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Marine myxobacteria as a source of antibiotics

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Abstract

Terrestrial myxobacteria have been deeply researched over last three decades and are known for production of bioactive substances. However, there is much less compounds known from marine myxobacteria and thus it is an interesting field for the search of antibiotics. In the study three marine myxobacteria strains, namely *Enhygromyxa Salina, Pseudenhygromyxa salsuginisis* and *Haliangium ochraceum* were cultivated and screened for bioactive secondary metabolites. The strains were successfully cultivated and the most effective media for their growth was determined. *Enhygromyxa Salina* showed the best growth in VY/2 SWS*, Pseudenhygromyxa salsuginisis* in E.S. and *Haliangium ochraceum* in VY/2 SWS. All strains were analyzed for bioactive compounds with serial dilution tests and HPLC-MS. The analysis revealed interesting results for *Pseudenhygromyxa salsuginisis.* To date, no substances from this strain were known, however it was discovered that *P. salsuginis* grown in VY/2 and E.S. media can produce enhygrolides. It is known that *E. salina* can produce this compound, which was also proved in this study. Also, *H. ochraceum* demonstrated production of haliangicin in this study. Haliangicin is a typical substance for *H. ochaceum*, thus it is a known substance from *H.ochraceum*. One more marine myxobacterial strain which was obtained for investigation was *Plesiocystis pacifica.* However, due to its slow growth and limitation of time frame of this Bachelor work, the strain had to be excluded from the experiments.

The second task of this study was to isolate myxobacteria from marine soil samples. Out of 33 samples obtained from HZI storage, nine strains managed to be isolated. The strains were identified with sequencing of 16SrRNA gene and nine strains could be divided into two dominant groups. These were terrestrial myxobacteria *Myxococcus fulvus* (halotolerant) and *Myxococcus xanthus.* The strains were screened for production of bioactive substance and only known myxalamid variants were discovered.

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Introduction

Myxobacteria are exceptional Gram-negative, rod-shaped bacteria and belong to the delta proteobacteria, order Myxococcales [1]. They have a unique life style which distinguishes them from the other prokaryotic organisms. Under unfavorable nutrient conditions vegetative cells are able to aggregate in mushroom-like cell colonies, called fruiting bodies, in which numerous vegetative cells develop to dry-resistant myxospores. These spores can be reactivated from soil samples on suitable solid media even after decades [1].

One interesting feature to mention is that myxobacteria are able to glide over surface. Bacterial gliding is a process whereby a bacterium can move under its own power. This process does not involve the use of flagella. The gliding mechanism is poorly understood, but one of the reasons could be related to the unusual structure of the cell wall [1]. In some parts of the cell wall peptidoglycan was found, this could be explanation of the flexibility of their cell wall. Also, it is known that gliding occurs only in presence of a surface, and the cells often glide against one another in masses rather than individually. Sometimes single bacteria can extend ahead of others at the colony margin, but they soon stop moving unless they are joined by other cells [1].

Myxobacteria can be divided in two groups, based on their nutritional requirements: two genera, *Sorangium* and *Byssovorax*, are able to utilizing cellulose, whereas all other genera are predators. Predation means establishing direct cell-cell contact with a prey (microorganisms like bacteria or fungi). Killing by myxobacteria involves secreting antibiotics to terminate the cells of the prey. After that, special enzymes are produced and lyse the killed cells [2].

The most interesting feature of myxobacteria is their enormous potential to produce various secondary metabolites, many of them with bioactivity. Among the described myxobacterial secondary metabolites approximately 30% have antibiotic activity [3]. Due to increasing resistance developments of many pathogenic bacteria, new antibiotics are urgently needed, which means that myxobacteria as well as other natural producers of bioactive metabolites like fungi or actinobacteria [3], are of high interest for medical research.

Terrestrial myxobacteria have been investigated over several decades and are known for their unique structural properties of their secondary metabolites which make them highly attractive for drug discovery[4]. There are valuable compounds discovered from these bacteria which revealed significant amounts of hits against infectious and non-infectious diseases. For instance, epothilone is one of the examples of these bio-active compounds. A derivative of epothilone – trade name ixabepilone - was approved as a drug for the treatment of breast cancer[3].

The very first step in the developing process of a new naturally produced antibiotic is the isolation and cultivation of the producing organism. It turned out that especially new species, genera and families are reliable sources for new secondary metabolites. Therefore, beside the establishment of innovative isolation techniques also new, neglected habitats can be a source for the isolation of new myxobacterial groups[3].

It is known that myxobacteria colonized a wide variety of environments. Soil, bark of trees, animal dung and plant detritus are the most typical habitats for these microorganisms[4]. But also marine environments a reliable sources for new myxobacterial genera like *Haliangium ochraceum* and *H. tepidum*[5]., *Plesiocysti spacifica* [11]., *Enhygromyxa salina*[6]. and *Pseudenhygromyxa salsuginis* [11], all from the Nannocystineae-suborder. All species were discovered from the coastal regions of Japan. However, it is hard to investigate marine species due to the fact that their isolation and cultivation is more difficult comparing to the terrestrial myxobacteria[1]. The main obstacle is the slow growth with the consequence that myxobacteria can be easily overgrown by other faster growing bacteria. Another problem is that marine myxobacteria cannot be cultivated in rich media which results in poor cell density[7].

To check the potential of the above mentioned marine myxobacteria to produce secondary metabolites they were screened for novel polyketide synthases (PKS) and/or non-ribosomal peptide (NRPS) genes *in silico* and these analyses revealed that marine strains also have the capability for the production of new polyketides [19].Peptides and polyketides are compounds often with bioactivity which could be isolated from myxobacteria in various forms. It is reasonable to expect that marine strains, as well as terrestrial, are an excellent source for new bioactive small molecules[5]. For example the fungicide haliangicin, and haliamide could be isolated from the marine myxobacterial genus *Haliangium* [5]. Salimabromide, salimyxins and enhygrolides, all with

weak anti Gram-positive activity, were described from *Enhygromyxa salina* [6]. Up to now, no substances are described from *Pseudenhygromyxa salsuginis* and *Plesiocystis pacifica*[11].

The search for myxobacteria over the last three decades has led to the isolation of strains from different environments, producing hundreds of new compounds. And one obvious prerequisite for discovering new strains is the ability to grow the bacteria under laboratory conditions[3]. One aim of this study was to cultivate marine myxobacteria from various marine soil and plant samples with the emphasis to establish the cultivation of marine myxobacteria in the working group MISG, HZI.

The second task was the screening of several marine myxobacteria, namely *Haliangium ochraceum*, *Pseudenhygromyxa salsuginis* and *Enhygromyxa salina* for production of bioactive compounds with antibiotic potential. The hitherto neglected strain *Plesiocystis pacifica* could not be investigated within this study because of weak growth. For this promising strain suitable growth conditions with sufficient cell mass production has to be found in the future.

Theoretical background

Classification of marine myxobacteria can be done according to salt requirements for growth [8]: (I) halotolerant strains which are able to grow under conditions of high salinity; and (II) halophilic bacteria which are unable to grow without sea salt. Bacteria of group first may be obtained from halotolerant terrestrial myxobacteria whose myxospores had been washed into the marine environment and adapted to grow with sea salt, and bacteria from the second group, which may be of truly marine origin[8].

Since the beginning of studies on myxobacteria, the isolates from marine habitats were thought to be halotolerant terrestrial myxobacteria [9]. *Myxococcus fulvus* strain HW-1 (Picture 1) is a typical halotolerant myxobacterium obtained from coastal samples. In this case, salt concentration not only affects growth of *Myxococcus fulvus*, but also benefits myxobacterial motility systems as well as fruiting body formation [10], whereas, in the absence of salt both capabilities were diminished.
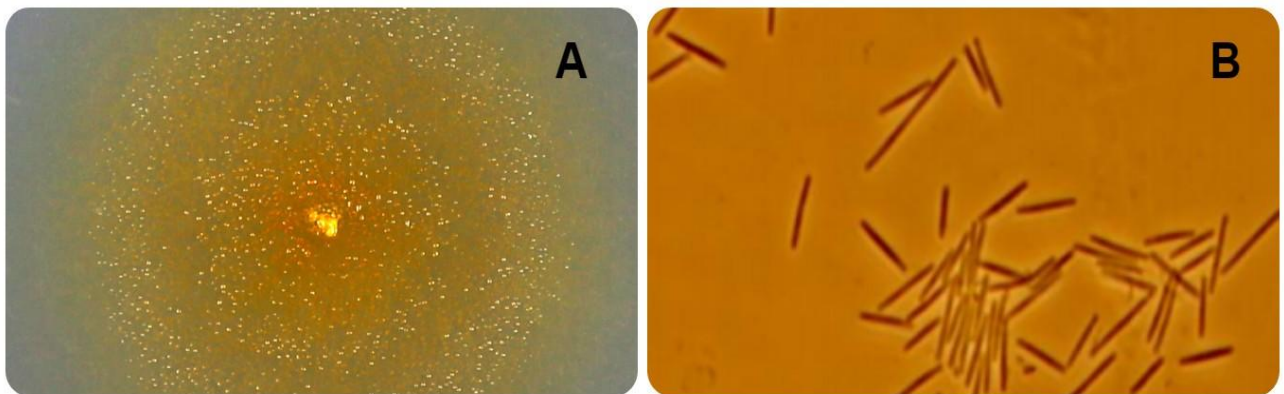
Picture 1. Colony and cell morphology of *Myxococcus fulvus* on agar (A) and under microscope (B)

The opinion that myxobacteria are absent in marine habitats prevailed until Fudou and Iizuka [11-14] discovered and described the first halophilic myxobacterial genera which required strictly sea-like salinity conditions for its growth, namely *Enhygromyxa*, *Haliangium*, and *Plesiocystis*, all members of the Nannocystineae suborder (Picture 2). Few years later, Iizuka and co-workers discovered a further genus of marine myxobacteria: *Pseudenhygromyxa.*
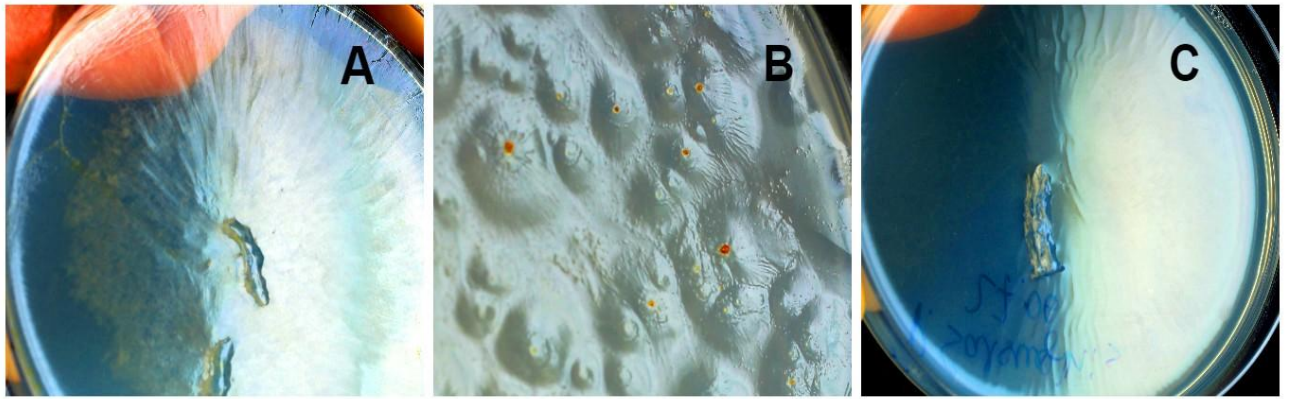
Picture 2. Example of growth on agar of (A) *Haliangium ochraceum*, **(B)** *Enhygromyxa salina*, **(C)** *Plesiocystis pacifica*

The following sections summarize features of four marine bacterial taxa clustered in the suborder Nannocystineae that were used in this study: *Haliangium, Enhygromyxa, Plesiocystis,* and *Pseudenhygromyxa*. The description of strains includes information from the literature about strain isolation, culture conditions, phylogeny, and secondary metabolites. This chapter also includes description of the techniques of molecular biology and analytical chemistry used in this study, namely, polymerase chain reaction, high-performance liquid chromatography, mass spectrometry and gel electrophoresis.

The genus *Haliangium*

The genus *Haliangium* comprises two species, namely *H. ochraceum* and *H. tepidum*[15]. The first halophilic myxobacteria isolated from an ocean by Fudou in 2002 were *Haliangium ochraceum* and *H. tepidum*. Until now no related myxobacteria have been found in terrestrial environments. *Haliangium* is rod-shaped and moves by gliding, like all other known myxobacteria. The cells are pale yellow and approximately 0.5-0.6 μm in diameter and 4.0-4.5 um in length [15].Recently, the 9.4 Mb genome of *H. ochraceum* was completely sequenced and published and therefore this is the first marine myxobacterium, which full genome has been determined [16].

H. ochraceum and *H.tepidium* were isolated from sea weed and sea grass samples, respectively, obtained from a sandy beach in Japan and by Fudou et al. in 2002 [11]. The optimal parameters for the species growth under laboratory conditions were salt concentrations between 2 to 3% NaCl, pH of 7.5. Yeast media with artificial seawater solution was used for cultivation. Due to its salt requirements, the species *H. ochraceum* and *H. tedium* were proposed to be indigenous marine myxobacteria. *Haliangium* was intensively screened for production of bioactive secondary metabolites [5]. One antibiotic isolated from *H.ochraceum* is haliangicin, which was the first myxobacterial metabolite of true marine origin. One interesting fact to mention is that Fudou discovered direct dependency of NaCl on production of this antibiotic [17]. The optimal salt requirements for production of haliangicin are the same as for optimal growth: 2–3% NaCl. Haliangicin revealed antibiotic activity against some fungal organisms such as *Aspergillus niger* and *Fusarium* species [15].

The genus *Enhygromyxa*

Only one species, *E. salina* is described within the genus *Enhygromyxa* so far. All *Enhygromyxa* strains isolated to date are halophilic and therefore this genus can be considered to be limited on marine environments. In 2003 Lizuka published the report on isolation, characterization and taxonomic classification of six strains of *E. salina*[13]. These myxobacteria were isolated respectively from mud, sand and algal samples collected in marine environments around Japan. Few years later, four additional strains of *E. salina* were isolated by the group of König from marine-intertidal sediment samples collected at the West Coast of the USA, at German coasts, and the Netherlands [18,19]. One may safely conclude that *Enhygromyxa* species are widely distributed throughout the world.

E.salina dwells in a wet or moist habitat. Cells are straight rods with rounded ends. Spherical myxospores with diameters 0.5–0.7 µm are formed. In the same way as terrestrial myxobacteria, *E. salina* cells move by gliding and tend to form fruiting bodies and myxospores. Fruiting bodies are brownish-orange. Optimal salt concentration for growth is between 1–2% NaCl (w/v),optimal pH is between 7.0–8.5 on yeast medium and optimal temperature is 28–30 °C [13].

Some *Enhygromyxa* strains are able to produce polyketide synthases (PKS)-type metabolites [19]. PKSs are a family of multi-domain enzymes or enzyme complexes that produce polyketides, a large class of secondary metabolites, which often possess pharmacologically important properties, involving antimicrobial and antifungal properties. To date, it is reported that *E. salina*SWB007 produces five structures of PKS metabolites classified as salimabromide [18, 20], as shown in Table 1.

The genus *Plesiocystis*

Two strains of *Plesiocystis pacifica*, the only described species of the genus *Plesiocystis*, were discovered from a sandy sample of a Japanese coastal area and from a piece of dried marine grass by Iizuka in 2003 [12]. Optimal parameters for growth of the strains is 2–3% NaCl (w/v) and a pH of 7.4 on yeast medium with artificial seawater solution at 28 °C. Cells of strains are rod-shaped and move by gliding. The strains are chemoheterotrophic and strictly aerobic. Up to date, no reports were published concerning biological testing of extracts or any metabolites isolated from the strains [12].

The genus *Pseudenhygromyxa*

*Pseudenhygromyxa*also consists of only one species, *P. salsuginis.* This genusand speciesis the most recent discovered halotolerant myxobacterium, and was published in 2013 by Lizuka [21]. *P. salsuginis* was isolated from mud samples of an estuarine marsh in a coastal area in Japan and termed SYR-2T. The strain showed ability to grow in the absence of salt, however, the optimal parameter for growth is 0.2–1.0% NaCl (w/v) and pH values about 7.0–7.5 on CY-S agar (bactocasitone,bacto yeast extract) between 30–35 °C [21].

Comparison of 16S rDNA sequences revealed that *P. salsuginis*SYR-2T showed only 96.5% and 96.0% identity to *Enhygromyxa salina* SHK-1T and *Plesiocystis pacifica*SIR-1T respectively [21]. Until now, no reports on metabolites derived from *P. salsuginis* were published. Because in the past it

turned out that especially new families, genera and species of myxobacteria are reliable candidates for new secondary metabolites, an intensive screen of *P. salsuginis* under various conditions is promising. Up to now, no genome sequence has been published, no analyses of PKS/NRPS-cluster, which could indicate the potential of this strain to produce secondary metabolites, is not possible at the moment [21].

Table 1. Metabolites reported to date from myxobacteria grouped into the suborder *Nannocystineae* and their known metabolites [21]

Genus and species	classification according to salt requirements for growth	known metabolites (references)
Haliangium	moderately halophilic	haliangicin [5] haliamide [5]
Enhygromyxa	halophilic	salimabromide [6] enhygrolide A, B [6] salimyxin A, B [6]
Plesiocystis	halophilic	no metabolites described
Pseudenhygromyxa	halotolerant	no metabolites described

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was developed in 1983 by biochemist Kary Mullis, who later was awarded with the Nobel Prize for his work. PCR is a unique method used in molecular biology to amplify selected section of DNA. It is a common technique used in medical and biological researches [22].

In PCR reaction five core components are used, namely: template DNA, two primers (forward and reserve), which match at the ends of a particular region of the template DNA which has to be copied, deoxynucleotide-triphosphates (dNTPs), the building blocks for construction of new DNA-

strands, Taq polymerase enzyme to add in new DNA nucleotides, and buffer solution to ensure right conditions for PCR [22].

The process includes three main stages: denaturing, annealing and extending. During denaturing stage the double-stranded template DNA is heated up to 94-95°C to separate the double strands into two single strands. The separation of strains occurs due to breakage of hydrogen bonds under high temperature. At this stage it is important that temperature is maintained high enough to ensure the complete separation [22].

Next stage is annealing, the temperature drops down to 50-56°C to enable attachment of DNA primers to a specific location on the single-stranded DNA template. The annealing temperature of primer is specific and depends on the GC-content of the primer and its length. The value is mentioned by the manufacturer when the primer is ordered or can be calculated online, for example with the annealing calculator provided by Thermo Fisher. The primers are designed to be complementary in sequence to specific regions of DNA on each end of sequence, which has to be amplified. Thus, primers are starting points for DNA synthesis; once primers bound with DNA, then enzyme is attached and new complementary strand of DNA can be synthesized. As a result, two separated strands of DNA are complementary and run in opposite directions [23].

Final step within a cycle is extending, the increasing of temperature results in a new double-strand DNA. Due to increased heat to 72°C, the optimal temperature for the Taq enzyme, the latter adds DNA nucleotides, and this way a new DNA-strand is synthesized. All three stages are repeated in cycle 20-40 times, each time the numbers of copies of DNA is doubled. The scheme of PCR process is presented in Picture 3 and Picture 4. After PCR is completed, electrophoresis is used to check the quality and quantity of produced DNA fragments [23].
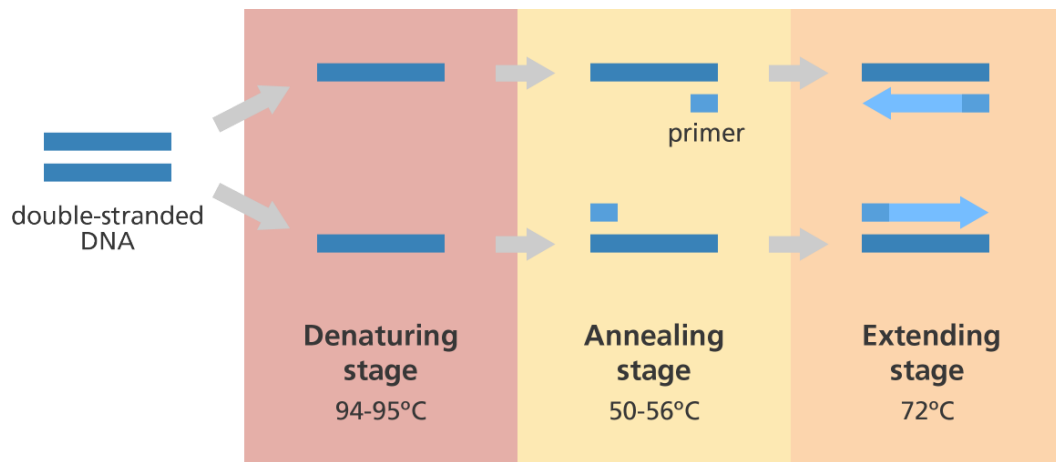
Picture 3. Main steps in polymerase chain reaction (PCR) [23]

cycle 1   cycle 2   cycle 3
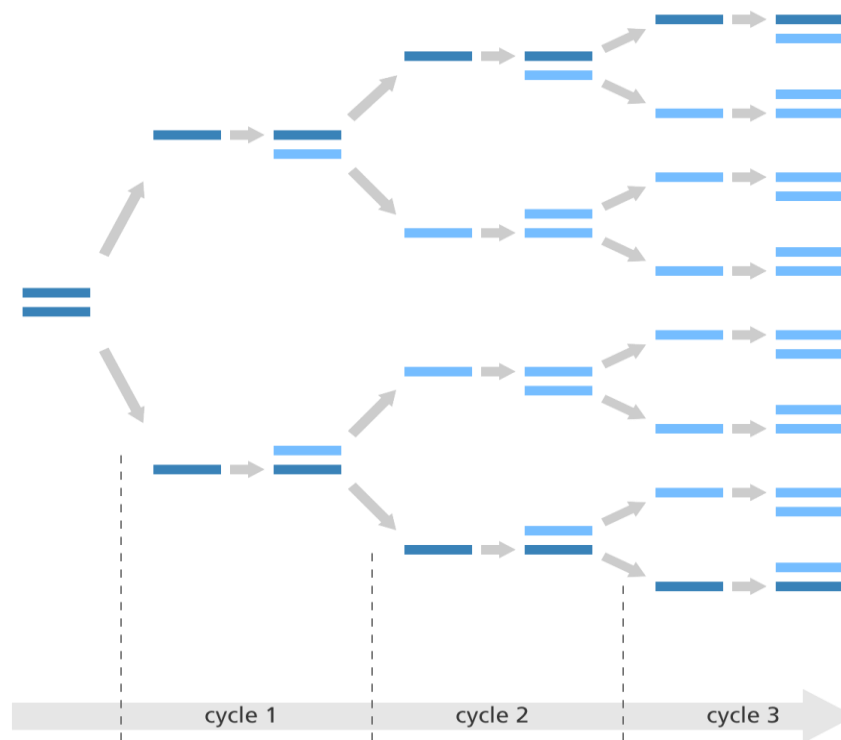
Picture 4. The scheme of production lots of DNA copies in polymerase chain reaction (PCR) [23]

Gel electrophoresis

Gel electrophoresis is a technique used to separate charged molecules like DNA, RNA and proteins in relation to their size. The method is based on the movement of charged molecules through a gel
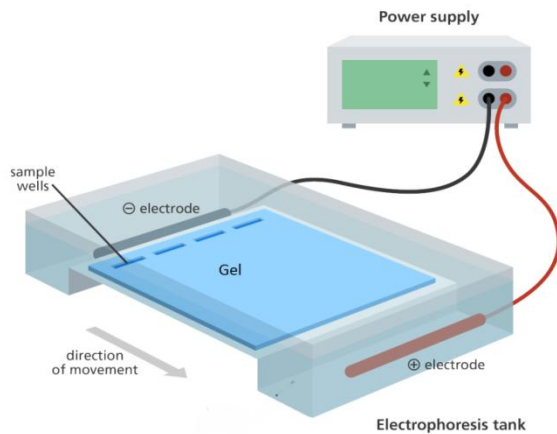
Picture 5. Gel electrophoresis [24]

under an electrical current. The current is applied across the gel, one end of the gel has negative charge and the other positively charged [24]. The gel consists of sieve-like matrix, thus molecules can move through. Migration of molecules occurs towards the opposite charge. As DNA is negatively charged, the products in the wells move from the cathode to anode. Once all DNA migrated far enough, so the trajectory of movement can be clearly distinguished, the electrical current is switched off (see Picture 5) [24].
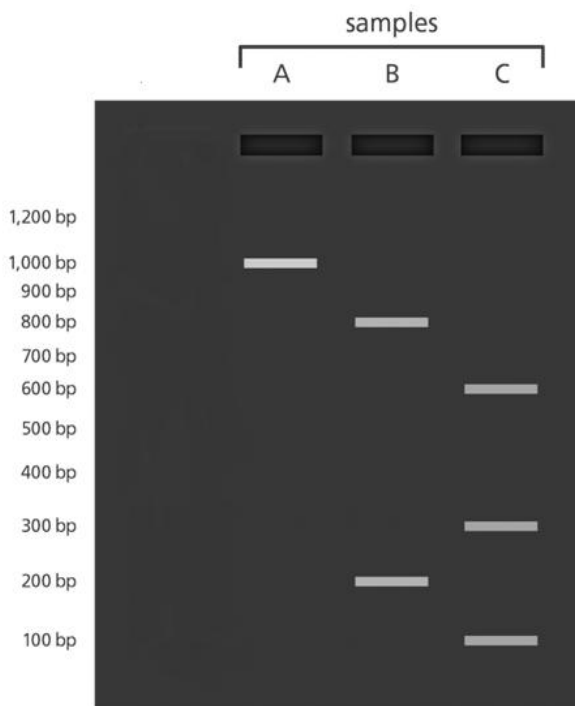
Figure 1. Illustration of DNA bands separated on gel [25]

To visualize the results and to weight the PCR product to prevent diffusion out of the gel pockets, a dye is added to the DNA before pipetting the samples in the pockets. After the migration is finished, the gel is removed and placed into ultraviolet illuminator which will reveal stained DNA as bright bands (Figure 1). It is possible to judge the size of DNA according to the distance which the molecule overcame. Large molecules move slower than smaller molecules, and thus migrate shorter on distance [25].

Role of 16S rRNA gene in phylogenetic analysis of bacteria

16S ribosomal RNA is a component of 30S subunit of prokaryotic ribosomes. It has several functions itself, but the major interest to this molecule is due to the features of its coding gene. First of all, the gene is present in almost all bacteria [36]. Secondly, the function of this gene is the same in all bacteria, and the existing differences in this gene are random in nature and accumulate

with time [37]. The rates of mutations in the 16S rRNA coding gene are also rather low, and it consists of conserved as well as of variable regions. It was quickly determined that conserved regions at the beginning and the end of the 16S gene were perfect binding sites for universal primers, which could be used for amplification and subsequent sequencing of almost the whole 16S gene (about 1500bp, depending on the species). In addition, in public databases (NCBI) hundreds of thousands of 16S rRNA-gene sequences are deposited and can serve as reference material for identification of self-isolated strains, for example, at least on the genus level [38]. With these reference sequences in addition to the sequences of own isolates it is also possible to calculate phylogenetic trees to determine the phylogenetic position of an unknown strain with appropriate computer programs. But it has to keep in mind, that reliable phylogenetic analyses are only possible with full length 16S rRNA-gene sequences of perfect quality and that for several genera (*Bacillus* spp., *Myxococcus* spp.) further house-keeping genes have to be analyzed for reliable classification [37]. All of these make 16S rRNA gene a perfect genetic marker in phylogenetic of prokaryotes. Currently recommended similarity threshold values for classification of bacterial isolates are 95% for genus and 97 % for species, however, these values do not serve as an ultimate criteria and caution should be exercised [38].

There is a number of universal primers, both forward and reverse, which are referred to by the position on *E. coli* gene, which they match. The primers, used in the Bachelor thesis are given in Table 2.

Table 2. List of primers applied to myxobacterial 16S rRNA gene throughout the study [39]

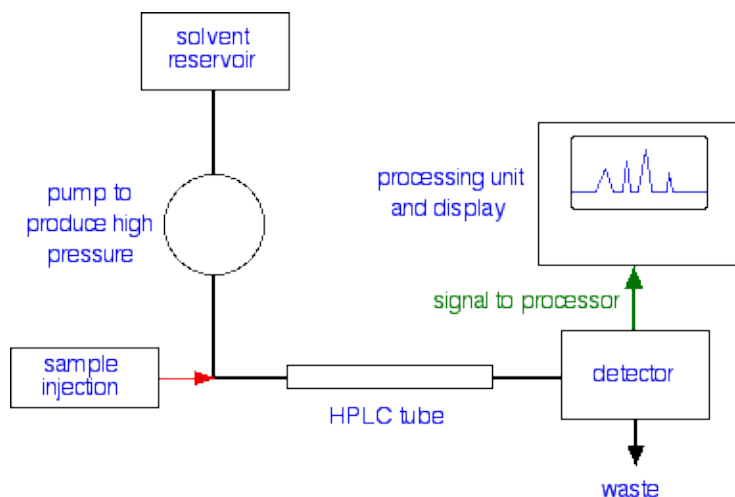| Primer	Sequence (5'-3')
16F27	AGAGTTTGATCMTGGCTCA [39]
16S1100R	GGGTTGCGCTCGTTG [40]
16R518	GTATTACCGCGGCTGCTG G[39]
16R1525	AAGGAGGTGWTCCARC[39]

High-performance liquid chromatography

Figure 2. HPLC flow scheme

High Performance Liquid Chromatography (HPLC) is a separation method of great importance to the chemical, pharmaceutical and biotechnological industry [26]. Figure 2 represents the flow scheme of HPLC. The principle is that a sample with one or many substances is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped under pressure through the column. The separation of substances is based on differences in rates of migration through the column arising from different affinity of the substances to the stationary and the mobile phases [26]. Depending on the affinity behavior of the different types of substances, these will elute at different times from the column outlet. In the MISG-group a method is established which turned out to be suitable for the detection and separation of most bacterial secondary metabolites. The mobile phase consists of a gradient (acetonitrile/water) starting with high concentration of water (polar) and increasing concentration of acetonitrile (nonpolar), resulting in an early elution of polar substances and increasing elution of nonpolar substances, like fatty acids [26]. At the point of elution a detector determines flow of separated compounds, which is typically done by analyzing the change in UV light absorption in case of organic compounds. The time required for a compound to travel through the column and reach the detector is known as retention time, which is under appropriate settings unique for each analyzed compound. For further analysis of the eluted compounds, particularly their mass and possible structure, mass spectrometry is used [26].

Mass spectrometry detector

Mass spectrometry (MS) is an analytical tool used for determination of mass of one or more molecules present in a sample. For organic molecules, mass spectrometers can be used to identify

unknown compounds via molecular weight determination, to quantify known compounds, and to determine structure of molecules by comparing its spectrum to other known substances in a database[27].

Normally MS is preceded with separation of a mixture into pure substances with HPLC. After that separated compounds are directed to MS module (see Figure 3). Within the MS they are bombarded with electrons to get the charged ions. Then the ions are accelerated by magnetic or electric field and are send towards detector. The time of flight or trajectory of molecules depends on the mass of the ion. This time is recorded and the mass of the ion is calculated. During bombarding there could be fragmentation of ions, these fragments have weights less than the original molecule. Their occurrence is also recorded and thus mass spectrum of compound is formed, where the ion with highest mass is the original molecule. This pattern of fragments with different mass is unique for each molecule and thus allows identification [28].
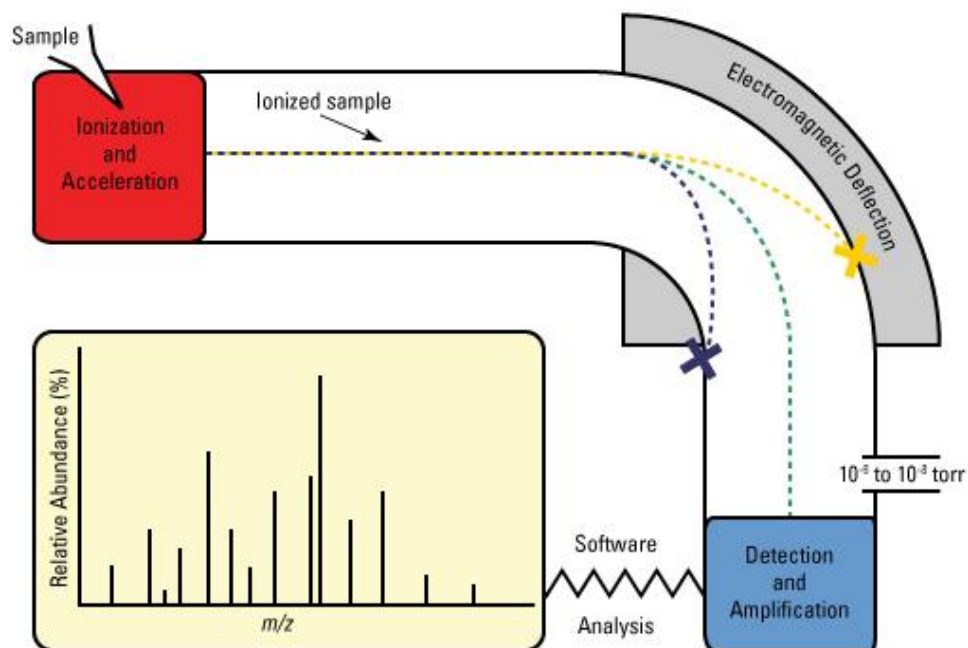
Figure 3. The scheme of trajectory of the sample, from its injection to generation of mass spectrum [28]

Methods

In this chapter the description of the methods which were performed during the laboratory work are presented. The described methods can be divided into two sections. In the first part type strains of marine myxobacteria were cultivated and screened for production of bioactive secondary metabolites. In the second part, isolation of myxobacteria from marine soils, their identification and analysis of strain's potential to produce antimicrobial compounds was performed.

Activation of strains from the collection

In order to test type strains of marine myxobacteria for production of antimicrobial metabolites, three different myxobacteria of the Nannocystineae-family were chosen (Figure 4). The list of selected species can be seen in Table 3. Microbiological cultures were activated from the internal strain collection (MISG/HZI) cryo-conserves, then upscaled and used for production of raw extracts which were subsequently tested for bioactivity in serial dilution tests against various test organisms. Species-specific full and synthetic media for marine myxobacteria were prepared and used for cultivation.

Table 3. List of marine myxobacteria species taken from HZI internal storage

Genus/species	DSMZ
Enhygromyxa salina	15217
Pseudenhygromyxa salsuginis	14622
Haliangium ochraceum	14365
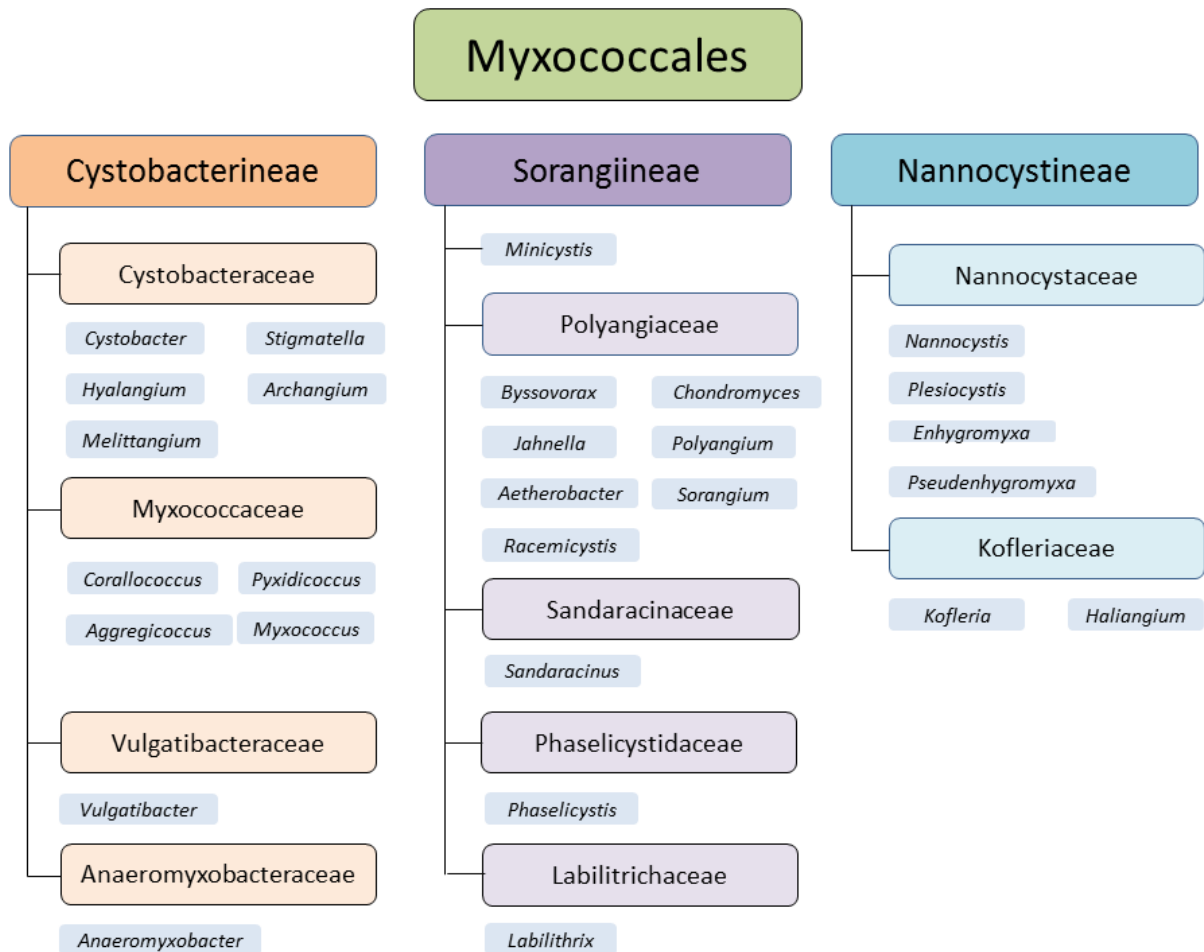
Figure 4. Phylogenetic tree of Myxococcales (picture provided by Corinna Wolf, HZI)

Preparation of full media

For the cultivation of marine myxobacteria different liquid media were used: VY/2-SWS, CY-SWS and *Enhygromya sasalina*-medium. The composition of all employed media is given in Table 4 below. After preparation of media, the broth was distributed to 250 ml glass flasks, 100ml per each, and autoclaved. After autoclaving vitamin B_{12} was added to each flask according to the recipe under sterile conditions.

For the agar media, the same recipe as for liquid media was used, with addition of Bactoagar (15 g/L). Media were also autoclaved for 20 minutes at 121℃. After autoclaving agar media were also supplemented with vitamin B_{12} according to the recipe and used to fill Petri dishes.

Table 4. Composition of different media used for cultivation

Media composition (per liter)			
Sea water solution (SWS)	CYSWS	VY/2 SWS	E.S.
8.0gMgSO$_4$·7H$_2$O 1.0gCaCl$_2$·2H$_2$O 0.5gKCl 0.16gNaHCO$_3$ 0,02gH$_3$BO$_3$ 0,08gKBr 0,03gSrCl$_2$ 10mg Ferrum(III)- citrate 1ml Trace elements solution 0,01gGlycerophosphat- 2Na	3gBactoCasitone 1gBacto Yeast Extract 20g NaCl	20ml Backer yeast 20gNaCl 15gBacto Agar	0,02g Ferric iron citrate 20mgH$_3$BO$_3$ 80mgKBr 30mgSrCl$_2$·6H$_2$O 2ml Trace elements solution 0,20gCaCl$_2$·2H$_2$O 1,60gMgSO$_4$·7H$_2$O 0,10gKCl 10gNaCl 0,50g Na-kaseinate 2gHEPES
dissolvedin 1L H$_2$O	Dissolved in 1LSWS	Disolved in 1LSWS	Dissolved in 1Ldistilledwater
	pH 7.5	pH 7.3	pH 7.2
	50mg/l B$_{12}$after autoclaving		

Activation and upkeepingof strains from collection

Three different strains of marine myxobacteria (Table 4) were obtained from internal collection of Microbial drugs working group (MISG), HZI, cryo-conserved liquid form (-80 °C). The strains were reactivated from the conserves on 20 ml agar plates and in 20 ml flask with liquid media. Both liquid and agar cultures were incubated at 30 °C with constant shaking at 160rpm.

When the liquid cultures were well-grown, they were upscaled to 100 ml in the appropriate media and subjected as working- and backup-culture by transferring 10 ml of the youngest culture into fresh 100ml medium with the same media. The procedure was done weekly. Thus, there were always two flasks, backup culture and the freshest one. This step was needed to avoid contamination and to support presence of grown culture. The cultures on the agar plates were also renewed regularly, depending on the individual growth of the strain. The upkeeping was done by transforming a small piece of the agar with the freshest culture onto a new agar plate with the same media.

Isolation of myxobacteria from marine soil samples

The isolation procedure was implemented using conventional myxobacterial isolation methods used in the MISG-group. The procedure involves several steps: isolation of culture on solid agar plates and repetitive series of purification steps until pureness of the culture is achieved. Pure culture is then transformed in liquid medium, 3x2ml are stored at -80 °C as cryo-conserves and the liquid culture is used for further investigations.

Selection of marine soil samples

Overall 33marine samples, collected in the intertidal area, were selected for the isolation procedure. All the samples were originating from different locations around the world (mostly Germany) and were provided to MISG by employees and scientific partners. The samples were dried at room temperature and then kept at 20 °C until use. The samples represent different types of marine soil and objects such as ooze, clay, sand, shells, sea grass and wood pieces. In Table 5, the areas and dates of collection are listed.

Table 5. List of samples used for isolation of myxobacteria

Sample	Area of collection	Date of collection	Sample	Date of collection	Date of collection
1303	Australia,S16°43'35''; E145°38'49'', Ellis Beach	November 2012	1897	Spain, Teneriffa, tidal Area	November 2015
1368	Germany, Döse, mud flat	October 2013	1923	Germany, Sylt, saltmarsh	March 2016
1369	Germany, Döse, mud flat	October 2013	1924	Germany, Sylt, Morsum cliff	March 2016
1370	Germany, Döse, Beach	October 2013	1925	Germany, Sylt, Morsum cliff	March 2016
1371	Germany, Döse, mudflat	October 2013	1926	Germany, Sylt, Morsum cliff	March 2016
1372	Germany, Döse, Beach	October 2013	1927	Germany, Sylt, Beach	March 2016
1373	Germany, Döse, mud flat	October 2013	1928	Germany, Sylt, Beach	March 2016
1555	Denmark; Saedding, Beach	August 2014	1929	Germany, Sylt, Beach	March 2016
1559	Germany, Sylt, cliff	August 2014	1930	Germany, Sylt, Beach	March 2016
1560	Germany, Sylt, cliff	August 2014	1932	Germany, Sylt, Beach	March 2016
1614	Darß Ort, Düne, sand	October 2014	1933	Germany, Sylt, Beach	March 2016
1685	Spain, Teneriffa, Playa Pareiso, beach	April2015	1942	Germany, Sylt, Beach	March 2016
1797	Denmark, Houvig, beach	August 2015	1943	Germany, Sylt, Beach	March 2016
1850	Netherlands, Texel, dried bladder wrack	September 2015	1991	Germany, Sylt, Beach	March 2016
1851	Netherlands, Texel, driedalgae	September 2015	1995	Germany, Sylt, Beach	March 2016
1852	Netherlands, Texel, dried bladder wrack	September 2015	1999	Germany, Sylt, Beach	March 2016
1895	Germany, Norderney	August 2015			

Preparation of media used for the isolation of myxobacteria

Modified versions of media for standard isolation procedure [45] were used. Seawater agar (instead of water agar used standard procedure) with living *E. coli* smeared in the form of cross on the surface of the plates and seawater-based Stan 21 media with cellulose paper on the agar surface. The seawater for media preparation was taken from the North Sea, Cuxhaven harbor, Germany and stored for no more than two weeks before use at 4 °C.

Sea water media

Basal media – seawater agar, with food organisms (*E.coli*) was used for isolation of bacteriolytic types of myxobacteria. This media contains no external carbon and nitrogen. Thus, *E.coli* served as source of both macronutrients, while most bacteria in environmental samples are not able to digest living *E.coli* cells. Therefore this medium is rather selective for myxobacteria. They can be distinguished by typical swarms and fruiting body development. Seawater as the basis of the media should approximate the natural conditions and enables growth of putative halophilic strains. In addition, cycloheximide, to inhibit the growth of fungi, and levamisol against nematodes were added. The composition of seawater media is shown in Table 6.

Table 6. Composition of seawater media (per liter)

Beforemediaautoclaving	After media autoclaving, per liter of solution
16 g Bacto Agar	1 ml Vitaminsolution
1 L SeaWater	2mlLevamisol
pH 7.2	200µlSoraphen
	500µlCycloheximid

The *E. coli* culture was preliminary cultivated on EBS agar media. First, the plates were incubated over night at 30 °C and stored subsequently at 4 °C. After the seawater media was prepared in Petri-dishes and solidified, crosses of living *E. coli* were smeared on the plates.

Stan 21 media

Stan 21 SWS was also used for screening of halophilic cellulose-degrading myxobacteria. Unlike seawater agar, this medium contains microelements and nitrogen inacceptable for cellulose-degrading myxobacteria form. However, carbon source is also missing and is introduced in form of cellulose paper filters placed onto the surface of plates. This restricts microbial growth only to certain types of microorganisms, mainly some types of myxobacteria and fungi.

In the Table 6, the composition of Stan 21 is shown. First, two different solutions were prepared separately and autoclaved and afterwards mixed and supplemented with vitamins and a set of antibiotics (fungicides, antihelmintic) were added. After the media was prepared, four preliminarily sterilized cellulose papers were put on each agar plate.

Table 7. Composition of Stan 21 media

Before media autoclaving		After autoclaving, per 1L
Solution 1, per 1L	Solution 2, per 1L	1 ml Vitaminsolution
15.76gBacto Agar	3.2gKNO$_3$	2mlLevamisol
3.2 g K$_2$HPO$_4$	3.2gMgSO$_4$7H$_2$O	200µlSoraphen
6.4ml 1% Yeastsolution	0.32gMnSO$_4$	500µlCycloheximid
	1.08gFeCl$_2$	
	3.2ml Trace elements solution	
	3.2gCaCl$_2$	

Isolation and purification of myxobacterial culture

water agar with living *E. coli*   Stan 21 agar with filter paper
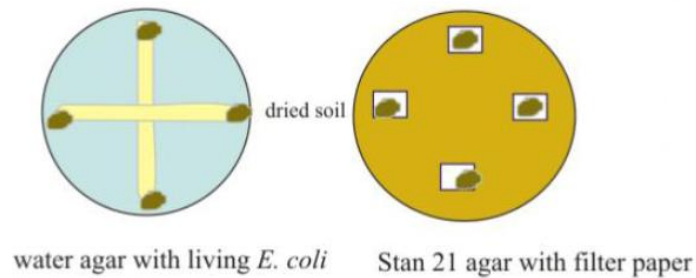
Picture 6. Scheme of sample output for screening of myxobacteria from marine material and sand samples using water agar with living E. coli-cross (left) and Stan21 with cellulose filter (right), (Mohr 2016)

Isolation process started with transferring sample material on the prepared media. For each marine soil sample from the list (Table 5), two plates of sea water agar and two Stan 21 plates were inoculated. Inoculation of seawater agar and Stan 21 media was done by placing a pea-sized marine soil sample onto the endings of a cross with living *E. coli* or onto the pieces of cellulose filters, respectively (Picture 6). One inoculated plate of each media type was then incubated a 30°C and the other one at 18°C.
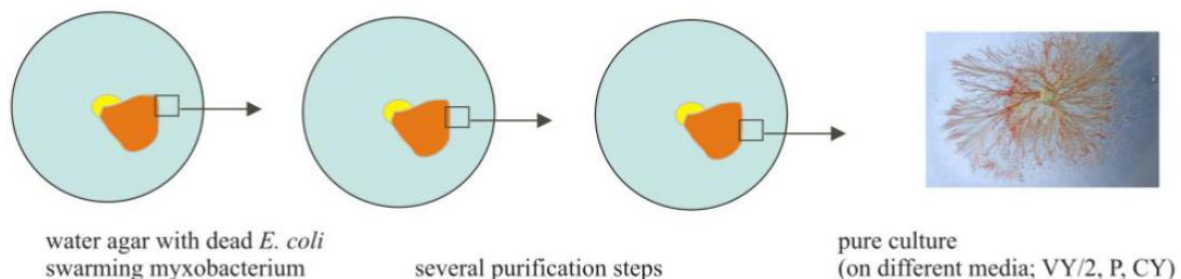
The plates with the soil samples were cultivated 18 ℃ and 30 ℃ degree and observed weekly under a dissection microscope. The occurrence of the myxobacteria was discovered after 2-4 weeks of incubation by the emergence of a swarm, formation of fruiting bodies or degradation of agar or cellulose paper (Picture 7). When the mentioned signs were identified, then the assumed myxobacterial culture was taken for purification.

a) Swarm   b)fruiting bodies   c) degradation of cellulose paper
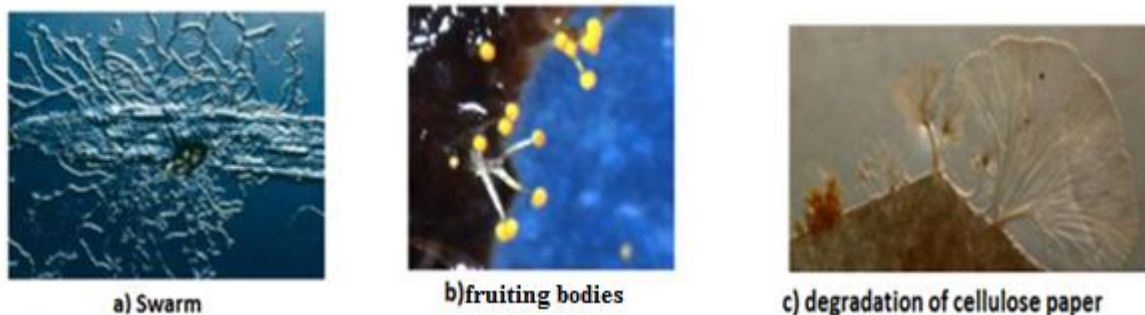
Picture 7. Occurrence of myxobacteria on agar plates (Mohr 2016)

To purify the culture, small piece of the agar, fruiting body or a piece of cellulose paper from the least contaminated areas was picked up with a sterile needle and transferred to the center of a fresh water agar plate with *E. coli* cross (Picture 8).

Picture 8. Scheme of purification steps of myxobacteria culture (Mohr 2016)

water agar with dead *E. coli*
swarming myxobacterium

several purification steps

pure culture
(on different media; VY/2, P, CY)

The procedure was repeated until an axenic culture was obtained. After that, a piece of agar with pure culture was transferred to a new agar plates with full media CY and VY/2. The composition of CY and VY/2 media is given in Table 8.

Table 8. Composition of CY and VY/2 agar media

CY-Agar	VY/2-Agar
18g Agar	20ml baker's yeast
10ml baker's yeast	20gNaCl
11.9gHEPES	15gBacto Agar
1gCaCl$_2$	
pH 7.0	
After autoclaving	
-	0.50 mg Vitamin B$_{12}$

If contaminations could be excluded, a piece of overgrown agar was transformed into 100 ml flask with 20 ml liquid medium and incubated for 2-3 days at 30°C and 160 rpm. CY-H media was used at this step. Purity of broth culture was checked under the microscope. If the culture looked axenic, it was upscaled up to 100 ml in H medium). After one week the grown culture was checked again.

Table 9. Composition of H and Myxovirescin media

H-Media	Myxovirescin– Media
0.2%Soy flour	1.0% Casein Pepton
0.2% Glucose monohydrate	0.005% $CaCl_2$ x 2 H_2O
0.8% Starch	0.025% $MgSO_4$ x 4 H_2O
0.2% Yeast extract (Marcortyp 9000)	1 mg/l $CoCl_2$
0.1% $CaCl_2$ x $2H_2O$	23.8gHEPES
0.1% $MgSO_4$ x 4 H_2O	pH 7.0
11.9 g/l HEPES	
8 mg/l Fe-EDTA	
pH 7.4	

Verifying purity of isolated cultures

In order to determine the purity of the isolated cultures two different techniques were used. First, observation under the microscope was done and then inoculation of selective media. First technique was performed by transferring a drop of liquid culture with an inoculation loop onto a microscopy slide and investigating it with a microscope. Chaotic movement of cells with different sizes and shapes was considered as sign of contamination, and such culture was dismissed because myxobacteria are able to glide over solid surfaces but not to move actively like other bacteria equipped with flagella. If the cells looked similar, the second technique was additionally applied.

To observe contamination possibly not detectable by microscopic observations, the following technique was applied. Agar plates with MYC media (identification of fungi growth) and EBS media (determination of foreign bacteria growth) were inoculated with wire loop by striping a drop of liquid culture on the plate. The plates were incubated over 1-2 days at 30 ℃. Myxobacteria are not able to grow on these media in such short period, thus, if there was growth, the culture was considered contaminated.

Identification of the isolated strains

When the pureness of cultures was ensured, identification of the isolated strains was performed by sequencing a part of 16S rRNA gene. The sequences were compared to data of a public database (NCBI) and revealed the next cultivated relatives. The process of identification involved several steps. At first, genomic DNA was isolated from the liquid culture. Then PCR procedure was performed with primer specific for 16S rRNA genes. The PCR products were checked for quality and quantity with agarose gel electrophoresis. PCR product of good quality were purified and sequenced.

DNA isolation

The identification of newly isolated strains started with genomic DNA isolation. Therefore 1 ml of a grown culture was transferred into a safe-lock tube and centrifuged at 11000 rpm for biomass concentration. The supernatant was removed. The biomass pellet was introduced to the "Invisorb Spin Plant Mini Kit" by Invitekt for DNA isolation. The procedure was done according to the instructions supplied by the kit producer.

PCR

Polymerase chain reaction (PCR) was used to amplify a certain region of chromosomal DNA, namely nearly the whole 16S rRNA gene, which was later used for genus identification. Eubacterial primers F27 and R1525 were used, which resulted in PCR products of about 1498 bp. The components, required for PCR, except water, primer and genomic DNA,as deoxynucleotide triphosphates (dNTP), polymerase enzyme, bivalent cations and buffer solution were provided in correct proportions in "JumpStartReadyMix" by Sigma-Aldrich. For one PCR reaction, 50 µl of all components, presented in Table 10 were required. First, a "master mix" of all components, except sample DNA, was prepared for the determined amount of samples by mixing JSRM, sterile water and the primers. The amount of master mix required was calculated based on the model composition of one PCR mixture of 50 µl. The master mix was distributed to 200µl tubes, 49µl per tube or each. Finally, sample DNAs were added to each tube, while one sample was filled with 1µl water to serve as a negative control to ensure that the mixture of the components was not contaminated with foreign DNA.

Table 10. Composition of PCR mixture and example calculation of master mix

	JSRM	Sterile water	Forward primer concentration	Reverse primer concentration	Sample DNA	Total
Recipe for one PCR reaction, µl	25	22	1	1	1	50
Master mix for 5 samples and 1 negative control	150	132	6	6		294

The PCR reaction involves several steps, namely initialization, denaturation, annealing, elongation, final elongation and final hold. Master cycler gradient device by Eppendorf was used for this process. In Table 11 summary of different steps and corresponding duration of each operation as well as corresponding temperature can be seen. The cycle of denaturation, annealing and elongation was repeated 34 times. After the PCR reaction was completed, the products were stored at 4 °C and subsequently checked by agarose gel electrophoresis.

Table 11. Setting for different PCR steps

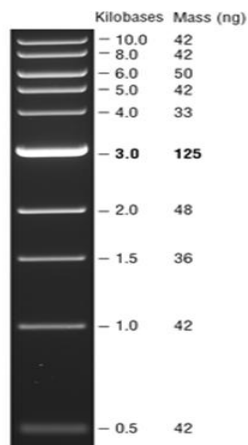|	Steps	Duration, *min*	Temperature, ℃
1	Initialization	5	95
2	Denaturation	0,5	94
3	Annealing	0,5	52
4	Elongation	2	72
5	Final elongation	10	72

Figure 5. Bands of 1 kb DNA Ladder by New England Biolabs

Electrophoresis

In order to check quality and quantity of PCR products gel electrophoresis was performed. Electrophoresis was done twice: after PCRs, and after purification of the product with the PCR clean-up kit. In order to assess the product's quality, one kilobase DNA Ladder by New England Biolabs was loaded as reference on the gel. This ladder can be used to indicate bands of 0.5-10.0 kilobases (Figure 6). Initially running buffer and agarose gel were prepared. 50 x *TAE* (Tris-Acetat-EDTA) was prepared according to the recipe (Table 12). 1 x TAE was used as the running buffer and as well as for agarose gel preparationand the 50 x stock

was diluted with water accordingly.

Table 12. Composition of TAE-buffer

TAE-buffer
(**T**ris-**A**cetat-**E**DTA; 1 L 50 x TAE buffer)
242 g Tris
57.1 ml acetic acid 100%
100 ml 0.5 M Na_2EDTA

For the preparation of agarose gel, agarose powder was dissolved in certain amount of 1 x TAE buffer to obtain 0.8% mass concentration of the former. The mixture was heated up in a microwave (R941INW, Sharp) in order to achieve complete dissolving of the powder. After that, the heated solution was directly used for electrophoresis.

The agarose solution was poured inside of a gel frame. In order to form vertical wells in the gel, a comb for 15 chambers was put into the gel mold. After approximately half an hour the gel was solidified and transferred to the electrophoresis device (PowerPac 300 by Biorad). Then, 1xTAE buffer was poured inside the apparatus, until the gel was entirely covered by the buffer solution. The comb was removed carefully and DNA samples were ready to be loaded into the formed wells.
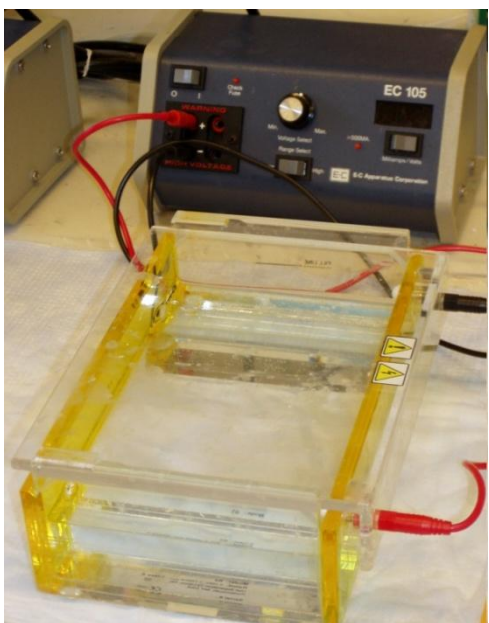
Picture 9. Photo of partly loaded gel inside electrophoresis apparatus

Prior to electrophoresis, all samples, ladders and negative control were mixed with loading dye (Gel Loading Dye, Purple by New England Biolabs). First, a $2\mu l$ droplet of the dye was released on a piece of a wax paper, one for each needed well. After that, $2\mu l$ of DNA solution were dropped onto the dye droplet and thoroughly mixed with a pipette. Then the mixture was transferred into the first well. The same procedure was implemented with the other samples and two ladders.

After all the samples and ladders were loaded and the gel frame was closed with a lid, the electrophoresis started. The time period of the procedure was one hour with the settings of 400mA and 80V. After the procedure was finished, the gel was removed, transferred into UV cabinet and the picture of the bands corresponding to dyed PCR products was done.

PCR product purification

After PCR quality was checked, the obtained product - amplified DNA fragments - had to be purified from the leftover molecules, such as salts and enzymes. Therefore the NucleoSpin® Extract II kit by Clontech Laboratories was used. The main principal of the kit is that the DNA molecules bind to silica membrane and are then washed with different buffers to remove the contaminants.

First, 48μl of sample was mixed with 96 μl of Buffer NT (provided by the kit) and transferred into the tube. A NucleoSpin® Extract II-column provided in the kit was loaded into collection tube. The mixture of buffer and sample was put into the column and centrifuged for one minute at 11000 rpm. DNA bound to the membrane and the filtrate was removed. For washing 700 μl Buffer NT3 (provided by the kit) were put on the column and centrifuged, the filtrate was dismissed. For membrane drying, the tube with the column was centrifuged for two minutes at 11000rpm and filtrate was removed. After that, the column was incubated at 70 ℃ on the incubation shaker to remove ethanol (NT3 Buffer component) from the membrane.

Finally 30μl of Buffer NE from the kit was poured inside the column to elute DNA. The tube was incubated for one minute under room temperature and centrifuged for one minute at 11000 rpm. Purified DNA concentrated in the filtrate was checked with electrophoresis.

Sequencing of 16S rRNA

The sequencing was performed in the Sequencing service of HZI with the Sanger method. The method can be characterized as a combination of PCR and gel electrophoresis. In the first part of the process double-strand DNA is denaturized and the selected primer binds to the known DNA region on one of the strands. After that DNA polymerase performs elongation of the compliment sequence with normal deoxynucleosidetriphosphates (dNTPs). In the solution also modified dNTPs are present, which are able to bind to the foregoing nucleotide, but then terminate the elongation process of the sequence they connect to. These modified dNTPs are also radioactively labeled, and each type of modified nucleotide (dATP, dGTP, dCTP or dTTP) has its own label. Once such nucleotide is adjusted to elongation sequence, a molecule is composed, which length ranges from the length of a primer plus one nucleotide to the length of the whole sequenced DNA. Each formed molecule holds radioactive marker of the final modified nucleotide. After elongation of all molecules is complete, the whole solution is than segregated by size with gel electrophoresis. The radioactive markers of the molecules of different sizes are detected with the sequencing machine. Finally, a chromatogram for the whole sequence is produced, which reflects rate of occurrence of different modified nucleotides at different positions.

Two primers were used with each sequenced sample, namely one forward primer F27 and one reverse primer R518 (Table 2). Each of them would provide information about 500-600 nucleotides. The overlap between the potential sequences was an approach to ensure correctness of the sequencing results. The sequences were obtained as a chromatogram files and processed in BioEditv.7.2.5 program. The most probable nucleotides were automatically assigned to each location. When a portion of chromatogram was not interpretable for the program's algorithm, ambiguity symbol "N" was assigned to these positions. First, the segments ambiguity symbols in the beginning and in the end of sequences were deleted. Then sequences, produced from forward and reverse primers, were aligned and consensus sequence was composed. The resulting consensus sequence was checked and all unclear positions were individually handled with the help of chromatograms.

The "cleaned" consensus sequence was saved in FASTA format and then submitted to Basic Local Alignment Search Tool (BLAST) by National Center for Biotechnology Information (NCBI) and compared to the database of 16S rRNA sequences. The results of the comparison gave a list of strains, which have high similarity of 16S rRNA gene with the submitted sequences.

Production of metabolite extracts

To bind the secondary metabolites excreted by the myxobacteria during the cultivation process in liquid media, the cultures were cultivated with XAD 16 adsorber resin. Addition of XAD has several advantages in comparison to cultivation without XAD: If the bacterium produces a potent antibiotic the strain will not be inhibited by this compound which would concentrate in the medium (feedback inhibition). Furthermore, the produced substances, which in some cases could serve as energy source when nutrients become scarce at the later cultivation phase, are saved. For the type strains *H. ocharaceum, E. salina* and *P. salsuginis* CY-SWS, VY/2-SWS and E.S.-media were used (Table 4). Each strain was cultivated in three different media (100 ml) and about 2 % XAD was added to each flask. The culture was incubated at 30℃ on a shaker at 160 rpm for the period of two weeks. For strains isolated from the soil samples, H-media and Myxovirescin-media were used. The composition of the media is presented in Table 9. Each isolated strain was cultivated in two media with XAD as described above but only for one week period.

To obtain a raw extract the well grown culture (with XAD) was sieved with a mashed sieve to separate the resin from the media. The resin was washed with deionized water and the culture-medium was discarded. The XAD resin, which was left on the sieve after washing, was transferred back inside the same flask with a spatula. After that, approximately 50 ml of acetone was added to the flask and incubated for at least one hour at room temperature.

After harvesting the solvent with XAD was sieved with a filter and dried in a rotary evaporator (Laborota 4003 control by Heidolph Instruments) with a water bath. There acetone intensively evaporated under reduced pressure, however, at the same time the conditions for the preservation of metabolites remained optimal.

After the solvent with XAD was incubated in acetone solution for at least one hour, the liquid culture was filtrated trough paper filter (Type 600P cellulose, pore diameter13 µm, by Roth GmbH) into a round-bottom flask. The flask was attached to the evaporator and the water bath was heated to 40°C before and the pressure was slowly decreasing automatically to prevent boiling of the acetone.

When the acetone was completely evaporated, the pressure was normalized (1 bar) and the flask was removed. To solve the dried extract 1 ml of methanol was added to the flask. The methanol extract was transferred into 1.5mlsafe lock-tube. The final concentration of the raw extract was 1:100. The extract was kept at -20 °C to prevent changes of secondary metabolites.

Picture 13. Rotary evaporator with vacuum system [32]

Analysis of secondary metabolites for bioactivity

With the raw extracts a series of different tests were implemented to discover and identify potentially new products with antimicrobial activity. The analysis involved serial dilution tests against fungal and bacterial test organisms, fractionation of the active extracts by high performance liquid chromatography (HPLC) with peak activity correlation and at finally identification of the mass of active substances by mass spectrometry analysis. If active compounds were discovered, identification of substances was done by comparing mass of the compound with the data from the HZI internal myxobacterial database (Myxobase).

Minimal inhibiting concentration test

Serial dilution tests (SDTs) were used to test raw extracts for presence of metabolites with antibiotic activities. The general idea of the tests was based on performing series of dilution of the extracts with different microbial cultures of the test organisms and hence, to check the presence of metabolites with potential antibiotic activities.

For this step, 20 µl of raw-extracts were used against several Gram-positive and Gram-negative bacteria as well as some species of fungi. In Table 13 below, the list of selected species of test organisms can be seen.

Table 13. List of test organism used in SDS and corresponding DSM numbers

Gram-negative bacteria	Gram-positive bacteria	Fungi
Escherichiacoli DSM1116	*Staphylococcus aureus*	*Mucorhiemalis* DSM 2656
E. coli TolC	*Micrococcus luteus* DSM1790	*Pichia anomala* DSM6766
Chromobacteriumviolaceum DSM30191	*Bacillus subtilis* DSM 10	*Candida albicans*DSM1665
Pseudomonas aeruginosa PA16		

To perform SDTs, 96 well plates (Techno Plastic Products AG) with 8 rows and 12 columns were used. One test strain with eleven raw extracts was tested on each plate. The procedure started with reactivation of bacterial test cultures from the storage -80 °C. Two different media were used: MYC for fungi and Müller-Hinton for bacteria. For every plate 20 ml of media inoculated with test organism from the cryo-conserve was prepared. The amount of organism's conserve used for media inoculation was different depending on the species and its growth rate. After the medium was prepared, the culture was inoculated on a plate. A multichannel pipette was used for this purpose. For the first row 280 µl of culture was added in each well, however for the other rows 150 µl were added. Then the rows from 1 to 11 were filled with 20 µl of the extract.

After that, a series of dilution from row A to row H were implemented with the help of a multichannel pipette. Series of dilution started with the filling of row A, the solution was thoughtfully mixed by pipetting it up and down and then 150 µl of the content of the first row were transferred to row B. In row B the procedure was repeated and 150 µl of the content were taken and transferred to the next row. This step was repeated for all rows, thus the concentration of the content was decreasing from row A to row H. In the final row 150 µl were taken out and dismissed, hence every raw contained equal amount of 150 µl with 8 different concentration of the extract. The procedure was performed for each extract.

After the plates were prepared, they were incubated on a shaker Titramax 1000 (by Heidolph Instruments GmbH) over 24 hours. All the plates were incubated at 30°C except *E. coli* and *E. coli* TolC, which were incubated at 37°C. After one day, the plates were checked to determine inhibition of the growth. The lowest row with no signs of microbial growth was noted, as it represented the lowest "concentration" of raw extract, which had inhibiting effect on the given tested strain.

Fractionation and mass spectrometry

With the raw extracts, which exhibited strong inhibiting activity (at least up to third dilution stage), fractionation was performed with the tested organisms, towards which the extracts were active. For fractionation purposes supernatant, resulting from centrifuging at 13000 rpm, was taken and transformed to Thermofisher HPLC-vial. Aligent 1100 set of devices, which included HPLC pump, autosampler, diode array detector and fraction collector modules, was used. Reverse-phase

chromatography with gradient elution (acetonitrile/water) was applied. The amount of fractionated raw sample was dependent on the strength of its activity against the selected test organism and was either 5 or 10 µl.

Fraction collector was set in a way that every 30 seconds a new well of bioassay plate was fed with eluate. After fractionation, the aeration with nitrogen was applied for about 45 minutes to remove water-acetonitril solvent from the wells. The plates were then inoculated with the selected test organisms, 150 µl of microbial culture pro well. The plates were then incubated for 24 hours at 30 °C or 37 °C, depending on the test organism. The wells, at which inhibition was present after incubation, were correlated with the retention time of the compounds, which were fed to this well. Thus, the chromatography peaks, related to inhibition of a test organism, were identified.

To exactly identify compounds behind the inhibition and to possibly find new compounds, mass spectrometry was applied in conjunction with HPLC. The compounds were first separated with HPLC and then sent to mass spectrometry device. The mass spectrum of separated compounds was found and their masses were determined. Based on the information about retention time, bioactivity, UV absorption graph, mass and mass spectrum, the antibiotic secondary metabolites in raw extracts were identified by comparison of the abovementioned parameters with the internal "Myxobase" database of Microbial drugs department.

Results

In the following chapter the results of conducted experiments are presented. The analysis of the results is then given in the discussion chapter.

## Isolation of myxobacteria from marine soil sample

A total of 33 environmental samples of marine origin (Table 14) were inoculated in each case on two plates of two different media: Stan 21 with filter and seawater media with *E. coli* and incubated at 18 ℃ and 30℃, respectively. On every plate four portions of a sample were placed: on four pieces of filter paper on a Stan 21-plate and at the ends of an *E. coli*-cross. Therefore in total, every sample was spotted 16 times (four spots on four plates each). The plates were observed for myxobacterial swarms and fruiting body-production every few days and after 2-4 weeks, 29 emerged colonies were identified and introduced into the purification process. In Table 14, the results of isolation can be seen. According to the appeared colonies, the best and fastest growth of myxobacteria was at 30 ℃. As is it can be judged from the Table 14 , the majority of isolated strains were originating from seawater media with *E. coli* crosses, rather than from cellulose-supplemented Stan21 media. Therefore, it can be assumed that among the isolated samples, there were more myxobacteria with bacteriolythic nutritional type rather than cellulose degraders.

Table 14. List of samples used for isolation of myxobacteria Stan 21(with cellulose filter); SM (seawater medium with *E. coli*-bait); emerged colonies of myxobacteria

Sample id	18℃ Stan21-1	30℃ Stan21-2	18℃ SM	30℃ SM	Sample id	18℃ Stan 21-1	30℃ Stan 21-2	18℃ SM	30℃ SM
1303					1897				
1368					1923	+	+		+
1369					1924		+		+
1370					1925				+
1371					1926				+

1372					1927				
1373		+		+	1928				+
1555					1929		+		+
1559					1930		+	+	+
1560					1932				
1614					1933		+		+
1685					1942				+
1797					1943	+	+	+	+
1850					1991	+		+	+
1851					1995				+
1852					1999		+		+
1895									

From 29 colonies introduced to the purification process and suspected to be members of the order myxococcales, nine strains could be purified within the time frame of this Bachelor thesis. All of these purified strains showed typical myxobacterial behavior like swarming and fruiting body formation, as shown exemplarily in Picture 10(A and B). Due to these morphological features, it could be assumed that all nine strains can be assigned to *Myxococcus* genus. The remaining 20 strains could not be purified, even after several purification steps due to high bacterial or fungal contamination. A culture was considered to be contaminated if the fruiting body or swarm had "milky" color, as seen in Picture 10, C.
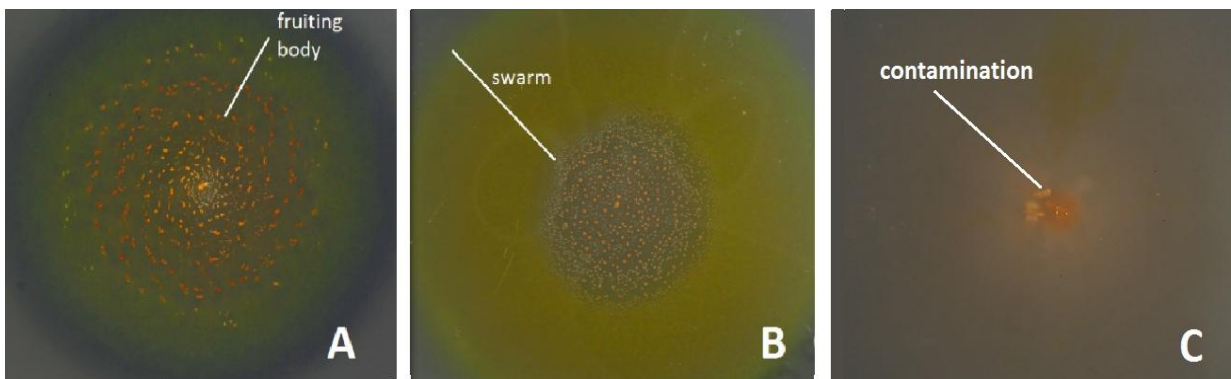
Picture 10, Cultures of isolated strains on agar plates, full media A) formation of fruiting bodies B) swarm C) contamination of culture (pictures were obtained during this study).

Identification of isolated myxobacteria by amplification and sequencing of the 16S rRNA gene

From all isolated myxobacteria, genomic DNA was extracted and a PCR for amplification of the 16S rRNA gene was conducted. PCR products as well as purified PCR products were checked for quality and quantity on agarose gels (Picture 11 A and B). As seen on the agarose gels isolation of myxobacterial DNA and subsequent PCR procedures were successful.
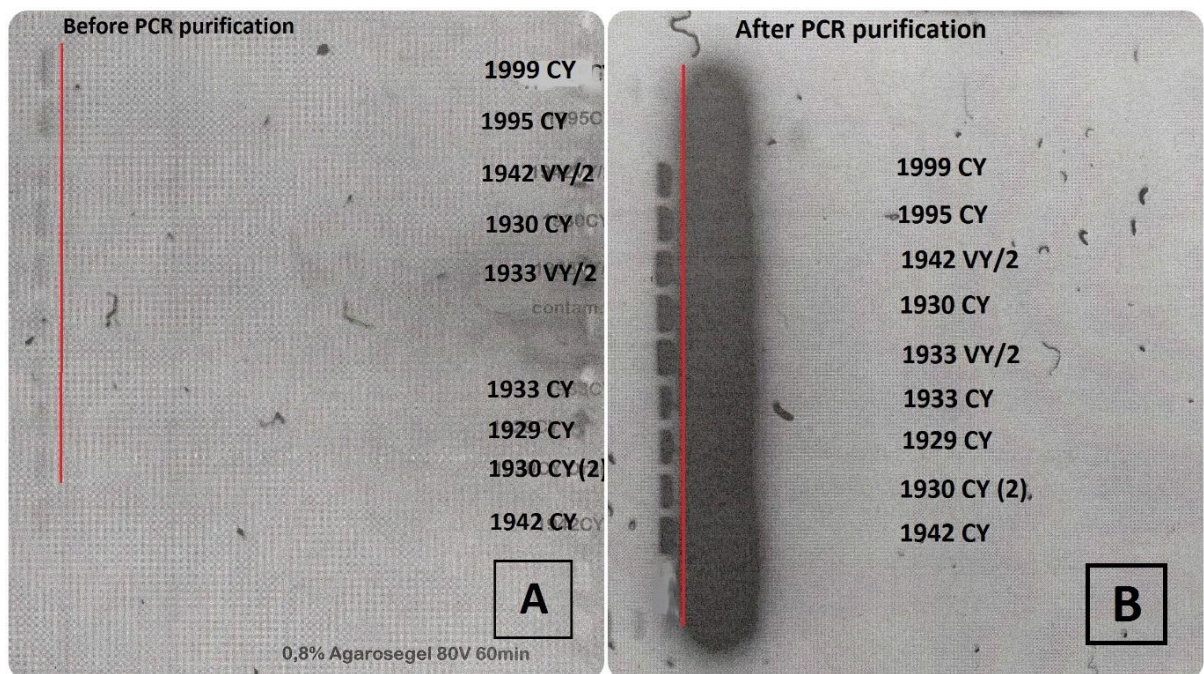
Picture 11. PCR products of the isolated strains (ladder is not seen on the picture owing to technical problems) Analyzed with gel electrophoresis A) Before purification B) After purification

Purified PCR products and primer were send to the sequence service and raw data were checked as described in the material and methods part. Comparison of 16S rRNA-sequences of the isolated strains with sequences of the NCBI-database (BLAST-tool), revealed that all isolated strains are closely related (>99,6%) to different species of *Myxococcus* (Table 15). The calculated degree of similarity of 16S rRNA gene shows, that no new species could be cultivated from the marine samples.

Table 15. List of isolated strains from marine soil samples, next relative (NCBI), similarity (%)

Sample	Nearest strain	Similarity (%)
1930 CY (2)	*Myxococcus fulvus* HW-1	99.6
1929 CY	*Myxococcus Xanthus* strainV3-1	99.6
1930CY	*Myxococcus fulvus*HW-1	100
1933CY	*Myxococcus fulvus* strain 0198-1	99.9
1933VY	*Myxococcus fulvus* strain 0198-1	100
1942CY	*Myxococcus xanthus*V3-1	99.6
1942VY	*Myxococcus fulvus* strain 0198-1	99.6
1995CY	*Myxococcus xanthus* strain V3-1	99.9
1999CY	*Myxococcus xanthus* strain V3-1	99.9

Activation and cultivation of strains from the MISG - collection

The reactivation and cultivation of three marine type strains obtained from the cryo-collection was performed in three different media, namely CY-SWS, VY/2SWS, and E.S.. The culture growth of these strains was very slow and the production of sufficient cell mass/well-grown culture for further experiments took approximately two months. However, the worst growing strain, *Plesiocystis pacifica*, could not be cultivated in sufficient amounts and had to be excluded from further analyses. In Table 16 the assessment of growth for the three different species is presented. The assessment was done visually according to formation of fruiting bodies (see Picture 12).

Table 16. Assessment of cultivation of strains in three different media (+) the poorest growth, (+++) the best growth

Activated strains	CYSWS	VY/2SWS	E.S.
Enhygromyxa salina	++	+++	+
Haliangium ochraceum	++	+++	+
Pseudenhygromyxa salsuginis	+	++	+++
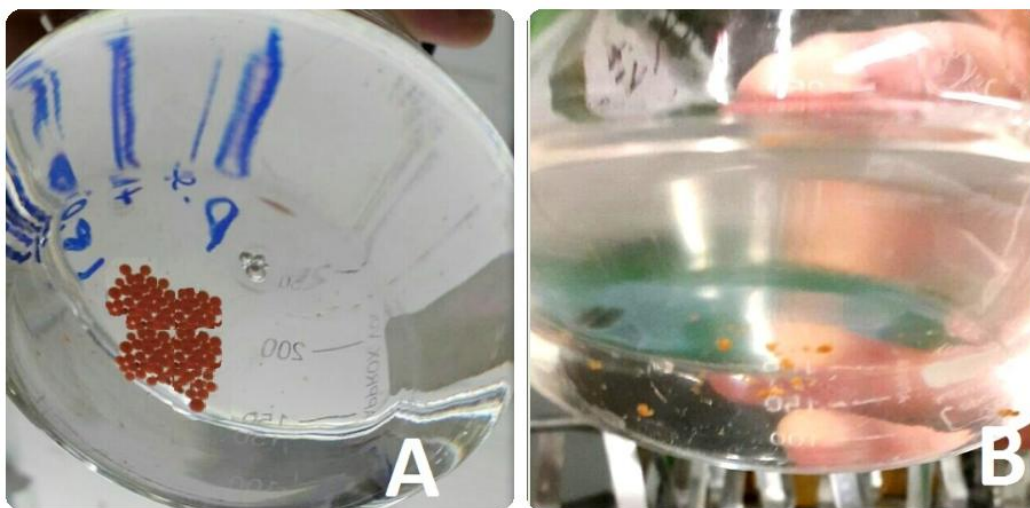
Picture 12. Example of assessment of the culture growth, best growth (+++) can be seen on the left (A); poor growth (+) can be seen on the right (B). On the left part the growth of _P.salsuginis_ (E.S. media) after 2 months, on the right the growth of _P.pacifica_ (E.S media) after 2 months is shown.

After all three strains were grown in sufficient amounts (Picture 12A), the three above mentioned media plus XAD adsorber resin were inoculated with each of the strains (nine cultures in total)and incubated over a period of two weeks. After harvesting the XAD, the resin was eluted in acetone over one night. In the next day, the raw extracts were obtained (filtered, dried, eluted in methanol 1:100) and used for further analysis.

Analysis of marine type strains obtained from MISG collection

These extracts were screened for bioactive secondary metabolites in serial dilution tests. In Table 17the results of SDTs for the type strains can be seen. No extracts showed activity against Gram-negative _E. coli_ and _P. aeruginosa_ (assuming that inhibition up to "A" is negligible) or against yeasts _P. anomala_ and _C. albicans_. However, all extracts show weak to strong activity against outer membrane defective and therefore highly sensitive _E. coli_ TOL C-strain as well as against Gram-positive _S. aureus_. Other Gram-positives, namely _B. subtilis_ and _M. luteus_ and filamentous fungi _M. hiemalis,_ were also inhibited by all tested strains (at least by one extract).

According to these results, the most active strains were _P. salsuginis_ (E.S. and VY/2 SWS) which showed inhibition against six test organisms and _E. salina_ (VY/2 SWS) which showed inhibition

against five test organisms. The weakest inhibition was shown by *E. salina* (CY SWS), *H. ochraceum* (E.S.) and *P. salsuginis* (CY SWS) only against three test organisms.

Several samples of the strains (Table 17) were selected for fractionation. But due to limitation of time of Bachelor work as well as a bottleneck caused by broken HPLC-fractionation apparatus at the MISG-working group, the selection of samples for fractionation was reduced. The primary objective of the selection was to coverall test-organism-groups, namely Gram-negatives, Gram-positives and filamentous/yeast, provided, that representatives of these groups were inhibited by a type strain extract. The cells which are marked with green color were considered to have the best inhibition and were selected for fractionation (Table 17).

Table 17. Results of serial dilution tests for the strains obtained from HZI storage. As higher the letter, as higher the inhibition (A: weak inhibition, H: strongest inhibition). The extracts/ test organisms of cells which are marked with green color were selected for fractionation.

Strain/ medium	E.coli TolC	C. violaceum	S. aureus Newman	M. luteus	B. subtilis	M. hiemalis
DSM number		30191		1790	10	2656
E. salina CY SWS	D	C	C	A	/	/
E. salina E.S.	B	A	D	/	C	/
E. salina VY/2 SWS	D	/	E	C	E	C
H. ochraceum CY SWS	E	/	D	E	/	E
H. ochraceum VY/2 SWS	F	A	E	C	H	/
H. ochraceum E.S.	E	/	C	/	/	B
P. salsuginis CY SWS	A	C	B	/	A	B
P. salsuginis E.S.	D	C	D	C	B	C
P. salsuginis VY/2 SWS	D	D	D	C	D	C

Enhygromyxa salina

Based on the results of serial dilution tests, all three extracts of *E. salina* showed weak to strong inhibitions against all test organisms. However, following extracts were chosen for fractionation andpeak-activity correlation, namely CY-SWS against *C. violaceum* and VY/2-SWS against *S. aureus* and *M. hiemalis.* After fractionation, drying the plate and inoculation with the former inhibited test

organism *E. salina* extracts showed inhibition against the selected organisms at retention times as shown in Table 18. It can be seen on Figure 6 that a lot of different peaks are visible on the chromatogram of VY/2 SWS-extract. The masses of compounds behind the corresponding peaks were analyzed with HPLC-MS and the already known substance enhygrolide was identified (Figure 6) in VY/2 SWS-medium. Due to too small amounts of the other compounds or overlapping chromatograms, the remaining activities could not be assigned to molecular masses/ known or new substances. Further measurements in buffer/acid system of the HPLC would have to be performed, but would disrupt the time frame of this thesis.

Table 18. Results of fractionation of different extracts from *E. salina*, injected volume, test organism, inhibition at retention time and results of HPLC-MS analyses

Medium	Volume injected	Test organisms	Inhibition (min)	identified masses/ substances (HPLC-MS)
VY/2 SWS	10 µl	*M. hiemalis*	21.5	-
VY/2 SWS	5µl	*S. aureus*	**21.0**	enhygrolide
			33.0	-
CY	10µl	*C. violaceum*	**24.0-24.5**	-
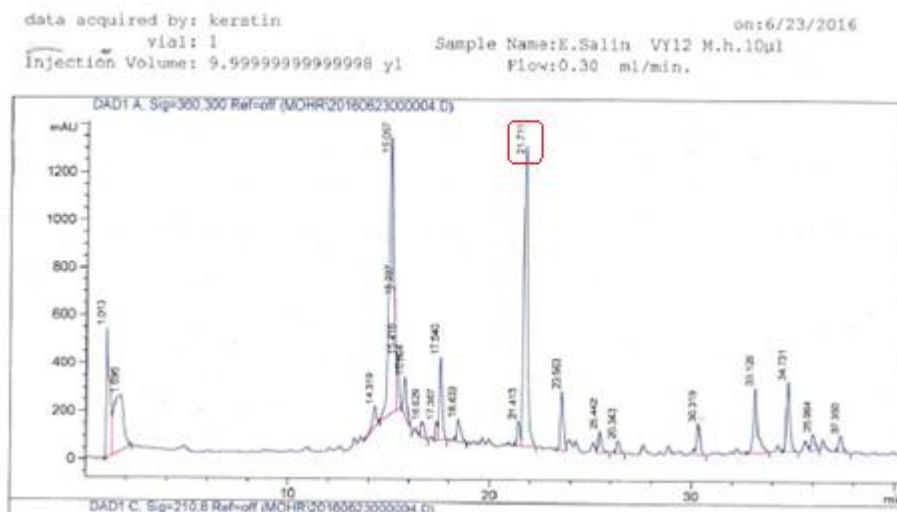
Figure 6. Chromatogram of *E. salina* VY/2 extract with peak activity correlation: activity against S. aureus at minute 21.7 could be identified in the HPLC-MS as enhygrolide (selected)

Haliangium ochraceum

The extracts of *H. ochraceum* revealed stronger inhibition against five different organisms, namely *B. subtilis, S. aureus, M. luteus, E. coli* TOLC and *M. hiemalis.* The CY SWS extract was chosen for fractionation against *M. hiemalis*, the extract VY/2 SWS against *B. subtilis* and *E. coli* TOLC. Thus, in the same manner as for *E. salina*, one Gram-negative, Gram-positive and fungus was selected, but only *E. coli* TOLC showed inhibition after this procedure (Table 19). The resulting chromatogram revealed several apparent peaks (Figure 7). The peaks were analyzed with HPLC-MS and only peak number one revealed haliangicin, still described from a *Haliangium* strain and known for antibiotic and antifungal activity was identified. No inhibition was revealed against other tests organisms. Due to the limited time frame the next consequent step, a repetition of fractionation of active extracts with higher volumes (10 µl) could not be performed within this thesis.

Table 19. Results of fractionation and HPLC-MS of the selected *H. ochraceum* extracts

Medium	Volume injected	Test organism	Inhibition (min)	identified masses/ substances (HPLC-MS)
VY/2	5µl	*Bacillus subtilis*	/	/
VY/2	10µl	*E. coli* TOLC	Min 21.0	haliangicin
CY	5µl	*Mucor hiemalis*	/	/
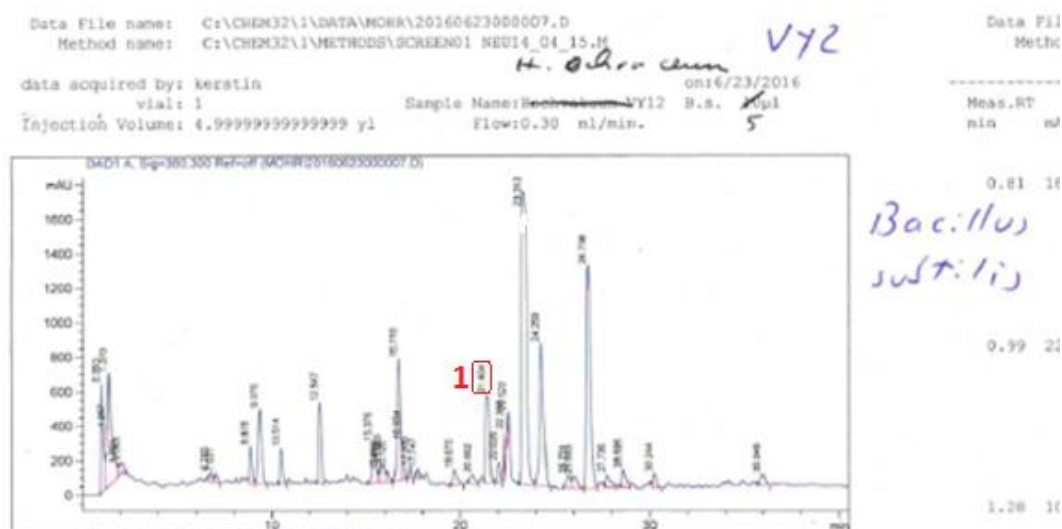
Figure 7. Chromatogram of H. ochraceum VY/2 extract, the peak #1 is haliangicin

Pseudenhygromyxa salsuginis

All three extracts from *P. salsuginis* (E.S., VY/2-SWS and CY-SWS), showed weak to good activity against all test organisms and E.S. and VY/2 SWS were selected for fractionation against different tests strains, namely: *M. hiemalis, E. coli* TOL C*, C. violaceum*, and *S. aureus.* After peak-activity correlation analysis with HPLC-MS, masses of the majority of peaks were identified and revealed some of them as already known enhygrolides (Table 20; Figure 8). However, to date, this compound was exclusively known from *Enhygromyxa salina*. This compound was detected in two extracts of *P.salsuginis*, E.S. and VY/2. The inhibition against *M. hiemalis* and *S. aureus*could not is assigned to masses/compounds by HPLC-MS due to too small amounts or overlapping peaks.

Table 20. Results of fractionation and HPLC-MS of the selected *P.salsuginis* extracts

Strain	Volume injected	Test organism	Inhibition (min)	Identified masses/substances (HPLC-MS)
E.S.	10 μl	*Mucor hiemalis*	33,5 min	no significant peak
		*E.coli*TolC	20,5 min	enhygrolide
		C. violaceum	24,5 min 25 min	enhygrolide
VY/2- SWS	10 μl	*C. violaceum*	23,5 min 24,5min	
CY - SWS	10 μl	*S. aureus*	32min	fatty acid
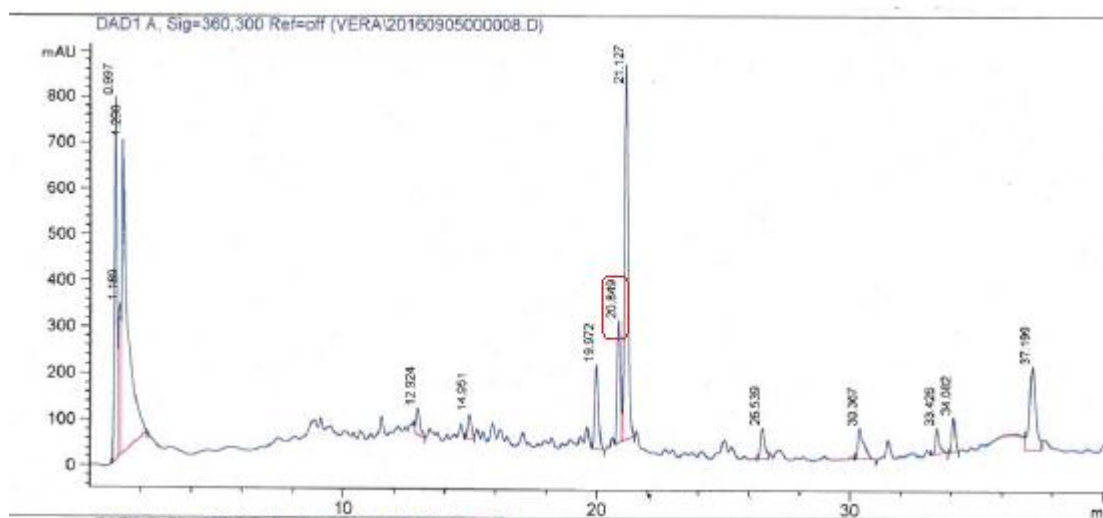
Figure 8. Chromotogram of P.salsuginis E.S.-extract with selected enhygrolide-peak

Analysis of strains isolated from marine samples

The nine self-isolated strains were cultivated in H- and Myxo-medium with XAD for seven days and raw extracts were produced.

Serial dilution tests with extracts of isolated strains grown in H-medium

The results of serial dilution tests for the raw extracts of H-medium are presented in Table 21. All strains showed the worst activity against *P.aeruginosa,* however against all other test organisms (with the exception of *E. coli*) all strains showed weak to very strong inhibition starting from A until H.

Table 21. Results of SDT of isolated strains grown in H-media. Extract of strains in pink colored fields were chosen for fractionation against test organisms of green colored fields.

Strains	*E. coli*	*E.coli TolC*	*C. violaceum*	*P. aeruginosa*	*S. aureus*	*M. luteus*	*B. subtilis*	*M. hiemalis*	*P. anomala*	*C. albicans*
DSM	**1116**		**30191**	**PA14**		**1790**	**10**	**2656**	**6766**	**1665**
1933VY/2	D	G	H	C	H	H	G	C	E	H
1999 CY	F	H	H	E	H	H	H	E	E	H
1942CY	/	F	/	/	F	F	H	F	F	G
1930CYorange	/	/	A	/	/	/	H	F	F	E
1942VY/2	/	F	A	/	E	E	H	E	E	G
1930CY	/	/	A	/	E	E	G	E	E	D
1933CY	D	G	H	/	H	H	H	H	H	H
1995 CY	E	H	H	/	H	H	H	F	F	H
1929VY/2	/	D	A	/	D	D	F	A	A	D
1929CY	/	C	A	/	D	D	F	/	/	D

In addition to serial dilution tests, all extracts were analyzed with HPLC and corresponding chromatograms were obtained. Based on the HPLC chromatograms, it was revealed that most chromatograms showed highly similar peak-composition with two dominant peak-groups (Figure 9 and Figure 10). Thus, it was decided to choose two "representative" strains. The strains were 1933

(VY/2) – 100 % similarity to *Myxococcus fulvus* strain 0198-1 and 1995 (CY), 99.9 % similarity to *Myxococcus xanthus* strain V3-1. Also these strains showed the strongest inhibition against majority of the test organism and thus one Gram-negative, one Gram-positive and one fungus were chosen. The cells which are marked with green color in Table 21 were chosen for fractionation.

Inhibition of *P. aeruginosa* by strains 1933VY/2 and 1999CY is of highly interest, because only very few substances are known, which inhibit this test strain. However, due to the limited time frame, this activity could not be processed within this thesis. Fractionation and peak-activity correlation will be performed by the MISG-group as soon as possible.

Serial dilution tests with extracts of isolated strains grown in Myxo-medium

All isolated strains were also grown in Myxo-medium with XAD. The results of SDTs with corresponding raw extracts are given in Table 22. There were no strains selected for fractionation since the chromatograms were very similar to the chromatograms of strains grown in H-media and the inhibitions were weaker.

Table 22. Results of SDT of strains grown in Myxo-medium

Strain	E. coli	E. coli TolC	C. violaceum	P. aeruginosa	S. aureus	M. luteus	B. subtilis	M. hiemalis	P. anomala	C. albicans
DSM	**1116**		**30191**	**PA14**		**1790**	**10**	**2656**	**6766**	**1665**
1933 (VY/2)	/	G	/	/	F	E	F	E	/	/
1999 (CY)	/	G	F	/	H	H	H	H	/	/
1942 (CY)	E	G	/	/	/	/	G	/	F	/
1930 (CY) orange	A	B	/	/	/	/	E	/	/	/
1942(VY/2)	/	G	/	/	/	/	H	/	/	/
1930 (CY)	/	C	/	/	D	/	G	/	/	/
1933 (CY)	/	D	/	/	H	H	H	H	C	/

1995 (CY)	/	H	F	/	H	H	H	H	C	/
1929 (VY/2)	C	C	/	/	C	C	D	C	/	/
1929 (CY)	/	C	/	/	/	/	D	/	/	/

1933 (VY/2) – H media

Serial dilution tests of extracts from strain 1933 (VY/2) revealed strong inhibition against all test organisms, but due to limitation of time of Bachelor work, as for the type strains one Gram-negative(*E. coli* TolC),one Gram-positive(*S. aureus*), one fungus (*P. anomala*)were chosen for fractionation. Fractionation (Table 23) and chromatogram (Figure 9) showed several bioactive peaks which could be identified with HPLC-MS, namely different myxalamid-derivates and fatty acids.

Table 23. Results of fractionation and HPLC-MS of 1933 (VY/2) extracts. In all cases 5 µl were fractionated.

Test organism	Inhibition (retention time)	Identified masses/substances (HPLC-MS)
S. aureus	20,5 min 21 min 22,5 min 28 min	myxalamid-variant myxalamid-variant myxalamid-variant fattyacid
E. coli TolC	21,5 min 22 min 22,5 min	myxalamid-variant myxalamid-variant myxalamid-variant
P.anomala	-	-
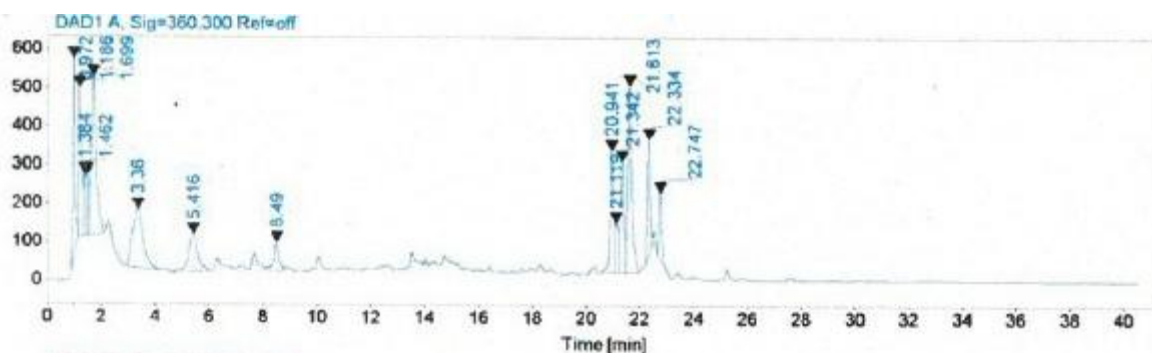
Figure 9. Chromatogram of strain 1933 VY/2 with peaks of different myxalamid-variants (min 20.5-22.5)

1995 (CY) - H media

The extracts of 1995 (CY) showed strong inhibition against nine test organisms, but in the same manner as for 1933 (VY/2) only *E. coli* TolC*, S. aureus,* and *P. anomala* were chosen for fractionation*.* The results of fractionation (Table 24) and chromatography (Figure 10) revealed several apparent bioactive peaks. The peaks were analyzed with HPLC-MS and different myxalamid variants could be identified. However, the other active peaks could not be identified clearly, although if masses were determined. Further measurements with HPLC-MS should be done, but were not possible within the given time frame.

Table 24. Results of fractionation and HPLC-MS of 1995 (CY) extracts. In all cases 5 µl were fractionated.

Strain	Test organism	Inhibition (min)	Identified masses/substances (HPLC-MS)
1995 (CY)	*P. anomala*	15,5 min	mass: 542
		16 min	mass: 542
		19 min	mass: 519
	E. coli TolC	5,5 min	mass 163
		6 min	mass 163
		8,5 min	mass: 608
		11,5 min	no significant peak
		21,5min	myxalamid-variant
		22 min	myxalamid-variant
	S. aureus	2,5 min	not identified
		8,5 min	mass: 608
		22 min	myxalamid-variant
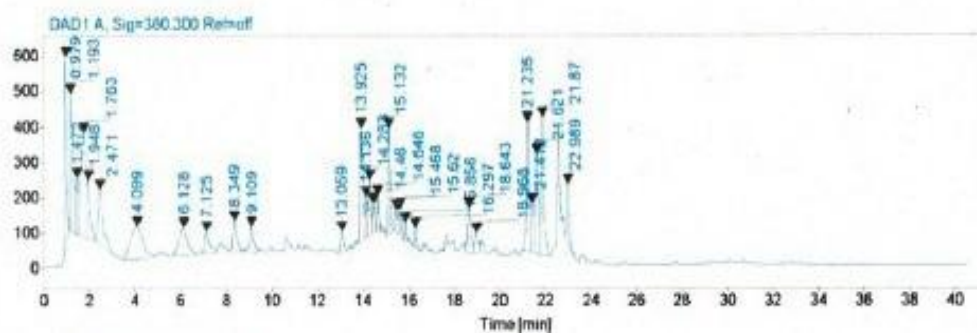
Figure 10. Chromatogram of 1995 CY-extract with peaks of different myxalamid-variants (min 21.5-22.0)

Discussion

In the following chapter the analysis of the results of this study are described. The chapter includes description of strains cultivation, both obtained from the HZI collection and isolated from marine soil samples. Also, the section contains the description and analysis of the compounds produced by the strains.

Analysis of the cultivation of marine myxobacteria obtained from the storage collection and their produced metabolites

In the beginning of the study four marine myxobacteria cultures were obtained from the HZI storage, namely *E. salina, H. ochraceum, P. salsuginis, and P. pacifica*. As for the first two in the list (*E.salina, H. ochraceum*) the strains are well researched and the cultivation method was successfully developed and published in papers during last few years. Additionally, the description of active compounds derived from secondary metabolites of the strains was done. As for the other strains from the list (*P. salsuginis and P. pacifica*), to date, no secondary metabolites and active compounds have been described, however, according to the literature sources successful cultivation strategies for cultivation of these species have been established.

Enhygromyxa salina

According to the published studies by Iizuka [40], the optimal parameters for *E. salina* growth are the temperature of 28–30 °C, NaCl concentration with the optimum at 1.0–2.0%. Besides NaCl, cations of seawater Ca^{2+} or Mg^{2+} are also required and the pH range for growth is 7.0–8.5. All requirements were met in this work and as a result the cultivation of the strain was successful. The medium which showed the best growth of *E. salina* was VY/2 SWS, the reason for that may be the most suitable concentration of NaCl for *E. salina* growth. Referring to the study of Iizuka [41], where the effect of NaCl on growth was investigated on VY/2-SWS medium, it was revealed that the best growth was at concentration of 2% NaCl. In terms of gliding motility and fruiting body formation, these bacteria show the typical features of terrestrial myxobacteria [40].

The screen of *E. salina* for secondary metabolites revealed no new compounds within this study. The reason for could be, that *Enhygromyxa salina* has already been investigated during the last decade and the possibility to discover unknown substances from this genus is not as high as from an totally uninvestigated genus/species [33]. Nevertheless, enhygrolide compound was revealed during the screening, showing that chosen culture conditions were suitable reproduction of

secondary metabolite production of this strain. Enhygrolides were the first natural products obtained from *Enhygromyxa* species [34]. These molecules resemble cyanobacteria-derived metabolites. Referring to the literature, enhygrolides inhibit the growth of Gram-positive bacteria [29]. In this study, the compound also showed strong inhibition again Gram positive *S. aureus*.

Haliangium ochraceum

Referring to Fudou [5], *H. ochraceum* was proposed to be of true marine origin according to its salt requirement for growth. Indeed, 2 to 3% NaCl (w/v) and a pH of 7.5 are optimal for growth; therefore these conditions were established for routine cultivation in this study. Also, *H. ochraceum* requires 2-3% of NaCl concentration for haliangicine production (*Figure 11*) [42]. The best growth of *H. ochraceum*, as well as for *E.salina*, was considered to be in VY/2-SWS media.
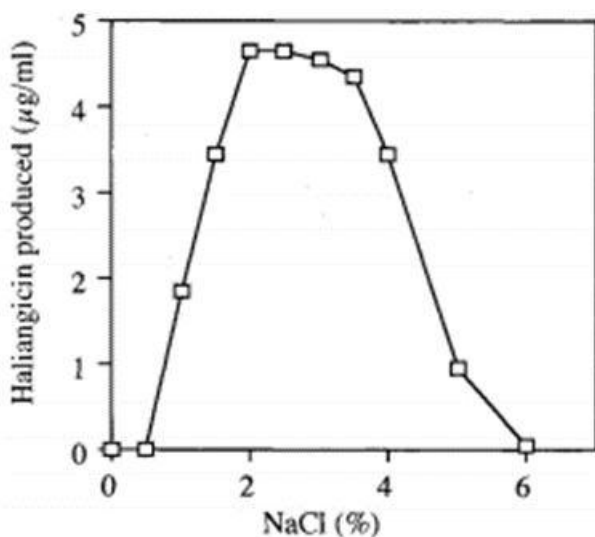
Figure 11. The effect of NaCl concentration on haliangicin production [42]

According to the literature [42], *H. ochraceum* is considered to be a producer of secondary metabolites with antibacterial and antifungal activities. The results of this study revealed antifungal antibiotic haliangicin. Surprisingly, haliangicin showed inhibition against *E. coli* TOLC, despite the fact that literature sources claim no activity of this compound against Gram-negative bacteria.

Pseudenhygromyxa salsuginis

Pseudenhygromyxa salsuginis the most recent example of a halotolerant myxobacterium, published in 2013 by Iizuka. Even though it is able to grow in the absence of salt, optimal growth occurs within a concentration range of 0.2–1.0% NaCl (w/v) and pH values from 7.0–7.5 in a

temperature range of 30–35 °C. To date, no reports on biological activities or any metabolites discovered from *P. salsuginis* have been published [41].

During the screening of *P. salsuginis,* enhygrolide was also discovered within the present study. *P. salsuginis* E.S. showed inhibition against *E. coli* TolC and *C. violaceum* and *P. salsuginis* VY/2 was active against *C. violaceum.* This compound was considered to be typical for *E. salina.* The production of this compound from *P. salsuginis* may have logical explanation. According to the literature, *P. salsuginis* has the genetic proximity to *E. salina* and the fact that it belongs to the group of myxobacteria suggests that this organism may also possess a high potential as a producer of active molecules.

Plesiocystis pacifica

Picture 13. Poor growth of *P. pacifica (yellow fruiting bodies)*

The last strain which was analyzed in this study was *Plesiocystis pacifica.*This strain requires 2–3% NaCl and a pH of 7.4 for optimal growth on yeast medium with artificial seawater solution at 28 °C [13]. In the present study, the same conditions were used for cultivation of this strain; however, the culture growth was very poor. For the period of two months culture was hardly growing, there were not enough cell mass produced [13].

The culture was upkept during the entire work and later, was given to Microbial Drugs department technicians for further cultivation and analysis of secondary metabolites.

The analysis of the available draft genome of *P. pacifica* strain was implemented and the presence of 12 NRPS, PKS and hybrid NRPS-PKS gene clusters were revealed. These results should motivate researchers to isolate some of the predicted metabolites [13]. To date, no active compounds derived from the secondary metabolite of the gene were detected.

Analysis of the cultivation of isolated myxobacteria from marine soil samples and their produced metabolites

As for the isolation of myxobacteria from marine soil samples, representatives of two species were isolated: *Myxococcus fulvus* and *Myxococcus xanthus.* Growth features of these strains made them rather easy to work with and this is reflected in high success rate of purification process. Those unidentified bacteria, which maybe showed no fruiting body formation, were not isolated successfully owing to little experience in this process. The isolation of the remaining samples failed due to contamination. In this case, the purification procedure had to be repeated to obtain pure cultures, but was stopped due to time limitation of the study**.**

In addition, it is interesting to notice that both halotolerant (*Myxococcus fulvus)* and generally not halotolerant (*Myxococcus xanthus)* species of one genus were isolated. This might suggest that intertidal area is suitable for both types of myxobacteria.

There are numerous of bioactive secondary metabolites which *M.xanthus* can produce. In this particular case only Myxalamid variants were discovered. Myxalamid variants are a well-studied group of myxobacterial antibiotics, which can be produced by both *Myxococcus* species. Myxalamid variants have antibiotic activity against molds, yeasts and several Gram-positive bacteria [43-44]. No other bioactive compounds from these strains were not discovered within the frame of this bachelor work.

Conclusion

The quantitative presence of myxobacteria in marine habitats can hardly be assessed. Based on the isolation success of marine myxobacteria, the occurrence of myxobacteria in marine environments is lower comparing to terrestrial habitats. One of the reasons for this could be simply not the best suitable conditions for isolation and cultivation which are used today. The currently applied protocols are based on those for terrestrial strains with slight modification in terms of salt content; however variation of such aspect as e.g., use of *Escherichia coli* as a prey was not still performed. Also, it's quite difficult to distinguish marine myxobacterial colonies after isolation. The fact is that their morphological features may differ from the ones of terrestrial strains and have not been well researched to date. To sum up, in order to use marine-derived myxobacteria as a source of bioactive metabolites, in-depth studies of their morphology and physiology are necessary.

Outlook

It could be possible to isolate more myxobacteria strains from marine soil samples. One of the methods which could be used to obtain new myxobacteria strains is to use different feed organisms rather than *E. coli*. According to the literature research, this method was not deeply investigated yet. Isolated strains from marine samples which showed inhibition against *P.aeruginosa* are in high interest and will be checked by MIC working group.

Also, marine myxobacterial strains of *P. salsuginis* and *P. pacifica* have interesting prospects for the future. As it was mentioned in the discussion part, *P. salsuginis* has close genetic proximity to *E. salina* and may reveal number of bio active compounds. Both strains will be further screened for the secondary metabolites production by laboratory technicians of Microbial Drugs department of HZI.

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List of abbreviations

Abbreviation	Meaning
B. subtilis	*Bacillus subtillis*
BLAST	Basic Logical Alignment Search Tool
Bp	Base pair (in DNA)
C. albians	*Candida albians*
C. violaceum	*Chromobacterium violaceum*
DNA	Desoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E. coli	*Eschrechia coli*
E. Salina	*Enhygromyxa Salina*
EDTA	Ethylenediaminetetraacetic acid
H.ochraceum	*Haliangium ochraceum*
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography – mass spectrometry
HZI	Helmholtz Centre for Infection Research (Helmholtz-Zentrum fur Infektionforschung *in german*)
JSRM	JumpStart^{TM} Taq ReadyMix^{TM} by Sigma
M.hiemalis	*Mucor hiemalis*
M.luteus	*Micrococcus luteus*
M. Xanthus	*Myxococcus xanthus*
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MSC working group	Microbial Strain Collection working group, HZI
P.aeruginosa	*Pseudomonas aeruginosa*
P.anomala	*Pichia anomala*
P.pacifica	*Plesiocystis pacifica*
P.salsuginis	*Pseudenhygromyxa salsuginisis*
PCR	Polymerase chain reaction
RNA	Ribonucleic acid

SDTs   Serial dilution tests

S.aureus   *Staphylococcus aureus*

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