

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

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19 Abstract: SeaBioTech is an EU-FP7 project designed and driven by SMEs to create  
20 innovative marine biodiscovery pipelines as a means to convert the potential of marine  
21 biotechnology into novel industrial products for the pharmaceutical, cosmetic, aquaculture,  
22 functional food and industrial chemistry sectors. To achieve its goals, SeaBioTech brings  
23 together leading experts in biology, genomics, natural product chemistry, bioactivity testing,  
24 industrial bioprocessing, legal aspects, market analysis and knowledge-exchange.

25 SeaBioTech targets novel marine endosymbiotic bacteria from unique and previously  
26 untapped habitats, including geothermal intertidal biotopes in Iceland, hydrothermal vent  
27 fields and deep sea oligotrophic basins of the Eastern Mediterranean Sea, and [under-explored](#)  
28 areas of Scottish coasts that are likely to be highly productive sources of new bioactive  
29 compounds. This chapter describes the four- years of activity in the SeaBioTech project,  
30 which resulted in a robust, validated workflow suitable for evaluating unexplored activities in  
31 marine samples to prioritise potential products for a biotechnological pipeline. An improved  
32 integrated methodology involving metagenomics and metabolomics were extensively utilised  
33 to prioritise five extremophiles as potential antibiotics, anti-cancer drugs and as novel drugs  
34 against metabolic diseases as well as new pharmaceutical excipients to the pipeline. A

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

5

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## 8 **1 Introduction**

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

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

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

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

### 8 **1.1 SeaBioTech has put together a marine biodiscovery pipeline using** 9 **an integrated approach.**

10 The project's innovation plan corresponded to the following scientific, technical and  
11 technological challenges [as shown in Figure 1](#):

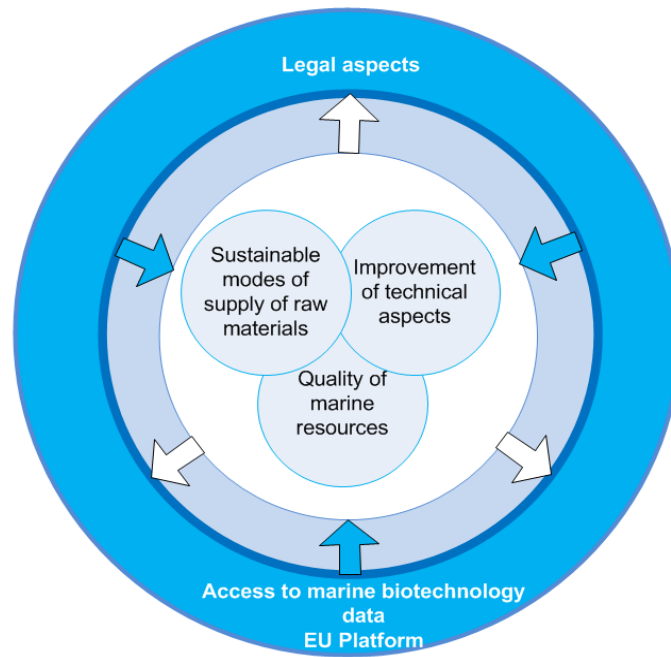
12 *Challenge 1:* The quality of marine resources: the approach to resource quality begun  
13 by standardizing the sampling process from unique and previously untapped habitats, which  
14 included geothermal intertidal biotopes in Iceland, hydrothermal vent fields and deep sea  
15 oligotrophic basins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

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

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

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

4



5

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

13

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

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

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

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

## 8 **1.2 SeaBioTech is an industry-driven project.**

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

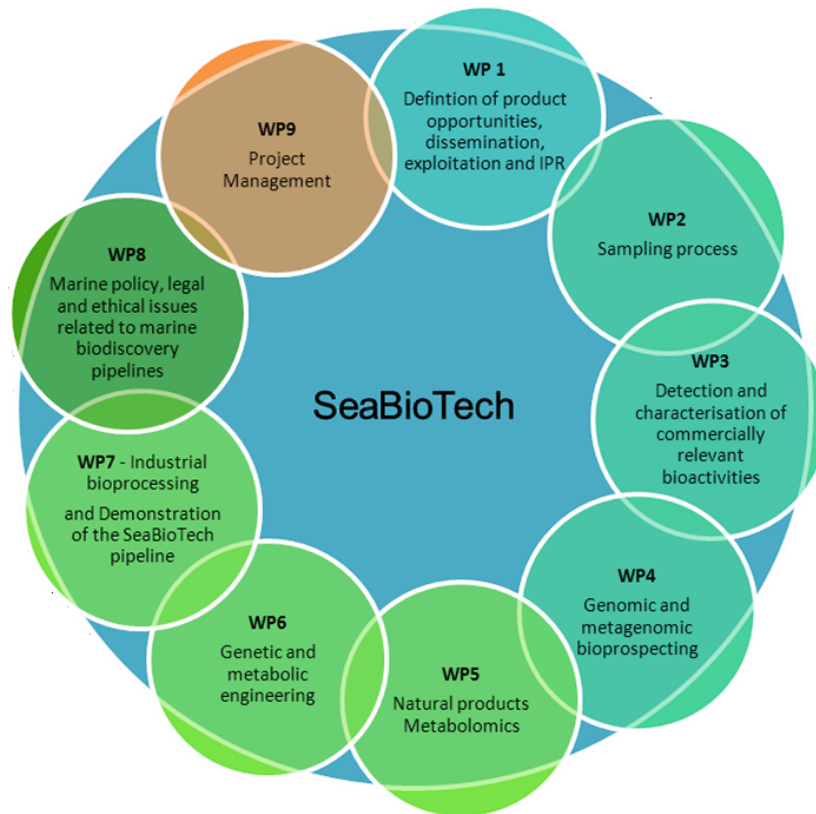
- 13 ❖ provide a pipeline of commercially viable products based on relevant  
14 bioactivity screening of samples of marine origin;
- 15 ❖ develop efficient standardized processes and methods across the biodiscovery  
16 pipeline;
- 17 ❖ introduce industrial bioprocessing methods suitable for commercial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

## 24 **1.3 Identification of industry needs: providing an industry-driven 25 project**

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

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

## 1 2 Methodology and Overall strategy



**Figure 2.** The SeaBioTech Work Packages

2

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



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



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

### 7 **2.3 The SME partners and their activities**

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

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

### 3 **2.13.1 Prokazyme (PKZ)**

4 PKZ has been engaged in commercialising enzymes that have advantageous  
5 biochemical properties over competing products. In this project, their work was focused on  
6 developing enzymes from extremophiles that were recognised as being potentially valuable in  
7 many applications. However, very few novel enzymes from the hundreds reported in the  
8 literature reach the market. Generally, the limitations in this area are difficulties in obtaining  
9 large enough supplies in a sustainable way and challenges in producing the enzyme to a high  
10 standard of purity in an economically attractive manner. PKZ saw opportunities in  
11 bioprospecting the unique genomic resources it had access to through the SeaBioTech gene  
12 banks and in the enormous, untapped marine biodiversity sampled through SeaBioTech. [The](#)  
13 [SeaBioTech project offered significant progress above the state of the art on new marine](#)  
14 [compounds \(particularly oligosaccharides\), as well as enzymes, increasing](#) PKZ existing  
15 offerings of specialized extremophilic enzymes for the R&D [market](#). PKZ has made strategic  
16 plans for future commercial production of enzymes on [an](#) economical large scale. As part of  
17 this future strategy, it is the intention that the production of enzymes shall be transferred from  
18 PKZ to a subsidiary company. PKZ and MATIS have initiated a large research proposal with  
19 a consortium consisting of 15 partners in Europe. The research proposal, “Virus-X: Viral  
20 metagenomes for Innovation Value”, has secured a EUR 8 million funding from the  
21 European Union under the Horizon2020 framework. PKZ will coordinate the project and  
22 within the project extend its collaboration with specific partners from the SeaBioTech  
23 project. A grant agreement was made during this period with the European Union for the  
24 funding and the project started on April 1st 2016 and will be continued until March 30th  
25 2020.

### 26 **2.23.2 PHARMAQ**

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

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

### 8 **2.33.3 Marine Biopolymers Ltd (MBL)**

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

### 27 **2.43.4 Ingenza (IGZ)**

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

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

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

### 17 2.53.5 **Horizon Discovery Ltd (HDL)**

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

### 30 2.63.6 **AXXAM**

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

1 functional assays that detect activities relevant to key diseases (infections, inflammatory  
2 diseases, chronic pain, etc.). AXXAM supported the hit discovery programmes of  
3 SeaBioTech by performing in total 11 screening campaigns on a comprehensive number of  
4 927 crude samples of marine origin on an array of cell-based and enzymatic assays, which  
5 was refined based on the obtained results to seven assays (TRPA1, TRPM8, TRPV1, PPAR $\alpha$ ,  
6 EL, HDAC6, HDAC2) suitable for high-throughput screening of complex extracts. These  
7 functional assays were developed to measure the activity of validated targets in three main  
8 disease indications: cancer (HDAC6 and HDAC2), metabolic syndrome (EL, PPAR $\alpha$ ) and  
9 pain (TRPA1, TRPM8, TRPV1). At the end of the primary screening activity, 287 crude  
10 extracts were confirmed as primary hits, distributed as follows: TRPA1 (12), TRPM8 (37),  
11 PPAR $\alpha$  (36), HDAC6 (81), HDAC2 (3), EL (118). In collaboration with WP2-WP5, 31 crude  
12 extracts derived from 17 marine microorganisms were prioritized and included in the  
13 SeaBioTech pipeline. A subset of 15 crude extracts was fractionated by WP5 and 629  
14 fractions were subjected to screening against the primary assays TRPM8, TRPA1, PPAR $\alpha$ ,  
15 HDAC6 and EL, respectively. The support to dereplication activities led to the identification  
16 of 148 fractions containing the sought bioactivity against the following primary targets:  
17 TRPA1 (9), TRPM8 (5), PPAR $\alpha$  (5), HDAC6 (76), EL (53). Remarkably, one series of 27  
18 fractions derived from the crude extract SBT0541 (*Algoriphagus marincola*) was confirmed  
19 to contain negative modulators of the catalytic activity of Endothelial Lipase (EL). Among  
20 them, 8 fractions contained pure compounds which were identified by WP5, which allowed  
21 the definition of a preliminary structure-activity relationship. This finding appeared  
22 consistent with the targeted enzyme Endothelial Lipase (EL), which physiologically releases  
23 fatty acids from phospholipids in HDL particles. The compounds displayed a dose-dependent  
24 inhibition on EL, with partial inhibition at the highest compound concentrations tested. The  
25 negative modulation of the EL activity identified by AXXAM has never been reported in  
26 literature.

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

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

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

### 13 **3.4 Addressing the Challenges through Scientific Breakthroughs**

#### 14 **3.4.1 Challenge 1: Access, sampling, storage and quality maintenance** 15 **of marine resources present in extreme environments and sponge** 16 **symbionts**

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



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

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

### 21 **3.1.14.1.1 Geothermal intertidal biotopes in Iceland**

22 Intertidal biotopes harbour a large diversity of ecologically and biotechnologically interesting  
23 organisms. This is a highly dynamic environment subject to constant periodic disturbances  
24 with steep gradients of temperature, mineral composition and salinity. The organisms need to  
25 tolerate periods of dryness and even exposure to harsh UV radiation during low tide.

26 Temperature gradients are manifested most clearly in the contrast between the hot fluid in  
27 geothermal coastal hot areas and the cold seawater, and the hot spring water may have high  
28 levels of sulphur compounds and toxic metals. These habitats have rich invertebrate fauna  
29 and often covered by a profusion of algal vegetation containing various complex recalcitrant  
30 polysaccharides that may be utilized by a variety of microbes, factors influencing the  
31 microbial diversity. Photosynthetic microbial mats are abundant in these areas and many hot  
32 springs may have both chemo-litho-autotrophic and photosynthetic organisms as primary  
33 producers, adapted microbes to unique conditions. Rare species in these areas include various



1 obligate heterotrophs, but their presence may be masked by the dominant primary producers  
2 and therefore they are not easily studied or accessible for biotechnological exploitation. The  
3 unique geothermal environments on the coasts of Iceland sustained a relatively high diversity  
4 of microorganisms and unique organisms not previously exploited as a resource for bioactive  
5 microbial metabolites or enzymes of industrial interest. Past studies revealed a great number  
6 of novel organisms indicating that geothermal habitats harbour an enormous diversity still to  
7 be isolated, characterized and exploited (Staley & Konopka 1985; Roszak 1987; Amann  
8 1995; Skirmisdottir 2000). Within the SeaBioTech project, a total of 49 samples were  
9 collected from coastal geothermal sites in Iceland, primarily from photosynthetic microbial  
10 mats and also from polysaccharide enrichments *in situ* and a total of 194 strains were  
11 isolated: 122 from Laugarvík, 47 from Yngingarlindir and 25 from Reykhólar. Numerous  
12 strains representing novel species and genera were isolated, especially from Yngingarlindir.  
13 Alginate degrading anaerobic isolates from Reykhólar were close to the genus *Clostridium*  
14 and five of them were selected for whole genome sequencing and genome annotation  
15 analyses in WP4. A preliminary study of the species composition of Cyanobacteria from the  
16 clone sequences from the YL samples was performed and the largest taxon contained several  
17 species representing distant (88-95% 16S rDNA similarity) relatives of *Geitlerinema* sp.  
18 within the Oscillatoriales. A similar study on the composition of Cyanobacteria in four of the  
19 Laugarvík biomat samples revealed the majority of sequences belonged to a filamentous  
20 *Leptolyngbya* sp highly related to a *Leptolyngbya* sp. found in arctic hot springs in  
21 Greenland. Results from culture independent biodiversity studies in Yngingarlindir and  
22 Laugarvík indicated novel species of Cyanobacteria. Seven Cyanobacteria strains were (M24-  
23 M36) isolated from mat samples and identified. Strains of interest (32) were selected for  
24 extractions in WP3. The extracts (62) and relevant control samples (6) were labelled and sent  
25 to the relevant partners for bioactivity screening. Based on novelty, 39 strains were selected  
26 for whole genome sequencing and annotations in WP4 & WP6. From the total of 39 strains,  
27 38 strains were sequenced and their genomes annotated.

### 28 **3.1.24.1.2 Deep sea oligotrophic basins and hydrothermal vent fields in the Eastern** 29 **Mediterranean Sea**

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

1 Mediterranean Ridge and deep basins as well as volcanism in the Hellenic Volcanic Arc.  
2 Major hydrothermal systems are found along the Hellenic Volcanic Arc at Methana, Milos,  
3 Santorini and Nisiros islands (Dando *et al.* 2000). Venting gases in these areas contain  
4 substantial amounts of CO<sub>2</sub>, H<sub>2</sub>, and H<sub>2</sub>S, thus providing the chemical environment for  
5 chemolithoautotrophic primary production (Dando *et al.*, 1995). Steep chemical and  
6 temperature gradients (Wenzhöfer *et al.*, 2000) create diverse niches for numerous microbial  
7 populations. Initial screening studies of microbial diversity indicated a high spatiotemporal  
8 variation in microbial community structure (Sievert *et al.*, 1999) combined with highly  
9 diverse bacterial communities, with less than 33% of 16S rDNA sequences being related at a  
10 90%, or higher, level to cultivated organisms (Sievert *et al.*, 2000).

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

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

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

### 11 **3.1.34.1.3 Coastal sites in Scotland with extreme conditions**

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

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

#### 3.1.44.1.4 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*, *Microtholunatus*, *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.

#### 3.1.54.1.5 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.

### 3.1.64.1.6 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*

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

14 Another strategy used by SeaBiotech to tap into the environmental DNA pool is by  
15 “sequence-driven metagenomics”. This approach has been undertaken by Venter and  
16 colleagues to yield a global genomic inventory of the oceans (Rusch *et al.* 2007). Other  
17 studies have employed sequence-driven metagenomics for example, to characterize the  
18 genomic repertoire of the microbial consortia of marine sponges (Fieseler *et al.* 2007;  
19 Thomas *et al.* 2011), of whale fall carcasses (Tringe *et al.* 2005) and the deep sea (Eloe *et al.*  
20 2011). The main outcomes of sequence-driven metagenomics are predictions on the  
21 metabolic repertoire of a given sample, to delineate metabolic pathways and to assess the  
22 potential of an environmental sample to perform specific, sought-after tasks. Single cell  
23 genomics based on whole genome amplification (WGA) is an emerging technology in the  
24 field of environmental microbiology, which is complementary to metagenomics (Hutchison  
25 & Venter, 2006; Ishoey *et al.* 2008). Owing to the experimentation and manipulation of  
26 single microbial cells, this technique allows promising genomic insights into complex  
27 environmental microbial consortia whose members are frequently resistant to cultivation  
28 (Siegl *et al.* 2011). Importantly, functional assignments of primary and secondary metabolism  
29 genes to specific bacterial genes of known phylogenetic identity are possible (Siegl &  
30 Hentschel 2010). Metagenomics and other –omics methods have opened new avenues for the  
31 sustainable production of marine enzymes/drugs that would otherwise be inaccessible by  
32 conventional microbiology techniques. By merging the scientific disciplines of molecular  
33 genetics, microbiology, chemistry and biochemistry, the promise that marine microorganisms



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

### 25 **3.1.74.1.7 Metagenomic bioprospecting**

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

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



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

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

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

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

### 3.1.84.1.8 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 butyrolactone, 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 *Rubrimicrobium 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

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

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

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

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

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

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

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

1 high species diversity can be maintained by periodic disturbance or by environmental  
2 fluctuations (i.e., nutrients, pH and temperature) (Buckling *et al.* 2000). Laboratory  
3 conditions that allow microbes to grow to high density in a short time are "unnatural" for  
4 many natural microorganisms that normally grow slowly, at very low and steady state  
5 concentrations of nutrients (Fry 1990). Re-creation of "nature-like" or natural, low nutrient or  
6 oligotrophic conditions have been attempted in a few cases but can only be done on a small  
7 scale and with great effort (Huber *et al.* 1998). Growing many oligotrophs in the laboratory  
8 on a large enough scale would be practically impossible. Different modifications of the  
9 enrichment concept have been developed in order to culture more novel organisms. These  
10 included serial-dilutions or pre-treatment of the sample. The purpose is to kill or dilute out  
11 numerically less numerous copiotrophic fast growing organisms in the sample before  
12 inoculating the enrichment medium (Grosskopf *et al.* 1998; Santegoeds 1996). Still another  
13 attempt towards "nature-like" enrichments is the technique of *in situ* enrichment or substrate  
14 colonization, which has been used in several environments. *In situ* enrichment is based on the  
15 principle of introducing one or few new factors into an existing "natural" environment.  
16 Techniques of *in situ* enrichments have been of interest to microbiologists ever since bacteria  
17 were found to colonize microscope slides submerged in aquatic environments (ZoBell *et al.*  
18 1943; ZoBell & Anderson 1936). Such techniques have been used in hot springs to obtain  
19 specific groups of microorganism, by using specific substrates such as cellulose. These  
20 techniques may be of special value for isolating or enriching species utilizing polysaccharides  
21 unique to the marine organism found in coastal areas. In the SeaBioTech project, the  
22 consortium developed selective enrichment methods, and serial dilutions for accessing rarer  
23 and potentially more interesting members of bacterial and protistan communities. The  
24 consortium was able to increase the overall diversity and phylogenetic depth of obtained  
25 strain collection for consequent screening for bioactive microbial metabolites and thus  
26 maximized the likelihood of obtaining novel bioactive lead-compounds. Enrichment methods  
27 were developed targeted towards certain metabolic types belonging to heterotrophic  
28 actinomycetes, thermophilic bacteria, marine and extremophilic cyanobacteria and rare  
29 coastal psychrophilic heterotrophs by using various cultivation methods and enrichment  
30 procedures. Special substrates such as complex recalcitrant polysaccharides or single carbon  
31 sources of predetermined type and structures were used often in conjunction with group  
32 specific inhibitory substances.

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

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

### 17 **3.24.2 Challenge 2: Improvement of technical aspects of the** 18 **biodiscovery pipeline**

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



### 3.2.14.2.1 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; Caretoni & 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



1 larger clinical trials) to the so-called “productivity gap” in pharmaceutical R&D, which has  
2 been posing major issues for the sustainability of drug development in the private and public  
3 sectors (Pammolli *et al.* 2011). Therefore, while the main technological improvements are  
4 still considered essential cornerstones for R&D, the basic paradigms of the process are  
5 currently under debate (Macarron 2006; Leeson and Springthorpe 2007; Harvey 2008; Mayr  
6 & Bojanic 2009). In particular, phenotypic screenings have been currently reconsidered as  
7 valid options along with target-based molecular assays, particularly for certain therapeutic  
8 areas (e.g. pathogenic infections, cancer and others) (Payne *et al.* 2007; Mayr & Bojanic  
9 2009). Moreover, emphasis has been given to highly validated targets, i.e. targets whose  
10 activity has been proven to be modulated by a chemical compound and with a direct  
11 causative link to the disease to be addressed. Therefore, highly innovative but poorly  
12 characterized targets were deprioritized (Gashaw *et al.* 2011). More recently, attention has  
13 been focused on the quality of the compounds in the chemical libraries, rather than on the  
14 number of compounds. In fact, retrospective analysis unequivocally clarified that early  
15 combinatorial chemistry produced large libraries with very limited diversity (Macarron 2006;  
16 Macarron *et al.* 2011). At present, investments in compound collections are not aimed at a  
17 numerical size increase, but at ensuring a constant stream of new chemotypes, meaning that  
18 natural products and mimetic derivatives are back into consideration (Harvey 2008; Macarron  
19 *et al.* 2011). This implies that drug discovery has to face well-known problems inherent to  
20 natural products, like supply at screening scale, purification, identification and structural  
21 complexity (Grabowski & Schneider, 2007; Ganesan, 2008). However, technical solutions  
22 have been rapidly developing to overcome these bottlenecks and in order to gain access to the  
23 potential of this valuable source of chemical diversity (Kennedy 2008; Rishton  
24 2008; Koehn 2008). Under this developing scenario, the SeaBioTech consortium integrated  
25 some of the most advanced technological applications with state of the art expertise in drug  
26 discovery research to identify bioactive compounds from libraries of marine origin. To  
27 increase the chances of a positive outcome of the screening campaigns, the assay types  
28 applied in SeaBioTech comprised a wide array of target-based and phenotypic assays.  
29 Some were configured in HTS-suitable formats to ensure a high processivity of large  
30 compound collections and of hit profiling; some will be performed as low-throughput assays  
31 to achieve a high level of information directly from primary screening (e.g., assays against  
32 sea lice affecting farmed salmon). Most importantly, all assays within SeaBioTech  
33 represented functional assays designed to provide unambiguous responses concerning their  
34 relevance for biomedical and biotechnological applications (e.g., isogenic X-MAN human

1 disease models from HDL). Having no pre-existing knowledge on the bioactivities present in  
2 the extract/compound collections obtained from underexplored marine sources, SeaBioTech  
3 members screened a very wide set of assays with relevance to diverse therapeutic areas,  
4 including cancer (AXXAM; HDL; MATIS), bacterial, viral and parasitic infections (SIPBS;  
5 AXXAM; UWUERZ), inflammation (SIPBS; MATIS), cardiovascular diseases (AXXAM;  
6 MATIS), metabolic disorders (SIPBS) and pain (AXXAM). Besides human health,  
7 SeaBioTech sought bioactive compounds to treat parasitic infections in aquaculture  
8 (PHARMAQ) and for food and cosmetics industry (MATIS). It is worth noting that discovery  
9 programmes in these fields are encouraged by the successful outcome of research projects  
10 using compounds of marine source, which have recently yielded molecular probes, pre-  
11 clinical candidates and therapeutic drugs in several clinical areas, including cancer  
12 (Napolitano *et al.* 2009; Dumontet and Jordan 2010; Galeano *et al.* 2011), bacterial, viral and  
13 parasitic infections (Mayer *et al.* 2011), inflammation (Folmer *et al.* 2010; Mayer *et al.*  
14 2011), Alzheimer's disease (Williams *et al.* 2010), and pain (Teichert & Olivera 2010; Mayer  
15 *et al.* 2011).

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

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

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

6         Second, SeaBioTech inherently enhanced the collaboration among different SMEs  
7 contributing at different stages of the project (identification of hit compounds, hit-to-lead  
8 phase, characterization of lead compounds), in order to define the chemical and  
9 pharmacological properties of the products. Thus, participation in SeaBioTech epitomised a  
10 valid opportunity for SMEs to establish collaborative partnerships with companies with  
11 contiguous expertise and complementary technologies. In parallel, the strategy adopted in  
12 SeaBioTech for bioactivity detection embodied an impressive improvement in terms of  
13 potential exploitation of the chemical diversity of the marine compound collections. Indeed,  
14 libraries of marine origin were subjected to screening campaigns against a panel of more than  
15 20 assays covering over 10 different therapeutic areas or biotechnological applications. This  
16 approach increased the probability that bioactive compounds are retrieved as positive hits,  
17 thus predicting a superior success rate compared to traditional screening on a few assays. In  
18 practice, the adopted strategy places SMEs and research institutes within

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

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

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

#### 4 **3.2.24.2.2 Metabolomics approach - Improving isolation and identification of target** 5 **compounds**

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

1 and NMR spectroscopy were the complementary analytical methods and were commonly  
2 employed in tandem as metabolomics tools. Mass spectrometry is sensitive even at  
3 femtogram levels but may not be reproducible between instrument types and ionization  
4 capability of the metabolites. While NMR data is reproducible, it may not be sensitive  
5 enough to detect metabolites at lower concentrations. Efficient high-throughput gradient flash  
6 and/or medium pressure chromatography, where gram quantities of a microbial extract can be  
7 loaded in a column, will be employed to isolate the bioactive natural products from microbial  
8 extracts. High-throughput gradient medium pressure chromatography is capable of delivering  
9 reproducible isolation schemes with high product yield, which is optimum in the purification  
10 of marine microbial extracts obtained from multiple batches and has great advantage over  
11 conventional column chromatography (Ebada, et al; 2008, Kjer et al; 2010). Structure  
12 elucidation was accomplished utilising pulse field gradient 2D NMR that would be able to  
13 provide high resolution data to determine the structure of complex molecules with multiple  
14 chiral centres as well higher molecular weight peptides and oligosaccharides (Murata *et al.*  
15 2006).

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

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

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



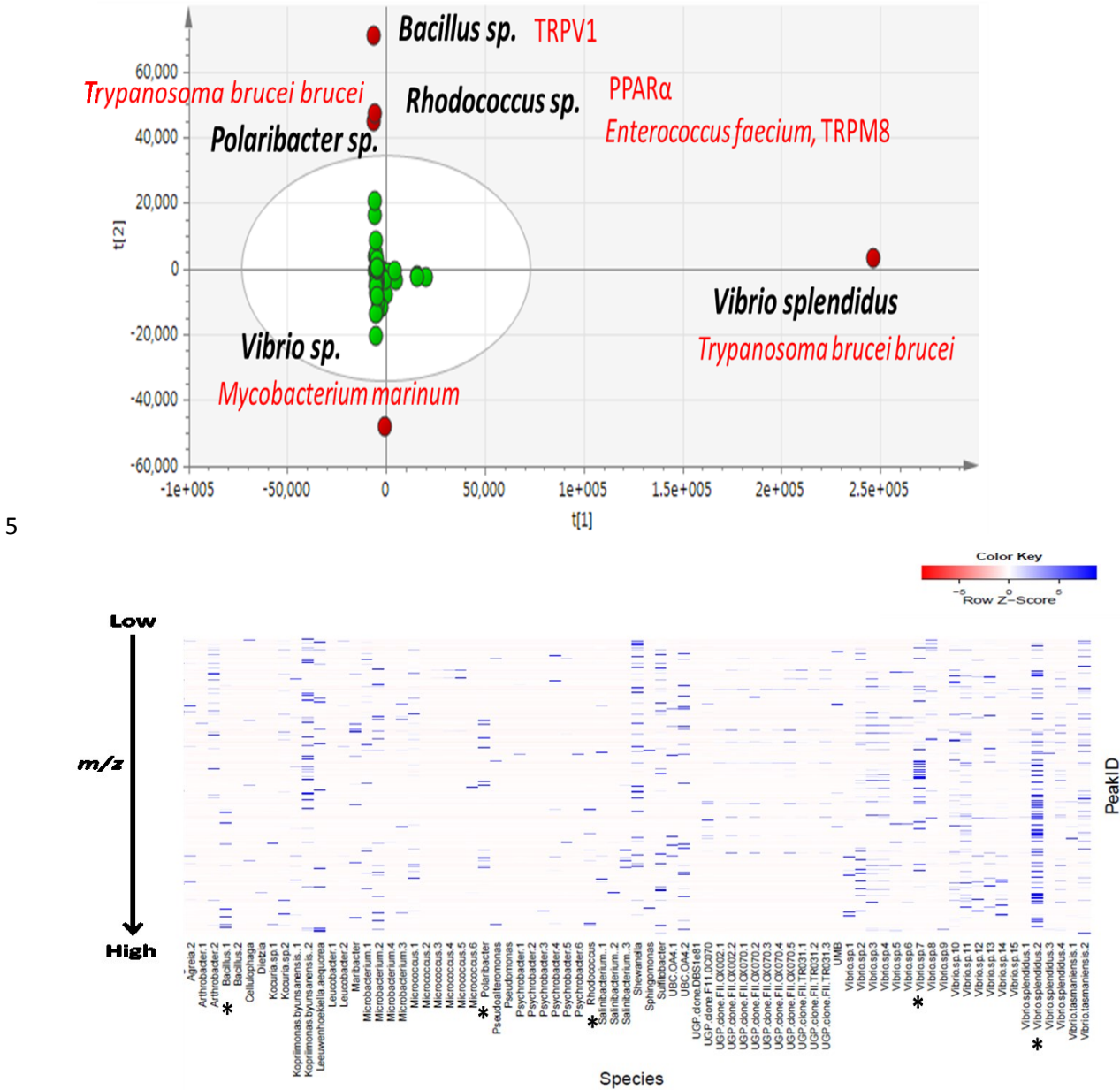
1 reductionist approach. Metabolomic tools enhanced the identification and dereplication steps,  
2 as in bioassay-guided isolation work (Yuliana *et al.* 2011). Metabolomics was applied to  
3 dereplicate the biosynthesis of the natural product at different development stages of their  
4 biological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

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



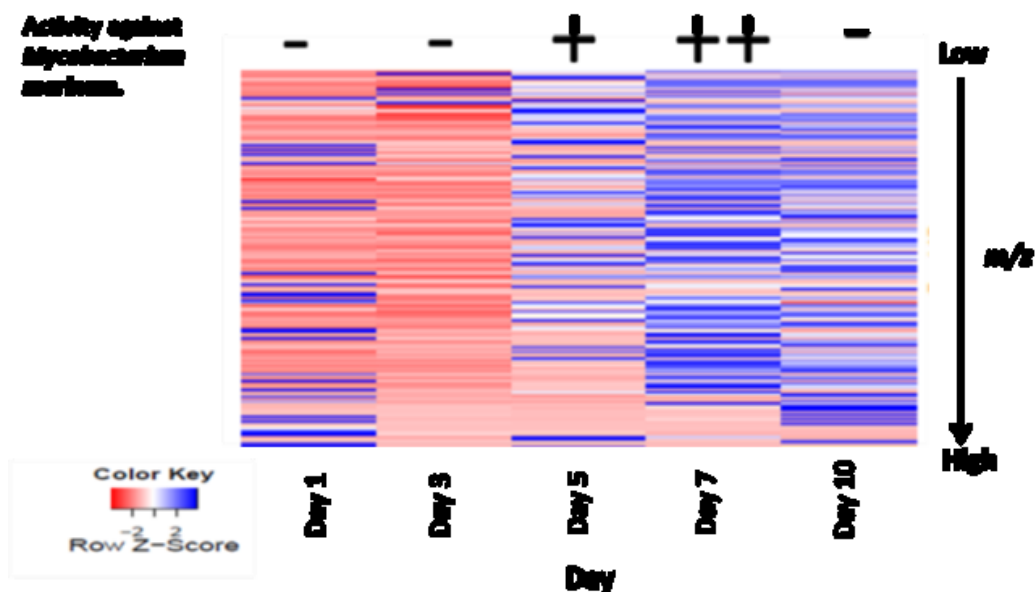
1 different physiological states. VTT optimised the much-needed algorithms to analyse the  
2 huge dataset generated by the dereplication study as well as metabolomics profiling in  
3 monitoring and exploring the relationship between culture methods, diversity, bioactivity,  
4 and metabolome evolution in selected marine isolates. Efficient cultivation and production  
5 processes at a small volume scale fermenter are developed through real time metabolomic-  
6 assisted optimization. Samplings were done in real time for detailed metabolome analysis to  
7 fully characterize intermediates, by-products and degradants. Applying metabolomics for real  
8 time analysis will in parallel check the stability of the production of the desired components  
9 when changing certain fermentation parameters prior to scale up. In addition, a chemometric  
10 study was accomplished to support and develop algorithms that was adapted and optimised to  
11 target the bioactive secondary metabolites. Metabolomics has become a powerful tool in  
12 systems biology and it allowed SeaBioTech to gain insights into the potential of natural  
13 marine isolates for synthesis of significant quantities of promising new agents, and guide the  
14 manipulation of the environment within fermentation systems in a rational manner to select  
15 the desired metabolome. Dereplication work was finalized for samples originating from  
16 Milos, Crete, and the geothermal vents of Iceland as well as those covering Scottish coastline  
17 and additional sample strains from the Antarctica region. Seventy-seven (77) bacterial  
18 samples were dereplicated from the NPMG-Orkney archive. A total of 34 bacterial extracts  
19 from Milos and Crete were analysed, yielding SBT348 and SBT687 as the candidate strains  
20 for further compounds isolation and purification. While based on mass spectrometry profiles  
21 of strains from the Scottish coastline and the Antarctica region, three isolates revealed distinct  
22 patterns, KP130 (an unidentified bacteria isolated from Maud Rise, Antarctica), KP044 (a  
23 *Streptomyces* strain isolated from St. Andrews sediment) and KP121 (a *Bacillus* strain from  
24 Bransfield Strait, Antarctica). The metabolites responsible for these unique profiles were  
25 identified using principle component analysis (PCA) and found to be a series of polymers  $m/z$   
26 363-1911 with spacing of 86 Da (KP130), a series of piscicides and antimycins known to be  
27 produced by *Streptomyces* spp. (KP044). These PCA outliers were also identified in the  
28 molecular network, demonstrating their complementary nature of metabolomic tools for  
29 secondary metabolite discovery. Metabolomic profiles have been documented into the  
30 SeaBioTech database. Metabolomes were dereplicated for priority strains while biosynthetic  
31 gene-based screening explored the presence of the genes for the respective secondary  
32 metabolite (WP4). However, bioactivity was used to prioritise strains for the WP7 pipeline  
33 (WP3). Extracts of priority strains were prepared from scale-up for further fractionation and  
34 isolation of bioactive secondary metabolites. Metabolomic-guided targeted isolation work

1 was done in parallel to and in support of the bioassay resulting to a quick identification of the  
 2 active metabolites. A total of 65 natural products have been elucidated and have been  
 3 documented in the SeaBioTech database which has been linked to Chempider and PubChem  
 4 databases ([http://spider.science.strath.ac.uk/seabiotech/pure\\_compounds\\_show.php](http://spider.science.strath.ac.uk/seabiotech/pure_compounds_show.php)).



6  
 7 Figure 3 – A) PCA scoring plot based on mass spectrometry data from all extracts.  
 8 (Taxonomic identification data and bioactivities of the outliers (red dots) are shown in the  
 9 plot.). B) Heat map based on mass spectrometry data. Outliers in PCA analysis plot are  
 10 annotated \*.

11



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

4

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

### 17 **3.34.3 Challenge 3: Sustainable modes of supply of raw materials**

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

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

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

### 20 **3.3.14.3.1 Sustainable production of macromolecules at the lab scale by metabolic** 21 **engineering**

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

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

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

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

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



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

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

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

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

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

### 3.3.24.3.2 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° C) (Burgaud *et al.* 2009) to thermophilic optimal temperatures (85° 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 °C (Matthews 2008), these unusual temperature requirements required significant re-design of plant in terms of heat removal and mass transfer (low O<sub>2</sub> 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<sub>2</sub>) (Nakagawa *et al.* 2005) which are unfamiliar to the mainstream fermentation industry, and dangerous, or unusual substrates or toxic by-products (e.g.H<sub>2</sub>S (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

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

#### 24 **3.44.4 Challenge 4: Legal aspects relating to access to marine** 25 **bioresources**

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

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

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

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

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

### 19 **3.54.5 Challenge 5: Improving access to marine biotechnology data** 20 **through an EU platform**

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



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

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

## 17 **5 Conclusion: Impacts and Future Insights**

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

### 22 **3.65.1 A reproducible quality of marine resources**

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

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

### 13 **3.75.2 An improved and integrated technology for drug discovery**

14 For the improvement in technical aspects, SeaBioTech integrated metabolomics-assisted  
15 methodology with systems biology and functional bioassays increasing the ability to divulge  
16 positive hits that proved to be affordable, innovative and efficient method (MacIntyre et al  
17 2014) to separate, elucidate the structure, and identify the bioactive metabolites. Novel and  
18 underexplored species of marine microorganisms were investigated for the first time as  
19 potential sources of novel therapeutics and they provide positive indications that lead  
20 compounds can be isolated and progressed to address significant unmet medical needs (e.g.,  
21 cancer, infections against, metabolic syndrome and inflammation) and threatening parasitic  
22 infections for aquaculture. WP3 partners in charge of the screening activities improved the  
23 performance and throughput of the assays, to comply with the requirement to process a  
24 remarkably high number of extracts, fractions and compounds of marine origin. Major  
25 improvements were obtained for the development of automated, high-throughput screening  
26 platform to provide cell-based assays for the detection of hits with anti-cancer activities, in  
27 particular for cell proliferation (HDL). Moreover, assay systems were modified to achieve a  
28 suitable robustness to screen complex marine extracts and subsequently to produce more  
29 accurate and reliable results (SIPBS, AXXAM).

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

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

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

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

### 24 **3.85.3 A sustainable production of raw materials**

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

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

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

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

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

### 3 **3.95.4 A harmonized legal position on marine bioprospecting**

4 SeaBioTech liaised closely with, and contributed to, common areas of activity dealing with  
5 legal/ethical aspects being undertaken in the parallel EU funded projects: MICROB3,  
6 BlueGenics and PharmaSea. An overarching group of experts was formed, i.e. the Advisory  
7 panel of policy and legal experts (APPLE). APPLE, an advisory board brought together the  
8 breadth of experience, legal, scientific and commercial, necessary to address the critical  
9 policy and legal barriers which currently hinder progress in innovative marine biotechnology  
10 in Europe. The projects have worked together on these aspects to avoid duplication of effort  
11 and enable a wider-reaching and more global approach of benefit to these consortia and  
12 beyond. During the lifetime of the project the legal implications to bioprospecting have  
13 changed status with the implementation of the Nagoya protocol, which became legally  
14 binding from the 12th of October 2014. An overarching, generic Material Transfer  
15 Agreement (MTA), conforming to the requirements of the Nagoya Protocol has been  
16 developed by Microbio3. This has, with minor adjustments, been applied across the projects.  
17 SeaBioTech contributed to the development, structure and content of the PharmaSea  
18 Deliverable on development of web-based, interactive, toolkit to assist Marine Genetic  
19 Resource (MGR) practitioners in navigating the different legal and policy regimes involved  
20 in access to MGR and associated benefit sharing. This area has rapidly developed and on-line  
21 resources associated with the CBD Clearing House are available to users/ potential users of  
22 biological resources. Work undertaken by APPLE, particularly the PharmaSea legal team, has  
23 resulted in considerable progress with respect to the developing of possible solutions to the  
24 implications of the collection of materials in areas beyond the Economic Exclusive Zone  
25 (EEZ) i.e. in Areas Beyond National Jurisdiction (ABNJ). These were presented at the UN  
26 HQ, New York on 16-20th June 2014 for consideration for possible future proposed changes  
27 to the UN Common Law of the Sea (UNCLOS).

### 28 **3.105.5 A centralized biobank repository and database of information**

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



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

27

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

<b>Milestones achieved to support project challenges</b>	<b>WP</b>	<b>Encountered confrontations and some strategies used to yield to the challenge</b>
<i>1) A reproducible quality of marine resources</i>		
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.
<i>2) An improved and integrated technology for drug discovery</i>		
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.
<i>3) A sustainable mode of supply of raw materials for the industries</i>		
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
<i>4) A harmonized legal position on marine bioprospecting</i>		
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.
<i>5) A centralised biobank repository and database of information</i>		
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

1

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 4 undergraduate students, research fellows and associates as well as technical assistants who  
 5 have worked rigorously with SeaBioTech. Without the input of these colleagues, putting the  
 6 results and legacy of SeaBiotech will not be possible.

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