

## Large-scale bioprospecting of cyanobacteria, micro- and macroalgae from the Aegean Sea

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### Abstract

Marine organisms constitute approximately one half of the total global biodiversity, being rich reservoirs of structurally diverse biofunctional components. The potential of cyanobacteria, micro- and macroalgae as sources of antimicrobial, antitumoral, anti-inflammatory, anticoagulant compounds has been reported extensively. Nonetheless, written records of biological activities of marine fauna and flora of the Aegean Sea has remained poorly studied when in comparison to other areas of the Mediterranean Sea. In this study, we screened the antimicrobial, antifouling, anti-inflammatory and anticancer potential of altogether 98 specimens collected from the Aegean Sea, including six species of cyanobacteria and 54 species of marine algae. Ethanol extract of diatom *Amphora cf. capitellata* showed the most promising antimicrobial results against *Candida albicans* while the extract of diatom *Nitzschia communis* showed effective results against Gram-positive bacterium, *S. aureus*. Extracts from the red alga *Laurencia papillosa* and three *Cystoseira* species exhibited selective antiproliferative activity against cancer cell lines and an extract from the brown alga *Dilophus fasciolaria* showed the highest anti-inflammatory activity as measured in primary microglial and astrocyte cell cultures as well as by. In summary, our study demonstrates that the Aegean Sea is a rich source of species that possess interesting potential for developing industrial applications.

### Keywords

Bioprospecting, Aegean Sea, biological activity, cyanobacteria, marine algae

## Introduction

Bioprospecting, a tool for biodiversity conservation, can be defined as the collection of biological material and analysis of its properties and/or its molecular, biochemical or genetic content for a purpose of developing a commercial product. However, special care must be given to ethical issues and conservation policies (Christoffersen and Mathur 2005; Beattie et al. 2011; Rose, Quave, and Islam 2012). Covering more than 70% of world's surface and with still many unknown habitats and species, the oceans and seas play an important role in the search for new solutions concerning human health. In recent years, many reports have been published about the biological activities of marine species (e.g., cyanobacteria, macro- and microalgae) and compounds isolated thereof (Burja et al. 2001; Pulz and Gross 2004; Raposo, de Moraes, and Bernardo de Moraes 2013; Barbosa, Valentão, and Andrade 2014; Wang et al. 2014).

The Aegean Sea is a saltwater body in the northeast part of the Mediterranean Sea. Being adjacent to the Mediterranean – a marine biodiversity hotspot (Coll et al. 2010) – makes this embayment an important site to study marine resources. However, the Aegean Sea has received little attention in bioprospecting studies, despite its economic and cultural importance. The Aegean Sea has endemic and rare species. The season in the Aegean Sea is characterized by the presence of two distinct periods (summer and winter) with spring and autumn relatively short and transitional.

Marine macroalgae, macroscopic and multicellular seaweeds, can be classified into three groups based primarily on their photosynthetic pigmentation: green (Chlorophyta), red (Rhodophyta) and brown (Phaeophyta) (Garson 1989). Each group is rich in a type of sulphated polysaccharide: ulvan (mainly in green algae), carrageenan (mainly in red algae) and fucoidan (mainly in brown algae). Many studies have shown that seaweeds provide a rich source of natural bioactive compounds, which have been shown to display antibacterial (Pierre et al. 2011), antitumoral (Ji et al. 2008), antiproliferative (Mhadhebi et al. 2014), antioxidant (Farasat et al. 2013; Zhang et al. 2013; Mhadhebi et al. 2014), and anti-inflammatory (Mhadhebi et al. 2014; Ribeiro et al. 2014), activities. For example, Abou Zeid et al. (2014) showed that polysaccharide contents of macroalgae *Dictyopterus membranacea* exhibited interesting antimicrobial and antitumoral activities (Abou Zeid et al. 2014).

Microalgae (or diatoms) are microscopic and unicellular, and normally classified not only by the presence or absence of cell nucleus, presence of flagella and cycle of life but also, as seaweed, by their photosynthetic pigmentation. Extracts from diatoms have been studied, for example, for their apoptotic effects (Lee et al. 2009; Prestegard et al. 2009) and biochemical composition (J. A.

Simental-Trinidad, M. P. Sánchez-Saavedra 2001). However, many aspects of diatom's metabolism and production of natural compounds remain unknown.

Macro- and microalgae are well recognized as valuable resources for industrial purposes. Cultivation of microalgae is known to be one of the most profitable business in the biotech industry, being used as nutritional supplements (Harmelen and Oonk 2006; West M. Bishop 2012), for CO<sub>2</sub> fixation (Sydney et al. 2010), in cosmetics (Spolaore et al. 2006; Tran et al. 2014), food colorants (Dufossé et al. 2005), and as biofuel (Singh and Gu 2010), among others. Macroalgae, crucial primary producer in oceanic aquatic food webs, is equally important ecologically and commercially. Their studied applications cover human food (MacArtain et al. 2007; Santos et al. 2015), pharmaceuticals (Smit 2004; Pereira et al. 2012), cosmeceuticals (Thomas and Kim 2013) and energy production (Aitken et al. 2014; Milledge et al. 2014).

The objective of this work was to carry out large-scale screening of antimicrobial, antifouling, anti-inflammatory and anticancer properties of a total of 98 specimens collected from the Aegean Sea and to evaluate their potential from biosprospecting perspective.

## **Results and Discussion**

A set of 98 specimens was collected from 21 collection sites located on the Turkish coast of the Aegean Sea such as Urla, Seferihisar, Urkmez, Burhaniye and Turgutreis (see Table S1 for details). The set consisted of seven cyanobacteria, 17 microalgae, 72 macroalgae and two seagrass samples, which belonged to six different phyla; Cyanobacteria (7%), Chlorophyta (27%), Haptophyta (1%), Ochrophyta (47%), Rhodophyta (16%) and Tracheophyta (2%). Collected macroalgal/seagrass specimen were washed and freeze-dried prior to extraction. Cyanobacteria and microalgal samples were first purified and then cultivated as monoalgal cultures in laboratory conditions to produce sufficient amounts of biomass for the extraction. Maceration with 80% ethanol was used for preparing crude extracts from the samples, which were then subjected to biological evaluation. As a primary screen, the extracts were evaluated at 50-100 µg/ml concentration by using a panel of 32 assays covering antimicrobial, anti-inflammatory and anticancer targets. By using >50% effect as a threshold, five extracts were identified active against pathogenic bacteria and/or fungi, and three against fouling bacteria (Fig. 1). Activity on breast and/or prostate cancer cells were detected for nine extracts and four extracts demonstrated activity in the anti-inflammatory evaluation. Interestingly, in most cases the activities were not overlapping between different assay categories. Altogether, 20% of the extracts were defined as potentially active. Majority (16) of the active samples were from macroalgae specimen, and from the phylum Ochrophyta (11), which was also

most abundantly represented within the collected set. The following sections describe the found activities in more detail.

#### *Antimicrobial and antifouling activity*

The inhibitory effects were tested against ten pathogenic strains: three Gram-positive (*E. faecalis*, *S. aureus* and *S. aureus* MRSA) and four Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *S. flexneri* and *V. cholerae*), and three fungi (*A. fumigatus*, *C. albicans* and *C. neoformans*). Extracts 10S014, 10S152 and 10S156 showed the most promising results, with growth inhibition above 50% (Fig. 1). Extract of *Amphora cf capitellata* (10S014) was found to fully inhibit *Candida* and to inhibit Gram-positive *E. faecalis* and *S. aureus* by 68% and 83%, respectively. Interestingly, *Nitzschia communis* (10S152) fully inhibited the growth of *S. aureus* at 100 µg/ml concentration. The present study also indicated that two macroalgal species of *Cystoseira* genus (10S022-1 and 10S027) as well as *Enteromorpha linza* (10S054), exhibited moderate inhibitory activity against fouling marine bacteria (*Alcanivorax* sp. and *Pseudoalteromonas* sp.).

#### *Antiproliferative activity*

The primary screen results obtained for antiproliferative effects of the extracts. In our study, the extracts of reproductive *Dictyopteris membranacea* (10S039) and *Hypnea musciformis* (10S049 and 10S053) displayed a general cytotoxicity against all four cell lines, with inhibition of growth in the range of 60 to 96%. However, extracts of species from the *Cystoseira* genus (10S035, 10S037, 10S038) and of red algae *Laurencia papillosa* displayed cytotoxic effects on the cancer cells (LNCa, PC-3 and MCF-7) but not on the non-tumorigenic cell line MCF-10, suggesting that these extracts might be interesting for further studies regarding their potential anticancer effects. The extract of macroalgae *Cystoseira stricta* (10S037) showed significant effects on human breast adenocarcinoma MCF-7 cells (inhibition of growth 87%) and similar effect has previously been reported for this species (Mhadhebi et al. 2014). Recent publications (Mikami and Hosokawa 2013; Mhadhebi et al. 2014) have provided evidence that some compounds and pigments present in brown algae are responsible for their antiproliferative activities.

#### *Anti-inflammatory activity*

Microglia and astrocytes are able to recognize harmful stimuli and respond by producing inflammatory cytokines such as TNF $\alpha$ , IL-6, IL-1 $\beta$ , and IFN- $\gamma$  (Boche et al. 2013). However, inflammatory factors produced by microglia and astrocytes can damage local tissue and can further increase inflammation and glial activation, leading to a persistent inflammatory cycle. This long-term inflammation can give rise to fatal consequences in the CNS, ranging from loss of synapses to

impaired cognition and overt neurodegeneration (Ward et al. 2015). The results obtained for anti-inflammatory effects of tested extracts are summarized in Figure 1. Extracts from *Chaetomorpha aerea* (10S018), reproductive *Cystoseira crinita* (10S038), *Dilophus fasciola* (10S043) and *Caulerpa racemosa* (10S020) clearly showed a reduction in the release of IL- $\beta$  (50 $\mu$ g/ml: 33%, 29%, 34%, 42%; 100 $\mu$ g/ml: 38%, 35%, 45%, 55%), IL-6 (50 $\mu$ g/ml: 29%, 36%, 35%, 34%; 100 $\mu$ g/ml: 42%, 47%, 45%, 54%), TNF- $\alpha$  (50 $\mu$ g/ml: 32%, 37%, 38%, 42%; 100 $\mu$ g/ml: 44%, 48%, 51%, 55%), and IFN- $\gamma$  (50 $\mu$ g/ml: 39%, 45%, 47%, 32%; 100 $\mu$ g/ml: 54%, 59%, 65%, 47%), pro-inflammatory cytokines when compared to positive controls. An increase in neuron survival was also observed at both concentrations: 10S018 (50 $\mu$ g/ml: 38%; 100 $\mu$ g/ml: 48%), 10S038 (50 $\mu$ g/ml: 49%; 100 $\mu$ g/ml: 55%), 10S043 (50 $\mu$ g/ml: 53%; 100 $\mu$ g/ml: 63%), and 10S020 (50 $\mu$ g/ml: 43%; 100 $\mu$ g/ml: 48%). The results of both microglial and astrocyte activation indicate a significant activity of 10S018, 10S038, 10S043 and 10S020 extracts (Figure 1), as well as that *Dilophus fasciola* (10S043) extract exhibit the highest anti-inflammatory activity. Taken together these results suggest that several marine extracts could be further investigated as therapeutic tools in experimental pathological conditions where microglial and astrocyte activation may contribute to disease.

## Conclusion

In this article, we presented the results of an extensive study on the bioactivity screening of several species living in the Aegean Sea, i.e., cyanobacteria, micro- and macroalgae. The Aegean Sea, considered as an arm of the Mediterranean Sea (biodiversity hotspot), is an interesting place for the study of marine pharmacology. Based on our results, several macro- and microalgae species showed stimulating results in antimicrobial studies against both pathogenic and fouling microbes. An alga belonging to phylum Rhodophyta, *Laurencia papillosa*, exhibited promising antiproliferative activity against cancer cell lines. Furthermore, an alga belonging to phylum Ochrophyta, *Dilophus fasciola* also showed significant anti-inflammatory activity. However, further work is required to fully elucidate the potential of these findings for the development of industrial applications.

## Materials and Methods

### Sample collection and preparation

#### *Collection of cyanobacteria, and micro- and macroalgae*

Altogether 98 cyanobacteria, micro- and macroalgae specimens were collected from the Aegean Sea, Turkey. Details on the collected species, collection sites and conditions are provided as Supplementary Information (Table S1). Macroalgae were collected from the seashore by hand

picking while cyanobacteria and microalgae were collected by plankton net (mesh size 20  $\mu\text{m}$ ). Samples were packed into clean nylon bags or polyethylene bottles, placed on ice, and taken to the laboratory within 48 hours. Microalgae and cyanobacteria species were checked and identified under microscope, and then diluted and plated onto plates to prepare monoalgal cultures.

#### *Isolation and purification of cyanobacteria and microalgae species*

Collected sample (1 ml) was inoculated into 9 ml of appropriate sterilized medium in a 15-ml tube. The tube was incubated for 7 days at 25°C at the light intensity of 30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The isolation was accomplished by streaking the sample across the agar surface. Isolated colonies were picked from the agar plate with disposable loop and then both re-streaked on a new agar plate and rinsed in liquid appropriate medium to suspend the cells. Isolates were incubated at 25°C at light intensity of 40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in 250-ml Erlenmeyer flasks for 14 days. Isolated species were added to the Ege University Microalgae Culture Collection (Ege-Macc; <http://www.egemacc.com>).

#### *Batch cultivation of cyanobacteria and microalgae*

The cyanobacteria and microalgae samples were monoalgal (non-axenic) and cultured in appropriate media at 22 $\pm$ 2 °C under continuous illumination (100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in 2-l sterile bottles for 23 days. Illumination was provided by standard cool white fluorescent lamps (18 W) from one side of the flask. Irradiance was measured in the center of the bottle with a quantum meter (Lambda L1-185). Air was continuously supplied to the culture at a flow rate of 1 l  $\text{min}^{-1}$  (1.25 vvm) by air pump.

#### *Preparation of extracts*

After the cultivation period of microalgae strains, the cells from the culture broth (> 5 l) were collected and separated using GEA Westafalin separator mineral oil systems GmbH (30,000 rpm, 30 min, 25°C) and the supernatant was removed. If the volume of culture broth was less than 5 l, the cells were separated by using Heichman centrifuge (5,000 rpm, 4 min, and 18°C) and the supernatant was removed. Collected cells were washed with distilled water and then dried using Alpha 1-2 /LD plus lyophiliser (kept at -20°C). Macroalgae specimen were washed with distilled water after collection and stored at -20°C. Then, the samples were dried using Alpha 1-2 /LD plus lyophiliser and powdered using porcelain mill after freezing with liquid nitrogen. Powdered material was macerated at RT for four hours with 80% ethanol (10 g/100 ml). The obtained extract was filtered through Whatman No. 1 filter paper. The residue from the filter paper was re-suspended in fresh 80% ethanol, re-extracted by maceration for two hours and filtered. Filtrates were combined and evaporated to dryness using rotary evaporator (at 45°C for 30 min) and lyophilisation if necessary.

For biological assays, the extracts were dissolved in DMSO to yield concentrations 20-50 mg/ml depending on the assay and stored at -20°C.

## **Biological assays**

### *Evaluation of antimicrobial properties*

Antimicrobial properties were evaluated against a panel of pathogenic bacteria and fungi as well as fouling bacteria (altogether 20 strains, see Table 1 for details) by using either agar diffusion or broth microdilution assays according to CLSI and EUCAST guidelines (Kahlmeter et al. 2006; Wikler et al. 2006).

For agar diffusion assays, sterile Whatman No. 1 filter paper (GF/C, ø6 mm) discs were impregnated with the sample (1mg/disc) and placed on Mueller Hinton agar (MHA) plates which had been previously surface-inoculated with uniformly spread test organism [approximately  $1.2 \times 10^8$  colony-forming units (CFU)/ml]. Standard antibiotic discs (10µg/disc) served as positive control (Table 1) and discs containing only solvent as negative control. MHA plates were incubated at 37°C for 24h (bacteria) or 48h (fungi) after which the zone of growth-inhibition observed around each disc was measured in millimeters. In broth microdilution assays, inoculum of  $5 \times 10^5$  CFU/ml in Mueller Hinton broth (MHB) was used for bacteria and  $2.5 \times 10^3$  CFU/ml in RPMI-1640 (with L-glutamine, w/o NaHCO<sub>3</sub> and supplemented with 2% glucose and 0.165 M MOPS, buffered to pH 7) for fungi. After incubation at 37°C for 24h (bacteria) or at 28°C for 48h (fungi), absorbance at 620 nm was used for evaluating antimicrobial activity by comparing with controls. Assays were carried out in triplicate.

### *Evaluation of anti-inflammatory properties*

#### *Primary neuron cell culture preparation and feeding*

Dissociated cortical cell cultures were prepared by papain digestion of embryonic-day-18 rat whole cortex. Timed-pregnant Wistar rats were euthanized by CO<sub>2</sub> inhalation, according to approved protocols for the care and use of lab animals. Embryos were removed, chilled on ice, and the cortex was micro-dissected under sterile conditions. Papain solution was prepared, quickly frozen by immersion into liquid nitrogen in 2-ml aliquots, stored at -15°C, and thawed at 35°C just before use. Cortex pieces (1 mm) were digested in 2 ml papain solution for 30 min at 35°C with gentle inversion every 5 min. The papain solution was aspirated and the pieces were triturated three times, each with 1 ml of medium. 20 000 to 50 000 cells per well were plated into 96-well plates in Dulbecco's modified Eagle's medium (DMEM) with 10% equine serum (Hyclone) and incubated at 37°C, 5% CO<sub>2</sub> for 48-72 hours. Since antibiotics and antimicrobics are reported to adversely affect neuron viability, they

were not used. Medium was used without glial conditioning, but was stored in a fluorinated ethylene-propylene (FEP) membrane-sealed flask in the incubator to equilibrate the pH and temperature before feeding. No attempt was made to control glial cell proliferation with antimetabolic drugs, because glial cells aid in neuronal survival and synaptic development. The cells were fed by completely replacing the media once per week.

#### *Primary astrocyte cell culture preparation and feeding*

Two-day old Wistar neonatal rat pups were sacrificed in a CO<sub>2</sub> chamber. Using micro-dissecting scissors, the skin was opened at the midline of the head, cutting from the base of the skull to the mid-eye area. After folding back the skin flaps with the scissors, the skull was cut at the midline fissure, without cutting into the brain tissue. The raised skullcap was removed with the curved forceps, applying slight pressure. The brain was then released from the skull cavity by running a microspatula underneath and along the length of the brain from the olfactory lobes to the beginning of the spinal cord. After gently transferring the brain to a 60-mm Petri dish, it was rinsed with modified DMEM/F12 culture medium, containing 10% FBS, 1% glutamine and gentamycin, to remove blood and to moisten the tissue. Using micro-dissecting forceps, each brain was transferred to an inverted lid of a 35-mm Petri dish, the cerebrum was separated from the cerebellum and brain stem, and the cerebral hemispheres were separated from each other. Cortices were dissociated into a cell suspension using mechanical digestion. After digestion, cells were plated in 75-cm<sup>2</sup> tissue culture flasks at a concentration of  $15 \times 10^6$  cells in 11 ml of medium and incubated at 37°C in a moist atmosphere with 5% CO<sub>2</sub> for 48-72 hours, allowing the cells sufficient time to adhere and multiply.

#### *Primary microglial cell culture preparation, feeding and experiments*

Primary microglial cell cultures were prepared from brains of newborn Wistar rats. The neocortex of one-day-old postnatal rats was aseptically dissected out and the meninges and blood vessels were carefully removed. Following cutting in a medium containing 20% horse serum, the suspension was filtered through a 40-mm filter and transferred as a single cell suspension to culture dishes, using 5 mL medium per dish. Cultures were incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Minimum Essential Medium Eagle, supplemented with L-glutamine, amino acids, vitamins, penicillin, gentamycin, sodium bicarbonate and 20% heat-inactivated horse serum was used as the medium and changed twice a week. After one week, the concentration of horse serum was reduced to 10%. After 16 days in culture, cells were treated with 1 µg/mL of lipopolysaccharide (LPS). After six hours, samples were added and plates were incubated for 72 hours at 37°C, 5% CO<sub>2</sub>. Positive controls were treated only with LPS while negative controls were treated with complete medium. After



incubation, cells were counted and 2.5 mL of supernatants were collected from each well and frozen at -40°C for cytokine analyses. All experiments were carried out in duplicate.

#### *MTT reduction assay*

For evaluating the effects on neuronal cells and astrocytes, a cell proliferation assay based on MTT was used. Cell lines ( $1 \times 10^5$  cells/ml) were incubated in 96 well plates with extracts for 24 hours. Ten  $\mu$ l of MTT (10 mg/ml) was added to each well and incubated further at 37°C for 4 hours. After incubation, MTT-formazan precipitate was dissolved in 100  $\mu$ l of DMSO and absorbance was recorded at 570 nm in an automatic plate reader (BioRAD instrument). Data are presented as percentage of cytotoxicity of treated versus untreated cells.

#### *Determination of cytokine concentration*

The supernatants obtained from microglial cell cultures were analyzed for TNF- $\alpha$ , IL-1 $\beta$ , IL-4, IL-6, IL-8, IL-10, and IL-18 using commercially available ELISA kits following the manufacturers' instructions. All samples were assayed in duplicate and equivocal results were repeated. The cytokine concentration was calculated from a standard curve of the corresponding recombinant cytokine.

#### *Evaluation of antiproliferative properties*

##### *Cell culture*

Two prostate cancer (LNCa, PC-3), a breast cancer (MCF-7) and non-tumorigenic epithelial(MCF-10A) cell lines were used for the evaluation of antiproliferative properties. LNCaP cells were obtained from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, PC-3 and MCF10A from ATCC (Manassas, VA, USA), and MCF-7 cells from Interlab Cell Line Collection (ICLC, Genova, Italy). LNCaP and PC-3 cells were grown in RPMI-1640, 10% fetal bovine serum, 1% penicillin/streptomycin, and 2mM L-glutamine.MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (1 g/l glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. MCF-10A were grown in 1:1 DMEM (4.5g/lglucose) and HAM F-12, 5% horse serum, 5  $\mu$ g/ml hydrocortisone, 20 ng/ml epidermal growth factor (EGF), 100ng/ml cholera toxin, 2mM L-glutamine, and 10  $\mu$ g/ml insulin. All cells were grown at 37°C/5% CO<sub>2</sub>.

#### *High throughput anticancer assays*

Sample stock solutions were transferred into 384-well master plates and subsequently processed with pipetting robot for 1:10 serial dilutions (20 mg/ml, 2 mg/ml and 0.2 mg/ml) for high-throughput

screening. 100 nanolitres of the stock solutions were transferred into white 384-well assay plates (Greiner) in four replicates for ready-made assay plates to be stored in the freezer until used for the screens (final assay concentrations 50, 5 and 0.5 µg/ml). Cell Titer Glo (CTG, Promega) proliferation assay was used for assessing anticancer effects of the extracts. DMSO and water were used as negative, and staurosporine and daunorubicine (5 µg/ml, 0.5 µg/ml, and 0.05 µg/ml of both), as positive controls. Cells (1500 MCF-10A, 1700 PC-3, 2000 MCF-7 and LNCaP cells/well) were plated in 40 µl of culture media into the assay plates on top of the samples. After 72 hours incubation at 37°C/5% CO<sub>2</sub>, 20 µl of CTG reagent was added, plates were placed in an orbital shaker for 30 minutes for lysis and the luminescence was measured with Envision Multilabel reader (Perkin Elmer). The screens were carried out in two biological replicates in two independent screen sets. The average signals for the four replicates in duplicate screens were calculated and compared to the averages of the control samples (DMSO or water). The screens were technically successful: DMSO controls showed less than 10% CV, the positive controls were very effective and the replicate samples showed very low variation. Due to the fact that the replicate biological screens correlated so well in early screens only one screen was carried out in the later experiments.

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### **References**

- [1] Christoffersen LP, Mathur EJ. Bioprospecting ethics & benefits: a model for effective benefit-sharing. *Ind Biotechnol* 2005;1:255–9. <http://dx.doi.org/10.1089/ind.2005.1.255>.
- [2] Beattie AJ, Hay M, Magnusson B, de Nys R, Smeathers J, Vincent JFV. Ecology and bioprospecting. *Austral Ecol* 2011;36:341–56. <http://dx.doi.org/10.1111/j.1442-9993.2010.02170.x>.
- [3] Rose J, Quave CL, Islam G. The four-sided triangle of ethics in bioprospecting: pharmaceutical business, international politics, socio-environmental responsibility and the importance of local stakeholders. *Ethnobiol Conserv* 2012;1:1–25. <http://dx.doi.org/10.15451/14>.
- [4] Burja AM, Banaigs B, Abou-Mansour E, Burgess JG, Wright PC. Marine cyanobacteria – a prolific source of natural products. *Tetrahedron* 2001;57:9347–77. [http://dx.doi.org/10.1016/S0040-4020\(01\)00931-0](http://dx.doi.org/10.1016/S0040-4020(01)00931-0).
- [5] Pulz O, Gross W. Valuable products from biotechnology of microalgae. *Appl Microbiol Biotechnol* 2004;65:635–48. <http://dx.doi.org/10.1007/s00253-004-1647-x>.
- [6] Raposo MFJ, Morais RMSC, Morais AMMB. Bioactivity and applications of sulphated polysaccharides from marine microalgae. *Mar Drugs* 2013;11:233–52. <http://dx.doi.org/10.3390/md11010233>.

- [7] Barbosa M, Valentaõ P, Andrade PB. Bioactive compounds from macroalgae in the new millennium: implications for neurodegenerative diseases. *Mar Drugs* 2014;12:4934–72. <http://dx.doi.org/10.3390/md12094934>.
- [8] Wang L, Wang X, Wu H, Liu R. Overview on biological activities and molecular characteristics of sulfated polysaccharides from marine green algae in recent years. *Mar Drugs* 2014;12:4984–5020. <http://dx.doi.org/10.3390/md12094984>.
- [9] Dussault D, Vu KD, Vansach T, Horgen FD, Lacroix M. Antimicrobial effects of marine algal extracts and cyanobacterial pure compounds against five foodborne pathogens. *Food Chem* 2016;199:114–8. <http://dx.doi.org/10.1016/j.foodchem.2015.11.119>.
- [10] Falch B, Koñig G, Wright A, Sticher O, Angerhofer C, Pezzuto J, et al. Biological activities of cyanobacteria: evaluation of extracts and pure compounds. *Planta Med* 2007;61:321–8. <http://dx.doi.org/10.1055/s-2006-958092>.
- [11] Gigova L, Gacheva G, Toshkova R, Yossifova L, Gardeva E, Ivanova N, et al. Effects of temperature on *Synechocystis* sp. R10 (Cyanoprocarvota) at two irradiance levels. II. Effect on antibacterial, antifungal and cytotoxic activities. *Genet Plant Physiol* 2012;2:38–49.
- [12] Coll M, Piroddi C, Steenbeek J, Kaschner K, Ben Rais Lasram F, Aguzzi J, et al. The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. *PLoS One* 2010;5:e11842. <http://dx.doi.org/10.1371/journal.pone.0011842>.
- [13] Garson MJ. Biosynthetic studies on marine natural products. *Nat Prod Rep* 1989;6:143. <http://dx.doi.org/10.1039/np9890600143>.
- [14] Pierre G, Sopena V, Juin C, Mastouri A, Graber M, Maugard T. Antibacterial activity of a sulfated galactan extracted from the marine alga *Chaetomorpha aerea* against *Staphylococcus aureus*. *Biotechnol Bioprocess Eng* 2011;16:937–45. <http://dx.doi.org/10.1007/s12257-011-0224-2>.
- [15] Ji H, Shao H, Zhang C, Hong P, Xiong H. Separation of the polysaccharides in *Caulerpa racemosa* and their chemical composition and antitumor activity. *J Appl Polym Sci* 2008;110:1435–40. <http://dx.doi.org/10.1002/app.28676>.
- [16] Mhadhebi L, Mhadhebi A, Robert J, Bouraoui A. Antioxidant, anti-inflammatory and antiproliferative effects of aqueous extracts of three mediterranean brown seaweeds of the genus *Cystoseira*. *Iran J Pharm Res* 2014;13:207–20.
- [17] Farasat M, Khavari-Nejad R-A, Nabavi SMB, Namjooyan F. Antioxidant properties of some filamentous green algae (*Chaetomorpha* Genus). *Brazilian Arch Biol Technol* 2013;56:921–7. <http://dx.doi.org/10.1590/S1516-89132013000600005>.
- [18] Zhang Z, Wang X, Mo X, Qi H. Degradation and the antioxidant activity of polysaccharide from *Enteromorpha linza*. *Carbohydr Polym* 2013;92:2084–7. <http://dx.doi.org/10.1016/j.carbpol.2012.11.096>.
- [19] Ribeiro NA, Abreu TM, Chaves HV, Bezerra MM, Monteiro HSA, Jorge RJB, et al. Sulfated polysaccharides isolated from the green seaweed *Caulerpa racemosa* plays antinociceptive and anti-inflammatory activities in a way dependent on HO-1 pathway activation. *Inflamm Res* 2014;63:569–80. <http://dx.doi.org/10.1007/s00011-014-0728-2>.
- [20] Abou Zeid AH, Aboutabl EA, Sleem AA, El-Rafie HM. Water soluble polysaccharides extracted from *Pterocladia capillacea* and *Dictyopteris membranacea* and their biological activities. *Carbohydr Polym* 2014;113:62–6. <http://dx.doi.org/10.1016/j.carbpol.2014.06.004>.

- [21] Lee S-H, Karawita R, Affan A, Lee J-B, Lee K-W, Lee B-J, et al. Potential of benthic diatoms *Achnanthes longipes*, *Amphora coffeaeformis* and *Navicula* sp. (Bacillariophyceae) as antioxidant sources. *Algae* 2009;24:47–55. <http://dx.doi.org/10.4490/ALGAE.2009.24.1.047>.
- [22] Prestegard SK, Oftedal L, Coyne RT, Nygaard G, Skjaerven KH, Knutsen G, et al. Marine benthic diatoms contain compounds able to induce leukemia cell death *New Biotechnology* 31 Volume 33, Number 3 May 2016 RESEARCH PAPER [www.elsevier.com/locate/nbt](http://www.elsevier.com/locate/nbt) 405 Research Paper and modulate blood platelet activity. *Mar Drugs* 2009;7:605–23. <http://dx.doi.org/10.3390/md7040605>.
- [23] Simental-Trinidad JA, Sa´nchez-Saavedra MP, Correa-Reyes JG. Biochemical composition of benthic marine diatoms using as culture medium a common agricultural fertilizer. *J Shellfish Res* 2001;20:611–7.
- [24] van Harmelen T, Oonk H. Microalgae biofixation processes: applications and potential contributions to greenhouse gas mitigation options. In: Report for the International Network on Bio-fixation of CO<sub>2</sub> and Greenhouse Gas Abatement with microalgae operated under the International Agency Greenhouse Gas R&D Programme; 2006.
- [25] West M, Bishop HMZ. Evaluation of microalgae for use as nutraceuticals and nutritional supplements. *J Nutr Food Sci* 2012;2. <http://dx.doi.org/10.4172/2155-9600.1000147>.
- [26] Sydney EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, Pandey A, et al. Potential carbon dioxide fixation by industrially important microalgae. *Bioresour Technol* 2010;101:5892–6. <http://dx.doi.org/10.1016/j.biortech.2010.02.088>.
- [27] Spolaore P, Joannis-Cassan C, Duran E, Isambert A. Commercial applications of microalgae. *J Biosci Bioeng* 2006;101:87–96. <http://dx.doi.org/10.1263/jbb.101.87>.
- [28] Tran D, Doan N, Louime C, Giordano M, Portilla S. Growth, antioxidant capacity and total carotene of *Dunaliella salina* DCCBC15 in a low cost enriched natural seawater medium. *World J Microbiol Biotechnol* 2014;30:317–22. <http://dx.doi.org/10.1007/s11274-013-1413-2>.
- [29] Dufosse´ L, Galaup P, Yaron A, Arad SM, Blanc P, Chidambara Murthy KN, et al. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends Food Sci Technol* 2005;16:389–406. <http://dx.doi.org/10.1016/j.tifs.2005.02.006>.
- [30] Singh J, Gu S. Commercialization potential of microalgae for biofuels production. *Renew Sustain Energy Rev* 2010;14:2596–610. <http://dx.doi.org/10.1016/j.rser.2010.06.014>.
- [31] MacArtain P, Gill CIR, Brooks M, Campbell R, Rowland IR. Nutritional value of edible seaweeds. *Nutr Rev* 2007;65:535–43.
- [32] Santos SAO, Vilela C, Freire CSR, Abreu MH, Rocha SM, Silvestre AJD. Chlorophyta and Rhodophyta macroalgae: a source of health promoting phytochemicals. *Food Chem* 2015;183:122–8. <http://dx.doi.org/10.1016/j.foodchem.2015.03.006>.
- [33] Smit AJ. Medicinal and pharmaceutical uses of seaweed natural products: a review. *J Appl Phycol* 2004;16:245–62. <http://dx.doi.org/10.1023/B:JAPH.0000047783.36600.ef>.
- [34] Pereira H, Barreira L, Figueiredo F, Custo´dio L, Vizetto-Duarte C, Polo C, et al. Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. *Mar Drugs* 2012;10:1920–35. <http://dx.doi.org/10.3390/md10091920>.
- [35] Thomas NV, Kim S-K. Beneficial effects of marine algal compounds in cosmeceuticals. *Mar Drugs* 2013;11:146–64. <http://dx.doi.org/10.3390/md11010146>.

- [36] Aitken D, Bulboa C, Godoy-Faundez A, Turrion-Gomez JL, Antizar-Ladislao B. Life cycle assessment of macroalgae cultivation and processing for biofuel production. *J Clean Prod* 2014;75:45–56. <http://dx.doi.org/10.1016/j.jclepro.2014.03.080>.
- [37] Milledge J, Smith B, Dyer P, Harvey P. Macroalgae-derived biofuel a review of methods of energy extraction from seaweed biomass. *Energies* 2014;7:7194–222. <http://dx.doi.org/10.3390/en7117194>.
- [38] EGEMACC. EGEMACC j Ege Uˆniversitesi Mikroalg Kuˆltuˆr Koleksiyonu n.d. <http://www.egemacc.com/> [accessed 01.08.15].
- [39] Kahlmeter G, Brown DFJ, Goldstein FW, MacGowan AP, Mouton JW, Odenholt I, et al. European Committee on Antimicrobial Susceptibility Testing (EUCAST) Technical Notes on antimicrobial susceptibility testing. *Clin Microbiol Infect* 2006;12:501–3. <http://dx.doi.org/10.1111/j.1469-0691.2006.01454.x>.
- [40] Wikler MA, Cockerill FR, Craig WA, Dudley MN, Eliopoulos GM, Hecht DW, et al. Performance standards for antimicrobial disk susceptibility tests; approved standard – ninth edition. *CLSI* 2006;26:1–35.
- [41] Oˆrdoˆg V, Stirk WA, Lenobel R, Bancˆrˆovaˆ M, Strnad M, van Staden J, et al. Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *J Appl Phycol* 2004;16:309–14. <http://dx.doi.org/10.1023/B:JAPH.0000047789.34883.aa>.
- [42] Mashjoor S, Yousefzadi M, Esmaili MA, Rafiee R. Cytotoxicity and antimicrobial activity of marine macro algae (Dictyotaceae and Ulvaceae) from the Persian Gulf. *Cytotechnology* 2015. <http://dx.doi.org/10.1007/s10616-015-9921-6>.
- [43] Mazur-Marzec H, Bˆaszczyk A, Felczykowska A, Hohlfeld N, Kobos J, Torunˆska-Sitarz A, et al. Baltic cyanobacteria – a source of biologically active compounds. *Eur J Phycol* 2015;50:343–60. <http://dx.doi.org/10.1080/09670262.2015.1062563>.
- [44] Najdenski HM, Gigova LG, Iliev II, Pilarski PS, Lukavskyˆ J, Tsvetkova IV, et al. Antibacterial and antifungal activities of selected microalgae and cyanobacteria. *Int J Food Sci Technol* 2013;48:1533–40. <http://dx.doi.org/10.1111/ijfs.12122>.
- [45] Rao M, Malhotra S, Fatma T, Rattan A. Antimycobacterial activity from Cyanobacterial extracts and phytochemical screening of methanol extract of *Hapalosiphon*. *Pharm Biol* 2007;45:88–93.
- [46] Tyagi R, Kaushik BD, Kumar J. Antimicrobial activity of some cyanobacteria. In: Kharwar RN, Upadhyay RS, Dubey NK, Raghuwanshi R, editors. *Microb. Divers. Biotechnol. Food Secur.*. New Delhi: Springer; 2014 . p. 463–70. [http://dx.doi.org/10.1007/978-81-322-1801-2\\_41](http://dx.doi.org/10.1007/978-81-322-1801-2_41).
- [47] Mikami K, Hosokawa M. Biosynthetic pathway and health benefits of fucoxanthin, an algae-specific xanthophyll in brown seaweeds. *Int J Mol Sci* 2013;14:13763–81. <http://dx.doi.org/10.3390/ijms140713763>.
- [48] Boche D, Perry VH, Nicoll JAR. Review: activation patterns of microglia and their identification in the human brain. *Neuropathol Appl Neurobiol* 2013;39:3–18. <http://dx.doi.org/10.1111/nan.12011>.
- [49] Ward RJ, Dexter DT, Crichton RR. Ageing, neuroinflammation and neurodegeneration. *Front Biosci (Schol Ed)* 2015;7:189–204.
- [50] Samarakoon KW, Ko J-Y, Shah MMR, Lee J-H, Kang M-C, O-Nam K, et al. In vitro studies of anti-inflammatory and anticancer activities of organic solvent extracts from cultured marine microalgae. *Algae* 2013;28:111–9. doi: <http://dx.doi.org/10.4490/algae.2013.28.1.111>.

**Table 1.** Microbial strains used for the evaluation of antimicrobial properties.

**Figure 1.** Dose-response results for selected extracts against tumorigenic (MCF-7) and non-tumorigenic (MCF-10) breast cell lines, and two tumorigenic prostate cell lines (LNCaP, PC-3) at 0.5 µg/ml (light grey), 5 µg/ml (mid-grey) and 50 µg/ml (dark grey) concentration.

**Figure 2.** Dose-response results for selected specimen against tumorigenic (MCF-7) and non-tumorigenic (MCF-10) breast cell lines, and two tumorigenic prostate cell lines (LNCaP, PC-3) at 0.5 µg/ml (light grey), 5 µg/ml (mid-grey) and 50 µg/ml (dark grey) concentration.

**Table 1.** Microbial strains used for the evaluation of antimicrobial properties.

	<b>Strain</b>	<b>Positive control</b>
<b>Pathogenic bacteria</b>		
<i>Enterococcus faecalis</i>	ATCC 29212 <sup>a</sup>	Ciprofloxacin
<i>Staphylococcus aureus</i>	ATCC 25923 <sup>a</sup>	Ciprofloxacin
<i>Staphylococcus aureus</i> (MRSA)	GMC <sup>b</sup>	Methicillin
<i>Escherichia coli</i>	ATCC 25922 <sup>a</sup>	Ciprofloxacin
<i>Klebsiellapneumoniae</i>	GMC	Streptomycin
<i>Shigella flexneri</i>	GMC	Trimethoprim
<i>Vibrio cholerae</i>	GMC	Chloramphenicol
<b>Pathogenic fungi</b>		
<i>Aspergillus fumigatus</i>	GMC	Amphotericin B
<i>Candida albicans</i>	ATCC 90028 <sup>a</sup>	Amphotericin B
<i>Cryptococcus neoformans</i>	GMC	Amphotericin B
<b>Fouling bacteria</b>		
<i>Aeromonashydrophila</i>	FCP <sup>c</sup>	Gentamycin
<i>Aeromonassalmonicida</i>	FCP	Gentamycin
<i>Alcanivoraxborkumensis</i>	FCP	Gentamycin
<i>Alcanivorax</i> sp.	FCP	Gentamycin
<i>Allivibriosalmonicida</i>	FCP	Gentamycin
<i>Erythrobacterlitoralis</i>	FCP	Gentamycin
<i>Planococcusdonghaensis</i>	FCP	Penicillin
<i>Pseudoalteromonassp</i>	FCP	Penicillin
<i>Pseudomonas mendocina</i>	FCP	Penicillin
<i>Vibrio furnissii</i>	FCP	Gentamycin

<sup>a</sup>Obtained from Microbiologics, Inc. (USA), <sup>b</sup>GMC obtained from Goa Medical College and Hospital; <sup>c</sup>FCP, fouled copper panels.





**Figure 2.** Dose-response results for selected specimen against tumorigenic (MCF-7) and non-tumorigenic (MCF-10) breast cell lines, and two tumorigenic prostate cell lines (LNCaP, PC-3) at 0.5 µg/ml (light grey), 5 µg/ml (mid-grey) and 50 µg/ml (dark grey) concentration.

