University of South Bohemia in České Budějovice
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Isolation of Quorum Sensing Inhibitory Compounds from Cyanobacteria

Bachelor thesis

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Annotation

Overuse of antibiotics has led to the development of resistance in many medically relevant human pathogens, posing a threat to human health. The recent decline in the discovery of novel antibiotics together with development of multi-antibiotic resistant strains demands a search of an alternative approach. Anti-virulence therapy poses significantly lower pressure on developing resistance given its focus on disarming the pathogen rather than eradicating it. Advancement in the field of bacterial cell-to-cell communication (Quorum Sensing, QS) and its molecular mechanism regulating the production of virulence factors in many clinically relevant human pathogens led to the discovery of Quorum Sensing inhibitory (QSI) molecules. Cyanobacteria are recognized as a prolific source of natural bioactive compounds with great pharmacological potential. The aim of this study was to screen cyanobacterial extracts for QSI activity in search of potential anti-virulence drugs. 45 cyanobacterial strains were randomly selected from the in-house culture collection of Centre Algatech, extracted, and fractionated to generate 1575 fractions for QSI evaluation. Strains 3, 16, and 113 exhibited the highest inhibitory potential against both used biosensors, *E. coli* pSB401 and *E. coli* pSB1075. The obtained results open a future prospect to isolate and elucidate the lead molecule responsible for QSI activity.

Keywords: Cyanobacteria, Quorum sensing inhibition, Antimicrobial resistance, Anti-virulence therapy, Anti-microbial activity, Hemolytic activity

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Content

1 INTRODUCTION

1.1 QUORUM SENSING (QS)

Bacteria form part of a complex ecosystem and tend to live among communities in the native environment. One common example is their ability to form biofilms, communities of surface-attached microbes covered in slime sheath made of polysaccharides, proteins, glycolipids, and DNA fragments, which provides protection against radiation or harmful substances to all members of the community. Biofilms are frequently produced by pathogens for protection against the immune system of their host. Once a population is formed, a need for a communication system that would ensure coordinated behaviour of all members arises (Willey, et al., 2013).

Quorum Sensing (QS) is a type of bacterial communication that allows bacteria to assess the density of the population in which they live (Willey, et al., 2013). This information is used to regulate expression of certain genes in cell density-dependent manner. Genes regulated by QS encode for traits that are advantageous when produced by a large population, but become pointless and unnecessarily expensive if expressed in a single cell. Expression of these genes is therefore induced only when the population reaches a certain density – a 'quorum' (Rutherford & Bassler, 2012).

Bacteria use QS to regulate processes like biofilm formation, bioluminescence, or production of virulence factors in pathogens, which require coordinated collective effort of the whole population in order to be effective (Mukherjee & Bassler, 2019). For example, *Vibrio fischeri*, a marine bacterium living in a symbiotic relationship with southern bobtail squid (*Euprymna scolopes*), uses QS to regulate bioluminescence. The squid is a night-time predator. As it passes in front of the moonlight, it creates a shadow, making it easy for the squid's predator to spot their prey. To combat this problem, the squid recruits *V. fischeri* and provides it with shelter in its light organ. Once the population of *Vibrio* grows sufficiently large, it starts producing luminescence, allowing the squid to hide in the moonlight without casting shadow. Light production by a single *V. fischeri* cell would be pointless and overly costly, and is therefore inhibited. However, a whole colony can easily produce enough luminescence to hide the squid while gaining a safe and nutrient-rich habitat (Willey, et al., 2013).

1.1.1 QS SIGNAL MOLECULES

QS is mediated by signal molecules called autoinducers (AIs) (LaSarre & Federle, 2013). There is a wide range of AIs used by different microbes. Gram-negative bacteria such as *Pseudomonas, Vibrio,* or *Escherichia*, use AI-1, acyl-homoserine lactones (AHLs); AI-2, a furanosyl borate diester, is used by both Gram-positive and Gram-negative bacteria; and Gram-positive bacteria such as *Clostridium, Bacillus,* or *Staphylococcus* use autoinducing peptides or pheromones (**Figure 1**).

Other classes of signal molecules include fatty acids used by *Xanthomonas* or *Burkholderia*, ketones in *Legionella* and *Vibrio*, epinephrine and norepinephrine used by enterohemorrhagic bacteria, *Pseudomonas* quinolone signals (Kalia, et al., 2019), diffusible signal factor (DSF) (LaSarre & Federle, 2013), or γ-butyrolactone, also known as A-factor, produced by *Streptomyces griseus* (Willey, et al., 2013). **Figure 1** shows several kinds of QS signal molecules produced by Gram-negative bacteria, out of which AHLs are the most widely studied.

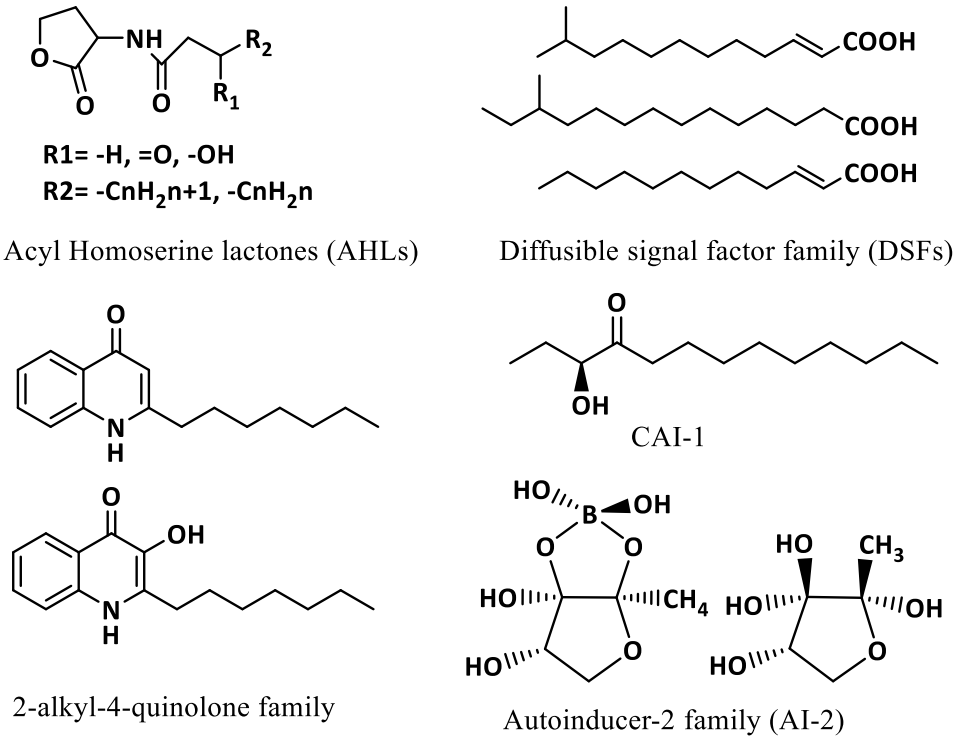


Figure 1: Quorum Sensing signal molecules produced by Gram-negative bacteria (created in ChemDraw 16.0).

1.1.2 AHL-BASED QS SYSTEM

AHLs, also referred to as autoinducer-1 (AI-1), are QS signal molecules frequently used by Gram-negative bacteria (Saurav, et al., 2017). As shown in **Figure 1**, AHLs are composed of a homoserine lactone ring with acyl side chain of variable length (4 to 19 carbon chains long (Saurav, et al., 2016)), saturation, and oxidation state (LaSarre & Federle, 2013). AHLs are produced and transported outside the plasma membrane along the diffusion gradient into the extracellular space, where they accumulate. Once the cell density reaches to quorum, the concentration of AHLs in the local environment rises until the diffusion gradient is reversed, causing the AIs to follow the flow back across the cell membrane (Willey, et al., 2013). Once the threshold concentration inside the cell is reached, the AHLs start binding to a receptor protein that controls the expression of QS-regulated genes (LaSarre & Federle, 2013) (**Figure 2**).

Most Gram-negative systems contain a *LuxI*/*LuxR* QS network homologs. AHLs are synthesized by AHL-synthases of the *LuxI* family using SAMO (S-adenosyl-L-methionin) and acyl-ACP as precursors. Each synthase usually produces one type of AIs. When AHL interacts with its specific LuxR-type regulator receptor protein, the DNA-binding ability of the protein changes and modifies the expression of target genes (LaSarre & Federle, 2013). Simultaneously, the AHL-protein complex stimulates expression of *LuxI*, thus increasing the production of signal molecules by creating an amplification loop (Lade, et al., 2014). To prevent premature activation of QS-regulating systems, AHLs are degraded rapidly when cell densities are low (Saurav, et al., 2016).

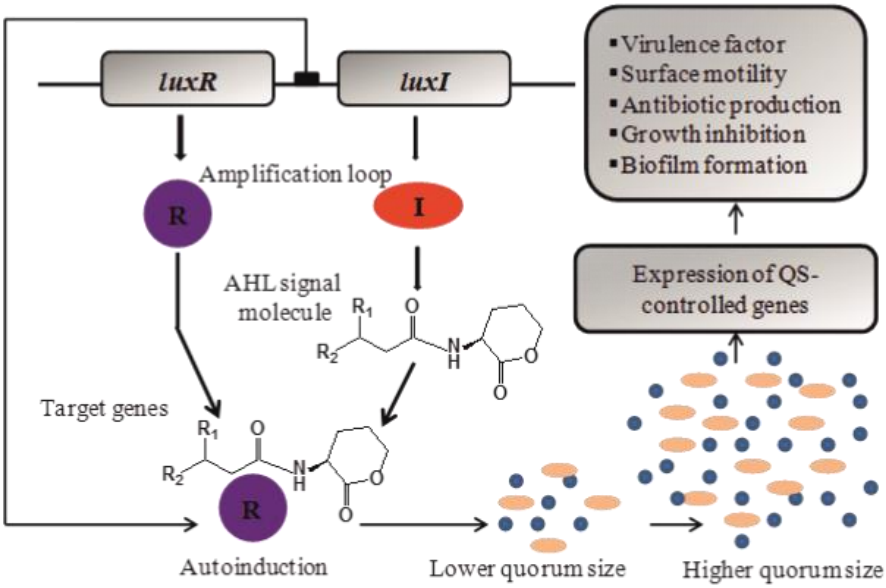


Figure 2: AHL-based QS *LuxI*/*LuxR* system in Gram-negative bacteria (Lade, et al., 2014).

1.1.3 QS AND PATHOGENESIS

Many bacterial pathogens use QS to regulate traits responsible for their virulence, such as production of toxins, proteases, formation of biofilms (LaSarre & Federle, 2013), sporulation, or cell competence (Rutherford & Bassler, 2012). Especially biofilm formation is a powerful tool since it allows bacteria to attach on basically any surface and provides protection against antibiotics and other harmful substances (Willey, et al., 2013). Biofilm-forming bacteria can tolerate up to 1000 times higher levels of antibiotics than free-living cells (Kalia, et al., 2019). Bacterial pathogens readily form biofilm on medical devices, eventually causing their failure and need for replacement, and since biofilms contain bacterial persisters, they can repopulate the host even after seemingly successful treatment (Willey, et al., 2013).

Quorum Sensing regulates virulence factor production in clinically relevant bacteria such as in *Staphylococcus aureus*, *Streptococcus pneumonia*, *Vibrio cholerae*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Serratia marcescens*, or *Pseudomonas aeruginosa*, among many others. For example, *P. aeruginosa*, an opportunistic human pathogen causing acute and chronic pulmonary infections in immunocompromised patients, uses at least three QS systems to regulate production of virulence factors: Two LuxI-LuxR systems (LasI-LasR and RhlI-RhlR), working in tandem, and a quinolone system (PqsABCD and PqsH-PqsR), along with accessory regulators which fine-tune the regulation **(Figure 3)** (Rutherford & Bassler, 2012).

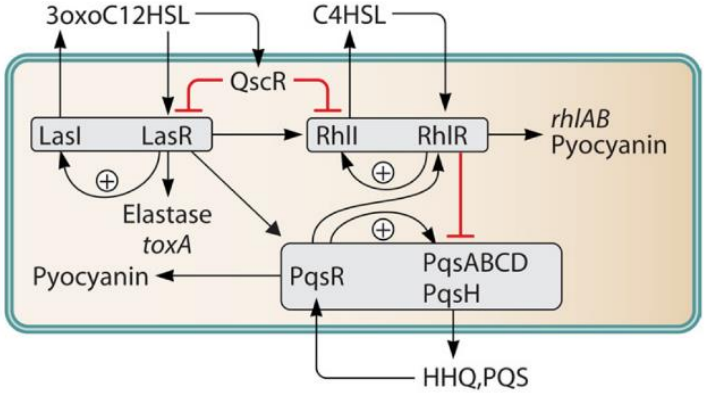


Figure 3: *P. aeruginosa* QS system regulating production of pyocyanin and elastase (LaSarre & Federle, 2013).

The networks function in hierarchical manner. Upon activation of the Las system, the remaining networks (Rhl and Pqs) are positively regulated, with Rhl also being upregulated by the Pqs system. The compounds used to signal the activation of each system are N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), N-butanoyl-L-homoserine lactone (C4-HSL), and 2-heptyl-3-hydroxi-4-quinolone for the Las, Rhl, and Pqs systems, respectively (**Figure. 3**) (LaSarre & Federle, 2013). Interconnection of the three systems ensures that virulence factors are only produced in the appropriate situation, since *P. aeruginosa* infection usually occurs when the immune system of its host is compromised (it frequently causes infection in cystic fibrosis patients). Virulence factors of *P. aeruginosa* regulated by QS include elastases, proteases, toxin pyocyanin, lectin, rhamnolipids, toxins, biofilm formation, and swarming motility (Rutherford & Bassler, 2012). A summary of QS systems of selected bacteria is presented in **Table I**.

Table I: Quorum-sensing systems utilized by selected bacteria and associated phenotypes (LaSarre & Federle, 2013).

Organism	Signal	Synthase(s)	Receptor(s)	Selected phenotypes
P. aeruginosa	C4-HSL	RhlI	RhlR	Exoenzymes, virulence, biofilm formation, motility, iron acquisition, pyocyanin
	3OC12-HSL	LasI	LasR, QscR	
	HHQ, PQS	PqsA to -D, PqsH	PqsR	
P. syringae	3OC6-HSL	AhlI	AhlR	EPS, plant colonization
E. carotovora	3OC6-HSL	CarI	CarR, ExpR, VirR	Carbapenem, exoenzymes, virulence
P. stewartii	3OC6-HSL	EsaI	EsaR	Adhesion, EPS, plant colonization
B. glumae	C8-HSL	TofI	TofR	Motility, toxoflavin, lipase, virulence
A. tumefaciens	3OC6-HSL	TraI	TraR	Ti plasmid conjugation, virulence
C. violaceum	C6-HSL	CviI	CviR	Exoenzymes, antibiotics, violacein
S. liquefaciens	C4-HSL	SwrI	SwrR	Swarming motility, biofilm formation
V. harveyi	3OHC4-HSL	LuxM	LuxN	Bioluminescence, siderophores, protease and EPS production, virulence
	AI-2	LuxS	LuxP	
	CAI-1	CqsA	CqsS	
V. cholerae	AI-2	LuxS	LuxP	Virulence, biofilm formation, EPS
	CAI1	CqsA	CqsS	
V. fischeri	3OC6-HSL	LuxI	LuxR	Bioluminescence, host colonization, motility
	C8-HSL	AinS	AinR	
	AI-2	LuxS	LuxP	
E. coli/S. typhimurium	3OC8-HSL[a]	NA[d]	SdiA	Motility, acid resistance, *lsr* operon expression (AI-2 uptake)
	AI-2	LuxS	LsrB	
	AI-3	Unknown	QseC	Virulence, motility, biofilm formation
S. auerus	AIP	*agrD*[b]	AgrC	Virulence, exotoxins, biofilm dispersal
E. faecalis	GBAP	*fsrD*[b]	FsrC	Gelatinase, protease
	cCF10	*ccfA*[b]	PrgX	Adhesion, conjugation
S. pneumoniae	CSP	*comC*[b]	ComD	Competence, virulence, autolysis
B. thuringiensis	PapR	*papR*[b]	PlcR	Exoenzymes
	NprX	*nprRB*[b,c]	NprR	Toxins, sporulation, necrotrophism
X. campestris	DSF	RpfB, RpfF	RpfC	Virulence, biofilm dispersal, EPS

[a] SdiA can also bind C4HSL, C6HSL, 3OC6HSL, and 3OC12HSL but does so with less affinity than 3OC8HSL.

[b] The peptide signal is genetically encoded by the indicated gene.

[c] The gene encoding NprX-containing propeptide is also referred to in the literature as nprX.

[d] NA, not applicable.

1.2 QUORUM SENSING INHIBITION

Quorum Sensing inhibition (QSI) is a widespread phenomenon in nature. Several reasons for bacteria to interfere with this communication system are known: apart from the products of degradation of signal molecules being used as energy source, inhibiting rival's communication may provide an advantage over competitors. Quorum Sensing inhibitors (QSIs) therefore play an important role in both intra- and inter-species bacterial interactions (Czajkowski & Jafra, 2009).

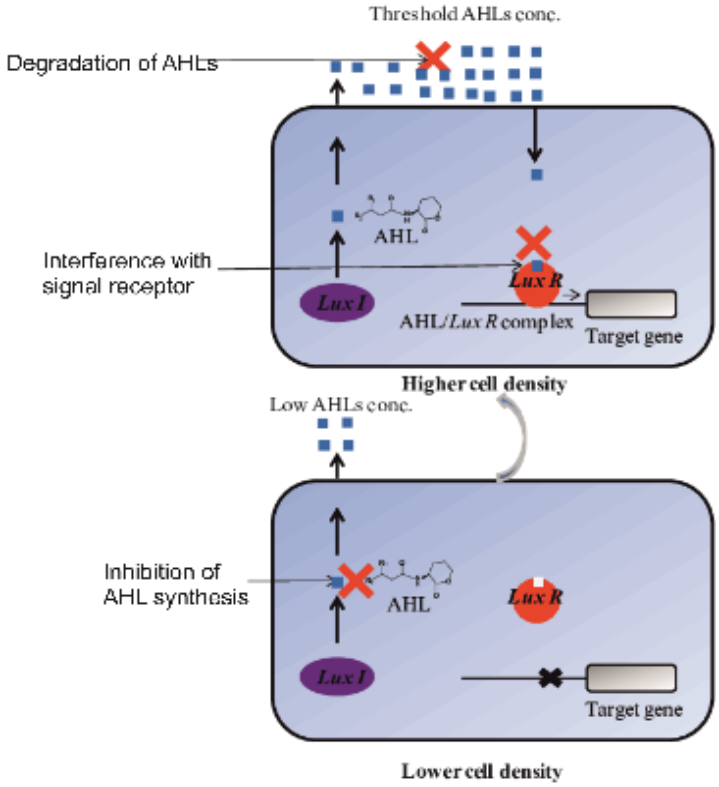


Figure 4: Mechanisms of QSI (Lade, et al., 2014).

Compounds that interfere with bacterial communication have been found to occur naturally in algae, plants, bacteria, or invertebrates, and have been effective in various host-microbe systems (Saurav, et al., 2017). They work by four main mechanisms depicted on **Figure 4:** inhibition of AIs synthesis, degradation or inactivation of the signal molecule, inhibition of signal detection, and inhibition of binding to gene promoters (Kalia, et al., 2019).

1.2.1 ANTIBIOTIC RESISTANCE

Resistance to antibiotics occurs naturally in bacteria via gene mutation when subjected to antibiotics. Antibiotics are targeted at complete elimination of pathogenic bacteria, thus posing strong selective pressure for resistance development. Resistant bacteria are able to tolerate higher levels of antibiotics and reproduce even in presence of the drug, forming increasingly resistant population. Moreover, bacteria are able to share genetic information via horizontal gene transfer, making resistance transmission even easier and faster. The emergence of resistant bacteria has been a problem ever since antibiotics were discovered in the last century, and recently, due to the irresponsible overuse of these drugs in healthcare as well as agriculture and livestock industry, it is starting to pose a serious threat to human and animal health. Since a higher amount of antibiotics is needed to combat less sensitive pathogens, there is an increased demand for antibiotics, further aggravating the problem. Although intensive research is addressing this issue, only few new antibiotics are produced, and the current strategy has to rely on modification of existing drugs (Zaman, et al., 2017). As we are barely able to keep up with development of resistance, alternative ways of fighting bacterial pathogens are acutely needed (Saurav, et al., 2017).

1.2.2 ANTI-VIRULENCE THERAPY

One of the alternative strategies to combat resistant pathogens is targeted at virulence factors responsible for the pathogenesis rather than elimination of the bacteria, therefore being referred to as the ‘anti-virulence therapy’. Anti-virulence therapy is designed to minimize the selective pressure on the microbes posed by the new drugs and ideally significantly slowing down the process of drug resistance development (LaSarre & Federle, 2013).

QS, regulating production of virulence factors and sometimes directly contributing to antibiotic resistance, is an ideal target for anti-virulence therapy. By disrupting the communication between the pathogens, production of virulence factors would be impaired and pathogenicity of the bacteria would be greatly reduced. Additionally, since QS signal molecules exhibit high species-specificity, drugs based on QS inhibition may offer greater precision in targeting specifically the pathogens causing the disease (LaSarre & Federle, 2013). While undoubtedly posing much lower selective pressure, there is evidence that development of resistance to QS-based anti-virulence therapy is possible, but arguably at much slower pace. Anti-virulence therapy therefore remains a promising way of therapeutic treatment (Saurav, et al., 2017).

1.2.3 BIOSENSORS

Several biosensor strains of QS-employing bacteria had been developed for efficient study of QS mechanics and its use in drug discovery. The most widely used biosensors are based on the AHL QS system. Biosensors are genetically modified to possess a functional receptor system, but a disabled AHL synthase, hence responding to stimulation of QS-regulated genes by externally provided signal molecules, but not producing their own AI. This way, the expression of QS-regulated genes can be manipulated by the researcher. Biosensors vary depending on the reporter trait regulated by QS, for example bioluminescence, violacein production, or Green Fluorescent Protein, as summarized in **Table II.** (Saurav, et al., 2017).

Table II. AHL-based biosensors used for the identification of active QS inhibitory molecules from the sea (Saurav, et al., 2017)).

Strain/Plasmid	QS System	Reporter Strain	Detection Range
Chromobacterium violaceum CV026	CviI/R	Violacein pigment	C_6-HSL, 3-oxo-C_6-HSL, C_8-HSL, 3-oxo-C_8-HSL
Chromobacterium violaceum CV017	CviI/R	Violacein pigment	3-oxo-C_6-HSL, C_8-HSL, 3-oxo-C_8-HSL
Escherichia coli pSB403	LuxI/R (*V. fisheri*)	*luxCDABE*	3-oxo-C_6-HSL, C_6-HSL, 3-oxo-C_8-HSL, C_8-HSL
Escherichia coli pSB536	AhyI/R (*A. hydrophyla*)	*luxCDABE*	C_4-HSL
Escherichia coli pSB401	LuxI/R (*V. fisheri*)	*luxCDABE*	3-oxo-C_6-HSL, C_6-HSL, 3-oxo-C_8-HSL, C_8-HSL
Escherichia coli pSB1075	LasI/R (*P. aeruginosa*)	*luxCDABE*	3-oxo-C_{12}-HSL, 3-oxo-C_{10}-HSL, C_{12}-HSL
QSIS2	LasI/R (*P. aeruginosa*)	*luxCDABE*	3-oxo-C_{12}-HSL, 3-oxo-C_{10}-HSL, C_{12}-HSL
Vibrio harveyi JMH 612	LuxPQ (*Vibrio harveyi*)	*luxQ*	3-OH-C_4-HSL
Agrobacterium tumifaciencens pZLR4	TraI/R (*A. tumefaciens*)	β-galactosidase	All 3-oxo-HSLs
Escherichia coli pKDT17	LasI/R (*P. aeruginosa*)	β-galactosidase	3-oxo-C_{12}-HSL, C_{12}-HSL, C_{10}-HSL, 3-oxo-C_{10}-HSL
QSIS1	LuxI/R (*V. fisheri*)	β-galactosidase	3-oxo-C_6-HSL, C_6-HSL, C_8-HSL, C_{10}-HSL
pAS-C8	CepI/R (*B. cepacia*)	*gfp*	C_8-HSL
pKR-C12	LasI/R (*P. aeruginosa*)	*gfp*	3-oxo-C_{12}-HSL, 3-oxo-C_{10}-HSL
Escherichia coli JB525	LuxI/R (*V. fisheri*)	*gfp*	3-oxo-C_6-HSL, C_6-HSL, C_8-HSL, C_{10}-HSL
QSIS3	LuxI/R (*V. fisheri*)	*gfp*	3-oxo-C_6-HSL, C_6-HSL, C_8-HSL, C_{10}-HSL
Tn5-Las	LasI/R (*P. aeruginosa*)	*gfp*	3-oxo-C_{12}-HSL, 3-oxo-C_{10}-HSL

1.2.3.1 PIGMENT-BASED BIOSENSOR:

Chromobacterium violaceum is a gram-negative proteobacterium producing a violet pigment violacein. Violacein production is regulated by AHL-based CviI/CviR QS system (**Figure 5-A)**, homologous to *Vibrio fischeri* LuxI/LuxR system. Apart from violacein production, *C. violaceum* also uses QS for regulating production of hydrogen cyanide, elastase, or biofilm formation. Biosensor strain *C. violaceum* CV026 is a mini-T5-mutant lacking AHL synthase so that violacein production can be controlled by supplying the signal molecules externally (**Figure 5-B**). CV026 is a widely used biosensor strain that led to identification of various QS inhibitors such as Maniwamycins produced by *Streptomyces* sp. TOHO-M025 (Kothari, et al., 2017).

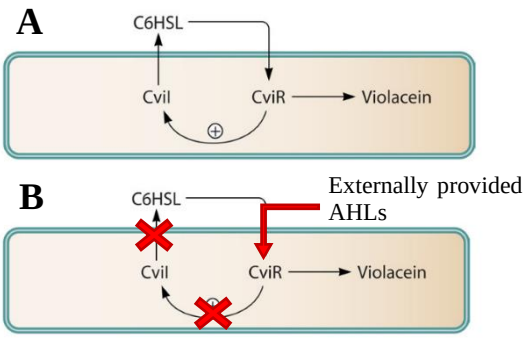


Figure 5: *C. violaceum* QS system

A. *C. violaceum* CviI/CviR regulation of violacein production.

B. Biosensor strain CV026 with disabled CviI synthase.

1.2.3.2 BIOLUMINESCENCE-BASED BIOSENSORS:

Thanks to the possibility of measurement of the emitted light by a luminometer, bioluminescence-based biosensor allows for easy quantitative evaluation of the pertinent QS activity of a given substance. Bioluminescence genes arranged in the *luxCDABE* operon encode for a substrate which in a reaction catalysed by *luxAB* luciferase releases light. Fragments of luminescence-encoding operons from *Photorhabdus luminescens* and *Vibrio fischeri/harveyi* (*V. harveyi* LuxI/LuxR-based QS system is shown on **Figure 6**) were used for construction of two reporter plasmids, pSB401 and pSB1075, which were transformed in *E. coli* to create two most widely used bioluminescence-based reporter strains. The main difference between the two is in what AHLs they recognize, with pSB401 responding to AHLs with shorter side chains, consisting of 6-8 carbons, and pSB1075 responding to AHLs with long side chains containing 10 and more carbons (Saurav, et al., 2017).

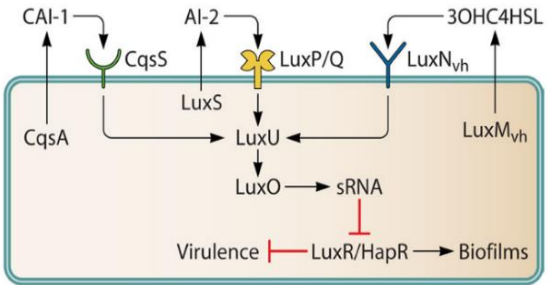


Figure 6: The Lux system in *Vibrio harveyi* (LaSarre & Federle, 2013).

1.3 CYANOBACTERIA IN NATURAL PRODUCT DISCOVERY

1.3.1 CYANOBACTERIA

Cyanobacteria, also known as blue-green algae, are autotrophic prokaryotic microorganisms belonging to the Bacteria domain (Singh, et al., 2005). They're one of the oldest organisms on Earth, with evidence of their existence dating as far as 3,5 billion years back (Schirrmeister, et al., 2011). Cyanobacteria are the only organisms to evolve oxygenic photosynthesis approximately 2,7 billion years ago (Schirrmeister, et al., 2015). This unique ability made them crucial for the development of the Earth, as cyanobacteria are responsible for oxygenation of the Earth's atmosphere during the so-called „Great Oxygenation Event", which allowed the evolution of other forms of life (Schirrmeister, et al., 2015). Photosynthesis was later adopted by heterotrophic eukaryotes via primary endosymbiosis with a cyanobacterium, resulting in the formation of plastids, organelles still present in modern plants and algae today (Ponce-Toledo, et al., 2017).

Given their long presence on the planet, cyanobacteria are immensely diverse and exhibit great adapting ability to various environments (Paerl & Fulton, 2006) - they can be found in almost every natural habitat, ranging from polar and marine to terrestrial environments and even thermal springs (Dvořák, et al., 2017). They are morphologically very diverse and can be found as unicellular, colonial, or filamentous with both false and true branching. The individual cells come in various shapes and sizes, and filamentous cyanobacteria, namely the order Nostocales, also form specialized cells called 'akinetes' and 'heterocytes' apart from vegetative cells. While vegetative cells perform photosynthesis, akinetes are reproductive cells packed with nutrients and heterocytes are cells primarily evolved to fix atmospheric nitrogen (Dvořák, et al., 2017). Cyanobacteria are one of the most important nitrogen fixers in oceans and freshwater as well as terrestrial environments, and as such have developed many different nitrogen fixing strategies with heterocytes being only one among many others (Shridhar, 2012). Another interesting adaptation of aquatic cyanobacteria is formation of aerotopes, or 'gas vesicles', which allow the cells to travel vertically in the water body by regulating the cell's buoyancy. This is advantageous in managing the cell's exposition to sunlight (Pfeifer, 2012). To further maximize the effectivity of photosynthesis, cyanobacteria are able to adjust the composition of their light-harvesting apparatus and photosynthetic pigments in a phenomenon known as „chromatic adaptation", which allows them to utilize a broad range of light spectra (Cohen & Gurevitz, 2006).

Many different life forms can be found in the domain, from free-living, planktonic, and benthic to terrestrial types (Kulasooriya, 2011). They often live in symbiosis with bacteria (bacterial mats),

fungi (lichens), bryophytes, or plants. For example, bacterial mats are complex surface-attached, vertically stratified microbial communities where cyanobacteria play a vital role in the development of the mat due to their nitrogen-fixing ability. Some microbial mat communities are able to mineralize organic matter and eventually form rock-shaped structures known as stromatolites (Stal, 1995). Fossil stromatolites dating back to 3,5 billion years found in Shark Bay, western Australia are considered one of the first evidence of life on the Earth, as modern cyanobacterial mats strongly resemble the ones from the Precambrian (Stal, 2012). Another example of symbiotic relationships of cyanobacteria are lichens, associations of a mycobiont (a fungus) and a photobiont. Cyanobacteria act as a photobiont in around 10% of lichen species with the majority coming from the genus Nostoc (Paulsrud & Lindblad, 1998). Thanks to their survival capabilities, cyanobacteria-containing lichens are often the first inhabitants of inhospitable habitats, acting as pioneer invaders (Kulasooriya, 2011). Perhaps the most prominent occurrence of cyanobacteria is in the form of algal blooms. Algal blooms are formed when cyanobacteria, such as *Microcystis*, *Cylindrospermopsis* or *Anabaena* (Kulasooriya, 2011), undergo explosive growth in eutrophied waters and rapidly increase their biomass due to over-presence of nutrients. This leads to increasing dominance of cyanobacteria in the algal community of eutrophied water bodies and, among other consequences, to a risk of production of toxins in concentrations potentially harmful to other algae, invertebrates, fish (Havens, 2008) and other organisms including humans (Kulasooriya, 2011).

1.3.2 CYANOBACTERIAL NATURAL BIOACTIVE COMPOUNDS

Cyanobacteria produce a substantial range of less abundant compounds frequently referred to as secondary metabolites (Croteau, et al., 2000). Generally, secondary metabolites in microorganisms have various purposes, from sunscreens to enzymes, inter- and intra-species signal molecules (for example AIs involved in bacterial Quorum Sensing (Mazard, et al., 2016)), vitamins, antioxidants, and toxins, many of which are biologically active (Abed, et al., 2009). Compounds isolated from cyanobacteria possess strong bioactive properties including antibacterial, antifungal, antiviral, immunosuppressant, anti-inflammatory, anticancer, anti-HIV, and other clinically relevant activities. Cyanobacteria are therefore regarded as one of the most promising sources of natural bioactive compounds with great pharmacological potential (Singh, et al., 2017). Besides potential drugs, compounds with biotechnological significance are also known, and various research projects conducted on cyanobacteria are focused on the development of food supplements (Singh, et al., 2005), bioplastics (Abed, et al., 2009) or biofuel (Ducat, et al., 2011).

Over 1100 cyanobacterial bioactive compounds had already been identified (Dittman, et al., 2015). They exhibit great structural diversity, ranging from lipopeptides, amino acids, fatty acids, macrolides, amides (Singh, et al., 2005), or alkaloids, to heterocycles (Hrouzek, et al., 2011). Highest

amount of metabolites is produced mainly by filamentous or colonial cyanobacteria (Méjean & Ploux, 2013), mostly the orders Oscillatoriales, Nostocales, and Chroococcales (Gerwick, et al., 2008), and their genome tends to be 5-6 Mb larger than that of unicellular species which do not produce secondary metabolites. Coincidentally, filamentous cyanobacteria use up to 6% of their genome for the production of bioactive compounds (Dittman, et al., 2015). Although most research was focused on aquatic, especially marine species (Burja, et al., 2001), more attention in natural compound discovery is nowadays driven towards terrestrial and symbiotic cyanobacteria (Méjean & Ploux, 2013), such as many strains of the genus *Nostoc*. Representatives of this genus can be found in freshwater habitats as well as terrestrial ones and often form symbiotic relationships with plants and fungi. Bioactivity is commonly present among *Nostoc* species, most prominently antifungal and toxic, less often antimicrobial activity, producing compounds such as cryptophycin A and nostodione A (Piccardi, et al., 2000).

The majority of these compounds are biosynthesised via Non-Ribosomal Peptide Synthesis (NRPS), Polyketide Synthesis (PKS), or hybrid systems (Méjean & Ploux, 2013). NRPS and PKS are modular synthase complexes which are able to use a variety of precursors, including non-standard amino acids, with quite low affinity to the substrate. This lack of specificity along with easy customizability of the domain sequence generates high variability even during synthesis of the same compound, resulting in many slightly different varieties of the same product. Additional diversity is created by auxiliary tailoring enzymes and, in case of ribosomal pathways, can be encoded in the corresponding genes (Dittman, et al., 2015).

The biological significance of secondary metabolites is not yet completely understood, but since the synthesis of such diverse molecules is quite costly, their production should be advantageous in some manner. It is therefore believed that secondary metabolites have evolved as a self-defence mechanism as well as a response to harsh conditions in extreme habitats (Singh, et al., 2017). It can be evidenced by the fact that cyanobacteria produce toxins continuously in conditions most favourable to them within their ecological niche, in other words, the production is not specific to only stress conditions. Other hypothesis suggest that secondary metabolites can replace the function of other compounds under non-standard conditions, or, since the type of compounds produced is influenced by habitat, that they can be used as weapons against competitors. Many compounds affect not only other microbes, but also fish and higher organisms, but since the genes encoding for these compounds are frequently older than eukaryotic organisms, it's unlikely to be the original purpose for their production. Some other possible explanations therefore suggest the toxins' function as iron chelators or signalling molecules (Sivonen, 2009).

1.3.2.1 CYANOTOXINS

Cyanobacteria have been known since the 19th century for their toxic effect on water, mainly for forming water bloom that poisoned cattle and was dangerous to drink. Cyanotoxins have since been quite thoroughly studied and had been discovered to possess various means of activity, among the most known, hepatotoxicity, neurotoxicity, endotoxicity, and cytotoxicity, as well as compounds triggering allergic reactions (Jaiswal, et al., 2011). Structurally, the toxins belong to both ribosomal and non-ribosomal peptides, polyketides, alkaloids, and lipopolysaccharides, and are found in cyanobacteria forming algal bloom (Kultschar & Llewellyn, 2018), predominantly produced by the orders Chroococcales, Oscillatoriales and Nostocales (Burja, et al., 2001). They can be found for example in *Microcystis, Anabaena,* and *Nostoc* species in fresh water (Kultschar & Llewellyn, 2018) and *Synechocystis, Oscillatoria*, or *Lyngbya* (*Moorea*) species in seawater (Burja, et al., 2001).

Most common toxins worldwide are cyclic hepatotoxic polypeptides found in both fresh- and seawaters (Jaiswal, et al., 2011). The most studied representatives of this group are microcystins and nodularins, hepatotoxins inhibiting protein phosphatases in eukaryotic cells (Burja, et al., 2001) by irreversibly binding to them (Jaiswal, et al., 2011), or anabaenopeptins (von Döhren & Welker, 2006).

1.3.2.1.1 MICROCYSTINS

Microcystins are cyclic heptapeptides produced by species of the genera *Microcystis*, *Anabaena*, *Nodularia*, or *Oscillatoria*, among others. They are synthesized via hybrid PKS/NRPS pathway and their structure is composed of seven-member peptide ring containing nonproteinogenic amino acids such as Adda (i.e., 3S-amino-9S-methoxy-2S,6,8S-trimethyl-10-phenyl-deca-4E,6E-dienoic acid) at position 5, or two amino acids derived from Asp and Glu at position 3 and 6, respectively, and two very variable positions (2 and 4), that serve as reference to the name of the variant derivatives. Microcystin-LR was the first identified and is the most commonly studied, and over 100 variants have already been discovered. Other commonly detected microcystins congeners are RR, YR and LA (**Figure 7)**. While the liver is the primary target of microcystins, it is also a skin, eye and throat irritant (Méjean & Ploux, 2013). The WHO has established a provisional guideline provisional of 1 ug/L for microcystin-LR (available online on https://www.who.int/water_sanitation_health/water-quality/guidelines/chemicals/microcystin/en/).

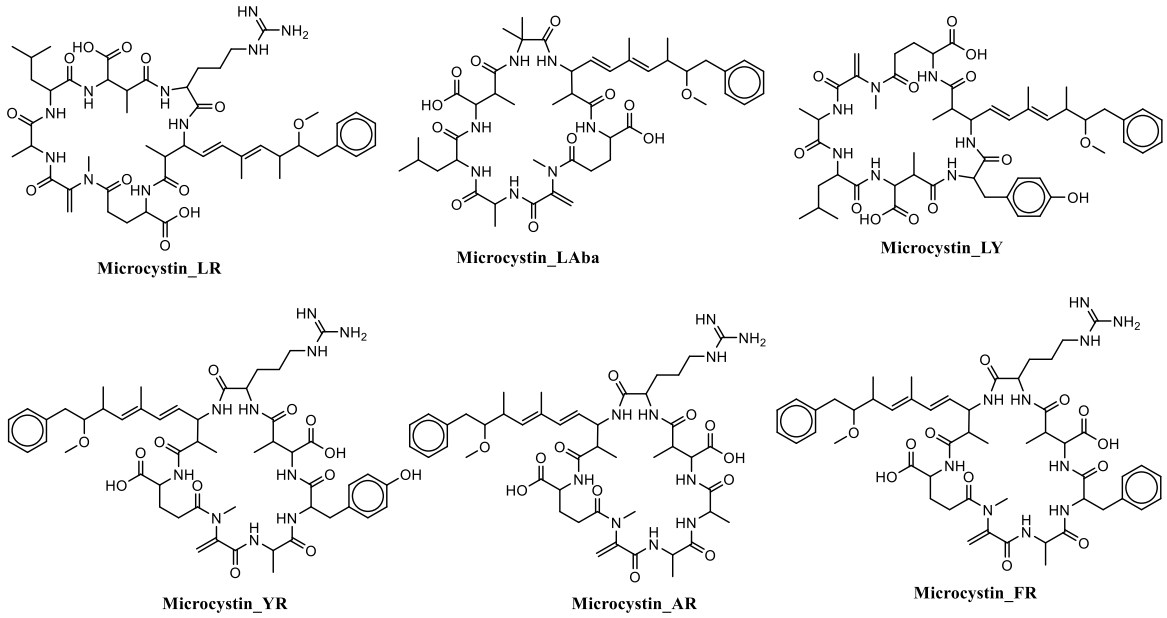


Figure 7: Six most common analogues of microcystins frequently observed (created in ChemDraw 16.0).

1.3.2.1.2 NODULARINS

Nodularins are hepatotoxic non-ribosomal cyclic pentapeptides found in the genus *Nodularia* and are also produced by a hybrid PKS/NRPS pathway (Méjean & Ploux, 2013). They are usually present in marine or brackish waters (Jaiswal, et al., 2011) and mostly isolated from *Nodularia spumigena*, a filamentous planktonic cyanobacterium. Much like microcystin, there are several isoforms of Nodularin (**Figure 8**) and the toxicity is dependent on the position that varies (Pearson, et al., 2010).

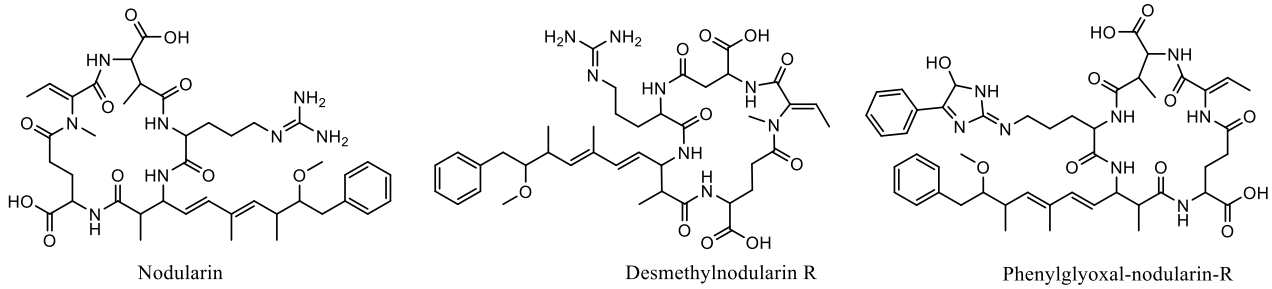


Figure 8: Common analogues of nodularins (created in ChemDraw 16.0).

1.3.2.1.3 ANABAENOPEPTINS

Anabaenopeptins are a highly diverse family of cyclic hexapeptides from diverse cyanobacterial taxa, first described in *Anabaena flos-aquae* NRC 525-17 (Harada, 1995). They are *N*-methylated and contain a conserved ureido linkage connecting the side-chain amino acid residue to the D-Lys. Except for the D-Lys on position 2, all the other positions are variable with members exhibiting masses between 750 and 950 Da, allowing for large structural variability (von Döhren & Welker, 2006). Some of the variants are depicted on **Figure 9**.

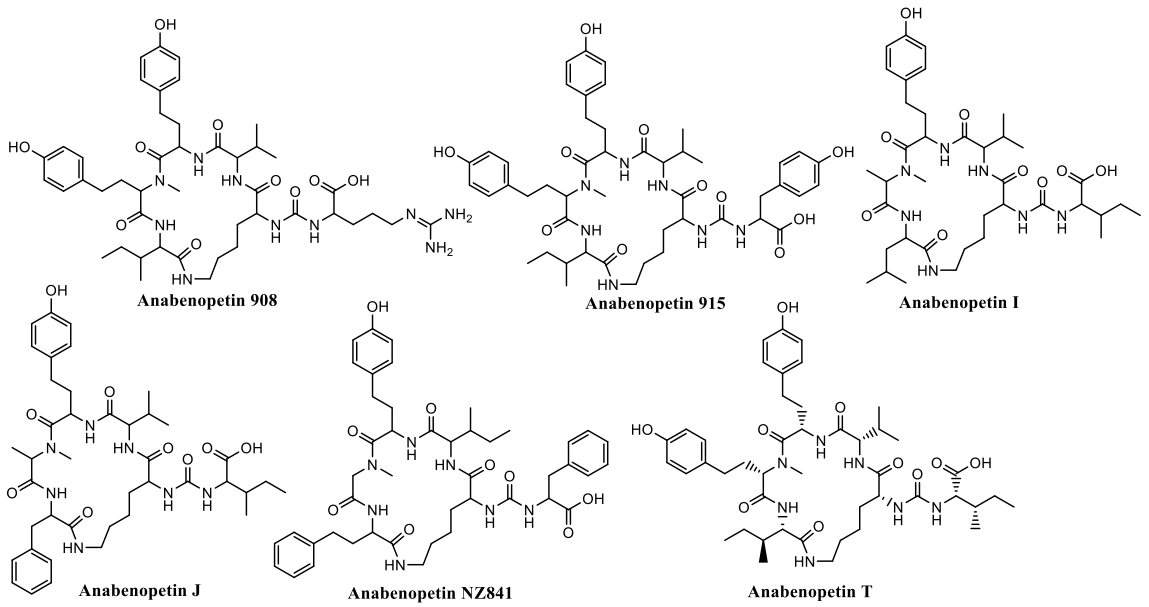


Figure 9: Common analogues of anabaenopeptins (created in ChemDraw 16.0).

1.3.2.2 METABOLITES WITH ANTICANCER ACTIVITY

Cyanobacterial metabolites with anticancer activity frequently target eukaryotic cytoskeleton, namely tubulin and actin filaments. Some examples of anti-cancerous compounds are Dolastatins 10 and 12 isolated from *Symploca* spp. and *Leptolyngbya* spp. (**Figure 10**); Curacin A and apratoxin from *Lyngbya majuscula*; Cryptophycin from *Nostoc*; or Calothrixins A and B from *Calothrix*. Anticancer activity has also been observed in microcystins and nodularins, which inhibit proteins phosphatases and lead to generation of reactive oxygen species (Dixit & Suseela, 2013).

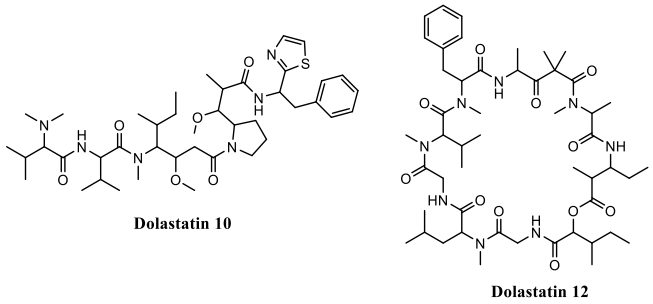


Figure 10: Structures of Dolastatin 10 and Dolastatin 12 (created in ChemDraw 16.0).

1.3.2.3 METABOLITES WITH ANTIMICROBIAL ACTIVITY

Cyanobacteria often produce potent antimicrobial compounds with antibacterial or antifungal activity. Many cyanobacterial extracts show antimicrobial activity, but only few compounds have been structurally characterized. These include for example noscomin isolated from *Nostoc commune*, norbietanes from *Micrococcus lacustris* (Singh, et al., 2011), ambiguine from *Fischerella ambigua* with antibacterial activity, and cryptophycins from *Nostoc* sp. (Dixit & Suseela, 2013) or hectochlorin from *Lyngbya majuscula (Moorea producens)* (Kehr, et al., 2011) with antifungal properties.

1.3.2.4 METABOLITES WITH OTHER ACTIVITIES

Various cyanobacterial compounds with a broad range of effects had been identified. For example, cyanoviridin-N inactivates the HIV (Singh, et al., 2005); Microcolin A isolated from *L. majuscula* exhibits immunosuppressive activity (Vijayakumar & Menakha, 2015); nostocarboline from *Nostoc sp.* was found to have antiprotozoal activity against *Trypanosoma* spp.; tumonoic acid I from *Blennothrix cantharidosmum* showed antimalarial activity; or protease inhibitors like microginins, which are already used to treat high blood pressure (Singh, et al., 2011).

1.3.3 QUORUM SENSING INHIBITORY COMPOUNDS FROM CYANOBACTERIA

Even though anti virulence therapy has not been given much attention so far and the search for QS inhibitors is still in its beginning, several compounds had already been identified in cyanobacteria (Saurav, et al., 2017). For example, just the well-studied marine cyanobacterium *Moorea producens*, previously referred to as *Lyngbya majuscula*, produces several compounds which exhibit QS inhibitory activity, including lyngbyoic acid (Kwan, et al., 2011), lyngbyastatin, or malyngamides (Dobretsov, et al., 2011) (Kwan, et al., 2011). Given that cyanobacteria often live in communities with other organisms, they have great potential to produce compounds targeted at interference with communication of other species such as proteobacterial communities (Dobretsov, et al., 2010), as evidenced by the discovery of lyngbic acid produced by marine cyanobacteria associated with Black Band Disease in corals (Meyer, et al., 2016). Examples of QSIs isolated from cyanobacteria are listed in **Table III** with structures depicted on **Figure 11**.

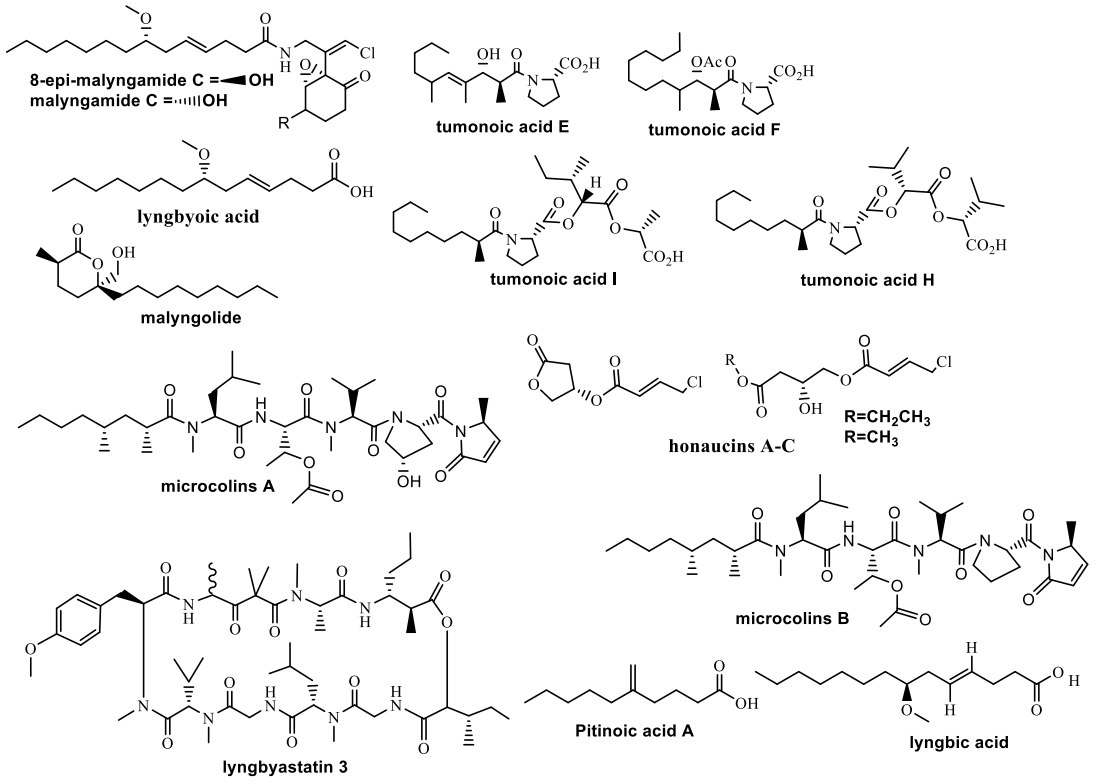


Figure 11: Structures of QSIs isolated from cyanobacteria (created in ChemDraw 16.0).

Table III: Quorum Sensing Inhibitory (QSI) compounds belonging to different classes isolated from various cyanobacterial species with their mode of activity.

COMPOUND	SPECIES	CLASS	BIOSENSOR	QSI EFFECT	REFERENCE
Malyngolide	*Lyngbya majuscula* (*Moorea producens*), widespread in various *Lyngbya* species		CV026	Inhibition of violacein production	(Dobretsov, et al., 2010)
Malyngamides A and B	*Lyngbya majuscula (Moorea producens)*	Amides	CV026	Inhibition of violacein production	(Dobretsov, et al., 2011)
Microcolin A	*Lyngbya* sp.	Peptide	pSB401, CV017	Inhibition of luminescence, inhibition of violacein production	(Dobretsov, et al., 2011)
Microcolin B	*Lyngbya* sp.	Peptide	pSB401, CV017	Inhibition of luminescence, inhibition of violacein production	(Dobretsov, et al., 2011)
Lyngbyastatin 3	*Lyngbya majuscula*	Peptide	CV017	Inhibition of violacein production	(Dobretsov, et al., 2011)
Honaucins A-C	*Leptolyngbya crossbyana*	γ-Butyrolactones	*Vibrio harveyi* BB120	Inhibition of luminescence	(Choi, et al., 2012)
Lyngbyoic acid	*Lyngbya majuscula*	Fatty acid	pSB1075	Inhibition of luminescence	(Kwan, et al., 2011)
8-*epi*-malyngamide C	*Lyngbya majuscula*		*E. coli* pSB1075	Inhibition of luminescence	(Kwan, et al., 2010)
Malyngamide C	*Lyngbya majuscula*		*E. coli* pSB1075	Inhibition of luminescence	(Kwan, et al., 2010)
Oleic and Palmitic acid	*Oscillatoria subliformis*	Fatty acids	*Pseudomonas aeruginosa*	Inhibition of biofilm formation	(LewisOscar, et al., 2015)
Lyngbic acid	Black Band Disease cyanobacteria		*Vibrio harveyi* KM413	Inhibition of luminescence	(Meyer, et al., 2016)
Pitinoic acid A	Cyanobacterium morphologically similar to *L. majuscula*	Fatty acid	*Pseudomonas aeruginosa*	Inhibition of pyocyanin production	(Montaser, et al., 2013)
Trikoramid A	*Symploca hydnoides*	Cyclic decapeptide	*Pseudomonas aeruginosa* lasB-gfp	Weak reduction in fluorescence	(Phyo, et al., 2019)
Sphaerocyclamide	*Sphaerospermopsis* sp. LEGE 00249	Cyanobactin	CECT494	Inhibition of violacein production	(Martins, et al., 2018)
Tumonoic acids E-H	*Blennothrix cantharidosmum*		*Vibrio harveyi*	Inhibition of luminescence	(Clark, et al., 2008)

2 AIMS AND OBJECTIVES

The emergence of antibiotic-resistant pathogenic bacteria calls for the development of new strategies to combat bacterial diseases. Anti-virulence therapy aims at interference with bacterial communication and at the ability of the pathogens to produce virulence factors which cause the infection. The main aim of this project was to attempt to find lead molecules with potential to interrupt bacterial communication (QS) or degrade signalling molecules and thus inactivate virulence genes. Extracts of filamentous cyanobacteria were screened for their QSI potential using genetically modified biosensors and fractionated based on the bioassay results to isolate and characterize novel QS inhibiting active compounds. In order to achieve this goal, the objectives were set as follows:

1. Growth optimisation of filamentous cyanobacteria from a large set of in-house culture collection and crude extract preparation.
2. Screening of crude extracts for their antimicrobial activity using various biosensor systems.
3. Screening for hemolytic activity of the crude extracts.
4. Fractionation of crude extracts to obtain numerous fractions.
5. High throughput screening of the fractions for their QSI activity.

3 MATERIALS AND METHODS

3.1 SCREENING FOR ANTIMICROBIAL ACTIVITY

3.1.1 CULTIVATION OF CYANOBACTERIAL STRAINS

A set of 34 cyanobacterial strains listed in **Table IX** were randomly selected from the in-house collection of Centre Algatech. Each strain was cultivated for 1 week in 300 mL glass columns in their respective medium (**Table IX**), Anabaena (Allen and Arnon Medium, pH±6,8 (Allen & Arnon, 1955)) or BG-11 ((10ml/L 100x BG-FCP, 1ml/L 1.000x ferric ammonium citrate, ml/L 1000x Na_2CO_3, 1ml/L 1000x K_2HPO_4; pH±7). The temperature was maintained at 28° C, illumination 50 μmol photon $m^{-2}s^{-1}$, and supplied with air enriched with 1.5 % CO_2. After 1 week, the biomass was harvested by centrifugation (3125 g), lyophilized (Scanvac CoolSafe Freeze dryer, Labogene), and stored at - 80° C until further use.

3.1.2 PREPARATION OF EXTRACTS AND SCREENING FOR ANTI-MICROBIAL ACTIVITY

100 mg of lyophilized biomass of each strain was ground with sea sand and extracted with 10mL of four different solvents: hexane (H), chloroform (C), chloroform:methanol (1:1) (CM), and 70% methanol in water (M). The extracts were sonicated in ultrasonication bath for 10 minutes, filtered through glass microfibre filter (1.2 μm), and the organic phase was dried in rotary vacuum evaporator (Laborota 4002 Rotary Evaporator, Heidolph). Dried extracts were resolubilized in 70% DMSO to a final concentration of 16 mg/mL and tested for their antimicrobial activity against five microbial and eight fungal isolates listed in **Table IV**, which were kindly provided by Engy Ahmed and Alica Chroňáková from the Biology Centre of the Czech Academy of Sciences, České Budějovice.

Table IV: Organisms used for testing of antimicrobial activity of cyanobacterial extracts.

Bacteria	**Strain**	**Fungi**	**Strain**
Staphylococcus aureus	CCM3824	*Candida friedreckii*	BCC020 2879
Bacillus subtilis	CCM1999	*Aspergillus fumigatus*	BCC020 2845
Streptococcus sanguinis	CCM4047	*Fusarium oxysporum*	BCC020 2866
Pseudomonas aeruginosa	CCM1959	*Trichoderma harzianum*	BCC020 0606
Escherichia coli	CCM2024	*Bipolaris sorokiniana*	BCC020 1571
		Monographella cucumerina	BCC020 2872
		Chaetomium globosum	BCC020 2527
		Alternaria alternata	BCC020 0609

The activity was tested by agar well diffusion assay. 20 mL of sterile Mueller-Hinton agar (1.5%) and Sabouraude dextrose agar (1.5%) for bacteria and fungi respectively was poured into petri dishes

and left to solidify in sterile conditions. Once solid, 100 µl of overnight cultures of the biosensors (bacteria cultured in Mueller-Hinton Broth and fungi in Sabouraude Dextrose Broth) were spread onto the agar and left to air-dry. Subsequently, wells (approximately 60 µl of volume) were cut into the agar layer and filled with 50 µl of cyanobacterial extracts. 50 µl of 70% DMSO was used as negative control and erythromycin (32 µg/mL) as positive control. The plates were sealed with parafilm and incubated at 37° C for 16 hr (for bacteria) and at 30° C for 48 hr (for fungi). Each extract was tested in triplicates. Antimicrobial activity of extracts was evidenced by the presence of inhibition zone surrounding the wells and expressed as the mean diameter of the inhibition zones in millimetres.

3.1.3 BIOASSAY-GUIDED FRACTIONATION

The strains which showed activity in antimicrobial tests were selected or further cultivation and fractionation. The two strains 5 and 142 were cultivated in their corresponding medium until 2 g of lyophilized biomass were obtained, extracted with methanol, and fractionated using Agilent 1260 Infinity instrument. Fractions were collected using automatic collector every 1.5 minutes. Fractionation was performed using reverse-phase Phenomenex Kinetex C18 column (250×21.2 mm, 5 µm). H_2O (A) and acetonitrile (B) acidified with 0.1 % HCOOH were used as a mobile phase in gradient described in **Table V**. The obtained fractions were dried in pre-weighted Eppendorf tubes and weighted again to ascertain the amount of compounds obtained, then resolubilized in 50 % DMSO in methanol to a final concentration of 5 mg/mL. The fractions were tested for their antimicrobial potential and the most active ones were further selected for determination of their minimum inhibitory concentration.

Table V: Solvent gradient and flow rate used for fractionation of crude cyanobacterial extracts.

Time [min]	A [%]	B [%]	Flow [mL/min]
0	85	15	15
4	85	15	15
45	0	100	15
51	0	100	15
55	85	15	15

3.1.4 MINIMUM INHIBITORY CONCENTRATION DETERMINATION

The most potent fractions were tested for their Minimum Inhibitory Concentration (MIC). The fractions were serially diluted in a transparent 96-well plate at concentrations of 250-0.122 µg/mL in Mueller Hinton broth and Mueller Hinton broth with 2 % glucose for bacteria and fungi respectively. 100 µl of overnight cultures of bacteria and fungi (final concentration approximately 5×10^5 CFU/mL) were added to each well containing the fractions, plates were sealed with parafilm,

and incubated at 37° C for 16 h (bacteria) and at 30° C for 48 h (fungi). 50 % DMSO as a negative control and antimicrobial agents as positive controls were serially diluted in the same way as the tested extracts: Erythromycin (32-0.0625 µg/mL) for *S. aureus* and *S. sanguinis*, gentamycin (32-0.0625 µg/mL) for *B. subtilis* and *P. aeruginosa*, and fluconazole (32-0.0625 µg/mL) for the fungal isolates. The well containing the lowest concentration of extract, but still exhibiting growth inhibition was marked as the MIC value for the respective organism. All the experiments were done in triplicates.

3.2 QUORUM SENSING INHIBITION AND HEMOLYTIC ACTIVITY TESTING

3.2.1 CULTIVATION AND CRUDE EXTRACT PREPARATION

The strains were cultivated in their respective media (Anabaena, BG-11, or "Z" (Zehnder & Staub medium (Zehnder & Staub, 1961)) to obtain 2 g of lyophilized biomass. The biomass was ground with sea sand, extracted with 25 mL of 70 % methanol in water (*v/v)*, sonicated for 10 min, and filtered through glass microfibre filter (1.2 µm). The organic phase was evaporated on rotary vacuum evaporator (MiVac Centrifugal Concentrator, Genevac) at 35° C and concentrated in 4 mL of 70 % methanol.

3.2.2 SCREENING FOR HEMOLYTIC ACTIVITY

Hemolytic effect of the cyanobacterial extracts was tested on sheep erythrocytes (RBCs) (Thermo Scientific Defibrinated Sheep blood). 500 µl of sheep blood was washed three times with sterile PBS (pH=7.4): first, 500 µl of blood were centrifuged for 3 minutes at 6000 rpm. The supernatant was discarded and pellet was resuspended in 1 mL of sterile PBS by gentle mixing with 1 mL pipette tip which has been cut to widen the opening to not damage the erythrocytes. The mixture was then centrifuged again for 3 min. at 6000 rpm. The washing was repeated 3 times and the final pellet was suspended with PBS to obtain a 2 % suspension. 100 µl of the erythrocyte suspension was kept in Eppendorf tubes along with 2 µl of the tested extracts and incubated at room temperature for 2 hours. 2 µl of 70 % methanol and 2 µl of PBS were used as negative controls and 1 µl of 0.1 % Triton-X as positive control. After incubation, the samples were centrifuged (10000 rpm for 10 min.), and 95 µl of supernatant was transferred to transparent 96-well plates. Absorbance of the supernatant was measured at 570 nm by Tecan Infinite F200 plate reader.

The level of hemolysis (%) was calculated using the following formula:

$$Hemolysis\ (\%) = \frac{100 \times (Sample - Negative\ control)}{(Positive\ control - Negative\ control)}$$

Where negative control is 70 % methanol and positive control is 0.1% triton X.

3.2.3 HPLC-HRMS/MS ANALYSIS OF THE ACTIVE STRAINS

Crude extracts were analysed on Thermo Scientific DionexUltiMate 3000 UHPLC (Thermo Scientific) equipped with a diode array detector (DAD) and high-resolution mass spectrometry with electrospray ionization source (ESI-HRMS; Impact HD Mass Spectrometer, Bruker). HPLC separation was performed on reversed phase Kinetex Phenomenex C18 column (150 × 4.6 mm, 2.6 μm) with H_2O/methanol containing 0.1 % HCOOH as a mobile phase. The flow rate and gradient information is listed in **Table VII**.

Table VII: Flow rate and solvent gradient used for UHPLC analysis of bioactive fractions.

Time [min]	A [%]	B [%]	Flow [mL/min]
0	85	15	0.6
1	85	15	0.6
20	0	100	0.6
25	0	100	0.6
30	85	15	0.6

The HPLC was connected to a high-resolution mass spectrometer (Bruker Impact HD) with settings listed below in **Table VIII.** The spectra were collected in the range 20-2000 m/z with spectra rate 2 Hz. Collision-Induced Dissociation (CID) was set as a ramp from 20 to 60 eV on masses 200-1200, respectively. Calibration was performed using LockMass 622 as internal calibration solution and CH_3COONa at the beginning of each analysis.

Table VIII: Parameters set for HRMS analysis of bioactive fractions.

Dry temperature	200° C
Drying gas flow	121/min
Nebulizer	3 bar
Capillary voltage	4500 V
Endplate offset	500 V

3.2.4 FRACTIONATION OF CRUDE EXTRACTS

The methanolic extracts were fractionated using an Agilent 1260 infinity instrument equipped with a diode-array detector. Separation of extracts was performed on a reversed-phase Phenomenex Kinetex C18 column (250 × 21.2 mm, 5 μm) using H_2O (A)/acetonitrile (B) containing 0.1% HCOOH as a mobile phase, at a flow rate of 15 $mL \times min^{-1}$. The gradient is shown in **Table VI.** The fractions were collected using automatic fraction collector with a time slices of every 1.5 min. All the fractions were dried (R- 114 Rotary Vap System, Büchi; Laborota 4002 Rotary Evaporator, Heidolph evaporator)

and weighed. Stock solution of the fractions were dissolved with 50 % DMSO in methanol to obtain a final concentration of 2.5 mg/mL. All fractions were tested for QSI activity using two biosensors.

Table VI: Solvent gradient used for fractionation of cyanobacterial extracts via HPLC.

Time [min]	A [%]	B [%]	Flow [mL/min]
0	85	15	15
4	85	15	15
45	0	100	15
51	0	100	15
55	85	15	15

3.2.5 QUORUM SENSING INHIBITION ASSAY: INHIBITION OF BIOLUMINESCENCE

The obtained fractions were tested for bioluminescence inhibitory activity against two biosensors, *E. coli* pSB401 (pSB401) and *E. coli* pSB1075 (pSB1075). 96-well plates with samples were prepared in duplicates to be tested with both biosensors independently. 5 µl of each sample were kept in 96-well plates with 5 µl DMSO as a negative control and 1 µl of penicillic acid (2 mg/mL) as a positive control. All plates were then stored at - 80° C and subsequently lyophilized. Biosensors pSB401 and pSB1075 were cultured overnight at 30° C with shaking (100 rpm) in LB medium (Luria Broth, HiMedia Laboratories, Mumbai, India) supplied with tetracycline and ampicillin respectively to a final concentration of 100 µg/mL. The overnight cultures were supplied with their cognate AHLs (AHL-C_6 and AHL-C_{10} for pSB401 and pSB1075 respectively) to give a final concentration of 200nM. 100 µL of the cultures were added into each well, the plates were sealed with parafilm, and incubated at 30° C. Luminescence reading was performed after 4 and 6 hours using Tecan Infinite F200 plate reader.

Activity of each fraction was calculated as mean percentage of decrease in bioluminescence emitted by the biosensor expressed as Relative Luminescence Units (RLU) relative to the average RLU value of negative control for each plate according to the formula given below:

$$Decrease\ (\%) = 100 - \left(\frac{100 \times mean\ of\ sample\ RLU}{mean\ of\ negative\ control\ RLU}\right)$$

To differentiate the QSI activity with growth inhibition, a growth-inhibitory activity of the extracts was performed parallelly, in another set of transparent 96-well plates. Absorbance at 570 nm was measured after 4 and 6 hours using Tecan Infinite F200 plate reader and compared with results from QSI assay to ensure the growth was uniform.

4 RESULTS

4.1 SCREENING FOR ANTIMICROBIAL ACTIVITY

4.1.1 CULTIVATION OF CYANOBACTERIA

The cyanobacterial strains selected for cultivation from the culture collection of Centre Algatech are listed in **Table IX** with their respective media. The average amount of lyophilized biomass obtained after 1 week of cultivation was 100-150 mg.

Table IX: Cyanobacterial strains tested for antimicrobial activity and their respective media.

Strain	Name	Medium	Strain	Name	Medium
1	*Nostoc sp.*	A	67	*Nostoc sp. VT-18*	A
5	*Nostoc calcicola*	A	68	*Nostoc sp.*	A
16	*Nostoc sp.*	A	84	*Nostoc sp.*	A
17	*Nostoc sp.*	A	85	*Nostoc sp. BR VI*	A
18	*Nostoc edaphicum*	A	87	*Nostoc sp.*	BG11
23	*Nostoc muscorum 85*	A	88	*Nostoc sp.*	A
25	*Nostoc sp.*	A	91	*Nostoc sp.*	BG11
26	*Nostoc muscorum*	A	111	*Nostoc sp.* Cr4 (Symbiont *Cycas revoluta*)	BG11
27	*Nostoc sp.*	A	112	*Nostoc sp.* Gm (Symbiont *Gunerra manicata*)	A
30	*Nostoc muscorum*	A	113	*Nostoc sp.* Mm (Symbiont *Macrozamia sp.*)	A
33	*Nostoc muscorum*	A	114	*Nostoc sp.* De (symbiont *Dioon edule*)	BG11
34	*Nostoc edaphicum*	A	116	*Nostoc sp.* 1LC27S01	A
43	*Nostoc linckia f. muscorum*	A	119	*Nostoc sp.* Cc3 (Symbiont *Cycas cyrcinalis*)	BG11
44	*Nostoc linckia f. piscinale*	A	122	*Nostoc sp.* TH1S01	BG11
52	*Nostoc sp.*	A	142	*Nostoc sp.* Cc2 (Symbiont *Cycas cyrcinalis*)	BG11
54	*Nostoc sp.*	A	183	*CALU* 1533	A
55	*Nostoc sp. CALU- 1191*	A	188	*Nostoc Viola*	A

4.1.2 PREPARATION OF EXTRACTS AND SCREENING FOR ANTI-MICROBIAL ACTIVITY

34 cyanobacterial strains were extracted with four different solvents with increasing polarity (hexane, chloroform, chloroform:methanol (1:1), 70% methanol) to capture wider range of extracted compounds. The activity was divided into three categories according to the diameter of inhibition zone: weak (5-8 mm), moderate (8-12 mm), and strong (≥ 12 mm), as tabulated in **Table X**. No samples extracted with chloroform showed any activity. Twenty-four chloroform extracts showed weak to moderate antibacterial activity, however, no antifungal activity was observed in any strain. When extracted with CM, twenty six strains exhibited antibacterial activity and eight extracts showed antifungal activity. The most active were extracts with 70% methanol, twenty of which showed moderate to strong antibacterial activity and twenty-five antifungal activity. 70% methanol was therefore selected for further extraction of the most active strains. Overall, approximately half of the strains showed activity against at least one bacterial isolate.

Strains 5 (*Nostoc calcicola*) and 142 (*Nostoc* sp. Cc2), exhibiting the most potent activity, were selected for further work. Strain 5 showed especially strong activity against *Streptococcus sanguinis* and *P. aeruginosa*, while strain 142 exhibited strong antifungal activity against *Candida friedreckii*, *Trichoderma harzianum*, and *Alternaria alternata*.

4.1.3 BIOASSAY-GUIDED FRACTIONATION

Extracts of strains 5 and 142 were fractionated into thirty five fractions, each tested for their antimicrobial potential by agar well diffusion assay in order to identify the fraction containing the active compounds. Multiple fractions, tested in concentration of 5 mg/mL, showed activity in both strains. In strain 5, fractions 14, 17-20, and 22-31 showed potent activity, while in 142, fractions 20-30 exhibited potent antifungal and weak antibacterial properties. UV spectra on **Figure 12** show the absorbance of the fraction at 290 nm. The spectra show clear peaks in individual active fractions, such as (most prominently) in the case of fractions 29 or 31 for strain 5 or 26 or 30 in the case of 142, indicating that the single fractions are responsible for the displayed bioactivity.

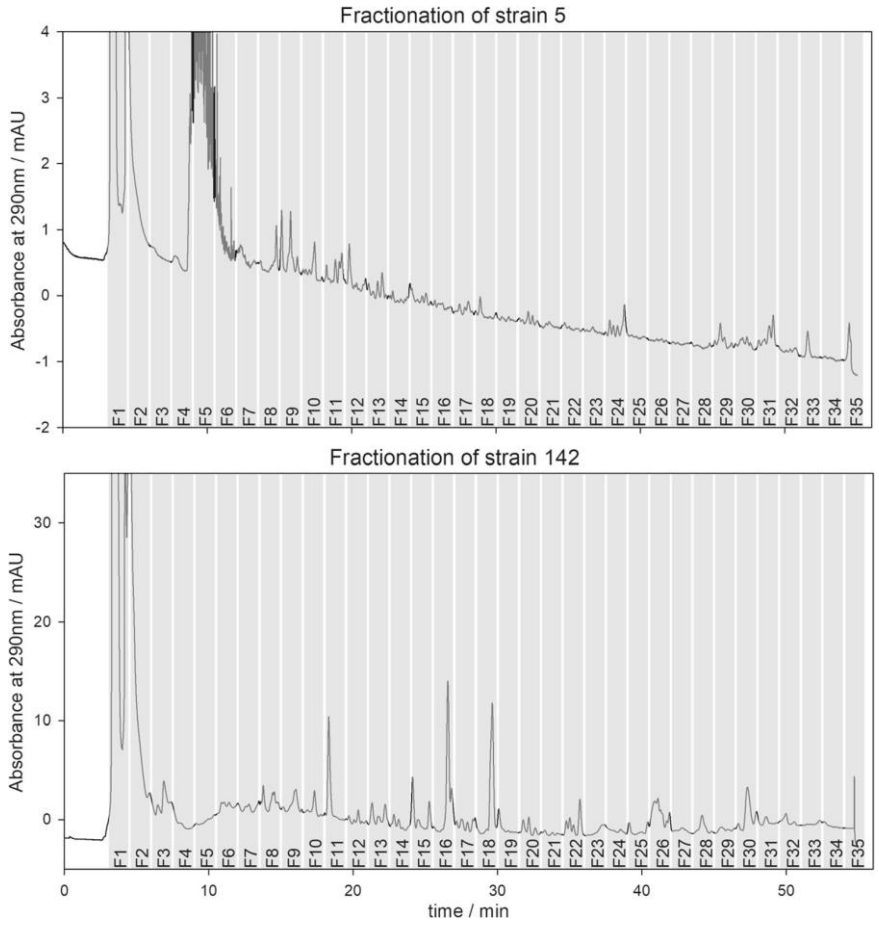
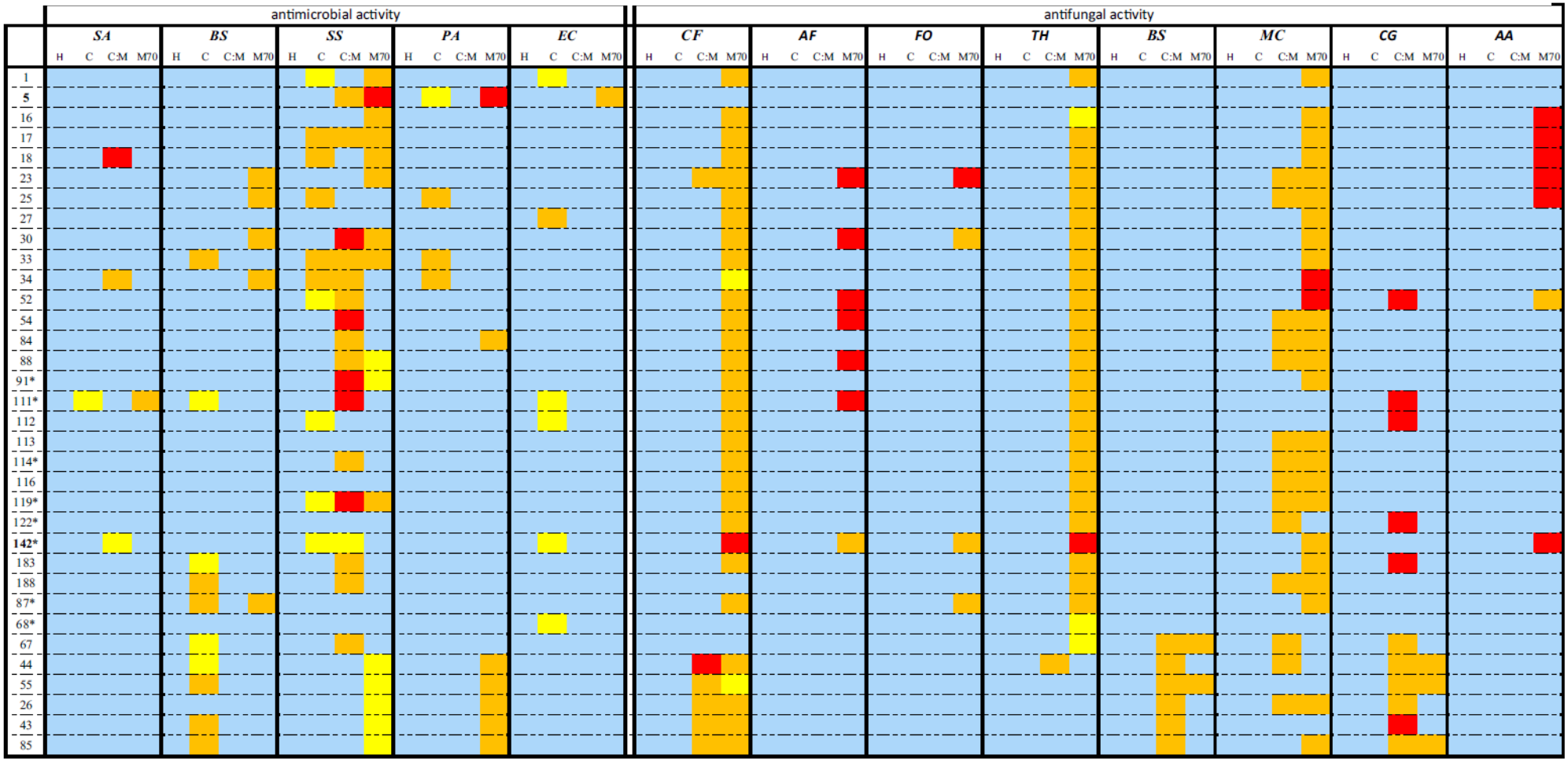


Figure 12: UV spectra of fractions of strains 5 and 142 measured at 290 nm.

Table X: Antimicrobial activity of the tested strains (SA = *Staphylococcus aureus*, BS = *Bacillus subtilis*, SS = *Streptococcus sanguinis*, PA = *Pseudomonas aeruginosa*, EC = *Escherichia coli*, CF = *Candida friedreckii*, AF = *Aspergillus fumigatus*, FO = *Fusarium oxysporum*, TH = *Trichoderma harzianum*, BS = *Bipolaris sorokiniana*, MC = *Monographella cucumerina*, CG = *Chaetomium globosum*, AA = *Alternaria alternata*) and solvents (H = n-hexane, C = Chloroform, C:M = Chloroform:Methanol (1:1), M = 70% Methanol). Blue = No activity, Yellow = Weak activity, Orange = Moderate activity, Red = Strong activity.



4.1.3.1 MINIMUM INHIBITORY CONCENTRATION DETERMINATION

Fractions of both strains which showed activity in well-diffusion assay were selected for tested of their MIC value. In strain 5, fractions 26-29 were the strongest with MIC values ranging from 0.48 to 1.95 µg/mL (**Table XI**). Fraction 26 was especially active against *P. aeruginosa* (MIC of 0.97 µg/mL) and fractions 27 and 28 showed strong antibacterial activity against *S. sanguinis* (0.48 µg/mL).

Table XI: MIC determination of selected fractions of strain 5. MIC value for standard antibiotic used as positive control is given in brackets (µg/mL). Strongest activities are given in bold.

Tested microorganisms	**Fractions MIC (µg/mL)**														
Bacteria	**14**	**17**	**18**	**19**	**20**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**
Staphylococcus aureus (2)	NA	NA	NA	1.95	NA	NA	NA	NA	3.9	7.8	NA	**1.95**	**1.95**	7.81	7.81
Bacillus subtilis (4)	NA	NA	NA	NA	NA	NA	NA	NA	NA	125	NA	NA	125	NA	NA
Streptococcus sanguinis (8)	NA	NA	NA	NA	NA	NA	NA	3.9	3.9	3.9	**0.48**	**0.48**	NA	NA	31.3
Pseudomonas aeruginosa (16)	125	NA	NA	250	250	62.5	31.3	NA	NA	0.97	3.9	7.81	NA	NA	NA
Escherichia coli (1)	NA	31.3	31.3	NA	NA	NA	NA	NA	NA	NA	3.9	3.9	NA	NA	NA

In case of 142, fraction 20 was the most active against *A. alternata* with lowest MIC value of 0.24 µg/mL and fractions 29 and 30 were most active against *A. fumigatus* and *M. cucumerina* respectively. MIC values for 142 fractions are listed in Table XII.

Table XII: MIC determination of fraction 20-30 of strain 142. MIC value for standard antibiotic used as positive control is given in brackets (µg/mL). Strongest activities are given in **bold**.

Tested microorganisms	**Fractions MIC (µg/mL)**										
Bacteria	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Staphylococcus aureus (2)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Bacillus subtilis (4)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Streptococcus sanguinis (8)	NA	NA	NA	NA	NA	125	250	NA	NA	NA	NA
Pseudomonas aeruginosa (16)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Escherichia coli (1)	NA	62.5	NA	125	NA	NA	NA	250	NA	NA	NA
Fungi											
Candida friedreckii (8)	62.5	62.5	62.5	125	250	NA	NA	NA	NA	NA	NA
Aspergillus fumigatus (32)	250	62.5	62.5	125	62.5	NA	NA	**1.9**	**0.97**	**0.48**	125
Fusarium oxysporum (8)	NA	NA	NA	NA	NA	NA	NA	NA	NA	62.5	NA
Monographella cucumerina (2)	250	250	125	250	NA	NA	NA	NA	NA	NA	0.48
Alternaria alternata (2)	**0.24**	7.81	62.5	**1.95**	**0.97**	**1.95**	**1.95**	**0.48**	31.25	7.81	**0.97**

4.2 QUORUM SENSING INHIBITION AND HEMOLYTIC ACTIVITY TESTING

4.2.1 CULTIVATION OF CYANOBACTERIAL STRAINS

Total of 44 strains listed in **Table XIII** randomly selected from the in-house culture collection were cultivated in their corresponding medium (**Table XIII**) for screening of their QS inhibitory and hemolytic activity.

Table XIII: Tested strains and their corresponding medium (A = "Anabaena", BG11).

Strain	Name	Medium	Strain	Name	Medium
3	*Nostoc* sp.	A	77	*Phormidium* sp., strain No. 52	BG11
5	*Nostoc calcicola*	A	84	*Nostoc* sp. BR V	A
10	*Nostoc* sp.	A	85	*Nostoc* sp. BR VI	A
16	*Nostoc* sp.	A	87	*Nostoc* sp. BROMEL	BG11
17	*Nostoc* sp.	A	88	*Nostoc* sp. BR RQII	A
23	*Nostoc muscorum*	A	91	*Nostoc* sp. NMB-21	BG11
25	*Nostoc* sp. CALU – 268	A	111	*Nostoc* sp. Cr4 (symbiont *Cycas revoluta*)	BG11
26	*Nostoc muscorum* CALU - 304	A	112	*Nostoc* sp. Gm (symbiont *Gunerra manicata*)	A
27	*Nostoc* sp. CALU – 327	A	113	*Nostoc* sp. Mm (symbiont *Macrozamia* sp.)	A
30	*Nostoc muscorum* CALU - 542	A	114	*Nostoc* sp. De (symbiont *Dioon edule*)	BG11
32	*Nostoc muscorum* CALU – 545	A	119	*Nostoc* sp. Cc3 (symbiont *Cycas cyrcinalis*)	BG11
34	*Nostoc edaphicum*CALU - 760	A	122	*Nostoc* sp. TH1SO1	BG11
36	*Nostoc* sp. CALU – 870	A	142	*Nostoc* sp. Cc2 (symbiont *Cycas cyrcinalis*)	BG11
39	*Nostoc* sp. CALU – 914	A	183	*Nostoc* sp. CALU - 1533	A
43	*Nostoc linckia* f. *muscorum* CALU – 981	A	221	3 SVA S 12	BG11
44	*Nostoc linckia* f. *piscinale* CALU – 982	A	248	TREB K 1/11 *Anabaena* sp. benthic	BG11
52	*Nostoc* sp. str. Gromov, CALU - 996	A	249	TREB K 2/7 *Anabaena* sp. benthic	BG11
55	*Nostoc* sp. CALU - 1191	A	250	VRES JH 11 *Anabaena* sp. benthic	BG11
58	*Amorphonostoc paludosu* CALU - 521	A	252	VRES L 2/3 *Anabaena* sp. benthic	BG11
67	*Nostoc* sp. strain VT - 18	A	253	VHAJ 4 *Anabaena* sp. benthic	BG11
68	*Nostoc linkia* Roth., strain VT - 19	A	254	DREN1 MII *Anabaena* sp. benthic	BG11
69	*Calotrix* sp., strain No.15	A	CTII	*Brasilonema* sp.	Z

4.2.2 SCREENING FOR HEMOLYTIC ACTIVITY

Fractions exhibiting more than 20 % of hemolysis were considered active. Out of the fourty four strains tested, three strains showed significant activity: 111, 248, and CTII (**Figure 13)**. All three strains hemolysed ±70 % of RBCs: 111 showed 70.673 % (SEM 10.9) activity, 248 exhibited 71.067 % hemolysis (SEM 0.82), and activity of CTII was 64.564% (SEM 4.187). Analysis was performed in GraphPad Prism 5.

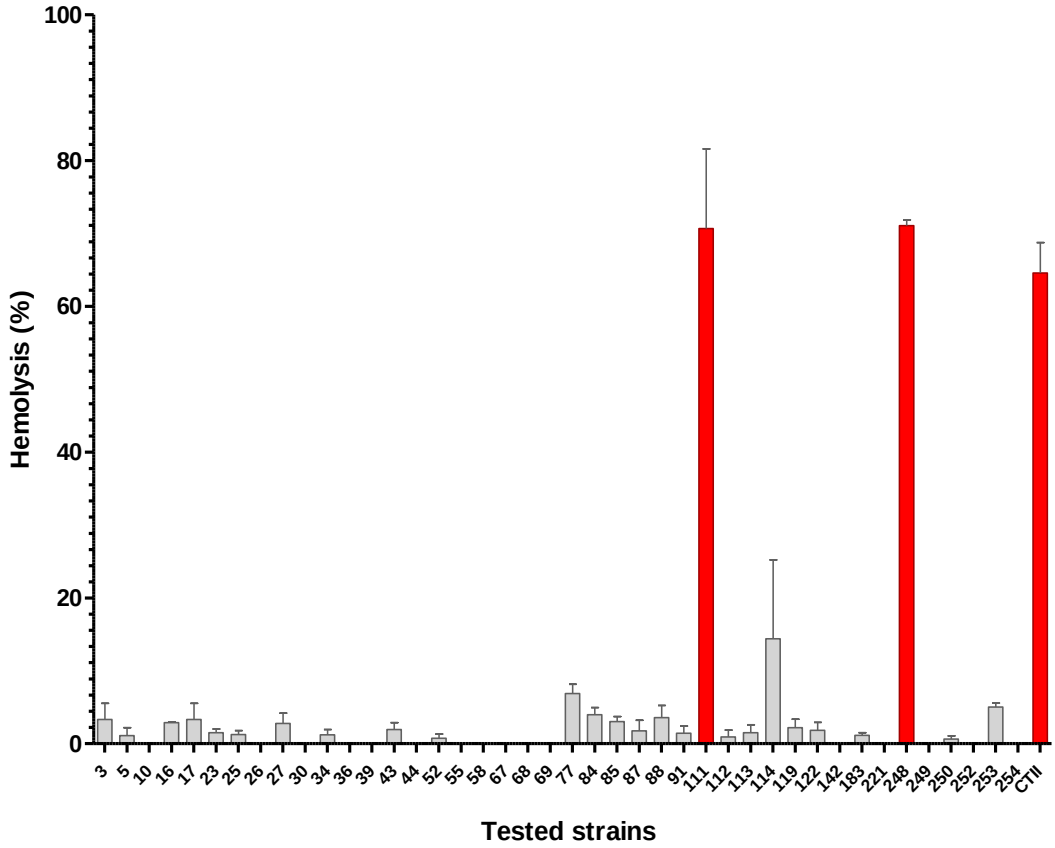


Figure 13: Strains which exhibited hemolytic activity. Extracts showing the strongest activity are marked red. Graph was plotted using GraphPad Prism 5.

4.2.3 HPLC-HRMS ANALYSIS OF THE ACTIVE STRAINS

All the crude extracts were analyzed to decipher the metabolites in their crude extracts. **Figures 18**, **19**, and **20** show the LC-HRMS UV spectra for three most potent strains (selected in section 4.2.4.). The spectra show the richness of the extract and suggests the need of the fractionation to increase the titre value of the metabolites present in low amount.

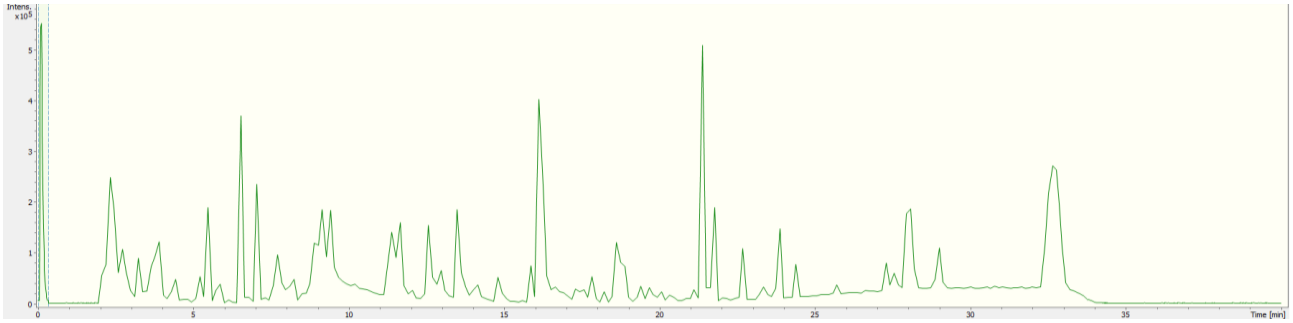


Figure 18: HRMS spectra of the crude extract from strain 3.

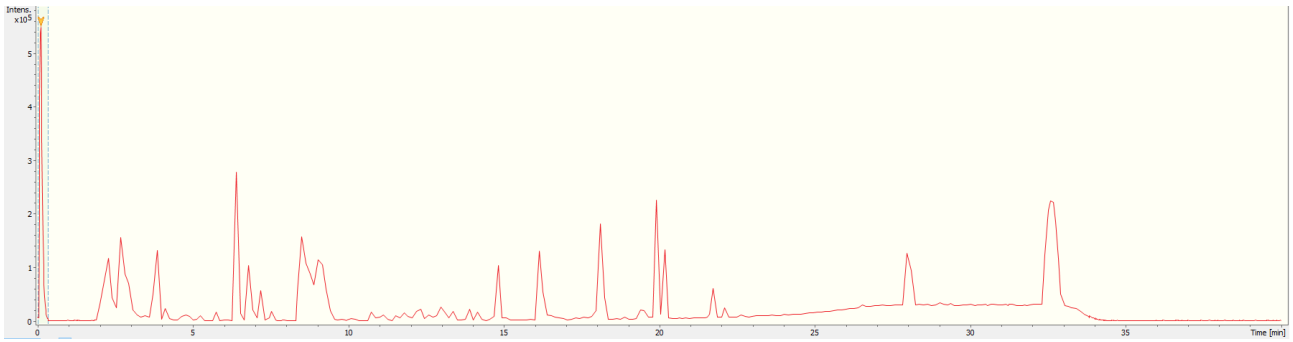


Figure 19: HRMS spectra of strain 16.

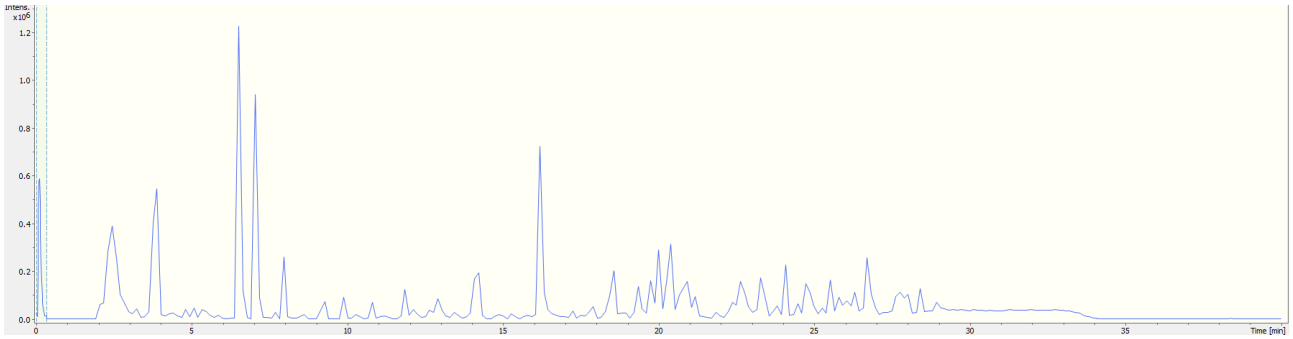


Figure 20: HRMS spectra of strain 113.

4.2.4 QUORUM SENSING INHIBITORY ACTIVITY: BIOLUMINESCENCE INHIBITION ASSAY

Bioluminescence inhibitory activity of the fractions was determined as significant decrease in emitted light compared to negative control. The activity was tested against two biosensors, pSB401 responding to AHLs with short sidechains and pSB1075 recognizing AHLs with long sidechains. Strains which showed less than 10 % decrease in light emitted by the given biosensor when compared to their respective negative control were not considered as active.

When tested against pSB401, forty out of the total forty-four strains reduced the amount of bioluminescence emitted by the biosensor by at least 10 % and seventeen strains showed more than 30 % reduction. Results are given in **Table XIV**. Eight strains (3, 16, 23, 43, 69, 111, 113, and 122 - **Table XVI**) exhibited prominent activity of more than 40 %. These strains were selected for further work.

Table XIV: Activity of tested strains against biosensor *E. coli* pSB401 expressed as reduction in bioluminescence emitted by the biosensor. Most active strains selected for further work are given in bold.

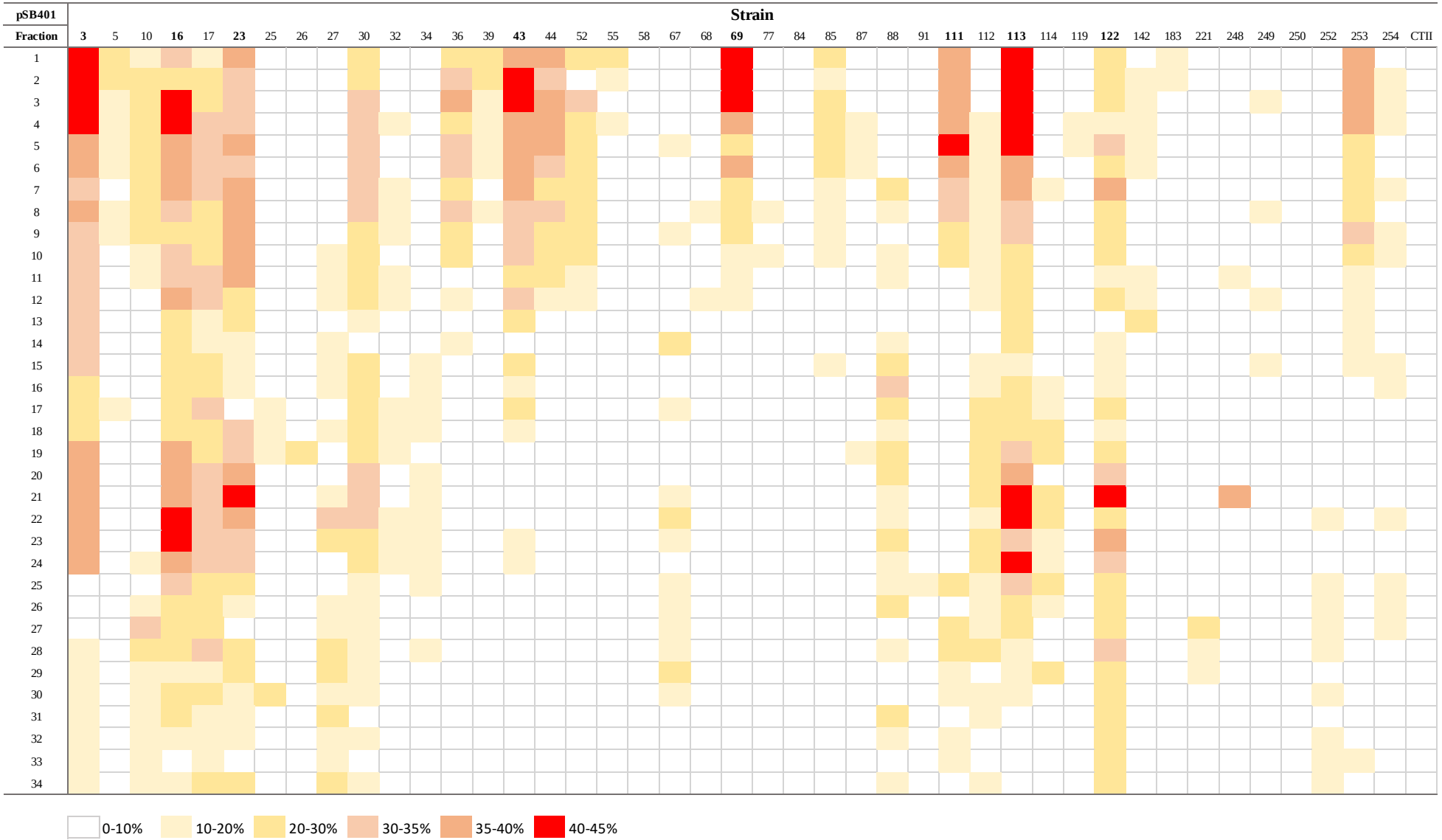
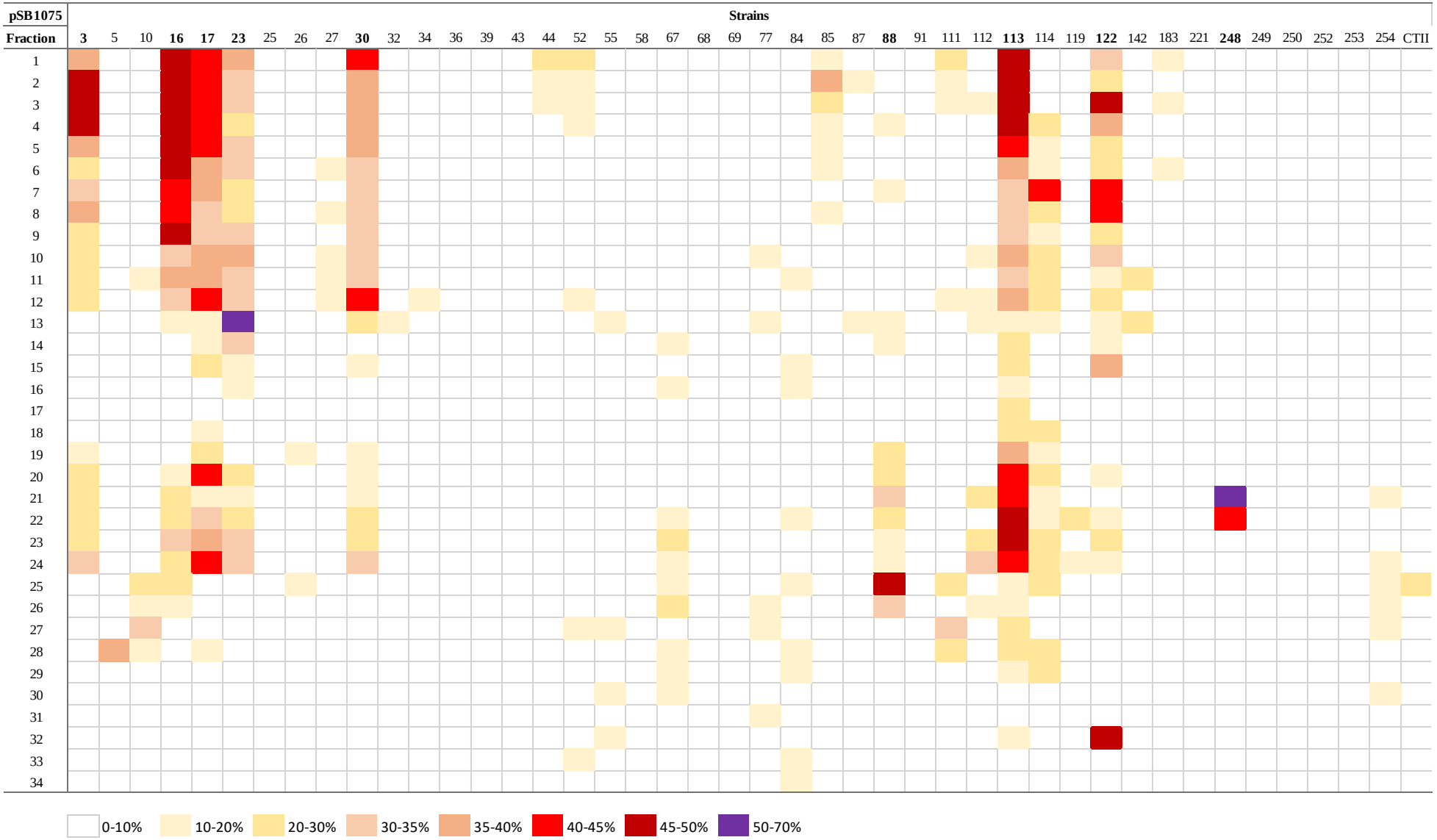


Table XV: Activity of tested strains against biosensor *E. coli* pSB1075 expressed as reduction in bioluminescence emitted by the biosensor. Most active strains selected for further work are given in bold.



Total of thirty-one strains showed at least weak (10-20 %) activity against pSB1075. Interestingly, although less strains proved active, the activity observed against this biosensor was overall stronger than that against pSB401, sometimes reaching 70 % inhibition (±30 % more than the most potent reduction observed against pSB401). Fractions of fifteen strains decreased bioluminescence by at least 30 %, out of which nine (3, 16, 17, 23, 30, 88, 113, 122, and 248 – **Table XV**) showed more than 40 % activity. The latter were selected for further work. One strain, 23 (*Nostoc muscorum*), showed exceptionally high activity, reducing bioluminescence by 74.8 %. Another strain, 248 (*Anabaena* sp.), exhibited reduction by 60 %, however, this strain was proven to be highly toxic in hemolytic assay and the activity can therefore be attributed to the toxicity rather than interference in QS.

Table XVI: Fractions of twelve strains with observed inhibitory activity higher than 40 % for both biosensors *E. coli* pSB401 and *E. coli* pSB1075.

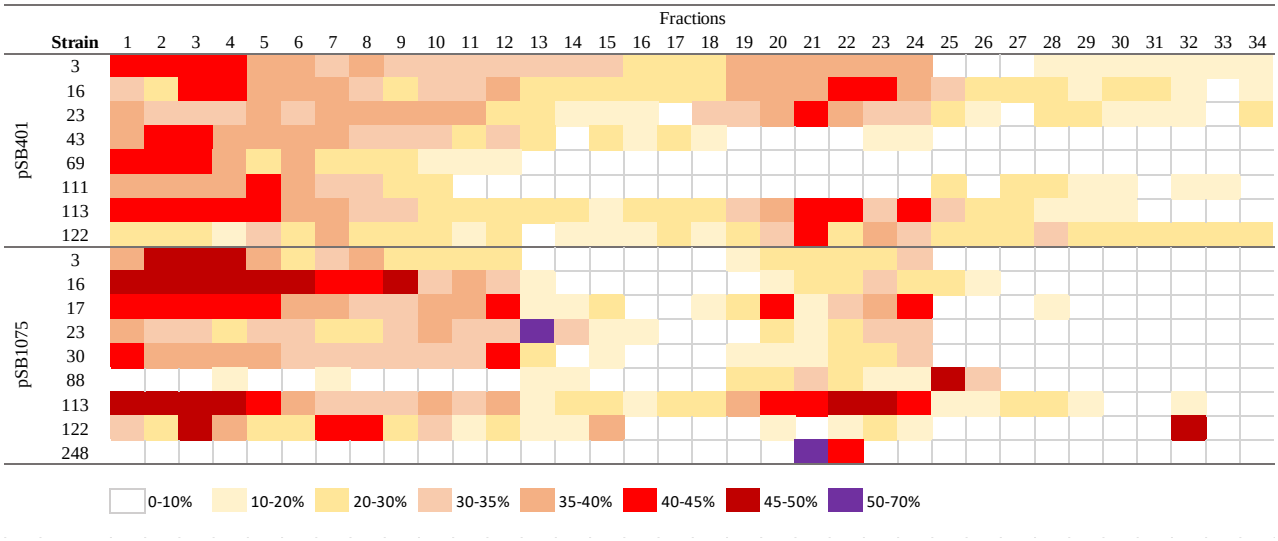


Table XVI lists the selection of strains which showed the highest inhibitory activity against each biosensor. As stated before, eight and ten strains showed more than 40 % activity against pSB401 and pSB1075 respectively. Five strains were common for both biosensors: 3, 16, 23, 113, and 122. Out of these, strains 3 (*Nostoc* sp.), 16 (*Nostoc* sp.), and 113 (*Nostoc* sp., symbiont *Macrozamia* sp.) showed the highest activity and number of active fractions against both pSB401 and pSB1075.

The values of the twelve selected strains were plotted in GraphPad Prism 5 software to show the overall activity profiles of each strain. In most of the strains, two main centres of activity can be observed, one in the initial fractions (in the first 10 to 15 minutes of retention time), then significant drop in activity (approximately 15-20 minutes), before the second peak at 20-25 minutes. These two centres correspond to mobile phase composition of approximately 15-80 % and 100 % of acetonitrile in water respectively (**Table VI**). In the twelve selected strains (except for strains 111 and 248),

fractions which showed activity higher than 40 % selected for further work are highlighted in the following figures in dashed red rectangles (**Figures 14-17**).
In strain 3, the most active fractions were 1-4 (pSB401) which showed reduction of 40-45 % with highest activity shown by fraction 3 (44.9 % reduction) and 2-4 (pSB1075) showing inhibitory activity of 45-50 % with most activity shown in fraction 2 (52.5 % reduction). In both biosensors, second area of activity was observed in fractions 19-24 inhibiting activity by at least 35 % (pSB401) and 20-24 by at least 20 % (pSB1075).

Strain 16 showed strong activity in similar fashion as strain 3. Fractions most active against pSB401 were fractions 3 (42.9 % reduction), 4 (40.4 %), 22 (44.2 %), and 23 (40.1 %). Fractions 5-7, 12, 19-21, and 24 showed inhibitory activity of 35-40 %. Strain 16, however, showed much more potent activity against pSB1075, with seven fractions (1-6 and 9) reducing bioluminescence by 45-50 %: fractions 1, 2, and 3 by 54.8%, 54.6 %, and 54.4 % respectively. Another centre of activity was observed in strain 16 in fractions 19-24 exhibiting activity of at least 30 %, in a similar manner as observed in strain 3.

Strain 113 was the most active among the three, with eight and ten fractions showing more than 40 % reduction against pSB401 and pSB1075 respectively. The active fractions were distributed in a similar fashion as in the other two strains. Fractions 1-5 (with highest activity of 49.9 % in fraction 1 and 48.3 % in fraction 2) and later 21 (41.5 %), 22 (41.0 %), and 23 (40.2 %) showed activity of more than 40 % against pSB401. Even more prominent activity was observed against pSB1075. Fractions 1-4 reduced activity by as much as 45.5 %, 50.1 %, 48.9%, and 46.9 % with highest activity in fraction 2. Fractions 5, 20, 21, and 24 reduced bioluminescence by at least 40 % against pSB1075 and fractions 22 and 23 even by 45.8% and 46.0 % respectively.

Given the strong activity they exhibited against both biosensors, strains 3, 16, and 113 were selected for analysis by HRMS and for further cultivation, extraction, and isolation of the active compound.

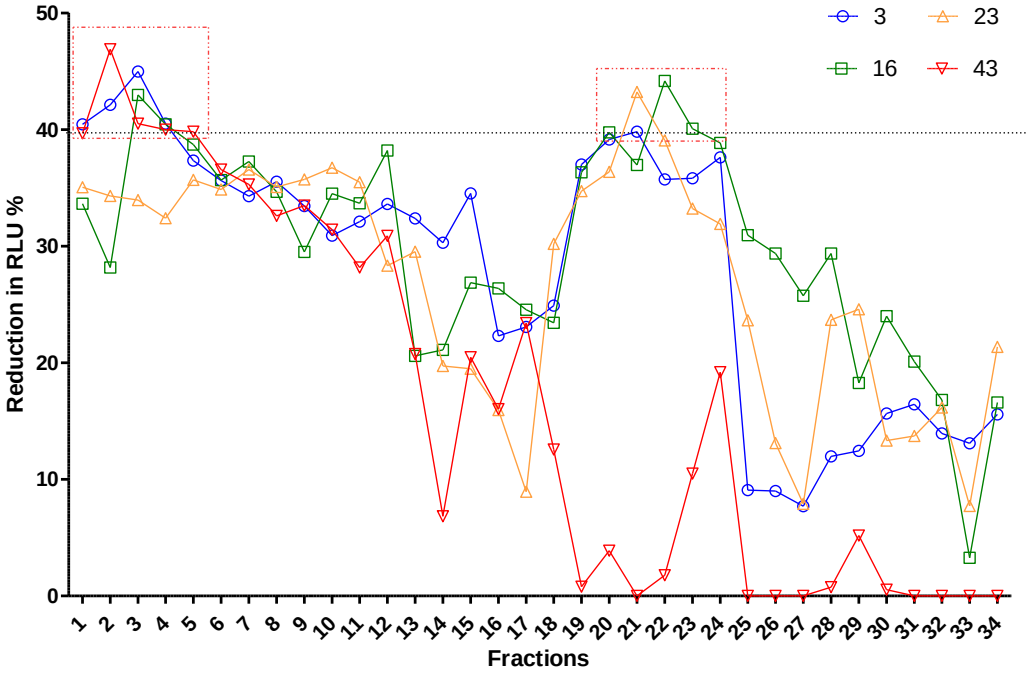


Figure 14: Activity of fractions of strains 3, 16, 23, and 43 against biosensor pSB401. Fractions were selected on the basis of activity exceeding 40 % threshold marked with dotted line. Selected fractions are highlighted in red dashed rectangles.

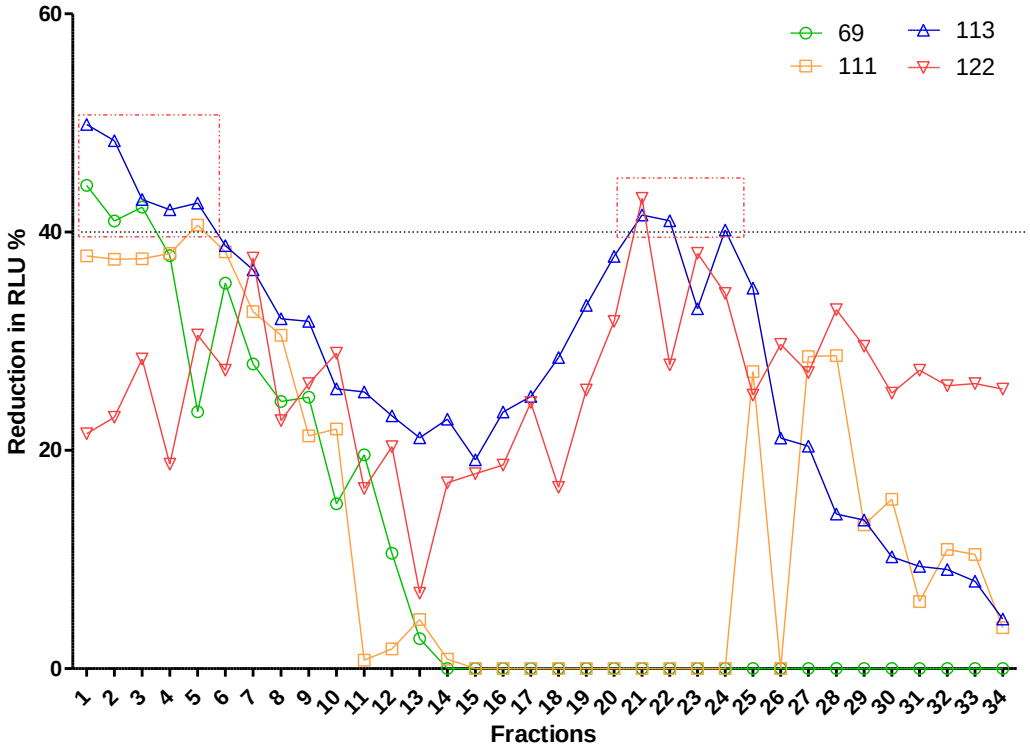


Figure 15: Activity of fractions of strains 69, 111, 113, and 122 against biosensor pSB401. Fractions were selected on the basis of activity exceeding 40 % threshold marked with dotted line. Selected fractions are highlighted in red dashed rectangles.

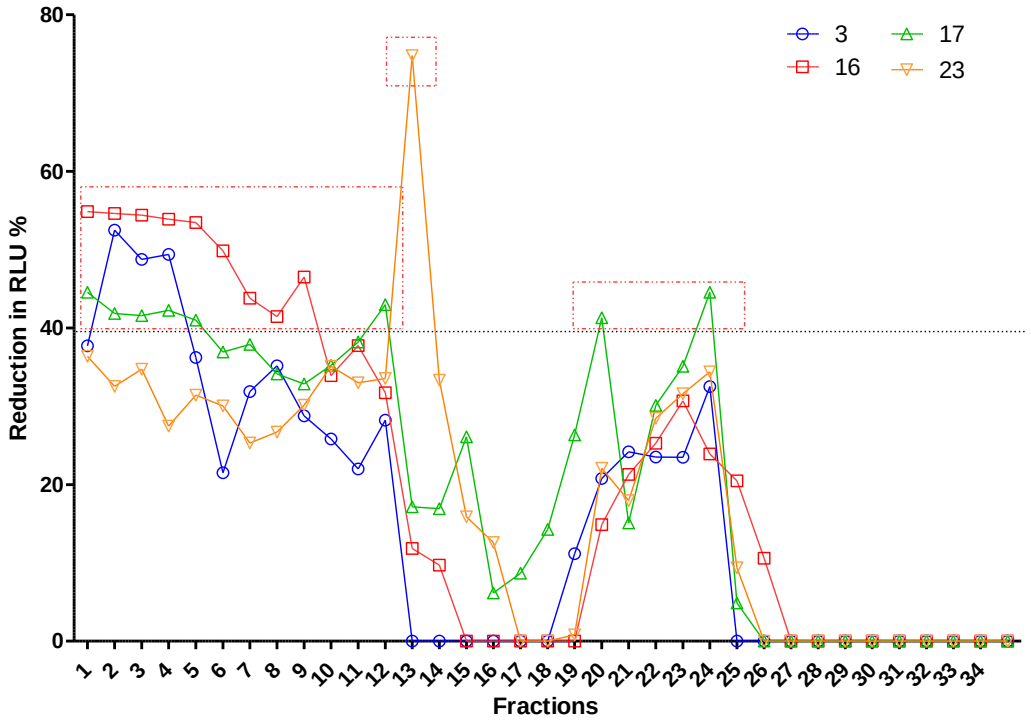


Figure 16: Activity of fractions of strains 3, 16, 17, and 23 against biosensor pSB1075. Fractions were selected on the basis of their activity exceeding 40 % threshold marked with dotted line. Selected fractions are highlighted in red dashed rectangles.

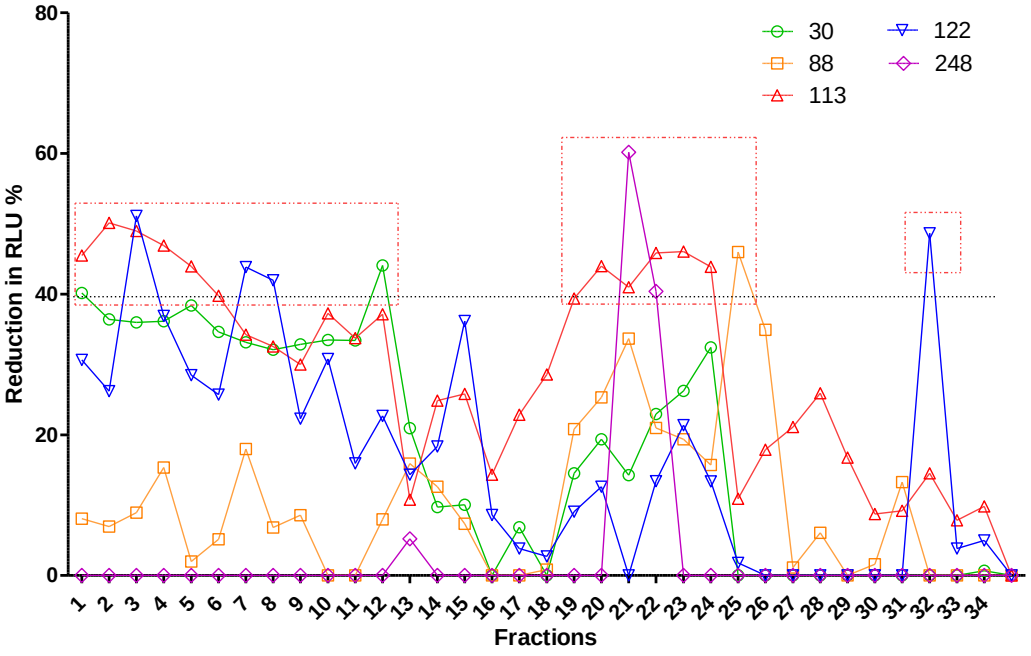


Figure 17: Activity of fractions of strains 3, 88, 113, 122, and 248 against biosensor pSB1075. Fractions were selected on the basis of their activity exceeding 40 % threshold marked with dotted line. Selected fractions are highlighted in red dashed rectangles.

5 DISCUSSION

The main purpose of this project is to screen cyanobacterial extracts for their potential of content of novel compounds useful in the combat against antibiotic-resistant bacteria. In order to do so, we are looking for compounds which interfere with bacterial QS. The main difference between traditional treatment with antibiotics and the alternative approach of anti-virulence therapy is in the effect each has on the pathogen causing the disease, with the former killing the pathogen while the latter tricking it into stopping the production of virulence factors (LaSarre & Federle, 2013). It is therefore extremely important to make sure the compounds we encounter actually posses QSI properties and the activity is not caused by possible antimicrobial or toxic effects of the compounds. In order to ensure the observed bioactivity can actually be attributed to QS inhibition, we tested the strains not only for their QSI activity, but also for antimicrobial as well as hemolytic activity. The strains selected for their prominent QSI activity had generally not been found to posses particularly strong antimicrobial (except for strain 16) or hemolytic activity. To further support the correct type of observed activity, QSI tests were measured for their optical density to detect possible growth inhibition indicating growth inhibitory activity, which would be undesirable in QSI activity. However, none of the selected strains exhibited growth inhibition in *E. coli* biosensors, ensuring the observed activity can be attributed to QSI.

5.1 SCREENING FOR ANTIMICROBIAL ACTIVITY

Antimicrobial activity of 34 cyanobacterial strain extracted with 4 different solvents was evaluated. The solvents were selected based on their increasing polarity (**Table XVII**): hexane, chloroform, chloroform:methanol (1:1), and 70 % methanol in water. Activity of the extract was increasingly more prominent following the rising polarity, with hexane extracts showing no activity whatsoever to methanolic extracts with the most activity observed. Another interesting correlation is in the type of activity exhibited, with antibiotic activity present in samples extract with C, CM, and M, but antifungal activity starting to show only after higher polarity level – in CM (8 strains) and M (25 strains). Since the active compounds were not extracted from the biomass by solvent of low polarity, it suggests the molecules being of more polar nature. Based on these results, methanol was selected as the most suitable solvent for extraction of active compounds for fractionation and more detailed testing. This decision was further supported by the fact that although both selected strains 5 and 142 showed activity in more than one solvent, the most potent activity always showed in samples extracted precisely with 70 % methanol **(Table X)**.

Tab XVII: Polarity of used solvents (mixtures of chloroform:methanol (1:1) and 70 % methanol in water were used for better polarity distinction) (FisherScientific.co.uk, 2020).

Solvent	Polarity index
Hexane	0.1
Chloroform	4.1
Methanol	5.1
Water	10.2

Another interesting correlation is in the type of activity exhibited (**Table XVIII**), with antibiotic activity present in samples extract with C, CM, and M, but antifungal activity starting to show only after higher polarity level – in CM (eight strains) and M (twenty-five strains).

Table XVIII: Number of strains exhibiting antibiotic and/or antifungal activity relative to the solvent used for extraction.

Solvent	**H**	**C**	**CM**	**M**
Antibiotic activity (strains)	0	24	26	20
Antifungal activity (strains)	0	0	8	25

Nearly all tested strains exhibited some level of antimicrobial activity, with 20 out of 34 strains (almost 60 %) showing strong activity against at least one tested microbial isolate. Gram positive bacteria were observed to be affected more than Gram-negative, being affected by 29 strains as opposed to 15 in the case of Gram-negative bacteria.

The strains which were selected for further testing exhibited strong activity against more than one isolate. Strain 5 was selected based on its potent antibacterial activity against opportunistic human pathogens, *Pseudomonas aeruginosa* (Gram-negative) and *Streptococcus sanguinis* (Gram-positive), while 142 was selected mainly for its strong antifungal activity against *Candida friedreckii*, *Trichoderma harzianum*, and *Alternaria alternata*. This difference in type of activity is especially interesting given the fact that both strains belong to the genus *Nostoc*, and might be attributed to the different environmental conditions in which they live – unlike *Nostoc* sp. strain 5 found in saline soil, strain 142 is a symbiont of a cycas species *Cycas cyrcinalis* (**Table IX**).

Since crude extracts of biomass contain a an overwhelming amount of different compounds, dividing the extract into fractions is a powerful tool of natural product discovery allowing for easier identification of the active compound (Sarker, et al., 2006). First step of fractionation in our case was extracting the biomass with four solvents of differing polarity, which already helped to at least roughly indicate the nature of the active compounds. In the selected strains, the crude compounds were further extracted and fractionated into 35 fractions using reverse-phase liquid chromatography.

Upon testing the obtained fractions, it had been observed that activity is influenced by specific fractions, indicating that the active compounds had been concentrated into one individual fraction rather than being spread over multiple aliquots. Apart from greatly simplifying the ensuing isolation process, this fact suggests strong chance of one particular compound being responsible for the observed antimicrobial activity rather than multiple compounds acting synergistically. The fractions causing the activity in fractionated strains were collected between 14 and 31 minutes of retention time, which correspond to mobile phase composition from approximately 35 to 70% acetonitrile in water (**Table V**). This further suggest the hydrophilic nature of the active compounds.

5.2 QUORUM SENSING INHIBITION AND HEMOLYTIC ACTIVITY TESTING

Out of the total forty-four tested strains, only three showed significant hemolytic activity: strains 111 (*Nostoc* sp., symbiont of *Cycas revoluta*), 248 (*Anabaena* sp.), and CTII (*Brasilonema* sp.) (**Table XIII**). Strain 114 (*Nostoc* sp., symbiont of *Dioon edule*, **Table XIII**) seemingly showed weak activity (**Figure 13)**, however, the SME of the calculated values was too big for the activity to be significant. The rest of the strains showed none to neglidgible activity, generally much lower than 10 %. In comparison with the results of screening for QSI activity, no strains selected for further work on QSIs showed any significant hemolytic activity. On the other hand, the strains which induced hemolysis in sheep erythrocytes were found to exhibit QSI activity in the QS bioassays in two out of three cases. This activity may be attributed to the toxic effect of these strains.
Strain 111, which showed over 70 % activity in the hemolytic assay, was also found to inhibit bioluminescence production in pSB401 by more 40 %. The results of strain 248 is particularly interesting since it showed exceptionally high QSI activity (60 % against pSB1075), but exclusively in fractions 21 and 22, suggesting the presence of the toxic (hemolytic) compound in these fractions. The third strain, CTII, did not show any activity at all in the QSI boiassay, the compound responsible for the observed hemolytic activity therefore probably does not have any inhibitory effect on the growth of the biosensors and does not interfere with the QS systems.

HPLC-HRMS analysis showed the large number of different compounds present in the crude extracts of strains 3 (**Figure 18**), 16 (**Figure 19**), and 113 (**Figure 20**). As mentioned earlier, fractionation is a necessary step for separation of different compounds in order to test their specific activity. Crude extracts of the tested cyanobacterial strains were therefore, separated using HPLC into thirty five distinct fractions, each one tested individually for their QSI activity.

Almost 40 % of the strains tested for QSI exhibited inhibitory activity stronger than 30% when tested against pSB401 and almost 35 % of them against pSB1075. Although slightly more strains inhibited production of bioluminescence in pSB401, the observed activity was significantly stronger against

pSB1075. Since pSB1075 QS system recognizes AIs preferably with side chains composed of ±10 carbons (Saurav, et al., 2017), the putative active compounds can be expected to posses structures resembling long side chains of AHLs should they be structurally similar to these signal molecules.

Total of twelve strains showed QS inhibitory activity greater than 40 % (**Table XVI**) including strains 111 and 248. Given the observed hemolytic activity, however, this result is not entirely indicative of the QSI activity of these strains, which would have to be ascertained before further progressing with work on these strains. Since they are not likely to contain the desired compounds, none of these strains were selected for the next steps.

Except for 3, 69, and 248, which were not tested, the twelve selected strains showed antimicrobial activity against several microbial isolates. Nevertheless, as stated before, these strains were not found to inhibit growth of the bacterial biosensors, and the observed QSI activity can therefore be regarded as valid.

5.3 FUTURE PROSPECTS

Out of forty-four initial cyanobacterial strains, three were selected for further work based on their QSI activity exhibited in bioassays. Strains 3, 16, and 113 exhibited the greatest potential for discovery of novel QSIs, the next step is therefore large-scale cultivation of these strains to obtain sufficient amounts of biomass in order to isolate lead molecule responsible for QSI activity. Cultivation of these strains has already been started. The biomass will be extracted based on the information about the nature of the active compounds gained during the screening described in this project. Once larger amounts of active fractions are available, molecular network will be constructed using the Global Natural Products Social Molecular Networking (GNPS) online workflow (Wang, et al., 2016) in order to compare obtained HRMS spectra with GNPS spectral library and try to identify compounds present in the extracts. Selected compounds will then be isolated and tested for their QSI potential to identify the exact compounds responsible for the activity observed during this screening.

The compounds which show activity will then be purified. After purification, structure elucidation via NMR will be attempted, for which pure compound in the order of milligrams is needed. MIC of the compounds will be determined in a similar fashion described in the present work. The precise anti-virulence properties of these compounds will be determined with established bioassays for **violacein inhibition, pyocyanin and rhamnolipids production, protease activity, biofilm formation, motility, and other traits known to be regulated by QS**.

Finally, the ultimate goal would be to discover the precise mechanism of action of the given compound, since three main mechanisms had already been described above (inhibition of AIs production, degradation of the signal molecules, and inhibition of signal retention). Should there not be any novel compound produced by the selected strains, characterisation of the responsible compounds can provide new knowledge on the biosynthetic properties of the tested strains, since search for new anti-virulence strategies using cyanobacteria is still in its beginnings. After the three selected strains will have been fully tested, the rest of the active strains will be studied in the same way to broaden the range of obtained knowledge.

6 CONCLUSION

In the present study, cyanobacterial extracts of 44 cyanobacterial strains were tested for their antimicrobial, hemolytic, and QSI properties.

Strains 5 and 142 showed potent antimicrobial activity, in the case of strain 5 antibacterial activity against opportunistic human pathogens *Pseudomonas aeruginosa* and *Streptococcus sanguinis* was observed, while strain 142 exhibited strong antifungal activity against fungal isolates *Candida friedreckii*, *Trichoderma harzianum*, and *Alternaria alternata*. One of the main achievements of the project was publication of the results obtained in screening for antimicrobial activity in Folia Microbiologica in 2019 (Saurav et al., Antimicrobial activity and bioactive profiling of heterocytous cyanobacterial strains using MS/MS-based molecular networking, Folia Microbiologica, 2019, https://doi.org/10.1007/s12223-019-00737-9),

Markéta Macho participated in the experimental designing, manuscript preparation, experimental set up and performance and finally the reviewing of the manuscript. She was solely responsible for conducting most of the experiment independently; cyanobacterial strains cultivation, crude extract preparation, antimicrobial screening, and minimum inhibitory concentration determination.

High throughput screening of cyanobacterial extracts for their QSI activity was successfully performed, revealing 10 strains with promising activity, out of which three strains 3, 16, and 113 had been selected for their exceptionally potent activity for further work.

Partial results from this study had been presented on the ISSNP conference in Naples, Italy in 2019 in form of a poster titled “Screening of cyanobacterial extracts for Quorum Sensing Inhibitory and anti-microbial activity”.

Work on the 3 most active strains has already begun in order to isolate the compounds responsible for the observed activity. Future work will focus on isolation, purification, and identification of the active compounds, aiming at discovery of novel QSIs with potential use in anti-virulence therapy as an alternative approach to treat bacterial infections as opposed to the current treatment with antibiotics causing dangerous development of bacterial pathogens resistant to known drugs.

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