SeaBioTech: From sea-bed to test-bed: harvesting the potential of marine biodiversity for industrial biotechnology.

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Abstract: SeaBioTech is an EU-FP7 project designed and driven by SMEs to create innovative marine biodiscovery pipelines as a means to convert the potential of marine biotechnology into novel industrial products for the pharmaceutical, cosmetic, aquaculture, functional food and industrial chemistry sectors. To achieve its goals, SeaBioTech brings together leading experts in biology, genomics, natural product chemistry, bioactivity testing, industrial bioprocessing, legal aspects, market analysis and knowledge-exchange. SeaBioTech targets novel marine endosymbiotic bacteria from unique and previously untapped habitats, including geothermal intertidal biotopes in Iceland, hydrothermal vent fields and deep sea oligotrophic basins of the Eastern Mediterranean Sea, and under-explored areas of Scottish coasts that are likely to be highly productive sources of new bioactive compounds. This chapter describes the four- years of activity in the SeaBioTech project, which resulted in a robust, validated workflow suitable for evaluating unexplored activities in marine samples to prioritise potential products for a biotechnological pipeline. An improved integrated methodology involving metagenomics and metabolomics were extensively utilised to prioritise five extremophiles as potential antibiotics, anti-cancer drugs and as novel drugs against metabolic diseases as well as new pharmaceutical excipients to the pipeline. A

centralised biobank repository, which included a database of information, was established for future bioprospecting activities. For future marine bioprospecting activities, a harmonised legal position was put together in collaboration with other EU-FP7 blue biotechnology projects.

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1 Introduction

The 48-month SeaBioTech project was designed and driven by SMEs to convert the huge potential from as yet underdeveloped marine biotechnology into novel bioactive pharmaceuticals (anti-cancer, anti-parasitic, antibiotic, and against metabolic diseases), cosmetic and food (antioxidant) as well as industrial chemistry (biocatalysts, reagents) sectors. The project made use of the biodiversity from marine extreme environments. Such environments are characterized by geochemical and physical conditions at the edges of the compatibility with life, and they are colonized by highly adapted organisms called extremophiles. These can provide unique chemicals and novel enzymes that have enormous potential because they maintain their performance even in harsh industrial process conditions. However, there are significant bottlenecks that presently restrict the marine biodiscovery pipelines relating to:

- limited availability of collections of marine extremophiles and little knowledge of their potential use in biotechnology (lack of qualitative and quantitative data with respect to the application performance)
- limited transfer of knowledge from fundamental research into technically realizable and cost-effective products and technologies
- technical hurdles with methods and processes, including in the cultivation and storage of organisms, and in extraction, isolation and characterization of bioactive components
- lack of industrial scale production techniques for marine substances, based on the limited understanding of the process physiology of the native producer microorganism

To develop efficiently marine biodiscovery pipelines and provide access to sustainable and economical production methods, SeaBioTech has tackled five key challenges (Figure 1) with an integrated approach combining access to unique marine biodiversity, innovative culturing

approaches, genomic and metagenomics analyses coupled with metabolomics, natural product chemistry, bioactivity evaluation and industrial bioprocessing along with legal aspects, market analysis and transfer of knowledge. SeaBioTech has not only increased the number of marine-based products but also their success rate for future commercialization. SeaBioTech's research and technological progress was completely within the framework provided by the participating SMEs relating to their definition of product opportunities and proof-of-concept demonstration activities.

1.1 SeaBioTech has put together a marine biodiscovery pipeline using an integrated approach.

The project's innovation plan corresponded to the following scientific, technical and technological challenges as shown in Figure 1:

Challenge 1: The quality of marine resources: the approach to resource quality begun by standardizing the sampling process from unique and previously untapped habitats, which included geothermal intertidal biotopes in Iceland, hydrothermal vent fields and deep sea oligotrophic basins of the Eastern Mediterranean Sea, and unsampled areas of Scottish coasts that are likely to be highly productive sources of new bioactive compounds. The marine resources also included the partners' existing biobanks (UK's Culture Collection of Algae and Protozoa, MATIS's Icelandic collection, Eastern Mediterranean Sea collections) as well as new *in situ* sampling. The SeaBioTech sampling process guaranteed the quality of marine resources for further industrial development, including identification of marine microorganisms and their variability based on genomics and metagenomics. This project also integrated the critical aspect of the maintenance of the sampled species with their intrinsic quality and their secondary metabolites, by developing special cultivation media and storage conditions.

Challenge 2: The improvement in technical aspects: to improve marine biodiscovery and reassure industries about its feasibility, SeaBioTech perfectly combined metabolomics assisted by systems biology and functional bioassays to increase the ability to disclose positive hits with an economical and faster approach: an affordable, innovative and efficient method to separate, elucidate the structure, and identify the bioactive metabolites.

Challenge 3: Sustainable modes of supply of raw materials for the industries: the last technical brick for industries is the sustainability of these newly discovered raw materials not only at lab scale but also at industrial scale. Thus, SeaBioTech benefited from the power of well-controlled metabolic engineering of interesting organisms (bacteria, microalgae,

cyanobacteria) increasing the yield of bioactive metabolites at lab scale and multiply this yield through fermentation technology at industrial scale to deliver promising enzymes, polymers and small molecules as industries need.

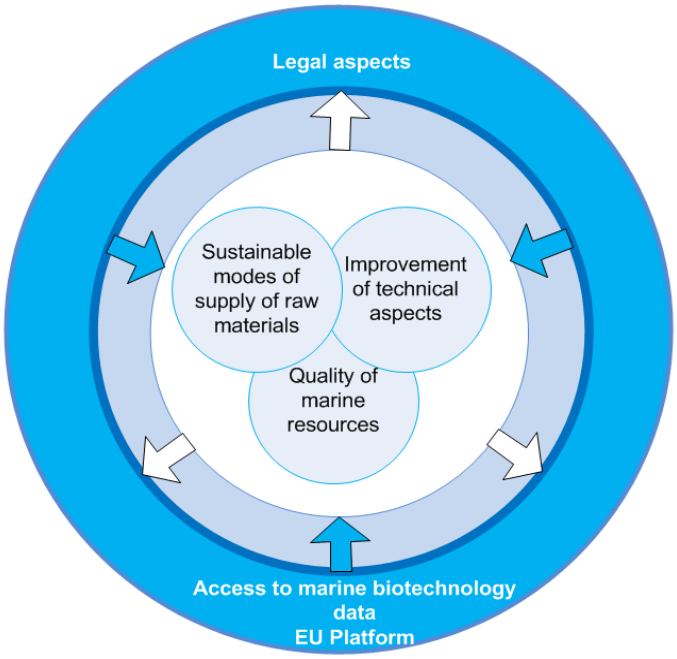

Figure 1. Concept of SeaBioTech, showing the interactions between the five key challenges to be faced in order to improve marine biodiscovery pipelines. The first three challenges in the inner most circle concerns the primary goals of the project that includes: 1) the quality of marine resources; 2) The improvement in technical aspects; and 3) a sustainable mode of supply of raw materials for the industries. The transversal activities involving challenges on 4) the legal aspects and 5) the access to marine biotechnology data are the second level represented on the outer circle.

The second level embraces the last two challenges as transversal activities: challenge 4, the legal framework was necessary to secure the access to marine resources, their sustainable use and their exploitation process; and challenge 5, the access to a marine biotechnology database and biobank.

Challenge 4: The whole biodiscovery process was completed by the clarification of all legal aspects to gain visibility and efficiency for industry. SeaBioTech coordinated the legal procedures with national, European and international authorities/stakeholders to propose

harmonization of the legal process related to marine bioprospecting, biodiscovery and marine biotechnology for commercial purposes.

Challenge 5: To crystalize this innovative approach, SeaBiotech created a centralized tool to describe the whole marine biodiscovery pipeline including available biobanks, the identified marine organisms, compounds and extracts, the cutting-edge methods in identification, elucidation, metabolic engineering to be further used for industrial purposes with all related procedures on legal process for companies, academia, and legal authorities.

1.2 SeaBioTech is an industry-driven project.

Contrary to previous approaches, SeaBioTech commenced by defining industry needs - more specifically SMEs'- across marine biodiscovery pipelines. To achieve the overall goal of making sustainable marine-based compounds more attractive for industries along with shortened time to market, the specific objectives of SeaBioTech are to:

- provide a pipeline of commercially viable products based on relevant bioactivity screening of samples of marine origin;
- develop efficient standardized processes and methods across the biodiscovery pipeline;
- introduce industrial bioprocessing methods suitable for commercial production of marine-sourced materials;
- clarify, harmonize and potentially simplify the legal aspects related to marine biodiscovery processes;
- create a central EU platform and biobank based on an integrated approach to biodiscovery pipelines for future use by other consortia, academia and companies.

1.3 Identification of industry needs: providing an industry-driven project

It is easier to put the key challenges for marine biotechnology into an addressable context by defining what the concrete output from marine biodiscovery pipelines might be. Therefore, SeaBioTech started with a thorough market analysis of the various industrial sectors that are relevant to the partners, particularly the SMEs. This clearly indicated where there is a need for products that go beyond the current state of the art. This in turn provided the perspective for the technical challenges and highlighted the needs for improvements. All SMEs within SeaBioTech were committed to the concept of marine bioprospecting as a strategy to provide them with key future products that are beyond the state of the art and

enhanced their business competitiveness. The SeaBioTech project was specifically designed to deliver to the SMEs' progress, eliminating or dramatically reducing bottlenecks to allow the SMEs to develop innovative products for the world market. It is important to note that success of the project must show other SMEs and industries what is technically feasible and economically attractive from marine biotechnology – SeaBioTech will represent a sustainable and reproducible model for the European biotech industry. The companies involved in SeaBioTech are focused on the biodiscovery pipelines of three compound categories: (1) polymers having bioactivities such as wound healing, optic turgor in lenses and pharmaceutical additives driven by Marine Biopolymers (MBL) and MATIS; (2) enzymes having bioactivities such as transaminases, reductases, etc driven by Ingenza, Prokazyme, Lund and MATIS; and (3) small molecules having bioactivities as therapeutics (antibiotic, anti-cancer, etc) driven by Axxam, HDL, PHARMAQ, SIPBS and UWUERZ. Each step of the biodiscovery pipeline related to these three types of compounds and the related target application explored to provide innovative ingredients for novel industrial products. The whole SeaBioTech biodiscovery process was informed by Rothwell's coupling model of innovation (Rothwell, 1992) so that there is a regular interplay between understandings of market needs and technical 'pull'.

2 Methodology and Overall strategy

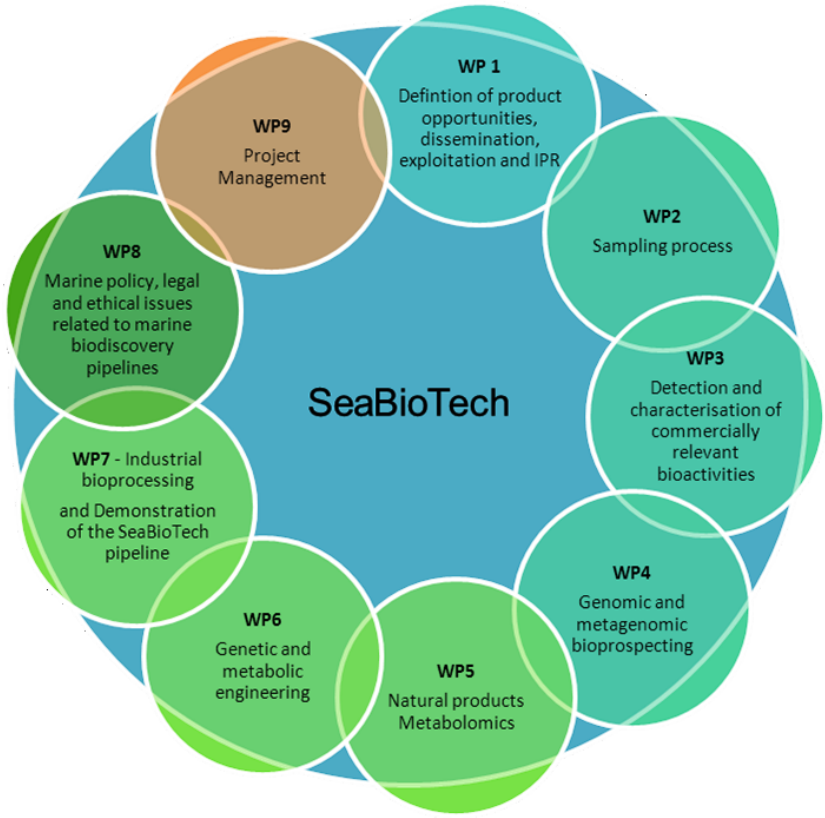

Figure 2. The SeaBioTech Work Packages

Work packages (Figure 2) were set up to organise a schematic flow of materials and data between partners. In order to achieve the desired outcomes of a greatly improved pipeline of products from marine biotechnology, the first step is to identify clearly the market opportunities for the companies involved in SeaBioTech and the precise bottlenecks they have to solve to target their respective markets. The target applications of the consortium involved pharmaceuticals, fish health, food, cosmetic, chemical and industrial in *WP1 (Definition of product opportunities, dissemination, exploitation plan and IPR).* WP1 formed the basis of the subsequent research activities in WP3-7. Through an understanding of market and technical requirements, several partners each contributed to the definition of demand statements for their own industrial sectors. In addition, WP1 integrated the IPR management. In parallel, *WP2* (*Sampling process*) created a huge collection of novel microbes and microbial consortia for genome and metagenomic analyses and to facilitate their biotechnological exploitation. WP2 led by HCMR collected information from all culture

collections available from partners, isolated novel microbes from several diverse environments and organisms and facilitated their exploitation at WP3/WP4 for bioactivity screening and genomic analyses. WP2 also prepared samples that can be screened in *WP3* (*Detection and characterisation of commercially relevant bioactivities)* led by AXXAM. WP3 was responsible for detecting bioactivities that were selected as priority commercial targets in WP1 by testing samples provided by WP2 and WP4. WP3 provided the detailed bioactivity assessments to guide isolation of substances with commercial potential. *WP4 (Genomic and metagenomic bioprospecting)* led by the University of Wuerzburg used molecular techniques to pinpoint novel enzymes of commercial interest and isolated the genes for synthetic pathways for novel small molecules for testing in WP3. This also allowed structural variations to be prepared as a mean to improve bioactivities. A number of high-throughput solid phase screening i*n vitro* and direct selection methods *in vivo* was applied in this work package to identify novel enzymatic activities of interest from metagenomic libraries constructed from genomic DNA derived from marine micro-organisms. *WP5 (Natural products metabolomic*s) led by SIPBS was the analytical arm of the consortium, undertaking dereplication studies on microbial extracts of interesting isolates from WP2 and those screened for the presence of biosynthetic clusters from WP4. WP5 isolated and structure elucidated the bioactive natural products determined in WP3. Along with WP6, their sustainable production by the microbial cultures was optimised through metabolomics tools. When interesting metabolites were confirmed in WP5 in collaboration with WP3, organisms could be engineered to guarantee sustainability of the interesting metabolites in *WP6 (Genetic and metabolic engineering)* also led by SIPBS. WP6 undertook the research that will allow organisms producing targeted substances to be maintained at the laboratory scale. It also performed genetic manipulation to produce structural variants of the target substances as a means to improving their commercial properties. When lab scale is validated, it is essential to integrate at industrial scale. *WP7 (Industrial bioprocessing)* led by MATIS focused upon developing rapid and robust methods for the industrial exploitation of microbial and enzyme based marine products. In order to achieve this, WP7 tasks will link very closely to those in WP5 (Metabolomics) and WP6. A sub-task in WP7 consisted of a series of projects conducted to test the viability of the outputs from earlier WPs as the basis for new commercial products relevant to various partners, including pharmaceutical, functional foods, novel enzymes, and research tools. *WP8 (Marine policy, legal and ethical issues)* ensured that the SeaBioTech project develops in accordance with all relevant national and international legislation governing bioprospecting and the marine environment. The WP's

main goal is to contribute to the on-going development of the legal framework for marine bioprospecting, and ensured dissemination of the project results to the scientific community, to public and political stakeholders. The key task was the creation of an EU platform allowing access to data from SeaBioTech and to physical samples in SeaBioTech's biobank. *WP9 (Project Management)* deployed and implemented management best practices through a clear focus on both strategic and operational administration.

~~2~~3 The SME partners and their activities

In this section we present the roles and contributions of SeaBiotech's partners from the industry as well as theSME's mutual gain from the consortium. With the analysis of market opportunities and the generation of an initial exploitation plan, the respective SMEs defined specific commercial goals, and strategies to reach these goals within SeaBioTech project. The exploitation plan also further underlined the importance of collaboration between the company and RTD partners as a key to the successful exploitation of the opportunities and potential of the SeaBioTech project. Through this EU-funded partnership, the SMEs made agreements with academic and research institutions in the consortium for the licensing of products that will be offered by the respective companies with shared revenues according to specific agreements. The strategy was to exploit potential collaborations with academic groups in SeaBioTech as a new business scheme for increased portfolio of products for the research laboratory market in Europe and elsewhere. SeaBioTech brought together significant members of the fields of marine biotechnology and biocatalysis experts for the first time and delivered industrially useful novel biocatalysts by developing highly innovative and powerful screening and selection technologies and novel, high yielding, scalable and economic enzyme production systems. Some of the SMEs had taken steps to further develop the successful strategy of alliance with its partners in the SeaBioTech project with continued collaboration that will extend well beyond the lifetime of the SeaBioTech project. Very good collaboration with both academia and SME's that will continue after the end of the project is one of the main and high impact results for the SMEs. SME partners have communicated with various potential end users and current market producers to develop collaborations for the future development of the compounds. As a next step to support the potential commercialization of bioactive compounds, further funding will be required to undertake the studies to better understand mechanism of action, develop a suitable patient stratification strategy and to assess tractability for conventional medicinal chemistry. However, one disadvantage for the

academic partners is that, a SME will not release any publication on the compounds for reasons of commercial sensitivity especially if patents are to be filed in the coming years.

~~2.1~~3.1 Prokazyme (PKZ)

PKZ has been engaged in commercialising enzymes that have advantageous biochemical properties over competing products. In this project, their work was focused on developing enzymes from extremophiles that were recognised as being potentially valuable in many applications. However, very few novel enzymes from the hundreds reported in the literature reach the market. Generally, the limitations in this area are difficulties in obtaining large enough supplies in a sustainable way and challenges in producing the enzyme to a high standard of purity in an economically attractive manner. PKZ saw opportunities in bioprospecting the unique genomic resources it had access to through the SeaBioTech gene banks and in the enormous, untapped marine biodiversity sampled through SeaBioTech. The SeaBioTech project offered significant progress above the state of the art on new marine compounds (particularly oligosaccharides), as well as enzymes, increasing PKZ existing offerings of specialized extremophilic enzymes for the R&D market. PKZ has made strategic plans for future commercial production of enzymes on an economical large scale. As part of this future strategy, it is the intention that the production of enzymes shall be transferred from PKZ to a subsidiary company. PKZ and MATIS have initiated a large research proposal with a consortium consisting of 15 partners in Europe. The research proposal, "Virus-X: Viral metagenomes for Innovation Value", has secured a EUR 8 million funding from the European Union under the Horizon2020 framework. PKZ will coordinate the project and within the project extend its collaboration with specific partners from the SeaBioTech project. A grant agreement was made during this period with the European Union for the funding and the project started on April 1st 2016 and will be continued until March 30th 2020.

~~2.2~~3.2 PHARMAQ

PHARMAQ specialises in vaccines and therapeutics for farmed fish. A key need for aquaculture is to have effective anti-parasitic agents that are potent and selective against the target parasite while having no damaging effects on the environment. Most of the anti-parasitic products available within aquaculture today are derived from known pesticides developed for terrestrial applications and some of these are limited by their toxicities. Hence, SeaBioTech offered a unique opportunity to search for bioactives from novel and unexplored sources, particularly by uncovering potential new therapeutics for aquaculture

applications and defining their suitability for commercial development. The SeaBioTech project yielded good results for PHARMAQ. SeaBiotech developed an HTS (high-throughput screening) assay directed against a target special for salmon lice. The assay was very valuable in screening large libraries in the search for new actives against one of the most devastating parasites in aquaculture. Some compounds with effect against salmon lice have been identified. Although the effect has so far only been identified at a relative high concentrations, the compounds were worthy of further examination.

~~2.3~~3.3 Marine Biopolymers Ltd (MBL)

MBL supplies chemicals derived from marine sources, including alginates and polyphenols. While the potential value of compounds such as alginate and fucoidan is well recognised, their widespread use is limited by technical problems: (i) low quality and low yields from existing extraction methods; (ii) lack of higher performance purification approaches to provide products at the 'fine chemical' standard; and (iii) incomplete analysis and characterisation of isolated components. MBL sees huge product opportunities arising out of the collaborative work in the SeaBioTech project. During the project's lifetime, MBL focused on defining the company's interests on polysaccharide compounds and their growing market demand. In addition to the polysaccharides, the sampling events of macro- and micro-epiphytes have presented interesting and new chemistry and bioactivity across a range of compounds. MBL continued sampling of key macroalgal species to develop seasonal metabolomic data. It has achieved commercially valuable improvements arising from the SeaBioTech consortium. An initial market analysis was also explored defining the potential market size/demand and market areas the compounds could feed into, whether that be as a stand-alone product or as an ingredient in a current or new formulation. Although MBL initially had a strong focus on polysaccharides, it was clearly observed that there are additional compounds that MBL now plan to commercialise over the coming years subject to the availability and success of appropriate follow-up funding mechanisms.

~~2.4~~3.4 Ingenza (IGZ)

SeaBioTech provided opportunity to discover new biocatalysts with industrially relevant substrate specificities for integration with IGZ's current bioprocesses for the manufacture of enantiopure chiral amines, unnatural amino acids and other chiral chemical platforms. The most promising identified biocatalysts were developed using economic and scalable fermentation and bioprocess systems. Further development and implementation of inABLE® which is IGZ's combinatorial genetics technology for the efficient and selective

assembly of DNA expression vectors, took place in the project. These technologies were key tools for improvement of strain construction and screening, and have been used and developed through SeaBioTech and the technology is of core importance to all of IGZ's commercial interests.

The screening of both alternative metagenomics libraries and those of the work package partners for new and novel enzymes of commercial interest to IGZ was carried out. This allowed expression constructs to be made and screens to be developed in WP6, which led to subsequent production processes in WP 7. These generic fermentation protocols which had been developed previously were then implemented to test the growth and expression of positive hits which were highlighted in the subsequent screening of the work package partner's databases. These novel marine enzymes were cloned into an industrially relevant *E. coli* strain using inABLE® compatible parts. Further optimisation of the expression of these strains has been carried out in shake flasks followed by activity assays of the successfully expressed enzymes. Based on these results, fermentation development has been implemented, linking into the deliverables required for WP7. A production process of the most successful enzymes was implemented and scaled up during the course of SeaBioTech.

~~2.5~~3.5 Horizon Discovery Ltd (HDL)

HDL has been developing new drug discovery opportunities in the cancer field through its creation of unique cell lines that are engineered to represent particular forms of cancers. HDL saw great application in screening marine-derived natural products from the project to therapeutically 'deorphan' the cancer genome. HDL's expertise on cell-based screens using genetically defined human disease models represented the ideal approach to directly find unexpected uses for naturally bioactive molecules from the project to such 'orphan-targets', where the full complexity of cell biology was screened in a rational manner to find novel cancer-selective agents. Ultimately, HDL was able to show that several fractions containing single compounds had a marked ability for specifically killing cancer cells via inducing apoptosis. This is proof-of-principle that bioactive compounds isolated from these particular classes of marine organisms may have at least some of the required characteristics for exploitation in the oncology arena.

~~2.6~~3.6 AXXAM

AXXAM is a lead compound discovery company that services the pharmaceutical, agrochemical and life sciences sectors. AXXAM was SeaBioTech's link to the mainstream of pharmaceutical development and marketing companies. AXXAM provided a panel of

functional assays that detect activities relevant to key diseases (infections, inflammatory diseases, chronic pain, etc.). AXXAM supported the hit discovery programmes of SeaBioTech by performing in total 11 screening campaigns on a comprehensive number of 927 crude samples of marine origin on an array of cell-based and enzymatic assays, which was refined based on the obtained results to seven assays (TRPA1, TRPM8, TRPV1, PPARα, EL, HDAC6, HDAC2) suitable for high-throughput screening of complex extracts. These functional assays were developed to measure the activity of validated targets in three main disease indications: cancer (HDAC6 and HDAC2), metabolic syndrome (EL, PPARα) and pain (TRPA1, TRPM8, TRPV1). At the end of the primary screening activity, 287 crude extracts were confirmed as primary hits, distributed as follows: TRPA1 (12), TRPM8 (37), PPARα (36), HDAC6 (81), HDAC2 (3), EL (118). In collaboration with WP2-WP5, 31 crude extracts derived from 17 marine microorganisms were prioritized and included in the SeaBioTech pipeline. A subset of 15 crude extracts was fractionated by WP5 and 629 fractions were subjected to screening against the primary assays TRPM8, TRPA1, PPARα, HDAC6 and EL, respectively. The support to dereplication activities led to the identification of 148 fractions containing the sought bioactivity against the following primary targets: TRPA1 (9), TRPM8 (5), PPARα (5), HDAC6 (76), EL (53). Remarkably, one series of 27 fractions derived from the crude extract SBT0541 (*Algoriphagus marincola*) was confirmed to contain negative modulators of the catalytic activity of Endothelial Lipase (EL). Among them, 8 fractions contained pure compounds which were identified by WP5, which allowed the definition of a preliminary structure-activity relationship. This finding appeared consistent with the targeted enzyme Endothelial Lipase (EL), which physiologically releases fatty acids from phospholipids in HDL particles. The compounds displayed a dose-dependent inhibition on EL, with partial inhibition at the highest compound concentrations tested. The negative modulation of the EL activity identified by AXXAM has never been reported in literature.

In addition, the collaboration between SIPBS, AXXAM and PHARMAQ has been reinforced throughout the SeaBioTech project to promote an integrated hit discovery program for the identification of marine compounds with anti-parasitic activity directed against *Lepeophtheirus salmonis*, a major threat for aquaculture. Three high-throughput assays made available by AXXAM (TRPA1, TRPV1 and voltage-gated Na-channel) were applied as pre-selection tools for the prioritization of crude extracts and fractions to be tested by PHARMAQ with the low-throughput phenotypic assay on living parasites. In total, AXXAM screened over 750 crude extracts for this purpose, which generated a list of 135 hits

prioritized for testing at PHARMAQ. A number of these hits were confirmed for their parasiticidal activity on *L. salmonis*, and further characterization is ongoing at PHARMAQ on a subset of fractions to identify the pure compounds responsible for the sought bioactivity.

Newly discovered and underexplored species of marine microorganisms were demonstrated to be effective sources of novel therapeutics to be progressed to address unmet medical needs and threatening parasitic infections for aquaculture. Thus, the availability of novel therapeutics for human health and aquaculture will directly contribute towards improving quality of life, health, employment and economic strength. In addition, the knowledge gained through SeaBioTech concerning the assay development and screening of complex marine extracts may directly or indirectly translate into new opportunities for the CROs to expand their potential market and for pharmaceutical and life science companies to undertake novel R&D projects.

~~3~~4 Addressing the Challenges through Scientific Breakthroughs

~~3.1~~4.1 Challenge 1: Access, sampling, storage and quality maintenance of marine resources present in extreme environments and sponge symbionts

The characterization of natural microbial communities in extreme environments has been a major challenge for microbial ecology. Considering that 71% of the earth's surface has an average depth of 3800 m, deep-sea environments have attracted much interest as niches of microbial life with considerable exploitation potential. Extreme environments are characterised by geochemical and physical extremes, at the edges of the compatibility with life. Many diverse extreme environments have been described, and they are colonized by highly adapted organisms called "*extremophiles*" (Rothschild & Mancinelli 2001). These organisms fall into a number of different classes that include thermophiles, acidophiles, alkalophiles, psychrophiles, barophiles (piezophiles) etc, depending on their ecological niche (Demirjian *et al.* 2001). Because of their unique metabolic adaptations to their environment, the extremophiles are considered to have an enormous potential for unique biotechnological applications because they allow the performance of industrial processes even in harsh conditions, under which conventional proteins are denatured or inefficient (Niehaus 1999, Rothschild & Mancinelli 2001). Consequently, these unique properties have resulted in several novel applications of enzymes in industrial processes. Similarly, the novel biochemistry of extremophiles is predicted to generate novel chemicals that are distinct in

structure from those from more conventional organisms. Hence, such compounds are likely to be useful in drug discovery applications. However, only a minor fraction of extremophile organisms has been exploited. Very few sources have been explored to date so that there was a rich potential for SeaBioTech to go beyond the state of the art so long as it is possible to obtain samples of a suitably high quality.

The first aspect of the 'quality of marine resources' challenge was simply to obtain access to extremophile samples from the marine environment. Companies seeking a wide range of biodiversity from extreme marine environments would struggle because such sources are not commercially available currently. The Australian Institute of Marine Sciences is no longer supporting access to its collections; MarBank in the University of Tromsø in Norway has a limited collection, which is not openly available; the National Cancer Institute in the USA provides access to a small number of marine-derived samples. There appears to be a single commercial source: Magellan Bioscience in the USA, which works collaboratively with other companies through offering access to its collection of marine microbes. However, few of these are extremophiles. Beyond that are the scattered 'ad hoc' collections found in some university departments and research institutes. The SeaBioTech project had access to several extreme environments that have not yet been explored for commercially relevant bioactivities and had capitalised on untapped resources associated with some of the participants, notably microbial symbionts from sponges and the UK's Culture Collection of Algae and Protozoa (CCAP). The benefits of these sources are explained below.

~~3.1.1~~4.1.1 Geothermal intertidal biotopes in Iceland

Intertidal biotopes harbour a large diversity of ecologically and biotechnologically interesting organisms. This is a highly dynamic environment subject to constant periodic disturbances with steep gradients of temperature, mineral composition and salinity. The organisms need to tolerate periods of dryness and even exposure to harsh UV radiation during low tide. Temperature gradients are manifested most clearly in the contrast between the hot fluid in geothermal coastal hot areas and the cold seawater, and the hot spring water may have high levels of sulphur compounds and toxic metals. These habitats have rich invertebrate fauna and often covered by a profusion of algal vegetation containing various complex recalcitrant polysaccharides that may be utilized by a variety of microbes, factors influencing the microbial diversity. Photosynthetic microbial mats are abundant in these areas and many hot springs may have both chemo-litho-autotrophic and photosynthetic organisms as primary producers, adapted microbes to unique conditions. Rare species in these areas include various

obligate heterotrophs, but their presence may be masked by the dominant primary producers and therefore they are not easily studied or accessible for biotechnological exploitation. The unique geothermal environments on the coasts of Iceland sustained a relatively high diversity of microorganisms and unique organisms not previously exploited as a resource for bioactive microbial metabolites or enzymes of industrial interest. Past studies revealed a great number of novel organisms indicating that geothermal habitats harbour an enormous diversity still to be isolated, characterized and exploited (Staley & Konopka 1985; Roszak 1987; Amann 1995; Skirnisdottir 2000). Within the SeaBioTech project, a total of 49 samples were collected from coastal geothermal sites in Iceland, primarily from photosynthetic microbial mats and also from polysaccharide enrichments *in situ* and a total of 194 strains were isolated: 122 from Laugarvík, 47 from Yngingarlindir and 25 from Reykhólar. Numerous strains representing novel species and genera were isolated, especially from Yngingarlindir. Alginate degrading anaerobic isolates from Reykhólar were close to the genus *Clostridium* and five of them were selected for whole genome sequencing and genome annotation analyses in WP4. A preliminary study of the species composition of Cyanobacteria from the clone sequences from the YL samples was performed and the largest taxon contained several species representing distant (88-95% 16S rDNA similarity) relatives of *Geitlerinema* sp. within the Oscillatoriales. A similar study on the composition of Cyanobacteria in four of the Laugarvík biomat samples revealed the majority of sequences belonged to a filamentous *Leptolyngbya* sp highly related to a Leptolyngbya sp. found in arctic hot springs in Greenland. Results from culture independent biodiversity studies in Yngingarlindir and Laugarvík indicated novel species of Cyanobacteria. Seven Cyanobacteria strains were (M24-M36) isolated from mat samples and identified. Strains of interest (32) were selected for extractions in WP3. The extracts (62) and relevant control samples (6) were labelled and sent to the relevant partners for bioactivity screening. Based on novelty, 39 strains were selected for whole genome sequencing and annotations in WP4 & WP6. From the total of 39 strains, 38 strains were sequenced and their genomes annotated.

~~3.1.2~~4.1.2 Deep sea oligotrophic basins and hydrothermal vent fields in the Eastern Mediterranean Sea

The Eastern Mediterranean Sea is a dynamic region with unique hydrographic and geomorphologic features (e.g. the Mediterranean Ridge, Hellenic Volcanic Arc, deep abyssal plains, seamounts, deep anoxic hypersaline basins, hydrothermal vent areas, submarine volcanoes and mud volcanoes, methane and hydrogen sulphide cold seep sites, etc.). The subduction of the African plate below Europe has resulted in the formation of the

Mediterranean Ridge and deep basins as well as volcanism in the Hellenic Volcanic Arc. Major hydrothermal systems are found along the Hellenic Volcanic Arc at Methana, Milos, Santorini and Nisiros islands (Dando *et al.* 2000). Venting gases in these areas contain substantial amounts of CO_2, H_2, and H_2S, thus providing the chemical environment for chemolithoautotrophic primary production (Dando *et al.*, 1995). Steep chemical and temperature gradients (Wenzhöfer *et al.*, 2000) create diverse niches for numerous microbial populations. Initial screening studies of microbial diversity indicated a high spatiotemporal variation in microbial community structure (Sievert *et al.*, 1999) combined with highly diverse bacterial communities, with less than 33% of 16S rDNA sequences being related at a 90%, or higher, level to cultivated organisms (Sievert *et al.*, 2000).

The deep eastern basin of the Mediterranean Sea is one of the world's most oligotrophic areas and is characterized by an overall nutrient deficit (Ignatiades 1969). As a result, only small amounts of organic matter reach the seafloor through the water column, resulting in low bacterial community growth and abundance (Danovaro 1999). Previous studies on the composition of microbial communities in these environments have shown that they are highly diverse, and the estimated total sequence richness has been found to be comparable to estimates for microorganisms inhabiting terrestrial ecosystems (Polymenakou *et al.* 2005; Polymenakou *et al.* 2009). Thus, these highly oligotrophic environments harbour a unique prokaryotic diversity, different from that described among other oxic and pristine marine sediments, and thus they can be considered as "bacterial hotspots" that deserve further investigation to assess their biotechnological potential.

For the first time, SeaBiotech was able to launch bioprospecting activities on organisms from these Mediterranean sources which were led by the Hellenic Centre for Marine Research (HCMR). Samples were collected in Santorini volcanic complex (Santorini caldera including the newly discovered Kallisti lakes, Kolumbo volcano, Aegean Sea, Greece) and in the deep-sea oxic Ierapetra basin, South Crete. Santorini volcanic complex is a part of the Hellenic Volcanic Arc characterized by a unique convergent setting and by a unique enrichment of polymetallic spires in As, Sb, Zn etc. Two major sampling events were organized by HCMR in September 2013 and in May 2014 in this volcanic complex with the Research Vessel Aegaeo and the remote operated vehicle of HCMR from which a large number of water samples (>100), pollymetalic active and inactive gas chimneys (>30 samples and subsamples) from the submarine Kolumbo volcano and microbial mat samples from Santorini caldera and Kolumbo volcano (>30) were collected and used for microbial strain isolation, community characterization and metagenomic libraries construction. In total, 280

microbial strains were finally isolated from the Kolumbo/Santorini samples for the other tasks and WPs, belonging to different species mainly within the Bacillales of Firmicutes phylum and within the Pseudomonadales of Gammaproteobacteria. Several novel species were also identified whereas additional strains isolated from the Milos sampling event of May 2013 are available also in MATIS strain collection. In addition a series of physicochemical parameters (e.g. gas analysis of the active vents, nutrients, organic carbon, metals, chloropigments etc) were also estimated in order to explain microbiological results and further evaluate the potential risks of the active submarine volcanoes of the Hellenic arc (Rizzo *et al.*, 2016). HCMR has created a collection of 280 strains from the extreme environments of the Hellenic Volcanic arc.

~~3.1.3~~4.1.3 Coastal sites in Scotland with extreme conditions

The west coast of Scotland and its outer islands provide a wide variety of extreme ecological niches including rock-pools, which undergo major shifts in osmotic potential and temperature, unusual niches such as the stratified, anoxic microzone at the head of Loch Etive, and highly polluted sites on the River Clyde estuary. These sources have not yet been explored for bioprospecting, but within SeaBioTech examination of the microbial diversity in these sites were undertaken. SAMS (Scottish Association for Marine Science) has created a unique collection of strains encompassing of a wide range of taxa including: a range of heterotrophic eubacteria, cyanobacteria and eukaryotic micro-algae. In total 480 biological isolates have been identified in the project and processed down the biodiscovery pipeline by SAMS, with 116 of these being identified by 18S rRNA gene sequence NCBI blast results in Period 3. Of these 310 biological isolates; were processed down the biodiscovery pipeline. Of the 310 samples processed, 246 were of bacteria identified in this project by molecular barcoding (16S rRNA gene) and 64 were algal, with identity confirmed by 18S rRNA gene sequence NCBI blast results. All the live microorganisms identified are held in the bacterial and protistan collections at SAMS. All bacterial isolates are held as frozen/ cryopreserved master stock-cultures at -80^{o}C, with glycerol (5% in medium) as cryoprotectant. The algal isolates are maintained by serial transfer and where practicable they are also held as cryopreserved master-cultures and stored at -196^{o}C in the CCAP Cryostore.

In addition, MBL has created a collection of 165 strains over 4 sampling sessions from Culzean bay and Oban. Of those strains which were isolated, the dominant members were affiliated within the class of Gammaproteobacteria and the phylum of Firmicutes.

~~3.1.4~~4.1.4 Microbial symbionts from sponges

Marine sponges often harbour dense and diverse microbial communities, with many of the microorganisms being specific to sponge hosts. These microbes, which can include bacteria, archaea and single cell eukaryotes, comprise up to 40% of sponge volume and may have a profound impact on host biology. For example, photosynthetically fixed carbon from cyanobacterial symbionts provides >50% of the energy requirements of certain tropical sponges, while other microorganisms may contribute to host defence via the production of biologically active metabolites. The latter also hints at the pharmacological potential of sponge-associated microorganisms. The group of Professor Ute Hentschel at the University of Wuezburg (UWUERZ) has a long experience in marine sponge microbiology, many of which have been collected from the Mediterranean Sea (Schmitt *et al.* 2011). Samples and background knowledge was made available to SeaBioTech from two collection efforts to the Greek islands yielded the following biomaterial: 64 unique actinomycetes were isolated from 12 different marine sponge species, which were affiliated to 23 genera representing 8 different suborders based on nearly full-length 16S rRNA gene sequencing ; 4 putatively novel species belonging to the genera *Geodermatophilus, Microlunatus, Rhodococcus, and Actinomycetospora* were identified based on a sequence similarity <98.5% to validly described 16S rRNA gene sequences; and 13 isolates showed antioxidant, antimicrobial, and antitrypanosomal activities.

~~3.1.5~~4.1.5 Existing collections

The marine resources exploited under SeaBioTech also included all culture collections available from partners. MATIS had amassed large strain collections of extreme organisms and also recently set up facilities and pipeline for eukaryotic microalgae collection and analysis. The Culture Collection of Algae and Protozoa (CCAP), located at SAMS, holds a uniquely diverse range of marine, freshwater and terrestrial protists (algal and protozoan) as well as prokaryotic cyanobacteria. Additionally, SAMS has collections of marine bacteria that are not replicated in any accessible Biological Resource Centre. HCMR has an existing microbial collection from deep sea sediments and from submarine volcanic sites in the Eastern Mediterranean. The Natural Products Metabolomics group at the Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS) also has a collection of marine microbes from the Northern Scottish coastlines of Orkney and Shetland. These collections have not been previously investigated for their potential to produce bioactive secondary metabolites and provided biotechnologically exploitable metabolites within SeaBioTech.

~~3.1.6~~4.1.6 Advances in the sampling and collection of extremophiles

From existing collections from different partner institutions, isolates have also been additionally generated from the following sources: Scottish sponge isolates (~150); Scottish & Antarctic sediment cores (~100 of which 54 have been processed); and polar Antarctic & Arctic sediment cores (~150). SeaBioTech partners shared their expertise in the successful sampling of extremophiles, and developed a common and efficient strategy to optimize the useful access to marine biodiversity. Targeted scientific and technological tools (ROV based technology) for deep sea sites and scientific diving for shallow sites for observing and sampling submarine ecosystems and collecting sponge samples were deployed to explore the series of diverse habitats described above. MATIS focused on geothermal coastal areas around Iceland and developed various methods isolating psychrophilic and thermophilic microbes relevant to the project. Specialized techniques were developed in the project for accessing rare species in order to increase the overall "phylogenetic depth" of the obtained strain collection. In addition to direct production of samples through cultivation methods (described in the next section),

SeaBioTech also employed molecular genetics, particularly a metagenomic sampling approach vastly increasing its access to relevant DNA from marine samples. The advent of molecular genetics in the 1970s prompted a major revelation in microbiology (Woese 1987). A huge pool of microbiota was discovered that had been previously missed because of their lacks of growth on laboratory media (Rappe & Giovannoni 2003). Several dozens of phyla have been discovered since then, encoding many novel metabolic functions and pathways (Achtman & Wagner 2008). Because of the sheer numbers of microorganisms in environmental samples, the limits of discovery have clearly not yet been reached: in addition to the 10^5-10^6 bacteria per ml seawater, an unimaginable number of microorganisms is associated with algal and animal surfaces, residing as commensals in the intestines of animals, or as symbionts in highly specialized organs, such as the cellulose degrading symbionts of wood-boring bivalves or the symbiotic microbial consortia of marine sponges. In order to access this largely untapped resource of marine microorganisms, metagenomic strategies were employed in the project. Metagenomics (or 'environmental genomics') involves the direct extraction of community high-molecular weight DNA from an environmental sample, and the cloning of the resultant DNA pool (called the 'metagenome') into suitable vectors (Grozanov & Hentschel 2007; Hugenholtz & Tyson 2008; Vieites *et al.* 2009). The cloning vectors have been designed to hold small, medium or large insert sizes. These vectors (fosmid, cosmid) are then propagated in surrogate host strains, such as *E. coli*

or specialized overexpression strains, such as *Streptomyces albus*, and others. With the generation of large libraries consisting of tens to hundreds of thousands of clones, the genomic complexity of the original microbial community can be maintained. These libraries were then screened, in what has been termed a "functional metagenomics" approach, for phenotypic activities and the responsible operon structures are sequence. In doing so, a number of enzymes (including esterases, lipases, cellulases, amidases, amylases), ribosomal operons, antibiotics and pigments have been recovered from environmental microbial communities whose large uncultured fraction would otherwise have been inaccessible (Lorenz & Eck 2005; Kennedy *et al.* 2010, 2011). Owing to the environment from which the enzymes had been isolated, they may have novel properties, such as increased stabilities under alkaline, acidic, or low or high temperature conditions. Functional metagenomics is thus a highly promising strategy for the recovery of biotechnologically relevant enzymes from the marine environment.

Another strategy used by SeaBiotech to tap into the environmental DNA pool is by "sequence-driven metagenomics". This approach has been undertaken by Venter and colleagues to yield a global genomic inventory of the oceans (Rusch *et al.* 2007). Other studies have employed sequence-driven metagenomics for example, to characterize the genomic repertoire of the microbial consortia of marine sponges (Fieseler *et al.* 2007; Thomas *et al.* 2011), of whale fall carcasses (Tringe *et al.* 2005) and the deep sea (Eloe *et al.* 2011). The main outcomes of sequence-driven metagenomics are predictions on the metabolic repertoire of a given sample, to delineate metabolic pathways and to assess the potential of an environmental sample to perform specific, sought-after tasks. Single cell genomics based on whole genome amplification (WGA) is an emerging technology in the field of environmental microbiology, which is complementary to metagenomics (Hutchison & Venter, 2006; Ishoey *et al.* 2008). Owing to the experimentation and manipulation of single microbial cells, this technique allows promising genomic insights into complex environmental microbial consortia whose members are frequently resistant to cultivation (Siegl *et al.* 2011). Importantly, functional assignments of primary and secondary metabolism genes to specific bacterial genes of known phylogenetic identity are possible (Siegl & Hentschel 2010). Metagenomics and other –omics methods have opened new avenues for the sustainable production of marine enzymes/drugs that would otherwise be inaccessible by conventional microbiology techniques. By merging the scientific disciplines of molecular genetics, microbiology, chemistry and biochemistry, the promise that marine microorganisms

hold for industry is becoming a manageable task. The advent of massive parallel DNA sequencing techniques has set the stage for the next level of genomic and metagenomic bioprospecting. This methodology provided the means for isolating genes directly from environmental DNA without cloning. In the SeaBioTech consortium, high throughput pyro-sequencing technology from Roche (the 454 genome sequencing platform) was the key instrument for metagenomics mining which was complemented upon demand by other sequencing technologies (e.g., Illumina). Importantly, sequence read lengths on the average of 700 bases were obtained with the 454 FLXplus platform, which resulted in higher numbers of informative sequences. The advantages of sequence-based metagenomics are many: this gave enzyme leads at least of an order of magnitude greater than other currently used screening techniques. A large number of genes were predicted to be detected that do not turn up using activity screening due to expression problems or the use of suboptimal substrates. And, as the genomic/metagenomic enzyme/gene discovery methodology is sequence-based, gene redundancy was eliminated very early in the process, which minimized the downstream analysis work. This was especially important for large-scale metagenomic sequencing projects as the sequence capture method reduced the need for as high coverage of sequencing for complete gene retrieval. The SeaBioTech methodology took the metagenomic mining out of the domain of large specialized companies and brought it into the field of small companies, universities and institutions. Hence, one of the most important contributions of SeaBioTech project was “affordable metagenomics”. Samples for metagenome libraries were made available from the project which, included strains from Yngingarlindir water samples in Iceland, microbial mats and sponges from Milos Island and Santorini volcanic complex in Greece; strains from Kallisti lakes water samples, strains collected from Kolumbo microbial mats covering the ocean floor and the polymetallic chimneys.

~~3.1.7~~4.1.7 Metagenomic bioprospecting

UWUERZ employed a metagenomic bioprospecting approach to unravel the differences in the functional gene repertoire between three Mediterranean sponge species, *Petrosia ficiformis*, *Sarcotragus foetidus*, *Aplysina aerophoba* and seawater, collected during a SBT sampling expedition (WP2). Microbial diversities were compared to those of other sponges within an EMP global sponge microbiome effort and contributed to the largest microbiology survey in sponges so far conducted (Thomas *et al.*, 2016).

With respect to gene function, different signatures were observed between sponge and seawater metagenomes with regard to microbial community composition, GC content, and

estimated bacterial genome size. The analysis showed further a pronounced repertoire for defense systems in sponge metagenomes. Specifically, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), restriction modification, DNA phosphorothioation and phage growth limitation systems were enriched in sponge metagenomes (Horn *et al.*, Frontiers in Microbiol, under review). These data suggest that the "defensosome" is an important functional trait for an existence within sponges that requires mechanisms to defend against foreign DNA from microorganisms and viruses.

With respect to secondary metabolism, the most abundant marker genes in the microbial metagenomes belonged to the groups of saccharides, bacteriocins, terpenes and fatty acids. Other indicator genes of secondary metabolism – linaridin, lantipeptides, ectoines, phosphonates, proteusin, polyketide synthases, nucleosides, microcins, siderophore or homoserine lactones - were found only in low copy numbers. Interestingly, while siderophores and homoserine lactone hits were only identified in seawater, lantipeptides, linaridines, and Type I Polyketide synthases were exclusively found in the sponge metagenomes. A total of 120 Type I PKS genes in the three sponge metagenomes were further identified. Phylogenetic analysis assigned the majority (109/120) to the symbiont ubiquitous *supA*-type PKS group. Most similar sequences from the sponge metagenomes were derived from bacterial symbionts of other sponge species. Most of the polyketide synthases in the *supA* clade of the tree resulted in a hit to epothilone with low to moderate sequence identities. Despite the variance of possible products in the FAS-like PKS clade, the order of the genes surrounding the polyketide synthase was highly conserved.

MATIS sequenced 34 novel bacterial strains from geothermal intertidal areas in Iceland, assembled and annotated for bioprospecting. An additional 4 strains that had been sequenced before SeaBioTech were also annotated at the beginning of SeaBioTech to allow bioprospecting to start. Of the 38 sequenced strains, 13 (34%) belong to the α-*Proteobacteria*, 10 (26%) to *Bacteroidetes*, 7 (18%) to Firmicutes, 6 (16%) to γ-*Proteobacteria* and one strain each to *Actinobacteria* and *Chloroflexi*. All strains are thermophiles or moderate thermophiles.

HCMR generated 2 metagenomic libraries from the Kallisti lakes in Santorini caldera characterized by high concentrations of metals and differences in pH, temperature and nutrient concentrations. HCMR also generated another 3 metagenomic libraries from a polymetallic spire located within the submarine Kolumbo volcano of the Hellenic Volcanic Arc. Each library has been constructed from different microbial mat layers of the spire characterized by differences in metal concentrations.

~~3.1.8~~4.1.8 Genome mining of bacterial isolates

UWUERZ provided draft genomes of 3 selected actinomycetes (Horn *et al.*, 2015). Metabolomic analysis in WP5 has shown the chemical richness of the sponge-associated actinomycetes *Streptomyces* sp. SBT349, *Nonomureae* sp. SBT364, and *Nocardiopsis* sp. SBT366 that had been isolated from sponges during a SBT sampling expedition. The genomes of these three actinomycetes were subsequently sequenced and draft genomes were mined using antiSMASH and NapDos. *Streptomyces* sp. SBT349 displayed the most diverse read-out. A total of 108 potential secondary metabolite gene clusters were predicted, encoding for 23 type I polyketide synthases (PKS), 11 non-ribosomal peptide synthetases (NRPSs), 2 terpenes, 21 saccharides, 3 siderophores, 3 lantipeptides, 1 butyrolacetone, 1 bacteriocin, 1 phenazine, 1 ladderane, and 1 linaridin, as well as 26 unidentified putative clusters. Furthermore, NaPDoS predicted the presence of natural products such as nystatin, rapamycin, rifamycin, epothilone, and tetronomycin. For *Nonomureae* sp. SBT364, NaPDoS predicted the presence gene clusters encoding for rifamycin, avermectin, avilamycin, concanamycin, and tetronomycin. Thirdly, for *Nocardiopsis* sp. SBT366, gene clusters encoding for pikromycin, alnumycin, amphotericin, and mycinamicin were predicted. In summary, UWUERZ efforts provided new insights into the genomic underpinnings of actinomycete secondary metabolism, which may deliver novel chemical scaffolds with interesting biological activities for the drug discovery pipeline.

An extremely high level of novelty was presented by this panel of novel strains. Based on 16S rRNA gene sequencing of the 38 genomes, 19 strains (50%) shared less than 94% similarity with their closest relative and are therefore considered novel species and novel genera. 10 (26%) shared between 94% and 97% similarity and are considered novel species and the remaining 9 strains (24%) shared more than 97% similarity with their closest relative. Strain MAT4553, which has 90% similarity with its closest relative *Rhodothermus marinus* (16S rRNA gene) was selected for further characterisation carried out by MATIS. It has been assigned the species name *Rubrimicrobum thermolitorum* and characterisation is still currently ongoing.

All 38 strains were annotated using subsystem annotation servers (RAST and MG-RAST), the genomes mined for novel genes of interest and analysed by antiSMASH for putative secondary metabolite gene clusters. A total of 2432 putative gene clusters were predicted, including 20 Non-Ribosomal Peptide Synthetase clusters and a total of 30 Polyketide Synthase clusters of Types I, II or III. A total of 64 genes encoding novel enzymes for applications in marine macroalgal biorefineries were identified and delivered for

cloning, expression and functional analysis in WP6 including, 51 carbohydrate active enzymes (CAE) 3 enzymes (oxidases) putatively active on polyphenols, 5 alcohol dehydrogenases, a sulfatase and 4 proteases. A total of 58 genes encoding novel enzymes including thioesterase, cyclic peptide related genes, and (3) lysine exporters , for application in synthesis of added value chemical and pharmaceutical were identified and delivered to IGZ for, cloning , expression in their proprietary inABLE® system and for further analysis and selection in WP6.

SAMS undertook whole genome sequencing of five bacterial strains and delivered a total of four draft whole bacterial genomes. The fifth bacterial genome was to be of the filamentous cyanobacterium, *Nodularia harveyana* CCAP 1452/2. This was advanced to the point of achieving an axenic culture (WP2) and development of a useable DNA extraction protocol based on mechanical tissue disruption without pre-digestion of the cell walls using the lysozyme, and purification using the quarternary ammonium detergent cetyl trimethyl ammonium bromide. However, significant quantities of polysaccharide were found to contaminate the DNA preparations, and refinements to the protocols were not successful in removing this. This meant the genome sequencing centre were unable to prepare the DNA library required for PacBio RSII genome sequencing.

All genome data was mined for enzymatic and secondary metabolite potential. In terms of carbohydrate active enzymes and xenobiotic degradation potential, *Colwellia* and *Rhodococcus* (SBT017), respectively, had the greatest potential of the four organisms. The *Colwellia* genome data will serve as an important resource for the scaling up and commercialisation of the gel-forming biopolymer this organism produces (WP7) during a PhD studentship working in conjunction with the multinational company, Unilever. The *Rhodococcus* genome is undergoing further analysis to link the secondary metabolite clusters identified with the metabolome of this organism fermented under different conditions (WP5 and WP7).

The Acidobacteria (Holophagales) genome showed an especially high number of novel secondary metabolite gene clusters belonging to the non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) classes. Metabolomic analysis (WP5) did not identify production any secondary metabolites putatively linked with these cluster, nor was any bioactivity identified (WP3). The lack of novel secondary metabolite production by the Acidobacteria is hypothesized to be a failure to induce the many cryptic secondary metabolite operons. This hypothesis is given some support by the observation that many signal transduction systems were found within or immediately adjacent to these clusters. This

suggests that these clusters are tightly regulated and are part of a signal transduction relay activated by specific signalling molecules or environmental stressors. In conclusion, this organism holds significant potential for secondary metabolite production. But, to achieve this though, further funding is required try to activate the cryptic secondary metabolite clusters, as well as continue to isolate and genome sequence new marine Acidobacteria from the environment.

Vibrio splendidus SBT027 produced a range of bisindoles, including the compound turbomycin. Several putative genes were identified that may be linked with turbomycin production. First, the biosynthetic pathway for the assumed precursor, L-tryptophan, was identified. Second, the enzyme 4-hydroxyphenylpyruvate dioxygenase had previously been identified as a part of turbomycin production, and this was identified in this genome. Third, inosine-5'-monophosphate dehydrogenase has been shown to be important in bisindole production previously, and this gene was also identified. However, as these genes are not organized in an apparent gene cluster, it is uncertain how these genes are involved in Turbomycin production by this *Vibrio*. Moreover, the above genes are all highly conserved and syntenic in all other *Vibrio splendidus* genome sequenced isolates. This suggests either, that all *V. splendidus* are capable of Turbomycin production, or that the main pathway for bisindole and/or Turbomycin production in *V. splendidus* SBT027 has not been correctly identified. Clearly, further work is required to identify this pathway.

The second aspect of the 'quality of marine resources' challenge is cultivation. Traditional techniques are often inadequate for accessing the microbial diversity of any given habitat. Studies from many extreme areas including MATIS' current and ongoing work in Iceland have demonstrated that cultivation of microorganisms living under extreme conditions is particularly difficult. The main reason for the low ratio of presently cultivatable microbial species is that their isolation takes place under both space and time limited by laboratory conditions. Other factors that explain the low ratio of cultivated species include unknown conditional or nutritional requirements or other important chemical components supplied to the species in their natural environment and missing in laboratory media. There could also be requirements for interdependent co-cultivation of two or more different species. A nutritionally rich laboratory medium is not a natural medium for many environmental microbes. Copiotrophic organisms therefore gain a competitive edge and out-compete oligotrophic species, although they may be more abundant in the habitat. Furthermore, rich medium may be growth inhibiting for oligotrophic species. Classical resource-competition theory maintains that highest diversity occurs when many resources are limiting. In addition,

high species diversity can be maintained by periodic disturbance or by environmental fluctuations (i.e., nutrients, pH and temperature) (Buckling *et al.* 2000). Laboratory conditions that allow microbes to grow to high density in a short time are "unnatural" for many natural microorganisms that normally grow slowly, at very low and steady state concentrations of nutrients (Fry 1990). Re-creation of "nature-like" or natural, low nutrient or oligotrophic conditions have been attempted in a few cases but can only be done on a small scale and with great effort (Huber *et al.* 1998). Growing many oligotrophs in the laboratory on a large enough scale would be practically impossible. Different modifications of the enrichment concept have been developed in order to culture more novel organisms. These included serial-dilutions or pre-treatment of the sample. The purpose is to kill or dilute out numerically less numerous copiotrophic fast growing organisms in the sample before inoculating the enrichment medium (Grosskopf *et al.* 1998; Santegoeds 1996). Still another attempt towards"nature-like"enrichments is the technique of *in situ* enrichment or substrate colonization, which has been used in several environments. *In situ* enrichment is based on the principle of introducing one or few new factors into an existing “natural” environment. Techniques of *in situ* enrichments have been of interest to microbiologists ever since bacteria were found to colonize microscope slides submerged in aquatic environments (ZoBell *et al.* 1943; ZoBell & Anderson 1936). Such techniques have been used in hot springs to obtain specific groups of microorganism, by using specific substrates such as cellulose. These techniques may be of special value for isolating or enriching species utilizing polysaccharides unique to the marine organism found in coastal areas. In the SeaBioTech project, the consortium developed selective enrichment methods, and serial dilutions for accessing rarer and potentially more interesting members of bacterial and protistan communities. The consortium was able to increase the overall diversity and phylogenetic depth of obtained strain collection for consequent screening for bioactive microbial metabolites and thus maximized the likelihood of obtaining novel bioactive lead-compounds. Enrichment methods were developed targeted towards certain metabolic types belonging to heterotrophic actinomycetes, thermophilic bacteria, marine and extremophilic cyanobacteria and rare coastal psychrophilic heterotrophs by using various cultivation methods and enrichment procedures. Special substrates such as complex recalcitrant polysaccharides or single carbon sources of predetermined type and structures were used often in conjunction with group specific inhibitory substances.

A third aspect of the ‘quality of marine resources’ challenge is accurate identification. Sampling of marine microbes from a range of environments was explored by 16S rRNA

and/or other candidate genes in order to assess the potential of the communities for industrial purposes and redirect new sampling. By 16S rRNA gene sequencing followed by phylogenetic tree construction analyses, the taxonomic identity of the bacterial isolates was determined. Colony lysates were amplified by PCR (Polymerase chain reaction) using the universal bacterial primers 27f and 1492r while PCR products were sequenced directly. DNA extraction protocols (i.e. for cells with hardy cell walls) and PCR conditions were optimized where necessary. Full 16S rRNA sequences of selected candidates were provided and phylogenetically isolated. Strain descriptions of novel species or genera were also undertaken. The genomic potential of bacterial isolates were assessed by PCR screening for genes indicative of secondary metabolism such as, polyketide synthases, non-ribosomally encoded peptide synthetases, halogenases and other genes of relevance for secondary metabolism. Likewise, metagenomics approaches were employed to assess the genomic potential of previously uncultivated marine microbial consortia. Biotechnologically relevant gene clusters were cloned into cosmid/fosmid vectors, sequenced and analysed with bioinformatic prediction tools. Full genome sequencing was performed for isolates of special interest using deep sequencing (454/Ilumina).

~~3.2~~4.2 Challenge 2: Improvement of technical aspects of the biodiscovery pipeline

Once samples from marine bioresources were obtained, they were explored for the presence of useful bioactivities. When activity was found, the component responsible was identified and characterised. The SeaBioTech project developed systems to enhance the efficiency and effectiveness of both bioactivity detection and compound isolation and characterisation. SeaBioTech focused on discovering useful marine components with enzyme activity, as biopolymers or with drug-like properties. The enzyme activities were predicted from analysis of metagenomic data followed by functional expression (Ferrer et al, 2016). Biopolymers were identified, quantified, as well as extracted and isolated. Development of biopolymers included progressive pharmaceutical screening as well as investigating the potential role of (isolated) algal endophytes in improving polymer and cultivated macroalgae resources, which are a well-established, but not fully developed, source of natural polymers. The next sections discussed the present state of ‘drug hunting’ and how SeaBioTech enhanced this process through improvements in screening and natural product chemistry.

~~3.2.1~~4.2.1 Bioactivity screening

The biodiscovery pipelines focused on the following categories: polymers, enzymes and small molecules used for drug discovery, functional foods or cosmetics. Drug discovery programmes seeking new bioactive compounds are driven by the existence of unmet therapeutic needs. In recent decades, advances in the understanding of the molecular basis of diseases and sequencing of the human genome and of pathogenic hosts have expanded the number of plausible therapeutic targets for the development of innovative drugs (Hopkins & Groom 2002; Russ & Lampel 2005). Therefore, a wealth of new technologies and paradigms has been established since the mid-1990s, with the initial expectation of generating novel drugs in a greater number and in a shorter time. Among others, cardinal roles were played by molecular biology, combinatorial chemistry and high-throughput screening (Drews 2000; Leland *et al.* 2003). First, genetic manipulation of expression host cells using molecular biology allowed the development of target based functional assays, in place of the traditional phenotypic systems (Drews 2000; Leland *et al.* 2003). In parallel, improvements in organic synthesis through combinatorial chemistry exponentially expanded the size of small-molecule compound collections (Chabal 1995). Consequently, natural products (which had been the basis of most previous drug discovery programmes) were progressively neglected. To confront the massive effort required to test the large number of newly identified molecular targets with huge chemical libraries, multiple areas of biology, chemistry, engineering, robotics, statistics and information technology were integrated to create high throughput screening (HTS). Hence, HTS has been established in large pharmaceutical companies as the technological platform able to screen compound collections containing over 1,000,000 molecules on biochemical and cellular assays in an automated manner and miniaturized format (Hüser *et al.* 2006; Carettoni & Verwaerde 2010). Subsequently, prominent academic institutions decided to exploit the potential of these technological advancements through initiatives to assemble centralized compound collections and screening facilities with the aim of identifying molecular probes with prospective applications in basic and applied biomedical research (Verkman 2004).

Although this pioneering approach to drug discovery has been successful in delivering innovative clinical candidates and marketed drugs (Macarron 2006; Macarron *et al.* 2011), it is undoubtedly true that the original expectations in terms of overall performance are far from being met and unlikely to be achievable. Rather, the increasing costs associated with the infrastructural and technological investments have contributed (within a framework of tackling more challenging diseases, higher scientific risks, increasing safety requirements and

larger clinical trials) to the so-called "productivity gap" in pharmaceutical R&D, which has been posing major issues for the sustainability of drug development in the private and public sectors (Pammolli *et al.* 2011). Therefore, while the main technological improvements are still considered essential cornerstones for R&D, the basic paradigms of the process are currently under debate (Macarron 2006; Leeson and Springthorpe 2007; Harvey 2008; Mayr & Bojanic 2009). In particular, phenotypic screenings have been currently reconsidered as valid options along with target-based molecular assays, particularly for certain therapeutic areas (e.g. pathogenic infections, cancer and others) (Payne *et al.* 2007; Mayr & Bojanic 2009). Moreover, emphasis has been given to highly validated targets, i.e. targets whose activity has been proven to be modulated by a chemical compound and with a direct causative link to the disease to be addressed. Therefore, highly innovative but poorly characterized targets were deprioritized (Gashaw *et al.* 2011). More recently, attention has been focused on the quality of the compounds in the chemical libraries, rather than on the number of compounds. In fact, retrospective analysis unequivocally clarified that early combinatorial chemistry produced large libraries with very limited diversity (Macarron 2006; Macarron *et al.* 2011). At present, investments in compound collections are not aimed at a numerical size increase, but at ensuring a constant stream of new chemotypes, meaning that natural products and mimetic derivatives are back into consideration (Harvey 2008; Macarron *et al.* 2011). This implies that drug discovery has to face well-known problems inherent to natural products, like supply at screening scale, purification, identification and structural complexity (Grabowski & Schneider, 2007; Ganesan, 2008). However, technical solutions have been rapidly developing to overcome these bottlenecks and in order to gain access to the potential of this valuable source of chemical diversity (Kennedy 2008; Rishton 2008; Koehn 2008). Under this developing scenario, the SeaBioTech consortium integrated some of the most advanced technological applications with state of the art expertise in drug discovery research to identify bioactive compounds from libraries of marine origin. To increase the chances of a positive outcome of the screening campaigns, the assay types applied in SeaBioTech comprised a wide array of target-based and phenotypic assays. Some were configured in HTS-suitable formats to ensure a high processivity of large compound collections and of hit profiling; some will be performed as low-throughput assays to achieve a high level of information directly from primary screening (e.g., assays against sea lice affecting farmed salmon). Most importantly, all assays within SeaBioTech represented functional assays designed to provide unambiguous responses concerning their relevance for biomedical and biotechnological applications (e.g., isogenic X-MAN human

disease models from HDL). Having no pre-existing knowledge on the bioactivities present in the extract/compound collections obtained from underexplored marine sources, SeaBioTech members screened a very wide set of assays with relevance to diverse therapeutic areas, including cancer (AXXAM; HDL; MATIS), bacterial, viral and parasitic infections (SIPBS; AXXAM; UWUERZ), inflammation (SIPBS; MATIS), cardiovascular diseases (AXXAM; MATIS), metabolic disorders (SIPBS) and pain (AXXAM). Besides human health, SeaBioTech sought bioactive compounds to treat parasitic infections in aquaculture (PHARMAQ) and for food and cosmetics industry (MATIS). It is worth noting that discovery programmes in these fields are encouraged by the successful outcome of research projects using compounds of marine source, which have recently yielded molecular probes, pre-clinical candidates and therapeutic drugs in several clinical areas, including cancer (Napolitano *et al.* 2009; Dumontet and Jordan 2010; Galeano *et al.* 2011), bacterial, viral and parasitic infections (Mayer *et al.* 2011), inflammation (Folmer at al. 2010; Mayer *et al.* 2011), Alzheimer's disease (Williams *et al.* 2010), and pain (Teichert & Olivera 2010; Mayer *et al.* 2011).

Since the final aim of SeaBioTech was the exploitation of the value of the new compounds, participants did not limit their investigation to the identification of hit compounds through primary screening, but also employed their competencies in more advanced stages of the drug discovery process, including studies on selectivity, mechanism-of-action, early toxicology and proof-of-principle in animal models. This guaranteed that the outcome of the bioactivity assessments were not just be compounds that 'hit' particular targets, but an activity profile of a bioactive substance and its drug-like properties. Such compounds represented potential development candidates, a critical step towards new medicines.

The organisational aspects of SeaBiotech also provided progress beyond what is normally achieved in drug discovery programmes in individual SMEs or in academic institutes. For SMEs, the successful outcome of large-scale drug discovery projects entails on extensive collaborations and partnership with public academic institutions. On the other side, access to advanced technological platforms, cost-sustainable exploitation of the results and interrelation between specific expertises, knowledge and competences were considered essential prerequisites to identify and progress novel molecular entities for biotechnological and biomedical applications. Hence, SeaBioTech was structured to promote and implement synergistic collaboration at two levels. First, extracts and compounds of marine origin collected and isolated by public research institutes will be made accessible to private

companies, which in turn will make available their technological platforms and market oriented approach to develop innovative products for human health and life sciences. In addition, SeaBioTech represented a valuable opportunity to synergistically link the public and the private sectors, offering the possibility to progress within an integrated partnership and providing common objectives through mutual connections.

Second, SeaBioTech inherently enhanced the collaboration among different SMEs contributing at different stages of the project (identification of hit compounds, hit-to-lead phase, characterization of lead compounds), in order to define the chemical and pharmacological properties of the products. Thus, participation in SeaBioTech epitomised a valid opportunity for SMEs to establish collaborative partnerships with companies with contiguous expertise and complementary technologies. In parallel, the strategy adopted in SeaBioTech for bioactivity detection embodied an impressive improvement in terms of potential exploitation of the chemical diversity of the marine compound collections. Indeed, libraries of marine origin were subjected to screening campaigns against a panel of more than 20 assays covering over 10 different therapeutic areas or biotechnological applications. This approach increased the probability that bioactive compounds are retrieved as positive hits, thus predicting a superior success rate compared to traditional screening on a few assays. In practice, the adopted strategy places SMEs and research institutes within

The screening method in SeaBioTech closely resembled a large pharmaceutical company, in which a proprietary compound collection was routinely screened against a series of disease-relevant assays. However, in SeaBioTech, costs and risks are shared among different participants, making the overall process more sustainable. In addition, as the consortium has access to an underexplored chemical diversity and the project focused also on products for aquaculture, food industry and cosmetics, in which a lower attrition rate is usually experienced, it then gave a remarkably high productivity for SeaBioTech. The central goal of the entire SeaBioTech consortium was the isolation and pharmacological characterization of novel lead candidates of marine origin. This goal was achieved through an integrated effort between WP2-WP5 with the six members of WP3 (SIPBS, AXXAM, HDL, PHARMAQ, UWUERZ, MATIS), who have made available comprehensively an array of 41 functional assays with relevance to 12 therapeutic and life science indications. The screening process and the bioactivity-assisted dereplication of crude extracts and fractions have led to the isolation and characterization of 35 pure compounds with promising therapeutic properties. Notable examples are the followings: (1) SBT0345 from *Streptomyces sp.* was fractionated by UWUERZ to yield three novel natural products, namely strepthonium A,

ageloline A and strepoxazine A. Strepthonium A inhibited the production of Shiga toxin produced by enterohemorrhagic *E. coli* at a concentration of 80 µM, without interfering with the bacterial growth (Cheng et al, 2016a). Ageloline A exhibited antioxidant activity and inhibited the inclusion of *Chlamydia trachomatis* with an IC_{50} value of 9.54 ± 0.36 µM without cytotoxicity towards human kidney 2 cells (Cheng et al, 2016b). Strepoxazine A displayed antiproliferative property towards human promyelocytic HL-60 cells with an IC_{50} value of 16µg/mL (Cheng et al, 2016c). Moreover, SBT0345 from *Streptomyces* sp. was yielded also the known compound phencomycin, which displayed cytotoxicity against colon cancer cell line SW48 at 30 µg/mL, and tubermycin B, which showed cytotoxicity against colon cancer cell lines DLD-1 and HCT116 at 30µg/mL. (2) SBT0348 from *Streptomyces* sp. was fractionated by UWUERZ to yield one novel compound, petrocidin A, exhibiting significant cytotoxicity towards the human promyelocytic HL-60 and the human colon adenocarcinoma HT-29 cell lines, with IC_{50} values of 3.9 and 5.3 µg/mL, respectively. (3) SBT0961 from *Polysiphonia lanosa* yielded three fractions, which were identified by HDL as active and selective for rapidly dividing cancer cells, with anti-proliferative properties strongly correlated with the induction of cell death via apoptosis. (4) MATIS identified from microorganisms collected from the Icelandic coastline 11 hits displaying high anti-oxidant activity, 9 hits that inhibited cell viability of breast cancer cell line and 13 hits that inhibited viability of intestine cancer cell line. (5) SIPBS isolated 13-methyltetradecanoic acid (SBT2309) from *Muricauda ruestringensis,* a compound with activity against PTP1B, a target to treat diabetes and metabolic syndrome. Remarkably, SIPBS isolated the same compound showing comparable activity against PTP1B at the end of an independent bioactivity-assisted screening campaign from extracts of another microorganism, *Algoriphagus marincola.* (6) SIPBS isolated a series of structurally related fatty acids from extracts of *Algoriphagus marincola*, which showed activity against PTP1B and allowed the definition of a preliminary structure-activity relationship on the basis of the relative potency. Remarkably, AXXAM isolated with an independent screening campaign for inhibitors of endothelial lipase, a validated target for atherosclerosis, a series of fatty acids derived from *Algoriphagus marincola* partially overlapping with the hits showing activity against PTP1B at SIPBS. This finding appears consistent with the targeted enzyme EL, which physiologically releases fatty acids from phospholipids in HDL particles. (7) SBT1997, a pure compound isolated by SIPBS from *Polysiphonia lanosa* as active against α-glucosidase, was identified as a known compound termed lanosol. Lanosol was documented in literature as an α-glucosidase inhibitor. (8) A series of bromophenyl homologous compounds have

been identified by PHARMAQ from *Polysiphonia lanosa* extracts and fractions having a potent parasiticidal activity against *Lepeophtheirus salmonis*, a major threat for farmed salmon in aquaculture.

~~3.2.2~~4.2.2 Metabolomics approach - Improving isolation and identification of target compounds

SeaBioTech used the state of the art approaches to isolation of bioactive compounds from extracts and microbial broths coupled with appropriate NMR (nuclear magnetic resonance) spectroscopy and mass spectrometry to elucidate chemical structures. Moreover, SeaBioTech pioneered the use of metabolomics as a new means to guide strain selection and the isolation of compounds (MacIntyre et al 2014; Cheng et al, 2015), as well as to help improve the productivity of downstream fermentation methods. Metabolomics is relatively a new field of 'omics', adopting to the system biology approach, with the goal of qualitatively and quantitatively analysing all metabolites contained in an organism at a specific time and under specific conditions. The metabolome is the complete set of small molecules found in a cell, tissue or organism at a certain point in time. Metabolomics is considered as the most functional approach in monitoring gene function and identifying the biochemical status of an organism (Yuliana *et al.* 2011). Metabolomics was utilised to confirm the results of the presence of biosynthetic gene clusters involved in the production of the biologically active components. This was accomplished with bioactive strains which, included the anti-mycobacterial *Vibrio splendidus* SBT-027 (MacIntyre et al, unpublished data) and *Rhodococcus* sp. SBT-017 found to be active against metabolic diseases (Hislop et al, unpublished data). Metabolomics in combination with genomics enhanced the production of important secondary metabolites which is one of the expressed phenotype in a living organism. Literature has shown that gene clusters are involved in every step of a biosynthetic pathway as in the production of biologically active polyketides (Moldenhauer et al 2007). With genomics, gene clusters can be manipulated to control a biosynthetic pathway. The procedure of employing metabolomics together with genomics to optimise a biosynthetic pathway to selectively produce biologically active secondary metabolites was explored during the project's lifetime. To identify and quantify the metabolites in natural product extracts is a massive job (Ebada, et al; 2008, Kjer et al; 2010). This is due to the fact that secondary metabolites have diverse atomic arrangements which results in variations in chemical and physical properties. They can also be found in wide range of concentrations. Reliable, robust, selective and high resolution analytical methods are therefore required in identifying and quantifying multiple chemical groups of natural products. Mass spectrometry

and NMR spectroscopy were the complementary analytical methods and were commonly employed in tandem as metabolomics tools. Mass spectrometry is sensitive even at femtogram levels but may not be reproducible between instrument types and ionization capability of the metabolites. While NMR data is reproducible, it may not be sensitive enough to detect metabolites at lower concentrations. Efficient high-throughput gradient flash and/or medium pressure chromatography, where gram quantities of a microbial extract can be loaded in a column, will be employed to isolate the bioactive natural products from microbial extracts. High-throughput gradient medium pressure chromatography is capable of delivering reproducible isolation schemes with high product yield, which is optimum in the purification of marine microbial extracts obtained from multiple batches and has great advantage over conventional column chromatography (Ebada, et al; 2008, Kjer et al; 2010). Structure elucidation was accomplished utilising pulse field gradient 2D NMR that would be able to provide high resolution data to determine the structure of complex molecules with multiple chiral centres as well higher molecular weight peptides and oligosaccharides (Murata *et al.* 2006).

Metabolomics provided statistical and computational tools to this standard approach of rapid HPLC (high-performance liquid chromatography) fractionation, which identified the active entities at an earlier stage (Abdelsohmen et al 2014, Cheng et al 2015). The goal of HPLC fractionation is to get to higher purity of active components which, however, is not achievable in the initial chromatographic isolation work. With metabolomics tools, it will be possible to pinpoint the active components at the first fractionation step as well as identify the functional groups involve in the bioactivity which would be present in a series of fractions as implied by the bioactivity screening results. This can be chemometrically achieved by such metabolomic/PCA approaches (principal component analysis) as shown in an example presented in figure 3. The use of metabolomics aided in prioritising the fractions that will go further for purification work, which should save time and resources in isolating the target compounds.

Within Seabiotech, metabolomics was used for quality control of the natural products and isolates to monitor the manifestation of a different metabolic profile between individuals, environmental alterations during growth and harvesting, post harvesting treatment, extraction and method of isolation, all of which can affect the efficacy of natural products.

Through the metabolomics approach, the link between chemical profile and bioactivity pattern of the secondary metabolites is correlated. Metabolomics was widely applied in the bioactive screening of natural products although it has several advantages over the

reductionist approach. Metabolomic tools enhanced the identification and dereplication steps, as in bioassay-guided isolation work (Yuliana *et al.* 2011). Metabolomics was applied to dereplicate the biosynthesis of the natural product at different development stages of their biological source as well as simultaneously screen for the bioactivity. By using combinations of different analytical methods, the bioassay-guided isolation route was shortened and rapid dereplication of known activities was rapidly delivered (Ebada, et al; 2008). The SeaBioTech consortium encompasses the expertise in metabolomics. VTT (Technical Research Centre of Finland) together with SIPBS developed MACROS and modified the algorithms of MzMine (version 2.10), a web-based differential expression analysis software (Pluskal *et al.* 2011) to efficiently detect the production of interesting secondary metabolites during the cultivation and production processes that would assist in maintaining or enhancing biosynthesis of the desired compounds (MacIntyre et al, 2015). An example is shown in Fig. 4. The results were integrated and coupled to an in-house database that includes DNP (Dictionary of Natural Products) and Antibase, a database of microbial secondary metabolites to further identify microbial secondary metabolites. Figure 4 presents the HRMS heatmap data as processed by Mzmine. The experiment analysed the production of anti-mycobacterial metabolites from a *Vibrio* sp. collected from the Atlantic coastline of the Northern Scottish Isles.

Within the SeaBioTech project, metabolomics was applied at two levels: first, to identify and track active compounds highlighted by screening assays and second, to optimise the biotechnological production of active compounds in the later stages of the pipeline. Dereplication of secondary metabolites from promising isolates was achieved by HRFTMS (high resolution Fourier transform mass spectrometry) using the LTQ-Orbitrap and high resolution NMR. Through multivariate analysis, this enabled Fourier transformation of FID (free induction decay) data of multiple samples to statistically validate the parameters in the production of pharmacologically interesting secondary metabolites. Metabolomes were identified with the aid of existing high resolution MS and NMR records from in-house databases like DNP and AntiBase. MZmine is a software that was utilized to perform differential analysis on the mass spectral data from a vast number of sample populations to find significant expressed features of complex biomarkers between parameter variables. This would be further validated through available reference standards and two-dimensional homonuclear NMR e.g., TOCSY (total spectroscopy) and *J*-resolved NMR experiments to classify unknown by-products or degradants which may affect the quality of the desired product. The NMR metabolomic software from MNova was employed for metabolome recognition as well as to statistically validate the occurrence of metabolic by-products at the

different physiological states. VTT optimised the much-needed algorithms to analyse the huge dataset generated by the dereplication study as well as metabolomics profiling in monitoring and exploring the relationship between culture methods, diversity, bioactivity, and metabolome evolution in selected marine isolates. Efficient cultivation and production processes at a small volume scale fermenter are developed through real time metabolomic-assisted optimization. Samplings were done inreal time for detailed metabolome analysis to fully characterize intermediates, by-products and degradants. Applying metabolomics for real time analysis will in parallel check the stability of the production of the desired components when changing certain fermentation parameters prior to scale up. In addition, a chemometric study was accomplished to support and develop algorithms that was adapted and optimised to target the bioactive secondary metabolites. Metabolomics has become a powerful tool in systems biology and it allowed SeaBioTech to gain insights into the potential of natural marine isolates for synthesis of significant quantities of promising new agents, and guide the manipulation of the environment within fermentation systems in a rational manner to select the desired metabolome. Dereplication work was finalized for samples originating from Milos, Crete, and the geothermal vents of Iceland as well as those covering Scottish coastline and additional sample strains from the Antarctica region. Seventy-seven (77) bacterial samples were dereplicated from the NPMG-Orkney archive. A total of 34 bacterial extracts from Milos and Crete were analysed, yielding SBT348 and SBT687 as the candidate strains for further compounds isolation and purification. While based on mass spectrometry profiles of strains from the Scottish coastline and the Antarctica region, three isolates revealed distinct patterns, KP130 (an unidentified bacteria isolated from Maud Rise, Antarctica), KP044 (a Streptomyces strain isolated from St. Andrews sediment) and KP121 (a *Bacillus* strain from Bransfield Strait, Antarctica). The metabolites responsible for these unique profiles were identified using principle component analysis (PCA) and found to be a series of polymers *m/z* 363-1911 with spacing of 86 Da (KP130), a series of piscicides and antimycins known to be produced by *Streptomyces* spp. (KP044). These PCA outliers were also identified in the molecular network, demonstrating their complementary nature of metabolomic tools for secondary metabolite discovery. Metabolomic profiles have been documented into the SeaBioTech database. Metabolomes were dereplicated for priority strains while biosynthetic gene-based screening explored the presence of the genes for the respective secondary metabolite (WP4). However, bioactivity was used to prioritise strains for the WP7 pipeline (WP3). Extracts of priority strains were prepared from scale-up for further fractionation and isolation of bioactive secondary metabolites. Metabolomic-guided targeted isolation work

was done in parallel to and in support of the bioassay resulting to a quick identification of the active metabolites. A total of 65 natural products have been elucidated and have been documented in the SeaBioTech database which has been linked to Chemspider and PubChem databases (http://spider.science.strath.ac.uk/seabiotech/pure_compounds_show.php).

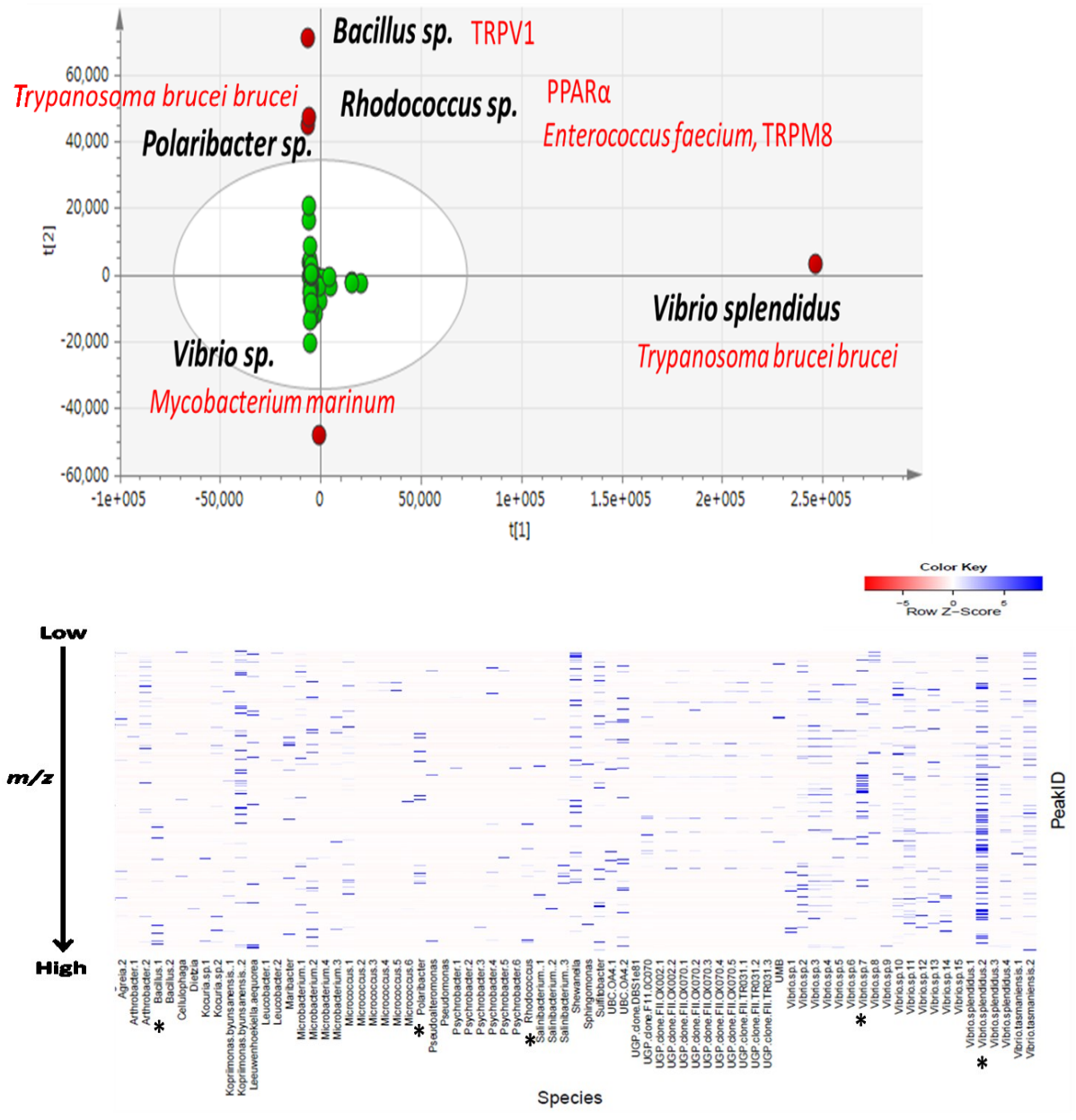

Figure 3 – A) PCA scoring plot based on mass spectrometry data from all extracts. (Taxonomic identification data and bioactivities of the outliers (red dots) are shown in the plot.). B) Heat map based on mass spectrometry data. Outliers in PCA analysis plot are annotated *.

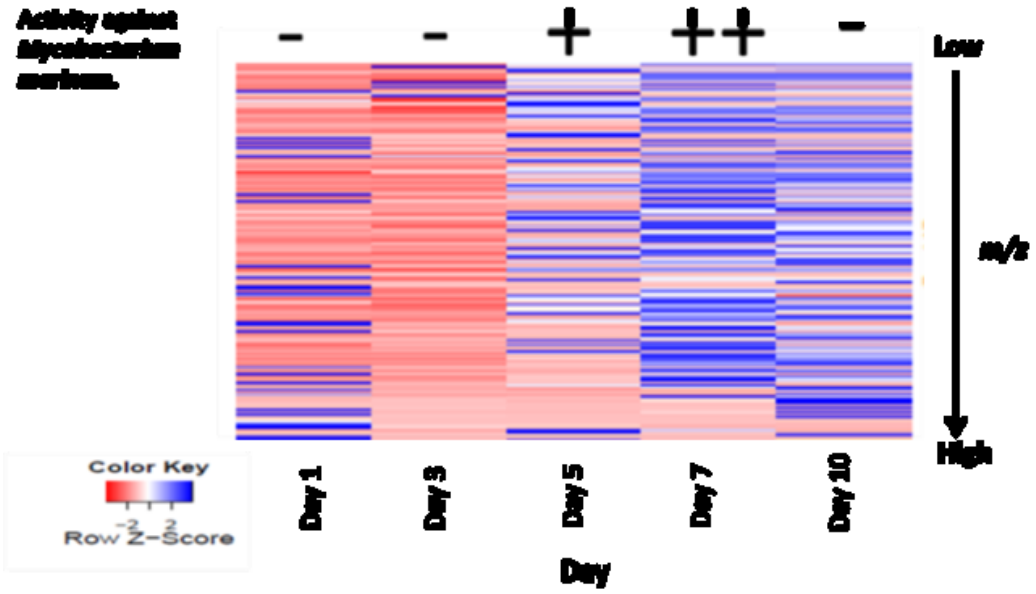

Figure 4 - Significant increase in bioactive metabolite production in *Vibrio splendidus* starts in Day 5 which steadily increases to Day10. However, only Day7 was found active against *Mycobacterium marinum*.

At VTT, an axenic *Euglena gracilis* microalga was introduced as a model organism for metabolic profiling. It was cultivated in 2 L stirred glass tank bioreactors in the presence of glucose under constant light or in the dark. The analyses showed that in light the glucose intake was delayed while the culture generated more biomass suggesting the contribution of photosynthesis. Lipidomic profiling by UPLC-QTof-MS in ESI+ mode (VTT) indicated that phosphatidylcholines were the prior lipid species, but in light cells accumulated large amounts of galactosyldiacylglycerols and ether-bonded lipids, while in dark medium-chain wax-esters were typically formed. LTQ-Orbitrap based metabolomic profiling (SIPBS), on the other hand, showed the richness of metabolites formed in dark especially, and numerous spectral library suggestions for terpenoids of marine origin were obtained. Bioactivity testing (AXXAM) was also indicating some HDAC6 and PPARα activities for the ethyl acetate extract of cells cultivated in the dark.

~~3.3~~4.3 Challenge 3: Sustainable modes of supply of raw materials

Marine organisms have provided many promising bioactive compounds with exciting therapeutic potential. However, their development has been severely curtailed because of the difficulties in obtaining adequate amounts. Examples include anticancer agents,

ecteinascidin-743 and bryostatin. Ecteinascidin 743 (trabectedin, marketed as Yondelis®), was first isolated from the sea squirt Ecteinascidia turbinata in 1984. However, yields from the sea squirt were extremely low and for further drug development, 1 tonne of animals was needed to isolate 1 gram of trabectedin. It was only after 15 years that the supply problem was resolved by a semisynthetic process of starting with safracin B, which was obtained by fermentation of the bacterium *Pseudomonas fluorescens*. In the case of bryostatin, laboratory colonies of the bryozoan *Bugula neritina* exhibited a reduced number of symbionts and a reduction of byrostatin content thus implicating those bacterial symbionts as the true sources of the byrostatins. When some macro-organisms were placed in aquaculture in attempts to scale up production of a bioactive compound, the active material was lost, almost certainly because of loss of associated microbial species in the transfer from the wild to the cultured environment.

SeaBioTech's goal is to avoid such problems by basing much of its scale-up on the knowledge gained through its genomic and metagenomic studies of the gene clusters involved in synthesis of bioactive small molecules. There is extensive information on manipulating genes for non-ribosomal peptides and for polyketides. In addition, SeaBioTech explored the biosynthesis of marine polysaccharides, for which much less is known. This aspect of the project, its background and the advances made by SeaBioTech, will be explained in detail in the following section.

~~3.3.1~~4.3.1 Sustainable production of macromolecules at the lab scale by metabolic engineering

Microbes in extreme environments often adapt through production of extracellular polysaccharides (EPS). They are highly hydrated, which helps to deter desiccation, and they mediate adhesion to inert surfaces or living tissues, which is important for colonization of host organisms and the formation of biofilms. Often these polysaccharides have novel and unusual characteristics and (Jia *et al.* 2007; Nicolaus *et al.* 2010) that can be exploited in various fields - in the food, pharmaceutical and cosmetics industries as emulsifiers, stabilizers, gel agents, coagulants, thickeners and suspending agents and in high value medical applications such as in tissue engineering and drug delivery (Wingender *et al.* 1999). Due to low production levels, few of these organisms have been exploited commercially. It follows that they are therefore basically untapped as a genetic resource for these activities. SeaBioTech explored the possibility of accessing these sources by developing a platform for production of tailor-made polysaccharides and oligosaccharides. While bioprospecting platforms have proven their value in mining natural genetic resources, the exploitation of

promising leads is often hampered by low production yields. This is especially true as regards complex carbohydrate molecules - oligosaccharides, polysaccharides and glycosides. These limitations can in theory be overcome by pathway engineering of the source organism. Biosynthetic pathways can be influenced at three different levels: synthesis of sugar nucleotide precursors; assembly of the repeating unit; and polymerization with concomitant export. By modifying the expression of single genes or groups of genes the conversion efficiency of the chemical entities involved can be increased, and therefore, enhance EPS yield (Ruffing & Chen 2006). While highly justifiable in many cases, such an approach necessitates the time-consuming work of developing genetic tools, selectable markers, transformation methods and ideally species-specific expression vectors for each organism. An alternative approach to make use of these genetic resources is to develop a versatile polysaccharide production microbe with suitable genetic tools for hosting genes from other organisms. Such genes could exert their effects in the biosynthetic pathway in variety of ways depending on the gene, by introducing novel mono-saccharide components and/or other substituents and by forming new linkages. Metabolic engineering of platform organisms for producing novel oligosaccharides and polysaccharides derivatives presents substantial challenges. Microbial polysaccharides are species-specific, highly heterogeneous polymers. These glycans include many unusual sugars not found in vertebrates, such as variously modified hexoses, non-carbohydrate substituents and an oligosaccharide sequence-repeating unit that can vary in size depending on the degree of polymerization. Besides requiring very complex synthetic machinery, the cellular context also matters, e.g. interference with energy metabolism, for synchronization/co-regulation of synthesis pathways, post-synthesis modifications and secretion mechanisms. Successful metabolic engineering of EPS-producing strains has been reported. In *Acetobacter xylinum,* the disruption of the diguanylate cyclase gene led to enhanced production of bacterial cellulose with altered structural properties (Bae *et al.* 2004) and it has been shown that inactivation of the C5 epimerase in *P. fluorescens* led to the production of the homopolymer polymannuronate. It is expected that continued effort in this field will open up an enormous potential for the biotechnological production of biopolymers with tailored properties suitable for various high value applications (Morea *et al.* 2001). The assembly of extracellular polysaccharide involves a set of genes that are often clustered in the bacterial chromosome in separate regions. This arrangement allows a simple mechanism for changing capsule types by merely swapping different gene cassettes. Genes in one particular region can encode enzymes involved in nucleotide sugar formation and capsule-specific transferases whereas genes in other regions

encode type independent transport activities required for movement of the polysaccharides across the inner membrane and periplasm. In other instances, synthases have formed membrane pores through which the polysaccharide is transported concomitantly with synthesis. This has been shown, for example, in hyaluronate synthetase in *Streptococcus* (Tlapak-Simmons *et al.* 2004) and suggested by MATIS as the most plausible model on synthesis/transport mechanism of periplasmic beta glucan oligosaccharides involving a multidomain glucosyltransferases in Proteobacteria (Hreggvidsson *et al.* 2011). Besides a region coding for a Leloir glucosyltransferase of family GT2, another region codes for a domain belonging to a Non-Leloir glucosyltransferase of family GH17, a trans-membrane domain is predicted to form a membrane pore through which the newly synthesized glucan chain, product of GT2, is transported. The periplasmic GH17 enzyme domain then further modifies the nascent β-glucan leading to the formation of branched and cyclic OPGs. The specific features of polysaccharide synthesis suggest that alteration of polysaccharide structure can be achieved by region, gene, and even domain swapping in their synthetic pathways and individual glucosyl transferases.

Glucosyltransferases are key enzymes in the anabolic polysaccharide biosynthesis, and microbial genome sequencing gives unprecedented access to this type of enzymes. They can now be systematically identified and compared with various bioinformatic tools. Valuable targets can be rationally selected and by appropriate molecular techniques they can be inserted into target production organisms and their effect on the biosynthesis pathway analysed by various methods. Glucosyltransferases catalyse the transfer of a glycosyl group from a high-energy donor or oligosaccharide to an acceptor. The Leloir type of enzymes utilize nucleotide phosphosugars (NP-sugar-dependent) as donors producing a nucleotide and saccharide as reaction products and it has been shown that microbial lucosyltransferases are more versatile than their eukaryotic counterparts.

A number of Leloir multigene families have been identified and an important focus of the bioprospecting effort was analysing and selecting an array of glucosyltransferase genes for cloning into a platform polysaccharide producing thermophile for expression, and structural and functional studies of the resultant effects on source oligosaccharide. The source polysaccharide was analysed for structural alteration including changes in monosaccharide composition linkage types, repeat structure and the degree of branching. Relevant accessory enzymes was also defined and co-expressed with selected transferases if needed. Of these, enzymes for generation of activated sugars are most critical. Their requirement is dependent

on which glucosyl transferase will be selected for expression in the hosting system and the inherent capabilities of the host.

The platform species envisaged for polysaccharide production needed to fulfil certain criteria. It should (a) produce polysaccharides in high quantities, (b) be able to import a variety of sugar to be used as acceptors, (c) produce a great variety of activated sugars, at least many important ones, and (d) produce few and low amounts of side products. The ideal strain chosen for such polysaccharide production is the thermophilic marine bacterium *Rhodothermus marinus,* which served as the "model organism" in this project. Under certain conditions, it produces large quantities of EPS. The organism has a broad substrate range, degrading a large variety of polysaccharides and growing on their constituent uronic acids, hexoses and pentoses. *R. marinus* showed diverse metabolic activity and is easily cultivated. The genome has been fully sequenced and various genetic tools and selectable markers have been developed in previous projects of the Matís group.

R. marinus belongs to the phylum Bacteroidetes and it was first isolated from the coastal geothermal area in Iceland. It is an aerobic heterotroph that grows at temperatures of up to 77°C (Bjornsdottir *et al.* 2006). It has been subject of considerable research much of which has been devoted to its thermostable enzymes on account of their biotechnological potential, particularly polysaccharide degrading enzymes. Interestingly, several of these enzymes are secreted and exhibit optimum activities at 80-100°C, which far exceeds the optimum for growth. Examples are cellulose, xylan and mannan degrading enzymes, some of which have been studied in great detail (Dahlberg *et al.* 1993; Hreggvidsson *et al.* 1996; Nordberg *et al.* 1997; Gomes & Steiner 1998; Abou Hachem *et al.* 2000; Politz *et al.* 2000; Wicher *et al.* 2001; Crennell *et al.* 2002; Abou Hachem *et al.* 2003). The work by MATIS has focused on developing gene transfer and genetic selection for the genetic engineering of *R. marinus* (Ernstsson *et al.* 2003; Bjornsdottir *et al.* 2005; Bjornsdottir *et al.* 2007; Bjornsdottir *et al.* 2011). *R.marinus* was considered suitable for genetic studies because of its aerobic nature, competence growth in the defined media. Importantly, it exhibited reproducible growth on solid media, and clonal populations were easily obtained. Restriction negative host strain has been established and expression vectors and selectable markers have been developed. Selectable markers, initially, biochemical and genetic properties of the species were poorly known and mainly restricted to single characterized proteins and genes, none of which could serve as a selective marker. The preferred antibiotic selection for thermophiles was based on the thermos-adapted kanR determinant, which was unsuitable for *R. marinus* because of its natural resistance to aminoglycosides. In continuing work, two

selective markers were identified, *trpB* and *purA*, which encode proteins of the tryptophan and adenine biosynthetic pathways, respectively. A restriction deficient *R. marinus* isolate was chosen as a recipient for gene transfer experiments (Bjornsdottir, *et al.* 2011). The endogenous *trpB* and *purA* were deleted from the chromosome of the recipient, making it compatible with both Trp+ and Ade+ selection. Moreover, the deletions prevented both the development of spontaneous revertants and unintended marker integration. *Expression vectors,* a small, cryptic *R. marinus* plasmid, pRM21, of 2935 bps (Ernstsson *et al.* 2003) served as the starting point for constructing *R. marinus–Escherichia coli* shuttle vectors (Bjornsdottir *et al.* 2005). They contained the *R. marinus trpB* gene expressed from the promoter of the *R. marinus groESL* operon. These vectors served as basis for the construction of cloning vectors and allowed for the cloning and expression of foreign genes as well as induced expression in *R. marinus* following temperature shifts. Two reporter genes were also identified, allowing for the investigation of *R. marinus* promoter activities *in vivo* (Bjornsdottir, *et al.* 2007). Both random and site-directed inactivation of *R. marinus* genes has been implemented. Unmarked deletions were generated resulting in a double mutant with the genotype Δ*trpB*Δ*pyrA*. Here, the marker carried by the vector, outside homologous sequences, is lost through resolution of co-integrate. Subsequently, in-frame deletions using the *trpB* and the *purA* marker genes have been introduced. The selection efficiency of the strain was e.g. demonstrated by insertional mutagenesis of the carotenoid biosynthesis genes *crtBI*. The resulting Trp+, CrtBI- mutants were colourless rather than orange-red (Bjornsdottir, *et al.* 2011). Also, marked deletions were obtained by performing gene replacements with linear molecules, which yielded double-crossover recombinants in a single step (Bjornsdottir, *et al.* 2011). The existence of selective markers and expression vectors enable rational genetic manipulation of *Rhodothermus,* which can result in altered metabolic pathways and novel products.

The extensive recombinant techniques available for *R. marinus*, existing genome sequence data, as well as broad substrate range and saccharide conversion features makes *R. marinus* feasible for metabolic engineering and eventually a versatile platform organism for production of structurally modified polysaccharide derivatives. By using metabolic engineering approaches, *R.marinus* was streamlined for production of complex molecules by eliminating the formation of side products by increasing gene dosages of critical genes, eliminating and/or modifying regulation mechanisms. By hosting appropriate genes from other organisms synthetic pathways can modified and consequently structure and properties of a target molecule can be altered.

~~3.3.2~~4.3.2 Sustainable production of secondary metabolites at the industrial scale

In traditional biotechnology, all industrial manufacturing processes began (and begin) with plate cultivation, followed by scale transfer to liquid culture and further scale-up. (Chen *et al.* 2011; Voulgaris *et al.* 2011; Finn *et al.* 2010; Fazenda *et al.* 2010; El-Sabbagh *et al.* 2008; Li *et al.* 2008). These steps, for some marine isolates, can be problematic and can be associated with a loss or reduction in the synthesis of desired metabolites or formation of unwanted by-products (Pettit 2011). The immense biodiversity apparent in the marine environment is a potentially rich source of novel antibiotics, other secondary metabolites, and metabolic potential (Pettit 2011), but in order to fully exploit this potential we must take interesting activities often noted under lab conditions and transfer them to industrial scale production. However, biomanufacturing using marine microorganisms presents several unusual challenges distinct from those encountered when manufacturing bioproducts from conventional terrestrial microorganisms. In part, these reflect the origins of the isolates themselves (source microbes). They include the use of media containing salt at moderate to high levels (0.43M to 2.5%) (Durand *et al.* 1993; Nakagawa *et al.* 2005; Slobodkina *et al.* 2008), which can present corrosion and wear issues on seals and bearings of fermenters. The range of temperature optima for cultivation of marine microbes also presents challenges to the biotechnology industry, with interesting bioactivities noted in marine microbes with psychrophilic (4^{o} C) (Burgaud *et al.* 2009) to thermophilic optimal temperatures (85^{o} C) (Andrianasolo *et al.* 2009). Since the biotechnology industry basically uses processes and fermenters designed for organisms with temperature optima from around 25 to 40 ^{o}C (Matthews 2008), these unusual temperature requirements required significant re-design of plant in terms of heat removal and mass transfer (low O_2 solubility as temperature rises). Even when isolation of interesting fungal microbes from marine sources using agar brine plate cultures rather similar to the industrial workhorse *Aspergillus* is successful, this does not easily lead to new industrial products due to some of the barriers discussed above (Raghukumar *et al.* 2008). Other hurdles to rapid industrial exploitation include the use of unusual energy sources (H_2) (Nakagawa *et al.* 2005) which are unfamiliar to the mainstream fermentation industry, and dangerous, or unusual substrates or toxic by-products (e.g.H_2S (Slobodkina *et al.* 2008)) also unfamiliar to the bioprocess industries and with significant safety implications. Such isolates usually are exposed to low levels of dissolved oxygen due to the sparing solubility of oxygen in seawaters, whereas the modern fermentation industry is geared up to deliver products largely from mesophilic cultures in highly aerated and agitated fermenters (Matthews 2008). On occasions, the early treatment or storage of natural isolates

leads to loss or reduction in metabolite synthesis on scale up. The perception of strain instability is a critical barrier. Despite the above, there is no fundamental reason why marine isolates should be inherently less stable than terrestrial, and even those from extreme environments have been shown to be amenable in some cases to cultivation under non-extreme conditions (Pettit 2011). Overall, though these hurdles and bottlenecks contribute to a less than certain and lengthier path to market for marine products when compared to terrestrial derived products arising from a narrower ecological range, and may well inhibit any further exploitation of an activity. The challenge is to match huge biodiversity in growth characteristics with a bioprocessing industry, which is largely based upon a very limited range of optimised process to effectively and efficiently scale up interesting activities from bench scale to industry volumes. One approach to this is simply to move novel activities from less tractable marine isolates to industrial workhorse organisms, which the bioprocessing industry is familiar with and accustomed to scaling up. Pathway and metabolic engineering is widely used in the biotechnology industry (Andersen & Nielsen 2009), and this may well overcome some of the challenges noted. Further, the path to industrial production for both source microbe-derived and novel construct-derived products can be made more certain and faster, by applying a combination of best industrial manufacturing practice for new fermentation products, together with novel in process real-time monitoring and multivariate analysis techniques (Chen *et al.* 2011, Roychoudury *et al.* 2007). These techniques would enhance the flow of process data in early development phase and put the physiology of these marine microbes and constructs on a sounder basis, hence ensuring the acceleration of industry exploitation (Chen *et al.* 2011, Roychoudury *et al.* 2007), faster delivery of marine products to markets, and safer and more predictable scale up.

~~3.4~~4.4 Challenge 4: Legal aspects relating to access to marine bioresources

Bioprospecting can be defined as commercially focused research and development that uses naturally occurring compounds. It includes steps from first discovery, through patenting, improvement, development and commercialisation. A simple breakdown of bioprospecting is: Phase 1: on-site collection of samples; Phase 2: isolation, characterization and culture of specific compounds; Phase 3: screening for potential uses, such as pharmaceutical or other uses; and Phase 4: product development and commercialization, including patenting, trials, sales and marketing (Leary, 2004). Bioprospecting using a country's genetic resources is covered by the United Nations Convention on Biodiversity (the CBD) (Harvey and Gericke

2011). This will extend to a coastal country's Exclusive Economic Zone (EEZ) and its Continental Shelf, as defined by the United Nations Convention on the Law of the Sea (UNCLOS – article 56(1) and article 77(1)). However, there is no international treaty that regulates bioprospecting in the water column above the continental shelf or in areas beyond national jurisdiction ('the deep sea', UNCLOS article 87(1)). Instead, each state is required to regulate the activities of its nationals in those areas, particularly with concern to avoid environmental damage. Aspects of the regulatory framework may distinguish between bioprospecting (as defined above) and the undertaking of scientific research without commercial motive (Leary, 2004). To summarise the present legal position in relation to marine bioprospecting: · Coastal States have the sovereign right to allow, prohibit, and regulate marine bioprospecting and/or scientific research in the water column of their EEZ, and on the seabed (including the subsoil) until the farther of either the limits of their EEZ or the outer edge of their continental shelf. · State regulation is subject to a number of international obligations incumbent upon coastal States, including in relation to the protection and preservation of the environment and to the conservation and sustainable use of marine genetic resources. Significantly, such regulation may also be impacted by access and benefit-sharing mechanisms established pursuant to the CBD;

· All States enjoy free access to marine genetic resources located seaward of other States' EEZs and continental shelf. They have jurisdiction to allow, prohibit, and regulate marine research and bioprospecting activities conducted by their nationals and/or vessels flying their flags; free access is subject to a number of international obligations incumbent upon coastal States, including in relation to the protection and preservation of the environment and to the conservation and sustainable use of marine genetic resources. Significantly, such free access is also subject to the duty of States to cooperate for the conservation of marine genetic resources. The mechanisms of benefit sharing and the related legal aspects of research on marine bioresources is a very important aspect of this project in collaboration with other marine biotechnology programmes that includes PharmaSea and MicroB3. Many marine ecosytems are still little studied, but their vast and novel biodiversity offers many possibilities for the discovery and development of novel industrial products. In spite of considerable previous work, particularly the CBD, many aspects remain unresolved. The discussion of equitable benefit sharing among interested parties often gets stuck because it tends to focus on percentages of a future income from possible blockbuster products. Another equally important aspect is to evaluate and discuss the mechanisms that can be used for more short term, more secure, and non-monetary ways of benefit sharing from bioprospecting activities,

as is highlighted in the Nagoya Protocol to the CBD. These are particularly important and relevant when it comes to sampling and research on novel ecosystems and unusual natural phenomena, particularly in the world oceans since they are still more underexplored than on land. SeaBioTech addressed the legal aspects in a concise way doing a direct evaluation of the legal and access issues connected to sampling in the project itself. Another task was to find and study some key cases of this sort that have come up recently, in particular in relation to novel marine ecosytems. Two such examples are the smectite geothermal cones north of Iceland and the Tufa columns in Greenland. SeaBioTech worked with other marine biotechnologically oriented projects to assist in the interpretation and application of best practice and conforming to current national, European and international legislations as well as the most recent Nagoya Protocol.

In addition to the close liaison maintained with the other KBBE Bioprospecting projects, SAMS, acted as a link between SeaBioTech and the ESFRI road map Research Infrastructures (RIs): EMBRC and MIRRI (MIcrobial Resource Research Infrastructure).This has involved relevant CBD related input to the development of the H2020 EMBRIC project. SAMS has also been responsible for providing advice to the government of the Republic of the Seychelles on building a Blue economy, including the need for managing access to MGR.

~~3.5~~4.5 Challenge 5: Improving access to marine biotechnology data through an EU platform

As highlighted in the recent position paper 'Marine Biotechnology: a New Vision and Strategy for Europe' (European Science Foundation, September 2010), there is a need for a "central European information portal, which provides a one-stop-shop for state-of-the-art reports on novel discoveries and success stories, challenges and applications". Currently, there are few sources of comprehensive information relevant to marine biotechnology. The Coordination and Support Action project under FP7, Marine 4Genomics Users created a "single entry-point to marine genomics knowledge". However, this did not encompass information relating marine samples to bioactivity test results, comparable to the US's NIH Roadmap initiative with results being openly available in the PubChem BioAssay database. For general information on marine biodiversity, there is the National Ocean Service, which is run by the US government agency, the National Oceanic and Atmospheric Administration, and there is MarineBio in the USA, which is a non-profit organisation that tries to provide a broad range of information relating to marine conservation and science. However, neither

covers details of species in particular environments or bioprospecting information. For extremophiles, there is a developing resource hosted by the Indian organisation, the Institute of Genomics and Integrative Biology, although this is not focused on marine species and does not cover bioprospecting. As described earlier, under challenge 1 (quality of marine resources), there is also very limited access to physical samples from marine environments. Hence, SeaBioTech developed and established both an information portal and a physical repository of samples for further genetic analysis and for use in additional bioactivity testing. SeaBioTech activity complemented other EU-funded projects such as FP5 MarGenes, FP6 Diatomics, FP6 Marine Genomics Network of Excellence, FP7 Micro B3 (Biodiversity, Bioinformatics, Biotechnology), and FP7 MAREX. It also linked to other projects funded under the present call. In that way, SeaBioTech provides a major contribution in achieving another recommendation of the ESF's position paper on marine biotechnology towards the creation of a virtual European Marine Biotechnology Institute.

SeaBioTech has provided input to the PharmaSea case studies: Role of biorepositories and impact of proposed EU regulation on ABS; the European blue biotech community's preparedness and response to the implementation of the Nagoya Protocol.

5 Conclusion: Impacts and Future Insights

In this section, we summarise the project's achievements to answer the challenges set by the consortium. The achieved milestones along with the encountered confrontations and some strategies used to yield to the challenges set by the SeaBioTech consortium are presented on Table 1.

~~3.6~~5.1 A reproducible quality of marine resources

Addressing the first challenge on quality of marine resources collected during the project's lifetime, the consortium was given the opportunity to investigate some of the unique environments/habitats on earth, isolate/characterize microbial species living there and create large strain collections for biotechnological exploitation. Some of the isolated strains were characterized by high novelty and biotechnological potential as they showed very low similarity with any other previously characterized bacteria. New knowledge was gained about gene diversity in extreme environments, as well as valuable information about environmental microbial functioning through the application of modern metagenomic deep-sequencing techniques. Genomic sequence data by UWUERZ has revealed the presence of a large fraction of putatively silent biosynthetic gene clusters in the genomes of actinomycetes that

encode for secondary metabolites that remain silent under standard fermentation conditions. Our work has provided here novel insights into actinomycete biodiversity as well as into the effects and consequences of elicitation of secondary metabolism in actinomycetes. Huge metagenomics datasets were created and used as a source for bioprospecting. WP2 served as the foundation of SeaBioTech discovery pipeline. By focusing on previously unexplored environments, WP2 attempted to increase the odds of discovering novel bacterial species that would contain novel bioactive compounds of potential economic interest. Indeed, WP2 supplied the other work packages with novel cultivable strains holding a great potential for the discovery of novel natural products of high-added value. In addition, through Seabiotech sampling campaigns knowledge on the activity of the extreme environments of the Hellenic Volcanic Arc was exploited demonstrating the need of a monitoring program for this dangerous environment (Rizzo *et al.*, 2016).

~~3.7~~5.2 An improved and integrated technology for drug discovery

For the improvement in technical aspects, SeaBioTech integrated metabolomics-assisted methodology with systems biology and functional bioassays increasing the ability to divulge positive hits that proved to be affordable, innovative and efficient method (MacIntyre et al 2014) to separate, elucidate the structure, and identify the bioactive metabolites. Novel and underexplored species of marine microorganisms were investigated for the first time as potential sources of novel therapeutics and they provide positive indications that lead compounds can be isolated and progressed to address significant unmet medical needs (e.g., cancer, infections against, metabolic syndrome and inflammation) and threatening parasitic infections for aquaculture. WP3 partners in charge of the screening activities improved the performance and throughput of the assays, to comply with the requirement to process a remarkably high number of extracts, fractions and compounds of marine origin. Major improvements were obtained for the development of automated, high-throughput screening platform to provide cell-based assays for the detection of hits with anti-cancer activities, in particular for cell proliferation (HDL). Moreover, assay systems were modified to achieve a suitable robustness to screen complex marine extracts and subsequently to produce more accurate and reliable results (SIPBS, AXXAM).

The personalised medicine market worldwide is estimated to be over €400 billion and the core diagnostic and therapeutic segment of the market is estimated at over €40 billion. The need to address this market and the benefit of doing so is supported by many facts, including a 75% increase in personalised medicine investment over the last 5 years and 30%

of all pharma companies now require compounds in R&D to have patient-relevant treatments. The potential novel marine products identified through the SeaBioTech consortium may enable such therapeutics to progress through the R&D process. In particular, prospective lead compounds have been isolated with a potential to address therapeutic indications for human health such as cancer, bacterial infections and metabolic syndrome, and to develop an effective treatment against the fish parasite *L. salmonis*, which represent a major threat for aquaculture. In addition, the knowledge gained through SeaBioTech concerning assay development and screening of complex marine extracts may directly or indirectly translate into new opportunities for the CROs to expand their potential market and for pharmaceutical and life science companies to undertake novel R&D projects. In addition, the phenotypic assay performed on the fish parasite of aquaculture plants *Lepeophtheirus salmonis* was also optimized to increase its capacity and processivity, thereby expanding the possibility to screen extracts and fractions of marine source (PHARMAQ). The lead compounds isolated at the end of the SeaBioTech collaboration have the potential to be evolved into novel therapeutics. The availability of novel therapeutics for human health and aquaculture will directly contribute towards improving quality of life, health, employment and economic strength.

Automated dereplication and chemical profiling aid screening for diversity and novelty were established in WP5. Marine invertebrate-associated symbiotic bacteria produce a plethora of novel secondary metabolites, which may be structurally unique with interesting pharmacological properties. Selection of strains usually relies on literature searching, genetic screening and bioactivity results, often without considering the chemical novelty and abundance of secondary metabolites being produced by the microorganism until the time-consuming bioassay-guided isolation stages. The development of a comprehensive metabolomics workflow pathway including an in-house developed Excel macro embedded with a database made it possible to rapidly dereplicate higher number of strains, providing putative identities of known metabolites in each extract. It is also shown that the dereplication results can also be correlated with bioassay screening results to support drug discovery efforts with the objective of both finding a bacterial isolate that has a unique diverse chemistry and is biologically active. Overall, this shows that metabolomics approaches are worthwhile for the selection of strains for the isolation of novel natural products and that this methodology reduces redundancy in drug discovery programs. Additionally, we have shown through PCA and heat map analysis that strains with nearly identical 16S rRNA sequences do not necessarily produce the same secondary metabolites.

Metabolomic-assisted isolation of target compounds efficiently improved the purification of the bioactive secondary metabolites. Multivariaye analysis that included Principal Component (PCA), hierarchical clustering (HCA), and orthogonal partial least square-discriminant analysis (OPLS-DA) were used to evaluate the HRFTMS and NMR data of crude extracts from different fermentation approaches. Statistical analysis identified the best culture one-strain-many-compounds (OSMAC) condition and extraction procedure, which was used for the isolation of novel bioactive metabolites. As a result, new natural products can be isolated from cultivated broth cultures (described under section 4.2.2). New natural products with novel mechanisms of actions were isolated. Biologically active compounds were isolated and purified from prioritized strains. SBT345 (*Streptomyc*es sp.) showed anti-oxidant, anti-cancer cell lines (DLD-1, HCT116) activities, and some activities in the enzymatic reactions. Compounds SBT1620 (phencomycin), SBT1621 (tubermycin B), SBT1186 (benzethonium), and SBT1187 (ageloline A, new compound) were isolated from SBT345. SBT1877 showed anti-oxidant and anti-*Chlamydia trachomatis* activities. SBT017 (*Rhodococcus* sp.) yielded 16 pure compounds after scale-up, one of which was elucidated as isohalobacillin B. SBT0027 (*Vibrio splendidus*) yielded 27 pure compounds, 7 of which are bis-indole analogues with strong to medium potency against *Mycobacterium marinum*. Three analogues are new. Other pure compounds from SBT0027 consisted of diketopiperazines, long chain amines, and hydroxylated fatty acids, the activities of which still need to be determined. SBT167 (*Polysiphonia lanosa*), an algal macro-epiphyte yielded the di-bromo-dihydroxylated-benzaldehyde as its major component. SBT167 was found to be active against parasitic sea lice and in several enzymatic assays against metabolic diseases. From the Icelandic collection, new BHA congeners bioactive against metabolic diseases were isolated.

~~3.8~~5.3 A sustainable production of raw materials

The last technical brick for the industry is the sustainability of the production of raw materials not only at lab scale but also at industrial scale. The program has developed standard operating protocols for the growth and exploitation of resources from both natural isolates and construct microorganisms, developed by identifying, isolating and genes of interest from marine species and inserting them into organisms which are regarded as industry work horses e.g. *Escherichia coli.* Scale up predictions for processes developed in WP10 were formulated by the fermentation group in SIPBS. Accelerated process development has been achieved either by utilizing powerful gene technologies to create construct organisms or by utilizing bioprocessing techniques with metabolomics with source

microorganisms to identify bottlenecks in the relevant catabolic pathways. Both of these techniques resulted in successful bioprocess intensification of the relevant target compounds or enzymes. Industrial partners identified appropriate target compounds, which allowed us to selectively mine the gene pool of the marine organisms for useful enzymes. Suitable protocols were then generated for the bioprocess and put together in a Process Manual.

Combining the novel gene technologies, metabolomics and ability to rapidly scale processes, using clearly defined standard operating procedures, is the unique aspect of the programme. This is of particular interest to industrial partners and significantly benefits both the companies involved in SeaBiotech and the scientific community in general. Many of the techniques can now be regarded as generic and could be exploited elsewhere on other projects and processes. Genes from source organisms, which express novel enzymes, have been successfully inserted into industry workhorse organisms and have been successfully scaled up. Such enzymes have novel capabilities and are successfully utilised by some industry partners. In particular generating new construct microorganisms has allowed the exploitation of enzymes, e.g. alginate lyases and thioesterases to name but two, capable of utilising different kinds of feedstocks and which allow processes which previously suffered from bottlenecks to work effectively and efficiently. This is a significant scientifc breakthrough as the potential for industry is great. A novel polymer was isolated from *Colwellia* sp. The organism has been successfully grown at scale in WP7 and a spin off project has developed between SAMS and Unilever. New bioactive compounds have been identified (WP3) and tested at scale in WP7. Initial trials have shown the organisms from which the bioactives are isolated can be grown at scale but research to improve the productivity of the bioactives continues.

The generation of new enzymes and polysaccharides will have considerable influence on the economies of the consortium partner companies and on the economy of the EU and also on global markets. The enzymes in particular have significant industrial capability and applications will be numerous. The ability to use new substrates , previously un-useable either because it was not scientifically possible or because process economics were not favourable, will have significant impact on increased process efficiency, improved supply chains (substrate choice increases) and reduction in upstream costs . As seen above impact will not just be industrial as IGZ see significant potential in the health care market where opportunities in drug discovery from marine derived biocatalysts are highly relevant to the

biosynthesis of compounds for the treatment of disease. The market share for companies who use SeaBiotech derived enzymes and compounds could expand rapidly.

~~3.9~~5.4 A harmonized legal position on marine bioprospecting

SeaBioTech liaised closely with, and contributed to, common areas of activity dealing with legal/ethical aspects being undertaken in the parallel EU funded projects: MICROB3, BlueGenics and PharmaSea. An overarching group of experts was formed, i.e. the Advisory panel of policy and legal experts (APPLE). APPLE, an advisory board brought together the breadth of experience, legal, scientific and commercial, necessary to address the critical policy and legal barriers which currently hinder progress in innovative marine biotechnology in Europe. The projects have worked together on these aspects to avoid duplication of effort and enable a wider-reaching and more global approach of benefit to these consortia and beyond. During the lifetime of the project the legal implications to bioprospecting have changed status with the implementation of the Nagoya protocol, which became legally binding from the 12th of October 2014. An overarching, generic Material Transfer Agreement (MTA), conforming to the requirements of the Nagoya Protocol has been developed by Microbio3. This has, with minor adjustments, been applied across the projects. SeaBioTech contributed to the development, structure and content of the PharmaSea Deliverable on development of web-based, interactive, toolkit to assist Marine Genetic Resource (MGR) practitioners in navigating the different legal and policy regimes involved in access to MGR and associated benefit sharing. This area has rapidly developed and on-line resources associated with the CBD Clearing House are available to users/ potential users of biological resources. Work undertaken by APPLE, particularly the PharmaSea legal team, has resulted in considerable progress with respect to the developing of possible solutions to the implications of the collection of materials in areas beyond the Economic Exclusive Zone (EEZ) i.e. in Areas Beyond National Jurisdiction (ABNJ). These were presented at the UN HQ, New York on16-20th June 2014 for consideration for possible future proposed changes to the UN Common Law of the Sea (UNCLOS).

~~3.10~~5.5 A c~~C~~entralised biobank repository and database of information

SeaBiotech created a centralized tool to organise the marine biodiscovery pipeline through a biobank repository and database of information for marine strains which included names of the identified marine organisms, compounds and extracts, their bioactivities, the cutting-edge methods in identification, elucidation, metabolic engineering to be further used for industrial purposes with all related procedures on legal process for companies, academia, and legal

authorities. The assembly of a centralized repository of marine extract and compounds of marine origin was amongst the major legacy of Seabiotech. The centralized repository contains at the end of the project 3209 samples of marine origin, including 1140 crude samples and 606 fractions plated in ready-to-screen format and 63 pure compounds. In addition, the repository contains samples which were received in a too small amount for general screening. Thus, they were stored and annotated in case further sample is obtained to ensure sufficient material is available for assaying. The annotation of samples, fractions and pure compounds stored in the centralized repository was managed through a database implemented by SIPBS and accessible in a secure manner through the SeaBioTech Portal to all partners involved in sampling, screening and dereplication activities (http://spider.science.strath.ac.uk/seabiotech/index.php). The SeaBioTech database sample submission portal ensures tracking of samples and transfer of data between partners ensuring CPD compliance. The detailed mechanisms to ensure access to the biological resources, and their associated data, beyond the lifetime of the project will agreed and implemented over the next 6-10 months. Each sample was assigned a unique SeaBioTech code and all information associated to each sample related to parental microorganism, genomics, LCMS, NMR data, bioactivity results and pharmacological profiling generated during the SeaBioTech collaboration was entered into the database. In addition, each sample was connected to its relevant negative control sample (e.g., culture media) that enabled validation and correct analysis of potentially active entities during bioactivity screening. The database played an essential role on the prioritization of samples, fractions and compounds for the SeaBioTech pipeline and represented a valuable asset for the prospective exploitation of the results obtained by SeaBioTech. The repository of extracts, fractions and pure compounds derived from underexplored marine microorganisms and the related information managed by the centralized database represents a valuable infrastructure for future R&D projects in diverse life science areas.

Table 1. Achieved milestones along with the encountered confrontations and some strategies used to yield to the challenges set by the SeaBioTech consortium.

Milestones achieved to support project challenges	WP	Encountered confrontations and some strategies used to yield to the challenge
1) A reproducible quality of marine resources		
Forty bacterial extremophiles were prioritised from a collection of *ca.* 3000 strains	WP2, WP5	Prioritised isolates were recollected at the same seasonal period of the initial collection for replication purposes for the repository.
Five best positive hits were identified during primary screening	WP3	Variation of chemical composition was encountered due to subtle changes in the laboratory conditions, which was monitored by metabolomics profiling.
2) An improved and integrated technology for drug discovery		
Availability of SOP for fraction dereplication, metabolomic profiling, and purification	WP5	Metabolomic and bioactivity profile preceded isolation work on prioritised extracts for the pipeline.
Construction of insertion modules and expression plasmids finished	WP4	Alternative expression systems or other systems for refolding of the proteins were used.
Small to medium scale cultivation optimised	WP5, WP6	Culture of each organism was cultivated under a variety of conditions that is metabolomic-guided to ensure replication of the original chemical profile or improvement in the concentration of the active constituents.
Biologically active compounds isolated and identified	WP5	If the selected targets were not affordable in the project time frame, suitable alternatives were selected as biology-driven construction of simpler assay models.
3) A sustainable mode of supply of raw materials for the industries		
Industrial scale cultivation optimised.	WP7, WP10	Mitigation of risk by metabolomics analysis and re-prioritisation of strains i.e. selection of alternative lead strains.
Carbohydrate structure data from mutants	WP4, WP6	Targeted gene transfer ensured close link between genetic changes to strains and subsequent polymer structure and function
4) A harmonized legal position on marine bioprospecting		
Legal aspects harmonised	WP8	The availability of additional academic expertise was enlisted
Central EU platform	WP1	A common board with Bluegenics, PharmaSea, Macumba, and SeaBioTech was set-up.
5) A centralised biobank repository and database of information		
Establishing a metabolomics and metagenomics database. The repository contains 3209 strains, 1140 crude samples and 606 fractions plated in ready-to-screen format and 63 pure compounds.	WP2 WP5, WP4	Genomic/metagenomics mining is iterative in nature: further rounds of sequencing generated leads was supported by metabolomic and bioactivity profile

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