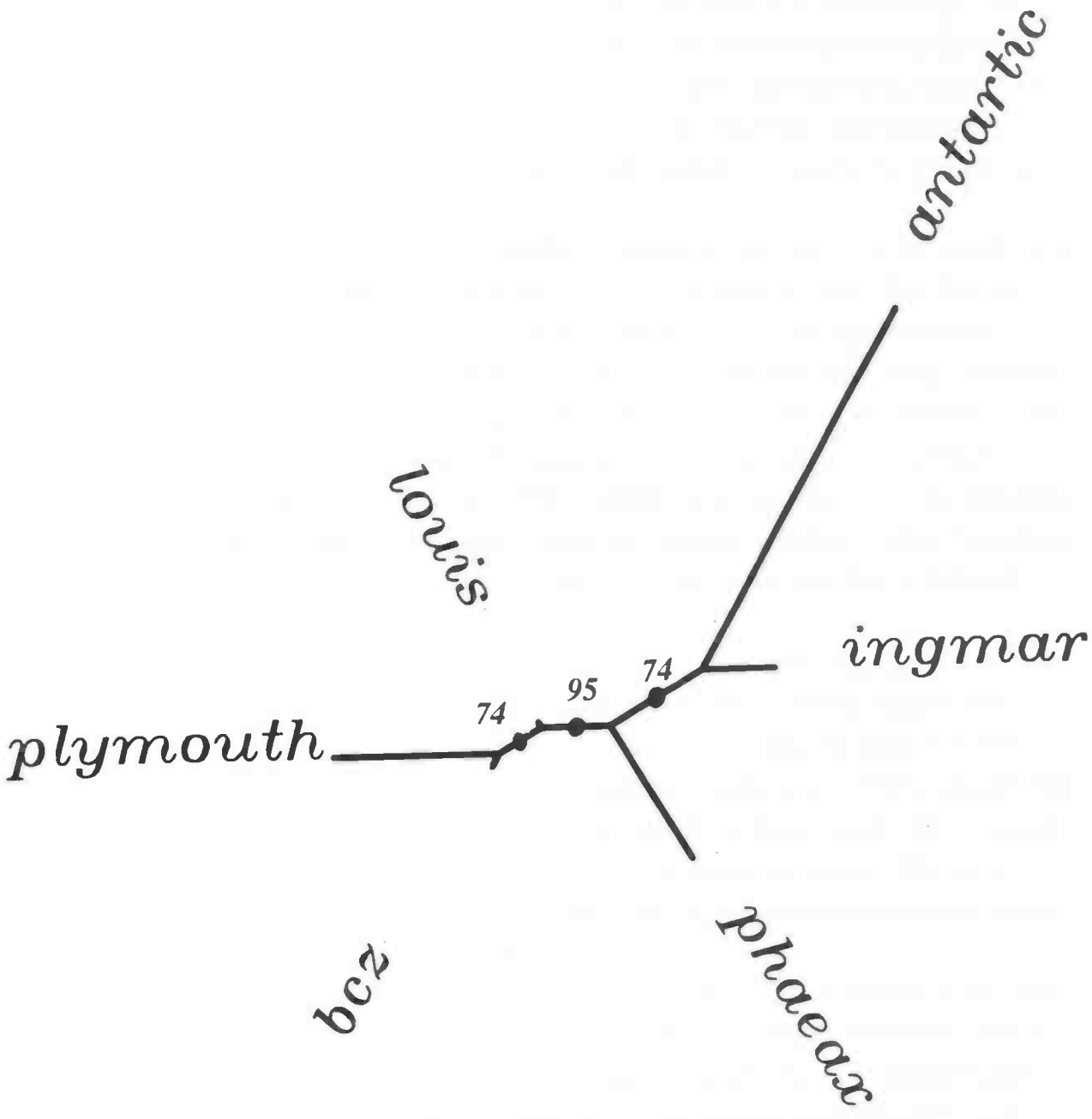



Fig. 10:Unrooted neighbour joining tree of the RAPD data. bootstrap values are indicated in the tree.

## 4. DISCUSSION AND CONCLUSIONS

Colony morphology (shape, distribution of cells) has been used as a criterium for *Phaeocystis* taxonomy by Jahnke and Baumann (1987). The present study indicates that percentage of cells in colonies is not a reliable criterium when distinguishing strains. There was daily variation and in every series of batch cultures the colonies were different in shape and size. The method tested for determining the proportion of cells in colonies was successful.

Presence or absence of colonies is probably coupled to life phase and can not be used as a taxonomical character because formation of colonies is unpredictable. The fact that strain Phaeo ax started to form colonies without changing culture conditions provides evidence to support this statement. Vaulot *et al* (1994) demonstrated that colony formation was connected with ploidy level and suggest that conditions for colony formation could be conditions necessary for ploidy change. Several authors have attempted to describe these conditions (Van Boekel, 1992; Peperzak, 1993; Veldhuis and Admiraal, 1987 and Riegman, 1992) but the triggering factors still remain unknown. Nutrient effects on colony formation are strain dependent (Vaulot *et al*, 1994) and the same conclusion can also be deduced from the present study.

Kornmann (1955) indicated a cell size range of 3 to 9 $\mu$m for *Phaeocystis* cells. The data for cell size from the present study falls within those values. Strains Ingmar and Phaeo ax were the smallest strains (4 and 3.9 $\mu$m), whilst the Plymouth cells were the largest (5.3 $\mu$m). The ranges of the cell sizes overlapped. Vaulot *et al* (1994) indicated that the average cell volume could vary two-fold during growth in batch culture and suggested that most of the variability could be attributed to growth phase and ploidy. This study demonstrates that size of cells should not be used alone for discrimination between strains but in combination with other characteristics.

Growth rates of the strains differed for all of the series of batch cultures of the same strain so in this study it has not been used as a taxonomical parameter. However, data of growth rates reviewed in Baumann *et al* (1994) indicate growth rate to be strain-specific. Examination of the growth rates of the strains used in the present study should be performed under the same culture conditions in further research in order to shed more light on the matter.

In the present study glucose varied in the different series of batch cultures between 30%-90% (Fig. 5b). The variation found in glucose content was first thought to be due to the change in seawater composition, i.e. nutrient effects. Combination of the data in Figs 6 and 8 demonstrates that there was more glucose per biomass when nutrients were limiting. However, during the second series of batch cultures when the seawater contained more

nutrients than in the other series, more glucose was found.

Sugar patterns presented in Fig. 5 indicate that the Plymouth strain behaved slightly different and that strains could vary their sugar composition due to different culture conditions (P.ax2 and P.ax3). A sugar fingerprint is recently demonstrated by Janse *et al* (1996) in *Phaeocystis* field material, and these authors suggest that variability in sugar patterns may reflect the presence of different strains. From the present study it could be concluded that sugar patterns can be variable. Caution should be exercised in making generalizations concerning sugar patterns even of one strain. In further research, the variation in the exponential phase of a batch culture of one strain should be examined.

Fingerprints from the filtered samples did not differ a great deal from the non-filtered samples (data not shown). This could be explained by the observation that mucus could slip through the 10 $\mu$m mesh size filter. The hypothesis behind the filtered samples was to find an enrichment of an intracellular glucose polymer in the filtrate. Glucose is the universal storage polymer for algae (Painter, 1983). By means of keeping the mucus on the filter and filtering the cells it was thought that the filtrate would enrich in glucose. Instead of filtering, the samples should be sedimented a few hours before harvesting. The colonies will then sink and the single cells can be poured out, leaving the colonies at the bottom of the flask.

The relationship between fluorescence of extracted samples and cell counts estimated in this study was not very accurate because it was based on 5 points only. Data from the stationary phase were excluded from Fig. 1. It has been demonstrated by Van Hall (1995) that plots of fluorescence against cell counts from a non-colony forming strain were linear in the exponential phase and that this relationship was lost in the stationary phase. Cell counts of colony-forming strains in this study were found to be too laborious and with limited accuracy. A disadvantage of cell counts is that cells can only be counted once a day due to the daily cell cycle. The fluorescence method was successful for colony-forming strains and in the time of writing this report an accurate correlation between fluorescence of extracted cells and cell counts has indeed been established (pers. comm. D.Noordkamp).

At the top of the bloom when nutrients are depleted, more than 80% of the photo-assimilated carbon has been shown to be devoted to the synthesis of exopolymeric substances. When nutrients are not limiting this percentage is 50% (Lancelot and Rousseau, 1994). Total sugar analysis (Figs 4a and 4b) showed that when the growth curve entered the stationary phase in this study, the amount of total sugars increased. This observation agrees with the previous statement. Bearing in mind that biomass estimated by means of fluorescence is not accurate in the stationary phase, actual results could be slightly different. Total sugars measured during the BCZ-experiment also indicate that when nutrients are limiting algae synthesise more sugars (Fig. 6).

The role of associated bacteria in *Phaeocystis* blooms is not yet understood. It is observed that colonies in early stages of blooms are almost entirely free of attached bacteria and that in late stationary phases of cultures and late bloom stages the opposite is true (reviewed in Thingstad and Billen, 1994). Van Boekel *et al* (1992) ascribe this sudden change to carbon-limitation of the microbial foodweb at the end of a spring bloom. In the present study an increase in bacteria in late stationary phase was measured (data not shown). Bacterial counts were not continued long after the stationary phase commenced so statements concerning sugar metabolism by bacteria can not be made.

Pigments can be applied as chemotaxonomical markers for *Phaeocystis* taxonomy (Gieskes and Kraay, 1986; Buma *et al*, 1991; Vaulot *et al*, 1994). Opinions differ on the reliability of this method. Buma *et al* (1991) found a variety of environmental factors to influence pigment content and ratio. On the contrary, Vaulot *et al* (1994) stated that culture conditions had little effect on pigment signature. A comparison of the pigmentation of several clones of *Phaeocystis* sp. isolated from different regions indicated that pigment composition and content is highly variable between clones (a.o. Gieskes and Kraay, 1986). Pigment analysis in this study did not show high variation between clones. Vaulot *et al* (1994) used 16 strains originating from different regions for pigment analysis and successfully performed a cluster analysis. The clustering was based on relative proportions of Fuco and Hfuc, on basis of these pigments results shown in this study fall in the North European cluster, ranging from the English Channel to The North Sea (near Texel). Pigment analysis did not appear as a useful tool for the purpose of this study. Although pigments seem to be less sensitive for changes in environmental conditions encountered in our studies, on the level of strain discrimination this method is not sensitive enough.

Analysis of the RAPD data revealed clear differences between the strains included in the analysis. Both parsimony analysis and neighbour joining procedures followed by bootstrap testing allowed the distinction of two separate clusters that overlap with the geographical origin of the strains. These clusters were called South and North. Within the South cluster the arrangement of the strains is not stable due to low bootstrap values. Strains Louis and BCZ in the South cluster both have very short branches, this is because the primers only amplified a few sequences of these strains. Van Oppen (1995) has demonstrated that in 16% of cases where a band that was scored as absent, it was shown to be present after Southern hybridizations using RAPD bands as probes. Accuracy in scoring of the banding patterns in the present study was achieved because two people performed the scoring independently which resulted in the same neighbour joining tree. Therefore, the network as presented in Fig. 10 is thought to be accurate. The separation of the strains in a North and South cluster is in concordance with the results from Janse *et al* (1996). These

authors based formation of a North and South cluster on differences in the sugar fingerprints using the sugar composition of *Phaeocystis* colonies.

Strain Ph'91 was excluded from the RAPD data analysis since it was a haploid flagellate and the other strains were diploid because colonies and flagellated cells that co-exist with colonies are diploid (Vaulot *et al*, 1994). Different ploidy levels can cause RAPD banding differences in the absence of genetic differences (Van Oppen *et al*, 1995).

Temperature ranges are commonly used to distinguish strains (i.e. Baumann *et al*, 1994). Due to practical reasons temperature tolerance was not investigated during this study. It was however noticed that the Plymouth strain could not be kept in stock on 5°C, only on 10°C. In further research it could be useful to add temperature tolerance as a taxonomical parameter.

The aim of the project was to describe clonal similarities or dissimilarities of the *Phaeocystis* strains. The main obstacle in doing so was the change of composition of the sea water which meant that obtained results could not be repeated. The different nutrient levels in the series of batch cultures gave rise to the observation that all strains reacted in a different manner. Overall, we have demonstrated that presence or absence of colonies, proportion of cells in colonies, pigment composition and growth rate did not appear to be good criteria to distinguish strains. Cell size and sugar fingerprints have potential to discriminate between strains. Analysis of the RAPD data allowed the strains to be grouped in a North and South cluster. The filter method was successfully used to measure amount of total sugar per cell. Autofluorescence of acetone extracted samples was achieved and showed to be a good biomass estimation of colony forming *Phaeocystis*.

## LITERATURE


Baumann M.E.M., C. Lancelot, F.P. Brandini, E. Sakshaug and D.M. John, 1994. The taxonomic identity of the cosmopolitan prymnesiophyte *Phaeocystis*; a morhological and ecophysiological approach. J. Mar. Syst. 5: 5-22.

Boekel, W.H.M. van, 1992. *Phaeocystis* colony mucus components and the importance of calcium ions for colony stability. Mar. Ecol. Prog. Ser. 87: 301-305.

Boekel, W.H.M. van, F.C Hansen, R. Riegmann and R.P.M. Bak, 1992. Lysis-induced decline of a *Phaeocystis* spring bloom and coupling with the microbial foodweb. Mar. Ecol. Prog. Ser. 81: 269-276.

Buma, A.G.J., N. Bano, M.J.W. Veldhuis and G.W. Kraay, 1991. Comparison of the pigmentation of two strains of the prymnesiophyte *Phaeocystis* sp. Neth. J. Sea Res. 27: 173-182.

Davidson, A.T. and H.J. Marchant, 1992. The biology and ecology of *Phaeocystis* (Prymnesiophyceae). Progress in Phycological Research, Vol. 8 (F.E. Round/D.J. Chapman, eds) Biopress Ltd.

Eberlein K., M.T. Leal, K.D. Hammer and W. Hickel, 1985. Dissolved organic substances during a *Phaeocystis pouchetii* bloom in the German Bight (North Sea). Mar. Biol. 89: 311-316.

Gieskes, W.W.C. and G.W. Kraay, 1986. Analysis of phytoplankton pigments by HPLC before, during and after mass occurence of the microflagellate *Corymbellus aureus* Green during the spring bloom in the northern North Sea in 1983. Mar. Biol. 92: 45-52.

Guillard, R.R.L. and J.A. Hellebust, 1971. Growth and production of extracellular substances by two strains of *Phaeocystis pouchetii*. J. Phycol. 7: 330-338.

Hall, P.J. van, 1995. Produktie en karakterisatie van het inwendige reservepolymeer in *Phaeocystis globosa*. Doctoraal scriptie RUG.

Hoek van den, C., 1978. Algen, 1st ed. Thieme Verlag, Stuttgart.

Jahnke, J. and M.E.M. Baumann, 1987. Differentiation between *Phaeocystis pouchetii* (Har.) Lagerheim and *Phaeocystis globosa* Scherffel. I. Colony shapes and temperature tolerances. Hydrobiol. bulletin 21:141-147.

Janse, I., M. van Rijssel, J. Gottschal, C. Lancelot and W. Gieskes, 1996. Carbohydrates in the North Sea during spring blooms of *Phaeosystis*: a specific fingerprint. Submitted to Aquat. Microb. Ecol..

Kamerling, J.P. and J.F.G. Vliegenthart, 1989. in Mass Spectrometry (Lawson, A.M., ed.) pp. 188-193, Walter de Gruyter, Berlin, New York.

Klerk, H., M.J.H. van Oppen and W.T. Stam, 1996. A comparitive study of the most frequently used diversity, similarity and distance used in RAPD analysis,

introducing DISTAN, a Personal Computer-program to reveal genetic relationships and variation within Random polymorphic DNA (RAPD) data sets (manuscript).

Kornmann, P. 1955. Beobachtungen an *Phaeocystis*-Kulturen. Helg. Wissens. Meeresunt. 5: 218-233.

Lancelot, C. and S. Mathot, 1985. Biochemical fractionation of primary production by phytoplankton in Belgian coastal waters during short- and long-term incubations with $^{14}$C-bicarbonate. II. *Phaeocystis pouchetii* colonial population. Mar. Biol. 86: 227-232.

Lancelot, C. and V. Rousseau, 1994. The structure and functioning of *Phaeocystis*-dominated ecosystems: the key role of the colony form. In: The Bioloy of the Prymnesiophyta (Ed. by J.C. Green and B.S.C. Leadbeater). Clarendon Press, Oxford.

Lancelot, C., P. Wassmann and H. Barth, 1994. Ecology of *Phaeocystis*-dominated ecosystems. J. Mar. Sys. 5: 1-4.

Liu, D., P.T.S. Wong and B.J. Dutka, 1972. Determination of carbohydrate in lake sediment by a modified phenol-sulfuric acid method. Wat. Res. 7:741-746.

Medlin, L.K., M. Lange and M.E.M. Baumann, 1994. Genetic differentiation among three colony-forming species of Phaeocystis: further evidence for the phylogeny of the Prymnesiophyta. Phycologia 33: 199-212.

Oppen, M.J.H. van, H. Klerk, M. de Graaf, J.L. Olsen and W.T. Stam, 1995. Assessing the limits of RAPD markers: an example from the red algae. In: Tracking trails by cracking codes, PhD thesis RUG, p.119-145.

Painter, T.J., 1983. Algal polysaccharides. In: Aspinall, G.O. (ed.) The Polysaccharides, Vol. 2. Academic Press, New York, p. 195-295.

Paul, J.P., 1982. Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. Appl. Environ. Microbiol. 43: 939-944.

Peeters, J.C.H. and L. Peperzak, 1990. Nutrient limitation in the North Sea: a bioassay approach. Neth. J. Sea Res. 26 (1): 61-73.

Peperzak, L., 1993. Daily irradiance governs growth rate and colony formation of *Phaeocystis* (Prymnesiophytceae). J. Plankton Res. 15: 809-821.

Putt, M., G. Miceli and D.K. Stoecker, 1994. Association of bacteria with *Phaeocystis* sp. in McMurdo Sound, Antarctica. Mar. Ecol. Prog. Ser. 105:179-189.

Riegman, R., A. Noordeloos and G.C. Cadeé, 1992. *Phaeocystis* blooms and eutrophication of the continental coastal zones of the North Sea. Mar. Biol. 112: 479-484.

Rousseau, V., S. Mathot and C. Lancelot, 1990. Calculating biomass of *Phaeocystis* sp. from microscopic observations. Mar. Biol. 107:305-314.

Rousseau, V., D. Vaulot, D. Casotti, R. Cario, V Lenz, J. Gunkel and M. Baumann, 1994. The life cycle of *Phaeocystis* (Prymnesiophyceae): evidence and hypotheses. J. Mar. Sys. 5:23-39.

Sambrook, J., E.F. Fritch and T. Maniatis, 1989. *Molecular cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York, 3vols.

Sournia, A. 1988. *Phaeocystis* (Prymnesiophyceae): how many species? Nova Hedwigia 47: 211-217.

Swofford, D.L., 1993. Paup: Phylogenetic analysis using parsimony, version 3.1. Lab. Mol. Sys. Sl, Washington DC 20560.

Thingstadt, F. and G. Billen, 1994. Microbial degradation of *Phaeocystis* material in the water column. J. Mar. Sys 5: 55-65.

Utermöhl, H., 1958. Zur Vervolkommnung der quantitativen Phytoplankton Methodik. Mitt. int. Verein. theor. angew. Limnol. 9:1-38

Vaulot, D., J.L. Birrien, D. Marie, R. Casotti, M.J.W. Veldhuis, G.W. Kraay and M.J. Chrétiennot-Dinet, 1994. Morphology, ploidy, pigment composition, and genome size of cultured strains of *Phaeocystis* (Prymnesiophyceae). J. Phycol. 30: 1022-1035.

Veldhuis, M.J.W., W. Admiraal and F. Colijn, 1985. Chemical and physiological changes of phytoplankton during the spring bloom, dominated by *Phaeocystis pouchetii* (Haptophyceae): Observations in Dutch coastal waters of the North Sea. Neth. J. Sea Res. 20: 49-60.

Veldhuis, M.J.W. and W. Admiraal, 1987. Influence of phosphate depletion on the growth and colony formation of *Phaeocystis pouchetii*. Mar. Biol. 95: 47-75.

Verity, P.G., T.A. Villereal and T.J. Smayda, 1988. I. Abundance, biochemical composition, and metabolic rates. J. Planton Res.

Verity, P.G., T.A. Villareal and T.J. Smayda, 1991. Photosynthetis, excretion and growth rates of *Phaeocystis* colonies and solitary cells. Polar. Res. 10: 117-128.

Villerius, L.A., R.E. Warnock, R. Bijkerk and W.W.C. Gieskes, 1996. Signal improvement by water packing of extracts in automated pigment analysis by HPLC (in prep).

**APPENDIX 1. Recipe acid lugol solution.**

Solution 1:
- 20 gr $I_2$
- 42.3 gr KI
- 80 ml $H_2O$

Solution 2:
12.5% acetic acid

Mix one part of solution 1 with 4 parts of solution 2 to obtain the acid lugol solution.

APPENDIX 2. Plot of cell counts versus autoflourescence of aceton extracted cells.