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Cambios fisiológicos, bioquímicos y ultraestructurales inducidos por el campo magnético en *Synechocystis aquatilis*

(Physiological, biochemical and ultrastructural changes induced by static magnetic field in *Synechocystis aquatilis*)

*Liliana María Gomez Luna

Universidad Luis Vargas Torres de Esmeraldas
*lilimagl@gmail.com

RESUMEN

Los estudios ultraestructurales en *Synechocystis* se centran principalmente en el ficobilisoma (PBS); sin embargo existen pocos informes sobre el efecto del campo magnético a este nivel. El objetivo de la investigación fue examinar los principales efectos fisiológicos, cambios bioquímicos y ultraestructurales inducidos por el campo magnético estático (SMF) en un rango moderado de inducción (β: 35-45 mT), con el fin de obtener evidencias para explicar la estrategia de respuesta a nivel celular y molecular de *S. aquatilis* (cepa local), explorando el potencial de SMF en el crecimiento y / o control de la toxicidad de esta cianobacteria ante mínimas variaciones del campo. Los cultivos control alcanzaron la máxima densidad celular el día 15, pero aquellos expuestos a 37,7 mT obtuvieron máximos el día 7, mientras que los expuestos a 44,3 mT el día 13, con diferentes valores de $k_{máx}$: 8 826,67 ± 538,40; 4 175,00 ± 459,62 y 3 943,75 ± 937,71, respectivamente (α = 0,05). De acuerdo con las evidencias ultraestructurales se observan modificaciones en la disposición de los tilacoides inducida por SMF, variando la distribución y las distancias entre los tilacoides y las membranas tilacoidales. Estos cambios deben tener implicación a nivel funcional, probablemente como respuesta celular para mantener la eficiencia fotosintética. Los resultados sugieren que el SMF (37,7 y 44,3 mT) induce cambios significativos en el crecimiento y la ultraestructura de esta cianobacteria planctónica, y cambios discretos moleculares que implican a la composición pigmentaria y el potencial tóxico.

Palabras clave: Cianobacterias, pigmento, potencial tóxico, el campo magnético.

ABSTRACT

Ultrastructural studies of *Synechocystis* mainly focus on phycobilisoma (PBS). There are few reports about the magnetic field effect at this level. The objective of this study was to examine the main physiological, biochemical and ultrastructural changes induced by static magnetic field (SMF) in a moderate strength magnetic field range (β: 35-45 mT). The purpose of this examination was obtaining evidence to explain the response strategy at the cellular and molecular level of *Synechocystis aquatilis* (local strain), even with minimal induction changes, exploring the SMF potential over the growth and/or toxicity control. Control cultures reach the maximum cell density at day 15, but with 37.7 mT at day 7; and, at with 44.3 mT at day 13, with different values of *kmax*: 8 826.67±538.40; 4 175.00±459.62 and 3 943.75±937.71, respectively (a=0.05). According to the ultrastructural evidence, there were modifications on thylakoid arrangement induced by SMF varying the distribution and distances between thylakoids and thylacoidal membranes. These changes should have implication at the functional level; probably as a resultant cellular response to maintain the photosynthetic efficiency. Results suggest that the SMF (37.7 and 44.3 mT) induces significant changes in growth and ultrastructure of this planktonic cyanobacterium, and discrete molecular changes which involve the pigment composition and toxic potential.

Keywords: Cyanobacteria, pigment, toxic potential, magnetic field.

INTRODUCTION

Exposure to magnetic fields has increased significantly over the years due to man-made activities and devices; for this reason the study of magnetic field effects (MFE) in biological systems, including humans, has gained interest, especially in the last two decades, mainly because of the understanding of the mechanism of action. Static or constant and pulsing magnetic fields and direct and alternating electric currents might be applied to regulate (stimulate or inhibit) cell proliferation or to monitor cell growth and other metabolic activities, increasing the use and benefits in microbial biotechnology (Elahee & Poinapen, 2006; Gómez, Menéndez, Álvarez, & Flores, 2009; Mihoub, El May, Aloui, Chatti, & Landoulsi, 2012; Velizarov, 1999; Wang, Zeng, & Gui, 2006).

Even when all life on earth has being influenced by electric and magnetic fields, it is still not fully understood the mechanism responsible for the magneto-sensitivity, and little is known about the influence of magnetic fields on microorganism ultrastructure.

*Synechocystis*is is an unicellular, photoautotrophic, facultative glucose-heterotrophic cyanobacteria that can survives and grows under a wide range of environmental conditions. *Synechocystis* has biotechnological interest, mainly to lipid extraction (Kaiwan-Arporn, Hai, Thu, & Annachhatre, 2012) and for large-scale purification of C-phycocyanin (Ramos, 2011; Ugwu, 2005). Nevertheless, because of a blooming capacity and toxicity of some species, it can become a problem in large scale cultures.

The majority of the studies about the magnetic field effect on living organisms, discuss one of the following theoretical mechanisms: the magnetite mechanism, not valid in *Synechocystis*, because of the lack of magnetosomes, and the radical pair mechanism (Y. Liu, 2008).The objective of the current research was to examine the main physiological, biochemical and ultrastructural changes induced by SMF in a moderate strength magnetic field range (β: 35-45 mT) in order to obtain evidences to explain the response strategy at cellular and molecular level of this cyanobacteria, exploring the SMF potential in the growth and/or toxicity control of *S. aquatilis*. These results were verified on growth kinetics, pigment content (chlorophyll *a* and C-phycocyanine), toxic potential measured as percentage of phosphatase inhibition and ultrastructural changes by transmission electronic microscope (TEM).

MATERIALS AND METHODS

Culture of Synechocystis cells: wild strain of the cyanobacteria *Synechocystis aquatilis* Sauvageau 1892: cxv (Synechococcales; Merismopediaceae) was isolated from a water reservoir (Chalóns, Santiago de Cuba) developing dense cultures in controlled experimental condition. The deposit code of the strain in the culture collection is CNEA-F140011-N-4014, culture collection subscribed to FELAC.

S. aquatilis was choose due to its structural simplicity and singularities, availabilty in aquatic (local) tropical ecosystems, ultrastructural studies previous reports, biotechnolgical interest, growth rate and toxic potential. It is important to remark that toxic cyanobacteria, their proliferation and toxin production strains is till nowadays a complex topic; cyanotoxins are one of the most toxic substances in the water mass, due to their distribution and toxic effect (Pérez, Soraci, & Tapia, 2008).

Asynchronic dense cultures of monospecific *S. aquatilis* were developed indoors during 15 days, in controlled laboratory conditions, using a 500 mL flask as photobioreactor with an initial concentration (Ci) = 500 x10^4 cell.mL^{-1}). Cells grew photoautotrophically in B12 (Shirai *et al*., 1989) liquid medium ($NaNO_3$ as main nitrogen source) in aerated cultures at 0. 45 L.min^{-1}, prefiltered air, using a glass microfiber syringe filter Midisart 2000 Sartorius 0. 20 µm and pH=7.5. Aeration allows an homogenous growth of the cells (Raven, 1988). The culture was supplied with 0, 3 % CO_2, favoring photoautotrophic metabolism and the culture buffering.

Culture temperature was around 20.00 ±1.0°C and the irradiance, 58.59 µ$Em^{-2}s^{-1}$; these

parameters fall on the range recommended by some authors (Boulay, Abasova, Six, Vass, & Kirilovsky, 2008; Ikeuchi & Tabata, 2001; Laczko-Dobos et al., 2008; Mattoo, Giardi, Raskind, & Edelman, 1999; Nascimento & Azevedo, 1999; Olive, Ajlani, Astier, Recouvreur, & Vernotte, 1997; Pawlik-Skowrofiska, 1997). Continuous light was supplied by means of PHILIPS DAYLIGHT, 32 W (TLD 32 W/865-NG) lamps. The light intensity was measured and corrected daily with a digital Voltcraft MS 1300 lux meter. Biomass was collected by centrifugation and immediately cooled at 4°C until the analyses.

Magnetic field arrangement and doses: static magnetic field treatment was applied to the whole culture during 1 h at two different induction values: 37.7mT and 44. 3 mT during the early exponential phase (day 7th). The β values were selected considering different criteria (Hirano, Ohta, & Abe, 1998; Y. Z. Liu, Wang, Ru, & Zhao, 2008; Singh, Tiwari, Abraham, Rai, & Rai, 1994; Zhi-Yong, Si-Yuan, Lin, & Miao-Yan, 2007). The reproducibility of all data in this study was confirmed by five repetitions.

Magnetos arrangement: two neodymium magnets arrangements were used to supply non-homogeneous magnetic fields. There were build and characterized in the National Centre of Applied Electromagnetism, Cuba. The β statistic in the work region (cylinder 8.4 cm diameter and 9. 7 cm length, collinear with magnet axis and centered on it) to arrangement A, have an average of 37. 7 mT; maximum value of 87. 9 mT and minimum 13 mT (SD: 10. 4 mT). To the arrangement B, the average is 44. 3 mT; maximum value of 103. 4 mT and minimum 15. 3 mT (SD: 12. 2 mT). In order to avoid any interference, cultures were developed in areas with values below 0. 25 µT. The low frequency electromagnetic interference (LFEI) (300 Hz) was measured with a Tester TES 1 390 (0. 01 y 1 999. 9 µT) in the culture chamber. The work range was 0.011 to 2. 27 µT, lower than the limit established to public environment exposure, according to World Health Organization, specifically the International Commission for No Ionizing Radiation Protection (ICNIRP): 83 µT (OMS, 2008).

Cells counting and growth parameters: Growth parameters were analyzed daily; other analyses were made at the end of the experiment (day 15). Algal densities were determined by daily counting triplicate samples in a Neuberger improved haematocytometer. Results were expressed in cell.mL^{-1}. Previous to the experiments with SMF, the *S. aquatilis* control culures (Ci= 500.00 ±56.55 x 10^4 cell.mL^{-1}) kinetic were studied.

The media, exponential (μ_{med}) and maximum growth rate ($\mu_{máx}$) were calculated from the number of cells values daily base. It represents the inverse of the duplication time (DT) which means the time that N cell needs to be transformed in 2N cells during the exponential phase. It was calculated using the following equation and expressed as divisions per day:

$$\mu \text{ (div. day}^{-1}) = (\log2 (N_t) - \log2 (N_0)) / (t_t - t_0)$$

Where: N_T and N_0 are cellular densities (cell.mL^{-1}) at time t and 0 respectively.

Phosphatase inhibition test: in order to evaluate the effect of SMF over the toxic potential of *S. aquatilis* cells, the phosphatase inhibition test was developed. The percentage of inhibition was calculated considering the maximum enzymatic activity. It was determined spectrophotometrically at 405 nm, during 3 min. A blank with bidistillated water was used. A positive control (buffer phosphate) was also prepared. The enzymatic activity was calculated with the following equation (Bessey, Lowry, & Brock, 1946; Kuwana & Rosalki, 1991):

$$\text{Activity (U L}^{-1}) = \Delta \text{ OD min}^{-1} \text{ x } 2\,750$$

Enzymatic activity factor: 2 750

Pigment analysis and chlorophyll a: C-phycocyanine ratio: To evaluate the effect of SMF over the cellular pigment composition in *S. aquatilis*, the ratio of the main pigments were analyzed (chlorophyll a: C-phycocyanine). It was evaluated considering their usefulness as indicator of culture status and to evaluate the environmental acclimation cell response strategy (Gómez, 1997). Cell pigments were extracted with 5 mL of methanol 99.8 % ac-

cording to modified ISO 10260 procedure (ISO, 1992) including 5 min of dark-iced disruption in an Ultrasonic chamber Scientz–IID, 20-25 Hz and 80 % of pulse ratio. Cells were resuspended by vortex-shaken for 10 s and centrifuged at 2 540 g for 10 min. The chlorophylls *a* were spectrophotometrically determined by recording the absorbance at 665 nm, using the molar extinction coefficient 74.46 mL $mg^{-1}cm^{-1}$ (Rivas, Fontes, Moreno, Rodríguez, & Vargas, 1992) with the equation:

Chlorophyll *a* (µg mL^{-1}) = 13. 43 x (A 665)* (V*Extract* / V*Culture*)

Pheopigments were determined by sample acidification with methanol extract (0. 1 mL HCl, 3 M), during 5 min. Corrections were made with the absorbance at 750 nm. Pheopigments were spectrophotometrically determined by recording the absorbance at 665 nm. Results were expressed in ng $cell^{-1}$.

The C-phycocyanine (C-PC) was extracted according to Boussiba y Richmond with 5 mL of buffer phosphate 100 mM (10.64 gL^{-1} de K_2HPO_4 y 5.29 gL^{-1} de KH_2PO_4 a pH 7) freezing at 4°C, overnight, in dark conditions (Boussiba & Richmond, 1979); then the cells were re-suspended by vortex-shaken for 10 s and centrifuged at 2540 g for 10 min. The spectrophotometric determination was made by recording the absorbance at 615, 652 y 730 nm. To calculate the concentration, the following equation was used (Sigelman & Kycia, 1978). Results were expressed as ng cell.$^{-1}$.

C-PC (µg mL^{-1}) = $(A_{615}-A_{730}-0.47(A_{652}-A_{730}))/5.34*(V_{Buffer}*1000)/V_{Culture})$

Statistical Analysis: growth parameters, toxic potential and pigment content were analyzed, considering 5 % of significance in the mean differences, by one-way ANOVA followed by Tukey-Kramer's HSD post-test, using ORIGIN 6.0 and/or SPSS PASW 18 software. A Pearson correlation was made to analyze the relation between growth and toxic potential.

Transmission electron microscopy and images analyses: cells were spun in 1 mL centrifuge vials at 13 000 x g for 5 min to form a pellet and rinsed 2 times with PBS. Approximately 20 µL aliquots of the cell pellet (PBS preparations) were fixed with 2 % glutaraldehyde in 0. 1 M dimethyl arsenic acid sodium salt trihydrate buffer pH 7. 2 overnight at 4°C. After glutaraldehyde fixation samples were rinsed 3 times 30 min 4°C with the same buffer and post fixated during 2-3 h 4°C. This step was followed by a rinsing sequence (3 times x 5 min) at room temperature with distilled water. After that, dehydration was developed with successive steps at room temperature: acetone 50 % (20 min); acetone 70 % (30 min); acetone 90 % (30 min); acetone 100 % (30 min) and acetone absolute (30 min). The samples were then rinsed in three changes of anhydrous acetone at −85 °C before being placed in a 1 % OsO_4 solution in acetone and slowly warmed to room temperature over a six-hour period (A. M. L. van de Meene, Hohmann-Marriott, Vermaas, & Roberson, 2006; A. M.L. van de Meene et al., 2012).

The cells were then rinsed in acetone and slowly infiltrated with and polymerized in Spurr's resin (Spurr, 1969). The impregnation was made in acetone: Spurr (1:1) overnight in rotator at room temperature and then embedding in rubber molds, polymerizing in the oven at 70°C, around 17 h.

Embedded cells were cut into serial 70 nm thick sections with an Ultracut R Microtome (Leica, Vienna, Austria) and collected on Formvar-coated copper slot grids. Sections were post-stained with 2 % uranyl acetate in 50 % ethanol for 5 min.

The grids were carbon-coated. At least 100 random cell images were taken from different samples, but only 25 images were recorded for images analyses. Electron microscopy was performed on a PHILIPS CM 12-STEM electron microscope operated at 120 kV at University of Hasselt, Belgium. Images were recorded under low dose conditions (a total dose ~25 e−/Å2) with a 4000 SP 4 K slow-scan camera (Gatan, Pleasanton, CA) at−390 nm defocus and at a magnification of 80.000. The pixel size (after binning the images) was 3. 75 Å at the specimen level and GRACE software was used for semi-automated

specimen selection and data acquisition. The image analysis was performed with the Image Tool v 3.0 Processing software on a PC cluster; 10 measurements were made by each picture. One-way ANOVA was used to compare mean differences at 5 % level of significance.

RESULTS AND DISCUSSION

Physiological changes (growth and survival): in control cultures, *S. aquatilis* cell reach the exponential growth early in day 3 until day 15, but at day 12 a slope change was visible (Fig. 1), beginning a decrease in the growth rate.

As result of the exposure to SMF, significant differences of cell density values (α=0. 05) were observed in those cultures in relation to controls. There was an inmediate cell density drop 24 h after the SMF application for both inductions. In cultures exposed at 37.7 mT cells population were decreased at 11 % (3 612.5 ±5 48.01x 10^4 cell.mL^{-1}) in relation to controls (4 048.67 ±466.89 x 10^4 cell.mL^{-1}). In those exposed to 44.3 mT there was a decrease of 25 % (3 040.63 ±181.51x 10^4 cell. mL^{-1}) (α=0.05). At the end of the experiment (day 15) control cell density value was 8 826. 67 ±538. 40 x10^4 cell.mL^{-1}. The minimum value was obtained at 37.7 mT (1650.00±212, 13 x10^4 cell.mL^{-1}) (Fig. 1, Table 1). The exponential and maximum growth rate (μ_{exp} and μ_{max}) are showed in table 1 as well as the maximum cell density to each treatment (k_{max}), and the cell density at the end of the exponential phase 15 (*k15*).

Values of exponencial and maximum growth rate in control cultures and those exposed to SMF had significant differences, being higher (α=0.05) in the SMF exposed cultures. Control cultures reach the maximum cell density at day 15, but at 37.7 mT in the day 7 and at 44.3 mT in the day 13, with different values of *kmax*: 8 826.67±538.40, 4 175.00±459.62 and 3 943.75±937.71, respectively (α=0.05).

- *Biochemical changes: toxic potential and pigments content*
- *Toxic potential*: There is no correlation between both parameters (r=0.82, Pearson; α=0.05) and no significant differences in the inhibition percentage (α=0.05) were obtained. It was higher than 50 % for all the treatments, but slightly higher than 55 % to 44.3 mT
- *Pigment content and ratio*: Pigment variation was not statistically significant (α=0.05); but minimum concentration was obtained at 37. 7 mT (22.59 ±0.21 ng $cell^{-1}$), and maximal C-phycocyanine concentration was reached at 44.3 mT (6.81 ±2.11 ng $cell^{-1}$) (Fig. 2).
- The ratio chlorophyll *a*:*C*-phycocyanin (Chl:Phy) was about 5.5 in control cultures(α=0.05) but it dropped when cultures were exposed to SMF (3≤Chl:Phy≤ 5).

Ultrastructure: some ultrastructural changes were verifyed in *S. aquatilis* cells exposed to SMF (Fig. 3).

The main differences were found in cell size, wall thickness and aparience, thylacoids arrangement and store granules presence. Cell size in control cultures and in cultures exposed to SMF is presented in table 2.

There were significant differences in both cell length and cell width, between all the treatments, being smallest the control cells, and largest those from cultures exposed to 44. 3 mT. These are evidences of ultrastructural changes in *Synechocystis aquatilis* cells produced by SMF and would be related with subcellular arrangement and cell viability and/or efficiency. There are visible differences on the outer layer of the cell wall; it is smooth and slimmer in controls, but thicker in those cells exposed to SMF.

Specifically at 44. 3 mT the cell wall seems wavy and rough, resembling a quiescent state. About the thylakoids membranes (TM) *S. aquatilis* control cells have a concentrically TM distributed on the cell periphery, but with the SMF this arrangement was modified (Fig. 3). There were a disaggregation of the staked thylacoidal membrane in SMF exposed cells and the cyanophycine granules displace it, at 37. 7 mT, modifying the initial appearance. The granules are conspicuous at 37.7 mT (around 0.5 µm); presumably

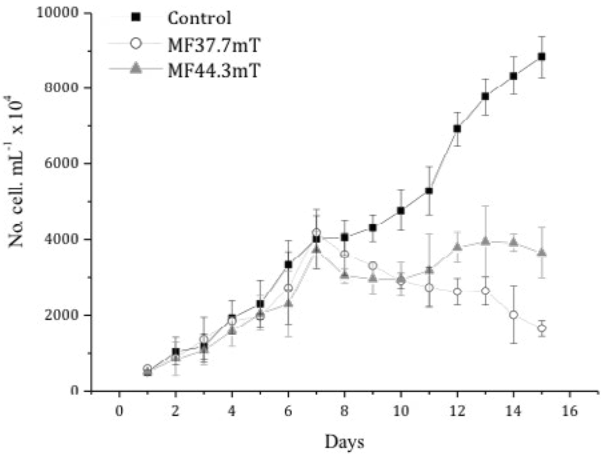

Fig.1. Growth kinetic of *S. aquatilis* control cultures and those exposed to SMF (b= 37.7 mT and 44.3 mT) (Media ± SE; n=5) at 58.59 $\mu Em^{-2}s^{-1}$.

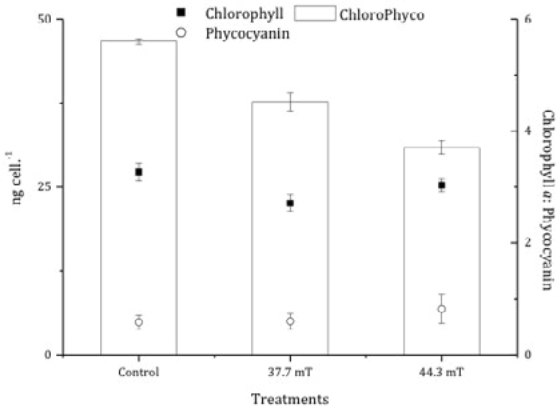

Fig. 2. Variation of the pigment concentration (chlorophyll *a* and C-phycocyanine) in *S. aquatilis* culture (day 15) at 58.59 $\mu Em^{-2}s^{-1}$, control culture and culture exposed to SMF: 37.7 y 44.3 mT and chlorophyll *a*: *C*-phycocyanin (Chl:Phy) ratio (Media ± SE; n=5).

Table 1. Exponencial and maximum growth rate, and cellular density in *S. aquatilis* cultures (control and SMF exposed cultures)

	μexp (div. day^{-1})	μmax (div. day^{-1})	*k*max (d*) (x10^4 cell. mL^{-1})	*k*15 (x10^4 cell. mL^{-1})
Control	0.2430±0.01^a	0.2694±0.02^a	8826.67±538.40$^{(15)a}$	8826.67±538.40^a
37.7 mT	0.4508±0.02^b	0.5399±0.01^b	4175.00±459.62$^{(7)b}$	1650.00±212.13^b
44.3 mT	0.4310±0.04^b	0.4343±0.03^c	3943.75±937.71$^{(13)c}$	3648.00±655.41^c

d*: day when the culture reach the maximum cell density
Diferent letters in columns indicate significant differences (α=0.05).
Values represent mean±SE.

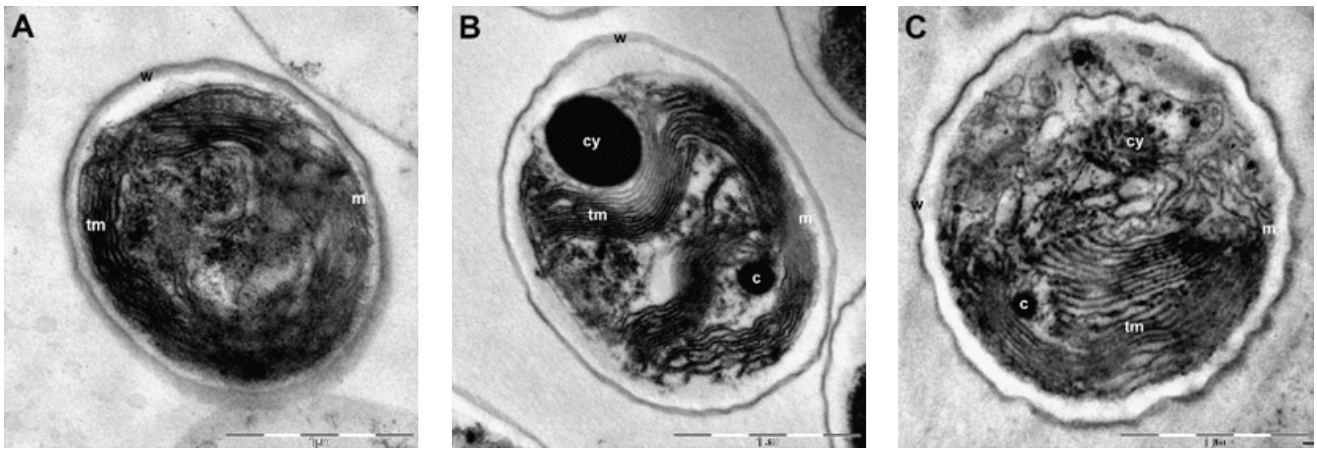

Fig. 3. TEM images of ultraestructural changes in *Synechocystis aquatilis;* control cultures (A) and cultures exposed to SMF: 37.7 (B) and 44.3 mT (C). C: carboxysome; CY: cyanophycine granule; tm: thylakoid membrane, m: cellular membrane.

these are formed by the accumulation of store substances like cyanophycine, which can content amino acids like arginine and aspartic acid (Bold & Wyne, 1985), and/ or pigments. Otherwise, carboxysomes or polyhedral bodies (around 0.5 µm) were visible in cells exposed to SMF, both at 37. 7 and 44. 3 mT.

Studies on the possible effects of SMF have

Table 2. Cell size measurements: media values and ES (control and SMF exposed cultures)

	μ_{exp} (div. day^{-1})	μ_{max} (div. day^{-1})	k_{max} (d*) (x10^4 cell. mL^{-1})	k_{15} (x10^4 cell. mL^{-1})
Control	0.2430±0.01^a	0.2694±0.02^a	8826.67±538.40$^{(15)a}$	8826.67±538.40^a
37.7 mT	0.4508±0.02^b	0.5399±0.01^b	4175.00±459.62$^{(7)b}$	1650.00±212.13^b
44.3 mT	0.4310±0.04^b	0.4343±0.03^c	3943.75±937.71$^{(13)c}$	3648.00±655.41^c

Diferent letters in ES columns indicate significant differences (α=0.05).

been conducted in different systematic groups and with diverse experimental conditions (Elahee & Poinapen, 2006). Even when detectable changes are often observed in exposed biological systems, the findings are usually statistically non significant comparing with controls, or there are contradictory effects described or erratic behaviors (Halpern, 1969; Pazur & Scheer, 1992).

According to the experimental evidences the number of cells of *S. aquatilis* decline inmediatily after the SMF exposure (both inductions). Furthermore, an apparent shortening of the exponential phase was observed, but cultures keep growing till day 15. Results showed a strong inhibitory effect of SMF over *S. aquatilis* cell growth, higher at 37.7 mT (α=0.01) than at 44.3 mT. The kinetic study showed that there are at least two responses: an inmediate system perturbation response R(t1) which was veryfied with a cellular decline 24h after the exposure =R(24h) and a late system recovery tendency response R(t2) which begun 96h till the final culture decline =R(96h). It was verifyied with the growth rate data analysis.

It seems that SMF gradient affects immediately the *S. aquatilis* kinetic when cultures were exposed for 1h to the magnet arrangement during the early exponential phase, inducing a strees response with two main components: R(24h) and R(96h). A culture aging and a drastically cell density decreasing was the final consequence of the SMF exposure. Probably, these two responses modulate the growth kinetic, depending of the induction, being 37.7 mT those that induces the highest growth inhibitory effect.

After 24h of SMF exposure, the cell population of cultures exposed to 37.7 mT decreases 11 % in relation to the control culture; but after 96h it decreases 14 %, in relation with those exposed to 44.3 mT SMF (2 737.50 ±512.65 x 10^4 cell.mL^{-1} *vs* 3 171.88 ±968.51 x 10^4 cell.mL^{-1}) and 48 % in relation to control cultures (5 291.67 ±630.53 x 10^4 cell.mL^{-1}) (α=0.05).

As a general view, the mortality rate was higher than the growth rate after the SMF exposure. Seemingly the exposition to a SMF at 37.7 mT did not allow the microorganisms recovery and R(24h) was the main component.

The pigment content and ultrastructure analyses confirmed an early senescence resulting in premature decline of cultures exposed to SMF, usually attributable to a strees response. The TM dispersion observed in cell exposed to SMF could be related with the integrity loss because of a permanent damage induced by SMF, which depend on the doses.

At ultrastructure level there were in some segments of the plasma membrane evidences of the integrity loss, confirming this explanation. In overall, these results suggest that the SMF into the study range (37. 7 and 44. 3 mT) induces significant changes in growth and ultrastructure of the planktonic cyanobacteria *Synechocystis aquatilis,* with molecular discrete changes which involve the pigment composition and toxic potential.

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