# Sustainable production of biologically active molecules of marine based origin

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**The marine environment offers both economic and scientific potential which are relatively untapped from a biotechnological point of view. These environments whilst harsh are ironically fragile and dependent on a harmonious life form balance. Exploitation of natural resources by exhaustive wild harvesting has obvious negative environmental consequences. From a European industry perspective marine organisms are a largely underutilised resource. This is not due to lack of interest but due to a lack of choice the industry faces for cost competitive, sustainable and environmentally conscientious product alternatives. Knowledge of the biotechnological potential of marine organisms together with the development of sustainable systems for their cultivation, processing and utilisation are essential. In 2010, the European Commission recognised this need and funded a collaborative RTD/SME project under the Framework 7-Knowledge Based Bio-Economy (KBBE) Theme 2 Programme 'Sustainable**

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**culture of marine microorganisms, algae and/or invertebrates for high value added products'. The scope of that project entitled 'Sustainable Production of Biologically Active Molecules of Marine Based Origin' (BAMMBO) is outlined. Although the Union is a global leader in many technologies, it faces increasing competition from traditional rivals and emerging economies alike and must therefore improve its innovation performance. For this reason innovation is placed at the heart of a European Horizon 2020 Strategy wherein the challenge is to connect economic performance to eco performance. This article provides a synopsis of the research activities of the BAMMBO project as they fit within the wider scope of sustainable environmentally conscientious marine resource exploitation for high-value biomolecules.**



## Introduction

Europe is well placed to maximize its marine biotechnology potential not only due to its 70,000 km coastline with ready access to diverse marine habitats but also its outermost regions and territories of Europe in tropical zones corresponding to a high marine biodiversity. The maritime economy of the EU comprises different sectors including blue biotechnology. Growth of the blue biotechnology sector is anticipated with the market projected to surpass €3.11 billion by 2015 [1]. The overriding objective of Sustainable Production of Biologically Active Molecules of Marine Based Origin (BAMMBO) is to develop a harmonious commercial relationship with the sea by applying sustainable environmentally kind practices to the valorization of high value added biomolecules (HVABs) from marine life. Such an approach will permit a conscientious means to maximize both human and economic benefits from the marine environment whilst creating new knowledge, processes, products and employment.

Marine ecosystems are biologically diverse competitive environments. Taxonomic groups of interest to BAMMBO are outlined as are the varied environmental conditions in which these organisms reside. Their unique ability to cope with harsh environments renders these organisms ideal candidates for novel sources of both pre-existing and unrecognised HVABs with potential for providing sustainable economic and human benefits [2,3]. A consistent supply of marine derived HVABs is the major limiting factor for biotechnological development. Unless there is a feasible alternative to harvesting, promising bioactive molecules will remain undeveloped.

Irrespective of the marine organism selected sustainable cultivation methods are essential and in development within BAMMBO. A lack of understanding of growth life cycle parameters, symbiotic relationships and nutritional requirements for cultivation of marine organisms has hindered progress in this area. Enclosed bioreactor systems for target marine organism cultivation with minimal environmental impact are essential to overcome bottlenecks associated with sustainable culturing. In terms of novelty marine life forms from diverse environments are also being evaluated as sources of known or novel HVABs. Selection of organisms and HVABs are aligned with industry stakeholder needs. Furthermore, existing biobanks and waste streams are bioprospected minimizing environmental impact and destructive *in situ* harvesting. Applied culturing and capture technologies are industrially sustainable and scalable.

Target compounds are considered in a life cycle context to attain a holistic evaluation encompassing environmental, health and economic aspects. A comprehensive life cycle assessment (LCA) ensures the environmentally conscientious sustainability of entire production pathways from organism to product. LCA also permits effective economic comparisons between HVABs produced by advanced technologies and alternatives currently in the market place.

## Sustainable production of marine HVABs

The main goal of sustainability is to raise the global standard of living without increasing the use of resources beyond globally sustainable levels. In utilizing the vast resources of the marine environment the challenge is cost effective production of HVABs from sustainable sources (Table 1). Industry guided HVAB targets feed into existing and developing markets (Table 2). The markets to which these resources feed are increasing and relevant to Europe.

Marine invertebrates are rich sources of bioactive molecules with a range of applications in pharmaceuticals, cosmetics, nutraceuticals and agrochemicals [4–8]. However, HVAB production by organisms often occurs in trace amounts so the natural abundance

**TABLE 1**

**High value added bio-molecule activities being assessed from different taxonomic groups of organisms. Targeted organisms are screened for the presence of specific classes of bio-molecules. The presence of multiple novel and/or pre-existing bioactivities in an organism enhances the cost benefit of production and underpins sustainability**

| Taxonomic group | Bioactivity or functionality | Status Pre-existing known HVAB[a] Novel HVAB |
|---|---|---|
| **Antarctic fungi** | Anticancer/antitumoural[b] | Novel |
| | Antielastase | Novel |
| | Antifouling | Pre existing + novel |
| **Bacteria** | Antihyaluronidase | Novel |
| | Phytase | Novel |
| **Epiphytic bacteria and fungi** | Antimicrobials[c] | Novel |
| | Antioxidants | Pre existing + novel |
| **Macroalgae** | Carotenoids | Pre existing + novel |
| | Cellular effectors[d] | Novel |
| **Microalgae** | Lignocellulases | Novel |
| | Lipases | Novel |
| **Mediterranean sponges** | Polyunsaturated fatty acids | Pre existing |
| | Spectrally active[e] | Novel |
| | Xanthan/Fucoxanthin | Novel |

[a] HVAB, high value added biomolecule.
[b] Lead and scaffold molecules for anticancer and antitumoural agents.
[c] Antimicrobial activity demonstrated against bacteria, fungi and viruses.
[d] Membrane receptor effectors, intracellular receptor effectors, secretory mechanism effectors, electrical effects and neuronal plasticity effectors.
[e] With unique absorbance, fluorescence and luminescence demonstrated properties.

**TABLE 2**

**Industrial sectors to which marine resources have the potential to feed are large by sale volume and increasing in value. The global marine biotechnology market from which these feeds would come was projected in early 2012 to surpass $3.92 billion (€3.11 billion) by 2015 [1]. Of specific interest is the non-United States segment which comprises the bulk of this market**

| Sector | Estimated World market value ($ million)[a] | HVAB[b] |
|---|---|---|
| **Pharmaceutical** | 1000 (2006) | Anticancer agents |
| | 23 | Anti HIV (Retrovir) |
| | 237 | Antiherpes (Zovirax) |
| | 200 (2010) | Microalgal ω3[c] PUFAs (EPA[d], DHA[e]) |
| | 1000 (2015) | Carotenoids |
| | 250 (2010) | Astaxanthin |
| | 250 (2007) | Beta carotene |
| | 190 (2009) | Lutein |
| **Cosmetic** | 38,300 (2006) | Skin care products |
| **Industrial** | 2500 (2010) | Industrial hydrolytic enzymes |

[a] Year of market report is indicated in parenthesis.
[b] High value added biomolecule.
[c] Omega3.
[d] Eicosapentaenoic acid.
[e] Docosahexaenoic acid.

supplied by the wild harvest will generally not support industrial HVAB production requirements [9]. Hence, feasible alternatives to harvesting to generate products on commercial scale are needed [10]. Specifically improved sustainable solutions overcoming bottlenecks associated with traditional culture systems are needed (Table 3). Options for sustainable marine resource provision are chemical synthesis, controlled harvesting, aquaculture, intensive production and transgenics [11]. Sustainability of these solutions needs to be measured. Quantification needs to take account of the ecosystem, goods and services [12,13]. Different measures were previously proposed ranging from climate change models, ecosystem services, biodiversity indicators to intactness indices [14]. Target compounds also need to be evaluated from a life cycle perspective permitting a holistic evaluation of a developed process's sustainability. In BAMMBO a 'cradle to gate' approach is applied to assess the sustainability of marine HVABs production (Fig. 1).

## Life cycle and cost benefit assessment

Sustainable development considers three main pillars (Fig. 2), that is, environmental, economic and social [16–21]. Collectively these pillars are referred to as the 'Triple Bottom Line' [22] and are a basis for sustainable development definitions [23,24]. Holistic process sustainability evaluations relating to HVAB from marine organisms therefore entail a comprehensive assessment of the 'Triple Bottom Line' [25,26].

**TABLE 3**

**Bottlenecks to sustainable production of HVABs from marine invertebrates and the mechanisms addressed. Many bottlenecks exist which limit the utilisation of marine invertebrates as sustainable sources of high value added biomolecules. In many cases multiple bottlenecks exist which need to be collectively addressed.**

| Bottleneck | Opening the bottleneck |
|---|---|
| **Macroalgae** | ✓ Macro-algal photobioreactor tissue culturing |
| Fragile | ✓ Screen for multiple HVABs in organism |
| Seasonal | ✓ Culture and screen associated epiphytes |
| Wild harvest with low abundance of HVABs[a] | ✓ Evaluate process using LCA[b] and CBA[c] |
| **Microalgae** | ✓ Novel bioreactor design, control and simulation |
| High biomass but low HVAB yield | ✓ Uniform light, food, gas, pH, heat distribution |
| Problems with system gas balance and light distribution | ✓ Environmental growth chamber recreation |
| | ✓ Proprietary reactor modular systems |
| | ✓ Auto-, hetero- and mixotrophic culturing |
| | ✓ Evaluate process using LCA and CBA |
| **Sponges** | ✓ Aquarium culturing system development |
| Slow sponge growth | ✓ Suspended seabed sponge farming |
| Recreating natural environment | ✓ Non destructive HVAB collection and extraction |
| Wild harvest low HVAB yield | ✓ Culture and screen associated epiphytes |
| Sustainable supply of sponge and HVAB | ✓ Evaluate process using LCA and CBA |
| **Process** | ✓ Solvent free supercritical fluid $CO_2$ extraction |
| Low extraction efficiency | ✓ Chemical waste and residues reduction |
| Reduced product stability | |
| Safety of HVAB | |
| Process scalability | |
| Practicality of process | |
| **Cost effectiveness** | ✓ Evaluate processes using LCA and CBA |
| Sustainable cost effective supply | ✓ Multiple bioactive productions from organisms |
| | ✓ Enhanced waste utilisation |
| | ✓ Alternative HVAB organism sources |
| | ✓ Reduced solvent use |
| | ✓ Efficient GREEN processes 'Organic' label |

[a] High value added biomolecules.
[b] Life cycle assessment.
[c] Cost benefit analysis.


**FIGURE 1**

A holistic approach is taken to assess the sustainability of a production process for marine high value added biomolecules. A cradle to gate (from extraction of raw material through production, distribution, use, recycling and waste treatment) approach permits an assessment from the growth of the organism through to ultimate disposal of the product.

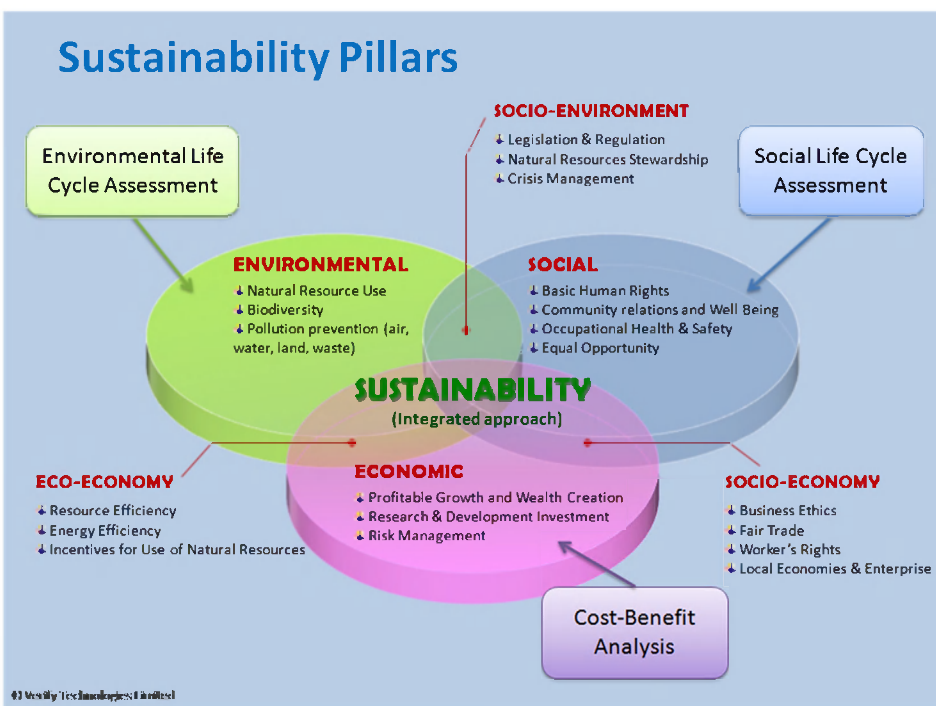


**FIGURE 2**

Life cycle assessment considers three pillars or dimensions of sustainable development, that is, environmental, social and economic [15]. Copyright Verify Technologies Limited.

A growing number of tools such as LCA have been developed [27,28]. Life cycle thinking represents the basic qualitative concept of considering the whole system life cycle [29]. Whilst carbon and water footprint analyses address single environmental issues LCA (Fig. 2) quantifies environmental impacts in different categories [30–32].

To date, LCA has been applied to the environmental evaluation of algae based biofuel production [33–39] and pharmaceuticals [40–44]. No LCA of HVABs from marine organisms has been published. The aim of LCA in the BAMMBO context is to advance the evaluation of such products using methodology required by EU legislation [45]. LCA will assist in decision making for process development and eco labelling [46,47].

Unlike environmental LCA, no social and socioeconomic assessment methodologies have been standardized yet [12]. However, the UNEP/SETAC Guidelines for Social Life Cycle Assessment of Products propose a set of inventory indicators grouped in specific impact categories [48]. Economic assessment will be supported by cost benefit analysis (CBA) allowing comparison of different processes according to their net profit [49]. CBA and LCA are widely combined for assessment of economic aspects and environmental impacts [50–53]. The technological developments of the project in development of *in vitro* production systems of high-valued molecules developed in this project will produce high-valued molecules without disturbing natural habitats. There is a growing understanding and focus that natural bio-active molecules have a significant role to play in the development of new pharmaceutical drugs. With such a growing market need for bio-active molecules from sustainable sources, the cultivation of marine organisms will have a leading role to play in the years to come. One of the major areas of research within the BAMMBO project deals with novel photobioreactor design for production of microalgal biomass for carotenoid production. The BAMMBO project is also developing an effective extraction procedure using supercritical carbon dioxide as a replacement solvent of bioactive solubilisation, extraction and enrichment. Furthermore, there is no net consumption of $CO_2$ in supercritical-extraction processes at a large scale as the total $CO_2$ flow is recycled within the system. The elimination of traditional solvents based extraction systems from the microalgal process has an obvious positive environmental societal and commercial impact. This will ultimately lead to exploitation of market-led opportunities driving the commercial partners within the BAMMBO consortium. Ultimately this should lead to greater social awareness of environmentally benign processes for value added products as well as job creation in satisfying the market needs for such products.

## High value added biomolecules of marine origin

Within the marine environment several biologically active molecules have been identified that have commercial as well as societal benefits. The following list identifies the specific high-value bioactivities which have been selected for in-depth analysis within the BAMMBO project. The research outcomes regarding overcoming the bottlenecks to sustainable production of these molecules will inform not only the BAMMBO project but also the wider scientific and industrial community as to the commercial viability of high-value biomolecule production from marine resources.

## Carotenoids and phycobiliproteins

Carotenoids are an important class of pigments found in plants, algae and photosynthetic bacteria. Several have been commercially exploited [54,55]. Carotenoids act as antioxidative scavenger complexes. Beta carotene, lycopene, astaxanthin, zeaxanthin and lutein are proven to remediate UV oxidative damage to the skin and retina [56,57]. The market for carotenoids was predicted to reach $1.2 billion by 2015 [55]. The largest growing carotenoid market is for lutein. The market for astaxanthin is dominated by low cost synthetic analogues.

Phycobiliproteins including phycoerythrin, phycocyanin, phycoerythrocyanin and allophycocyanin are water soluble chromogenic proteins. Initial application of these molecules was in the replacement of synthetic dye products [58,59]; however, they also have health, pharmaceutical and label applications [60,61]. Phycobiliprotein products range from $3–25/mg for native pigments and up to $1500/mg for cross-linked pigments.

## Polyunsaturated fatty acids (PUFAs)

Benefits of PUFAs are widely accepted. PUFAs play key roles in cellular metabolism, membrane fluidity, transport and eicosanoid precursors [62,63]. In the 1980s, the major source of PUFAs was fish oil. Declining fish stocks and contamination has led to use of alternative sources [64]. The market for omega 3 fatty acids was estimated to reach $13 billion in 2011 and expand up to 2016 [65]. Microalgae (Table 4) offer a sustainable non-polluted source of PUFAs [66]. Commercialisation of microalgal PUFAs is still in its infancy. Efforts are needed to identify optimal production strains with abilities to produce high PUFA yields, high cell densities and grow on cheap carbon sources. In 2011, the global average price for PUFAs was approximately $140/kg.

## Antioxidants

Antioxidants have wide ranging applications as sacrificial reducing agents. They elicit their benefits by preventing, delaying, or neutralising the effects of oxidative change and suppression and/or scavenging of free radicals. Antioxidants can be synthetic or natural. Physiologically, oxidative stress is implicated in pathology of ageing, atherosclerosis, cancer, malaria, neurodegenerative diseases and arthritis [67–71]. Recommendations of international organisations limit the use of synthetic antioxidants in products regionally. Antioxidants fit into several markets spanning food, feed, nutraceutical, pharmaceutical, cosmetic, health and packaging. Market reports for antioxidants show tremendous growth promise. The market for antioxidant products was predicted to approach $86 billion by 2016 [72].

## Antimicrobials

Bacterial related food spoilage causes substantial economic losses on a global scale.

Bacterial infections especially those associated with emerging multidrug resistance organisms are a primary health care problem [73]. Antibiotics have had widespread and diverse use as therapeutic agents, animal growth promoters and food and aquaculture additives. In the past 50 years only three classes of new antibiotics have been introduced to the market [74,75]. Marine invertebrates have unique antimicrobial mechanisms to cope with coexisting organisms with potential for discovery of novel antimicrobial agents.

**TABLE 4**

**Major commercially important microalgal derived polyunsaturated fatty acids (PUFAs) and their potential applications [60]**

| PUFA | Potential application | Source |
|---|---|---|
| **γLinolenic acid** (18:3*n*6) | Infant formulaessential fatty acid | *Arthrospira* sp. |
| | Cosmetics | |
| | Dietary supplements | |
| **Arachidonic acid** (20:4*n*6) | Premature Infant formula essential fatty acid | *Porphyridium* sp. |
| | Food additive | |
| | Cosmetics | |
| | Dietary supplements | |
| **Eicosapentaenoic acid** (20:5*n*3) | Essential fatty acid | *Nannochloropsis* sp. |
| | Food additive | *Phaeodactylum tricornutum* |
| | Aquaculture feed additive | *Nitschia laevis* |
| | Cosmetics | *Porphyridium purpureum* |
| | Dietary supplements | *Isochrysis galbana* |
| **Docosapentaenoic acid (DPA)** 22:5(*n*3) | Infant formula essential fatty acid | *Crypthecodinium* sp. |
| | Food additive | *Schizochytrium* sp. |
| | Chronic disease prevention/treatment | *Ulkenia* sp. |
| | Aquaculture feed additive | |
| | Cosmetics | |
| | Dietary supplements | |

Macroalgae are showing promise as sources of such compounds. Macroalgae have been linked to the production of secondary metabolites including phlorotannins and terpenoids that provide algae with defences against bacteria [76–78]. The antimicrobial market can be broken down by drug class, efficacy and product application. The antibiotic global market alone was projected to reach $40.3 billion by 2015 [79]. With respect to the number of candidates in pipeline manufacture antibacterials rank second in the antimicrobial market.

## Anticancer, antiviral, antifouling, antifungal and antitumoural HVABs

Several isolated compounds from marine organisms, in particular sponges, act as antitumour and anticancer agents having an effect, for example, on protein kinase C (PKC) and in the inhibition of fucosyltransferase [80–83]. In general these compounds can be divided into (i) nonspecific inhibitors of cell growth, (ii) specific inhibitors of cancer cells and (iii) inhibitors of specific cancer cell types (PKC inhibitors) [84].

Marine organisms are also sources of metabolites with antiviral properties (e.g. HIV inhibiting) arousing the interest of researchers as the precise mechanism of action by which these compounds function is not elucidated [84].

Natural marine antifouling molecules may provide less toxic and more specific antifouling promise inhibiting the settlement of barnacle larvae, fouling by macroalgae or repelling of blue mussels. Development of research on marine derived antifouling products is promoted by the International Maritime Organization convention as alternatives to antifoulant paints [85].

Many compounds of marine origin have antifungal activity [86] although their non-toxic effects to humans, animals and plants compared to fungicides have yet to be demonstrated.

Guanidine alkaloids such as Crambescidin 800 and 816 may play an important role in improved pathologies of neuronal degenerative diseases. It was reported that these compounds protected mouse hippocampal cell lines against glutamate induced oxidative stress [87]. Crambescidin 800 also protects both HT22 and neuroblastoma cells from oxidative stress induced by hypoxic conditions or nitric oxide insult. Crambescidin demonstrated a more potent $Ca^{2+}$ channel blocking activity than nifedipine, a recognised selective blocker of L type $Ca^{2+}$channels [88]. Crambescidins are patented for several applications associated with viral, tumour and cardiovascular disease treatment [89–91].

## Antielastase and antihyaluronidase HVABs

Skin ageing processes may be intrinsic or extrinsic in action [92]. Intrinsic ageing is due to skin elasticity changes over time whereas extrinsic ageing is mainly associated with UV exposure. Proteinaceous components of the extracellular matrix (ECM) are susceptible to proteolytic degradation by collagenase, metalloproteinase and elastase which affect skin elasticity. Elastases cleave elastin, collagen, fibronectin and other ECM proteins. Elastase and metalloproteinase activities are essential to the digestion of foreign proteins within the ECM tissue following repair and after phagocytosis. However, inhibitors of elastase offer the potential to maintain skin elasticity and thus reduce skin sagging.

Hyaluronidases are a class of enzymes produced by humans and some pathogenic bacteria. Hyaluronidase catalyses the hydrolysis of hyaluronic acid, a major constituent of the skin's interstitial barrier. Hyaluronidase reduces the viscosity of hyaluronic acid, thereby increasing tissue permeability. The enzyme is used in medicine to enhance uptake, dispersion and delivery of drugs. The most common application is in the combined use with local anaesthetics during ophthalmic surgery [93]. Inhibition of hyaluronidase activity offers the potential to act as a cosmetic additive for the alleviation of wrinkles [94]. Extracts derived from the BAMMBO invertebrate biobank are high throughput screened to identify HVABs with such applications.

## Cold active enzymes

Antarctic microbiology is a recent science and little is known about its nature and genetic resources. Psycrophilic enzymes are ten times more active at low to moderate temperatures, have lower $E_a$ values and are inactivated at lower temperatures than the catalytic optimal temperature of most mesophilic homologues [95]. Moreover, because of the significant heat lability of their active sites these enzymes are rendered inactive before protein unfolding. These enzymes have attracted increased attention due to their commercial potential. Cold active microbial lipases are carboxyl ester hydrolases of the $\alpha/\beta$ hydrolase super family which catalyse the hydrolysis and synthesis of long chain acylglycerols. These enzymes have high specific activities in processes that occur at low and moderate temperatures, especially in the production of detergents used in 'washing cold' and in chemical, pharmaceutical, food and agricultural applications [96]. Drivers of this technology are energy savings, salinity tolerance and open environment activity. The global enzyme market is growing and projected to reach $4.4 billion by 2015. Lipases are particularly being watched due to their activity in organic solvents and application in biofuels [97].

## Bromoditerpenes

Macroalgae are rich sources of halogenated compounds such as cyclic terpenes [98]. Diterpenes are widespread secondary metabolites of terrestrial plants and brown algae. They are derived from geranylgeranyl pyrophosphate and have cytotoxic effects with applications in cancer treatment, for example, Taxol. Ecological pressures including competition for space and predation prevention in part explains the diversity of terpenoids to intertidal species [99]. Some halogenated metabolites show high antibacterial and antitumour activities [78,100].

## Sesquiterpenes

Marine organisms produce a variety of terpenoids, for example, diterpenoids, triterpenoids and sesquiterpenoids [101,102]. Terpene units attached to core prenylated naphthoquinones, hydroquinones and alkaloids play important roles in organism survival. This biological activity may be pharmacologically exploited. Bioactive terpenoids with biomedical potential are currently in preclinical and clinical development. Structural classes such as cembrane, chamigrene, amphilectane skeletons and terpenes with isonitrile, isothiocyanate, isocyanate, dichloroimine and halogenated functional groups occur predominantly in marine metabolites. Sesterterpenes are common metabolites in marine organisms with sponges being considered one of the prime sources of these $C_{25}$ terpenoid compounds [103].

## Coenzyme $Q_{10}$

Coenzyme $Q_{10}$ ($CoQ_{10}$) is ubiquitously found in organisms. $CoQ_{10}$ is an obligatory cofactor in the electron transport chain. It is a membrane bound redox active molecule also involved in disulphide bond formation, detoxification of reactive oxygen species, control of redox flux, generation of cell signals and gene expression [104]. $CoQ_{10}$ is orally administered for cardiomyopathy, diabetes, Parkinson's and Alzheimer's. $CoQ_{10}$ is popular in cosmetics owing to its antioxidant properties [105]. Increased demand has resulted in a variety of nonnative commercial process developments, that is, chemical synthesis [106], semi-chemical synthesis [107] and microbial conversion. Wild type strains and mutants of *Agrobacterium*, *Rhodobacter*, *Paracoccus*, *Candida*, *Rhodotorula* and *Saitoella* spp. have been reported as $CoQ_{10}$ producers in patent applications [108]. Promising $CoQ_{10}$ producers were screened from 500 White Sea Coastal Zone species with potential indicated from *Altererythrobacter*, *Rhodobacter* and *Paracoccus* spp. The US and European $CoQ_{10}$ market was projected to exceed $133.3 million by 2015 [109].

## Phytases

Phytases are phosphatase enzymes catalysing the hydrolysis of phytic acid/phytates to phosphorylated myoinositol and inorganic phosphate. Four phytate degrading enzyme classes are found in a range of organisms [110]. Phytic acid is a component of plant seeds constituting 1–3% by weight of cereals and oil seeds and typically accounting for 60–90% of the total phosphorus. Phytic acid phosphorus in feed is practically unavailable to monogastric or agastric aquatic animals due to the lack of intestinal phytase. Unassimilated phosphorus is excreted into waterways leading to environmental problems. Phytates also bind to minerals, starch and proteins reducing bioavailability in feed [111]. The nutritive value of feeds may be enhanced by exogenous phytate hydrolysis [112]. Phytases are produced commercially from a variety of microorganisms generally by recombinant DNA technology [111]. In BAMMBO recombinant phytase producers active at pH 7 are being constructed from White Sea derived genetic resources of *Bacillus*, *Pseudomonas*, *Shewanella* and *Arthrobacter* spp. The phytase world market is increasing with estimates of size for 2012 at $350–400 million.

## Cellular active compounds

The therapeutic potential of purified cellular active compounds specifically those with the capability to dramatically affect mechanisms within a variety of healthy and diseased mammalian cell lines is being assessed from targeted marine organism extracts. Compounds are evaluated based on their external cell effect (channels, membrane receptors) and on their internal effect (cellular signalling, gene expression). Assessment of pure compounds permits a greater understanding of structural functional relationships and reveals potential as lead drug molecules based on their toxicity and mechanistic performances. High throughput screening is revealing promising applications as antineurodegenerative, anticancer, immunomodulatory and metabolic modulating agents [113].

## Marine organisms

The previous section outlines several high-value bioactivities that form the basis for the BAMMBO research project. Below we describe several target marine invertebrate organisms within the project that have been identified with potential for sustainable commercial production of thee bioactivities.

## Microalgae

*Phaeodactylum tricornutum* is a coastal diatom [114] and a model organism for microalgal studies [115]. *P. tricornutum* produces high concentrations of EPA (5% dry weight) and low amounts of other PUFAs [116,117]. The average EPA content is 29.8% of total fatty acids [118]. Under phototrophic conditions *P. tricornutum* is capable of producing enough EPA [119] to render processes competitive to fish oil derived EPA. Alternative fermentation based processes are competitive and as yet a market challenge.

*Scenedesmus obliquus* is a common green freshwater alga recognized for high growth rate [120,121]. It is a prolific producer of lutein, astaxanthin and beta carotene [122,123]. *S. obliquus* is used in feeds to enhance egg and chicken skin colour. It is a model phototrophic system organism with limited information available on heterotrophic or mixotrophic cultivations on low cost carbon sources.

*Cylindrotheca closterium* is a common coastal, mainly benthic diatom with promise in the nutraceutical and therapeutic sectors. High proportions of EPA were confirmed [124] but dry weight levels are too low for industrial production. *C. closterium* contains high concentrations of fucoxanthin with reported antioxidant, antiinflammatory, anticancer, antiobesity, antidiabetic, antiangiogenic and antimalarial bioactivities [125,126]. Bioavailability of brown seaweed fucoxanthin, the highest fucoxanthin producers known, is low in humans [126].

*Haematococcus pluvialis* is a green alga found in temperate regions known to accumulate high levels of antioxidants such as beta carotene, lycopene, astaxanthin, zeaxanthin and lutein. The organism is a reference strain for model microalgal growth and production of astaxanthin.

*Dunaliella salina* has an exceptional ability to survive hypersaline conditions [127]. *D. salina* synthesises high concentrations of beta carotene (14% dry weight) and glycerol, protecting the organism from the extreme environment. *D. salina* was the first microalga cultivated in large scale for beta carotene. Cultivation is facilitated by hypersalinity reducing contamination although productivity is low.

*Gambierdiscus toxicus* is a dinoflagellate that produces the largest biomolecule in nature, maitotoxin [128]. In addition to maitotoxin, it is the source of other highly interesting pharmacological compounds [129,130]. Maitotoxin is the most toxic natural compound and an interesting lead drug molecule. Development of sustainable culture systems for this molecule broadens its therapeutic possibilities as a calcium channels effector.

## Macroalgae

Recent trends in macroalgal natural drug research are revealing the biomedical potential of macroalgae in human disease treatment [131]. *Fucus spiralis* is an intertidal brown alga with high levels of protective polyphenolic phlorotannins [76,132,133]. Phlorotannins have potential human health benefits associated with their antioxidant, antibacterial and antitumoural properties [76,132]. *Sphaerococcus coronopifolius* is a red macroalga which grows from low to deeper seawater level extremes. *S. coronopifolius* produces halogenated metabolites with antibacterial and antitumour activities [78,100]. The main bottleneck associated with the production of macroalgal bioactive compounds is a stable tissue culture permitting controlled continuous production not affected by seasonality.

## Sponges

*Crambe crambe* is a red orange encrusting toxic marine common throughout the Mediterranean area. They are found at depths ranging between 5 and 35 m. Toxicity is associated with bioactive crambescidins but the role of the crambescins remains unclear. BAMMBO chemo diversity work isolated 8 new analogues of crambescins [134]. Three approaches to produce sustainably large quantities of crambescins are (i) larvae and juvenile sponge cultivation, (ii) bioreactor design and optimisation of sponge explant cultivation and (iii) unique total synthesis of crambescidins (patent application). Nevertheless all these approaches are not practical today for a large scale production of these therapeutically important alkaloids.

*Sarcotragus spinosulus* is a large shallow water Irciniidae sponge common in the Western Mediterranean. *S. spinosulus* supports a symbiotic bacterial community with a bacterial biomass representing up to 40% of sponge biomass [135]. Terpenoids, sterols and hydroquinones were isolated from *Sarcotragus* species. *S. spinosulus* compounds exhibit cytotoxic, antiinflammatory, antiviral [136,137], antifouling [138,139] and neuroprotective activities [140].

## Epiphytic sponge bacteria

Symbiotic systems of marine eukaryotes and their associated bacteria are known to produce bioactive compounds [141] such as antibiotics and antifungals [142]. Uncertainty exists as to whether an identified bioactive compound is produced by the epiphyte or the organism on which it lives, as is the case of manzamine, an alkaloid with antimalaria properties originally believed to be produced by the sponge *Acanthostrongylophora ingens* which is actually produced by the bacterial symbiont *Micromonospora* sp. [143]. Not all associated microbes are culturable when isolated and even when cultured they do not always continue to produce the anticipated bioactive compound [2]. Towards the development of systems for the sustainable production of bioactive compounds from epiphytic bacteria, cultures from the surface of the marine sponges *C. crambe* and *S. spinosulus* were collected, isolated and screened for the production of bioactive compounds. Novel reactor cultivation strategies, such as those developed for mammalian and insect cell culture, are being adapted for the scalable production of these cultures.

## Epiphytic macroalgae bacteria

Seaweeds defend themselves against microbial attack via production of secondary metabolites which prevent attachment and growth of such microbial colonisers [144]. Studies have demonstrated that epibiotic bacteria from macroalgal surfaces produce antimicrobial metabolites [145–147]. The contribution of such bacteria to the identified bioactivities evidenced from the targeted macroalgae is being studied in BAMMBO. Bottlenecks associated with scale up of epiphytic macroalgal bacteria monocultures are averted using stable immortalised macroalgae cultures cell lines.

## Antarctic marine fungi and yeasts

Filamentous fungi and yeasts were isolated from Antarctic marine samples with the intent of discovering low temperature active lipolytic enzymes. Notable lipolytic producers belong to the phylum Basidiomycota. Sea urchin derived *Cryptococcus laurentii* and a macroalgae *Palmaria decipiens* derived *Geomyces* sp. were notable lipase producers from screening assays. Both fungi are psychrotolerant and associated with cold tundra, arctic, Antarctic and desert soil environments [148–151]. Lipases produced by *C. laurentii* and *Geomyces* sp. are under experimental design investigation within BAMMBO to evaluate carbon sources, nitrogen sources and physicochemical parameter conditions required for optimal enzyme production.

## Sustainable bioreactor culturing systems for micro and macroalgae

Production of HVABs from micro and macroorganisms is strongly dependent on the ability to efficiently control appropriate levels of culture $CO_2$, light and nutrients. Overcoming such bottlenecks requires an integrated approach identifying optimal enclosed production systems and physiochemical parameters specific for the production of the target organism and compound. Light is typically the most difficult substrate to deliver although mixing and aeration are also known production bottleneck contributors. The high initial investment and operating costs of enclosed photobioreactors present the greatest barrier to the development of phototrophic cultivation systems [3]. Novel bioreactor systems with increased light and mixing with reduced operating costs through minimized power consumption for aeration and illumination are eliminating the need for mechanical pumping. Affordable reactor designs are in development. Scalability is provided through use of multiple enclosed photobioreactor modular units.

## Immortalised macroalgae lines

Multicellular organism tissue cultures are ideal for producing complex natural metabolites with antioxidant, antitumoural and anticoagulant activities [152–154] or for production of complex metabolites induced by genetic transformations. Novel macroalgal plantlet tissue cultures from *S. coronopifolius* and *F. spiralis* are being developed in BAMMBO as a new biotechnology platform. Plantlet cultures can be grown axenically in photobioreactors under controlled environmental and nutritional conditions required to produce high yields of HVABs [155]. Phototrophic growth reduces the risk of contamination with production levels on the same order of magnitude as heterotrophic plant tissue systems [156].

Methods employed in seaweed tissue culture are essentially the same as for higher plant tissues involving axenic explant generation and culture on solid agar medium enriched with nutrients and growth regulators [157]. Several macroalgae were reported to have been regenerated from tissue or callus culture; however, true cell suspension cultures from seaweeds are rare [158]. Transfer of cell suspensions to a photobioreactor offers a sustainable means to produce high value macroalgae secondary metabolites (*in vitro*). The main advantage of this photobioreactor cultivation is the avoidance of seasonality production barriers [155,158].

## Sustainable culturing of sponges

Experimental farms have been developed for natural product formation worldwide. The sustainable culture of sponges remains a bottleneck towards their commercialisation for HVABs. Production of sponge biomass through *ex situ* cultivation is complicated by slow growth and essential micro fauna associations which render such applications a challenge for sustainable non-destructive production of HVABs by sponges.

Sponge culture systems for HVABs may be sea, land or cell/primmorph culture based [159,160]. Sea based culturing is an old method for sponge culturing for medicaments [161]. It is considered the only reliable and feasible biotechnological tool to provide large biomass of sponges for the production of HVABs [160–164]. Sponge farming is a cheaper, more eco sustainable solution to exploit the natural tendency of sponges to perform clonal proliferation simply by fragmentation. BAMMBO studies the growth performances of target sponge species for HVAB production under mariculture conditions with varied biotic and abiotic factors (e.g. light, current, sedimentation and food availability) and farming procedures [165,166]. A second approach based on an *ex situ* process will also be investigated to better control the conditions for optimising the production of HVABs. Knowledge on the ecology and growth performances of the target species has a primary relevance for farming scale up [160]. Sponge health, survival and metabolite production are influenced by symbiotic associations which must be taken into consideration during cultivation [167].

## Sustainable extraction methodologies

Efficient extraction, enrichment and purification strategies are essential for sustainable marine HVAB production. Widely used methods include chemical disruption of the organism material, enzymatic hydrolysis, solvent extraction (soxhlet/microwave assisted) and mechanical disruption/pressing. Such methodologies are disadvantageous as they can result in the exposure of targeted molecules to microbes, oxygen and elevated temperatures with negative influence on product yield, stability, quality, safety and organoleptic attributes. Additionally, such processes are expensive in terms of capital investment, time and have a relatively large carbon footprint. A variety of solvent extraction processes have been widely utilised and investigated for the extraction of HVABs from natural materials. These solvents may be used at low temperatures to solvate the PUFAs and carotenoids but this adversely affects the product yield. Accelerated microwave assisted solvent extraction is a scalable process which has demonstrated ability to increase yields of PUFAs and carotenoids but the elevated temperatures and microwave energy required to reduce the time element has a detrimental effect on the HVABs stability. This is a significant barrier. Furthermore solvents are expensive, not environmentally friendly and require expensive recovery systems. Other concerns centre on green labelling, capital investment, other processing steps and residual solvents limit applications. Within BAMMBO comparative supercritical fluid $CO_2$ extraction processes are being evaluated to address many of these barriers (with the exception of the capital investment).

## Conclusion

BAMMBO is screening and identifying target marine organisms from diverse global locations for their potential as sustainable producers of HVABs. The aim is to provide innovative solutions to overcome bottlenecks associated with developing economically sustainable, environmentally friendly and scalable culturing methodologies designed to produce high yields of value added products from marine resources for the pharmaceutical, cosmetic and industrial sectors. Novel analytical methods for the extraction, purification and enrichment of targeted bioactive compounds are in development. Life cycle analyses of the production pathways are being undertaken to attain an environmentally holistic perspective of the sustainable production potential of HVABs from marine organisms where possible knowledge and technologies developed during the project will be exploited and transferred to achieve maximum impact on relevant research, policymakers and industrial stakeholders. In adhering to the European Strategy for Marine and Maritime Research BAMMBO's aim is to encourage capacity-building, integration and synergies across all marine sectors. BAMMBO outcomes will increase the competitiveness of the EU economy based on the capacity to create high value added knowledge based goods and services and foster the sustainable economic development of the marine sector.

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