4.1 Final publishable summary report

Executive Summary

The ocean covers more than 70% of the surface of the earth and represents the largest continuous habitat with a volume of nearly 1.5 billion cubic kilometres. It has been estimated that 1.2×10^{29} bacteria live in the ocean representing the unseen majority that forms 90% of the total biomass in this habitat. Up to one billion (10^9) bacteria may be present in every litre of ocean water! These numbers contrast sharply with the approximately 10,000 bacteria that have been described and cultivated. The microbial diversity is immense and this is particularly the case in the ocean where in each millilitre thousands of different microbial 'species' may thrive. Moreover, the ocean and seas contain a plethora of (sub)habitats for microorganisms such as marine sediments, the deep biosphere, hydrothermal vents, cold seeps, and deep-sea brines, while all animals and marine plants contain their own specific microbiota. Most ('99%') of this diversity has not been taken into culture and often referred to as 'unculturable'. However, this concept is likely incorrect as the organisms seem to be viable and clearly multiply in nature. Culturing the 'uncultured' depends on finding the right conditions and growth media and this may prove extremely time consuming due to the nearly infinite possibilities of different media compositions and culture conditions, let alone that microorganisms need to be grown in defined consortia or at least need growth factors produced by other microorganisms.

Microorganisms have been used in the biotech industry for decades and have been a source of enzymes, antibiotics and a range of pharmaceuticals. Indeed it is anticipated that the biotechnological potential of new or newly cultured organisms is huge. Their genetic diversity is near to infinite as it has evolved during an almost 4 billion year history. This genetic diversity of bacteria holds promise for bio-prospecting. Additionally, microscopic algae possess high biotechnological potential, as they act as miniscule photosynthetic factories that use sunlight as the source of energy, and could mitigate the impact of greenhouse gas emissions, namely CO_2. The short generation times of microorganisms and their high potential growth yields make them attractive agents for the blue biotechnology industry. Since freshwater is a limited resource and is expected to become scarce in the near future, marine biotechnology should be the preferred way to go forward.

MaCuMBA investigated and developed a plethora of novel approaches and methodologies, including high-throughput procedures for the isolation of new marine microorganisms, using a wide variety of samples from different marine environments. **MaCuMBA** cultivated marine microorganisms under conditions that mimic or closely match their natural environment, thereby improving the strain isolation and cultivation efficiency. **MaCuMBA** combined the various approaches and methods and whenever possible developed automated procedures.

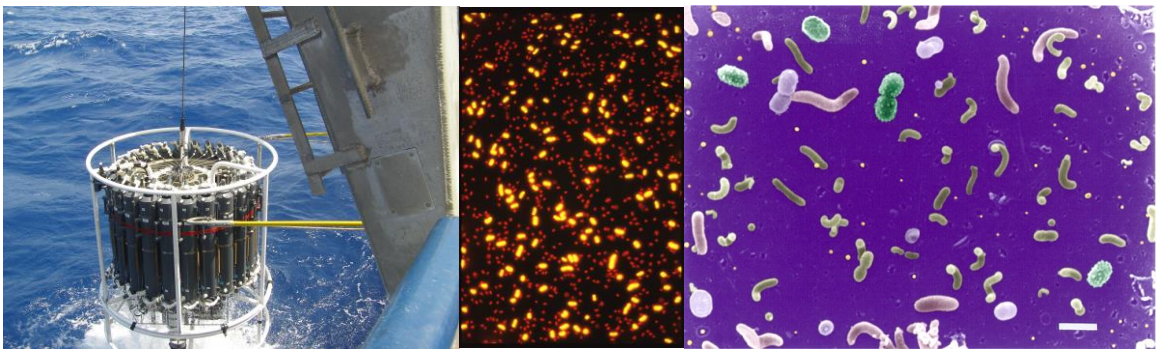

Figure 1. Sampling seawater, microscopic image of a mixed culture of phototrophs, and high diversity on a filter

Summary description of project context and objectives

The 10 aims of **MaCuMBA** are summarised as follows:

1. To increase the success rate of isolation of marine microbes
2. To isolate numerous novel marine microorganisms
3. To improve the cultivation efficiency of biotechnological relevant marine microorganisms
4. To increase the production rate of new biomolecules with high added value
5. To develop high throughput culturing methods that mimic natural conditions
6. To use cell-to-cell communication to improve cultivation efficiency and bioactive production
7. To develop robotics for high-throughput isolation and cultivation of marine microorganisms
8. To develop cultivation and genetic strategies to enhance bio-prospecting
9. To integrate bio-prospecting strategies towards industrial applications
10. To implement, disseminate and exploit the results and to develop outreach and education

The bacteria isolated in the **MaCuMBA** project could be of high interest for biotechnological applications. These might include the production of novel antibiotics or other pharmaceuticals, novel enzymes and polymers, or could provide positive applications for biofuel production. Our society has an urgent demand for these novel products to improve health and contribute towards achieving a more sustainable world.

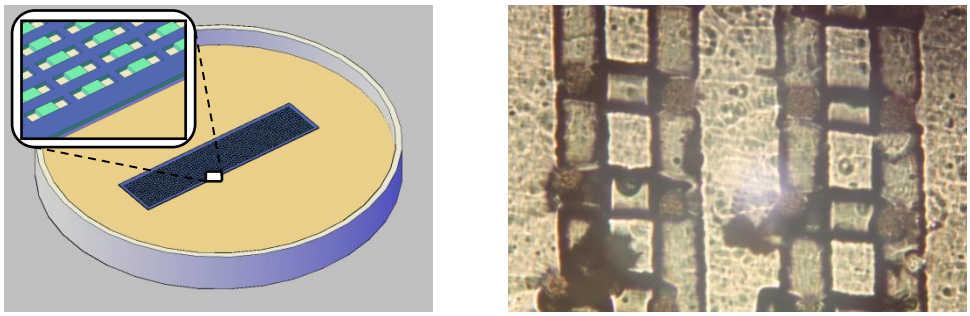

Figure 2. MicroDish Culture Chip (MDCC) for high throughput co-culturing different microorganisms

Description of main S & T results/foregrounds

MaCuMBA has organised the work into 9 work packages (WP) of which 7 were RTD. WP-2 comprised all the work related to sampling, isolation and cultivation, while WP-3 encompassed the work related to improving the cultivation efficiency with the aim of enhancing bioactivity. For the latter, selected strains isolated in WP-2 or that are available in the culture collections of the consortium members (WP-4) were used after screening for their biotechnological relevance in WP-7 (screening) or WP-6 (genome analysis). WP-6 also involved the work of genome analysis to search for clues that may improve culture efficiency. The isolated strains are stored in culture collections (depending on the type of organism), and were geno- and phenotypically identified and described (WP-4). WP-5 comprised all the work related to cell-to-cell communication. WP-8 developed the hardware that is necessary for the high-throughput isolation and cultivation of microorganisms. **MaCuMBA** had a comprehensive strategy for exploitation, dissemination, implementation and education (WP-9). Finally, WP-1 ensured the appropriate overall management of the project.

WP-1. The aim of WP-1 was to coordinate and manage the project. The coordinator did the overall management of the project and the financial administration and maintained the contact with the Commission. Several committees were established, including an active international scientific advisory board (SAB) that attended all the project meetings and provided valuable advice. The coordinator organised the project and board meetings, guided the work package leaders and reviewed all deliverables before he submitted them to the Commission. The kick-off meeting in the new Eye photo- and movie museum in Amsterdam (Netherlands) got **MaCuMBA** started. The yearly General Assemblies (GAs) were organized in the format of scientific meetings where PhD students, post-docs and principal investigators (PI's) presented their results of **MaCuMBA** related research results. SAB members alternatingly presented the keynotes. The coordination of the project was efficient so that only a minimum of time was used during the GA for administrative business. The yearly GAs were held in Roscoff (France), Cadiz (Spain), and Reykjavik (Iceland). **MaCuMBA** ended with an Industry conference face-to-face with an Open Science conference in Berlin (Germany). The coordinator kept in contact with all partners in the consortium and linked to other Knowledge Based BioEconomy (KBBE) projects. WP-1 collaborated closely with WP-9 for all dissemination and exploitation activities.

WP-2. The aim of WP-2 was to develop and apply a range of different approaches for enriching and isolating bacteria that have not been isolated before. WP-2 coordinated the sampling of various marine environments, including unusual extreme environments. A 'survival box' was developed for sampling and transporting phototrophic microorganisms. Cryogenic protocols were established for preservation of samples for transport to the laboratory. WP-2 developed protocols for the isolation of single cells with different high-throughput approaches, such as the MicroDish Culture Chip (MDCC), the microdrop technique and flow cytometric cell sorting. Also developed and implemented were the dilution to extinction and the microencapsulation methods. Using these new approaches, **MaCuMBA** has been able to isolate many new strains, including novel ultra-small nanobacteria. WP-2 has developed strategies to mimic environmental parameters accurately in order to increase the success rate of isolation of novel microorganisms. These included incubators that changed light, temperature, pressure, and CO_2. Other approaches used signalling compounds, natural substrates, and various surfaces for the development of biofilms. Novel approaches were also developed and tested for the isolation and purification of parasites, symbionts, and epibionts. Several thousands of cultures have been obtained in this way. This work package was the cornerstone of **MaCuMBA**, because it provided the input, *i.e.* microbes, for other work packages.

WP-3. The aim of WP-3 was to develop innovative methods to improve the cultivation of marine microorganisms, using both previously isolated microorganisms and those isolated by **MaCuMBA** WP-2. The strategies tested include the design of new culture media, the optimisation of growth conditions in bioreactors and the development of culture systems mimicking natural conditions. Phototrophic microorganisms were cultivated using novel LED light sources. Much effort was required for co-cultivation, but good results were obtained. For instance, marine eukaryotic phototrophic microorganisms were cultivated with their viruses and predators. FACS and MicroDish were applied to improve cultivation success when several different microorganisms were cultured. It was very elegantly shown that the cyanobacterium *Synechococcus* survived much longer in batch culture with the 'helper' bacterium *Ruegeria pomeroyi.* The latter degraded a substance produced by the cyanobacterium. Natural environments such as deep-sea hydrothermal vents and for deep-sea hypersaline basins were mimicked using devices that control pressure and temperature. A heat-stable matrix was designed that allowed growth of hyperthermophiles by immobilisation. New protocols were put into place that minimized the 'viable but non culturable' (VBNC) state of marine microorganisms, resulting in a much higher yield of isolates. In order to circumvent the highly diverse and complex marine microbial mat systems, two approaches were successfully applied. Dilution to extinction resulted in much

less complex but still functional microbial mats. The second approach used isolates that were co-cultivated as an artificial microbial mat. Media were optimised to improve the production of secondary metabolites. Media optimisation was done by developing and implementing genetic algorithms in order to circumvent the infinite variations. Improving the cultivation of marine microorganisms is not only useful for demonstrating their biotechnological potential: cultivating microorganisms is the best way to assess their metabolic and physiological properties and thus understand their fundamental role in the ecosystem they originate from and in the global biogeochemical cycles on Earth.

WP-4. The aim of WP-4 was to deposit strains in collections together with their descriptions and to develop long-term preservation protocols. Core collections received the most important strains and preserved them for the long-term. These core collections were: 'Deutsche Sammlung für Mikroorganismen und ZellKulturen' (DSMZ) (Braunschweig, Germany), Université de Bretagne Occidentale Culture Collection (UBOCC) (Brest, France), Roscoff Culture Collection (RCC) (Roscoff, France), and Culture Collection Yerseke (CCY) (Texel, Netherlands). Core collections compile all information available for the strains, allowing an on-line search. In total more than 1800 strains of microorganisms have been deposited to core collections. For example, CCY transferred all cyanobacteria (>300 strains) to DSMZ. These research collections are more limited in scope and hold strains isolated by individual partners during the project. Several long-term preservation protocols have been established. Different cryo-protectants have also been tested. Cryo-preservation included storage at -80^{o}C, in liquid nitrogen, or in air that is cooled by liquid nitrogen. In the latter method cross contamination through the liquid is prevented and result in a better resuscitation of the organisms. The strains isolated within **MaCuMBA** have been entered in the Bacterial Diversity database to secure and preserve their phenotypic and genetic information.

WP-5. The aim of WP-5 was to use cell-to-cell communication to improve cultivation efficiency. Many isolates have been tested for the presence of molecules involved in cell-to-cell communication, including the classical AHL-type molecules that are characteristic of many bacterial families. Additional signalling molecules and biotic/abiotic factors that may potentially contribute to the culturability of marine isolates were also sought and identified. Several important results have been achieved, not least the profiling of signalling compounds and other lead molecules that are likely involved in promoting or antagonising the growth organisms that do not produce these compounds. In the cyanobacterium *Synechococcus* – *Roseobacter* co-cultures an exo-metabolite was identified that served as an inter-communication molecule that 'senses' the stationary phase of the cyanobacterium. Living quorum-sensing (QS) microorganisms were succesfully used to promote the growth and isolation of sponge associated bacteria. The living QS bacteria grew separated by a membrane from the sponge bacteria. The stability of N-Acyl homoserine lactones (AHLs) in molten, cooled agar was evaluated. It was demonstrated that AHL promoted the growth of a range of microorganisms.

WP-6. The aim of WP-6 was to use genome and metagenome sequencing and bioinformatics to help develop isolation strategies and bioactive compound production. Protocols were developed to allow for some uniformity across studies of nucleic acid isolation and sequencing. A genomic/metagenomics workshop was held to improve the knowledge of **MaCuMBA** partners. This workshop was also open for scientists outside the **MaCuMBA** consortium. Pipelines were established to ensure that all the **MaCuMBA** partners were able to analyse their sequence data in an appropriate manner. These included genomic sequencing from cultured microbes, metagenomic sequencing of environmental samples, and transcriptomic studies for functional analysis. Among others, a novel type III polyketide synthase (PKS) was discovered. The online service JSpeciesWS was launched for pairwise genome comparison. An ocean-sample-to-sequence protocol was developed and tested that showed that water filtering is critical but that sample storage and cell sorting have less effect.

Many metagenomes from various marine and oceanic samples have been obtained and were being analysed. They revealed a range of different genetic markers and genes for quorum sensing, bioactive compounds, and bacterial metabolisms. They helped understand the meaning of natural units of diversity in the ocean by defining the concept of 'Ecologically Significant Taxonomic Units'. Novel key modulators of bacteria-bacteria host interactions were discovered. The role of flexible genomic islands in the evolution of bacteria has been disclosed detecting different speeds of change, allowing the establishment of metaclonal populations in the sea. Metatranscriptomics revealed a delicate equilibrium between primary producers, nitrogen fixers, and heterotrophs in the ocean. Several sequencing-based studies were initiated and many are still ongoing, which will produce a wealth of new data in the coming years.

WP-7. The aim of WP-7 was to develop high-throughput screening methods of marine microorganisms for the identification of bioactive compounds. Various bio-assays have been developed or optimised. Several groups have sent microorganisms isolated by them or extracts of those cultures for screening for bio-actives. Microorganisms have been screened and tested for antibacterial and antifungal activities and several positive strains were detected. Of the 183 deep-sea fungi screened against 19 bacterial pathogens, 14 possessed activity and new secondary metabolites. Of the 2,277 cyanobacterial extracts 362 showed antibacterial activity. The minimal inhibition concentrations were determined in a new MTP format. More than 1000 marine bacteria were screened for quorum quenching activities with the aim of searching for compounds that can stop biofilm formation or the virulence of pathogens. Supernatants of quorum quenching sponge isolates showed antifouling activity and prevented biofilm formation. Novel extracellular polysaccharides and lipids from marine microorganisms have been extracted and were characterised. None of the extracted and analysed EPS possessed antifouling activity. Two strains were found that possessed the interesting potential to produce polyhydroxy alkanoates (which is used for the production of biodegradable plastic). Two lipases were found that possess novel functionality. Many interesting enzymes were detected in Deep Hypersaline Anoxic Basins (DHABS). Actinobacteria were screened for anticancer activity and 47 out of 1000 strains have been selected for further study. Novel algicides were isolated from *Ruegeria mobilis* strains that were isolated and cultured from surface waters around the globe. Also the novel antibacterial compounds are likely produced by the marine bacterium *Pseudoalteromonas luteoviolacea* S4054 has been reported to produce at least two bioactive compounds with antimicrobial activity: violacein and indolmycin. Knockout mutants were investigated and a violacein knockout mutant produced three times as much indolmycin. Thus a third unrelated compound is responsible for the major part of the antimicrobial activity in this organism. Overall, the genetic manipulation of *de novo* isolated marine bacteria proved to be very difficult.

WP-8. The objective of WP-8 was to develop the hardware for high-throughput isolation of microorganisms. The Cocagne platform was further developed so that gel microdroplets (GMDs) containing cells could be sorted by flow cytometry and dispensed in microplate wells by the pipetting robot. Also the GMDs have been modified to increase their stability at high temperature. The components for robotic colony picking and transferring microalgal colonies into microtiter plates was developed. A robotised colony picker system has been designed for the recovery of cells from the wells of the MDCC. The isolation of single cyanobacterial cells using the optical tweezer was demonstrated, and the cells were brought into culture. The optical tweezer system is now in a box and can be used in a general laboratory environment. The optical tweezers has been fully automated in order to make it user friendly. In addition, an optical tweezer with fluorescence imaging was developed. This system has been used to image and isolate fluorescent and non-fluorescent cells. A microfluidic device with optical waveguides was developed aiming at the selective isolation of single microbial cells from a stream of cells using optical pressure. WP-8 has demonstrated that it is possible to fabricate a MDCC in which cells are co-cultured in individual wells separated by a hydrogel

layer. The fabrication process for the co-culture chambers with a gel diffusion barrier was optimised. Testing of the device in liquid medium showed significant convective transport through the nanoporous aluminiumoxide membrane at the bottom of the device. The MDCC has been adapted for in situ cultivation and has been tested in a natural marine environment and in the lab. Two new versions of the MDCC have been created to increase the functionality of culture chips. These are the MDCC100HEX.10 and MDCC50HEX.10. These have been fabricated for a variety of applications, particularly: (1) high throughput screening, and (2) printing and blotting

WP-9. The objective of WP-9 was to ensure effective external communication, dissemination and optimal outreach of **MaCuMBA**'s results and applications leading to optimal exploitation of its research, as well as raising awareness of the potential of the European marine biotechnology industry. From the start of the project, **MaCuMBA** carried out external communication and dissemination by means of the factsheet, press releases, the public website, social media such as Facebook, LinkedIn and Twitter, a quarterly newsletter, and presentations at external events. The website was continuously updated with news, events and results as they became available. WP-9 organised 3 industry seminars, the last one face-to-face to the final Open Science conference in Berlin. WP-9 together with WP-1 organized the yearly GA's. An introductory video on the project has also been produced and features on the homepage of the website. Another short video was produced as advertisement for the Berlin Industry- and Open Science Conference, and a main 15 minute video was produced to explain what **MaCuMBA** is about. **MaCuMBA** has established strong linkages with other projects and initiatives in the European marine biotechnology area by numerous different means including co-organising several industry events. Transfer of the current knowledge and application of state-of-the-art techniques took place during the **MaCuMBA** Summer School on isolation and cultivation of marine microorganisms (two weeks in July 2015 at the Royal Netherlands Institute for Sea Research, NIOZ. The main event was the Berlin Industry- and Open Science Conference on the 'Marine Microbiome' took place in the last week of June 2016, marking the end of the **MaCuMBA** project.

Potential impact and main dissemination activities and exploitation results

WP-1. This work package succeeded in an efficient and well-coordinated and –managed project. All agreed products were delivered in time and the finances of the project remained within the budget although some budget transfers were necessary. WP1 arranged well-organised scientific and administrative meetings that facilitated the active exchange and networking within the consortium. Especially the yearly GAs, which were in the format of a scientific congress and the three Industry Conferences, helped developing close liaisons between the industrial and academic partners. The coordinator developed tight links with other EU projects operating in the same area.

WP-2. This work package coordinated the sampling of a wide range of different marine environments and developed sampling strategies, including preservation and transport of samples. WP-2 developed successful novel and high-throughput methods and strategies for the isolation of marine microorganisms. The scientific community and industry will use these methods to increase the number of isolates that can be screened for biotechnological applications. **MaCuMBA** isolated numerous new isolates.

WP-3. This work package developed successful novel media and culture conditions that allow better growth of existing and of hitherto poorly growing marine microorganisms, which is a requirement for their biotechnological use. This includes co-culturing of microorganisms in defined consortia, conditions that mimic the natural environment of the organism, and the

use of signalling compounds that are involved in cell-to-cell communication. These new insights are crucial for the biotechnological application of marine microorganisms.

WP-4. This work package ensured that the strains isolated during the course of the project are properly deposited in culture collections, described, and taken up in internet-searchable databases. For these strains, protocols for appropriate long-term storage have been developed and the strains stored accordingly. This work package also ensured that the strains remain available for the scientific community and industry.

WP-5. This work package identified signalling molecules produced by marine microorganisms and involved in cell-to-cell communication. It investigated the conditions under which these compounds are produced and used these products to increase cultivation efficiency and the production of bioactive compounds. These results are critical for the biotech industry when using new marine microorganisms.

WP-6. This work package used (meta)genomics for two reasons. Firstly, the genome of cultivated or uncultivated microorganisms contains information that can be used to improve the efficiency of cultivation. The genome contains information on the potential of synthesis of bioactive or other compounds of interest as well as how to trigger their production. Secondly, metagenomes of consortia of marine microorganisms or of certain marine environments contain information on the presence of potentially interesting microorganisms and the clues of how to isolate and grow them. Metagenomes also give information on the presence of biotechnologically interesting organisms. The information fed into the WPs 2-7 and therefore played a central role in **MaCuMBA** but may also provide further crucial information for the biotech industry and the scientific community. Analysis of various (meta)genomes and (meta)transcriptomes is still on-going. The sequences are deposited in public databases.

WP-7. This work package screened the isolated microorganisms or their extracts so that the most biotechnologically interesting strains received priority and appropriate attention in WPs 2-6. This work package also developed and improved the bioassays necessary for the screening. The outcome of this work package is therefore directly relevant to the biotech industry.

WP-8. This work package developed a variety of hardware that became critical instruments for the large-scale, high-throughput isolation of microorganisms and will be critical for the biotech industry when searching for novel bioactive compounds. The instruments developed by this work package will have a wide application and are not be limited to the marine environment.

WP-9. This work package disseminated and highlighted **MaCuMBA**'s efforts to strengthen the awareness of marine biotechnology results and applications across European industries, particularly among the SME sector. This was done by publishing 16 project newsletters, the organization of three international industry events targeting the European marine biotechnology sector, and producing numerous dissemination materials such as press releases, articles, posters and three movies; various scientific and technical papers, publications and conference proceedings that informed scientists about **MaCuMBA**'s work and discoveries, several policy briefs targeted towards EU policy makers and a **MaCuMBA** legacy brochure. Effective dissemination of novel research discoveries in the field of marine biotechnology greatly improved Europe's capacity to generate new commercial opportunities. As part of the outreach this WP organised a Summer School for young scientists. The Summer School was a great success and it may become a recurrent teaching event (legacy of **MaCuMBA**). **MaCuMBA** produced a special education supplement newsletter. The final event, the 'face-to-face' Industry- and Open Science Conference on the 'Marine Microbiome' held in the last week of June 2016 in Berlin was a great success and will probably have a follow-up.

Address of project public website and relevant contact details

The website address is as follows: www.macumbaproject.eu
MaCuMBA was also active on Facebook (https://www.facebook.com/MaCuMBAProject) and Twitter (https://twitter.com/MaCuMBAProject)

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