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Influence of cultivation conditions and age of the culture on the production of cytostatic secondary metabolite 2505 and its natural analogues

Bachelor thesis

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Annotation

The aim of this thesis was to investigate the effect of different cultivation conditions and age of the culture on the production of cytostatic secondary metabolite 2505 and its natural analogues in terrestrial cyanobacterium *Desmonostoc* sp.

Affirmation

I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section.

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Zuzana Gajarská

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Abstract

Cyanobacteria are considered to be a rich source of secondary metabolites with unique chemical structures and interesting biological activities. Thanks to these properties, many compounds produced by different cyanobacterial genera have gained a promising potential in the pharmaceutical field as immunosuppressant, antimicrobial or anticancer agents. Previously, two metabolites possessing a cytostatic effect against HeLa and PaTu cell lines were detected in the crude extract of *Desmonostoc muscorum*, with metabolite designated as 2504 (in this work designated as 2505 according to its neutral molar mass) having the most profound effect. The aim of this thesis was to investigate the production of this metabolite, as well as its five natural analogues in two types of photobioreactors and different cultivation conditions including the light, temperature and nitrogen availability. Unfortunately, the production trends revealed in one type of photobioreactor, the small tubular photobioreactor, were not repeated in the large scale flat photobioreactor and thus universal optimal conditions in the production of 2505 and its analogues cannot be concluded. Additionally, production of biomass in the individual experiments was studied, revealing several culture growth trends.

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1 Introduction

Cyanobacteria are simple prokaryotic microorganisms belonging among the earliest inhabitants of our planet. Thanks to their ability of oxygenic photosynthesis, they have played an important role in the formation of oxygen levels in the Earth's atmosphere and until this day remain to be an important factor in their maintenance (Schopf and Packer, 1987). Cyanobacteria significantly contribute to the bio-geochemical cycles of carbon and nitrogen, as apart from oxygenic photosynthesis, some cyanobacterial genera are also capable of nitrogen fixation (Karl et al., 1997). During the later process, inert atmospheric nitrogen is converted into various nitrogen compounds available for uptake by other organisms within the natural ecosystems. Despite their relatively simple structure, cyanobacteria have reached quite remarkable degree of morphological diversity (Komárek, 2006). They are able to survive in broad range of habitats including the extreme ones, such as deserts, hot springs or freezing environments (Kulasooriya, 2011).

In recent years, noticeable amount of attention was paid to these microorganisms mainly in relation to their possible applications as food supplements, biofertilizers, biofuel producers and small factories of molecules with unique bioactivities. As this thesis is related to the last application, in the following text a small overview on the topic will be given.

1.1 Secondary metabolites

Secondary metabolites are organic compounds of diverse chemical structures, which are not essential for normal growth, development or reproduction of an organism. Their roles are usually not known, but it is expected that they give some advantage to their producers in the complex ecosystem (Mejeán et al., 2013). It was therefore suggested that they might play a significant role in the interspecies competitions, reproductive processes, provide protecting mechanisms against stress, or serve as organism's nitrogen storage molecules (Mandal and Rath, 2014).

1.2 Biosynthetic pathways

The unique chemical features and wide diversity of secondary metabolites originates in the nontraditional ways of their biosynthesis. These include primarily large multimodular enzymatic

systems of non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS) and hybrid NRPS-PKS pathways (Wase and Wright, 2008).

1.2.1 Non-ribosomal peptide synthetases (NRPS)

Secondary metabolites of peptide structure are generally produced by two types of pathways: the non-ribosomal peptide synthetases or the traditional ribosomal synthesis followed by post-translational modifications and processing. The first mechanism is described in more detail, as it is the more prevailing one (Kehr et al., 2011).

The NRPS system is encoded by large gene clusters, which are translated into multi-enzyme complexes followed by post-translational modifications (Welker & Von Döhren, 2006). In contrast to traditional ribosomal synthesis, the NRPS system operates nucleic acid-free, and the whole process is based on the protein templates (Finking and Marahiel, 2004; Sieber and Marahiel, 2005). The NRPS assembly line is composed of protein modules, which are responsible for incorporation of a single building block (Weber and Marahiel, 2001; Schwarzer et al., 2003; Finking and Marahiel, 2004). Each protein module is further composed of catalytic domains accountable for single reactions taking place during the biosynthesis, such as activation of amino acids or amino acid condensation. While minimal protein module consists of three main domains – adenylation (A), thiolation (T)/peptidyl carrier protein (PCP) and condensation (C) domain; other main or tailoring domains might be present. These mostly introduce modifications of the amino acid substrates thereby contributing to the overall uniqueness of the final product.

The main role of the adenylation domain is the specific recognition of substrate and its activation for further reactions in the pathway, which is achieved by the substrate's adenylation at the expense of an ATP molecule. The activated intermediate is then covalently bound by the 4'-phosphpantetheine arm of the peptidyl carrier protein via thioester linkage, which enables its interaction with other main or modifying domains. The elongation of the peptide chain is catalyzed by the condensation domain, which receives two activated intermediates bound to PCPs of the adjacent modules and facilitates the formation of peptide bond. Another main domain, the heterocyclization (Cy) domain, gives rise to heterocyclic moieties within the metabolite by catalyzing the cyclization of the cysteine, serine or threonine side chains. Terminal steps of the NRPS assembly line are catalyzed by the thioesterase (Te) domain and depending on the way they proceed, they result in formation of linear or cyclic product (Welker & Von Döhren 2006).

Apart from core NRPS domains, NRPS embedded modifying enzymes and external associate enzymes can be involved in the synthesis of natural products. These enzymatic units are often responsible for incorporation of unique residues hardly encountered in the products of classical biosynthetic pathways. Examples of those include the D-amino acids produced by epimerization (E) domains or external amino acid racemases, *N*-methylated and formylated intermediates originating from *N*-methyl transferase and formyl-transferase domains, respectively and halogenated residues produced by halogenases (Hur et al., 2012; Welker & Von Döhren 2006).

1.2.2 Polyketide synthases (PKS)

Some cyanobacterial secondary metabolites possess polyketide or fatty acid side chains originating from PKS pathways. Similarly to NRPS, polyketide synthases of modular type are large multifunctional assemblies organized into repeated units catalyzing gradual condensation of primitive building blocks (Dittmann et al., 2001).

Each polyketide module is responsible for recognition, activation and modification of single substrate. The PKS models are further composed of domains, with each domain performing single reaction in the process of overall synthesis. Minimal PKS domain consists of a kethosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP); however, additional auxiliary domains responsible for substrate modifications are often present. These include ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER). In analogy to NRPS, the last domain in the PKS assembly lines is the thioesterase (TE) domain, which catalyzes the hydrolysis or cyclization of the final product. The type and organization of PKS modules predetermines the final structure of secondary metabolite (Staunton and Weissman, 2001).

Like adenylation domains in the NRPS, the acyltransferase domains of PKS serve as gate keepers of the module. They recognize the starter, extender or intermediate acyl unit and covalently bind it to the prostetic group of the acyl carrier protein. Alternatively, the fatty acid precursors can be activated by adenylation catalyzed by the fatty acyl-AMP ligase (FAAL) enzymes and acylated onto the ACP of the polyketide synthases (Mohanty et al., 2011). In analogy to peptidyl carrier protein in NRPS systems, acyl carrier protein possesses the 4'-phosphopantetheinyl moiety that acts as flexible arm enabling the interactions of the acyl intermediates with other domains and is thus the main transportation principle of the PKS. The extension of the acyl chain is catalyzed by the kethosynthase domain. In this process, decarboxylative Claisen condensation between a

neighboring ACP-bound malonate derivative and an ACP-bound acyl thioester takes place (Chan et al., 2009).

The KR domain, DH domain, and ER domain are auxiliary domains increasing the diversity of PKS products. As its name suggests, the *β*-ketoreductase domain catalyzes the reduction of *β*-keto group of the acyl intermediate, using NADPH as cofactor (Bonett et al., 2013). The dehydratase domains generate an *α*,*β*-unsaturated thiol esters upon reaction in which water molecule is lost and the enoyl reductase domains catalyze the final reduction to full intermediate saturation (Cane et al., 1999).

1.2.3 Hybrid NRPS-PKS pathways

NRPS and PKS pathways share some structural and catalytic features and possess a very similar approach in their strategy for the biosynthesis of natural products. Both pathways are large multifunctional systems composed of smaller compartments. They use the same transportation principle based on the 4'-phosphopantetheinyl arm of the peptidyl/acyl carrier protein, which is attached to these domains post-translationally by 4'phosphopantetheinyl transferases. Another shared feature of the two machineries is the release of the peptide/polyketide product catalyzed by the thioesterase domain located at the *C*-terminus of the assembly lines (Du et al., 2001).

These similarities suggested the existence of mixed NRPS-PKS systems, which were proven to be present in many secondary metabolite producing organisms and are a major trait of cyanobacterial pathways. Based on the way the two pathways interact and cooperate with each other, the mixed systems can be divided into two classes. The first one contains systems in which direct functional hybridization of the pathways is present, further divided into hybrid NRPS-PKS and hybrid PKS-NRPS pathways, depending on the chemical character of the precursor and extending units. The second group comprises of systems involving some other, indirect mechanisms of hybridization, e.g. coupling of the individual NRPS and PKS products by ligases (Kehr et al., 2011).

1.3 Secondary metabolites of cyanobacteria and their screening

The uniqueness and large diversity of the chemical structures produced by cyanobacteria via secondary metabolism has attracted the attention of many scientific groups and was leading to their investigation at different levels. Up to date, cyanobacteria from different taxa and geographic

origins have shown to be producing metabolites with interesting biological activities, ranging from antimicrobial and immunosuppressant to anticancer and anti-HIV (Wase & Wright 2008).

Along the compounds with potential pharmacological implications, cyanobacteria have been long known to produce toxic secondary metabolites (Krishnamurthy et al. 1986; Codd 1992). In the past, cases of sickness and death of cattle, horses, pets and wildlife occurring upon ingestion of water containing toxic cyanobacterial cells or the toxins themselves were reported (Francis 1878; Carmichael & Bent 1981).

The vast majority of cyanobacterial secondary metabolites are synthesized via hybrid NRPS-PKS pathways (Mejeán *et al.*, 2013). The great diversity of the structures originates in the contributions from both already very unique pathways, making it possible to combine proteinogenic amino acids with non-proteinogenic amino acids and fatty acids of large variety. Additionally, fatty acids have often noticeable influence on physico-chemical properties of peptides, for example by influencing their hydrophobicity (Welker & Von Döhren 2006).

Apart from polyketide and non-ribosomal peptide metabolites, other groups like alkaloids, terpenoids, glycosides or ribosomal peptides have been reported. Last, but not least, each metabolite can have several variants, which are synthesized by the same cluster. Common variations include change in amino acid residue, methylation, hydroxylation and others. These can be introduced by tailoring domains or by NRPS/PKS domains with relaxed specificity (Mejeán *et al.*, 2013).

Rising discovery of secondary metabolites produced by cyanobacteria is very closely related with the rapid advance in development of bioinformatic and analytical tools. Up to date, large range of techniques have been used to detect and identify these natural products. Main approaches include the individual one at a time screening of extracted metabolite and the complex genomic screening of an organism (Mandal & Rath 2015).

In the genomic approach, the so called genomic mining is performed. Upon this process, the screening of cyanobacterial genomes for presence of genes and gene clusters related to the biosynthesis of natural products takes place. The screening is based on the alignment of the sequence under investigation with the sequences of characteristic enzymes involved in the specific biosynthetic pathway, such as PKS or NRPS. Nowadays, some genome-screening programs like ClustScan, NRPS-PKS and others can be used to predict the presence and location of these genes and gene clusters within the cyanobacterial genome. However, the prediction of the exact structure of natural products is sometimes not possible, mainly due to non-predictability of the post-

assembly modifications, skipping of the domains, ambiguous cyclization patterns or noncollinearity of some enzymes within the pathway (Mandal & Rath 2015).

Another approach is the analytical one. Up to date, various analytical tools have been used in the studies of cyanobacterial secondary metabolites, allowing for formation of rapid, selective and highly sensitive procedures for their detection. High performance liquid chromatography (HPLC), mass spectrometry (MS), nuclear magnetic resonance (NMR) and their combinations belong to the tools most widely used for the screening of natural products, allowing for their separation, detection, quantification and structural elucidation (Lee & Shen, 2004).

1.3.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) has become a very powerful and widespread technique adapted for analysis of secondary metabolites of diverse chemical natures. Regarding the detection, methods such as ultraviolet (UV) detection and photodiode array detection (DAD) belong to the most common ones. In recent years, the so called hyphenated techniques coupling HPLC system with mass spectrometer (MS), tandem mass spectrometer (MS-MS) or nuclear magnetic resonance (NMR), have become of great importance in the field (Wolfender 2009).

HPLC is a very versatile technique, which enables efficient separation of natural products from crude extracts. With continuous advance in technology, HPLC has undergone a great development, leading to increase in the speed, sensitivity, applicability to a wide range of sample types and many other factors. Nowadays, raw mixtures of samples or samples enriched in a metabolite by solid phase extraction (SPE) or liquid-liquid extraction (LLE) are commonly injected into HPLC systems. The separations of analytes usually take place in the reversed-phase C18 or C8 columns with the use of methanol-water or acetonitrile-water mobile phases in gradient elution modes.

Hyphenation of the HPLC system with the mass spectrometry ensures exceptional sensitivity and selectivity for the analysis of natural products in the biological matrices. Additionally, the mass spectrometry detection provides important structural information about the metabolite, such as its molecular weight and characteristic fragments crucial for the metabolite identification and characterization. In the case of HRMS, the molecular formula of the metabolite can be obtained as well.

In the HPLC-MS coupled systems, three main ionization modes are used: electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), all occurring at the atmospheric pressure.

In the case of ESI, the sample eluted from the HPLC column experiences nebulization performed by a high voltage field (3-5kV). This results in the formation of charged eluate droplets, which are directed towards the mass analyzer. During this process, the so called "ion evaporation" takes place resulting in the formation of individual ions, which are subsequently separated by the MS system according to their *m/z*.

In APCI, the vaporization of eluent takes place by applying heat. Then, corona discharge is used for ionization of solvent particles, which are subsequently used for production of analyte ions in the process of chemical ionization.

Similarly to APCI, APPI starts with vaporization of the eluent by heat. However, the formation of ions is based on the process of photoionization, in which an UV lamp producing photons with energy of 10 eV is involved. Depending on the sample, either ionization of mobile phase, or ionization of dopant added to effluent occurs, which is followed by ionization of analyte.

Aforementioned modes of ionization are recognized as soft methods, usually leading to formation of protonated molecules $[M+H]^+$ in positive-ion mode (PI) or deprotonated molecules $[M–H]^-$ in negative-ion mode (NI). Formation of different adducts $[M+Na]^+$ (PI) or $[M+HCOO]^-$ (NI) is also possible and depends on the solutes and modifiers used in the analysis.

The ions produced are subsequently analyzed by the MS systems. Nowadays, various kinds of mass spectrometers are available, covering range of resolutions. Single quadrupole (Q) mass spectrometers possess low resolution and are commonly the choice when expenses have to be considered. The high resolution (HR) mass spectrometers with high mass accuracies, such as the time of flight (TOF) and triple-quadrupole (QQQ) have become very popular. The QQQ systems are widely used in bioanalytical assays mainly due to their ability of specific detection as they can provide useful information about structure-specific fragments (Mandal & Rath 2015).

1.3.2 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance provides information about the structure, atomic connectivity and stereochemistry of the analyte, which mass spectrometry cannot. In order to obtain these information, various methods developed over years of NMR existence are used.

Skeletal connectivity of the metabolite can be proposed by combining data from homonuclear (COSY) and heteronuclear (HSQC and HMBC) correlation spectroscopy. These data can be supplied with the information about stereochemistry obtained from the Nuclear Overhauser Effect (NOE) correlations between proton-proton and proton-carbon coupling constants. Gathering and interpretation of all these data yields complex information about the natural product structure (Kwan & Huang 2008).

1.4 Principles of cyanobacterial cultivation

Basic principles of microbial cultivation can be applied to cultivation of cyanobacteria, with additional factors to be considered.

1.4.1 Culture modes

Generally, two main modes of cultures are recognized: batch and continuous cultures.
The batch cultures belong to the most common ways of cyanobacterial cultivation due to their ease of operation and simple system. In this culture type, limited amount of cyanobacterial inoculum and medium are placed in a cultivation vessel and incubated in the desired conditions. Changes in biomass and environment are reflected by different growth phases of the culture.
Lag phase is the initial phase of the culture, in which growth lag occurs. This might be caused by presence of the non-viable cells or spores in the inoculum as well as by the physiological adjustment of the inoculum to change in culture conditions (Lee and Shen, 2004).
When cells adjust to the new environment they enter the phase in which their growth and division is an exponential function of time, the so called exponential or log phase. The time necessary for doubling of the number of viable cells in the exponentially growing culture is called the doubling time and the process can be mathematically described as:

$$N_t = N_0 2^n = N_0 2^{\frac{t}{t_d}}$$

where N_t is the number of cells in the exponential phase after some time of incubation t, N_0 is the initial number of cells, n corresponds to number of doublings in the time of incubation t and t_d is the doubling time of the culture. As biomass can be determined more accurately than the number of the cells, the aforementioned equation is usually expressed in terms of the biomass. The culture

resides in the exponential phase as long as nutrients and light energy are saturated (Lee and Shen, 2004).

Last phase of the culture is the linear or stationary phase, in which biomass accumulates at constant rate until some factor becomes limiting. This phase occurs when certain concentration of cells is reached in which all photons reaching the culture surface are absorbed (Göbel, 1978; Lee and Shen, 2004).

In the case of continuous cultures, homogeneously mixed culture is supplied with fresh medium and removed continuously, sustaining the cell growth over time. This allows to maintain the culture in the pre-determined cell growth rates, allowing the cells to fully adjust to the environmental factors. Five basic types of this culture mode are distinguished: chemostat, cyclostat, turbidostat, fed-batch culture and cell recycled culture (Lee and Shen, 2004).

1.4.2 Light requirements

When dealing with photoautotrophic organisms, light is a very important factor determining the productivity of biomass as well as the productivity of secondary metabolites. The light energy received by the culture is dependent on the photon flux density (PFD) arriving to its surface. The extent of photons absorbed by cells is affected by several factors, such as rate of culture mixing, cell density and length of the optical path of the reactor. Excess photons are either dissipated as heat or reflected. When the culture density reaches a value, in which all the photosynthetically available photons are absorbed, the biomass accumulates at constant rate until light per cell or some other factor reaches a limiting value (Richmond, 2004).

Additionally, it was discovered that for each temperature, there is a specific light intensity at which the maximum of photosynthetic rate is reached (Collins and Boylen, 1982). At low irradiance levels, high temperatures of the cultures significantly decreased the photosynthetic rate. Generally, the optimal temperature for photosynthesis increases with increasing irradiance (Richmond, 2004).

1.4.3 Nutrient requirements

Besides the light energy needed for the reduction of CO_2, photoautotrophic organisms require specific amounts of nutrients in form of inorganic mineral ions. These are known as macro- or micronutrients, depending on the amount necessary to be supplied to the organisms, such that their

normal growth and reproduction is sustained. The most important macronutrients for autotrophic growth include carbon, nitrogen and phosphorus (Vonshak, 1986).

As supply of CO_2 is crucial for high rates of autotrophic production, carbon is the most important nutrient contributing to the biomass produced. Since the CO_2 supply from atmosphere is insufficient to satisfy the carbon needs of the cyanobacterial production systems especially in higher light densities, constant gassing of the cultures with air enriched in CO_2 is usually performed. Additionally, the bicarbonate-carbonate buffer system is very important for the control and maintenance of the pH levels optimal for the cultivation. In case of mixotrophic species, carbon can be supplied in the form of various organic substances, such as sugars, acids or alcohols (Grobbelaar, 2004).

The second most important macroelement is nitrogen. The nitrogen content in the biomass can range from 1 to 10%, depending on the species and on the nitrogen supply and availability. When nitrogen becomes limited, the cultures tend to respond by discoloration due to lowered chlorophyll and increased carotenoid content and by accumulation of various compounds, such as polysaccharides or polyunsaturated fatty acids (PUFs) (Becker, 1994). Nitrogen is most often supplied in the form of nitrate (NO_3^-), but other sources such as ammonia (NH_3) or nitrogen-containing organic compounds can be used. Additionally, some cyanobacteria are capable of atmospheric nitrogen fixation. In general, the nitrogen supply should be regulated according to the purpose of the culture. If high biomass yields are to be obtained, sufficient amounts of nitrogen, such that it never becomes a limiting factor, should be supplied. However, for some applications such as production of β-carotene, carbohydrates or oils, nitrogen is supplied in limiting concentrations on purpose (Ben-Amotz and Avron, 1989; Borowitzka, 1988)

As phosphorus is part of many important biomolecules, its supply is essential for normal growth of the culture. It is usually supplied in the form of orthophosphate (PO_4^{2-}). Due to its ease of precipitation from the medium upon binding with other ions, phosphorus belongs to one of the most important limiting factors in the cultivation. Additionally, the ratio of nitrogen to phosphorus in the medium predetermines the productivity as well as the type of species dominating the culture (Grobbelaar, 2004).

Examples of other important macro- and micronutrients are: S, K, Na, Fe, Mg, Ca, B, Cu, Mn, Zn, Mo, Co, V and Se. Many trace elements are important cofactors of various enzymes (Kaplan et al., 1986).

1.5 *Desmonostoc muscorum*

The cyanobacterial genus *Nostoc* includes wide range of species with various morphology, biotic relations and habitat distribution. Some species are free-living, while others enter symbiosis with broad range of different land plants or fungi, mainly due to their ability to fix atmospheric nitrogen. Cyanobacteria belonging to this genus are able to survive repeated freeze-thaw cycles and remain in the desiccated state for longer periods of time, coming back to their activity within hours to days after rehydration, which enables their survival in several extreme environments (Dodds et al, 1995). While some *Nostoc* species have been reported to cause nuisance to humans (Wnorowski 1992), other species have been considered as a delicacy and recognized for their herbal values for more than 2000 years (Gao et al, 1998).

Based on recent phylogenetical and morphological studies, *Nostoc muscorum* and related strains were delimited into a novel cyanobacterial genus *Desmonostoc* including both free-living and symbiotic representatives (Hrouzek et al., 2013).

Desmonostoc muscorum is a free-living cyanobacterium inhabiting terrestrial as well as aquatic environments. In recent years, it was studied in relation to its ability of production of the polyhydroxybutyrates (PHBs), which have gained relatively large popularity as biodegradable and biocompatible plastics (Sharma and Mallick, 2005). Very recently, the production of two secondary metabolites with interesting cytostatic activity was described from *Desmonostoc muscorum* CALU 456 (Vicková, 2015).

1.6 Targeted cancer therapy drugs

Close investigation of the unique molecular changes underlying the development of certain cancer type led to a new generation of cancer treatment, generally recognized as targeted cancer therapy. As the name suggests, these therapies interfere with specific target genes or proteins crucial for promotion of the cancer growth, rather than having universal mode of action, which affects the rapidly dividing cells as well as normal cells of certain type. In general, two groups of targeted cancer therapy drugs are recognized, including the monoclonal antibodies and small molecule inhibitors. While monoclonal antibodies target antigens located on the cell surface, small molecules penetrate the membrane of the cell and interact with target molecules within the cell (Baudino, 2015).

Relatively large number of drugs belonging to the group of small molecules was obtained from the natural resources, either by structural modification of the natural product or by synthesis of new compounds with natural compound serving as a model for the synthesis. Some examples of those are paclitaxel first isolated from the Western yew *Taxus brevifolia*; docetaxel, a semisynthetic analogue of paclitaxel; vinblastine and vincristine originally isolated from the pink periwinkle plant *Vinca rosea*; camptothecin produced by tree of happiness *Camptoteca acuminate* and podophyllotoxin isolated from a resin produced by the plant of *Podophyllum* genera (Gordaliza, 2007).

1.6.1 Cyanobacterial secondary metabolites with anticancer activity

As stated previously, cyanobacteria possess large diversity of unique secondary metabolites with interesting biological activities of different kind, including the anticancer activity. Some of the cyanobacterial products and their synthetic derivatives are already being used in the preclinic and clinic studies, examples of which include cryptophycins and dolastatins.

Cryptophycin-1 was originally isolated from the terrestrial cyanobacterium *Nostoc* as a potent antifungal agent. Upon its studies, a high cytotoxicity towards cancer cell lines was detected, which was later explained by its attack on the tubulin microfilaments of the eukaryotic cells, thereby hindering their division and reproduction (Vijayakumar and Menakha, 2015). Thanks to this activity, cryptophycin-1 has entered the clinical trials. So far, many cryptophycin analogues differing in their physical-chemical properties and anticancer activities have been obtained by isolation or chemical synthesis. Some of the most prominent examples are cryptophycin-5 and cryptophycin-8 (Liang et al., 2005; Corbett et al., 1996).

Another cyanobacterial secondary metabolite showing an anticancer activity is dolastatin 10. Its production was first attributed to a marine mollusk *Dolabella auricularia* (Kamano et al., 1987), however, it was later discovered, that the producer of this compound is not the mollusk itself, but rather a cyanobacterium *Symploca* sp. eaten by it. Dolastatin 10 is a pentapeptide, which binds to tubulin affecting thus the microtubule assembly and leading to incapability of the cell to go from G2 to M phase of the mitotic division (Vijayakumar & Menakha 2015). Different compounds have been derived from dolastatin 10, among which monomethyl auristatin E is of great importance. It is covalently linked to a monoclonal antibody directed to antigens overexpressed in tumor cells

and generally included within the group of antibody-drug conjugates (ADCs) (Bouchard et al. 2014).

Most of the aforementioned examples of naturally derived (cyanobacterial) compounds possess a rather general cytotoxic/cytostatic effect. However, their specificity and use in targeted therapy can be reached by subsequent chemical modification and binding to monoclonal antibodies.

2 Aims of the work

- To study the biomass production of the terrestrial cyanobacterial strain *Desmonostoc muscorum* CALU 456 in different cultivation conditions (temperature, light, nitrogen availability)

- To investigate the effect of different cultivation conditions (temperature, light, nitrogen availability) and the age of the culture on the production of the cytostatic secondary metabolite 2505 and its natural analogues

3 Materials and methods

3.1 Chemicals

Acetonitrile (LC-MS grade, VWR)
Formic acid (LC-MS grade,VWR)
Methanol (LC-MS grade, VWR)
Methanol (p.a., VWR)
Sea sand (VWR)

All chemicals used for preparation of the BG-11 medium (see chapter 3.2.1) were purchased at VWR.

3.2 Methods

3.2.1 Preparation of cultivation media

BG-11 cultivation medium

The BG-11 cultivation medium was prepared according to the Stanier et al., 1971. The medium was prepared by dissolving the stock solutions (100 times concentrated) in distilled water and autoclaved for 20 min at 120°C.

Table 1: Final concentrations of chemicals in the BG-11 medium

Chemical	Concentration / mg L^{-1}
$NaNO_3$	1496.000
$MgSO_4 . 7 H_2O$	74.800
$CaCl_2 . 2 H_2O$	36.000
$C_6H_8O_7$	6.000
NaEDTA	0.940
H_3BO_3	0.572
$MnCl_2 . 4 H_2O$	0.362
$ZnSO_4 . 7 H_2O$	0.044
$Na_2MoO_4 . 2 H_2O$	0.078
$CuSO_4 . 5 H_2O$	0.058
$Co(NO_3)_2 . H_2O$	0.010
Na_2CO_3	20.000
$K_2HPO_4 . 3 H_2O$	41.000
$C_6H_8O_7.xFe^{3+}.yNH_3$	6.000

Nitrogen-free BG-11 cultivation medium

For the preparation of the nitrogen-free BG-11 medium, the same procedure as for preparation of basic BG-11 was used, except for the addition of $NaNO_3$.

3.2.2 Cultivation and harvest of cyanobacterial biomass

For all the experiments, the strain of terrestrial cyanobacterium *Desmonostoc muscorum* CALU 456 (denoted as strain 33 in the collection of Laboratory of Algal Biotechnology in Algatech Centre, Institute of Microbiology, The Czech Academy of Sciences) was used.

Cross-gradient experiment

For the cross-gradient experiment, 20 mL of homogenized cyanobacterial inoculum and 180 mL of sterile BG-11 cultivation medium were transferred into 9 similar Erlenmayer flasks placed on the cross-gradient table. Along the *x* axis, temperature gradient (from x °C to y °C) was established such that three different temperature conditions (30, 23 and 15 °C) were obtained. Along the *y* axis, three different light intensities (5, 50 and 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were established by partly shielding the incoming light by filtration paper. As a result, 9 batch cultures with different temperature and light conditions were obtained (Tab. 2, Fig. 1). The strains were cultivated in a static way – without shaking or mixing with air enriched in CO_2. After 10 days from the beginning of the experiment, the individual biomasses were harvested by centrifugation (Sorvall Evolution RC, 16 264 *g*, 15 min), stored at -80 °C and freeze-dried.

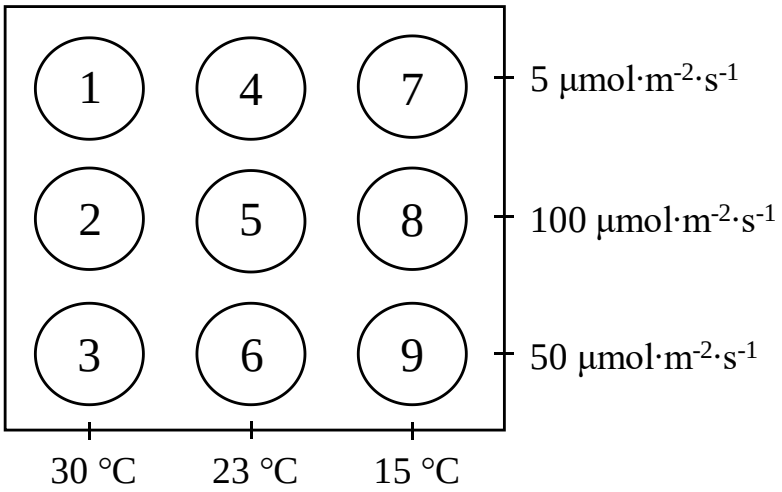

Figure 1. Cross-gradient experiment cultivation conditions: x axis – Temperature (°C), y axis – Light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$).

Table 2. Cross-gradient experiment cultivation conditions.

Sample	Temperature / °C	Light intensity / $\mu mol \cdot m^{-2} \cdot s^{-1}$
1	30	5
2	30	100
3	30	50
4	23	5
5	23	100
6	23	50
7	15	5
8	15	100
9	15	50

Dynamic experiment I

For the first dynamic experiment, 20 mL of homogenized inoculum and 280 mL of the corresponding cultivation medium were transferred into 12 cylindric photobioreactors with volume of 350 mL. This time, four triplicate sets with different cultivation conditions (Tab. 3) were obtained by establishing two different light intensity values (L1 = 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, L2 = 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and two different nutrient conditions (full BG-11 and nitrogen-free BG-11 medium). The strains were cultivated nine days at constant temperature of 29 °C and were bubbled with air enriched in 2% CO_2. The sample (10 mL) of each batch culture was taken every 24 hours, the biomass was separated from medium by centrifugation (Sorvall Evolution RC, 24 303 *g*, 10 min), stored at –80 °C and freeze-dried.

Table 3. First dynamic experiment cultivation conditions (N+ = *N*-containing BG-11, N- = *N*-free BG-11).

Set of triplicates	Sample	Medium	Light intensity / $\mu mol \cdot m^{-2} \cdot s^{-1}$
1	1	N+	250
	2	N+	250
	3	N+	250
2	4	N-	250
	5	N-	250
	6	N-	250
3	7	N+	100
	8	N+	100
	9	N+	100
4	10	N-	100
	11	N-	100
	12	N-	100

Dynamic experiment II

For the second dynamic experiment, approximately 10 L of cyanobacterial inoculum and 50 L of the BG-11 medium were transferred into 60 L flat photobioreactor. The strain was cultivated for 12 days at temperature 29 °C, light intensity 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and constant bubbling with air enriched in 0.7% CO_2. Every 24 hours, 0.5 L sample was taken from the homogenized culture, biomass was separated from the medium by centrifugation (Sorvall Evolution RC, 24 303 *g*, 10 min), stored at –80°C and freeze-dried.

For the first repetition of this experiment, approximately 10 L of previously grown culture were left in the photobioreactor and diluted with 50 L of BG-11 medium. The cultivation conditions were kept the same, except for the overall time of the experiment, which was reduced to 10 days.

For the second repetition of the experiment, the photobioreactor was sterilized by ethanol and approximately 10 L of cyanobacterial inoculum and 50 L of BG-11 medium were transferred into it, resulting in approximately the same biomass concentration as in the previous two experiments in 60 L flat photobioreactor. The strain was cultivated for 10 days with the same temperature, light and aeration conditions as in the original experiment.

3.2.3 Extraction of cyanobacterial biomass

After lyophilization in plastic Petri dishes the exact dry weight of biomass was determined with analytical balances. Subsequently, approximately 10 mg of the biomass was transferred into 2 mL Eppendorf tube, into which 70% methanol and sea sand were added. Ratio of the extraction solvent to biomass was kept constant, using 1 mL of the solvent per 10 mg of biomass. The mixture was homogenized by a pestle and the extraction time was set to minimum of 1 hour, after which centrifugation step was performed (Eppendorf centrifuge, 1920 *g*, 10 min). The supernatant was analyzed by HPLC-HRMS directly or stored at –80 °C and analyzed afterwards.

3.2.4 HPLC-HRMS analysis of the extracts

Crude extracts were analyzed for the presence of 2504 cytostatic secondary metabolite (in this work designated as 2505 according to its neutral molar mass) detected previously (Vicková, 2015). For the analysis, HPLC system Dionex UltiMate 3000 (Thermo Scientific) with diode array detection (DAD) hyphenated with Impact HD mass spectrometer with an ESI source (Bruker) was

used. The separation of the sample components was performed on the Phenomenex Kinetex (150 x 4.6 mm; 2.6 µm) C18 column, with constant flowrate of 0.6 mL·min^{-1} and gradient elution, for which the water (A)/acetonitrile (B) system (both enriched in 0.1 % HCOOH) was used as a mobile phase (Tab. 4). Following operating parameters of the mass spectrometer were set: flowrate of drying gas 12 L· min^{-1}, temperature of drying gas 210 °C, pressure of nebulizing gas 3 bar and spray needle voltage of 3.8 kV.

Table 4. Gradient elution summary.

Time / min	%A	%B
0	85	15
1	85	15
20	0	100
25	0	100
30	85	15

4 Results and discussion

4.1 Results

During previous studies on cytotoxicity of crude extract of the terrestrial cyanobacterium *Desmonostoc muscorum* CALU 456 (in the collection denoted as strain 33), a cytostatic effect towards PaTu (human pancreatic adenocarcinoma) and HeLa (human cervix adenocarcinoma) cell lines was detected. This activity was attributed to a substance with *m/z* of 2505, as it was present in all active sub-fractions of the crude extract. Another substance proposed to have a similar cytostatic effect was reported as 2519 based on its *m/z* (Vicková, 2015).

Mass spectra of both compounds showed similar features – presence of two intensive isotope clusters, which were attributed to $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ion upon studying the differences between *m/z* values of individual isotopologues within the cluster, as these were equal to 0.5 and 0.33, respectively. Upon closer investigation of the base peak chromatogram, four more substances with these features were detected (Fig. 2). As the $[M+H]^{+}$ ions of these substances were absent, their mass to charge ratios as well as neutral molar masses were obtained by deconvolution, for which the *m/z* value of $[M+3H]^{3+}$ ion was used (Table 5). This choice was based on the fact that peaks of the isotope cluster belonging to triply charged ion were generally higher in intensity compared to the doubly charged ion.

Table 5: Summary of *m/z* values, retention times and neutral monoisotopic masses of detected metabolites.

Compound	**$[M+3H]^{3+}$**	**$[M+2H]^{2+}$**	**$[M+H]^{+}$ (calculated)**	**M / g·mol^{-1}**	**retention time / min**
2449	817.2841	1225.4345	2449.8363	2448.8283	12.5
2463	821.9557	1232.4398	2463.8511	2462.8431	13.0
2477	826.6307	1239.4501	2477.8761	2476.8681	13.7
2491	831.2992	1246.4582	2491.8816	2490.8736	14.4
2505	835.9738	1253.4643	2505.9054	2504.8974	15.0
2519	840.6477	1260.4814	2519.9271	2518.9191	15.5

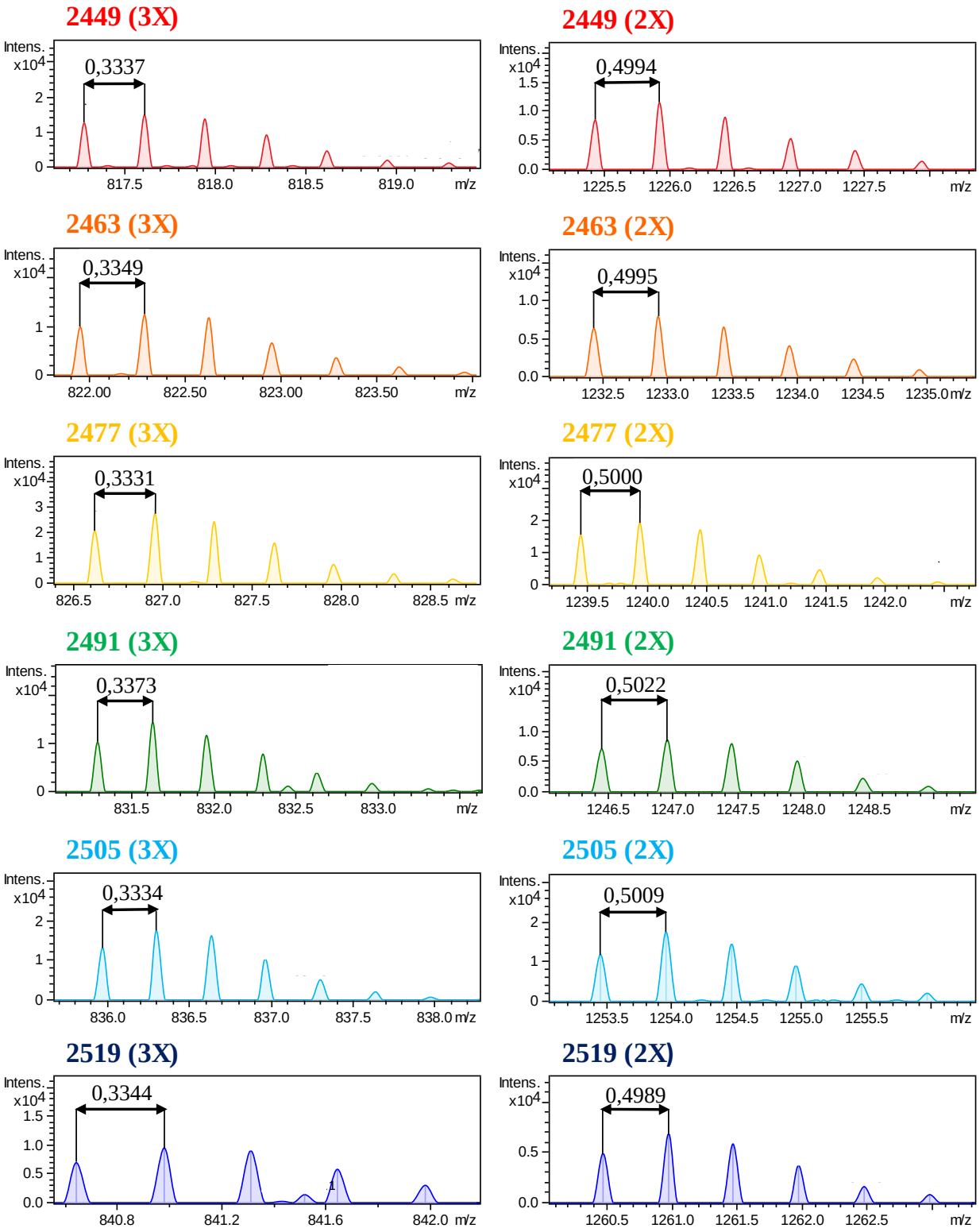

Fig. 2: Isotope clusters belonging to $[M+3H]^{3+}$ (left) and $[M+2H]^{2+}$ (right) of the detected secondary metabolites; first peak on each picture corresponds to monoisotopic mass of the cluster.

Experimentally determined monoisotopic masses of $[M+2H]^{2+}$ and $[M+3H]^{3+}$ were used to obtain the extracted ion chromatograms (EIC) of the corresponding substances.

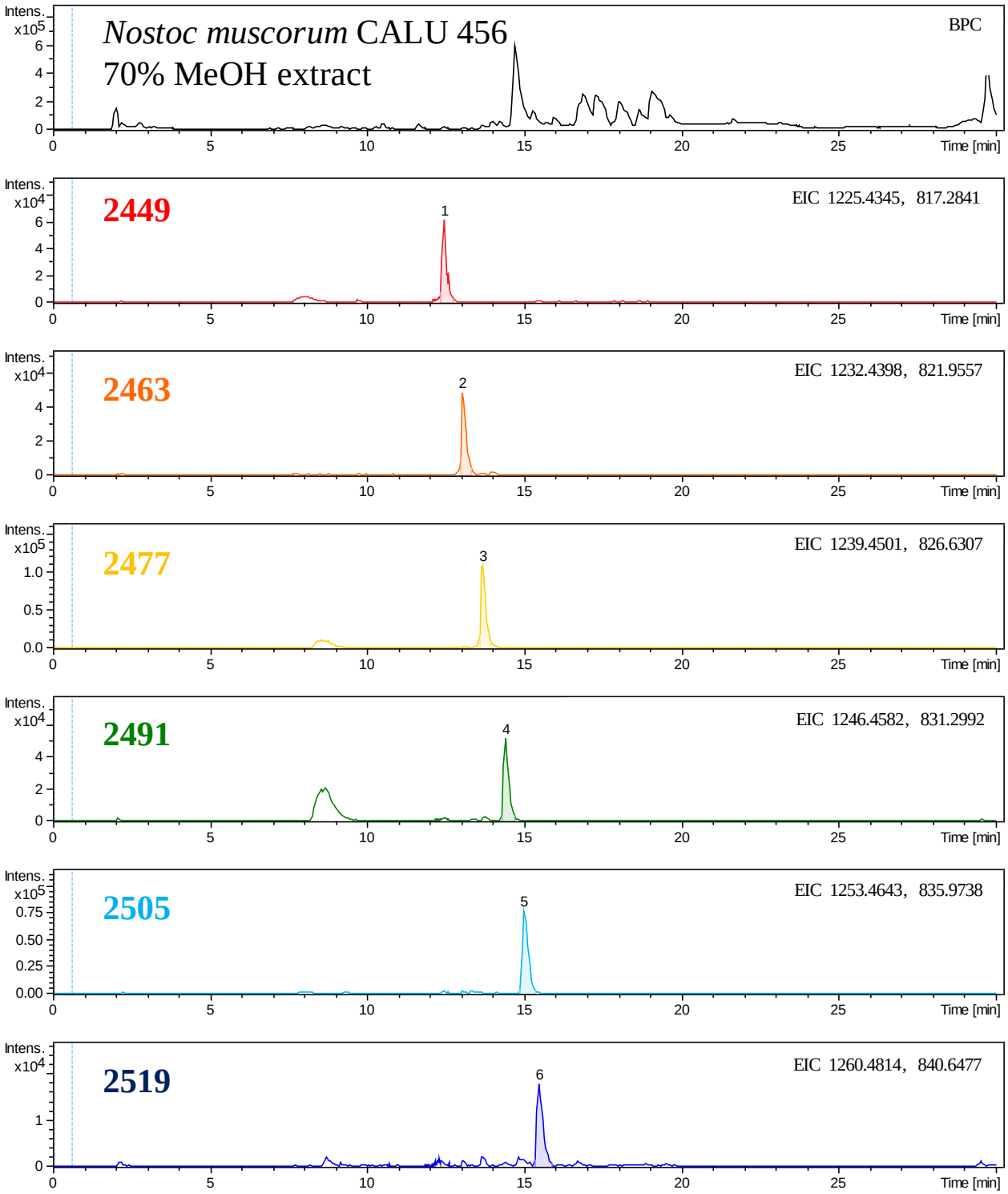

Fig. 3: Base peak chromatogram of Desmonostoc muscorum CALU 456 crude extract and extracted ion chromatograms of the individual metabolites.

The EIC overview (Fig. 3) showed a presence of relatively constant shifts in the retention times. By calculating the differences between the *m/z* of $[M+H]^+$ ions of individual metabolites, values in the range from 14.0055 to 14.0217 were obtained. According to these differences, it was suggested that the individual metabolites could differ by presence of methylene group, $-CH_2-$, with average neutral mass of 14.0266 $g \cdot mol^{-1}$ and therefore represent the natural analogues of the metabolite 2505. By relating the *m/z* of $[M+H]^+$ of the metabolites with their retention times, a linear trend was obtained (Fig. 4).

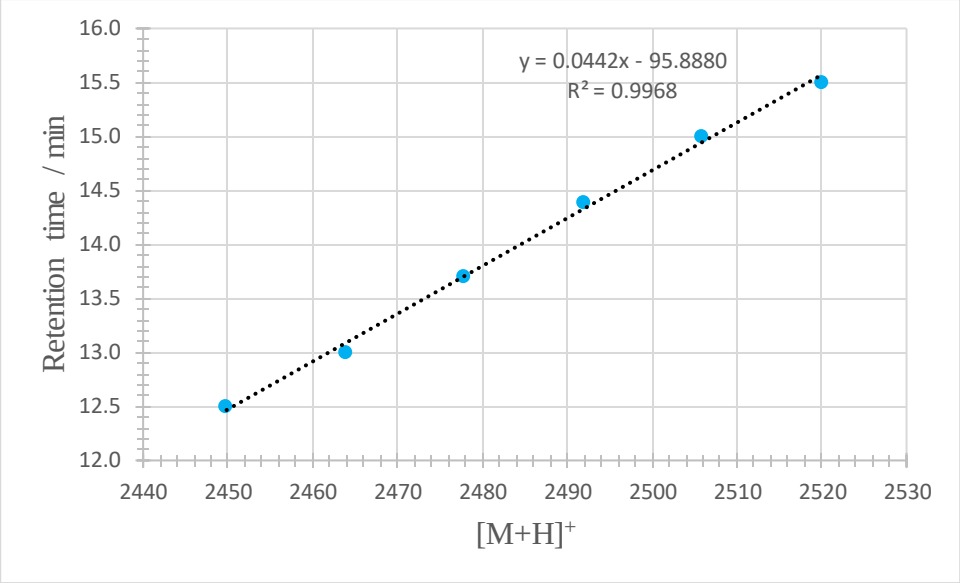

Fig. 4: Relation between the m/z of $[M+H]^+$ of the six metabolites produced by Desmonostoc muscorum CALU 456 and their retention times.

During the practical part of the work, the study of the influence of different cultivation conditions and age of the culture on the production of cyanobacterial biomass as well as the metabolite 2505 and its natural analogues was performed.

Cross-gradient experiment

Note: T stands for temperature (°C), L stands for light intensity ($\mu mol \cdot m^{-2} \cdot s^{-1}$)

In the cross-gradient experiment, the effect of nine different temperature/light combinations was studied on the non-aerated batch cultures of *Desmonostoc muscorum* CALU 456.

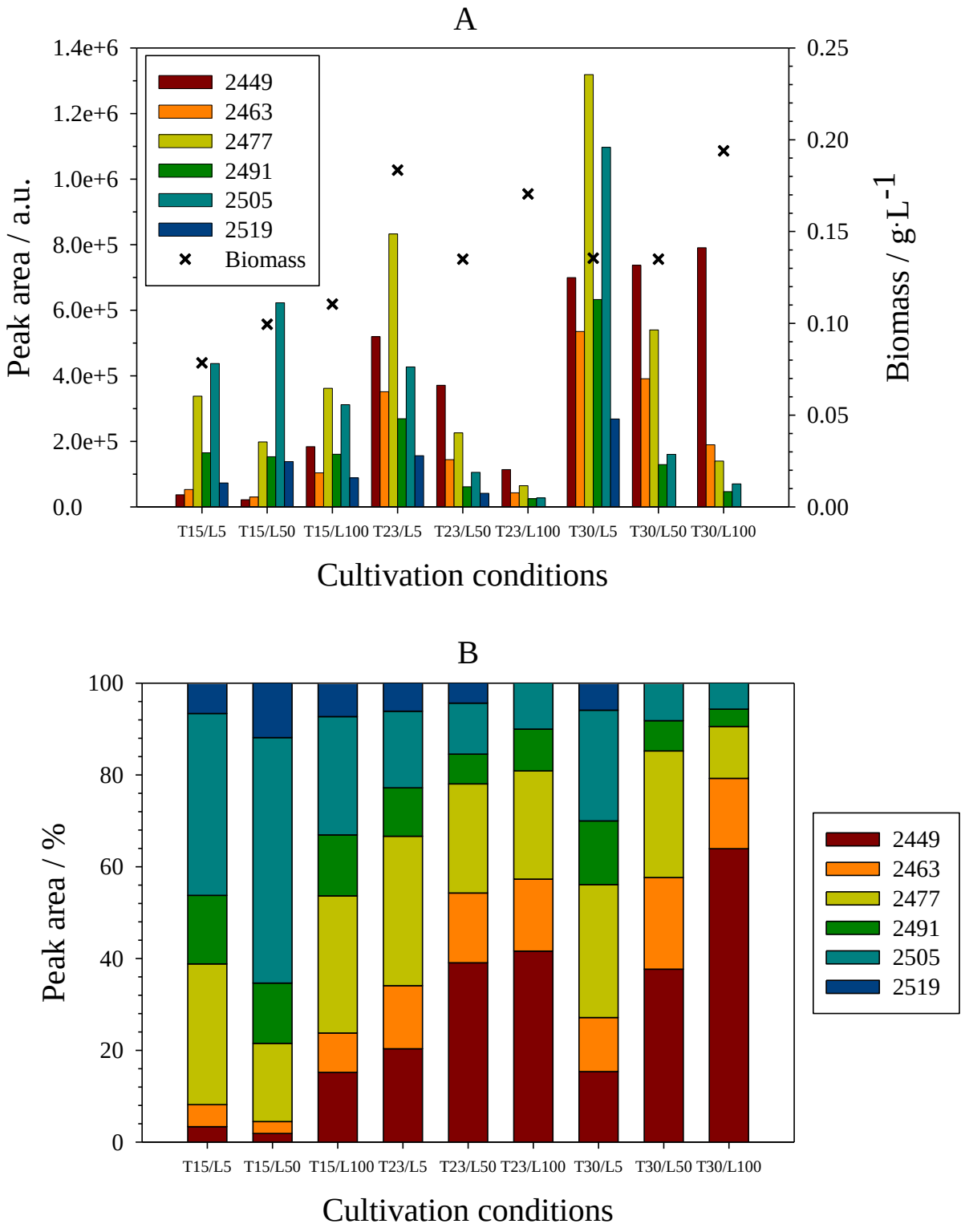

Fig. 5: Figure A shows the peak area of 2505 and its analogues (y-axis, left) and biomass concentration in each Erlenmayer flask after 10 days of cultivation in specific conditions (y-axis, right); figure B shows the relative abundance of the 2505 and its analogues in Desmonostoc muscorum CALU 456 biomass.

The biomass resulting from 10 days of cultivation in the specific conditions was generally reaching values from ~0.08 g $\cdot L^{-1}$ to ~0.20 g $\cdot L^{-1}$ (Fig. 5, A). The highest amount of biomass was produced at temperature 30 °C and light intensity 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, while the lowest amount was produced at 15 °C and 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Regarding the production of the metabolite 2505 and its analogues, the highest content was observed in conditions T30/L5, while the lowest one in conditions T23/L100. As the data corresponding to first three sets of columns (representing the temperature 15 °C and three different light intensities (Fig. 5, A)) show, the analogues of lower mass (2449, 2463 and 2472) had the same trends of absolute abundance content, reaching minimum at L50 and maximum at L100, analogue 2491 was produced in approximately the same amounts and compounds 2505 and 2519 had similar regularity of production with maximum at L50. In second set of columns representing 23 °C and three different light conditions, the metabolite 2505 and the analogues seem to be following the same trend of decreasing metabolite production with increasing light intensity. The third set of columns also showed the trend of decreasing production of the individual analogues with increasing light intensity, with the exception of analogue 2449, which showed an opposing trend and thus its production slightly increased with increasing amount of light.

Depending on the conditions, one of the compounds 2505, 2449 or 2477 was dominating the relative abundance (Fig. 5, B). Generally, the analogue 2449 was prevailing in the higher temperatures (23 and 30 °C), while metabolite 2505 was more abundant in the lower temperatures (15°C). The least abundant analogue of all was the 2519.

Dynamic experiment I

Note: L stands for light intensity, N+ stands for nitrogen-containing and N- for nitrogen-free BG-11 cultivation medium

During this experiment, the effect of nitrogen availability and its depletion was studied on the batch culture of *Desmonostoc muscorum* CALU 456 in two different light conditions.

As inferred from the data, the nitrogen availability had a profound effect on the culture growth. In the case of nitrogen-free condition, the increase in the biomass was smaller with the biomass concentration reaching a value of ~3 g$\cdot L^{-1}$ after 9 days of cultivation, which is comparingly lower than the biomass produced in the nitrogen-containing medium, where the final concentration was ~6 g $\cdot L^{-1}$. The effect of light was not that significant, however, the final biomass concentration was slightly higher in the L2 condition, corresponding to the light intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Regarding the production of 2505 and its analogues, similar trends have been detected in all four conditions. The lower molecular weight analogues (2449, 2463, 2477 and 2491) were produced prevailingly in the first days of the experiment, with analogues 2477 and 2491 reaching their production maximum at the biomass concentration of approximately 2 g· L^{-1}. The production of analogue 2505 was increasing rather quickly at the beginning of the experiment, in most cases leading to a static or decreasing phase in later days. The production of analogue with highest molecular weight, namely the 2519, was gradually increasing from the beginning till the end of the experiment, mostly reaching maximum in the last day of cultivation.

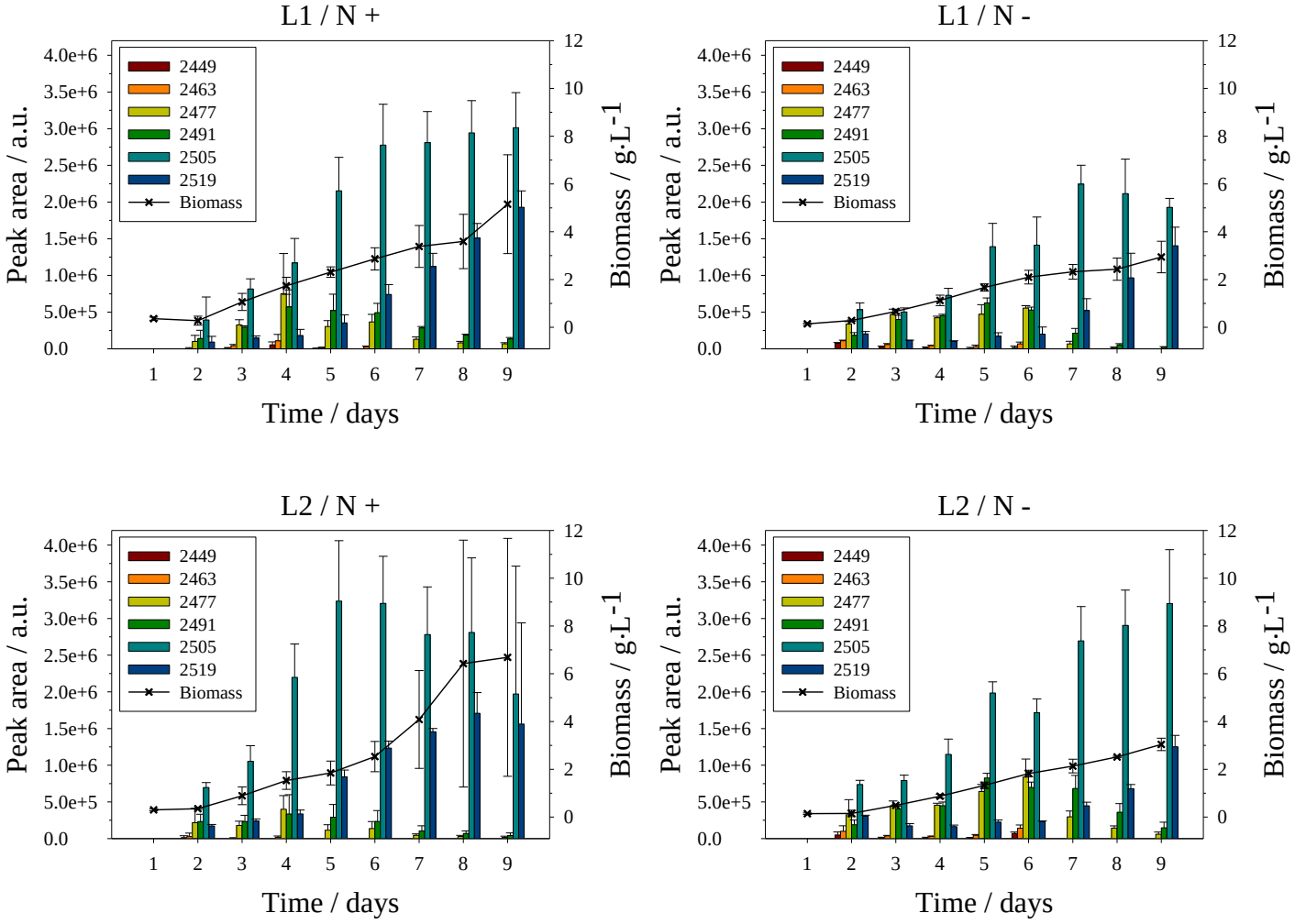

Fig. 6: Individual plots represent the peak area of 2505 and its analogues (y-axis, left) and the biomass concentration (y-axis, right) produced by Desmonostoc muscorum CALU 456 during its cultivation in specific conditions; N- stands for nitrogen-free BG-11 medium, N+ stands for full BG-11 medium, L1 = 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, L2 = 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Regarding the relative abundance content of the individual compounds (Fig. 7), 2505 seemed to generally dominate the production in all conditions. The contributions of the minor analogues 2449, 2463, 2477 and 2491 were higher in the first days, having thus an opposite trend of share compared to metabolite 2519, which contribution was significantly increasing during the final phases of the experiment.

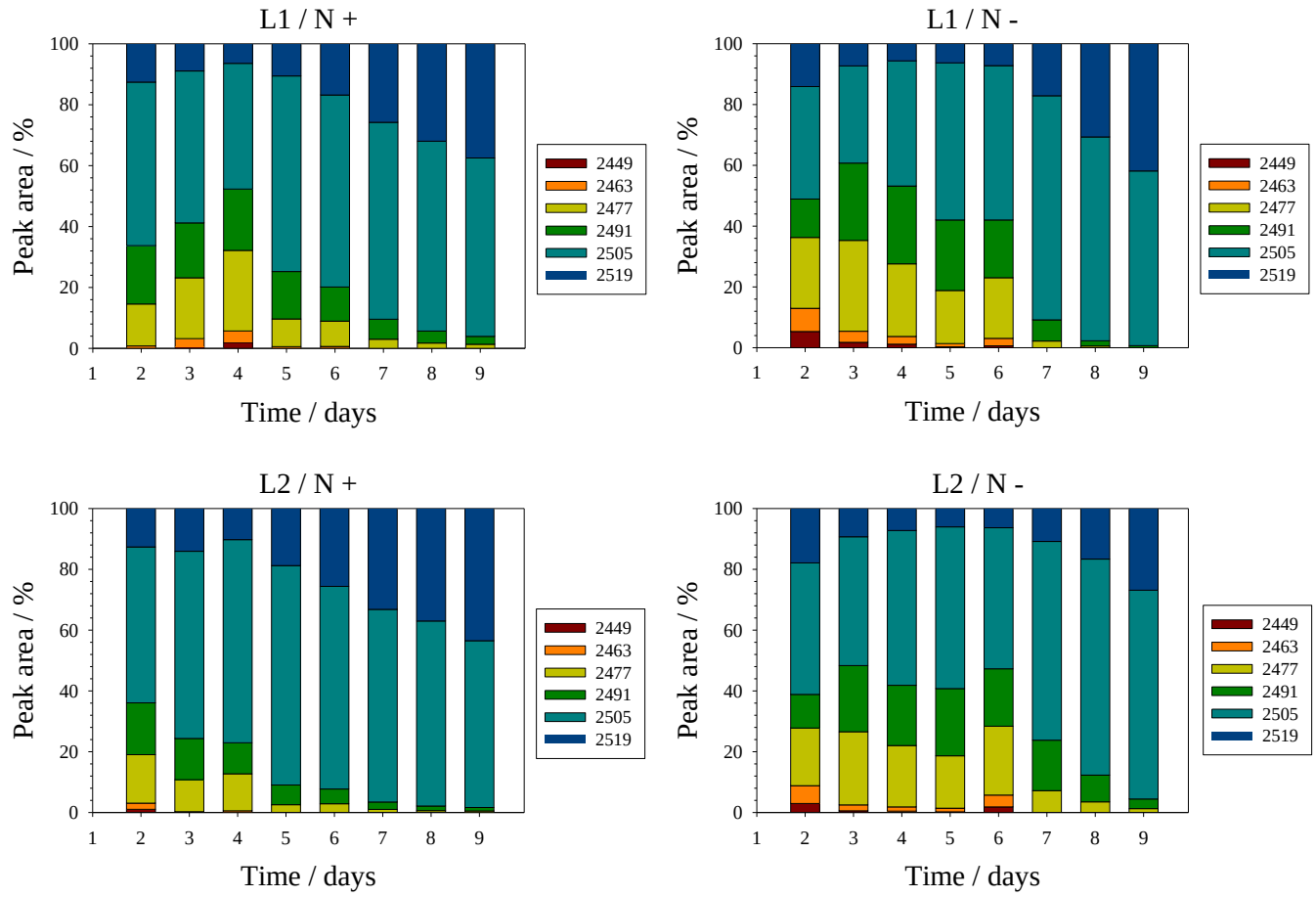

Fig. 7: Individual plots show the relative abundance of the 2505 and its analogues in Desmonostoc muscorum CALU 456 during its cultivation in specific conditions; N- stands for nitrogen-free BG-11 medium, N+ stands for full BG-11 medium, L1 = 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, L2 = 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Dynamic experiment II

In this experiment, the studies on *Desmonostoc muscorum* CALU 456 biomass and 2505 analogues production were performed in the 60 L flat photobioreactor with constant temperature, light and aeration conditions. The experiment was performed three times.

Regarding the biomass production (Fig. 8), the trends in the individual experiments were rather different and are thus to be described individually. As it is obvious from data representing the 60 L (I) experiment, the culture retained in the lag phase until approximately fifth day of the cultivation. This was followed by an exponential phase, which gradually reached a plateau in the last two to three days of the experiment with the biomass concentration of ~1.2 g· L^{-1}.

The second culture arose from the dilution of the final biomass of the 60 L (I) experiment and despite the approximately same initial biomass concentration and cultivation conditions, the culture growth was significantly slower, resulting in the biomass concentration of ~0.6 g ·L^{-1} after 10 days of cultivation.

The third culture was established from newly cultivated inoculum in the thoroughly sterilized 60 L flat photobioreactor. The overall biomass production trend was similar to that of the 60 L (I) experiment, however, the lag phase lasted shorter and the stationary phase was not reached, most probably due to shorter time of the experiment.

Despite the production of the individual compounds differed throughout the repetitions, the metabolite 2505 dominated the production in all of them. The 60 L (I) experiment showed a relatively similar trend to the one described in the dynamic experiment I, however, the lowest molecular weight analogues, namely the 2449 and the 2463 were completely absent. These were occasionally present in the second cultivation, in which the overall production of secondary metabolites was rather fluctuating. The analogues 2449 and 2463 were again absent in the third experiment and the production of other 2505 analogues differed from the first cultivation.

These observations were also visible from the relative abundance of the individual metabolites (Fig. 9). The major contribution to the production was attributed to 2505. The contribution of minor analogues in the first cultivation was similar to the one observed in the dynamic experiment I, while their contribution in the second cultivation was rather random. The relative abundance content of individual 2505 analogues in the third experiment was approximately constant over time.

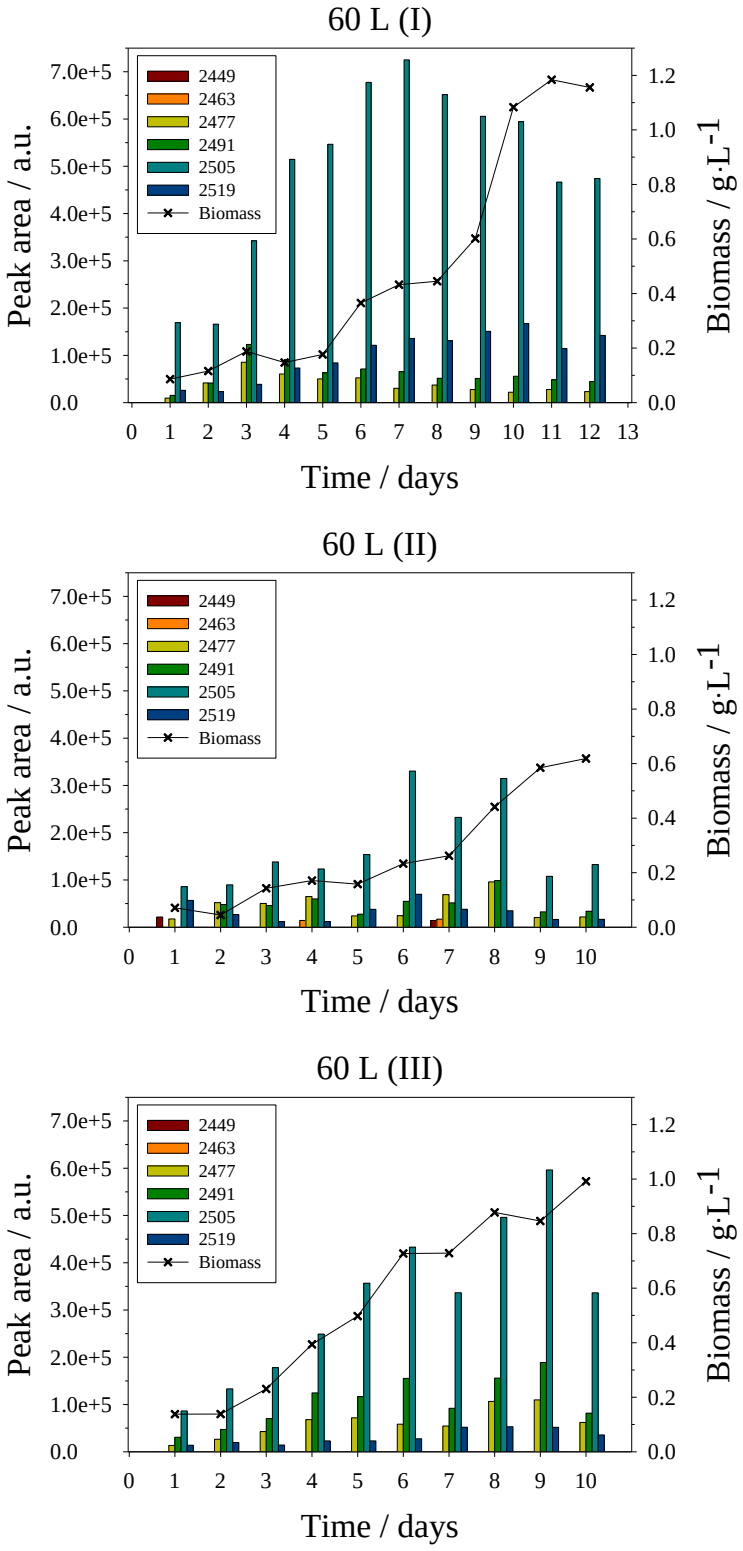

Fig. 8: Individual plots show the peak area of particular 2505 analogues (y-axis, left) and biomass concentration (y-axis, right) produced during cultivation of Desmonostoc muscorum CALU 456 in the 60 L flat photobioreactor; number in the brackets represents order of the individual experiments (I – original, II – first repetition, III – second repetition).

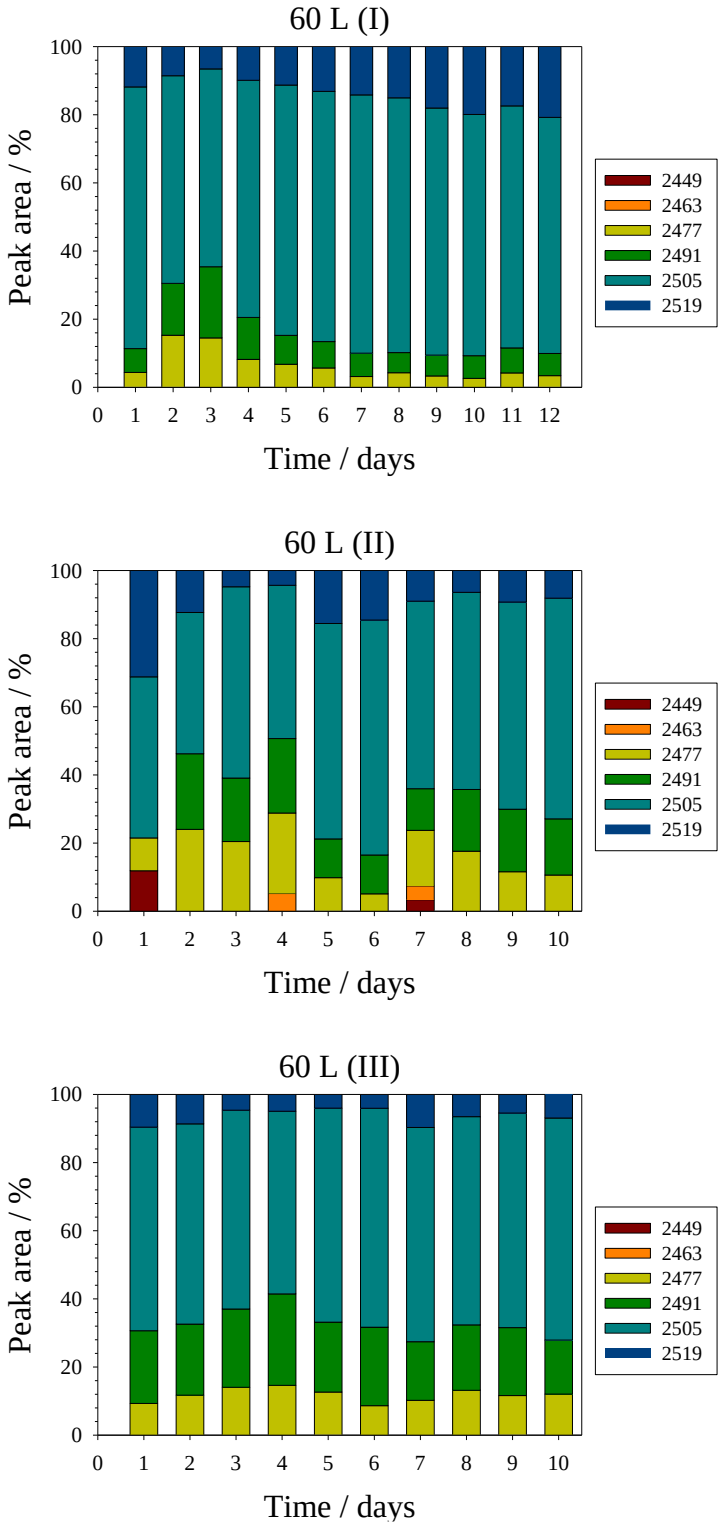

Fig. 9: Individual plots show the relative abundance of the 2505 analogues in Desmonostoc muscorum CALU 456 during its cultivation in 60 L flat photobioreactor; number in the brackets represents order of the individual experiments (I – original, II – first repetition, III – second repetition).

Doubling times

For the dynamic experiments, the doubling times of the individual cultures were calculated using the exponential phases of the biomass concentration curves (Tab. 6). This was not done for the cross-gradient experiment, as in this case, the initial concentration as well as the time span of the exponential phase were not known.

Table 6: Summary of doubling times.

Experiment	Culture	Doubling time / days	Average doubling time / days
Dynamic I N+	L1/N+	1.90	1.34
	L2/N+	0.79	
Dynamic I N-	L1/N-	1.73	1.79
	L2/N-	1.86	
Dynamic II	60 L (I)	2.46	2.40
	60 L (II)	2.66	
	60 L (III)	2.09	

4.2 Discussion

As shown in the results section, in addition to metabolites described previously (Vicková, 2015), four other metabolites with similar mass spectra features were found to be produced by the strain of terrestrial cyanobacterium *Desmonostoc muscorum* CALU 456. The elution of the individual metabolites from the HPLC column was regular, showing a trend of increasing retention time with increasing *m/z* of metabolite, suggesting possible relation between the analytes. Studies of the *m/z* differences between $[M+H]^+$ ions of individual metabolites showed constant mass difference attributed to the methylene group ($-CH_2-$). The metabolites were therefore considered to be the natural analogues of the previously detected metabolite 2505.

Production of structurally closely related analogues is well evidenced in cyanobacteria (e.g. reviewed by Welker et al., 2006). Moreover, it is known, that in the case of NRPS, the structural variability of one chemical scaffold can be caused by multispecificity of a certain adenylation domain, as for example several closely related amino-acids (Ile/Leu, Ser/Thr, Asn/Gln) can be activated by one domain. The exchange of these closely related amino-acid residues in one position typically generates mass differences of one or more CH_2 groups (Δ14, Δ28 etc..) among analogs.

However, a single or multiple exchange of structurally similar amino-acid residues does not have a profound effect on the compound polarity and thus also does not affect the retention time in the reversed-phase chromatography considerably. Thus, the linear increase of the retention time with the increasing *m/z* ratio in 2505 analogs is probably caused by other structural features of the molecule than by substitutions of closely related amino-acids. Moreover, preliminary trials to determine the structure of 2505 did not prove its peptidic character, as only one amino-acid residue (Glu) was obtained by acid hydrolysis (Hrouzek, personal communication). On the other hand, presence of fatty acid chain of various length within the molecule would result in such a relation of m/z of the molecule with its retention time. This was recently demonstrated in the case of cyanobacterial lipopeptides (Urajová et al., 2016). From biosynthetic point of view, the enzyme fatty-acyl AMP ligase (FAAL) directly linked to PKS modules was previously demonstrated to be of incorporation of fatty acids with different length in various NRPS/PKS clusters (e.g. Mareš et al. 2014). From these and other preliminary data, we presume that 2505 and its analogues will have origin in some large biosynthetase combining FAAL and PKS units.

The cross-gradient experiment was an initial experiment performed in order to investigate set of different conditions on the production of the individual 2505 analogues. Interestingly, both light and temperature affected the production of the 2505 analogues in static conditions, as the production of lower molecular weight analogues was higher in the case of highest temperature condition (30 °C) and generally highest yields of all analogues were obtained in low light conditions. However, the results of this experiment were not repeatable in the large scale cultivation. Despite the highest analogue productivity obtained in the lowest light condition, higher light intensities were chosen for the dynamic experiments as they generally result in faster growth of the biomass and thus higher biomass concentration obtained in shorter period of time.

The data obtained from the dynamic experiment I showed an interesting trend of production of individual compounds, with lower molecular weight analogues (2449, 2463, 2477 and 2491) reaching the production maximum in the first half of the cultivation period and higher molecular weight analogues (2505 and 2519) reaching the maximum of their production in the second half of the cultivation period.

However, the experiments in the 60 L flat photobioreactor showed a deviation from this trend and rather irregular production of the individual analogues was detected in the first repetition of the experiment (60 L (II)), prepared by dilution of the original (60 L (I)) inoculum in the 60 L photobioreactor. As the conditions in this type of photobioreactor are semi-sterile, it is not

excluded, that this irregularity in production was might have been caused by presence of contamination.

The nitrogen depletion did not seem to have a significant effect on the analogue production. The doubling times corresponding to the dynamic experiment I suggest faster growth of the inoculum in the nitrogen containing medium compared to the nitrogen-free medium. This conclusion would be in accordance with the one based on the general appearance of the biomass concentration curves and is fully in agreement with the need of the cyanobacterium to compensate for the absence of nitrates by nitrogen fixation.

5 Conclusions

Recently, two metabolites possessing a cytostatic effect towards HeLa and PaTu cell lines were detected in the crude extract of *Desmonostoc muscorum* CALU 456, with metabolite designated as 2504 (in this work designated as 2505 according to its neutral molar mass) having the most profound effect.

During this work, four additional secondary metabolites were detected to be produced by the aforementioned strain along with the two previously described. By studying the relationship between the 2505 and other five metabolites, it was proposed that the individual compounds differ by presence of methylene group, $-CH_2-$ and were considered to be natural analogues. It was further proposed that this is caused by the formation of fatty acid chain of differing length depending on the amount of methylene groups, rather than by substitution of the structurally similar amino-acid residues among the individual analogues.

Further aim of the thesis was to study the influence of different cultivation conditions and age of the culture on the production of the metabolite 2505 and its analogues. Despite an interesting production trend obtained from cultivation of *Desmonostoc muscorum* CALU 456 in the small tubular photobioreactor, the universal optimal conditions for the production of 2505 and its analogues cannot be concluded, as this trend was not repeatable in the large scale flat photobioreactor. Additionally, the nitrogen depletion did not have any significant effect on the production of the individual compounds.

6 References

Baudino, T. (2015). Targeted cancer therapy: the next generation of cancer treatment. *Current drug discovery technologies*, *12*(1), 3-20.

Becker, E. W. (1994). Nutrition. *Microalgae: biotechnology and microbiology*, 196-250.

Ben-Amotz, A., Shaish, A., & Avron, M. (1989). Mode of action of the massively accumulated β-carotene of Dunaliella bardawil in protecting the alga against damage by excess irradiation. *Plant Physiology*, *91*(3), 1040-1043.

Bonnett, S. A., Whicher, J. R., Papireddy, K. et al. (2013). Structural and stereochemical analysis of a modular polyketide synthase ketoreductase domain required for the generation of a cis-alkene. *Chemistry & biology*, *20*(6), 772-783.

Borowitzka, M. A., Borowitzka, L. J. (1988). Fats, oils and carbohydrates. In *Micro-algal biotechnology*, 257-287.

Bouchard, H., Viskov, C., & Garcia-Echeverria, C. (2014). Antibody–drug conjugates—a new wave of cancer drugs. *Bioorganic & medicinal chemistry letters*, *24*(23), 5357-5363.

Cane, D. E., & Walsh, C. T. (1999). The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. *Chemistry & biology*, *6*(12), R319-R325.

Carmichael, W. W., Bent, P. E. (1981). Hemagglutination method for detection of freshwater cyanobacteria (blue-green algae) toxins. *Applied and environmental microbiology*, *41*(6), 1383-1388.

Chan, Y. A., Podevels, A. M., Kevany, B. M., Thomas, M. G. (2009). Biosynthesis of polyketide synthase extender units. *Natural product reports*, *26*(1), 90-114.

Codd, G. A., Edwards, C., Beattle, K. A., Barr, W. M., & Gunn, G. J. (1992). Fatal attraction to cyanobacteria? *Nature*, *359*(6391), 110-111.

Collins, C. D., & Boylen, C. W. (1982). Physiological responses of Anabaena variabilis (Cyanophyceae) to instantaneous exposure to various combinations of light intensity and temperature. *Journal of Phycology*, *18*(2), 206-211.

Corbett, T. H., Valeriote, F. A., Demchik, L. et al. (1996). Preclinical anticancer activity of cryptophycin-8. *Journal of experimental therapeutics & oncology*, *1*(2), 95-108.

Dittmann, E., Neilan, B., Börner, T. (2001). Molecular biology of peptide and polyketide biosynthesis in cyanobacteria. *Applied microbiology and biotechnology*, *57*(4), 467-473.

Dodds, W. K., Gudder, D. A., Mollenhauer, D. (1995). The ecology of Nostoc. *Journal of Phycology*, *31*(1), 2-18.

Du, L., Sánchez, C., Shen, B. (2001). Hybrid peptide–polyketide natural products: biosynthesis and prospects toward engineering novel molecules. *Metabolic engineering*, *3*(1), 78-95.

Finking, R., Marahiel, M. A. (2004). Biosynthesis of nonribosomal peptides 1. *Annu. Rev. Microbiol.*, *58*, 453-488.

Francis, G. (1878). Poisonous Australia lake. *Nature*, *18*, 11-12.

Gao, K. (1998). Chinese studies on the edible blue-green alga, Nostoc flagelliforme: a review. *Journal of Applied Phycology*, *10*(1), 37-49.

Göbel, F. (1978). Quantum efficiencies of growth. *The photosynthetic bacteria*, *899*, 90-125.

Gordaliza, M. (2007). Natural products as leads to anticancer drugs. *Clinical and Translational Oncology*, *9*(12), 767-776.

Grobbelaar, J. U. (2004). Algal Nutrition–Mineral Nutrition. In A. Richmond (Eds.), *Handbook of microalgal culture: biotechnology and applied phycology*, 95-115.

Hrouzek, P., Lukešová, A., Mareš, J., Ventura, S. (2013). Description of the cyanobacterial genus Desmonostoc gen. nov. including D. muscorum comb. nov. as a distinct, phylogenetically coherent taxon related to the genus Nostoc. *Fottea*, *13*(2), 201-213.

Hur, G. H., Vickery, C. R., Burkart, M. D. (2012). Explorations of catalytic domains in non-ribosomal peptide synthetase enzymology. *Natural product reports*, *29*(10), 1074-1098.

Kamano, Y., Kizu, H., Pettit, G. R. et al. (1987). Structure of the marine animal antineoplastic constituent dolastatin 11 from Dolabella auricularia. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu*, *29*, 295-300.

Kaplan, D., Richmond, A. E., Dubinsky, Z., Aaronson, S. (1986). Algal nutrition. *Handbook of Microalgal Mass Culture*, 147-198.

Karl, D., Letelier, R., Tupas, L., Dore, J., Christian, J., Hebel, D. (1997). The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. *Nature*, *388*(6642), 533-538.

Kehr, J. C., Picchi, D. G., Dittmann, E. (2011). Natural product biosyntheses in cyanobacteria: a treasure trove of unique enzymes. *Beilstein journal of organic chemistry*, *7*(1), 1622-1635.

Komárek, J. (2006). Cyanobacterial taxonomy: current problems and prospects for the integration of traditional and molecular approaches. *Algae*, *21*(4), 349-375.

Krishnamurthy, T., Carmichael, W. W., Sarver, E. W. (1986). Toxic peptides from freshwater cyanobacteria (blue-green algae). I. Isolation, purification and characterization of peptides from Microcystis aeruginosa and Anabaena flos-aquae. *Toxicon*, *24*(9), 865-873.

Kulasooriya, S. A. (2011). Cyanobacteria: pioneers of planet earth. *Ceylon Journal of Science (Biological Sciences)*, *40*(2), 71-88.

Kwan, E. E., Huang, S. G. (2008). Structural elucidation with NMR spectroscopy: practical strategies for organic chemists. *European journal of organic chemistry*, *2008*(16), 2671-2688.

Lee, Y. K., Shen, H. (2004). Basic culturing techniques. In A. Richmond (Eds.), *Handbook of microalgal culture: biotechnology and applied phycology*, 40-55.

Liang, J., Moore, R. E., Moher, E. D., Munroe, J. E. et al. (2005). Cryptophycins-309, 249 and other cryptophycin analogs: preclinical efficacy studies with mouse and human tumors. *Investigational new drugs*, *23*(3), 213-224.

Mandal, S., Rath, J. (2014). Anticancer Drug Development from Cyanobacteria. *Extremophilic cyanobacteria for novel drug* development, 63-73.

Mareš, J., Hájek, J., Urajová, P., Kopecký, J., Hrouzek, P. (2014). A hybrid non-ribosomal peptide/polyketide synthetase containing fatty-acyl ligase (FAAL) synthesizes the β-amino fatty acid lipopeptides puwainaphycins in the Cyanobacterium Cylindrospermum alatosporum. *PloS one*, *9*(11), e111904.

Méjean, A., Ploux, O. (2013). Genomic View of Secondary Metabolite Production in Cyanobacteria. *Genomics of Cyanobacteria*, *65*, 189-234.

Mohanty, D., Sankaranarayanan, R., Gokhale, R. S. (2011). Fatty acyl-AMP ligases and polyketide synthases are unique enzymes of lipid biosynthetic machinery in Mycobacterium tuberculosis. *Tuberculosis*, *91*(5), 448-455.

Richmond, A. (2004). Biological principles of mass cultivation. In A. Richmond (Eds.), *Handbook of microalgal culture: biotechnology and applied phycology* 125-177.

Schopf, J. W., Packer, B. M. (1987). Early Archean (3.3-billion to 3.5-billion-year-old) microfossils from Warrawoona Group, Australia. *Science*, *237*, 70-74.

Schwarzer, D., Finking, R., Marahiel, M. A. (2003). Nonribosomal peptides: from genes to products. *Natural product reports*, *20*(3), 275-287.

Sharma, L., Mallick, N. (2005). Accumulation of poly-β-hydroxybutyrate in Nostoc muscorum: regulation by pH, light–dark cycles, N and P status and carbon sources. *Bioresource technology*, *96*(11), 1304-1310.

Sieber, S. A., Marahiel, M. A. (2005). Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. *Chemical reviews*, *105*(2), 715-738.

Staunton, J., Weissman, K. J. (2001). Polyketide biosynthesis: a millennium review. *Natural product reports*, *18*(4), 380-416.

Urajová, P., Hájek, J., Wahlsten, M., et al. (2016). A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides. *Journal of Chromatography A*, *1438*, 76-83.

Vicková, K. (2015). *Cytotoxic effect of some cyanobacterial crude extracts and metabolites against selected human cancer cell lines, especially focusing on pancreatic cancer cell line PaTu* (Bachelor thesis, in Czech). Retrieved from https://theses.cz/id/l1ph6n

Vijayakumar, S., Menakha, M. (2014). Prediction of new cyanobacterial drug for treating lung cancer. *Biomedicine & Aging Pathology*, *4*(1), 49-52.

Vonshak, A. (1986). Laboratory techniques for the cultivation of microalgae. *Handbook of microalgal mass culture*, 117-145.

Wase, N. V., Wright, P. C. (2008). Systems biology of cyanobacterial secondary metabolite production and its role in drug discovery. *Expert opinion on drug discovery*, *3*(8), 903-929.

Weber, T., Marahiel, M. A. (2001). Exploring the domain structure of modular nonribosomal peptide synthetases. *Structure*, *9*(1), R3-R9.

Welker, M., Von Döhren, H. (2006). Cyanobacterial peptides—nature's own combinatorial biosynthesis. *FEMS microbiology reviews*, *30*(4), 530-563.

Wnorowski, A. U., & Scott, W. E. (1992). Incidence of off-flavors in South African surface waters. *Water Science and Technology*, *25*(2), 225-232.

Wolfender, J. L. (2009). HPLC in natural product analysis: the detection issue. *Planta medica*, *75*(07), 719-734.