PROJECT FINAL REPORT

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4.1 Final publishable summary report

- **An executive summary**

Biodiversity in the seas is only partly explored, although marine organisms are excellent sources for many industrial products. Through close cooperation between industrial and academic partners, it is possible to successfully collect, isolate and classify marine organisms, such as bacteria, fungi, micro- and macroalgae, cyanobacteria, and marine invertebrates from the oceans and seas globally. Classification of marine organisms exceeds 230,000 species; in the European seas over 36,000 species are known and 5,000-20,000 species are estimated to be still undiscovered. The maritime territory of the European Union is the world's largest with an economic zone covering 25 million km². The Atlantic Ocean, the Mediterranean Sea, the Aegean Sea, the Tyrrhenian Sea, the Adriatic Sea, and the brackish Baltic Sea can be regarded as the most important ones for the EU. All these seas have been accessible through the partners of our **MAREX** Consortium, in which also Pacific Ocean, Indian Ocean and Arabian Sea have been included through the Beneficiaries from four international collaboration partner countries (ICPC).

The marine environment is a wealthy source of plants, animals and micro-organisms, which due to their adaptation to this unique habitat, produce a wide variety of primary and secondary metabolites with significant biological activities. The intraspecific variation can produce differences in compounds and in their concentrations in different marine environments. It is thus essential to study organisms from various locations, as has been done in the **MAREX** project that brings together a strong combination of interdisciplinary academic and industrial researchers from Europe, Western Asia, India and South America.

Through close co-operation between industrial and academic partners, the **MAREX** project has collected, isolated and classified marine organisms, such as micro- and macroalgae, cyanobacteria, sea anemones, tunicates and fish. The ultimate objective of **MAREX** was to isolate, characterise, and sustainably exploit new compounds from extracts prepared from marine organisms that have been harvested from the seas and oceans, culture collections, or acquired from institutional, local, national, and commercially available collections. The extracts have been subjected to a highly representative and diverse panel of multidimensional bioactivity assays such as anticancer, anti-inflammatory, ion channel modulation, antimicrobial, etc. assays, and most promising extracts showing bioactivities have been subjected to chromatographic separation. Isolated marine compounds have been characterized thoroughly in terms of analytical purity and chemical structure. The most interesting hits have been produced via sustainable biotechnological processes or structurally optimized in a sustainable medicinal chemistry program with the aim of developing therapeutic agents to the stage where they can be available for thorough early-ADME and toxicological evaluation. Furthermore, the extracts prepared from marine organisms as well as isolated fractions and pure compounds have been screened and tested for other than pharmaceutical and medicinal applications. These industrially interesting application areas include nutraceuticals (*e.g.* marine lipids and antioxidants), cosmetics (*e.g.* antimicrobial preservatives, UV-absorbing compounds), agrochemicals (*e.g.* plant growth regulators, nutrients, biocides, herbicides, algicides, antifoulants, antibiofilm agents) and other specialty chemicals (*e.g.* antibiofilm agents, antifoulants). One of the objectives was also to develop biosensors for monitoring the quality of marine organism-based food and the biotechnological production of targeted bioactive compound for process control purposes. The research was expected to offer novel marine-based lead compounds for industries and strengthen their product portfolios related to pharmaceutical, nutraceutical, cosmetic, agrochemical, food processing, material and biosensor applications. These aspects have been met in **MAREX**.

- **A summary description of project context and objectives**

Background and General Context

The maritime territory of the European Union is the world's largest with an economic zone covering 25 million square kilometers. The most important seas and oceans for the European Union are the Atlantic Ocean, the Mediterranean Sea, the Aegean Sea, the Tyrrhenian Sea, the Adriatic Sea, and the brackish Baltic Sea. If we count all economic activities depending on the sea, then the so-called blue economy of the European Union represents 5.4 million jobs and a gross added value of just under €500 billion *per annum*. More specifically, 75% of Europe's external trade and 37% of trade within the EU is seaborne. The sea and the coastal regions are significant drivers of the economy (please see, for example, *Blue Growth Opportunities for Marine and Maritime Sustainable Growth; EU COM (2012) 494; DOI: 10.2771/43949*).

Until now, the explorations of marine biodiversity in the seas have just "scratched the surface". Most of the animal phyla can be regarded as exclusive to sea environment. There are 230,000 known marine species, and a predicted total number of undiscovered marine species exceeds two million. In the last decades, the natural product research has been focused on the marine environment: indeed, it is a rich source of plants, animals and micro-organisms, which due to their adaptation to this unique habitat produce a wide variety of primary and secondary metabolites as biologically active compounds. Many of them have demonstrated significant activities against, e.g., cancer and inflammation, as well as shown anti-analgesic, immunomodulatory, anti-allergic and antiviral properties. The specific habitat where an organism is growing has influence on the chemistry of the marine primary and secondary metabolites. The intraspecific variation can produce differences in the chemical structures of compounds and their concentrations in different marine environments. Therefore, it is essential to study different strains of the same organism from various locations. It is worth mentioning that biodiversity in the sea can be considered as a synonym to the chemical diversity of the marine compounds. Over thirty thousand compounds of marine origin are listed in *The Dictionary of Marine Natural Products* and *MarinLit database* (http://pubs.rsc.org/marinlit/) at the moment, and about 1,200 new marine compounds are reported yearly, only 5% of them having European origin. The potential of the marine environment as a source of novel bioactive compounds remains still relatively unexplored field of research.

*The **MAREX** Project*

Through a close co-operation between industrial and academic partners, the **MAREX** project (*Exploring Marine Resources for Bioactive Compounds: From Discovery to Sustainable Production and Industrial Applications*, No. 245137, 1.8.2010-31.7.2014) collected, isolated and classified marine organisms, such as micro- and macroalgae, cyanobacteria, sea anemones, tunicates and fish from the Atlantic, Pacific and Indian Oceans as well as from the Mediterranean, Baltic and Arabian Seas. Extracts and purified compounds of these organisms were studied for several therapeutically and industrially significant biological activities, including anticancer, anti-inflammatory, antiviral and anticoagulant activities as well as for ion channel/receptor modulation and plant growth regulation. Chromatographic isolation of bioactive compounds was followed by structural determination. Sustainable cultivation methods for promising organisms and biotechnological processes for selected compounds were developed, as well as biosensors for monitoring the target compounds. The work entailed sustainable organic synthesis of selected active compounds and new derivatives, and development of selected hits to lead compounds. The **MAREX** project successfully expanded marine compound libraries.

Innovations of the project were targeted for industrial product development with the aim of improving the growth and productivity of European marine biotechnology. Furthermore, the **MAREX** project aimed at better understanding of environmentally conscious sourcing of marine biotechnology products and increased public awareness of marine biodiversity and potential. Finally, **MAREX** offered novel marine-based lead compounds for European industries to strengthen their product portfolios related to pharmaceutical, nutraceutical, cosmetic, agrochemical, food processing, material and biosensor applications.

*Objectives of the **MAREX** Project*

The ultimate objective of the **MAREX** project was to isolate, characterize, and sustainably exploit new compounds from extracts prepared from marine organisms that have been harvested from the seas and oceans, culture collections, or acquired from institutional, local, national, and commercially available collections.

The specific objectives of the project were the following.

- to collect, isolate and classify marine organisms, such as dinoflagellates and other microalgae, macroalgae, cyanobacteria, sea anemones, tunicates, ascidians etc. from the Atlantic, Pacific, and Indian Oceans, and from the Mediterranean, Baltic, and Adriatic Seas, as well as from existing culture collections
- to ensure compliance with national, EU and international legislation and guidelines governing the collection of marine organisms
- to prepare crude extracts and fractions from the collected marine organisms for generating a sample library for studies on biological activity
- to carry out primary screening of the Consortium samples to evaluate a comprehensive selection of biological and pharmacological activities (incl. anticancer, anti-inflammatory, antimicrobial, antifouling and anticoagulant, assays, effects on ion channels/receptors/protein phosphatases, etc.)
- to select samples for further investigations and isolation through bioactivity-guided processes
- to fractionate crude extracts and to isolate bioactive compounds produced by marine organisms
- to determine chemical structures and stereochemistry of bioactive compounds
- to determine chemical diversity of bioactive compound producing cyanobacteria based on chemotaxonomic studies
- to develop sustainable biotechnological production protocols and long-term storage methods for promising cultures
- to generate and replenish marine compound libraries as well as promote taxonomy and cataloguing of collected marine organisms
- to develop suitable and sustainable cultivation and harvesting processes for selected organisms (including basic analysis for quality control purposes)
- to use larger scale cultures to provide sufficient biomass for the isolation of pure compounds by bioactivity-guided fractionation for strains from which extracts showing promising bioactivity have been obtained
- to select an aptamer against a bioactive compound
- to carry out biosynthetic pathway experiments using isotopically labelled metabolites
- to design and synthesize derivatives and analogues of isolated and characterized marine bioactive compounds in amounts needed for characterization and biological testing
- to carry out structure-activity relationships analyses

- to design and synthesize mimetics of most promising compounds
- to design and synthesize lead compounds
- to create standardized sample logistics for the **MAREX** Consortium
- to establish and update **MAREX** database containing information on collected and processed samples, isolated compounds and derivatives, as well as results from the bioactivity evaluations
- to standardize data formats and data processing
- to ensure a widespread dissemination of results and activities of the Consortium
- to create guidelines and instructions for storing project samples and research data *beyond the span of the Project* to facilitate a widespread dissemination of results and activities of the Consortium
- to ensure fair and equitable benefit sharing, and the proper handling of IPR
- to provide excellent and high-quality training to doctoral students and post-doctoral fellows
- to grant access rights to additional third parties, including SMEs
- to establish and implement the organizational structure for the consortium
- to ensure timely initiation of the project according to the contract and Consortium Agreement
- to ensure efficient administrative and financial co-ordination
- to ensure that the overall activities of the project are met
- to co-ordinate Work Packages and the activities of the project partners
- to solve potential problems raised during the project according to the consortium agreement
- to organize a Consortium Meeting of all partners once a year
- to organize the meetings of Steering Committee
- to compile the final project report

- **A description of the main S&T results/foregrounds**

The research in the **MAREX** project was carried out in six Work Packages and the main achievements of each Work Package are summarised in the following sections. **MAREX** was a collaborative effort of University of Helsinki (**UHEL**, Finland), University of Ljubljana (**UL**, Slovenia), University of La Laguna (**ULL**, Spain), University of Gdansk (**UG**, Poland), Katholieke Universiteit Leuven (**KULEUVEN**, Belgium), Åbo Akademi University (**ABO**, Finland), American University of Beirut (**AUB**, Lebanon), University of Antofagasta (**UANTOF**, Chile), University of Strasbourg (**UDS**, France), Ege University (**EGE**, Turkey), University of Naples Federico II (**USNF**, Italy), Catholic University of the North (**UCN**, Chile), Technical Research Centre of Finland (**VTT**, Finland), National Institute of Oceanography (**NIO**, India), Acreo AB (**ACREO**, Sweden), Xention (**XENTION**, UK), BioVico (**BIOVICO**, Poland), Ebiotec S.A. (**EBIOTEC**, Spain) and BiotechMarine (**BIOTECHM**, France).

WORK PACKAGE 1: Sourcing and bioprospecting

WP1 was responsible for sourcing, bioprospecting and extraction of marine organisms from European, Asian and South American marine and brackish ecosystems. One of the main objectives was to ensure that the collections comply with relevant legislation and guidelines. This WP aimed at collecting and extracting a vast number of marine organisms (such as micro- and macroalgae, dinoflagellates, sponges and marine invertebrates) from several different locations.

The research group at **ULL** developed and scaled up the culturing of dinoflagellate strains obtained from the Culture Collection of the Spanish Oceanographic Institute at Vigo (IEO-Vigo). A total of 27 microalgal strains out of the initial 47 were cultured by **ULL** and periodically maintained in standardised manner due to their chemical and pharmacological interest as well as their potential amenability for scaled up culturing. Considering the activity results and growing conditions, eight microalgal species were selected to be scaled up to continue the process. Both small and large-scale cultures were concluded and the resulting extracts (cellular and media) were included into the **MAREX** pipeline for further processing. Also, seven species of marine macro-organisms were collected from the coastal waters of the Canary Islands (Atlantic Ocean). Extracts of these organisms were prepared and included into the **MAREX** pipeline for further processing.

UG actively participated in the preparation of procedures for sampling and extraction of biological material in WP1. The work was focused on collection of microalgae bloom samples and isolation of cyanobacterial, green-algae, dinoflagellate, diatom strains from the Southern Baltic Sea. During bloom events, the high biomass of cyanobacteria (*Nodularia spumigena*), diatom (*Coscinodiscus granii*) and dinoflagellate (*Alexandrium ostenfeldii*) were collected. Altogether, 59 strains of cyanobacteria from Chrococcales (10), Oscillatoriales (24) and Nostocales (25) orders, three strains of green algae, two strains of dinoflagellates and one diatom were isolated and purified. The isolates were morphologically characterized using light microscopy. Genetic identification and phylogenetic analysis based on 16S rRNA gene and PC-IGS sequencing were done for 35 strains. LC-MS/MS-based metabolite profiles were determined for nine strains of *Nodularia spumigena*, 24 other cyanobacterial isolates and two strains of saxitoxin-producing dinoflagellate – *Alexandrium ostenfeldii*. All strains isolated during the **MAREX** project are being maintained in the Culture Collection of Northern Poland at **UG**. Additionally, 59 strains of cyanobacteria and three strains of green algae maintained in the collection were cultured for high biomass. A total number of 301 crude extracts were prepared with different solvents in order to yield the highest amount of bioactive compounds. The material of 32 strains (altogether 109 extracts) was sent to seven partners (**UHEL**, **KULEUVEN**, **VTT**, **NIO**, **ACREO**, **EBIOTEC**, **BIOVICO**), the rest of the extracts were analysed

at **UG**. Furthermore, three fosmid-based libraries of cyanobacterial metagenomes were constructed at **UG**. They were prepared using genomic DNA isolated from a biological material derived from: (a) a bloom of *Nodularia* sp. which occurred in Gdańsk Bay, Baltic Sea (Poland) in June 2009; library 1; (b) a bloom of *Nodularia* sp. and *Aphanizomenon flos-aquae* which occurred in Gdańsk Bay, Baltic Sea (Poland) in May 2011; library 2; and (c) cultures of five marine cyanobacteria, isolated from Baltic Sea (deposited in Culture Collection of Northern Poland, CCNP, at University of Gdańsk, Poland) and cultured in laboratory: *Microcystis aeruginosa* CCNP 1101, *Microcystis aeruginosa* CCNP 1102, *Microcystis* aeruginosa CCNP 1103, *Anabaena* sp. CCNP 1406, *Synechocystis salina* CCNP 1104; library 3. Considering average sizes of cyanobacterial genome (reported to be between 1.44 and 9.05 Mb), it was calculated that about 600 clones in the fosmid-based library (with average size of the insert of 40 kb) should cover efficiently a whole single genome. The obtained clones in each library (from 2.000 to 30.000) were, therefore, sufficient to cover at least several cyanobacterial genomes. As indicated above, two libraries (no. 1 and 2) were constructed with the use of DNA isolated from environmental samples of biological material, whereas library 3 contained DNA isolated from a mixture of five strains (to increase variability of the library) of cyanobacteria cultured in laboratory after their isolation from a natural habitat. Cultivation of cyanobacteria under laboratory conditions enhanced efficiency of metagenomic library construction (~30.000 clones vs. ~2.000 clones). We suggest that this might arise from contaminations of the environmental samples, which could interfere with efficiency in DNA isolation and purification, and/or cloning procedures.

ABO established a culture collection of Baltic Sea organisms. Microalgae and cyanobacteria were collected from diverse brackish habitats on the Åland Islands (Baltic Sea) during three large sampling events. A broad range of locations were chosen in order to get a diverse set of secondary compounds, and e.g. semi-enclosed brackish lakes, sub-ice, coastal inlets, open sea and rock pools were included. Cultures (64 cultures of 31 species) were established from the collected material. In order to get enough crude extracts for chemical isolation and characterisation (WP2) and bioactivity analyses (WP3), culture volumes of selected species were increased. Species showing fast growth or containing comparatively high compound concentration and amount per protein content (protein assays, HPLC-DAD and LC-MS analyses) were selected for large-scale cultures. The volumes of 27 cultures were increased to 5-25 litres. Biomasses from the large-scale cultures were harvested by filtration or centrifugation and freeze dried. The duration for attaining on average 110 mg freeze-dried material/l was 40 days. The freeze-dried biomasses were extracted with 80% ethanol, and a standard simplified crude extraction protocol was developed. The produced crude extracts were divided and sent to **MAREX** beneficiaries for bioactivity analyses (WP3), and also kept for own analyses. Methods for establishing axenic cultures were tested.

By **AUB,** sea cucumbers and sponges were freshly collected of the Lebanese shore in compliance with the legislation and guidelines provided. The samples were kept in plastic ventilated container filled with seawater. A total of 43 sea cucumbers and two sponges were collected. Samples were rinsed with distilled water and cleaned of all visible surface debris. Initially, sea cucumbers were used individually and later they were used as pooled batches. For the data generated in this project, **AUB** focused our study on sea cucumbers. The work involved processing of the samples for extraction. The samples were dissected into 2x2x2 cm pieces, rinsed with distilled water, snap frozen in liquid nitrogen, freeze-dried, pulverized, and extracted with 80% ethanol. After extraction, the supernatant of samples was collected, and lyophilized. They were either re-extracted with PBS (10% DMSO) and used as crude extract or fractionated for bioactive compounds/molecules.

UANTOF prepared extracts of ten macroalgae, two tunicate species and one sponge. The work concentrated on bioguided fractionation and purification of the chloroform extracts of *Pyura chilensis* and *P. stolonifera* or *praeputialis* (tunicate species). The fractions of *Pyura stolonifera* or

praeputialis showed interesting apoptotic (screening was performed on a new human hepatocyte cell line), anticancer (human prostate cancer and human breast cancer cell lines), antifungal (*Candida albicans*) and antibacterial (*Staphylococcus aureus, Enterococcus faecalis*) activities. From *Pyura stolonifera* or *praeputialis* the chemical structures of five known metabolites were identified: the nucleosides 2'-deoxyadenosine, 2'-deoxyguanosine, and ionosine, together with homarine and taurine. Also, the monoacylglycerol fatty acids, 3-palmitoyl-sn-glycerol and 1-monopalmitoeoyl-glycerol were identified. In addition, macroalgae *Ceramium rubrum* (antibacterial against *Staphylococcus aureus*), *Ulva lactuca* and *Chondrus canaliculatus* (anti-inflammatory activity; microgial activation), and *Ulva nematoidea* (antiviral activity in Chikungunya virus replicon model) and sponge *Clionopsis platei* (antifungal against *Candida albicans*) are examples of organisms studied by **UANTOF**.

During the project, **EGE** collected and isolated 28 microalgae specimens and 157 field specimens from Aegean and Mediterranean Seas. 28 collected microalgae specimens were cultivated for bioactivity studies. 28 cultivated (microalgae) and 147 field (macroalgae) specimens (total 185 specimens) were extracted using 80% ethanol to produce extracts for WP3 experiments. 161 (14 cultivated and 147 field specimens) of 185 extracted specimens were sent to seven different partners. Additionally, 24 different extracted specimens [four cultivated (microalgae) specimens and 20 field (macroalgae) specimens] were sent to **USNF** and four cultivated specimens were sent to **ULL**. A total of 1227 deliveries among partners were achieved by cargo. New website related to Ege Microalgae Culture Collection (EGE-MACC) was prepared. In this website (http://egemacc.com), the photographs, information, culture conditions of specimens, as well as **MAREX** brochure and publications are presented.

UCN collected Chilean endemic species of sea anemones. These species were firstly frozen and subsequently lyophilized, and extracted with three types of organic solvents of different polarities (*n*-hexane, dichloromethane and methanol) and crude extracts were prepared.

VTT focused on the cultivation of Baltic microalgae. 18 species and four non-Baltic microalgae species were included for further investigations in WP4. **VTT** microalgae were maintained in liquid cultures and regularly sub-cultured. All together 40 cultivations were carried out in different cultivation vessels. The production processes fulfilled our tasks for optimization and up-scaling cultivation processes in WP4, and the biomass produced was extracted by **MAREX** extraction protocol and exploited in bioactivity tests by project partners. In order to study microalgae under controlled, defined conditions, **VTT** aimed to establish axenic cultures of Baltic microalgae besides cultivation of the native microalgae cultures. The protocol developed for removing algae-associated bacteria and methods for confirming the absence of bacterial contaminants were introduced. Finally **VTT** established axenic microalgae culture of *Euglena gracilis*, proven by microscopic evaluation, bacterial plate tests and PCR analysis. The following microalgae species were purified from bacterial co-cultures in the early stages of this project, but recent experiments showed still bacterial growth: *Chlorella pyrenoidosa, Scenedesmus obliquus, Thalassiosira pseudonana* and *Nitzschia microcephalia.* In addition, *Alexandrium ostenfeldii, Kryptoperidinium foliaceum, Melosira* sp. and *Isochrysis* sp. have been under several treatments with antibiotics to reach the axenic status with no positive result. Protoplast technique was studied for establishment of axenic cultures of *A. ostenfeldii*, *Isochrysis* sp. and *K. foliaceum*. As a result, some spheroplasts of *K. foliaceum* and protoplasts of *A. ostenfeldii* were observed, but there was no regrowth of the treated cultures.

NIO collected 179 marine organisms such as sponges, soft corals, gorgonians, macroalgae and conus species from Indian Ocean and Arabian Sea. 15 different *Conus* sp. were collected and the venom glands and ducts were isolated for chemical studies. The processing of the samples included taxonomic classification, documentation (including voucher specimens), preparation of database of

collected marine organisms, preservation, freezing/freeze-drying, storage and extraction according to the needs of specific samples and project guidelines. 153 extracts obtained from these organisms were submitted for screening to the following laboratories: **UHEL**, **KULEUVEN**, **UDS**, **VTT**, **ACREO** and **EBIOTEC**. In addition to the crude extracts, fractions, subfractions and compounds were sent to **UDS** and **UHEL** for screening. Bulk extracts were also submitted to **USNF** for chemical investigations.

The research at **EBIOTEC** focused on marine organisms possessing interesting lipid compositions, which make them attractive as a source for bioactive compounds. The main polar lipids found in these organisms include monogalactosyl diacylglycerols, digalactosyl diacylglycerols, and phosphatidylglycerols. These lipids show several functional activities, but are mainly referenced in the literature for their anti-inflammatory activities. The lipid fraction consists primarily of the polyunsaturated fatty acids (PUFAs) that are well documented as essential for human health. The composition and extraction of PUFAs from algae, fish, fish by-products, and other marine sources have been extensively studied. Other bioactivities, associated with sterols, include anti-inflammatory and antiaterogenic activity. **EBIOTEC** has been working during the last 20 years on the identification of marine organisms with potential medical use. For this reason it was decided that **EBIOTEC**'s contribution to the WP1 was to collect specimen from the Atlantic Galician coast. All samples were processed following the shared instructions and sent to **MAREX** partners for bioactivity analyses.

The aim of **BIOTECHM** in WP1 was the selection of samples from the company libraries and prospection of new samples for the biological screening program of the Consortium. This included: 1) Collection and/or selection and preparation of samples, which were sent to WP3 partners for screening and 2) Exchange of information with WP3 partners about the specificity of the purposed assays and targets. **BIOTECHM** made also a significant contribution to the development of a standard procedure for the preparation of crude extracts for biological screening. In addition to the existing library samples, **BIOTECHM** carried out a collection of new algae or halophytes to be engaged in the project. To achieve this, different selection criteria such as the availability of the material, were used in accordance with the respect of environmental charts. The presence or absence of application patents and scientific publications on chemical and/or biological activities, use in cosmetics, pharmacy, and nutrition were also considered. For example, some seaweeds were selected because of their negative impact on the environment in Brittany, region in France.

WORK PACKAGE 2: Isolation and chemical characterization

WP2 focused on processing further the crude extracts produced in WP1 in order to isolate and chemically characterize the bioactive compounds discovered. Main objectives of this WP included fractionation of extracts, isolation of compounds by different chromatographic techniques and the determination of chemical structures and stereochemical and conformational properties of bioactive compounds produced by marine organisms. All these objectives were addressed and properly and effectively achieved. Partners **USNF**, **ULL**, **UG**, **ABO**, **AUB**, **UANTOF**, **UCN** and **NIO** contributed to the research carried out in WP2.

In particular, the crude extracts from collected and cultivated marine organisms were selected mainly on the basis of the pharmacological activity exhibited in the primary screening. The selected extracts belong to different Phyla including macro- and microalgae, sponges, soft corals, sea cucumbers, tunicates, and sea anemones. The crude extracts were first fractionated through extraction with solvents with increasing polarity, following a general procedure concerted between different partners in the early stage of the Project's start.

The enriched extracts from preliminary fractionation were subjected to further fractionation through modern chromatographic techniques. When possible, a bioassay-guided fractionation was made, in order to isolate the compounds responsible for the biological activity of the crude extracts. MPLC adsorption chromatography on silica gel and size-exclusion chromatography on Sephadex LH-20 columns were utilized for the preliminary fractionation of the crude extracts. After a series of further fractionation, pure compounds were obtained, using normal phase and reverse phase HPLC as final purification step.

The structures were determined by interpretation of extensive spectroscopic and spectrometric data (MS, ^{1}H and ^{13}C NMR, COSY, HSQC, HMBC). For all known compounds, their identity was secured by comparison of their NMR and MS with those reported in literature. The relative configuration of novel marine natural products isolated within WP2 of **MAREX** Project was solved through innovative approaches combining NMR spectroscopic techniques and quantum mechanical calculation of chemical shift parameters. In some cases the stereochemical assignments were supported by biogenetic considerations.

A total of 35 crude extracts from marine macro- and microorganisms were subjected to chromatographic fractionation. This study resulted in the isolation of about one hundred new compounds together with about eighty already known compounds.

In addition, the diversity of peptides produced by more than 25 strains of the cyanobacterium *Nodularia spumigena* from various geographical regions, and collected in different seasons, was analyzed through liquid chromatography-tandem mass spectrometry techniques.

Relevant results are summarized here:

- Four new polyether triterpenoids were isolated from the red algae *Laurencia viridis* and were demonstrated to possess antiproliferative activity against sarcoma and leukemia tumor cells in the μM range. The capacity of the above compounds to inhibit cell proliferation was likely due to their ability to induce apoptosis, as assessed by the appearance of a sub-G1/G0 subpopulation in cell cycle analysis, indicative of DNA breakdown.
- From the same algae a new oxasqualenoid, nivariol, was isolated. The flexibility of this molecule and the high number of quaternary carbon atoms that it contains make its configurational assignment very difficult. The complexity of the molecule represented a case study to solve the stereochemical relationship through an innovative approach that involved NMR spectroscopy and DFT calculations.
- Chemical investigation of an Atlantic variety of *Zoanthus* sp. led to the isolation of two new metabolites, zoaramine and zoarenone. These compounds which feature a new skeleton resemble the structure of norzoanthamine alkaloids, now considered one of the most promising drug candidates for the treatment of osteoporosis, a disease with an increasing worldwide prevalence. As the compounds were isolated in scarce amounts, their antiosteoporotic activity was not determined. A re-collection of the biological material have been planned to solve the problem.
- A novel 25-membered polyketide-derived macrocycle, belizentrin was isolated from cultures of the marine dinoflagellate *Prorocentrum belizeanum*. This metabolite is the first member of an unprecedented class of polyunsaturated and polyhydroxylated macrolactams. Pharmacological assays with cerebellar cells showed that belizentrin produces important changes in neuronal network integrity at nanomolar concentrations.
- The analysis of the crude extracts from the Indian soft coral, *Sinularia kavarattiensis*, selected for its promising activity in Chikungunya virus replicon model, resulted in the isolation of six known norcembranoids and of one new derivative, named kavaranolide with

an unprecedented carbon skeleton. Whereas kavaranolide was proved to be inactive in the antiviral assays, two known compounds epi-sinuleptolide and sinuleptolide showed inhibition of the viral replication, even if they also revealed cytotoxic properties. The isolated compounds were also studied in an *in vitro* model of neuroinflammation and the study disclosed sinuleptolide as the first marine compound able to modulate the progression of neodegeneration.

- The analysis of the apolar extracts from the same Indian soft coral *Sinularia kavarattiensis* resulted in the isolation of five conventional 3*β*-hydroxysteroids. As an interesting finding, one component, (24*S*)-ergosta-5-en-3*β*-ol, was for the first time disclosed as potent agonist of the metabolic nuclear receptor PXR. PXR is a transcriptional factor involved in the detoxification of xenobiotics, but also well recognized as interesting target for the development of new drugs for the treatment of intestinal inflammation and of other immune-mediated dysfunctions in humans.
- The content of cyanobacterial non-ribosomal peptides was analyzed in 20 strains of *N. spumigena* from different geographical regions. 47 non-ribosomal peptides (NRPs), including nine new congeners of spumigins, four aeruginosins and 12 anabaenopeptins (nodulapeptins) were identified. These results corroborate the potential of cyanobacteria as a rich source of NRPs, which can be tested for various bioactivities and potentially chosen as lead compounds for drug development. In addition, 14 anabaenopeptins, including 4 new variants were isolated from cyanobacterial bloom samples. The isolated peptides showed strong inhibition against protein phosphatase 1, carboxypeptidase-A; two of them inhibited the activity of thrombin.
- The venom of *Conus longurionis* was subjected to chemical analysis, that resulted in the isolation of an 18-amino acid peptide named α-conotoxin Lo1a, which is active on the Nicotinic acetylcholine receptor nAChRs. To the best of our knowledge, this is the first characterization of a conotoxin from this species. The peptide was characterized by electrophysiological screening against several types of cloned nAChRs expressed in *Xenopus laevis* oocytes. The three-dimensional solution structure of the α-conotoxin Lo1a was determined by NMR spectroscopy. Lo1a, a member of the α4/7 family, blocks the response to acetylcholine in oocytes expressing α_7 nAChRs with an IC_{50} of 3.24 ± 0.7 μm. Furthermore, Lo1a shows a high selectivity for neuronal *versus* muscle subtype nAChRs. Because Lo1a has an unusual C terminus, two mutants, Lo1a-ΔD and Lo1a-RRR, were designed to investigate the influence of the C-terminal residue. Lo1a-ΔD has a C-terminal Asp deletion, whereas in Lo1a-RRR, a triple-Arg tail replaces the Asp. They blocked the neuronal nAChR α_7 with a lower IC_{50} value, but remarkably, both adopted affinity for the muscle subtype $\alpha_1\beta_1\delta$□.

WORK PACKAGE 3: Screening and application development

In WP3, marine extracts, fractions and isolated or synthesized compounds supplied by WP1, WP2, WP4 and WP5 were studied for their potential use in pharmaceutical, cosmetic, food/nutraceutical, agricultural and other specialty chemicals applications.

Inflammation and cancer. Although the association between inflammation and cancer has long been suspected, the precise mechanism of inflammation-induced malignant transformation is not fully understood. Chronic inflammation is characterized by an ongoing and sustained release of reactive oxygen and nitrogen species and of pro- and anti-inflammatory cytokines. Such inflammatory mediators are considered as major pathogenic factors of carcinogenic malignant transformation of human intestinal epithelial cells during chronic inflammatory diseases (i.e. ulcerative colitis). Epigenetic changes have emerged as one of the most consistent molecular

alterations in various neoplasms and have been implicated in mechanisms of cancer progression such as DNA damage and repair, apoptosis, and cell cycle control. In WP3, anticancer effects were studied on human cell-based models of cancer from different organs (liver, testis, pancreas, gut, blood). Two tumour-derived cancer stem cell (CSC) cell-lines (testis and pancreas) compared to non-tumorigenic transformed cell lines were used to identify cancer-specific cellular responses. Apoptosis, also known as programmed cell death, is a mechanism regulating cell growth and proliferation. Apoptosis disrupts cellular membrane integrity. Changes in cellular membrane are early markers of apoptosis. Micro-volume cytometry was used to evaluate, in a first step, the potential of the **MAREX** extracts against human liver cancer cells and so to monitor hepatotoxicity. Then a model gut cancer was tested.

At the mid-point of the project, nine partners (**UHEL**, **UL**, **ULL**, **UG**, **EGE**, **USNF**, **UCN**, **NIO** and **BIOTECHM**) had delivered to **UDS** 652 crude or purified samples to be tested for their biological activities on human cell lines. Of these, 126 extracts came out as positive hits (110 for pro-apoptotic effects and 16 for anti-inflammatory): an unexpected high rate showing that the partners had done an inspired choice of the origin of the extracts.

Apoptosis, cell growth and toxicity are typical endpoint parameters for cell-based primary screening assays. Micro-volume cytometry was used for pro-apoptotic readouts. Caspase activation routes were added to the previous read-outs for, *e.g.*, synthetic compounds from **UL**. When considering cancer stem cell lines from testis or pancreatic origins, effects at micromolar concentration were observed for four **UL** synthetic analogs of clathrodin showing a high anticancer potency for these molecules. On the other hand, **VTT**'s final set of extracts based on bioguided selection was highly promising with an impressive (>50%) hit rate, which was obtained for hepatocyte cells with equivalent activities on a resistant metastatic colorectal cancer cell line. Further studies will be conducted to elucidate the nature of the active molecules present in **VTT**'s library. In addition, results obtained with **EGE** library (25% of the extracts showed important pro-apoptotic activities on either hepatic or colorectal cancer cell lines) are in the process of creating a joined publication during the coming year.

VTT screened samples with proliferation assays on two human prostate cancer cell lines and one human breast cancer cell line for assessment of anticancer effects. All the screens were technically successful: DMSO controls showed less than 10% standard deviation, the positive controls were very effective and the replicate samples showed very low variation. The samples were qualified as anti-proliferative hits when they inhibited cell viability by at least three standard deviations from the average of the controls. The screens resulted in 99 compounds (out of 186 compounds screened), which were anti-proliferative hits in any of the three cell lines. Many of the samples in the last screen were sub-fractions of hit samples from earlier screens.

Research at **BIOVICO** was aimed at finding the most immuno-active and anti-inflammatory components from marine algae extracts. Around 30 different extracts were prepared for biological activity studies. In the inflammation tests, both on human and mouse immune cellular models, none of the tested fractions showed cytotoxic activity. **BIOVICO** observed that the anti-inflammatory activity was pronounced for some extracts based on protein and gene expression, which showed an inhibition of the Cox-2 expression in mice.

Microglial activation may be a response to neuronal loss as well as to neuronal dysfunction and their extremely low activation threshold results in their involvement in several CNS diseases. In neurodegenerative diseases, widespread microglial activation may reflect widespread neuronal dysfunction in addition to the more apparent alterations of synapses. Although, under different situations, microglia may directly affect neuronal survival, this event is, however, rare and a

secondary phenomenon. The regulation of microglial activation by using *in vitro* studies with microglial, neuronal and mixed microglial and neuronal cells for the identification of both anti-inflammatory and neurotrophic molecules was investigated at **EBIOTEC**. Morphology, apoptosis, activation and release of pro-inflammatory cytokines were analyzed after exposure to the extracts obtained from **MAREX** partners. During the project, a total of 406 samples were analyzed at **EBIOTEC**, and more than 5,500 results were obtained. Some of the samples showed interesting anti-inflammatory properties in the primary microglial, neuronal and astrocyte cell culture models. In addition, the analysis of pro-inflammatory cytokine release also showed that some extracts and identified compounds may regulate the immune response.

Ion channels. The demonstrated implication of ion channels in physiological phenomena such as neuronal excitation, excitation-contraction coupling or stimulation-secretion coupling makes not surprising that a number of organisms have developed channel-specific toxins as mechanisms for self-defence or for capturing prey. A rich, non-exhaustive collection of natural toxins selectively target the ion channels of neurons and other cells, and represent valuable tools for studying the function of cellular ion channels. Despite a growing interest, the use of toxins for developing drug leads is still a niche research area. Electrophysiology studies carried out by **KULEUVEN** using standard voltage-clamp setups suited for heterologous expression of cDNAs encoding ion channels and receptors in oocytes of *Xenopus laevis*, and a high-throughput screening setup where 96 oocytes can be voltage-clamped automatically. Available cDNA clones (encoding voltage-gated ion channels and receptors) at **KULEUVEN** are today: voltage-gated Na channels; voltage-gated K channels; voltage-gated Ca channel; voltage- and nucleotide gated pacemaker channels; capsaicin receptor; opioid receptors; 2-pore background channels; inward rectifier K channels; G-protein coupled inward rectifier K channels; P2x receptors; nicotinic alpha-7 acetylcholine receptor.

Electrophysiology experiments in the voltage- and/or patch clamp mode were executed by **KULEUVEN** and **XENTION**. **KULEUVEN** used the heterologous *Xenopus laevis* oocyte expression system, while **XENTION** focused on mammalian cells. 592 samples were tested and 26 fractions/extractions/compounds were found to be active, and 11 novel peptides were identified.

In collaboration with **KULEUVEN** and **UL**, **XENTION** decided to focus on voltage-gated sodium channels as targets for marine-derived and synthetic molecules. Impaired function of specific isoforms of sodium channels can lead to various pathologies across different regions of the human body, such as neuropathic pain in the nervous system, hyperkalemic periodic paralysis in skeletal muscle, and Brugada syndrome in the heart. **XENTION** expanded the previous work of profiling natural product modulators of sodium channels to **UL** collection of small molecules against the Nav1.3 sodium channel designed as hybrid structures of the marine product clathrodin and Nav1.3 inhibitors described by Pfizer-Icagen. The effect of previous clathrodin analogues against a panel of potassium channels was as well tested. In both cases, the activity of marine products or small molecule analogues against these classes of ion channels could yield important and novel leads for further drug discovery development in the fields of pain and autoimmune disease. A total of 38 **UL** synthesized clathrodin analogues were tested. Ten compounds showed potent activity (<3µM) including 6 with sub-µM activity. In addition to high potency, compounds tested showed preference for binding to the inactivated state, displaying >3-fold selectivity for block of inactivated channels. Molecules with this profile offer better therapeutic potential in the treatment of pain, as inactivated state blockers are better able to block pain signals transmitted from repetitively firing damaged neurons. From the results of the Nav1.3 screen, 15 hit compounds were selected for testing against the Nav1.5 channel, an important channel in the generation of cardiac action potential. All 15 compounds tested were inactive against the Nav1.5 channel up to 10µM, thereby displaying good selectivity (3-300 fold) over the Nav1.3 channel. In summary, the **UL** clathrodin hybrid molecules

tested showed a range of promising potency and state-dependency for inhibition of Nav1.3 channels with good selectivity over the TTX-r cardiac sodium channel Nav1.5.

Finally, **XENTION** determined the mechanism-of-action of the active clathrodin analogues against Kv1.3 channels. Voltage-dependent channels are opened by changes in voltage across the cell membrane, moving from 'closed' to 'open' and then 'inactivated' states. Inhibitors could bind to and modulate the stability of channels in the closed, open or inactivated state, and **XENTION** can detect these different mechanisms biophysically by patch clamp recordings. The obtained data indicates that most of the active clathrodin analogues preferentially block the "peak" current (transition from closed to open state) compared to the "end" (inactivated states).

In conclusion synthesis and biophysical characterization of novel small molecule scaffolds derived from the marine product clathrodin reveal potent and selective modulators of Nav1.3 and Kv1.3 channels, with indications of a binding mechanism that interferes with channel inactivation. These novel compounds could expand the repertoire of medicinal chemistry leads suitable for development of treatments for chronic pain and autoimmune disease.

Antimicrobial and antiviral properties. There is a great demand for discovery of new antimicrobial agents due to the fact that the increasing number of emerging resistant bacterial strains is becoming a limiting factor in the usefulness of antibiotics currently available. However, antimicrobial drug discovery has been highly unattractive to pharmaceutical companies mainly due to short antibacterial drug lifecycles and the acute, rather than chronic, nature of antibacterial therapy. As a result, new classes of antibiotics are nowadays hardly discovered. During **MAREX**, over 6000 experiments were carried out by partners **UHEL**, **NIO**, **UCN**, **UG**, **ABO** and **ACREO** on different microbial strains, including Gram +/Gram- bacteria and fungi. This work lead to the identification of several samples with interesting antimicrobial properties.

To summarise the screens performed at **UHEL**, more than 1100 samples, including extracts, fractions, subfractions, isolated and synthetic compounds, were provided by the project partners. With the threshold >50% inhibition, 7% of the samples were active in the primary screening against one or several microbes. For example, extracts of marine organisms provided by **ULL** and **UANTOF**, were detected to possess antifungal activity against *Candida albicans*. Two compounds isolated from marine organisms by **BIOTECHM**, were shown to fully inhibit the growth of *Staphylococcus aureus* at low µg/ml concentration. A number of synthetic compounds from **UL**, **ULL** and **UHEL** were also studied, and of these, the clathrodin analogues were found to be the most promising set. Secondary evaluation, such as dose-response and selectivity analyses were carried out for the most promising primary hits.

At **NIO**, samples were tested, for example, against *Shigella flexneri*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Staphylococcus aureus* (MRSA), *Aspergillus fumigatus* and *Cryptococcus neoformans*. As examples of obtained results, promising activities were detected from extracts of marine organism collected from the Indian Ocean by **NIO** and from the Mediterranean Sea by **EGE**. Of crude extracts from **UG**, three samples showed inhibition against *Shigella flexneri*. Methicillin- resistant *S. aureus* (MRSA) was found to be sensitive to four **EGE** samples. Mild antifungal activity was also shown by two other samples against *Aspergillus fumigatus*. Of **ABO** samples, one extract was shown to be selectively active against *Vibrio cholerae*. Samples were also evaluated for their activity against a panel of laboratory strains of ten known antifouling pathogens. Crude samples from **UG**, when screened for antifouling activity, revealed five samples significantly active against seven fouling bacteria strains. From **EGE** samples, antifouling activity was detected for six crude extracts. Of the samples from **ABO**, three were found to be mildly active.

Studies at **UCN** included marine organisms, such as sea anemones, *Anemonia alicemartinae*, *Actinia papillosa*, and *Phymanthea pluvial*, which were evaluated against, e.g., *Candida albicans*, *Aspergillus fumigatus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The experiments showed that only the methanolic extract of *Actinia papillosa* displayed a low inhibition of both fungi species at 300 μg/ml of concentration. In general, the results showed that the great majority of tested extracts did not show activity. However, the methanolic and dichloromethane extracts showed a mild antibacterial and antifungal activity against some of the strains.

The extracts of 200 clones from the expression of gene libraries, randomly taken from previously constructed cyanobacterial metagenomic libraries, were assessed for their effects on growth of various bacterial strains by **UG**. Although no dramatic inhibition of growth was found, it is important to note that concentration of the extract was relatively low (1%). Thus, it is likely that identification of the inhibitory agent should allow cloning of appropriate gene(s) in an expression vector, its production in large amounts, and its potential use to achieve significant inhibition of bacterial growth. In collaboration with **UG**, **ABO** studied the antibacterial properties of cyanobacteria and microalgae crude extracts. The effect of 20 ethanol crude extracts was tested on four pathogenic bacteria, and the effect of one cyanobacteria crude extract was tested on ten pathogenic bacteria. The growth of *Enterococcus faecium* was significantly inhibited by three diatom crude extracts and weakly inhibited by one cyanobacteria crude extract.

Though for medical application it is useful to identify antibacterial activities, for biotechnological purposes, it is often desirable to stimulate growth of bacteria, which are employed to express recombinant genes and synthesize particular products. Therefore, **UG** also tested the effects of the clones from the libraries on growth of host *E. coli* cells. Since it is known that the presence of plasmids, especially relatively large constructs, may influence bacterial metabolism, physiology and growth, in control experiments, a strain bearing a plasmid analogous to those containing fragments of cyanobacterial genomes, but carrying a fragment of *E. coli* genome (the 105-2 clone), was used. Surprisingly, it was found that in the presence of two clones the growth of *E. coli* host was stimulated. Generation time at mid-exponential phase of growth was 36 min for the control strain, while it was calculated as 28 min for cells bearing either 123-3 or 129-3 clone. This indicates that expression of some cyanobacterial genes may enhance growth of *E. coli* cells, which may be potentially useful in construction of new host strains to be used as microbial cell factories for biotechnological production of certain compounds. The clone 123-3 appeared interesting as growth of bacteria bearing this clone was stimulated. Stimulation of protein and nucleic acid syntheses might likely enhance growth rate of host bacteria. DNA of 129-3 code for several putative proteins involved in biosynthetic pathways of proteins and lipids and their transport. Therefore, one may speculate that stimulation of anabolic processes might positively affect growth rate of *E. coli* host cells, which can have a potential impact on biotechnological production.

For the studies of bacterial adhesion, **ACREO** used a method of staining the vacuoles, caused by VacA toxin from the bacteria *Helicobacter pylori*, with neutral red dye. The setup, which is typically done with epithelial cells and free VacA toxin, was altered by using the bacteria for delivering the toxin via a contact dependent mechanism. A decrease in vacuolization is then in direct correlation to a decrease in adhesion of the bacteria. Extracts of collected marine samples provided by **ULL**, **UG**, **UANTOF**, **EGE**, **NIO**, **BIOTECHM** and **EBIOTEC** were evaluated in the model, but no confirmed reduction of vacuole formation was observed.

MAREX samples were evaluated by **UHEL** also for their antiviral activities by using two different model systems: a stable baby hamster kidney cell line expressing Chikungunya virus (CHIKV)

replication proteins and a replicon model based on hepatocyte cell line expressing hepatitis C virus (HCV) replication proteins. Promising anti-CHIKV leads were further tested *in vitro* against a related alphavirus, Semliki Forest virus. Altogether more than 850 antiviral screens were carried out within the **MAREX** project. As a result, a number of extracts from **NIO** showed potent activity in CHIKV model, and were studied further. Of the synthetic compounds from **UL** and **UHEL**, interesting activities especially in the HCV model were observed. The secondary testing carried out showed that five of these compounds are of interest for further studies, based on their dose-dependent inhibition of the HCV replicon with low IC_{50}-values, and no significant cytotoxic activity. A newly synthesized compound by **UHEL** was also shown to inhibit the HCV replicon in a dose-dependent manner, without significant toxicity against the host cell. This compound will be further studied in the CHIKV replicon model and possibly also for activity against infectious Semliki Forest virus. During the last months, the focus of antiviral bioactivity studies was on in-depth studies of hit samples identified during earlier stages of the project (**NIO** and **ULL**). In addition, **UHEL** developed and optimized a novel imaging-based assay in 96-well plate format based on a cell-cultivating platform with integrated optics. The developed assay was used to study antiviral properties of a collection of compounds isolated by bioassay-guided chemical fractionation (**USNF**) from an Indian Ocean soft coral (**NIO**).

Biofilms. A biofilm is any group of microorganisms in which cells stick to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance. Biofilm extracellular polymeric substance is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides. Biofilms may form on living or non-living surfaces and can be prevalent in natural, industrial and hospital settings. Often microorganisms exist in the environment as multicellular communities, so-called biofilms. Among bacteria, only a little is known about chemical defense against bacterivorous consumers. According to one hypothesis sessile bacterial communities organized as biofilms serve as bacterial refuge from predation. By testing growth and survival of common bacterivorous nanoflagellates, evidence has been found that chemical mediated resistance against protozoan predators is common among biofilm populations in a diverse set of marine bacteria. Chemical communication is essential in biofilm populations to coordinate their behaviour and respond to environmental pressure. Recent research has been unravelling a complex web of chemical crosstalk mediating microbial symbiosis, competition and defence against predators and pathogens. The unique properties of bacterial biofilms call for the development of reliable and specific research methods, different to the ones optimized for planktonic bacteria. To fill the void of biofilm assays suitable for screening, a statistically robust platform of assays had to be developed.

ACREO focused on a measurement setup since a vital step in studies of bacterial adherence and testing extracts from marine samples is to optimize the assay for biofilm formation monitoring. The conclusion was that the best way to perform the assay is to do the measurements without any media exchanges and then to follow the biofilm formation during 48 hours. During this time period the chamber of the developed device has to remain untouched to reduce background noise. **ACREO** also put together a switch to make it possible to measure two magnetoelastic resonance (MER)-films in two different chambers, during the same time period, exposed to the same environmental conditions. Subtracting the signal of the reference film from the signal of the second will give a signal, which (mostly) corresponds to the biofilm on that MER-film. To establish this, **ACREO** would need to do some configurations of the software and calibration work. The method developed for analyzing biofilm formation using MER-technology also proved to be more challenging than expected and therefore time consuming.

Effects on mitochondrial functions. The status of mitochondria activity was studied by **ACREO** by following the changes in NADH concentration. NADH can be distinguished from NAD+ by fluorescent analyses. The effect of marine extract samples and standard references were studied using multiple duplicates of cells in 96-well plates. The cells were exposed to reference standards (known to affect the cells activity) at different dilutions in cell growth media. After six hours of incubation, readings were done using a fluorescent plate reader. The excitation/emission peak studies revealed that the optimal excitation wavelength was about 325 nm and the emission peak at about 440 nm. But difficulties detecting the fluorescent signal from the NADH using available instrumentation for screening halted the analyses of marine extract samples. **ACREO**'s more sensitive fluorescence spectrophotometer (FluoroMax) would have the necessary performance but can only handle one sample at a time, thus making large scale experiments impossible.

Anticoagulant and antiaggregatory activity. Inhibition of thrombin and factor Xa was used to evaluate anticoagulant activity, and inhibition of trypsin to assess the selectivity of potential anticoagulants for coagulation enzymes. Binding affinities to the integrin GPIIb/IIIa receptor (fibrinogen receptor) involved in platelet aggregation were measured by a solid-phase competitive displacement assay as an indication of potential antiaggregatory activity. Over 200 **MAREX** samples were evaluated during the project by **UL** and **UG**. As an example of the results, compounds isolated from cyanobacteria (especially *Nodularia spumigena*, *Nostoc edaphicum*, *Anabaena* sp. and *Spirulina subsalsa*) through activity-guided fractionation by **UG** and **ABO**, showed strong activity against thrombin and/or trypsin (as well as against chymotrypsin and carboxypeptidase-A).

Inhibition of protein phosphatases. Inhibition of protein phosphatase (PP) leads to hyperphosphorylation of proteins and disrupts cellular homeostasis. The activity of microalgal and cyanobacterial extracts, fractions and isolated compounds were tested against PP type 1 and 2a by **UG** and **ABO**. Interesting activity was detected, for example, for an isolated compound from the **BIOTECHM** library, and extracts and fractions prepared by **ULL**, **EGE** and **ABO**.

Protection against UV radiation. Exposure to UV radiation may cause sunburns, skin cancer, oxidative stress as well as photo aging. UV opacity is the property of sunscreens to absorb and/or block UV radiations. In this task, the UV absorbing properties of a selected set of **MAREX** samples were evaluated by using spectral scanning at the wavelengths covering UV-A, UV-B and UV-C regions. The aim here was to evaluate the potential of **MAREX** samples as innovative UV protective agents, which could be adapted for cosmetic and other applications.

Results obtained demonstrated that the **UHEL** set of synthetic compounds show UV-absorbing properties that might be potentially interesting to study further for the purpose of cosmetic applications. The absorbance potential is most prominent in the UV-C region, but four of the compounds display absorbance also in the UV-B region. This region, together with UV-A region, is considered to be the most important for protection against erythemally effective solar UV. Results by **NIO** demonstrated that among all the extracts tested for UV-absorbing properties most interesting were five macroalgal extracts. Only three sponge extracts showed activity but to a less significant extent. All the extracts, except one, showed absorption at the UV-A, UV-B and UV-C region indicating their effectiveness in producing UV absorbing compounds.

Agrochemical and insecticidal applications. Algal extracts have been used in agriculture as plant growth regulating agents for many years. They are known to stimulate chlorophyll synthesis and growth of shoots and roots of fruit-trees, vegetables and rice. The application of natural components of algae and cyanobacteria as materials used for the control of plant growth constitutes an attractive alternative for chemical pesticides. In the project, studies into the potential use of cyanobacterial

extracts as agrochemicals were performed, and thus **UG** focused on the assessment of the activity of cyanobacterial extracts against higher plants and microalgae. For example, the effects of cyanobacterial extracts and bloom samples (collected from different locations in the Gulf of Gdansk) on the growth of young roots and shoots of *Sorghum saccharatum* were tested. Both inhibitory (down to 0% of control) and stimulatory activities (up to over 160% of control) were observed. In the case of the algicidal activity, ethanol extracts from cyanobacterial were analyzed and the strongest inhibition was recorded for 80% EtOH extract obtained from *Nodularia spumigena* CCNP1403 (down to 20% of control).

In addition, **KULEUVEN** identified a peptide toxin with potential use as agrochemical and insecticide due to the fact that this toxin preferentially blocks insect Kv channels, as opposed to being active on vertebrate Kv's (and hence also mammalian Kv's), which is quite unique and different from the classical insect target, the Nav channel in insects. In this way, this toxin has insect selectivity exerted via a novel pathway/target.

WORK PACKAGE 4: Biotechnological production and biosensing for sustainable industry

The focus of WP4 was pointed towards biotechnological production and biosensing. Both activities aimed at providing tools to allow sustainable industrial exploitation. While the objectives concerning biotechnological production were the production of biomass by means of scaling and optimisation of culture conditions including harvesting and long-term storage, the objectives of biosensing were directed to safety and quality control and could be more generic in nature.

Biotechnological production. While microalgae are generally grown as autotrophic cultures, mixotrophic or heterotrophic modes can be beneficial in providing higher cell densities and higher growth rates. Since quantitative data on the interrelationships e.g. between the utilisation of organic and inorganic carbon are specifically missing for continuous cultures, *Chlorella protothecoides* was investigated at **VTT** during steady states in well-defined conditions. To this end *C. protothecoides* was cultivated in a Sartorius BioStat B 2.5 l glass bioreactor at a working volume of 1.5 l in defined medium either under light (mixotrophic) or without light (heterotrophic). Steady states were identified based on constant biomass concentration, dissolved oxygen tension, CO_2 evolution rates, and O_2 uptake rates. Continuous flow cultures were successfully established. Mixotrophic growth of *C. protothecoides* under nitrogen-limited conditions produced up to 57% lipid in its biomass i.e. more than reported earlier in heterotrophic batch cultures (3%). Organic carbon and CO_2 was co-utilised and therefore CO_2 output was decreased in mixotrophic conditions compared to hetereotrophic conditions.

Medium composition, nutrient supply and various process parameters influence biomass and product formation and therefore these factors were broadly investigated at **EGE**. A set of four microalgae specimens (*Dunaliella* sp.-10S008, *Nannochloropsis* sp.-10S010, *Chlorella* sp.-10S011 and *Nitzschia* sp. -10S013) was selected based on growth rates and bioactivity. These were cultured in appropriate media at 22±2 °C in both 2-l sterile bottles and 5-l and 9-l sterile plastic bags for 20 days. The maximum biomass was reached for *Nitzschia sp.* at 16.10 g in 5-l and 9-l sterile plastic bag productions.

Optimum physical conditions were also determined for the growth of seven selected microalgae strains (*Chlorella* sp.-10S009, *Chlorella* sp.-10S011, *Dunaliella salina*-10S008, *Nannochloropsis* sp.-10S010, *Picochlorum* sp.-10S147, *Prasinococcus* sp.-10S146, *Tetraselmis striata*-10S148) based on 42 response surface curves and 14 second order polynomial equations (14 mathematical models)

with chlorophyll-a and protein amounts as a function of light intensity (µmolphotons m^{-2} s^{-1}), temperature (°C) and agitation speed (rpm).

Macroalgae growth was mainly affected by light and temperature conditions (**EGE**). Strong illumination (10,000 lux) caused higher extraction yield and higher growth rate for *Chodium fragile*. For *Enteromorpha compressa*, darkness caused higher extraction yield, whereas the growth rate was increased by higher temperature levels (30 °C). For *Halopteris scoparium*, nitrogen free f/2 medium resulted in higher extraction yield, whereas growth rate was increased by higher nitrogen (200 %). For *Jania rubens*, the biomass density of 5 g/l increased the extraction yield and the growth rate. For *Chladophora prolifera*, darkness caused higher extraction yield, whereas growth rate was increased by the addition of aeration (3 vvm) into the vessels.

Dinoflagellates are notoriously difficult to cultivate. *Alexandrium ostenfeldii* culture showed very strong activity as ethanol and water extracts (50 µg ml^{-1}) especially against human prostate cancer cells, and in apoptose activity with both human hepatocyte (HepPS) and human colorectal adenocarcinoma (SW 620) cells. However, the cultivation season was very important for the production of biomass and compound composition. A yield of 1.5 g/l independent of lighting conditions was achieved at **VTT** from early spring to late autumn in Wave bags without mixing. Another species, *Prorocentrum micans*, was grown in Wave bioreactor but did not produce okadaic acid.

The biogenesis of two new dinoflagellate toxins, DTX5c and belizeanolic acid, was investigated by biosynthetic experiments using ^{13}C-enriched precursors at **ULL**. Thus sodium acetate, sodium glicolate, methionine and glycerol were added as metabolic precursors into axenic cultures of *Prorocentrum belizeanum*. The labelling pattern of both metabolites was determined by nuclear magnetic resonance (NMR) spectroscopy. Based on these results, a polyketide biogenesis was proposed for both metabolites.

Further photobioreactors (bag, panel and bubble column) were compared by **EGE** for the growth of other species. *Cylindrotheca closterium*-10S149 cells were cultured at 21±2 °C in the temperature-controlled incubator under the continuous illumination (50 µmol photons m^{-2} s^{-1}) at a flow rate of 2 l min^{-1} in 1.5-l sterile reactors containing 1 l f/2 medium for 7 days. The maximum specific growth rate of 0.21 day^{-1}, corresponding to the doubling times of 3.30 day, was obtained in the bag reactor at a light intensity of 50 µmol photons m^{-2} s^{-1}. Growth in the bag reactor was 25% higher than in the bubble column reactor.

Diatoms *Nitzschia* sp. and *Cylindrotheca closterium* cultivated in air-lift photobioreactors in f/2 medium at 56 µmol photons $m^{-2}s^{-1}$ showed maximum specific growth rates of 0.181 and 0.209 day^{-1}, respectively, corresponding to doubling times of 3.831 and 3.315 day. The dominant fatty acids in *Nitszchia* sp and *Cylindrotheca closterium* were palmitoleic acid methyl ester (C16:1) and heptadecanoic acid methyl ester (C17:0) constituting 48.99% and 32.50% of the total fatty acid contents, respectively. *Nitzschia communis* 10S152 showing promising bioactivity exhibited good growth in a flat-plate photobioreactor. The production process was scalable to six liters without problems.

In order to facilitate long- term storage of production strains several methods such as cold storage and cryopreservation were examined at **VTT**. Growth and storage on solid f/2 medium at 4°C was very good for all species (*Alexandrium ostenfeldii, Chaetoceros muelleri, Chlorella pyrenoidosa, Dunaliella salina, Euglena gracilis, Kryptoperidinium foliaceum, Nitzschia microcephalia, Phaeodactylum tricornutum, Scenedesmus obliquus*) tested on plates. Successful cryopreservation

strategies in liquid nitrogen were developed for the following monoalgal cultures: *C. prothecoides, E. gracilis, Melosira* sp. *Dunaliella salina, Chaetoceros muelleri* and *P. tricornutum. P. patelliferum* was the only native strain in this study which did not recover from the freezing procedure. Axenic strains of *C. pyrenoidosa* and *S. obliquus* exhibited good tolerance for cryopreservation. The capture of the cells in glycerol beads is operationally supreme, as the procedure includes no time-consuming or labour-intensive steps.

Biosensing. The aim of this part of the Work Package was to develop a generic sensing platform, based on aptamers and microparticles to facilitate product detection (**KULEUVEN**, **ACREO**). The original target molecule, okadaic acid, had to be changed into nodularin, a nonribosomal peptide produced by the cyanobacterium *Nodularia spumigena.* A large-scale culture of nodularin-producing *Nodularia spumigena* was initiated to supply the necessary amount. Several protocols, essential for performing aptamer selection against small molecule, were first optimized and subsequently implemented. In magnetic bead-based SELEX, magnetic microbeads were used as carriers of nodularin. Nine rounds of SELEX were performed, and DNA that showed enrichment in potential nodularin binding sequences was cloned and sequenced. After extensive analysis of 45 sequences both for their sequence and structure similarity, 12 candidates were chosen for further testing. The interaction with nodularin was tested by qPCR bead-based assay. Results showed that at least two selected sequences showed improved binding towards nodularin compared to other DNA sequences tested, including three other aptamers used as negative controls in this experiment. These two candidate aptamers were further characterized for their binding affinity and specificity using reference technologies, such as Biacore™ and FortéBIO Blitz® platform. However, detecting interactions between nodularin and its bioreceptors (being either aptamers or antibody) failed on both platforms due to the small size of this peptide.

The next aim of **KULEUVEN** was to develop an aptamer-based bioassay suited for online monitoring of nodularin production in a bioreactor. However, because selected aptamers against nodularin could not be characterized using reference technologies due to the small size of nodularin, a stepwise approach was followed for developing a proof-of-concept bioassay using other target molecules as model system (IgE or synthetic DNA target*)*. The first step in the bioassay (competition between capture probe and target molecule for binding with aptamer) was demonstrated using IgE and well characterized aptamer against this protein. The second step in the bioassay (labelling of capture DNA probes with detection probes and enzyme needed for generating the fluorescent signal) as well as the third bioassay step (ultrasensitive DNA detection on DMF platform using super paramagnetic beads) were demonstrated with synthetic DNA target. In this competitive assay, the most essential step is the releasing of aptamer only in the presence of the target molecule, i.e. IgE. This was achieved by optimizing several parameters, including the right combination of buffers for hybridization between aptamer and capture probe as well as for incubation of aptamer with the target molecule.

As an alternative approach the possibility of using a magnetic AC susceptometer, "DynoMag" developed by **ACREO**, was evaluated. The DynoMag system measures the dynamic magnetic response from thermally blocked magnetic nanoparticles exposed to a magnetic field that changes in frequency. Magnetic nanoparticles of different diameters and with various surface chemistries were tested for optimizing the surface functionalization protocol and stability of the beads. Streptavidin-biotin chemistry proved to be the most suited for this system and was used in experiments. Although the major aim of this task was to develop a bioassay for nodularin detection, the two approaches have been successfully developed only when using model systems, i.e. synthetic DNA target and IgE with a well described aptamer against this protein. This was mainly due to the small size of nodularin, which (i) demands optimization of an indirect setup of the DynoMag measurements and (ii) limited

in depth characterization of aptamers selected against this target molecule, which was essential for their further implementation in the bioassay development on DMF platform. Despite these limitations, several important achievements have been realized in this task using these model systems: (i) development of ultra-sensitive digital bioassays with a limit of detection in the range of expected nodularin concentrations in the bioreactor; (ii) implementation of these bioassays on a DMF, a platform that can support low sample consumption, complete automation and high-throughput of a bioassay, thus offering desirable features for online monitoring of compound production in bioreactor; (iii) proof-of-concept competitive assay between DNA capture probe and the target molecule for aptamer binding, which is essential step in the designed bioassay; (iv) application of the DynoMag system in detection of proteins when using aptamers as bioreceptors.

A prototype lab-on-a-chip i.e. DMF platform was designed (**KULEUVEN**) and microfabricated for performing ultra-sensitive bioassays and therefore quantification of molecules down to atomolar levels. The bottom plate of double plated DMF chip was microfabricated to contain actuation electrodes of 2.8 x 2.8 mm^2, which allow electrowetting-on-dielectric (EWOD) actuation of micro- to nanoliter sized droplets. The top plate was designed with an array of 62500 femtoliter-sized microwells, which together with the EWOD actuation and magnetic attraction of superparamagentic beads dispensed in the droplet, leads to fast (less than a minute), efficient (98% of filled wells) and precise (single bead per well) printing and sealing of beads into these wells. Biotinylated β-galactosidase enzyme was used as a model system for establishing the proof-of-concept bioassay and demonstrating capacity for the DMF single-molecule detection. Therefore, this platform offers the possibility to perform a bioassay in a completely automated fashion and in a small sample volume, while achieving extremely low sensitivity, and was used, as already described, for developing proof-of-concept aptamer-based competitive bioassay.

Several elements important for coupling of bioreactor to the biosensor were developed. First, the growth of *Nodularia spumigena* cultures in bioreactor was optimized since these cultures served as a source for target sample, namely nodularin (**ABO**, **VTT**). Moreover, sample preparation protocol for extracting both intracellular and extracellular nodularin from these cultures was adapted towards simplified method more suitable for automation of sample delivery (**ABO**). Next, a system was built (**KULEUVEN**), consisting of bioreactor, syringe pump, T valve, tubing and microfluidic platform, that can ensure automated delivery of a sample from the bioreactor to the biosensor. In this system, the sample delivery is performed by (1) drawing the sample into the tubing through the T valve toward the syringe pump, (2) switching the T valve to close the bioreactor tube when the sufficient amount of sample has passed the valve and (3) opening the microfluidic platform tube which is simultaneously followed by change of pump operation into the pushing mode to deliver the sample toward the microfluidic platform. The microfluidic platform that can be used as a biosensor is either DMF or segmented flow microfluidics. The latter, similarly to DMF, allows for bioassay performance in confined reaction volume and in a fully automated fashion. Such PDMS segmented flow chips can be used for droplet splitting in combination with magnetic particles as well as for extraction of target molecules from samples.

WORK PACKAGE 5: Sustainable synthetic methodology, compound optimization and medicinal chemistry

The WP5 focused on medicinal chemistry and synthesis of the selected promising compounds in amounts needed for the work of the consortium. More specifically, the main objectives of WP5 were to design and synthesize derivatives, analogues and mimetics of marine bioactive compounds, carry out structure-activity and structure-property relationships analyses, identify pharmacophores and develop the most promising hits to lead compounds.

Synthesis of clathrodin, oroidin and hymenidin. Marine alkaloids oroidin, clathrodin and hymenidin, isolated from sea sponges of genus *Agelas* are important members of a pyrrole-2-aminoimidazole (P-2-AI) marine alkaloids family which represent also the biosynthetic building blocks for whole P-2-AI family of alkaloids. They were reported in 1995 to possess a broad spectrum of bioactivity, with reduction inward sodium currents through sodium channels into the cell and antibacterial activity being most important.

With a desire to examine their effects on voltage gated sodium channels (Na_v) as well as on other biological targets, we developed an optimized, well reproducible and economical synthesis of clathrodin, oroidin and hymenidin and their analogs possessing a saturated or unsaturated linker moiety. The key intermediates were obtained through two different synthetic pathways starting from L-ornithine and benzyl pyridine-1(2*H*)-carboxylate, respectively, using an innovative combination of Weinreb amide strategy with di-Boc protection and benzyl pyridine-1(2*H*)-carboxylate based strategy. A convenient access to key 2-aminoimidazole amines which was developed as a part of this synthesis is crucial for the synthesis of libraries of clathrodin, oroidin and hymenidin analogs. Clathrodin thus prepared was reinvestigated in WP3 for its potency and Na_v channel subtype selectivity on a broad range of Na_V channel isoforms, both in voltage clamp and patch clamp conditions and, contrary to previous reports it was not found to exert any Na_v modulating activity.

First total synthesis of marine steroids solomonsterol A and solomonsterol B with PXR agonistic activity. Among the existing hits and identified key structures of marine origin from the participating beneficiaries, solomonsterols A and B, recently isolated and characterized from the marine sponge *Theonella swhinoei* in the laboratories at University of Naples, were selected as target molecules to be developed in the WP5. Solomonsterols represent the first example of natural marine agonists of PXR, a master gene orchestrating the expression of a wide family of genes involved in uptake, metabolism and disposal of a number of endo- and xenobiotics, including drugs, bile acids, steroid hormones and metabolic intermediates in mammalian cells. As the solomonsterols were isolated from natural sources in scarce amount, total synthesis was necessary to provide enough amount of material for further pharmacological experimentation.

The first total synthesis of solomonsterol A was completed in ten steps starting from commercially available hyodeoxycholic acid, with an overall yield of 40 percent. This route was efficient enough to prepare sufficient quantities of solomonsterol A to proceed to pharmacological tests. Solomonsterol B features a C_{23}-nor side chain shorter of one carbon atom with respect to solomonsterol A whereas the functionalization of the steroidal core is identical. The most convenient route toward solomonsterol B was planned to involve a divergent approach in which elaboration of the side chain would follow the functionalization of steroidal ring but, however, this approach failed to give appreciable results. Therefore we turned to alternative strategy in which the one carbon degradation of the side chain was realized in an early phase, through a second-order Beckmann rearrangement of hyodeoxycholic acid 3,6-diformate and then the obtained C_{23} norcholanic acid derivative was subjected to the same synthetic sequence elaborated for solomonsterol A, to afford synthetic solomonsterol B.

Synthesis of purpurealidin I. Synthesis of purpurealidin I, a new bromotyrosine alkaloid isolated from sponge *Psammaplysilla purpurea* by a **MAREX** partner **NIO** was initiated by **UHEL** and the synthesis for the left-hand acid-part of the molecule has been developed while synthesis of the right-hand part is still on-going. **UHEL** made progress also in the synthesis of aplysamines and purpurealidin E, alkaloids structurally similar to purpurealidin I which have been shown to have antibacterial and anticancer activities.

Clathrodin analogues, derivatives and mimetics obtained by targeted rigidification of clathrodin molecule, as new voltage-gated sodium and potassium channels modulators. A broad synthetic modification plan involving stepwise rigidification of clathrodin, oroidin and hymenidin in different regions of the molecule was elaborated towards a library of analogs, derivatives and mimetics to be tested in electrophysiological assays as well as in other assays offered by WP3. The design strategy of analogs and mimetics involved rigidification of the parent molecules in the western, an eastern part, replacement of the pyrrole ring with other heterocyclic moieties and methylation of the amino group of aminoimidazole moiety. Compounds containing several unnatural spacers like piperazine, 1,3-diphenyl and 1,4-diphenyl were prepared and synthetic strategies for compounds containing rhodanine spacer and compounds rigidified in eastern part of the molecule were explored.

Altogether, over 100 new compounds were designed and synthesized and submitted to WP3 for electro-physiological testing on voltage-gated sodium channels. Evaluation of a series of conformationally restricted clathrodin analogues incorporating the 4,5,6,7-tetrahydrobenzo[*d*]thiazol-2-amine moiety for their modulatory activities on human voltage-gated sodium channel isoforms $Na_v1.3$, $Na_v1.4$ and $Na_v1.7$, as well as for their selectivity against cardiac isoform $Na_v1.5$ showed that they act as state dependant modulators of $Nav_{1.3}$, $Nav_{1.4}$ and $Nav_{1.7}$ with IC_{50} values in the lower micromolar range for the open-inactivated state of the channels. (*S*)-*tert*-butyl 2-[((S)-2-amino-4,5,6,7-tetrahydrobenzo[*d*]thiazol-6-yl)carbamoyl]pyrrolidine-1-carboxylate with IC_{50} value of 8 µM against $Nav_{1.4}$ represents a novel selective state-dependent $Nav_{1.4}$ channel modulator. Among analogs containing a 1,3-phenylene or 1,4-phenylene linker, several compounds exhibited promising activities on different Na_V channel isoforms in the medium micromolar range and some of the compounds showed also moderate isoform selectivities. The most promising results were obtained for the $Na_V1.3$ channel, for which four compounds were found to possess IC_{50} values lower than 15 µM. All of the active compounds of this series were found to bind to the open-inactivated states of the channels and therefore act as state-dependent modulators.

Based on crystal structures of the voltage-gated sodium channel from bacteria *Arcobacter butzleri* published in 2011 and of the open bacterial voltage-gated sodium channel from *Magnetococcus marinus* MC-1 published in 2012, we built homology models of human $Nav_{1.4}$ channel in both open and closed form*s* as well as homology models of human $Na_v1.3$ and human $Na_v1.7$ channels in their closed and open conformations in order to enable structure-based design of clathrodin analogues. Further clathrodin related compounds for screening were identified by similarity search in ZINC library of druglike compounds based on well performing synthetic hits and subsequent docking of resulting sub-libraries into local anesthetic-binding site of the built homology models. Using this approach, *N*-(3-(1*H*-pyrazol-5-yl)phenyl)benzofuran-2-carboxamide and *N*-(3-(1*H*-tetrazol-1-yl)phenyl)-4-fluorobenzo[*b*] thiophene-2-carboxamide that blocked sodium permeation in $Na_v1.7$ with IC_{50} values of 7 and 9 µM, respectively, are among the most potent clathrodin analogs discovered so far and represent novel scaffolds for the discovery of human $Na_v1.7$ modulators.

Design and synthesis was also performed in the field of potential $Na_v1.3$ modulators, possessing structural elements of our previous clathrodin-based series combined with structures of potent $Na_v1.3$. channels modulators reported in the patent literature. Synthetic efforts complemented by similarity searches led to identification of nanomolar $Na_v1.3$ blockers selective against cardiac isoform $Na_v1.5$.

A promising blocking activity of voltage gated potassium channels was observed for several direct clathrodin analogues and detailed studies are still underway.

The screening of a sub-library of 96 compounds selected among the prepared clathrodin analogs in WP3 revealed 3-chloro-, 5-fluoro-,5-methoxy- and *N*'-methylamino derivatives of 2-amino-4-(3-(1*H*-indole-2-carboxamido)phenyl)-1*H*-imidazole as potent inducers of apoptosis in HepG2 and THP-1 cell lines with EC_{50} values in the low micromolar range, highlighting these oroidin analogues as interesting candidates for further evaluation of their anticancer activity.

Clathrodin and oroidin prepared by the synthesis described above were evaluated for their antimicrobial activity against three bacterial strains (*Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli*) and one fungal strain (*Candida albicans*), and oroidin was found to possess promising Gram-positive antibacterial activity. Using oroidin as a scaffold, 34 new analogues were designed, prepared and screened for their antimicrobial properties. Of these compounds, 12 exhibited over 80% inhibition of the growth of at least one microorganism at a concentration of 50 μM. The most active derivative was found to be 2-amino-4-(3-(5-(benzyloxy)-1*H*-indole-2-carboxamido)phenyl)-1*H*-imidazole, which exhibited MIC_{90} values of 12.5 μM against the Gram-positive bacteria and 50 μM against *E. coli*. Several novel synthetic indole-based analogues exhibited antibiofilm activities in the low micromolar range against the Gram-positive biofilm-forming strains of MRSA and *Streptococcus mutans* and therefore represent a novel class of 2-aminoimidazole-based inhibitors of *S. aureus* biofilm formation and, to the best of our knowledge, the first reported 2-aminoimidazole-based inhibitors of *S. mutans* biofilm formation.

Design and synthesis of a library of synthetic derivatives of solomonsterol A, souvanine and theonesterol as modulators of nuclear receptors PXR and FXR. Through structural changes of steroidal core or side chain in the intermediates in the synthesis of solomonsterol A, we built a library of synthetic derivatives of this natural lead for the treatment of inflammatory bowel disease. The regio- and stereoselective introduction of selected functional groups in the A/B rings of the steroidal nucleus or in the side chain where the sulfate function at the C-24 was replaced with a carboxy, hydroxy or ester functions were prepared and tested for PXR agonistic activity. 5-Aminosalicylic acid derivative was also prepared with the aim of obtaining compounds acting as dual PXR PPARγ agonists.

A library of ten semisynthetic derivatives of theonesterol from *Theonella swinhoei* which was recently disclosed by **USNF** group as the first example of natural highly selective FXR antagonist, demonstrating its pharmacological potential in the treatment of cholestasis, was prepared in order to gain insight into the structural requirements for antagonistic activity. The transactivation assays on FXR showed that the introduction of a hydroxyl group at C-4 as well as the oxidation at C-3 with or without concomitant modification at the exomethylene functionality preserve the ability of theonellasterol to inhibit FXR transactivation caused by CDCA. Suvanine, a furano-sesterterpene sulfate from the marine sponge *Coscinoderma mathewsi* was also found to display potent antagonism against FXR and a library of its semi synthetic derivatives was prepared to study their binding mechanism to farnesoid X receptor and reveal a strategy to forestall drug modulation by nuclear receptors.

USNF also designed and synthesized a family of new steroidal derivatives, analogues of bile acids, that can act as dual modulators of GP-BAR1 and FXR receptors, two receptors with overlapping activities in the maintenance of bile acid homeostasis and in the protection of gastric and intestinal mucosal integrity.

Starting from a serendipitous discovery that two quite conventional steroids isolated from the **NIO** marine sample were endowed by an agonistic activity on PXR, the synthesis of a library of 3*β*-hydroxy-steroids was realized in order the explore the molecular requisites for the 3*β*-hydroxysteroidal

scaffold for modulation of the activity of this nuclear receptor. Evaluation on PXR in a luciferase reporter assay on a human hepatocyte cell line (HepG2 cells) revealed natural (24*S*)-ergost-5-en-3β-ol as the most potent PXR agonist.

The semi synthetic derivatives of conicasterol from *Theonella conica* were prepared and tested for PXR activation with the double aim to develop PXR agonists based on the natural conicasterol with better safety profile, and to explore the role of punctual functional group in the 4-methyliden-steroid scaffold for PXR activation. The study indicated that the substitution of the exomethylene function, present a C_4 of natural conicasterol with a methyl appendage or with a polar group causes a loss of the activity. The only active component of the library acted as a PXR agonist, inducing the expression of three genes, CYP3A4, MDR1 and MDR2, regulated by PXR.

Stimulated by the observed agonistic activities of solomonsterols A and B on PXR receptors, beneficiary **UL** designed and synthesized a series of steroidomimetic analogs of solomonsterols A and B by replacing the steroid core of the natural leads with a steroidomimetic bazedoxifene scaffold. This resulted in the discovery of novel series of PXR antagonists among which 4-(5-hydroxy-3-methyl-1*H*-indol-2-yl)benzene-1,2-diol and 4-(5-(3-hydroxypropoxy)-3-methyl-1*H*-indol-2-yl)benzene-1,2-diol possessed IC_{50} values of 11 and 14 μM, respectively, for the inhibition of rifaximin-induced PXR transactivation. These compounds stand out as an important new structural class of PXR antagonists offering good opportunity for further optimization. The suppression of the PXR master target gene CYP3A4 highlights these two compounds as PXR antagonists with the capacity to attenuate PXR-regulated phase-I drug metabolism *in vitro*. Finally, they represent unique examples of PXR antagonists that can downregulate the expression of PXR.

The replacement of the bazedoxifene scaffold of these PXR antagonists with the scaffold of diethylstilbestrol, as well as the incorporation of the structural motifs of solomonsterols A and B resulted in novel PXR modulators with the ability to alter PXR-regulated CYP3A4 expression. The methylated diethylstilbestrol derivative *(E)*-4,4'-(hex-3-ene-3,4-diyl)bis(methoxybenzene) displayed potent PXR agonistic activity with an EC_{50} value of 10.5 μM, whereas *(E)*-2,2'-((hex-3-ene-3,4-diylbis(4,1-phenylene))bis(oxy))diethanol exhibited PXR antagonistic effects with IC_{50} of 27.4 μM. The PXR modulatory effects of the synthesised diethylstilbestrol derivatives were further confirmed by the induction of PXR-regulated CYP3A4 expression with *(E)*-4,4'-(hex-3-ene-3,4-diyl)bis(methoxybenzene**,** as well as by the inhibition of the rifaximin-promoted up-regulation of CYP3A4 expression with *(E)*-2,2'-((hex-3-ene-3,4-diylbis(4,1-phenylene))bis(oxy))diethanol.

Oroidin-related inhibitors of gyrase B. Identication of a striking structural similarity between some recently described inhibitors of bacterial gyrase B and some **UL** clathrodin and oroidin-related Na_v channels modulators, stimulated us to test oroidin and some prepared clathrodin analogs for gyrase B inhibition. Some promising micromolar hits were identified and this information formed a basis for an intensive structure based design and synthesis campaign at **UL** in which over 200 compounds from 8 structural classes comprising tetrahydrobenzothiazole series, 5,6,7,8-tetrahydroquinazolin-2-amine series, *N*-phenyl-1*H*-pyrrole-2-carboxamide series, 3-(1,2,4-oxadiazol-5-yl)-aniline series, *N*-2-acyloroidin series, 1,4-benzoxazin-3-on series, piperazine/pyrimidine series and 6-(aminomethyl)benzothiazol-2-amine series were designed, synthesized and tested for *E. coli* gyrase B inhibition. Among them some very potent nanomolar gyrase B inhibitors, *e.g. (S)*-3-((6-(4,5-dibromo-1*H*-pyrrole-2-carboxamido)-4,5,6,7-tetrahydrobenzo[*d*]thiazol-2-yl)amino)-3-oxopropanoic acid were discovered. The pharmacophoric features responsible for gyrase B inhibition were identified and formed a basis for design of lead compounds. Several low nanomolar leads were identified and synthesized in quantities required for antibacterial testing which is still ongoing. X-ray crystal structures of two inhibitors, 2-((2-(4,5-dibromo-1*H*-pyrrole-2-carboxamido)benzo[d]thiazol-

6-yl)amino)-2-oxoacetic acid and (4-(4,5-dibromo-1*H*-pyrrole-2-carboxamido)benzoyl)glycine in complex with gyrase B were solved and they provide an excellent basis for structure based design of potent gyrase B inhibitors with antibacterial activity.

Analogues of spumigins and aeruginosins. A library of analogues of spumigins, protease inhibitors from *Nodularia spumigena* from the Baltic sea, based on mimicked *D*-Phe-Pro-Arg sequence, was designed, synthesized and biologically evaluated for thrombin inhibitory activity. (2*S*,4*S*)-4-methylproline central core of spumigins was replaced with indoline as a more hydrophobic or (*S*) proline as a more flexible central core, while the configuration of amino acids in spumigins structures was retained. The most potent thrombin inhibitor derived from this series was ((*R*)-2-((*R*)-2-hydroxy-3-(4-hydroxyphenyl)propanamido)-4-phenylbutanoyl)-L-prolyl-L-arginine methyl ester with a K_i of 3 μM and it could be concluded that in the investigated series of spumigin *L*-proline is the most optimal central scaffold for thrombin inhibition.

Potential inhibitors of protein phosphatases based on the structure of okadaic acid. A library of 15 compounds based on the structure of okadaic acid were designed and synthesized by partner **ULL** in order to obtain inhibitors of protein phosphatases, which are still under biological evaluation.

WORK PACKAGE 6: Sample logistics and data management

The aim of this work package was to ensure efficient sample and data flow between project partners. The main objectives were to create Consortium level recommendations for standardisation of logistics, sample collection, preparation and handling related to field and cultivated specimen as well to fractions, isolated compounds and synthetic derivatives.

In addition, this WP focused on standardisation of the data collection from the multidimensional work carried out during the project as well as on enhancing data sharing between the beneficiaries by creating a relational database that allows secured, on-line access to the Consortium members. The **MAREX** database contains detailed data on five different levels from the following sample types: collected specimen, crude extracts, fractions, subfractions, and isolated/synthesised compounds. From each of the sample types detailed information on processing as well as on biological testing were collected. At the end of the project, the database holds over 38 000 records.

During the last project period, guidelines and instructions also for storing project samples and research data beyond the span of the project were discussed and described. A good example of such efforts is a website established by EGE University, http://www.egemacc.com/index.php. This website includes an electronic catalogue of microalgae cultures available at EGE, and includes their photos as well as information on the origin and main characteristics.

- **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**

There is a perpetual need for new chemotherapeutics for the treatment of human diseases, especially in the anti-infective, anticancer and anti-inflammatory fields. The discovery and development of new drugs from natural products (NPs) has played a significant role over the last few decades. In this context, the exploration of still untapped marine environment represents a great potential for the discovery of new chemical entities for example for pharmaceutical, nutraceutical, cosmetic, and agrochemical applications. Therefore, the main objective of **MAREX** was to isolate, characterize and sustainably exploit new compounds from extracts prepared from marine organisms. The screening of **MAREX** consortium samples for therapeutically and industrially significant biological activities was an important task of the Project and the results of these activities will undoubtedly have great potential to have significant impacts on several fields. Despite the abundance of marine organisms and the diversity of valuable compounds derived from them, direct exploitation of these resources is increasingly problematic due to environmental pollution and extinction of species. One of the solutions for this dilemma as explored in **MAREX** is the sustainable cultivation and controlled maintenance of various micro- and macroalgae allowing reproducible extraction of valuable compounds. Coupled with this approach is the need to elucidate the biosynthetic pathways to these metabolites and the need to monitor their accumulation in cultures.

The following section describes the potential of impact of the **MAREX** project in more detail by highlighting examples from each Work Package.

The potential impact of the activities in WP1 (Sourcing and bioprospecting) are the following:

- Over 600 marine organisms were collected from the Atlantic, Pacific, and Indian Oceans, and from the Mediterranean, Baltic, Adriatic, and Arabian Seas. These organisms included dinoflagellates and other microalgae, macroalgae, cyanobacteria, sea anemones, tunicates, ascidians etc., and the collections and related information are available for scientific and potential commercial exploitation.
- Improved processes for the collection, isolation and extraction of marine organisms were developed during the project, which will potentially increase their amenability for industrial use.
- Techniques for maintenance of collected and isolated microalgal strains and cryopreservation methods for long-term preservation were also developed. Thereby these strains will be continuously available for future studies as well as for possible collaborations.
- A new website (***http://egemacc.com***) that allows accessing photographs, information and cultivation conditions of specimens related to the **MAREX** was established.

The potential impact of the activities in WP2 (Isolation and chemical characterization) can be summarised as follows:

- A wide number of crude active extracts from different marine macro- and micro-organisms were subjected to a complete chemical characterization.
- More than one hundred new secondary metabolites were chemically identified. Some of them feature very complex or unprecedented molecular architectures confirming the enormous potential of the marine environment as source of new chemical entities.
- Eighty known compounds were re-isolated. When subjected to pharmacological investigation, some were found to possess pharmacological activities not previously described. Surely the preliminary results obtained in the pharmacological investigation should

represent a precious starting point for the scientific community to investigate in detail the mechanism of action and the therapeutic potential of the isolated compounds.

- Some innovative approaches to the stereochemical characterization of complex flexible molecules were developed.
- Novel HPLC methods based on reversed-phase chromatography and acidic/neutral mobile phases were developed for the chemical decodification of the complex mixtures of peptides from cyanobacteria.

The research carried out in WP3 (Screening and application development) resulted in the following findings with potential impact:

- Apoptosis, cell growth and toxicity are typical endpoint parameters for cell-based primary anticancer screening assays. When considering cancer stem cell lines from testis or pancreatic origins, micromolar values were observed for selected synthetic analogs of clathrodin showing a high anticancer potency for these molecules. For other cancerous cell lines of pancreatic, hepatic, or hematopoietic origins similar potency were obtained. Kinetic differences in the action of the analogs, suggest a difference in targets involved in the cell death of monocytic cells considering all other tested cell types were responding as the testis cancer stem cells. Thus, these synthetic analogs of clathrodin represent promising pro-apoptotic compounds as pharmacological tools on one side but also as putative new anticancer drugs.
- A screen with proliferation assays on two human prostate cancer cell lines and one human breast cancer cell line for assessment of anticancer effects resulted in 99 anti-proliferative hits. Many of the samples were sub-fractions of extracts originating from marine organisms collected during the project.
- Some of the samples showed interesting anti-inflammatory properties as observed in the primary microglial, neuronal and astrocyte cell culture models. In addition, the analysis of pro-inflammatory cytokine release has also shown that some extracts and identified compounds may regulate the immune response.
- Synthetic analogues of the marine product clathrodin resulting from computational and medicinal chemistry design in WP5 showed nice activity against therapeutically important targets, specifically voltage-dependent sodium (Nav) and potassium (Kv) ion channels involve in pain and autoimmune disease. In conclusion synthesis and biophysical characterization of novel small molecule scaffolds derived from the marine product clathrodin reveal potent and selective modulators of Nav1.3 and Kv1.3 channels, with indications of a binding mechanism that interferes with channel inactivation. These novel compounds could expand the repertoire of medicinal chemistry leads suitable for development of treatments for chronic pain and autoimmune disease.
- **MAREX** samples with significant antimicrobial activity against bacteria, fungi and viruses were identified during the project. For example, samples strongly reducing the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) were discovered making these potentially useful leads. Samples active against several other bacteria, such as *Vibrio cholerae,* were also identified. Evaluation of antiviral activities on Chikungunya virus and hepatitis C virus replicon models lead to the discovery of promising antiviral leads. Antimicrobial and antifouling activity testing showed potential inhibition crude extracts of cyanobacteria and microalgae on pathogenic bacteria when tested on different bacteria strains (e.g., three diatom crude extracts significantly inhibited growth of the gram+ *Enterococcus faecium*).
- Stimulation of bacterial growth may be desirable for biotechnological purposes, for example, to express recombinant genes and synthesize particular products. One may speculate that stimulation of anabolic processes by fragments of cyanobacterial genomes issued from the

MAREX project, might positively affect growth rate of *E. coli* host cells, which can have a potential impact on biotechnological production.

- **MAREX** extracts were studied also for their potential use agricultural applications i.e. for plant growth regulation. Cyanobacteria samples studied showed effects on higher plant growth (*e.g.*, sorghum), but most interestingly, high activity of *Nodularia* extracts against marine microalgae growth was detected (IC50 < 0.2 mg/ml), suggesting that compounds from this species could be developed as marine algicides.
- A new predictive model of idiosyncratic hepatotoxicity, in which extracts are administered to a human hepatic cell line, was developed as hepatotoxicity commonly results from drug-induced hypersensitivity
- A method for analyzing a biofilm formation using magnetoelastic resonance (MER)-technology was developed and will be useful for studies of bacterial adherence in the near future.
- A novel imaging-based screening assay for identifying inhibitors of Chikungunya virus replication on a cell-cultivating platform with integrated optics.

The potential impact of the activities in WP4 (Biotechnological production and biosensing for sustainable industry) can be summarised as follows:

- Extracts and fractions of a wide variety of species have been made available for screening and bioactivity testing in order to initiate lead and development of products for human benefit
- Cost-effective cultivation protocols to produce valuable bioactive molecules with high productivity for industrial use were developed.
- Long-term storage of important strains was developed in order to ensure future exploitation.
- The biosynthesis of marine toxins responsible for different seafood poisonings with important consequences for human health and great economic impact in the fishery industry were elucidated.
- Detailed methods allowing the setup of biosensors with applications in environmental and process monitoring were developed.

Activities of WP5 (Sustainable synthetic methodology, compound optimization and medicinal chemistry) lead to the following findings with potential impact for the future:

- The results obtained in WP5 will contribute to better availability of bioactive compounds of marine origin and strenghten the potential of European pharmaceutical and chemical industry in drug discovery and production of fine chemicals.
- The developed efficient syntheses of *Agelas* alkaloids clathrodin, oroidin an hymenidin, marine steroids solomonsterol A and solomonsterol B as well as the marine alkaloid purpurealidin I can be regarded as contribution to overcome the bottleneck of limited availability of these compounds from natural sources.
- About 400 new synthetic compounds designed, synthesized and evaluated as derivatives, mimetics and analogs of these natural leads to interact with various biological targets involved in channelopaties, cancer, bacterial infections, inflammation, thrombosis, drug metabolism and chemoresistance are an important contribution to a search for new agents for alleviating these diseases and processes contributing to drug resistance. The obtained results validate the approach of using marine natural products driven chemistry for drug discovery starting points and represent a good foundation for future design of more potent compounds.

Main dissemination activities and exploitation of results

One of the **MAREX** Work Packages, WP7 (Training, dissemination, industrial feasibility and technology transfer) was dedicated for ensuring a widespread dissemination of results and activities of the Consortium, to ensure fair and equitable benefit sharing and the proper handling of IPR, and to provide excellent and high-quality training.

To summarize, in total 333 dissemination activities were done during the project including oral presentations to civil society (especially for the school pupils) and scientific audience, scientific posters, articles in popular press, press releases and TV, radio and newspaper interviews. Also several workshops and conferences were organized and the project was presented in exhibitions.

62 scientific publications in peer-reviewed journals (27% of them in open access journals), three book chapters, and one conference proceeding were published (see the list below for further details). Additionally 21 scientific publications are submitted to the journals or books but not yet published. Final exploitation plan for the project results has been made.

MAREX webpage was established www.marex.fi and a brochure "MAREX: Explores marine biodiversity for a healthier future" for general public was published and it can be downloaded from the webpage www.marex.fi.

Three thematic workshops were held during the project. The themes were "Sustainable Cultivation of Marine Organisms" in La Laguna, Tenerife, 2011, "Young scientists' session" in Naples, Italy, 2012 and **"**Marine organisms and their collection" in Goa, India, 2013.

MAREX has also made an important contribution to training of young researchers on several research fields: 38 theses (PhD and Master's) have been finalized during the project. The theses cover a plethora of topics, such as sustainable production of marine bioactive compounds and medicinal chemistry approaches for their progression towards lead compounds, bioassay development for screening of marine samples, microfluidics, and structural and chemotypic characterisation of marine compounds. This is an important socio-economic impact of **MAREX**.

There were also a number of researcher exchanges between **MAREX** partner laboratories, encompassing junior and technical staff undertaking training or transfer of new techniques, to senior researchers forging scientific collaborations and presenting their work at partner sites.

List of MAREX publications

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