UNIVERSITY OF SOUTH BOHEMIA
FACULTY OF BIOLOGICAL SCIENCES

OSMOPROTECTANTS IN TWO STRAINS OF CYANOBACTERIA: *SYNECHOCYSTIS* CF. *SALINA* (NaCl) AND *ARTHRONEMA AFRICANUM* ($MgSO_4$)

MASTER THESIS

Eliška Hořejší
2007

Supervisor: Ing. Jiří Kopecký CSc.

Guarantor: Prof. RNDr. Jiří Komárek, DrSc.

I hereby state that the following master thesis is of my own original work, any literature or other sources used are fully referenced and listed in the References.

Prohlašuji, že svoji diplomovou práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své diplomové práce, a to v nezkrácené podobě, biologickou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

Datum.

Podpis studenta

“How surprising it is that any creatures should be able to exist in a fluid, saturated with brine, and that they should be crawling among crystals of sulphate of soda and lime!”

Charles Darwin

from OREN (2002)

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

First of all I am grateful to my supervisor, Ing. Jiří Kopecký CSc., whose advice was a great help for me, also for teaching me so much about osmoprotectant analysis, for granting me all equipment necessary and for devoting his time to me and my work. Furthermore I am very indebted to my dear parents for standing by me all during my studies. Many thanks belong also to my boyfriend for his patience. My thanks also to Zac for the proofreading. And,last but not least, I want to thank all the team in our laboratory (namely Pavel and Jana), as well as our lab assistant Lada for her help with cultivation treatment.

AIMS OF THE STUDY

I. Estimation of the optimal concentration of $MgSO_4$ for *Arthronema africanum* by the method of microassay; assessment of cultivation conditions suitable for experiments of osmoprotectant detection in *Synechocystis* cf. *salina* (in addition of NaCl) and *A. africanum* (in addition of $MgSO_4$).

II. Establishment of suitable method for extraction, detection and identification of osmoprotectants in *S.* cf. *salina* and *A. africanum*; analysis of intracellular extracts from various concentrations of NaCl (*S.* cf. *salina*) and $MgSO_4$ (*A. africanum*) for osmoprotectants.

III. Comparison of osmoprotectants in the two chosen strains of cyanobacteria under NaCl and $MgSO_4$ stress; observation of the amount of osmoprotectants along increasing salt concentrations and along growth curves.

IV. Appraisal of morphology of *A. africanum* along the $MgSO_4$ gradient.

I. INTRODUCTION

I. 1. SECONDARY METABOLITES IN OSMOTIC STRESS; COMPATIBLE SOLUTES

The fact that cells of various organisms under osmotic stress synthesize various compounds to protect themselves against hyperosmotic shock has been well known for a long time. These substances are either stock piled inside cells or emited into the surrounding environment. The function of these cell metabolites is not necessarily just single osmoprotection, they can often take over various other key roles in cell survival.

Compatible solutes (CS) (OREN,1999, 2002) is one of the most important terms used by the majority of authors for characteristic group of compounds involved in cell osmoprotection. CS are soluble chemical substances of an organic character, enhancing osmotic value of cytoplasm. The intracellular concentration of anorganic salts remains at low level. No adaptation of internal cell systems is needed. Small organic low molecular molecules are synthesized not only inside the cell itself but also taken, if possible, from surrounding medium. General definition of CS: soluble substances allowing the effective function of enzymes, even when present in high concentrations.

There is a wide diversity among the types of these compounds. The main groups of CS are; sugars and their derivatives (especially alcohols), amino-acids and derivatives and also methylamines. Various types of CS are utilized by organisms in osmotic stress, from bacteria to vertebrae. Many halophilic/halotolerant organisms are able to synthesize and use more than one CS. So there is a mixture of osmoprotectants in their cytoplasm and the regulation of synthesis is optimized by exact needs of the cell. However, they primarily prefer one CS.

The term “compatible solutes” is most widely used. However, there are some authors disputing that this term is, in many cases, obsolete and not exact considering their influence on macromolecules. YANCEY (2005) suggests that more appropriate term would be “counteracting solutes” or GILLES’s (1997) term “compensatory solutes”. WELCH & BROWN (1996) call these substances “chemical chaperones” in the respect of their assay (more information further in the part I. 4.)

I. 2. COMPATIBLE SOLUTES IN CYANOBACTERIA

The preference of certain compatible solute by cyanobacteria depends largerly on the degree of their halotolerance as rewieved by many authors (e. g. OREN,1999, 2002, MARGESIN & SHINNER, 2001, YANCEY, 2005). A wide range of substances of specific properties from chemical groups mentioned above occur in cyanobacterial cells. Further minutely described CS represent the most important of them which are the main osmoprotectants in all halophilic/halotolerant cyanobacteria studied thus far. Others are just part of the CS "cocktail" in the cells. From these minor osmoprotectants we can mention for example ectoine, proline or fructose (OREN,1999, 2002, SALERNO et al., 2004, YANCEY, 2005).

A) Sugars and derivatives

Saccharose and trehalose (SACH/TRE) are the main CS in the fewest halophilic/halotolerant types of cyanobacteria. This has two, most probable, reasons. Firstly, these two compounds are energetically one of the most "expensive". Secondly, SACH/TRE do not supply the activity of salt-sensitive enzymes sufficiently when present at high concentrations. They are synthesized in the cell by coupling of UDP-glucose with fructose-6-phosphate and glucose-1-phosphate with the assistance of specific enzymes. These enzymes complexes can also be used as a proof of the presence of these compounds in the cells.

For example, the activity of enzymatic complex saccharose-phosphate synthase/saccharose-phosphate phosphorylase was documented by PORCHIA & SALERNO (1996) in *Anabaena* sp. This genus produce also so called "fructose-containing oligosaccharides" as its CS (SALERNO et al., 2004). Besides *Anabaena*, the production of saccharose as CS is well documented in the species *Nostoc muscorum* (BLUMWALD & TEL-OR, 1982; PANDEY & CHATTERJEE, 1999), as another example of saccharose accumulation we can mention the genus *Synechococcus* (BLUMWALD et al., 1983). Saccharose and trehalose together with glucosyl glycerol are used as osmoprotectants also in *Synechocystis* sp., strain PCC6803 (MIKKAT et al., 1997, MARIN et al., 2004).

Cyanobacteria living in higher salt concentrations (marine and slightly halophilic) accumulate glucosyl glycerol (GG) inside their cells. This compound is formed through reaction of ADP-glucose and glycerol phosphate, with the help of enzymes GG-phosphate synthase/phosphatase. The production of GG is well documented in the strain *Synechocystis* PCC 6803; including exact path of reaction in the cell (ERDMANN et al., 1992, HAGEMANN et al., 1996, SCHOOR et al., 1996, MATSUDA et al., 2004). KEBEDE (1997) presents the occurence

of this compound, for example, in the species *Spirulina platensis* (=*Arthrospira fusiformis*). This CS can be, together with saccharose, utilized also by *Synechococcus* (OREN & SECKBACH, 2001, KARANDASHOVA et al., 2002, FERJANI et al., 2003). JUNGBLUT et al. (2005) mention that glucosyl glycerol together with trehalose is synthesized even by one antarctic cyanobacterial species of *Microcoleus*.

B) Amines

Cyanobacteria living in hypersaline environments protect their cells against osmotic stress by the synthesis of quaternary amine glycin betain formed by the oxidation of choline. Examples of such osmoprotection are; *Aphanothece halophytica* (LALOKNAM, 2006), *Halospirulina* (MOORE et al., 1987, OREN & SECKBACH, 2001), *Synechococcus* and *Arabidopsis* (WADITEE et al., 2005).

I. 3. OTHER SUBSTANCES SYNTHESIZED BY CYANOBACTERIA UNDER OSMOTIC STRESS

Interesting group of compounds often formed by halophilic cyanobacteria are mycosporines, also called mycosporin-like amino acids, or MAAs. These are water soluble, low molecular weight substances. Most probably their main aim is to protect the cell against UV radiation (GARCIA-PICHEL & CASTENHOLZ, 1993, PORTWICH & GARCIA-PICHEL, 1999, CASTENHOLZ & GARCIA-PICHEL 2000, QUESADA et al., 2001). They work mainly as UVB shield (PORTWICH & GARCIA-PICHEL, 1999, SINHA et al., 2003). However, considering their chemical properties, the possibility that they can also serve as CS cannot be excluded (PORTWICH & GARCIA-PICHEL, 1999,OREN, 1997, 2000, 2002).

At present, some studies suggest that some algae and cyanobacteria synthesize abscisic acid (ABA) under osmotic stress. Yet the function of this substance in such groups of organisms remains unclear. It is possible that the emittion of ABA into surrounding medium is a stress signal for the other cells (MARŠÁLEK et al., 1992). This substance could also influence the amount of Ca^{2+} in the cells and number of heterocytes (POTTS, 2000).

I. 4. APPLICATION OF COMPATIBLE SOLUTES

Compatible solutes are renowned as stabilizers of membranes and enzymes. Not only can they protect the cells against salt stress but they can also serve as cryoprotectants

(YANCEY, 2005), thermostabilizers (BORGES et al., 2002) or protect the cells during desiccation (OREN,1999, 2002).

There are many studies trying to explore these unique substances in order to apply them to a wide range of important branches including; cosmetics and pharmaceutical industry, cell engineering and agriculture. Under focus are not only the CS of cyanobacterial origin but also those from other organisms (algae, bacteria, Animalia) which are usually on a very similar basis chemically.

WELCH & BROWN (1996) examined CS for their ability to stabilize proteins in their natural conformation. They call these substances "chemical chaperones" and suggest that CS could be used in human medicine as "rescue" substances for some mutant regulator proteins, since they correct protein folding abnormalities in human diseases.

In agriculture CS also have a promising future. Genetically modified crop plants with some genes from CS producing organisms have an improved ability of salt and drought tolerance (CUSHMAN, 2001). Halotolerant N-fixing cyanobacteria are also used as ecological fertilizers, especially at rice fields (WHITTON, 2000, BHARGAVA et al., 2003). They can also efficiently help to ameliorate soil salinity during crop growth (MARGESIN & SHINNER, 2001); in this respect the use of *Anabaena torulosa* was proposed by APTE & THOMAS (1997).

Some assays suggest CS application in science itself. These compounds, for example, improve the crystalization of proteins (JERUZALMI & STEITZ, 1997). CS could improve and enhance production of the required substances by bacteria or enzymatic complexes through improving the conformation properties and stability of enzymes in various conditions (YANCEY, 2001). This can be useful in many scientific branches including medical science and molecular biology.

Immobilised cyanobacterial cells are suitable to produce exopolysaccharides (EPS), for this purposes *Aphanocapsa halophitica* and some strains of *Synechococcus, Anabaena* and *Cyanothece* were previously utilized (MARGESIN & SHINNER, 2001).

Besides renowned taurin (also a compatible solute from marine invertebrae) glycine-betaine and inositol are sometimes added to energy drinks (YANCEY, 2005).

All of the above mentioned features sound really promising, yet the exact principles of CS protective functions remain unrevealed in many cases so far. Also biotechnological production of CS by cyanobacteria and their wider application is at the very beginning. To utilize cyanobacteria in the comercial production of CS it is necessary to optimize all requirements, costs and profits; this challenge still remains unsolved in most cases.

I. 5. ANALYSIS OF COMPATIBLE SOLUTES

CS analysis brings some difficulties since these compounds have no UV-chromophores. Detection by spectrophotometric methods is therefore useless until some kind of derivatisation is applied (e.g. KUNTE et al., 1993, MADAJ et al., 1993, KUHLMAN & BREMER, 2002). However, the effort to simplify CS assesment is obvious. If we want to avoid time-consuming derivatisation step, a method to determine compounds with oxidable groups such as –OH;-NH2;- SH; -SR is advantageous.

Furthermore in some kinds of assays high content of salt in samples can be a serious problem and interfere. It is clear that the selection of proper analysis method is crucial to successfully assess the whole range of osmoprotectants and their content in cyanobacterial cells.

The solution extracted from natural material contains a mixture of compounds which have to be split up first. For this purposes the chromatographic methods are widely used. Especially utilized are those of HPLC (KUNTE et al., 1993, SCHOOR et al.,1995, HALLSWORTH & MAGAN 1997, BOUCHERAU et al., 1999, KUHLMAN & BREMER 2002, TOLSTIKOV & FIEHN, 2002 and many others) The greatest advantage of this method is efficiency with no need of derivatisation. Some authors however prefer anion exchange chromatography (HPAEC) (e.g. RIIS et al. 2003) or, the more protracted, gas chromatography (GC), where derivatisation step is necessary (e.g. ADAMS et al., 1999, FIEHN et al., 2000a).

After separation, the individual substances should be identified. That is usually performed by the coupling of HPLC (or other separation method) to the chosen detection method. At such a point we have more options. One of the best choices is, surely, mass spectrometry (MS) (FIEHN et al.,2000b, TOLSTIKOV & FIEHN, 2002, WOOD et al., 2002). SHAMSI & MILLER (2004) also use MS as a detection step, except in former tandem with CZE (capillary zone electrophoresis). Sensitive MS offers the ability to both quantify known target analytes whilst identifying unknown metabolites in the same run. At the same time MS is convenient for a wide range of metabolites with various chemical and physical properties. A good choice could be also that of HALLSWORTH & MAGAN (1997) who used pulsed amperometric detection (PAD) after HPLC. One very exact and specific method of determination is also NMR (BERNARD et al., 1993, KUHLMAN & BREMER, 2002), however, this method is very demanding on the purity of the substance.

II. MATERIALS AND METHODS

II. 1. STRAINS CHOSEN FOR EXPERIMENTS

We used *Synechocystis* cf. *salina* strain MARKLE/1430-3 (planktonic species from uknown locality; the salt tolerance properties previously decribed in HOŘEJŠÍ (2005)) and *Arthronema africanum* strain LUKAVSKÝ 1980/1 (benthic species of salt marsh, Kuwait; NaCl tolerance properties as previously described in HOŘEJŠÍ (2005)); both from the CCALA culture collection, Třeboň.

II. 2. MICROASSAY ON SEROLOGICAL PLATES **(LUKAVSKÝ, 1992)**

This method was used to estimate the optimal $MgSO_4$ concentration for the growth of *A. africanum*. The plates used were 12x8 sockets, with frame sockets filled with distilled water to eliminate dessication. The experimental sockets in the sterile plate were filled with 0.2 ml of a sterile media BG11 concentrated with $MgSO_4$ and inoculated by 0.01 ml of *A. africanum* solution (OD 750 nm = 0.4) in a laminar box. The chosen $MgSO_4$ concentrations gradient was following: 0-5-10-15-20-25-30-35-40-45 g $MgSO_4$/l BG11 media. Two experimental plates were inoculated to acquire 12 replicates. These inoculated plates were cultivated as described in LUKAVSKÝ (1992) and OD 750 nm was measured three times in each plate by Labsystems iEMS reader MF, type 1401. The data obtained were processed by Kruskal-Wallis ANOVA and Median test in software STATISTICA version 9.0.

II. 3. EXPERIMENTAL CULTURE CONDITIONS

Both strains were grown in BG11 media (for content of nutrients see Tab. 2., part VI, Supplementary materials) with the addition of NaCl (*S.* cf. *salina*) or $MgSO_4$ (*A. africanum*). First they were pre-cultivated in a concentration of 10 g NaCl/l (*S.* cf. *salina*) and 5 g $MgSO_4$/l (*A. africanum*) to eliminate hyperosmotic shock after long term cultivation in culture collection. After matching the stationary phase of growth, these pre-cultures were used as inocula.

Four experimental concentrations of salts were estimated for each strain. *S.* cf. *salina*: 0 (control), 15, 30, 45 g NaCl/l BG11 media; *A. africanum*: 0 (control), 10, 25, 30 g $MgSO_4$/l BG11 media (conversion into molarity further in the text below). All chemicals used were from Sigma in p. a. quality. 5 ml of each strain suspension was inoculated into 300 ml of BG 11 (either pure or with addition of salts) in sterile conditions.

Conversion of concentrations used (g/l-molarity):

NaCl: 15 g/l…….0.263 M
30 g/l…….0.526 M
45 g/l……0.789 M

$MgSO_4$: 10 g/l……0.096 M
25 g/l……0.24 M
30 g/l……0.288 M

Both strains were maintained in batch cultures in glass cylinders (volume 350 ml) in a water bath with a thermostat (cultivation temperature 28 °C), iluminated constantly by white fluorescent tubes panel (70 $W.m^{-2}$) and bubbled with 2% CO_2 in air. During the growth the evaporated volume of media was refilling with deionized water in order to keep the salts concentration at constant level. After each harvesting of part of the culture, the new level of culture volume was marked. OD 750 nm was measured (TECAN, model Sunrise) in the early growth phase every day, then later every couple of days.

II. 4. SAMPLING AND EXTRACTION OF INTRACELLULAR COMPOUNDS

Four samples of each strain in each concentration of NaCl/MgSO4 were collected along the growth curve. The first sample ,matching the end of exponential growth (or early linear growth), was harvested on the volume of 100 ml of culture solution, the second sample, matching linear growth, on the volume of 80 ml of culture solution, the third sample, matching late linear or early stationary phase of growth, on the volume of 50 ml of culture solution, as well as the fourth sample, matching stationary phase of growth. As the growth rate varied in some concentrations, we had to collect samples in various days of growth to match the recquired phase of growth. The sampling days were as following:

A. africanum		*S.* cf. *salina*	
$MgSO_4$ concentration	sampling days	NaCl concentration	sampling days
0 g/l	10, 14, 17, 28	0 g/l	5, 7, 12, 17
10 g/l	10, 14, 17, 28	15 g/l	5, 7, 12, 17
25 g/l	10, 14, 17, 28	30 g/l	5, 7, 12, 17
30 g/l	17, 21, 28, 32	45 g/l	10, 12, 17, 20

Samples were harvested by centrifugation (4500g, 10 min for *S*.cf. *salina* and 5000g, 15 min for *A. africanum*), the sedimented cells were washed with distilled water and centrifuged again, this was repeated three times. The final cells sediment was frozen to minus 60°C for a couple of days and than lyophilised. The dry mass was weighted to evaluate the dry mass-OD relationship. For dry mass-OD relationship one extra sample (volume 10 ml)

was collected during early growth (third day in *S.* cf. *salina*, fifth day in *A. africanum*), treated as described above and weighted.

5 ml of 80 % ethanol was added to 500 mg of lyophilised sample and left to extract for 30 minutes. Then the suspension was heated on 85°C for 10 minutes. Samples were centrifuged (4500g for 10 min). Supernatant was collected and the sediment was exctracted once again with the same procedures. Both supernatants were put together and evaporized under reduced pressure. The residues were redissolved in 1 ml of distilled water, centrifuged and the supernatant was used for HPLC/MS analysis.

II. 5. HPLC/MS ANALYSIS

The HPLC/MS system consisted of Agilent 1100 series liquid chromatograph with MSD SL ion trap mass-spectrometer. The system was operated under the ChemStation™ software. The analysis were run according to TOLSTIKOV & FIEHN (2002). Briefly, HPLC was performed using acetonitrile (A) and 6.5 mM ammonium acetate (pH 5.5, adjusted by acetic acid) (B) as the mobile phase. As the stationary phase the column TSK Gel Amide 80, 4.6 mm i.d. x 250 mm. Gradient of A/B was as following: 5 min of isocratic run with 0% B, a gradient to 15% B concluded at 10 min and than gradient to 55% B completed at 60min.

MS^n experiments were carried out with Agilent MSD SL ion trap mass-spectrometer equipped with an electrospray ion source (ESI). The spray needle was at a potential of 4.5 kV, and a 5000 kPa coaxial flow of nitrogen was used to stabilize the spray. The stainless-steel capillary held at a potential of 10 V and tube-lens offset was 65 V. Helium gas was introduced into the ion trap at a pressure of 1 mtorr to improve the trapping efficiency of the sample ions introduced into the ion trap. The helium gas also served as the collision gas during the collisionally induced dissociation (CID). The range of collision energy was 0.75–1.4 eV. Spectra were acquired in the mass-profile mode, and the number of scans ranged from 2 to approximately 30, depending on the relative abundance of the precursor ions, the number of stages of CAD, and the nature of the compound analysed.

II. 6. APPRAISAL OF MORPHOLOGY OF *A. AFRICANUM* ALONG $MGSO_4$ GRADIENT

The samples of volume 0.2 ml were fixed by addition of 0.012 ml 30% formaldehyde for morphology measurements. The samples were collected at the same phase of growth from all $MgSO_4$ concentrations (0-25 g/l: day 17; 30 g/l: day 26).

The parameters measured were: filament length, filament width, number of involution cells (for an explanation of this term see KOMÁREK & LUKAVSKÝ, 1988). For each parameter 100 mesures were carried out with the help of microphotography software OLYMPUS DP SOFT (version 3.0). The data obtained were logaritmicaly transformed and processed by ANOVA-one way method with the software STATISTICA (version 9.0).

III. RESULTS

III. 1. GROWTH CHARACTERISTICS

A) *Synechocystis* cf. *salina* strain MARKLE/1430-3
(planktonic species from uknown locality)

The optimum NaCl concentration for this strain in the medium BG 11 was previosly evaluated as 30 g/l (HOŘEJŠÍ 2005). This strain did not show significant tolerance against $MgSO_4$. The growth curve during osmoprotection experiments is shown in Fig. 1. The calibratin line for the relationship between OD 750 nm and dry mass is shown in Fig. Ia, in the part Supplementary materials. The exponential growth of the culture lasted until the fifth day of cultivation in concentrations 0 – 30 g NaCl/l, in the concentration 45 g/l the exponencial growth prevailed for 10 days of cultivation (graph of relationship between time and $\log_2$ OD 750 ($x10^{3)}$ is presented in Fig.Ib in Supplementary materials).

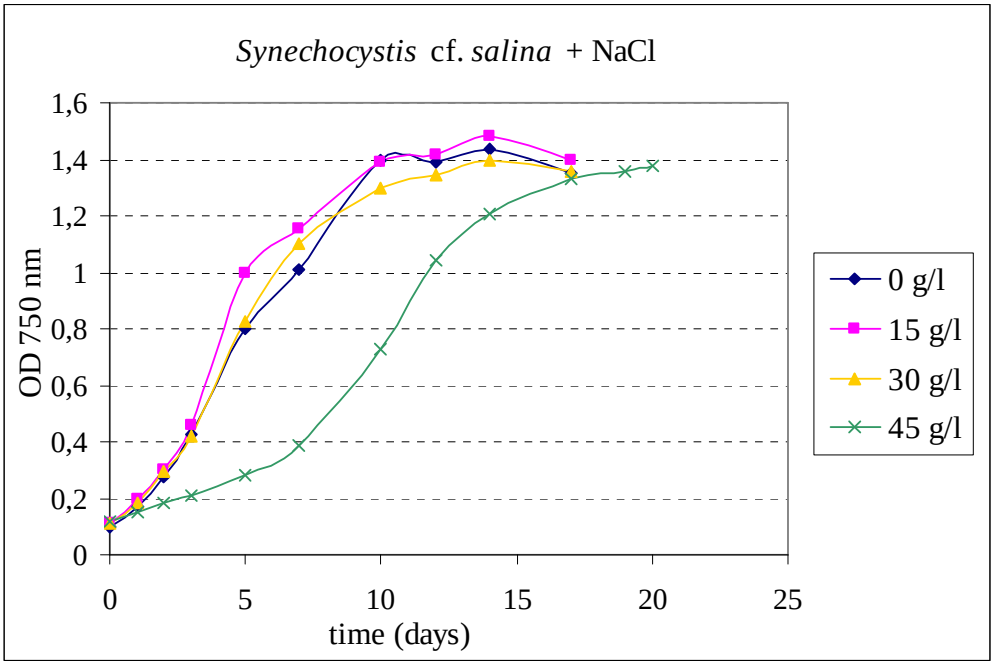


Fig. 1.
A growth curve of *Synechocystis* cf. s*alina* in medium BG11 treated with various NaCl concentrations.

B) *Arthronema africanum* strain LUKAVSKÝ 1980/1

(benthic species of salt marsh, Kuwait)

The optimum $MgSO_4$ concentration in the medium BG 11 was evaluated as 25 g/l. This strain does not show significant NaCl tolerance as previosly discussed in HOŘEJŠÍ (2005). The results of microassay for optimum $MgSO_4$ concentration are presented in Fig 2. together with the growth curve during osmoprotection experiments. The exponential growth prevailed for 7 days in concentration 0 g $MgSO_4$/l, for 10 days in 10 g $MgSO_4$/l and for 12 days in 25 and 30 g $MgSO_4$/l (graph of relationship between $\log_2$ OD 750 ($x10^3$) is presented in Fig. IIb, Supplementary materials). The dry weight calibration line is presented in Supplementary materials, Fig IIa.

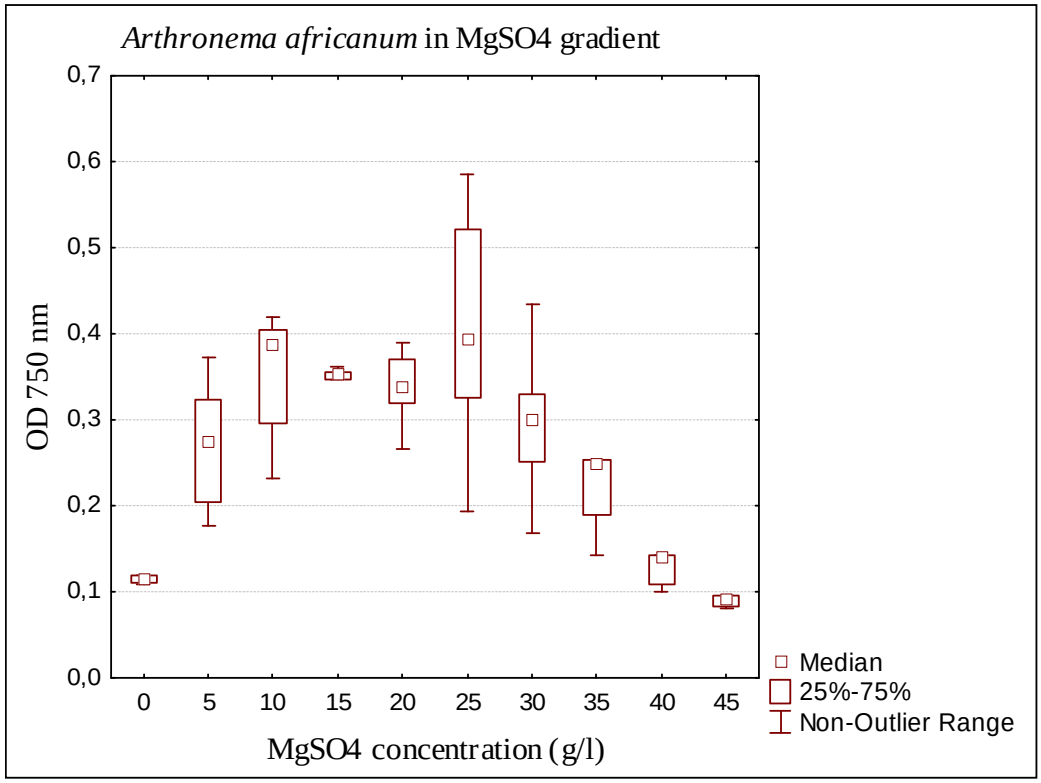


Fig. 2a.

The results of *Arthronema africanum* microassay; gradient of $MgSO_4$ added to the medium BG11 and its relationship with OD 750 nm. Optimal growth at 25 g/l $MgSO_4$. Chi-square = 32; df = 5;

p = 0,0002.

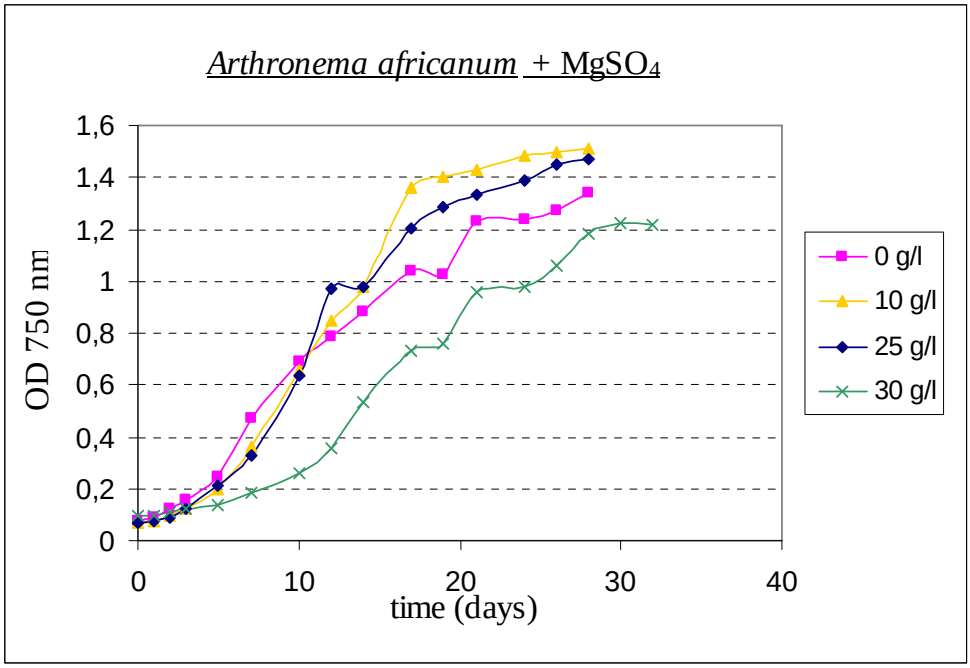


Fig. 2b. A growth curve of *Arthronema africanum* in medium BG11 treated with various NaCl concentrations.

III. 2. ANALYSIS RESULTS

A) *Synechocystis* cf. *salina* strain MARKLE/1430-3
(planktonic species from uknown locality)

All compounds detected were assigned on the basis of their molecular weight (MW) in positive mode ($[M+H^+]^+$), MW of their typical adducts and the presence of characteristic fragments. In this strain we detected the presence of saccharose/trehalose (SACH/TRE) with $[M+H^+]^+ = 343$, the prevailing sodium aduct $[M+Na^+]^+=365$ and typical fragmentation on *m/z* = 185 (sodium-fructofuranosyl/glucopyranosyl) and *m/z* = 203 (sodium aduct of glucose/fructose). SACH/TRE was eluted at the time of 37.1 min. From the MS spectra it is impossible to asses whether the compound of $[M+H^+]^+ = 343$ is saccharose or trehalose since these two substances have exactly the same molecular weight. Saccharose is composed of glucose (MW = 180.16) and fructose (MW = 180.16) and trehalose is composed of two glucose units. After which we identified glucosyl glycerol (GG; $[M+H^+]^+ = 255$, sodium aduct $[M+Na^+]^+ = 277$) with typical fragmentation on *m/z* = 75 (rest of molecule after neutral loss of glucose (MW = 180)) *m/z* = 93 (glycerol) and *m/z* = 163 (glucopyranosyl). The retention time (t_R) of GG was 31.8 min. And, last but not least, glycine betaine ($[M+H^+]^+ = 118$, t_R = 32.6 min) with its typical fragments *m/z* = 72 (rest of N-trimethylglycine after splitting of carboxylic group) and *m/z* = 45 (carboxylic group) as well as the rest of molecule after loss of water (*m/z* = 100) was detected. Other interesting solutes which were detected are: ectoin (t_R =

36.5 min) and prolin (t_R = 34.0 min). An overview of compounds detected, as well as their characteristic fragments and structures, is shown in Table I in the part Supplementary materials. Chromatograms for three main osmoprotectants are shown in Fig. 3, MS spectra are in Fig. 4, 5, 6.

The presence of these substances was indicated in all samples yet their amount differed in samples with different NaCl concentrations. The influence of sampling time on the amount of certain substances was also observed. The exact influence of salt concentration and sampling time for three main osmoprotectants, will be described below.

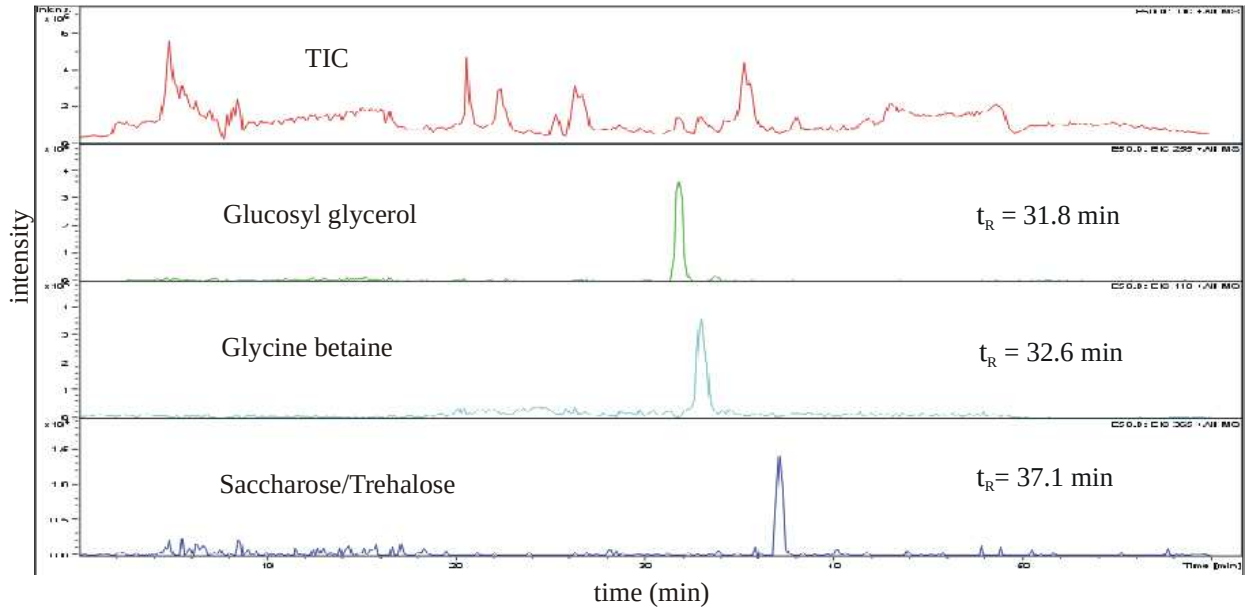


Fig.3.

Total ion chromatogram and chromatograms of 3 main osmoprotective compounds (GG $[M+H^+]^+$ = 255, gly-bet $[M+H^+]^+$ = 118 and SACH/TRE $[M+Na^+]^+$ =365) in positive mode. Chromatograms are from the concentration 30 g NaCl/l, 4th sampling time.

Saccharose/trehalose

Increasing the salt concentration has an obvious negative influence on the amount of this compound. The highest SACH/TRE level is in culture cells treated with pure BG11 and the lowest level we can observe at the concentration of 45 g NaCl /l. The amount of SACH/TRE decreases during stationary growth phase. In the control culture and the concentration 15 g NaCl/l is the highest amount we can observe at the second sampling time (linear phase of growth). The graph of intracelullar SACH/TRE amount, calculated for the

corresponding dry mass, in various NaCl concentrations is presented in Fig.4 together with MS spectra.

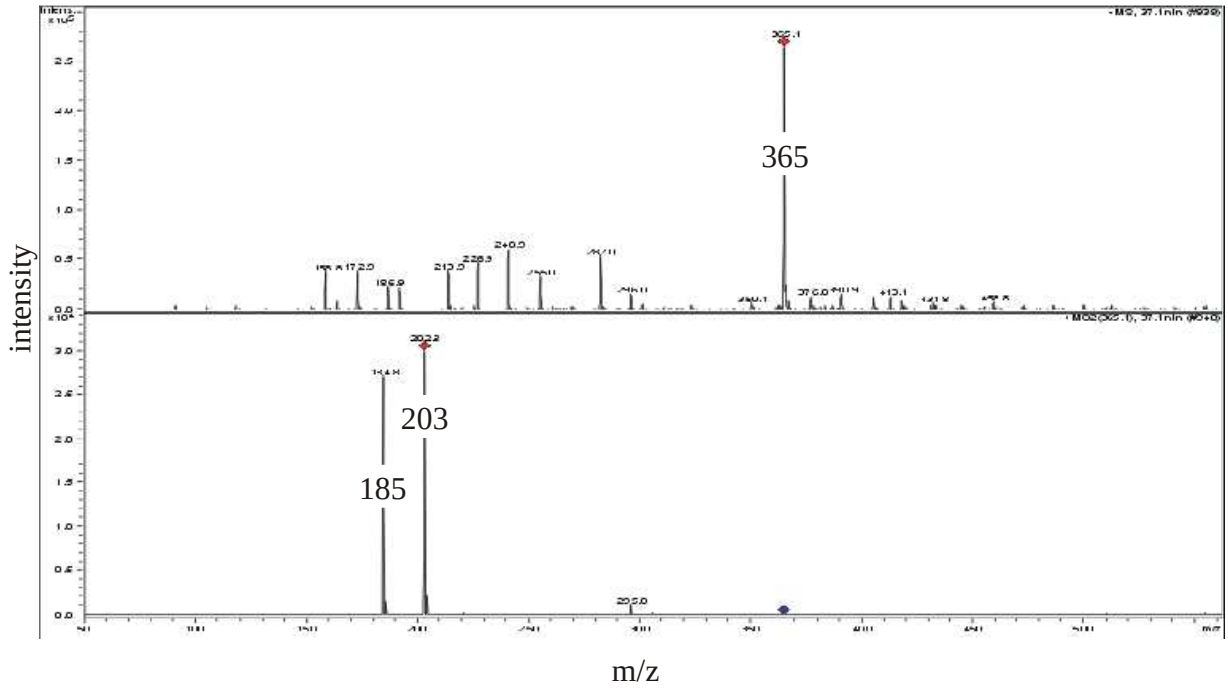


Fig. 4a.

The MS spectra of saccharose/trehalose (upper box: full scan mass spectrum; $[M+Na^+]^+$ = 365; t_R = 37.1 min) and sodium aduct fragmentation (lower box) providing characteristical fragments: *m/z* = 203 and *m/z* = 185.

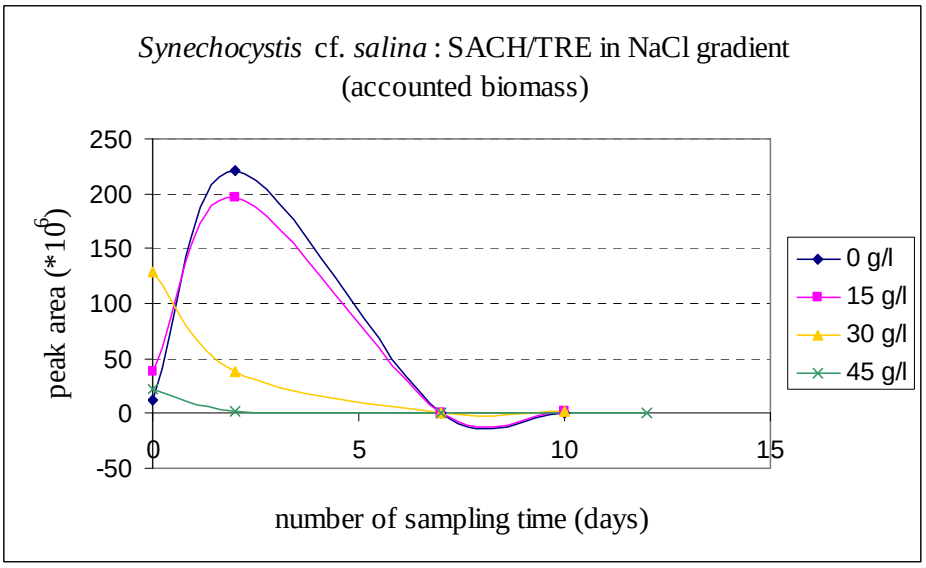


Fig. 4b.

The Amount of saccharose/trehalose, calculated for dry weight, changes during salt gradient and growth phases; negative influence of NaCl concentration.

Glucosyl glycerol

This osmoprotectant prevails in NaCl concentrations of 15 and 30 g/l in the overall point of view. Values at samples from pure BG 11 are obviously lower during all the time of growth and those from a concentration 45 g NaCl/l are lower in all growth phases except of exponential growth. The trend of lowering the amount of solutes is obvious; similarly as in SACH/TRE. The MS spectra and the graph of intracelullar GG amount in various NaCl concentrations are presented in Fig.5.

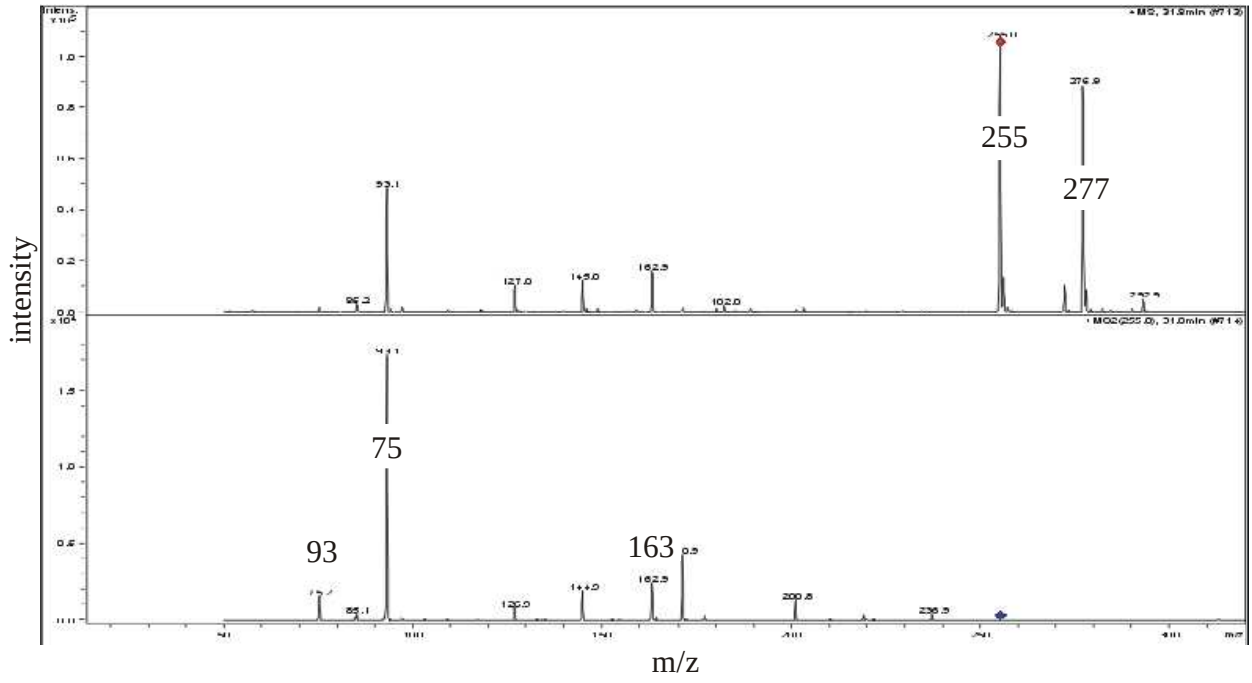


Fig. 5a.

The MS spectra of glucosyl glycerol (upper box: full scan mass spectrum; $[M+H^+]^+$ = 255, $[M+Na^+]^+$ = 277; t_R = 31.8 min) and the fragmentation of the protonated molecule (lower box) providing characteristical fragments: *m/z* = 75, *m/z* = 93 and *m/z* = 163.

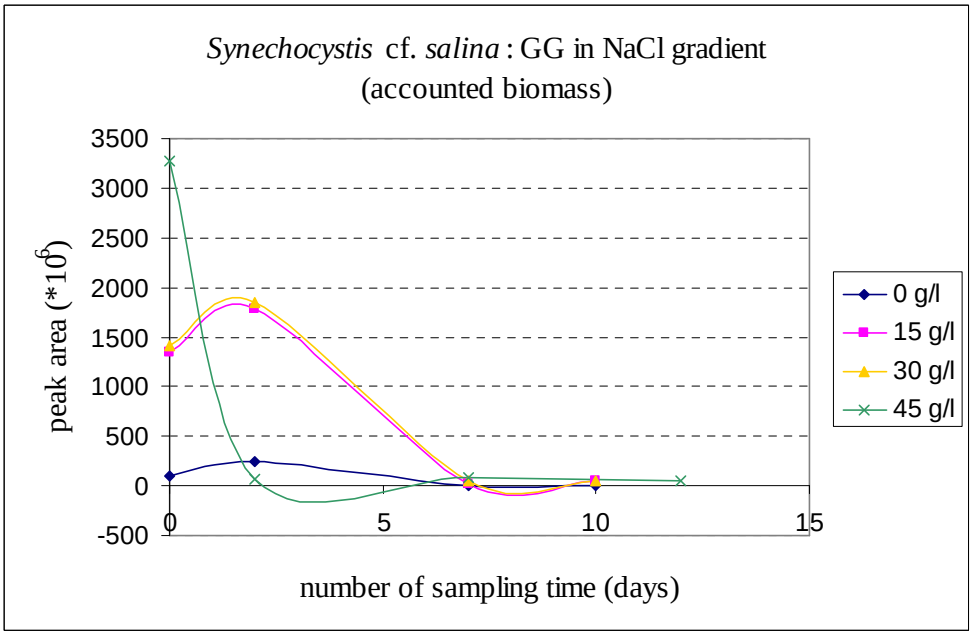


Fig. 5b.
The amount of glucosyl glycerol, calculated for dry weight, changes during salt gradient and growth phases; The prevelation in concentrations 15 and 30 g NaCl/l.

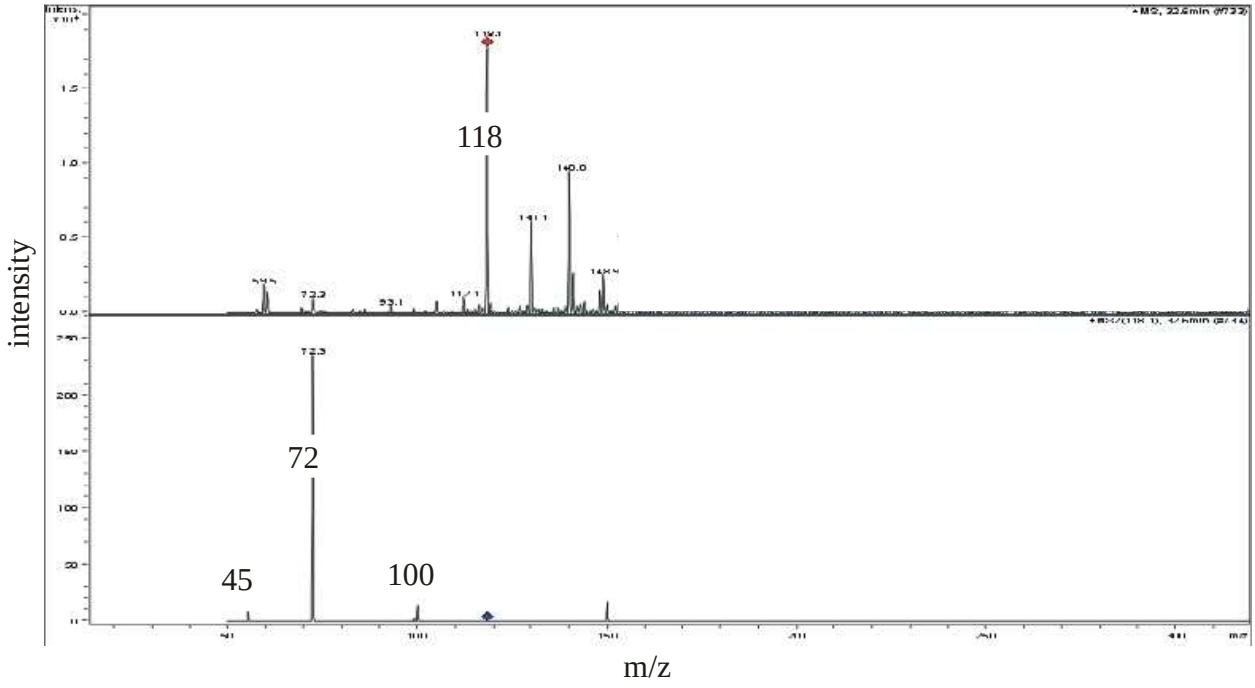


Fig. 6a.
The MS spectra of glycine betaine (upper box: full scan mass spectrum; $[M+H^+]^+$ = 118, t_R = 32.6 min) and the fragmentation of the protonated molecule (lower box) providing characteristical fragments: *m/z* = 100, *m/z* = 72 and *m/z* = 45.

Glycine betaine

The prevelation of this osmoprotectant was observed at the highest salt levels (30 and 45 g NaCl/l). The curves of peak areas for 0 and 15 g NaCl/l are lower. The amount of gly-bet lowers sharply during growth in the concentration 30 g NaCl/l. The curve for highest concentration rises slightly in the linear phase of growth and than slowly lowers into the stationary phase yet still remains above all others. The MS spectra and the graph of intracelullar glycine betaine amount in various NaCl concentrations are presented in Fig.6.

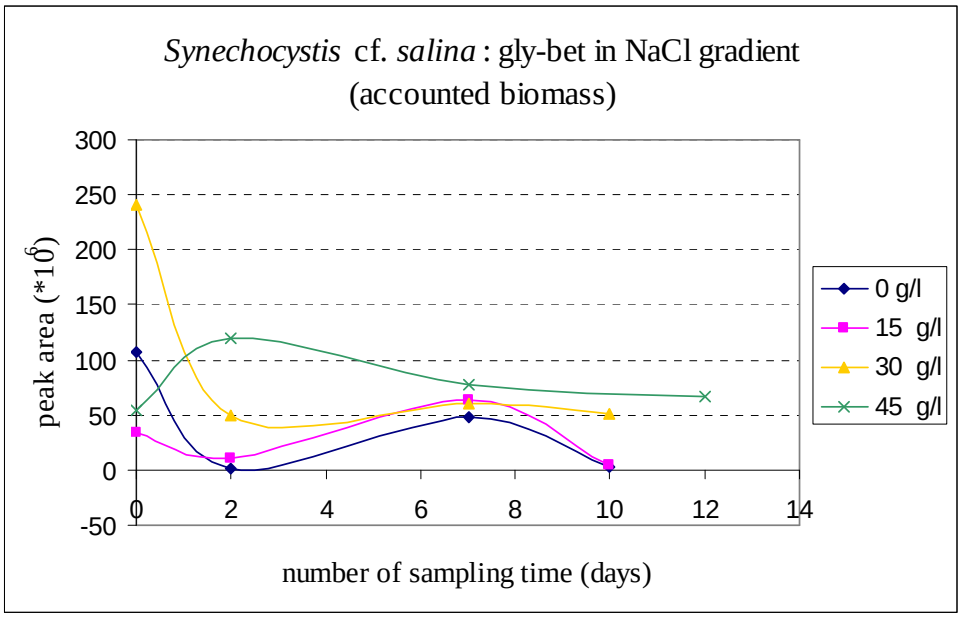


Fig. 6b. The amount of glycine betaine,calculated for dry weight, changes during salt gradient and growth phases; The prevelation in concentrations 30 and 45 g NaCl/l.

B) *Arthronema africanum* strain LUKAVSKÝ 1980/1
(benthic species of salt marsh, Kuwait)

None of the osmoprotectants so far known from NaCl tolerant strains was present in *Arthronema* samples. We are able to identify two entirely new compounds in this strain at this time. Acetylserine ($[M+H^+]^+$ = 148, t_R = 37.0 min) is split up into fragmets: *m/z* = 130 (neutral loss of H_2O), *m/z* = 102 (loss of carboxylic group) and *m/z* = 84 (rest of the molecule after the loss of carboxylic group and NH_4). The second compound detected and identified was Acetylglucosamine ($[M+H^+]^+$ = 222, t_R = 18.7 min) with its fragment *m/z* = 165 (protonated molecule of glucopyranosyl) The intracellular amount of these two compounds along the salt gradient will be described below. Chromatograms for acetylserine and acetylglucosamine are

presented in Fig. 7. Overview of compounds detected, their characteristic fragments and structures, is shown in Table I in Supplementary materials.

There are some other compounds which occured in *Arthronema* samples. However, we are not able to identify them accuratelly at the moment. We detected two prevailing substances: *m/z* = 284 and *m/z* = 198. Both of these substances show a strong absorption at 220 nm. We suppose that the substance "*m/z* = 198" should act as an osmoprotectant since its intracellular amout changes along $MgSO_4$ gradient; it prevails in the concentration of 25 g $MgSO_4$/l (Fig. IIIa in Supplementary materials.). However, other metabolical roles cannot be ruled out until this substance is reliably determined. The influence of $MgSO_4$ content on the amount of the "substance *m/z* = 284" is not obvious and therefore we cannot assume its osmotic role with doubt (Fig. IIIb in Supplements).

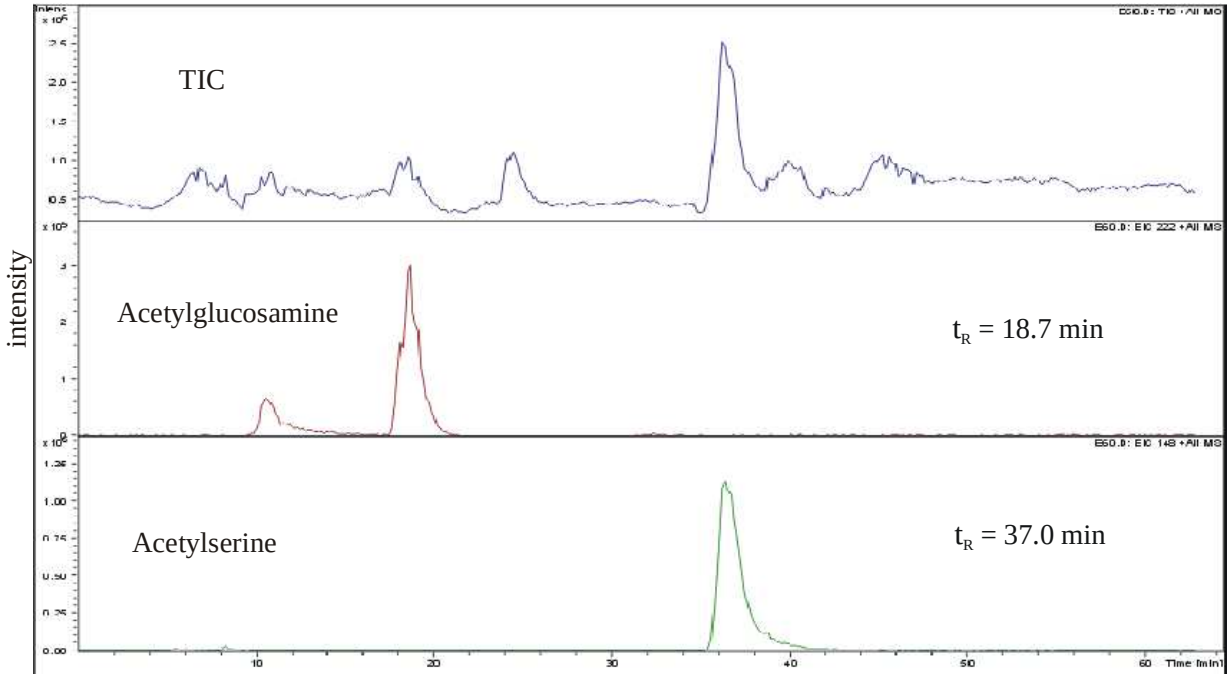


Fig.7.

Total ion chromatogram and chromatograms of acetylglucosamine ($[M+H^+]^+$ = 222) and acetylserine $[M+H^+]^+$ = 148 in positive mode. Chromatograms are from concentration 30 g NaCl/l, 4th sampling time.

Acetylserine

We suppose that this substance has a significant role in *Arthronema* osmoprotection against $MgSO_4$. As is obvious in the graph in Fig. 8b, the concentration of sulphate significantly influences the intracellular amount of acetylserine. The highest amount of this compound is apparent in the concentration of 30 g $MgSO_4$/l; the curve of this concentration prevails above all the others. The amount of acetylserine in the cells slowly decreases with the number of the sampling time (or lenght of cultivation).

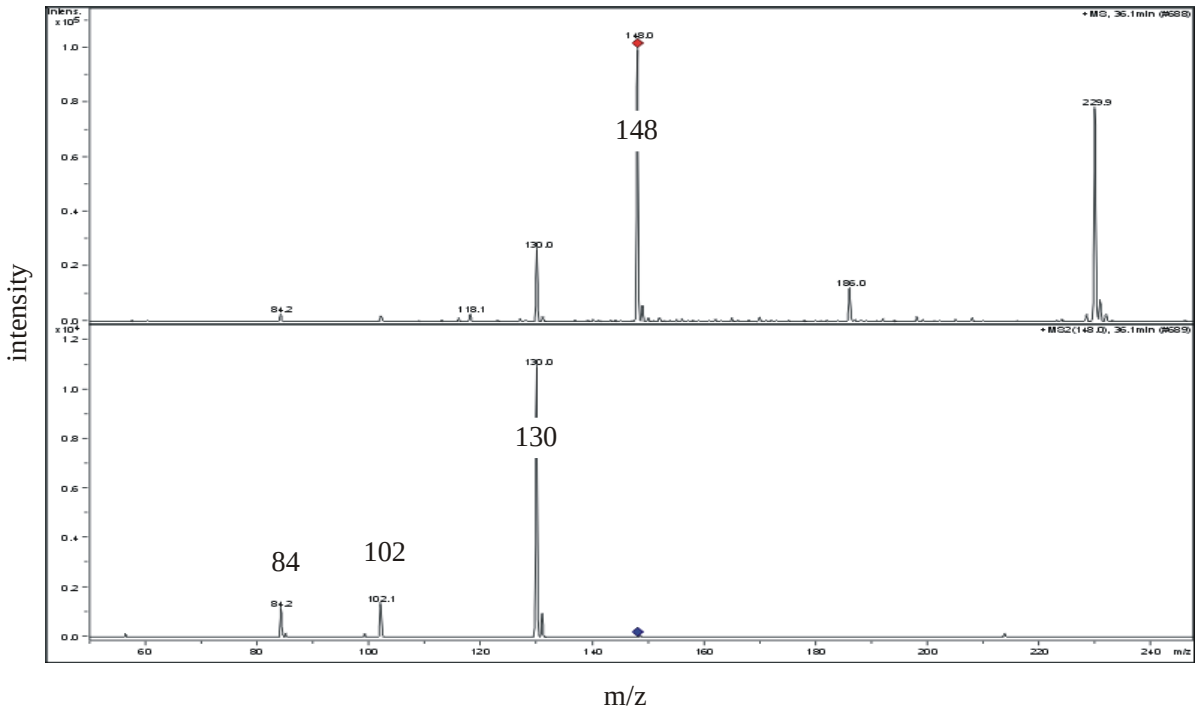


Fig. 8a.

The MS spectra of acetylserine (upper box: full scan mass spectrum; $[M+H^+]^+ = 148$, $t_R = 37$ min) and the fragmentation of the protonated molecule (lower box) providing characteristical fragments: *m/z* = 130, *m/z* = 102 and *m/z* = 84.

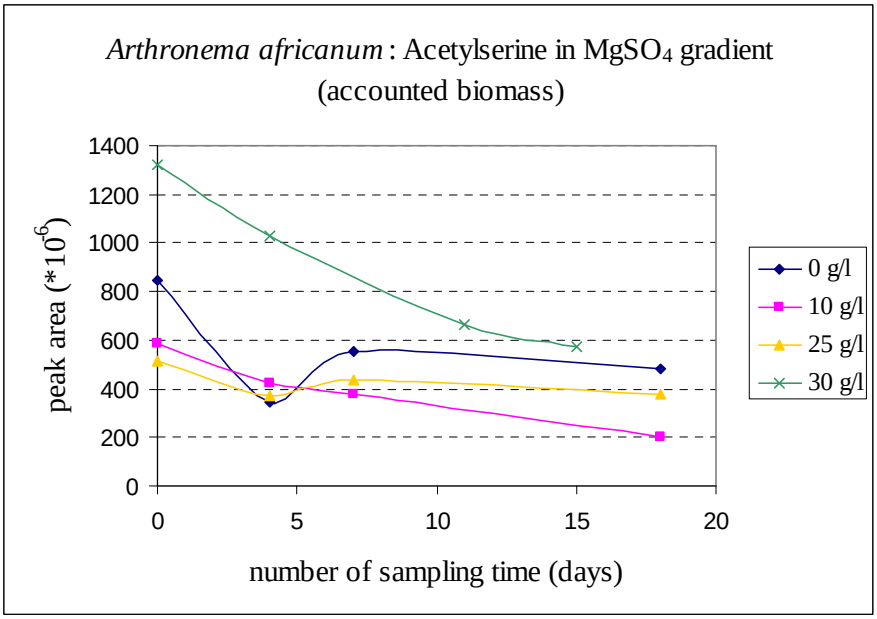


Fig. 8b.
A graph showing acetylserine intracellular amount changes along $MgSO_4$ gradient. Acetylserine is present mostly in the highest $MgSO_4$ concentration.

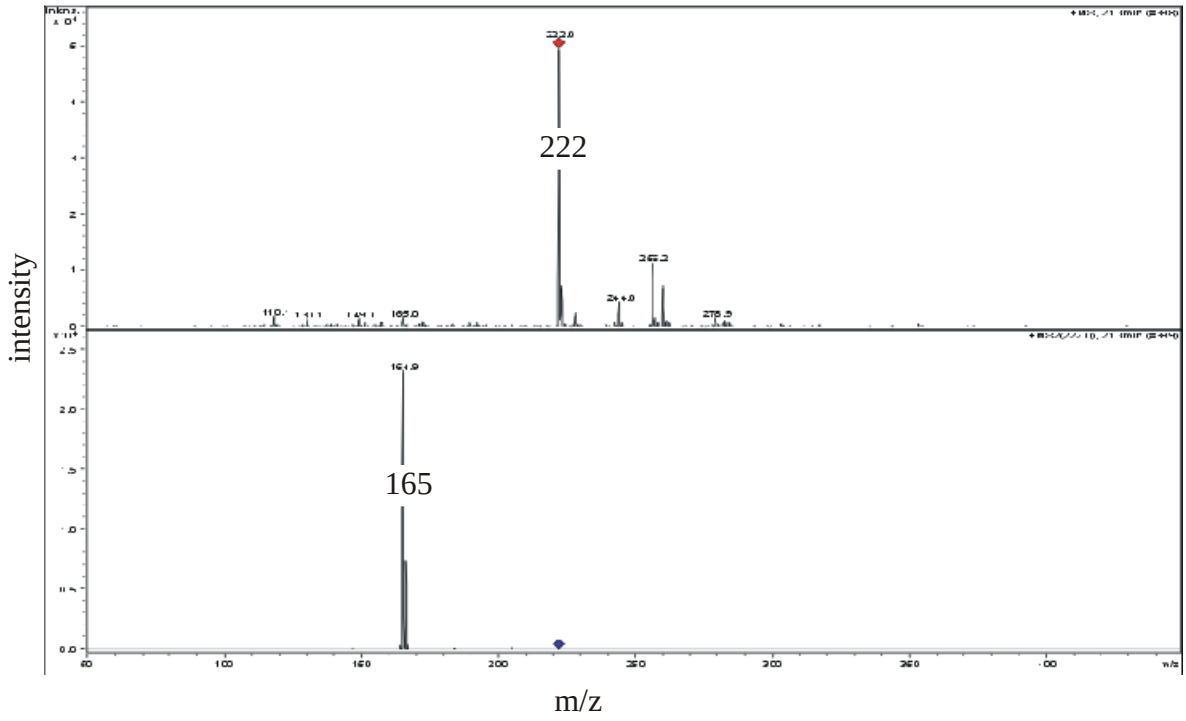


Fig. 9a.
The MS spectra of acetylglucosamine (upper box: full scan mass spectrum; $[M+H^+]^+ = 222$, t_R = 18.7 min) and the fragmentation of the protonated molecule (lower box) providing characteristical fragment: *m/z* = 165.

Acetylglucosamine

The osmoprotective function of this substance is not significant since its amount in zero addition of $MgSO_4$ seems to be one of the highest. In the graph in Fig 9a. we can see an interesting fact that the production of acetylglucosamine is the lowest in the optimal concentration of $MgSO_4$ (25 g/l).

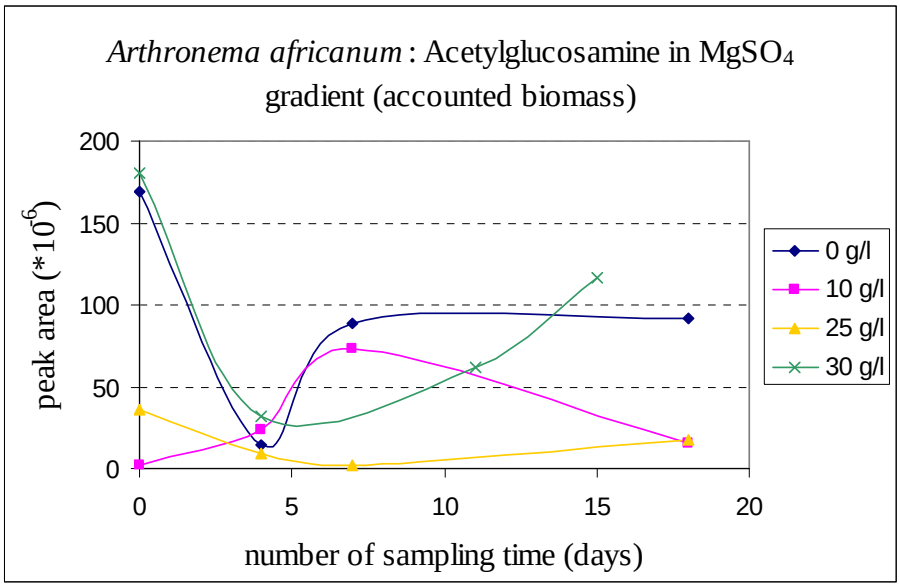


Fig. 9b.
A Graph showing acetylglucosamine intracellular amount changes along $MgSO_4$ gradient. The influence of rising MgSO4 concentration is not apparent.

III. 3. MORPHOLOGY ALONG SALT GRADIENT

A) *Synechocystis* cf. *salina* strain MARKLE/1430-3
(planktonic species from uknown locality)

Morphology along NaCl gradient was previously discussed in HOŘEJŠÍ (2005). The parametres observed were; the diameter of cells and the percentage of dividing cells. The diameter of cells tend to decrease significantly with increasing salt in the media. The percentage of dividing cells tend to rise with increasing NaCl concentration.

B) *Arthronema africanum* strain LUKAVSKÝ 1980/1
(benthic species of salt marsh, Kuwait)

Filament length (μm) differs significantly (DF = 3, F = 86.691, $p<10^{-6}$) along the $MgSO_4$ gradient. The Tukey test (Fig. 10) shows that there are significant differences among all groups (i.e. $MgSO_4$ concentrations). In the graph in Fig. 10 we can see that the filaments tend to break mostly in pure BG 11. Conversely *Arthronema* form the longest filaments in a concentration of 10 g $MgSO_4$/l.

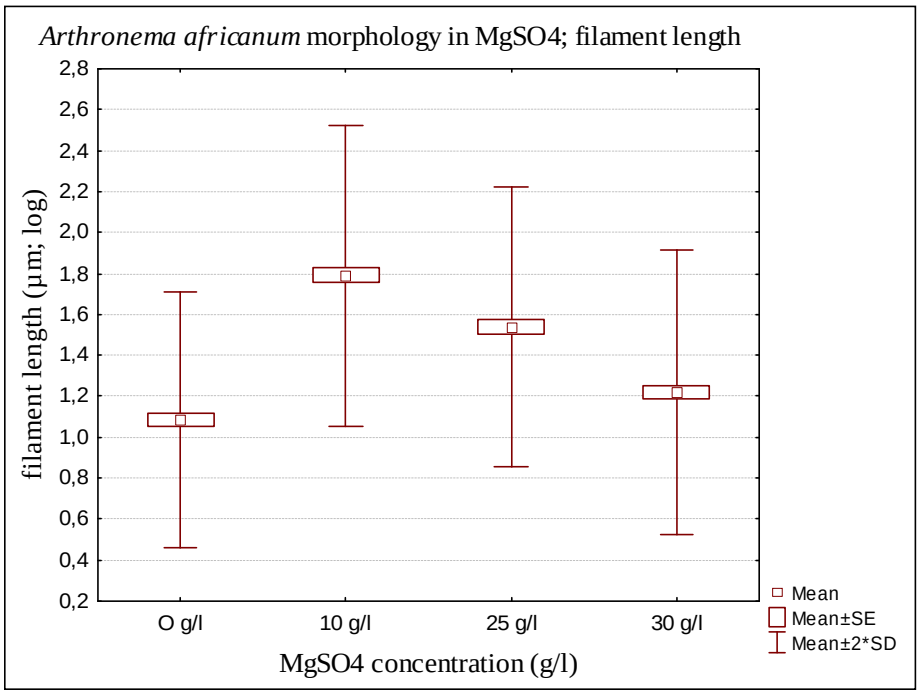


Fig. 10a.
A graph showing the dependency of filament length on $MgSO_4$ concentration in *Arthronema africanum.*

Filament length (µm; log)	O g/l	10 g/l	25 g/l	30 g/l
O g/l		0,000008	0,000008	0,027143
10 g/l	0,000008		0,000009	0,000008
25 g/l	0,000008	0,000009		0,000008
30 g/l	0,027143	0,000008	0,000008	

Fig. 10b.
The Tukey test results proving dissimilarity among all tested concentrations of $MgSO_4$ in *A. africanum.*

Filament width (µm) differs significantly (DF = 3, F = 187.0154, $p<10^{-6}$) along the $MgSO_4$ gradient. The Tukey test (Fig. 11) shows that there are significant differences among all studied $MgSO_4$ concentrations. The widest filaments are present at the highest concentration (45 g/l), the thinnest filaments were observed at the concentration 10 g $MgSO_4$/l as shown in the graph in Fig. 11.

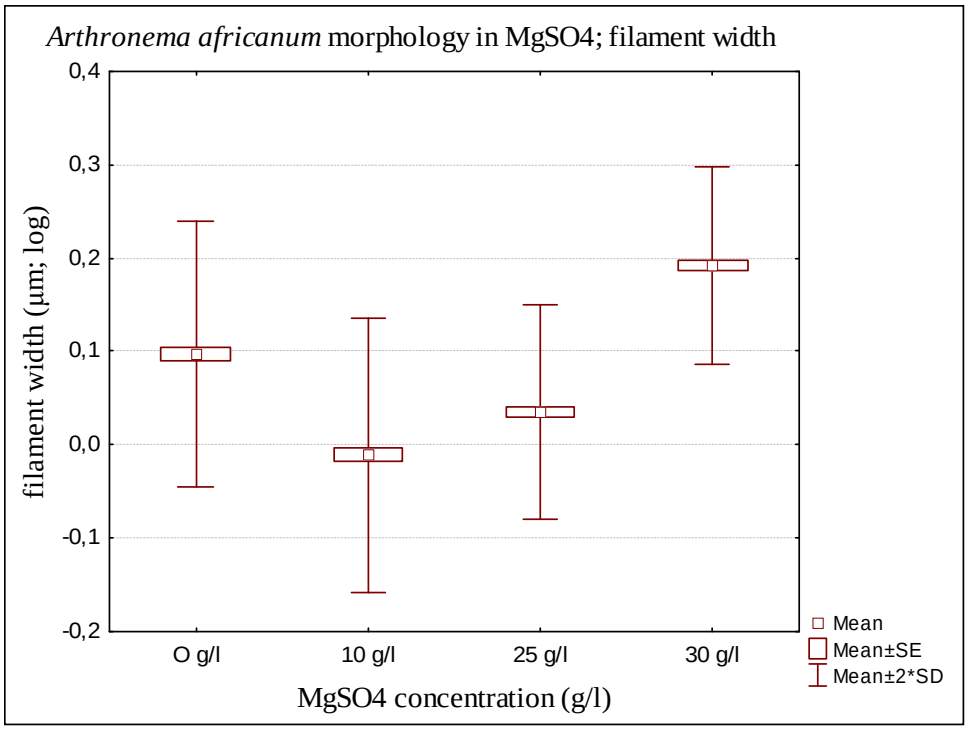


Fig. 11a.
A graph showing the dependency of filament width on $MgSO_4$ concentration in *Arthronema africanum.*

filamenth width (µm; log)	O g/l	10 g/l	25 g/l	30 g/l
O g/l		0,000008	0,000008	0,000008
10 g/l	0,000008		0,000010	0,000008
25 g/l	0,000008	0,000010		0,000008
30 g/l	0,000008	0,000008	0,000008	

Fig. 11b.
The Tukey test results proving dissimilarity among all tested concentrations of $MgSO_4$ in *A. africanum.*

The number of involution cells per filament differs significantly (DF = 3, F = 65.16070, $p<10^{-6}$) along the $MgSO_4$ gradient. The Tukey test (Fig.12) shows that there are no significant differences between 0 and 10 g $MgSO_4$/l (p = 0.948149) and between 25 and 30 g $MgSO_4$/l (p = 0.887075). Also from the graph in Fig. 12 we can see that this morphological parameter of *Arthronema africanum* forms two "groups" along $MgSO_4$ gradient; The number of involution cells in filament differs significantly between the "group" 0 and 10 g $MgSO_4$/l and the "group" 25 and 30 g $MgSO_4$/l. However, there is quite high variability in 10 g $MgSO_4$/l.

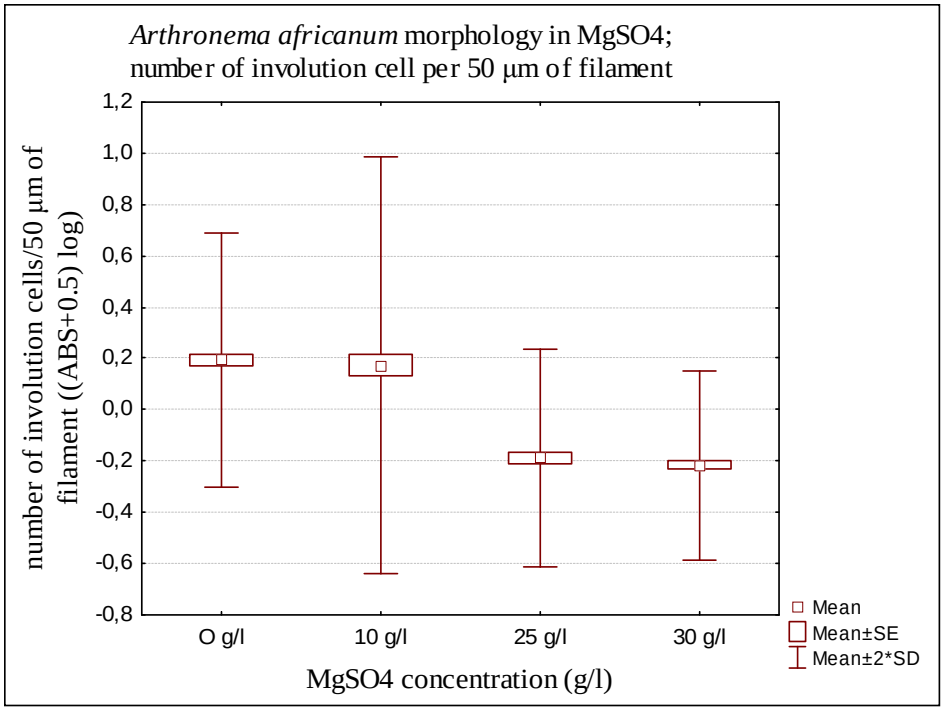


Fig. 12a.
A graph showing the dependency of the number of involution cells per filament on $MgSO_4$ concentration in *Arthronema africanum.* (ABS means absolute value)

number of involution cells per 50 µm of filament ((ABS+0.5) log)	O g/l	10 g/l	25 g/l	30 g/l
O g/l		**0,948149**	0,000008	0,000008
10 g/l	**0,948149**		0,000008	0,000008
25 g/l	0,000008	0,000008		**0,887075**
30 g/l	0,000008	0,000008	**0,887075**	

Fig. 12b.
The Tukey test results proving that the number of involution cells do not differ between MgSO4 concentrations 0 and 10 g/l and between 25 and 30 g/l in *Arthronema africanum* (thick font).

IV. DISCUSSION

IV. 1. MATERIALS AND METHODS

A) Assesment of the optimal growth concentration of $MgSO_4$ for *Arthronema africanum*

We used the method of microassay, instead of usual (flask) assays, as this method provides many advantages when compared to flask assay. It is a very rapid and simple assay providing a precise overview of the growth demands of chosen cyanobacteria (LUKAVSKÝ, 1992, ROJÍČKOVÁ ET AL., 1998, LUKAVSKÝ & SIMMER, 2001). For our purposes this microassay was very suitable as the first overview of $MgSO_4$ tolerance before beginning of the main experiments for the determination of osmoprotectants.

As there are no leads about cyanobacteria tolerance against $MgSO_4$ in literature, the gradient of $MgSO_4$ for microassay was estimated with the steps of 5 g/l until the concentration 45 g/l, which showed to be sufficient.

B) Strains chosen for experiments

From the strains studied in HOŘEJŠÍ (2005) were chosen two of appropriate properties of good tolerance against NaCl/MgSO4. *Arthronema africanum* tolerates $MgSO_4$ while showing poor tolerance against NaCl while *Synechocystis* cf. *salina* shows opposite salts demands. The use of two strains in two salts brings the question of the species specifity in intracellular substances production. However, such a sharp diferences between the osmoprotectants produced by *Synechocystis* in NaCl and *Arthronema* in MgSO4 are unlikely to be just species specific. From the previous assays of many authors (see the review, part I.) we have enough reasons to suppose that cyanobacteria are quite “conservative” with compatible solutes production in NaCl stress. In general, all authors present that cyanobacteria under NaCl stress accumulate the three main osmoprotectants that we have also found in our experiments with *Synechocystis* (SACH/TRE, GG, gly-bet). On the basis of numerous previous studies we have a good reason to believe that *Arthronema* would produce the same compounds if treated with high NaCl concentration (if it were able to cope with raised NaCl amount). Therefore, the fact that *Arthronema*, treated with $MgSO_4$, accumulates completely different compounds should not be caused by the species specifity.

To completely eliminate the question of species specifity it would be necessary to find a strain which tolerates both NaCl and $MgSO_4$ and to prove differences between compounds accumulated under different salts stresses in such strain.

IV. 2. ANALYSIS RESULTS

A) *Synechocystis* cf. *salina* strain MARKLE/1430-3
(planktonic species from uknown locality)

As one of the three main osmoprotectants (glucosyl glycerol) is not commercially produced, we could not acquire its standart for analysis and the quantification of its exact amount in extracts. Because of this fact, all amounts of three main osmoprotectants (SACH/TRE, GG, gly-bet) are presented just in units of peak areas. The peak area reflects the quantity for each osmoprotectant on its own, however it is impossible to compare the substances with each other.

The identification of osmoprotectants in *S.* cf. *salina* is significant enough even without use of their standarts since we have sufficient previous records about compatible solutes in cyanobacteria in literature (see the literature rewiew in part I.). Furthermore, the characteristical fragmentation of these substances in MS is obvious.

For the futher work it would be necessary to gain the standart of glucosyl glycerol to compare exact amounts of the three main osmoprotectants with each other. It is possible to prepare GG through a sequence of enzymatic reactions. Yet this is quite complicated procedure and it needs good experiences with this kind of reactions. Alas, our laboratory is not appropriately equipped for such procedures.

The assay was carried out at once (with no repeats). Therefore the results are not supported by statistical processing, which is quite disadvatage. This was mainly due to insuficient time and culture space. For further study it would be necessary to repeat these experiments at least twice more. The results show that preference of the three main osmoprotectants changes along the salt gradient. SACH/TRE is prefered in the lowest concentrations, GG in medium concentrations tolerated and gly-bet in highest concentrations. This fact is widely supported by previous studies on other halotolerant/halophilic cyanobacteria (see the review in part I.). As the observed preference of osmoprotectants in our strain appears as expected and our results are well supported in literature, the absence of statistical processing in this case is not such a great challenge.

Saccharose/trehalose

This osmoprotectant is prefered in the lowest concentration of NaCl (15 g/l). However the highest amount we can observe in pure BG11 (control). This fact could be caused by the possbility that SACH/TRE can be also facultatively utilised as a source of energy, when the salt stress is not present (VAN LAERE , 1989, HERZOG et al., 1990, OREN, 1999, 2002). In the graph in Fig 4b, part III. 2., we can also see the obvious increase of the SACH/TRE amount in the second sampling time (linear growth) in 15 g NaCl/l, which can be also caused by the stocpiling of this substance as an energy resource as well as just its osmoprotective function. In the concentration of 15 g NaCl/l the osmoprotective function is starting to be passed over to GG as discussed further.

Glucosyl glycerol

This osmoprotectant is prefered in medium concentratins of NaCl (15 and 30 g/l). As written above, this fact has is well supported in literature. We observed a high amount of GG in the first sampling time of the concentration of 45 g NaCl/l (Fig. 5b., part III. 2.). GG is more energy-demanding to produce than gly-bet (OREN, 1999, 2002). So it is possible that the cells can afford to produce GG as an osmoprotectant in the highest NaCl concentration just in exponential phase (first sampling time) of growth when no limitation of nutrients and light is present. Than (in further growth phases), obviously they have to pass over to "cheaper" gly-bet (as discussed further).

Glycine betaine

This osmoprotectant is prefered in the highest concentrations of NaCl (45 and 30 g/l). As written above, this fact is well supported in literature. We observed slightly lower amount of gly-bet in the first sampling time in the cocentration of 45 g NaCl/l (Fig. 6b, part III. 2.). This lowering probably corresponds with the rise of GG in the same sampling time in this concentration. This fact also supports the above explanation, that in the exponential growth phase, with no limitation of nutrients and light, *Synechocystis* can afford to produce "more expensive" GG instead of (or as well as) "cheaper" gly-bet.

B) *Arthronema africanum* strain LUKAVSKÝ 1980/1
(benthic species of salt marsh, Kuwait)

The identification of newly detected substances is challenging as there are so few leads describing osmoprotection topic in other salts than NaCl in literature. There are no articles available about the topic of osmoprotectants against $(SO_4)^{2-}$ stress in cyanobacteria. It

is possible to find just a few assays describing the ecology of cyanobacteria living in various salts environments. The little literature available are mostly studies of $MgSO_4/(SO_4)^{2-}$ tolerance in agriculture plants (KLAGES et al., 1999, WU et al., 2003, TURAN & AYDIN, 2005, WILSON ET AL., 2006) or some other salt-tolerant plants (TOBE et al., 2004, WIRTZ et al., 2004). In these studies the authors suggest the accumulation of following substances: proline (WU et al., 2003), myo-inositol (KLAGES et al., 1999), acetylserine (WIRTZ et al., 2004) and HILL et al. (1999) report glucosaminoglycans (including acetyglucosamine). From these compounds we proved acetylserine and acetylglucosamine in *Arthronema* samples.

Other two compounds detected (*m/z* = 284 and *m/z* = 198) remain unidentified for the present. However, for the osmoprotective function, there are some necessary features of these compounds, which could help to exclude some of the numerous possible substances. In general they should fulfill the parameters of compatible solutes described above in the part I. 1.

Also the assay for *Arthronema* osmoprotectants was carried out just once similarly as in *Synechocystis*. However, the support of literure is very poor in *Arthronema*'s case. So it is crucial to repeat the procedures at least twice more to declare results as fully provable. Until results are supported with statistical processing their value can be named just as a first screening.

Acetylglucosamine

Our results indicates that the osmoprotective function of this substance is rather insignificant. The curves of concentrations have no obvious tendencies proving relationship of acetylglucosamine and $MgSO_4$ addition.

In the graph in Fig 9a., part III.2., we can see interesting fact that the production of acetylglucosamine is lowest in the optimal concentration of $MgSO_4$ (25 g/l). There is a possibility that this substance could play a role in the cell stress response in general and is formed in suboptimal conditions; either through a lack or excess of sulphate. Yet this theory must be further verified. We also observed that in all samples the lowest amount of acetylglucosamine is in the second sampling time (14th day of cultivation) matching the linear growth phase. Yet we have no satisfactory explanation for this observation at the moment as the metabolic role of this compound is unclear.

IV. 3. MORPHOLOGY ALONG SALT GRADIENT

A) *Synechocystis* cf. *salina* strain MARKLE/1430-3

(planktonic species from uknown locality)

The morphology of this strain in NaCl was discussed previously in HOŘEJŠÍ (2005).

B) *Arthronema africanum* strain LUKAVSKÝ 1980/1

(benthic species of salt marsh, Kuwait)

The results of the morphology parameters measurements show a very good significance. However, in first two parameters (filament length, filament width) there was not observed any salt depent tendency. It means that the results did not show any linear relationship between the addition of $MgSO_4$ and the filament length/width as could be expected. The concentration of 10 g NaCl/l appears as a turning point in filament length/width (see Fig. 10a, 11a, part III. 3.). In this concentration (10 g NaCl/l) the filaments are the longest (i.e. they have the least tendency to break). In the control concentration, the breaking of filaments is frequent and also from "turning point" of 10 g $MgSO_4$/l with further arise of NaCl amount in media (concentrations 25 and 30 g $MgSO_4$/l) the filaments are breaking more often. The concentration of 10 g $MgSO_4$/l appears as a turning point also for filament width, where the observations are just oposite to those of filament legth; in 10 g $MgSO_4$/l the filaments are the thinnest and in both directions from this concentration the width of the filaments is rising.

The number of involution cells has a tendency to decrease with increasing $MgSO_4$ in media (see Fig. 12a, part III. 3.), which is interesting as KOMÁREK & LUKAVSKÝ (1988) propose that these cells should play some role in osmoprotection. Also in HOŘEJŠÍ (2005), where *Arthronema* was tested for morphology parameters changes in NaCl, the number of involution cells was the lowest in maximal concentration tolerated (10 g NaCl/l BG11).

V. CONCLUSIONS

I. We estimated the optimal concetration of MgSO4 for *Arthronema africanum* at the value of the addition of 25 g $MgSO_4$/l media BG11.

II. We succesfully established a suitable method of extraction, detection and identification of osmoprotectants in *Synechocystis* cf. *salina* and *A. africanum*. We also succesfuly analysed intracellular extracts from various concentrations of NaCl (*S.* cf. *salina*) and $MgSO_4$ (*A. africanum*) for osmoprotectants.

III. We compared osmoprotectants in two chosen strains of cyanobacteria under NaCl and $MgSO_4$ stress. These two strains chosen, under different salts stress, produced entirely different substances.

A) *Synechocystis* cf. *salina* under NaCl stress formed three main osmoprotectants (saccharose/trehalose, glucosyl glycerol and glycine betaine). The preference of each osmoprotectant appeared to be dependent on the degree of salt stress. SACH/TRE is prefered in the lowest concentration (0 g NaCl/l (possibility of utilization as energy source) and in 15 g NaCl/l), GG in intermediate concentrations (15,30 g NaCl/l) and gly-bet in the highest concentration (45 g NaCl/l). These results correspond with previous observations of osmoprotectants and their preference by other cyanobacteria in various salt concentrations which have been presented in literature.

B) We succesfully identified two entirely new substances for the species *Arthronema africanum*: acetylserine and acetylglucosamine. However, only acetylserine appears as a compound with an osmoprotective function. The other two unidentified substances observed were; the substance "*m/z* = 198" and the substance "*m/z* = 284". However, only the substance "*m/z* = 198" appears as a compound with an osmoprotective function.

C) All compounds observed (except of acetyglucosamine) in both strains showed the tendency to decrease their intracellular amount along the time of cultivation.

D) All procedures, especially in *A. Africanum*, must be repeated at least twice more to acquire sufficient support in statistical processing of the collected data.

IV. We observed the morphology of *A. Africanum* along the $MgSO_4$ gradient succesfully. All paremeters observed change significantly along the sulphate gradient.

REFERENCES

ADAMS, M. A., CHEN, Z. L., LANDMAN, P. & COLMER, T. D. (1999): Simultaneous determination by capillary gas chromatography of organic acids, sugars, and sugar alcohols in plant tissue extracts as their trimethylsilyl derivatives. Analytical Biochemistry. 266:77−84.

APTE, D. E. & THOMAS, J. (1997): Possible amelioration of coastal soil salinity using halotolerant nitrogen-fixing cyanobacteria. Plant and soil189: 205-211.

BERNARD, T., JEBBAR, M., RASSOULI, Y., HIMDI-KABBAB, S., HAMELIN, J. &BLANCO, C. (1993): Ectoine accumulation and osmotic regulation in Brevibacterium linens Journal of General Microbiology 139:129–136.

BHARGAVA, S., SAXENA, R. K., PANDEY, P. K. & BISEN, P. S. (2003): Mutational engineering of the cyanobacterium Nostoc muscorum for resistance to growth inhibitory action of LiCl and NaCl. Current Microbiology 47: 5-11.

BLUMWALD, E. & TEL-OR, E. (1982): Osmoregulation and cell composition in salt-adaptation of *Nostoc muscorum*. Archives of Microbiology 132: 168 –172.

BLUMWALD, E., MEHLHORN, R. J. & PACKER, L. (1983): Studies of osmoregulation in salt adaptation of cyanobacteria with ESR spin-probe techniques. Proceedings of the National Academy of Sciences 80: 2599-2602.

BORGES, N., RAMOS, A., RAVEN, N. D. H. (2002): Comparative study of the thermostabilizing properties of mannosylglycerate and other compatible solutes on model enzymes. Extremophiles 6: 209-216.

BOUCHEREAU, A., DUHAZÉ, C., JOSETTE, M.-T., GUÉGAN, J.-P. & LARHER, F. (1999): Improved analytical methods for determination of nitrogenous stress metabolites occuring in *Limonium* species. Journal of Chromatography A 836: 209-221.

CASTENHOLZ, R. W. & GARCIA-PICHEL, F. (2000): Cyanobacterial responses to UV-radiation, pp. 591-611 In: Whitton, B., A. & Potts, M. (Eds), The Ecology of Cyanobacteria - Their Diversity in Time and Space. Kluwer Academic Publishers, Dordrecht.

CUSHMAN, J. C. (2001): Osmoregulation in plants: Implications for agriculture. American Zoologist 41:758–769.

ERDMANN, N., FULDA, S. & HAGEMANN, M. (1992): Glucosylglycerol accumulation during salt acclimation of two unicellular cyanobacteria. Journal of General Microbiology 138: 363-368.

FERJANI, A., MUSTARDY, L., SULPICE, R., MARIN, K., SUZUKI, I., HAGEMANN, M. & MURATA, N.(2003): Glucosylglycerol, a compatible solute, sustains cell division under salt stress. Plant Physiology 131: 1628-37.

FIEHN, O., KOPKA, J., TRETHEWEY, R. N. & WILLMITZER, L. (2000a): Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. Analytical Chemistry 72: 3573-3580.

FIEHN, O., KOPKA, J., DÖRMAN, P., ALTMAN, T., TRETHEWEY, R. N., & WILLMITZER, L. (2000b): Metabolite profiling for plant functional genomics. Nature Biotechnology 18: 1157-1161.

GARCIA-PICHEL, F. & CASTENHOLZ, R. W. (1993): Occurrence of UV-absorbing, Mycosporine-like compounds among cyanobacterial isolates and an estimate of their screening capacity. Applied and Environmental Microbiology 59: 163-169.

GILLES, R. (1997): „Compensatory“ organic osmolytes in high osmolarity and dehydration stresses: history and perspectives. Comparative Bichemistry and Physiology, Part A 117: 279-290.

HAGEMANN, M., SCHOOR, A., ERDMANN, N. (1996): NaCl acts as a direct modulator in the salt adaptive response: Salt-dependent activation of glucosylglycerol synthesis in vivo and in vitro. Journal of Plant Physiology 149: 746-752.

HALLSWORTH, J. E. & MAGAN, N. (1997): A rapid HPLC protocol of polyols and trehalose. Journal of Microbiological Methods 29: 7-13.

HERZOG, R. M., GALINSKI, E. A. & TRÜPER, H. G. (1990): Degradation of the compatible solute trehalose in Ectothiorhodospira halochloris: isolation and characterization of trehalase. Archives of Microbiology 153: 600-606.

HILL, W. G., HARPER, G. S., ROZAKLIS, T. & HOPWOOD, J. J. (1999): Enhanced channelling of sulphate through a rapidly exchangeable sulphate pool in response to stimulated glycosaminoglycan synthesis in pancreatic epithelial cells. Biochimica et Biophysica Acta-Molecular Basis of Disease 1454:174 – 182.

HOŘEJŠÍ, E. (2005): Závislost vybraných kmenů halofilních řas na koncentraci NaCl, bachelor’s thesis. Fakulty of Biological Science, University of South Bohemia, České Budějovice, Czech republic.

JERUZALMI, D. & STEITZ, T. A. (1997): Use of organic cosmotropic solutes to crystallize flexible proteins: Application to T7 RNA polymeras and its complex with the inhibitor T7 lysozyme. Journal of Molecular Biology 274:748–756.

JUNGBLUT, A. D., HAWES, I., MOUNTFORT, D., HITZFELD, B., DIETRICH, D. R., BURNS, B. P. & NEILAN, B. A. (2005): Diversity within cyanobacterial mat communities in variable salinity meltwater ponds of McMurdo Ice Shelf, Antarctica. Environmental Microbiology 7: 519-529.

KARANDASHOVA, I., ELANSKAYA, I., MARIN, K., VINNEMEIER, J. & HAGEMANN, M. (2002): Identification of genes essential for growth at high salt concentrations using salt-sensitive mutants of the cyanobacterium Synechocystis sp. strain PCC 6803. Current Microbiology 44: 184-188.

KEBEDE, E. (1997): Response of *Spirulina platensis* (=*Arthrospira fusiformis*) from Lake Chitu, Ethiopia, to salinity stress from sodium salts. Journal of Applied Phycology 9: 551-558.

KLAGES, K., BOLDINGH, H. & SMITH, G. S. (1999): Accumulation of myo-inositol in *Actinidia* seedlings subjected to salt stress. Annals of Botany 84: 521-527.

KOMÁREK, J. & LUKAVSKÝ, J. (1988): *Arthronema*, a new cyanophyte genus from Afro-Asian deserts. Archiv für Hydrobiologie, Suppl. 80: 249-267.

KUHLMANN, A. U. & BREMER, E. (2002): Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* related *Bacillus* spp. Applied and Environmental Microbiology 68: 772-783.

KUNTE, H. J., GALINSKI, E. A. & TRÜPER, H. G. (1993): A modified FMOC-method for the detection of amino acid-type osmolytes and tetrahydropyrimidines (ectoines). Journal of Microbiological Methods 17: 129-136.

LALOKNAM, S., TANAKA, K., BUSBOOCHA, T., WADITEE, R., INCHAROENSAKDI, A., HIBINO, T. & TAKABE, T. (2006): Halotolerant cyanobacterium *Aphanothece halophytica* contains a betaine transporter active at alkaline pH and high salinity. Applied and Environmental Microbiology 72: 6018-3026.

MADAJ, J., WISNIEWSKI, A., SKORUPOVA, E. & SOKOLOWSKI, J. (1993): Capillary gas chromatographic separations of a multicomponent mixture of polyalcohol compounds. Journal of Chromatography 655: 267-273.

MARGESIN, R. & SHINNER, F. (2001): Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 5: 73-83.

MARIN, K., KANESAKI, Y., LOS, D. A., MURATA, N., SUZUKI, I. & HAGEMANN, M. (2004): Gene expression profiling reflects physiological processes in salt acclimation of *Synechocystis* sp. Strain PCC 6803. Plant Physiology 136: 3290-3300.

MARŠÁLEK, B., ZAHRADNÍČKOVÁ, H. & HRONKOVÁ, M. (1992): Extracellular Abscisic acid

produced by cyanobacteria under salt stress. Journal of Plant Physiology 139: 506-508.

MATSUDA, N., KOBAYASHI, H., KATOH, H., OGAWA, T., FUTATSUGI, L., NAKAMURA, T., BAKKER, E. P. & UOZUMI, N. (2004): Na-dependent K uptake Ktr system from the cyanobacterium *Synechocystis* sp. PCC 6803 and its role in the early phases of cell adaptation to hyperosmotic shock. The Journal of Biological Chemistry 279: 54952 54962.

MIKKAT, S., EFFMERT, U. & HAGEMANN, M. (1997): Uptake and use of the osmoprotective compounds trehalose, glucosylglycerol, and sucrose by the cyanobacterium Synechocystis sp. PCC6803. Archives of Microbiology 167: 112-118.

MOORE, D. J., REED, R. H. &. STEWART, W. D. P (1987): A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine synthesising cyanobacteria. Archives of Microbiology 147: 399-405.

OREN, A. (1997): Mycosporine-like amino acids as osmotic solutes in a community of halophilic cyanobacteria. Geomicrobiology Journal 14: 231-240.

OREN, A. (1999): Bioenergetic aspects of halophilism (Rev.). Microbiology and Molecular Biology Reviews 63: 334-348.

OREN, A. (2000): Salt and Brines, pp. 281-306 In: Whitton, B., A. & Potts, M. (Eds.), The Ecology of Cyanobacteria-Their Diversity in Time and Space. Kluwer Academic Publishers, Dordrecht.

OREN, A. (2002): Halophilic Microorganisms and their Environments, Kluwer Academic Publishers, Dordrecht.

OREN, A. & SECKBACH, J. (2001): Oxygenic photosynthetic microorganisms in extreme environments, pp.13-31. In: Elster, J., Seckbach J., Vincent, W. F., Lhotský, O. (Eds), Nova Hedwigia - Algae and extreme environments, Beiheft 123, Gebr. Borntraeger, Berlin.

PANDEY, U. & CHATTERJEE, C. (1999): Response of two strains of *Nostoc muscorum* to metal stress and salinity. Annals of Applied Biology 134: 259-263.

PORCHIA, A. C. & SALERNO, G. L. (1996): Sucrose biosynthesis in a prokaryotic organism: Presence of two sucrose-phosphate synthases in Anabaena with remarkable differences

compared with the plant enzymes. Proceedings of The National Academy of Sciences of The United States of America 93 (24): 13600-13604.

PORTWICH, A. & GARCIA-PICHEL, F. (1999): Ultraviolet and osmotic stresses induce and

regulate the synthesis of mycosporines in the cyanobacterium *Chlorogloeopsis* PCC 6912. Archives of Microbiology 172: 187-192.

POTTS, M. (2000): *Nostoc*, pp. 465-504 In: Whitton, B., A. & Potts, M. (Eds), The Ecology of Cyanobacteria - Their Diversity in Time and Space. Kluwer Academic Publishers, Dordrecht.

QUESADA, A., SÁNCHEZ-CONTRERAS, M. & FERNÁNDES-VALIENTE, E. (2001): Tolerance of Antarctic cyanobacterial mats to natural UV radiation, pp. 275-290 In: Elster, J., Seckbach J., Vincent, W. F., Lhotský, O. (Eds), Nova Hedwigia - Algae and extreme environments, Beiheft 123. Gebr. Borntraeger, Berlin.

RIIS, V., MASKOW, T. & BABEL, W. (2003): Highly sensitive determination of ectoine and other compatible solutes by anion-exchange chromatography and pulsed amperometric detection. Analytical and Bioanalytical Chemistry 377: 203-207.

SALERNO, G. L., PORCHIA, A.C., VARGAS, V. A.& ABDIAN, P. L. (2004): Fructose-containing oligosaccharides: novel compatible solutes in Anabaena cells exposed to salt stresss. Plant Science 167: 1003-1008.

SCHOOR, A., ERDMANN, N., EFFMERT, U. & MIKKAT, S. (1995): Determination of the cyanobacterial osmolyte glucosylglycerol by high-performance liquid chromatography. Journal of Chromatography A 704: 89-97.

SCHOOR, A., HAGEMANN, M. & ERDMANN, N. (1996): Non-radiometric assay for glucosylglycerol-synthesizing enzymes involved in the cyanobacterial salt adaptation. Journal of Microbiological Methods 27: 139-145.

SINHA, R. P., AMBASHT, N. K., SINHA, J. P. & HADER, D. P. (2003): Wavelenght-dependent induction of mycosporine-like amino acids in a rice-field cyanobacterium *Nostoc commune*: role of inhibitors and salt stress. Photochemical and Photobiological Sciences 2: 171-176.

TOBE, K., LI, X. M. & OMASA, K. (2004): Effects of five different salts on seed germination and seedling growth of *Haloxylon ammodendron* (Chenopodiaceae). Seed Science Research 14: 345-353.

TOLSTIKOV, V. V. & FIEHN, O. (2002): Analysis of highly polar compounds of plant origin: combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. Analytical Biochemistry 301: 298-307.

TURAN, M. & AYDIN, A. (2005): Effects of different salt sources on growth, inorganic ions and proline accumulation in corn (Zea mays L.). European Journal of Horticulture Science 70: 149-155.

VAN LAERE, A. J. (1989): Trehalose: reserve and/or stress metabolite? FEMS Microbiology Reviews 63: 201-210.

WADITEE, R., BHUIYAN, N. H., RAI, V., AOKI, K., TANAKA, Y., HIBINO, T., SUZUKI, S., TAKANO, J., JAGENDORF, A. T., TAKABE, T., TAKABE, T. (2005): Genes for direct methylation of glycine provide high levels of glycinebetaine and abiotic-stress tolerance in Synechococcus and Arabidopsis. Proceedings of The National Academy of Sciences of The United States of America 102 (5): 1318-1323.

WELCH, W. J. & BROWN, C. R. (1996): Influence of molecular and chemical chaperones on protein folding. Cell Stress & Chaperones 1: 109-115.

WHITTON, B. A. (2000): Soils and rice fields., pp. 233-255 In: Whitton, B., A. & Potts, M. (Eds), The Ecology of Cyanobacteria - Their Diversity in Time and Space. Kluwer Academic Publishers, Dordrecht.

WILSON, C., LIU, X., LESCH, S. M. & SUAREZ, D. L. (2006): Growth response of major US cowpea cultivars. I. Biomass accumulation and salt tolerance. Hortscience 41: 225-230.

WIRTZ, M., DROUX, M., HELL, R. (2004): O-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in *Arabidopsis thaliana*. Journal of Experimental Botany 55: 1785-1798.

WU, L. Q., FAN, Z. M., GUO, L., LI, Y. Q., ZHANG, W. J., QU, L. J. & CHEN, Z. L. (2003): Over-expression of an Arabidopsis delta-OAT gene enhances salt and drought tolerance in transgenic rice. Chinese Science Bulletin 48: 2594-2600.

YANCEY, P. H. (2001): Water stress, osmolytes and proteins. American Zoologist 41:699–709

YANCEY, P. H. (2005): Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. The Journal of Experimental Biology 208: 2819-2830.

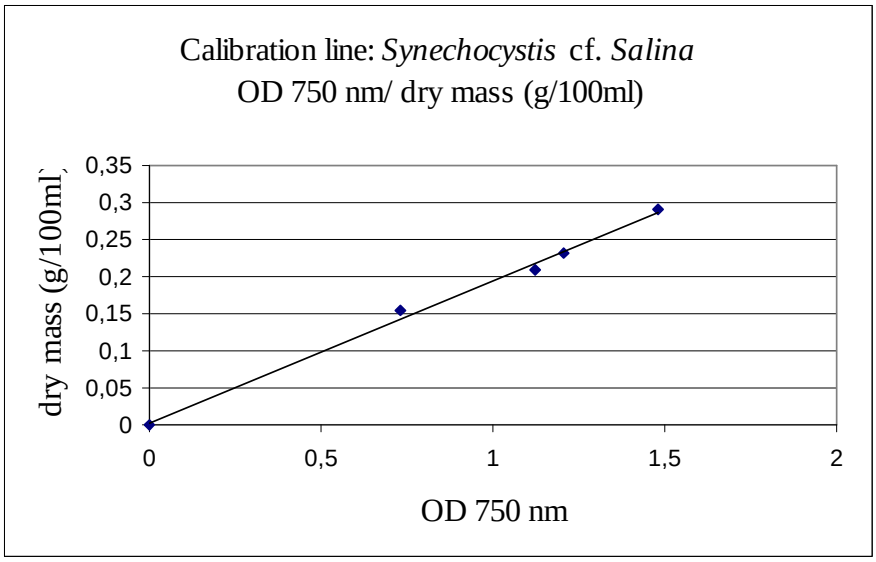


Fig. Ia.
The calibration line for relationship between OD 750 and dry mass in *S.* cf. *salina*
y = 0.1925x + 0.0026

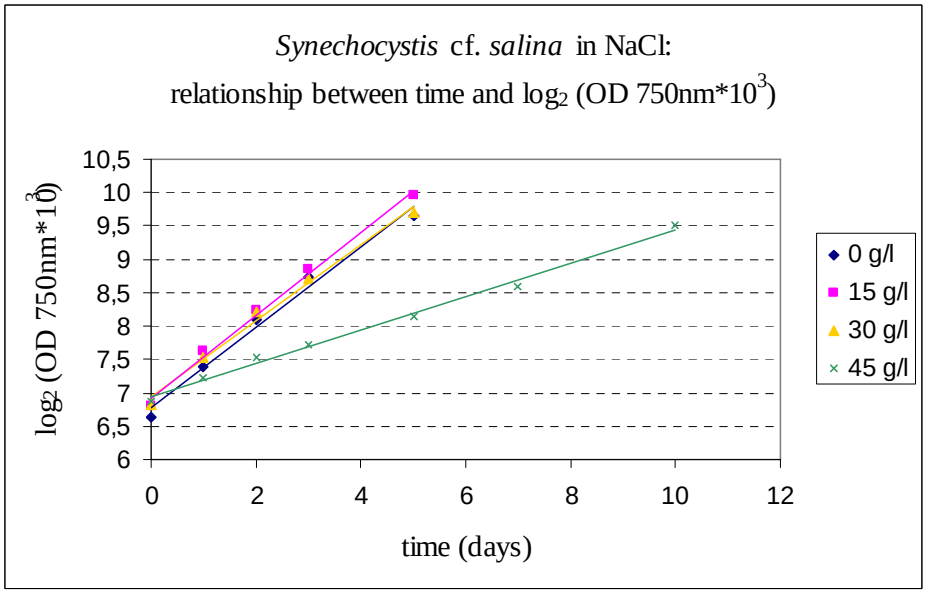


Fig. Ib.
The relationship between time and $\log_2$ (OD 750nm*10^3) in *S.* cf. *salina* in NaCl gradient and its growth parameters.
Exponential growth obvious until 5th day of cultivation in concentrations 0-30 g/l and until 10th day in 45 g/l.

0 g/l:
y = 0.6021x + 6.7756
15 g/l:
y = 0.6221x + 6.924
30 g/l:
y = 0.568x + 6.9437
45 g/l:
y = 0.2508x + 6.9377

Reproductive rate (c)				
NaCl concentration	0 g/l	15 g/l	30 g/l	45 g/l
gen/day	0.6021	0.6221	0.568	0.2508
gen/hour	0.025088	0.025921	0.023667	0.01045

doubling time (T)				
NaCl concentration	0 g/l	15 g/l	30 g/l	45 g/l
hours	39.86049	38..57901	42.25352	95.69378

specific growth rate (μ)				
NaCl concentration	0 g/l	15 g/l	30 g/l	45 g/l
g/g/h	0.017389	0.017967	0.016404	0.007243

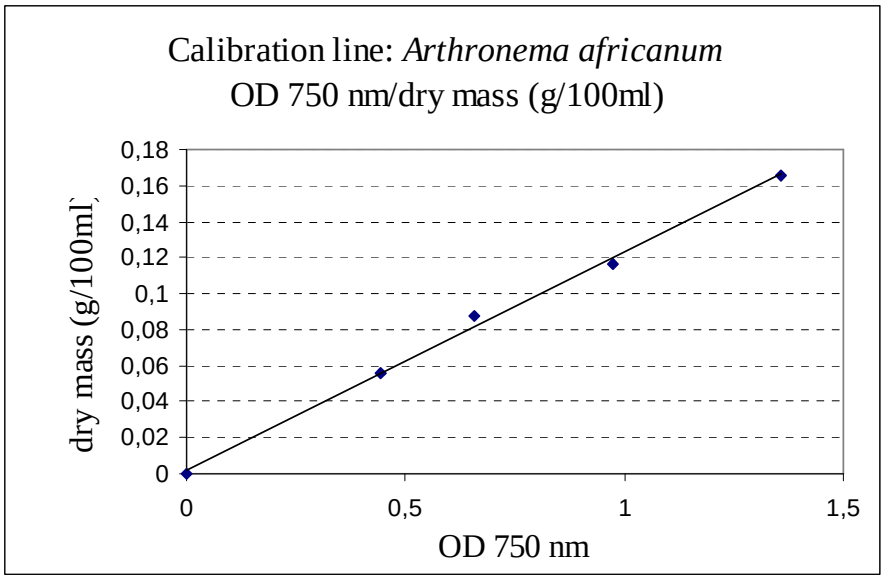


Fig. IIa.

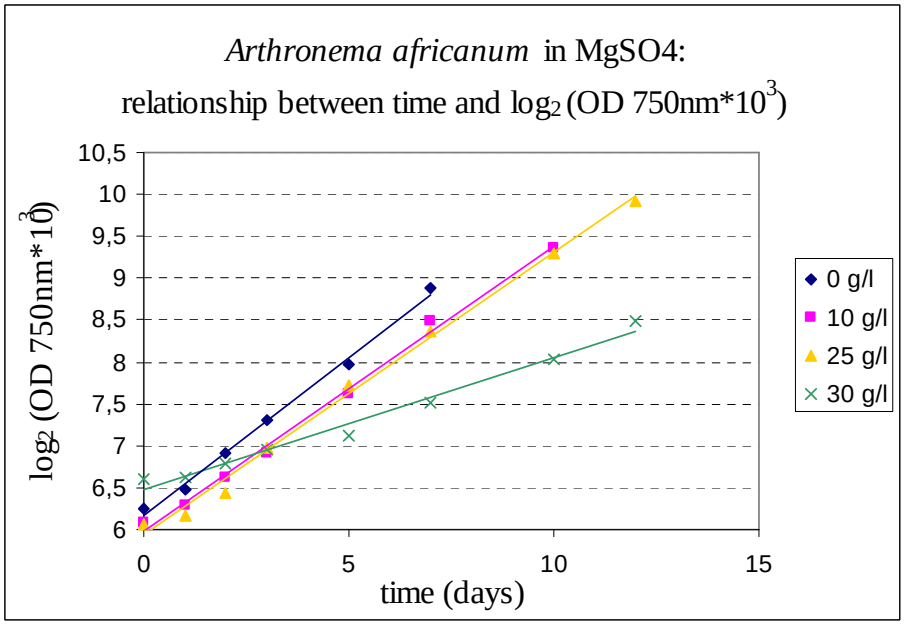
The calibration line for relationship between OD 750 and dry mass in *A. africanum*.

y = 0.1207x + 0.0023

Fig. IIb.

The relationship between time and $\log_2$ (OD 750nm*10^3) in *A. africanum* in $MgSO_4$ gradient and its growth parameters.

Exponential growth obvious until 7th day of cultivation in concentration 0g/l, until 10th day in 10 g/l and until12th day in 25 and 30 g/l.



0 g/l:
y = 0.3772x + 6.1636
10 g/l:
y = 0.3408x + 5.9734
25 g/l:
y = 0.3368x + 5.9367
30 g/l:
y = 0.1571x + 6.4792

Reproductive rate (c)				
$MgSO_4$ concentration	0 g/l	10 g/l	25 g/l	30 g/l
gen/day	0,3772	0,3408	0,3368	0,1571
gen/hour	0,015717	0,0142	0,014033	0,006546

doubling time (T)				
MgSO4 concentration	0 g/l	10 g/l	25 g/l	30 g/l
hours	63,62672	70,42254	71,25891	152,7689

specific growth rate (μ)				
MgSO4 concentration	0 g/l	10 g/l	25 g/l	30 g/l
g/g/h	0,010894	0,009843	0,009727	0,004537

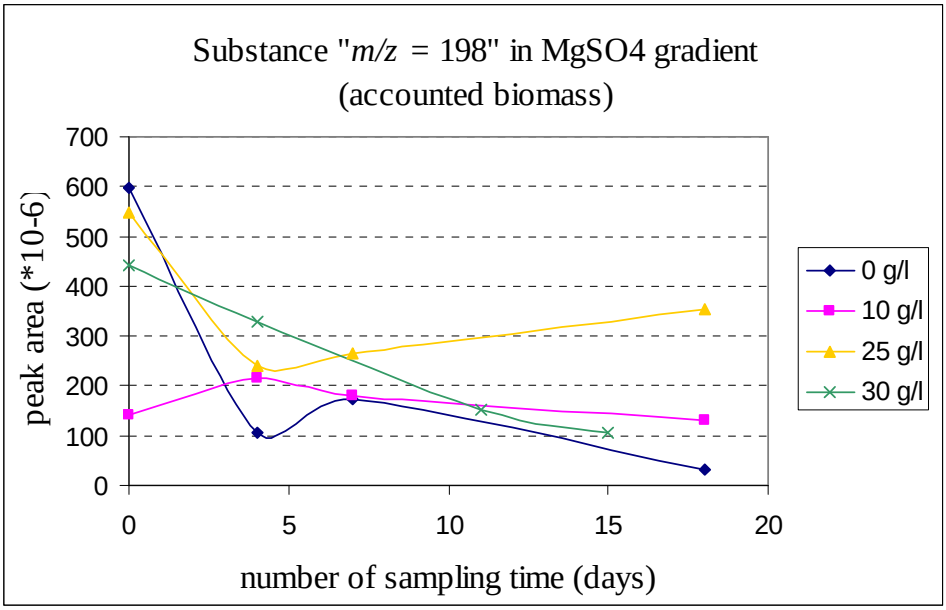


Fig IIIa.

Graph showing substance "*m/z* = 198" intracellular amount changes along $MgSO_4$ gradient; possible influence of $MgSO_4$ concentration is obvious.

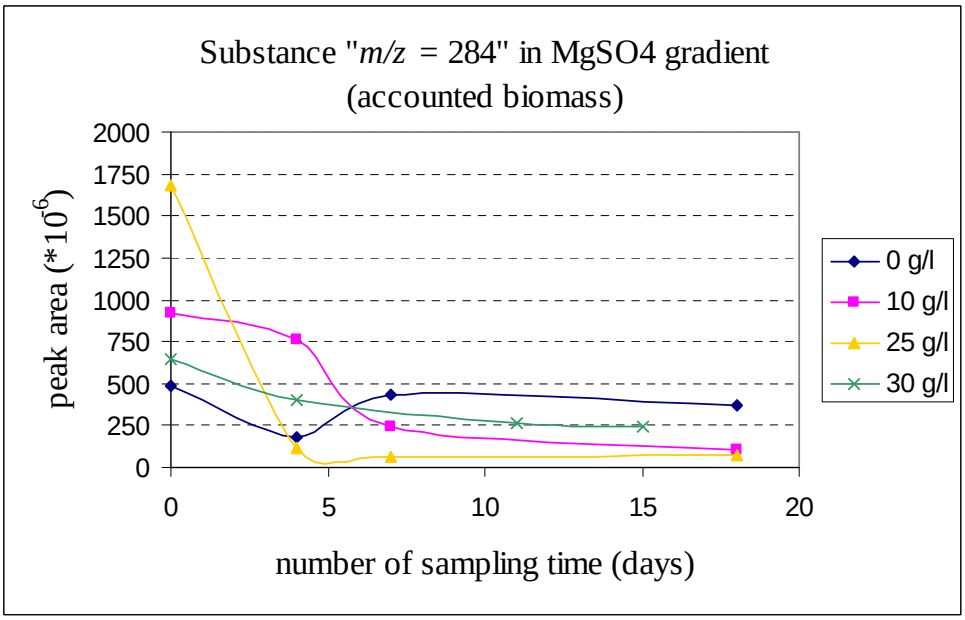


Fig. IIIb.

Graph showing substance "*m/z* = 284" intracellular amount changes along $MgSO_4$ gradient; influence of $MgSO_4$ concentration is not apparent.

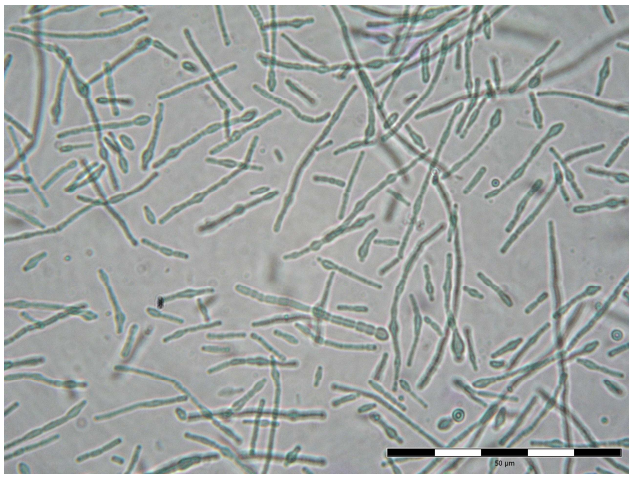


Fig. IVa.
The photograph of *Arthronema africanum* in 0 g $MgSO_4$/l; magnification: 1000x

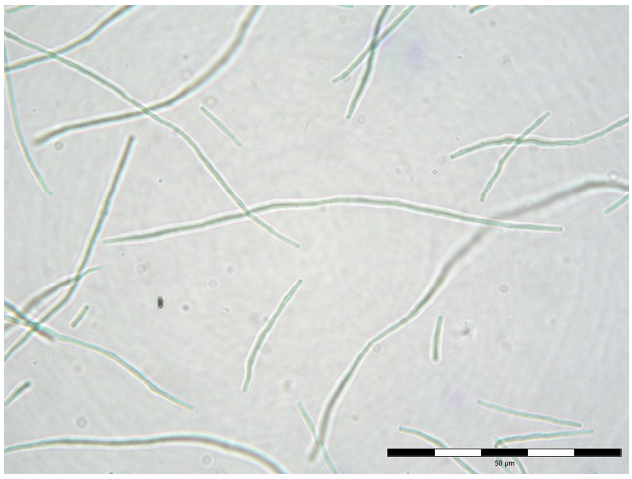


Fig. IVb.
The photograph of *A. africanum* in 10 g $MgSO_4$/l; magnification: 1000x

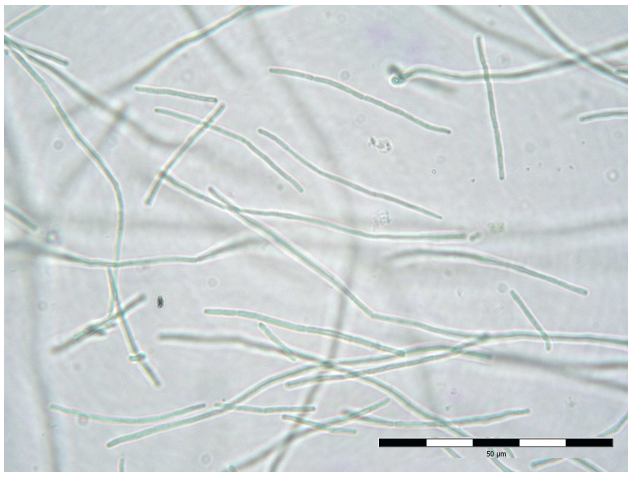


Fig. IVc.
The photograph of *A. africanum* in 25 g $MgSO_4$/l; magnification: 1000x

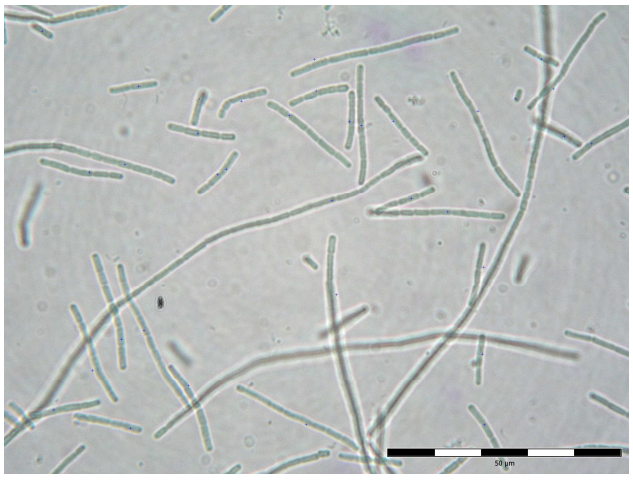
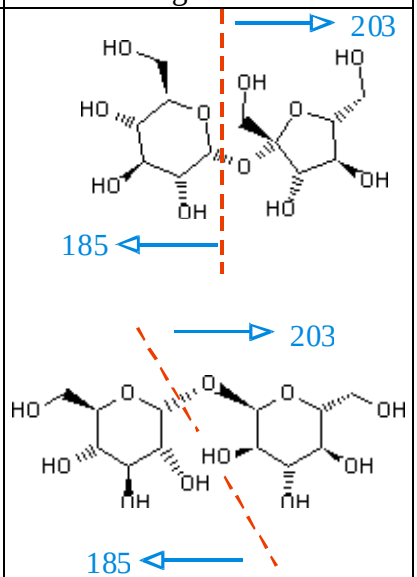
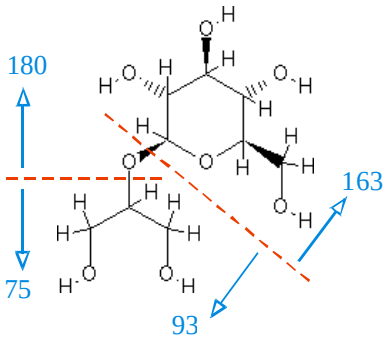
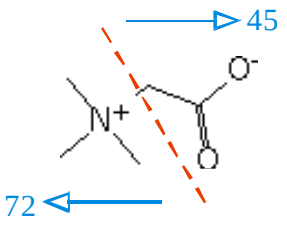
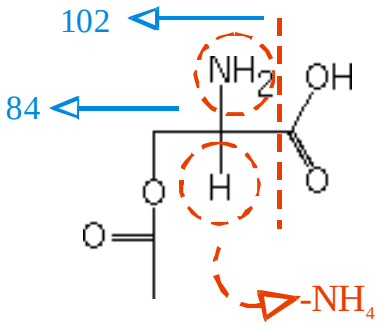
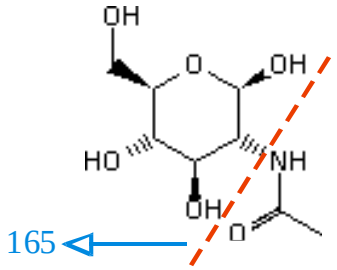


Fig. IVd.
The photograph of *A. africanum* in 30 g $MgSO_4$/l; magnification: 1000x

Tab.I. Overview of compounds detected in *Synechocystis* cf. *Salina* and *Arthronema africanum* (divided into two parts)

Main osmoprotectants detected in *Synechocystis* cf. *salina* strain MARKLE/1430-3					
compound name	MW	t_R	aducts	characteristic fragments	Formula + graphic depiction of fragmentation
Saccharose/ trehalose	342.3	37.1	$[M+H^+]^+ = 343$ $[M+Na^+]^+ = 365$ $[M+K^+]^+ = 381$ $[M+NH_3^+]^+ = 360$	185 (163+Na^+) 203 (181+Na^+)	
Glucosyl glycerol	254.24	31.8	$[M+H^+]^+ = 255$ $[M+Na^+]^+ = 277$ $[M+K^+]^+ = 293$ $[M+NH_3^+]^+ = 272$	75 (180-neutral) 93 163	
Glycine betaine	117.15	32.6	$[M+H^+]^+ = 118$ $[M+Na^+]^+ = 210$ $[M+K^+]^+ = 226$ $[M+NH_3^+]^+ = 205$	45 72 100 (loss of H_2O)	

Second part of Tab. I.

Main compounds detected in *Arthronema africanum* strain LUKAVSKÝ 1980/1					
compound name	MW	t_R	aducts	characteristic fragments	Formula + graphic depiction of fragmentation
O-acetylserine	147.13	37.0	$[M+H^+]^+ = 148$ $[M+Na^+]^+ = 170$ $[M+K^+]^+ = 186$ $[M+NH_3^+]^+ = 165$	130 (loss of H_2O) 102 84	
N-acetyl-glucosamine	221.21	18.7	$[M+H^+]^+ = 222$ $[M+Na^+]^+ = 244$ $[M+K^+]^+ = 260$ $[M+NH_3^+]^+ = 239$	165	

BG11: The media suitable particularly for cyanobacteria cultivation.	
STANIER, R.Y., KUNISAWA, R., MANDEL, M & COHEN-BAZIRE, G.(1971): Purification and properties of unicellular blue-green algae (Order Chroococcales). – Bacteriol. Rev. 35: 171-205.	
Stock solutions (dle návodu CCAP):	
1. $NaNO_3$	15,0 g/l000 ml
2. K_2HPO_4.	2 g/500 ml
3. $MgSO_4.7H_2O$	3.75 g/500 ml
4. $CaCl_2.2H_2O$	1.80 g/500 ml
5. Kys. citonová	0.30 g/500 ml
6. Citronan železito-amonný (zelený)	0.30 g/500 ml
7. EDTA-Na_2	0.05 g/500 ml
8. Na_2CO_3	1.00 g/500 ml
9. Roztok mikroprvků – do 1000 ml	
H_3BO_3	2.86 g
$MnCl_2.H_2O$	1.81 g
$ZnSO_4.7H_2O$	22 g
$Na_2MoO_4.2H_2O$	0.39 g
$CuSO_4.5H_2O$	0.08 g
$Co(NO_3)_2.6H_2O$	0.05 g
Smícháme 10 ml zásobního roztoku č.1, 10 ml každého ze zásobních roztoků č.2 až 8 a 1 ml zásobního roztoku 9. Doplníme deionisovanou vodou na 1000 ml.	

Tab.II.
The medium BG11 composition, from http://www.sinicearasy.cz