1.) Executive Summary

Objective	Proposed	Achieved
Dereplicated microbial strains	2 500	>13 000
Dereplicated extracts	18 000	>15 000
Biological assays	-	>130 000
Bioactive extracts	540	>600
Molecular families	115	90
Drug leads in animal trials	2	**5**

The PharmaSea project has exceeded its stated global aim of producing two compounds at larger scale and advancing them to preclinical evaluation for infection or central nervous system diseases. In total five marine derived compounds progressed to animal models for central nervous system diseases. Two marine sponge derived compounds showed positive results in a behavioural model for Alzheimer's disease, were non-toxic and showed 'drug-like' properties. Analogues of these compounds are being synthesised by a US collaborator and patent protection will be applied by the PharmaSea partners and the US collaborator. For epilepsy, three different compounds have proceeded to animal trials. Of these, two compounds from a marine fungus reduced seizures significantly in the gold-standard animal model for epilepsy. These compounds were also shown to drug-like and non-toxic, and will be patented by the PharmaSea partners involved and advanced to the next stage. In the area of anti-infectives many of extracts contained known antibacterials, but work will be continued on at least 20 promising extracts showing activity and chemical novelty as part of the MSCA ITN consortium 'MarPipe'.

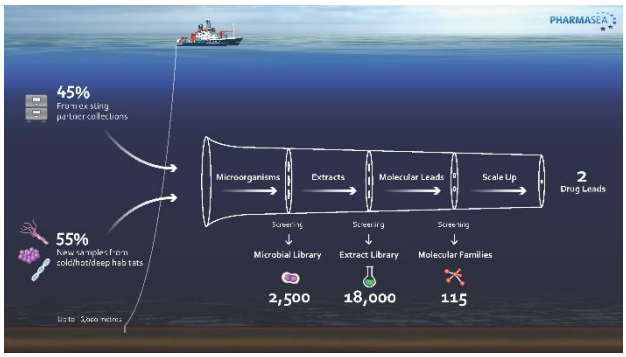
PharmaSea also attained its major objectives by reducing the bottlenecks present in the marine biodiscovery pipeline. High-quality taxonomically distinct marine microorganisms were isolated from a range of marine environments including sediments the Arctic (Barents Sea) and Antarctic (South Shetland Trough). Improved methods for strain isolation, cultivation and elicitation of new compounds were developed. The genome of many strains was studied leading to the discovery of new compounds, their expression in surrogate hosts and an understanding of the ecological role. New tools for heterologous expression were generated and a full genome scale model of a marine bacterium was created to study its metabolism. Microorganisms were cultivated under a broad range of conditions, and the majority of extracts were generated using standardised conditions. Extracts were screened for biological activity and chemical profile, and a combined approach was taken to prioritise those for further studies. Biological assays ranged from cell based (bacteria, fungi), enzymatic (anti-inflammatory) to zebra fish and covered infection, inflammation, epilepsy and neurodegeneration. Extracts that were deemed to have potent and selective activity, few off-target effects, low toxicity and showed a unique chemical profile were progressed to the next stage. Chemo-informatics tools were developed highlighting unique chemistry at the extract stage.

Mid-scale re-cultivation of promising microorganisms yielded sufficient material for structure determination and secondary biological assays. Structure determination was assisted by the PharmaSea databases and dereplication tools that speeded up the discovery of known compounds, pinpointing unknown compounds and accelerating the complex process of their structural clarification. Compounds prioritised by the PharmaSea prioritisation committee were tested in a variety of ways to determine their 'drug-likeness', several then progressing to animal trials based on the assembled evidence and others undergoing further work after the project end.

In the policy-sphere PharmaSea was very active and worked with policy makers from the UN, the EU and other national blocs such as the African Union and the G77 amongst others, taking part in over 25 events at the UN and internationally. PharmaSea provided policy options based on scientific good practice for the UN Preparatory Committee on the Sustainable Use of Marine Biodiversity in Areas Beyond National Jurisdiction. Good practice guidelines have been developed and will be put online in June 2017. Case studies dealing with complex situations covering access and benefit sharing of marine genetic resources will be published soon. Media coverage of PharmaSea was excellent, with TV coverage on, amongst other, CNN and Al Jazeera, a large online and print media presence. (http://www.pharma-sea.eu/)

2.) Description of Project Context and Objectives.

2.1) The PharmaSea Concept



The ocean environment covers a multitude of ecological niches and is home to more living organisms, especially microorganisms, than any other environment. This drives the concept of "blue biotechnology", where unique and novel biological compounds or principles from the marine environment are harvested and exploited for the benefit of humankind. Despite the tremendous potential, exploitation, particularly at a commercial scale, has been hampered by a number of constraints. These relate to sampling extreme environments, genetics of the organisms, compound isolation, structure elucidation, early reliable validation of biological activity and best mechanisms of flow-through into exploitation. PharmaSea has solved some of these chronic bottlenecks by developing essential actions beyond the state of the art and linking them with best practice and appropriate pragmatic approaches towards a robust pipeline structure. PharmaSea has processed a wide genetic basis including marine microbial strain collections held by partners and new strain collections from extreme marine environments and has produced new products with desirable characteristics for further development by partners. The main disease areas that were addressed have included microbial infection, diseases of the central nervous system and inflammation, the last area is also of possible interest for nutritional and personal care/cosmetic uses.

2.2) Objectives of PharmaSea

PharmaSea aimed to provide a decisive impetus to enable industrial use of marine bioresources, focusing on the improvement of the efficiency and effectiveness of the key stages and links in the biodiscovery pipeline. **The global aim of PharmaSea was to produce two compounds at larger scale and advance them to preclinical evaluation for infection or CNS diseases**. The high-level PharmaSea objectives were to:

1.) Generate a high-quality library of taxonomically distinct marine microorganisms from a broad range of phyla derived from unique habitats and assessed for their ability to produce novel chemistry.

2.) Improve methods and tools for extracting, isolating, screening, dereplicating and identifying small molecules with desired biological and physical characteristics.

3.) Generate a library of novel chemical entities with high bioactivity in antimicrobial, anti-inflammatory and CNS screens for further development.

4.) Deliver sustainable ways to supply novel chemical entities and analogues for further evaluation in a number of markets, by PharmaSea partners or via licensing to industry. Relevant markets include, but are not limited to, microbial infection, inflammation, CNS diseases, nutrition and personal care.

5.) Create databases for maintaining strain, assay and compound data with integrated methods to grant access to end users and licence IP to relevant industry.

6.) Provide recommendations and solutions to address legal/policy barriers to the access and sustainable use of marine genetic resources for biotechnology research and commercialisation as well as the equitable sharing of the benefits resulting from this usage based on a multi-stakeholder analysis.

7.) Disseminate project outputs to relevant end users via targeted means, including increasing industry awareness of the benefits of using marine biotechnology.

8.) Produce enhanced alignment with existing EU infrastructure and initiatives relevant to PharmaSea's mission.

As targeted objectives, PharmaSea promised to deliver:

1.) A curated network of highly annotated libraries of microbial strains, extracts and pure compounds leading to scale up and preclinical evaluation of a number of leads (Figure 1):

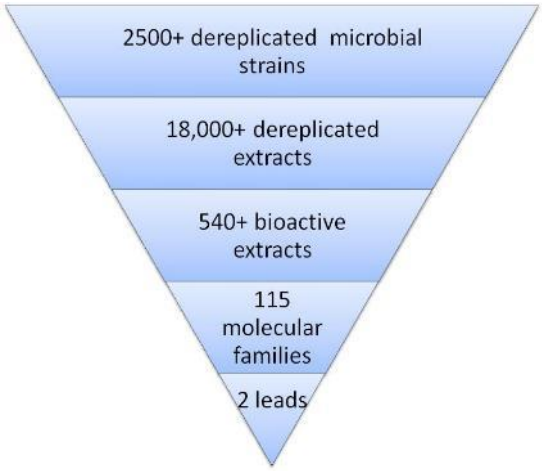


Figure 1 PharmaSea libraries and products.

   a. 2500+ taxonomically dereplicated strains (45% strains newly isolated by PharmaSea, 55% part of partners' current collections), together with culture conditions and a database containing information on biosynthetic capacity of isolates and metagenomes.

   b. Methods that increase expression of biosynthetic capacity of strains.

   c. 18,000+ dereplicated extracts for screening.

   d. 540 extracts active in antimicrobial, anti-inflammatory or CNS assays progressed to chemical dereplication.

   e. Discovery of 45 new molecular families (15 with novel skeletons) and 70 known molecular families with new biological activity.

   f. Scale-up and follow-up testing of a number of candidates in the above disease areas.

   g. Two (2) new chemical entities (leads) for development and licensing as treatments for microbial, inflammatory or CNS diseases.

   h. Protocols and a European focal point for PharmaSea and other marine biodiscovery resources (data, strains, extracts, compounds) available to the biomedical, pharmaceutical and agrochemical research communities.

2.) Chemometric tools, software, databases and datamining procedures:

   a. To determine chemical diversity and novelty at the extract and fraction stage.

   b. Dereplication approaches using NMR and MS data-integrating software and instruments using predicted spectra for most known natural products.

   c. Simplified workflows to accelerate structure elucidation.

   d. Laboratory information management system containing data on PharmaSea libraries integrating as a minimum, for each tested extract and isolated molecule, data on strains, genetics, bioactivity, analytical and spectroscopic data, structures and predicted properties.

3.) Improved, targeted methods to isolate bioactive and structurally novel compounds.

4.) Robust stakeholder advisory mechanism and interaction fora for stakeholder dialogue, including a series of workshops, to identify and analyse key policy and legal barriers towards the access, sustainable use and equitable sharing of the benefits of marine genetic resources.

5.) Selected case studies and best practices on access and benefit sharing, property rights and intellectual property associated with marine genetic resources.

6.) A 'PharmaSea Toolkit' for marine biotechnology researchers, industrial users and interested parties on the legal frameworks and practical aspects of how to secure access to marine genetic resources.

7.) A communication strategy to improve uptake of marine biodiscovery products by industry.

2.3) Beyond the State of the Art

The PharmaSea project moved beyond state-of-the-art in critical aspects of the biodiscovery pipeline that are bottlenecks to flow-through of bioactives into industrial use. Table 1 lays out some of these, and how the consortium worked to overcome them.

Table 1 Widening the Bottlenecks in the Marine Biodiscovery Pipeline – the aims of PharmaSea

Bottleneck	**How PharmaSea will solve this**	**Work Package**
Access to bioresources	1) Develop deep sea sampling technology	WP1
	2) Develop legal framework for EU-wide A&BS	WP6
	3) Resolve IP issues inherent in different legal regimes	WP6
	4) Produce best practice guidelines	WP6
Quality of marine resources	1) Habitat selection leading to novel microbial strains	WP1
	2) Selective cultivation leading to novel microbial strains	WP1
	3) Phylogenetic analysis of strains to ensure diversity/quality	WP1
	4) Genome scanning to uncover biosynthetic capacity of strains	WP1
Extract generation	1) Media development to realise biosynthetic capacity of strains	WP2 WP2
	2) Stress/elicitation to realise biosynthetic capacity of strains	WP2 WP2
	3) Heterologous expression of biosynthetic genes	
	4) Extraction technology/Robotics/Automation	
Extract dereplication	1) Chemometrics for dereplication and prioritisation of extracts	WP2 WP3
	2) Innovative high content assays/assay technology	
Isolation & Purification	1) Explorative solid phase extraction to develop isolation protocols	WP4 WP4
	2) Targeted chromatography	
Chemical dereplication	1) Liquid chromatography-mass spectrometry	WP4
	2) Novelty screening using NMR techniques	WP4
	3) Datamining using predicted NMR & MS properties	WP4
Structure determination	1) Low volume probes/cryomicroprobes	WP4
	2) Computer aided structure elucidation	WP4
Hit selection	1) Innovative MOA screens (zebrafish) & counterscreens	WP3

	2) Property prediction of compounds for ADMET/PK/PD	WP5
	3) Rapid *in-vivo* evaluation	WP5
Supply issue	1) Use of microbial strains	WP5
	2) Process intensification	WP5
	3) Scale-up in saline media	WP5
	4) Heterologous expression	WP5
Uptake of technology	1) Data management	WP7
	2) PatentBox	WP6/7
	3) End user panel	WP6/7
	4) Inventory of assets/outputs	WP7
	5) Targeted technology transfer briefs	WP7

The work strategy in PharmaSea has followed a clear structure to address the concept and objectives.

As key achievements, PharmaSea has been able to address three key areas to improve the flow-through the marine biodiscovery pipeline:

a.) High quality libraries of strains, extracts and pure compounds evaluated at each step to increase their value and prioritise those that are structurally novel and have potent and selective bioactivity.

b.) Acceleration of the workflow from strain to pure compound by the use of novel dereplication strategies, isolation techniques and software tools for datamining and structure elucidation.

c.) Addressing the legal, intellectual property and policy aspects that affect marine biodiscovery to make it simpler to access marine bioresources and increase industry uptake.

The PharmaSea process resembles a pharmaceutical industry pipeline but improves upon it in many aspects by widening the bottlenecks through a multilayered selection procedure to prioritise those samples with high probability of chemical novelty and potent and specific bioactivity.[1] The involvement of industry and not-for-profit partners and external industrial advisors, with extensive experience in the area of natural product based drug discovery, is instrumental in defining the workflow from strain to lead compound and has been used to shape the overall workplan strategy. The assembling and evaluation of biodiversity has received the most intensive effort to ensure that the quality of extracts going forward to screening and compound isolation and identification is maximal, and to generate a lasting resource for the marine biodiscovery community. PharmaSea also recognised the pressing need for development of new approaches to the integration of analytical data from diverse sources, to speed structural identification of complex natural molecules and dereplication.

PharmaSea dedicated WP6 to bringing together legal and policy experts with practitioners in the field of marine biodiscovery to resolve issues with the legal aspects of marine biodiscovery. The WP6 team and project coordinator led two structured workshops, a large number of side-meetings and briefings at UN level and 4 meetings of an advisory panel of policy and legal experts (APPLE), to assess the landscape and provide reports and tools to assist practitioners in navigating the different legal regimes involved in access to marine genetic resources and associated benefit sharing. The project objectives also included dissemination and communication to diverse stakeholders in industry, policy, government in a number of formats to inform, educate and increase industry uptake of marine biodiscovery products.

3.) A description of the main S&T results/foregrounds.

3.1) Achieving the Objectives:

Global Aim: PharmaSea exceeded its global aim of producing two compounds at larger scale and advancing them to preclinical evaluation for CNS diseases. The exceptional work done by PharmaSea partners on compounds active in epilepsy and Alzheimer's disease based screens means that 2 compounds have advanced to small animal trials for Alzheimer's disease, and 3 compounds progressed the epilepsy *in vivo* model. The work on Alzheimer's disease showed the compounds were active in a mouse behavioural model and showed excellent pharmacokinetic properties. PharmaSea has been working with a US research group to develop analogues of this compound family and is working towards a joint publication and patent. One compound derived from a Greenland marine sediment fungus was shown to have seizure-reducing activity *in vivo*, but with a narrow therapeutic window. The activity and pharmacokinetic properties of this compound and its analogues will be further studied with a view to intellectual property protection. Two related compounds derived from a marine fungus reduced seizures significantly in the gold-standard animal model for epilepsy. These compounds were also shown to have excellent pharmacokinetic properties, and will be patented and advanced to the next stage by the PharmaSea partners involved.

3.1.1) A library of marine microorganisms assessed for their ability to produce novel chemistry.

a.) *2500+ taxonomically dereplicated strains*. PharmaSea has used 9,849 legacy strains and isolated a further 3,840 strains from sediments and marine sponges collected by PharmaSea partners in deep and cold environments.[2-5] Existing collections include bacterial, fungal and microalgal strains from a range of locations, such as the Arctic, Antarctic and deep water off the coast of the Republic of Ireland, South Africa and Argentina. For the new strains isolated, the highlights are 1285 isolates from South African sediments which have yielded more than 30 with anti-bacterial activity. Nearly 1000 strains have been isolated from Antarctic sediments from which 20 isolates were identified as strongly antibacterial. Genome sequencing of 29 actinobacteria identified a large number of potential biosynthetic clusters, in addition to potential clusters present in the genome sequences obtained for another *ca*. 3000 strains.[6] The microbiota of four individual deep water sponges has been analysed, and are being mined *in silico* for the presence of secondary metabolite gene clusters and metagenomics clones are being expressed in a surrogate host.

b.) *Proprietary methods were used to increase expression of biosynthetic capacity of strains to generate 18,000+ dereplicated extracts for screening.* A number of approaches were taken to activate cryptic genes including chromatin remodelling experiments (HDAC and DMAT inhibition) for fungi and co-cultivation experiments for bacteria. Using a special co-cultivation vessel showed additional peaks appearing in the extract when compared to control fermentations and that antibacterial activity improved (Figure 2).

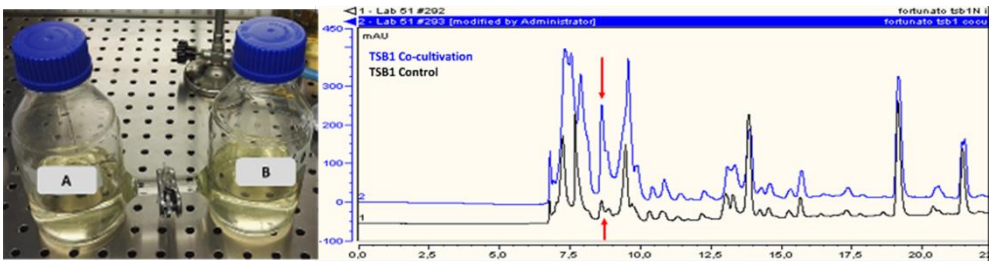


Figure 2. The use of a co-cultivation vessel (left) shows that the LC-MS chromatogram changes from the control culture (right, black trace) and when subjected to challenge by a second strain (right, blue trace) showing the appearance of new, bioactive peaks (red arrow)

3.1.2) Improved methods for extracting, isolating, screening, dereplicating and identifying bioactive molecules.

a.) *Improved, targeted methods to isolate bioactive and structurally novel compounds.* In order to efficiently process the large sets of extracts and compounds, a high-throughput photomotor response (PMR) based pre-screen in zebrafish embryos was set up to identify substances with a CNS-active profile before following up with specific anti-seizure testing (AST). The hyphenated combination of PMR/AST turned out to be an efficient and time-saving approach to identify extracts and compounds of interest. The PMR of zebrafish embryos was previously reported as a robust behaviour that is useful for high-throughput neuroactive drug discovery and mechanism prediction. Given the complexity of the PMR however, there was a clear need for rapid and easy analysis of the behavioural data. Thus we developed an automated analysis workflow using the KNIME Analytics Platform (https://www.knime.org/knime-analytics-platform) and made it publicly accessible. This workflow allowed us to simultaneously calculate a behavioural fingerprint for all analysed compounds and to further process the data. The methodology was also described in detail in a published article.[7]

b.) *To determine chemical diversity and novelty at the extract and fraction stage.* Implementing this approach would enable the prioritisation of extracts on the basis of bioactivity, sample complexity and degree of novelty in the extract, thus reducing effort on samples of limited interest. PharmaSea achieved the implementation of a workflow using the ACD/Labs database software. Initial approaches relied on peak identification in the LC-MS trace of an extract followed by the removal of all peaks present in the media and in the RSC MarinLit database. The final approach taken was to compare the LC-MS data to the RSC ChemSpider database and determine the proportion of peaks above a threshold not previously identified, given a score of 0-1 with 0 indicating no new peaks and 1 showing an extract with only previously unidentified peaks. Added to this was a score to show the diversity of the extract using a Shannon entropy approach which weights knowns/unknowns by abundance. The combination of these scores can be used to view the novelty and complexity of any extract and used to prioritise extracts for further work. The workflow only became available late in the project so has only been tried in a handful of cases. It was used to identify novelty in a family of invertebrate extracts which led to the discovery of a range of previously unreported natural products. The approach and results are currently being written up for publication.

c.) *Dereplication approaches using NMR and MS data integrating software and instruments using predicted spectra for most known natural products.* The ACD/Spectrus Database allows storing and searching for live spectra, spectral similarity, subspectral similarity, chemical structures, substructures, and associated metadata. In order to speed up both the dereplication and the structure elucidation process, these features were implemented as follows, taking into account the specifics of dealing with microbial natural products: Any components present in 24 PharmaSea standard culture media are stored in a matrix database, containing mass spectrum, retention time and MS/MS spectrum (Figure 3). This will act as a "blacklist", against which every component identified in a microbial culture extract will be searched. In a second step, components identified as unique, i.e. not also present in the culture medium, will be checked against a list of known marine natural products, i.e. the PharmaSea database, using molecular formulae, retention times as well as predicted MS/MS spectra as exclusion criteria. After the matrix and PharmaSea components have been removed from the list of sample components a list of unique components will remain. This list may contain 10 to 100 unique components. Rather than trying to identify each component this allows the prioritisation of a large number of sample results so that any follow up work can be focused on the most 'interesting' samples.

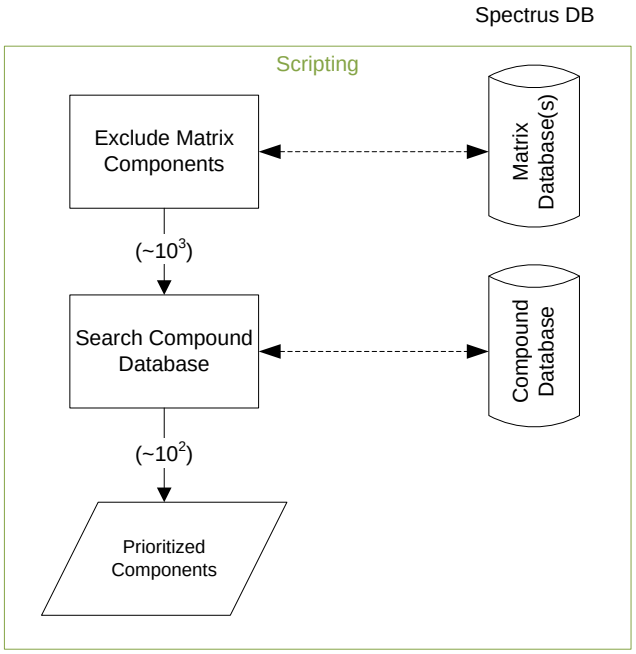


Figure 3. The dereplication workflow developed by ACD/Labs for PharmaSea.

d.) *Simplified workflows to accelerate structure elucidation*. The core functionality in ACD/StrucEluc (Structure Elucidator Suite) which has been developed over the past decade has been further modified in the PharmaSea project with new functionality to meet specific needs that better facilitate workflow requirements stated by users. This substantially simplifies the use of the software and supports the characterization of unknown structures and the construction or expansion of reliable databases, in particular the PharmaSea DB. The new ACD/MS Structure ID tool enables a user to identify or reduce the number of potential structures for an unknown component when no library spectra are available. It uses a mass search to return a list of potential structures. The potential structures are then theoretically fragmented and the observed MS/MS spectrum for the unknown component is matched against each theoretically fragmented spectrum. A score for each comparison is then calculated with a perfect match being 1 and no matches being 0. The ACD/MS Structure ID tool uses a local version of ChemSpider which currently contains approximately 23 million compounds.

e.) *540 extracts active in antimicrobial, anti-inflammatory or CNS assays progressed to chemical dereplication.* The use of the laboratory information management system NAPIS (http://whitepointsystems.com/manual/napis_l/index.php) implemented and curated at UiT allows the prioritisation of extracts and fractions based on bioactivity. Broadly, the approach taken was to prioritise those extracts and fractions that have specific and potent activity, that is, only have activity in a single screen above a certain threshold, without off-target effects or toxicity. Over 600 such bioactive extracts were identified using this methodology and advanced to dereplication. Bioactive hits were subjected to LC/MS dereplication using different platforms available at partners UNIABDN, DTU and MEDINA. These platforms included low and high resolution mass spectrometers and different detection modes (TOF, Orbitrap). Expertise in the dereplication of microbial compounds according to their origin (fungi, bacteria or actinomycetes) was also complementary between the three different sites. The development of new tools for dereplication (see 3.1.2 b/c) has also accelerated the prioritisation of extracts. The PharmaSea prioritisation panel had monthly teleconferences to

discuss which extracts and fractions should be advanced to scale-up for further work such as pharmacokinetic assessment and *in vivo* studies.

3.1.3) Generate a library of novel chemical entities with high bioactivity in antimicrobial, anti-inflammatory and CNS screens.

a.) *Discovery of 45 new molecular families (15 novel skeletons) and 70 known molecular families with new biological activity.* A large number of known, new and novel bioactive compounds were discovered during the PharmaSea project. The total projected figure at the end of PharmaSea was 105 families of chemical entities with biological activity (70 known; 20 with new structural features; and 15 novel), while the actual figure reached over the lifetime of the PharmSea project stands at 90 families (Figure 4, 56 known; 25 with new structural features; and 9 novel). Some examples are listed in the reference section.[8-13] These were from a diverse range of structural families (Figure 5) and many showed potent and specific biological activity. In fact, the 5 compounds that were taken forward to animal trials were all known compounds, or known compounds with new structural features. These 5 compounds had previously unreported bioactivity and favourable ADMET profiles and were therefor taken forward into the relevant animal models at the advice of the External Advisory Board.

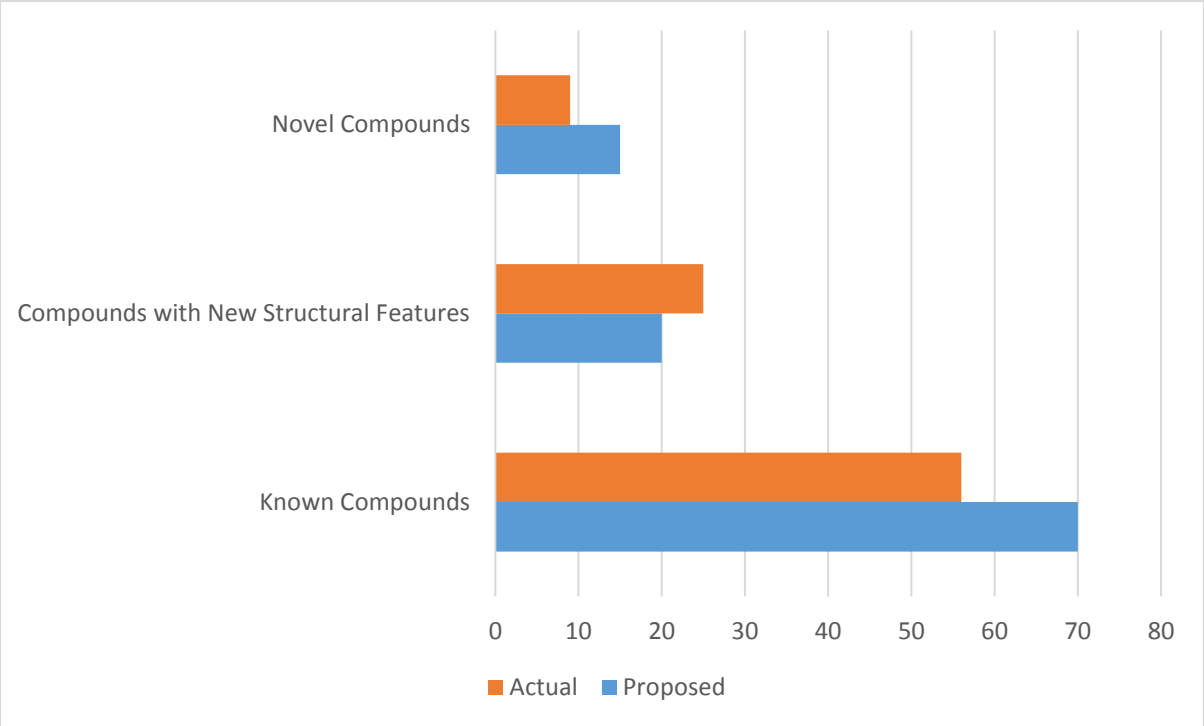


Figure 4. Actual *vs*. proposed numbers of compounds in various categories produced during PharmaSea

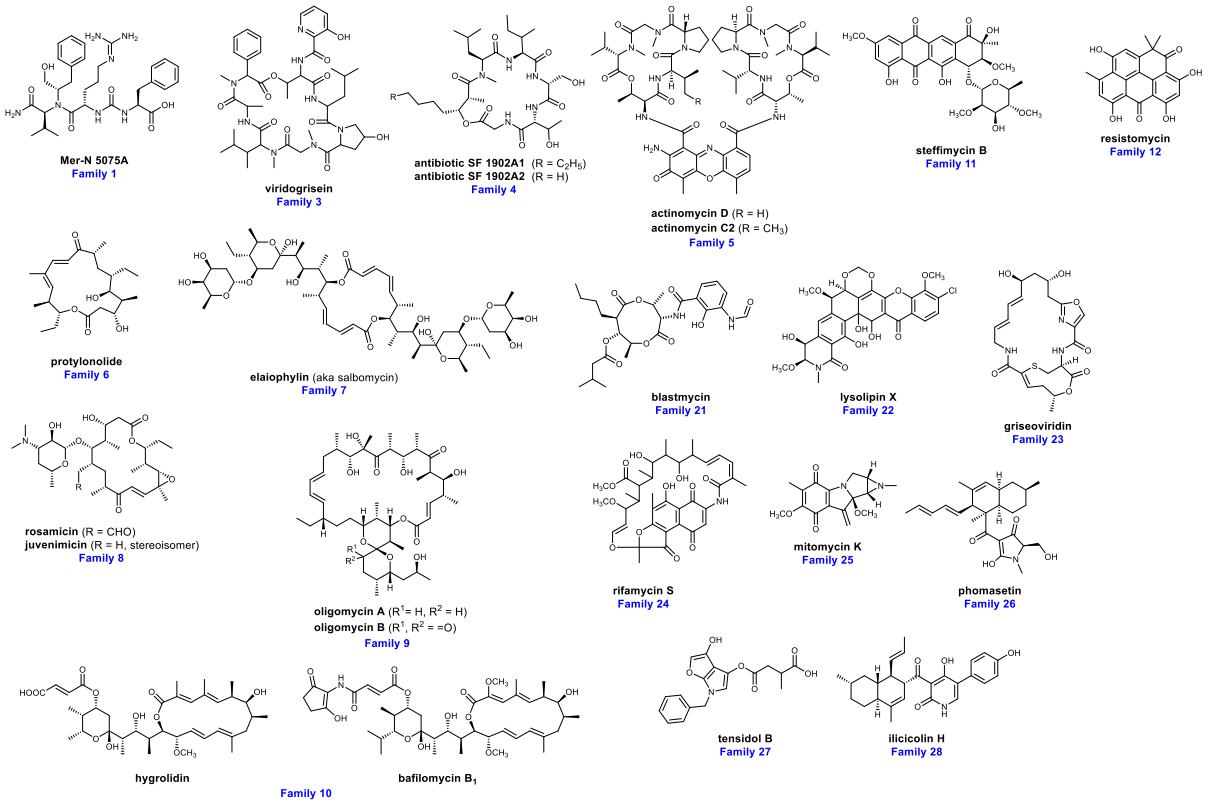


Figure 5. An indication of the chemical diversity of the compounds produced by PharmaSea.

3.1.4) Deliver sustainable ways to supply novel chemical entities and analogues for further evaluation in a number of markets.

a.) *Scale-up and follow-up testing of a number of candidates.* A total of 52 extracts active in the different bioassays were scaled-up at volumes ranging between 1L and 3L for isolation of the bioactive principles. When required, additional fermentations were performed to obtain more material of interesting hits.

b.) *Two (2) new chemical entities (leads) for development and licensing as treatments for microbial, inflammatory or CNS diseases.* More than 75 marine organism-derived compounds from the UNIABDN compound library were tested in a neuronal model for neurodegenerative diseases. These assays tested the effect of the compounds in the mitochondria and their antioxidant potential in SHSY5Y human neuroblastoma cells.[14, 15] The most promising compounds were then tested in a specific cellular model for Alzheimer´s disease. This model is based on the measurement of the main hallmarks of Alzheimer´s disease in the SHSY5Y transfected cell line that carries two mutations, V337M and R406W with the resulting overexpression of relevant Tau phosphorylated residues (Ser 181, Ser 199, Thr 231, Ser 393) and the contribution of different kinases like JNK, GSK-3, ERK and CDK 1/5. Two molecules of *Streptomyces* origin[16] and four derived from sponges gave outstanding results in the antioxidant assays and were selected for further Alzheimer's disease assays. Two sponge derived compounds, Gracilin H and L gave the best results and were studied in advanced ADME tests to evaluate their bioavailability and pharmacokinetics.[17] The intraperitoneal route of administration was used instead of the oral route due to the compound scarcity. No cardiotoxicity was shown for either compound. Both compounds were tested in an Alzheimer´s disease animal model (3xTg-AD mice) and an additional mouse model of inflammation due to the interaction of these compounds with the cyclophilins, a protein involved in the functioning of the mitochondrial membrane pore and highly associated with

inflammation.[18] Analogues of the Gracilins have been synthesised by a US collaborator and we are currently re-testing these compounds with a view to protecting and then publishing this family of compounds with the potential for the treatment of Alzheimer´s disease.

Out of the 2009 marine extracts investigated using a zebrafish-based methodology, 332 were neuroactive and non-toxic and 43 of these also showed anti-seizure activity.[7] Based on availability, anti-seizure efficacy and concentration dependency, four hit extracts were prioritized and their constituents isolated and investigated for anti-seizure activity. This has led to the discovery of two novel anti-seizure compounds isolated from a shallow sea marine fungus, and three novel anti-seizure compounds (two isoquinolines and a diketopiperazine) isolated from a marine fungus collected from a Greenland marine sediment. Compounds also tested positive in a relevant standard *in vivo* model for drug-resistant focal seizures, the 6-Hz (44 mA) mouse seizure model. Good druggability was observed for the compounds isolated from the shallow sea fungus by ADMET analysis. Scale-up and follow-up testing of the compounds is ongoing. One of the Greenland compounds showed good ADMET properties but had a narrow therapeutic window in the animal model. Further work will be carried out on this compound and its analogues. The partners involved intend to file for intellectual property protection for several of these compounds.

3.1.5) Create databases for maintaining strain, assay and compound data.

a.) *Laboratory information management system containing data on PharmaSea libraries integrating as a minimum data on strains, genetics, bioactivity, analytical and spectroscopic data, structures and predicted properties.* The laboratory information management system (NAPIS) was in place at UiT from the end of the first reporting period and data was entered and curated throughout the project to allow prioritization and selection of the most active and novel extracts for downstream processing. From the outset UiT defined a spreadsheet template to be used to share the information on samples between partners and to upload into the NAPIS LIMS. The database NAPIS Enterprise was installed at UiT, and UiT entered and curated the data from all the other partners. Partners were given access to view data in PharmaSea-NAPIS through a web-interface (OpenNAPIS), and key personnel from all relevant partners have been added as users to OpenNAPIS (http://opennapis.org/dokuwiki/doku.php). NAPIS has been instrumental in tracking the progress of all samples throughout the biodiscovery pipeline and has allowed the prioritization of extracts and fractions for downstream processing. To date NAPIS contains data on 130,000 bioassays for the 15,000+ extracts and fractions generated.

3.1.6) Provide recommendations and solutions to address legal/policy barriers to the access and sustainable use of marine genetic resources.

a.) *Robust stakeholder advisory mechanism and interaction fora for stakeholder dialogue, including a series of workshops, to identify and analyse key policy and legal barriers towards the access, sustainable use and equitable sharing of the benefits of marine genetic resources.* PharmaSea established an expert group, the APPLE (Advisory Panel of Policy and Legal Experts), and held 4 meetings involving this group and external experts on marine genetic resources, as well as 2 workshops focusing specifically on Access and Benefit-Sharing for Marine Genetic Resources in Areas Beyond National Jurisdiction. PharmaSea was also highly-influential in international discussions on future legislation through initiatives with United Nations bodies. This included 7 contributions at the Preparatory Committee discussing the Conservation and Sustainable use of Biodiversity in Areas Beyond National Jurisdiction, either as side events or workshops. In addition, WP6 partners contributed to at least 8 intersessional workshops on this topic, taking place between the meetings of the preparatory committee. The results of these meetings were combined into an article in the *International Journal of Marine and Coastal Law* entitled '*Mare Geneticum*' which offers a science-based pragmatic

approach to address the complex issues around marine genetic resources faced by the UN Preparatory Committee.[19] This information was also translated to the scientific community at 7 events. This was strengthened by a short article in *Natural Product Reports* on legal obligations for scientists involved in bioprospecting.[20] PharmaSea partners contributed to the IUCN Matrix – a web based tool to assist negotiators in this UN process.

b.) *Selected case studies and best practices on access and benefit sharing, property rights and intellectual property associated with marine genetic resources.* The overall aim in developing the case studies was to support the production of guidelines and best practice that will have a lasting impact beyond the end of the project and wider than the consortium itself. The PharmaSea Executive Committee discussed the approach to Marine Genetic Resources (MGR) and Access and Benefit-Sharing (ABS). Given the opportunity of alignment with activities in other EU-funded projects including SeaBioTech, BlueGenics and MicroB3, the decision was taken to focus more on governance issues in MGR/ABS, within national and beyond-national zones of jurisdiction, without detracting from case studies that illuminate best practice. During the first half of the PharmaSea project, the current thinking about ABS, in terms of the proposed European Commission regulation on ABS in the EU and a possible UN implementing agreement on ABS of MGR in ABNJ, also needed to be taken into account. Discussions at the Advisory Panel of Policy and Legal Experts covered the potential topics and shortlisted those case studies which informed current debates on access and benefit sharing of MGRs as well as their traceability. PharmaSea intends that the case studies will be published in the near future.

The case studies were:

i. Impact of the Nagoya Protocol on access to MGR and the role of biorepositories

ii. Realistic monetary benefits from ABS of MGR

iii. Non-monetary benefits in ABS

iv. 'Piggy-backing'- use of samples from other activities for marine bioprospecting – fishery, mining-prospecting, oceanography *etc*.

v. Legacy issues in ABS of MGR

vi. Traceability of MGRs and genomic tech/synthetic biology

b.) *A 'PharmaSea Toolkit' for marine biotechnology researchers, industrial users and interested parties on the legal frameworks and practical aspects of how to secure access to marine genetic resources.* PharmaSea has developed a dynamic internet-accessible web-based marine genetic resources (MGR) information toolkit for users (see Figure 6 and www.marinegeneticresources.org), hosted by the Flanders Institute of the Sea (VLIZ), to ensure maintenance and longevity after the end of the PharmaSea project. The purpose of the toolkit is primarily to provide an online resource to assist marine scientists in complying with legal and policy frameworks governing access to marine genetic resources and secondly provide links to relevant organisations and entities such as biorepositories, collections and research vessel operations. The legal landscape surrounding the sampling and utilisation of MGRs is changing rapidly. For this reason, the Toolkit has been created in such a way that it can be easily maintained and integrated into a longer-term initiative after the end of the PharmaSea project. The Toolkit contains 5 menu items linking to:

i. A brief introductory page to the PharmaSea project and the rationale behind the toolkit

ii. A web page with background info on the legal instruments

iii. A step-by-step guideline for users of MGR to be ABS compliant

iv. A list of biorepositories

v. Resources: an overview of additional useful resources for users of MGR

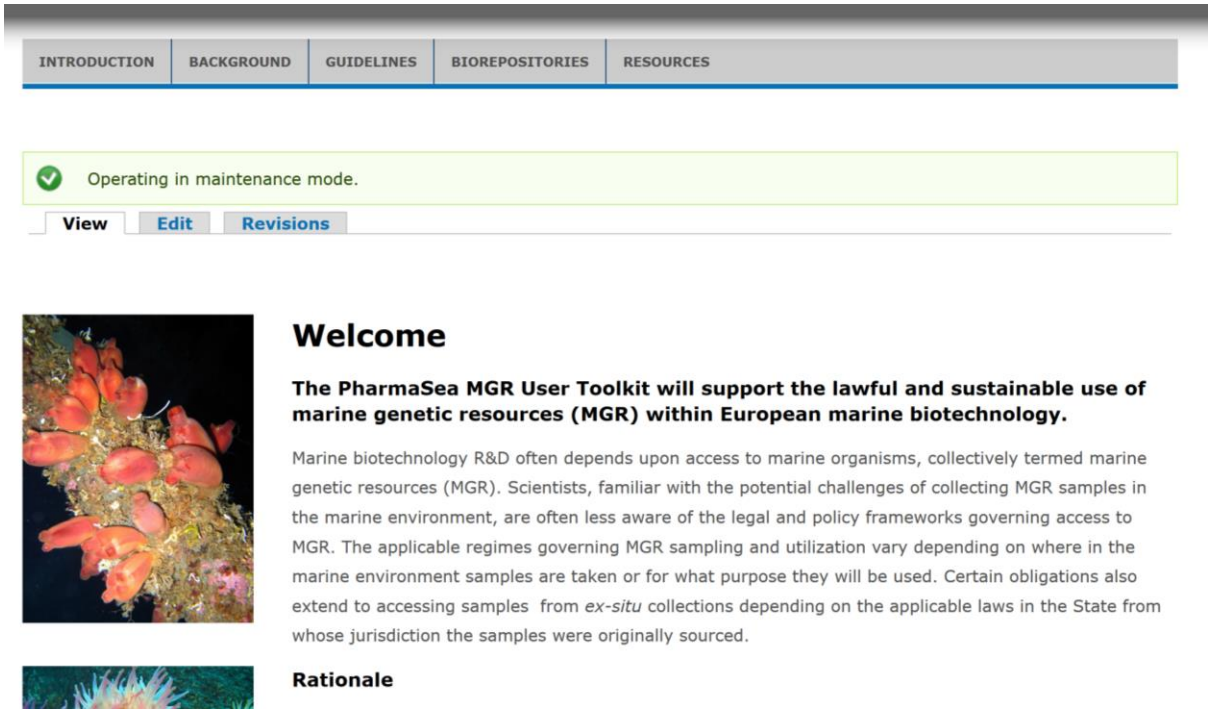


Figure 6. The layout of the PharmaSea web-based toolkit.

3.1.7) Disseminate of project outputs to relevant end users via targeted means.

A communication concept was set up in the beginning of the project and helped to disseminate project results in a targeted manner.

On the basis of the overall communication plan, a strong core branding for the PharmaSea project was created, with a logo and branding style and templates for all external communications. Communication material such as flyers, posters, brochures and slide-presentation templates were distributed to all partners and were accessible on the PharmaSea website to foster a uniform appearance of the project and increase the recognition value of the project.

The external communication of PharmaSea addressed the general interested public (consumers, politicians) and the professional public (science community, industry). The actual results of the project work were communicated embedded in information about current challenges facing society, the use of renewable resources for different purposes and, more specifically, the potential of marine resources for drug research and the advancement of new therapeutic and diagnostic approaches.

The main communication instruments were the PharmaSea website, news, press releases and events. All of these instruments were used in an integrated manner. The PharmaSea website was established as an information platform to present the overall objectives of the research network, the participating partners in science and industry as well as results of the different research projects conducted under the roof of the project. Press releases and news proved to be suitable communication instruments to raise awareness of the project and to stress major progress, as well as highlight results. Participation at events proved to be a good tool to inform and engage with the professional public. Flyers, leaflets and posters supported the dissemination activities. The general public was reached through coverage by general media, such as CNN, BBC or die Deutsche Welle and the project video.

In total, more than 70 media impacts were achieved, including printed, news-channel, general television and others. The PharmaSea team's efforts were recognised by the CommNet Impact Award for best Citizen Engagement. The PharmaSea video was produced in the 6-months' extension period, to summarise the project and its achievements. In addition, members of the consortium organised direct engagement and dissemination events with members of the public and other stakeholders. Several partners including UNIABDN, CNR-IBP and MEDINA were involved in a range of public engagement events such as schools lectures, European Researchers' Nights and other open days.

Overall, the project succeeded in reaching the general public, as can be seen from the numerous articles and interviews in the general media, as well as the professional public, as can be seen from publications in renowned journals and presentations in front of policy experts, such as at the UN headquarters (see 3.1.6.a and in the Impact section under WP6/7).

3.1.8) Integration with existing EU infrastructure and initiatives relevant to PharmaSea's mission.

a.) *Protocols and a European focal point for PharmaSea and other marine biodiscovery resources (data, strains, extracts, compounds) available to the biomedical, pharmaceutical and agrochemical research communities.* A discussion was held with the director of EU-OPENSCREEN Dr Ronald Frank on 22 June 2015 to determine whether PharmaSea's extract and compound libraries as well as associated data could be maintained in OPENSCREEN facilities beyond the lifetime of PharmaSea. The main issues with incorporating the PharmaSea extract/fraction and compound libraries with OPENSCREEN revolved around the inability of OPENSCREEN to accept extracts/fractions and that pure compounds needed to be provided in quantities that were potentially unattainable for natural products. In addition OPENSCREEN was developed as a non-commercial platform and this might not meet the commercial end goals of PharmaSea. It was therefore decided not to pursue the discussions with OPENSCREEN, but rather to find other avenues to explore. At the final PharmaSea GA, a representative of EMBRIC (European Marine Biology RIC) mentioned that they will accept biological, gene and extract materials as well as pure compounds. Since there are a number of extracts/fractions and compounds that are still being actively pursued by PharmaSea partners after the end of the project, it was decided that discussions with EMBRIC will continue for 12 months after the project end date.

3.2) Overcoming the Bottlenecks:

3.2.1) Access to bioresources

a.) *Develop deep sea sampling technology.* The innovative Deep Water Fibre Rope Deployment and Recovery System by DeepTek, was not completed. The main components of the system were assembled, apart from the reeler control and system integration and testing. The deployment of the system did not take place in the Chilean waters of the Atacama Trench as planned, mainly due to a limited availability of suitable vessels to charter and consequent prohibitive costs, as well as medical and financial problems suffered by DeepTek. DeepTek subsequently withdrew from the project after month 30. PharmaSea has nevertheless been able to obtain samples from the unique environment of the South Shetland Trough (5200 m deep, -2^{o}C) in the sub-Antarctic. This was facilitated by several PharmaSea partners taking part in the Eurofleets II 'PharmaDeep' expedition, allowing deep sediment coring and plankton collections which were delivered to partners in April 2016.

b.) Develop legal framework for EU-wide A&BS. The Nagoya Protocol of the Convention on Biodiversity (CBD) provides a legal framework for implementing the third objective of the CBD, i.e. access to genetic resources and the sharing of benefits arising from the use of genetic resources. The Protocol entered into force in October 2014. All Parties to the Protocol are obliged to monitor the use of genetic resources within their jurisdiction. Under the Protocol, access to genetic resources is subject to the prior informed consent of the Party providing the resources, unless that

Party determines otherwise. The process of signing and ratifying the Nagoya Protocol in the EU was already well advanced at the start of PharmaSea and members of the consortium took part in joint discussions with other EU FP7 consortia and PharmaSea convened its own workshops on the matter. Representatives from MicroB3, PharmaSea, SeaBioTech and BlueGenics participated in a stakeholder workshop organised by MicroB3 in Feb 2013. The aim of this workshop was to present a model material transfer agreement (MTA) which had been prepared by the MicroB3 partners. This model MTA aimed to provide a legal framework to help facilitate the transfer of samples obtained during the MicroB3 Ocean Sampling Day and could be further adopted to facilitate sample transfer in a Nagoya compliant way.

The first meeting of the PharmaSea Advisory Panel of Policy and Legal Experts (APPLE) took place in Vigo Spain in Sept 2013 with the main topic being Access and Benefit-sharing of Marine Genetic Resources within national jurisdiction. There was general agreement that the Nagoya Protocol will be positive for biodiversity-based R&D. It will facilitate access to genetic resources, provide the R&D sector with legal certainty and encourage the use of benefits for the conservation and sustainable use of biodiversity. However, the consensus of the APPLE was that PharmaSea's efforts were best focused on access and benefit sharing of marine genetic resources in areas beyond natural jurisdiction (ABNJ) and subsequent APPLE meetings included this topic as well as reviews of the Case Studies and their progress. The APPLE consensus led to PharmaSea's involvement as part of the IUCN delegation to the UN Preparatory Committee discussing the conservation and sustainable use of biodiversity in areas beyond natural jurisdiction. Several of the concepts put forward by PharmaSea, such as the use of a notification-based access regime, have found traction with several key States, and a possible agreement on this principle could prove to be key for fostering marine scientific research in Areas Beyond National Jurisdiction.

c.) *Resolve IP issues inherent in different legal regimes.* The main conflict in this regard is between the UN Convention on the Law of the Sea (UNCLOS) and the World Patent Treaty. UNCLOS clearly states that "no state shall claim sovereignty over any part of the area or its resources" with the area being defined as the seafloor in ABNJs and that this is contrary to the idea of protecting intellectual property. In addition living resources (marine genetic resources) are not mentioned in UNCLOS. These issues were discussed at the second APPLE meeting in Leuven in May 2014 on "Options for an Access and Benefit-Sharing Regime for Marine Genetic Resources from Areas Beyond National Jurisdiction", but no concrete suggestions were made regarding the inherent IP differences between these legal regimes. However, the report from this meeting was used to develop the PharmaSea '*Mare Geneticum*' concept which was presented at the UN Preparatory Committee to allow them to generate evidence-based policy for incorporation into the UNCLOS implementing agreement on biodiversity in areas beyond national jurisdiction. This concept would allow a pragmatic solution to access and benefit sharing for Marine Genetic Resources in areas beyond national jurisdiction based on current scientific best practice.

d.) *Produce best practice guidelines.* EU Regulation 511/2014 notes that best practices need to be put in place to ensure effective implementation of the Nagoya Protocol and that users need to develop these to achieve appropriate due diligence measures. The intention of the PharmaSea Best Practice Guidelines is to set out a series of steps for marine biodiscovery that will inform and support the process of sampling MGR and the broader one of bioprospecting, especially for early career researchers. The approach taken by PharmaSea is therefore to provide overall guidance based on understanding of ABS and policy, and practical points gained from field experience. The principles and practices set out are designed to fully support those in the field of marine biodiscovery in complying with ABS legal and ethical requirements, and aim to guide users through negotiation of Prior Informed Consent (PIC) and Mutually Agreed Terms (MAT) with Providers. PharmaSea's Best Practice Guidelines apply to Marine Genetic Resources (MGR) that are accessed, i.e. acquired from a Provider Country, after the entry into force of the Nagoya Protocol (NP). It is important to note that additional national or regional laws might have been in place and

applicable before 2014 and these need to be respected by all users in the biodiscovery process. Legislation might also exist that requires a scientist to acquire specific additional permits, for instance to sample in national parks or protected areas. For these reasons, before undertaking any biodiscovery or bioprospecting activity, the full legal requirements of the Provider Country/ies must be checked. The PharmaSea Best Practice Guidelines give practical guidance for the day-to-day work of Users of MGR and their institutions. The Best Practice will be available online on the PharmaSea User Toolkit in a very user-friendly format.

3.2.2) Quality of marine resources

a.) *Habitat selection leading to novel microbial strains.* At UCC 154 bacterial isolates were obtained from 4 deep sea sediment samples (at depths of 1214m, 1480m, 1880m and 2400m). Sediment samples were collected from the Barents Sea during the Barkut expedition at different locations and depths using a multicorer device. Samples were also obtained from the PharmaDEEP EuroFleets cruise to the South Shetland Trench on the Spanish Vessel BIO *Hesperides* involving five sampling stations from 2000 to 5200m in deep and cold water habitats, using a multitude of sampling equipment. Four sediment samples were collected from the top layer of gravity core, which was sampled on 2000, 3000, 4000 and 3500 m, respectively.

b.) *Selective cultivation leading to novel microbial strains.* At SZN Culture conditions were developed for different phytoplankton species that had previously displayed anti-grazing or anti-proliferative activity on crustacean copepods. These conditions involved fermentation/growth, in semi-continuous batch cultures on a 12:12 h light:dark cycle. At IBP-CNR growth conditions were developed for *Pseudoaltermonas* strains of Antarctic origin involving growth under both nutrient rich and depleted conditions and at different temperatures.[10] At UCC three different inoculation methods involving dry-stamping, heat-treated liquid inoculum and non-heated liquid inoculum on 3 different media together with media supplemented with antibiotics were employed to culture microbial strains from deep sea sediments and sponges. For the sediment samples from the Barents Sea, two different isolation growth media were used at two different temperatures, increasing the salt concentration, from 3% to 25% in order to select for halophiles. Single colonies were selected on the basis of different morphology, color and size. From a total of 9 sea-sediment samples 75 bacterial and 10 fungal strains were isolated. For samples from the PharmaDEEP cruise four different growth media were employed yielding a large number of strain, many of which will be followed up after the end of PharmaSea. An advanced isolation technology, based on the MicroDish Culture Chip (MDCC), was used in collaboration with MicroDish, Netherlands to cultivate 40 bacterial strains from Antarctic sediments. The MDCC contains a large number of miniaturized “Petri Dishes on-a-chip” made out of porous aluminium oxide (PAO) with a pore size of about 0.2 µm each of which is inoculated with microorganisms. When it is placed in contact with environmental sediments, an exchange of nutrients and signal molecules occur, allowing the isolation of previously uncultured organisms on the chip. Using this system strains belonging to different species (*Psychrobacter, Pedobacter, Gelidobacter, Micrococcus, Shewanella, Pseudomonas, Aequorivita),* were identified. This work involved a collaboration with MicroDish who were a partner in the MaCuMBA project, to improve the cultivation methodologies in PharmaSea. A selection of strains isolated during the PharmaSea project is shown in Figure 7.

Figure 7. A selection of strains isolated during the PharmaSea project

c.) *Phylogenetic analysis of strains to ensure diversity/quality.* At MEDINA a total of 174 strains were selected based on 16S rRNA sequence, geographic origin, sample type (sediment, type of marine invertebrate) and/or the isolation method. These strains were collated and subjected to fermentation in a suitable selection of marine media according to their taxonomy. At UCC phylogenetic affiliations of 70 of the deep sea isolates were determined by 16S rRNA analysis. Phylogenetic analysis of the microbiome of three deep sea sponge species, *Inflatella pellicula*, *Poecillastra compressa* and *Stelletta normani* was performed.[21, 22] Phylogenetic trees constructed from the sequences obtained for several non-ribosomal peptide synthase biosynthetic gene cluster domain fragments shows the novel hidden biological potential of microbial populations associated with these deep sea sponges. These gene fragments form clades which were clearly distinct from those of known antibiotic related gene clusters. This indicates that potential novel biodiversity with respect to marine natural products is likely to be present in these deep sea sponge microbiomes.

d.) *Genome scanning to uncover biosynthetic capacity of strains.* A number of *Streptomyces* genomes together with several *Pseudoalteromonas* genomes were *"*mined" for biosynthetic gene clusters using antiSMASH (https://antismash.secondarymetabolites.org).[23] Thirteen *Streptomyces* spp. strains isolated from shallow water and deep-sea sponges that display antimicrobial activities against a number of clinically relevant bacterial and yeast species were analysed at UCC. Draft genomes were assembled and the genomes were compared to each other and to publicly available *Streptomyces* spp. genomes from terrestrial sources. The strains appear to host abundant secondary metabolite gene clusters which encode polyketides, non-ribosomal peptides, siderophores, bacteriocins and lantipeptides.[24] Many of the secondary metabolism gene clusters show high degrees of novelty with 40% of clusters identified showing no similarities to known clusters in the antiSMASH database. Three *Streptomyces* strains isolated from deep sea sponges in particular appear to be enriched with gene clusters encoding non-ribosomal peptide synthases. Genome scanning was also performed on eighteen marine *Streptomyces* strains, which possessed potent bioactivity profiles at UWC. Subsequently a number of biosynthetic pathways were identified and selected for heterologous expression using the Transformation-Associated recombination cloning strategy involving *Saccharomyces cerevisiae.* Genome scanning of *Streptomyces* sp strain 219807 identified the gene cluster responsible for the biosynthesis of halichoblelide D,[11] while scanning of *Streptomyces qinglanensis* strain 172205 predicted 28 gene clusters related to secondary metabolites. Clusters for enterocin, qinlactone A, qinlactone B and qinlactone C were targeting isolated and identified by using the OSMAC, high expressed gene cluster deletion strategies.

Genome-scale models were also employed to maximize production of metabolites in *Salinospora* tropica CNB-440. A manually curated *genome-scale* metabolic model for *Salinispora tropica* strain CNB-440 was constructed (Figure 8). This enabled characterization of the metabolic capabilities

for understanding and modeling the cellular physiology of the strain. The genome-scale metabolic model, which was based on physiological and biochemical information of primary and specialised metabolism pathways, was used to study strain-specific capabilities in defined minimal media involving 41 different minimal growth-supporting environments. The incorporation of modifications led to increased accuracy in predicting the outcome of growth/no growth experiments. This model can thus be used to define the metabolic capabilities of *S. tropica*, guiding and enhancing the production of specialised metabolites such as Salinosporamide A and B as well as Sporolide A and B. A generic model that can be used for all known *Salinispora* and also two specific models for *Salinispora arenicola* CNH-643 and *Salinispora pacifica* CNR-114 (iCC926) was also constructed that contained the genes shared by all 92 sequenced strains and a few non-conserved genes associated with essential reactions. *In silico* growth predictions were simulated using the models on different carbon, nitrogen, phosphorous, and sulfur sources, with over 450 different growth-supporting environments being analyzed using phenotype microarray data. The models which have been developed can be used to systematically analyze the essential growth capabilities of *Salinispora* metabolism that delineate the adaptation process and aid researchers to guide and enhance the production of specialised metabolites. In addition the full-scale Genome Scale Models can also be adapted to other Actinomycetes.

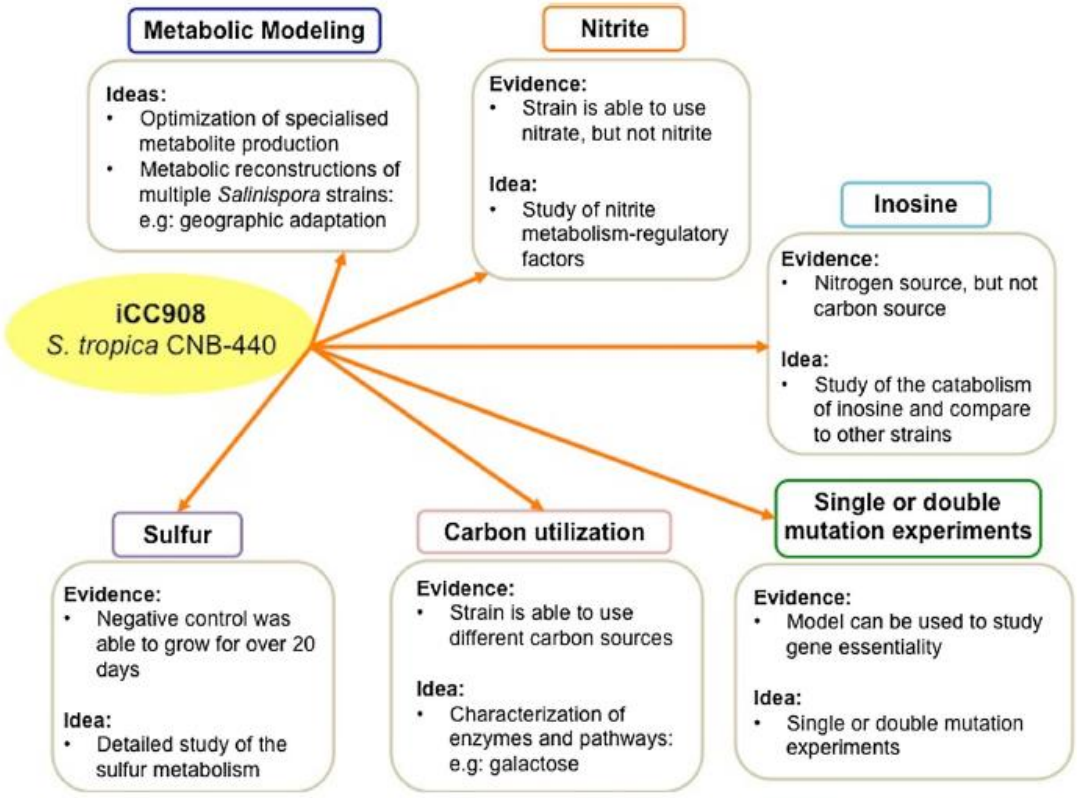


Figure 8. The development of the full genome-scale metabolic model for *Salinispora tropica.*

3.2.3) Extract generation

a.) *Media development to realise biosynthetic capacity of strains.* Isolation media were developed for the isolation of microbial strains from deep-sea sediment samples.[25] Oligotrophic media together with colloidal chitin as a carbon source were employed. Addition of antibiotics such as nalidixic acid was used to enrich for Gram-positive isolates, while novobiocin was used to enrich for actinobacterial isolates. Plates were incubated for up to two months at 28°C and 70% humidity. A total of 138 unicellular strains were isolated in this fashion and their axenic condition checked. For

other deep-sea sediment samples growth media used included marine broth amongst others. Filtered seawater was included into the broths with nystatin and cyclohexamide being added in some media to avoid fungal contamination and streptomycin to other media to prevent bacterial growth. To enrich for halophiles from sediment samples, growth media with increasing percentages of NaCl were employed (up to 30%). Additionally for deep sea sponge and sediment samples bacteria were isolated on media containing starch, yeast extract, peptone artificial seawater and fungi were isolated media composed of yeast extract, peptone, glucose-artificial seawater. Two plating methods were employed (a) plating serial dilutions of the samples (b) stamping dessicated samples onto petri dishes.

b.) *Stress/elicitation to realise biosynthetic capacity of strains.* Growth media was developed for numerous microalgal species, including diatoms and flagellates. These media included nitrogen- and phosphate –starved media to mimic stress conditions, which are known to induce secondary metabolite production. Cultures were kept in a climate chamber at 19°C on a 12:12h light:dark cycle at 100 µmol photons m^{-2} s^{-1}. These culture conditions were found to influence bioactivity in the majority of the species tested, with anti-inflammatory, anti-infective and anti-neurodegeneration activities varying depending on the specific culture conditions employed.

c.) *Heterologous expression of biosynthetic genes.* Three different heterologous expression systems were developed, namely pCCERI, pQG022 and pET43.1a. These vector systems allow for interspecies conjugation in different hosts, such as *Escherichia coli*, *Pseudomonas putida*, *Streptomyces lividans, Pseudomonas sp, Bacillus subtilis and Streptomyces sp.* Inducible expression of ectoine, hydroxyectoine, prodigiosine, xanthan and carotenoid was achieved. In addition an overexpression system involving the positive regulatory gene *ectR3* involved in ectoine biosynthesis in *Streptomyces* sp. 219808 was also developed to further upregulate both ectoin and hydroxyectoine in this system.

d.) *Extraction technology/Robotics/Automation.* A common library 96-well screening plate format was agreed by the consortium in which 80 samples/plate are included, leaving columns 1 and 12 empty for the allocation of controls and blanks, and distributing samples by columns. A spreadsheet template was also defined to be used to share the information of samples between partners. Semi-automated procedures have been set-up and applied to the solid phase extraction (SPE) of small volume cultures in saline media, and vials, extracts and assay plates have been handled with the help of robotic systems and LIMS traceability (Figure 9).

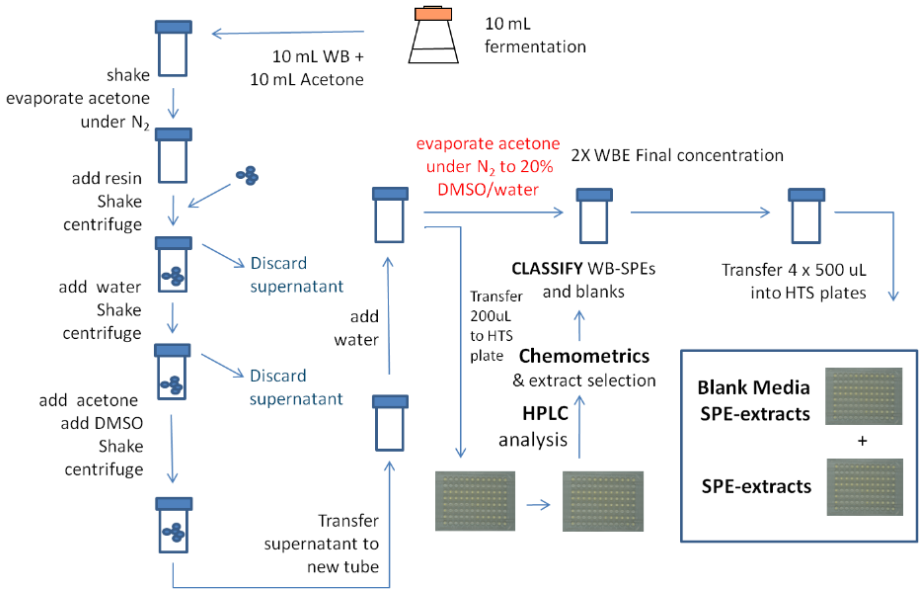


Figure 9. The PharmaSea extraction and sample preparation protocol.

3.2.4) Isolation & Purification

a.) *Explorative solid phase extraction (E-SPE) to develop isolation protocols.* Analysis of the ACD database reported that nearly half of all natural products contain an ionisable functionality. Consequently there is a great potential for incorporating ion-exchange chromatography as an integral part of the separation procedure. Before scaling up separation using semi-preparative ion-exchange columns, small commercial pre-packed solid phase extraction columns containing either anion or cation exchange material can be used to test small amount of aliquots, to see whether the bioactive principle can be retained. The E-SPE approach offers several advantages when dealing with complex extracts. By using a multi-column strategy, including i) cation-exchangers for collection of amine containing compounds, or ii) strong or mixed mode anion exchangers for collection of compounds with carboxylic acids and weaker acids such as phenols, we have managed to design preparative purification steps that selectively retained the given target candidate (and related compounds) or remove unwanted components. Altogether, the E-SPE procedure has been used with great success during the PharmaSea project, such as for the isolation of helvolic acid amongst other target compounds.

b.) *Targeted chromatography.* Preferentially separating target molecules containing a specific element has advantages for the isolation of marine natural products which often contain elements such as sulphur and the halide ions (chloride, bromide, iodide). We exemplified this technology for PharmaSea by focusing on marine natural products containing a sulphur atom. We investigated a range of natural and chemoenzymatically-produced heterocycle-containing cyclic peptides called the cyanobactins.[26] To achieve this we used a unique combination of LC coupled in parallel to electrospray ionisation MS (ESI-MS) and inductively -coupled plasma MS (ICP-MS). ICP-MS is a sensitive analytical tool for elemental analysis with advantages of having species independence and high ionization efficiency for most elements in the periodic table and high sensitivity of parts per billion to parts per trillion levels. Application of ICP-MS allows the quantification of elements independent of their molecular form. Molecular information obtained from ESI-MS enables the compound identification simultaneously with its quantification. We were able to use this methodology for the discovery and quantification of known and new cyanobactins in organism extracts and chemoenzymatic reaction mixtures.

3.2.5) Extract dereplication

a.) *Liquid chromatography-mass spectrometry.* In order to accelerate the isolation and characterisation of structures of new or novel metabolites it is crucial to develop efficient strategies that prioritise samples with greatest promise early in the project so that resources can be utilised in a more efficient and cost-effective way. A strategy developed by PharmaSea accelerates the identification of known compounds in *Streptomyces* extracts that can be applied in the discovery of natural products.[27] The strategy incorporates information in StrepDB, a database of 5,098 known compounds from *Streptomyces* sp. containing the molecular mass, molecular formula, structure and predicted LC retention time (t_R). Crude extracts are screened for these compounds by the LCMS data processing algorithm. Compounds that satisfy both requirements, HRESIMS (high-resolution ESI-MS) and t_R, are colour-coded for easier identification. The database is demonstrated in Figure 10 where it identifies known and unknown compounds in a *Streptomyces* extract. Complementary dereplication approaches were reported by MEDINA[28] and DTU[29].

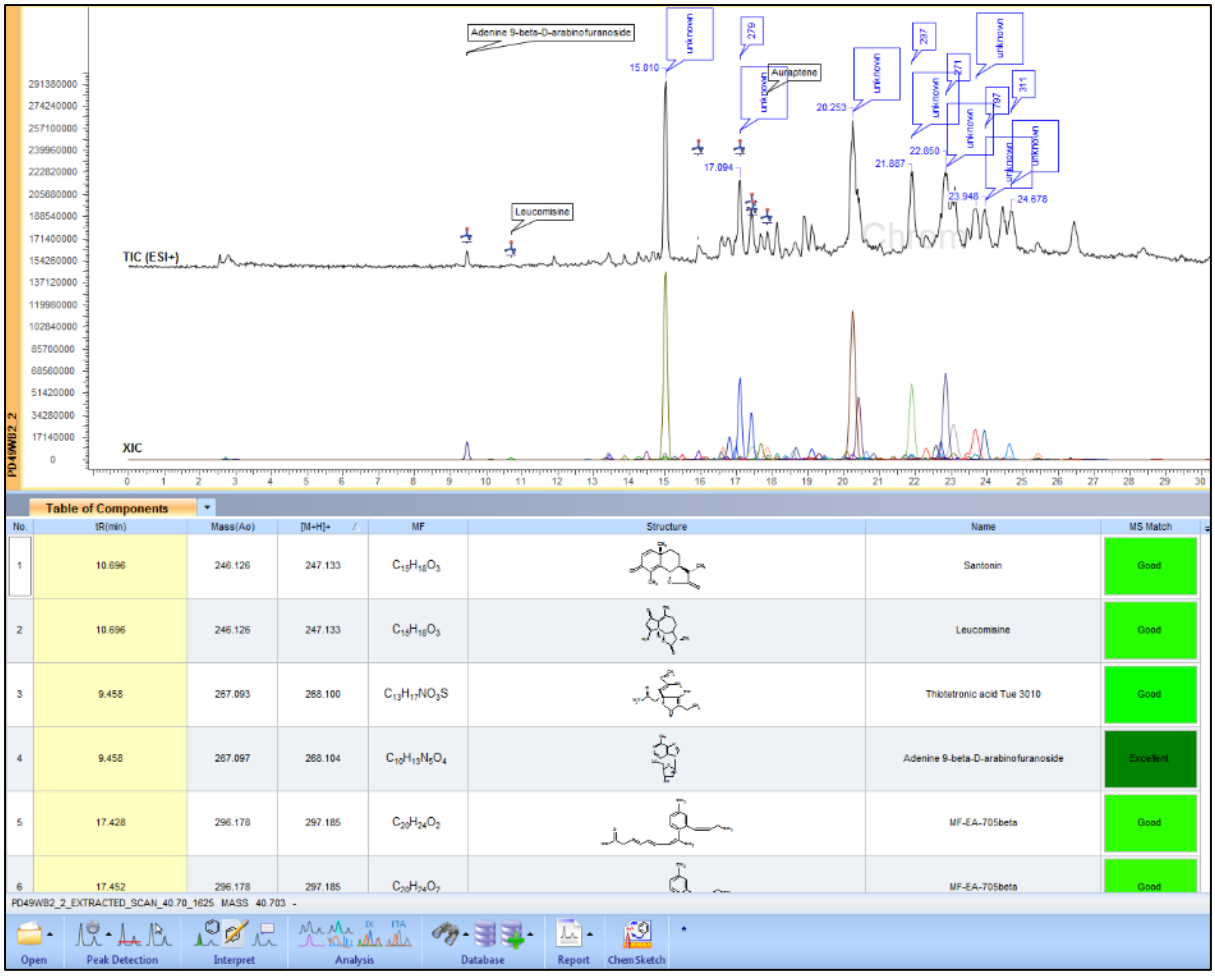


Figure 10. The PharmaSea StrepDB used to identify known and unknown peaks in an LC-MS trace.

b.) *Novelty screening using NMR and MS techniques.* A prioritisation approach has been developed based on the screening of 25,000 marine natural products held in the database MarinLit using HR-LCMS data. Each sample is evaluated and allocated a score by the software algorithm based on number of new masses (novelty), ion intensities (productivity), and compound complexity (spread of LC retention time). Samples are then ranked and prioritized based on these scores.

This approach relied on the identification of **chemical novelty**, using a measure of the proportion of peaks not previously identified, detected in a given dataset at a particular time of analysis; ranges between 0 (not novel at all) and 1 (very novel), and **chemical diversity**. For the latter, a chromatogram containing a number of peaks (denoted i) are searched in ChemSpider. In this example a number of peaks (j) are found in the database whereas the remainder (k peaks) are not found (in all cases i = j + k). For the set of peaks (i) found in the database there will be information on mass, retention time and abundance. This information is then used to calculate diversity by calculating a Shannon index (*H'*) in which each component present in the database is weighted by proportional abundance (p_i) calculated from available height or area values. An example of the use of the PharmaSea Chemical Novelty and Diversity indices is shown in Figure 11.

$$H' = -\sum p_i \ln(p_i)$$

To validate this approach we analysed extracts from 21 marine tunicates and sponges collected from the Fiji Islands to determine if the top prioritized samples yielded new compounds. The results showed the three top prioritised samples yielded 6 new compounds illustrating the effectiveness

of this approach. All structures were elucidated by spectroscopic techniques such as 1D and 2D NMR, MS, MS/MS. Structures were further confirmed by Computer Assisted Structure Elucidation methods (CASE) using the ACD/StrucEluc Suite.

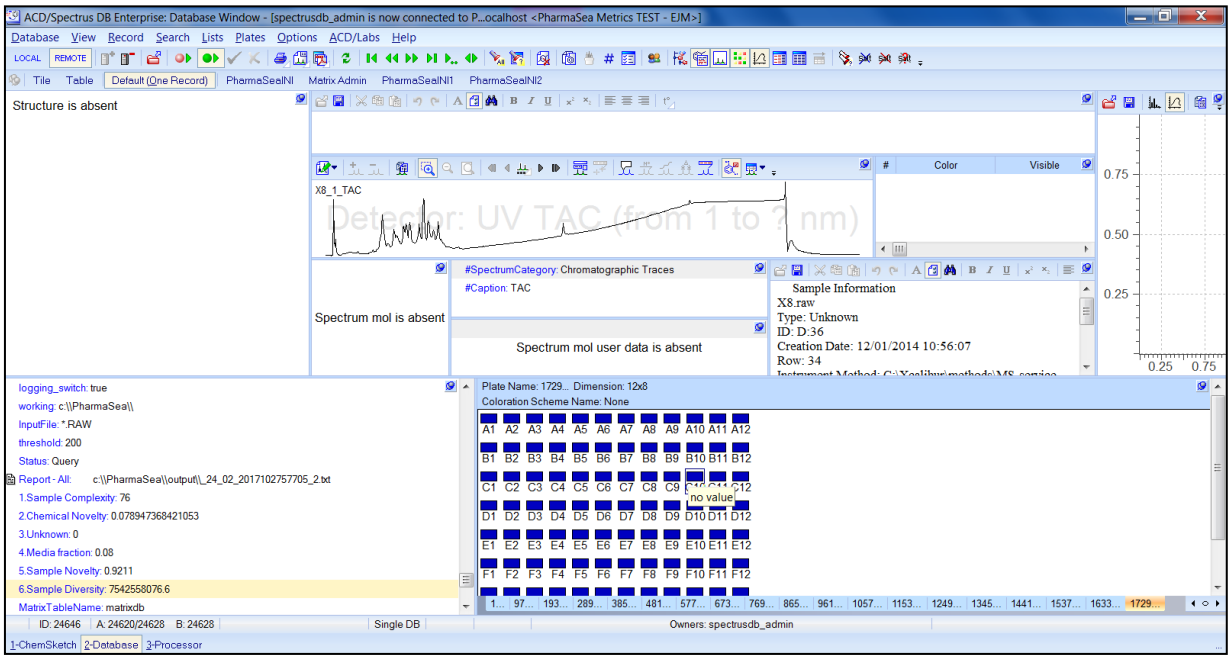


Figure 11. Chemical novelty and chemical diversity analysis on an extract LC-MS dataset.

c.) Datamining using predicted NMR & MS properties. In addition, a spectral database named MBC-DB was generated using ACD/Spectrus DB Platform (Figure 12). MBC-DB contains 665 natural products with structures, experimental HRESIMS, MS/MS, UV, and NMR spectra. StrepDB was used to dereplicate a mutant *Streptomyces albus* extract that led to the identification and isolation of two new compounds, legonmaleimides A and B, the structures of which were elucidated with the aid of MBC-DB and spectroscopic techniques.[27] The structures were confirmed by CASE methods using ACD/StrucEluc. The developed methodology provides and improved pipeline approach to the dereplication of extracts and discovery of novel natural products.

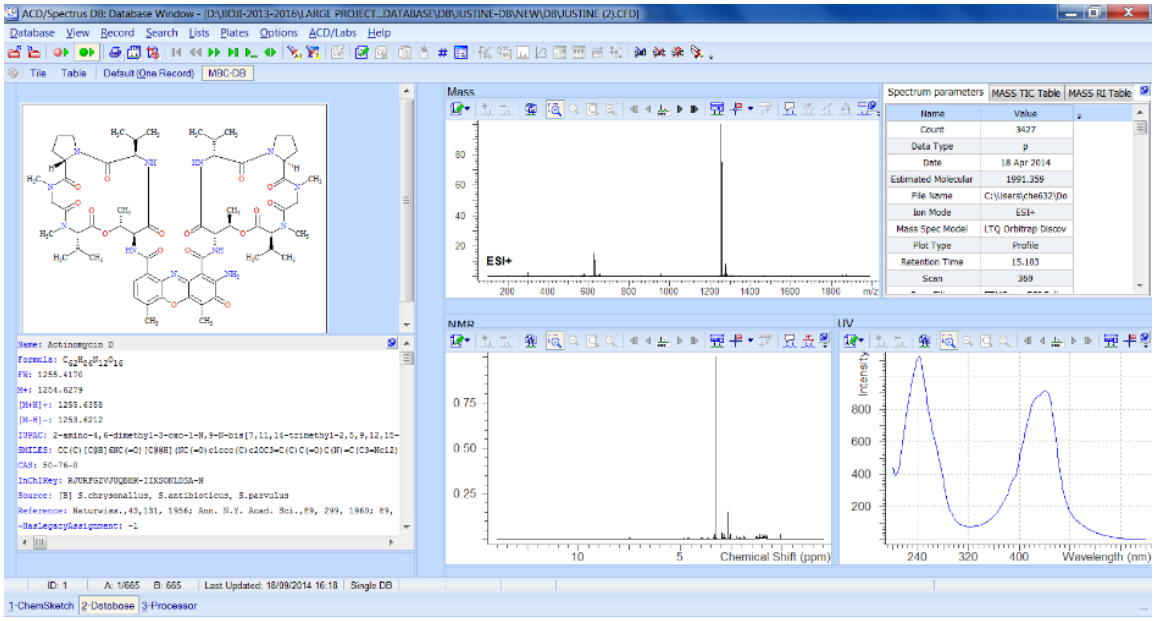


Figure 12. The PharmaSea spectroscopic database showing the searchable data available.

3.2.6) Structure determination

a.) Low volume probes/cryomicroprobes. The availability at MEDINA of a 500 MHz NMR spectrometer equipped with a 1.7 mm low volume micro-cryoprobe has been essential for the dereplication and structural elucidation of new molecules isolated in sub-milligram amounts. The elucidation of the structures of large depsipeptides belonging to the callipeltin family and other small microbial metabolites isolated in amounts as low as 200 μg has been successfully completed employing this technology.[9] Additionally, this approach has also accelerated the process of identification of interesting hits, allowing the early structural characterization of structures with minute amounts of material isolated, and eliminating the requirement of big isolation efforts.

b.) Computer aided structure elucidation. The powerful ACD/Labs StrucEluc Suite was provided and proved a powerful adjunct to solving complex structures rapidly. An example of the software in action is shown in Figure 13. Specific PharmaSea requested modifications were made to the software. These included amongst others: Improved assignment tools; Ability to transfer assignment to spectra of similar structures; A new auto-assignment algorithm takes into account the same parameters as before (shifts, integrals, multiplicity), but adds correlation determined through cross peaks from 2D spectra (COSY, HMBC etc); Ability to show assignment from different atoms at the same frequency; Automatic resolution of ambiguous connectivities; Simultaneous assignment of ^{13}C and ^{1}H for diastereotopic CH_2 groups; New Combined Concurrent verification – the ability to generate most "NMR-similar" structural isomers from a proposed structure, to evaluate them against an NMR dataset and to highlight structural differences between proposed structure and generated isomers; The ability to take multiple substructures supplied by a user; For all generated isomers the software keeps the MF and the number of Cs, CHs, CH_2s and CH_3s the same; The ability to move heteroatoms in cycles and in chain. As a final improvement the HSQC spectrum can be predicted for all generated isomers and is compared with the predicted HSQC spectrum of the proposed structure. The isomers are ranked by HSQC similarity and the software takes the best of them to verify against the NMR dataset. The software was used on a number of occasions to solve complex structures for PharmaSea.

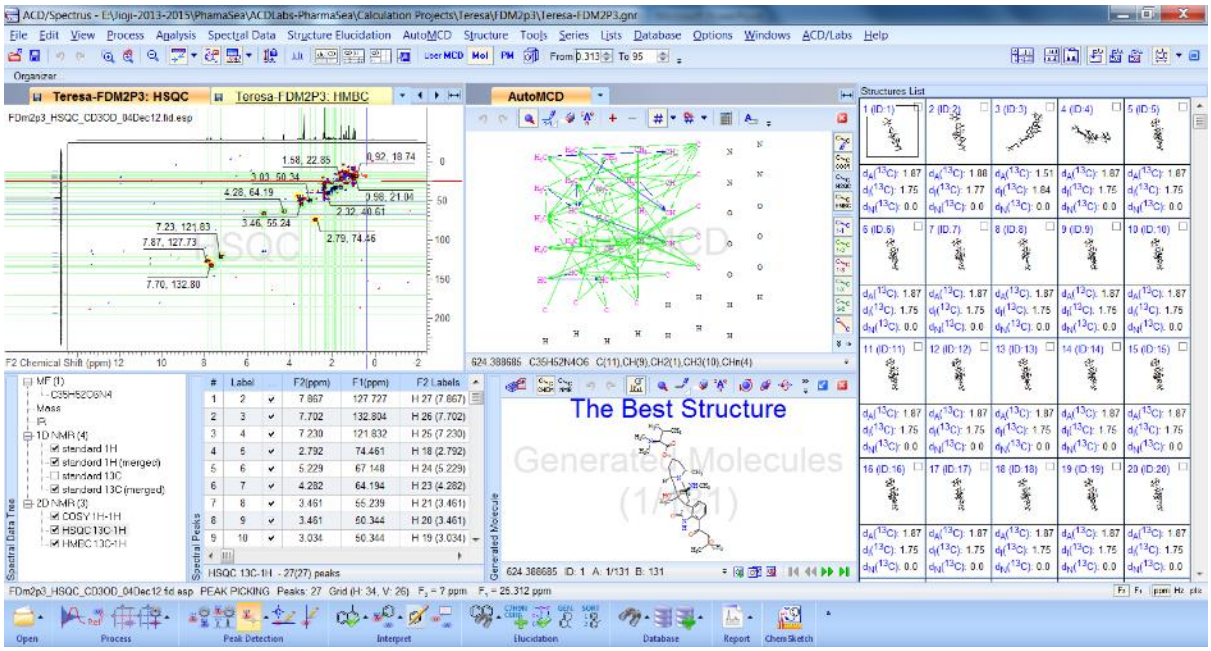


Figure 13. ACDLabs StrucEluc software demonstrated on a compound from a marine derived fungus.

3.2.7) Hit selection

a.) *Innovative MOA screens (zebrafish) & counterscreens.* The original idea behind the innovative mode-of-action (MOA) screen was to use the photomotor response (PMR) assay for rapid target identification of the isolated anti-seizure compounds. To that end, we performed PMR analysis of 767 neuroactive compounds covering 14 different receptor classes using the KNIME workflow. In summary, ligands from the following classes of receptors were observed to induce a distinct PMR phenotype: adrenergics, dopaminergics, serotonergics, metabotropic glutamatergics, opioids, and ion channel ligands. This means that sigma ligands, cholinergics, histaminergics, melatonin ligands, ionotropic glutamatergics, GABAergics, purinergics, and adenosines seem to fail to induce a distinct PMR phenotype despite their strong PMR-modifying effects. These data suggest that PMR is useful for mechanism prediction only within the above first-mentioned neurological pathways. Since the PMR fingerprints therefore did not allow a robust and comprehensive identification of a wide-range of targets, the methodology was not applied in the context of target finding of the isolated anti-seizure compounds. The data were published.[7]

Counterscreens are often used to eliminate false positives from a screen, and ideally this is an assay against another member of the target family under identical assay conditions. In this project, the screening data from the different assay categories (anti-infectives, anti-inflammatory and neuroactive) were used as a tool (counterscreen) to guide the selection of hits. Even though completely different assays were used, the screening results when evaluated simultaneously reveal whether the extract/fractions is a frequent hitter or actually shows a specific bioactivity. Hit selection was the carried out using combined information from the assays and counterscreens prioritising those extracts/fractions and compounds that showed potent and selective activity without off-target effects. Extracts and fractions were then dereplicated using LC-MS and datamining techniques and the combined assay/dereplication results discussed by the PharmaSea prioritisation committee who monitored progress of samples on the list (Figure 14).

b.) *Property prediction of compounds for ADMET/PK/PD.* ACD/Labs provided the Percepta suite of software which is able to predict relevant ADMET/PK/PD properties. Development work on the physicochemical prediction modules continued at ACD/Labs during the PharmaSea project, implementing improved features relevant to the research work for PharmaSea. This software allowed the prediction of lipophilicity (logD and logP values) and ionization constants (pKa), important parameters to estimate the molecule behaviour in different environments. Three different mechanisms of log*P* prediction were available (Classic, GALAS, and Consensus) as well as two different approaches to estimate pK_a values (Classic and GALAS) and any of these can be selected as default algorithms for corresponding log*P* and pK_a calculations used to derive log*D* values. These multiple options, each with its own strengths and limitations, maximized flexibility and user choice. The software was not used to prioritise compounds, as rapid *in vitro* assays were available at two partners to accurately define the ADMET/PK/PD properties of the compounds prioritised for animal studies.

c.) *Rapid in-vivo evaluation*. PharmaSea developed a zebrafish-based drug discovery platform employing an hyphenated PMR/AST (photomotor response/anti-seizure testing) paradigm to screen marine natural products for promising anti-seizure activity. This discovery platform allowed us to test thousands of extracts, fractions and compounds in a short period of time. This allowed PharmaSea to prioritize samples of interest in a short period of time, selecting highly promising compounds for further testing in preclinical mouse models. Antioxidant and Alzheimer´s disease human cell models were used as a first screening for compounds. Highlighted compounds from these two models were tested for cardiotoxicity in a rapid automatic platform for electrophysiological measurements in human transfected cells. The use of these cellular models allowed us to minimize the number of compounds to be tested in the animal models.

CHEMISTRY_ID	ASSAY_CATEGORY	ASSAY_NAME	DEREPLICATIO	SCALE_UP	ISOLATION	BIOACTIVITY PROFILE
SK0107	NEUROACTIVE	PMR-ASSAY				
BMR_1a, BMR_1E and MBR_1J	ANTICONVULSANT	PTZ-assay				
MDNBC-04359218F	ANTI-INFECTIVE	Abs_MB5393 (MRSA)				
MDNBC-04349594C	ANTI-INFECTIVE	Abs_MB5393 (MRSA)				
MDNBC-04330831T	ANTI-INFECTIVE	Abs_MB5393 (MRSA)				
MDNBC-04366071T	ANTI-INFECTIVE	TUBERCULOSIS/ MRSA				
MDNBC-04451741N	ANTI-INFECTIVE	TUBERCULOSIS/ MRSA				
MDNBC-04496513D	ANTI-INFECTIVE	TUBERCULOSIS/ MRSA				
MDNBC-04349590K	ANTI-INFECTIVE	TUBERCULOSIS/ MRSA				
01STGROWTH37-1	ANTI-INFECTIVE					
MDNBC-04849719P	ANTI-INFECTIVE	TUBERCULOSIS/ MRSA				
MDNBC-05165354J	ANTI-INFECTIVE	TUBERCULOSIS/ MRSA				
KB13-8-ZBA-F04	ANTI-INFLAMMATORY	TNF_THPAIF				
KB12-38-ZBA-C06	ANTI-INFLAMMATORY	TNF_THPAIF				
PE08-15-ZBA-H7	ANTI-FUNGAL					
UCC_E128_B_03	ANTI-INFLAMMATORY	TNF_THPAIF				
CHEMFE2/1	ANTI-INFLAMMATORY	TNF_THPAIF				
X0127A-1-04	NEUROACTIVE	PMR-ASSAY				
UCC_E104	ANTI-INFECTIVE	ABS_MB2884 (E.COLI)				
UCC_E360	ANTI-INFECTIVE	ABS_MB2884 (E.COLI)				
USCF025B	ANTI-INFLAMMATORY	TNF_THPAIF				
X0613A-1-05	ANTI-INFLAMMATORY	TNF				
MDNBC-04346468C	ANTI-INFLAMMATORY	TNF_THPAIF				
DONE						
ONGOING						

Figure 14. Current prioritization list

3.2.8) Supply issue

a.) *Use of microbial strains*. Over 13,689 strains have been worked on, including over 9,849 legacy strains have been used together with and a further 3,840 strains from sediments and marine sponges collected by PharmaSea partners in deep and cold environments. Existing collections include bacterial, fungal and microalgal strains from a range of locations, such as South Africa and Argentina, the Arctic, Antarctic and deep water sediments and sponges in deeper waters in the EEZ of the Republic of Ireland. For the new strains isolated, the highlights are 1285 isolates from South African sediments which have yielded more than 30 with anti-bacterial activity. Nearly 1000 strains have been isolated from Antarctic sediments from which 20 isolates were identified as strongly antibacterial. Genome sequencing of over 35 Actinobacteria has resulted in the identification of a large number of potential biosynthetic clusters, in addition to potential clusters present in the genome sequences obtained for another ca 3000 strains. The microbiota of four individual deep water sponges has been analysed, and are being mined *in silico* for the presence of secondary metabolite gene clusters and metagenomics clones are being expressed in a surrogate host.

b.) *Process intensification*. Medium scale fermenters and strategies were available at some partners (c-Lecta, MEDINA) for use in the scaling-up of the production of interesting hits for further biological testing. Media were manipulated to obtain optimal production of the desired compounds. Fortunately, the amounts required of the most interesting hits were obtained from cultures performed in flasks at a scale of 1-3 L

c.) *Scale-up in saline media* A total of 52 fermentations were performed for the scaled-up production of those extracts identified as bioactive in assays performed in WP3 included in the anti-infective, anti-inflammatory and neuroactivity panel. Extracts included in this process were selected using the dereplication tools and approaches developed in WP4 by the partners DTU, MEDINA and UNIABDN, and contained molecules that were identified as new natural products or, alternatively,

known compounds with previously unreported bioactivity. Media composition, pH, temperature, and aeration conditions employed in the small-scale production of extracts were adapted to the new fermentations. The production in the scaled-up cultures of the original molecules found in the original extracts was confirmed by LC/HRMS. Extraction of these new fermentations was adapted to the particular fermentation conditions for marine microorganisms (saline media) with regular use of SPE resins to capture the metabolites of interest. Most scale-up fermentations could be performed in shaker flasks and the use of larger scale fermenters was not required.

d.) *Heterologous expression.* Three different heterologous expression systems have been developed. One system, developed at UCC, involves the multi-host fosmid pCCERI which is based on a BAC/fosmid shuttle vector. It contains an *ori*T which allows interspecies conjugation in three different hosts, namely *Escherichia coli*, *Pseudomonas putida* and *Streptomyces lividans.* The next system developed by c-LEcta was based on a commercial BAC system involves pQG022 which contains selectable hygromycin and kanamycin markers together with different origins of replication which allows interspecies conjugation in four different hosts namely, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas* sp and *Streptomyces* sp. Inducible expression of ectoine, prodigiosine, xanthan and carotenoid has been achieved with different constructs. Finally a pET43.1a vector system was developed in WHU, which facilitated the heterologous expression of both the ectoin and hydroxyectoine biosynthetic gene clusters following cloning in *Escherichia coli* BL21 (DE3) and subsequently transfer to *Streptomyces lividans* TK24. In addition an overexpression system involving the positive regulatory gene *ectR3* involved in ectoine biosynthesis in *Streptomyces* sp. 219808 was also developed to further upregulate both ectoine and hydroxyectoine in this system.

3.2.9) Uptake of technology

a.) *Data management.* The implementation of the laboratory information management system 'NAPIS' allowed the tracking of all materials through the biodiscovery pipeline. A spreadsheet template was used to input and curate data to NAPIS. The database NAPIS Enterprise was installed at UiT, and UiT uploaded data to the database through Citrix clients. Partners are able to view data in PharmaSea-NAPIS through a web-interface (OpenNAPIS), and key personnel from all relevant partners were added as users to OpenNAPIS. Protocols were prepared for the partners to submit data to UiT, who in turn validated the data and uploaded them to NAPIS. The system was fully operational at the end of the first reporting period, allowing retrieval of information from samples and screening results for all partners of the consortium throughout the project period. Data management via NAPIS was essential to permit the PharmaSea prioritization committee to make reliable decisions regarding which extracts/fractions and compounds should progress to the next stage of the screening cascade.

b.) *Patentbox.* The PatentBox concept, as envisaged by the original project description, was the intention to create a pool of intellectual property in those cases where project partners did not wish to exercise their right of first access. This is a speculative concept dependent on generating IP over and above whatever would be taken forward by the consortium and its immediate exploitation contacts. In the event, the PatentBox concept was not needed.

c.) *End User Panel.* The End user panel was intended to bring together a select group of contacts in industry who could first of all advise on the exploitation potential of project achievements, and second, be candidates for taking forward commercial opportunities. In the event, the Industry Advisory Group members were able to provide general internal advice, and the actual outputs of PharmaSea with commercial potential did not need this broad approach to decide how best to take them forward. A community list was developed as a deliverable for use within the consortium. This includes contact details of almost 1000 potential interested parties and remains available for use by project partners in connection with developing and exploiting the PharmaSea outcomes in future.

d.) *Asset Inventory.* A format for an inventory of assets/outputs was developed, in the event that the project outcomes could be widely disseminated to gauge attractiveness to industry and other potential partners during the lifetime of the project. The actual outputs of the project, including organisms, extracts, chemical data, software for integrating analytical data, characterized molecules and bioactive leads, did not however require this approach during the project. The format was adapted for use in the final reporting of outputs and achievements.

e.) *Targeted technology transfer briefs.* Targeted technology transfer briefs were an intended output, in the event of needing to inform interested parties about the availability of licensable project outcomes. The actual output of the project did not however need these during the project period. A format for disseminating information is available for future use.

4.) The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results.

WP1 Bioresources:

A number heterologous expression systems have been developed, which will facilitate the future production of gene clusters encoding novel small molecules or molecular scaffolds in *Escherichia coli*, *Pseudomonas putida*, *Streptomyces lividans* and *Bacillus subtilis* hosts. An overexpression system involving the positive regulatory gene *ectR3* involved in ectoine biosynthesis in *Streptomyces* sp. 219808 has been developed to further upregulate both ectoine and hydroxyectoine in *Streptomyces lividans.*

New growth media has been developed for microalgal, bacterial and fungal strains, together with the development of a novel MicroDish Culture Chip (MDCC) system to cultivate bacterial strains from Antarctic sediments. This work involved a collaboration with MicroDish who were a partner in the MaCuMBA project, to improve the cultivation methodologies in PharmaSea.

Genome mining approaches have been employed to identify a number of biosynthetic gene clusters in marine *Streptomyces* strains that display antimicrobial activities against a number of clinically relevant bacterial and yeast species. Up to 40% of clusters identified showing no similarities to known clusters indicating potential novel chemistry. A number of biosynthetic pathways have been identified and selected for heterologous expression using the Transformation-Associated recombination cloning strategy involving *Saccharomyces cerevisiae.* Heterologous expression of these clusters is likely to uncover novel small molecule chemistry.

Genome-scale models have been developed to maximize production of metabolites in *Salinospora tropica* CNB-440. The model system which was based on physiological and biochemical information of primary and specialised metabolic pathways was used to study strain-specific capabilities in defined minimal media, involving 41 different minimal growth-supporting environments. This model can be used to define the metabolic capabilities of *S. tropica*, guiding and enhancing the production of specialised metabolites such as Salinosporamide A and B as well as Sporolide A and B.

A generic model that can be used for all known *Salinispora* and also two specific models for *Salinispora arenicola* CNH-643 and *Salinispora pacifica* CNR-114 (iCC926) has also been constructed. The models which have been developed can be used to systematically analyze the essential growth capabilities of *Salinispora* metabolism that delineate the adaptation process and aid researchers to guide and enhance the production of specialised metabolites. In addition the full-scale Genome Scale Models can also be adapted to optimize production of other interesting small molecules in other Actinomycetes.

WP2 Libraries:

Semi-automated methodologies for the preparation of microbial extracts were established and implemented for the creation of a central repository of marine microbial extracts and library containing more than 15,000 extracts and fractions has been created (Figure 15). It includes extracts obtained from a wide taxonomical diversity of microbial strains, covering marine fungi, bacteria, actinomycetes, dinoflagellates and diatoms. The library is annotated with data on bioactivity in antimicrobial, anti-inflammatory and anticonvulsant hits. Additionally, bioactive hits have been chemically profiled and dereplicated by LC/MS and data on the composition of each individual extract are also available.

A copy of these extracts and fractions is stored in a central repository created at KULeuven. They are prepared in 96 deep-well plates, following a format agreed at the beginning of the project by all partners. Plates are available for further biological testing by all the consortium partners and/or other EU screening initiatives and traceability to the original microorganism, culture media and conditions used in the original fermentation, bioactivity and chemical profiling data of bioactive extracts is secured through the information stored in NAPIS. Some of the strains and biological activity data will be the starting point for future research in the area, carried out under the Ocean Medicines and MarPipe H2020 EU funded projects, where partners IBP-CNR, SZN, UABDN, KULeuven, UCC, MEDINA, eCoast and UWC are members of the consortia.

To extend the use of the resources generated by PharmaSea (e.g. strains, extracts, fractions, pure compounds and associated data) we have sought for a solution that allows long term curation of these materials and data. We have started a conversation with EMBRIC (European Marine Biology RIC) who will accept biological, gene and extract materials as well as pure compounds. Since there are a number of extracts/fractions and compounds that are still being actively pursued by PharmaSea partners after the end of the project, it was decided that discussions with EMBRIC will continue for 12 months after the project end date.

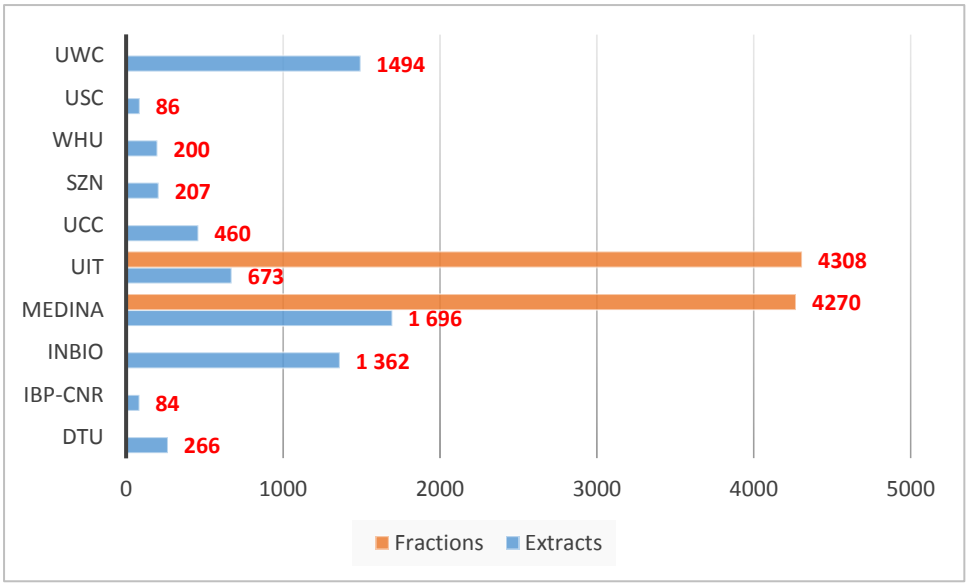


Figure 15. Extracts and fractions generated by the different PharmaSea partners.

WP3 Bioassays:

During the PharmaSea project over 130,000 primary assays were carried in the target disease areas of antibacterial activity, antifungal activity, epilepsy and inflammation (Figure 16). The overall hit rate was just above 4%, but this was skewed by the high neuroactivity hit rate in the photomotor response assay (Figure 17). The latter was reduced to a manageable 2% by a number of secondary screens including toxicity and more indicative zebrafish screens. In addition to this large number of primary screens a very large number of secondary screens and toxicity screens (>5000 with a hit rate of 2.74%) were also carried out to trace the activity of the extracts and fractions as they followed a purification cascade to pure compound. The main impact of WP3 was to prioritise samples for further work for WP4 and WP5. If a compound was prioritised by the PharmaSea prioritisation committee, further scale up was carried out followed by structure elucidation (WP4) and bioactivity studies (WP5). Compounds progressed to this stage were either new/novel with bioactivity or were known structures with exceptional activity, in particular in the area of epilepsy and neurodegenerative diseases, the data for

which is reported in WP5. WP3 highlighted the activity of a range of compounds with potent and specific activity. Several were progressed for anti-CNS activity in WP5 and will be pursued after the end of PharmaSea by generating families of analogues and a data package to allow the creation of a spin-out or licensing of the compound to a pharmaceutical company.

In the area of anti-infectives many of extracts contained known antibacterials, but partner MEDINA will continue the work on at least 10 promising extracts as part of the follow-on MSCA ITN consortium 'MarPipe'. Similarly partner UNIABDN will continue to work on more than 3 anti-infective extracts as part of MarPipe. In the area of inflammation, 7 samples are progressing through follow-on assays as part of the relevant partners' work or as follow-on projects in MarPipe. The most exciting results were obtained in the area of neurodegeneration and epilepsy. The former are reported in detail in WP5 as the compounds concerned were provided to USC from the UNIABDN compound library to allow USC to carry out relevant assays and animal studies. One fungal extract from DTU yielded 3 known compounds that are active in the KULeuven zebrafish epilepsy assay. All of these are being scaled up and one has had activity confirmed in an animal model (WP5). A further two known compounds discovered at UNIABDN were scaled up, isolated at MEDINA and tested in an animal model of epilepsy at KULeuven. These compounds were active (WP5) and had no toxicity associated with them and presented a good ADMET profile (WP5). The technology transfer office at KULeuven has been notified with the intention to file for intellectual property protection. Thus it is clear that WP3 played a pivotal role in identifying and progressing bioactive candidates with a successful outcome. Continued impact from WP3 will be obtained from the detailed datamining of the combined assay results and the eventual public release of the dataset from which any data with commercial possibilities has been removed. As specified in WP2, PharmaSea hopes to lodge this data with EMBRIC one year after the end of the PharmaSea project.

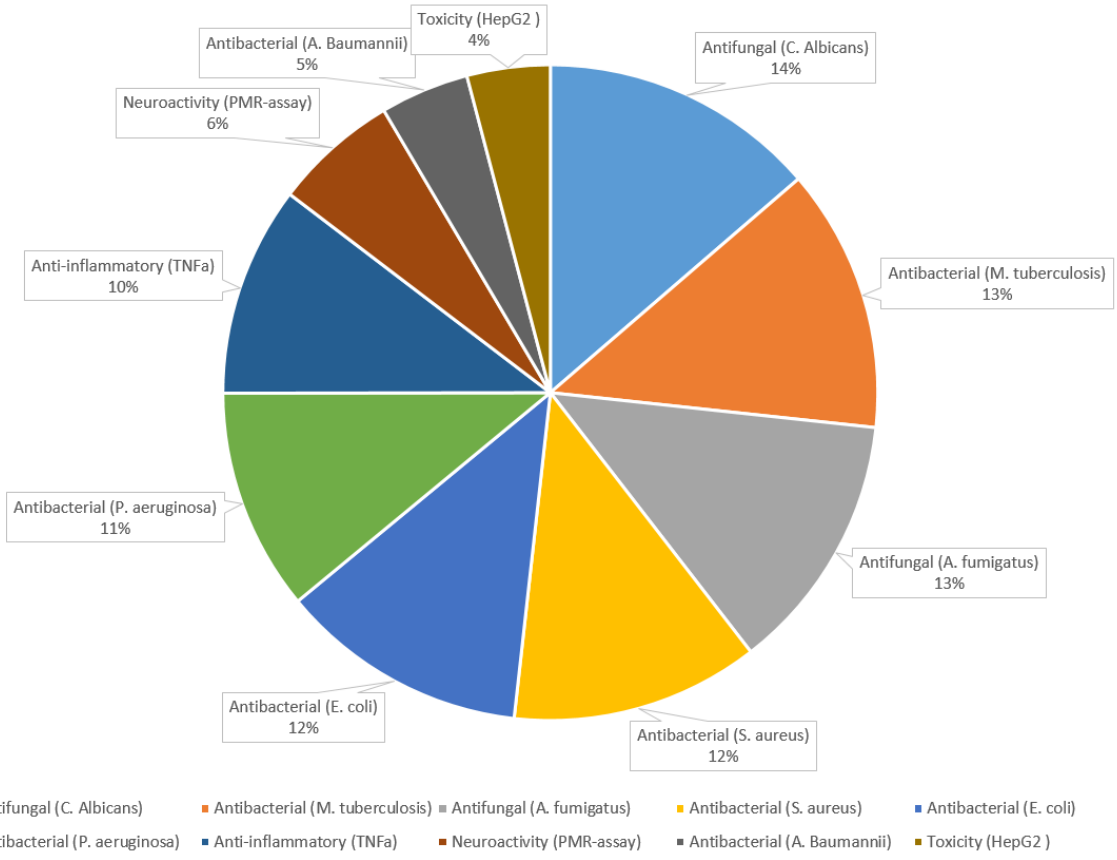


Figure 16. Primary screening events against different targets (N=130369)

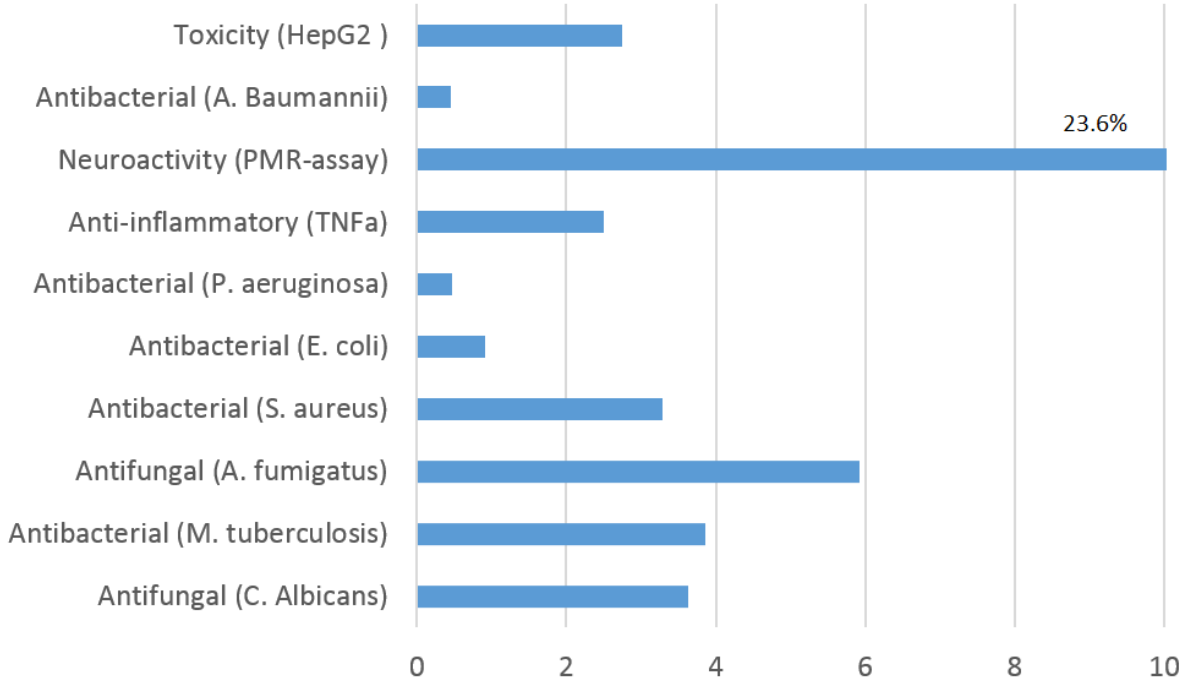


Figure 17. PharmaSea primary screening hit rates. In total 130369 assays were carried out with 5372 actives, giving an overall 4.12% hit rate.

WP4 Chemistry:

The primary role of WP4 has been the production of chemical diversity, but in addition to this, a range of methods, tools and databases has been developed which will continue to have an impact in the wider field of natural product biotechnology. A large number of known, new and novel bioactive compounds were discovered during the PharmaSea project with the total being 90 molecular families (56 known; 25 with new structural features; and 9 novel). Several of families of known compounds or known families with new structural features have been pursued to the point of animal trials for CNS diseases due to their potent and selective activity and desirable ADMET characteristics. Several other families with activity against bacterial and fungal infections will be pursued beyond the end of PharmaSea (Figure 14).

The methods developed during PharmaSea accelerate the isolation of compounds and aid their rapid structural characterisation. The explorative solid phase extraction protocol was successful in the development of preparative purification steps that selectively retain a given target candidate (and related compounds) with concomitant removal of unwanted components. Targeted chromatography using a unique parallel coupling of molecular specific electrospray mass spectrometry and element specific inductively coupled plasma mass spectrometry has been able to target sulphur containing compounds in an extract whilst simultaneously quantifying them. Improvements in the ACDLabs structure elucidation package have improved the speed at which complex natural product structures can be solved and will have an impact on the whole natural product community.

Several different tools and linked databases were developed by PharmaSea partners led by ACDLabs to accelerate the throughput of materials in the marine biodiscovery pipeline. These tools act at different stages of the process, and assist in rapidly uncovering known compounds either at the extract or pure compound stage and enable the assessment of an extract to allow it to be prioritised for further work. Dereplication, the process of discovering whether a compound has previously been described in the literature, should be carried out as early as possible in the biodiscovery pipeline, preferably at the extract stage. Many different protocols exist and the first PharmaSea solution to this

problem was a specific database containing data on all known *Streptomyces* products (e.g. species, structure, mass, retention time). After removal of blank and media components from the extract LC-MS by the software, all components in the database are searched for amongst the extract LC-MS peaks, a process known as targeted dereplication. This is successful when you can be certain that your organism belongs to the Streptomycetes, and allows rapid identification of unknown compounds in an extract. A second strategy is broader, but also accommodated by this software, and relies on removal of blank and media components as well as the entirety of the RSC MarinLit compound structure database. The remaining LC-MS peaks are then searched in the RSC open access ChemSpider database to identify possible novel peaks in a process known as untargeted dereplication. This process can be inefficient, which is why the PharmaSea partners have developed an innovative process to rapidly prioritise extracts based on potential novelty and diversity. Each sample is evaluated and allocated a score based on the number of new peaks present in the LC-MS chromatogram (novelty), ion intensities (productivity), and compound complexity (spread of LC retention time). This is a novel approach which will be validated via collaboration with other natural product labs globally. Finally, a database has been generated that contains all spectroscopic data on a pure compound (MS, 1D & 2D NMR, UV), containing data for over 650 natural products to date. This database can be queried with spectroscopic data resulting a match or near match. The latter is of interest because it would point to the query compound belonging to a particular structural family. The main impacts of WP4 are therefore the new bioactive chemistry provided to WP5 and also the methods and tools that widen the technical bottlenecks in the marine biodiscovery pipeline. The commercialisation of the software tools is still being discussed with ACDLabs, but we hope that they will become available for use by other biodiscovery researchers to accelerate the process and thus widen PharmaSea's impact.

WP5 Demonstration:

Neurodegenerative diseases: Neurodegenerative diseases including Alzheimer´s disease (AD), are a growing problem in industrialized countries. Alzheimer's disease affects millions of people worldwide due to the rise of life expectancy with a concomitant high socioeconomic impact. This disease was reported more than 100 years ago but, despite this, the exact cause and pathological mechanism are still not clear. Several hypotheses have been proposed and have been investigated in the search for a therapeutic strategy to halt this devastating disease. To date all the attempts to find a disease modifying treatment have result in failure. Initially research focused on abnormal processing of β-amyloid peptide (βA) as the cause of this pathology, and in consequence the most likely therapeutic target. Recent evidence shows that inflammatory and mitochondrial dysfunctions play a key role in the progression of AD even before of the appearance of the characteristics hallmarks and clinical symptoms.

Despite a major global effort to develop effective AD treatments, only palliative drugs have reached the clinic to date. Cholinesterase inhibitors, such as donepezil, galantamine or rivastigmine and memantine, a N-methyl-D-aspartate antagonist are the most often prescribed clinically. These treatments have some effect in mild to moderate AD cases but are ineffective in severe AD and do not stop disease progress. Therefore the search of new molecules is very important for the discovery of disease modifying treatments in AD. Marine natural products are a rich source of chemical novelty with a potential impact for the discovery of effective treatments for neurodegenerative diseases with unprecedented modes of action.

Pharmasea has tested the effect of several marine sponge and bacteria-derived compounds on AD targets *in vitro* and *in vivo* with excellent results. Initially, compounds were tested in a neuronal *in vitro* neurodegenerative disease model based on the antioxidant and mitochondrial protection potency of the compounds. Two sponge-derived compounds, Gracilins H and L, and one *Streptomyces* derived compound were tested in an animal model of Alzheimer disease (3xTg-AD mice). *In vivo* experiments confirmed the capacity of Gracilin H and L to inhibit the hallmarks of AD and to improve

behavioural tests in AD mice. The neuroprotective and antioxidant abilities of the Gracilins in cells and more inhibition of specific AD targets like BACE1 and ERK correlated with a decrease of Aβ and hyperphosphorylated tau levels (another hallmark of AD) in the animal model. The Gracilins were shown to have desirable pharmacokinetic and toxicity properties making them excellent starting points for the development of multi-target treatments of AD.

Although the Gracilins have been previously published and some of this family are known to be cytotoxic, neither of the non-toxic Gracilins tested in the PharmaSea project have patents associated with them for neurodegenerative diseases. Consequently we have freedom to operate and are currently working with a global leader in the field of chemical synthesis of this family of compounds to take this project to the next stage. The research group of Professor Daniel Romo at Baylor University, Texas, have synthesized several families of Gracilin analogues which we are currently patenting and anticipate publishing after intellectual property protection has been applied for.

Epilepsy: Approximately 65 million people suffer from epilepsy worldwide and an additional 2.4 million are diagnosed each year. The predominant treatment to control seizures is drug therapy. Currently there are 25 anti-seizure drugs on the market. Major players of the epilepsy drugs market include CB Pharma; Sanofi S.A.; Pfizer, Inc.; Johnson & Johnson; Abbott Laboratories, Inc.; Novartis AG; Eisai; GlaxoSmithKline PLC.; and Shire PLC. The largest market for anti-seizure drugs is the US, followed by Japan and Europe's 5 major EU markets (Figure 18, France, Germany, Italy, Spain and the UK). The sales for epilepsy drugs in these regions totaled $4.4bn in 2013 and this is forecast to grow to $5.4bn at a compound annual growth rate (CAGR) of 2.3% in 2022.

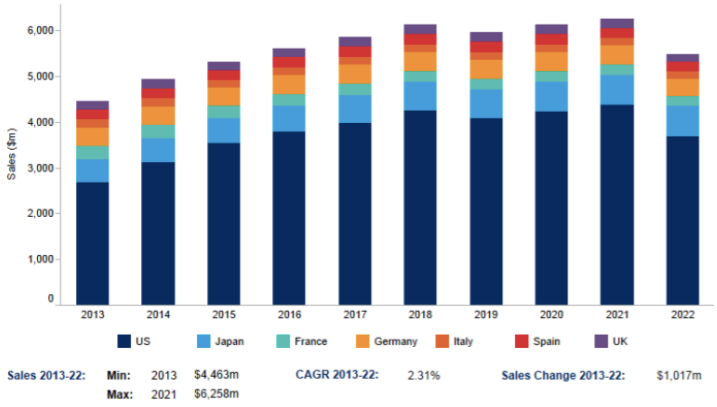


Figure 18. Anti-seizure drug sales value in US, Japan and 5 major EU markets ($m) 2013-2022.

The greatest unmet need in epilepsy is that current anti-seizure drugs fail to control seizures in 30% of the patients due to drug-resistance. This equates to >1.1 million patients across the US, Japan, and the 5 major EU markets. Drug-resistant patients tend to be prescribed the same drugs as patients responding to seizures, but as combination therapy in an attempt to reduce the seizures.

Levetiracetam (UCB Pharma) is the most prescribed drug for this patient group (50.6%). The market value of this patient group was an estimated 35% market share amounting to $1.6bn in 2013. Only 3 of the 12 anti-seizure drugs in Phase III trials are attempting to target drugs towards resistant seizures (against genetic child epilepsy Dravet syndrome). There is hence an urgent need and a significant market opportunity for anti-seizure drugs targeted towards drug-resistant epilepsies.

The CNS discovery program developed within Pharmasea has led to the discovery of two novel anti-seizure compounds isolated from a shallow sea marine fungus, and three novel anti-seizure

compounds isolated from a marine fungus collected from a Greenland marine sediment. So far three compounds (two isolated from the shallow sea sample, and one from the Greenland sample) were tested in the 6-Hz (44 mA) mouse seizure model. The results unambiguously show that the compounds have a potent anti-seizure effect. The other two compounds will be tested soon.

Searches in SciFinder and patent databases (Orbit-Questel) reveal that none of the compounds are under patents regarding epilepsy and hence freedom-to-operate is demonstrated. Based on these highly promising results, we aim to develop novel and proprietary classes of anti-seizure candidates (ASD) to the drug lead stage. To reach this aim we are assembling an interdisciplinary team (including PharmaSea partners) bringing together complementary expertise from drug discovery, medicinal chemistry, animal models and molecular cell biology that will generate a comprehensive data-package establishing not only the increased potency and druggability of newly generated analogues, but also a structure-activity relationship (SAR), a comprehensive *in vitro* ADMET profile and a clear view on the *in vivo* pharmacokinetics. The efficacy of promising hits will be studied in a panel of zebrafish and acute mouse models of drug-resistant seizures, whereafter a drug lead will be selected. The molecular target(s) of the lead drug will be identified in a chemical Yeast 3-Hybrid (Y3H) system, sampling the human proteome, and finally will be further evaluated in chronic (drug-resistant) rodent models. The partners involved intend to file for intellectual property protection for several of the hit compounds.

WP6 Policy:

One of the most successful and influential aspects of the PharmaSea project was its interaction with policymakers and legal experts via a variety of means. PharmaSea's Advisory Panel of Policy and Legal Experts (APPLE) became a trusted neutral forum for practitioners and policymakers to come together to discuss issues around access and benefit sharing of marine genetic resources from within and beyond national jurisdiction. The 4 APPLE meetings covered different topics on each occasion and were attended by developed and developing country scientists, intellectual property lawyers, intergovernmental organisations such as the International Union for the Conservation of Nature, EU DG-MARE and DG-ENV, United Nations Bodies such as the Division for Ocean Affairs and the Law of the Sea, The Convention on Biodiversity and the Convention on Migratory Species. Attendees at the 4th APPLE meeting have indeed requested some mechanism for this activity to continue. The APPLE reports were cited by a number articles and policy papers, but most importantly the establishment of the APPLE led to the invitation of PharmaSea participants at a large number of EU and UN as well as independent events to provide information on scientific best practice to an audiences of policymakers.

The initial interaction with EU policy makers was at a technical meeting of the member states taking part in the UN open ended working group to studies issues pertaining to the conservation and sustainable use of biodiversity in areas beyond national jurisdiction. The delegates were provided with an information paper ahead of time and two short presentations at the meeting were followed by 2½ h of questions. This meeting led to the invitation to stage a side event at the UN open ended working group in June 2014 which was attended by ca 80 delegates to the process. Following the UN General Assembly resolution 69/292, a preparatory committee ('PrepCom') was established to develop an international legally binding instrument under UNCLOS on the conservation and sustainable use of marine biological diversity of areas beyond national jurisdiction. PharmaSea staged 3 side events at the UN PrepCom and organised 3 Saturday workshops on different topics. All these events were attended by 60-100 delegates seeking independent information to use in developing evidence based policy. Additional events during the PrepCom (breakfast/weekend meetings with national blocs) and intersessional events with blocs such as the African Union, G77 and Small Island Developing States were useful to provide information tailored to these audiences and to answer specific questions. Much of these discussions has been distilled into the '*Mare Geneticum*' concept, recently accepted for publication in the *International Journal of Marine and Coastal Law*. PharmaSea partners will continue

their involvement with the UN PrepCom process beyond the end of PharmaSea to provide information on scientific best practice to incorporate in the implementing agreement to UNCLOS.

The legacy of WP6 will be achieved by the paper *Mare Geneticum*, the Best Practice Guidelines and the associated web-presence via VLIZ, the Case Studies to be adapted as publications and the Recommendations for further action for policymakers, industry, culture collections and general that came out of the Case Studies.

WP7 Dissemination:

Highlights in this section include the external communication of the project, the close cooperation with other projects and the successful outreach to policymakers.

The PharmaSea project obtained extensive media coverage, including specialist, but particularly also general media. Overall, PharmaSea and its objectives have been covered by more than 70 magazines, TV and radio channels and news websites all over the world – among others BBC, Al Jazeera, CNN, Daily Mail, Reuters, Radio Canada, Deutsche Welle and the Irish Times. Through these channels, consortium members achieved reach-out to the general public internationally, to raise awareness about the increasing number of drug resistant pathogens and communicate what is done for the discovery of new drug compounds. One further highlight is the PharmaSea movie, created in the final 6-months' extension period, which has – six weeks after upload – already received more than 1160 viewings on YouTube.

Five EU-funded consortium projects, PharmaSea, SeaBioTech, BlueGenics, MicroB3 and MaCuMBA successfully worked together and pooled resources and expertise to organise several workshops and conferences (selection):

- A half day workshop "Streaming Marine Biotechnology through into business - overcoming bottlenecks and generating innovation" was organised at the 6th EFIB, 2013, by PharmaSea (BioBridge) with participation of representatives from MicroB3 and BlueGenics
- A full day workshop "Building Blue Biotech Capacity in Europa" was organised at the 7th EFIB, 2014, by PharmaSea (BIOCOM) with participation of representatives from MicroB3, SeaBioTech and MaCuMBA
- The 9th European Conference on Marine Natural Products (ECMNP) was co-organised and a pre-conference workshop organised 2015 by the PharmaSea consortium with contributions by SeaBioTech and BlueGenics. The ECMNP was a success with more 188 participants from all over the world.

An impression of the extensive collaboration of the projects can be seen in Figure 19. PharmaSea has throughout its life had a strong outward-facing policy of collaboration, and this was again highlighted at the Final General Assembly of PharmaSea, in which members of the MarPipe and Ocean Medicines projects, which have both been inspired by the achievements of PharmaSea and the synergies of consortium members, were actively involved and participated in order to be able to follow-up with the PharmaSea results, and where representatives of other EU-funded projects and infrastructures including TASCMAR, Marine Fungi, EMBRIC/EMBRC, COLUMBUS and the SUBMARINER Network took part in a round-table on lessons and opportunities for the future in blue discovery.

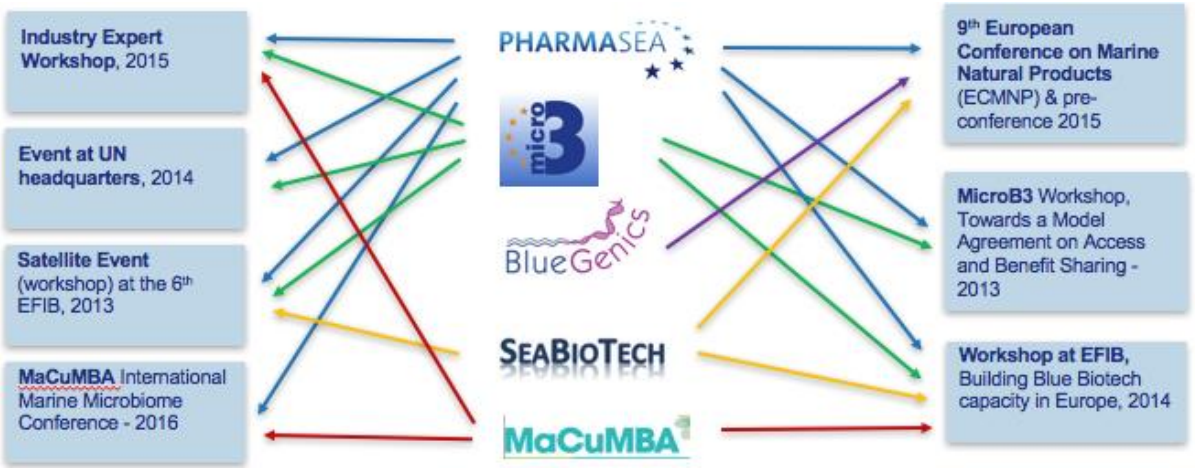


Figure 19. Common dissemination and communication activities

Impact on SMEs:

SeaLife Pharma: SeaLife Pharma was a marine biotechnology company that was located in Tulln and joined PharmaSea in 2014 by replacing one of the SME partners which had dropped out due to closure. For SeaLife Pharma, a company focused on the development of natural anti-infective compounds from the marine organisms, PharmaSea was an excellent opportunity to enlarge its marine biotechnology network around Europe. Taking the role of screening and compound development partner matched SeaLife Pharma's core business and was thus an excellent fit. Beside the financial contribution, PharmaSea gave SeaLife Pharma the possibility to make contact with different experts in the field, to evaluate a large number of marine compounds for their bioactivity and to get a good view of the whole marine biotechnology scene in Europe. SeaLife Pharma changed its development strategy at the end of 2014 to finalize its biocidal development, thus PharmaSea offered a good opportunity for SeaLife Pharma to remain up-to-date in the pharmaceutical field. By the middle of 2015 SeaLife Pharma started a mergers and acquisition process for its main product "SeaLife Polymer - SLP0901" and sold the assets at the end of 2016. Being a partner in PharmaSea and other EU FP7 projects was a great asset in the sale of SeaLife Pharma by verifying our international awareness and co-operation in the field. In the course of merger and acquisition process SeaLife Pharma had to stop all other activities in the middle of 2015 and cancelled all external projects.

eCoast: For eCOAST, PharmaSea was the first large project in which it could develop its new field of marine law & policy. The size of the project, in terms of funding, duration and consortium, turned out to be of crucial importance to the development of eCOAST from a very small ecological monitoring company into an SME that serves clients on all aspects of the sustainable use of marine resources.

Leading the work package on legal and policy aspects of marine biodiscovery (WP6) gave eCOAST the chance to increase its visibility on a global stage. This was partially thanks to the ideal timing of the project, with the coming into force of the Nagoya Protocol in 2014 and the ongoing negotiations for the biodiversity in areas beyond national jurisdiction process at the UN. Together with the other WP6 partners and with the scientific leader of PharmaSea, we quickly made a name as a project on the international policy scene, and ended up being highly respected advisers to different states, amongst which was the European Commission itself.

The policy work on access and benefit sharing became so important to eCOAST, that in 2015 this work - which outgrew the marine scene – was given a new brand name: ABS-int. This brand name was developed into a spin-off company in 2017, and is now serving government and private clients all over the world, including some of the largest pharmaceutical companies on the planet. It is very unlikely this would have happened without the involvement of eCOAST in PharmaSea.

Personal Note from the Scientific Leader:

I have been privileged to guide the work of the researchers in PharmaSea and would like to thank the hard work of all those involved. All workpackages have produced multiple exciting results which we will take forward through a variety of partnerships in the future. The presentations at the final general assembly made it clear PharmaSea has achieved a lot over the past 4.5 years despite a number of serious setbacks and obstacles. PharmaSea has performed beyond our expectations in the policy sphere and policy makers are appreciative of our efforts with many requests for further input.

The success of PharmaSea should be celebrated by all the partners and we should be proud of what we have achieved. The incorporation of partners from a range of organisations and countries, including non-EU countries has been incredibly successful to make a friendly, cooperative and productive consortium.

I would like to thank all of the PharmaSea participants for their hard work and friendship over the last 4.5 years, and hope our relationships will continue to grow both personally and scientifically. I would like to particularly thank Tine Heylen for guiding us through the intricacies of EU rules with good grace and humour and our administrators Meg Foster and Monika Slezak for turning our sometimes chaotic discussions into coherent minutes and actions.

The executive committee are thanked for steering the project through sometimes difficult conditions to a successful outcome. All of you took your tasks seriously, worked hard and complained little – elements of a great team. The external advisory board's advice was valuable in order to allow us to make the right decisions at difficult points, and are thanked for their friendly support throughout the project. Your encouragement and patience is appreciated.

Most of all I am proud of the researchers who carried out all of the work, some conducting PhDs, others postdocs, all with the involvement of other partners through the exchange of methods, practices and data. The outputs in terms of discoveries and publications has been phenomenal.

Marcel Jaspars, Scientific Leader, PharmaSea EU FP7 Consortium

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