University of Akureyri
Faculty of Business and Science
Department of Natural Resource Science

Bioprospecting for antimicrobial activity at the hydrothermal vent site in Eyjafjörður

Submitted for a partial fulfillment for a Master of Science degree in Biotechnology

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Bioprospecting for antimicrobial activity at the hydrothermal vent site in Eyjafjörður

Arnheiður Eyþórsdóttir

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Statements

I hereby declare that I am the only author of this thesis and it is the work of my own research.

Arnheiður Eyþórsdóttir

It is hereby confirmed that this masters thesis is satisfactory to an M.Sc. degree from the Faculty of Business and Science at the University of Akureyri.

Dr. Hjörleifur Einarsson, professor

Abstract

Nature has provided mankind rich resources of bioactive compounds, many of which have not been exactly defined. Novel natural products can have many valuable utility possibilities, such as drugs, functional compounds for foods, cosmetics, agriculture or aquaculture. Research in this field has been richer in terrestrial sites, but the marine environment has recently gained more interest and led to discoveries of several thousands novel bioactive compounds from various organisms including microbes.

A submarine area with geothermal activity and chimney or cone structures – Arnarnesstrýtur - has been discovered at relatively shallow waters in Eyjafjörður. The site appears to host a rich biosphere. The aim of this study was to isolate bacteria from invertebrates at this site and screen for antimicrobial activity, with the ultimate goal of isolating antimicrobial compounds. Samples were collected by diving, cultivable bacteria isolated using selective media for actinomycetes and antimicrobial assays were carried out for the isolates. A total of 1866 bacterial isolates were retrieved from the samples, more than half of them from sponges. Ninety-six isolates from various organisms were found revealing antimicrobial activity against one or more test strains and the inhibition pattern varied. One of the active isolates, (101-54) was selected for studying the activity by growth experiments of test strains. Ethyl acetate extract of this isolate inhibited growth of four test strains, but different concentrations of extract were needed for inhibition.

Future work will include attempts to isolate and refine the active compounds, determine their mode of action as well as classifying the microbial producers.

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1 Introduction

1.1 Natural products and bioactivity

Mankind has from early evolutional times not only developed various methods to collect proper food from nature, but also to find health remedies from natural resources. This can even be observed by the behaviour of other animals such as wild ape species that are normally seen using certain leaves to cover wounds. Nature has provided rich resources of bioactive compounds, many of which have not been exactly defined. Diseases and accidents have been around humans from the very beginning and although the reasons for illness or infections were not known, people found their ways to fight them. It is therefore reason to believe that antimicrobials or other biologically active compounds are widely distributed in nature, but they can be hidden treasures. Novel antimicrobials and other cell inhibitory compounds are examples of natural bioactive compounds which have gained increased interest. The search for bioactive natural products started in the scientific world many years ago and logically went on mostly on land, but late in the 20th century, the oceanic world started to raise interest and has already revealed many novelties. This chapter gives a broad overview of bioactivity in nature, placing special emphasis on exploiting bioactive compounds of marine origin, especially antimicrobials.

1.1.1 Traditional herbal medicine

Starting more than 3000 years ago, ancient communities used natural sources for healing purposes, mostly as “teas” or infusions (for oral intake) or salves (external application) from land plants. These “botanical healing” traditions were passed on through the generations for ages, where some of them are still practiced (Fenical 2006). As an example, around 80% of the population in Ethiopia still rely on such traditional medicine for health problems and in that country around 800 plant species are used for treating over 200 diseases (Teklehaymanot et al., 2007). In the later years, increasing effort has been made to document these traditional medical uses of plants around the world, as the knowledge is gradually falling by younger generations. An example is a study of one province in Turkey, where 126 plant species were recorded for traditional

medical use. Usually, teas were made of the plants and the treatments were mostly for wounds, cold and stomach problems, but many other disorders were also mentioned (Kültur, 2007). De-la-Cruz et al. (2007) collected medicinal plants and information from inhabitants in one province in Peru, where the use of plants is still very common for medicinal purposes. They reported 87 plant species used to treat health problems, mostly cooked for teas or broths for bathing and massaging parts of the body. Ethiopian herbal treatments include drinking teas or juices, chewing plant parts and inhaling smoke of roasted plants powder (Teklehaymanot et al., 2007).

In Iceland, similar traditions exist on herbal teas and salves or even alcoholic plant extracts, used drop wise (Bjarnason, 1983). Documents originated from the 13th century on plant medicines from Denmark and Iceland are still conserved (Steffensen, 1990). For the last decades, more attention has been brought to these traditions, resulting in fabrication of commercial products based on them, mostly as various types of skin-care creams and oils.

1.1.2 Bioactive natural products

Based on the healing traditions, scientists gained interest in screening natural organisms for bioactive compounds. Terrestrial organisms, preferably plants have been extracted and the extracts screened for various types of bioactivity, although the main focus has been on cytotoxic and antimicrobial activity. As examples of well documented natural plant anticancer agents, vincristine and camptothecin could be mentioned. Vincristine and vinblastine are vinca alkaloids, derived from *Catharanthus roseus,* a perennial herb originated from Madagascar (Madagascar periwinkle) and was used for treating diabetes in traditional medicine. The compounds and their analogues are now used as drugs for treatment of many cancer types, such as leukemia, lymphomas and lung cancer (Cragg and Newman, 2005). Camptothecin is a quinoline based alkaloid, derived from *Camptotheca acuminata* - a tree originated in China where it had a record in traditional medicine (Cyberbotanica, 1998). Chemical drug derivatives of camptothecin are used for treating ovarian and lung cancer (topotecan) and colorectal cancer (irinotecan). One of the most "famous" natural anticancer drugs is probably paclitaxel (Taxol), which is particularly used for treatment of breast and ovarian cancer. Paclitaxel is a complex molecule, originally isolated from the bark of the Pacific yew tree (*Taxus*

brevifolia). It was discovered as a plant extract in a big programme initiated by the U.S. National Cancer Institute in the 1960´s, where enormous efforts were put into biological screening of diverse plant extracts for anticancer activity (Cragg and Newman, 2005). The compound itself was not isolated until 1971. The research and clinical trials on the compound were ongoing for many years, costing millions of US dollars and hundreds of tons of the tree bark, actually almost drying up the resources of the plant. Later, a method to semi synthesise taxol from a related compound in the leaves of another plant - *Taxus baccata*, - was developed. Finally, Taxol made it to the commercial market in 1993 after 31 years of research, "from bark to business" (Stevenson, 2002). Many paclitaxel derivatives (taxanes) have now been developed and have either already become drugs or are currently in the state of clinical trials (Cragg and Newman, 2005).

Many studies have reported antimicrobial activity of extracts or essential oils from all kinds of plants. These include well known herbs such as basil (Opalchenova and Obreshkova, 2003, Suppakul et al., 2003), salvia (Tepe et al., 2004), lemongrass and oregano (Hammer et al., 1999), thyme and black pepper (Dorman and Deans, 2000), garlic and clove (Arora and Kaur, 1999), only to name a few. Work on isolating the active compounds is continuously ongoing and has resulted in lots of isolated compounds such as alkaloids, flavonoids, sesquiterpene lactones, diterpenes, triterpenes or naphtoquinones, among others (Rios and Recio, 2005).

The animal kingdom is certainly also an object of studies on bioactive compounds. A big variety of antimicrobial peptides apparently exists and they have been found in all complex species. Insects have been the biggest source to date, a few randomly picked examples being the house-fly *Musca domestica* (Liang et al., 2006) the Australian bull ant *Myrmecia gulosa* (Sheridan, 2006), the silk moth *Hyalophora cecropia* and the fruit fly *Drosophila melanogaster* (Vilcinkas and Gross, 2005). The major groups of these compounds are cecrofins, proline-rich and glycine-rich peptides and the antifungal defensins, (Liang et al., 2006). The multitude of antimicrobial peptides isolated from natural sources exceeds 400 compounds, the biggest group originated from insects (Bachère, 2003). Maggots – larvae of the fly *Phaenicia sericata* are used in the so-called Maggot therapy which is actively used in some hospitals to treat seriously infected wounds. The live larvae are put right onto the wound to stay there for days. Not only do they often manage to overcome the infection, but also clean dead and infected tissue and

have considerable healing effects. Supported by research studies, it has been suggested that the maggots literally eat the infecting bacteria which then are killed in the maggots' intestines by a potent antimicrobial, possibly a peptide. Studies on excretory enzymes from the larvae are also been carried out to study the wound healing effects (Nigam et al., 2006).

In a review of naturally originated and approved drugs since 1981, Newman and Cragg (2007) list a grand total of 1010 compounds commercially available. The list is divided into 60 categories according to type of action, the biggest groups being antibacterial, anticancer, antihypertensive, anti-inflammatory and antiviral compounds. The authors' conclusion is to strongly recommend paying more attention to microbes and microbial interaction in terms of future research in drug development and new natural compounds.

Novel natural products do have many other interesting utility possibilities than drug development, although that part is quite voluminous in the literature. Functional compounds for foods and cosmetics, herbicides, insectides or growth factors for agriculture and nutritional components for feed in aquaculture are potentials for development. In these sectors, the cost and time in development and trials can be considerably less than in the pharmaceutical sector

1.1.3 Microbial metabolites and bioactivity

Microbial metabolites have played a very important role in natural product drug discovery since the end of World War II (Figure 1). After Alexander Fleming's discovery of penicillin in 1929 a very fruitful discovery period of new antibiotics followed, lasting until around 1970 and resulting in most of the widely known antibiotic drugs currently in use (Strohl, 2004). One bacterial genus carries head and shoulders above all the others when it comes to bioactive metabolite production, as more than 70% of the isolated compounds in this period, were actually produced by *Streptomyces sp*. (Bérdy, 1995). The genus *Streptomyces* belongs to the class Actinobacteria and order Actinomycetales (commonly called actinomycetes) and is a quite dominating genus, so that the most of the other genera in this order have sometimes been referred to as "rare actinomycetes" (Figure 1). The compounds discovered from these microorganisms differ widely both in structure and activity and apart from antibacterial activity, they exhibit antifungal, antitumor, antiparasitic or immunosuppressive activity. Among the well known

antimicrobial compounds derived from *Streptomyces* sp. are streptomycin, kanamycin, chloramphenicol, tetracyclines, novobiocin, cyclocerine and vancomycin (Strohl, 2004).

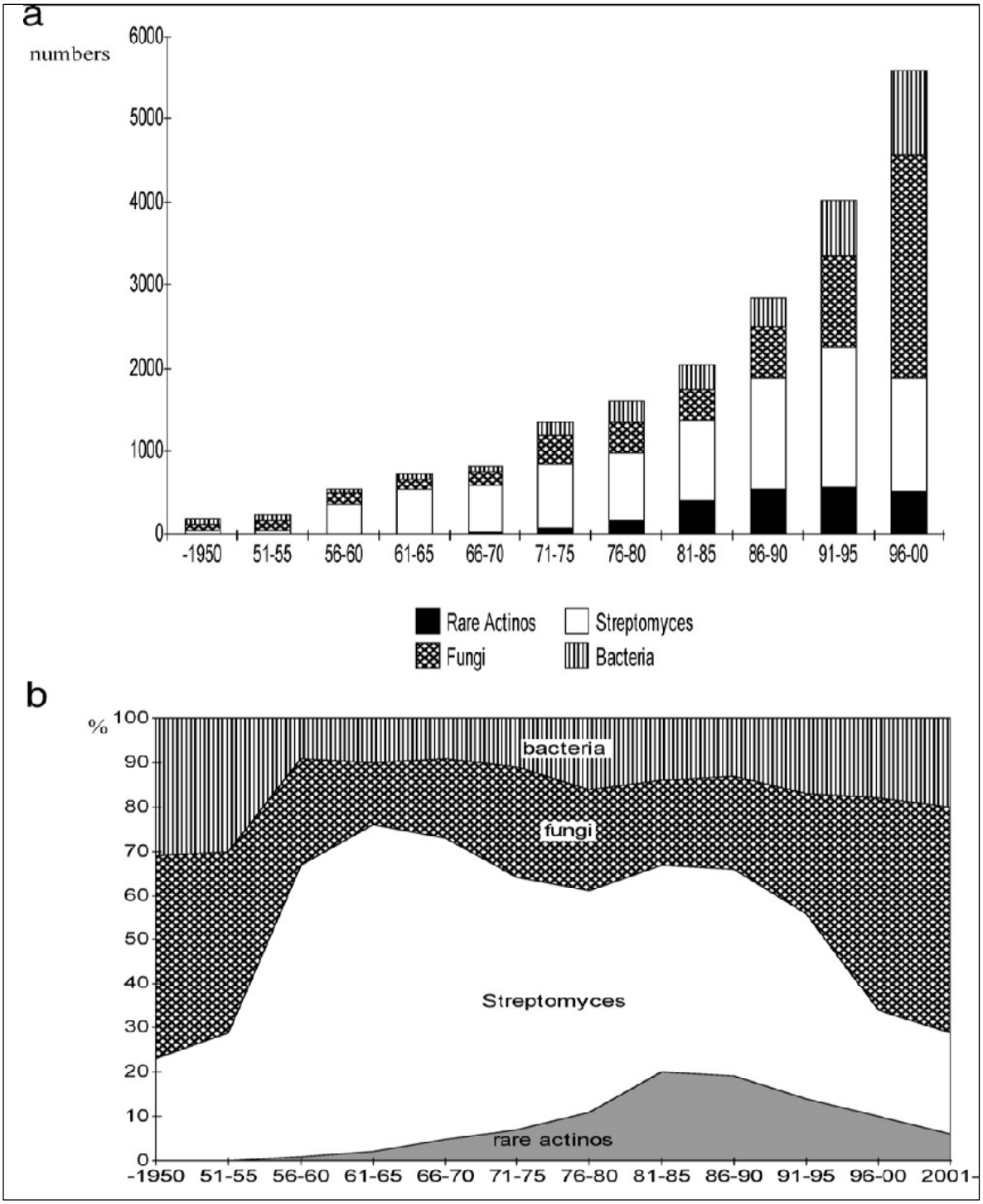

Figure 1 *Graphical view of the discovered antibiotics for the later half of the 20th century. Note that in this figure, the actinomycetes are counted as a separate group outside bacteria and the term antibiotic is defined as microbial metabolites with antimicrobial, antiviral or antitumor activities.* ***Source: J. Bérdy (1995).***

The rate of new discoveries of antimicrobials has somewhat ceased for the last two decades, but by no means come to a halt. It has in fact been estimated that although the order Actinomycetales has already been the source of around 3000 known antibiotics, more than 95% of the existing ones are still left to be discovered (Clardy et al., 2006). New microbiologically derived antibiotics include daptomycin, an actinomycetes produced compound recently approved by the FDA and with broad activity against Gram positive bacteria (Pham, 2004). Although the Actinomycetales is somewhat dominating in this field, antimicrobial compounds have also been found in other bacteria types such as *Bacillus* (Yan et al., 2003) and *Pseudomonas* sp. (Matthijs et al., 2007). Lyostaphin, a compound currently in clinical trials for staphylococcal infections, was isolated from *Staphylococcus simulans* (Clardy et al., 2006). Antitumoral agents such as daunorurubicin and aclacinomycin A were both originally isolated from *Streptomyces* sp. and are in use as cancer drugs (Fiedler, 2004). Bleomycins and actinomycin D, also originated from *Streptomyces* sp. are widely used in cancer chemotherapy, such as Hodgkin's disease and prostate cancer (Janin, 2003). An antiparasitic macrolide – avermectin was isolated from *Streptomyces avermitilis* and was later chemically modified to produce ivermectin. Ivermectin is a potent nematocidal compound and is used as a drug for humans, treating various parasite caused diseases, mainly in Africa. It is also of use in agriculture, where it has a role in eliminating gastrointestinal nematodes in domestic animals and as an insecticide (Fiedler, 2004).

1.1.4 Natural product studies in Iceland

Lichens are one group of organisms known to have been used in natural medicine for centuries. Icelandic lichen metabolites have been extensively studied at the Faculty of Pharmacy, University of Iceland, in terms of bioactivity, structure, separation and molecular mode of action. Among their published findings are *in vitro* activity of protolichesterinic acid isolated from Iceland moss (*Cetraria islandica*) against *Helicobacter pylori* (Ingólfsdóttir et al., 1997) and *in vitro* immunological activities from lichen-derived polysaccharides (Ómarsdóttir et al., 2006a and references therein). Ingólfsdóttir et al. (2000) collected 29 lichen species and screened their extracts with various bioassays for cancer chemopreventive activity. Nine extracts showed some kind of activity, the most significant being specific enzyme induction in *Xanthoria elegans* and

Alectoria nigricans. In the recent years, this work has been expanded to Icelandic plants, mainly looking at plants with known use in nature medicine. Ólafsdóttir and Halldórsdóttir (2006) found acetylcholinesterase inhibition in alkaloids isolated from the club moss *Lycopodium annotinum*, an activity which is possibly of interest in treating Alzheimer ´s disease.

Furthermore, Bergsson et al. (2005) isolated four antimicrobial proteins from epidermal mucus of Atlantic Cod, three ribosomal proteins and one histone. These compounds inhibited both representatives of Gram positive and Gram negative bacteria as well as *Candida albicans*, but the activity varied with different salt levels in the solutions. Stoffels et al (1992) isolated a new bacteriocin – carnocin UI49 - from *Carnobacterium* sp. originated from fish. Carnocin UI49 is a large peptide belonging to the bacteriocines termed lantibiotics type A (Stoffels et al., 1992, Nes and Tagg, 1996).

Cold-adapted trypsins from Atlantic cod have been extensively studied at the Science Institute, University of Iceland. These enzymes are already used in skin care products and in seafood flavourants intended for food processing (Gudmundsdottir and Palsdottir, 2005).

1.1.5 Commercial use of antimicrobial compounds

There is definitely still a growing demand for development of new specialised antimicrobial compounds. The most urging reason is the rapidly growing number of antibiotic resistant strains of bacterial and fungal pathogens. The generous use of traditional antibiotics for the last half a century has created a selective pressure for increased resistance and the resistant strains are no longer limited to hospitals. One of the biggest health threats facing the global population remains the multidrug resistant strains of the tuberculosis bacteria - *Mycobacterium tuberculosis*. Methicillin resistant *S. aureus* (MRSA), vancomycin resistant *Enterococci* (VRE) and β-lactam resistant *Streptococcus pneumoniae* are already creating huge medical problems and a few years ago a vancomycin resistant MRSA appeared (Strohl, 2004). Resistance for traditional antibiotics will only grow larger for the years to come, creating greater threats and propulsive need for new antibiotics. Resistance spreads rapidly and continuous work has to go on in this field. As an example, in only two years time from the launching of methicillin – a penicillinase-stable penicillin- the first MRSA strains emerged (Bernan et al. 2004). Furthermore, there are also possibilities that previously non-virulent bacteria

are turning into pathogens by picking up virulent genes from other strains in their environment, which makes the situation even more complicated (Fernandes, 2006, Strohl, 2004). Another concern is certainly the serious discussion on the bioterrorism threat, where new compounds will be needed to fight a possible bioterror attack.

From an industrial point of view, antimicrobials continue to be of great interest and development work on new preservatives for food, feed and cosmetics industries remains a noteworthy issue. Increasing incidents of allergic reactions to various food preservatives are drivers to find new ones and the term "natural" remains a marketing moment within this sector, as the trend is still towards health foods and natural ingredients (Sloan, 2006).

The cosmetic and toiletries sector is a very important market for antimicrobials. The cosmetic industry is huge, with global sales more than 250 billion USD a year and still fast growing (Davies, 2006). Preservatives are standard additives in cosmetics and toiletries such as skin care, sun screens, make up foundations and hair care products. Incidents and even outbreaks of infections traced to make up or skin care products have been recorded and both the US and European Pharmacopoeia have a microbiological criteria for such products (Schwarb et al. 2001, Campana et al. 2006). These consumer products are usually touched by hand a number of times, but are all the same supposed to keep for a relatively long time in microbially friendly conditions (damp and warm bathrooms). Parahydroxy benzoic acid compounds (parabens) are widely used as preservatives in these products, but with a raising voice of criticism. Parabens have been connected to breast cancer due to oestrogenic activity, but hard scientific evidence for such relations is not available. Regardless of toxicity, the trend seen in the cosmetics and toiletries sector is similar as in the food industry, it is moving towards natural and organic (Davies, 2006) and this could be considered as an opportunity for new antimicrobials of natural origin.

1.2 Marine resources of bioactivity

1.2.1 Bioprospecting in the oceans

In historical perspective, using organism from marine sources for specific purposes like healing or traditional medicine has not been very common. However, there are source materials indicating use of sponges for remedies already at the times of Hippocrates

(Müller et al. 2004b). The most logical reason is the difficulty of exploring and collecting organisms from the oceans, hence less knowledge was built up about the marine environment. There are however, vague descriptions of marine natural medicine in southern China (Fenical, 2006) and the use of algae in traditional Chinese medicine is known. In Iceland, dulse (*Palmaria palmata*) was widely used for human consumption, usually cooked for porridge and areas where dulse was available were considered valuable. Seaweed or kelp was also traditionally used as a kind of fertilizer when growing potatoes, as it was meant to improve the plant growth and health (Gunnarsson et al., 1998).

Around 70% of the earth ´s surface is covered by oceans and they possess twice as many life phyla as the terrestrial systems. General biological research has been much richer in land-based environment than in the oceans, although the marine biosphere has a longer history. Considering this fact, it is not surprising that many secrets are still kept in the seas. For the last decades, interest in marine research in a broad sense has grown significantly. This has led to discoveries of several thousands novel bioactive compounds from marine invertebrates, algae and microorganisms. Among these organisms are sponges, ascidians (tunicates), bryozoans, sea anemones, molluscs, crustaceans, seaweeds, cyanobacteria and actinomycetes. The classes of these compounds are diverse and include peptides, polyketides, sterols, terpenes, alkaloids and fatty acids (Faulkner, 2000, 2001, 2002, Mayer and Lehmann, 1999, Mayer, 2000, Mayer and Hamann, 2002). For many of these compounds, molecular formula and molecular mode of action have been defined, though their activity is a continuous subject of research (Monks et al., 2002, Mayer and Hamann, 2004, Blunt et al., 2003 and 2006).

1.2.2 Bioactive compounds from marine invertebrates or plants

One of the most promising resources for bioactivity is marine sponges. These organisms as a whole have already achieved an excellent record in this field, considering the number of studies and new isolated metabolites documented. These novel metabolites exhibit various types of bioactivity including anti-cancer, antimicrobial (fungi or bacteria), antiviral, anti-inflammatory, analgesic, anti-malarial or antifouling activities. All these factors are important in the constant search for new drugs or treatments for known diseases, as well as industrial additives. Sponges are quite interesting organisms as they are really ancient creatures, their history on earth covers more than 500 million years

(Carté, 1996) and an individual sponge is believed to be able to live for more than 1500 years (Belarbi et al., 2003). At least 10,000 species of sponges have been described, most of them from marine habitats (Osinga et al., 1999). They are sessile filterfeeders with a relatively simple body and function. In spite of that, they have survived drastic evolutional changes in the biosphere over millions of years and have been able to defend themselves against a variety of predators and threats through history. Apparently, they foster some more highly advanced defending mechanisms, than previously accounted for (Müller et al., 2004a). This is supported with the fact that thousands of novel bioactive metabolites have been found in sponges.

Table 1 lists a few reported examples of bioactive metabolites isolated from sponges.

Table 1 – Part I *Examples of bioactivity revealed by sponges*

Genus	Compound	Function	Reference
Aaptos sp.	Aaptamine	Anti- fouling	Diers et al., (2006)
Arenosclera sp.	Arenosclerine Haliclonamides	Antimicrobial, cytotoxic	Torres et al., (2002)
Discodermia dissoluta	Discodermolide	Cytotoxic Immunosuppressive	Salomon et al., (2004) Carté (1996)
Dysidea avara	Avarol /Avarone	Antimicrobial, cytotoxic, antiviral	Müller et al., (2004a)
Luffariella variabilis	Manoalide	Anti-inflammatory and analgesic	Kijoa and Sawangwong, (2004)
Lissodendoryx sp.	Halichondrin B	Antimicrobial	Salomon et al.,(2004) Hill, (2004)
Theonella swinhoei	Swinholid A Papuamides	Cytotoxic Antimicrobial Antiviral Cytotoxic	Sipkema et al., (2005) Müller et al., (2004c) Janin, (2003)
Cryptotethya crypta	Arabinofuranosyl- adenine (Ara-A) Arabinofuranosyl- cytosine (Ara-C)	Antiviral (herpes) Cytotoxic	Kobayashi, (2000)

Table 1 – Part II *Examples of bioactivity revealed by sponges*			
Genus	**Compound**	**Function**	**Reference**
Oceanapia sp. *Plakortis* sp. *Acanthella* sp.	Phloeodictine Plakortin Axisonitrile-3 /Kalihinol A	Antimalarial	Laurent and Pietra, (2006)
Mycales hentscheli	Peloruside A	Cytotoxic	Page et al., (2005)
Halichondria okadai *Discodermia calyx*	Okadaic acid	Cytotoxic	Kobayashi, (2000)
Nine different sponge genera	Manzamines	Cytotoxic Antimalarial	Urban et al., (2000)
Cacospongia sp *Hyatella* sp *Fasciospongia rimosa* *Dactylospongia* sp	Laulimalide	Cytotoxic Antifungal	Newman and Cragg (2006); Simmons et. al (2005)

The screenings of marine organisms have by no means been focusing narrowly on sponges, although they appear quite extensive in the literature. Antimicrobial proteins have for example been found in many marine vertebrates (fish) or crustacians. Such proteins exist in the mucous secretion of teleost fish and the antimicrobial lysozyme is found in many organs of fish. Hemocytes from different crustacians show antimicrobial activity and squalamine, a antimicrobial steroid was isolated from shark (Patrzykat and Douglas, 2003). Investigations of different phyla of lower invertebrates have also gained more interest and led to plenty of discoveries of new and active metabolites. Ascidians offer some good examples thereof, as they have been widely studied, yielding among other things potent compounds for cancer drug development, antimicrobial proteins and compounds inhibiting *Plasmodium falciparum* (anti-malarian). Novel bioactive metabolites have as well been isolated from bryozoans, sea anemones, sea snails and corals, to name a few.

In Table 2 a few known compounds originated from various marine invertebrates are listed.

Table 2 *Examples of marine invertebrates revealing bioactivity*

Genus	Compound	Function	References
Aplidium albicans (tunicate)	Dehydrodidemnin (aplidine)	Cytotoxic	Kijoa and Sawangwong, (2004)
Elysia rufescens (mollusc)	Kahalalide F	Cytotoxic	Janin, (2003)
Ecteinascidia turbinata (tunicate)	Ecteinascidin-743	Cytotoxic	Simmons et al., (2005)
Dolabella auricularia (sea hare)	Dolastatin 10	Cytotoxic	Kijoa and Sawangwong, (2004); Simmons et al., (2005)
Bugula neritina (bryozoan)	Bryostatin	Cytotoxic	Kijoa and Sawangwong, (2004)
Conus magus *Conus* sp. (cone snails)	Ziconotide Other conotoxins	Analgesic	Peng et al., (2006); Salomon et al., (2004)
Pseudopterogorgia elisabethae (coral / gorgonian)	Elisabethadione and other pseudopterosins	Anti-inflammatory	Proksch et al., (2002)
Perinereis aibuhitensis (clamworm)	Perinerin (peptide)	Antimicrobial	Pan et al., (2004)
Strongylocentrotus droebachiensis (sea urchin), *Cucumaria frondosa* (sea cucumber) *Asterias rubens* (starfish) *Modiolus modiolus* (horse mussel)	Peptide(s) not isolated	Antimicrobial	Haug et al., 2002 and 2004

Seaweeds and other macroalgae were one of the first marine organisms to be screened for bioactivity, probably due to being the easiest ones to collect. Since the research interest for the marine biological environment widened and better technology was available, the number of studies on algae has diminished (Carté, 1996). This is not due to lack of findings, since a number of compounds with antimicrobial and cytotoxic activity have been isolated from macroalgae (Freile-Pelegrín and Morales, 2004, Xu et al., 2004, Carté, 1996).

Table 3 displays some examples of algae and identified bioactive compounds.

Table 3 *Examples of algae revealing bioactivity*

Genus	**Compound**	**Activity**	**Reference**
Various brown algae, e.g. *Scytosiphon* sp.	Fucoxanthin	Antioxidant Cytotoxic	Mori et al., (2004)
Fucus vesiculosus (seaweed)	Fucophlorethol	Antimicrobial	Sandsdalen et al., (2003)
Bryopsis sp. (green algae)	Kahalalide F	Cytotoxic	Simmons et al., (2005), Janin (2003)
Laurencia intricata (red algae)	Laurenditerpenol	Cytotoxic	Mohammed et al., (2004)
Portieria hornemannii (red algae)	Halomon	Cytotoxic	Carté, 1996

Many of the above mentioned compounds have been a long term objects of studies and a few of them play an important role in drug development. A well known example is the cyclic macrolide bryostatin 1, which was first isolated from the bryozoan *Bugula neritina* in 1968, but first chemically characterized in 1982. An enormous effort has been put into developing a cancer drug with this compound as the active ingredient. Recent studies also indicate effects on biochemical pathways in the brain, leading to possible use in treatment of Alzheimer ´s disease or depression (Sun and Alkon, 2006). Bryostatin 1 has reached phase II clinical trials, but has proven to be relatively ineffective unless used in combination with other cancer drugs and probably has still some way to go before it is brought to the market (Kijoa and Sawangwong, 2004).

Ecteinascidin 743 (ET-743) is a tetrahydroisoquinoline alkaloid isolated from the Caribbean tunicate Ecteinascidia turbinata. An extract of this organism was first reported with an antitumor activity in 1969 (Faulkner, 2000, Urban et al., 2000). The compound itself was isolated many years later and has exhibited a broad anti-cancer activity. ET-743 is currently in phase III clinical trials and is expected to be launched as a commercial drug (Yondelis® or trabectedin) in the very near future (Anon, 2006, PharmaMar, 2006). Another example is Dolastatin10, member of a group of cytotoxic peptides named dolastatins and are originally isolated from the sea hare *Dolabella auricularia*. Dolastatin 10 has been undergoing phase II clinical trials for cancer, but toxic effects are of major concern, which has now led to a derivative of the compound (TZT-1027), which still reveals potent anticancer activity but reduced toxicity (Rawat et al., 2006, Simmons et al., 2005).

Didemnin B is a cyclic peptide isolated from the tunicate *Tridemnum solidum* and has shown both cytotoxic and antiviral activity. Didemnin B was the first marine natural compound to enter clinical trials in the 1980´s, but as the phase II trials revealed some serious toxic effects, it was not feasible to continue this work (Faulkner, 2000, Rawat et al., 2006). However, didemnin B can be oxidised to another compound – dehydrodidemnin B-, which as well has been isolated from yet another tunicate (*Aplidium albicans*). This compound revealed six times stronger anti-cancer activity and reduced toxic side effects. Dehydrodidemnin B is commonly known as aplidine (Aplidin®, plitidepsin) and is still the subject of clinical trials (Faulkner 2000, Janin 2003, Kijoa and Sawangwong, 2004).

The conotoxins are yet another example of drug potentials, but they affect pathways in the neurological systems, that has raised expectations for treatments of diseases like Parkinson's disease and epilepsy (Mayer and Hamann, 2002), but many questions are still unanswered on these matters. Their analgesic activity is more promising, as one of these compounds, - Ziconotide - derived from a conus snail (*Conus magus*) is the first marine natural compound to become to a commercial drug, Prialt® (Fenical, 2006). This drug is used for treating severe chronic pain and was launched in USA in January 2005 (Elan Corp., 2007).

1.2.3 Supply of raw material

The story of taxol is a descriptive example of the complications in harvesting bioactive compounds from natural resources either for research purpose or commercial production. Regarding marine products, the problem of collection adds to the system, many of the organisms can only be collected by diving, which both limits the collection efficiency and the working depth. Sustainable supply is though by far the biggest concern in these matters. The metabolites are often only present in trace amounts in the organisms, even as low as 10^{-6} % of weight (Proksch et al., 2002). This has led to enormous harvesting of the interesting organisms, although only for research purposes. In a two years period around 1990, thirty-eight metric tons of the bryozoan *Bugula neritina* were harvested off the coast of California just for the preclinical trials of bryostatin. Other examples are similar; one ton wet weight of *E. turbinata* is needed for isolation of one gram of ET-743 and 300 mg of a mix of two halichondrins could be isolated from one ton of the *Lissodendoryx* sponge (Proksch et al., 2002). The extreme example is probably the 12 years collection period of 2 tons of *D. auricularia* to retain 20 mg of dolastatin 10 (Harrigan and Goetz, 2002). Vague information are available on the ecology of these organisms, such as distribution, growth and regeneration rate, so discussions on overharvesting and other environmental issues are likely to rise. Doubtlessly, regular wild harvesting of marine invertebrates for large scale metabolites production will not be a feasible future option.

There are a few alternatives to consider in this matter. Once the compound in question has been chemically and structurally identified, chemical synthesis should be one of them. Unfortunately, many of the novel natural compounds have turned out to be structurally complex and chemical synthesis is a difficult and labour intensive task. Syntheses of many such compounds have been described, but can include a multitude of steps, expensive reagents and low yield. Accordingly, the process with big and complex molecules is seldom economically feasible. Bioactive peptides and smaller molecules give more hope on this subject. Ziconotide, (Prialt) the first marine drug on the market, is a peptide based compound and is successfully produced by chemical synthesis (Donia and Hamann, 2003). Furthermore, if a complete molecular mode of action of a certain compound has been found, the opportunity to synthesise more simplified analogues arises. Chemists are then able to build a compound including the active part of the natural

"mother molecule". Halichondrin B is an example where a simplified analogue called E7389 was successfully synthesised without losing the activity (NCI, 2005).

Another way to approach the supply of product is to use biotechnology and molecular methods to clone the responsible gene cluster into microorganisms for large scale fermentation. These methods demand the absolute knowledge of the biosynthetic pathways for a given metabolite and that the gene clusters are identified. For many of the marine natural products, these pathways are not well understood yet (Proksch et al., 2002).

A lot of discussion has been ongoing regarding mariculture or aquaculture of marine invertebrates for metabolites production and some quite massive farming experiments have been carried out. Trials with *E. turbinata* to retrieve ET-743 and *B. neritina* for bryostatin 1, both proved economically acceptable in open sea cultures (Mendola, 2000 and 2003). However, ET-743 is now partially synthesised from the bacterial metabolite safracin B (Proksch et al., 2002, PharmaMar, 2007) and the future of bryostatin 1 still seems to be too uncertain for investing in *Bugula neritina* farming (Mendola, 2003). Most of the aquaculture studies have dealt with sponges, usually performed by placing small explants of a wild sponge onto a substrate, either a rope line or mesh trays in the open sea. The recorded growth and survival rate is very variable as well as production of the desired compound and maximal growth does not necessarily correlate with optimal metabolite production (Page et al., 2005, de Voogd, 2007, Hadas et al., 2005, Osinga et al., 1999). Furthermore the studies were carried out on diverse species, which probably behave differently. Temperature, depth, nutritional availability, harvesting season and flow rate are critical factors and different sponge species react differently to these factors. Levels of bioactivity in farmed sponges have been reported higher, lower or similar in comparison to wild sponges, depending on species (Duckworth and Battershill, 2003, Munro et al., 1999). Page et al. (2005) estimated that a yearly explants production of 6.2 mt of the sponge *Mycale hentscheli* could yield 10 kg of peloruside A, a cytotoxic compound found in this specie. However, in their farming experiments, peloruside A was not present in all individuals (either wild or farmed) and in variable amounts, although the sponge grew excellently, so the recoveries can be hard to predict. Open sea ranching is always susceptible to environmental conditions, such as storms or temperature changes, predators or fouling. *In vitro* culturing of sponges has also been studied, but in general much more knowledge is needed regarding optimal culture conditions for metabolite

production. This refers to feed, temperature, oxygen, light, possible growth factors and media design (Belarbi et al., 2003). In short, aquaculture of marine organisms for metabolite production could be promising for certain species, but there is still much more knowledge to be gained on the subject.

Microbial symbionts and their possible role in metabolite production, have gained increased attention the last years. Indeed, there are strong reasons to believe that microbes might be playing a great part in this production or that they are in many cases the actual producers. The microbial community in or on a benthic organism individual, such as a sponge, has proven to be both big and diverse, which obviously can be a factor of influence. Accordingly, when metabolites are extracted directly from a marine invertebrate, it is not known whether they are produced by the invertebrate, symbiotic microorganisms or both. It is therefore of great interest to dig deeper into the world of marine microorganisms and gain more understanding on what they are capable of doing. Large scale microbial fermentation is a known method for production of various compounds and where applicable, this would be an ideal method for production of bioactive natural compounds. Consequently, it is important to identify the original producer of each compound and if they turn out microbial - ultimately find ways to cultivate them. To get these organisms in culture might be the biggest barrier to cross, but as cultivating methods are gradually improving, progress in these matters can be expected.

1.3 Microbial metabolites

1.3.1 Marine microbes and bioactivity

There is still a long way to go until the microbial communities in the oceans will be completely understood and unravelled. Many bacterial genera seem to be able to have their habitats both in terrestrial and marine environments (Holmström and Kjelleberg, 1999). On the other hand, obligate marine bacteria – requiring seawater for growth - have also been discovered and marine extremophiles can as well be found. From the extremophilic group, hyperthermophiles (at deep-sea hydrothermal vents) and barophiles (at greater than 10,000 m depth) are examples. In recent literature on marine natural products, microorganisms have apparently acquired more interest in regard to production of promising bioactive compounds. It has to be considered of great value, if cultivable

bacteria are found to produce bioactive metabolites, creating the option of direct production with the well documented method of microbial fermentation. As previously mentioned, such findings can not bee seen as straight forward since only a small proportion of the marine bacteria are cultivable so far.

Table 4 displays a few examples of such microbial producers already discovered. These microorganisms are not only associated with marine invertebrates, but have also been isolated from seawater or sediments. Many more representatives are already available, for example where Jensen and Fenical (2000) list more than 70 compounds that have been isolated from marine bacteria or fungi.

Table 4 *Examples of microorganisms producing bioactive compounds*

Genus	**Compound**	**Activity**	**Reference**
Micromonospora sp. (isolated from sponges or sediment)	Manzamin Thiocoralin	Antimalarial Cytotoxic	Laurent and Pietra, (2006); Simmons et al., (2005)
Micromonospora lomaivitiensis	Lomaiviticins	Cytotoxic Antimicrobial	Blunt et al., 2003
Salinispora sp. (isolated from sediment)	Salinosporamid A	Cytotoxic	Simmons et al., (2005)
Pseudoalteromonas sp. (isolated from marine algae or invertebrates)	Several compounds, not fully identified	Antimicrobial Antifouling	Holmström and Kjelleberg, (1999)
Ampelomyces sp. (marine fungi)	3-chloro-2,5-dihydroxybenzyl alcohol	Antimicrobial Anti-fouling	Kwong et al., (2006)
Prorocentrum spp. (dinoflagellates) *Dinophysis spp.* (diatoms)	Okadaic acid	Cytotoxic	Hill (2004), Proksch et al., (2002)
Microbacterium sp.	Glucosylmannosyl-glycerolipid	Cytotoxic	Lang et al., (2004)
Marinomonas mediterranea	Marinocine	Antimicrobial	Lucas-Elío et al., (2005)

1.3.2 Microorganisms as symbionts or diet

Marine invertebrates, especially filterfeeders like sponges, tunicates and bryozoan are loaded with bacteria that in most cases do not seem to be of any nuisance for the host (Hentshcel et al., 2006, Kennedy et al., 2007). Whether this bacterial load is primarily made up from the animal feed or symbionts is somewhat unclear, but most probably both topics have some significance. Prokaryotic organisms make up the backbone of the diet for sponges and other filterfeeders, but some sponges also harbour loads of bacteria in their mesohyl (Hentschel et al., 2006, Piel, 2006). Sponges can pump up to 24 m^3 of seawater pr kg own biomass through their canal systems daily and 40% -50% of the animal biomass can be composed of bacteria (Kennedy et al., 2007). Considering these facts, some of the active compounds already reported as sponge metabolites, might in fact be of microbial origin (Faulkner et al., 2000, Proksch et al., 2002, Hill, 2004). All the same, the invertebrate hosts should probably benefit from these metabolites to improve their defensive systems. There are a few leads to suspect microbial symbionts to be responsible for the production of active compounds, apart from the great record of terrestrial bacteria and fungi on the matter (see 1.1.3). One of them is where the same compound can be isolated in invertebrates of more than one genera and another when the metabolite production is found to differ in the same genus at different locations. Furthermore, the majority of the cytotoxic compound derived from marine organism can be classified as polyketides or nonribosomal peptides and the enzymes responsible for producing such compounds are so far only known in microorganisms (Piel, 2006). Microbial production has already been shown for some of the most studied marine natural products. Returning once again to bryostatin 1, it is now assumed to be produced by a microbial symbiont of Bugula neritina. Although not cultivated, the presence of this symbiont – "*Candidatus* Endobugula sertula"- has been confirmed and by methods of molecular biology, a gene cluster involved in the bryostatin production process has been cloned and partly classified as from bacterial DNA (Hildebrand et al., 2004). Furthermore, by using antibiotics to reduce or eliminate "*Candidatus* Endobugula sertula" from the *B. neritina* larvae, the production of bryostatin could be reduced or eliminated without affecting the larvae growth (Davidson et al., 2001). Links to microbial symbionts have also been found for more of the compounds previously mentioned in Table 1 to

Table 3. Okadaic acid, first discovered in more than one *Halichondria* specie, has now been shown to be produced by dinoflagellates of the genus *Prorocentrum* which appear in the sponge diet, but have no symbiotic relationship with the sponge (Faulkner et al., 2000). Furthermore, there are indications that bacteria associated with the dinoflagellates could be responsible for the okadaic acid production (Wiens et al., 2003), although other studies support the dinoflagellates origin (Piel, 2004). The anti-cancer agent dolastatin 10, was first found in the sea hare *Dolabella auricularia*, but a very close analogue, symplostatin (with one added methyl group) was found to be produced by the cyanobacterium *Symploca hydnoides*, which is known to be in the diet of *D. auricularia* (Shimizu, 2000). Dolastatin 10 itself has also been detected in another marine *Symploca* sp. (Proksch et al., 2002). The antifungal compound theopalauamide originally found in the sponge *Theonella swinhoei* has now been linked to a novel bacteria ("*Candidatus* Enthotheonella palauensis") belonging to the δ – proteobacteria (Faulkner et al., 2000). In the case of ET-743 originally discovered in the tunicate *E. turbinata*, a closely related compound, safracin B is produced by a *Pseudomonas fluorescens* strain and this compound now serves as a precursor for synthesis of ET-743, although it takes 21 synthetic steps (Piel, 2006).

Proving the microbial origin of bioactive marine compounds can be extremely difficult, as most of the symbiotic bacteria are not yet cultivable. Obtaining production of the desired compound from a pure bacterial culture obviously points out the producer without doubt. However, only 0,1 - 1% of the bacterial community can so far be cultivated, which complicates these matters considerably. The diversity of bacteria associated with representatives of sponges has been unravelled and mapped by culture-independent molecular methods, and bacteria have been localised by electron microscopy, but bacterial production of compounds is difficult to confirm completely unless it is isolated from a pure culture of the bacteria in question (Faulkner et al., 2000, Hill, 2004). Some progress has been made by using genomic sequencing methods to identify the genes controlling the metabolite production, as in the bryostatin case. This was also done successfully while studying the symbiosis of the ascidian *Lissoclinum patella* and the cyanophyte *Prochloron didemni*, where the gene clusters involved in the production of the cytotoxic cyclic peptides called patellamides could be linked to the cyanophyte rather than the ascidian. By cloning the specified cluster into *E. coli* , patellamide production could

be confirmed in culture (Schmidt et al., 2005). The cellular localisation of the compound is another way of approaching the subject and this has been reported by several studies, mostly on sponges (Piel, 2004, Giller et al. 2000). By chemical or fluorescent markers followed by elaborate microscopic examination or using cell sorters to discriminate between invertebrate and symbiont cells, it is possible to localise the given compound cellwise. These methods were used to identify the theopaulamide producing δ-proteobacteria in *T. swinhoei* and also to localise dercitamide only in the sponge cells of *Oceanapia sagittaria*, but not in any bacterial cells (Faulkner et al. 2000). However, scientists disagree on whether finding a certain metabolite inside a sponge (invertebrate) cell confirms the producer. It has been pointed out that these compounds might be stored in the invertebrate cell although produced and excreted by a symbiont or that the producers could be ingested in the diet leaving the compound to accumulate in the invertebrate cells. The invertebrate would probably benefit from these metabolites, being the reason for the accumulation. It has as well been argued to be hardly likely that metabolites are stored in relatively large quantities inside cells that do not produce them and this is supported be studies on sponge cell culturing (Erpenbeck and van Soest, 2007). One example of such contradictions is the toxin latrunculin B, a compound found in many sponges, but has been extensively studied in the Red Sea sponge *Negombata magnifica* (Hill, 2004). Fish avoid grazing on this branching bright-coloured sponge and the reason has been shown to be various toxic effects from latrunculins. In vitro studies of these compounds have furthermore shown potent cytotoxicity (Gillor et al., 2000, Hadas et. al. 2005) Latrunculin B. was confidently localised in cell vacuoles of the sponge, using immunolocalising methods with latrunculin-specific antibodies (Gillor et al., 2000). On the other hand, the biosynthetic pathways for the production of latrunculin B are more likely to be the bacterial type, as the compound's structure points to complex polyketide synthase pathways, which are so far only known in bacteria (Piel, 2004).

Studies on the pseudopterosins are illustrative on how tracking marine bioactive products to their producers can be complicated. Pseudopterosins are a group of tricyclic diterpene glycosides isolated from a coral - the Caribbean sea whip *Pseudopterogorgia elisabethae* (Table 2) . The compounds have shown potent anti-inflammatory activities and analgesic activity and they are licensed to a pharmaceutical company which has conducted preclinical trials for drug development (Kijoa and Sawangwong, 2004). The

success of the pseudopterosins is however in the cosmetic sector, as partially purified extracts of *P. elisabethae* are used as additives in skin-care products from Estée Lauder (Resilience® – line), providing anti-inflammatory properties, reducing allergic responses and sun irritation effects (Fenical, 2006, Kijoa and Sawangwong, 2004, Faulkner, 2000). The supply of *P. elisabethae* is a greatly limiting factor for the production and the synthesis of pseudopterosin has proven difficult. Mydlarz et al. (2003) presented their results on studying the symbiosis of *P. elisabethae* and a dinoflagellate *Symbiodinium* sp. By identifying the *Symbiodinium* cells inside the coral, they could demonstrate a strong correlation between the number of *Symbiodinium* cells and pseudopterosins pr mg of coral tissue. Further biosynthetic studies confirmed the involvement of the symbiont in the compounds production (Mydlarz et al. 2003). The same group continued their studies on corals following this lead, working with different corals with similar *Symbiodinium* symbionts, recovering further terpene metabolites such as fuscol and eunicol. By molecular methods (PCR amplification with specific primers) it was possible to discriminate between DNA from the coral, the symbiont and bacteria, which drew the attention to the bacterial community. Mixed cultures of these bacteria revealed production of fuscol and eunicol and further studies identified a Gram positive coccus, which is regarded a potential producer of these compounds. Following these findings, the *Symbiodinium* cultures from *P. elisabethae* were treated with antibiotics, resulting in normal growth of the dinoflagellate, but no pseudopterosins, which raises the question of bacterial production of pseudopterosins (Newman and Hill, 2006).

1.3.3 Marine actinomycetes and new metabolites

For a period of time it was generally assumed that actinomycetes found in marine environment were all of terrestrial origin, being dormant spores washed into the sea (Lam, 2006). It was therefore not expected to find any different species from this group in the oceans. Studies from the last decade or so, have shown that the case is not that simple, as indigenous marine actinomycetes of different genera have indeed been isolated. The best example is probably the novel genus *Salinispora* of the family *Micromonosporaceae* and order *Actinomycetales* (Mincer et al. 2002, Maldonado et al. 2005b). As the marine environment creates completely different and harsher conditions from the terrestrial ones, it might indicate that marine actinomycetes should be a rich source of novel bioactive

compounds and new antibiotics. This becomes especially valid as the terrestrial actinomycetes have a remarkable track record of bioactive metabolites (Figure 1).

Actinomycetes have been found in the sea surface layer and in marine aggregates (Ward and Bora 2006, Bull et al., 2005), but so far mostly from sediments at a range of depths, from tidal areas to more than 10,000 m depth (Colquhoun et al.1998, Mincer et al., 2002, Bull et al., 2005, Pathom-aree et al. 2006). Only a few of the isolated species appear to be obligate marine in the sense of requiring certain salinity, but anyhow, both new representatives of known genera and novel genera have been found in the marine environment. Examples of reported cultivable marine actinomycetes include members of *Streptomyces*, *Rhodococcus*, *Micromonospora*, *Verrucosispora*, *Actinomadura*, *Pseudonocardia*, *Gordonia*, *Williamsia*, *Dietzia* and *Salinispora* (Lam, 2006, Maldonado et al., 2005a). Novel cultivable species have as well been discovered, an example being three groups within the *Micromonosporaceae* family, recently isolated from sediments from Papua New Guinea, where two of these groups are likely to be new genera (Magarvey et al., 2004). The groups had salt tolerance of 4-5%, but did not need salt for growth and members of the lot showed promising bioactivity, both in antimicrobial and anticancer assays. The proposed genus names for the two supposed new groups are "Solwaraspora" and "Lamerjespora" respectively (Magarvey et al., 2004, Newman and Hill, 2006). Reports are also emerging on actinomycetes isolated from marine invertebrates. Kim et al. (2005) isolated *Salinispora* strains from the Great Barrier Reef sponge *Pseudoceratina clavata* and found indications of other uncultivable actinomycetes by molecular methods. Zhang et al. (2006) found cultivable representatives from 7 actinomycetes genera from the Yellow Sea sponge *Hymeniacidon perleve* and a new *Micromonospora* sp. (*M. lomaivitiensis*) was isolated from the ascidian *Polysyncraton lithostrotum* along with a *Salinispora* sp. (Blunt et al., 2003, Jensen and Mafnas, 2006). Jensen et al. (2005) collected 288 marine samples, mostly sediments around the island of Guam in the Pacific Ocean. Yielding more than 6,000 actinomycete colonies and almost 1000 pure cultures, more than half of them turned out as *Salinispora* (58%). A few of these samples were sponges, but only four actinomycete colonies were retrieved from those, indicating that actinomycetes from sponges might be more difficult to recover than from sediments. It is however rather ambiguous to draw many conclusions from these cases as the studies available on microbial communities associated with sponges show

huge differences between sponge species, whether based on culture-dependent or culture-independent methods (Webster and Hill, 2001, Webster et al., 2001, Burja and Hill, 2001, Hentschel et al., 2002, Montalvo et al. 2005).

Actinomycetes from the marine environment have not been studied for a long time, but have already provided new bioactive compounds. The genus *Salinispora* was discovered fairly recently, but is already well documented (Mincer et al., 2002, Maldonado et al., 2005b). It is the first reported actinomycete requiring seawater for growth, with two species already described formally, *S. arenicola* and *S. tropica* and the third one being proposed as ´*S. pacifica*´ (Jensen and Mafnas, 2006). The *Salinispora* strains found in the sponge *P. clavata* fall within these species based on the 16S rRNA sequencing, although differences in spore arrangement and sodium requirements were noted, which might in the end classify them as new species (Kim et al. 2005). The genus has been isolated from sediments at various sea bed sampling sites and depths around the world, although so far only from tropical or subtropical latitudes (Mincer et al. 2005). *S. tropica* produces a potent and highly cytotoxic β-lacton-γ-lactam, which has been given the name salinosporamide A (Feling et al., 2003). The compound's activity is based on proteasome inhibition, which is considered a highly promising target in cancer treatment. Salinosporamide A is currently in the state of Phase I clinical trials and is produced by large scale fermentation of *S. tropica* (Gullo et al. 2006). *S. arenicola* and *S. tropica* only grow in the presence of seawater and more specifically, are Na^+ dependent. The *Salinispora* isolates retrieved from a sponge, however, grew equally well whether the salt cation was K^+ or Na^+. Furthermore, those isolates grew considerably faster on the isolation media, than reported for the sediment strains. Organic extracts of the sponge isolates exhibited inhibiting activity against other bacterial isolates from the same sponge and also against *Staphylococcus* sp. (Kim et al., 2005). Another group of obligate marine actinomycetes species from sediments is currently being studied and has the suggested genus name "Marinispora" (family *Streptomycetaceae*). This genus has yielded a new group of antibacterial and anticancer metabolites, the marinomycins (Jensen et al., 2005, Fenical, 2006, Lam, 2006, Ward and Bora, 2006). Marine *Streptomyces* sp. have also been found to produce interesting compounds. A *Streptomyces* strain obtained from the Gulf of Mexico yielded gutingimycin, which has shown strong antibacterial activity against a range of test strains and trioxacarcin D, a potent antiplasmodian compound also

exhibiting antitumor activity (Blunt et al. 2006). Strong antimicrobial activity against MRSA and VRE clinical isolates was observed in a culture of a marine *Streptomyces* isolated from a cyanobacterium in Puerto Rico. The active compounds were isolated and classified as bisanthraquinones, which led to successful semi-synthetic active derivatives (Socha et al., 2006). Diazepinomicin was isolated from a *Micromonospora* sp. obtained from the ascidian *Didemnum proliferum* and exhibits antimicrobial, anti- inflammatory and antitumor activity. It is currently in preclinical development as a drug for cancer treatment (Gullo et al., 2006). A study by Imada (2005) describes testing for enzyme inhibition in marine actinomycetes strains, using different enzymes which play important roles in diseases like cancer or diabetes. A few *Streptomyces* strains were retrieved which showed such inhibition, but the search demanded very extensive screening. Only one *Streptomyces* strain was found to inhibit amylase after screening of almost 5000 isolates. A new member of the *Verrucosispora* genus from a sediment origin was isolated from the Sea of Japan and found to produce abyssomicins, a group of polycyclic polyketides (Fiedler et al., 2005). Abyssomicins inhibit *para*-aminobenzoic acid pathways involved in folic acid biosynthesis which happens in plants, fungi, procaryotes and certain parasites including *Plasmodium* – but not in vertebrates. This nature creates an interesting opportunity for development of new antibiotics. One abyssomicin producing strain was found after screening of 930 extracts from 201 actinomycetes isolates (Riedlinger et al., 2004).

Large scale fermentation of actinomycetes for metabolite production has been successfully established for some of the desired compounds. Diazepinomicin is produced with this method for preclinical drug trials. By studying the organism, selecting streptomycin resistant clones and using mutations and media optimisation, the fermentation yield has been increased from 1 – 3 mg/L to more than 300 mg/L. Likewise for salinosporamide A, the yield in the lab was originally 4 mg/L, but after various optimising operations, the yield in a 1,500 L fermenting tank under GMP production conditions reached 240 mg/L (Gullo et al., 2006).

1.4 Hydrothermal marine sites

1.4.1 Hydrothermal vents

Deep-sea hydrothermal vents were first discovered in 1977 on the Galapagos Rift in the Eastern Pacific. This finding and the following studies created new understanding of life forms down in the deep where primary production cannot be based on photosynthesis (Van Dover and Lutz, 2004). Such vent sites have since then been found at several mid-ocean ridge locations like in the Pacific or Indian Oceans, as well as on the Mid-Atlantic ridge. The superheated hydrothermal fluid (200 - 400°C) flowing up from the vents is usually acidic and rich with sulphides and minerals (Little and Vrijenhoek, 2003). These systems are commonly known as black smokers where the fluid is dominated with iron sulphides or similar metallic sulphides, and white smokers issuing fluid with higher proportion of calcium sulphate (anhydrite) and sicilium. Precipitations around the fissure openings sometimes build up to form spectacular chimneys, but these are usually not seen at depths less than 2000 m. Submarine hot springs or geothermal activities are also widely distributed at shallow waters and have been known to exist for a much longer period of time. Shallow-water hydrothermal vents usually do not build up any "landscape" or chimneys. The exact depth distinction between shallow-water and deep-sea vents is not very sharp and figures from 200 to 1000 m have been mentioned. By comparing 76 defined submarine areas from 0 to 4000 m deep, Tarasov et al. (2005) concluded to draw their line at 200 m depth, based mostly on biology and ecology. The main differences they found were a) the endemic fauna (vent specific) dominating at deep sea vent sites, whereas shallow-water vent fauna does not differ much from the surroundings and b) the forming and constant changes of landscape, e.g. chimneys occurring around the deep-sea vents. It should also be mentioned that at least one submarine hydrothermal field is known, being a completely different system, not driven by volcanic activity. This is a site called Lost City, discovered in year 2000 at a depth of 750 – 900 m, where white carbonate tower structures rise up to 60 m from the sea floor. The hydrothermal activity in the Lost City is caused solely by continuous exothermic chemical reactions and issues alkaline fluid at temperature around 50 – 100°C (Kelley et al., 2005).

Iceland is a part of the Mid-Atlantic ridge system and hydrothermal vents in Icelandic waters have been reported at least at the Reykjanes ridge, the Kolbeinsey ridge (south of Kolbeinsey) and east of Grímsey. The main site of the Grímsey vent field is at a depth of 400 m, where temperature has been reported up to 250°C as well as formation of small anhydrite and talc chimneys (Hannington et al., 2001). At the Kolbeinsey site, Fricke et al. (1989) observed chimneys, measured temperature up to 89°C and found indication of temperature around 180°C, according to pressure at approximately 100 m depth. Thermophilic bacteria were isolated and various samples of invertebrates collected. It was noticed that the fauna at the Kolbeinsey vent site was not exceptionally different from the surroundings (Fricke et. al., 1989). Apart from these hydrothermal vents sites, numerous sites of hot springs are known at tidal areas in Iceland, connected to land based geothermal systems (Benjamínsson, 1988).

1.4.2 Hydrothermal structures in Eyjafjörður

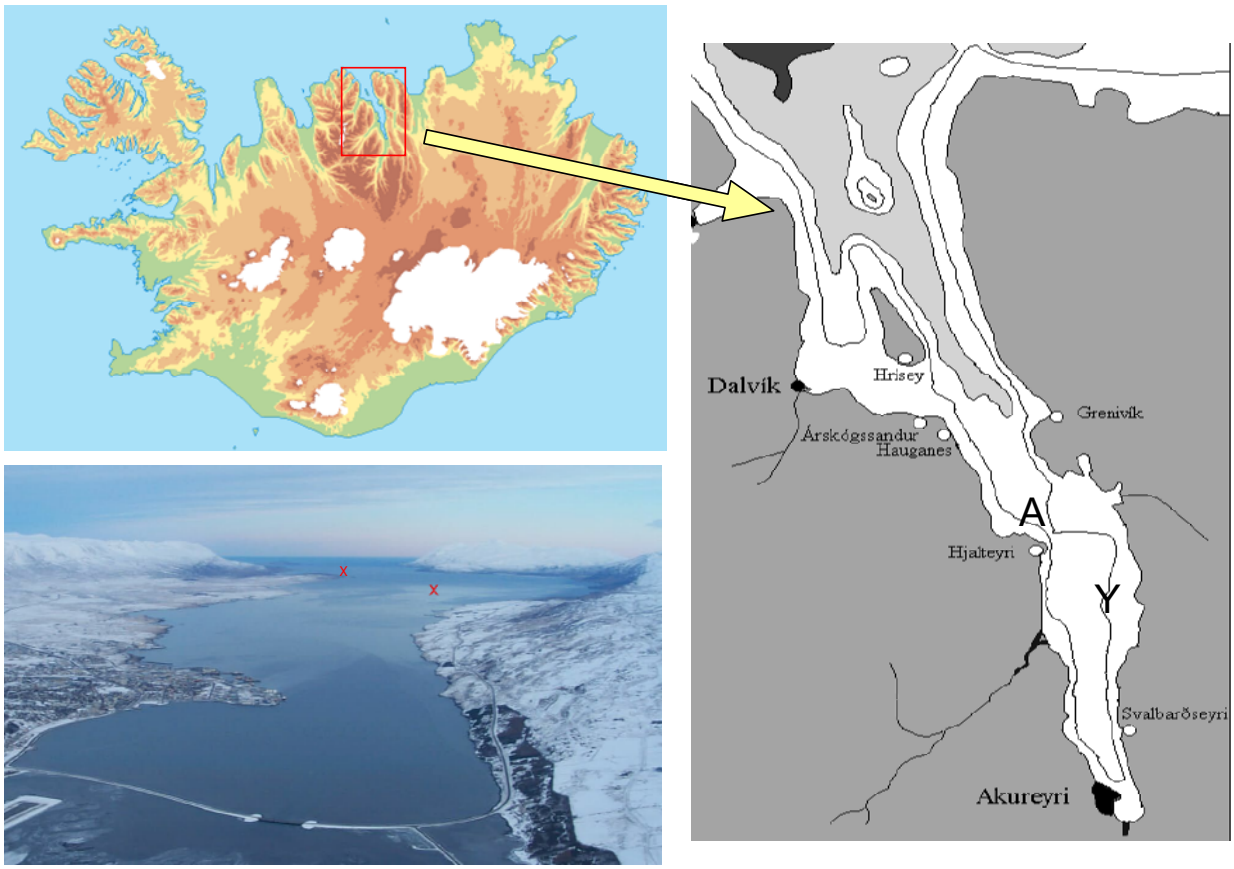

Figure 2 *Eyjafjörður in Iceland. Locations of the cones are marked on the map as: A: Arnarnesstrýtur and Y: Ystuvíkurstrýtur*

In the 1990´s three submarine hydrothermal vent cones rising 25 - 45 m from the seafloor, were discovered at a depth of 65 m in eastern Eyjafjörður. These cones are apparently a part of a land based geothermal system and as such are defined by location as submarine hot springs. However, the chimney formation is distinctive and resembles more the deep-sea chimneys like the black or white smokers. The geology and microbiology of these cones were studied in 1997 and 1998. Fifty strains of thermophilic microbes were isolated from either the fluid or the structures, some of which turned out to be novel species (Marteinsson et al., 2000). Although the structure formation resembled deep-sea chimneys, the chemical composition of both the cones and the hydrothermal fluid was similar to common precipitations or geothermal waters ashore. The fluid is fresh water at pH 10 and the cones themselves are made of magnesium silicate clay called smectite (Geptner et al., 2002). The area of these cones has been given the name "Ystuvíkurstrýtur".

Figure 3 *Top openings on of one of the Arnarnesstrýtur chimneys*. ***Photo: E. Bogason***.

In August 2004 another submarine area with geothermal activity and chimney structures was located in the west side of the fjord. This site now known as "Arnarnesstrýtur" (Figure 2) and is located at 25 – 40 meters depth. Arnarnesstrýtur is a larger hydrothermal area with many cones of various sizes, but not exceeding 25 m in height. The cones follow an almost straight line on a slightly sloping plateau at the sea floor. Fresh water at pH 10 is flowing from these chimneys and temperature at the openings is 75- 77°C (Figure 3). X-ray diffraction (XRD) analyses revealed that the cones are made of a poorly crystalline clay (Bjarni Gautason, Iceland GeoSurvey, pers. communication). By comparison with the similarly formed hydrothermal mounds at Ystuvík it can be assumed that the chief constituent of the cones is a poorly-crystalline saponite (Geptner et al. 2002).

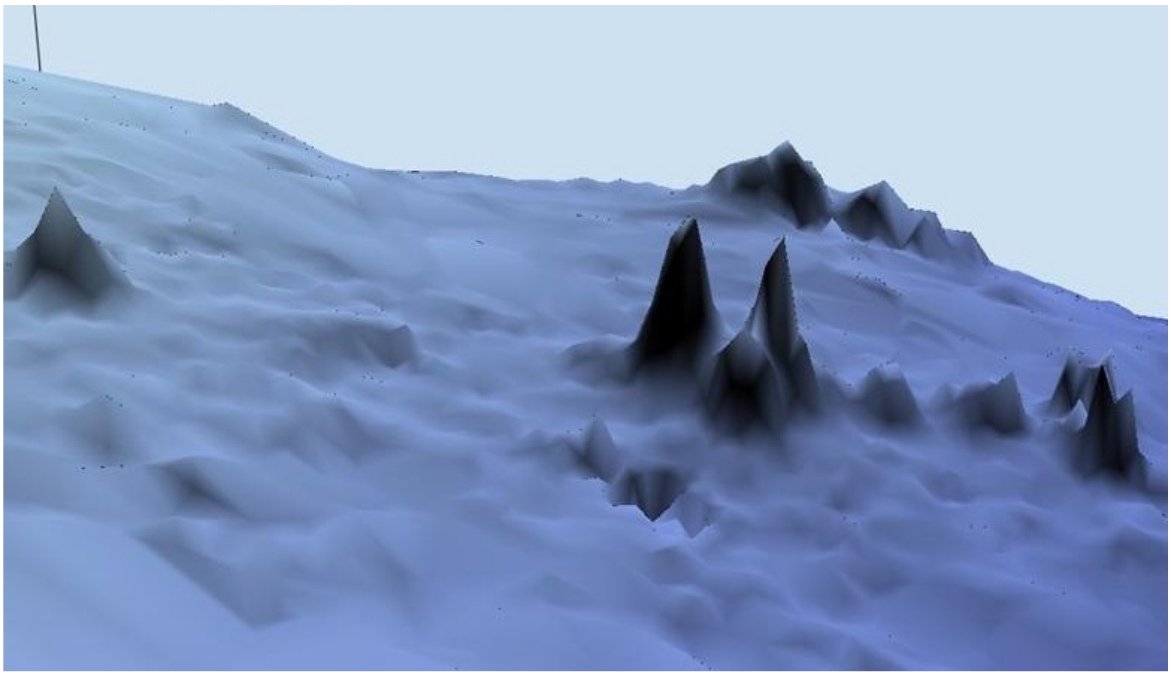

Figure 4 *A 3D-diagram of the Arnarnesstrýtur area, as plotted with an ecchosounder. The highest structures are approximately 25 m high.* ***Source: Marine Research Institute, 2005.***

As the chimney site is relatively easily accessed by SCUBA diving, great opportunities are created for exploring. Several excursions have been made down to the site both by SCUBA diving and a submersible ROV (remotely operated vehicle) to film and photograph. The area has also been plotted in 3D by an ecchosounder (Figure 4).
The pictures and videos taken at the site indicate a rich biodiversity, consisting of benthic organisms and algae totally covering the cones, except at the venting top part where no organisms are visible. The unique position of this ecosystem is the combination of seawater, fresh water, light and temperature, as well as the shallow water location making it easier to approach than most of similar hydrothermal vent sites. This exceptionality makes this site an interesting subject of geological and biological research in general. Possibly, the biota hosted by the cones has adapted to these circumstances or these chimneys in some way provide benefits for the ecosystem, referring both to invertebrates and symbiotic microorganisms.

1.5 Aim of the study

New discoveries of antimicrobial or other bioactive compounds remain of great value as they might be of various practical use. In addition, marine microorganisms are an interesting and probably underestimated source of bioactive compounds, whereas they might not only be producing undiscovered compounds, but also be the actual producers of some of the compounds previously assumed to be made by higher marine organisms. It is therefore of interest to study marine microorganisms at different environmental conditions. Placing emphasis on actinomycetes is logical, considering the multitude of compounds that have been discovered from terrestrial members of this group. Production of bioactive compounds by marine organisms have often been discovered where biodiversity is relatively rich, coral reefs being one example. From that point of view, the hydrothermal cones site should be an interesting ecosystem for bioprospecting. It is also a well defined and limited area, where sample collection is relatively easy and identifying organisms and screening for bioactive compounds can be a part of a multidiscipline research on an exceptional site. The aim of this study was to combine these viewpoints on bioprospecting in a marine environment to search for antimicrobial compounds.
The objectives of the study were the following:

- to collect samples of invertebrates on and around the *Arnarnesstrýtur* hydrothermal cones in Eyjafjörður
- to isolate bacteria which are capable of producing antimicrobial compounds, from these samples
- to make some effort to refine these compounds, with the final goal to isolate them completely and study their activity

2 Materials and methods

2.1 Samples collection

Five sampling trips to the hydrothermal cones site "*Arnarnesstrýtur*" were made on the Marine Research Institute research boat "Einar í Nesi" during the time period February 2005 to July 2006. The trips were reported as follows:

- EN-2-2005 in February 2005
- EN-12-2005 in August 2005
- EN-20-2005 in December 2005
- EN-4-2006 in June 2006
- EN-7-2006 in July 2006

All samples of invertebrates were collected by SCUBA diving by the professional diver Erlendur Bogason. Underwater, each individual sample was cut from its site and placed in a clean plastic bag along with seawater. The sample bags were kept in a seawater bin on the way back to the laboratory where they were kept refrigerated until further processing. Time from sampling until processing varied, as some experiments were made on conserving methods. Due to these experiments, not all the collected samples ended up as applicable for the processing described in section 2.2 and no bacteria were isolated from the EN-20-2005 samples (see 3.1). Three to fifteen samples were collected during each sampling trip, focusing mainly on sponges, but not entirely. Types of samples are summarised in Table 5.

Table 5 *Distribution and types of samples collected in different field trips.*

Trip ID	No. of samples	Types of samples
EN-2-2005	1	Unidentified (Figure 5)
	3	Sponges
EN-12-2005	10	Spo nges
	1	Sea anemone
	1	Unidentified
	1	Coralline algae (Figure 6)
	1	Egg strand from a nudibranch
EN-20-2005	7	Sponges
	1	Algae (growing on a sponge)
	2	Unidentified
EN-4-2006	11	Sponges
	2	Sea anemones
EN-7-2006	1	Mixed sample, containing sea anemone larvae, nudibranch and skeleton shrimps (*Caprella*)
	1	Sponge (Figure 8)
	2	Ascidians (Figure 8)
	1	Sea anemone
	1	Algae growth on cones (Figure 7)
Total	**47**	

A few examples of samples are shown in figures 5 – 8. Figure 6 and Figure 7 are underwater photos showing representing sites corresponding to actual samples collected.

Figure 5 *Unidentified sample from the sampling trip EN-2-2005.*

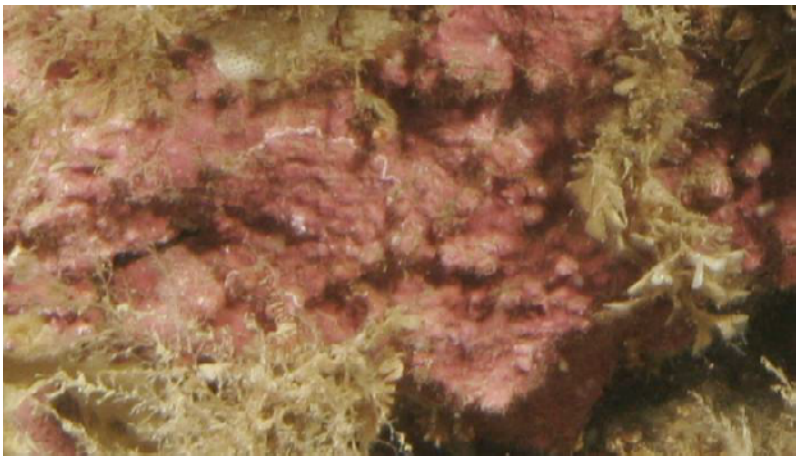

Figure 6 *Coralline algae on the cone surface (**Photo: E. Bogason, 2005**)*

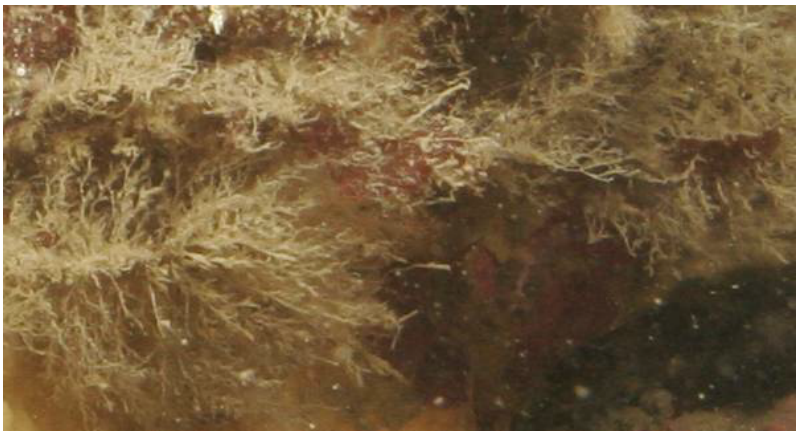

Figure 7 *Algae growth on a cone surface. (**Photo: E. Bogason, 2005**).*

Figure 8 *Samples at the lab after rinsing. Two sponge samples and one ascidian (middle).*

2.2 Culture and isolation

Each sample was washed three times in sterile natural seawater. Approximately 1cm^3 was aseptically cut from the tissue and weighed in a sterile mortar. The samples were minced with mortar and pestle with 9 mL of sterile natural seawater. Inoculations were made by surface spreading with serial dilutions on the following agar media plates:

- *ISP2 Medium* (BD Diagnostics) with 2% salt added.
- *Actinomycetes Isolation Agar* (BD Diagnostics) with 2% salt added.
- *R2A Medium* (BD Diagnostics) with 2% salt added.
- *Marine Agar 2216* (BD Diagnostics).
- *Starch Casein Agar*: 1 g casein dissolved in 10 mL 1,0 M NaOH, 10 g soluble starch, 0,5 g K_2HPO_4, 20 g NaCl, 20 g agar, 980 mL distilled water, 10 mL 1,0 M HCl, (Webster et al., 2001).

After the first sample collection trips, the procedure was modified to further inhibit Gram negative bacterial and fungal growth and make more effort to grow actinomycetes. Accordingly, for the samples collected in 2006, all the media types were supplemented with cycloheximide (10 µg/mL); nalidixic acid (10 µg/mL) and nystatin (25 µg/mL) after autoclaving at 121°C for 20 minutes. The main objective with the media and antibiotics selection was to stimulate growth of actinomycetes bacteria.

All samples were incubated at 15°C, which has been a standard temperature for culturing marine bacteria from Eyjafjörður or from seawater aquaculture in other studies

(Björnsdóttir et al., 2006). Besides, the samples collected in 2006 were also incubated at 23°C (room temperature), as the temperature at the sampling site is higher than in the surrounding seawater. Incubation time varied from 7 to 14 days. The plates were kept in incubation for additional 6 – 8 weeks and then examined for additional colonies.

Single colonies were selected from each medium for isolation, picking a few representatives of different morphologies. Colonies considered as potential actinomycetes were prioritised, e.g. visibly sporulating, often with tough and dry texture, as well as ones with bright yellow, orange or red pigments. Slow growing organisms, appearing after weeks of incubations were also considered of interest, as some marine actinomycetes are known to be very slow growers. Selection of colonies was however, not entirely limited to these groups. Selected colonies were streaked on ISP-2 medium, but in case of no growth, further attempt was made by streaking on Marine Agar or R2A agar. Cultures of these isolated colonies are referred to as bacterial *isolates* in this text.

The samples collected in the trip EN-20-2005 were treated differently to experiment with handling and culturing. Most of the sponges collected in this trip had relatively undamaged bodies and an attempt was made to keep a few of them alive in a aquarium for one or two weeks. The aquarium was rinsed and filled with fresh seawater and a controlled continuous oxygen flow was set up in it. The sponges were positioned on the pebbly bottom of the aquarium. The rest of the samples (3 organisms) were put directly into 200 mL of Marine broth in a sterile plastic bag which was then incubated at 15°C.

2.3 Culture extractions

2.3.1 Supernatant of liquid culture

Pure cultures from isolated colonies were inoculated into 100 mL of liquid ISP2 medium and incubated until dense growth was visible, usually 7 to 15 days. Marine broth was used as an alternative in case of colonies that were uncultivable on ISP2 agar. A sample of the broth culture was centrifuged at 5000 g for 10 minutes in a microcentrifuge (Sorvall-Heraeus Microfuge pico) and the resulting supernatant used immediately for antimicrobial susceptibility tests. Every culture retrieved successfully from the samples collected in 2005 was tested by this method. Testing of cultures from the year 2006 was

prioritised somewhat differently (see section 2.4.3), so this method was not used as extensively for that lot.

2.3.2 Organic extracts of liquid cultures

Fifty mL of ethyl acetate were added to approximately 100 mL of each broth culture, and the mixture left at room temperature for 2 - 7 days with occasional swirling. The two phases were separated in a separatory funnel after vigorous shaking and the aqueous phase was further washed with additional 50 mL of ethyl acetate. The organic phases were combined and the solvent left to evaporate completely at room temperature. Finally, 1,5 mL of ethyl acetate was added for redissolving and the resulting solution used for agar disc diffusion tests. A relatively small part of the total bacterial isolates was tested by this method on the whole.

2.4 Antimicrobial susceptibility tests

2.4.1 Indicator test strains and antibiotics

Each sample was tested by the agar diffusion test against eight indicator strains from DSMZ (Deutsche Sammlung for Mikroorganismen und Zellculturen, Braunschweig, Germany). The indicator strains were selected by two main motives. Five of them are special test strains for antibiotic susceptibility testing:

Enterococcus faecalis (DSM 2570)
Escherichia coli (DSM 1103),
Pseudomonas aeruginosa (DSM 1117),
Staphylococcus aureus (DSM 1104)
Staphylococcus aureus (DSM 2569)

Three of the strains are commonly used in quality testing of preservatives in cosmetic products:

Staphylococcus aureus (DSM 799)
Pseudomonas aeruginosa (DSM 1128)
Candida albicans (DSM 1386)

Five of these strains were also used in the direct streaking test and additionally a strain of *Listeria monocytogenes* (kindly provided by Jón Jóhannesson, ProMat ehf) and in some of the samples also a methycillin resistant *S. aureus* (DSM 111729) – referred to as MRSA.

Ready made antibiotic discs (Oxoid) served as positive controls for each bacterial indicator strain as follows:

Chloramphenicol 30 µg for *P. aeruginosa* DSM 1128 and *E. coli*

Tetracyclin 30 µg for *P. aeruginosa* DSM 1117

Rifampicin 5 µg or Erythromycin15 µg for *S. aureus* DSM 2569 and DSM 1104

Penicillin 10 units or Erythromycin 15µg for *S. aureus* DSM 799

Penicillin 10 units for *E. faecalis*

In the direct streaking test, the following test strains were used:

P. aeruginosa DSM 1128

S. aureus DSM 799

E. faecalis DSM 2570

E. coli DSM 1103

C. albicans DSM 1386

L. monocytogenes (a strain originally isolated from a food sample).

Streakings were repeated for all the strains showing inhibition.

2.4.2 Agar diffusion assay

Agar diffusion assay was used for testing antimicrobial susceptibility. The assay was carried out as follows: A fresh (16-20 h) broth culture of each indicator strain was diluted to approximately 10^5 pr. mL and 100 µl surface spread onto Mueller-Hinton agar (BD Diagnostics) plates. After drying at room temperature for approximately 30 minutes, 20µl of the isolate culture supernatant was added as a drop onto the plate surface. As a negative control, 20µl of the respective culturing broth (after centrifuging) was placed onto one plate of each indicator strain. For the ethyl acetate extract, 20 µl were transferred onto 6 mm sterile filter paper discs and allowed to dry for a few minutes. Equal volume of pure ethyl acetate served as a negative control on a corresponding disc. The discs were positioned on the inoculated Mueller-Hinton plates.

The plates were incubated at 35°C for 24 h and examined for inhibition zones in the growth lawn. Inhibition zones were measured as mm from the edge of the disc or droplet to the visible growth lawn of the indicator strain.

Two sponge samples from the EN-20-2005 collection were used to test a variation of the agar diffusion test. In this case, slices were aseptically cut from the inner tissue of the sponge and these slices put directly onto the surface of inoculated Mueller-Hinton plates. The purpose was to see whether antimicrobial compounds would diffuse directly from the sponge tissue and inhibit the test strain growth.

Figure 9 *Direct streaking test. The isolate is the vertical line on the plate and the test strains are streaked horizontally. This isolate does not show any inhibition. The black lines are drawn on the plate to indicate precisely where the streaks are.*

2.4.3 Direct streaking test

After screening of more than 900 isolates from two sampling trips in 2005, by the agar diffusion test with very low findings of activity, it was decided to change the screening method and switch to direct perpendicular streaking. Each isolate was then applied as a single streak onto an ISP2 agar plate and incubated for 5 – 10 days at the relevant temperature for the isolate, or until dense growth was visible. Six or seven test strains were then streaked perpendicular to the isolate line, each as a single streak (Strahl et al. 2002, Ivanova et al.1998). Test strains streaked on a similar plate without an isolate in the middle, served as controls. Plates were incubated at 35°C for 24 h and examined for test strain inhibition in presence of the isolate strain. This test was carried out on all isolates from the sample collections in 2006 (EN-4-2006 and EN-7-2006). The method is referred to as *direct streaking test* in this text.

2.5 Growth studies

2.5.1 Isolates for further work

Eight isolates displaying different inhibition in the susceptibility tests, were chosen for further investigation. Of them, three showed substantial antimicrobial activity in repeated tests. All these isolates were frozen in 30% glycerol and are kept at -70°C for

further work. The isolate 101-54 (EN-12 2005) was selected for creation of growth curves according to its growth inhibition of more than one test strain in the direct streaking test as well as showing inhibition both in a culture supernatant and with an ethyl acetate culture extract.

2.5.2 Growth studies

Growth studies were carried out in a Bioscreen microplate reader (Growth Curves Ltd, Finland), using 100-wells flat bottom honeycomb microplates. Ethyl acetate extract from isolate 101-54 (EN-12 2005) was used with 5 test strains, to find out whether the extract inhibited growth. Extracts were made from 2 litres of Marine Broth culture, extracting 200 mL at a time with double volume of ethyl acetate. The acetate phases were then combined, filtered through Whatman no.1 filter paper and the solvent allowed to evaporate completely before redissolving in 36 mL of ethyl acetate. Six different final concentrations of extract were prepared in Nutrient Broth: 10%; 5%, 2.5%, 1,25%, 0.5% and 0.1%, respectively, all with a final volume of 25 mL. Fresh cultures (16 – 20 h) of 5 test strains – *S. aureus* (DSM 799), MRSA, *L. monocytogenes*, *E. faecalis* and *C. albicans* were used as inoculates, where 0.1 mL of diluted culture was added to each of the extract supplemented Nutrient Broth. The dilution was aimed at a final culture density of 10^3 – 10^4 pr. mL. These mixtures were used as samples for the microplates. The microplate wells were loaded with 400µl of sample in quadruplicates for each test strain. *S. aureus* and *C. albicans* were additionally run with 0.02% extract. Pure ethyl acetate was used as a control for all the concentrations and all the strains in duplicates and a control sample with pure Nutrient Broth was also run. Cultures of each test strain in Nutrient Broth served as negative controls (control cultures). The growth studies were carried out at 35°C with optical density (OD) reading at 600nm at 20 minutes intervals for 96 hours.

The OD readings recorded for pure Nutrient Broth medium were subtracted from all measurements (stable values of around 0.11 over the whole experiment) plotted on the growth graphs. Normally, the OD values for the extract cultures were averages of four measurements. A high extract concentration in the cultures e.g. 5 % and 10 % did not produce curves that could be logically interpreted and created suspicion that the solvent itself was interfering. Solvent control runs confirmed solvent interference as growth inhibition was recorded both with 5% or 10% solvent in cultures. Consequently, the

highest extract concentration shown on the graphs presented is 2.5% (Figure 17 to Figure 20).

3 Results

3.1 Yield of samples and bacterial isolates

All the sampling trips were made as planned and no major complications were faced with collection of samples or transport to laboratory. Cultures on agar media and isolations of colonies were made from all the sample organisms, except from the EN-20-2005 trip (see below). However, in the cases where samples were kept refrigerated in seawater for more than 48 h, the recovery was poorer, due to heavy growth and isolation difficulties. Attempt to keep sponges (collected in EN-20-2005) alive in an aquarium at the lab was not successful. In two or three days, the sponges started to float up to the surface and in a short time, they were rapidly decaying. For that reason, no cultures were made out of these samples.

A total of 1866 bacterial isolates were retrieved from the samples collected at the sampling sites. Thereof, 1087 were isolated from sponges (58.3%), 197 from sea anemones (10.6%), 68 from a nudibranch (3.6%), 107 from ascidians (5.7%), 198 from algae (10.6%) and 209 from other organisms (11.2%). Due to a difference in processing, the isolates are divided into two separate lots by sampling periods (years 2005 and 2006). The number of isolates obtained in 2005 was 949 and isolates obtained in 2006 were 917 in total. Normally, each sample yielded 40 to 70 isolated colonies, but the outcome from sample EN-4-2006-202 (sponge) was only one isolate, whereas from sample EN-7-2006-305 (algae), 198 isolates were retrieved.

Apart from the above mentioned, 87 more colonies were isolated from the first sampling trip (EN-2-2005), most of them from sponges. Work on these isolates was not carried out to the end; hence they are not counted in the calculations above.

3.2 Culture and isolation

Best results for bacterial cultures were achieved by processing the samples as soon as possible, preferably within 24 h. Longer keeping time resulted in overgrowth of plates and difficulties in picking isolated colonies. The agar media used were all except Marine Agar selective for actinomycete to some extent, but on most of them quite diverse growth

appeared. Actinomycete Isolation Agar has the strongest selective components and developed least growth, whereas Marine Agar provided the most. Plate counts from Marine Agar were made on some of the samples collected in EN-12-2005, to get rough ideas on the total cultivable count. Most samples resulted in $10^5 – 10^6$ pr g. In the samples collected in 2006, antibiotics were added to the media to increase the selectivity. The overall look of the plate growth seemed visually different after this change was made, one example being colonies with morphology pointing to actinomycetes more frequently seen. Plate counts from three different media were made on some of the samples collected on the trip EN-4-2006, resulting in counts of approximately $10^4 – 10^5$ pr g. Total number of obtained isolates was slightly less in 2006 (917) than in 2005 (949).

A few small sized samples collected in EN-20-2005 were placed directly into Marine broth and incubated. The objective was to find out whether growing the whole bacterial population in enrichment would result in better recovery of antibacterial compounds. These liquid cultures turned completely black in a few days and created strong and unpleasant smell of decay, but were all the same used for susceptibility tests (supernatant of culture in an agar diffusion assay). No antimicrobial activity was detected in these samples. Additionally, two sponge samples collected in this same trip, were cut into small slices which were positioned directly onto inoculated Mueller-Hinton plates without any further processing. No inhibition zones were detected on any of these plates by using this method and no further such experiments were done.

When a new sampling period started in year 2006, incubating at room temperature (relatively stable at 23°C) was additionally tried for the samples, to look for indication of a different bacterial flora at a slightly higher temperature. As a general visual appearance of colonies was somewhat different between temperatures, it was decided to use the two incubation temperatures in the proceedings, so all samples collected in 2006, were incubated both at 15°C and 23°C. Plain results on whether this has made any difference are not available, until some classifications on the isolates have been made. However, cultures from both incubating temperatures provided interesting isolates.

Not all the isolates grew in subculture or restreaking. Thus, the yield in the direct streaking test was calculated as the proportion of isolates growing on the test plate, enabling completion of the test. The yield was 74% and 81% respectively for the two

sampling lots in 2006 (EN-4 and EN-7). Similarly the yield for the agar diffusion assay is counted as the proportion of isolates growing in broth culture.

3.3 Antimicrobial susceptibility

3.3.1 Agar diffusion assay

Agar diffusion assay from culture supernatant was carried out on 927 isolates in total. Attempt was made to carry out the test on all the 949 isolates from the year 2005, but not all the isolates grew up in broth culture and the final yield was 829 or 87.4%. Ninety-eight isolates from the samples collected in the 2006 period, were tested by this method. As the emphasis was placed on the direct streaking test for that lot, yield figures are not available for the agar diffusion assay in the year 2006 samples.

Table 6 shows the results for the isolates with recorded activity in the culture supernatant. The inhibition zone was not significantly wide in any of these tests, but all the same a clear inhibition was observed. Other isolates did not respond positively in this test.

Table 6. *Antimicrobial activity observed in testing supernatant of broth cultures by agar diffusion assay. Inhibition zones are shown (mm) as measured from the edge of the droplet to a visible growth lawn of the test strain. These isolates were all obtained at incubation temperature of 15°C.*

Isolate no. (sampling trip) / Test strains	**101-54 (EN-12-2005) mm zone**	**105-41 (EN-12-2005) mm zone**	**206-38 (EN-12-2005) mm zone**
P. aeruginosa DSM 1117	**0**	**0**	**0**
P. aeruginosa DSM 1128	**0**	**0**	**0**
E. coli DSM 1103	**0**	**0**	**0**
E. faecalis DSM 2570	**1**	**1-1.5**	**0**
S. aureus DSM 2569	**< 0.5**	**0.5**	**0**
S. aureus DSM 1104	**1.5**	**0.5-1**	**< 0.5**
S. aureus DSM 799	**4**	**4.5**	**0.5**
C. albicans DSM 1386	**0**	**0**	**0**

3.3.2 Direct streaking test

As the recovery of active isolates from the agar diffusion screening turned out rather poor, the decision was made to change the screening method to direct streaking on

agar plates. The most important difference between these methods, apart from higher throughput with the streaking, is that the direct streaking method allows each isolate to be tested while in full growth and there is almost a direct contact between the isolate and the test strain. Figure 10 shows examples of two different isolates revealing positive responses, where parts of the test strains are inhibited, while others are unaffected.

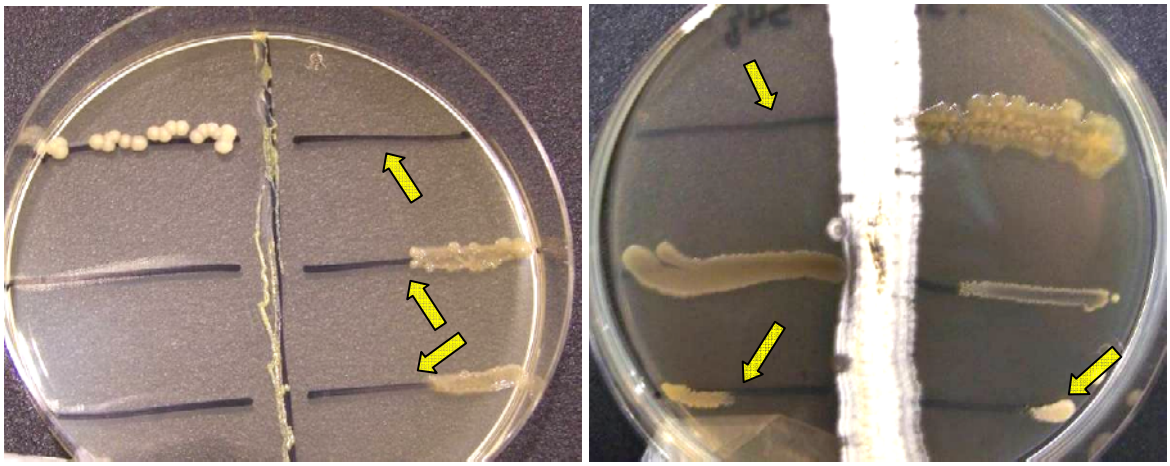

Figure 10. *Antimicrobial activity test by direct streaking. On the left side plate, the two upper test strains to the left (C. albicans and E. faecalis) are growing normally, where as the other five are all inhibited to some extent by the isolate line in the middle. On the right side plate, C. albicans is completely inhibited and the two bottom strains (L. monocytogenes and E.coli) are also inhibited, but to a lesser extent.*

The direct streaking test was carried out on all the 917 isolates from samples collected in 2006 and one of the three positives isolates from year 2005 (Table 6). The method yielded results for 704 isolates (77 %), whereas the rest did not grow on the testing plates. A total of 96 isolates revealed antimicrobial activity against one or more test strains by this method The following figures are based solely on results from the direct streaking tests and therefore only refer to isolates retrieved in 2006.

On Figure 11 the isolates which revealed inhibition of test strains are shown in connection with their sample type origin. Almost one third of the positive isolates were of sponge origin (30), reflecting the majority of collected samples being sponges. On the other hand, the 13 positive isolates from algae all come from the same sample. The mixed sample was small, but contained at least three different species (Table 5) and resulted in 17 bacterial isolates revealing antimicrobial activity.

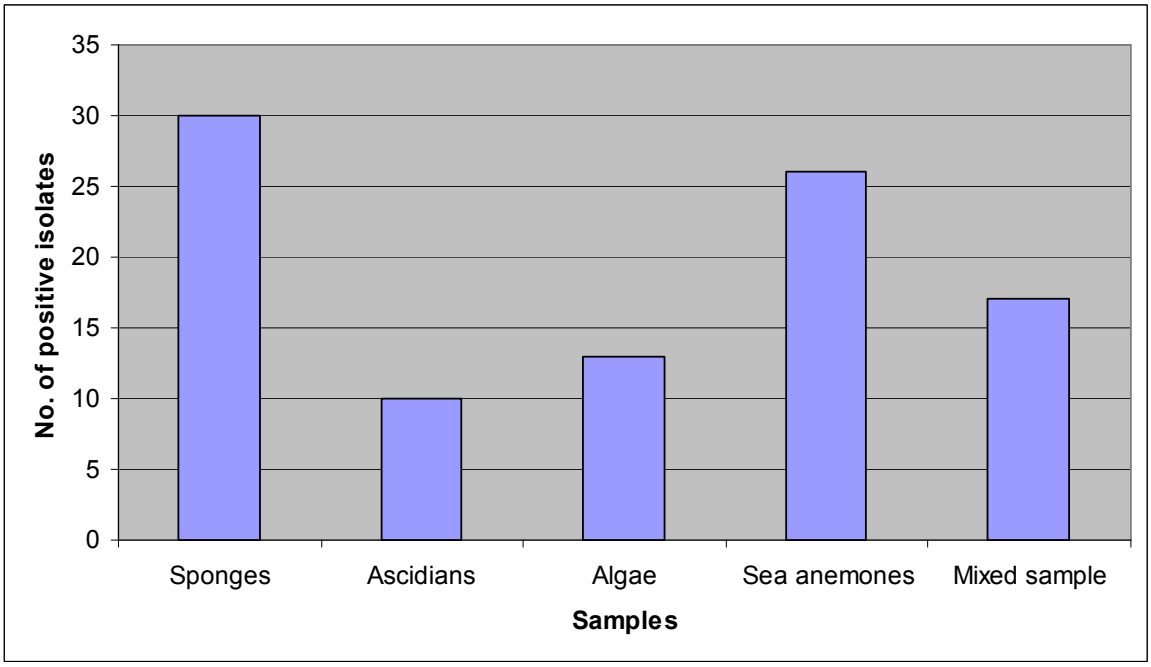

Figure 11. *Sample organisms hosting the bacterial isolates with detected antimicrobial activity in the direct streaking test.*

The isolates revealing activity are shown in Figure 12 as a proportion of the total number of isolates from each sample type. All the sample types in the lot provided isolates showing inhibition of one or more test strains. The frequency varied between groups, whereas 21% of the strains isolated from the mixed sample revealed inhibition and 17% of the sea anemones isolates showed inhibition. Although sponges hosted the highest number of active isolates, the active proportion of the total sponge isolates run in this test, was not particularly high or 8%.

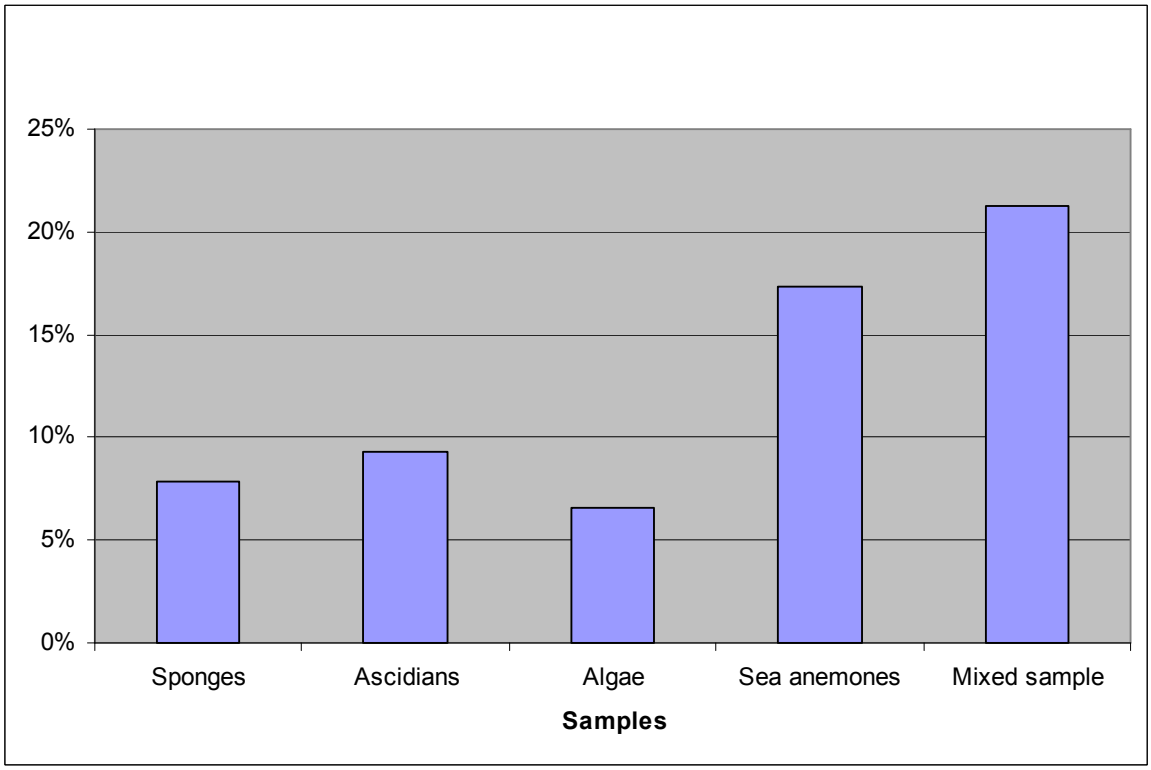

Figure 12 *Isolates with antimicrobial activity as a proportion of total isolates from each sample type. Active isolates refer to isolates revealing inhibition in the direct streaking test.*

The isolates revealing activity, also acted in different ways, as some of them inhibited one test strain whereas others showed a wider inhibition. Figure 13 divides the active isolates by the number of test strains inhibited. Forty-seven isolates revealed activity against one test strain and one isolate inhibited all the six of them. Twenty-two isolates inhibited three or more test strains. About a half of the isolates (47) acted against one of the strains, most frequently that strain turned out to be *C. albicans* or in 29 cases.

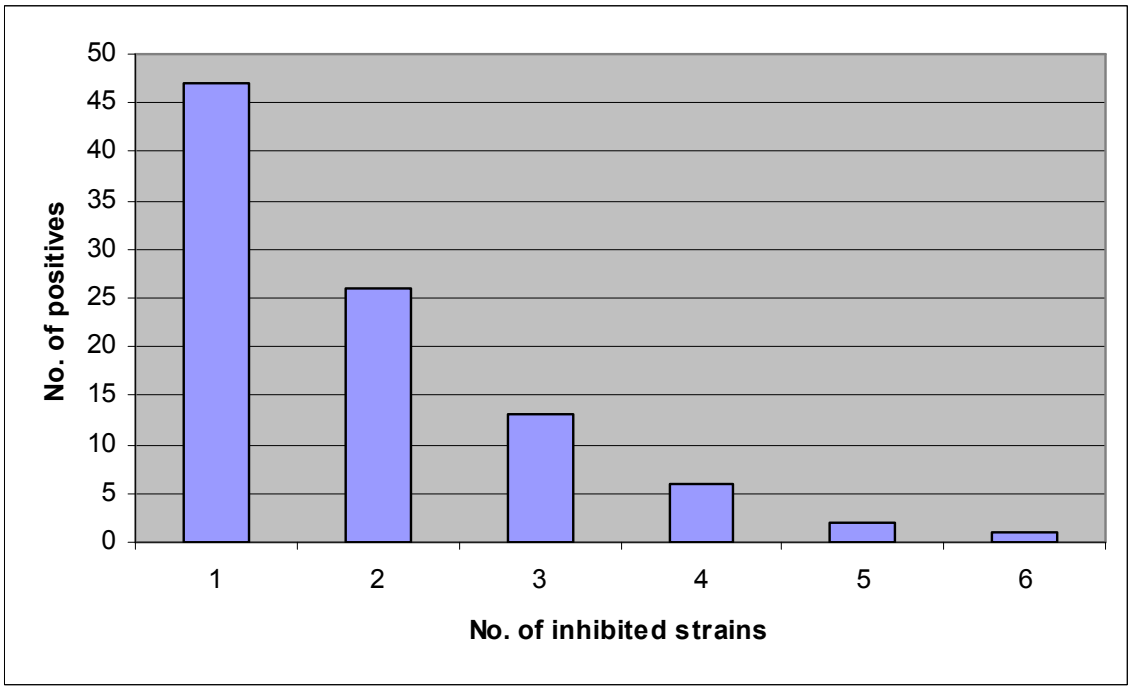

Figure 13. *Broadness of the antimicrobial activity detected by the direct streaking test, e.g. number of test strains inhibited by each isolate.*

The total score for each test strain is demonstrated in Figure 14, where inhibition of one strain is counted as one positive response and inhibition of three strains are counted as three positive responses etc. Growth of *C. albicans* was most often inhibited on the whole, as 56 isolates showed activity against it. Inhibition of *E. coli* growth was recorded in 42 incidents, but growth of *E. faecalis* had the lowest inhibition frequency or in 8 incidents.

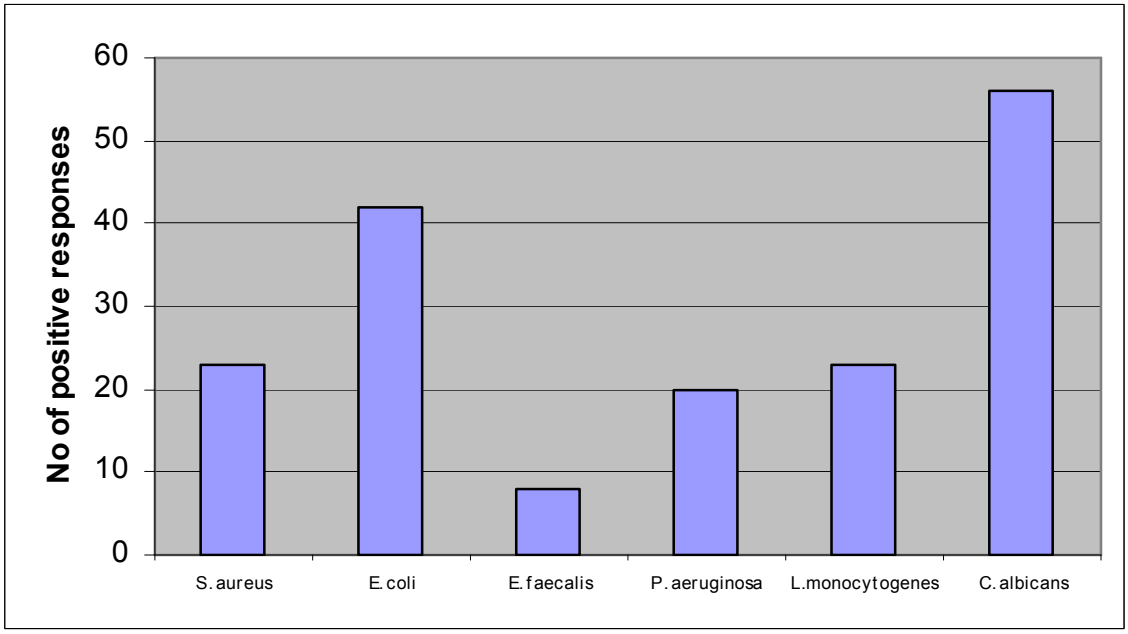

Figure 14. *Frequency of inhibition measured by direct streaking, against each of the test strains.*

As a first step in isolating the active compounds from the broth cultures, positive isolates were picked out for retesting by agar diffusion assay. Both culture supernatant and an ethyl acetate extract of each culture were used for this test (Figure 15).

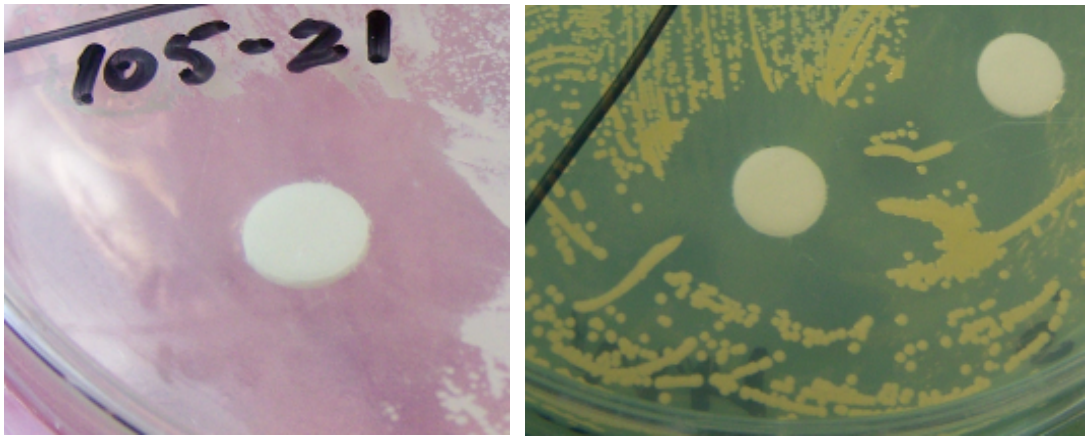

Figure 15 *Antimicrobial activity on an agar disc diffusion assay. Test strains are C. albicans to the left and S. aureus to the right.*

The assay was carried out for 79 isolates, including repetition of the three isolates from year 2005, listed in Table 6. Two of the isolates from year 2005 displayed stronger activity with the acetate extract, than with the supernatant previously, but the third one did not respond. None of the tested isolates from year 2006 displayed activity when the culture supernatant was tested. Ten isolates from that lot responded positively with the acetate extract, but solely against *C. albicans*. The results are summarised in Table 7,

showing only the isolates that did reveal activity. In this test 12 isolates out of 79 tested (15%) revealed activity. It is therefore obvious that the susceptibility observed with the streaking does not necessarily appear in the agar diffusion assay.

Table 7. *Antimicrobial activity observed in ethyl acetate extracts of broth cultures by agar diffusion assay. Inhibition zones are shown (mm) as measured from the edge of the filter disc to a visible growth lawn of the test strain. Isolates were obtained at incubation temperature of 15°C.*

Test strains / Isolates (sampling trip)	*P. aeruginosa* DSM 1117	*P. aeruginosa* DSM 1128	*E. coli* DSM 1103	*E. faecalis* DSM 2570	*S. aureus* DSM 2569	*S. aureus* DSM 1104	*S. aureus* DSM 799	*C. albicans* DSM 1386	*L. monocytogenes*
101-54 (EN-12-2005)	-	-	-	**2**	-	**1**	**5**	-	**1-2**
105-41 (EN-12-2005)	-	-	-	**3**	-	**1**	**6**	-	**2**
101-29 (EN-4-2006)	-	-	-	-	-	-	-	**6**	-
105-13 (EN-4-2006)	-	-	-	-	-	-	-	**7**	-
105-21 (EN-4-2006)	-	-	-	-	-	-	-	**6**	-
301-10 (EN-7-2006)	-	-	-	-	-	-	-	**6**	-
301-30 (EN-7-2006)	-	-	-	-	-	-	-	**9**	-
303-4 (EN-7-2006)	-	-	-	-	-	-	-	**6**	-
305-2 (EN-7-2006)	-	-	-	-	-	-	-	**10**	-
305-61 (EN-7-2006)	-	-	-	-	-	-	-	**5-6**	-
305-65 (EN-7-2006)	-	-	-	-	-	-	-	**7-8**	-
305-70 (EN-7-2006)	-	-	-	-	-	-	-	**5**	-

3.4 Growth studies with an extract of isolate 101- 54

The effects of ethyl acetate extract (EAE) from the culture of isolate 101-54 on the growth of the various test strains are shown in Figure 16 to Figure 20. The growth, measured as optical density (OD) at 600 nm in a Bioscreen microplate reader, is plotted against time. The growth studies were done using *S. aureus*, MRSA, *L. monocytogenes, E. faecalis* and *C. albicans*, respectively with or without the isolate culture extract. Solvent controls consisting of culture with pure solvent (EtOAc) added, but without extract, were

also run for each concentration to test for interference of the solvent. Result for all the test strains showed that the 5% and 10% EtOAc concentration affected the growth significantly (data not shown). Accordingly, the results for 5% and 10% EAE were regarded as interfered by the solvent and are not presented on the following graphs. Controls were cultures of each strain in Nutrient Broth without any addition and they are usually referred to as control cultures. The OD graphs indicate both biomass formation (height of curve) and growth rate (slope of curve). The time period before the OD values start to rise, e.g. the horizontal parts of the curves at the beginning of the time scale, are referred to as the *optical density lag phase (OD lag phase)*.

The effect of EAE on the growth of *S. aureus* in Nutrient Broth at 35°C is shown in Figure 16. Without addition of EAE the bacteria grew well with a short optical density lag phase around 5 h and reached a maximum OD-value in 42 h. Addition of EAE even at a low concentration (0.02%) slightly prolonged the OD-lag phase. Increasing the EAE concentration further resulted in greater inhibition. No growth was observed (in 96 h) when 0.5%, 1.25% and 2,5% EAE was added, whereas slow growth started after 80 h in the 0,1% EAE culture.

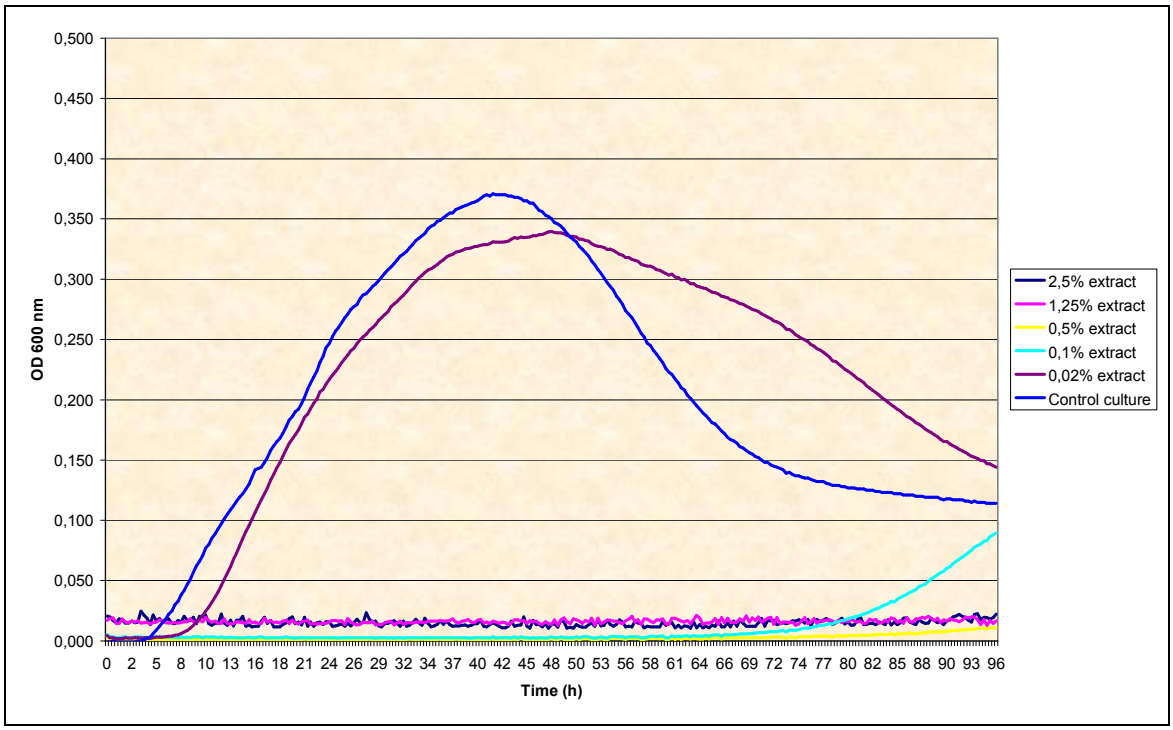

Figure 16 *Effect of different concentrations of EtOAc extracts from bacterial isolate **101-54** from a sponge (see M&M) on the growth of S. aureus in Nutrient Broth at 35°C The control culture (no addition) is shown in blue.*

The effect of EAE on the growth of MRSA in Nutrient Broth at 35°C is shown in Figure 17. Without addition of EAE the bacteria grew very well with an optical density lag phase around 15 h and reached maximum OD-value in 60 h. Addition of low concentrations (0.1%, 0.5% and 1.25%) of EAE shortened the OD lag-phase and increased the growth although slower growth rate was observed as concentration increased. An EAE concentration of 2,5% inhibited the growth by increasing the lag-phase but the apparent growth rate was higher than that of the control.

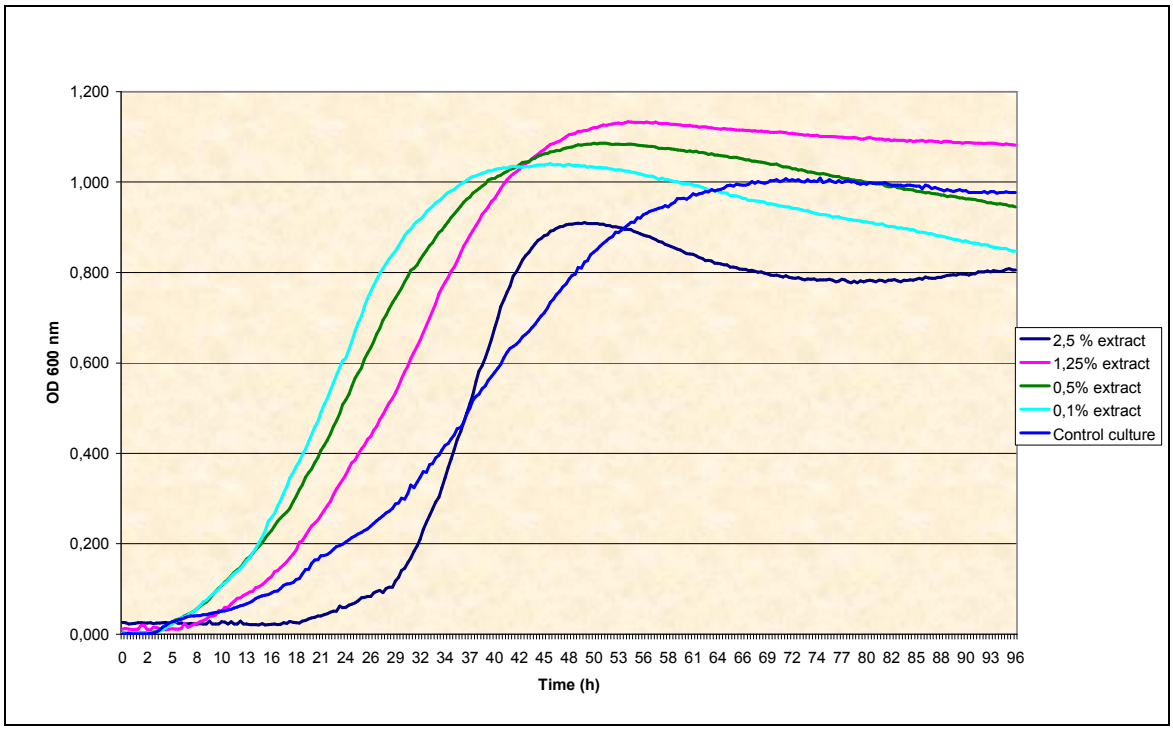

Figure 17 *Growth of MRSA strain in presence of ethyl acetate extract of isolate **101-54** in different concentrations. A control culture (blue), represents MRSA in Nutrient Broth without addition.*

Growth of *L. monocytogenes* with and without the presence of EAE in Nutrient Broth at 35°C is shown in Figure 18. The bacterial growth without addition of EAE was relatively poor in this case, as the OD value never exceeded 0,14 after allowing for media turbidity. However, the bacteria had an optical density lag phase around 7 h and stationary phase in 24 h. Addition of 0.1% or 0.5% of EAE had no effect on growth, but higher concentrations (1,25% and 2,5%) inhibited it.

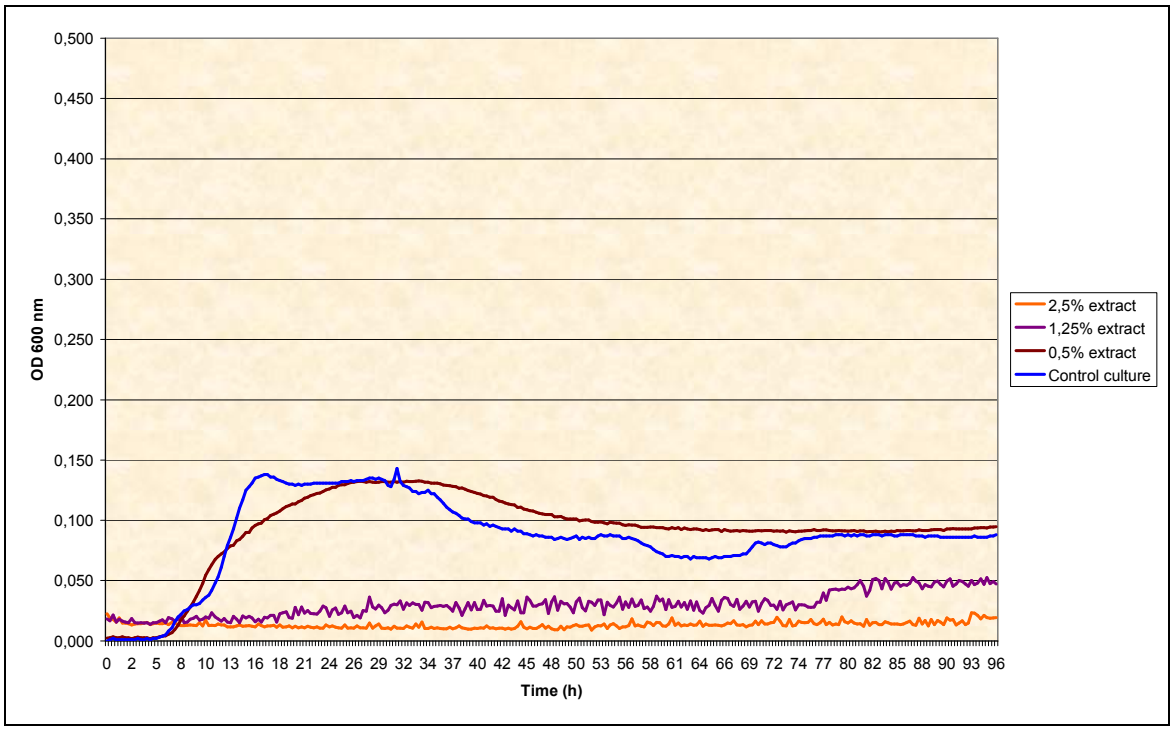

Figure 18 *Growth of L. monocytogenes in presence of ethyl acetate extract of isolate **101-54** in different concentrations. The control culture (L. monocytogenes in Nutrient Broth) is shown in blue.*

The effect of EAE on growth of *C. albicans* in Nutrient Broth at 35°C is presented in Figure 19. The yeast grew well, but very slowly and first reached the OD stationary phase in 96 h or at the end of the experiment. Addition of 2.5% EAE, clearly inhibited growth of C. albicans, whereas the 1.25% EAE culture exhibited much denser growth than the control, a difference of one third in OD value.

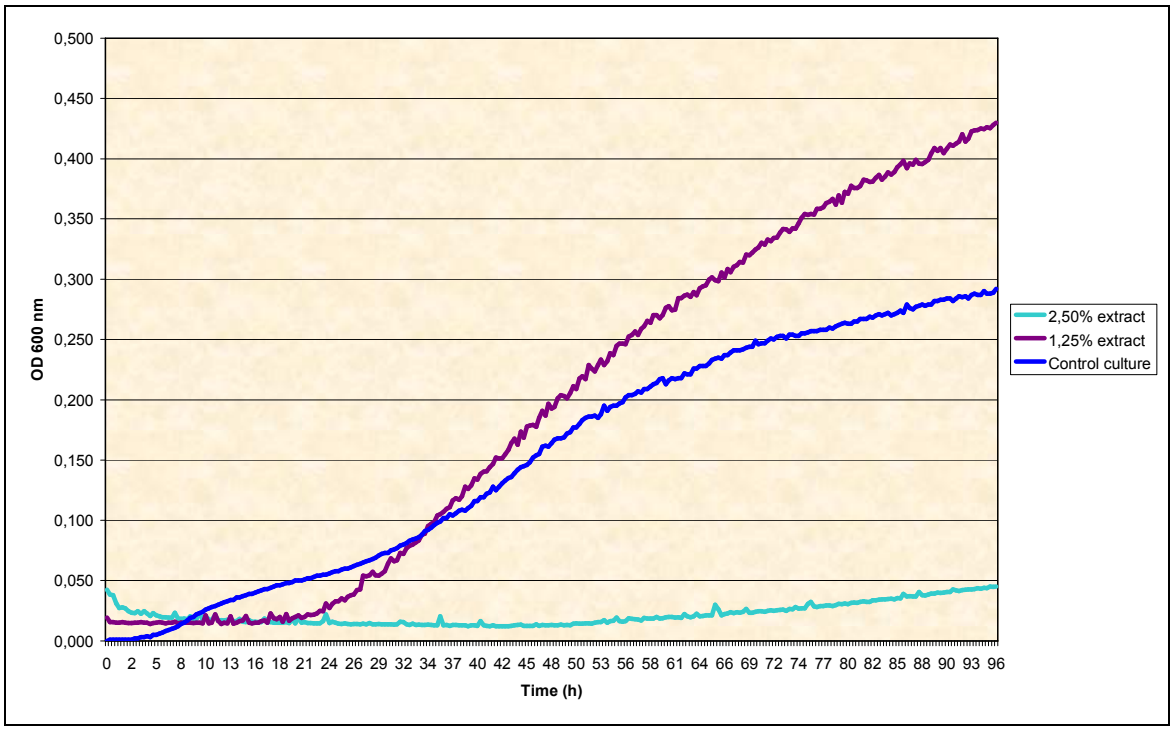

Figure 19 *Growth curves for C. albicans in presence of ethyl acetate extract of isolate **101-54** in different concentrations. The control culture is shown in blue and solvent controls in red (5 %) and green (2, 5 %).*

The growth of *E. faecalis* without or in presence of EAE in Nutrient Broth at 35°C is shown on Figure 20. The bacteria grew poorly under these conditions and the OD values for the culture were recorded below 0.1 throughout the experiment after allowing for media turbidity. An optical density lag phase lasted 8 h and stationary phase was reached in approx. 18 h. Addition of EAE in low concentrations decreased the growth and prolonged the optical density lag phase considerably, to 44 h with 0,1% EAE and 62 h with 0,5% EAE. Higher concentrations of EAE (1,25% and 2,5%) inhibited growth for 96 h or throughout the experiment.

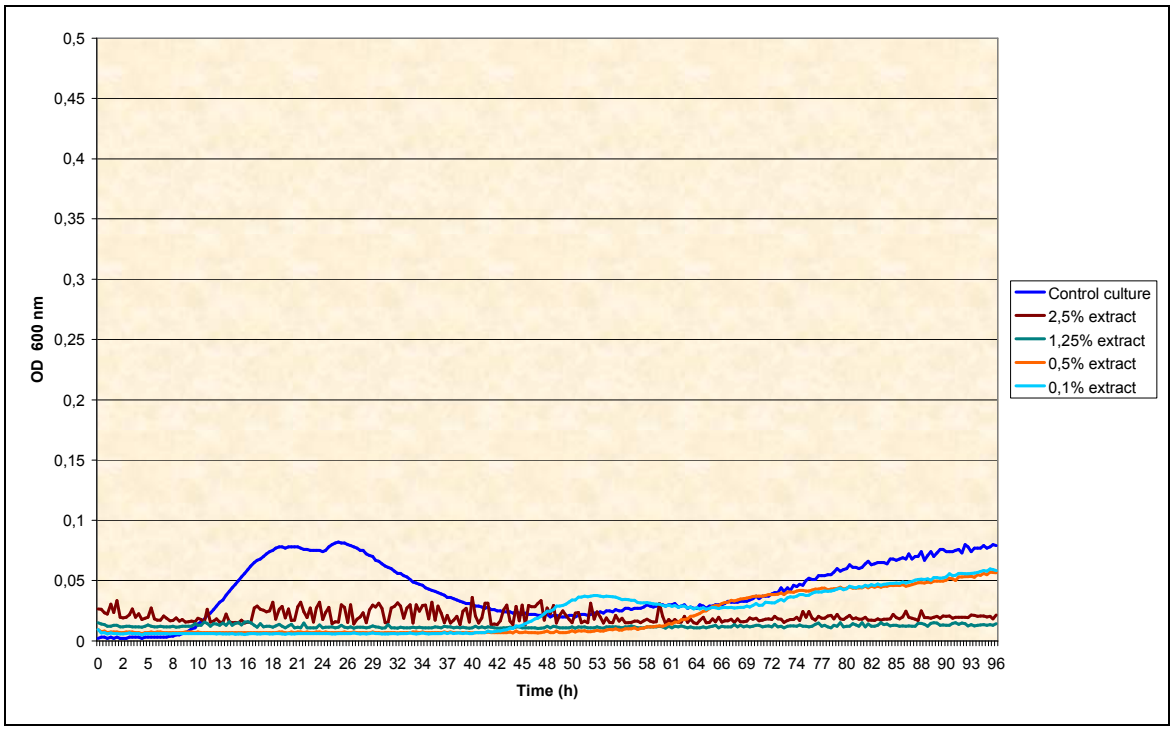

Figure 20 *Growth curves for E. faecalis in presence of ethyl acetate extract of isolate **101-54** in different concentrations.. The control culture is shown in blue and a culture with pure solvent in red.*

To summarise the growth studies' results, the extract from isolate 101-54 affected growth of all the test strains except methycillin resistant *S. aureus* (MRSA) where no inhibition was observed (Figure 17). Growth inhibition was seen for extract concentration as low as 0.1% for *S. aureus* (Figure 16) and *E. faecalis* (Figure 20). The growth curves looked very different for each strain and in the case of *L. monocytogenes* and *E. faecalis*, the growth as interpreted from the OD graph, was poor in general. All the same, the EAE effects were relatively clear, either it had no effect on growth, or it completely inhibited it.

4 Discussion

4.1 Sampling and culturing

Collection of invertebrates at the hydrothermal cones site by SCUBA diving was made without facing any major difficulties. The site is located very near to the lab, so a typical sampling trip was made in 5 – 7 h. On the other hand, attempts to keep sponges alive for days, in a small aquarium were not successful. Müller et al. (2004a) have successfully kept sponges in 1000 L tanks for months, for research purposes, but probably in more elaborate and controlled conditions. One of many things to be taken into consideration is that our aquarium had a gravelly bottom and no special sticking or fastening features were used (glue or thread). This might prevent the sponges' ability to form a stable contact. Such contact has been shown to form in 2 – 3 days on a metallic or plastic surface, but with attachment aid (Müller et al., 1999). Exposure to air during transport and handling could be another reason, as air in the sponge canal system will result in blocking of the sponge pumps (Osinga, 1999). No further attempts were made on these matters, as that was not according to the main aim of this project.

As the cones are a part of a geothermal area, it might seem logical to use an incubating temperature considerably higher than 15°C for the bacterial isolates. The 15°C incubation has been used in microbial studies on seawater aquaculture in the Eyjafjörður area (Björnsdóttir et al., 2006). The highest temperature in the cones area is 75-77°C, on top of the cones, where the hydrothermal fluid flows out (Figure 3) and this fluid probably contains extremophile bacteria (Marteinsson et al., 2001). However, the sample organisms were not located in any extreme conditions, where they were either sitting on the cones themselves or on rocks beside them. Temperature at the collecting sites varied, but was normally around 6 – 8 °C higher than the surrounding sea water. Hence, the conclusion was to add room temperature incubating without dropping the 15°C incubation. The available literature usually reports incubation temperature around 28°C (varying from 25 – 37°C), but these studies are normally performed on samples from warmer oceanic sites.

Culture and isolation of bacteria from the sampling trips in 2005 was carried out without use of any antibiotics or stronger selectivity than the types of culturing media offered. By adding antibiotics, the yield of isolates with antimicrobial activity increased significantly. Cultures from EN-4-2006 were made with cycloheximide and nystatin in the media and to the cultures from EN-7-2006, nalidixic acid was also added according to Webster et al. (2001). Accordingly, the yield of isolates with antimicrobial activity was 0.3 % in samples collected in 2005, but 14 % from samples collected in 2006 (10 % from EN-4-2006 and 17 % from EN-7-2006). Furthermore the total yield of growing isolates was also better from EN-7-2006 than EN-4-2006, or 81% compared to 72%, but no obvious reason can be pointed out to explain that.

The literature does not offer a great deal of comparable screenings, as most of the studies deal with extracts of invertebrates. However, in the studies available, yield of active isolates is rather variable. Chelossi et al.(2004) isolated bacteria from the sponge *Petrosia ficiformis* in the Mediterranean Sea off the coast of Northern Italy. Total isolates with antimicrobial activity were 5 out of 57 or 9 %. Zheng et al. (2005) isolated bacteria from the sponge *Hymeniacidon perleve* in the Eastern China Sea. Eight isolates showed antimicrobial activity of 29 tested (28%). Hentschel et al. (2001) tested 238 isolates form *Aplisina* sp. sponges collected in the Mediterranean Sea and found 27 with antimicrobial activity (11%). Anand et al. (2006) tested 75 isolates from four species of sponges collected off the coast of South East India and found 16 revealing activity or 21 %. Ivanova et al. (1998) tested 491 bacterial strains originated from marine invertebrates or seawater in the Pacific Ocean and found 126 with antimicrobial activity (16%). Li and Liu (2006) isolated sponge-associated bacteria from *Craniella australiensis* collected in the South China Sea. The isolates were identified and 23 actinomycetes isolates were tested for antimicrobial activity, resulting in 15 active ones (65%). Zheng et al. (2000) collected 3 species of marine invertebrates and 3 species of seaweeds off the Chinese coast in the Taiwan Strait. Thirty-nine identified actinomycetes isolates were tested and 17 strains exhibited antimicrobial activity (44%). Finally, one study could be found where the yield of antimicrobially active isolates was 100%. Those isolates were retrieved by four different methods, from wound tissues on the brittlestar *Amphipholis gracillima* after amputating one of the arms of the organism. The resulting isolates were 59 and they all exhibited some antimicrobial activity, when tested against 21 test strain (Strahl et al.,

2002). These studies are not directly comparable, as they use different conditions, selectivity and different test strains, due to difference in objectives. However, in general the active isolate yield of 14% in this study, is considered approximately similar.

4.2 Antimicrobial activity tests

The screening for antimicrobial activity was most efficient with the direct streaking, where the test strain is inoculated onto medium where the isolate is already in vivid growth. With this method, the test was completed for close to 80 % of the isolates started off with, whereas in most of the remaining incidents, the isolates did not grow on the test plate. The method made screening possible of all the isolates from samples collected in 2006. Higher efficiency in the later stages also probably depends on other reasons, most noteworthy the use of antibiotics in the media where the goal was to stimulate the competitiveness of actinomycetes. The cultivable bacterial flora was visibly different than from previous samples after these changes had been made. Higher frequency of colonies with morphology pointing to actinomycetes e.g. visible filamentous mycelium or aerial hyphae was noticed. A total of 96 bacterial strains with antimicrobial activity were isolated, whereas the sample lot collected in 2005 resulted only in three positive isolates after screening more than 800 isolate culture supernatants by the agar diffusion test. This test is carried out with supernatants of broth cultures, assuming that the bacteria have excreted the active substance which is then left in the suspension. For the three isolates which responded positively with the supernatant (Table 6) some further tests were tried (results not shown). By directly testing the culture, instead of the supernatant, a stronger response e.g. wider inhibition zone appeared. Furthermore these three cultures were run through a few steps to eliminate the bacterial cells – centrifuge, a 0.45 µm filter and finally a 0.22 µm filter. After each step, a similar agar diffusion test (see 2.4.1) was carried out. In this case the antimicrobial activity faded out along the way and after 0. 22 µm filtering, the activity had vanished. This might point out that some active compounds are bound to the cells and will be lost with them when a supernatant of the culture is tested. Disrupting the cells before centrifuging might be a more effective procedure for the agar diffusion assay.

Testing the culture supernatant without concentrating the solution, could also be a reason for poor yield of activity. An active compound could be present in a very low

concentration – too low to exhibit any growth inhibition. Compared to the ethyl acetate extraction, where in this study an extract of 100 mL was reduced to 1.5 mL, the concentration difference is significant. The results from the agar diffusion assay with the culture supernatant do not indicate that water soluble antimicrobial compounds are present (with one exception – the isolate 101-54). Still, it remains interesting to examine this further, as for practical uses there are considerable advantages to work with water soluble compounds e.g. in cosmetics or foods and use of organic solvents in such products are in many cases not acceptable.

As mentioned before, not many studies are available on antimicrobial screening of marine microorganisms. The methods reported in these studies are the following:

a) Agar diffusion or agar overlay with direct use of culture (Chelossi et al., 2004, Ivanova et al., 1998, Li and Liu, 2006, Anand et al., 2006).
b) Agar diffusion with concentrated organic extracts of cultures (Thakur et al., 2005, Zheng et al., 2005)
c) Agar diffusion with culture supernatant (Mearns-Spragg et al.,1998)
d) Direct streaking (Strahl et al., 2002, Zheng et al., 2000)

These methods, all except one, involve either direct contact between culture and test strains, or concentrated extracts, so comparison to direct use of culture supernatant is difficult. However, in one of these studies (Zheng et al., 2005), supernatant of the culture is used for extraction in ethyl acetate, resulting in 28 % of the isolates being antimicrobially active. Mearns-Spragg et al.(1998) used culture supernatant for the agar diffusion assay, but the total yield is not mentioned.

The direct streaking method creates somewhat different conditions for the isolates while testing for antimicrobial activity that might have some value in explaining the results difference compared to the agar diffusion assay. Direct streaking allows the isolated strain to grow on the media it was originally isolated on, e.g. in its preferred environment. It is in full growth and generally good shape when introduced to the test strain. Possibly the presence of a test strain has some influence on the antimicrobial compound(s) release. Clearly, the compounds are not tightly bound to the cells in this case as they diffuse very well through the agar media resulting in growth inhibition (Figure 10). It is also interesting how easily this diffusion occurs, the medium being an aqueous substance, which indicates that the antimicrobials are not hydrophobic. This contradicts

the general indications from the agar diffusion and needs further investigation. In their experiment with isolated epibionts from seaweed and a nudibranch, Mearns-Spragg et al. (1998) found that antimicrobial activity was induced by the presence of terrestrial *S. aureus cells* (hospital isolates) and one of the marine isolates did not exhibit activity except under these conditions. The same group has also reported similar effects, when marine bacteria were exposed to a cell-free bacterial supernatant (Burgess et al., 1998). However, media factors might also interfere the results and cannot be ignored, e.g. the isolates might simply cause pH change or nutritional depletion in their nearest medium environment, creating growth inhibition of some of the test strains and accordingly false indications of antimicrobial compounds.

The test results for the antimicrobial susceptibility test using ethyl acetate extract were somewhat disappointing, as this solvent has been used successfully in other studies to retrieve marine metabolites (Zheng et al., 2005, Anand et al., 2006, Jensen and Fenical, 2000). Interestingly, the activity detected with these extracts was almost entirely against *C. albicans*, although important exceptions are recorded (Table 7). These extracts were originated from isolates which often revealed activity not only against yeast, but also bacterial test strains in the direct streaking test. This might indicate that more than one antimicrobial compound is present in the culture, whereas only one of them can be extracted with ethyl acetate. General assumptions from these results are that many of the antimicrobial compounds in question are neither extractable into aqueous solution nor ethyl acetate and therefore have to be reached by other extraction methods.

The wide susceptibility of *C. albicans* is interesting and rather unexpected, as it is generally considered a pathogen relatively difficult to fight (Jensen and Fenical, 2000). In total, 56 isolates of the 96 active inhibited *C. albicans* (58%). Zheng et al. (2000) reported 4 isolates inhibiting *C. albicans* in a total of 17 revealing antimicrobial activity. Anand et al.(2006) found 4 isolates which inhibited *C. albicans* out of 16 showing activity and Ivanova et. al. (1998) reported 28 isolates active against *C. albicans* out of 126 revealing activity. According to that, this frequency of inhibition for *C. albicans* is high. It should though be taken into consideration here that the isolates haven not yet been identified so the multitude of duplicates is not known. Apart from the recorded activity against *C. albicans*, several isolates appeared to slow down the growth of this strain. Those results should not be entirely counted out, until further looked into, although not accounted for

here. No apparent pattern could be figured out, when looking at the results for *C. albicans*, but some variations were noted. Many of the isolates that acted against *C. albicans* in the direct streaking test did not respond in the agar diffusion with acetate extract. Two incidents were also recorded where the response was positive by agar diffusion with the extract, but not in the direct streaking test. It was however noticeable that many of the isolates strongly inhibiting *C. albicans* in the direct streaking test, were potential representatives of actinomycetes. One example can be seen in Figure 10 (right).

It is also interesting to notice that *P. aeruginosa* was susceptible to the presence of 20 isolates (Figure 14). *P. aeruginosa* is an opportunistic pathogen to humans, often responsible for various infections f. ex. in severe burn wounds and it is resistant to many of the commonly used antibiotics and antibacterial agents (Hitchins et al., 2001). In the US and European Pharmacopoeias the absence of *P. aeruginosa* is required in cosmetics, as infections from such products have been documented (Campana et al. 2006, Schwarb et al. 2001). A compound inhibiting growth of this organism is therefore of interest for further research, not the least with regard to possible use in the cosmetic industry.

4.3 Growth studies

Measuring inhibition zones in agar diffusion assays can under standardised conditions give both quantitative and qualitative results. Inhibition zones give very little if any information on the nature of the inhibition whereas growth curves can. In this study, measurement of inhibition zones only gave qualitative results as the quantity of antibacterial agent was unknown. For further studies it is important to isolate, quantify and identify the active compound(s). For that purpose the compound must be extracted from the isolated bacteria strain or its growth medium and tested for activity preferably by studying growth.

One isolate, (101-54) revealed activity in the agar diffusion assay, both with the culture supernatant and ethyl acetate extract. The response was stronger in the acetate extract, where the inhibition zone was wider. This particular isolate inhibited four Gram positive test strains in the agar diffusion assay (with EAE), two of which were different *S. aureus* strains. In the direct streaking test, three of these same strains were likewise inhibited (the fourth was not tested), as well as a strain of MRSA. Following the broad

antimicrobial activity response, a culture extract of the isolate 101-54 was a first choice of a candidate for growth studies. The growth studies revealed clearly that antibacterial compound(s) could in this case, easily be extracted from the culture and tested in a semi-quantitative way. They also showed somewhat different results than the two inhibition zone tests. In the growth studies the MRSA was almost totally insensitive while the non-MRSA was very sensitive. The MRSA test strain was clearly inhibited by this isolate in the direct streaking test (14 mm clear zone), but was not tested by agar diffusion so no comparison is available there. On the other hand *C.albicans* was not affected in the agar diffusion assay, but the growth curve indicates inhibition by EAE. However, this might indicate more than one active compound, where one of them can be retrieved by ethyl acetate.

The Staphylococci test strains grew relatively well under the growth studies test conditions, as well as the *Candida* strain although it grew very slowly. In the cases of *E. faecalis* and *L. monocytogenes*, growth was extremely poor. These test strains have been grown repeatedly for the other assays, using 12-16 hours cultures, with no growth retardation experienced. The experiment was carried out in Nutrient Broth, but *C. albicans* and *E. faecalis* have usually been grown in slightly different media (Tryptic Soy Broth for *E. faecalis* and DSM Universal Medium for Yeasts for *C. albicans*), but had been tested in Nutrient Broth before the experiment. The different media might be a part of the explanation for these two strains, especially regarding the slow *C. albicans* growth. The honeycomb microplates were not shaken in the Bioscreen reader, which might affect the OD readings. If the bacterial cells tend to sink and accumulate at the bottom of the wells, as a result the OD will not coincide with the actual growth density. This might also explain to some extent the shape of the result curves e.g. the fairly quick drop of the OD values for most of the strains and the second rise in OD for *E. faecalis*. However, the growth experiments were repeated for three of the test strains, *S. aureus, E. faecalis* and *L. monocytogenes*, with shaking of the microplates before each measurement (data not shown). The results were very similar, *L. monocytogenes* and *E. faecalis* grew rather poorly, but the inhibiting effect of the extract was stable, indicating that the plate shaking is not a significantly important factor.

Although the overall growth was variable between different test strains, a clear growth inhibition was observed, without much intermediate responses. In other words, for

all the test strains, EAE addition either had no effect or it prevented growth almost completely, rather than retarding it. This was confirmed in the repetition experiment.

As the results from the growth experiments are rather inexplicit, it is recommended to repeat them, both for this particular isolate and other active ones. Some action might have to be taken to improve the bacterial growth for some of the test strains, possibly by changing the media. An ethyl acetate extract concentration higher than 3-4 % should not be used, as that will create too much solvent interference. The aqueous phase remaining after the ethyl acetate extraction, could be used in more concentrated form to test for antimicrobial activity, either by growth studies or agar diffusion assays.

5 Conclusion

Antimicrobial compounds are produced by an apparently diverse group of cultivable marine bacteria, hosted by organisms living on the hydrothermal cones in Eyjafjörður. Selective media for actinomycetes are preferable to detect these groups of bacteria. The hosts are different organisms such as algae, sea anemones, ascidians or sponges. Antimicrobial activity from these bacteria has been observed against both Gram positive and Gram negative bacteria as well as yeast, indicating that there might be several compounds behind the activity. There are still many questions unanswered so further studies are needed to follow up the results of this project. Future work should include further attempts to isolate and refine the active compounds and determine their mode of action, classify the microbial producers, perform more growth experiments and test the active compounds in commercial products.

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