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LIPID PRODUCTION FROM ALGAE GROWN IN SALINE WATER USING LOW INTENSITY CULTURE TECHNIQUES

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ABSTRACT

Many arid regions of the United States have high levels of incident solar radiation and ample supplies of saline groundwater. This project's goal was to determine the feasibility of growing algae for lipid production in saline water using low intensity management techniques in the southwestern United States. After preliminary trials, dry matter production, lipid content, lipid production, and lipid characteristics were determined for *Chaetoceros gracilis, Monoraphidium sp., Tetraselmis suecica* and *Navicula sp.* to select promising species for pilot scale outdoor trials. *Tetraselmis* and *Chaetoceros* exhibited superior performance in these trials.

In outdoor, open tank field trials designed to simulate low intensity management as would be found in farm ponds, *Chaetoceros* performed poorly and was replaced routinely by "weedy" species. *Tetraselmis* performed at a satisfactory level, producing approximately 4.5 $g \cdot m^{-2} \cdot day^{-1}$ ash-free dry material and 0.7 $g \cdot m^{-2} \cdot day^{-1}$ lipid during a 30-day mid-summer trial. *Tetraselmis* had only moderate weed resistance and was also replaced by weed algae. A weed alga, strain MA1, produced dry matter and lipid at about the same rate as *Tetraselmis*.

Unfortunately, these rates are one-third or less of the productivity of intensely managed outdoor algae cultures. It is probable that intensive management techniques will need to be used to justify the capital investment in algae harvesting and lipid processing equipment.

Key words: algae, brackish water, plant lipids

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INTRODUCTION

Problem Statement

New Mexico's fresh water use currently equals its renewable supply. For a short while, economic growth can be based on a fresh water supply expanded by mining. In the long run, fresh water use will be limited by the state's precipitation and compact entitlements to surface water flows. The ultimate limitation of economic activity by a lack of fresh water is a problem New Mexico shares with many of the region's arid states. One way to relieve some of the pressure placed on the region's economic activity is to utilize its abundant supplies of saline groundwater in a productive manner. Because desalinization is so costly, the most feasible uses of saline groundwater are those which use the water as it comes from the well with little or no modification. The production of algae for biomass and oils is such a use. Preliminary calculations indicated that low input growth procedures, could provide algal yields sufficiently high to be profitable provided that these yields were realized in practice and that reasonable by-product recovery could augment income from algal oils. This project investigated the possibility of using a low inputs/low technology approach for growing microalgae as a feedstock for oil or petrochemical production.

Related Research

In 1979, the US Department of Energy (DOE) began serious study into the possibility of using microalgae as an alternative, renewable source of hydrocarbon fuel. The assumption underlying this initiative was that lipids from microalgae could be extracted and converted to gasoline and diesel-like hydrocarbon fuels for a cost of $1.60 to $2.00 per gallon by the year 2010 [Neenan et al. 1986]. While the recent depressed state of the oil and gas industry made this price range seem unrealistically

high, the current political instability in the Middle East and the finite nature of fossil fuel resources indicate that the long-term price trend will be upward.

DOE targeted the southwestern U.S. desert for its research effort and as the prime location for commercial endeavors for a number of reasons including:

- High incident solar radiation
- An abundance of flat and relatively inexpensive land
- Few competing land uses
- Large quantities of unused saline groundwater

These factors combine to minimize the cost of fuel production. In a study designed to identify locations with the best combination of these factors, southeastern New Mexico was one of the priority sites [Maxwell et al. 1984]. More recent studies have investigated a series of sites in New Mexico to determine their suitability for the location of 1000 ha algae production facilities based on the DOE criteria listed above. The study found two sites in the Tularosa Basin and one in the Crow Flats Basin of south central New Mexico which met all DOE criteria. In addition, it found that the Roswell area would be suitable for facilities smaller than the 1000 ha DOE target [Lansford et al. 1986].

Roswell's feasibility as a production site for both blue-green filamentous, and diatomaceous microalgae has been demonstrated [Goldstein 1986a; Goldstein 1986b]. *Spirulina* production in covered, outdoor raceways gave average yields of $13 g \cdot m^{-2} \cdot day^{-1}$. *Chaetoceros gracilis* strain ss14 grown in the same raceway system for oyster food yielded densities of $250 mg \cdot m^{-2} \cdot day^{-1}$. These figures translate to about 20 metric $tons \cdot acre^{-1} \cdot year^{-1}$ [B. Goldstein, personal communication 1989].

There is an extensive literature on the potential of halotolerant (saline water tolerant) algae in producing chemical feedstocks such as glycerol and carotenoids. This literature has been reviewed by Ben-Amotz [Ben-Amotz and Avron 1983]. The review indicates that halotolerance confers an advantage in addition to the ability to utilize the saline water resource. The problem of unwanted "weed" algae growth is minimized because many of the algal propagules which blow into the outdoor culture facility are unable to tolerate the saline conditions [Ben-Amotz and Avron 1983].

Under DOE contract, the Solar Energy Research Institute (SERI) maintains a culture collection of strains with potential for oil/lipid production [Microalgal Technology Research Group 1984]. The culture listing includes some information on lipid composition and limited information on growth-condition induced composition changes [Microalgal Technology Research Group 1984]. A review of this document and other recent papers on algal lipid synthesis [Maxwell et al. 1984; Ben-Amotz et al. 1985; Wolf et al. 1985, Chirac et al. 1985; Piorreck et al. 1984; Smith and Harwood 1984; Sargent et al. 1985; Fried et al. 1982] allows one to draw conclusions about factors which increase cell lipids as a percent of cell biomass. In general, the following factors stimulate lipid production.

- Nitrogen starvation
- Low light intensities
- Low environmental temperatures
- Salt stress

Unfortunately, these factors also serve as limits to algal growth. In any production facility, a careful balance between limiting growth and stimulating lipid levels will have to be reached. Some of these factors are relatively easy to manage. By

manipulating fertilization and feed water, fairly constant nitrogen and salt levels are obtainable. To a degree, light intensity to the cells can be controlled by manipulating the harvesting rate. Few economically feasible steps can be taken to manipulate the water temperature [Neenan et al. 1986]. SERI's recent study [Neenan et al. 1986] summarizes state-of-the-art engineering of outdoor algae production and harvesting facilities. The study identifies the major costs and limitations of biofuels production and provides access to a computer model designed to estimate input costs.

Because of their oxygen content and physical properties, algal lipids will probably have only limited direct applicability as liquid fuels. Usually, algal lipids will have to serve as feedstocks for conversion to gasoline-like fuels by catalytic conversion or diesel-like fuels by transesterification [Neenan et al. 1986]. Though generally more complex mixtures, algal lipids resemble vegetable and seed oils. Much of the information on the direct use of these oils as fuels and their conversion to conventional liquid fuels can be extended to algal lipids [Ryan et al. 1984; Freedman and Pryde 1982; Kusy 1982; Pryde 1982]. In addition, the use of specific algal products in fuel production has been assessed [Hill and Feinberg 1984; Voltz 1976; Weisz 1979; Feinberg 1984].

METHODS

Culture Procedure

Screening Studies

Initial pilot studies to determine the best species were carried out in 250 ml flasks using full and 50% strength SERI type II-25 artificial groundwater amended with nitrogen, phosphorous, and PII trace metal mix. Flasks were grown under an illumination of 250 and 75 $\mu Einst \cdot m^{-2} \cdot sec^{-1}$, at 12 °C, 22 °C, and 34 °C. Species tested in initial studies were: *Chaetoceros gracilis* (CHAET9), *Phaeodactylum tricornutum* (PHAEO1) , *Monoraphidium sp.* (MONOR1), *Navicula sp.* (NAVIC1), *Botryococcus braunii* (BOTRY1) and *Tetraselmis suecica* (TET1). All strains tested originated with the SERI Micro Algae Culture Collection and were selected to provide a good taxonomic mix.

There was no difference in growth at the two salinities and growth of all species was very poor at the lower light intensity. Therefore, an illumination level of 250 $\mu Einst \cdot m^{-2} \cdot sec^{-1}$ and a salinity level of half strength SERI type II-25 water (amended with nitrogen, phosphorous, and PII trace metal mix (II-25/2)) were chosen for the screening study. This mixture was further amended with Na_2SiO_3 for the diatoms tested. PHAEO1 and BOTRY1 were discarded from the study prior to the screening due to extremely poor growth. Cultures were screened at three temperatures (12 °C, 22 °C, and 34 °C) and at nitrogen levels of 0.5 mM (1xN) and 2.5 mM (5xN). Data presented represent the average of three determinations.

Tank Studies

A small pilot facility was assembled in the Biology Annex Enclosure on the New Mexico State University (NMSU) campus. The facility consisted of two growing

tanks with a surface area of approximately 0.75 m^{-2} each and a depth of 0.25 m. Water of the appropriate salinity and nutrient level was pumped from a pair of holding tanks. The water was mixed in a pair of mixing tanks. This paired system allowed the comparison of the tested strains in two different water chemistries under the same light and temperature regimes. Cells in the growth tank were mixed by a submersible pump on the tank bottom with the outlet directed upward at approximately a 30° angle to mix the tank contents and drive cells at depth back to the surface.

Tanks were inoculated with 20 to 40 l of culture grown in the laboratory and the population allowed to reach a stable density. Fresh feed water (II-25/2) containing the appropriate nitrogen level was pumped in at rates ranging from 15 to 40 l per day in three equal pumping intervals. Fresh water was added daily in quantities to make up for evaporation. Harvesting was accomplished by overflow. To collect cells, a container was placed under the harvesting port and the valve opened. At all other times, the overflow was discharged to a drain system. The tank facility is illustrated in Figure 1.

Sample Analysis

Harvest

Cells were pelleted by centrifugation, resuspended to wash them free of excess salt and centrifuged in a preweighed tube to pellet them again. In all cases, the cell pellet was weighed. To determine dry weights, wet samples were weighed and dried at room temperature to constant weight. Ash-free dry weights (AFDW) were determined by combusting dry samples and subtracting ash weights.

Lipid Extraction

Total lipids were extracted from a known weight of cell pellet by multiple

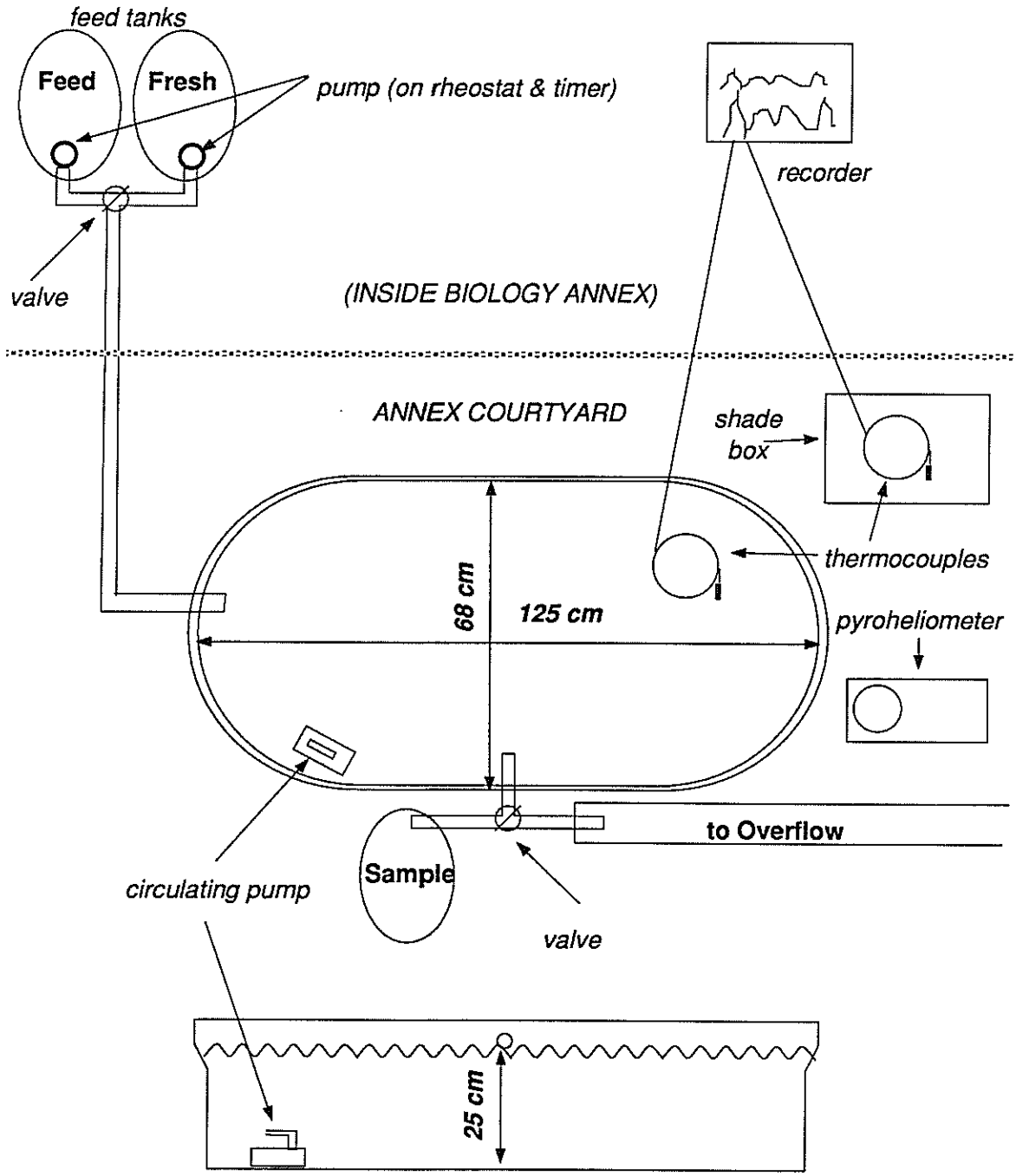

Fig. 1. Small pilot scale tank facility located in the courtyard of the Biology Annex on the New Mexico State University campus.

chloroform-methanol-water extractions using the method of Bligh and Dyer [1959] as modified by Kates [1964]. Phases were separated and the organic phases from the repeated extractions pooled. The volume of the pooled organic phases was reduced by rotary evaporation and transferred quantitatively to pre-weighed tubes. The remaining chloroform/methanol was removed under a stream of dry, oil free N_2. Tubes were further dried under vacuum and lipid weight determined gravimetrically. All samples were stored at -80 °C.

Lipid Fractionation

Pre-fractionation. Gross fractionation into major lipid groups was carried out by sequential extraction. A weighed sample of the total Bligh-Dyer lipid was applied to a silicic acid column (1 g per 10 mg lipid). The columns were eluted sequentially using the solvent sequence, hexane, benzene, chloroform, acetone, and methanol [Ben-Amotz and Avron 1983]. This yielded fractions containing the following major constituents acyclic hydrocarbons (hexane); cyclic hydrocarbons, acyclic unsaturated hydrocarbons, fatty acid methyl esters, sterols, and carotenoids (benzene); mono-, di- and tri-glycerides, free fatty acids, and carotenoids (chloroform); glycolipids, chlorophylls a+b, and carotenoids (acetone); phospholipids, chlorophyll c (methanol). In all cases, the solvent fractions were reduced in volume by rotary evaporation, quantitatively transferred to sample storage tubes, dried under a stream of N_2 and stored at -80 °C.

Fraction fingerprinting. Fractions were fingerprinted on two different TLC solvent systems. The first was chloroform:methanol:water:acetic acid, 73:25:1.5:0.5. The second was chloroform:methanol:ammonia(30%):water, 60:35:5:2.5 [Christie 1982].

Identification and quantitation of fatty acids. To determine the fatty acid composition, total lipid fractions were further analyzed by gas chromatography (GC) [Ben-Amotz and Avron 1983; Wolf et al. 1985]. The lipid sample was saponified in 20% KOH in 50% methanol and methylated with ethereal diazomethane. Analysis of these fatty acid methyl esters was carried out on an SP-2330 high polarity 2 m packed column using a temperature program from 175°C to 220 °C as previously described for fungal lipids [Herman and Herman 1985]. Detection was by flame ionization detector (FID) and fatty acid quantities estimated from the integrated area under the peak for each fatty acid.

FINDINGS

Screening Studies

Growth Studies

The results of the microalgae growth screening are summarized in Figure 2. Data are normalized to let the maximum growth equal 100%. *Tetraselmis* grown at 22 °C in the 5xN treatment yielded the highest growth (715.11 ± 57.2 mg·l^{-1} · 10 days^{-1}). All mean yields and standard error of the mean (SEM) values were divided by 715.11 to obtain the plotted values. Several patterns emerged from these data. First, all algal strains tested grew far better at 22 °C than at either 12 °C or 34 °C regardless of the nitrogen level. Second, growth was always higher in the 5xN treatment than in the 1xN treatment, though not always significantly so. Finally, species often changed their relative performance rank under the different growth conditions. *Tetraselmis* was the best performer at 22 °C followed by *Chaetoceros*, *Monoraphidium* and *Navicula* at both nitrogen levels. At 12 °C, there was little difference in growth among the species tested. All grew poorly when compared to their performance at 22 °C. *Chaetoceros* was the best performer at both nitrogen levels but not significantly so at either. The relative performance of *Tetraselmis* fell sharply at reduced temperature, dropping from first to fourth rank. *Chaetoceros* showed the highest growth performance at 34 °C at both nitrogen levels. It also showed the strongest response to the increased nitrogen levels at this temperature. *Tetraselmis* again performed poorly when compared to 22 °C, dropping from first to third rank.

These data suggest that *Tetraselmis* is likely to be the best performer in the field in a narrow range of conditions while *Chaetoceros* and *Monoraphidium* are likely to be good candidates under a broader variety of field conditions.

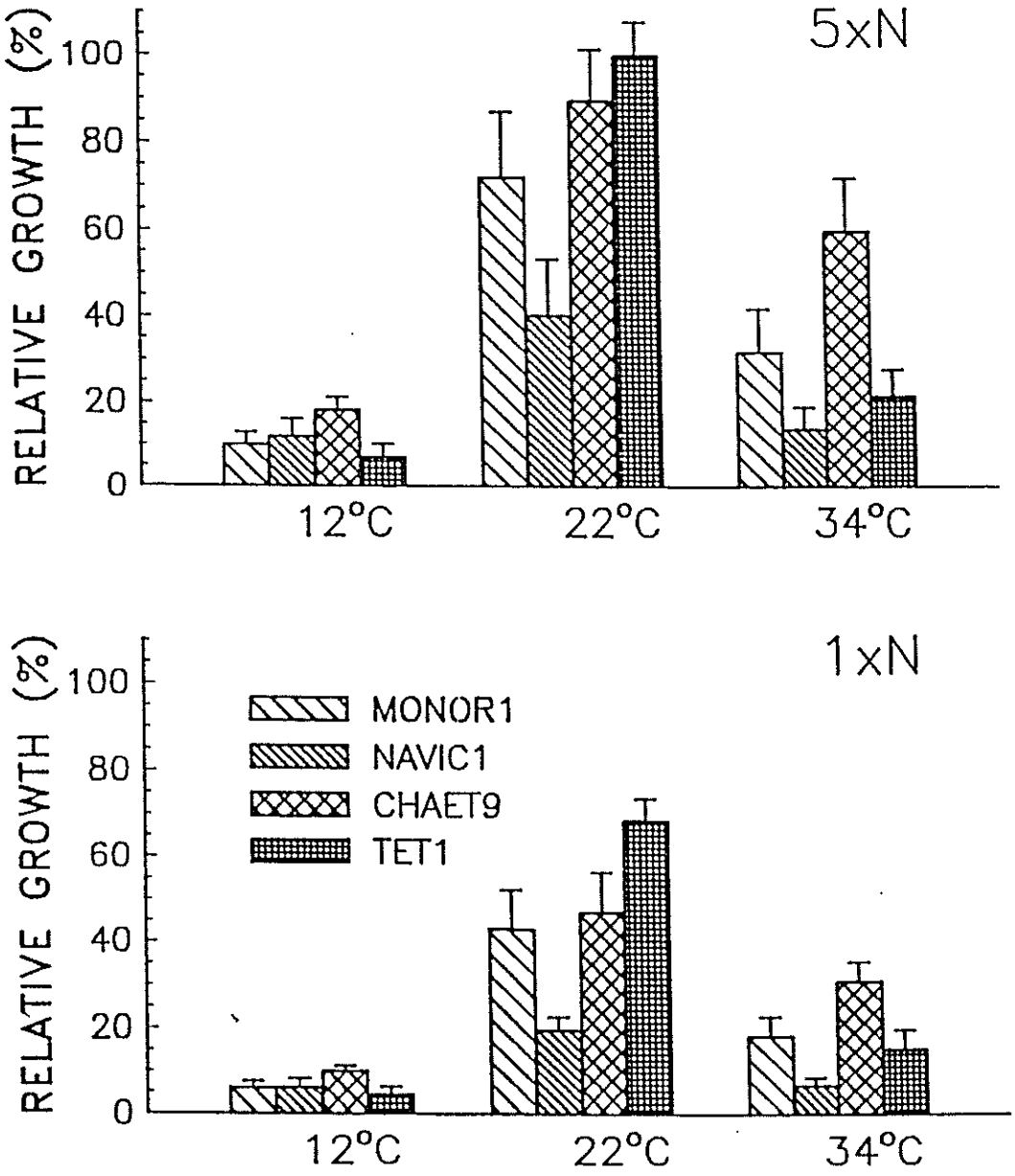

Fig. 2. **Relative growth of microalgae under laboratory conditions. Data are normalized to allow 715.11 mg·l^{-1} to represent 100 %.**

Lipid Production Studies

The lipid composition of each species tested at both nitrogen levels is presented in Figure 3. All data represent total lipid extracted by exhaustive Bligh-Dyer extraction from fresh frozen tissue expressed as a percentage on an ash-free dry weight basis. *Chaetoceros* had the highest percentage lipids at all conditions tested, followed by *Tetraselmis*. Both *Monoraphidium* and *Navicula* had consistently lower lipid contents. The lipid content was generally higher in cells grown at 1xN and at the extreme temperatures, though not significantly so. These observations are consistent with the well known phenomenon of stress-induced increases in lipid content [Microalgal Technology Research Group 1984; Piorreck et al. 1984].

As the goal of this project was to maximize lipid output, lipid yield was determined. The data in Figure 4 represent the relative lipid yields of the four species tested. Data are normalized to let the maximum lipid yield equal 100%. *Chaetoceros* grown at 22 °C in the 5xN treatment yielded the highest growth (120.19±8.50 $mg \cdot l^{-1} \cdot 10\ days^{-1}$). All yields and SEM were divided by 120.19 to obtain the plotted values. As with total ash-free dry matter production, *Tetraselmis* was a leading performer at 22 °C and suffered in performance at lower and higher temperatures. *Chaetoceros* was again relatively high-yielding species over a broader range of conditions than *Tetraselmis*. *Monoraphidium* dropped in relative rank because, while a consistent dry matter producer, it had a relatively low lipid content.

An interesting pattern emerges from these data. The small increase in lipid content on a percentage basis obtained by nitrogen or temperature stress is unable to offset the loss in productivity caused by these same stresses. The best strategy to maximize lipid yield on a per area or volume basis is to maximize growth.

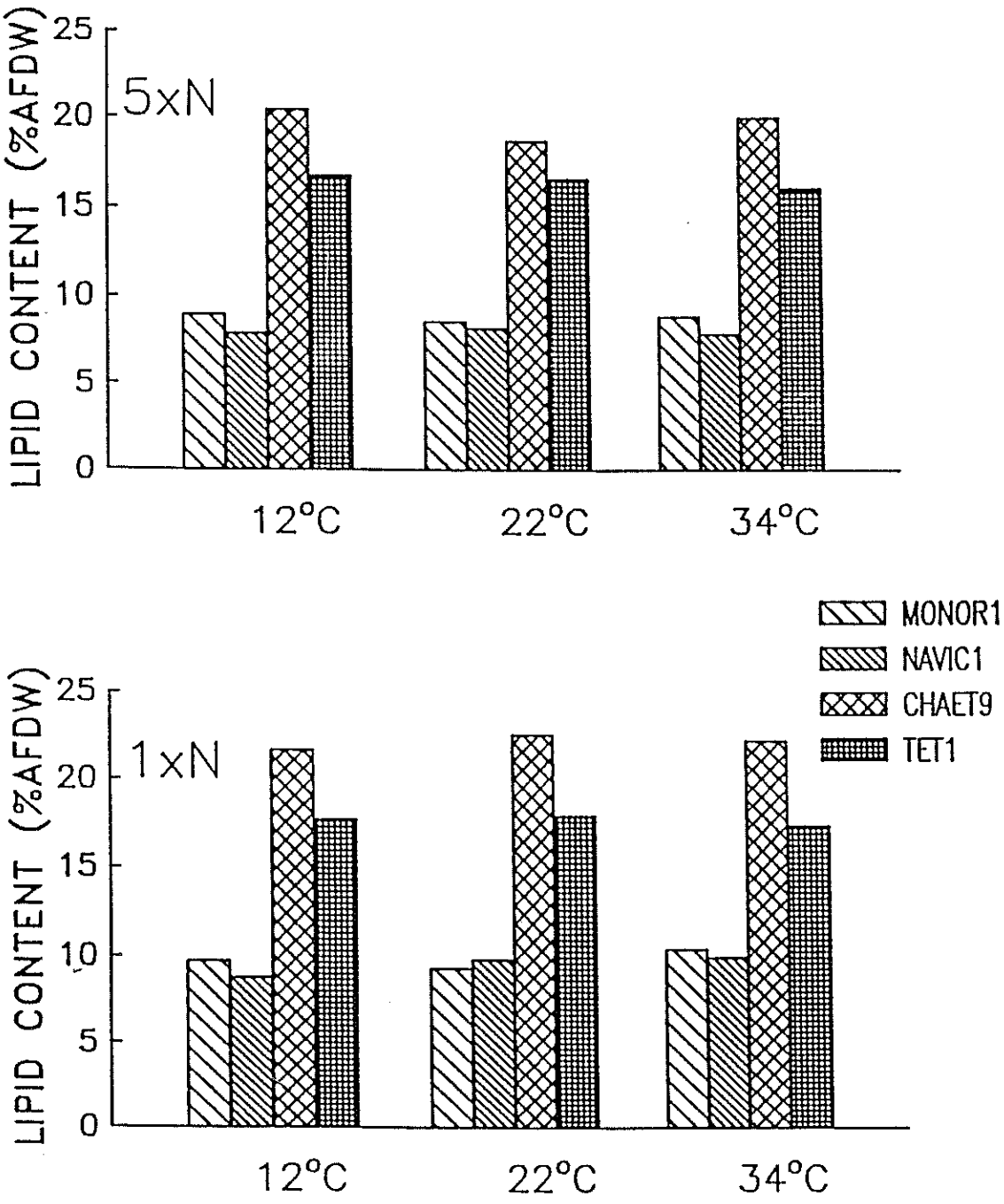

Fig. 3. **Lipid content of microalgae under laboratory conditions. Data are expressed as percent lipid on an ash-free dry weight basis.**

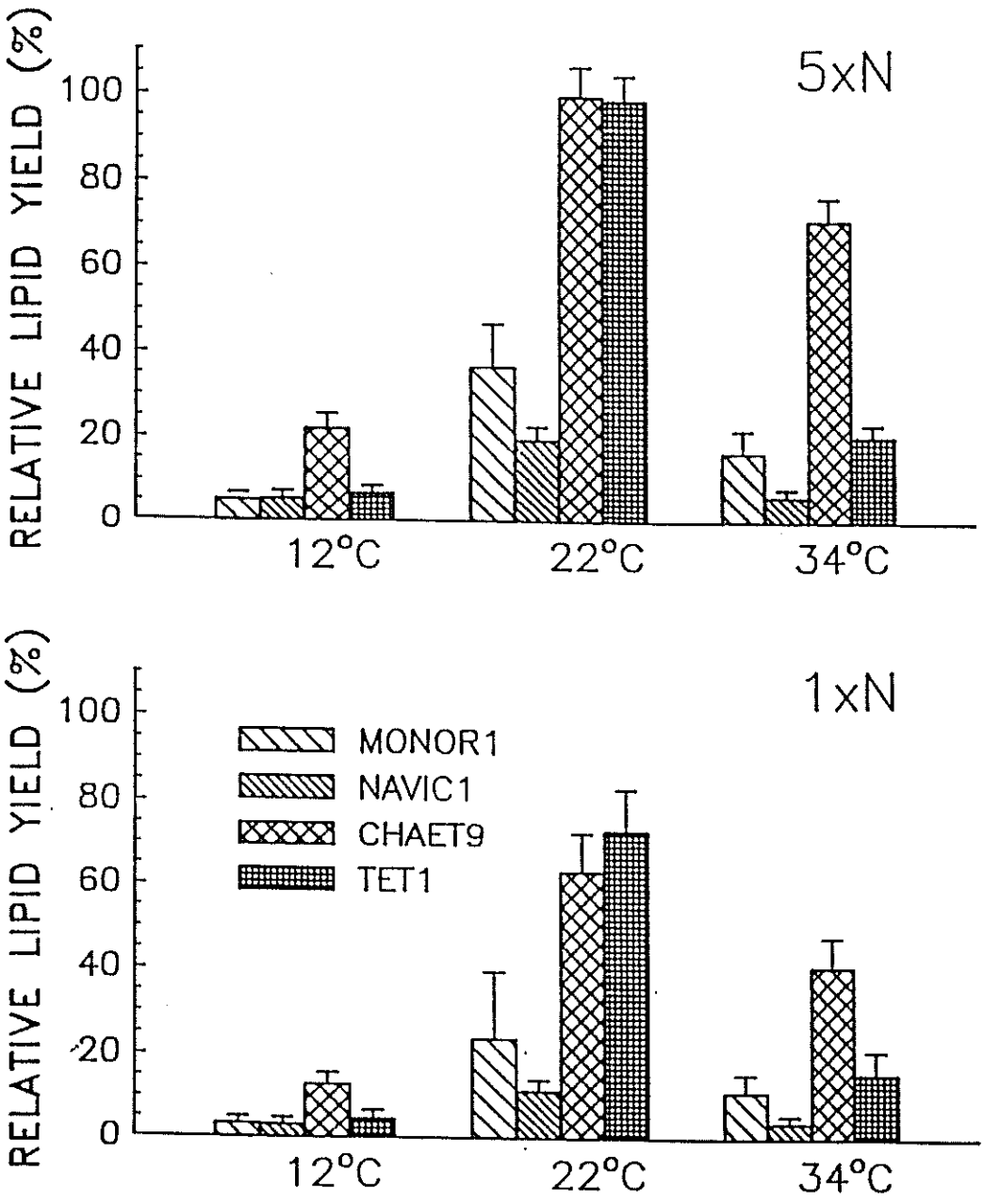

Fig. 4. **Relative lipid production of microalgae under laboratory conditions. Data are normalized to allow 120.19 mg·l^{-1} to represent 100%.**

Lipid samples from the screening study were subjected to silicic acid column chromatography followed by thin layer chromatography (TLC) to obtain a "fingerprint" and by gravimetric analysis of the fractions to determine the total lipid contained in each elution fraction. Table 1 shows the elution profile for two of the species, the green alga *Monoraphidium* and the diatom *Navicula.*

TABLE 1
Lipid Composition of MONOR1 and NAVIC1 Total Lipid Extracts
(Mean Percent Total Lipid ±SEM)

Eluent	MONOR1	NAVIC1
Hexane	9.6 ± 1.4	6.2 ± 0.9
Benzene	13.1 ± 2.1	22.1 ± 4.2
Chloroform	15.3 ± 2.3	33.4 ± 6.7
Acetone	44.7 ± 6.2	23.5 ± 5.1
Methanol	17.3 ± 3.3	14.8 ± 3.3

Figure 5 gives a typical "fingerprint" obtained (*Tetraselmis*, 22 °C, 5xN). The TLC fingerprints obtained from all samples were similar qualitatively, though they did vary in the density of individual spots. In no case was the resolution obtained by TLC fingerprinting great enough to detect differences between different treatments of the same species. As a result, this procedure was not routinely carried out on tank-grown cultures in subsequent experiments.

Total lipid samples were also saponified, methylated and chromatographed by gas chromatography (GC) to obtain a total fatty acid profile to determine changes in

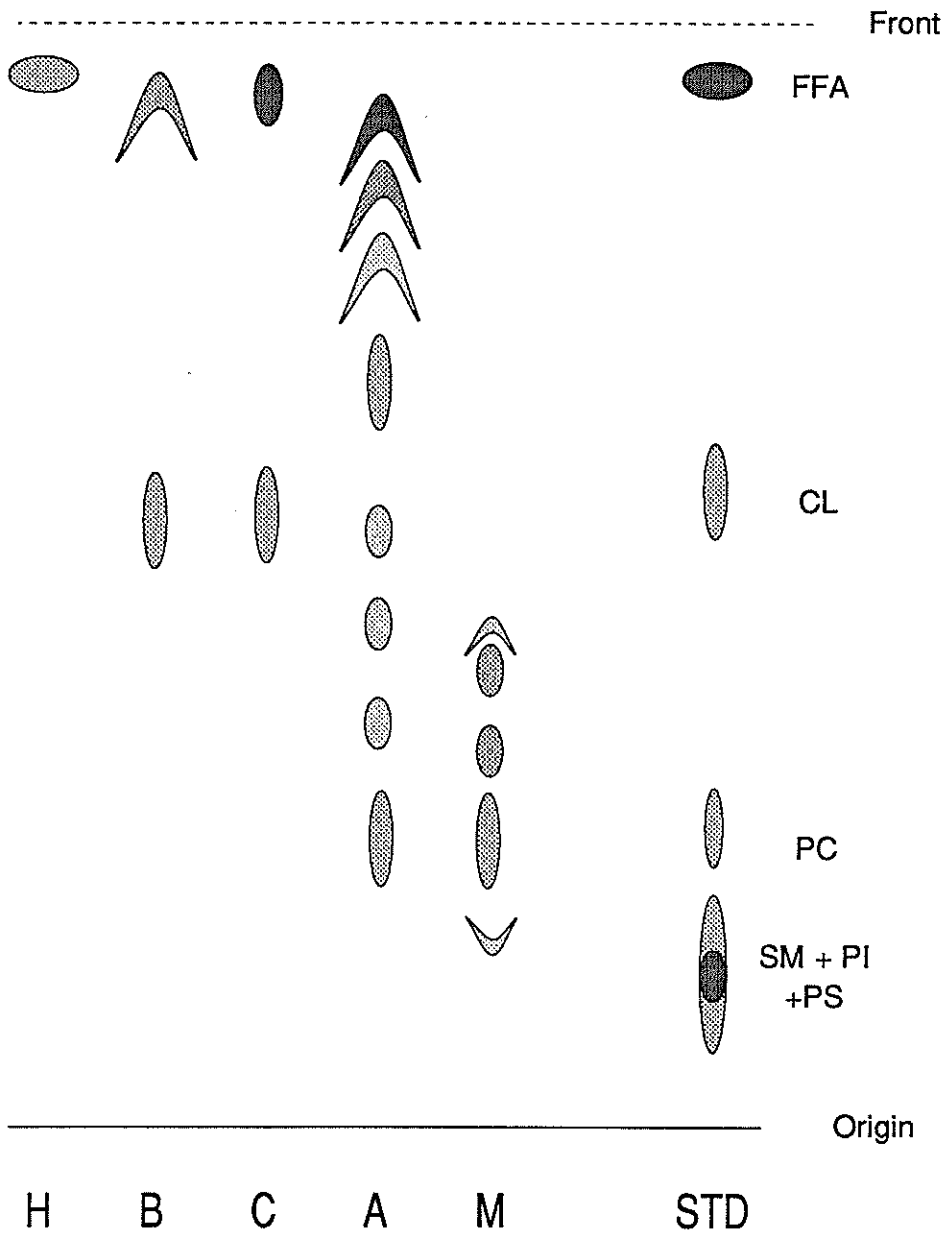

Fig. 5. **Sample chromatographic fingerprint of fractionated total lipids from *Monoraphidium* grown at 22 °C, 5xN. H, B, C, A, M represent the hexane, benzene, chloroform, acetone and methanol column fractions respectively. STD represents the standard track containing fatty acids (FFA), cardiolipin (CL), phosphotidyl choline (PC), serine (PS), inositol (PI), and sphingomylin (SM).**

fatty acid composition due to environmental conditions. These analyses were also useful in assessing the presence of high value fatty acids. For example, the ω-3 fatty acids, particularly 5,8,11,14,17 *z,z,z,z,z*-eicosapentaenoic acid (EPA) and 5,8,11,14,17,20 *z,z,z,z,z,z*-docosahexaenoic acid (DHA), have been suggested as dietary supplements for preventing cardiovascular disease. The extraction of EPA and DHA from algal lipids prior to refining in a fuels option could augment the algae crop's total value if these fatty acids are present in sufficient quantities. Figure 6 shows the fatty acid profile obtained from the green alga *Monoraphidium* and the diatom *Navicula* grown at 22 °C, 5xN. In general, diatoms showed a higher proportion of long chain polyunsaturated fatty acids than green algae. *Navicula* contains significant quantities of EPA (# 12) while this ω-3 fatty acid was absent in *Monoraphidium. Monoraphidium*, on the other hand, contains two short chain fatty acids absent in *Navicula.* These fatty acids (#16 and #17) were unidentified. Fatty acid #16 and #17 both had mobilities between palmoleate (16:1) and sterate (18:0). Fatty acid #16 is possibly a 16:2 isomer.

There were consistent but non-significant increases in chain length and degree of unsaturation in cultures grown at colder temperatures. The lack of obvious homeoviscous adaptation in total lipid samples may be due to the large storage lipid component. Storage lipids might not be expected to respond to decreasing temperatures with the same degree of chain elongation and unsaturation as membrane lipids as the need to maintain fluidity in stored lipid is not as great as in membrane lipid. From a practical point of view, there would be little or no economic benefit to chilling cultures to increase long-chain, polyunsaturated fatty acid production as an adjunct to producing lipids for fuel use.

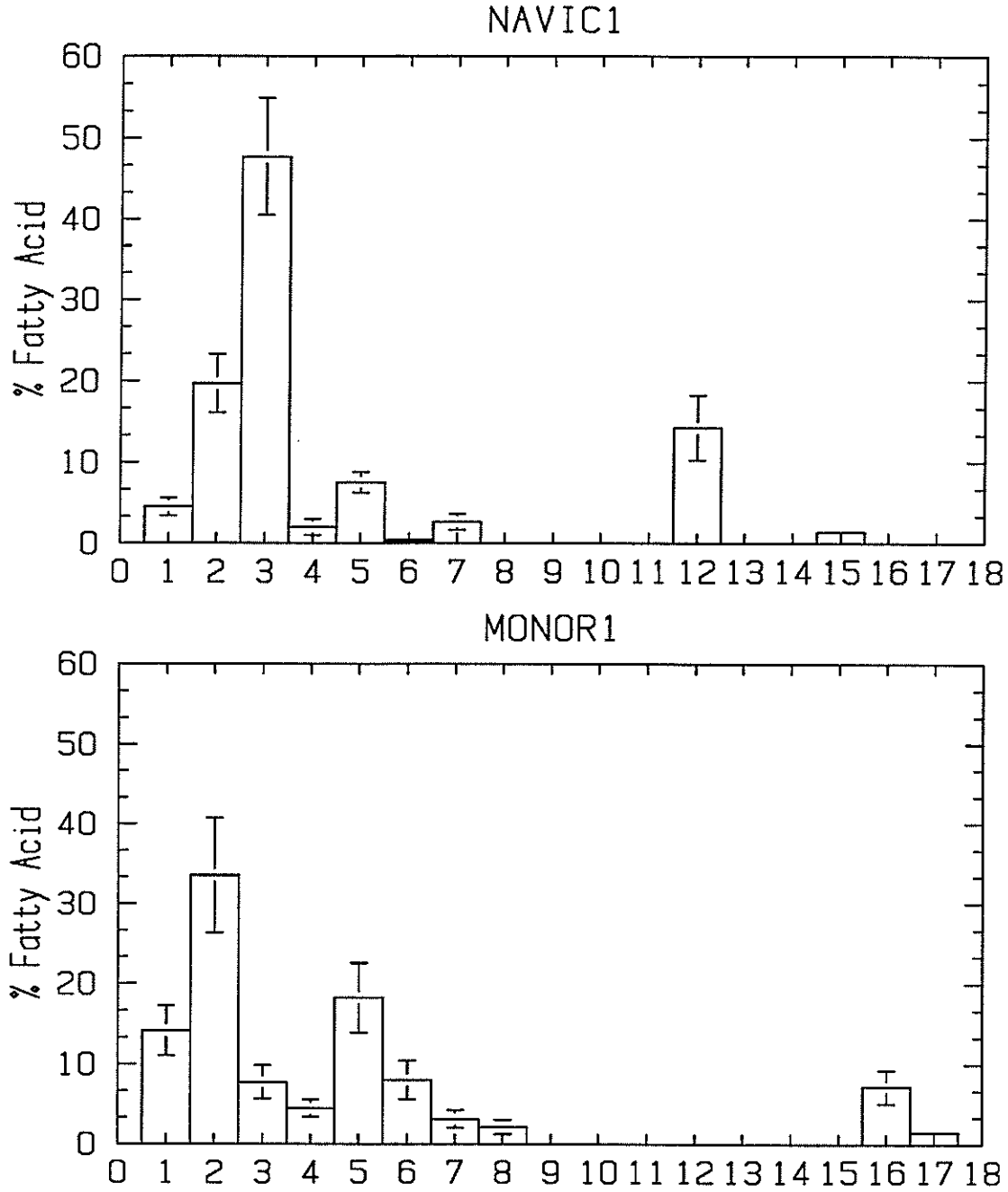

Fig. 6. **Fatty acid profile of *Navicula* and *Monoraphidium* grown in flask culture. Key to fatty acids:**

1 myristate (14:0)
2 palmitate (16:0)
3 palmitoleate (16:1)
4 stearate (18:0)
5 oleate (18:1)
6 all octadecadienoates
7 γ-linolenate (18:3)
8 α-linolenate (18:3)
9 arachidate (20:0)
10 dihomo-γ-linolenate (20:3)
11 arachidonate (20:4)
12 eicosapentaenoate (20:5)
13 docosatetraenoate (22:4)
14 docosapentaenoate (22:5)
15 docosahexaenoate (22:6)
16 Unknown 1 (probable 16:2)
17 Unknown 2

Pilot Scale Tank Experiments

Pilot scale experiments were initiated in the late summer of 1988. Initial trials compared *Chaetoceros* grown under high (5xN) and low (1xN) conditions. This comparison was designed to check the operation of the tank facility. Figure 7 shows the standing crop and productivity of *Chaetoceros* expressed as wet weight in $g \cdot m^{-2}$. Productivity was calculated by computing the difference between the standing crop on day *n* and day *n-1* and adding the weight of the material harvested in the interval. Thus, "negative productivity" can be obtained if the drop in standing crop is greater than the material harvested. In general, the design goal was to keep the standing crop constant so that productivity and the overflow harvest were equivalent.

As can be seen in Figure 7., this goal was not obtained in this set of experiments. Both standing crop and productivity of *Chaetoceros* were generally greater in the 5xN treatment than in the 1xN treatment. However, the fluctuation in both parameters was great, particularly in the 5xN treatment. The experiment was terminated due to invasion of the 5xN tank and subsequently, the 1xN tank by "weed" algae, particularly a green unicell, which was soon replaced by mixed cyanobacterial blooms.

After some changes in plumbing and tank position, outdoor trials were resumed in the winter of 1989. *Chaetoceros* was chosen again as the test species because of its relatively good performance under cold conditions during the screening procedure. Figure 8 shows the productivity obtained during this 36-day trial. Again both standing crop and productivity exhibited a high degree of variability, though not as great as in the preliminary trials. There was no apparent difference in standing crop or productivity between nitrogen treatments.

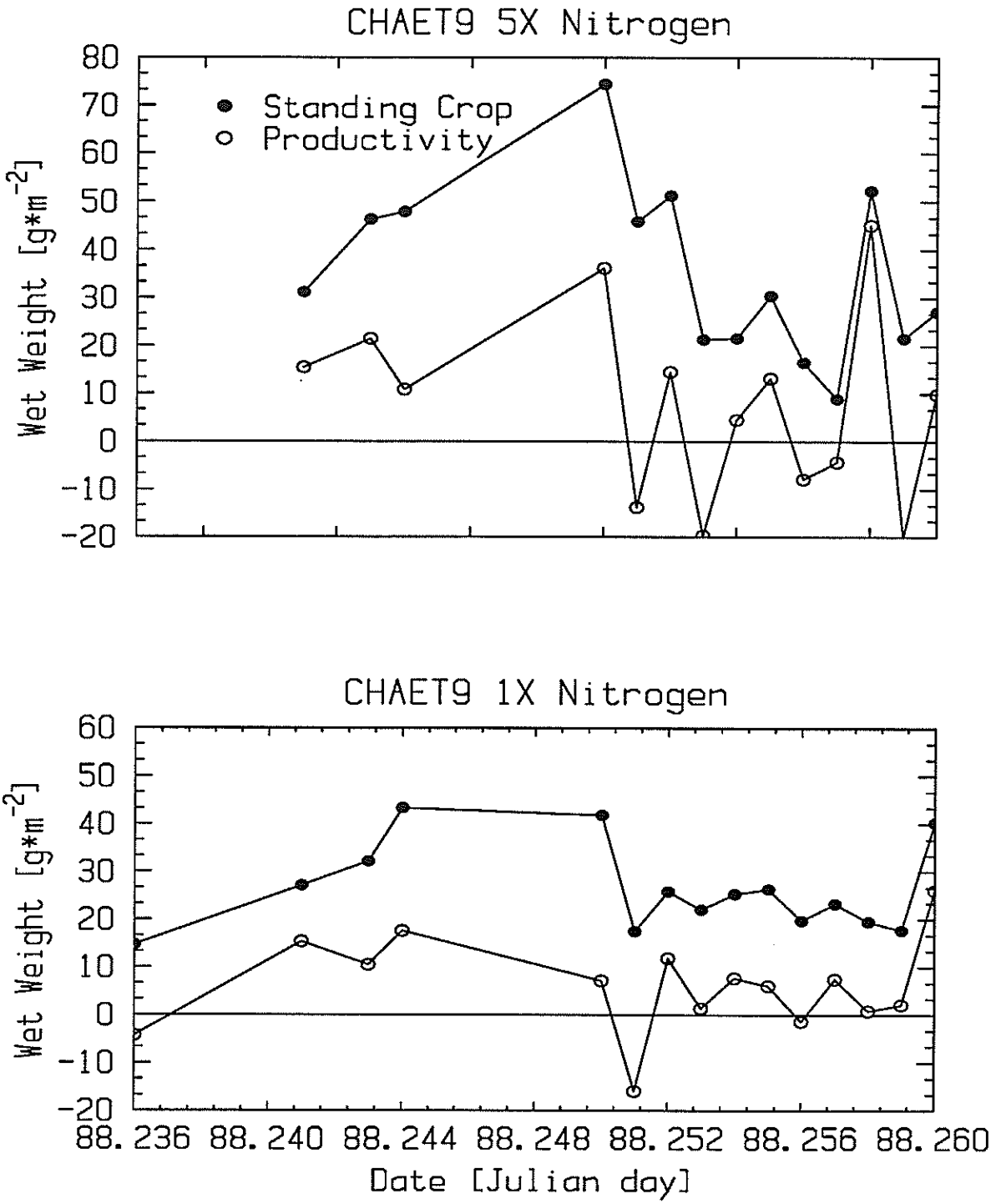

Fig. 7. **Standing crop and productivity of *Chaetoceros* expressed as wet weight in g·m^{-2}. First trial.**

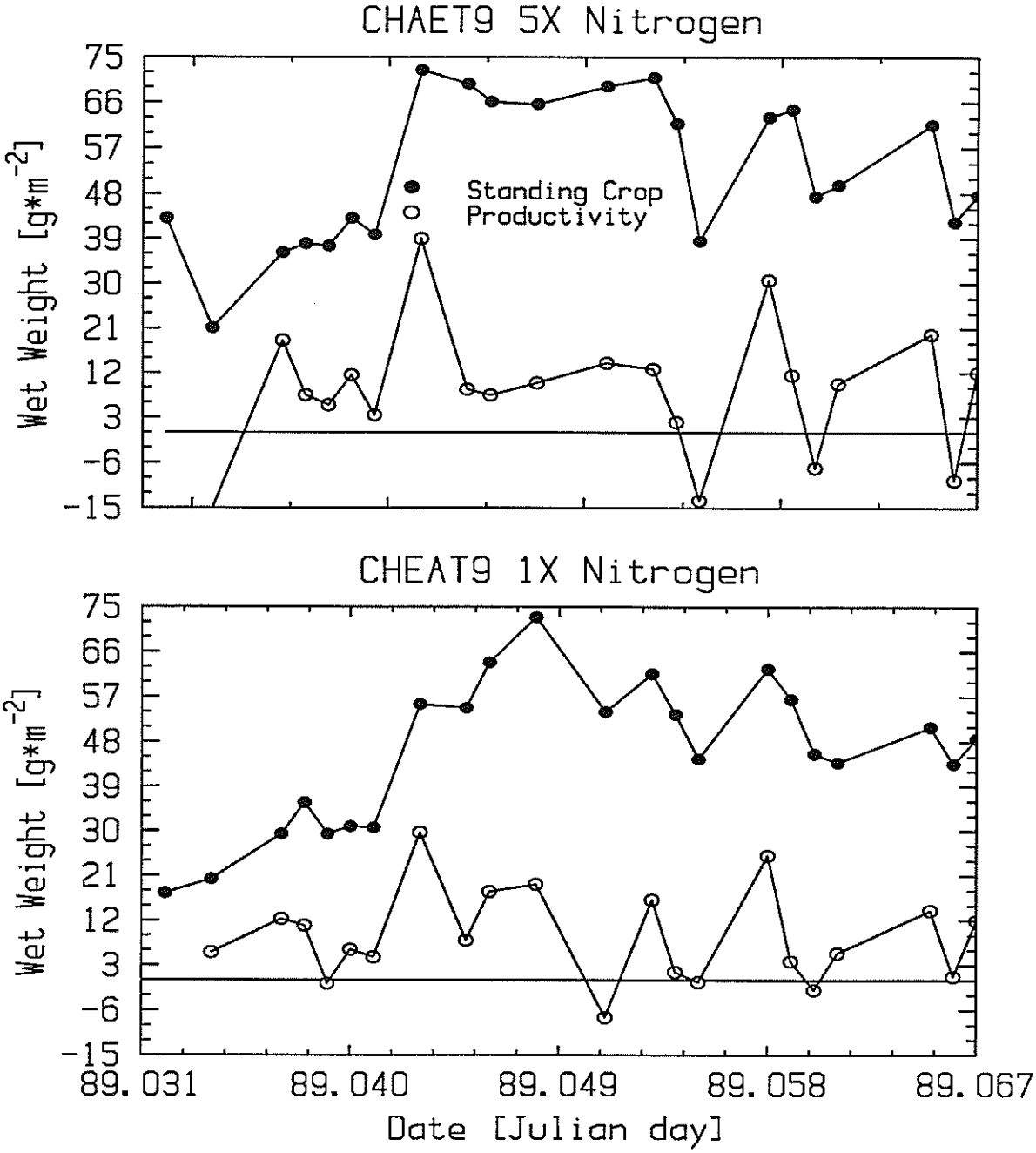

Fig. 8. **Standing crop and productivity of *Chaetoceros* expressed as wet weight in g•m^{-2}. Second trial.**

As in the first *Chaetoceros* trial, the tanks ultimately were infested with a green "weed alga" and the *Chaetoceros* measurements were terminated. In both *Chaetoceros* trials the field performance was lower than would be predicted by the laboratory screening trials. The high productivities predicted for summer temperatures were not obtained. The winter field performance was also lower than expected. In addition, "weed resistance" in the open tanks was poor. Because the tanks had been thoroughly washed and disinfected prior to use, the source of the contaminating alga was assumed to be air borne material.

Ash-free dry weight and lipid percentage was calculated on periodic samples obtained from each of the *Chaetoceros* trials. These mean lipid content values, and the error associated with them, were used to calculate lipid productivity on a $g \cdot m^{-2}$ basis. Productivity, on a wet weight basis was multiplied by the wet weight/AFDW ratio and the lipid weight/AFDW ratio to obtain the measure of lipid production. These data are shown on Figures 9 and 10 for the first and second *Chaetoceros* trials respectively.

The total fatty acid profiles obtained from both *Chaetoceros* field trials and the initial laboratory screening trials were similar. Figure 11 shows the profile obtained from the second field trial, 5xN treatment. *Chaetoceros* fatty acid profiles were dominated by myristate (14:0), palmitate (16:0), and palmoleate (16:1) with relatively low quantities of the C-18 fatty acids stearate (18:0), oleate (18:1) and linoleate (18:2). There was no detectable γ or α-linolenate (18:3ω-6, and 18:3ω-3, respectively). Interestingly, *Chaetoceros* produced significant quantities of the ω-3 fatty acid eicosapentaenoate (EPA, 20:5) and smaller quantities of docosahexaenoate (DHA, 22:6 ω-3). Both DHA and EPA have potential market value as dietary supplements.

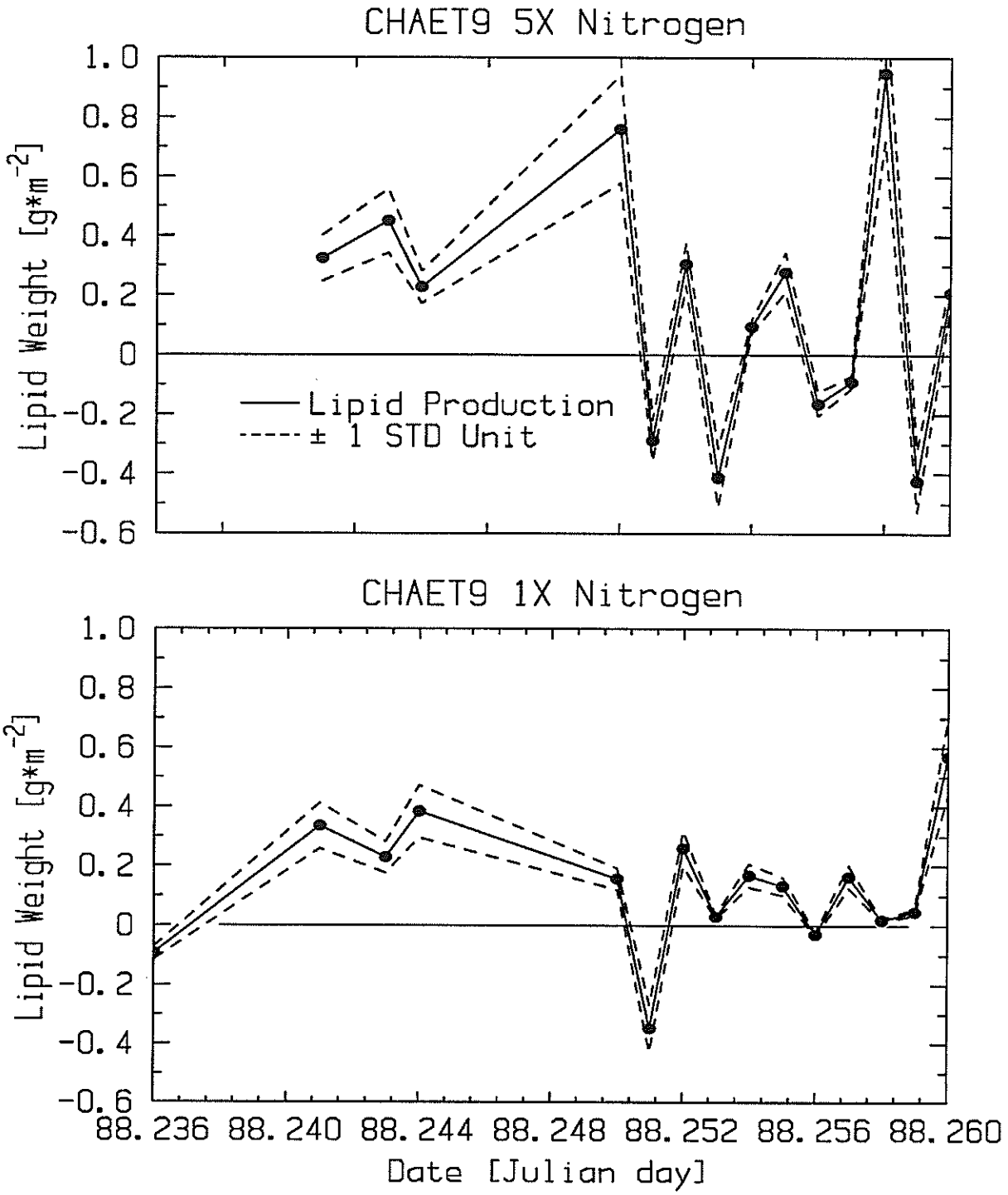

Fig. 9. **Lipid production of *Chaetoceros* grown in pilot scale tank culture expressed as wet weight in g·m^{-2}. First trial.**

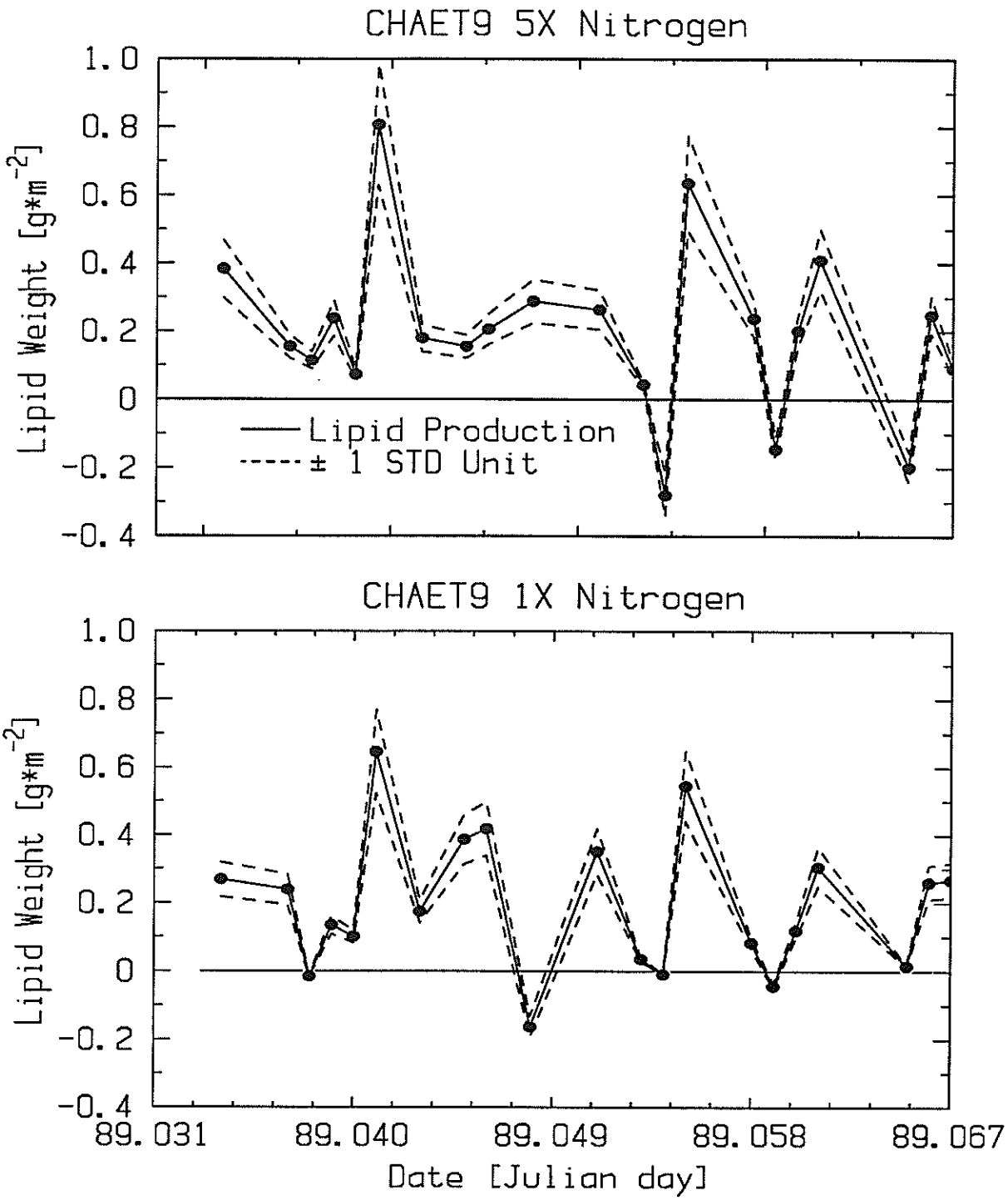

Fig. 10. **Lipid production of *Chaetoceros* grown in pilot scale tank culture expressed as wet weight in g·m^{-2}. Second trial.**

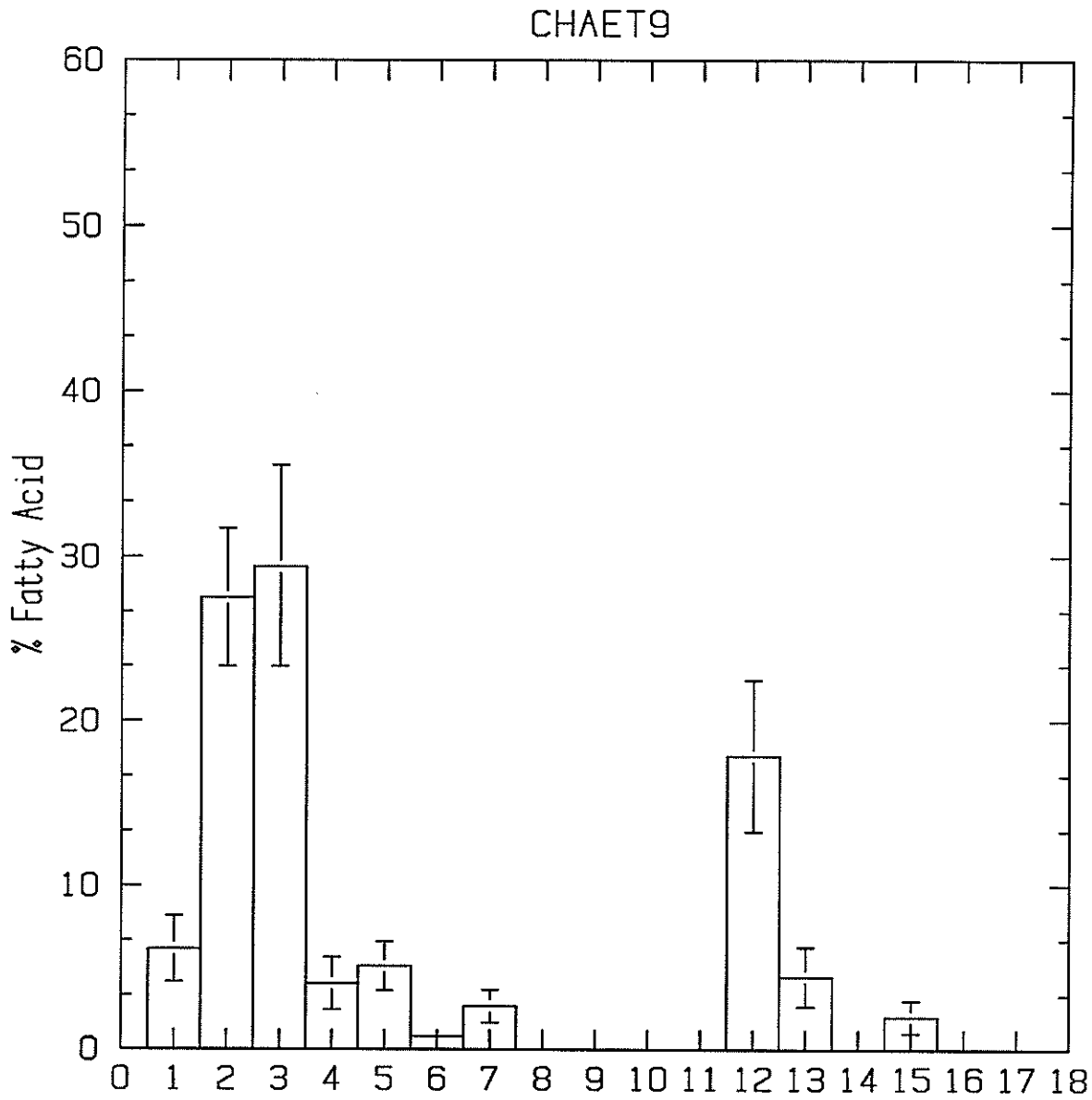

Fig. 11. Fatty acid profile of Chaetoceros grown in pilot scale tank culture, 5xN. Second trial. Key to fatty acids:

1 myristate (14:0)
2 palmitate (16:0)
3 palmitoleate (16:1)
4 stearate (18:0)
5 oleate (18:1)
6 all octadecadienoates
7 γ-linolenate (18:3)
8 α-linolenate (18:3)
9 arachidate (20:0)
10 dihomo-γ-linolenate (20:3)
11 arachidonate (20:4)
12 eicosapentaenoate (20:5)
13 docosatetraenoate (22:4)
14 docosapentaenoate (22:5)
15 docosahexaenoate (22:6)
16 Unknown 1 (probable 16:2)
17 Unknown 2

The distribution of *Chaetoceros* total lipids into silicic acid column fractions was variable within a given trial but not different between field trails and the preliminary screening. Table 2 shows the distribution of total lipids into silicic acid column fractions from the second pilot scale tank culture, 5xN treatment.

TABLE 2
Lipid Composition of CHAET9 Total Lipid Extracts
(Mean Percent Total Lipid ±SEM)

Eluent	CHAET9
Hexane	4.1 ± 2.3
Benzene	23.5 ± 9.4
Chloroform	31.6 ± 9.7
Acetone	28.8 ± 9.1
Methanol	11.6 ± 4.7

Because of the same green unicell's persistent invasion, studies were undertaken to investigate its potential for lipid production in saline water. The strain was labeled MA1 and tentatively identified as a *Chlorella sp.* Samples were taken from the overgrown *Chaetoceros* tanks and streaked on II-25/2-5xN agar plates to obtain unialgal cultures. These plates were saved as reference cultures. The overgrown *Chaetoceros* tanks were allowed to become virtually unialgal in MA1 and the sample collection was carried out as described. This process was repeated a second time when MA1 overgrew a *Tetraselmis* trial in the late summer.

Figures 12 and 13 show the standing crop and productivity for the first and second MA1 trial, respectively. Productivity and standing crop were fairly high and

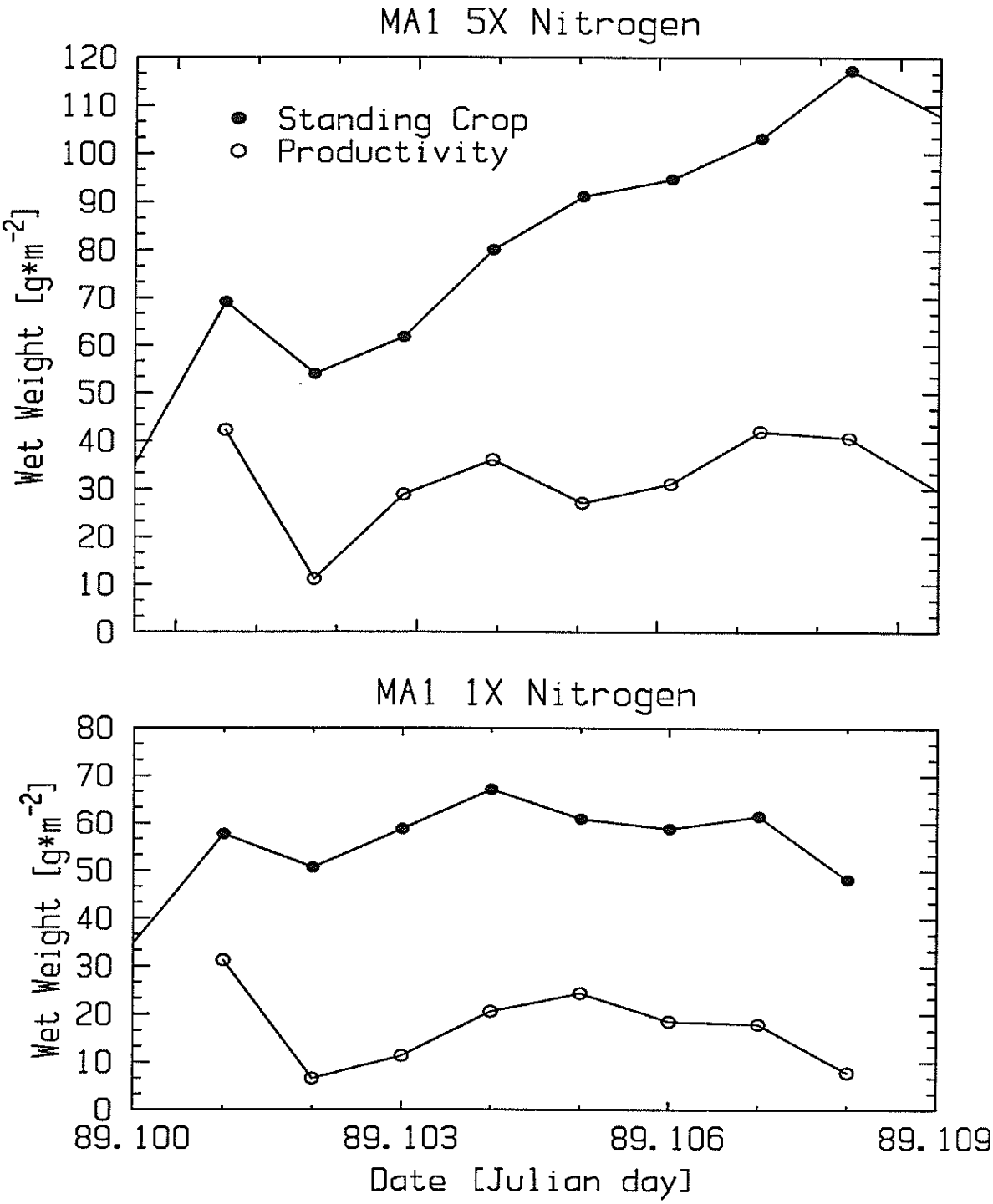

Fig. 12. **Standing crop and productivity of strain MA1 expressed as wet weight in $g \cdot m^{-2}$. First trial.**

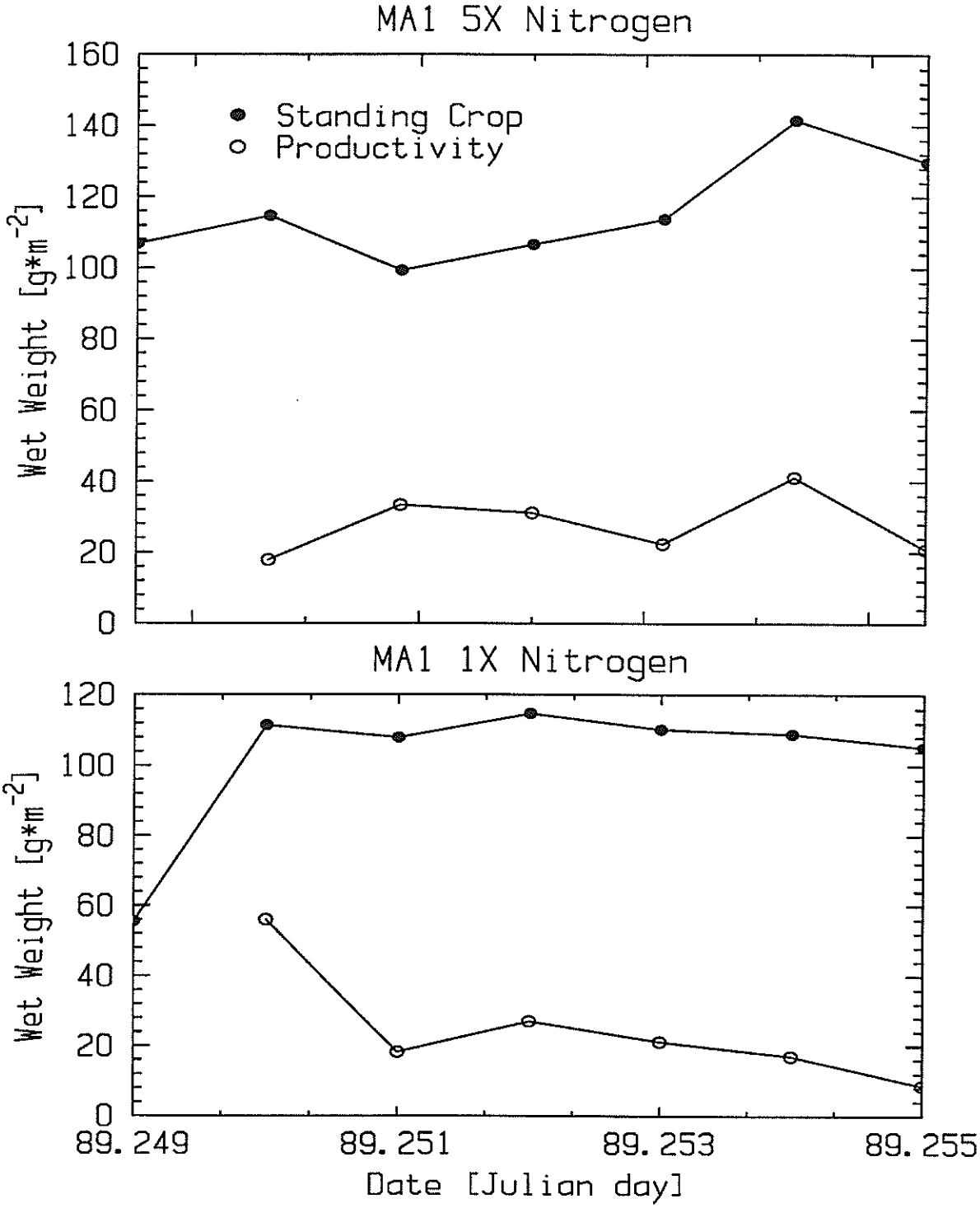

Fig. 13. **Standing crop and productivity of strain MA1 expressed as wet weight in $g \cdot m^{-2}$. Second trial.**

consistent in both trials. Ash-free dry matter represented about 14% of the graphed wet weights. Ash-free dry matter production fluctuated around approximately 3 $g \cdot m^{-2}$ in the 1xN treatment and 5 $g \cdot m^{-2}$ in the 5xN treatment for both trials, though the standing crop was somewhat higher in the second trial.

Figures 14 and 15 show the lipid production by strain MA1 in the two field trials. As with all the strains tested in the screening trials, MA1 did not show a significant increase in lipid content on an AFDW basis as a result of the lower nitrogen level. Thus, lipid yield closely followed overall productivity with the 5xN treatment yielding more lipid on an area basis than the 1xN treatment.

The fatty acid profiles of the total lipid fraction were variable within treatments and trials but were similar between treatments and trials. The profile obtained for the 5xN treatment of the first trial is presented in Fig. 16. The profile is dominated by C-16 and C-18 fatty acids (fatty acids #2 through 8). The profile is missing any significant quantities of long chain fatty acids. As with the fatty acid profile, the silicic acid column profiles of MA1 total lipids showed a high degree of variation within trials and treatments but no significant variation between treatments or trials. Table 3 shows the silicic acid column profile for the 5xN treatment, first trial.

TABLE 3
Lipid Composition of MA1 Total Lipid Extracts
(Mean Percent Total Lipid ±SEM)

Eluent	MA1
Hexane	4.1 ± 2.3
Benzene	23.5 ± 9.4
Chloroform	31.6 ± 9.7
Acetone	28.8 ± 9.1
Methanol	11.6 ± 4.7

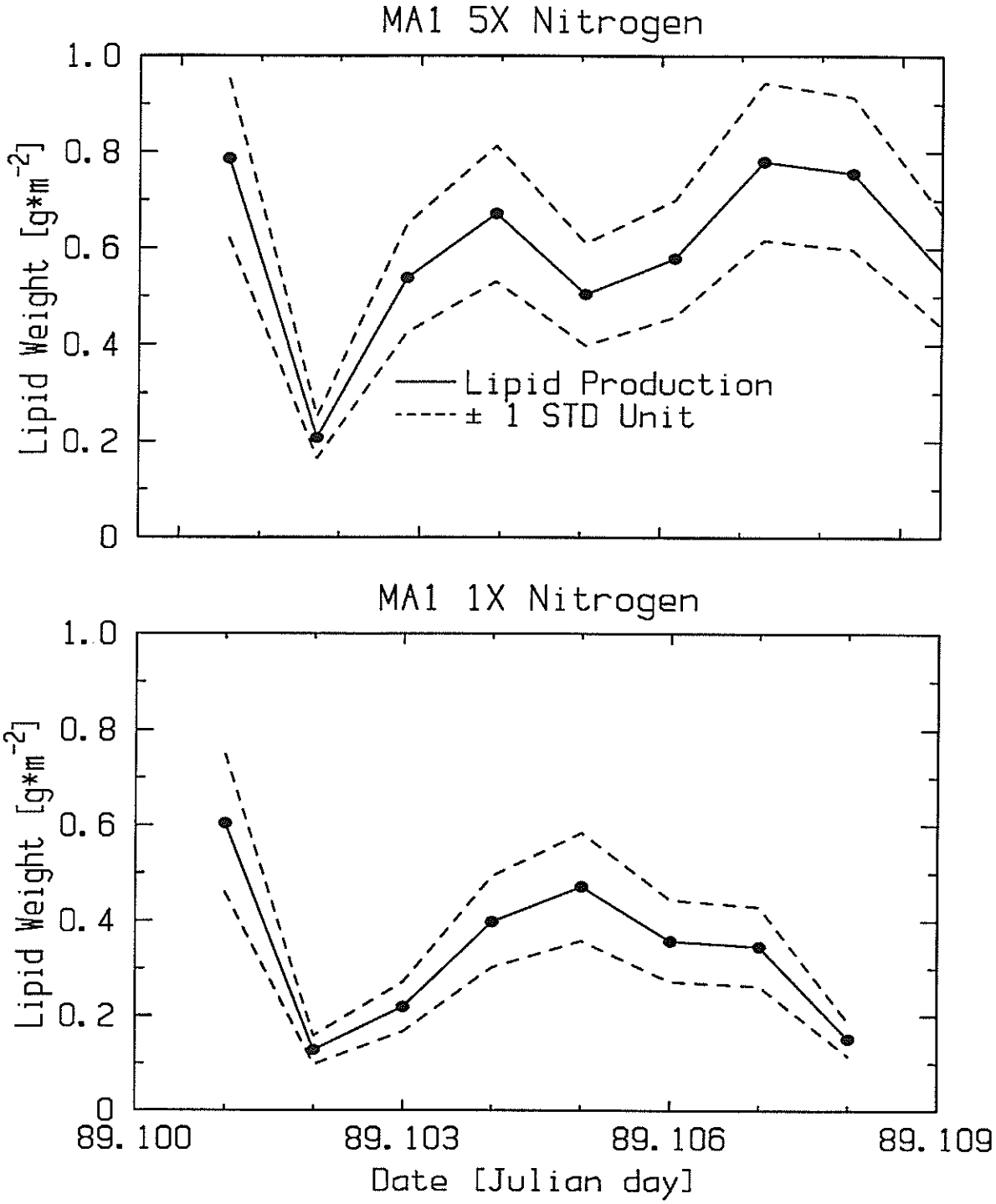

Fig. 14. **Lipid production of MA1 grown in pilot scale tank culture expressed as wet weight in g·m^{-2}. First trial.**

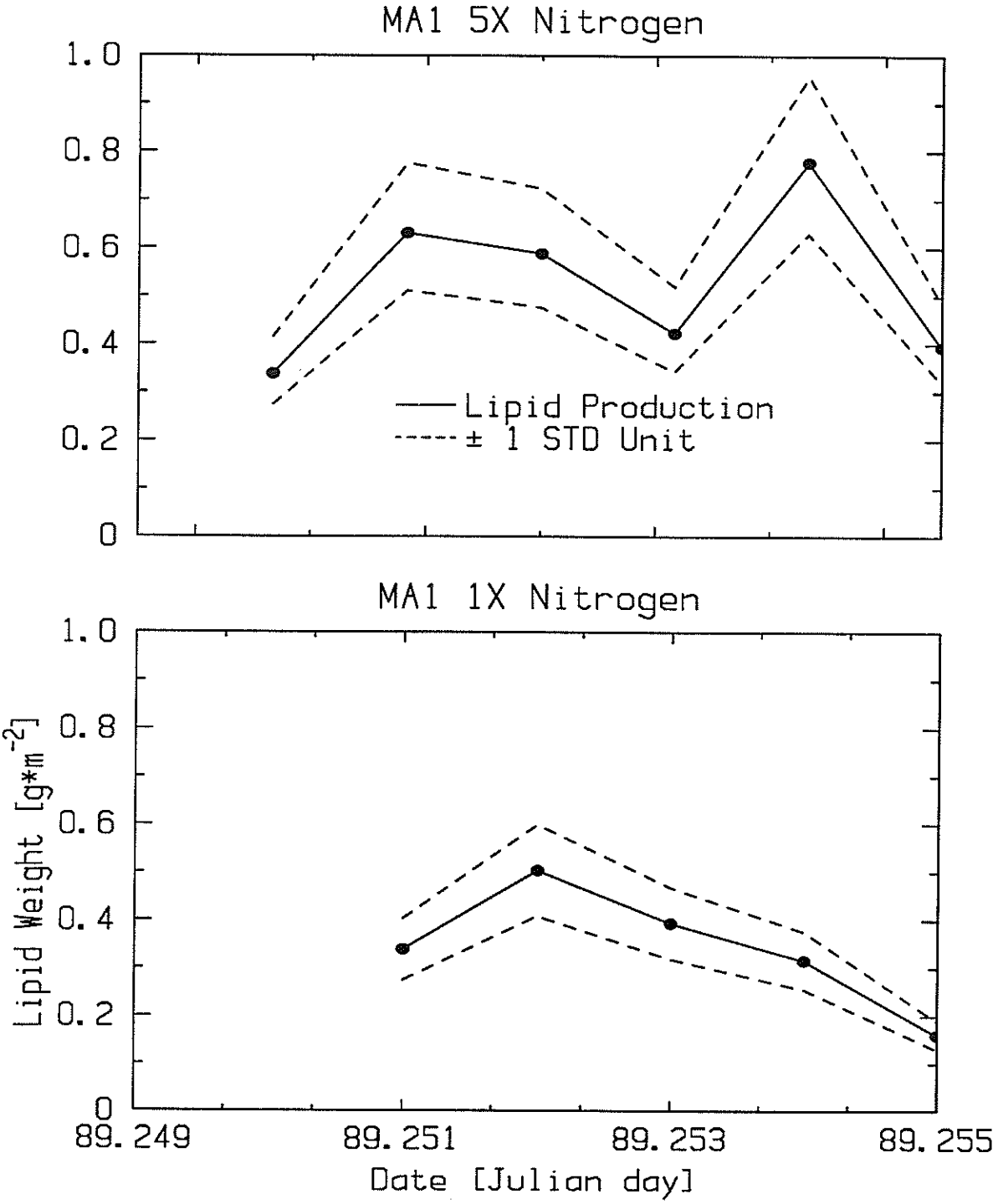

Fig. 15. **Lipid production of MA1 grown in pilot scale tank culture expressed as wet weight in $g \cdot m^{-2}$. Second trial.**

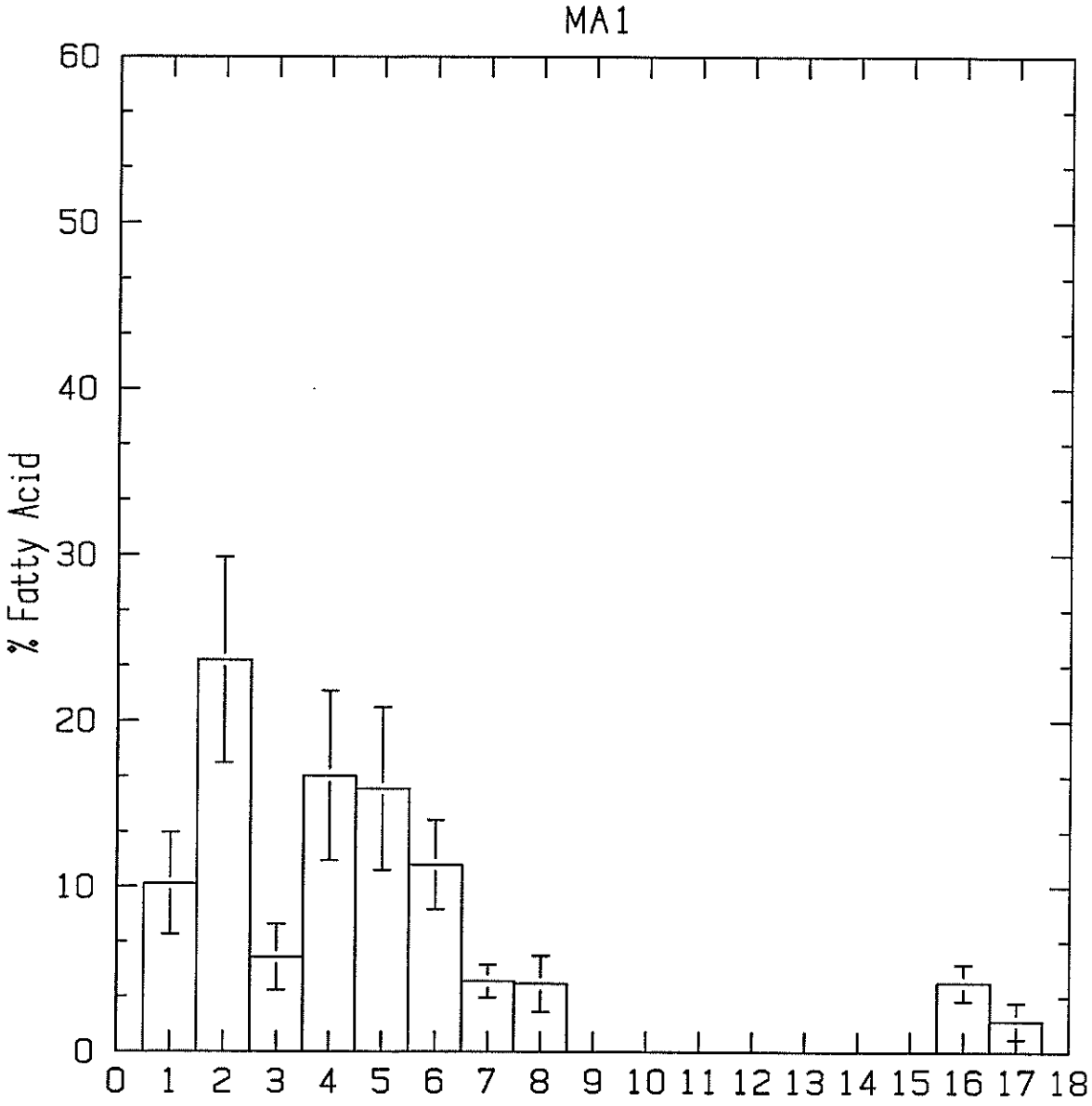

Fig. 16. Fatty acid profile of MA1 grown in pilot scale tank culture, 5xN. First trial. Key to fatty acids:

1 myristate (14:0)
2 palmitate (16:0)
3 palmitoleate (16:1)
4 stearate (18:0)
5 oleate (18:1)
6 all octadecadienoates
7 γ-linolenate (18:3)
8 α-linolenate (18:3)
9 arachidate (20:0)
10 dihomo-γ-linolenate (20:3)
11 arachidonate (20:4)
12 eicosapentaenoate (20:5)
13 docosatetraenoate (22:4)
14 docosapentaenoate (22:5)
15 docosahexaenoate (22:6)
16 Unknown 1 (probable 16:2)
17 Unknown 2

In both trials, the MA1 culture was ultimately overgrown by a mixed culture of cyanobacteria resulting in abandonment of the trial. The source of contamination is assumed to be airborne material.

The final field trial was carried out with *Tetraselmis*. This trial was initiated in July 1989. The standing crop and productivity performance from this trial are presented in Figure 17. *Tetraselmis* generated relatively high and consistent standing crop and productivity. Productivity fluctuated around 4.5 $g \cdot m^{-2}$ on an AFDW basis for the 5xN treatment and 3 $g \cdot m^{-2}$ for the 1xN treatment. *Tetraselmis* showed the highest consistency of all strains tested, until it became contaminated by MA1. Figure 18 shows the lipid productivity of *Tetraselmis*. As with other strains tested, lipid productivity closely followed biomass productivity. Figure 19 displays the fatty acid profile of the total lipid fraction from *Tetraselmis*. As with the other green algae tested, the profile is dominated by fatty acids of 18 or fewer carbons. A small amount of DHA (fatty acid #15) was detected. Also as found previously, the variation within treatments and trials was greater than among them. Also as before, this variation pattern was true of the silicic acid column fractions. The silicic acid profile for the 5xN tank trial is shown in Table 4.

TABLE 4
Lipid Composition of TET1 Total Lipid Extracts
(Mean Percent Total Lipid ±SEM)

Eluent	CHAET9
Hexane	4.7 ± 2.1
Benzene	18.4 ± 5.3
Chloroform	25.4 ± 7.4
Acetone	41.8 ±11.1
Methanol	9.9 ± 3.6

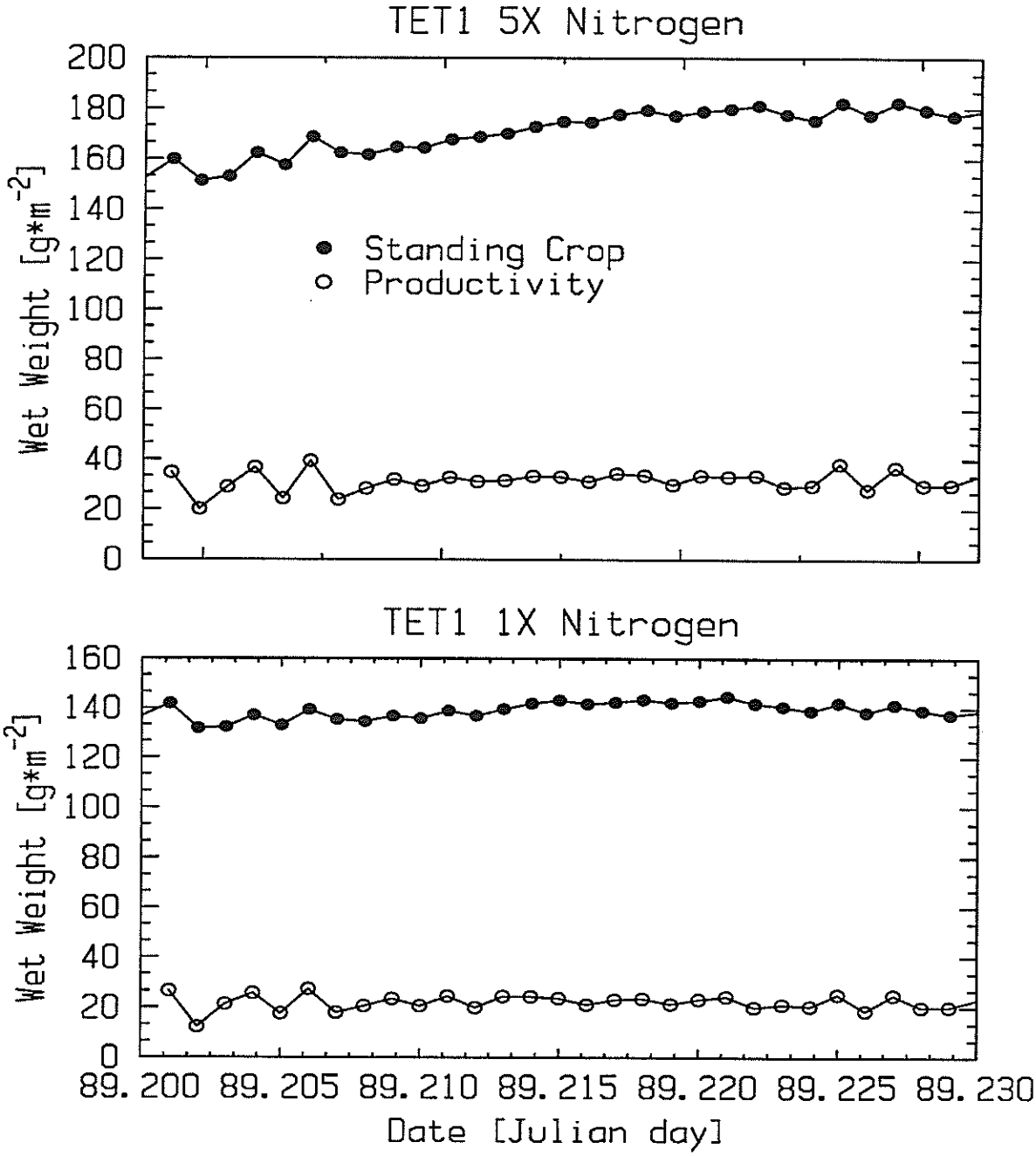

Fig. 17. **Standing crop and productivity of *Tetraselmis* expressed as wet weight in g·m^{-2}.**

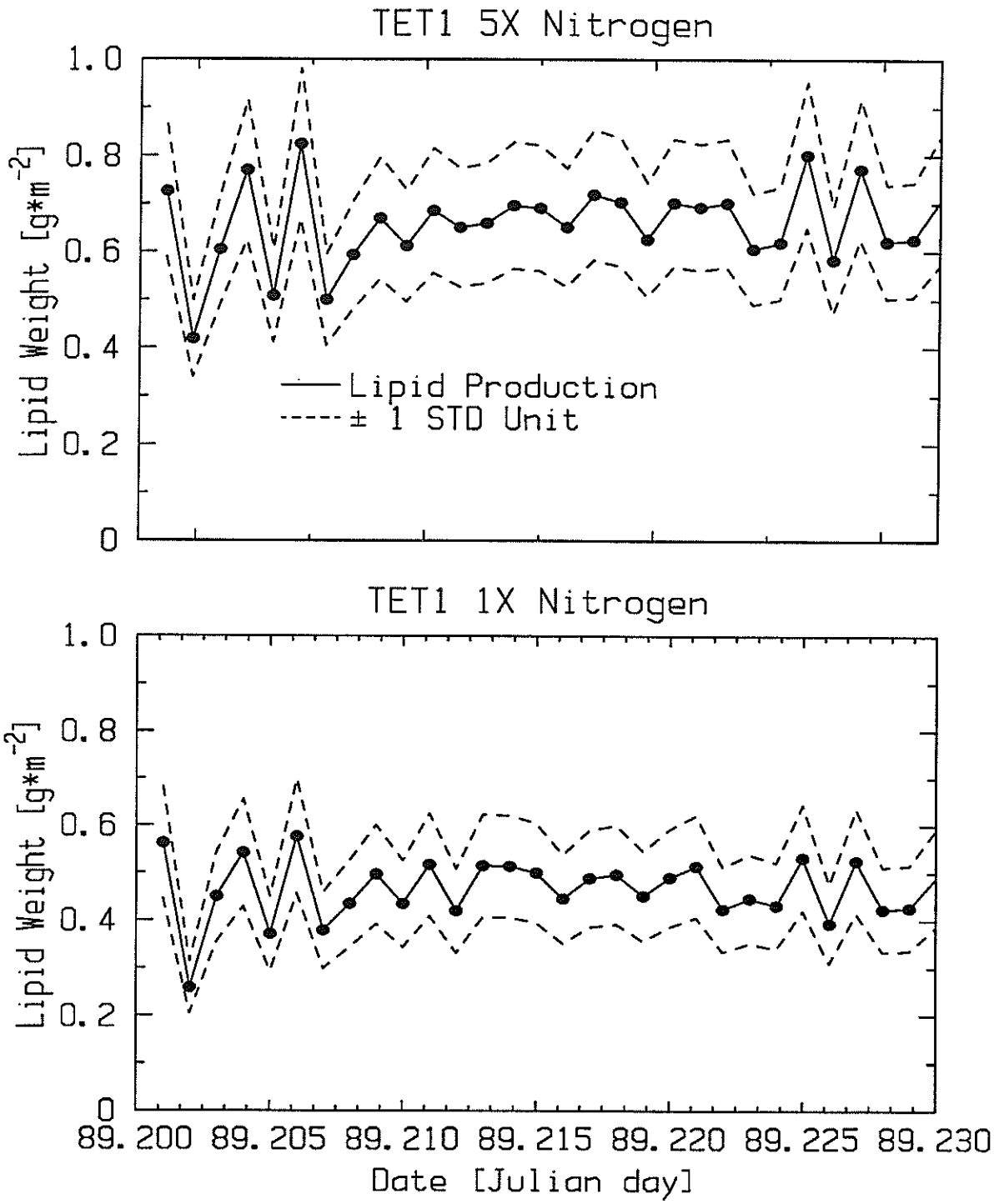

Fig. 18. **Lipid production of *Tetraselmis* grown in pilot scale tank culture expressed as wet weight in $g \cdot m^{-2}$.**

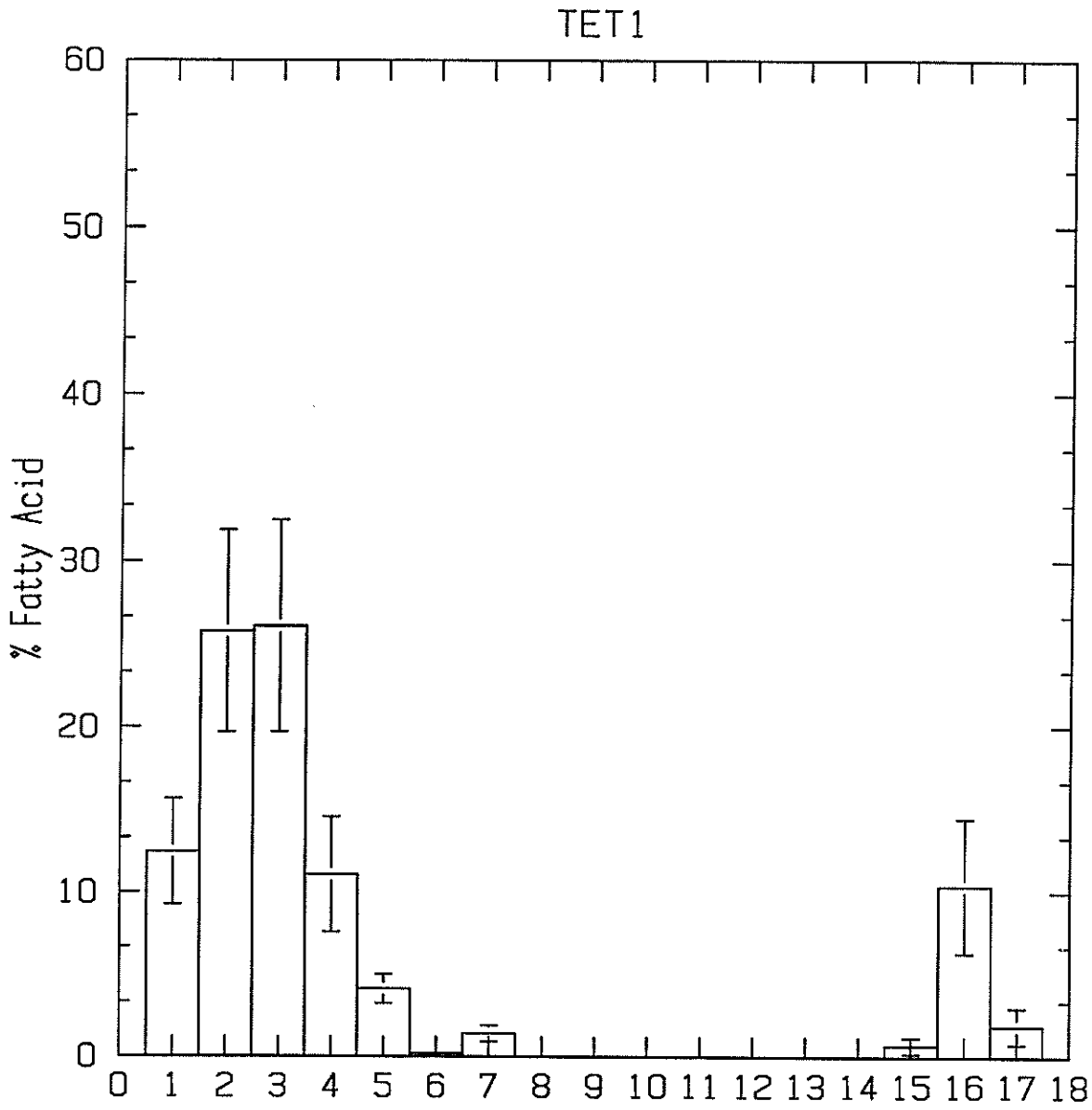

Fig. 19. Fatty acid profile of *Tetraselmis* grown in pilot scale tank culture, 5xN. Key to fatty acids:

1 myristate (14:0)
2 palmitate (16:0)
3 palmitoleate (16:1)
4 stearate (18:0)
5 oleate (18:1)
6 all octadecadienoates
7 γ-linolenate (18:3)
8 α-linolenate (18:3)
9 arachidate (20:0)
10 dihomo-γ-linolenate (20:3)
11 arachidonate (20:4)
12 eicosapentaenoate (20:5)
13 docosatetraenoate (22:4)
14 docosapentaenoate (22:5)
15 docosahexaenoate (22:6)
16 Unknown 1 (probable 16:2)
17 Unknown 2

SUMMARY AND CONCLUSIONS

1. ***Laboratory trials were not an entirely accurate predictor of algal field performance.***

Laboratory trials led to the conclusion that *Chaetoceros* would be a high level performer in a relatively wide range of field conditions. In the screening trials, it was consistently at or near the top in both lipid and biomass production at all conditions tested. This prediction was not confirmed under relatively uncontrolled field conditions. Where *Chaetoceros* has been successfully grown in desert settings it has been under more controlled and intensively managed conditions [Goldstein 1990]. On the other hand, the screening predicted that *Tetraselmis* would be a good producer of lipid and biomass, at least in environmental conditions near its growth optima. This was confirmed by field testing. *Tetraselmis* was the least variable producer of biomass and lipid tested in the field trials. It is important to recognize that low intensity field culture conditions are much less regulated than the procedures used in laboratory screening with the potential for discrepancies between laboratory and field performance.

2. ***Nitrogen and temperature stress at levels practical in low intensity field culture increase the proportion of lipids in algae to only a limited degree.***

The small increases in the percent dry matter comprised of lipid was more than offset by losses in overall productivity. Thus, to maximize lipid production of a given algal strain on a per unit area or volume basis, maximize biomass production. There are clear species differences among algal species so that strain selection is also an important factor in maximizing lipid production.

3. ***"Weed" control is an important factor in successful outdoor algae culture for lipid production.***

All outdoor trials experienced "weed" invasion problems to a greater or lesser degree. In some cases, more than one month elapsed before invasion

problems became serious. In others, "weed" overrun occurred more quickly. This presents a severe problem for two reasons. First, invading species may not be as good a lipid or biomass producer as the chosen strain, resulting in an overall loss of productivity. Second, some weed species are highly undesirable due to unacceptably low lipid production or toxic by-product production. This is especially true of cyanobacteria. These blue-green algae often produce both toxic compounds and a gelatinous material necessitating the complete breakdown and cleaning of the production facility.

On the other hand, some "weeds" can be a useful resource. The weedy strain MA1, which invaded the field trial tanks on a regular basis, was a relatively good lipid producer, certainly better than the *Chaetoceros* and comparable to *Tetraselmis*, which it replaced. Ironically, even this weedy strain was not immune to "weed" invasion. It is possible that some sort of covering to reduce airborne contamination would be required for maximum culture stability.

4. ***Low intensity culture procedures can only produce a fraction of the algal yield of intensively managed algal production facilities.***

The best sustainable biomass production in these experiments was in the 4.5-5 $g \cdot m^{-2} \cdot day^{-1}$ range. This represents only one quarter to one third of the levels obtained in covered raceways with CO_2 enrichment [Goldstein 1990]. These rates were sustained only during the best growing seasons of the year. It is likely that production rates for at least three months of the year would not be sufficiently high to justify facility operation.

It is probable that the yield increases and season elongation obtainable with intensive culture conditions such as covered structures and CO_2 enrichment will more than offset their increased costs and produce yields great enough to justify the capital outlays for harvesting and lipid extraction equipment. It is unlikely that the yields obtainable without such measures can sustain a viable algal oil industry.

REFERENCES

Ben-Amotz, A. and M. Avron. 1983. Accumulation of metabolites by halotolerant algae and its industrial potential. *Ann. Rev. Microbiol.* 37:95.

Ben-Amotz, A., T. G. Tornabene, and W.H. Thomas. 1985. Chemical profile of selected species of microalgae with emphasis on lipids. *J. Phycol.* 21:72.

Bligh, E.G. and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911.

Chirac, C., E. Casadevall, C. Largeau, and P. Metzger. 1985. Bacterial influence upon hydrocarbon production of the green alga *Botryococcus braunii. J. Phycol.* 21:380.

Christie, W.W. 1982. *Lipid Analysis.* Second ed. Permagon Press, Oxford.

Feinberg, D.A. 1984 *Fuel options from microalgae with representative chemical compositions.* SERI/TR-231-2427. Solar Energy Research Institute. Golden, CO.

Freedman, B. and E.H. Pryde. 1982. Fatty esters from vegetable oils for use as a diesel fuel. In *Proc. of the Int. Conf. on Plant and Vegetable Oils as Fuels.* ASAE. St. Joseph, MI.

Fried, A., A. Tietz, A. Ben-Amotz, and W. Eichenberger. 1982. Lipid composition of the halotolerant alga, *Dunaliella bardawil. Biochim. Biophys. Acta* 713:419.

Goldstein, B. 1986a. Saline groundwater aquaculture I. The growth of *Spirulina spp.* in the saline groundwaters of New Mexico. In *Proceedings of the World Mariculture Society.* Reno, NV.

Goldstein, B. 1986b. Saline groundwater aquaculture I. The growth of *Crassostrea spp.* in the saline groundwaters of New Mexico. In *Proceedings of the World Mariculture Society.* Reno, NV.

Goldstein, B. 1990. *Microalgae production and shellfish feeding trials at the Roswell test facility.* New Mexico Water Resources Research Institute, Technical Completion Report No. 249, New Mexico State University, Las Cruces, New Mexico.

Herman, R.P. and C.A. Herman. 1985. Prostaglandins or prostaglandin-like substances are implicated in normal growth and development in oomycetes. *Prostaglandins* 29:819.

Hill, A.M. and D.A. Feinberg. 1984. *Fuel from microalgae lipid products.* SERI/TR-231-2348. Solar Energy Research Institute. Golden, CO.

Kates, M. 1964. Simplified procedures for hydrolysis or methanolysis of lipids. *J. Lipid Res.* 5:132.

Kusy, P.F. 1982. Transesterification of vegetable oils as fuels. In *Proc. of the Int. Conf. on Plant and Vegetable Oils as Fuels.* ASAE. St. Joseph, MI.

Lansford, R.R., J.W. Hernandez, P. Enis, and C.L. Mapel. 1986. *Evaluation of avalable saline water resources for the construction of large-scale microalgae production facilities in New Mexico.* New Mexico Water Resources Research Institute, Technical Completion Report No. 218, New Mexico State University, Las Cruces, New Mexico.

Maxwell, E.L., A.G. Folger, and S.E. Hogg. 1984. *Resource evaluation and site selection for microalgae production systems.* SERI/TR-215-2484. Golden, CO. Solar Energy Research Institute.

Microalgal Technology Research Group. 1984. *Microalgae culture collection 1984-1985.* SERI/SP-231-2486. Golden, CO. Solar Energy Research Institute.

Neenan, B., D. Feinberg, A. Hill, R. McIntosh, and K. Terry. 1986. *Fuels from microalgae: Technology status, potential, and research requirements.* SERI/SP-231-2550. Golden, CO. Solar Energy Research Institute.

Piorreck, M., K-H. Baasch, and P. Poh. 1984. Biomass production, total protein, chlorophylls, lipids, and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. *Phytochemistry* 23:207.

Pryde, E.H. 1982. Vegetable oil fuel standards. In *Proc. of the Int. Conf. on Plant and Vegetable Oils as Fuels.* ASAE. St. Joseph, MI.

Ryan, T.W.,III, L.G. Dodge, and T.J. Callahan. 1984. The effects of vegetable oil properties on injection and combustion in two different diesel engines. *J. Am. Oil Chem. Soc.* 61:1610.

Sargent, J.R., H.C. Eilertsen, S. Falk-Petersen, and J.P. Taasen. 1985. Carbon assimilation and lipid production in phytoplankton in northern Norwegian fjords. *Mar. Biol.* 85:109.

Smith, K.L. and J.L. Harwood. 1984. Lipid metabolism in *Fucus serratus* as modified by environmental factors. *J. Exp. Bot.* 35:1359.

Voltz, S.E. 1976. *Development studies on the conversion of methanol and related oxygenates to gasoline.* ERDA Report FE-1773-25. Mobil Research and Development Corp. Paulsboro, NJ.

Weisz, P.B. 1979. Catalytic production of high-grade fuel gasoline from biomass compounds by shape-selective catalysis. *Science* 206:57.

Wolf, F.R., A.M. Nonomura, and J.A. Bassham. 1985. Growth and branched hydrocarbon production in a strain of *Botryococcus braunii* Chlorophyta. *J. Phycol.* 21:388.