Ostfalia
Hochschule für angewandte Wissenschaften

Faculty of Supply Engineering

Bachelor thesis

to obtain the academic degree „Bachelor of Engineering“ (B.Eng.)

Induction of secondary metabolites production in myxobacteria under conditions of heavy metal stress

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	Faculty of Supply Engineering
	Study Program Bio- and Environmental Engineering
Written in	Helmholtz Centre for Infection Research
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Submitted on	09.09.2016
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ACKNOWLEDGMENT

In the first place I would like to thank my supervisor Dr. Kathrin I. Mohr for her patience, openness, professionalism and all the personal effort she has put into guiding me throughout this work.

I also want to express my gratitude to Prof. Dr. Wilharm. Without her help, supervision and handling of the official procedures this work would not be possible.

Besides my supervisors, I am also very grateful to the members of the Microbial Drugs Department for all-around support, friendly attitude and patience. In particular, I want to thank Wera Collisi for her technical guidance and enthusiasm, Sabrina Karwehl and Cäcilia Schwager for analyses of extracts with HPLC-MS, as well as PD Dr. Joachim Wink, Klaus Conrad, Birte Trunkwalter, Jolanta Lulla, Stephanie Schulz, Simon Nwakor and Romy Schade, who were always ready to help.

I also want to thank Daniel Rückamp and Elke Fries from Federal Institute for Geosciences and Natural Resources, Hannover for analyses and supply of soil samples for this work.

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ABSTRACT

No studies have been performed so far, which would deal with induction of bioactive secondary metabolites of myxobacteria by exposure to high concentrations of heavy metals. Considering the fact that this approach has been tried with other groups of antibiotic producing microorganisms to activate sleeping gene clusters, and promising results have been obtained, this study was performed to investigate the issue with the focus on myxobacteria. A number of type strains from different myxobacterial groups were taken and the range of tolerance towards selected heavy metals (iron, lead, cobalt, copper, zinc and nickel) was tested on full agar media and in full liquid media. Additionally, a number of myxobacterial strains were isolated from heavy metal contaminated soils and the same tests were performed. Strains from the both test groups, which showed relatively high tolerance, were selected and their metal-spiked cultures were cultivated. The produced secondary metabolites were analyzed and their profiles and amounts were compared to those of the not metal-spiked cultures. No unknown myxobacterial metabolites were found in metal-supplemented cultivation broth. However, secondary metabolites extracts of some strains, grown in the presence of a metal salts, showed increased production of some known myxobacterial compounds, as well as the presence of some unidentified derivatives. Additionally, a putative new antibiotic metabolite of *Labilithrix luteola* was discovered, which was present both in metal-spiked and not metal-spiked cultures. This study suggests that increased production of certain myxobacterial secondary metabolites could be a response to metal-induced stress, and the strategy might be applied to other myxobacterial strains.

TABLE OF CONTENTS

1. INTRODUCTION

Natural products have played crucial role in the establishment and development of antibiotic drug discovery. Penicillin and its potent antibacterial activity, together with its relatively low toxicity to humans, has sparked wide interest in search of similar compounds. Natural products, namely naturally occurring metabolites of various organisms like fungi and bacteria, have become the predominant source of novel classes of antibiotics. [1]. Among 148 compounds, which were used or under clinical investigation in 2008, 66% were either natural products themselves, or could be considered the derivatives of the natural products [2, 3].

However, the golden age of antimicrobial drugs discovery is now the part of 20th century history, when within the period from 1930 to 1962 twenty novel classes of antibiotics were discovered [4]. Since then only two novel classes (oxazolidinone and daptomycin) of antibiotics have entered the market. Isolation and cultivation of antibiotics producing microorganisms, as well as subsequent screening for bioactivity and search for sufficient conditions for large scale production are very time- and cost-intensive processes. In the meantime, nearly all big companies withdraw from this basic research and mainly focus on development of derivatives of known compounds. However, this strategy, despite of its success, has limited prospective, considering the rapid emergence of bacterial resistance [5].

Antimicrobial resistance (AMR) has become an alarming issue in the last few years [6]. It concerns both Gram-positive and Gram-negative bacteria. Some of the most significant types of AMR for public health worldwide include resistance or decreased susceptibility to third-generation cephalosporins among *Escherichia coli* and *Klebsiella pneumonia*; to fluoroquinolones among *Salmonella* spp., *Shigella* spp. and *E. coli*, and methicillin and vancomycin resistance of some *Staphylococcus aureus* strains [7]. Another phenomen is the emergence and rise of multi-drug resistant and even pandrug-resistant bacteria, with the latter term meaning non-susceptibility to all compounds in all applied categories of antibiotics [8] [9]. Considering the fact that 20 classes of antimicrobial compounds were marketed between 1930 and 1960, some authors suggest that another 20 classes are needed to ensure the needs of public health for the coming 50 years [10].

One prominent source of bioactive natural products are myxobacteria, as they are known to produce structurally diverse secondary metabolites with various biological activities [11]. Myxobacteria (described further in the section 2.1) are Gram-negative bacteria, which commonly inhabit soil environment and animal dung [12]. Up to now more than 100 secondary metabolites,

many with bioactivity, and more than 500 derivatives have been isolated from myxobacteria. However, in comparison to actinobaceria or *Bacillus* spp., isolation and cultivation of myxobacteria is challenging. Therefore, screening of myxobacteria for secondary metabolite production has been established only in a few microbiological laboratories. Due to this fact, only about 5% of all known compounds from microorganisms were attributed to myxobacteria in 2003 [13]. The metabolic targets of currently known antimicrobial compounds of different myxobacteria include RNA polymerase, protein biosynthesis, respiratory chain, biofilm formation, topoisomerase inhibitors and signal peptidase LspA [14]. Some of these targets are highly bacteria-specific (LspA peptidase), which makes the corresponding compounds good candidates for drug development [15].

Owing to this potential, myxobacteria remain a promising object of research. One of the world's leading institutions for isolation and investigation of myxobacteria and screen for their potential to produce secondary metabolites is the Helmholtz Center for Infection Research (HZI), Braunschweig. HZI is the part of the Helmholtz Association, Germany's largest scientific organization, which consists of 18 research centers. In particular the department Microbial Drugs (MD) and the working group Microbial Strain Collection (MSC) are focused on the isolation and identification of new strains (myxobacteria, actinobacteria and fungi) and their screening for natural products. Myxobacteria have been the topic of research in HZI (former GBF) for over 30 years, and currently the center possesses the largest collection of myxobacteria isolates (more than 8.500) and the biggest database on myxobacteria secondary metabolites.

Some of the most prominent compounds discovered in HZI are epothilon and soraphen. While both of them originate from *Sorangium cellulosum* strains, their bioactivity and structure are very different. Epothilon shows activity against tumor cells and was further used as a lead structure to develop the drug Ixabepilone, which was marketed in US as chemotherapeutic medication for cancer [16]. Soraphen, on the other hand, demonstrates high antifungal activity. Owing to its ascertained teratogenic activity, development of this lead structure was stopped [17]. However, the compound is currently used in HZI to suppress fungal growth in media for isolation of myxobacteria. It is also used to study possible mechanisms of HIV and hepatitis C virus inhibition [18].

There are two general approaches in the search of new natural products of microorganisms, both of which apply well to myxobacteria. The first approach is to study the metabolome of already discovered strains for PKS/NRPS gene clusters, while the second approach is isolation and

screening of new strains. Both of them have potential to reveal previously unknown compounds and structures. Regarding the first approach, genome analyses revealed that number of potential secondary metabolites which could be produced by a strain is significantly higher, than the number which is detected during standard cultivation approaches. Therefore it can be assumed that most genes are not active under laboratory fermentation conditions [19]. For example, *in-silico* analyses of the genome of *Myxococcus xanthus* strain DK1622, which is known to produce only five different bioactive metabolites, showed that there are 18 gene clusters, coding for secondary metabolites. The same founding concerns myxobacterium *Sorangium cellulosum*, and the pattern can be explicated on other types of myxobacteria [20]. This shows that there is hidden potential in myxobacterial metabolism which is inaccessible under standard cultivation conditions.

In order to reveal this big potential of myxobacteria, one option is to use varying cultivation parameters. Typical approaches to induce or increase secondary metabolite production include altering media composition, pH, temperature, aeration, culture vessel, addition of enzyme inhibitors, feeding of pre-cursors (if the biosynthetic pathway of the wanted compound is known), and creation of other stress conditions like, for example, by addition of salts or heavy metals. Using the so-called one strain-many compounds approach (OSMAC), many strains were made to produce previously unexpressed secondary metabolites [21]. The possible mechanisms of action of different parameters on natural product synthesis are summarized in the Figure 1.

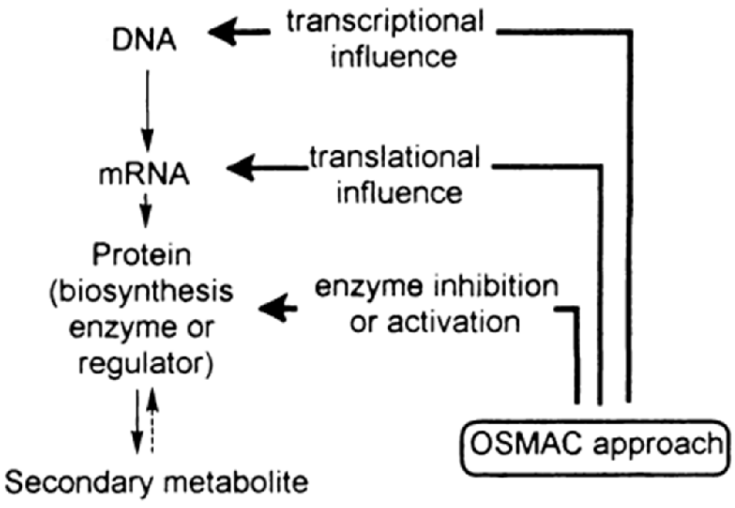


Figure 1. Illustration of possible mechanisms of action of OSMAC approach (Bode et al. 2002).

A few studies have been conducted [22], which investigate possible use of heavy metals as inducers of secondary metabolites' production by, for example, actinobacteria (see section 2.2.3). Some of these investigations give promising results, and in many studies treatment with heavy metals was routinely applied along with other stress factors. However, no published study was

found, about treatment with heavy metals to induce/increase secondary metabolite production in myxobacteria. Therefore the present study is innovative and of highly interest and could open up urgently needed new ways to weak up some of the numerous existing but silent gene clusters.

Isolating myxobacteria from previously neglected environments and screening their metabolites is another possible approach to find new bioactive compounds. Traditionally myxobacteria research mostly focused on mesophilic bacteria living in soil environments with neutral pH and under aerobic conditions [12]. However, at certain point the focus has shifted towards attempts to isolate myxobacteria from unconventional environments. Marine myxobacteria are good example of such promising source, which already provides information about natural products with new carbon skeletons [23].

One of these uncommon environments, which has not been studied as a source of new myxobacteria strains so far, are heavy-metal contaminated soils. While some studies claim that microorganisms, adapted to life in hazardous environments, are generally no good producers of antimicrobial compounds [24], other studies came to contrary results as shown by [22] who investigated actinobacteria. Therefore in this study myxobacteria from heavy-metal contaminated soils were screened for their potential to produce secondary metabolites with focus on novel bioactive compounds.

Considering apparent lack of research on the topic of myxobacteria and heavy metals, and keeping in mind two possible approaches to find new microbial natural products (OSMAC and strains from unconventional environments), it was deemed promising to conduct complex investigation of heavy metals' effect on myxobacteria with the focus on secondary metabolism. Therefore four major targets were pursued within this Bachelor-thesis.

In the beginning nothing was known about inhibiting concentrations of different heavy metals on myxobacteria. Therefore the first goal was to:

1. get a general overview about the tolerance of selected myxobacteria against various concentrations of different heavy metals.

Several type strains of myxobacteria from different families were taken from HZI collection and cultivated both on agar and in liquid media in presence of different concentrations of the selected metals. The growth inhibition was recorded, and based on these results several type strains showing relatively high tolerance were selected for:

2. checking, whether treatment of these myxobacteria with different concentrations of selected heavy metals would induce sleeping gene clusters or increase productivity of already known secondary metabolites.

Selected type strains were cultivated under determined metal concentrations and the produced secondary metabolites were extracted. Antibiotic activity of these metabolite extracts was tested against various test organisms. Then bioactive compounds were correlated to retention times/peaks by fractionation with high-performance liquid chromatography (peak-activity correlation) and subsequently the molecular masses were identified with liquid chromatography-mass spectrometry method. Comparison of mass, retention time, UV-spectrum and bioactivity information with data of the internal HZI database ("Myxobase") was used to determine the corresponding substance as known or unknown. To compare productivity of myxobacteria at different concentrations of metals, the composition of extracts was compared by performing HPLS with each used metal-concentration combination.

In order to investigate heavy metal - polluted soils as a source of (new) myxobacterial strains, another target was:

3. the isolation and identification of myxobacteria from the selected environment.

For isolation of (new) myxobacterial strains standard techniques were applied (section 2.1.4). The isolated strains were identified by 16S RNA-gene sequencing. Heavy metal tolerance of the self-isolated strains was tested, and the results were used to develop experiment design for the last target of the work:

4. the screen of isolated strains from heavy metal - polluted soils for production of antimicrobial compounds, analysis of the effect of heavy metals on productivity and comparison of these results to those of the type strains

Procedures used for analysis of secondary metabolites were the same as for the type strains. The results for metal spiked and non-spiked cultures were compared to each other, as well as to those of the type strains.

2. THEORETICAL BACKGROUND

This chapter provides basic theoretical information about the subjects, relevant to this Bachelor thesis. It gives overview on the current knowledge about the order Myxococcales with the focus on the aspects, which form basis for cultivation and isolation approaches, used during the practical work. In addition, more detailed description of the myxobacteria, used in this study, is given. Very limited information on heavy metals' influence on myxobacteria and their secondary metabolism is found in the literature. For this reason, information about bacteria's mechanisms of heavy metal tolerance in general, as well as their role in metabolism, is cited. Finally, such sophisticated techniques of molecular biology and analytical chemistry, used at different stages of this work, as polymerase chain reaction, high-performance liquid chromatography and mass spectrometry are described.

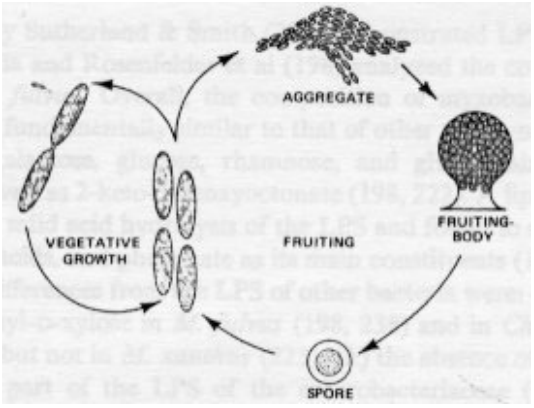
2.1. MYXOBACTERIA

Myxobacteria are Gram-negative rod-shaped unicellular microorganisms, which belong to the order of Myxococcales. Results of 16S RNA gene analysis revealed myxobacteria to be members of the deltaproteobacteria class [25]. Myxobacteria possess a number of distinguishable features, such as particular way of moving on solid surfaces – gliding, myxospore-development and formation of fruiting bodies. Myxobacteria can be divided in two types, based on their nutritional behavior: two genera, *Sorangium* and *Byssovorax*, are cellulose decomposers. All other genera are predators and degrade other living or dead microorganisms in the environment like bacteria, yeasts, or filamentous fungi by collective excretion of enzymes, as described later. All of these features are attributed to the high degree of cooperation within myxobacteria colonies, which makes these bacteria one of the most "social" representatives of the microbial world.

2.1.1. Key features

Formation of fruiting bodies represents the most sophisticated life cycle known in prokaryotes. It happens under starvation conditions or can be triggered artificially by addition of certain chemicals in laboratory. Cooperative morphogenesis of hundreds or thousands of cells first leads to their aggregation and formation of large mounds and finally yields the so-called fruiting bodies. This species-specific aggregates have different degrees of complexity in different strains with the size ranging from 50 to 500 µm. Inside the fruiting bodies vegetative myxobacteria cells transform into short, optically refractive myxospores, which are able to endure unfavorable conditions over decades. The simplest lifecycle of myxobacteria is shown on Picture 1, while some strains have

more complex fruiting bodies differentiated into sporangioles, which germinate separately. In any case, fruiting bodies are formations, which allow convenient isolation of many strains from samples. [26]



Picture 1. Simple scheme of myxobacterial lifecycle [27]

Another distinguishable feature of myxobacteria - gliding motility – is defined as ability to move on surfaces without the use of flagella. This feature fits well into the notion of soil and vegetation as a typical environment for myxobacteria, where motion through liquid with flagella has little benefits for those organisms. Also gliding is the key features which allows hunting and feeding on pray organisms in soil, it is also used as basis for myxobacteria isolation (paragraph 2.1.4). It has been identified, that two distinct types of gliding exist within one cell. Mechanism of type S (S stands for "social") gliding has been relatively well elucidated, and its oversimplified principal is mutual repelling of cells with pili. For this reason this kind of movement is only possible in presence of other myxobacteria in close proximity. The other type of motility – type A – doesn't require community of neighbor cells and is presumably based on ejection of a polysaccharide slime, though the mechanism is not fully understood. Both gliding types are present simultaneously in the microbial colonies of well-studied myxobacteria, such as *Myxococcus xanthus*. The "wave" of bacteria moving together and excreting polysaccharide slime results in the typical "swarming" colonies on agar plates and solid surfaces [28].

2.1.2. Natural occurrence

While soil, manure and decaying plant material are typical habitats for most myxobacterial species, occurrence of these organisms is not restricted to them. In addition to already mentioned marine myxobacteria, there are psychrophilic bacteria from Antarctic soils and arctic tundra, thermophilic strains from tropical rain forests, as well as anaerobic and alkaliphilic strains from other habitats [29].

However, caution should be exercised, when isolation of myxobacteria from extreme environments is in question, since myxobacterial spores can survive conditions, under which normal growth is not possible. Thus, it is worth testing whether the isolated organisms are able to grow under the conditions, to which they are presumably exposed in the environment [26]. This consideration is applicable to this Bachelor thesis, since a part of it deals with isolation of myxobacteria from heavy metal contaminated soils.

The majority of isolated strains are believed to have optimal growth at 30 °C. However, extreme values as high as 46 °C and as low as 4 °C were also described for some strains [26]. In respect to the pH, in most cases myxobacteria were isolated from samples with pH lying in the range from 6 to 8 [26]. Typical optimal temperature and pH are reflected in the techniques for myxobacteria isolation (paragraph 2.1.4) and in standard cultivation conditions (paragraph 3.2).

2.1.3. Nutrition and energy metabolism

Development of isolation techniques for myxobacteria would not be possible without solid understanding of their metabolism and nutrition. While all myxobacteria decompose and then consume various macromolecules, their metabolism can be divided into two distinct groups (omitting facultative anaerobic *Anaeromyxobacter dehalogenans*). The first group can be characterized as cellulose decomposers. Their cellulase production allows them to equally feed well on cellulose and on sugar monomers, such as glucose. As for the nitrogen source, this group of myxobacteria are able to obtain nitrogen both in organic (e.g. peptone) and in inorganic (NH_4^+ or NO_3^-) forms. Media, which contain suitable defined sources of nitrogen, carbon and energy are, for example, yeast media (VY/2), peptone media (CY), as well as various carbohydrates-containing media. [26]

The majority of myxobacteria belong, however, to the second metabolic group which is characterized by bacteria's dependence on amino acids as the only acceptable form of nitrogen and preferable source of carbon and energy. As for other nutrients, their uptake is ensured by enzymatic decomposition of compounds with nucleases, lipases, glycanases, and cell wall lytic enzymes. Cellulose decomposition does not exist in this group, but many myxobacteria are able to decompose starch and quite a few are potent chitin degraders. However, *Myxococcus*-species, for example, are ineffective in using sugar as primary carbon source.

This group of myxobacteria relies on lysis and consumption of other bacterial cells. For this reason the term "micropredator" has been coined for this type of myxobacteria. However, "predatory"

behavior is not obligatory and myxobacteria from this group can act as scavengers. As for cultivation in laboratory, either peptone or yeast are essential components of suitable media (CY and VY/2), though many bacteria in this group are able to grow on starch-based or chitin-based media [26].

2.1.4. Isolation

Based on the knowledge about soil myxobacteria, such as their optimal growth conditions (section 2.1.2), nutrition (section 2.1.3) and distinct features (section 2.1.1), several isolation techniques have been developed [26]. Two commonly used ones, which are also utilized in the MSC working group, HZI, are isolation of predators with bait organisms and isolation of cellulose decomposers with filter paper (*Sorangium* and *Byssovorax*).

The first method is based on bacteriolytic properties of myxobacteria. Cross-streaks of living food organisms on water agar are used as baits for myxobacteria, while the environmental samples (usually soil, sand, dead wood, plant residues, etc.) are placed on the ends of the crosses. Water agar is a basal medium, which means that it lacks any sufficient sources of microbiologically available carbon and nitrogen. Predator behavior of most of the myxobacteria strains and their gliding movement gives selective advantage compared to other, often faster growing soil organisms. *Saccharomyces cerevisiae* yeast, *Micrococcus luteus* and *Escherichia coli* are suitable as food organisms, however the latter one is applied most commonly. Subsequent swarming growth of myxobacteria over and above the underlying bacteria, as well as fruiting body production, are visually distinguishable, and thus microbial culture can be further transferred.

There are several challenges, encountered during isolation process. Other gliding bacteria, such as Cytophaga-like or flexibacters can also grow on the bait. However, normally, unlike myxobacteria, they are not able to swarm far away from the food source. Swarms of slime producing myxobacteria, like *Cystobacter* or *Archangium*, are often contaminated with undesired accompanying bacteria and can sometimes only be purified by performing numerous purification steps or by using antibiotics.

Isolation of cellulose decomposing myxobacteria is also based on the metabolic characteristics of this group. Basal medium ST21 agar is used, which has no carbon source, but contains NO_3^-, which is a perfect inorganic source of nitrogen. Cellulose, applied in form of sterile filter paper placed on agar, serves as selective substrate for cellulose-degraders from a sample.

Unless the samples come from extreme environments, 30 °C are typically used for incubation. Experiments with parallel cultures showed no strong quantitative difference in myxobacteria yield from typical soil samples, when incubated under room temperature and at 30 °C, while myxobacteria development happened faster at the higher temperature [26]. With the swarms method it is common that myxobacteria appear after 2-3 days, while cellulose degraders are rarely expected before 8-10 days of incubation.

2.1.5. Description of the strains used in this study

To study the effect of metals on myxobacterial growth and secondary metabolite metabolism, a number of type strains from each suborder (Cystobacterineae, Sorangiineae, Nannocystineae) was chosen, which covers the wide range of myxobacterial diversity. Representatives of different families of myxobacteria were selected (Table 1, section 3.2), based on the current phylogenetic tree of myxobacteria (Figure 2). More detailed description of the strains is given below.

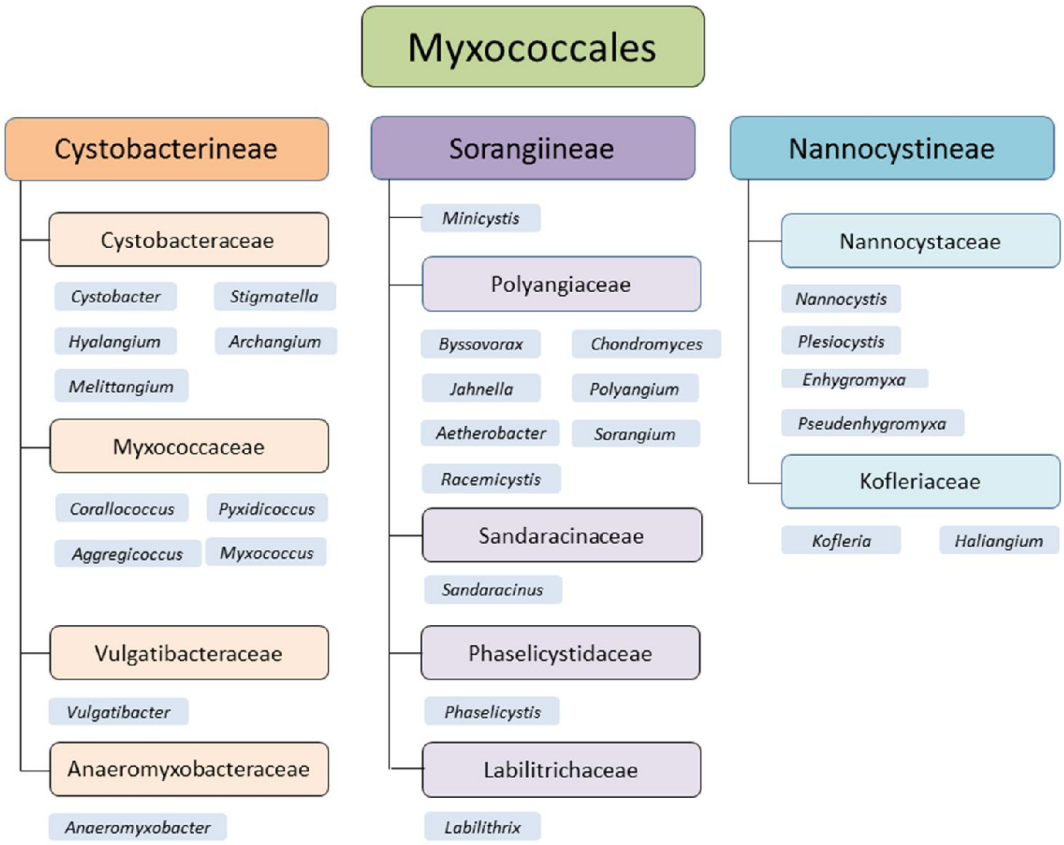


Figure 2. Phylogenetic tree of Myxococcales order (figure provided by Corinna Wolf, HZI)

*Labilithrix luteola (*DSM 27648) is a recently described species of myxobacteria, isolated from soil in Yakushima Island, Japan. Phylogenetic analysis based on 16S rRNA gene, supported by analysis of fatty acid composition, suggested that the new species can be attributed to a new family *Labilitrichaceae*, genus *Labilithrix*. The strain is capable of typical myxobacteria swarming, but has not demonstrated neither fruiting body formation nor microbial lysis or cellulose decomposition. Production of antimicrobial metabolites has not been reported. [30]

Kofleria flava (DSM 14601) is also a sole species in its genus and family. All strains of this species have been isolated from soil, dung, or bark, while metagenomic analysis of freshwater sediments showed their presence in this habitat as well. Fully formed fruiting bodies have not been identified. The species is capable of swarming and lysis of food organisms. It can also decompose starch and chitin, but not cellulose. No secondary metabolites with antibiotic activity have been identified so far, which might be due to still a small number of isolated strains [31].

Sandaracinus amylolyticus (DSM 53668) was isolated at HZI from soil sample originating from India. The strain has bacteriolytic type of nutrition, but can also degrade starch [32]. *S. amylolyticus* produces unique compounds - indiacen A and B, which are active against Gram-positive and Gram-negative bacteria, as well as against fungus *Mucor hiemalis* [33].

Cystobacter velatus (DSM 14718). The *Cystobacter* genus was first described already in 1886. Its representatives are common in soil and herbivore dung. They are bacteriolythic and able to hydrolyze glycogen, but not other carbohydrates [34]. A species of *Cystobacter* produces novel natural products, cystobactamides, with activity against Gram-negatives (*E. coli, P. aeruginosa*, and *A. baumannii*), as well as Gram-positive bacteria [35].

Nannocystis pusilla (DSM 14622). Bacteria of this genus have been isolated from dung, soil, lake mud and marine sand. They have bacteriolytic and non-cellulolytic nutrition [36]. Different strains of *N. pusilla* produce a number of bioactive secondary metabolites, such as: phenylnannolone A, which can reverse certain drug resistance in cancer cells, pyrronazol A (with weak antifungal activity) and 1,6-phenazine-diol (with weak antibacterial and antifungal activity), as well as nannozinones with antibacterial and antifungal activity [37‑39].

Myxococcus xanthus (DSM 16526) is one of the most well-studied myxobacteria species, which serves as model organism for the whole Myxococcales group. *Myxococcus* possesses all typical features of this order, like fruiting body formation, gliding and bacteriolysis. However, *Myxococcus* cannot utilize carbohydrates as energy source. Large number of *M. xanthus*-strains has been

isolated, many of them produce secondary metabolites like, for example, myxalamids, myxochromids, myxochelins, DKxanthenes, myxovirescin and cittilin, just to name a few [40]. In addition, *in-silico* analysis revealed *M. xanthus* to have a number of cryptic genes, whose products still have to be identified.

2.2. HEAVY METALS IN BACTERIAL METABOLISM

Prior to addressing the topic, a clear distinction should be made, which metals are included in the category of heavy metals. According to IUPAC, the term "heavy metal" has no formal universal definition and thus is often meaningless and misleading. Heavy metals can be defined according to their minimal density, atomic weight or even toxicity [41]. In the context of this work, metals with density of more than 5 g/cm^3 are attributed to heavy metals, which is a common practice in scientific literature [42].

Little is known about myxobacteria and heavy metals [43–45]. In this section a general overview of the current knowledge about bacteria and metals will be given. It has been well elucidated, that many metals, included in the "heavy metal" definition, have important biological role and are often defined as essential metals, while other elements do not take part in normal metabolism, and are referred to as nonessentials [46]. Naturally both, essential and nonessential heavy metals, have certain degree of toxicity at different concentrations. In this section first the roles of essential heavy metals in microbial metabolism will be discussed, followed by bacteria mechanisms of heavy metal tolerance. Finally, the studies are reviewed, where heavy metals were used for induction of sleeping genes.

2.2.1. Role of essential heavy metals in bacterial metabolism

Among all heavy metals **iron** is the most abundant one in cells of nearly all organisms. Its ability to readily alternate between Fe^{2+} and Fe^{3+} forms, as well as to take different spin states, made this metal crucial component of many essential enzymes, where it acts as a part of reaction center or as electron carrier. Iron is part of such essential cellular mechanisms as synthesis of deoxyribonucleotides (ribonucleotide reductase enzymes) and antioxidant defense (superoxide dismutase). Its most common metabolic form is heme, which is Fe^{2+} ion bound by organic ring. In this form iron serves as electron carrier in transmembrane proteins, involved in bacterial respiration [47]. Under aerobic conditions iron is poorly soluble, and in order to supply adequate amount of ions, specific iron-uptake mechanisms have been developed by bacteria, including myxobacteria, which produce order-specific compounds myxochelin [48].

After iron, **zinc** is the second most abundant transitional element in living cells [49]. It is a constituent of more than 200 known enzymes in prokaryotic and eukaryotic organisms. At the same time, it also serves as stabilizer of various macromolecules, membranes and transcription factors [50]. Myxobacteria-specific extracellular matrix-based metalloprotease *FibA,* which contains zinc, is believed to regulate motility and fruiting body formation of myxobacteria colonies [51].

One of the most prominent roles of **cobalt** in organisms, ranging from bacteria to mammals, is its role as central metal ion in corrin ring of B_{12} vitamin. As a coenzyme, this vitamin is involved in a vast amount of biochemical reactions [52]. There is a hypothesis that vitamin B_{12}, and particularly cobalt ion inside its structure, may be the reason for myxobacteria light sensitivity. By changing the oxidation state of cobalt, and thus properties of the coenzyme, light may trigger various processes (e.g. biosynthesis of carotenoids) in some myxobacteria [53]. In addition to $B_{12,}$ there is a number of other metalloproteins, which have cobalt as their constituent.

Currently more than 30 types of **copper**-containing proteins are known. Prominent example is cytochrome c oxidase, which acts as the last acceptor of electrons in respiratory chain of aerobic organisms [54].

On the other hand, **nickel** ions typically play important role in anaerobic metabolism. There have been identified operons, which are controlled by nickel-binding proteins. The operons are believed to regulate production of enzymes, responsible for anaerobic processes. In addition, a number of these enzymes also contain nickel as a component of active center [55]. No information was found about role of nickel in myxobacteria metabolism.

2.2.2. Mechanisms of heavy metal tolerance

Toxicity of both essential and non-essential heavy metals arises from their ability to inactivate enzymes, to act as antimetabolites, to produce toxic radicals and to form intracellular precipitates [56]. There are two general mechanisms by which heavy metals pass cell membranes. The first one is facilitated by non-specific transporters, which are driven by chemiosmotic gradient. The transporters act even in the cases, when a cell already has excess of a certain ion, which causes destructive processes inside the cell [50]. Another mechanism of metal transport is active only under certain conditions, when a cell lacks some metal for normal metabolism. This type of mechanism is usually slower, than the non-specific one, but it targets certain ion or a group of

ions. A big number of various specific transporters have been elucidated, which target all known essential metals [57].

Owing to the fact, that cells are permeable for metal ions via the non-specific transporters, two types of malignant scenarios are possible: 1) essential metal may reach supra-optimal concentrations and become toxic and 2) toxic non-essential metals may get inside the cell. There are five types of approaches, developed by microorganisms in order to prevent such scenarios [50]:

1. reducing uptake of metal by decreasing permeability of membrane,
2. efflux pumping of ions out of the cell,
3. intracellular sequestration of ions by binding with specific proteins (e.g. metallothioneins),
4. extracellular binding of ions,
5. transformation of metal's toxic form into non-toxic (e.g. changing the oxidation level).

A number of different protection mechanisms can be present in one species. Some microorganisms show extraordinary heavy metal tolerance attributed to several metal resistance clusters. These clusters are mostly coded in plasmids [58]. Some strains, isolated from mines have developed resistance as high as 30 mM to 40 mM to Ni^{2+}, 20 mM to 30 mM to Zn^{2+}, 5 mM to 10 mM to Co^{2+} and 5 mM to Cu^{2+} [59]. However, heavy metal tolerance of more "common" species is significantly lower. In that way, growth of *E. coli* is inhibited at 1mM of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and at 5 mM of Pb^{2+} [60].

In respect to myxobacteria, no inhibiting concentrations of metals or specific mechanisms of metal tolerance have been elucidated. However, some works provide information on myxobacteria and heavy metals interaction in other aspects:

In experiments with dry and wet biomass of *M. xanthus*, high efficiency in biosorption of lead, uranium and lanthanum was demonstrated. In both cases metals were mostly found in polysaccharide slime and at lesser degree in the cell walls. Dry biomass adsorption was 1.28 mM of lead/g and 2.4 mM of uranium/g [43 - 45]. In a similar study with saline myxobacterium *Myxococcus* sp.NUSTO3, adsorption value for copper was 0.36 mM of Cu^{2+} per gram of dry biomass, which was higher than for nickel (value not mentioned) [61].

Other data related to the possible mechanism of myxobacteria metal tolerance were obtained with bioinformatics tools. Full genome sequencing of *Myxococcus xanthus* DK1622 and its

consequent analysis have revealed presence of putative cobalt-zinc-cadmium efflux transporter encoding gene [51].

2.2.3. Heavy metals as inducers of metabolite production

As it was shown in the section 2.2.1, heavy metals can be part of transcription factors, as well as various other enzymes. According to the OSMAC approach (Figure 1), it means they have potential to be involved at all three stages of secondary metabolites production: at transcription, translation or at further biosynthesis. No exact mechanism of heavy metal – induced synthesis of antimicrobial metabolites has been described so far. However, it has been shown that biological function of many products of actinomycetes is to scavenge heavy metal ions from the environment [62]. For example, antimicrobial compound isatin, produced by *Streptomyces albus*, was found to act as chelating agent towards some trace elements [63]. Another example is gamma-actinorhodin, produced by *Streptomyces coelicolor*. The compound with antibiotic activity has shown ability to actively bound iron from the environment [64].

One extensive study has been conducted to investigate potential of heavy metals to induce production of antimicrobial metabolites in actinobacteria. Ten strains were isolated from metal contaminated and non-contaminated environments. The strains were cultivated in standard and in metal-supplemented (nickel or cadmium) media and their metabolite patterns were analyzed. The results have shown that some bioactive metabolites achieved higher concentrations in metal-supplemented media, and some were even solely produced under the influence of the metals. It was hypothesized that addition of metals to fermentation media of heavy metal – tolerant strains induces synthesis of metabolites, which are responsible for metal tolerance. At the same time this metabolites can demonstrate antimicrobial activity. [22]

In fact, exposure to elevated concentrations of heavy metals is not a rare approach to reveal concealed metabolites of a strain. Heavy metals are used routinely as a stress factor, along with other types of extreme treatment [19, 65].

2.3. POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is an indispensable technique in biological and medical research, forensics and clinical medicine. PCR is used to amplify across several orders of magnitude a selected portion of DNA sequence. The reaction is based on activity of an enzyme, triggered by repeated changes of temperature in solution with components, which define and facilitate the process. The first essential reagent of PCR is double-strand DNA template containing the region,

which needs to be amplified. Template is required only in low amounts, which can assure success of the first few cycles of the reaction. Another basic component are primers, namely oligonucleotides, which define the boundaries of the DNA's fragment, which needs to be amplified. The so-called forward primer should be complement to the 3' end of the fragment on the antisense-strand, while the backwards primer has to be complement to the 3' end on sense-strand. The primers define points, at which the third component, DNA polymerase, start to elongate the DNA strand. Typically thermostable polymerases with a temperature optimum at around 70° C are used. In order for DNA polymerase to start elongation, DNA's building blocks - deoxynucleoside triphosphates (dNTP) – are required as the fourth component. The rest of the PCR mixture is composed of buffer solution with optimal chemical environment for the polymerase and mono- and bivalent cations (typically K^+ and Mg^{2+}). Typically small reaction volumes in the range 10–200 µl are used [66].

For PCR cyclic alterations of temperature are required, which are facilitated by device called thermal cycler. This device ensures rapid change of temperature according to set program. Each temperature and time, during which it is applied, corresponds to different PCR steps. Normally there are initial and final steps with 20-40 repeated temperature cycles in between, each cycle consisting of 3 steps (Figure 3), [66].

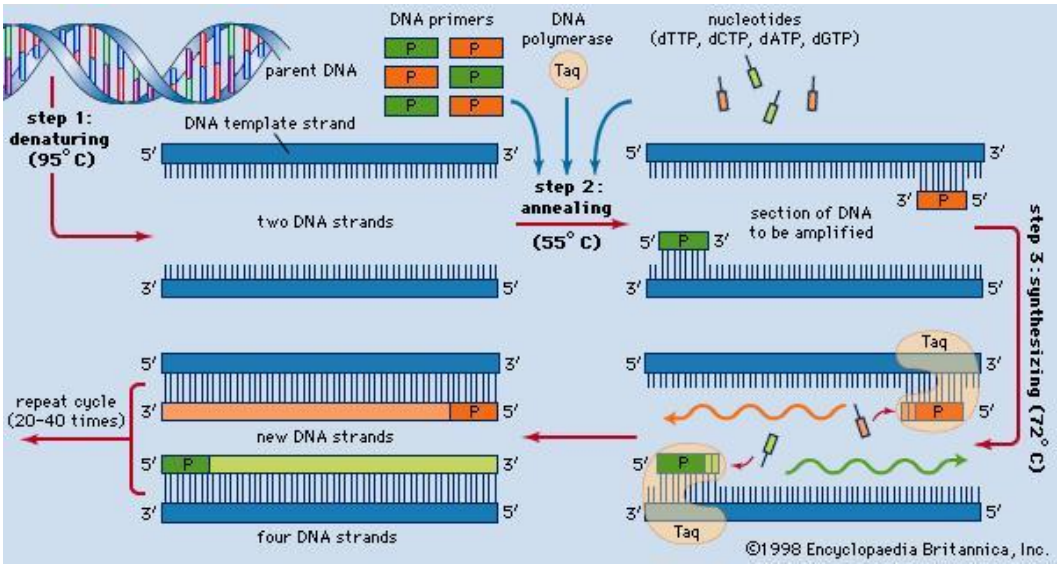


Figure 3 Schematic representation of PCR process. [67]

Initialization step is performed first, in order to activate polymerase at about 95 °C. The first regular cycle starts with denaturation step, at which hydrogen bonds of double-strand DNA brake, which results into a mixture of single strands. Temperature of 94 -98 °C is required for period of

20–30 seconds. The next step within the cycle is annealing, at which primers have to bind to single strands at correct locations. For correct annealing, the set temperature has to be low enough for binding, and high enough for specificity, so that only well-matching fragments (the primers with the corresponding DNA fragments) can bind together. The annealing temperature is normally within the range of 50–65 °C and depends on the length of the primers and their composition (GC-content). Every cycle is completed with an elongation step, at which polymerase adds dNTPs to the annealed primers. Added nucleotides are complementary to the template and are attached in 5' to 3' direction in respect to the template. The elongation is conducted at temperature, which is optimal for activity of the applied polymerase. The required time depends on the performance of the utilized polymerase and on the length of the amplified fragment (usually 1 kilobase/minute). After elongation the temperature cycle starts again with the denaturation step. Theoretically, with each new cycle the number of amplified fragments doubles, and thus follows exponential growth. Finally, when a set number of cycles was performed (usually 25-35), final elongation step is used to ensure that no single-strand fragments are left in the final mixture. Typically, when successful setting for the given primers, type of initial DNA template and for the polymerase are found, they do not have to be further adjusted [66]. Quality (correct length, single product) and quantity of PCR products can be checked by agarose gel electrophoresis in the presence of a standard ladder showing bands of known size and quantity.

2.4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) is a technique which can be used to separate components of a mixture, based on their affinity to adsorbent material, as well as to simultaneously identify and quantify them with the help of supplementary detectors. The basic principle of HPLC is the following: a sample mixture together with special solvent (called mobile phase) is pumped under pressure through a column, which is composed of adsorbent material (stationary phase). The flow rate of the components diverges owing to slightly different interaction with the stationary phase. In the end the separated compounds leave (or elude) chromatography column at different retention times. Upon leaving chromatography the compounds are either simply fractionated, or are, with the help of supplementary detectors, identified, quantified, further analyzed, or are subject to a combination of the above mentioned. Retention time and corresponding UV-chromatogram of a substance can be used for identification of known, characteristic substances when HPLC is conducted under strictly fixed conditions (buffer, gradient,

type of column). However, for an absolutely certain identification HPLC is usually not enough. For this purpose the extract has to be analyzed with HPLC-mass spectrometry [68].

There are several types of HPLC techniques, and their core difference is the nature of stationary and mobile phases used. For analysis of natural products typically so-called reversed-phase chromatography (RP-HPLC) is applied. It is characterized by a non-polar stationary phase and moderately polar aqueous mobile phase. Stationary phase is normally composed of modified silica with straight chain alkyl group. In this case natural products, which are usually non-polar compounds, have strong affinity to the silica and elute later than polar components of a mixture, while also separating from each other by the degree of their polarity. In case of reversed-phase chromatography the retention time of non-polar components can be adjusted by altering the composition (and thus polarity) of the mobile phase [68]. This is useful when the purpose of chromatography is fractionation of a mixture, which is one of the methods used for identification of the active compound among several metabolites.

2.4.1. Diode Array Detector

Diode array detector (DAD) is a detector, which is used to determine and quantify separate components in HPLC eluent, as well as to determine their light absorbance at different wavelength of UV spectrum. Schematically, DAD contains four crucial components: UV light source, flow cell, diffraction grating and photodiode array (Figure 4).

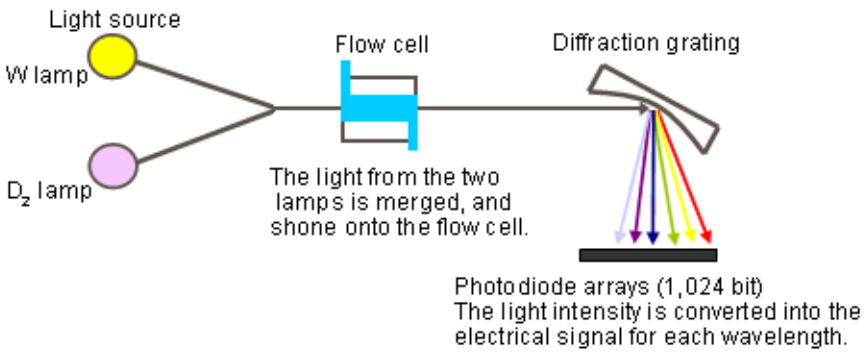


Figure 4. Schematic depiction of diode array detector (Hitachi High-Technologies Co.)

First, UV radiation source directs light via a slit through the flow cell, which hosts constant flow of eluent from the HPLC unit. A portion of the UV light is absorbed by the components of the flow. The light leaves the flow cell and reaches the diffraction grating, which disperses the beam into individual wavelengths. Finally, this spectrum of monochromatic light reaches an array of photodiodes. Each photodiode receives its own band of wavelengths and measures its intensity.

Depending on the used spectrum and the number of diodes, each diode collects data from 0.5 to 1 nm. The intensity, which reaches the diodes is compared with the reference intensity of the light source and the value, called absorbance unit (AU), is found for each band of wavelengths. Since both the flow and the detector's monitoring are constant, UV absorbance is recorded for the whole duration of HPLC run [69].

In the end of chromatography, graphs of absorbance versus retention time are constructed. The wavelengths typically chosen for the graphs are those, which are not absorbed by liquid phase and at the same time are suitable for detection of organic compounds. If absorption peak of the sought-for compound is already determined, this wavelength can be used for analysis. The resulting graphs are analyzed by software and the peaks of absorption at different wavelengths are recognized as distinct compounds.

The need for analyzing several wavelengths arises from the fact that the resulting peaks might be drastically different and some compounds might not be identified with certain wavelengths [70]. For example, Figure 5 shows two graphs for a HPLC run of one sample. Graph **A** shows absorption readings for wavelength 360±300 nm (basically the whole measured spectrum) and graph **B** for 210±8 nm (narrow range). As it can be seen, the compound 1 has higher absorption at specific 210 nm, than at the whole spectrum. Compounds 2 and 3, clearly seen on the graph B, are almost indistinguishable, when the absorption at the whole spectrum is analyzed (graph a).

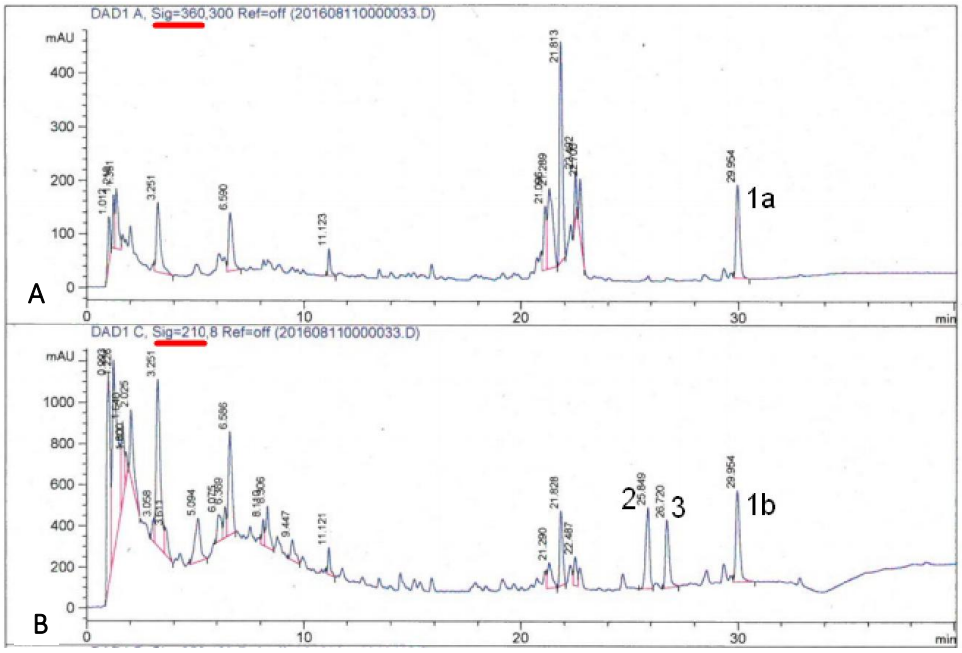


Figure 5. Graphs constructed for the same HPLC run with different wavelengths' absorptions

The calculated area below the peaks (designated as mAU*sec) is directly proportional to the amount of compound and is used for its quantification. But it has to keep in mind, that big peaks do not mean big quantities. Substances with ring systems, double-or triple-bounds, for example, show very good adsorption even if only little substance is present in an extract. On the other hand substances with low UV-activity like many peptides can produce small peaks, even if they are present in large amounts. To determine the exact amount of a compound, a standard-curve has to be established. This is done by analyzing different known concentrations of the pure substance (for example 1mg/ml, 500 µg/ml, 250 µg/ml and 100 µg/ml) and calculating the standard curve. Subsequently, the amount of the relevant compound can be determined in raw extracts, provided that the HPLC conditions are the same. However, under fixed HPLC conditions, the mAU*sec value of the same compound can still be compared and used to determine relative concentration difference between different raw samples [69].

In addition, for each identified separate compound a graph of light absorption (the example in Figure 6) is constructed across the whole measured spectrum. Together with the retention time, this graph can be used for identification of the compound, though these characteristics are not usually enough for identification of components from an unknown mixture. Just like the retention time, absorption pattern can alter with changes of sample's solvent, pH and temperature [70]. Thus, another parameter – mass – has to be determined with mass spectrometry detector.

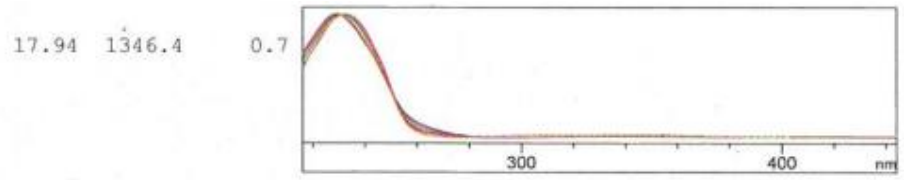


Figure 6 Example of a light absorption graph produced with DAD detector

2.4.2. Mass spectrometry detector

Mass spectrometry (MS) is a method, which allows the determination of molecular mass of an unknown compound. In case of organic molecules it also allows to elucidate the structure of the compound or to possibly identify the compound by comparing its mass spectrum to other known substances in a database [71].

In case if analyzed sample is a mixture of compounds, mass spectrometry is preceded by HPLC. Afterwards, separated compound is bombarded with electrons in order to obtain charged ions. By this process organic molecule can break into fragments, one of which always carries a positive

charge +1, and the others are neutral molecules. With some molecules of the compound, bombarding will not cause breakage of the structure, while still producing positive ions [71].

The resulting ions are transported further and accelerated by electric or magnetic field of fixed strength. After this step different MS methods use different approaches, which however share the same core idea. Molecules of the same charge but different mass will have different acceleration, when field of fixed strength is applied. With this acceleration ions eventually reach a detector, which either records the time of flight (TOF analyzer), or the trajectory of flight under magnetic field (sector MS). These parameters are then used to find the mass-to-charge (m/z) ratio of the corresponding ions. Since a number of different fragments is created by ionization, there can be a graph produced (Figure 7), which shows the rate of occurrence of different m/z values. The peak with the highest m/z value, also called molecular ion peak, corresponds to the not fractionated, whole compound itself. The occurrence of the other m/z values, which correspond to fragments of the original compound, can give idea about the structure of the latter and can be used as unique identifying characteristic [71].

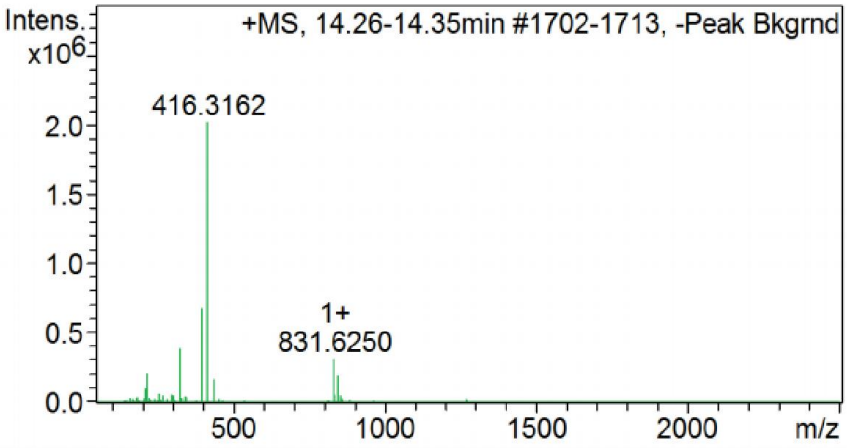


Figure 7. Example of a mass spectrum graph.

3. MATERIALS AND METHODS

This chapter describes the methods, which were applied during the practical part of the Bachelor thesis. For description purposes the chapter is divided into several parts. In the section 0 general aspects of synthetic media preparation are discussed. After that, acquisition of pure cultures of selected myxobacteria type strains is described in the section 3.2. Myxobacteria from contaminated soils were obtained by means of isolation and culture purification described in the section 3.3. These isolated wild strains were identified by extracting bacterial DNA and sequencing 16s RNA coding gene, which is described in the section 3.4. Both type strains from the collection and isolated strains from the soil samples were subject to metal tolerance tests (section 3.5). After that a number of strains, both from collection and from the set of newly isolated bacteria, were cultivated in media with addition of heavy metals, and the extracts, containing excreted secondary metabolites, were prepared (section 3.6). Finally, antibiotic activity of the extracts was tested and the effect of metals on secondary metabolites production was investigated (section 3.7). Flowchart of the work is given in the Figure 8.

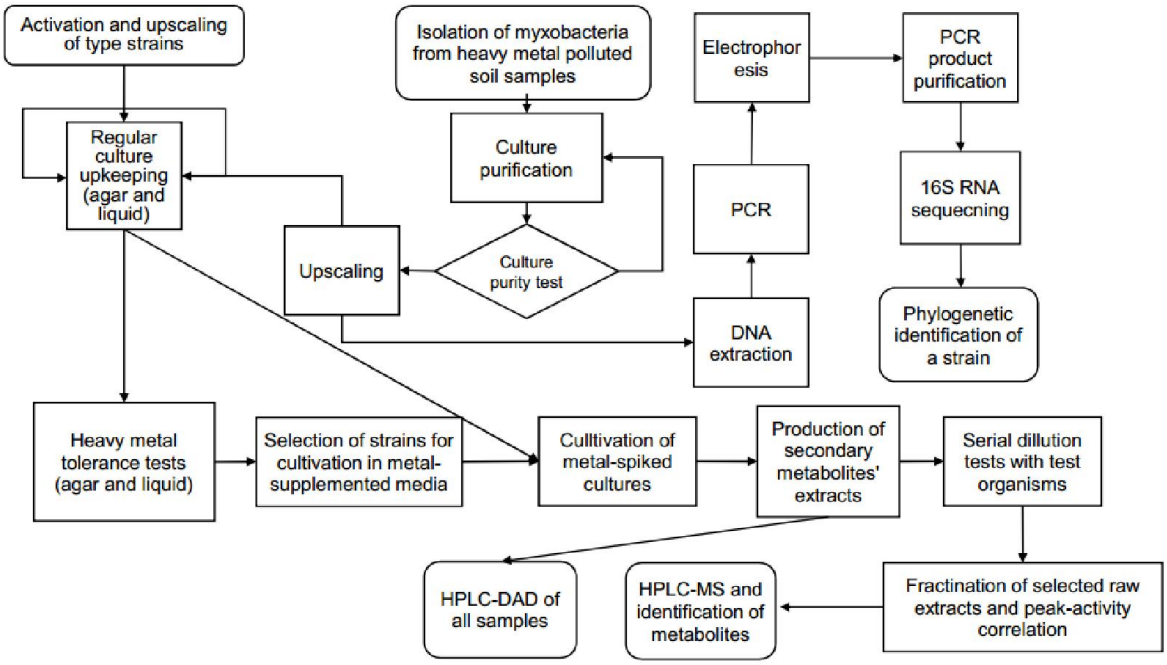


Figure 8 Flowchart of the practical part of the thesis

3.1. MEDIA PREPARATION

Working with myxobacteria required utilization of a number of cultivation media types at different stages. Their composition is given in the appendix. Deionized water was used for solution preparation. For different purposes either solid or liquid forms of the same media were used. In the most cases the only difference in composition between these two forms was addition of agar powder for media solidifying. For autoclaving media were exposed to 121°C for 20 minutes. Media were either prepared independently or provided by the laboratory staff of the MS department. In the first case detailed information about preparation procedures is given in the relevant chapters.

3.2. ACTIVATION OF COLLECTION STRAINS AND UPSCALING OF MICROBIAL CULTURE

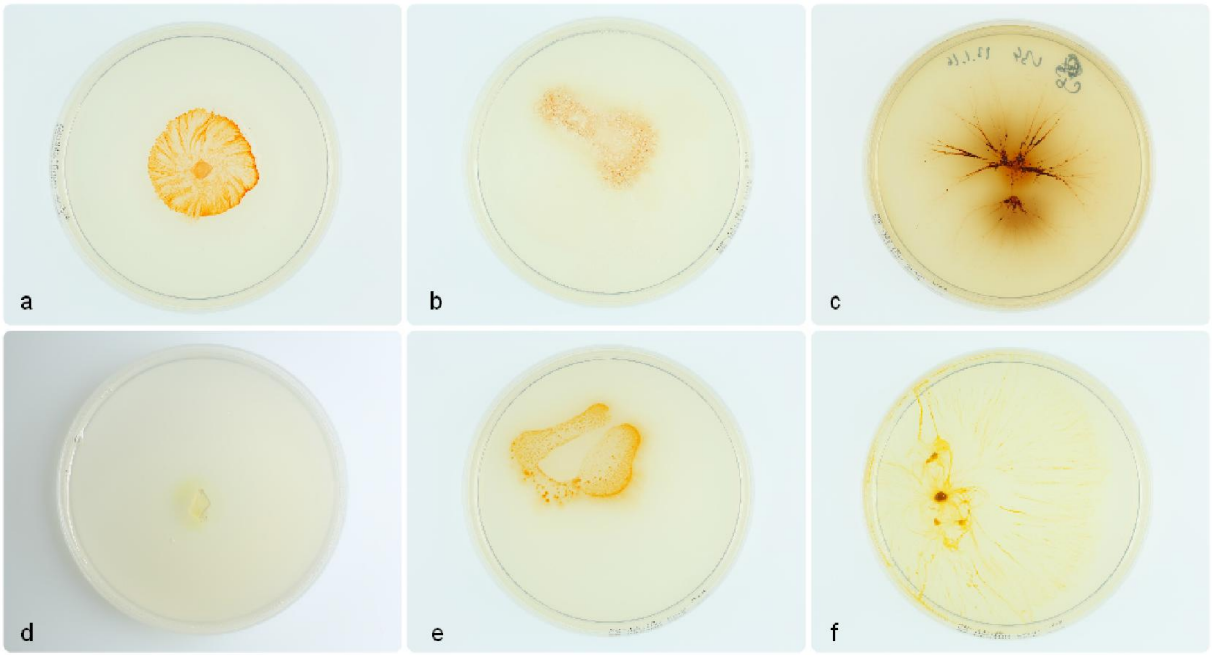
A number of type strains from each suborder (Cystobacterineae, Sorangiineae, Nannocystineae) was chosen (Table 1) to study the effect of metals on myxobacterial growth and production of antimicrobial compounds. The description of each chosen species is given in the section 2.1.5. The strains were obtained in the form of conserves, and then activated, upscaled and maintained. For the whole course of the laboratory work it was used as stock microbial culture for conduction of the experiments.

Table 1. List of myxobacteria strains, used for investigation of myxobacteria tolerance towards heavy metals and their influence on secondary metabolites production

Genus/species	Internal strain name	DSMZ number
Sorangium cellulosum	So ce1871	14627
Nannocystis pusilla	Na p29	14622
Cystobacter velatus	Cb v34	14718
Myxococcus xanthus		16526
Sandaracinus amylolyticus	No So4	53668
Kofleria flava	Pl vt1	14601
Labilithrix luteola		27648

3.2.1. Activation of strains from MD department internal collection

The set of type strains (Table 1) was taken from the internal collection of the working group MISG (Microbial Strain Collection), HZI Braunschweig. All strains are also available at the German Collection of Microorganisms and Cell Cultures (DSMZ). Reactivation of strains from conserves (from liquid medium stored at -80° C) was done by inoculation of 100 µl from the thawed conserve on agar plates (CY- and VY/2- media) (Picture 2), as well as inoculation of 1.8 ml in 20 ml liquid media CY+H (in 100ml flasks). Well-grown liquid culture was upscaled to 100 ml in CY+H media and was used as stock microbial culture for conducting the experiments. Both liquid and agar cultures were incubated at 30° C in an incubation room. The flasks with liquid culture were kept on a shaker with set rotation speed of 160 rpm.



Picture 2 Activated strains from MISG collection on agar culture: a) Sorangium cellulosum (VY/2 media), b) Nannocystis pusilla (VY/2 media), c) Cystobacter velatus (CY media), d) Myxococcus xanthus (VY/2 media), e) Sandaracinus amylolyticus (VY/2 media), f) Kofleria flava (CY media)

3.2.2. Maintenance of the microbial culture

Working with the type stains, as well as with the self-isolated ones, required regular renewal of the existing culture to prevent contamination and to assure constant presence of well-grown culture for the experiments. The renewal was performed weekly and was done by transferring 10 ml of the freshest culture into a new 250ml flask with 100ml of CY+H media. The transfer was performed with a 10ml pipet with truncated tip (wide opening XAD pipet) under sterile conditions. During the whole practical work, the previous culture was kept as a backup so that there were always two flasks from one strain: the freshest one and the backup, while the older stock cultures

where autoclaved. If contamination of working culture was suspected, its purity was controlled as described in the paragraph 3.3.3.

The microbial culture on agar plates was also renewed regularly by transforming a piece of overgrown agar onto the center of a new plate of the same medium (either CY or VY/2). The frequency of this procedure was dependent on individual growth rates of the specific bacteria.

3.3. ISOLATION OF MYXOBACTERIA FROM SOIL SAMPLES

In search of new myxobacteria strains, which could show adaptation towards high concentrations of heavy metals in the environment, soils with high metal content were examined. The soil samples were taken from the areas in Harz Mountains and in Hessen region, which, owing to mining activities taking place in the past, demonstrate elevated content of heavy metals [72]. The procedure of sample collection and their chemical analysis were performed by the Federal Institute for Geosciences and Natural Resources, Hannover. The samples were provided by the institute together with broad data about the origin of the samples, results of their pedological (sand, silt and clay content) and chemical (pH, organic carbon content and metal content) analysis. The list of the samples, the region of their origin, essential soil characteristics and the main metal contaminants for each sample are listed in the Table 2. The whole extensive information about the samples, as it was provided by the Federal Institute for Geosciences and Natural Resources, Hannover is given in the appendix (on CD).

Table 2. List of samples, used for isolation of myxobacteria

Sample ID	Region of origin	Soil organic carbon	pH_{water}	Sand content, %mass	Silt content, %mass	Clay content, %mass	Metals, present at elevated concentrations (not specified)
HS14/1	Hessen	6.01	5.47	51.6	34.4	14	Cr, Ni
HS3/1	Hessen	6.34	7.35	69.3	12.6	18.1	Zn
Ni-47-2	Lower Saxony	4.89	3.7	23	57.9	19.1	Pb
Ni-48-2	Lower Saxony	5.45	4.2	34.4	45.7	19.9	Pb
Ni-49-2	Lower Saxony	6.43	4.1	11	64.9	24.1	Pb
Ni-50-2	Lower Saxony	3.18	4	14	62.2	23.8	Pb
Ni-51-2	Lower Saxony	5.43	3.8	13	61.9	25.1	Pb
Ni-53-2	Lower Saxony	4.69	4.2	22.1	50.8	27.1	Pb
ST20/1	Saxony-Anhalt	2.78	6.23	15.5	48.9	35.6	Hg, Zn
ST23/1	Saxony-Anhalt	1.99	7.05	9.4	62.4	28.2	Hg
ST62/1	Saxony-Anhalt	2.01	6.03	19.7	60.6	19.7	As

The employed methods for myxobacteria isolation were the standard ones used in the MSC working group, HZI. The theoretical background of these methods is given in section 2.1.4. It is a process, which consists of initial culture isolation and consequent repetitive steps of purification. The final objective of the procedure is to obtain pure culture on agar plate and to consequently transform the culture into liquid media, while preserving microbial culture's purity.

3.3.1. Preparation of media used for myxobacteria isolation

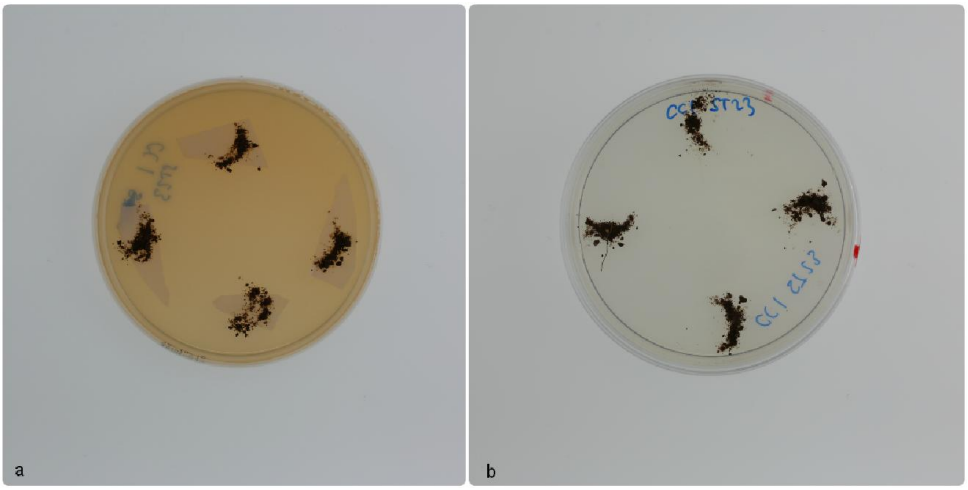
Two different types of media were employed for isolation: water agar with *Escherichia coli* crosses striped over the media's surface as bait for predators and Stan 21 media with cellulose paper filters on agar for the cellulose decomposers. Additionally, vitamin mixture solution, which promotes better growth of myxobacteria and gives advantage over other faster-growing bacteria was added. To minimize the growth of fungi and nematodes on the plate, fungicides cycloheximid and soraphen A, as well as anthelmintic levamisol were added.

First, water agar media was prepared, autoclaved and supplemented with the vitamins and antibiotics according to the recipe. The resulting mixture was poured into petri dishes before solidifying. In order to produce bait organisms, *E. coli* culture was pre-grown on EBS agar media. After that, crosses with living *E. coli* cell culture were drawn on the surface of each plate with wire loop under sterile conditions.

The Stan 21 medium was produced by preparing and autoclaving two different solutions separately (see the recipe in the appendix) and mixing them after autoclaving. Afterwards, the same set of vitamins and antibiotics was added and the petri dishes were filled with the media. Finally, four preliminarily autoclaved cellulose paper pieces were put with forceps on each plate.

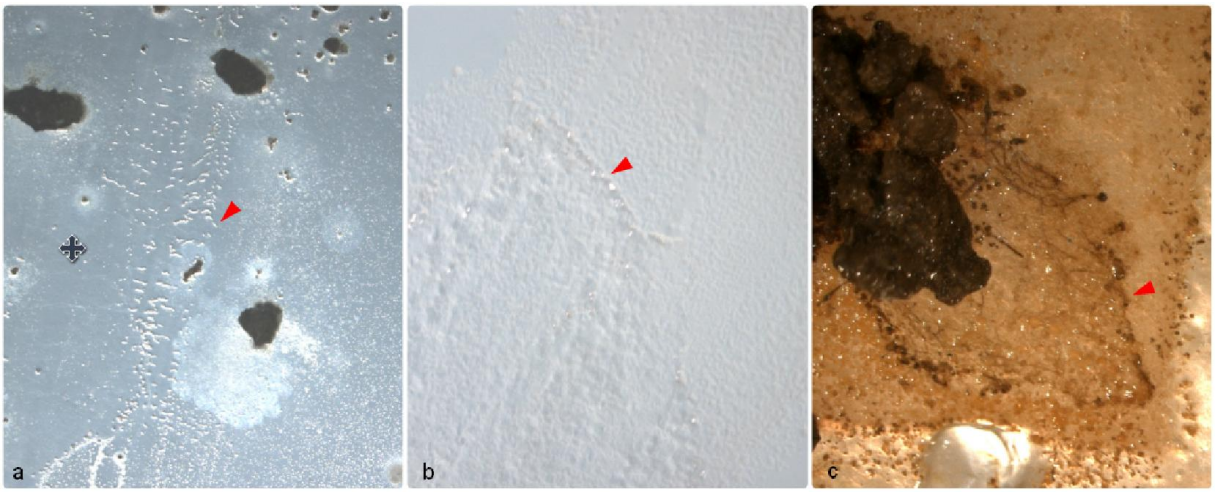
3.3.2. Isolation process

Isolation process itself started with transferring a small amount of soil with a spatula onto either the ends of the *E. coli* crosses, or onto the pieces of filter paper (Picture 3). In the first run two plates with modified water agar and two plates with Stan21 agar were inoculated for each soil sample from the list (Table 2). After inoculation, the plates with soil samples were stored at 30° C inside a plastic box in an incubator.



Picture 3. Start of the isolation process. a) Stan 21 agar with soil sample ST23-1 on cellulose paper. b) Water agar with E. coli cross and soil sample ST23-1

Approximately seven day after the inoculation the plates were examined for myxobacterial growth with an Olympus binocular SZX10. Myxobacterial growth could be observed as fruiting body development (Picture 4a), myxobacterial swarming (Picture 4b) or as degradation of cellulose (Picture 4b and c).

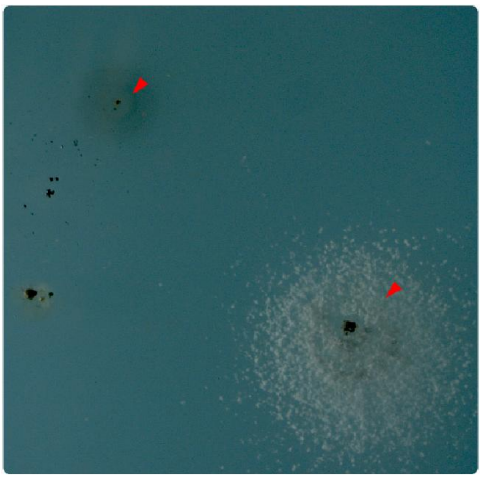


Picture 4. Typical signs of myxobacteria growth. a) Formation of fruting bodies of Corallococcus coraloides (ST23-1 sample) b) Colony growth by "swarming" and agar degradation of an Unidentified strain (Ni-49-2 sample) c) Cellolose and agar degradation by an Unidentified strain (HS3-1 sample)

In case of myxobacterial growth, either a piece of agar, a single fruiting body or a piece of cellulose paper with myxobacterial cell mass was transformed with a sterilized needle onto the center of an *E. coli* cross on a new antibiotics-containing water agar plate. The new so-called purification plate was incubated under the same conditions as the original.

Those raw plates with soil samples, which showed no signs of myxobacterial growth at first examination, were further incubated and checked in the same manner regularly 1-2 times a week. If no myxobacteria were identified on the plates with soil sample after the period of over 2 months, the plates were discarded and new plates were inoculated with the same sample.

Each myxobacteria culture, which was isolated from the soil samples, was purified in a series of purification steps. Material from swarm edge or fruiting bodies was transferred to a fresh water agar plate (*E. coli*) 2-3 times a week. The older plates were kept as a backup. Water agar with fungicides was used as long as fungal growth was observable (Picture 5). As soon as the transformed culture was free of fungal growth, water agar plates without fungicides were employed.



Picture 5. Fungi colonies on water agar media.

When the cultures appeared free of contaminations, pieces from the very swarm edge was transformed onto full medium agar plates (VY/2). At myxobacterial swarm edges the possibility is high that associated contaminants were outpaced. If the observed cultures seemed to be free of contamination after 3-4 days, overgrown pieces of agar were transformed into 100 ml flasks containing 20 ml CY/H medium. The liquid cultures of new isolated strains were incubated under the same conditions as described for the type strains from internal collection (paragraph 3.2.1). After the lapse of 4-5 days the purity of well grown cultures were checked with the methods described in the section 3.3.3 and further upscaled to 100 ml.

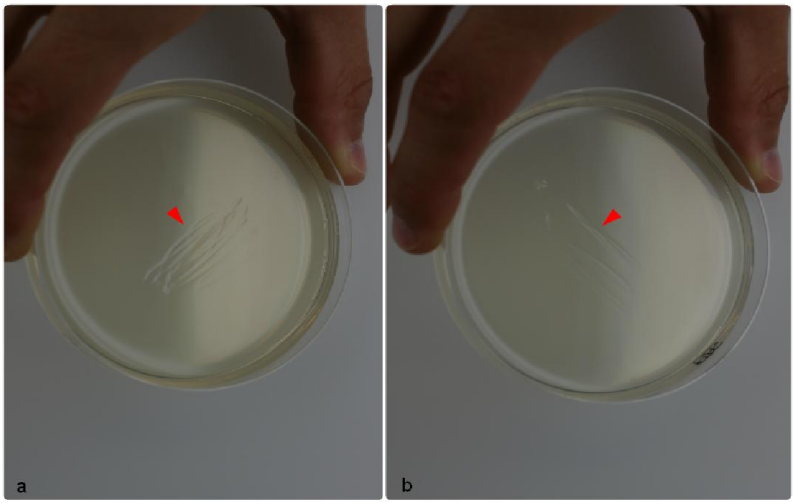
When the purity of the culture was proven, conserves were made in order to preserve the strain. For each newly isolated strain 3 x 1.8 ml of cell culture with cell mass was transferred into 2ml Eppendorf Safe-Lock tubes and were cryo-preserved at -80 °C.

3.3.3. Purity testing of cultures

A pure microbial culture is a population, emerged from one single cell. It is a prerequisite for all microbial experiments. In the case of the highly "social" myxobacteria, cultivation of colonies which emerged from one single cell is not possible for most species. Pureness of these bacteria has to be ensured by checking morphological characters of a colony on agar plate as well as via microscopic observation.

For the microscopic observation an inoculation loop was used to transfer small volume of media from the examined flask onto microscopy slide, which was then covered with a cover slip. The culture was observed with Zeiss standard optical microscope under 400x optical magnification. Myxobacteria are rod shaped and are not able to move actively in liquid. Also, older cells are shorter and convert to round optically refractive myxospores. If with microscopic observation no contamination was noticed, the culture was used for further processing. The contaminated cultures were discarded and the purification was repeated.

In order to more safely determine pureness of the culture, inoculation of selective media was used, according to [73]. These media were MYC for determination of fungi presence and EBS for revealing other faster growing bacteria. Agar plates with these media were inoculated by striping small volume of liquid culture across the plate with a wire loop. The plates were then incubated for 1-2 days at 30 °C. Myxobacteria are not able to grow on this media within a few days, so that in case of microbial growth the culture was considered contaminated. If finally no growth was noticed, the culture was assumed to be pure (Picture 6).



Picture 6. Checking purity of myxobacteria culture after 24 hours incubation on a) MYC media (for fungi) and b) EBS (for bacteria). The tested culture is isolated CC1 from ST23-1 soil sample

3.4. IDENTIFICATION OF THE ISOLATED STRAINS

Identification of the newly isolated strains was done by sequencing a part of the 16S rRNA gene. First, genomic DNA was isolated and PCR with primers, matching nearly the whole 16S rRNA gene was performed. Quality and quantity of PCR products were checked using gel electrophoresis. The PCR products were purified with a commercial kit and the first part of the 16S rRNA gene (up to 800 bp) was sequenced using three different primers. The sequences were compared to those available in the public database NCBI (National Center for Biotechnology Information).

3.4.1. DNA isolation

In order to obtain the genomic DNA of the isolated strain 1ml of well-grown culture was transferred into a safe-lock tube. The biomass was concentrated by centrifugation at 10000 rpm for 1 minute. The supernatant was discarded with a pipette and the pellet was used for DNA extraction using the "Invisorb Spin Plant Mini Kit" by Invitek.

The first step was the disruption of bacterial cells by addition of 100 µl lysisbuffer P to the tube and briefly vortexing the mixture. Then the tube was incubated at 95°C in a shaker for one minute for enzymatic and thermal cell disruption. The second step was separation of DNA from other biochemical components of the cell mass. Therefore, 300 µl lysisbuffer P and 20 µl proteinase K* were added to the sample. The resulting mixture was incubated for 30 minutes at 65°C on a shaker. During this time proteinase K* was acting as both exo- and endopeptidase for chromosomal proteins.

Tube filter, called PreFilter, was put into a 2 ml Receiver Tube (provided in the kit) and the incubated solution was poured inside. The locked tube was centrifuged for 1 min at 12 000 rpm and the PreFilter was discarded. Afterwards 20 µl of RNase A-solution was added to the filtrate, briefly vortexed and left at room temperature for 5 minutes. During this time the enzyme was splitting the single-strand RNA's. Consequently 200 µl of Binding Buffer was added to the tube and vortexed again.

SpinFilter, provided by the manufacturer was put into a new Receiver Tube, and the solution, resulted from the previous steps was poured inside the filter. The tube was then incubated for 1 minute and centrifuged at 12000 rpm for 1 minute. The DNA from the sample was detained on the filter. The filtrate was discarded and the filter was put again into the Receiver Tube.

Then 550 µl of Washing Buffer I was added into the filter, which was then centrifuged at 12000 rpm for 1 minute. The filtrate was dismissed and the same procedure was repeated with Washing

Buffer II. After removing the resulting flow-through, the filter was put back into the tube and centrifuged again for 2 minutes in order to get rid of the remaining washing buffer. Finally SpinFilter with bound DNA was transferred into a new 1.5 ml Receiver Tube and 30 µl of pre-warmed Elution Buffer D was added. The incubation period comprised 3 minutes, after which the tube was centrifuged at 10 000 rpm for 1 minute. As a result of this procedure the DNA extract was concentrated on the bottom of the Receiver Tube, while the filter could be discarded. The tubes with DNA solution were stored at 4°C

3.4.2. PCR

The purpose of PCR application was to amplify a specific region, namely a part of 16S rRNA gene. Further sequencing of this gene is a common approach for genus and often even specie identification. The primers used to amplify the DNA fragment were universal primers F27 and R1525, whose nucleotide sequences are given in the Table 5. The primers match *E. coli* 16sRNA gene positions 27 and 1525, and are suitable for most bacterial orders. Successful application of these primers in PCR gives DNA sequence of about 1498 base pairs (bp) [74].

Standard PCR procedure, as described in the section 2.3, was performed. All required reagents of PCR, except for DNA sample, primers and water, were supplied as the components of the Sigma-Aldrich "JumpStartReadyMix" (JSRM). PCRs were conducted in volumes of 50 µl per reaction with the composition as in the Table 3. First, a "master mix" of all components, required for the chosen number of PCRs, except genomic DNA, was created. Example composition of master mix for 5 samples is also given in the Table 3. The "master mix" was then distributed in portions of 49 µl in 200µl tubes. After that 1 µl of sample DNAs were added to the PCR tubes. In each run of PCR reaction one negative control tube with 1 µl sterile PCR water instead of genomic DNA was used to ensure that the "master mix" was not contaminated with external DNA.

Table 3. Composition of PCR mixture and example calculation of master mix.

	One PCR reaction(µl)	Master mix (6 reactions including 1 negative control
JSRM	25	150
Sterile water	22	132
Forward primer	1	6
Reverse primer	1	6
Sample DNA (or PCR water for negative control	1	
Total	50	294

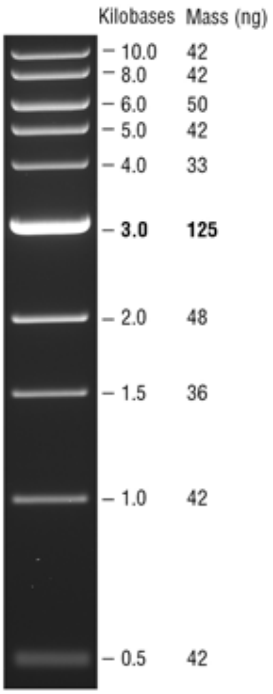
The PCRs were performed in thermal cycler "Matercycler gradient" by Eppendorf. Standard settings, suitable for the given primers, commonly used in the Microbial drugs department, HZI, were applied. The reaction consisted of initialization, 34 cycles and final elongation. The corresponding temperatures, as well as the duration of each step are summarized in the Table 4. After the PCR, the products were stored at 4 °C and later checked by gel electrophoresis.

Table 4. Summary of the used PCR program settings

		Duration, min	Operational temperature, °C
	Initialization	5	95
34 cycles	Denaturation	0.5	94
	Annealing	0.5	52
	Elongation	2	72
	Final elongation	10	72
	Final hold	Until the samples are withdrawn	10

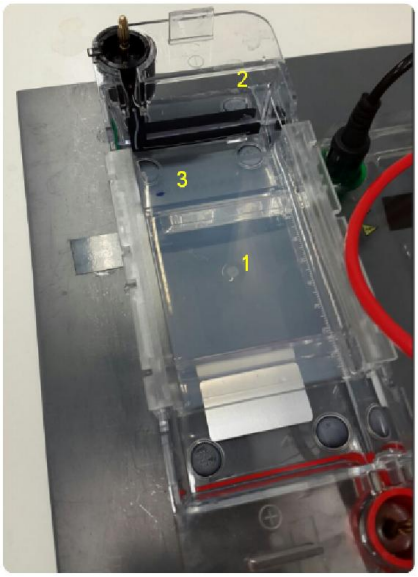
3.4.3. Electrophoresis

Electrophoresis was used for quality and quantity check of the PCR products. It was performed twice: first, after the PCR reaction, and the second time after purification of the products (section 3.4.4). To check, whether PCR products have the expected quality (correct size and proper bands), a standard ladder (New England Biolabs 1 kb DNA Ladder) was also loaded on the gel. This ladder is designed to indicate bands corresponding to the 0.5-10.0 kilobases (Picture 7). For electrophoresis both running buffer and the agarose gel had to be prepared first. TAE (Tris-Acetat-EDTA) buffer was used as the running buffer as well as for preparation of agarose gel. The recipe of TAE can be found in the attachments.



Picture 7. Bands of 1 kb DNA Ladder by New England Biolabs. Mass values are given for the case of 0.5 µg/gel lane loading.

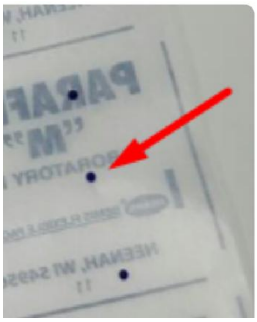
Agarose gel for electrophoresis was prepared with mass concentration of agarose powder of 0.8% by dissolving the required amount in TAE buffer. Complete dissolving of the powder was achieved by heating the suspension up to the boiling point several times. The prepared solution was used immediately for electrophoresis procedure. Gel mold was fixed in a press frame and leveled off. The agarose solution was poured inside of it and a comb for 15 chambers was put into the frame to form vertical wells. After approximately 30 minutes the gel solidified and was transferred into the electrophoresis apparatus (PowerPac 300 by Biorad). The device was filled with TAE buffer up to the point when the gel was completely submerged. After that, the comb was removed carefully from the gel and the device was ready to be loaded with DNA samples (Picture 8).



Picture 8. Photo of partly loaded gel inside electrophoresis apparatus. 1. - Agarose gel; 2. – Frame of the electrophoresis apparatus; 3. – Loading wells in the gel, formed by the comb.

Before electrophoresis, all samples, negative control and ladders had to be mixed with the loading dye (Gel Loading Dye, Purple by New England Biolabs). First, 2 µl of the dye were dropped with 5 µl pipette on a piece of waxed paper, one droplet for each potentially loaded well (see Picture 9). Then 2 µl of either DNA solution, negative control or ladder were picked with a pipette, released onto one of the droplets and mixed together by pipetting the mixture up and down. After that the same pipet was immediately used to uptake the whole volume and transfer it into one of the wells inside the gel. The same procedure was done with the other samples and with the ladders. For loading of every well a new tip was used.

After all the samples and two ladders were loaded, the rack was closed with a lid and the electrophoresis was launched. The current of 400mA and 80 V was applied for 60 minutes. After the lapse of the set time the electricity supply was turned off, the lid was removed and the gel was put inside a UV cabinet. The picture of the gel with bands corresponding to dyed PCR products was taken by a build-in camera under ultraviolet radiation.



Picture 9. Preparation of loading dye mixture with samples.

3.4.4. PCR product purification

For PCR solution with amplified DNA fragments suitable for sequencing, all the contaminants and leftover molecules had to be removed. Among the components subject to removal are salts and enzymes from the JSRM solution. In order to clean the samples from the mentioned contaminants, commercial NucleoSpin® Extract II kit by Clontech Laboratories was used. The basic principle behind the kit functioning was binding DNA molecules to the silica membrane of the provided filters, followed by washing out unwanted components, and completed with final elution of uncontaminated DNA fragments.

All the procedures described further were done under non-sterile conditions. In the beginning, required chemical conditions for DNA binding were created by mixing one volume of a sample (48 µl) with two volumes (96 µl) of Buffer NT from the kit.

Meanwhile, the provided NucleoSpin® Extract II column was put inside a 2 ml collection tube. The mixture of sample and Buffer NT was loaded inside the column and the tube was centrifuged for 1 minute at 11000 rpm. The flow-through was dismissed and the column was put back into the tube. As a result, the DNA fragments were bound to the tube's silica membrane.

After that the contaminants were washed out from the membrane, while DNA fragments were preserved. To do so 700 µl of preliminary prepared Buffer NT3 from the kit were put inside the NucleoSpin® Extract II column and the whole tube was again centrifuged at 11000rpm for 1 minute. The resulting liquid at the bottom of the tube was disposed and the column was put back into the tube. To dry the membrane the tube was again centrifuged for 2 minutes at 11000 rpm and the flow-through was disposed. In order to completely eliminate one of the NT3 Buffer components – ethanol, from the membrane, the latter was incubated for 5 min at 70 °C on a shaker.

The final step of the protocol was elution of the DNA components. The silica filter with bound DNA fragments was put into a new 1.5ml tube. Then 30 µl of Buffer NE from the kit were poured into the column and left for 1 minute at room temperature. Finally the tube was centrifuged at 11000 rpm for 1 minute. As a result, purified fragments of DNA were concentrated in the flow-through solution inside the tube. This solution was further checked with electrophoresis (section 3.4.3) and prepared for sequencing (section 3.4.5).

3.4.5. Sequencing and phylogenetic analysis

For sequencing 16S rRNA genes of the isolated strains, Sanger method was used, which was performed by the sequencing service group of HZI. The method requires providing starting points for the reaction in form of primers. Thus, sequencing was performed with one forward and two reverse primers matching at *E. coli*-positions F27, R518, and R1100 (Table 5). Each of the three single sequences gives information about approximately 500-600 nucleotides in the corresponding direction. The final consensus sequence largely consists of reliable information from one forward and two backward sequences from different points, and can reach lengths of around 1000 bp. The schematic diagram of the used primers and the resting sequences in respect to the sense strand of DNA is shown in the Figure 9.

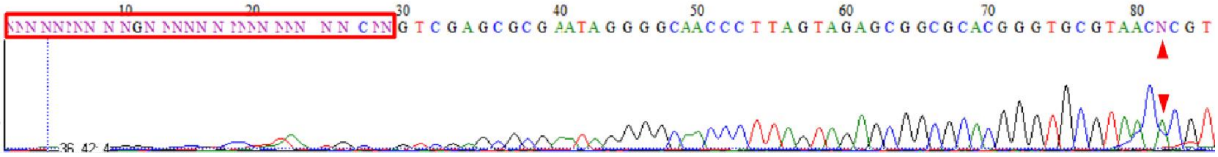
Figure 9. Schematic representation of the 16S RNA gene sequencing with three different primers.

Table 5. List of primers used in PCR reaction with references

Primer	Sequence (5‘-3‘)	References
16F27	AGAGTTTGATCMTGGCTCA	[74]
16S1100R	GGG TTG CGC TCG TTG	[75]
16R518	GTATTACCGCGGCTGCTG G	[74]
16R1525	AAGGAGGTGWTCCARC	[74]

In order to meet the technical requirements of the sequencing device, the selected primers had to be diluted. For each primer, 8 µl of its stock solution (10 pmol/l) were mixed with 42 µl of so-called PCR water, which is free of DNA contaminants. For each isolated bacteria, 10 µl solution of amplified and purified 16sRNA coding gene fragments were required to perform sequencing. Together with the prepared primers' solutions, they were submitted to the HZI sequencing service.

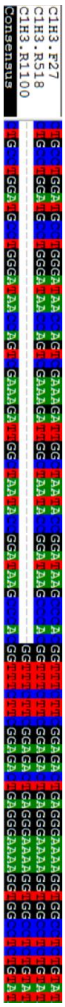
The resulting raw data (three sequences per culture) was provided as chromatogram files in the .ab1 format. They displayed the chromatography peaks of the four different nucleotides and the corresponding most probable nucleotide types (Picture 10). When probable nucleotide could not be assigned by the program owing to low quality of the data, "N" sign was shown. The files were processed with BioEdit v.7.2.5 program (free available in the Internet).



Picture 10. Chromatogram of DNA sequence displayed by BioEdit program. Bases 0-29 and 82 are selected.

In order to prepare the sequences from one sample for alignment, the regions with low quality of data had to be cut off manually. In the shown example (Picture 10), the bases 0-29 had to be removed, since the chromatographic peaks are not distinguishable in this fragment. Base 82 also cannot be identified by the program, but a clear "A"-peak is visible and thus can be assigned manually. The regions with low quality of data were mostly in the beginning and at the end of the sequence.

After all three partial sequences were edited, the reverse sequences, which basically corresponded to antisense strands, had to be transformed into sense strand. This was done by using build-in tool in BioEdit program, called "Reverse complement". After R518 and R1100 sequences were reversed, the whole set was ready for alignment.



Alignment was done by ClustalW Multiple alignment algorithm implementation in BioEdit. As a result of running the algorithm, all three sequences were spaced in respect to each other so, that the matching fragments were spatially corresponding to each other. The resulting consensus strand (Picture 11) was saved as fasta-file and used for searching the next relative strains using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A list of strains with the highest degree of similarity was given as the result of performing the search (Table 6). "Raw" sequences produced with three primers, as well as consensus sequences for each strain are given in the appendix (on CD).

Table 6. List of sequences, which produce significant alignments with the sequence from the strain CC1 (ST23-1 sample). Corresponding microorganisms and the degree of alignment are given in the columns.

Description	Max score	Total score	Query cover	E value	Ident
Corallococcus coralloides 16S rRNA gene, strain DSM 52500	1901	1901	100%	0.0	99%
Corallococcus coralloides 16S rRNA gene, strain DSM 52501	1895	1895	100%	0.0	99%
Corallococcus coralloides 16S rRNA gene, strain DSM 51625	1895	1895	100%	0.0	99%
Corallococcus coralloides 16S rRNA gene, strain DSM 51619	1895	1895	100%	0.0	99%
Myxococcus coralloides 16S rRNA gene, strain Cc c494	1884	1884	100%	0.0	99%
Corallococcus coralloides 16S rRNA gene, strain DSM 51643	1868	1868	100%	0.0	99%
Corallococcus coralloides 16S rRNA gene, strain DSM 51639	1868	1868	100%	0.0	99%

Picture 11. Processed and aligned sequences with consensus sequence.

CC1 strain from ST23-1 soil sample.

3.5. METAL TOLERANCE TESTS

It has not been found in the literature, which concentrations of different heavy metals exert inhibiting effect on myxobacteria. The purpose of the conducted tests was to fill this knowledge gap. Both different strains from the collection (Table 1), representing different suborders, and some self- isolated strains (Table 12) were tested. More specifically, the experiments had the following targets:

- to get a general overview about the tolerance of myxobacteria against various concentrations (0.25, 0.75, 1, 5 or 10 mM) of different heavy metals both on solid agar and in liquid media (VY/2 and CY), as well as to compare the growth of metal spiked and non-spiked cultures of the same strain

- to find out if myxobacteria, which were isolated from heavy metal contaminated soils, show different tolerances against metals in comparison to the strains without previous intense exposure to these substances;
- to use this information for designing secondary metabolite production experiments with and without metal exposure during the production phase (section 3.6)

Two different types of media, VY/2 and CY, were used. In general, both media types are sufficient for myxobacteria nutrition, while VY/2 agar fosters faster propagation of colonies on agar plates [26]. On the other hand, CY broth media has an advantage of having better transparency due to suspension of its components, and thus is a perfect media for performing liquid tests.

3.5.1. Preparation of stock solutions of metal salts

In order to prepare both solid and liquid metal-supplemented media, stock solutions of these metals were used. For each metal 50 ml of 1 M solution was prepared by dissolving its hydrate salt in water. Dissolving was aided by heating up the solution and intensive vortexing. The list of metals, chosen for performance of the tests, as well as the corresponding (mostly hydrate) salts and their amounts, used for preparing the stock solutions, are shown in the Table 7.

Table 7. List of metal cations for testing, their salts and corresponding amounts, used for stock solution preparation.

Metal ion	Compound used for solution	Molecular mass, g/mol	Amount of compound used for 50 ml solution, g
Pb^{2+}	$(CH_3COO)_2Pb\ 3H_2O$	379.3	18.96
Fe^{3+}	$FeCl_3\ 6H_2O$	270.3	13.52
Co^{2+}	$CoCl_2\ 6H_2O$	237.93	11.9
Cu^{2+}	$CuCl_2\ 2\ H_2O$	170.48	8.52
Ni^{2+}	$NiSO_4\ 6H_2O$	262.84	13.14
Zn^{2+}	$ZnCl_2$	136.28	6.81

3.5.2. Measuring pH of metal-supplemented media

Before preparation of metal-enriched media for the experiments, effect of the stock solutions on pH of the resulting broth was tested. First, pH of both media (VY/2 and CY) was adjusted according to the recipe. Then the media were distributed to six 100ml beakers. The amount of stock metal solution, which was needed to prepare 1 mM, 5 mM and 10 mM solutions was calculated according to the Formula 1 and comprised 100 µl, 500 µl and 1 ml correspondingly. Thus, for each

metal salt, first 100 µl were added and the resulting pH was measured. Then another 400 µl and 500 µl were added in order to check the pH at 5 mM and 10 mM concentrations correspondingly. Changes of pH at salt concentrations of 0.25 mM and 0.75 mM were not tested owing to their negligibility.

3.5.3. Metal tolerance tests on solid media

Metal tolerance tests on solid media were used as a relatively fast method to initially determine the ranges of metal concentrations, at which myxobacteria were able to grow. It was used instead of minimal inhibiting concentration or similar tests, since it was more approximate for slow-growing myxobacteria. In addition, it provided overview of possible growth variations, caused by metal supplements, which could either foster or hinder microbial colony's growth.

$$V_{stock\ solution} = \frac{V_{medium} \times C_{molar,\ metal\ in\ medum}}{C_{molar,\ stock\ solution}}$$

Formula 1. Calculation of stock solution amount for preparation of metal-supplemented medium.

Solid media preparation

Two types of agar media were prepared for the tests, namely CY and VY/2, and were subject to the initial pH adjustment. Glass bottles with volume of 1 liter were filled with 500 ml of media. The required amounts of metal stock solutions were added in order to achieve the metal concentrations, which were chosen for testing. These amounts are shown in the Table 8. Media without addition of metal was also prepared at the same time.

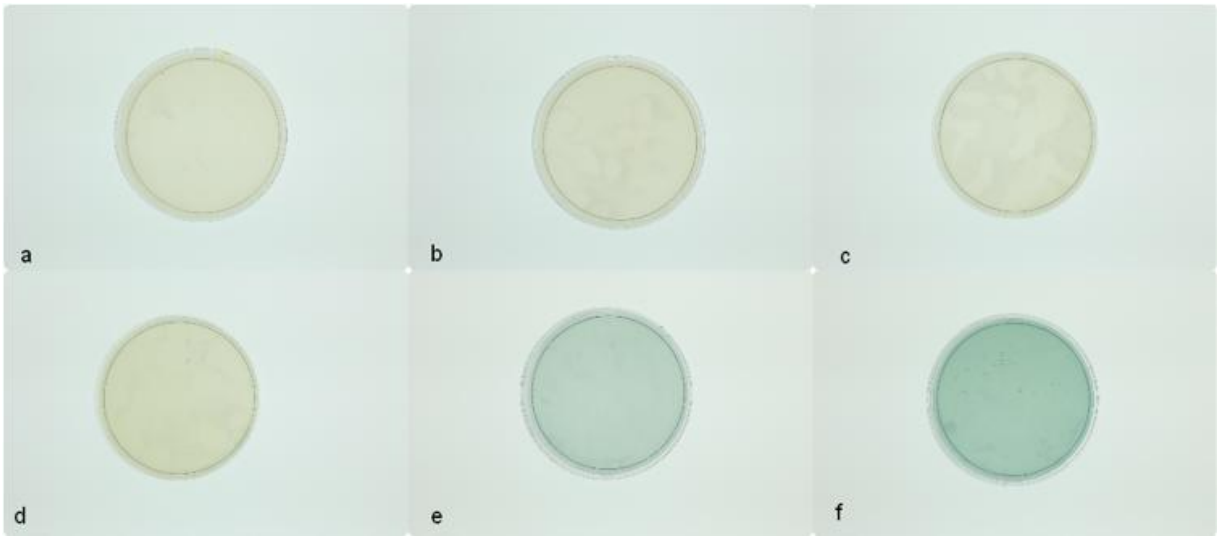
Table 8. Amounts of stock solution required to produce corresponding metal concentrations.

Concentration, mM	Amount of stock solution added to 500ml media, ml
0.25	0.125
0.75	0.375
1	0.5
5	2.5
10	5

After addition of metal salts to the media, pH of some solutions was re-adjusted, based on the results of the pH measurements (see appendix). The necessity of secondary adjustment of pH

raised in cases, when addition of stock solution would shift the initially set medium's pH below the point of 6.8 and thus affect the standard growth conditions of myxobacteria.

Finally, bottles with media were autoclaved. After autoclaving, vitamins were added according to the recipe. Directly before distributing the media to petri dishes, the bottles were thoroughly mixed by magnetic stirrer. Afterwards, about 20 ml of media with different metal concentrations (including zero-concentration) was poured manually into 100 ml petri dishes. Outer surface of agar plates were marked with a colored bar code to provide information about the media, the supplemented metal (if any) and its concentration. After solidifying, the agar plates were stored at 4 °C.



Picture 12. Not inoculated VY/2 agar plates with different $CuCl_2$ concentrations: a) 0 mM (control plate), b) 0.25 mM, c) 0.75 mM, d) 1 mM, e) 5 mM, f) 10 mM

Inoculation and conduction of the experiment

Prior to the start of the experiments, the bacteria, chosen for testing, were grown on agar plates in order to obtain enough microbial culture for the experiments. For each strain a total of four plates of CY and VY/2 agar were inoculated with myxobacteria from regularly renewed agar plates (see section 3.2.2). A small piece of overgrown agar was placed upside down in the middle of a new plate. These pre-culture agar plates were incubated at 30 °C for 1 to 2 weeks until the plates were overgrown.

For inoculation, the edges of bacterial swarms on the agar pre-cultures were cut into equally sized squares (Picture 13a). This was done to obtain pieces with equal amount of actively growing bacteria culture and because cell population tends to migrate towards outer regions of a swarm. The squares were then used for inoculation of CY and VY/2 agar plates with various concentrations

of different metals (0 mM, 1 mM, 5 mM and 10 mM in the first run for the type strains and 0 mM, 0.25 mM and 0.75 mM in the second run for both type strains and the self-isolated strains). Plates were inoculated with the pieces of pre-culture from the same media as the test plates. Each combination of media, metal and its concentration for each strain were done in duplicate. Media without metals supplement were inoculated in both runs of the experiment to serve both as a reference and as a control plate. For example, to test tolerance of *S. amylolyticus* (NOSo4) towards iron, the experiment design shown in the Figure 10 would be used.

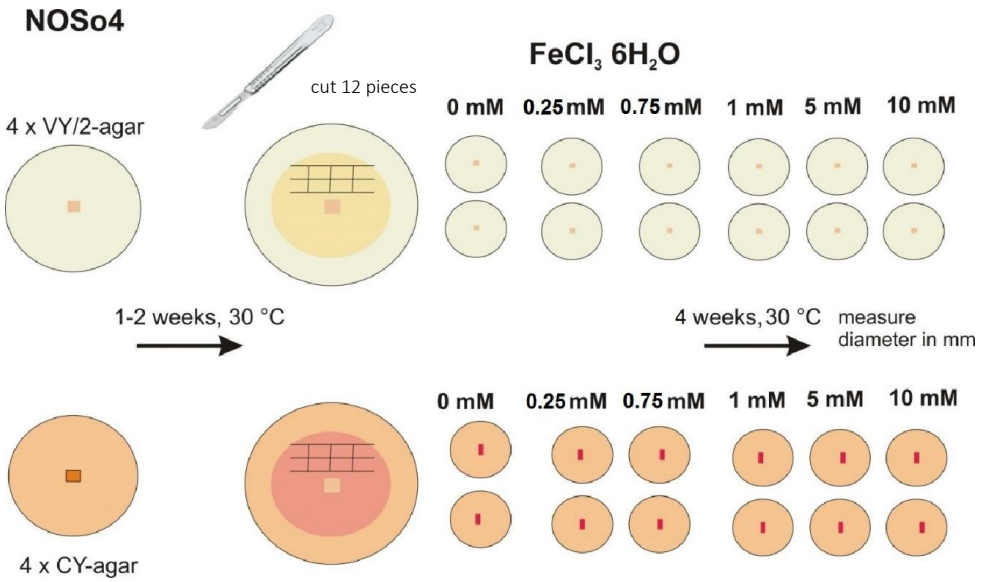
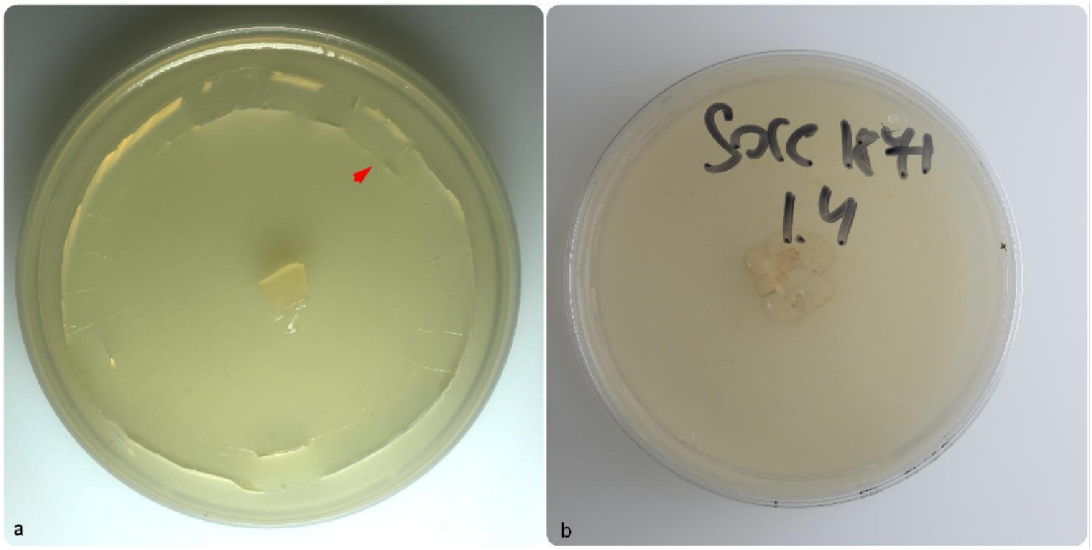


Figure 10. Experiment design for testing tolerance of S. amylolyticus towards iron



Picture 13.a) Equally-sized squares of pre-culture used for inoculation (L.luteola on VY/2 agar). b) Growth of S.cellulosum colony during the heavy metals tolerance test (0.75 mM Pb VY/2 agar)

The bacteria were incubated for a total period of one month after the start of each experiment. Weekly the growth diameters of cultures were noted by measuring the swarm of the myxobacteria

(Picture 13b) with a ruler. In case of non-circular shape of the swarm, the distance between the most remote edges was measured. In case if control plates showed no growth, the experiment was repeated using new pre-cultures. In case if light contamination on the surface of a plate was observed, the plate's results were still recorded, as long as the contamination did not inhibit the myxobacterial swarm.

3.5.4. Metal tolerance tests in liquid media

Evaluation of myxobacteria ability to grow in metal-enriched environments was also tested in liquid media in order to determine possible differences in comparison to the growth on solid media. Additionally, these results were used to select candidates for the external metabolite production tests. While the seven strains from the collection (Table 1) were tested for heavy metal tolerance in broth and on agar plates, the self-isolated stains were only tested on solid media, due to the restrictions of the given timeframe.

Media preparation

For conduction of metal tolerance tests in broth, the same two types of media were chosen as for the tests on agar plates, namely CY and VY/2. In order to test all the strains with one metal in both media, at least 560 ml of each media type was made. Prepared media was subject to initial pH adjustment. Then these 560 ml were spread equally among 4 beakers, resulting in 140 ml medium in each beaker. In three beakers metal concentrations of 1 mM, 5 mM and 10 mM were created by adding the metal stock solution. The required amount of the stock solution was calculated using the Formula 1. The forth beaker was left without metal supplement. After addition of metal salts, pH of the media was adjusted again, if necessary.

Finally, the media from all of the beakers were equally distributed in preliminary marked 100 ml flasks, in portions of 20 ml. The flasks were then autoclaved and stored at 4 °C until the start of the experiment. For one metal a total of 56 flasks was prepared for testing seven strains with four different metal concentrations (0, 1, 5 and 10 mM) in two media. In the first run, Pb^{2+}, Fe^{3+} and Co^{2+} cations were tested (168 flasks), followed by Cu^{2+}, Ni^{2+} and Zn^{2+} in the second run of the experiment.

Inoculation and conduction of the experiment

Before the experiment 100ml of pre-culture of each tested myxobacterial strain was grown in CY and VY/2 media. Inoculation was done with 10 ml of test strain from the regularly renewed stock cultures (section 3.2.2) and incubated until sufficient cell mass and density was reached.

Before the experiment with each metal, the flasks were taken from the cool storage. To avoid temperature shock for bacteria, cooled flasks were left for at least one hour at room temperature before inoculation. For each combination of "metal-media-strain" four different concentrations of 1 mM, 5 mM, 10 mM and 0 mM were tested, with the latter serving as positive control and as a reference for growth assessment. First, vitamin B_{12} and vitamin solution (Schlegel; 1 ml/l) were added. Then inoculation was performed by adding 2 ml of well-grown pre-culture with tip-cutted XAD-pipette (because of clumpy cell growth) under sterile conditions. The flasks were incubated for one week at 30 °C at 180 rpm. Model experiment design for testing tolerance of *S. amylolyticus* towards iron in liquid media is shown in Figure 11.

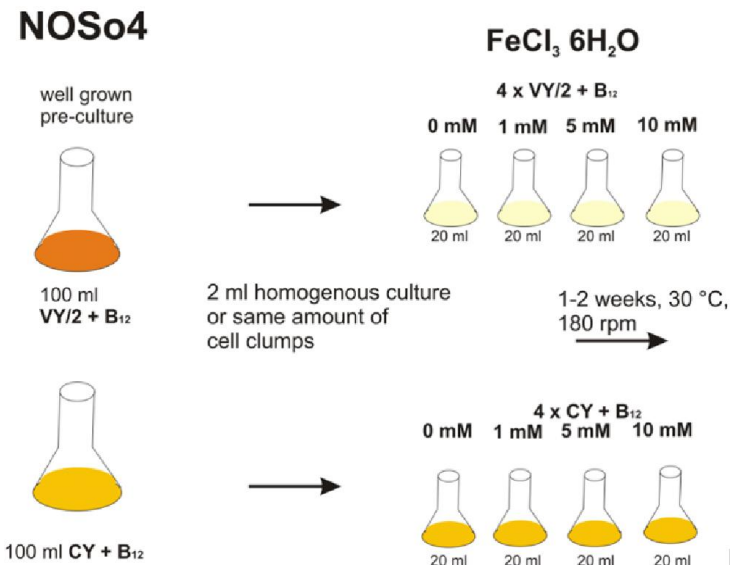


Figure 11 Example of experiment design for testing metal tolerance in liquid media

Assessment of growth

After the lapse of seven days the growth of metal spiked and non-spiked cultures was assessed (Picture 14). Since myxobacteria in liquid medium tend to form biomass agglomerates and clarify the medium during the growth, such standard techniques for culture density assessment, as optical density measurement and cell counting would not give reliable data about the growth of the myxobacteria. For this reason own empirical visual system for growth assessment was used.

Picture 14. Comparing the growth of Nannocystis pusilla strain in VY/2 medium with 4 different concentrations of $(CH_3COO)_2Pb$. (10mM, 5mM, 1mM and 0mM from left to right). The growth in the control flask (0mM concentration) was assessed with grade 3 and the growth in flask with 1mM – with grade 2. In other flasks no growth could be detected visually or with a microscope.

Grades from 0 to 4 were given to describe the growth of myxobacteria in the test flasks with different metal supplements. The grade 3 was corresponding to the growth in flasks without metal supplementation and served as a reference. If visually assessed growth was better in one control medium (CY or VY/2 with 0mM concentration) compared to the other, or in metal-supplemented medium, the grade 3 was given to the flask with the worse growth, and the grade 4 - to the flask with the better growth.

In case of no growth (determined via visual assessment and microscopic observations), grade 0 was given. In case if no growth was observed visually, but could be determined with microscopic observations, the initial flask was used to inoculate (with 2 ml) a new flask of the same medium without metal. After the lapse of one week, this new flask was examined. If growth could be visually observed, then slight growth (0.5 grade) under the initial metal and media conditions was noted. The list of the used grades with short description is summarized in the Table 9.

Table 9. List of grades used to assess microbial growth in liquid media.

Grade	Description
0	Growth seen neither with naked eyes nor under microscope
0.5	Growth visible under microscope in the original flask and after re-inoculating control media growth was also visible with naked eyes
1	Minimal growth visible without microscope
2	Significant growth, which is however worse than in reference flask
3	Reference growth grade, similar to reference flask
4	Growth is better than in the reference flask

3.6. SECONDARY METABOLITE PRODUCTION EXPERIMENTS AND EXTRACT PREPARATION

One of the core targets of this Bachelor thesis was to discover the influence of elevated heavy metal concentrations on production of secondary metabolites of myxobacteria. For this purpose those bacteria from the collection (Table 1) and self-isolated strains (Table 12) were chosen, which showed relatively high tolerance towards certain metals. The selection was done based on the results of solid and liquid media tests.

For the experiment the myxobacteria were cultivated in media with various concentrations of the selected metals, as well as in media without the metals, which served as a reference and were also used for secondary metabolite production. Two different concentrations were selected for each of the tested metals (except lead, for which only one concentration was tested) to observe the effect of increasing metal content on secondary metabolism. In case of cobalt, zinc and copper concentrations were used, which were lower than those, determined to be tolerable. This was done in order to minimize the inhibiting effect of the metals on bacteria's growth and to allow better comparison of the productivity between two concentrations and with the reference media. The selected metal-tolerant strains with the corresponding metals and tested concentrations are given in Table 17. All flasks in the experiment were supplemented with Amberlite® XAD-16 hydrophobic adsorber resin by Sigma-Aldrich. This cross-linked polystyrene copolymer is typically used for binding soluble organic compounds from liquids [76], and is also employed in the MD department, HZI for extraction of bacterial secondary metabolites.

This chapter describes the conduction of the experiments, as well as the methods, used to obtain media extracts with metabolites. Analytical techniques, applied to the extracts, are described in section 3.7.

3.6.1. Media preparation

CY and Pol medium were used for cultivation of the selected strains. VY/2 liquid medium was not used for this experiment, because it is sufficient for cultivation of most myxobacteria, but usually unsuitable for secondary metabolite production. CY is also not a perfect medium for this purpose, however, it was used for better comparability with the previous heavy metal tolerance tests. On the other hand, Pol is a medium, which is known to be adequate for metabolite production for many species of myxobacteria.

The media were prepared in beakers according to the recipes and the pH was adjusted. The amount of required initial media of one type (CY or Pol) for one strain was calculated according to the Formula 2.

$$V_{total,one\ media\ type} = N_{metals\ tested\ with\ the\ strain} \times N_{tested\ concentrations\ pro\ metal} \times 100\frac{ml}{flask} + 100\,ml_{reference\ media}$$

Formula 2. Calculation of media amount, required for secondary metabolite production experiment with one strain

This medium was distributed among the flasks in equal portions of 100 ml. In every flask the required amount of metal stock solution, calculated with the Formula 1, was added. Because of low resulting concentrations of the salts, only pH of the media with addition of $FeCl_3$ $6H_2O$ (both 5 mM and 10 mM) had to be re-adjusted. The flasks were then filled with 2 ml of XAD resin solution and autoclaved.

3.6.2. Inoculation and incubation

Preliminary grown pre-cultures were used for inoculation of the test flasks. For each strain, which was chosen for metabolite analysis, one flask of each medium, CY and Pol, was first inoculated with stock microbial culture, and then left for incubation for approximately a week.

Before inoculation, vitamin B_{12} and vitamin solution (Schlegel) were added to both CY and Pol flasks according to the recipe. Finally, 7 ml of the pre-culture were transferred with XAD-pipet in the experiment flasks. Inoculation was conducted according to the experiment design, summarized in Table 17 and shown in Figure 12. Cultivation of one strain started on the same day with all different metals and concentrations. This was done to archive quasi-synchronous microbial culture for the course of the experiment.

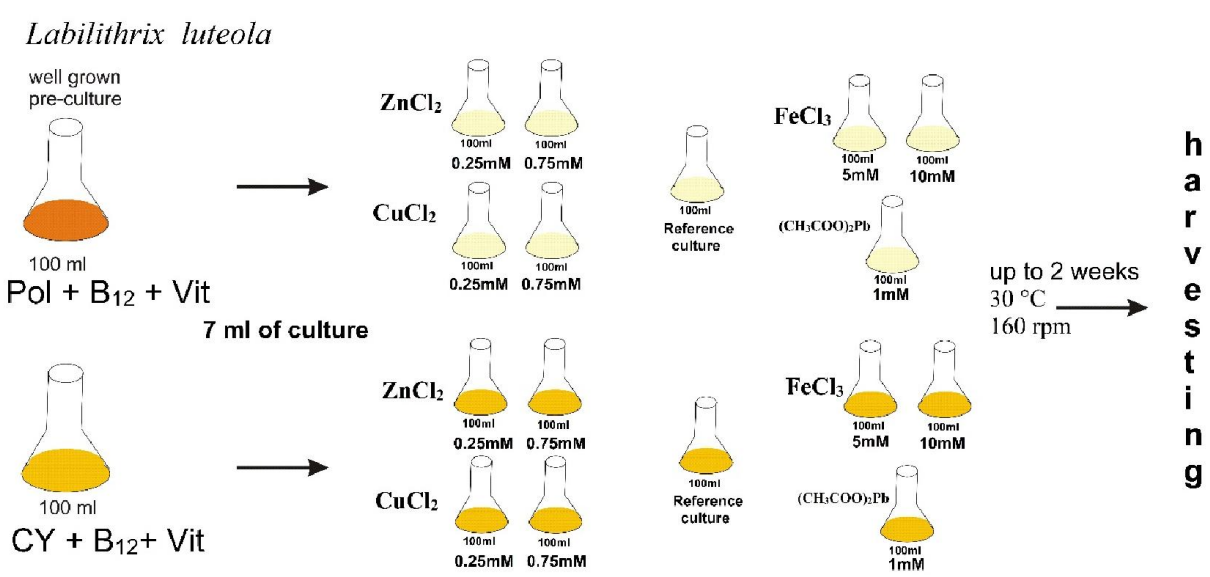


Figure 12. Model design of secondary metabolites production test for L. luteola.

The inoculated flasks were incubated at 30 °C (160 rpm) for up to two weeks. The incubation period varied across different strains. However, the metabolites of one strain were harvested strictly on the same day. Harvesting was performed when the slowest growing culture of one approach reached near-stationary phase, observed by microscopic observations.

3.6.3. Harvesting and extraction of metabolites

First, the microbiological culture was sieved with 200 µm sieve, separating XAD resin from the medium. Inside the sieve the resin was thoroughly flushed with deionized water to get rid of microbial biomass. With a spatula the XAD resin was transferred back into the flask and approximately 70 ml of acetone were added. The flasks were incubated at room temperature for at least one hour in order to allow metabolites get into the solvent.

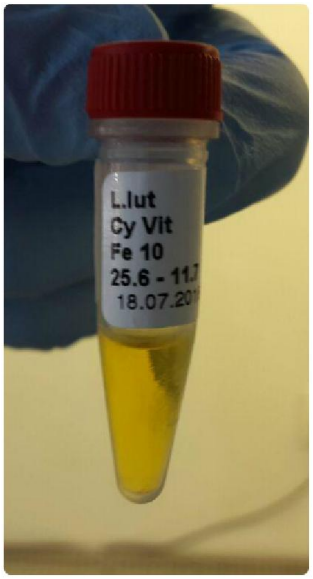
3.6.4. Rotary evaporator

The next step after harvesting the metabolite adsorbing resin and re-solving the metabolites in acetone was to increase the concentration of metabolites in the final extract. The relative concentration of an extract, typically used for testing unknown samples, is 100:1 in respect to the initial volume of media. This means that for 100 ml of microbial culture, used for metabolite production, extract with volume of 1 ml had to be produced. Methanol was used as solvent in the final extract.

To produce the extract, a rotary evaporator with a water bath (Heidolph Instruments Laborota 4003) (Picture 15) was used. This device allows evaporating acetone under constantly decreasing pressure at high rates, while keeping the conditions mild, in order to preserve the metabolites.

Picture 15.Rotary evaporator with vacuum system (1), water bath (2), condenser column (3) and rotation unit (4)

After incubation, the acetone solution with XAD was filtered through paper filter with pore diameter 13 µm (Carl Roth, Type 600P cellulose) into a round-bottom glass flask to separate the resin and the remaining microbial biomass from the solvent. The flask was then fixed at the evaporator, the water bath temperature was set to 40 °C and the pressure was decreased steadily to avoid boiling of the acetone and thus loss of the metabolites. After all acetone in the flask was evaporated under vacuum, atmospheric pressure was reestablished in the flask and it was detached. 1ml of methanol was added into the flask and the solid sediments at the inner surface of the flask were dissolved. Finally, the methanol raw extract was collected with a pipette and transferred into a 1.5 ml safe-lock tube (Picture 16). The final extract was kept at -20 °C in order to increase the stability of potentially sensitive and unstable secondary metabolites.



Picture 16. Methanol raw extract from L. luteola, cultivated for 14 days in CY media with 10 mM concentration of $FeCl_3$

3.7. ANALYSIS OF SECONDARY METABOLITE PRODUCTION

A number of tests were performed with the raw extracts, the purpose of which was to find and identify possibly new natural products with antimicrobial activity, as well as to see the influence of metals on composition of secondary metabolite profiles.

The analysis of the raw extracts consisted of serial dilution tests in 96 well plates against different bacterial and fungal test organisms, fractionation of active extracts with subsequent peak-activity correlation by HPLC and elucidation of molecular masses of active or dominant substances by HPLC-MS. Identification of interesting substances was performed by comparing the detected masses with data from the internal myxobacterial database “Myxobase”,

which includes all known substances, derivatives, masses and UV-spectra of secondary metabolites produced by myxobacteria (> 800). HPLC-MS measurement and subsequent data analysis was performed by Sabrina Karwehl and Cäcilia Schwager from the MD department.

Comparison of productivity of the same strain under different metal concentrations was done by analyzing the extracts by HPLC with diode array detector and comparing the metabolites' peaks as described in the section 2.4.1.

3.7.1. Serial dilution test

In order to check raw extracts for presence of metabolites with antibiotic activities, serial dilution tests (SDT) were performed. The principle of the test is based on performing a series of dilutions of the raw extract with microbial culture of different test organisms. If SDT is performed with a pure substance of known concentration, for example 1mg/ml, a minimal inhibition concentration (MIC) value can be determined. This value indicates the minimal concentration of a bioactive compound, at which no microbial growth is visible with bare eyes or measurable with a plate reader. Since neither quantitative nor qualitative composition of the extracts is known, no MIC value can be determined. However, the serial dilution test performed with raw extracts show, whether the contained microbial metabolites inhibit the chosen test organisms.

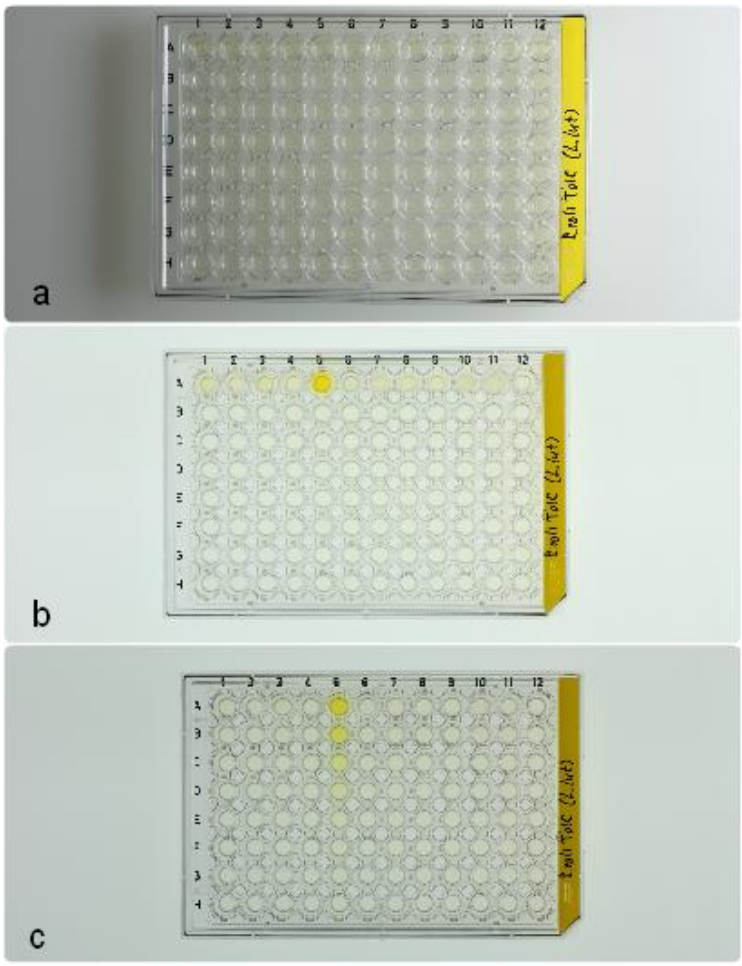
Bioassay plates (by Techno Plastic Products AG) with 8 rows and 12 columns, in total 96 wells, were used to perform series of dilutions. On each plate one strain was tested with eleven extracts, which had decreasing concentrations from the top to the bottom of a column.

As test organisms, the following species were chosen: Gram-negative bacteria, Gram-positive bacteria and fungi (yeast and filamentous). The set of organisms, used for SDT tests, is shown in Table 10 together with a short description of the microorganism and the specific strain.

Table 10. List of test organisms in SDTs with short descriptions

Type of microorganism	Name	Short description
Gram-negative bacteria	*Escherichia coli* DSM1116	Non-pathogenic strain of *E.coli* [77]
	E. coli TolC	
	Chromobacterium violaceum DSM30191	Rare human pathogen, causing skin lesions, sepsis, and liver abscesses [78]
	Pseudomonas aeruginosa PA16	Pathogenic in animals and plants. Human opportunistic pathogen. The strain is a human clinical isolate. [79]
Gram-positive bacteria	*Staphylococcus aureus* Newman	Opportunistic pathogen of human skin and respiratory tract, prone to multiple resistance. The most common cause of postoperative wound infections. [7]. The strain was isolated from human infection. [80]
	Micrococcus luteus DSM1790	Actinobacteria, normally nonpathogenic [81]
	Bacillus subtilis DSM 10	Normally present in soil. Also found in human gut flora. [82]
Fungi	*Mucor hiemalis* DSM 2656	Emerging mycosis pathogen. [83]
	Pichia anomala DSM6766	Yeast form, opportunistic pathogen in immunocompromised hosts. [84]
	Candida albicans DSM1665	Present in yeast or filamentous form. One of the most common opportunistic yeast pathogens among humans. [85, 86]

First, aliquots of bacterial culture, taken from cryogenic storage at -80 °C, were used for inoculation of appropriate media. MYC media were used for cultivation of fungi and Müller-Hinton for bacteria, correspondingly. Approximately 20 ml of media had to be inoculated with the test-train to prepare enough culture for one 96 well plate. The amount of aliquot, used for inoculation, varied between different test-organisms. The exact amount for each strain was based on the required initial density of the culture, which would, considering individual growth rate of a microorganism, lead in 24 hours to distinguishable growth in the wells. These amounts were set by the MSC group's standard procedures. After preparation, the microbial culture was distributed across a plate with the help of a multichannel pipette. In the first row 280 µl of the tested organism culture was added, while the other rows were filled with 150 µl (Picture 17a). After that, the wells 1 to 11 in the first row (row A) were additionally filled with 20 µl of different raw extracts. The 12th well in the first row was filled with 20 µl of methanol to serve as a positive control (Picture 17b), since methanol-based extracts were tested.



Picture 17. Bioassay plate, filled with a)microbial culture of a test organism, b)additionally with the tested extracts in the first row, c)with exracts, diluted from top to the bottom

After the first row was loaded with the extracts, a series of dilutions from row A to row H was performed with a multichannel pipette. First, the content of row A was mixed by pipetting it up and down. Then 150 µl from row A was taken and transferred to row B, where it was mixed with the initial 150 µl of the microbial culture. The resulting solution was then transferred into the next row and the procedure continued until the row H. Finally, the wells in row H were mixed and the extra 150 µl were sucked up with the pipette and disposed, leaving the whole plate with 150 µl in each well. As the result, the plate had 8 different concentrations of each of 11 raw extracts and

methanol (Picture 17, c). The concentration of the extract in lower row was always one half of the concentration in the upper row.

When plates for each tested organism were prepared, they were incubated on vibrating shaker Titramax 1000 (Heidolph Instruments GmbH) at 160 rpm. Test organisms *E. coli* and *E. coli* TolC were incubated at 37 °C, while the others were kept a 30 °C. After 24 hours the inhibition of growth was checked. Relative concentration of raw extract was considered inhibiting, if no signs of growth were present in the corresponding well. The inhibited row with the lowest relative concentration of an extract was noted for the given microorganism. For example on the Picture 18, the extract 1 inhibits the test organism *E.coli* TolC up to the row B, while the extracts 9 to 11 have stronger inhibition up to the row C. If strong inhibition was present in the column with methanol, the test with the organism had to be repeated.

Picture 18. Serial dilution test with different raw-extracts of L. luteola (A 1-11) against test strain E. coli TolC. Raw 12 was not inoculated with raw extract and serves as control.

3.7.2. Fractionation and identification of antimicrobial products

Fractionation of extracts with HPLC and subsequent peak-activity correlation is normally employed with the extracts, which give strong inhibition up to at least line C (third dilution stage). Since the number of the produced extracts was rather high (56), overall 119 significant inhibitions were identified (one test organism inhibited by one extract). In order to efficiently proceed with the target, which was to see whether metal-spiked myxobacteria cultures produce new compounds, only a portion of these inhibitions was chosen for fractionation. The choice was based on the type

of the test organism inhibited by the extract and the degree of inhibition in comparison to other extracts (the most strongly inhibiting extracts were chosen).

First, selected raw extracts (given in the section 0 for each tested strain) were centrifuges at 13000 rpm and 100 µl of supernatant were transferred into Thermofisher HPLC-vial. These samples were used for fractionation with HPLC-DAD set (Agilent 1100 Series with fractionation collector), which employed reversed-phase chromatography with diode array detector (see section 2.4). Depending on the strength of the inhibiting activity of a raw extract, either 5 or 10 µl of a sample were fractionated.

Gradient elution was applied, which means that the composition of the mobile phase was changing with time. First water-acetonitril with high concentration of water was supplied to the column, while later the composition shifted towards higher acetonitrile content. Thus, eluent gradually changed from polar to nonpolar, which facilitated faster elution of nonpolar metabolites in the end of HPLC.

Every 30 seconds eluate was collected in a new well of bioassay plate (the same as used in SDT). After finishing the fractionation process the plates were aerated with nitrogen for approximately 45 minutes in order to remove water-acetonitril solvent. Finally, the plates were inoculated with the formerly inhibited test organism. In each well 150 µl of microbial culture were added and plates were incubated for 24 hours (under conditions described in section 3.7.1). The wells with growth inhibition were noted and correlated with the retention time and absorption peaks on the corresponding chromatogram.

Finally, the extracts were subject to investigation with HPLC-MS (as described in the section 2.4.2). This was done to determine the masses of the active or interesting compounds responsible for the peaks. In addition to peaks of bioactive compounds, identified by HPLC-fractionation, also other significant peaks with uncommon UV-chromatograms were identified, if present. All information like bioactivity, retention time, mass and UV absorption patterns of compounds were compared with data from the in-house “Myxobase” database to elucidate if compounds are known or new.

3.7.3. HPLC for comparison of productivity

In order to compare final concentrations of different myxobacterial compounds in the cultivation broth at the end of the secondary metabolites production experiments, all produced extracts were analyzed by HPLC with DAD. Therefore all raw extracts were centrifuges and 100 µl of each was transferred into separate HPLC-vials (Thermofisher). The same HPLC setting were used, as in case

of fractionation (section 3.7.2), but with 2 µl instead of 5 or 10 µl as used for fractionation. Chromatograms at different wavelengths were produced and the mAU*sec values (see section 2.4.1) for the relevant peaks were compared.

4. RESULTS

In the following chapter the results of conducted experiments and procedures are presented in a summarized manner. Consequent analysis of the results is then given in the Discussion chapter.

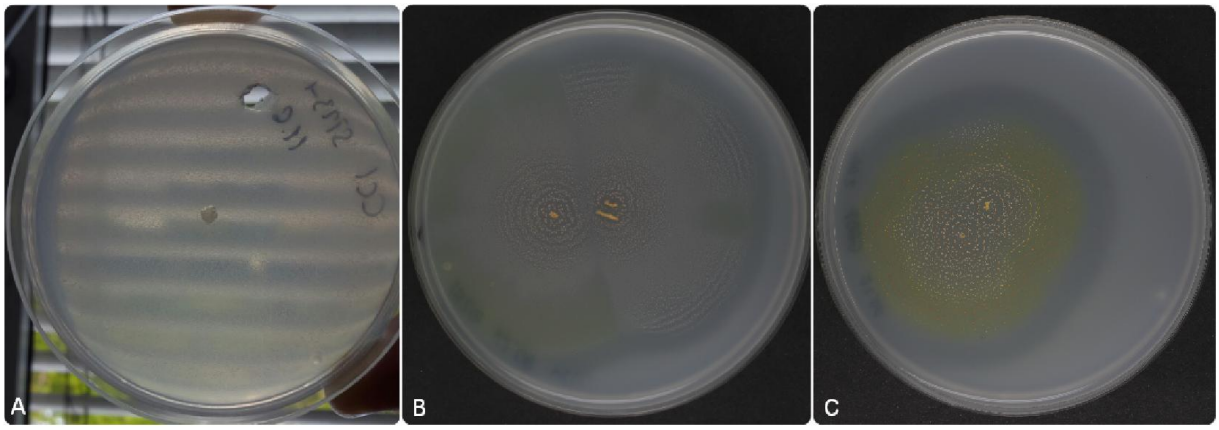
4.1. ISOLATION OF MYXOBACTERIA FROM HEAVY METAL CONTAMINATED SOIL SAMPLES

After inoculation of plates with soil samples a total of 22 emerged microbial colonies were identified as myxobacteria and were introduced to the purification process (Table 11). If the strains could be assigned to common myxobacteria genera due to morphological features of colonies and/or fruiting bodies, they were named as following: CC for *Corallococcus*, Mx for *Myxococcus*, Soce for *Sorangium cellulosum*, Na for *Nannocystis* and Pol for *Polyangium.* If morphological features did not enable to unambiguously classify the strain, abbreviation Myxo was used.

Putative myxobacteria were isolated from most of the obtained samples except from four, namely HS14/1, Ni-47-2, Ni-49-2 and Ni-50-2. Several isolation attempts were undertaken with these samples with standard methods, but all attempts failed. From the remaining samples, both nutritional types, cellulose degraders and micropredators were isolated. In most of the cases, if strains were isolated from water agar, the counterpart Stan21-approach also showed signs of myxobacterial (assumed *Sorangium*) growth.

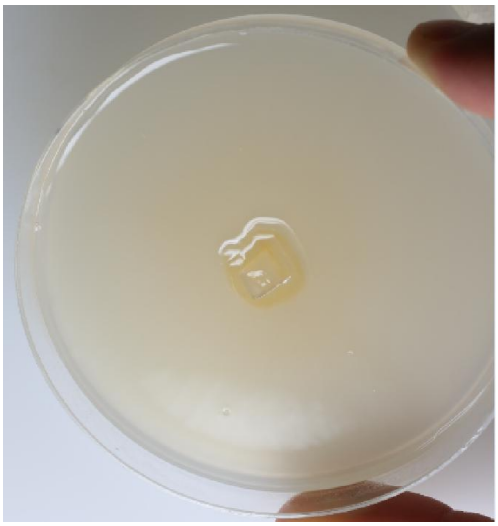
Table 11. Strains, identified as myxobacteria, which have reached the purification stage

Sample ID	Type of metal contamination	Stan21 + filter	Water agar + *E.coli*
HS14/1	Cr, Ni	No myxobacterial growth	
HS3/1	Zn	Mx1, Myxo3, CC1	Myxo1, Mx2
Ni-47-2	Pb	No myxobacterial growth	
Ni-48-2	Pb	Soce1, Myxo 1	Myxo3
Ni-49-2	Pb	No myxobacterial growth	
Ni-50-2	Pb	No myxobacterial growth	
Ni-51-2	Pb	-	Na1
Ni-53-2	Pb	Soce1	-
ST20/1	Hg, Zn	CC1	Pol1, Myxo2, Na1
ST23/1	Hg	Soce1	Myxo1, Myxo2, CC1, Na1,
ST62/1	As	Soce1, Soce2	Myxo3



Picture 19 Agar plates (VY/2) with pure cultures of isolated strains A) CC1 from ST23/1 sample B) Mx1 from HS3/1 and C) Mx2 from HS3/1

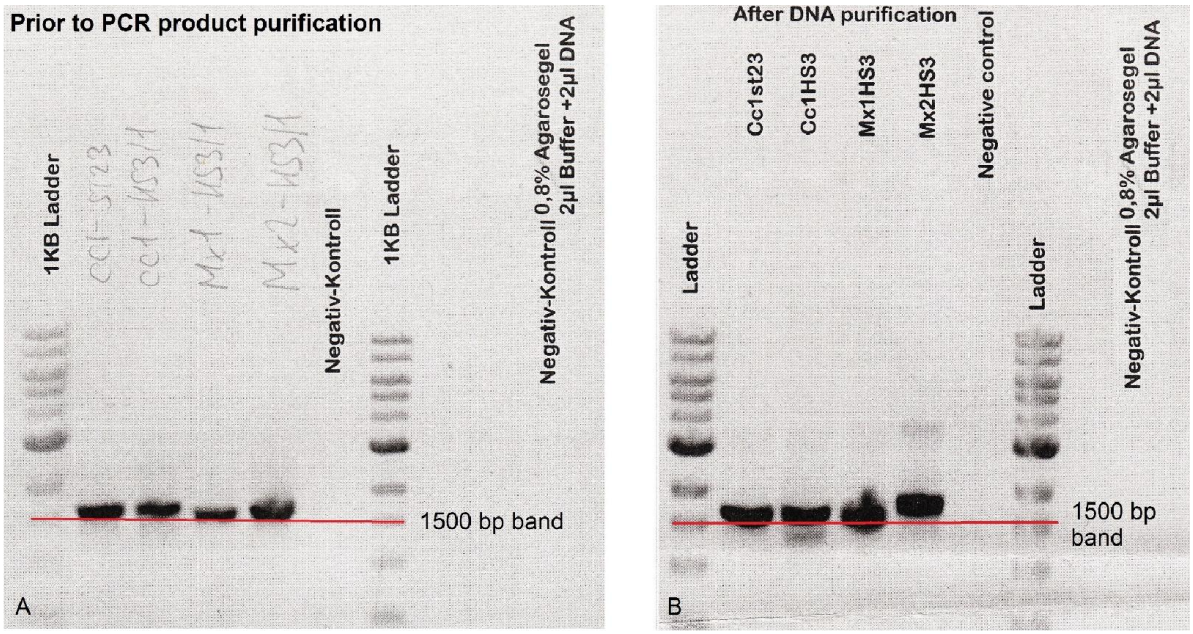
From 22 strains introduced to the purification process, only 4 (Table 12) strains could be purified within the time frame of the Bachelor thesis (see Picture 19). All putative *Myxococcus-,* and two of three *Corallococcus* strains were successfully purified. The majority of the strains at purification stage were growing as swarms, but as soon as a piece of swarm was transferred on full media, contamination was apparent (Picture 20). Some of the isolated strains are still in purification process, while the other were dismissed because of heavy contamination.



Picture 20. Failed attempt to cultivate isolated strain on full medium (CY). 'Milky' type of swarming is a sign of contamination.

4.2. IDENTIFICATION OF ISOLATED MYXOBACTERIA BY PCR AND SEQUENCING OF THE 16S RRNA GENE

The PCRs with annealing temperature 52 °C were performed successfully and DNA fragments of around 1500 bp were obtained, as it was determined with the gel electrophoresis (Picture 21). The results of sequencing and comparison of 16S RNA genes of the isolated strains with BLAST database proved the initial guess about their phylogenetic affiliation. The similarity values showed that all isolated strains belong to known species.



Picture 21. PCR products of the isolated strains show expected length (16S RNA gene), analyzed with gel electrophoresis A) Before purification B) After purification

Table 12 List of isolates, origin, next relative (NCBI), similarity (%) to the next relative and the sequence length (bp) used for search

Soil sample	Internal strain name	Next relative (NCBI)	% similarity	Sequence length (bp)
HS3/1	CC1 HS3/1	Corallococcus coralloides DSM 51615	100	1044
ST23/1	CC1 ST23/1	Corallococcus coralloides DSM 52500	99.6	1041
HS3/1	Mx1 HS3/1	Myxococcus xanthus DK1622	100	1044
HS3/1	Mx2 HS3/1	Myxococcus xanthus DK1622	99.9	1037

4.3. HEAVY METAL TOLERANCE TESTS

The heavy metal tolerance tests were performed with all type strains (Table 1), both of the isolated *Corallococcus*-strains (CC1 ST23/1 and CC1 HS3/1) and with one of the isolated *Myxococcus xanthus* strains (Mx2 HS3/1). Owing to small sample size (2 metal-supplemented plates of certain concentration and 1 reference plate for each tested metal), no statistical processing was done. The results in raw format are given in the appendix (on the supplement CD). Analysis of notable patterns and visual representation of the data are given below for each tested metal.

General tendency was that all the strains were spreading faster on VY/2 media agar, than on CY. The only exception was the *S. amylolyticus* strain, which grew better on CY media in every experiment run. In broth media the opposite situation was observed and all strains demonstrated better biomass formation and higher metal tolerance in CY media.

Morphology of the cells, growing in metal-supplemented media (agar or liquid), was not distinguishable from their counterparts growing in control media. The only exception was the cultures growing on lead-supplemented agar (both media types), which is described further.

4.3.1. Lead

Tests, performed during the first run of the experiment (with concentrations 1 mM, 5 mM, 10 mM) showed that none of the strains could grow on agar at 5 mM and 10 mM of lead on neither CY nor VY/2 media. At 1 mM on CY agar, however, all strains except *S. cellulosum* were able to grow within 2 weeks (see Figure 13), with some strains *N. pusilla*, *S. amylolyticus* and *L. luteola* showing even faster swarming at 1 mM of lead.

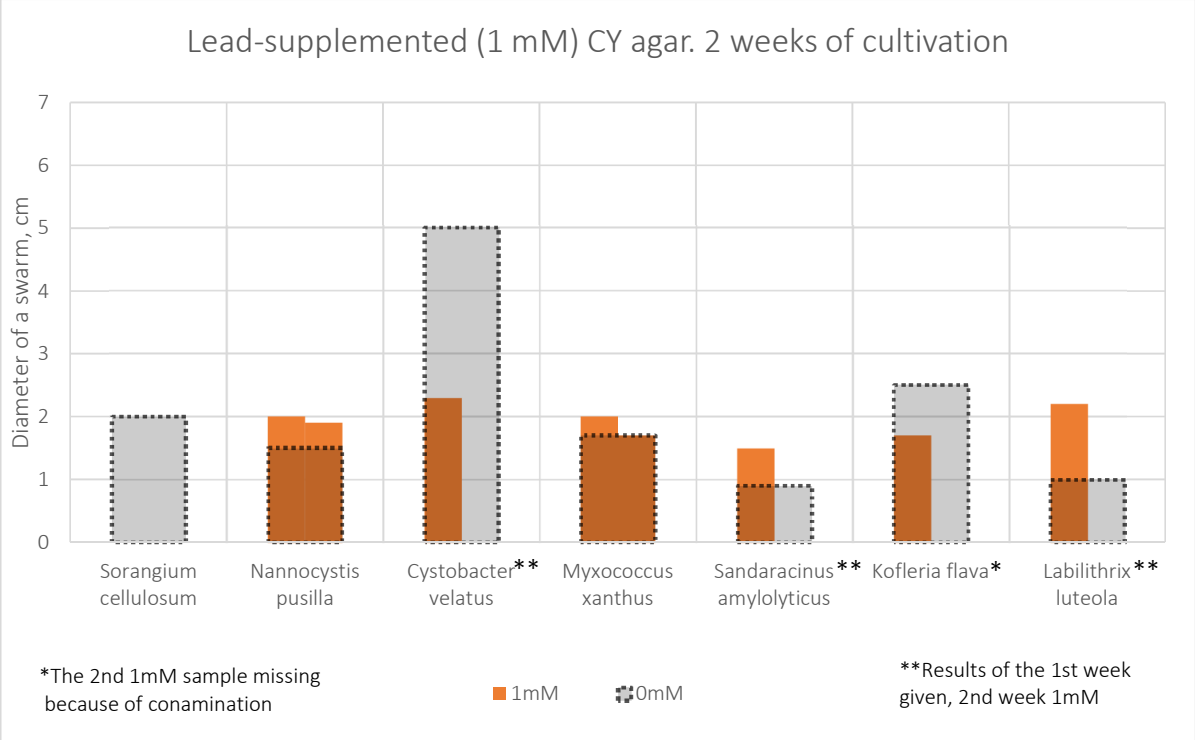
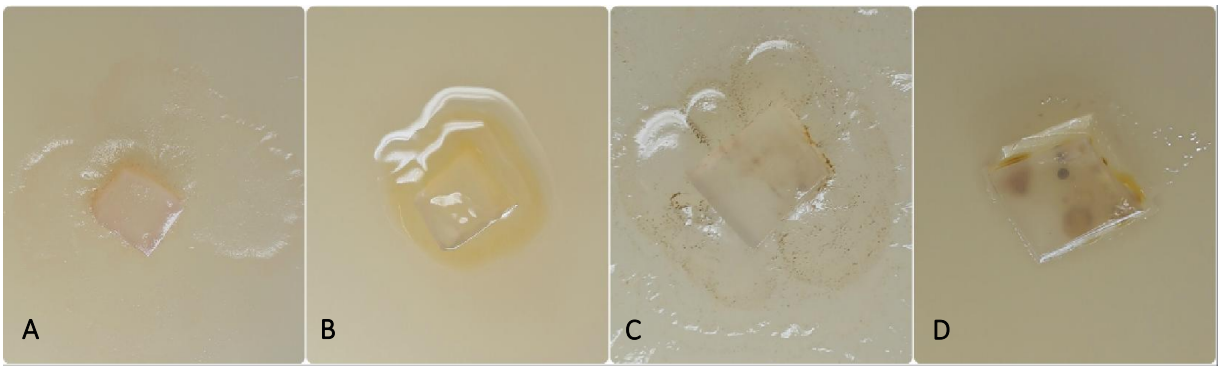


Figure 13 Diameters of myxobacterial colonies after 2 weeks of cultivation on lead-supplemented (1mM) CY agar

Propagation of colonies on VY/2 agar. Only *N. pusilla* and *C. velatus* showed very minor growth within 2 weeks (ca. 1.5 cm). For this reason the test was repeated on VY/2 media with concentrations of 0.25mM and 0.75mM with each strain except *C. velatus,* since the strain showed significant growth already at lead concentration 1mM. According to the results (Figure 14), all strains could grow at 0.25mM with almost no inhibition. However, only *N. pusilla* and *L. luteola* gave swarming colonies at 0.75mM within 2 weeks and *S. amylolyticus* – with longer lag phase in 3 weeks (not shown on the graph). The swarming of some strains on lead-enriched media had distinguishable darker pigmentation, comparing to their counterparts on reference plates, despite the media being not colored (see Picture 22).



Picture 22 Myxobacterial growth on lead-supplemented media. A) N. pusilla with 0.75 mM B) S. amylolyticus with 0.75 mM C) *S. cellulosum* with 0.75mM D) *M. xanthus* with 0.75mM

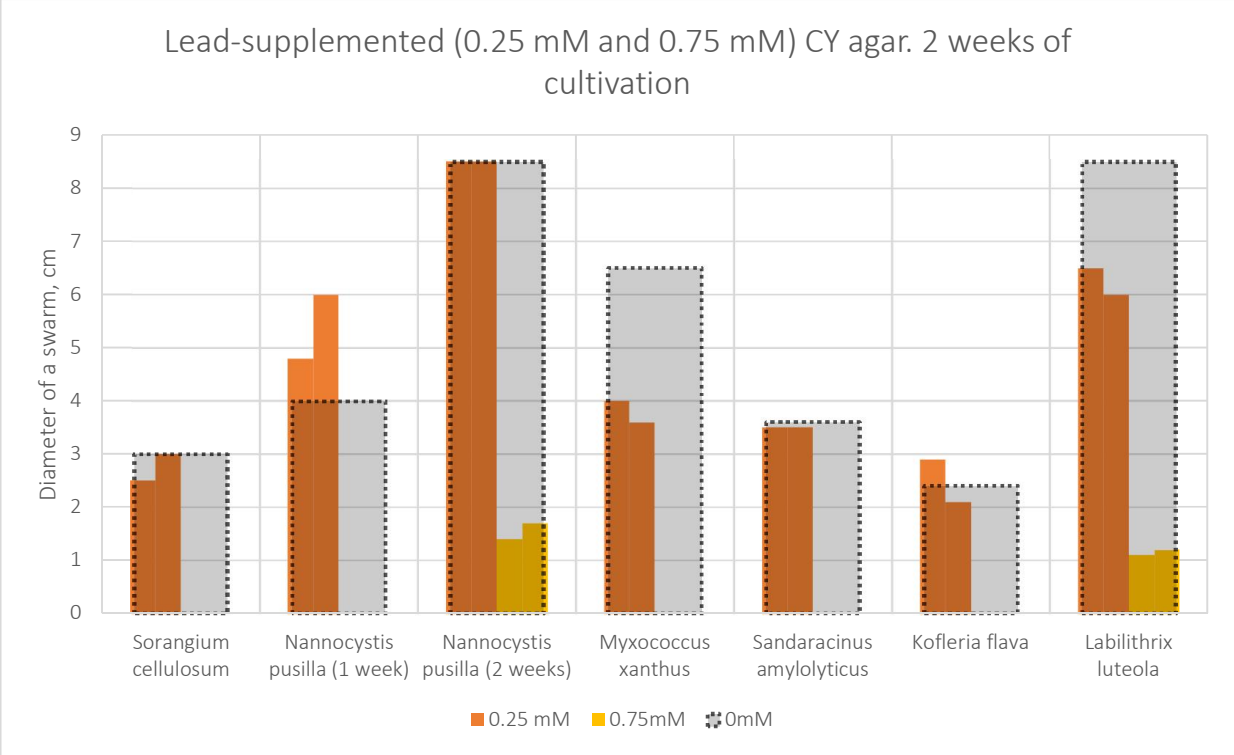


Figure 14 Diameters of myxobacterial colonies after 2 weeks of cultivation on lead-supplemented VY/2 agar

Based on the results of broth tests (see Table 13), none of the myxobacteria could grow at 5mM and 10mM. In CY media at 1mM all strains except *S. cellulosum* showed good biomass formation, unlike in VY/2 medium, in which no strain could grow at 1mM concentration of lead.

Table 13 Assessment of myxobacterial growth in lead-supplemented liquid media. 0 – no growth, 0.5 – healthy cells seen under microscope, 1 – minimal growth seen with bare eyes, 2 – significant growth but less than in reference flask, 3 – growth like in reference flask, 4 - growth better than at reference

Media	Concentration	Sorangium cellulosum	Nannocystis pusilla	Cystobacter velatus	Myxococcus xanthus	Sandaracinus amylolyticus	Kofleria flava	Labilithrix luteola
CY	0 mM	3	3	3	4	3	3	3
	1 mM	0.5	3	3	3	3	3	3
VY/2	0 mM	3	2	3	3	2	2	2
	1 mM	0	0	0	0	0	0	0

As a result of the tolerance tests both in agar and in liquid media, strains ***N. pusilla*** and ***L. luteola*** were chosen for testing induction of metabolites production in presence of lead.

4.3.2. Cobalt

No growth was observed in the first run of agar tests with cobalt concentrations 1 mM, 5 mM and 10 mM. Only *M. xanthus* grew up to 0.6 cm after 2 weeks on 1mM CY agar (comparing to 1.5 cm on reference plate) and to 1 cm on 1 mM VY/2 agar within 3 weeks. For this reason another series of tests was performed with 0.25 mM and 0.75 mM on VY/2 media. Self-isolated strains were also subject to this tests.

Only five of the tested strains could tolerate cobalt concentration 0.25mM (see Figure 15), four type strains and one of the self-isolated ones. In addition both *Myxococcus xanthus* strains – the type strain and the self-isolated were able to grow at 0.75mM concentration after long lag phase (4 weeks for type strain and 3 for Mx1HS3). None of the strains could grow at any tested concentrations (1, 5 or 10 mM) in broth media. However, *M. xanthus* and *L. luteola* were able to reproduce after re-inoculation and thus were given grade 0.5 assessment.

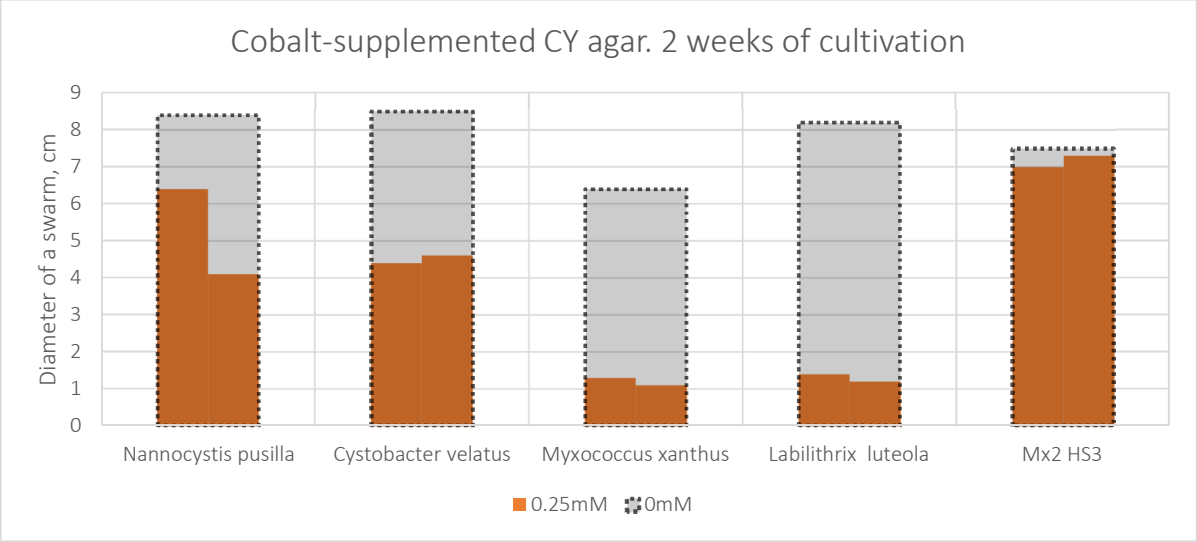


Figure 15 Diameters of myxobacterial colonies after 2 weeks of cultivation on cobalt-supplemented VY/2 agar

Since ***M. xanthus*** showed minor growth at 1mM in both agar media it was chosen for metabolite production tests with cobalt to see whether the response to stress would be production of bioactive compounds. Also self-isolated **Mx2HS3** was chosen for the same reason, and since its growth was not inhibited at 0.25mM and it was able to grow at 0.75mM.

4.3.3. Copper

The only myxobacterial strain, which was able to grow during the first run of the experiment (with 1, 5, and 10 mM) was *N. pusilla* on CY media supplemented with 1mM copper. However, it might me an error, since the second plate showed no growth. Second run was performed with 0.25mM and 0.75mM concentrations and included the self-isolated strains.

Only three strains could grow at concentration as low as 0.25mM on VY/2 agar (Figure 16). By the end of the experiment also *N. pusilla* on 0.75 mM had signs of growth after 3 weeks of lag phase.

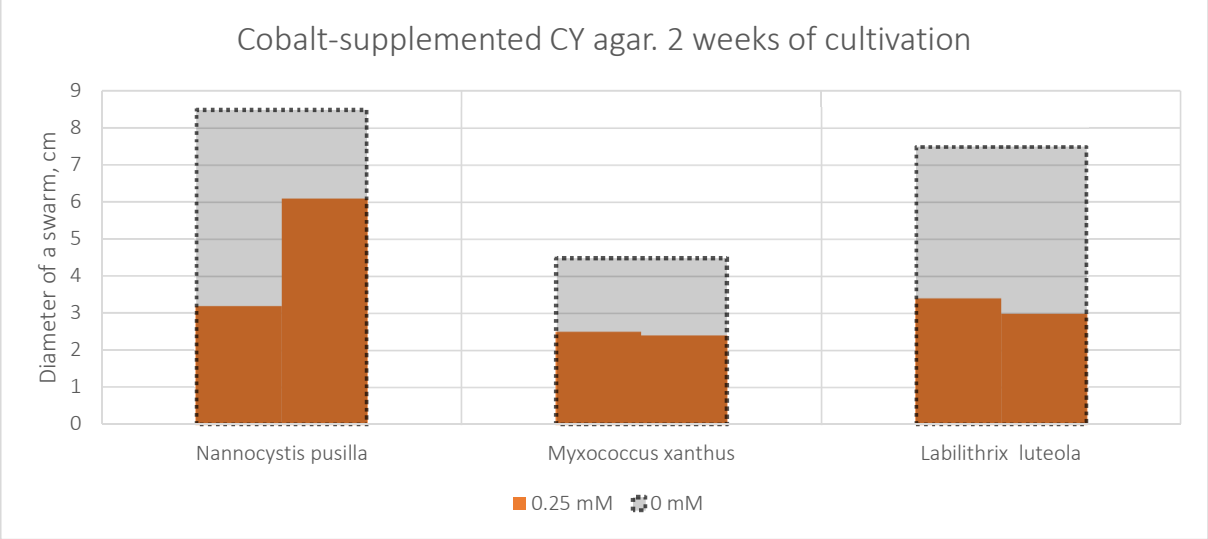


Figure 16 Diameters of myxobacterial colonies after 2 weeks of cultivation on cobalt-supplemented VY/2 agar

Observation of liquid media under microscope showed presence of living cells after 1 week of cultivation in 1 mM copper in CY media for ***N. pusilla*** and ***L. luteola***. For this reason, also considering growth on agar media, these two strains were chosen for cultivation in metabolite production tests with copper.

4.3.4. Nickel

Neither on CY nor on VY/2 mediacould tested myxobacteria strains tolerante concentrations between 1 -10 mM. For this reason in the second run concentrations 0.25 mM and 0.75 mM were tested, also with the self-isolated strains. At 0.75 mM no strain could grow. At 0.25 mM only three strain showed growth within 2 weeks (see Figure 17). Two other strains (*S. amylolyticus* and *M. xanthus* type strain) showed minor swarming only after lag phase of 2-3 weeks. No strain could survive in liquid media with the tested concentrations. Due to minor tolerance of this heavy metal by myxobacteria no experiments for secondary metabolite production were performed with nickel.

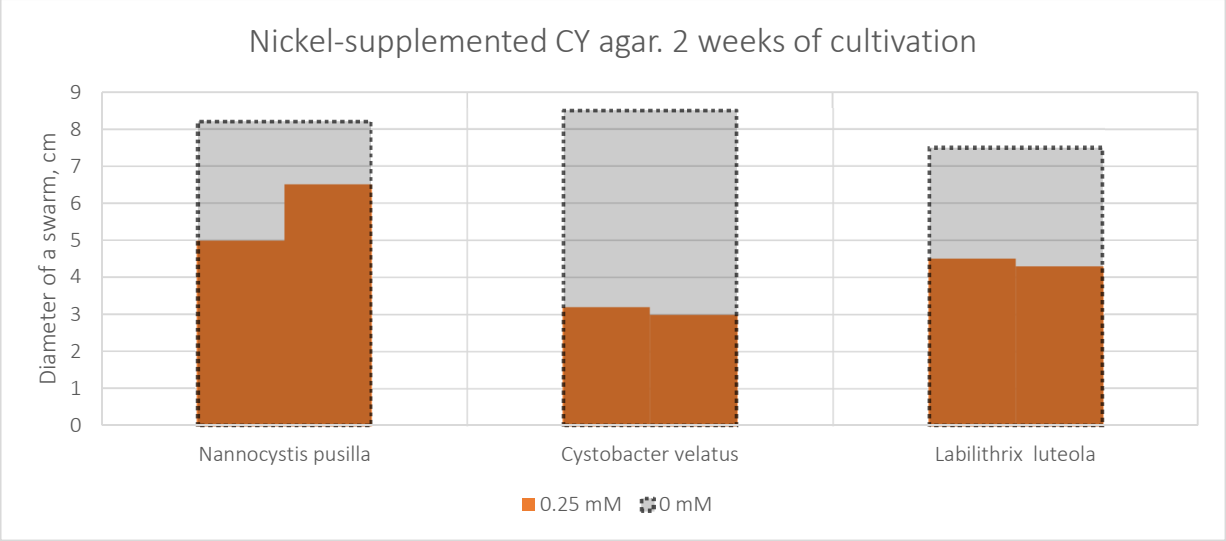


Figure 17 Diameters of myxobacterial colonies after 2 weeks of cultivation on nickel-supplemented VY/2 agar

4.3.5. Zinc

In the first run of the experiment only *L. luteola* could grow slightly on CY agar in presence of 1 mM zinc. Therefore the other type strains and the self-isolated strains were subsequently tested again with concentrations 0.25 mM and 0.75 mM on VY/2 media. As a result, the following strains could not tolerate presence of zinc at any concentration: *Sorangium cellulosum*, *Kofleria flava*, Mx2 HS3 and CC1 HS3. The results of the remaining strains after 2 weeks of cultivation are shown in the Figure 18.

Tests in liquid media proved that ***N. pusilla*** and ***L. luteola*** are able to tolerate zinc concentrations of about 1 mM (Table 14). For this reason these two strains were selected for metabolite production test with zinc. In addition, the **CC1 ST23** isolate, was chosen for testing, as it was the only self-isolated strain, which could tolerate zinc at the tested concentrations.

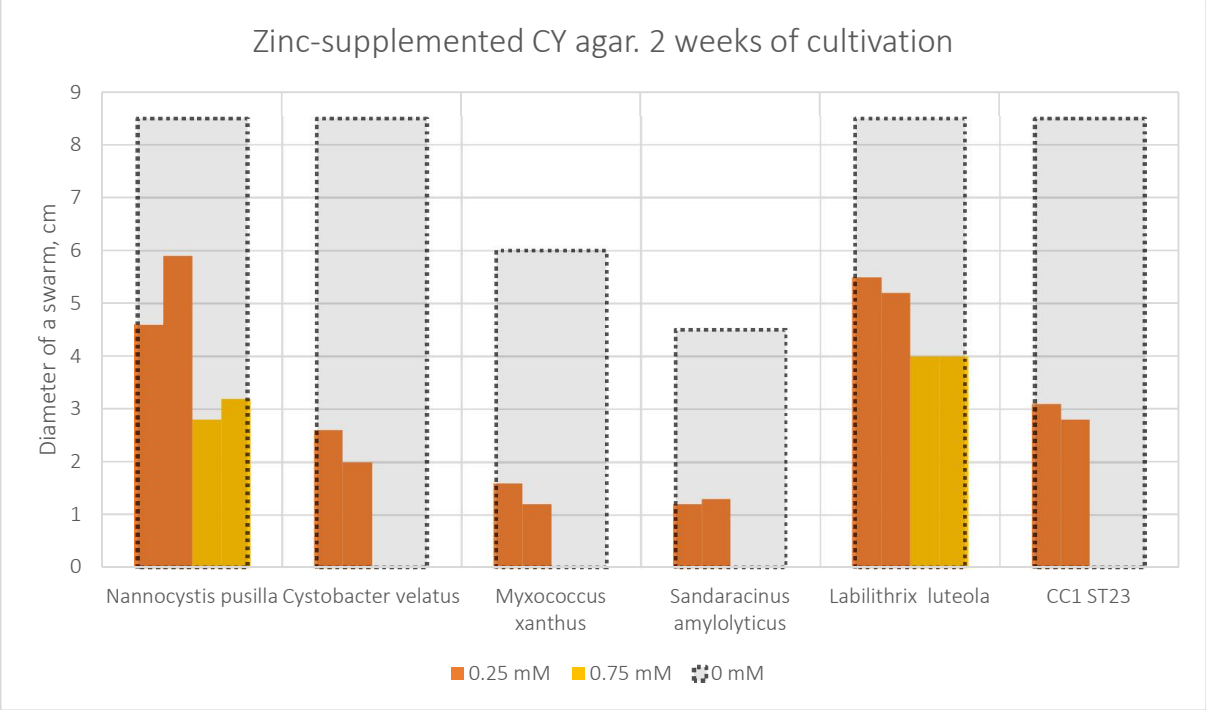


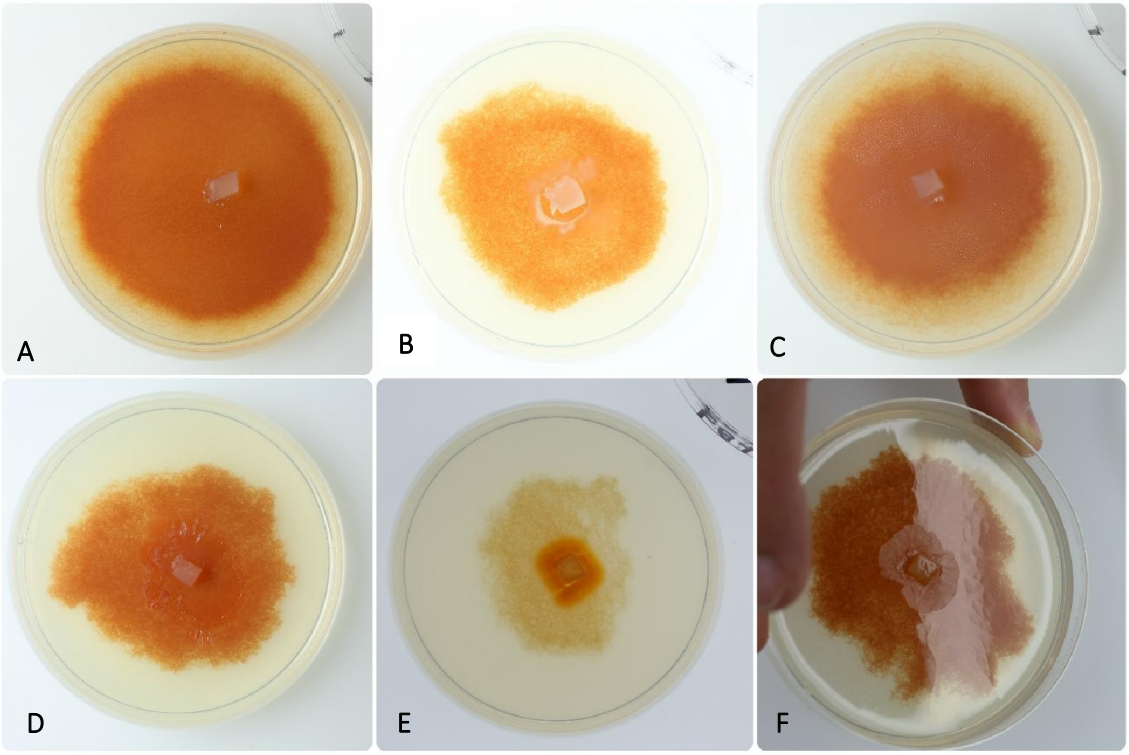
Figure 18 Diameters of myxobacterial colonies after 2 weeks of cultivation on zinc-supplemented VY/2 agar

Table 14 Assessment of myxobacterial growth in zinc-supplemented liquid media

Media	Concentration	*Sorangium cellulosum*	*Nannocystis pusilla*	*Cystobacter velatus*	*Myxococcus xanthus*	*Sandaracinus amylolyticus*	*Kofleria flava*	*Labilithrix luteola*
CY	0 mM	3	3	3	4	3	3	3
	1 mM	0	1	0	0	0.5	0	1
VY/2	0 mM	2	2	3	3	2	3	2
	1 mM	0	2	0	0	0	0	0

4.3.6. Iron

Only type strains were tested for tolerance against iron. All tested strains showed noticeable growth after one week of cultivation on both media. Myxobacteria were growing in form of swarms and showed no fruiting bodies formation or any unusual features (Picture 23).



Picture 23. Myxobacterial growth on iron-supplemented media. A) C. velatus on VY/2 Fe 10 mM, B) L.luteola VY/2 Fe 5 mM, C) M. xanthus VY/2 Fe 5 mM D)N. pusilla CY 5 mM E) S. amylolyticus CY Fe 1 mM F) S. cellulosum CY Fe 5 mM

In Figure 19 and Figure 20 the results of cultivation after 2 weeks on VY/2 and CY media are shown, respectively. As a general pattern, all of the tested strains tend to swarm faster on VY/2 agar than on CY agar. It can be observed, that either no inhibition took place for some strains and media combinations, or it only happened at concentrations of 5 mM or 10 mM. In some cases (*L. luteola* on VY/2 and CY media, and *M. xanthus* and *K. flava* on CY media) colony expansion was more rapid at 1 mM and 5 mM than at the reference plate.

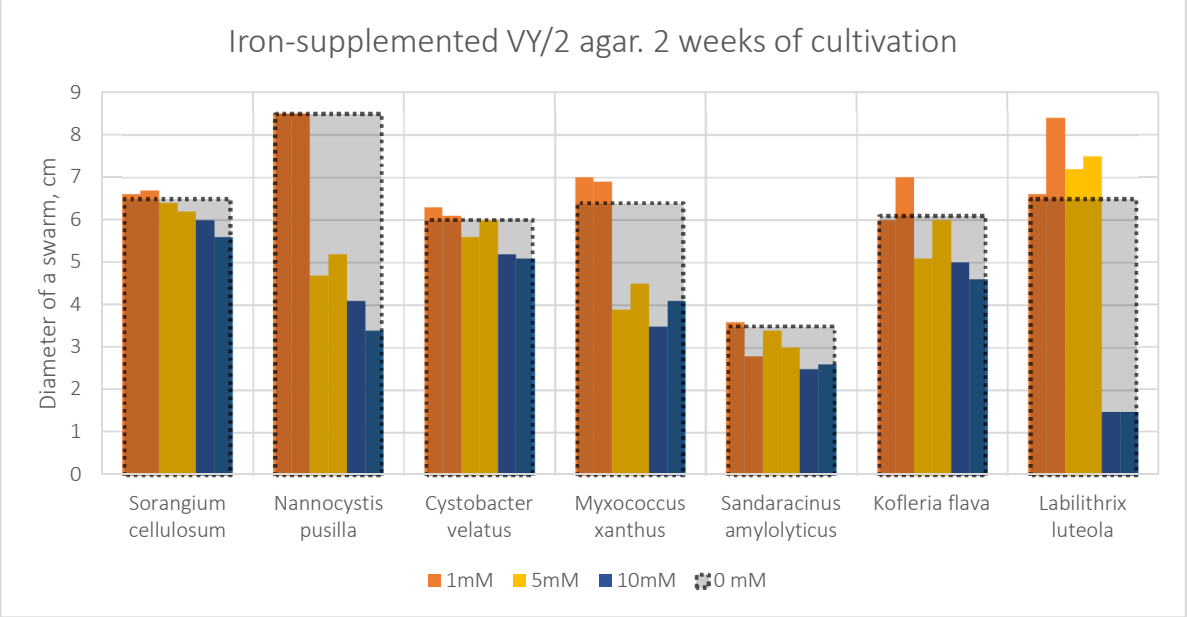


Figure 19. Diameters of myxobacterial colonies after 2 weeks of cultivation on iron-supplemented VY/2 agar

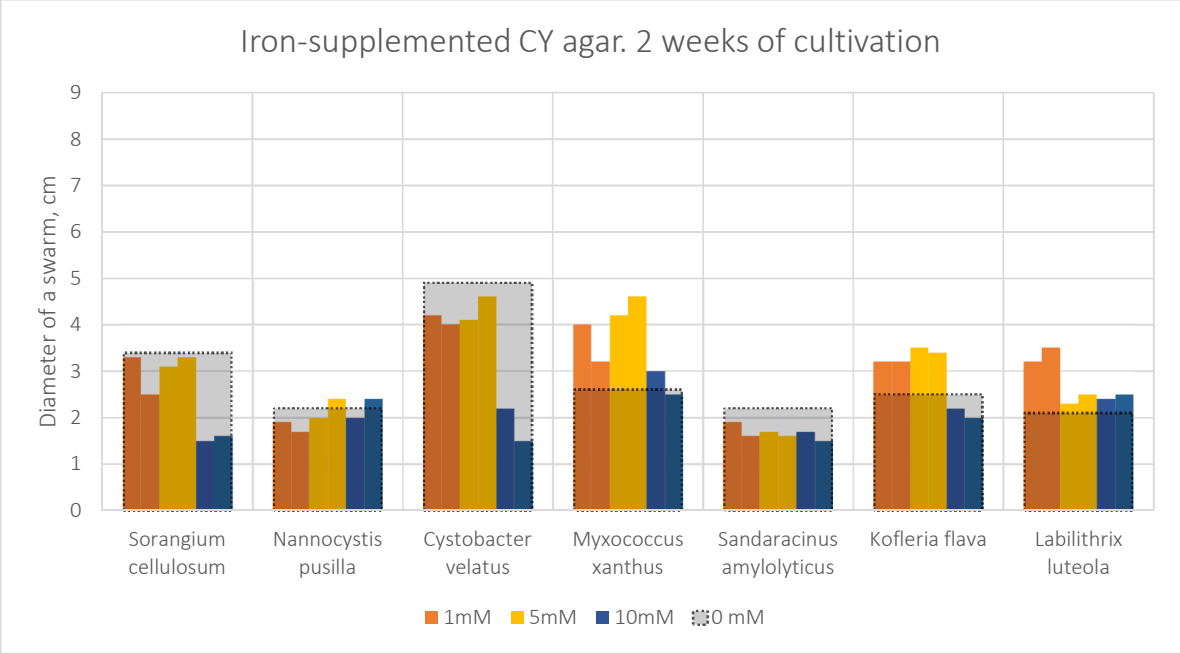


Figure 20. Diameters of myxobacterial colonies after 2 weeks of cultivation on iron-supplemented CY agar

The results of metal tolerance tests (Table 15) in liquid media also show that all tested type strains were able to grow at concentrations as high as 10mM. In all cases growth became apparent via formation of cell biomass. Since many tested strains were well growing at high iron concentration, for secondary metabolites production test with iron two already selected strains - ***N. pusilla*** and ***L. luteola*** - were chosen, to see the effect of this metal in comparison with the other two (zinc and copper), tested with these strains.

Table 15 Assessment of myxobacterial growth in iron-supplemented liquid media

Media	Concentration	Sorangium cellulosum	Nannocystis pusilla	Cystobacter velatus	Myxococcus xanthus	Sandaracinus amylolyticus	Kofleria flava	Labilithrix luteola
CY	0 mM	3	3	3	4	3	3	3
	1 mM	3	4	4	3	4	3	4
	5 mM	2	3	4	2	4	2	2
	10 mM	1	2	2	1	3	1	2
VY/2	0 mM	3	2	3	3	2	2	2
	1 mM	4	2	3	3	2	3	3
	5 mM	4	1	3	2	2	3	2
	10 mM	2	1	1	1	1	2	1

4.3.7. Summary of the heavy metal tolerance tests

The summary of the maximum concentrations regardless of the media, at which myxobacterial growth was observed is given in the Table 16 for each tested strain and metal. The results are based on agar media tests, since they cover all strains and concentrations of 0.25mM and 0.75 mM. It is worth mentioning that some of the maximum concentrations were based only on one plate's result and may be caused an error.

Table 16. Highest concentrations, at which myxobacterial growth was observed on agar media.

Strain	Lead, mM	Cobalt, mM	Copper, mM	Zinc, mM	Nickel, mM	Iron, mM
Sorangium cellulosum	0.25	0	0	0	0	10
Nannocystis pusilla	1	0.25	1	0.75	0.25	10
Cystobacter velatus	1	0.25	0	0.25	0.25	10
Myxococcus xanthus	1	1	0.25	0.25	0	10
Sandaracinus amylolyticus	1	0	0	0.25	0	10
Kofleria flava	1	0	0	0	0	10
Labilithrix luteola	1	0.75	0.25	1	0.25	10
Mx2 HS3	Not tested	0.75	0	0	0	Not tested
CC1 HS3	Not tested	0	0	0	0	Not tested
CC1 ST23	Not tested	0	0	0.25	0	Not tested

4.4. Screening for secondary metabolites and productivity comparison

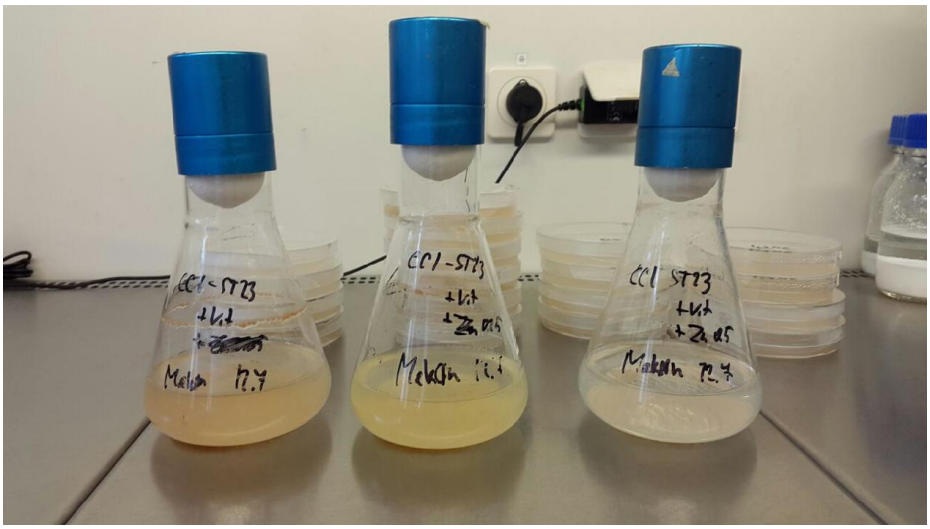
Based on the results of heavy metal tolerance tests with liquid medium and agar cultures, the strains given in Table 17 were chosen and tested for:

1. Possibility of **induction of unknown** metabolites production with high concentrations of heavy metals
2. Effect of the metals on **productivity** of strains with respect to known secondary metabolites

Table 17. List of the strains, chosen for secondary metabolites screening at different metal concentrations

Tested strain	Metal cation	Tested concentrations, mM
Labilithrix luteola	Zn^{2+}	(0), 0.25, 0.50
	Cu^{2+}	(0), 0.25, 0.50
	Fe^{3+}	(0), 5, 10
	Pb^{2+}	(0), 1
Nannocystis pusilla	Zn^{2+}	(0), 0.25, 0.50
	Cu^{2+}	(0), 0.25, 0.50
	Fe^{3+}	(0), 5, 10
	Pb^{2+}	(0), 1
Myxococcus xanthus	Co^{2+}	(0), 0.25, 0.50
CC1 ST23	Zn^{2+}	(0), 0.25, 0.50
Mx1 HS3	Co^{2+}	(0), 0.25, 0.50

Cultivation of metal-spiked *Myxococcus* cultures (both the type strain and the self-isolated Mx1 HS3) lasted 10 days and in the end at each concentration dense cultures were achieved. However, in case of other strains the growth period was extended to 14 days, since some cultures at high metal concentrations (0.75 mM of Zn and Cu or 1 mM of Pb) could not reach near-stationary phase. Such culture, which failed to grow at comparable with the reference culture rate, were dismissed (see Picture 24).



Picture 24.Example of culture (CC1 ST23 in Pol medium with 0.75mM of zinc ion, the flask on the right), which was not used for extract preparation owing to little growth.

The results of SDT, fractionation, HPLC-MS and comparison of chromatograms on the subject of productivity are given further and grouped by strains. SDT results are given only for those test organisms, which showed considerable inhibition by the myxobacterial extracts.

4.4.1. Nannocystis pusilla

In case of *N. pusilla,* cultures, growing at 1 mM of lead and 0.75 mM of zinc in POL medium and in CY medium, respectively didn't reach near-stationary phase and thus from these approaches no extracts were produced. The results of the SDT tests are given in the Table 18. Since extract of Cu 0.25 mM CY media showed stronger inhibition then other extracts against *E. coli* TolC and *B. subtilis*, and Cu 0.25 mM POL strongly suppressed growth of *B. subtilis*, these two extracts were chosen for fractionation.

Table 18 SDT results of N. pusilla extracts

Media	CY							POL						
Metal conc. (mM)	Reference	Zn 0.25	Cu 0.25	Cu 0.5	Fe 5	Fe 10	Pb 1	Reference	Zn 0.25	Zn 0.5	Cu 0.25	Cu 0.5	Fe 5	Fe 10
E. coli TolC	B	A	C	B	B	B	B	B	B	B	B	C	C	B
S. aureus	A	A	B	B	-	-	B	D	D	D	D	D	D	C
M. luteus	B	C	B	D	-	B	C	B	C	C	C	C	B	B
B. subtilis	B	C	D	D	A	-	B	E	F	F	F	F	E	E

Table 19 Results of fractionation and HPLC-MS of the selected N.pusilla extracts

Media	Metal	Inhibited strains	Activity (min)	Identified significant peaks
CY	$CuCl_2$ 0.25	E. coli TolC	no inhibition	Nannochelin A (768.2) (?)Nannochelin complex with metal (792)
POl	$CuCl_2$ 0.25	B. subtilis	25.5; 31.5-33.5	-

As a result of fractionation (Table 19), no inhibition of *E. coli* TolC was observed. However, on the resulting chromatogram there were two distinguishable peaks, which were analyzed with HPLC-MS. As a result, the masses of the compounds were identified as 768.2 and 792. The first one corresponded to the known metabolite of *N. pusilla* with antibiotic and iron-chelating properties, namely Nannochelin A, while the second compound could not be identified, but most probably was a derivative of Nannochelin with a metal because of its similar absorption pattern and slightly higher mass. Fractionation of Cu 0.25 mM Pol extract showed inhibitions, corresponding to 3 peaks (in 210 nm band). However, closer look at the absorption graphs (Figure 22) showed either no organic compound or a mixture of various compounds, which mass could not be identified with MS.

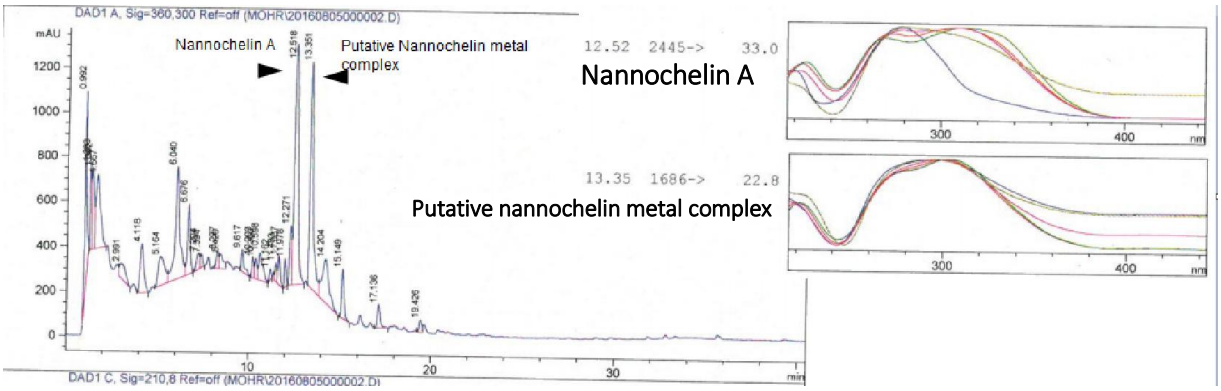


Figure 21 Chromatography of Cu 0.25mM CY extract of N. pusilla and absorption graph of two significant peaks

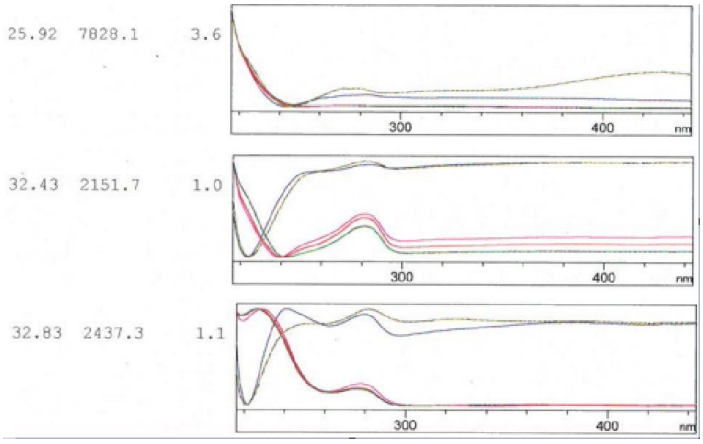


Figure 22 Absorption graphs of the peaks corresponding to B. subtilis inhibition. (Cu 0.25mM CY extract of N. pusilla)

Since fractionation of $CuCl_2$ 0.25 CY brought no results, the two peaks, corresponding to nannochelin A and unknown nannochelin derivative were chosen for comparison of *N. pusilla* productivity in CY media with different metal concentrations. It could be observed that the amounts of both compounds decreased in metal-spiked cultures (zinc and copper). In extract from iron-supplemented culture nannochelin A was absent completely, however, its derivative was still present and its amount increased from concentration 5 mM to 10 mM of iron.

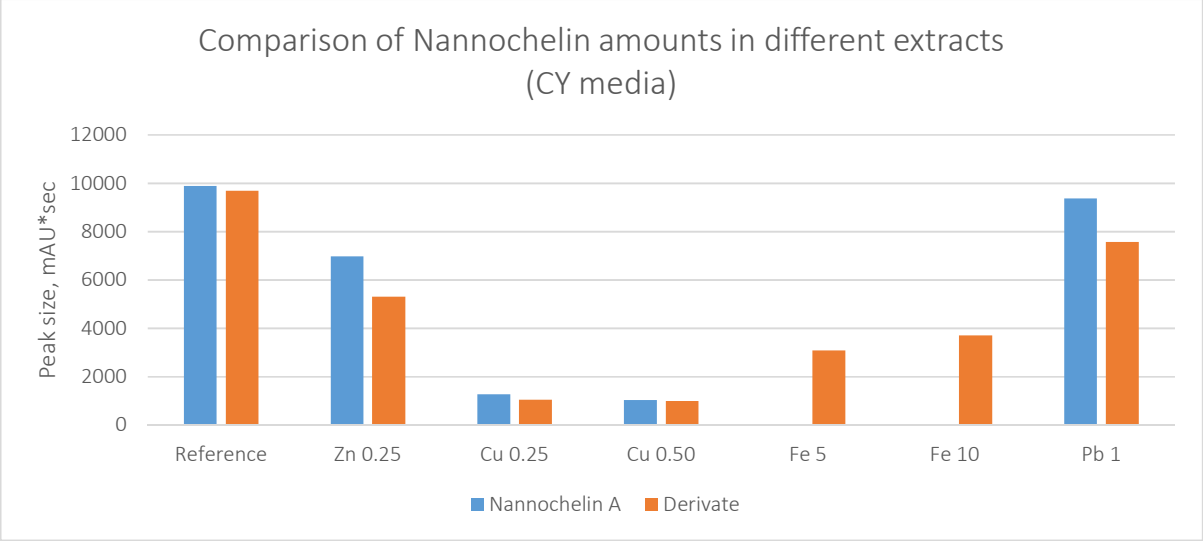


Figure 23 Comparison of nannochelin amounts in different extracts based on CY media

In respect to Pol medium extracts, an interesting observation was a peak with the size of 6125 mAU*sec, observed in the extract of copper-supplemented (0.50 mM) culture (Figure 24A) at 13.40 min. The same peaks are present in other Pol extracts, but at much lesser size (in reference POL medium – 1245 mAU*sec and in copper 0.25 mM – 1820 mAU*sec). According to the absorption diagrams, it probably corresponds to a mixture of compounds, which are, however, similar to putative nannochelin derivative, identified from CY media extracts.

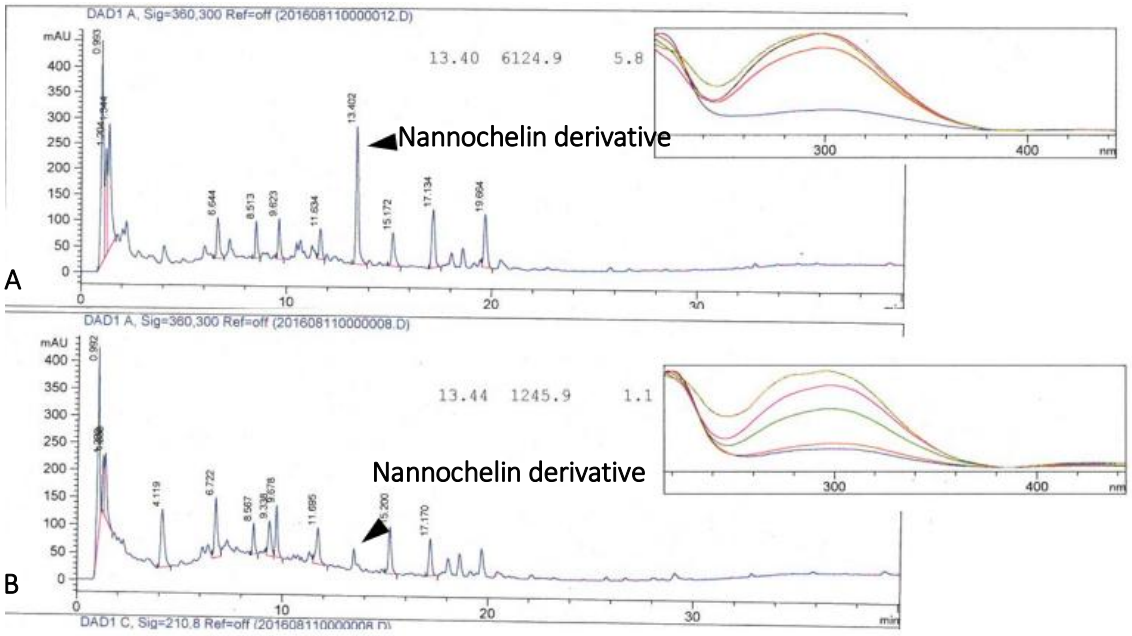


Figure 24 Chromatograms of A) Cu 0.50 mM Pol extract and B) Pol control extract with selected peaks, corresponding to putative nannochelin derivative, and nannochelin absorption graphs

4.4.2. Myxococcus xanthus

SDTs results (Table 20) showed inhibition of many test organisms by raw extracts of *Myxococcus xanthus.* To limit the number of performed fractionation tests only three extracts were chosen, which had rare inhibition in general (against Gram-negative *C. violaceum*) and high activity in comparison to the other tested extracts.

Table 20 SDT results of the extracts of M. xanthus

Media	CY			Pol		
Metal conc (mM)	-	Co 0.25	Co 0.5	-	Co 0.25	Co 0.5
E. coli TolC	G	H	H	H	H	H
C. violaceum	C	C	F	F	F	B
S. aureus	D	C	D	D	D	B
M. luteus	D	B	D	E	E	B
M. hiemalis	C	B	C	E	D	B
B. subtilis	B	B	B	B	A	A

Table 21 Fractionation results for the selected Myxococcus xanthus raw extracts

Media	Metal	Inhibited strains	Activity (min)	Identified significant peaks
CY	$CoCl_2$ 0.5	*C. violaceum*	no inhibition	Myxochromid A3 (845.46) Myxalamids
CY	$CoCl_2$ 0.5	*M. luteus*	31.5-32.0; 33.0	Myxochromid A3 (845.46) Myxalamids
Pol	control	*M. hiemalis*	19.5-20.0	Myxovirescin (609)

Fractionation of $CoCl_2$ 0.5 mM CY extract (Table 21) showed no inhibition of the test organism *C. violaceum*. However, a number of apparent peaks on the corresponding chromatogram (Figure 25, A) were recognized and identified as myxochromid A3 and a group of various antibiotic metabolites called myxalamids. Inhibition of *M. luteus* by fractionated $CoCl_2$ 0.5 mM CY was analyzed, but no peaks could be correlated with the inhibiting activity. Inhibition of *M. hiemalis* by fractionated Pol extract could also not be correlated with chromatographic peaks (Figure 25, B), however, antibiotic myxovirescin was identified by MS. In addition, the spike at 21.8 min was closer observed and the absorption pattern revealed peak at 411 nm, which is the same peak known for myxochromid A3. However, the MS could not give reliable results, since the peak was probably

contaminated by other compounds. In addition to all mentioned compounds, antibiotic althiomycin was probably present in small amounts in the raw extracts, but its corresponding peaks were rather contaminated to prove this claim with applied HPLC technique.

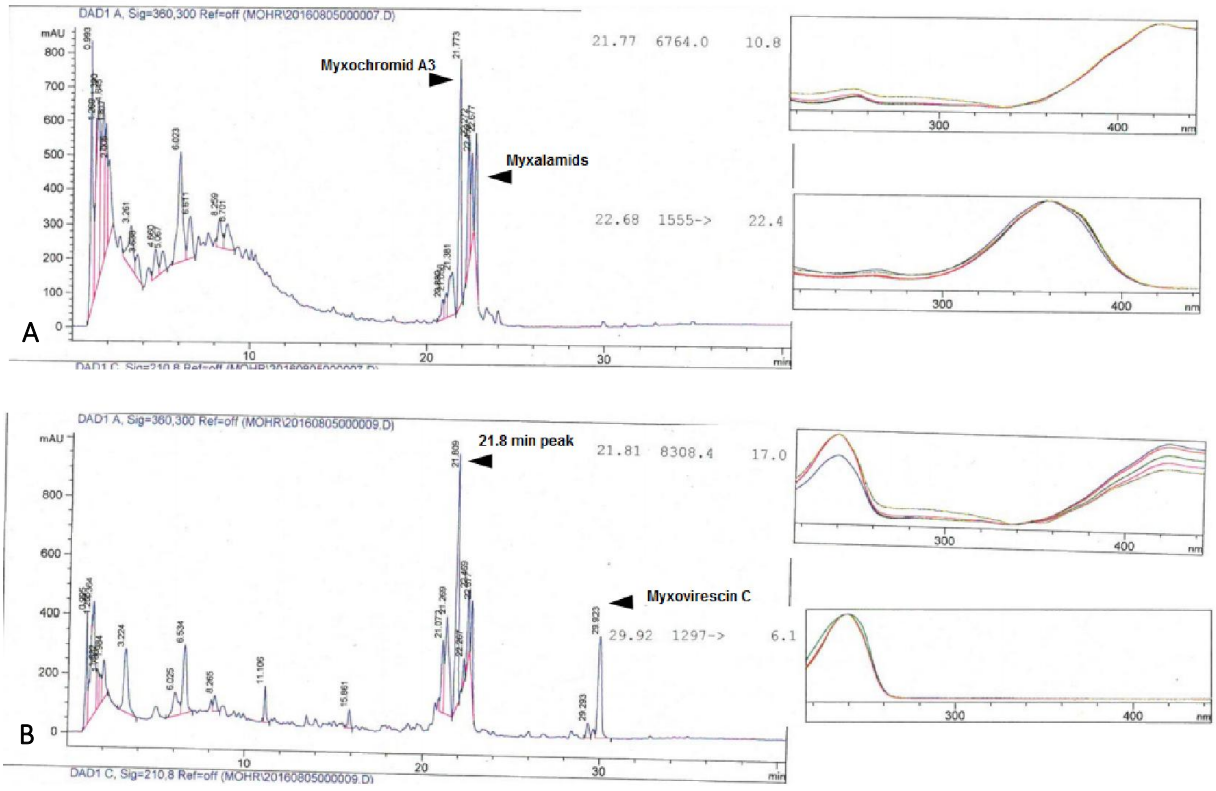


Figure 25. Chromatograms of fractionated Co 0.5 mM CY and Pol reference extracts produced from Myxococcus xanthus cultures

For comparison of productivity of metal-spiked and not metal-spiked cultures the following compounds (or peaks) were chosen: myxochromid A3 for CY media, and myxovirescin C and min. 21.8 peak for Pol medium. It is to be observed that the amounts of myxochromid A3 and the compound(s) behind 21.8 peak in Pol medium increase with increase of cobalt concentration within the cultivation media. On the contrary, the peak size of myxovirescin C drops from 5671 mAU*sec to 1404 mAU*sec as the concentration of cobalt in the cultivation media, corresponding to the raw extracts, increases.

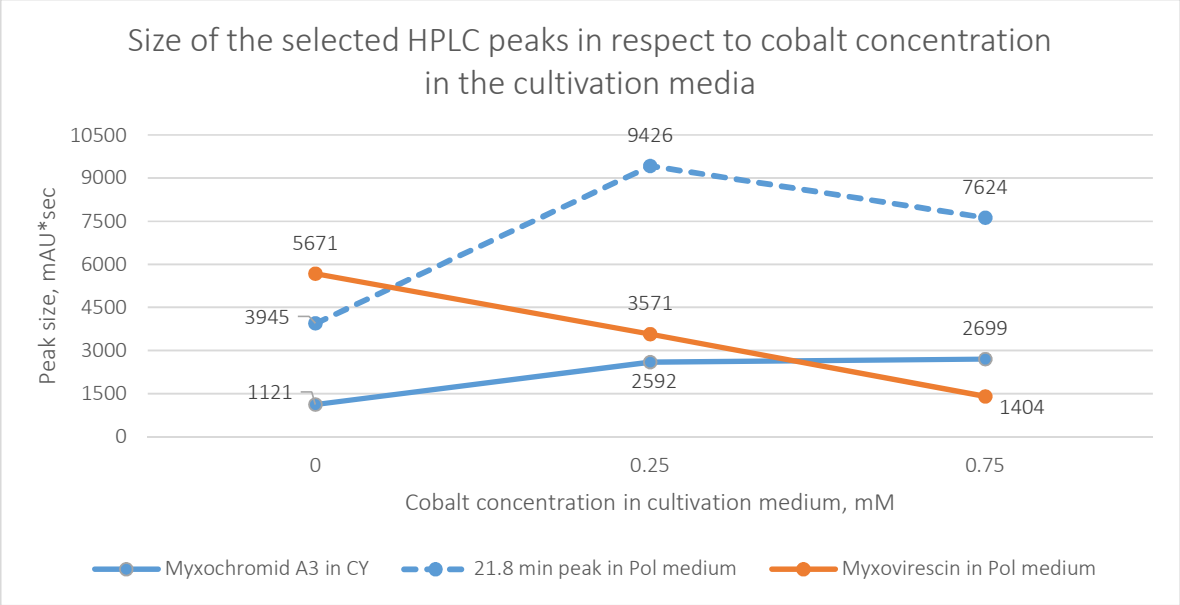


Figure 26 Comparison of Myxococcus xanthus productivity at different cobalt concentrations in respect to several compounds

4.4.3. Labilithrix luteola

Raw extracts of *L. luteola* showed activity against both Gram-positive and Gram-negative bacteria, as well as against fungi (see Table 22). For each group of test organisms, one representative was chosen to perform fractionation test with the most active extract.

Table 22 SDT results of L.luteola extracts

Media	CY							Pol							
Metal conc. (mM)	-	Zn 0.25	Zn 0.50	Cu 0.25	Cu 0.5	Fe 5	Fe 10	-	Zn 0.25	Zn 0.5	Cu 0.25	Cu 0.50	Fe 5	Fe 10	Pb 1
E. coli TolC	B	B	B	A	B	C	C	D	C	C	C	B	C	B	A
S. aureus	C	B	C	C	C	B	C	C	C	D	C	E	E	D	A
M. luteus	C	B	C	C	C	B	C	C	C	D	C	C	D	C	-
M. hiemalis	B	B	A	C	A	C	C	C	B	B	B	B	B	B	A
C. albicans	A	-	-	C	-	C	B	-	-	-	-	-	-	-	-
B. subtilis	C	C	C	C	C	B	B	E	E	F	E	E	G	E	B

Table 23 Fractionation results for the selected Myxococcus xanthus raw extracts

Media	Metal	Inhibited strains	Activity (min)	Identified compound behind peak activity	Identified significant peaks
Pol	control	*E. coli* TolC	19.0	New metabolite (19min)	New metabolite (16.5min)
Pol	FeCl3 5	*B. subtilis*	31.5-32.0; 33.0	-	New metabolite (16.5min) New metabolite (19min)
CY	CuCl2 0.25	*C. albicans*	19.5	New metabolite (19min)	New metabolite (16.5min)

Fractionation of control Pol medium extract (Table 23) revealed inhibiting activity against *E. coli* TolC by a compound, corresponding to the peak at minute 19. Consequent analysis of the compound with HPLC-MS showed, that the metabolite is not recorded in the "Myxobase". The same compound was active against *C. albicans*, as the fractionation showed. *B. subtilis* was not inhibited by the assumed new metabolite. The area of chromatogram, corresponding to the inhibited wells of *B. subtilis*, contained no distinguishable peaks. All fractionated samples, in addition to the new metabolite at minute 19, contained a chromatographic peak at minute 16.5 (see Figure 27), which had very similar absorption pattern. Additionally, chromatography of all raw extracts showed, that some of them contain 3rd unknown peak at minute ~25.5, which absorption pattern also resembles the one of bioactive new compound (minute 19). The following samples contained the 3rd peak (the amounts are given in mAU*sec): CY reference (1418), CY Fe 5 mM (2292), Pol Zn 0.25 mM (2039), Pol Zn 0.50 mM (2274) and Pol Fe 5 mM (1913). Comparison of the productivity in terms of bioactive compound (minute 19) was not possible owing to too big peaks and related technical limitations of the detector.

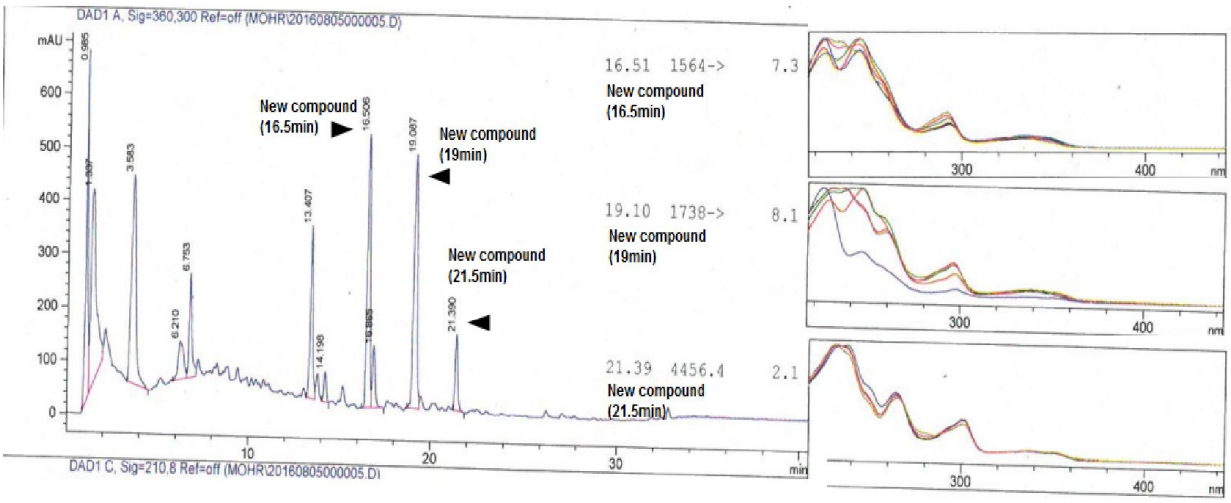


Figure 27 Chromatogram of Fe 5 mM Pol media extract of L. luteola, containing three peaks of unknown compounds with similar absorption patterns

Mass spectrometry of the compound, eluting at minute 16.5 on chromatogram, was not possible because of high contamination of the peak. However, the bioactive metabolite (19min) could be analyzed. Its mass comprised 319 g/mol and the two likely general formulas, suggested based on the mass spectrometry, were $C_{18}H_{38}O_4$ and $C_{19}H_{34}N_4$.

4.4.4. Isolated strain Mx1HS3/1

Similar to the type strain, raw extracts of isolated *Myxococcus xanthus* strain Mx1HS3/1 had inhibiting activity against vast variety of test organisms. The difference from the type strain was that the isolated strain showed no activity against *C. violaceum*, but instead gave inhibition of *C. albicans*. Extracts produced from CY media showed higher inhibition than those from Pol media. Again, three different test organisms were selected for fractionation test with the most active raw extracts.

Table 24 SDT results of extracts from isolated Mx1HS3/1 strains culture

Media	CY			Pol		
Metal conc. (mM)	-	Co 0.25	Co 0.5	-	Co 0.25	Co 0.5
E. coli TolC	E	D	C	B	D	C
S. aureus	F	D	A	A	E	D
M. luteus	F	E	C	B	D	D
M. hiemalis	C	B	B	B	B	A
C. albicans	D	-	-	-	-	-
B. subtilis	E	E	C	C	G	G

Table 25 Fractionation results for the raw extracts of the isolated strain Mx1HS3/1

Media	Metal	Inhibited strains	Activity (min)	Identified compound behind peak activity	Identified significant peaks
CY	control	*M. luteus*	5.5-6.5; 7.5; 20.5-23.0; 28.5; 31.5-32.0; 33.0	5.5-6.5: - 7.5: – 20.5-23.0: myxalamids 31.5 – 32.-0: - 33.0: -	Myxochromid
CY	control	*C. albicans*	19.5	-	Myxochromid, myxalamids
POL	$CoCl_2$ 0.25	*E. coli* TolC	6.0; 25.5	6.0: althiomycin 25.5: fatty acid	Myxochromid, myxalamids

The inhibitions, corresponding to the low retention time regions 5.5-6.5 min and 7.5 min in CY control extract against *M. luteus*, were not fully elucidated. One of the compounds could be althiomycin, which was also present at this retention time in $CoCl_2$ 0.25 mM Pol extract, however, the absorption graphs of this region are rather contaminated and MS could not give reliable results. The inhibition at the late region also could not be correlated with any peaks. However, it was clear by observation of the absorption graphs that inhibition corresponding to minute 20.5-23.0 retention time region was caused by the compounds from the group of myxalamids. Inhibition of *C. albicans* by CY control extract could not be correlated with chromatographic peak.

It was determined that $CoCl_2$ 0.25 Pol extract contained antibiotic althiomycin (see Figure 28) with retention time around 6.0-6.2min, which suppressed growth of *E. coli* TolC. Another compound, responsible for inhibition of the test organism was identified as a fatty acid.

It was not deemed possible to compare sized of althiomycin peaks in different extracts, since they were highly contaminated. Since clear myxochromid peaks (not analyzed with MS) were identified on chromatogram of each raw extract, their comparison is shown in the Figure 29.

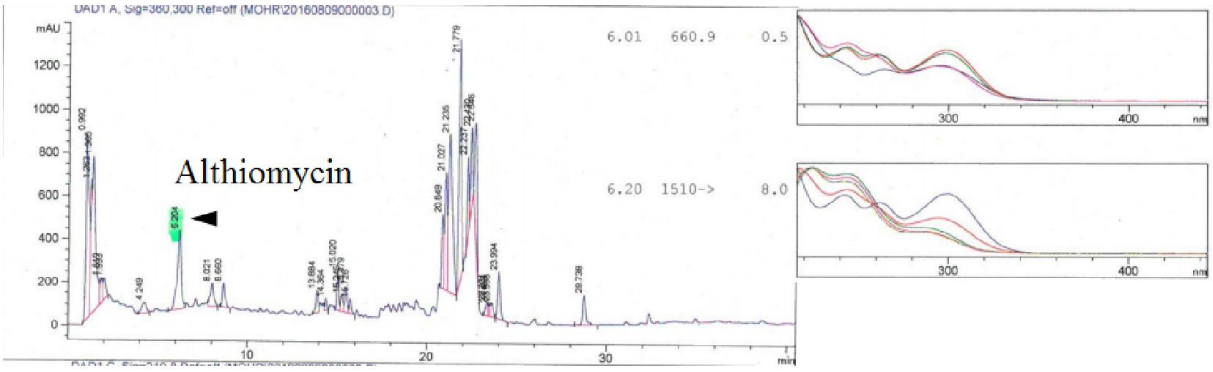


Figure 28 Chromatogram of Co 0.25mM Pol media extract of isolated strain Mx1HS3/1, containing the peak for althiomycin and the corresponding absorption graphs

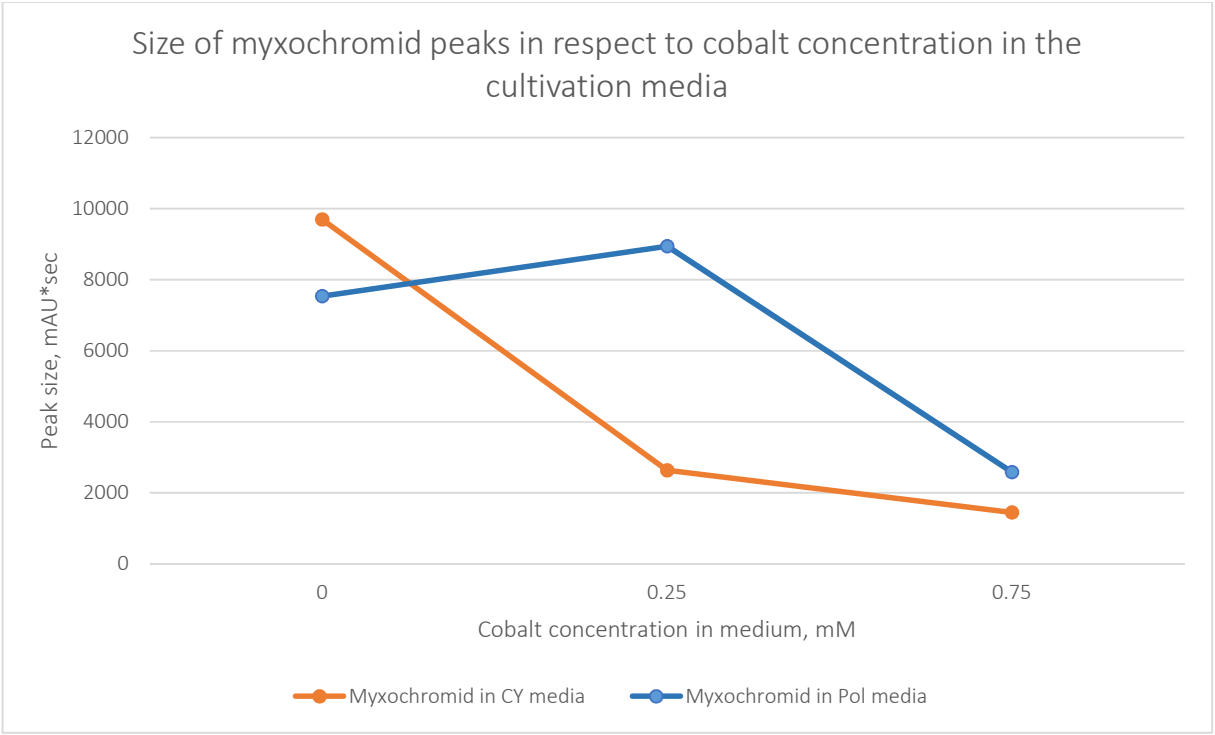


Figure 29 Comparison of isolated Mx1HS3/1 strain's productivity at different cobalt concentrations in respect to supposed myxochromid

4.4.5. Isolated strain CC1ST23

Based on the results of SDTs with raw extracts of CC1ST23 (Table 26), the isolated strains produced bioactive compounds against a number of test organisms. Generally, extracts of POL media, particularly supplemented with zinc, had much higher inhibiting potential against selected organisms than extracts produced from CY broth. One Gram-positive bacteria *B. subtilis* and Gram-negative *E. coli* TolC were selected for peak-activity correlation test with CY control and Zn 0.25 mM POL raw extracts correspondingly.

Table 26 SDT results of extracts from isolated CC1ST23 strains culture

Media	CY			Pol	
Metal conc. (mM)	-	Zn 0.25	Zn 0.5	-	Zn 0.25
E. coli TolC	A	-	A	B	C
S. aureus	A	-	A	D	E
M. luteus	-	-	-	C	D
B. subtilis	D	A	A	F	G

Table 27 Fractionation results for the raw extracts of the isolated strain CC1ST23

Media	Metal	Inhibited strains	Activity (min)	Identified compound behind peak activity	Identified significant peaks
CY	control	*B. subtilis*:	31.5-33.5	-	-
POL	$ZnCl_2$ 0.25	*E. coli* TolC	25.5	Fatty acids	Fatty acid (19.1m)

Fractionation of CY control extract (see Table 27) showed inhibition against *B. subtilis*, which corresponded to the region in the end of the respective chromatogram. However, no corresponding peaks could be identified. Inhibition of *E. coli* TolC by fractionated Zn 0.25 mM POL extract revealed that the bioactive compound(s) of the raw extract were fatty acids. One distinguishable peak with retention time 19.1m (see Figure 30) was also identified as fatty acid by MS and absorption pattern. The sizes of the peaks were significantly different in zinc-supplemented and not supplemented Pol media extract, with the former having much higher concentration of the both compounds (see Figure 31).

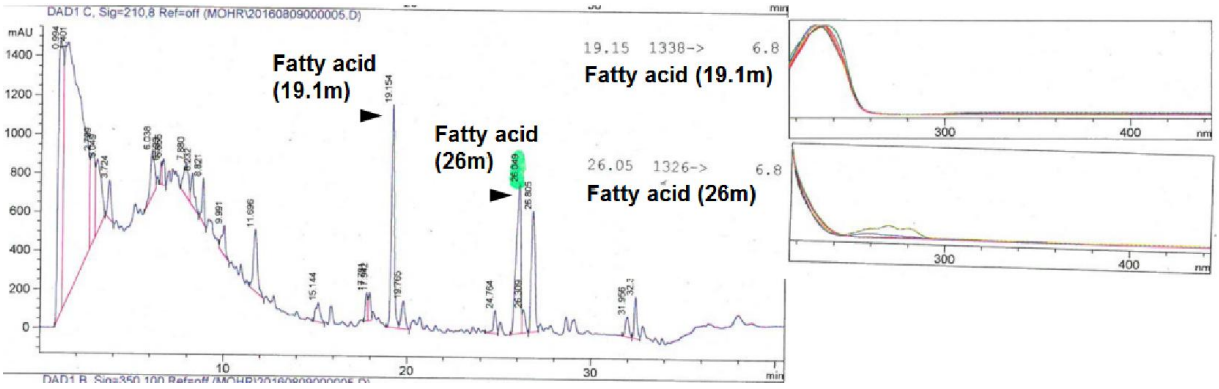


Figure 30 Chromatogram of ZnCl2 0.25 mM Pol media extract of CC1ST23 strain culture, containing two major peaks of fatty acids with the corresponding absorption graphs

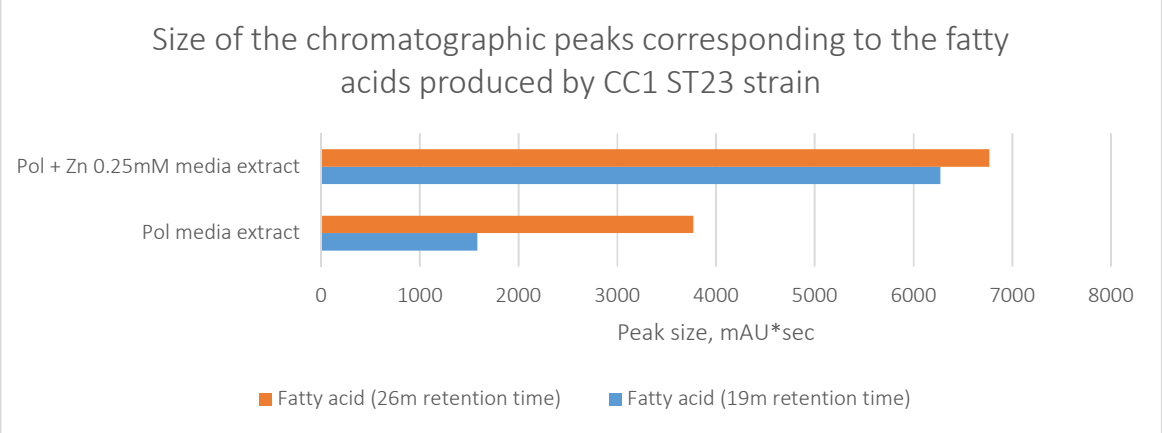


Figure 31 Comparison of peaks, corresponding to fatty acids, produced by CC1ST23 strain in Zn 0.25 mM Pol and Pol control media

5. DISCUSSION

In the following section the results of strain isolation, metal tolerance tests and the effect of metals on production of metabolites are discussed, also in the context of existing literature on these topics.

5.1. ISOLATION OF MYXOBACTERIA FROM HEAVY METAL CONTAMINATED SOIL

Isolation of supposed myxobacterial strains from the soil samples was performed with rather fruitful results. In order to check, whether the samples, which have yield no swarming colonies or fruiting bodies, in fact contain myxobacteria, genetic analysis of the samples could be performed. The composition, origin and metal content of the samples, which produced no myxobacterial colonies, was not found to have distinguishable features from the other samples. It might be speculated that more myxobacteria were isolated from soil samples with pH closer to neutral (>6), however sample Ni-48-2 with pH 4.2 also produced many swarming and fruiting bodies.

The probable reason for small number of strains, which reached pure culture was contamination with Cytophaga-like bacteria or flexibacters, the bacteria which can also form swarms. Also, some other not swarming bacteria could overgrow the myxobacterial swarms and then be transferred from one purification plate to another. It is particularly the case for slow growing *Sorangium cellulosum*, which often contains accompanying bacteria within the slime is one of the most challenging genera with regard to purification.

All four purified cultures were isolated rather easily from fruiting bodies and transferred to full media in maximum 4 purification steps. However, none of the cultures, initially isolated from a swarm was purified, which means they are more prone to contamination than the fruiting bodies, which is in accordance with the literature [26]. One possible explanation for small amount of purified samples is the lack of experience in this process, reflected in picking not the cleanest parts of a swarm or not adhering to sterility conditions.

5.2. HEAVY METAL TOLERANCE TESTS RESULTS

The first thing to mention regarding the heavy metal tests, is that they were not designed to claim statistically significant results. Instead, they were rather an intermediate step to determine the strains, with which to perform further investigations. Also, agar tests results were prone to strong variations, since the growth was very dependent on the amount of living cells by inoculation. This

is the reason why the cultures on reference plates had significantly difference growth in the two separate approaches (concentrations >1 mM and <1 mM). Some records (corresponding to 1mM concentrations) are based only on results from one plate, since the other one showed no growth. This casts doubt on the assumption that all strains, potentially capable of grow at certain higher concentrations, really did so and no underestimation of heavy metal tolerance took place.

One weak point of the experiment design was not testing metal tolerance on CY agar media with concentrations of 0.25mM and 0.75mM, since it was assumed that all strains have better heavy metal tolerance on VY/2 media. For this reason, the results, related to these concentrations, are based only on VY/2 media observations, which could have lead to underestimation of tolerance for some strains. One fault of the experiment was not testing lead and iron tolerance of the self-isolated strains, which could have revealed increased tolerance in comparison to the type strains. Despite the mentioned drawbacks, some hypothesizing can be done based on the obtained results.

It seems clear that none of the tested type strains, as well as no self-isolated strain possess extremely high tolerance, as those common to bacteria isolated from mines or other polluted environments. The inhibiting values for most of the tested strains are in the same order of magnitude as those of common Gram-negative bacteria *E. coli* [60] (see section 2.2.2).

In fact, propagation of a swarm is not necessarily proportional to the growth rates of myxobacteria on agar. This is reflected in the fact that most of the strains had faster colony spreading on VY/2 agar, but grew better in CY broth. One prominent exception from this pattern was lead-supplemented CY agar (also copper-supplemented CY agar in case of *N. pusilla*), on which strains were able to tolerate slightly higher lead concentration than on VY/2 agar. One reason for that might be that since VY/2 is scarcer in nutrients, myxobacteria have to swarm more to obtain sufficient energy for living. However, this process is hindered by lead, which can interfere with external polysaccharides of slime and prevent colony growth. In case of CY media vital swarming is not important at such extend, since the media has higher nutrients content. Thus, as it was seen in the experiments, CY agar culture slowly spreads across agar at high lead concentrations, while VY/2 culture lacks nutrients and dies off.

Extracellular polysaccharides and other excreted compounds seem to play important role in metal tolerance of myxobacteria. Noticeable change of color of myxobacterial slime on lead-supplemented agar and pigmentation of biomass in liquid media could be caused by interaction of extracellular compounds with the metal ions. One observation is that tolerance of *M. xanthus*

to different heavy metals correlates with the potency of its biomass to perform biosorbtion of this ions. In other words, some studies [43, 44, 61], mentioned in the section 2.2.2, claim that biomass of *M. xanthus* is capable of biosorbtion of metal ions in the following order: 1.28 mM of lead/g biomass, 0.36mM of copper/g biomass and smaller value for nickel (not found). This correlates with the observations of heavy metal tolerance tests on agar media, which showed that the type strain can tolerate 1 mM of lead, 0.25 mM of copper and no tested concentration of nickel. If this is true, than some strains, which have greater tolerance towards some heavy metals (e.g. *N. pusilla* towards copper) might be good candidates for biosorbtion studies.

In respect to *M. xanthus*, another noteworthy observation is that it was the only species (both type strain and the self-isolated strain), which could tolerate relatively high concentrations of cobalt (0.75mM), in comparison to other strains. It is not clear, what mechanism is behind this unique tolerance for myxobacteria. It might be related to the cobalt‑zinc‑cadmium efflux transporter, revealed by mining of *M. xanthus* genome (see section 2.2.2), or as well be caused by produced extracellular compounds.

For another classic myxobacterial species, *Sorangium cellulosum,* low heavy metal tolerance is an apparent pattern, found during the tests. This issue, however can be further investigated by choosing other media, which contain carbohydrates as the energy source (glucose-containing CK6 or cellulose-containing CEL3 [26]). This might trigger production of external enzymes, which could contribute to metal tolerance. Considering the rich metabolom of *S. cellulosum* such attempts may bring fruitful results.

No increased tolerance of myxobacterial strains, isolated from heavy metal contaminated soils, was detected by the tests. One reason could be that with the strains isolated from the HS3/1 sample, which is contaminated with mercury, no tests with this metal were performed and thus the tolerance was not revealed. However, the strain CC1 from ST23 sample, contaminated with zinc, showed no growth at even relatively small concentrations of this metal, which is a clear sign of lack of acquired tolerance (unless error was made). One reason for that could be that usually gene clusters, responsible for metal tolerance, are located at plasmids and overwhelming majority of myxobacteria are not capable of bearing and replicating non-chromosomal genes [87]. Also, low tolerances of isolated strains from heavy metal contaminated soils arise question, whether these bacteria were growing in vegetative form in the soil or it were fruiting bodies/myxospores, which were not able to live normally at high metal concentrations.

Another interesting observation is that for example, isolated Mx1HS3/1 strain has low (<0.25 mM) tolerance towards copper and zinc in comparison to the *Myxococcus* type strain. This might be a sign of variability of tolerance between different strains of one species.

5.3. Secondary metabolites production investigation

There are two major imperfections, related to production of extracts, which might have affected the consequent results and implications. The first one is that the amount of cells, used for inoculation of different liquid media, was subject to variations. The reason was that the density of the culture was not measured owing to the features of myxobacterial growth, which do not easily allow exact measurements with conventional techniques.

Another issue was different growth rates of metal-spiked and not metal-spiked cultures. As it was shown by the metal tolerance tests on agar, at the concentrations, found to be acceptable for growth, the development of myxobacterial colonies was still suppressed, in many cases significantly. To tackle this issue, lower concentrations were chosen for cultivation of metal-spiked cultures. However, it was still apparent that many strains grew less rapidly in the media with metals, which is the factor affecting the final concentrations of metabolites in broth and thus in the analyzed extracts. Keeping this in mind, it may be safely assumed, that if the amount of a secondary metabolite in an extract of metal-spiked culture is higher, than in reference extract, then it is due to increased productivity, since the amount of cells in metal-spiked culture was always smaller.

In possible further experiments for investigation of some revealed patterns it would be suggested to pick even lower concentrations of heavy metals to allow better comparability of the reference media with metal-supplemented parallels. Also, smaller steps between concentrations could be used to investigate the possible patterns.

Further in this section strain-specific issues are discussed and the results are examined in the context of existing literature.

5.3.1. Nannocystis pusilla

Production of family of siderophores called nannochelins by some *N. pusilla* strains is a well-known phenomenon and the structures of the three compounds within the group - nannochelin A, B and C have been elucidated. There minor antimicrobial activity against several Gram-positive bacteria has been attributed to their ability to bind iron from the environment [88]. The performed SDTs

with *N. pusilla* extracts have also shown inhibition of Gram-positive bacteria, but consequent fractionation did not relate this affect to nannochelins, which might be owing to their low concentration in the corresponding wells of the fractionation plate. Only nannochelin A was identified, while another peak with slightly higher mass and retention time could be attributed to nannochelin complex with metal or some other compound, which could explain messy graph of UV absorption.

The previous studies suggested that low iron levels in cultivation media do not trigger higher production of nannochelins and media with standard metal content was used to produce these compounds. Indeed, as shown by the conducted tests, high concentrations of iron in CY media leads to absence of nannochelin A in the produced raw extracts and as a result only putative nannochelin complex was present at rather low amounts (Figure 23). In POL medium nannochelins were not identified owing to very small chromatographic peaks. However, at concentration of copper 0.50mM putative nannochelin-metal complex emerged on a choromatogram. This might suggest, that nannochelins are not only responsible for iron transport to the cells, but also for protection against high concentrations of other metals, such as copper. In fact, such alternating role of some siderophores is a known phenomenon [47].

5.3.2. Myxococcus xanthus

All of the identified secondary metabolites of type stain *Myxococcus xanthus* DSM16526 are already rather well studied. They were present in both metal-spiked and not metal-spiked cultures. Thus, no new metabolites seem to be induced in this strain with cobalt. Interesting observation is that production of myxochromid A3 might be increased by addition of cobalt to the cultivation media. Myxochromid A3 is a myxobacterial lipopeptide pigment, which is present in the majority of *Myxococcus xanthus* strains [40] and which structure is well elucidated [89]. No antimicrobial activity was identified for the compounds. Since biosynthesis of some pigments of myxobacteria involves complex regulatory circuits [90], it might be that cobalt plays some role in the processes (see the section 2.2.2 on possible role of cobalt in light sensitivity of some myxobacteria). On the other hand, one study showed 23 fold increase in production of myxochromide A in iron-depleted media [91], which might as well mean that production of this compound is a response to stress conditions.

In respect to myxovirescin, it is not clear, whether cobalt reduces the production of this antibiotic, or it is general inhibition of growth at high concentrations of cobalt, which resulted in lower amounts of the compound in the extract. Another myxobacterial metabolite to focus on would be

myxochelin A - iron-chelating compound [92], which also has some antimicrobial and antitumor properties. No information was found in the literature, whether this siderophore is able to perform defense mechanism against high heavy metal concentrations. This compounds was not identified on the chromatograms owing to restricted timeframe.

5.3.3. Labilithrix luteola

Owing to the fact that *Labilithrix luteola* is a recently discovered species (see section 2.1.5) and no screening for bioactive metabolites was previously performed, it was not unexpected to find two new myxobacterial metabolites. The further steps to elucidate the exact structures will be cultivation of the strain in bigger volumes (10 liters) to produce greater amount of the metabolite, its purification and consequent analysis with spectroscopy, followed by elucidation of cytotoxicity of the compound and its exact activity spectrum. Presence of two (or three in some cases) peaks on the chromatograms with similar UV absorption patterns might either mean 2 (or 3) different derivatives of the same base structure, or breakage of the main compound due to exposure to media components, light or to other factors. Connection between the emergence of the 3rd peak and the heavy metal presence in the media could be a subject of investigation, once the structures of all three derivatives are elucidated.

5.3.4. Isolated strain Mx1HS3/1

Interesting aspect about this isolated wild strain was production of althiomycin – antibiotic with very wide antibacterial spectrum against both Gram-positive and Gram-negative bacteria [93]. Only a small portion of strains *M. xanthus* strains are able to produce this compound [40]. Unfortunately, production of this antibiotic could not be compared between metal-spiked and not metal-spiked cultures owing to unsuitability of the used HPLC settings. According to SDTs results, however, no major difference in inhibition of Gram-positive test organisms was observed between different raw extracts. Comparison of productivity of the wild strain and the type strain, which also produced althiomycin would be another thing to discover.

The decrease of myxochromid content with increase in cobalt concentration in cultivation media is an unexpected trend, which is opposite to the results of the type strain observations. It might be attributed to an error in the experiment, or to some adaptation of the wild strain towards constant presence of heavy metals in the environment, which might take a form of decreased pigment production. Absence of myxovirescin A in the secondary metabolites' extracts of the wild strain can be attributed to the fact, that production of this compound is not inherent to all strains (39 out of 98 tested in the study of [40]).

5.3.5. Isolated strain CC1ST23

There are currently known two families of bioactive secondary metabolites, produced by *Corallococcus coralloides* strains – corallopyronins [94] and corallorazines [95]. Neither one of them was found in the isolated wild strain CC1ST23. Inhibition of *E. coli* TolC by certain unidentified fatty acid is not an unusual thing, since some fatty acids are known for their antimicrobial properties against both Gram-negative and Gram-positive bacteria [96], as well as against fungi [97]. Increased production of the fatty acid in zinc-supplemented POL medium can be the reason for the higher antimicrobial activity of its raw extract in comparison to the one of the control POL medium. Increased production of the fatty acid might be attributed to a mechanism of protection. One study has shown increased ω-Cyclohexyl undecanoic acid production by zinc-tolerant bacterium in Zn-enriched medium [98]. To determine, whether it is the case with the isolated *Corallococcus* strain, further investigation with higher sample size could be performed.

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7. LIST OF ABBREVIATIONS

Abbreviation	Meaning
A. baumannii	*Acinetobacter baumannii*
AMR	Antimicrobial resistance
B. subtilis	*Bacillus subtillis*
BLAST	Basic Local Alignment Search Tool
bp	Base pair (in DNA)
C. albicans	*Candida albicans*
C. velatus	*Cystobacter velatus*
C. violaceum	*Chromobacterium violaceum*
DAD	Diode array detector
DNA	Desoxyribunukleinsäure (desoxyribonucleic acid)
dNTP	Deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GBF	Gesellschaft für Biotechnologische Forschung
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography - mass spectrometry
HZI	Helmholtz Centre for Infection Research (Helmholtz-Zentrum für Infektionsforschung in german)
JSRM	JumpStart™ Taq ReadyMix™ by Sigma
K. flava	*Kofleria flava*
L. luteola	*Labilithrix luteola*
M. hiemalis	*Mucor hiemalis*
M. hiemalis	*Mucor hiemalis*
M. luteus	*Micrococcus luteus*
M. xanthus	*Myxococcus xanthus*
MD department	Microbial Drugs department, HZI
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MSC working group	Microbial Strain Collection working group, HZI
N. pusilla	*Nannocystis pusilla*

NCBI	National Center for Biotechnology Information
P. aeruginosa	*Pseudomonas aeruginosa*
P. aeruginosa	*Pseudomonas aeruginosa*
P. anomala	*Pichia anomala*
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase-chromatographie
S. amylolyticus	*Sandaracinus amylolyticus*
S. aureus	*Staphylococcus aureus*

8. LIST OF FIGURES

9. LIST OF PICTURES

10. LIST OF TABLES

11. APPENDIX

Schlegel vitamin solution:

Component	Amount per 100ml of distilled water
p-Aminobenzoate	1.0mg
Biotin	0.2mg
Nicotinic acid	2.0mg
Thiamine-HCl x 2 H2O	1.0mg
Ca-pantothenate	0.5mg
Pyridoxamine	5.0mg
Vitamin B12	2.0mg
Distilled water	100.0ml

Stan21 agar medium composition:

Before media autoclaving		After media autoclaving, per liter of solution
Solution 1, per liter of solution	Solution 2, per liter of solution	1 ml Vitamin solution
57.6g Bacto Agar	3.2g KNO3	2ml Levamisol
3.2 g K_2HPO_4	3.2g MgSO4 7H2O	200µl Soraphen
6.4ml 1% Yeast solution	0.32g MnSO4	500µl Cycloheximid
	1.08g FeCl	
	3.2ml Spurenelementlösung	
	3.2g CaCl2	

Water agar medium composition

Before media autoclaving, per liter of solution	After media autoclaving, per liter of solution
16 g Bacto Agar	1 ml Vitamin solution
1,5 g $CaCl_2$	(2ml Levamisol)*
1,5 g $MgSO_4$	(200µl Soraphen)*
11.5g HEPES	(500µl Cycloheximid)*
pH 7.2	
	* components only to the special antibiotic-containing modification of the media

Composition of used full cultivation media

Before autoclaving, per liter	After autoclaving, per liter
CY	
18 g Agar 3 g Casiton 1 g Yeast extract 11.9 g HEPES 1 g $CaCl_2$ pH 7.2	
VY/2	
18 g Agar 5 g Baker's yeast 11.9 g HEPES 1 g $CaCl_2$ pH 7.0	0.50 mg Vitamin B_{12}
POL	
3 g Probion 3 g Starch 2g $MgSO_4$ 0.5 g $CaCl_2$ 11.9 g HEPES	

pH 7.2	
Cy+H	
Mixture of two media: 0.5 l Cy medium 0.5 l H medium	0.50 mg Vitamin B_{12}
H	
2 g Soyamehl 2 g Glukose 8 g Starch 2 g yeast extract 1 g $CaCl_2$ 1 g $MgSO_4$ 11.9 g HEPES 8 g Fe-EDTA pH 7.4	
EBS	
5 g Peptone Marcor 5 g glucose 1 g meat extract 1 g yeast extract 11.9 g Hepes pH: 7.0	
MYC	
10 g Phytone Pepton 10 g Glukose 11.9 g Hepes pH 7.0	

TAE buffer composition, per liter:

242 g TrisBase

57.1 mL glacial acetic acid

100 mL of 500 mM EDTA

pH 8.0

List of metal-supplemented media, which pH has to be adjusted.

Metal salt	Concentration, mM	Media	pH
$FeCl_3$ $6H_2O$	5	Cy	6.52
$FeCl_3$ $6H_2O$	10	Cy	4.22
$FeCl_3$ $6H_2O$	5	vy/2	5.62
$FeCl_3$ $6H_2O$	10	vy/2	3.56
$CuCl_2$ 2 H_2O	10	Cy	6.10
$CuCl_2$ 2 H_2O	5	vy/2	6.40
$CuCl_2$ 2 H_2O	10	vy/2	6.20

The raw results of HPLC analysis, 16S rRNA sequencing, soil samples analysis, metal tolerance tests, SDTs and fractionation are stored on the digital supplement (CD disk).