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GROWTH CHARACTERISTICS AND FATTY ACID COMPOSITION OF APHANIZOMENON SP LABORATORY CULTURES GROWING AT 15°C AND 28°C.

BY

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ABSTRACT

*In this study the thermal adaptation of cyanobacteria, nature, and changes in membrane lipid fatty acids during the adaptation process were investigated. **Aphanizomenon sp.** Cultures were grown at thermally different incubation temperatures in the laboratory, i.e.28°C and 15°C, for 25 days, where growth characteristics – as determined by the level of chlorophyll a, caroteniods and dry weight – as well as fatty acid compositions were followed up during the growth period. Results of this study can be summarized as follow: 1) The growth characteristics of **Aphanizomenon sp.** grown at either temperature were very similar. At both temperatures a lag phase of about 168 h was observed after inoculation before the cultures enter their exponential phase. At both temperatures a doubling in time of 20 h was observed. These characteristics showed that the organism adapts its physiology to the different growth temperatures. 2) At both temperatures the main composition of unsaturated fatty acids was 16:0, 16:1, 16:3, 18:0, 18:1, 18:2, and 18:3 after when the organism was grown at 15°C, also the organism responded to the low temperature by a 3.7 fold increase in the proportion of polyunsaturated fatty acids (18:3 and 16:3). An aspect which indicates that increasing the unsaturation of fatty acids may play a role (at least partially) in the adaptation of **Aphanizomenon sp.** to low temperature.*

INTRODUCTION

Marine *Aphanizomenon sp.* are major contributors to the N_2 cycle in the Baltic ecosystem Evans *et al.*, (2000). They are also major contributors to cyanobacterial blooms which are nuisance and toxic or potentially toxic to man and livestock Kononen and Nomman (1992). In the Baltic, *Aphanizomenon sp.* and other cyanobacteria are exposed during the summer to a wide range of temperatures from a maximum of 30°C whilst floating at the surface under calm conditions, to a minimum of 10°C when they sink to the thermocline following a wind-induced mixing event. These organisms also need to adapt to longer temperature changes as the temperature falls to its typical late summer value for the upper layer of 17°C - 18°C Kononen and Nomman (1992) and much lower temperature during the winter.

Recent research has focused on the cellular membrane as the primary site of cold injury. Fatty acids play an important role in determining the physicochemical properties of membrane lipids. The amounts of membrane fatty acids may be altered by changes in temperature Cronan, (1978). Less is known as to whether the capability of an organism to adapt to low temperature really reflects predetermined to alter the relative fatty acid composition of membrane lipids Neidleman, (1987). The mechanisms of temperature adaptation probably involve many cellular function components, but membrane fluidity and its maintenance can hardly be ignored.

To elucidate the nature and significance of changes in membrane lipid fatty acid compositions of cyanobacteria in the Baltic Sea, the growth characteristics and fatty acid composition of *Aphanizomenon sp.* cultures grown thermally at 28°C representative of the summer surface of the Baltic, and at 15°C representative of the temperature at the base of the water column of the thermocline were investigated.

MATERIALS AND METHODS

Microorganism and Growth Conditions

A culture from a strain of *Aphanizomenon sp.* originally isolated from the Baltic Sea was obtained from the Biochemistry Research Group, University of Wales, Swansea, henceforth referred as *Aphanizomenon sp.*, and was grown photoautotrophically in modified ASM-1iquid medium lacking a fixed nitrogen source. The medium ingredients were mixed thoroughly and the pH of the medium was adjusted by adding small volume of 0.1M HCl. Volumes of the medium were transferred into conical flasks, typically to occupy 1/2-2/3 of the flasks volume and sterilized in an autoclave at 121°C at pressure of 1.5 kg f /cm^2 for 15 minutes. When the flasks attained room temperature, they were inoculated. Normally, 10% volume of inoculation was used from a culture which had previously been grown for 10-12 days. Inoculation was carried out by sterile transfer in console safety cabinet. Inoculation flasks were placed in an illuminated refrigerated orbital incubator for 12 h light (90 υ E m^{-2} s^{-1}) /12 h dark cycle; the speed of shaking was 120 rpm and normally cultures were maintained at growth temperature of 28°C Jones, (1999).

Growth characteristics and fatty acid composition of *Aphanizomenon sp.* grown in laboratory culture for 25 days in a fixed nitrogen source free medium were studied under two different temperatures. The selected temperatures for this study were 28°C (high temperature) corresponding to the temperature to which *Aphanizomenon sp.* colonies floating at the surface of the Baltic Sea and 15°C (low temperature) corresponding to the temperature at the Baltic Sea thermocline. Samples of the culture were collected at 24 h interval during the growth period of the culture grown up to 25 days at 28°C and 15°C for growth and fatty acid analysis Kononen and Nomman (1992).

Growth Curve Determination

The growth characteristics were investigated by determination of two markers for Cyanobacterial biomass; chlorophyll a and carotenoids, which are present in the lipid bilayer of cyanobacteria membranes Fay, (1983). Growth of the cultures was also determined by measuring the dry weight of the cells contained in aliquots of culture media sampled over the growth period Holton *et al.*, (1964).

Cyanobacterial culture (duplicate, 5 ml samples) was centrifuged at 3,000 xg for 10 min, and left for 15 h at room temperature in the dark. Chlorophyll a was determined by measuring the absorbance at 665 nm of the resultant ethanolic solution in a scanning spectrophotometer Stal *et al.*, (1984). Carotenoids concentration was determined by measuring the absorbance of the ethanolic solution at 431nm Fork *et al.*, (1979).

Fatty Acid Composition

Total lipid was extracted from *Aphanizomenon sp.* cells which had been harvested by centrifugation, normally at 3,000 xg and essentially according to the method of Bligh and Dyer (1959) as modified by Sato and Murata (1980). Fatty acid methyl esters (FAME) were prepared by transesterification of total lipid extracts according to the method of Christie (1982). The methyl ester samples were purified by preparative thin layer chromatography (TLC) on either 20 cm x 20 cm or 10 cm x 20 cm Whatman 60A silica gel-G coated plates. Ten µl authentic solution of methyl ester standard (5 mg/ml) was used. A solvent system of diethyl ether: petroleum ether (1: 9 v/v) was used to develop the chromatogram.

The recovery of FAME from transesterification and preparative TLC was estimated by adding a known amount of internal standard heptadecanoic acid to representive total lipid extract before transesterification. An apparent recovery of 73% was routinely obtained.

Fatty acid methyl esters recovered from TLC were identified and quantified by GLC equipped with a flame ionization detector using a high 'polar' capillary column (BPX70, 0.25µm film, 2.5m x 0.22mm id). Oven temperature was programmed from 130°C to 210°C at the rate of 20°C min^{-1}. Injection was made in the split injection mode (10:1) at an injector temperature of 160°C using helium as a carrier gas at flow rate of 10 ml min^{-1}. Individual components were identified by comparing their retention times with authentic fatty acid methyl ester standard and by comparison with a GLC-MS total ion chromatography determined under similar conditions. The relative % composition of fatty acid was calculated from area of the individual peaks of interest in the chromatogram. Absolute quantification of fatty acid methyl ester was obtained by injection of known amounts of an appropriate authentic FAME standard and determination of the peak area. The amount of unknown FAME was then calculated by proportionation.

RESULTS AND DISCUSSION

Growth Characteristics

Growth in *Aphanizomenon sp.* Cultures at 28°C and 15°C was measured by determining the concentration of chlorophyll a, carotenoids and the dry weight over a period of 28 days (0–600 h) following incubation.

At 28°C (Fig.1a) the growth curves determined from chlorophyll a and carotenoids are almost identical and composed of four phases; lag period from (0–160 h), an exponential phase (168 h–263 h) followed by a second lag period (288 h–384 h) then a further exponential phase from (408 h–553 h). The growth curve determined from dry weight is relatively differed from that determined from chlorophyll a and carotenoids. As shown in fig.1a the dry weight did not increase during the first 120 h after incubation, after which the dry weight increased gradually between 144 h and 192 h, and then more rapidly between 216 h–336 h. After this period however there was no further significant increase in dry weight. This observation suggests that the high levels of chlorophyll a and carotenoids seen between 430 h–600 h may represent increased accumulation of these pigments within *Aphanizomenon sp.* cells rather than an increase in the number of the pigment cells.

In view of the unexpected data from of the growth curve, the experiment was repeated over 14 days growth period (0–336 h) under the same conditions. A similar pattern in the growth curves was observed in the growth parameters (Fig.1b), where the chlorophyll a and carotenoids levels indicating the presence of an early lag phase, a growth phase, whilest there was no significant increase in dry weight associated with the sharp increase in pigment concentration seen between 264 h and 312 h.

The growth characteristics of Aphanizomenon sp. culture grown at 15°C was presented in Fig.1c. The growth curve based on chlorophyll a shows a relative constant concentration for the first 168 h after inoculation and then underwent a continuing substantial increase until the culture was 576 h old. The growth curve based on carotenoids concentration indicates a lag phase period between 0–148 h, followed by an exponential phase growth between 148 h–276 h and a second phase of sustained carotenoids accumulation was observed between 456 h and 552 h. The curve based on dry weight indicates an apparent lag period for 144 h following incubation, followed by an increase between 144 h–366 h, after which the dry weight level remains relatively constant during an apparent stationary phase from 384 h–600 h.

Results of this study indicated that growth characteristics of *Aphanizomenon sp.* grown at either temperature were very similar. At both temperatures a lag phase of about 168 h was observed after inoculation before the culture entered their exponential phase. Under both conditions a doubling time – determined from the slope of growth curve in the exponential growth phase – of approximately 20 h was observed, which although within the range reported for marine cyanobacteria Mitsui et al., (1986). It is considerably shorter than the 66h previously reported Jones, (1999) for *Aphanizomenon sp.* cultures grown in the same medium at 20°C in aerated 20 L batch cultures These characteristics together with the observation that the maximum culture density observed at the growth temperature were very similar showed that the organism adapts its physiology to the different growth temperatures. The accumulation of both chlorophyll a and carotenoids at the end of a stationary phase which is not associated with an increase in dry weight of the cells in the culture, suggested that this accumulation of pigments may be a response to stress, possibly associated with depletion of micronutrients. Changes in pigment composition in

response to nitrogen deficiency have been observed in *Pseudoanabaena sp.* and the *Oscillatoria splendida* De Loura *et al.*, (1987). Another stress factor in the 28°C culture may be the prolonged exposure to the relatively high temperature Vigh *et al.*, (1998).

Fatty Acid Composition

Figure 2 a& b show the relative percentage composition of fatty acids present over the time course when the *Aphanizomenon sp.* was grown for 25 days at either 28°C or 15°C. At both temperatures the main fatty acids included are C-16 and C-18, both monoenoic and polyunsaturated fatty acids. In the monoenoic fraction two C-18 isomers, 18:1 (9) and 18:1(11) together with 16:1(9) were identified whilst the major polyunsaturated fatty acids were identified as 18:2(9,12) and 18:3(9,12,15) and 16:3. Besides these major unsaturated fatty acids, a small amount of 16:2 was detected.

At 28°C (Fig. 2a), 16:0 was the most abundant fatty acid present throughout the 25 days of the study. There was no significance change in the proportion of the C-16 during the 25 days, although there is some fluctuation in its level during this period. It appeared to be a small gradual increase in the monoenoic fatty acids (16:1 and18:1) after 384h, then the proportion of 18:3 showed a significant decrease.

When the cells were grown at 15°C, the organism responded to low temperature by characteristic changes in the relative proportions of the fatty acids. During the first 144h after inoculation there was a decrease in 16:0 concomitant with an increase in both 18:3 and 16:3, but there no significant change in 16:1 in the same period (Fig. 2b). After this period of growth, the fatty acid characteristics of culture grown at 15°C appear to be established, in which 18:3 is predominant (35 to 40%) with substantial amounts of 16:3 (about 14%) whilst the relative level of 16:0 is about (23.5%).

Comparison of the ratio of saturated: monoenoic: polysaturated fatty acid present after 240 h growth at 28°C (1: 0.66: 0.58) with that after 240 h growth at 15°C (1: 0.66: 2.13) indicates that the proportion of saturated fatty acid was unaffected by growth temperatures, but there was a 3.7 fold increase of the proportion of polyunsaturated fatty acid at lower temperature. These results are in agreement with those of Sato *et al.* (1979). They reported that when *Anabaena viriabilis* grown isothermally at 38°C and 22°C, the average number of double bonds in the lipid molecules was increased from 1.82 to 2.71 at the lower growth temperature.

This first systematic study of the fatty acid composition of *Aphanizomenon sp.* indicated that the organism should be classified as group 2 cyanobacterium as defined by Kenyon (1972) and Kenyon *et al.* (1972) as it contains 16:1, 16:2, 18:2 and α18:3 but not γ 18:3 or 18:4.

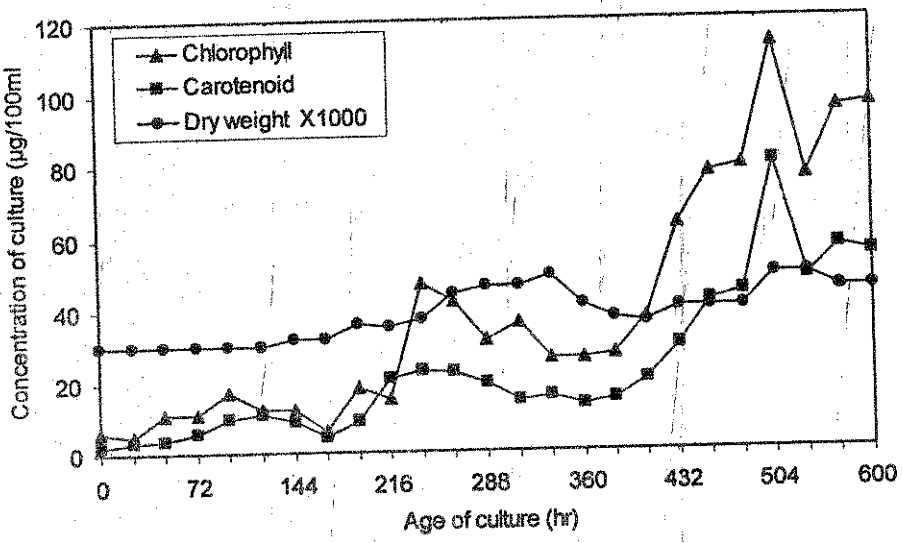

Figure (1a): Concentration of chlorophyll, carotenoid and dry weight from culture grown at 28°C for 25 days.

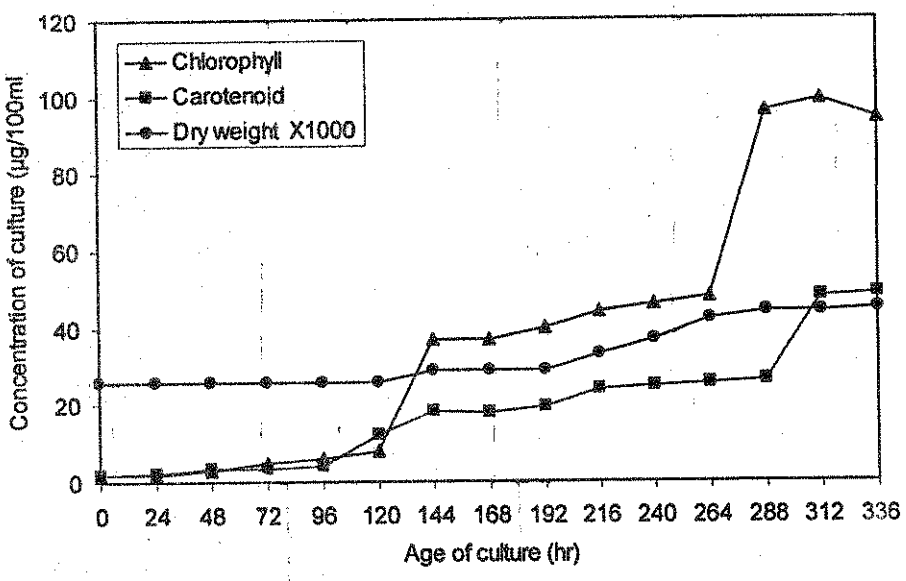

Figure (1b): Concentration of chlorophyll, carotenoid and dry weight from culture grown at 28°C for 14 days.

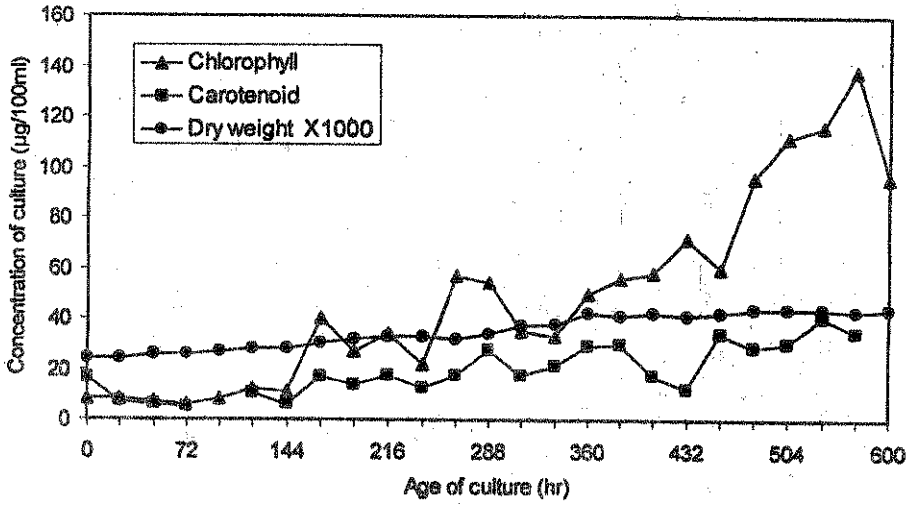

Figure (1c): Concentration of chlorophyll, carotenoid and dry weight from culture grown at 28°C for 25 days.

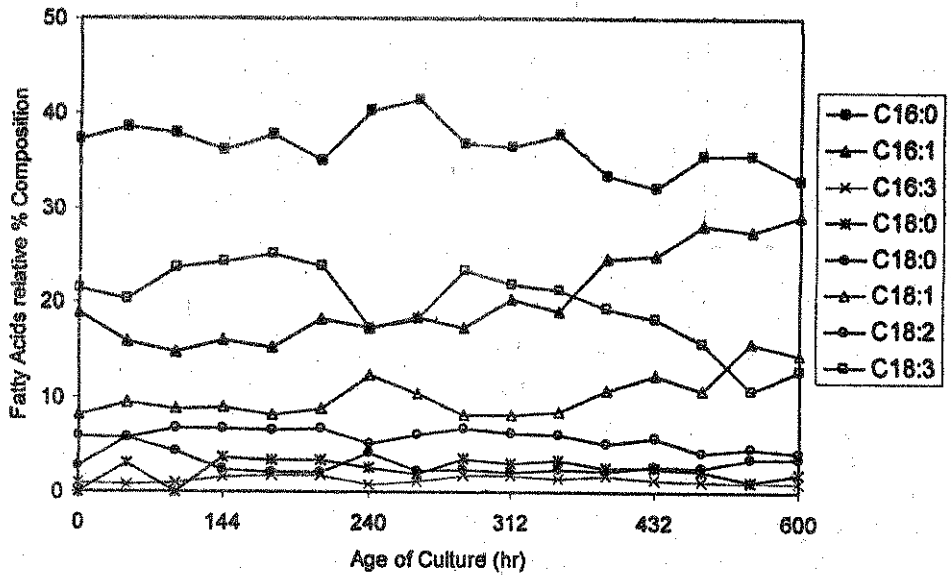

Figure (2a): The level of the most abundant fatty acids present in *Aphanizomenon sp.* from culture grown at 28°C for 25 days.

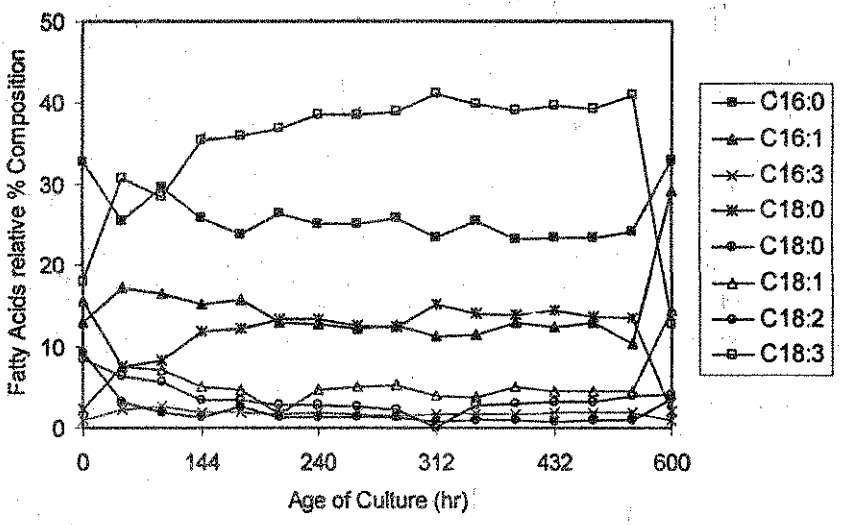

Figure (2b): **The level of the most abundant fatty acids present in *Aphanizomenon sp.* from culture grown at 15°C for 25 days.**

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ARABIC SUMMARY

خصائص النمو ومكونات الأحماض الدهنية في نوع من أنواع السيانوبكتيريا الأفانيزومينون في بيئات معملية درجات حرارتها ١٥ و ٢٨.

للسادة الدكاترة

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يتم في هذه الدراسة معرفة التكيف الحراري للسيانوبكتيريا (الأفانيزومينون) في درجات حرارة مختلفة بحيث يتم تنمية هذا النوع من السيانوبكتيريا لمدة ٢٥ يوم في درجات حرارة عالية (٢٨) ثم في درجات حرارة منخفضة (١٥) حيث يتم أثنائها متابعة النمو من خلال قياس كمية الكلوروفيل (a) والكروتينات وكذلك قياس الوزن الجاف للبكتيريا خلال تلك الفترة.

كذلك تتضمن هذه الدراسه تتبع التغيرات في غشاء الخلية لمكونات الأحماض الدهنية في فترة النمو المقترحة (٢٥يوم) ويمكن تلخيص نتائج الدراسة كما يلي:

١- لقد وجد أن خصائص النمو لهذا النوع من السيانوبكتيريا متشابهة في درجات الحرارة المرتفعة و المنخفضة. وبينت الدراسة أنه في درجة حرارة ٢٨ أو ١٥ منذ بدء عملية تلقيح البيئة وحتى ١٦٨ ساعة أنه لا يوجد أي تغير ملحوظ في نمو البكتيريا (lag phase) وبعد هذه الفترة وجد أن عملية النمو تدخل في مرحلة التمدد والتزايد (exponential phase) . ومن خصائص النمو هذه تبين أن هذا الكائن يستطيع أن يكيف نفسه وينمو في درجات حرارة مختلفة.

٢- في درجات الحرارة ٢٨ و ١٥ وجد أن الأحماض الدهنية الأساسية هي: ١٦: ٠، ١٦: ١، ١٦: ٢، ١٦: ٣، ١٦: ٠، ١٨: ١، ١٨: ٢، ١٨: ٣.

وقد لوحظ أنه في درجة الحرارة المنخفضة (١٥) زيادة نسبة الأحماض الدهنية الغير مشبعه بنسبه 3,7 وخاصة ١٦: ٣ و١٨: ٣.

هذه النتائج تؤكد أن الزيادة في تركيز الأحماض الدهنية الغيرمشبعة تلعب دورًا رئيسيًا في عملية التكيف بالنسبة لهذا النوع من السيانوبكتيريا (الأفانيزومينون).

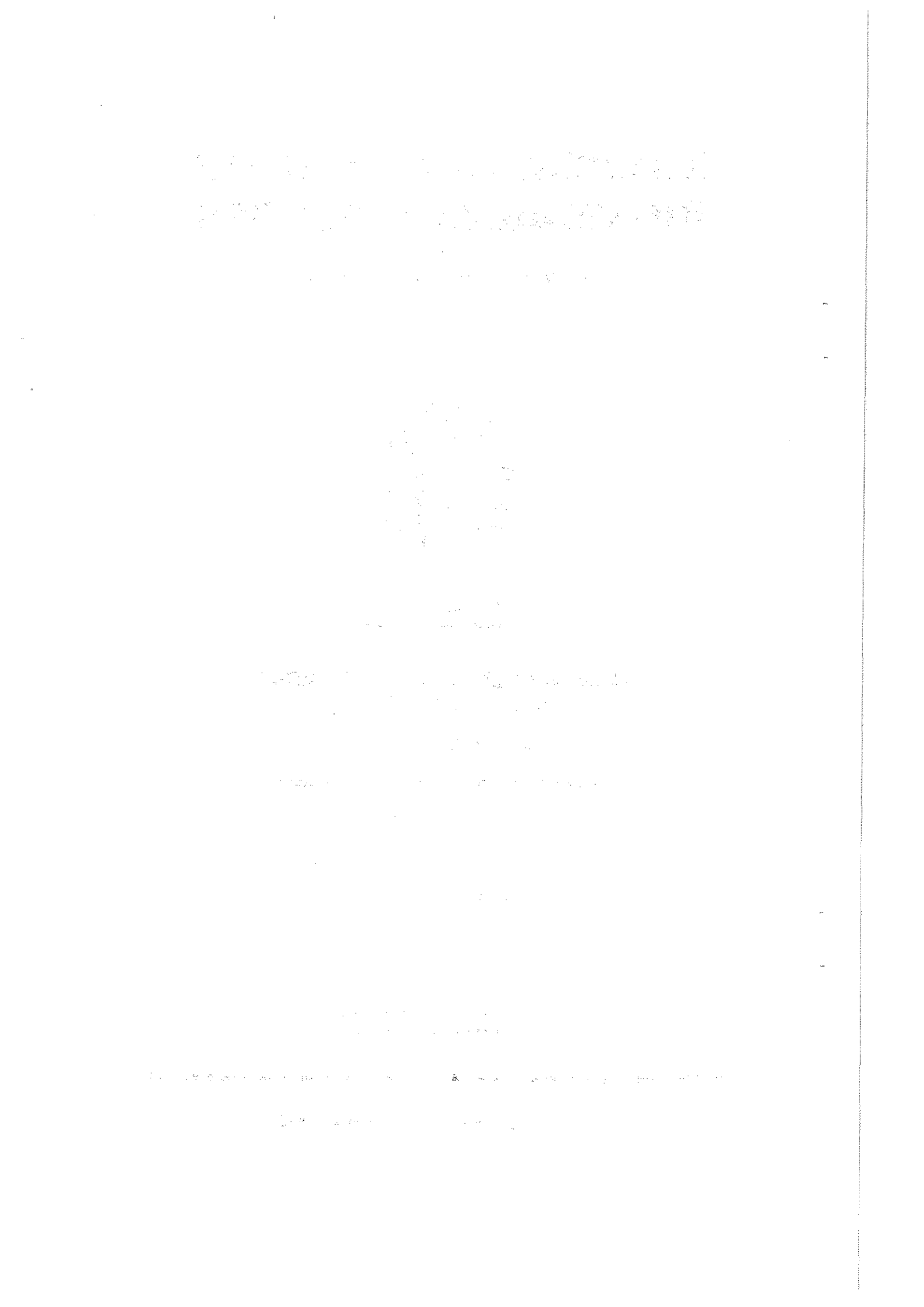
المجلة المصرية للعلوم الطبية الحيوية

تصدرها الجمعية المصرية للتكنولوجيا الحيوية

المشهرة برقم ٤٢٠٦ لسنة ١٩٩٥

الترقيم الدولى ٦٣٧٩ ـ ١١١٠

العدد التاسع عشر نوفمبر ٢٠٠٥



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