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Physiological, Morphological and Ultrastructural to Combination Responses of Cyanobacterium *Fischerella* sp. FS 18 Effects of Extreme Conditions

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ABSTRACT

Fischerella sp. FS 18 has been characterized physiologically, biochemically and morphologically at the combination of extremely low and relatively high irradiance (2 and 300 $\mu E.m^{-2}.s^{-1}$), different pHs (5, 7, 9) and extremely limited carbon dioxide concentration. Results showed that neutral condition and limited irradiance cause maximum biomass production. The amount of oxygen liberation normalized to dry weight showed higher rate at neutral and then alkaline conditions. Chlorophyll content wassignificantly higher at neutral but the rate of photosynthesis increased at alkaline condition especially at fourth day after inoculation. Both phycobilisome length and PSII/PSI ratio were significantly higher at pH 7. Photosynthetic-irradiance curves showed that although maximum photosynthesis normalized to chlorophyll unit seem higher at neutral conditions, degree of adaptation to limited irradiance and consumed energy need reaching to maximum photosynthesis increase at this condition. Difference in pH, irradiance and DIC concentration could not change photoinhibition resistance pattern even at extremely high light intensities (more than $2000\mu E.m^{-2}.s^{-1}$). Nitrogenase activity pattern were similar to photosynthesis. Biometrical analysis showed that except diameter of the heterocyst at the branches, decline of irradiance cause decrease in cell dimensions regardless of acidity and alkalinity. In low light intensity, there was no hormogonium production at different pHs.. TEM photomicrographs showed relatively linear and parallel configurations of thylakoids at pH 7, extremely limited irradiance and carbon dioxide concentrations. SEM photomicrographs showed holes and ornamentations belong to –especially – vegetative cells of the main axis at both pHs 7 and 9 regardless of light intensity.

KEY WORDS: Acclimation, Cyanobacteria, Dissolved inorganic carbon, Irradiance, pH

Abbreviations: APC allophycocyanin, Chla chlorophyll a, CCM carbondioxide concentrating mechanism, DIC dissolved inorganic carbon, PBP phycobiliproteins, PC phycocyanin, PE: Phycoerythrin, PSI, PSII photosystems I and II.

INTRODUCTION

Species of *Fischerella* have been reported from North and South of Iran (Soltani et al., 2011, Siahbalaie et al, 2012, Soltani et al., 2012). Rice in North and petroleum in South play main roles in our economy. Until now, studies of soil microalgae and cyanobacteria of Iran, have been limited to disperse reports and a few publishedpapers. Regarding necessity of local studies for the next decade applied programs including agricultural and petroleum biotechnologies, characterization of native microalgae and cyanobacteria seem essential forfuture basic-applied projects. Programs that must be planned carefully start regularly and organized by intelligence management systems. Characterization of potent cyanobacteria for agriculture (North) and petroleum (South) biotechnology seems basic at this time. Regarding this, morphological, taxonomical, ecological and ecophysiological characterization of native algae seems essential especially at this decade for our country.

Conspicuously there are environmental differences between North and South of Iran but there are some similarities. Necessity of acclimation to light, pH and carbon dioxide concentration fluctuations is an example of these common aspects. Choosing ecophysiological approach with agricultural point of view, rice-fields are special chaotic habitats with a combination of pH, irradiance and carbon dioxide concentrations fluctuations (Poza-Carion et al., 2001). During the rice cultivation cycle, not only underwater light intensity, but also pH show considerable variations during the season and days (Soltani et al., 2006). Water logging cause limitation of carbon dioxide diffusion ability and alkaline soils cause high bicarbonate production (Shokravi et al., 2011). Carbon dioxide concentration mechanism induction seems essential at such a situation by cyanobacteria (Müller et al.,1993). At low external Ci, most freshwater cyanobacteria induce protein complexes associated with carbon-concentrating mechanism (CCMs) to actively increase the internal concentration of Ci and release dissolved CO_2 in the direct vicinity of their primary carboxylation enzyme, RUBISCO (Burns et al.,2005). We

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have some experiments on *Hapalosiphon* sp. FS 56 (Shokravi et al.,2011), *Hapalosiphon* sp. FS 44 (Shokravi et al.,2011), and *Nostoc* sp. JAH 109 (Shokravi et al., 2006) which showed the importance of carbon dioxide on the results of pH and irradiances effects on cyanobacteria. Researches on *Nostoc* sp. UAM 205 (Fernandez-Valiente and Leganes, 1989) and *Nostoc* sp. UAM 206 (Poza-Carion et al.,2001) emphasized on the role of carbon dioxide concentrations at such similar situation too.

From the other side, extremely limited irradiance especially at the time of maturing the rice produce high shading conditions (more than 98% comparing bare soil) (Shokravi et al., 2011). Acclimation to light intensity has been the topic of numerous physiological, ultrastructural and biochemical studies on a variety of cyanobacterial strains (Soltani et al., 2006). As with other photosynthetic organisms cyanobacteria are able to adapt to variations in light intensity by modulating their photosynthtetic apparatus and physiological activities such as dinitrogen-fixation and photosynthesis (Soltani et al., 2006). Furthermore, in each of these habitats, widely fluctuating environmental parameters, including light level and quality, temperature and mineral nutrient availability, interact to influence growth, molecular resource allocation, and photosynthesis through complex acclamatory strategies (Burns et al., 2005). Hypoliths are the best examples of acclimation to extremely low irradiances in cyanobacteria (Where they photosynthesis at irradiance levels less than 0.1% of the incident light) (Thomas, 2005). Therefore cyanobacteria of North of Iran which will possibly be candidate for agricultural biotechnology (biofertilizer, soil-conditioners and so on) must be completely powerful to acclimate to these tensions.

At oil polluted soils of South, this condition seems more or less similar. Hard pollution of soil with petroleum especially at recent years and extension of oil polluted lands cause hard bioenvironmental problem for soils of South. Petroleum extraction industry (as our first economical source) cause serious damages to the soils (Soltani et al., 2012). Pollution of microbial mats with a layer of oil polluted water is one of these harmful effects. Carbon dioxide limitation, pH fluctuations and sharp decline in receiving light seem more or less the same as paddy-fields of North. Microbial layer must acclimate to the complex of these tensions. Using bio and phytoremediators for purification and removing of pollutions at oil polluted habitats seems a serious demand for petroleum land of South of Iran and research for finding and selection of the most potent strains have been started.

Stigonematalean cyanobacteria including *Fischerella*spp. have been found and reported from both rice fields of North and soil polluted soils of South of Iran (Siahbalaie et al.,2012, Soltani et al., 2012). Regarding this it seems logical to look at these strains especially Fischerella as suitable candidates using in both regions. In addition Fischerellashow unique potentialities including antibacterial (Ghasemi et al., 2003), biotransformation and allelopathic features (Gross et al.,1991, Falch et al.,2003, Ghasemi et al.,2004, Tabatabaii Yazdi et al.,2005). Beside these, nitrogen fixation ability and nitrogen compound liberations to soil, in addition of special morphology and spatial topology cause soil stability and fertility are the other positive properties of this species. Until now we have published data about taxonomy, ecophysiology, medical and pharmaceutical aspects of *Fischerella* which has been restricted to North. Recent floristic researches introduce *Fischerella* sp. FS 18 from south of Iran which show high frequency especially at oil-polluted soils (Soltani et al., 2012).

The aim of this research is ecophysiological and morphological characterization of common species *Fischerellaambiguae* (Strain FS18) collected from North and South of Iran in relation to combination of extremely low irradiance and carbon dioxide limitation at pH fluctuations in the border usually found in soils of both regions. It has been tried to consider basic elements of ecophysiology (growth, biochemistry, photosynthesis and nitrogenase activity) beside pigment system versatility (photosynthetic units and phycobilisome flexibility). The effect of such treatments on morphology and ultrastructure of the species has been studied too. Regarding complexity of morphological variation of stigonemataleas as a whole and Fischerella specially, beside taxonomical evaluation, topological configuration and ultrastructure features may be connected to ecophysiological acclimations and applied future purposes (agricultureand petroleum). Until now the same reports about Fischerella are rare and we could not find reports about the effects of such treatments on morphology and ecophysiology of this genus. Albertano et al (2000) have studied the effect of photosynthesis on pH variation in cyanobacteria biofilms from Roman catacombs made by Scytonema (and a little Fischerella) species using potentiometric microsensors. Results have shown that both morphological and physiological patterns changes with pH and irradiance. They have collected cyanobacteria at photosynthetic photon flux density (PPFD) below 2.5 µmol photon m^{-2} s^{-1} .As noticed before we have published some papers about taxonomy (molecular and traditional) and ecophysiology (light and pH acclimation but not combination) (Soltani et al., 2006, 2007, 2008, 2011). In this paper we used some of the previous results but prefer to choice a holistic view and avoiding repetition of details which have been published in the past.

MATERIALS AND METHODS

Isolation of strain

*Fischerella*sp. FS 18was isolated from endaphic and epilithic form in Khuzestan province (Khark Island, South of Iran and near the Persian Gulf).Complete description about the stations and their geographical and environmental conditions have been reported in Soltani et al.(2012). Epilithic forms were collected from bricks

and cement blocks which were submerged in oil-polluted waters. The collected samples were cultured by ordinary methods (Kaushik, 1987). After colonization and isolation, the cyanobacteria *Fischerella* sp. FS 18was purified and became axenic (Kaushik, 1987). Identification was done according to Geitler (1932), Desikachary (1959), Prescott (1962), Tiffany and Britton (1971), Komárek and Anagnostidis (1988), and John et al. (2003). Stock cultures were grown in N-free medium. $BG11_0$Solid medium used for culturing (materials for $BG10_0$ medium: $MgSO_4 \cdot 7H_2O$, 0.3 mM; $CaCl_2 \cdot 2H_2O$, 0.25 mM; $K_2HPO_4 \cdot 3H_2O$, 0.18 mM; $Na_2MgEDTA$, 0.003 mM, Citrate ferric 0.02 mM; Acid Citric, 0.029 mM; Na_2CO_3 0.188 mM; microelements 1 $ml \cdot L^{-1}$). The pH was then raised to 8.2 by the addition of NaOH and the solution was autoclaved. Axenicity was controlled microscopically (Shokravi and Soltani, 2011).

Culture conditions

Stock cultures were grown in the $BG11_0$ culture medium. Temperature was maintained at 30 °C and cultures were incubated under a constant light intensity of 60 µmol photon.m^{-2}.s^{-1} supplied by three florescent lamps. Cells in logarithmic phase of growth were collected from stock cultures and used as inoculate for experiments. Cells from stock culture were inoculated in 300 ml of $BG11_0$ medium in 500 ml erlenmeyer flasks stoppered with cotton plugs. Culture media were buffered with 25 mMMes (for pH 5), 2.5 mM HEPES (for pH 7) or 10 mM BTP (for pHs 9) and adjusted to the pHs with HCl or KOH. Cultures were illuminated via different numbers of nets between light source and flasks. Illumination was supplied with 40 W cool white fluorescent tubes to obtain a desired of low irradiance (2 µmol photon.m^{-2}.s^{-1}). For high irradiances (300 µmol photon.m^{-2}.s^{-1}) we move the cultures and near toward the light source. Light measurements were made with Licor LI-1000 Datalogger equipped with quantum sensor. Aliquots were taken and used for determinations, when cells adapted to light regime and pH in logarithmic phase. Finally we compared cultures without supplementary aeration or stirring (standing condition, extremely DIC limitation) (Shokravi and Soltani, 2011, Shokravi et al., 2006, Soltani et al., 2006).

Analytical methods

Growth measurements and Pigment composition

Growth was estimated as the increase in chlorophyll, as described by Anand et al (1990). Growth rate were calculated as Soltani et al. (2006). Chlorophyll content was determined spectrophotometrically at 665 nm according to Marker (1972) Phycobiliproteins were extracted after osmotic shock and measured spectrophotometrically at 652, 615, and 562 nm (Soltaniet al., 2007).

Oxygen exchange and Nitrogenase activity

Oxygen exchange was measured with a Hansatech O_2 electrode. Two ml aliquots of cell suspensions were placed in water-jacketed, temperature-controlled cuvette and placed in dark or illuminated with quantum flux densities of 2 and 300 µmol photon m^{-2} s^{-1}(which were supplied with florescent lamps). Nitrogenase activity was determined by acetylene reduction in 15 ml aliquots of cell suspensions placed in stoppered 25 ml vials. Prior to incubation 10 % of the air inside the vial was replaced with the same volume of acetylene. Cells were incubated for 1 hour under the same conditions as they were cultured. After incubation 0.5 ml of gas samples were taken and ethylene concentration was determined in a Shimadzu GC-8 gas chromatograph (Soltani et al., 2006).

Morphological variations

Morphological observations were made in liquid as well as on solid media. Thallus growth, filament structure, types of branching, position of the heterocysts, multiplication, in addition of biometrical information were recorded (Guggerand Hoffmann, 2004, Shokravi et al., 2007). Colony formation and cells shapes were evaluated by binocular and light microscope (in addition phase contrast, epifluoresscence, and electron microscopy) each day in two week's periods. For scanning electron microscopy (SEM), samples were fixed in 2.5% glutaraledehyde and washed in buffer phosphate. They were then centrifuged and dehydrated in successively increasing concentrations of methanol (10%, 30%, 50%, 70% and 100%). Finally, all samples were mounted on metal stubs and coated with a layer of gold (Soltani et al.,2010, 2012).

Statistical analysis

Data are the means and standard deviation of at least four replicates. Statistical differences were examined using the ANOVA test.

RESULTS AND DISCUSSION

DIC limitation could not change the pattern of biomass productionin *Fischerella* sp. FS 18 (dry weight and chlorophyll, dry weight not shown).pH 7 and limited irradiance (beside extremely limited DIC), caused the maximum biomass production (Fig.1) similar to relatively limited and non-limited DIC conditions (Soltani et al., 2006). The role of pHwas more outstanding than irradiance. pH 9 caused decline in biomass production. At relatively limited DIC, growth at pH7 and pH9 were almost the same regardless of irradiance. Extremely limited

DIC changed the patternand difference between biomass production between pH 7 and 9 turned to significance (ANOVA, $p<0.05$). We have no information about biomass production at the first day after inoculation. Figure 1 shows that at pH 7,cyanobacteriumhas had no problem for adaptation to both irradiances and DIC limitation. pH 9 possibly caused problems at this special time. This is not compatible with Soltani et al (2006). Naturally this strain could not survive at acidic conditions. Irradiance and DIC fluctuations had no effect on this. This is compatible with our previous results (Soltani et al.,2006; Soltani et al.,2011).

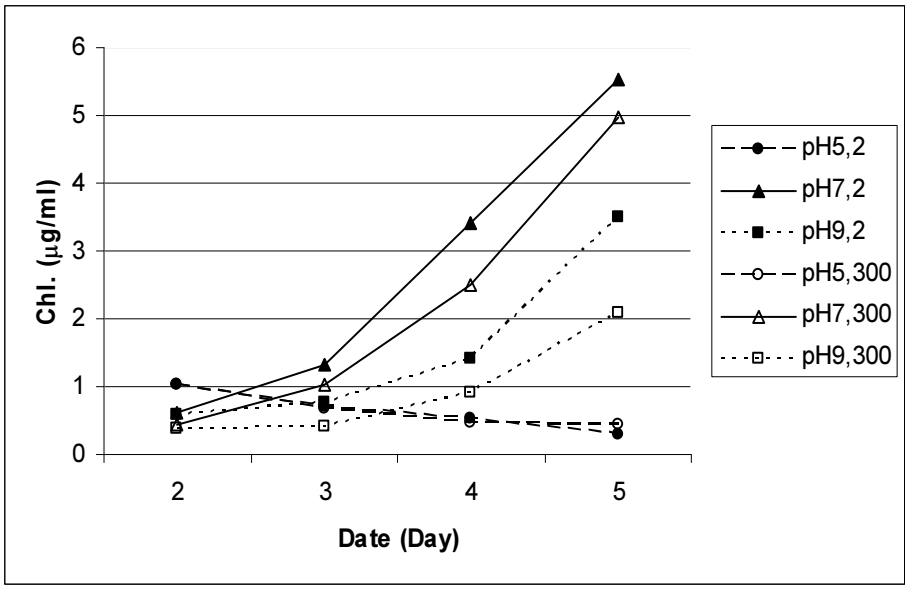

Figure 1. Comparison of different growth curves in different light intensities.

Sensitivity of pigment production and photosynthesis apparatus to extremely limited irradiance seems noticeable. In Soltani et al., (2006) the amount of chlorophyll production at relatively limited DIC (aeration condition) was about 11.99 ug.mg dw^{-1} (at 3 $\mu E.m^{-2}.s^{-1}$) and 8.32 ug.mg dw^{-1} (at 300 $\mu E.m^{-2}.s^{-1}$). Decreasing irradiance to 2 (instead of 3 $\mu E.m^{-2}.s^{-1}$) and limitation of DIC to non-aeration conditions caused different amount of chlorophyll production especially at pH 9 (Fig.2). We tested behaviorsat 24 $\mu E.m^{-2}.s^{-1}$ for better understanding the patterns but could not see significant difference between 2 and 24 $\mu E.m^{-2}.s^{-1}$. So sensitivity to DIC and irradiance seems more dependent to pH than irradiance (Fig 2.). Beside this, the pattern of chlorophyll production were different with on limited and relatively limited (Soltani et al., 2006) Fig 2. shows that the higher amount of chlorophyll production at pH7 belong to $2\mu E.m^{-2}.s^{-1}$ when DIC is not limited but to 300 $\mu E.m^{-2}.s^{-1}$ when DIC is extremely limited. This was also true for pH9. We could hypothesized that DIC concentration not only affect theamount of chlorophyll production but also ondegrees of the influence of pH and irradiance. It may be possibly species specific characteristics of photosynthesis machinery which help dominance of the strain at DIC concentration tensions especially at different environmental conditions include light and alkalinity (Deblois et al., 2013).Taylor et al (2004) showed that at *Synechococcus elongates* cells grown bubbled with air (approximately 370 mmolCO2 mol) induced a high-affinity CCM with a Km of14 mmolCi, which maintained growth rates nearlyas high as *S. elongatus* cells grown bubbled with50,000 mmol CO2 mol21 air, which had a Km of 281 mmolCi. Thus, synthesis and maintenance of the CCMrequires significant investments and rearrangementsfor cells growing in low-Ci environments, but nonetheless, under steady light and nutrient supplies, low-Ci cells can maintain photosynthesis and growth at levels comparable to high-Ci cells without the sameenergetic and metabolic constraints of the inducedCCM. They hypothesized that the induced CCMs in low-Cicells would, however, constrain the rate and amplitudeof light acclimation (Taylor et al.,2004).

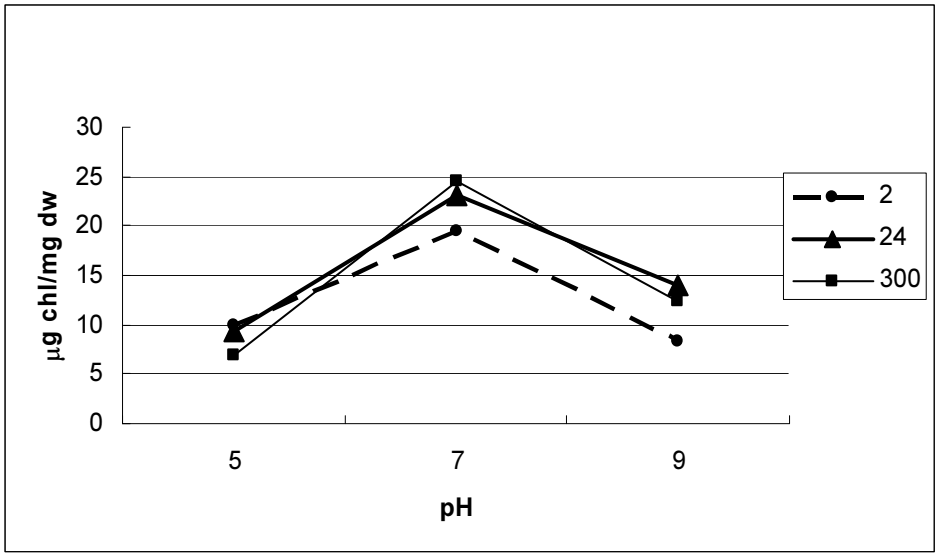

Figure 2. Chlorophyll concentration at pHs and different light intensities.

As we know among soil properties of paddy field, pH is a very important factor in growth, establishment and diversity of cyanobacteria which have generally been reported to prefer neutral to slightly alkaline pH for optimum growth (Poza-Carion et al., 2001;Soltani et al., 2006Soltani et al.,2011, Pakzad et al.,2012). Soil pH is also known to have a selective effect on the indigenous algal flora(Padhi et al., 2010). We have no special information about behaviors of different strains of Fischerella to pH fluctuations but specialty (at species level) has been reported for other cyanobacteria. For example Padhi et al (2010) have studied the effect of pH on biomass and NR activity of different species of *Anabaena* and have shown almost special behaviors of each species. Howevermost of cyanobacteria include *Fischerella* sp. FS 18 prefer neutral to alkaline environments (Poza-Carion et al, 2001; Soltani et al., 2006,2011). The amount of oxygen liberation normalized to dry weight at the first days after inoculation at different pHs has been shown in table1. Increase in production may be influenced by pH combined with irradiancesand time except acidic conditions that showedlower rates comparing neutral and alkaline conditions. At the second day after inoculation, cyanobacterium showed high amount of photosynthesis compatible with absence of lag phase in growth curve (Fig.1). Neutral condition caused relatively regular increase but behavior at alkaline condition was not regular and declined at third day after inoculation though matter production ability was higher at the second comparing fourth day after inoculation. Long time photosynthetic rate of this strain whichwas more than other cyanobacteria collected from South of Iran at combination of DIC, pH and irradiance (data not shown) reveal that adaptation potentiality.

Table 1.Photsynthesis contents in different pHs at 2 µmol photon $m^{-2}s^{-1}$ (nmol O_2/mg dw.min).

Date	pH 5	pH 7	pH 9
2th	4.18±2.14	12.53±0.3	26.16±12.4
3th	4.55±3.5	18.74±9.4	9.6±4.5
4th	4.903±3.1	4.71±4.1	27.92±12.7

Position of light harvesting complex especially phycobilisomes (Table5) reveal that phycobillins (collectively) and each part of phycobilisome (separately) was higher at neutral condition (Fig.5). It was noticeable that increasing irradiance although cause decrease of phycobiliprotein production, but at extremely limited DIC concentration, the role of pH was more prominent than relatively available situation (Soltani et al., 2006). Difference between PBP at neutral condition was not significant at both low and high irradiances. At alkaline condition (pH9) the amount of PBP decreased significantly and difference between low and high irradiances turned to significant (Fig.5). The lowest rate of PBP belonged to acidic condition (pH5) which is compatible with the survival and growth behavior at this condition. This pattern is more or less similar with two strains of native rice-field cyanobacteria *Nostoc* sp. JAH 109 (Shokravi et al.,2006) and*Hapalosiphon* sp. FS 44 (Shokravi et al., 2010), but not with another native one *Hapalosiphon* sp. FS 56 (Shokravi et al., 2011) and so may be regarded as a species specific trait.

Beside chlorophyll, the size of antenna and number of phycobilisomes per unit area of thylakoid membrane are essential for photo acclimation as well as relationships between photosystems (Tandeau et., 1993; Reuter and Muller, 1993; Yamamaka and Glazer 1981).Chlorophyll contentwas – significantly- higher at neutral but the rate of photosynthesis increased at alkaline condition especially at fourth day after inoculation. We

considered two possibilities. The first, chlorophyll production rate may be changed by time and place. At the second, photosynthesis unit and reaction centre production of this strain may be versatile and flexibleinfluencedby time and environmental conditions and would be rearranged continuously depending on the conditional changes. Rode and core parts showed higher amount at these conditions. Beside this, both the length of phycobilisomses and PSII/PSI ratio were significantly higher at this (neutral) condition comparing alkaline. Considering this, both possibilities seem logical (Tables 3 and 4). At neutral condition the amount of photosynthetic units and meanwhile the length of units increased which causedincrease of efficiency of photosynthesis, both reaction centers and light harvesting complex systems. This increase is not compatiblewith decrease of acclimationability at neutral condition and light harvesting systems (and photosynthesis units) seem higher than the other cyanobacteria which collected from South (not shown). This may be the main reason of preserving survival ability and photosynthesis power at this special (neutral) condition which may be reflected in growth curve. In Kromkamp et al (2001) it has been emphasized that upon transfer of *Planktothrixrubescens* to high light, the quantum requirement increased which was interpreted as inactivation of PSII units which is compatible with our results.

Better understanding of this may be possible using photosynthetic-irradiance curves. Results (Figs.3 and 4) showed that although maximum photosynthesis normalized to chlorophyll was higher at neutral condition, degree of adaptation with limited irradiance and consumed energy need reaching to maximum photosynthesis increased at this condition.(Table2). Result of studies per biomass (not shown) revealed similarly thatdegree of adaptation to limited irradiances (reaching to highest degree of photosynthesis) was higher at neutral condition. It seems that efficiency of photosynthesis system increases at alkaline condition.We could not observe photoinhibition even at extremely high light intensities (more than 2000 $\mu E.m^{-2}.s^{-}$). This is the same as relatively limited and non limited DIC concentration conditions at different pHs, irradiances and even nitrogen sources (Soltani et al, 2010, 2009). So may be regarded as a stable physiological trait in this strain and may be a revolutionary trait which returns to the past (possibly at the ancestors which may live at completely different environmental conditions).

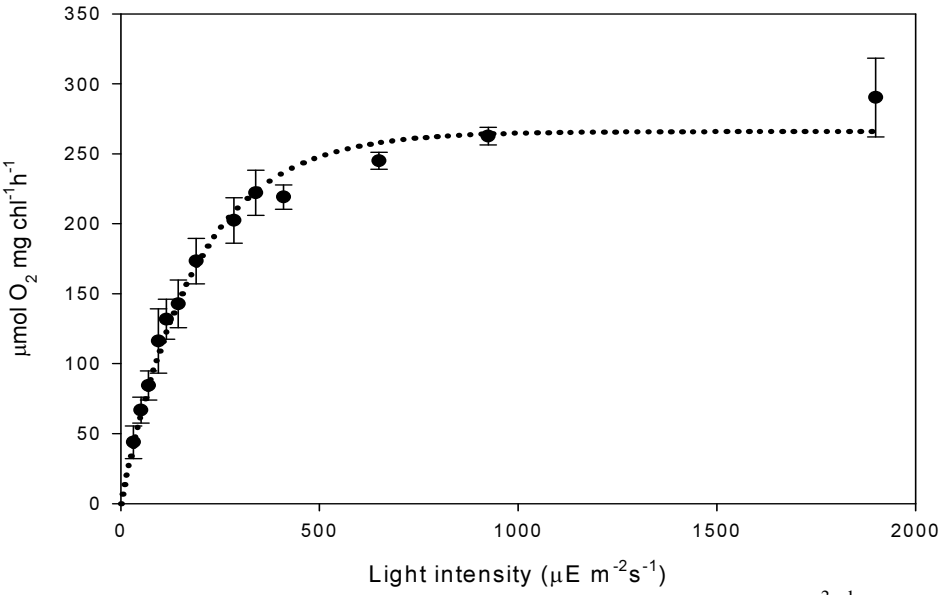

Figure 3. Photosynthesis rate vs light intensities at pH7 and 2 $\mu E\ m^{-2}s^{-1}$.

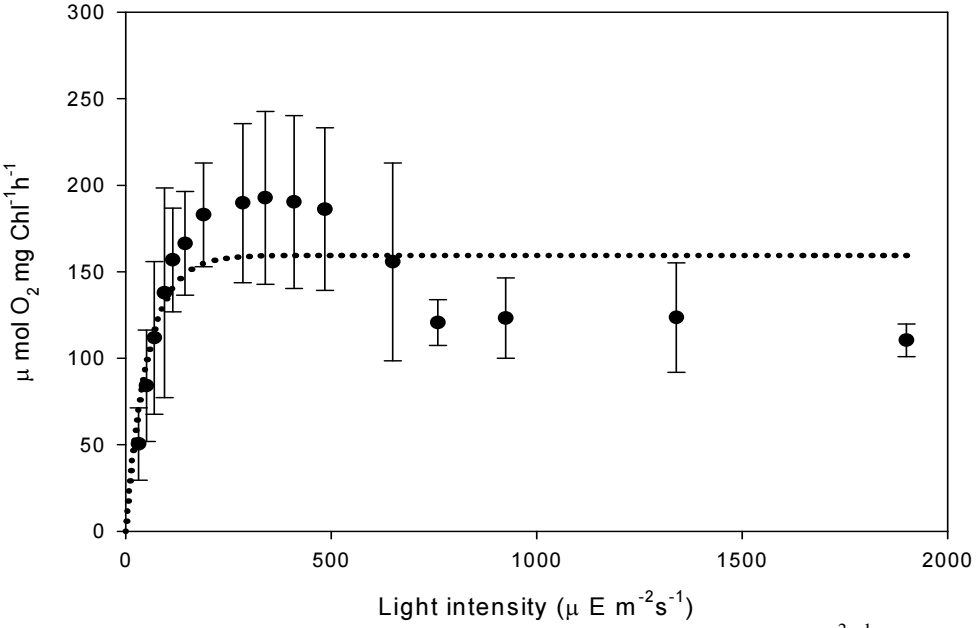

Figure 4. Photosynthesis rate vs light intensities at pH9 and 2 μE $m^{-2}s^{-1}$.

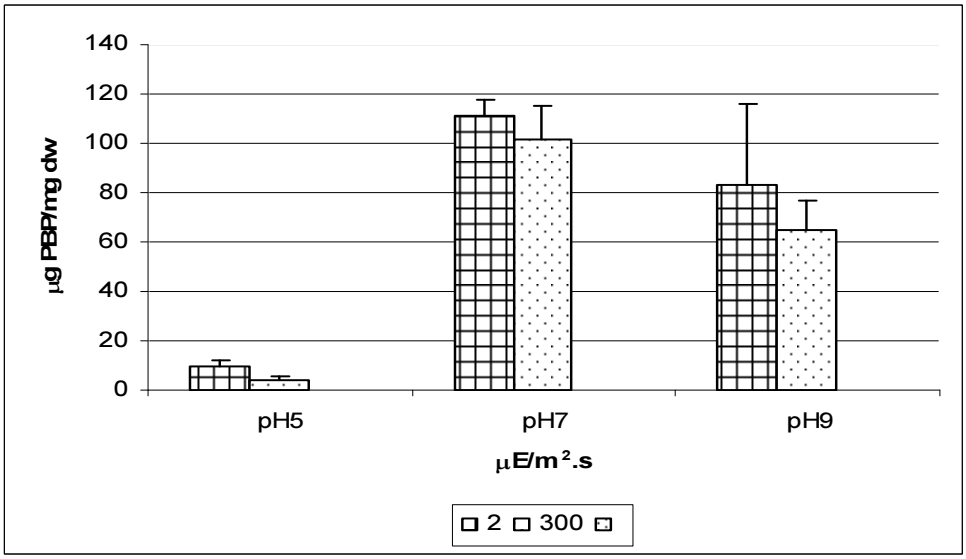

Figure 5. Phycobiliprotein concentration at pHs and different light intensities

Table 2. Effect of combination of two pH valμEs (7, 9) on photosynthetic parameters of *Fischerllaambigua* strain FS18 grown under 2 μmol photon $m^{-2}s^{-1}$. Data are means of three experiments ± SD.

pH	Irradiance μmol photon $m^{-2}s^{-1}$	Pmax μmol O_2 mg $chl^{-1}h^{-1}$	α	I_k
7	2	265.9±10.4	1.4±0.1	**189.9**
9	2	159.5±10.2	2.9±0.8	**55**

Table 3.Phycobiliproteins contents in different pHs at 2 μmol photon $m^{-2}s^{-1}$ (μg/mg dw).

	pH 5	pH 7	pH 9
APC	0	12.43 ± 1.41	7.805±9.43
PC	14.99 ± 1.14	91.02 ± 6.10	65.68 ± 21.44
PE	0	7.73 ± 1.03	5.71 ± 4.9

Table 4. Effect of combination of two pH values (7, 9) and two irradiances (2,300 µmol photon $m^{-2}s^{-1}$) on (PC+PE)/APC and APC/Chlorophyll ratios, of *Fischerellaambigua* strain FS18 grown under the above conditions.

Culture conditions		(PC+PE)/APC	APC/Chla
pH	µmol photon $m^{-2}s^{-1}$		
5	2	-	-
7	2	7.98 ± 0.48	0.51 ± 0.03
9	2	4.74 ± 3.70	2.19 ± 1.97

Pattern of nitrogenase activity especially at alkaline condition was similar to photosynthesis (table5). It seems that 4th day after inoculation may be considered as a special period of time. Both nitrogenase and photosynthesis activity increased at this day at alkaline condition. It is not complex from physiological point of view. Nitrogenase activity needs equipments and compartments which must be provided by photosynthesis and significant increase of photosynthesis activity may be regarded as a vast source of energy and reluctant production for nitrogenase activity. It is considerable that both photosynthesis and nitrogenase activity turned to the higher rates at the second day, declined at third day and then increased again reaching to significant higher amount at the fourth day. This pattern is almost at the opposite of neutral condition. Acclimation of nitrogenase activity was considerably more difficult at the second day after inoculation. This may root in the higher velocity of making different parts of nitrogenase equipment's and compartments at limited irradiance and it need no more time for making and arrangements. This may be compatible with absence of lag phase in the growth curve.

Table 5. Comparison of nitrogenase contents between pHs 7 and 9 at 2 µmol photon $m^{-2}s^{-1}$ (nmol C_2h_4/mg dw.h^{-1}).

Date	pH 7	pH 9
2th	242.1±26.5	88.618±17.5
3th	54.8±9.31	85.07±22.1
4th	44.2±10.9	212.79±113.7

However from applied point of view, high nitrogenase activity at alkaline condition, extremely limited DIC and irradiance may be regarded as a box of benefits for this strain. Considering that high amount of nitrogenous compounds must be provided by ammonium production and liberation pathway from atmospheric nitrogen, and considering essentiality of nitrogenous compounds for ecosystem equilibrium, it seems logical to believe that cyanobacterium has no problem at limited irradiance, DIC and alkalinityfluctuations which show its potentiality. Regarding border of alkalinity of Iranian soils (paddy-fields at North and oil polluted regions at the South); this ability may be noticed as a positive applied pointfor future purposes including phycoremediation and biofertilizer technologies approaches (Subashchandrabose et al., 2013, Soltani et al.,2006).

Acclimation to low or high light involves short to long term adjustment of the photosynthetic apparatus as well as changes in cell ultrastructure (Deblois, 2013) and morphology (Gao et al., 2007, Andressen et al.,2010).Gao et al (2007) studied the effects of solar radiation on growth, photosynthesis activity, trichome morphology and heterocyst differentiation of *Anabaens* sp. PCC 7120,emphasized that there was no effect on cell size but changes in filament length were observed and heterocyst formation was enhanced under elevated PAR levels that stimulated quantum yield and growth after an initial inhibition. In Andressen et al (2010), *Trichodesmiumerythraeum* ISM 101 has been considered physiologically, biochemically, biophysically and briefly morphologically at high and low irradiances. Results showed different appearances of the cultures and Individual cells. The color of aggregations and dimension of the trichomes and the cells has been related to high and light irradiances. In addition it has been shown that cells acclimated to extremely low irradiances were less vacuolated and filled with more thylakoids instead. In Albertano et al. (2000) it has been noticed that morphological and ultrastructural analysis of the microbial communities developed on mineral surfaces of Roman including Fischerella must be noticed beside physiological and ecophysiological studies. In Tezano Pinto and Litchan (2010), morphological variations of *Aphanizomenongracile*, *Anabaenaminderi* and *Anabaena torques-reginae*, has been studied briefly beside analyzing ecophysiological responses of the strains to light and has shown that there is strong relationships between acclimation to irradiances and morphological variations.

Species of stigonemataleancyanophytes (cyanobacteria) are distributed allover the world but mainly scarcely and strictly in special (extreme) biotopes (Anagnostidisand Komarek, 1990). Consequently, they are traditionally considered cosmopolitan micro-organisms with remarkable capabilities to acclimatize to broad ranges of environmental conditions (Geitler, 1932). Genera of the stigonematales exhibit the highest degree of morphological complexity and differentiation within the cyanobacteria (AnagnostidisandKomarek, 1990, Castenholz, 2001). Many populations of stigonematales show considerable morphological variation, even at one

site (John et al. 2003). The complex variety of forms or developmental stages exhibited by Fischerella, which may include primary and secondary trichomes, hormogonia, unicells and amorphous cell aggregates. As we know this variations is not confined to the strains (Castenholz, 2001).However, morphology based classification may provide insufficient taxonomic resolution and cyanobacteria with similar or identical morphology may have significantly different physiology (Soltani et al., 2011). In recent years, the analysis of 16S rRNA gene sequences has demonstrated that morphological groupings of cyanobacteria in some cases correspond to phylogenetically coherent taxa (Shokraviet al.2011), whereas in others the traditional classification drastically underestimates extant diversity (GuggerandHoffmann2004). In Soltani et al (2011) we proposed a new description of this strain using multidisciplinary approaches including molecular and physiological analysis. Since our morphological data at that paper had been based on relatively DIC limitation (not extremely limited), salinity and temperature fluctuations; in this paper we try to add morphological data considering combination of extremely DIC limited conditions beside pHs and Irradiances flactuations which may complete the previous one. Although difference in vegetative cell dimensions may be affected by irradiance, acidity and alkalinity but this effect is more obvious at the length of the cell. Vegetative cell diameter may be affected less both at main axis and branches by irradiance. We can say length of vegetative cell decrease by irradiance but it has no affect on diameter. Acidity or alkalinity has no effect on this pattern except possibly at high irradiance. At low irradiance cellswere more stable and less affected by pH fluctuations. This pattern seemed true for heterocyst of the main branches with slight differences. Higher irradiance caused increase of the length of heterocysts but had no significant effect on the diameter. This was true for branches although at neutral conditions they showed the highest length. Collectively biometrical results showed that comparing neutral and alkaline conditions, except diameter of the heterocyst at the branches, decrease of light intensity cause decrease in cell dimensions regardless of acidity and alkalinity (Table 8).

Table6. Biometrical data (Length x Diameter by micrometer) of vegetative cells of main axis (VCM), branches (VCB), heterocysts of the main axis (HCM) and branches (HCB) at different pHs and Irradiances (2 and 300 µE $m^{-2}s^{-1}$).

	PH5	PH7	PH9
VCM (300)	17 x 8	12.5 x 6.5	9 x 8
VCM (2)	9.2 x 4	9.5 x 6.5	10 x 6.5
VCB (300)	16 x 5.6	12.5 x 4	15.5 x 4
VCB (2)	10.7 x 4	10 x 5	11.25 x 4
HM (300)	16 x 6	14 x 6.5	13.5 x 7
HM (2)		11.5 x 6.75	10.25 x 6
HB(300)	14.5 x 6	12.5 x 4.5	12 x 5
HB(2)		14 x 4	11.75 x 5

It seems that during 4th day after inoculation, pH fluctuations caused noticeable changes in the morphology of the organism. The highest and lowest pHs (pH9 and pH5) seem the points of highest variations. On solid medium, like other stigonematalean isolates, this strain showed creeping growth behavior. This was in agreement with other papers (Guggerand Hoffmann, 2004). In high acidic condition (pH5), at the first week (4th day after inoculation), conspicuous morphological changes were seen in both irradiances (Fig.6). In low light condition (2 µE $m^{-2}s^{-1}$), cells were going to degenerate very soon, but the rate of degeneration seemed slower in comparison with high (300 µE $m^{-2}s^{-1}$)irradiances. At these light conditions, cells were going to change their form and we had no swelling of the cells.In the second week, irradiances could not avoid degeneration even in high acidic condition. At the last days of the second week(pH 5); the cell shapes were changed and formed banana shapecells (Fig7). The cytoplasm in these cells were small and located in center of thecells. Also there could be seen some enlarged and round cells which deformed and their cytoplasms are decreased. In the case of cell shapes, in pH 5, at the end of second week, 70%of filaments were degenerated and their colors changed to yellow. There was no indication of swelling in germination placements. Also hormogonia were not seen. These are completely the same as relatively DIC limitation experiments (Soltani et al., 2011).

Figure 6.Degeneration of cells in pH 5.

Figure 7. Degenerated cells with banana shape.

At pH 7; cells were in normal condition at the first week. Germination were complete (Fig.10). Hormogonia were seen and even some of them were released and it seemed that the sehormogonia formed branches after maturation (Fig8). At the second week, 300 µE m^{-2} s^{-1}irradiance condition, caused absence of hormogonia and this may be considered as Young filaments germination. At pH 9, the cells were enlarged and granulated and their cytoplasm was gathered in peripheral zone or centre of cells. In some sections of branches, cell sizes were decreased and formed hormogonia. In future these would be released in medium. Alsonecridia were formed in some of the filaments (Figs9,11). This was more or less similar with relatively limited DIC concentration conditions (Soltani et al.,2011).

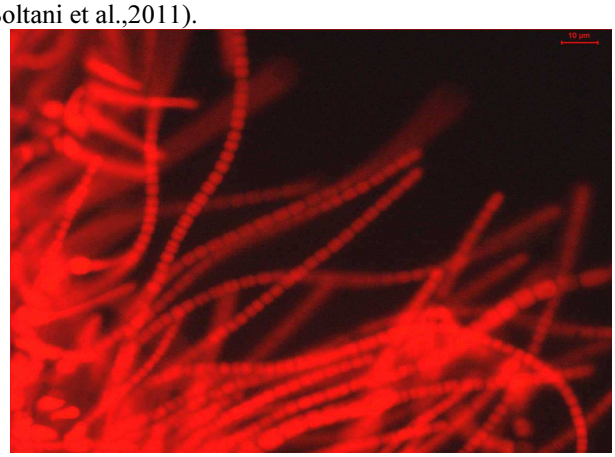

Figure 8. Hormogonia release in pH 7.

Figure 9.Necridia formed in filaments

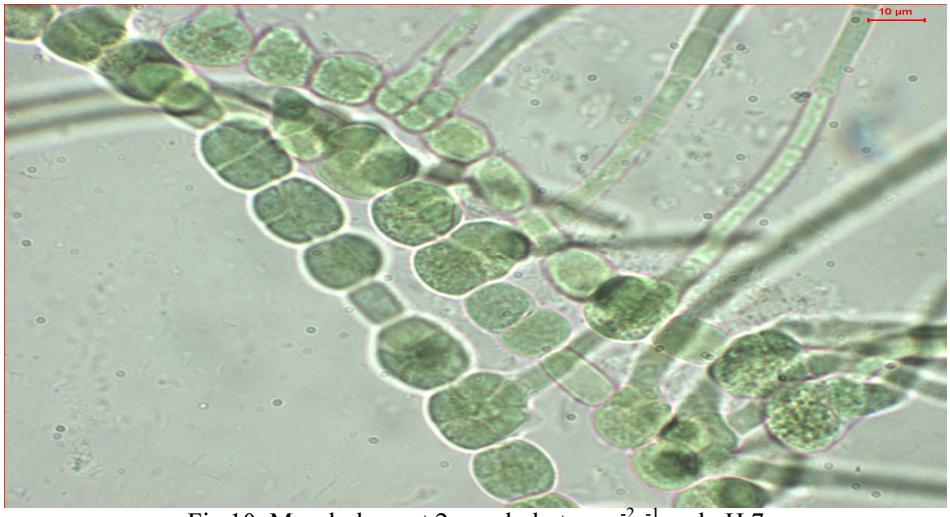

Fig 10. Morphology at 2 µmol photon $m^{-2}s^{-1}$ and pH 7

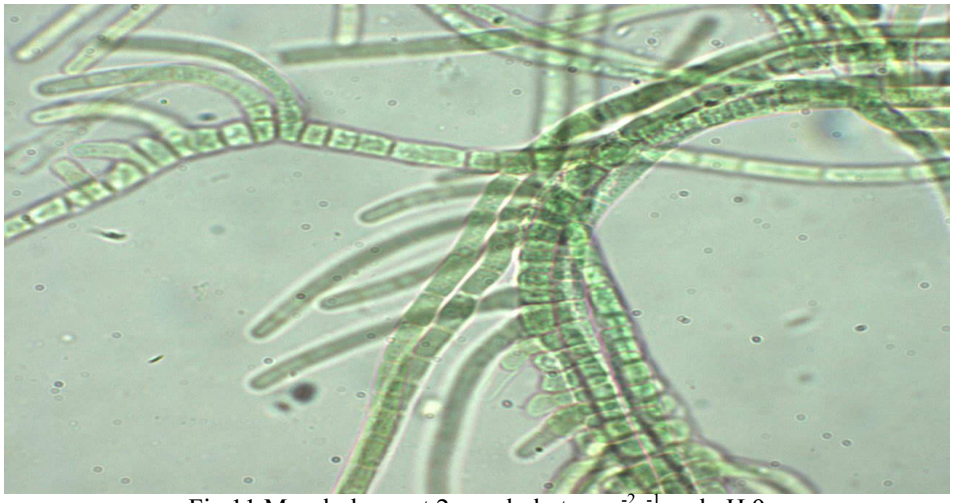

Fig 11.Morphology at 2 µmol photon $m^{-2}s^{-1}$ and pH 9

According to colonization, there was no colony (just a little) at pH 5 and 300 µE m^{-2} s^{-1}. With increasing pH, the growth and colonization rate increased. Same results were achieved in altered light intensities. As other cyanobacteria *Fischerella* sp. FS 18 prefer shadow. Decreasing light intensity caused better colonization. In branches, significant differences among width of heterocysts (ANOVA $p<0.05$) could be seen in 300µE m^{-2} s^{-1} and pH9 conditions. High acidic condition had a remarkable inhibitory effect on the ability of germination.In pH 5, for instance, there was no growth (but slightly in conditions like 300 µE m^{-2} s^{-1}).In low light intensities, there was no hormogonium production at different pHs. This was true for both the first and second weeks. It has been emphasized that when mature cultures are inoculated on agar-solidified medium motile hormogonia are readily formed and easily isolated after migration on agar (Castenholz, 2001), but this situation could not be seen in all the acidities and irradiances. It seems that the potential of branch producing (especially main axes) decreased sharply in these conditions. In comparison with growth curves, it seemed that growth of the organism at least in neutral acidity, was not compatible with hormogonium production ability. In 4th day, low light intensities caused more growth (or at least equal) than high light intensities. It is in agreement with Castenholz (2001), who emphasized that in nonthermophilic strains of *Fischerella*, hormogonia were not always formed under favorite conditions and sometimes the multiseriate axis was rare or lacking.

TEM photomicrographs analysis of cyanobacteria is not common in Iran. Until now only Soltani et al. (2011) and Pakzad et al (2012) have used this kind of studies forultrastracture variation analysis of some strains of stigonemataceae and nostocaceae. However result of this research showed relatively linear and parallel arrangements of thylakoids at pH 7 extremely limited irradiance and carbon dioxide concentrations (Fig.12). This pattern was more or less different at other conditions (Fig.15), but need more researches to draw a regular pattern for thylakoid arrangements at different treatments. Thylakoids tended to move toward the edge of the cell and this behavior is not compatible with the low irradiances. So it may be reflection of the combination of treatments but as pointed above it seems soon for any judgment. In Soltani et al (2011) the situation of thylakoids was more or less similar but theirdistribution tends toward central parts of the cell even at extremely

limited irradiances. So it is logical to think about the effect of DIC concentration on the position of thylakoids and their spatial distribution. SEM photomicrographs showed some holes and ornamentations at–especially – main axis at pHs 7 and 9 (Figs 13, 14) at both high and low irradiances.

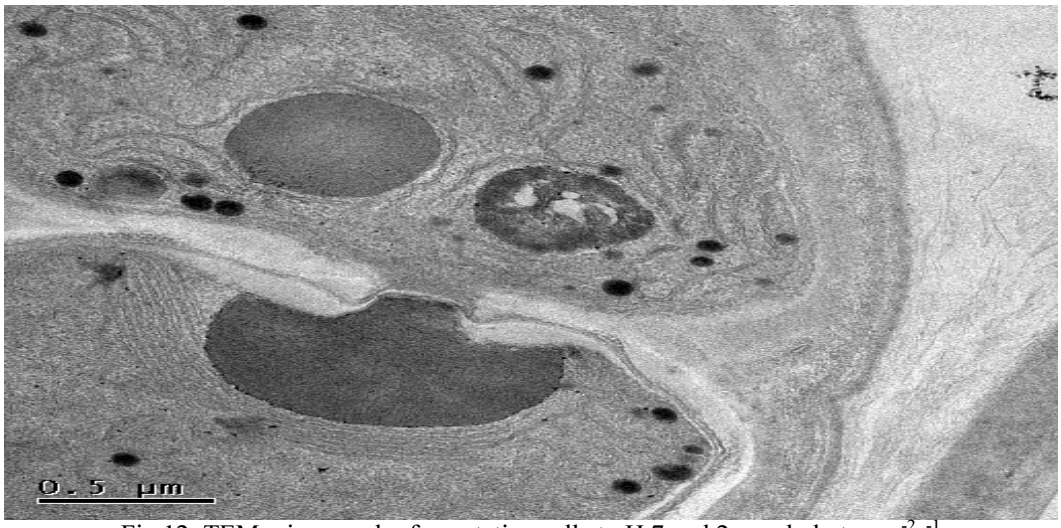

Fig 12. TEM micrograph of vegetative cell at pH 7 and 2 µmol photon $m^{-2}s^{-1}$

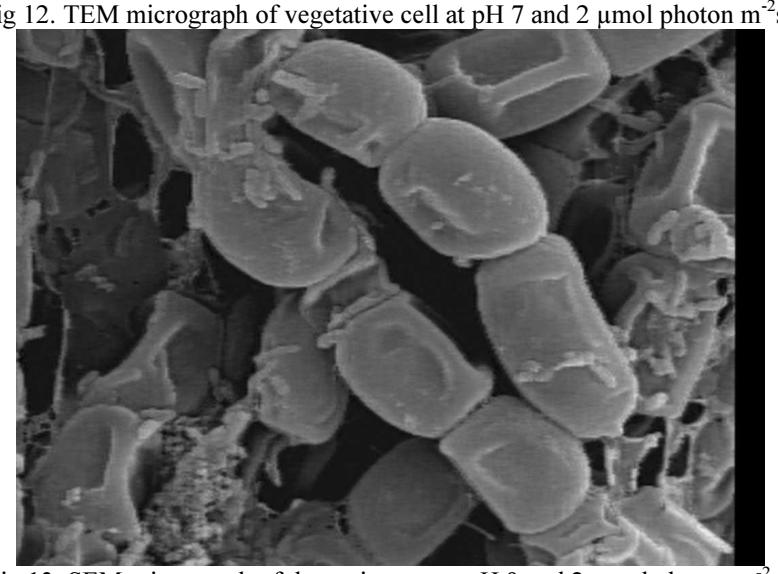

Fig 13. SEM micrograph of the main axes at pH 9 and 2 µmol photon $m^{-2}s^{-1}$

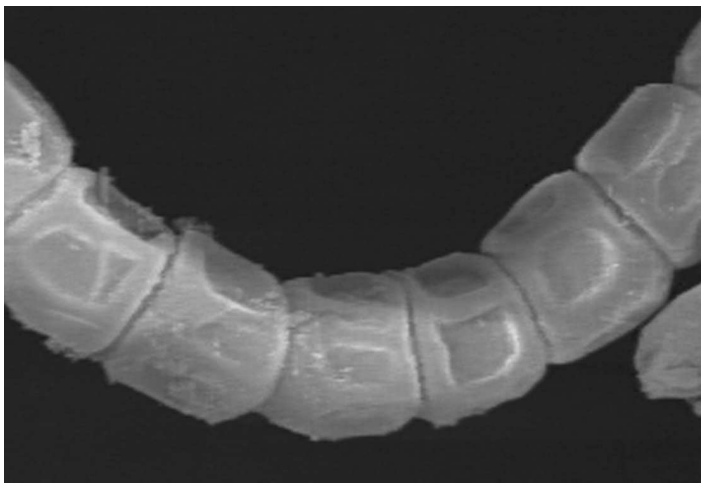

Fig 14. SEM micrograph of the main axes at pH 7 and 300 µmol photon $m^{-2}s^{-1}$

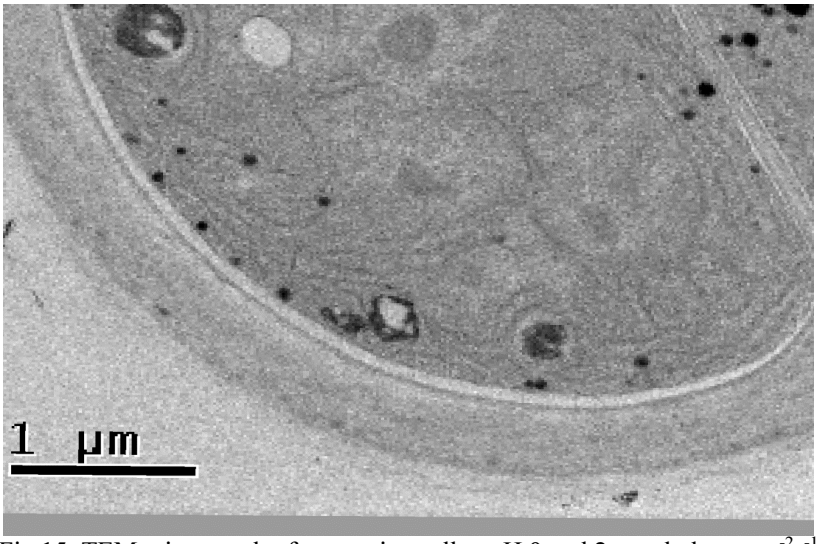

Fig 15. TEM micrograph of vegetative cell at pH 9 and 2 µmol photon $m^{-2}s^{-1}$

Vegetative cells at both conditions were not uniform and have their special ornamentations and topology. In Iranshahi et al (unpublished data) the effect of salinity and petroleum on the vegetative cell shapes were different and at high degree of salinity cells tended to select oval-cylindrical and oval shapes that is different with the shape influenced by pH and irradiances. However we guess this kind of holes and configuration of vegetative cell must be a constitutive trait because seems similar in North and South strains and apparently may not be influenced by geographical and environmental differences. This is a new finding and it seems soon to discuss about it. However we could observe this pattern in many micrographs at different conditions but until now has not reported about the other strains of Stigonematales (Shokravians Soltani, 2011) and even *Fischerella* sp. FS 18 at not limited carbon dioxide concentration (Soltani et al.,2011). The role of DIC at least at the first step seems basic but need time and more researches for any discussion.

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