XXX Ciclo di Dottorato in Scienze del Mare

PhD Thesis

Genova, May 2018

Structure and functioning of *Cystoseira* (Fucales) populations

An applicative study to assess their relevance for marine coastal ecosystems and for human well-being, in a conservation and restoration perspective

Struttura e funzionamento dei popolamenti a *Cystoseira* (Fucales)

Uno studio applicativo per valutare la loro importanza per l'ecosistema marino costiero e per il benessere umano, in una prospettiva di conservazione e ripristino

Gina De La Fuente Mancebo

Supervised by:

Prof. Mariachiara Chiantore

Dr. Paolo Vassallo

Table of Contents

General introduction

Marine macroalgae are plant-like organisms that mainly live attached to hard substrata in coastal areas in the different latitudes and along the different bathymetric levels of the euphotic layer. Macroalgae differ from plants because they have not organs such as roots, stem or leaves, instead they are formed by a thallus, which completely developed acquires a characteristic morphology such as: leaf-shape, round-shape or filamentous shape, which help to identify each species. However, colour is the main feature that may be detected, so macroalgae are classified in three big groups depending on their pigments content. Chlorophyll a is responsible of the photosynthesis process; therefore, it is present in all species, being present in higher quantity in the green algae classified as Chlorophyta. Red algae form the Rhodophyta, thanks to the additional abundant pigment phycobilin. In the case of brown algae, which belong to Heterokontophyta (Phaeophyta), the most abundant pigments in addition to chlorophyll are carotenes and xanthophylls.

Along history, macroalgae have been used for many different purposes, in particular in the Eastern Countries (Japan, China, Korea) forming part of their culture, although, the earliest archaeological evidences suggest that in Monte Verde (Southern Chile) macroalgae was harvested and used for food and medicinal purposes around 20,000 years ago (Dillehay et al., 2008). Further archaeological findings have also demonstrated the human and macroalgae interactions around the Neolithic period (Ainis et al., 2014; Erlandson et al., 2015). Nevertheless, the first written records are from 1700 years ago, where Chinese people used the blad of red algae (Bangiales order) as food and for pharmaceutical purposes (Yang et al., 2017).

In Europe, Greeks used macroalgae as medicine and Romans used macroalgae (Columella and Paldeus), as fertilizers (Henderson, 2004; Fleurence and Levine, 2016). From pre-Christian times, in the Mediterranean, some red algae were used as source for dyeing agents and as a medicine to treat parasitic worms (Stein & Borden, 1984). In the Northern Europe, early farmers in Ireland and Iceland harvested *Palmaria* (red algae) for human consumption (Guiry and Blunden, 1991).

The uses of macroalgae have changed along time, In fact, while in earlier times, the most common purposes were for domestic use as food and feed, later industrial uses emerged (Delaney et al., 2016). Now, the most common uses of macroalgae include food for human consumption, cosmetics, fertilizers, and extraction of industrial gums and chemicals (Pereira, 2011; Anis and Hasan, 2017). Global demand for macroalgae has been growing together with increase in usage beyond former traditional applications (Hafting et al., 2015). In 2014, the largest macroalgal producers by far were China and Indonesia with 85.7% of the world production (>23 million tonnes; FAO, 2014, 2016). The top seven most cultivated macroalgae taxa, three red algae, were used mainly for hydrocolloid extraction (*Euchema* spp, *Kappaphycus alvarezii* and *Gracilaria* spp.). Instead, one red algae (*Pyropia* spp.) and three brown algae (*Saccharina japonica*; *Undaria pinnatifida* and *Sargassum fusiforme*) were the most important for human consumption (FAO, 2014, 2016).

In addition, macroalage have the potential to be used as a source of long- and short-chain chemicals with medicinal and industrial uses (Guiry, 2018). Consequently, there is an increasing evidence of the concept about the consumption of algal food/feed products may have health and nutritional benefits (Plaza et al., 2008).

As far as brown algae, around 2000 species, mostly marine, have been identified, which vary in form and size from small filamentous epiphytes (*Ectocarpus*) to complex giant thallus can reach tens of meters in length (Laminariales). Thirty five percent of brown algae (Fucales and Laminariales; Guiry and Guiry, 2018) contribute to the structure of the coastal landscapes playing a very important role as ecosystem engineers, providing an important value as natural capital. Kelps and fucoids are complex canopy-forming brown algae that form reliable marine forests, due to their three-dimensional morphology, provide habitat for many other associated species (algae, invertebrates and fish), affecting the structure, biodiversity and functioning of their habitats (Thompson et al., 1996; Chemello and Milazzo, 2002; Christie et al., 2007; Airoldi et al., 2015). Around the world, kelp forest (i.e. *Macrocystis*, *Lessonia* and *Laminaria*) constitute some of the most important habitats, distributed worldwide throughout temperate and polar coastal oceans (Steneck et al., 2002; Villegas et al., 2008). In the Mediterranean Sea, *Laminaria*, *Sacchoriza*, *Phyllariopsis* and *Sargassum* genus, also play a role as foundation species in some specific locations but the canopy-forming brown algae of the *Cystoseira* genus are the most important being amongst the largest and longest-living in this zone (Rodriguez-Prieto et al., 2013). The Mediterranean Sea is a hot spot of this genus, where several of its species are endemic.

Besides the evident ecological interest, brown algae are a potential natural source of valuable compounds providing, among others, antimicrobial, antiviral, anti-inflammatory and antioxidant properties (Berteau et al., 2003; Li et al., 2008; Jiao et al., 2011; Milledge et al., 2016). Especially, antioxidant properties have been reported being higher in brown algae than in red and green algae, in relation to their phenolic content (Balboa et al., 2013). In particular, the species-specific biological activities of the *Cystoseira* genus has been also widely studied: *Cystoseira myrica* (Indian coast; Mandal et al., 2007), *Cystoseira compressa*, *Cystoseira crinita* and *Cystoseira sedoides* (Tunisian coast; Mhadhebi et al., 2012), *Cystoseira humilis* (Moroccan Atlantic coast; Belattmania et al., 2016) or *Cystoseira tamariscifolia* (Brittany coast; Zubia et al., 2009). As well as for *Sargassum* species: *Sargassum horridium* (Baja California, Mexico; Tenorio-Rodriguez et al., 2017), *Sargassum muticum* (British coast; Milledge et al., 2016) and *Sargassum vulgare* (Brazil; Dore et al., 2013).

Large canopy-forming brown algae are exposed to multiple disturbances that have caused a decline in their abundance in many coastal areas of the world (Steneck et al., 2002; Airoldi et al., 2008; Coleman et al., 2008; Connell et al., 2008; Mineur et al., 2015). The main pressures affecting these valuable ecosystems are sedimentation (Perkol-Finkel & Airoldi, 2010), low water quality (Sales et al., 2011), anthropization (Mangialajo et al., 2008; Sales & Ballesteros, 2010) overgrazing by herbivores (Verges et al., 2014) and harvesting (Zhao et al., 2008).

In order to prevent further deterioration of the environment and to protect the natural heritage, the European Union has adopted a wide legislative framework that directly or indirectly protects the coastal environment, its associated biodiversity and other natural resources. Since 1973, the European Commission has developed seven environmental action programmes (EAP) with the aim to introduce common environmental policies addressing different objectives and key actions to be taken into account by each Member States. Under the first EA programmes, Ramsar, Bonn, Bern and Barcelona Conventions were adopted, which were addressed to provide protection and conservation to marine habitats and species. Afterwards, the EU Habitats Directive and the Natura 2000 network complemented conservation efforts of the previous legislation.

Regarding brown algae, most of *Cystoseira* species are strictly protected by the Bern and Barcelona Convention. Additionally, the *Cystoseira*/*Sargassum* beds with a mixture of other red algae (Gelidiales, Ceramiales), brown algae (Dictyotales) and green algae (Siphonales, Siphonacladales) are listed as protected habitat in the Annex I of the Habitats Directive (code 1170 “reefs”).

In the late 90s, the 5th EAP aimed to sustainable development, which differed from previous programmes, setting longer term objectives and focusing on a more global approach. For instance, the Ambient Air Quality Directive (96/62), the IPPC Directive (1996/61) or the Water Framework Directive (WFD; 2000/60) are examples of the integration of previous legislations in a holistic approach.

In particular, the WFD aimed to maintaining and improving the aquatic environments (inland surface waters, transitional waters, coastal waters and groundwater) and committed the Member States to reach and maintain the “good” Ecological Status (ES) within 2015. As far as the coastal water bodies, the WFD proposed the use of the following four quality biological elements (BQE): benthic invertebrates, phytoplankton, macroalgae and marine angiosperms, to estimate their ecological status. In this framework, the CARLIT index (Ballesteros et al. 2007) was created to assess the coastal water quality in the Mediterranean Sea using macroalgae as a BQE. The CARLIT Index is based on the cartography of the commonest littoral and upper-sublittoral macroalgal communities along rocky shores. Macroalgae are sessile organisms, therefore they integrate the effects of long-term exposure to different anthropogenic pressures resulting in decrease or even disappearance of the most sensitive species and their replacement by highly resistant, nitrophilic and/or opportunistic species (i.e. Borowitza, 1972; Diez et al., 1999). For that reason, the CARLIT methodology classifies the macroalgal communities in different sensitivity levels, where the highest sensitivity values refer to the canopy-forming brown species of *Cystoseira*.

In the same line, the 6th EAP promotes full integration of environmental protection requirements into all Community policies and actions and provides the environmental component of the Community's strategy for sustainable development. Regarding the marine environment, the Marine Strategy Framework Directive (MSFD) was adopted in 2008 in order to achieve Good Environmental Status (GES) by 2020 for the European marine waters, integrating elements such as, e.g., noise, litter or aspects of biodiversity, which were not assessed before by WFD. Moreover, MSFD is the first EU legislative instrument related to the protection of marine biodiversity, as it contains the explicit regulatory objective "biodiversity is maintained by 2020", as the keystone for achieving the GES that is supported by the Biodiversity strategy 2020.

Both legislations (MSFD and Biodiversity strategy 2020) aim to maintain biodiversity and ecosystem services of the marine environment by protection and conservation and through restoration actions in appropriate cases. Today and accordingly with the commented above, marine environment restoration gains strength as new action in the environmental policies to enhance biodiversity and ecosystem functioning. As examples, the *Marine Ecosystem Restoration in Changing European Seas* (MERCES) and the *Promoting biodiversity enhancement by Restoration Of Cystoseira POPulations* (ROC-POP-LIFE) are European projects that have been launched focusing on the restoration of the different degraded marine habitats such as: shallow soft bottom habitats (e.g. seagrasses), coastal shallow hard bottom habitats (e.g. canopy-forming brown algae) and deep-sea habitats.

The last EAP have as the first priority objective to protect, conserve and enhance the Union's natural capital. Natural capital can be understood as all natural resources, or, the stock of material and information that exists in a given moment originated from natural environments, such as solar energy, soils, trees, minerals, fossil fuels, ecosystems and atmosphere (Costanza et al., 1997; Daly and Farley, 2004). Ecosystem goods and services (simplified as ecosystem services *sensu* Costanza et al., 1997) represent the benefits that human populations derive, directly or indirectly, from ecosystem functions. According to the Common International Classification of Ecosystem Services (CICES) ecosystem services are classified as following: production services (food, water, timber, and fiber); regulation services (affecting climate, floods, disease, wastes, and water quality) and cultural services (providing recreational, aesthetic, and spiritual benefits).

In the last decade, a growing interest in the quantification of the economic value of ecosystems has been observed, to raise awareness of the public towards the preservation of biodiversity, and support the policy makers in the process of creating new legal instruments (Braüer et al., 2006; Beaumont et al., 2008). Consequently, different marine ecosystems have been widely evaluated economically: the coral reefs, the wetlands, the mangroves, the seagrasses (Groot et al., 2010; TEEB, 2010) and the coastal zone habitats (Zuo et al., 2004; Vassallo et al. 2007, 2013; Tilley & Brown, 2006; Paoli et al., 2008).

References

Ainis, A. F., Vellanoweth, R. L., Lapeña, Q. G., & Thornber, C. S. (2014). Using non-dietary gastropods in coastal shell middens to infer kelp and seagrass harvesting and paleoenvironmental conditions. *Journal of Archaeological Science*, *49*, 343-360.

Anis, M., & Hasan, S. A. (2017). Algae as nutrition, medicine and cosmetic: the forgotten history, present status and future trends. *World J Pharm Pharm Sci*, *6*(6), 1934-1959.

Airoldi, L., Balata, D., & Beck, M. W. (2008). The gray zone: relationships between habitat loss and marine diversity and their applications in conservation. *Journal of Experimental Marine Biology and Ecology*, *366*(1), 8-15.

Airoldi, L., Ballesteros, E., Buonuomo, R., van Belzen, J., Bouma, T. J., Cebrian, E., De Clerk, O., Engelen, A.H., Ferrario, F., Fraschetti, S., Gianni, F., Guidetti, P., Ivesa, L., Mancuso, F.P., Micheli, F., Perkol-Finkel, S., Serrao, E., Strain, E.M., Mangialajo, L. (2015, January). Marine forests at risk: solutions to halt the loss and promote the recovery of Mediterranean canopy-forming seaweeds. In 5th Mediterranean Symposium on Marine Vegetation.

Balboa, E. M., Conde, E., Moure, A., Falqué, E., & Domínguez, H. (2013). In vitro antioxidant properties of crude extracts and compounds from brown algae. *Food chemistry*, *138*(2), 1764-1785.

Ballesteros, E., Torras, X., Pinedo, S., García, M., Mangialajo, L., & De Torres, M. (2007). A new methodology based on littoral community cartography dominated by macroalgae for the implementation of the European Water Framework Directive. *Marine pollution bulletin*, *55*(1), 172-180.

Beaumont, N. J., Austen, M. C., Mangi, S. C., & Townsend, M. (2008). Economic valuation for the conservation of marine biodiversity. *Marine pollution bulletin*, *56*(3), 386-396.

Belattmania, Z., Reani, A., Barakate, M., Zrid, R., Elatouani, S., Hassouani, M., Eddaoui A., Bentiss F., Sabour B. (2016). Antimicrobial, antioxidant and alginate potentials of Dictyopteris polypodioides (Dictyotales, Phaeophyceae) from the Moroccan Atlantic coast. *Pharma Chem.*, *8*, 216-226.

Berteau, O., & Mulloy, B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. Glycobiology, 13(6), 29R-40R.

Borowitzka, M. A. (1972). Intertidal algal species diversity and the effect of pollution. *Marine and Freshwater Research*, *23*(2), 73-84.

Bräuer, I., Müssner, R., Marsden, K., Oosterhuis, F., Rayment, M., Miller, C., & Dodoková, A. (2006). The use of market incentives to preserve biodiversity. ECOLOGIC, 51p.

Chemello, R., & Milazzo, M. (2002). Effect of algal architecture on associated fauna: some evidence from phytal molluscs. Marine Biology, 140(5), 981-990.

Christie, H., Jørgensen, N. M., & Norderhaug, K. M. (2007). Bushy or smooth, high or low; importance of habitat architecture and vertical position for distribution of fauna on kelp. Journal of Sea Research, 58(3), 198-208.

Coleman, M. A., Kelaher, B. P., Steinberg, P. D., & Millar, A. J. (2008). Absence of a large brown macroalga on urbanized rocky reefs around Sydney, Australia, and evidence for historical decline. *Journal of phycology*, *44*(4), 897-901.

Connell, S. D., Russell, B. D., Turner, D. J., Shepherd, S. A., Kildea, T., Miller, D., Airoldi, L., & Cheshire, A. (2008). Recovering a lost baseline: missing kelp forests from a metropolitan coast. *Marine Ecology Progress Series*, *360*, 63-72.

Costanza, R., d'Arge, R., De Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton. P., & Van Den Belt, M. (1997). The value of the world's ecosystem services and natural capital. *Nature*, *387*(6630), 253-260.

Daly, H., & Farley, J. (2004). Ecological economics: principles and practice. *Island, Washington, DC, USA*.

De Groot, R. S., Alkemade, R., Braat, L., Hein, L., & Willemen, L. (2010). Challenges in integrating the concept of ecosystem services and values in landscape planning, management and decision making. *Ecological complexity*, *7*(3), 260-272.

Delaney, A., Frangoudes, K., & Ii, S. A. (2016). Society and seaweed: understanding the past and present. In Seaweed in Health and Disease Prevention (pp. 7-40).

Díez, I., Secilla, A., Santolaria, A., & Gorostiaga, J. M. (1999). Phytobenthic intertidal community structure along an environmental pollution gradient. *Marine Pollution Bulletin*, *38*(6), 463-472.

Dillehay, T. D., Ramirez, C., Pino, M., Collins, M. B., Rossen, J., & Pino-Navarro, J. D. (2008). Monte Verde: seaweed, food, medicine, and the peopling of South America. *Science*, *320*(5877), 784-786.

Dore, C.M.P.G., Alves, M.G.C.F., Costa, T.G., Sabry, D.A., Rêgo, L.A.S., Accardo, C.M., Rocha, H.A.O., Filgueira, L.G.A., Leite, E.L. (2013). A sulfated polysaccharide, fucans, isolated from brown algae Sargassum vulgare with anticoagulant, antithrombotic, antioxidant and anti-inflammatory effects. Carbohydrate polymers, 91(1), 467-475.

Erlandson, J. M., & Braje, T. J. (2015). Coasting out of Africa: The potential of mangrove forests and marine habitats to facilitate human coastal expansion via the Southern Dispersal Route. *Quaternary International*, *382*, 31-41.

FAO (2014). The State of World Fisheries and Aquaculture 2014 (SOFIA).

FAO (2016). The State of World Fisheries and Aquaculture 2016 (SOFIA).

Fleurence, J., & Levine, I. (Eds.). (2016). *Seaweed in health and disease prevention*. Academic Press.

Guiry, M. D., & Blunden, G. (1991). *Seaweed resources in Europe: uses and potential*.

Guiry, M. D. (2018). Seaweed site. World-wide electronic publication, National University of Ireland, Galway.

Guiry, M.D. & Guiry, G.M. 2018. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 16 January 2018.

Hafting, J. T., Craigie, J. S., Stengel, D. B., Loureiro, R. R., Buschmann, A. H., Yarish, C., Edwards, M.V., & Critchley, A. T. (2015). Prospects and challenges for industrial production of seaweed bioactives. *Journal of phycology*, *51*(5), 821-837.

Henderson, J. (2004). *The Roman book of gardening*. Psychology Press.

Li, B., Lu, F., Wei, X., & Zhao, R. (2008). Fucoidan: structure and bioactivity. Molecules, 13(8), 1671-1695.
Jiao, G., Yu, G., Zhang, J., & Ewart, H.S. (2011). Chemical structures and bioactivities of sulfated polysaccharides from marine algae. Marine drugs, 9(2), 196-223.

Mandal, P., Mateu, C.G., Chattopadhyay, K., Pujol, C.A., Damonte, E.B., & Ray, B. (2007). Structural features and antiviral activity of sulphated fucans from the brown seaweed Cystoseira indica. *Antiviral Chemistry and Chemotherapy*, *18*(3), 153-162.

Mangialajo, L., Chiantore, M., & Cattaneo-Vietti, R. (2008). Loss of fucoid algae along a gradient of urbanisation, and structure of benthic assemblages. *Marine Ecology Progress Series*, *358*, 63-74.

Mhadhebi, L., Dellai, A., Clary-Laroche, A., Said, R.B., Robert, J., & Bouraoui, A. (2012). Anti-inflammatory and antiproliferative activities of organic fractions from the Mediterranean brown seaweed, Cystoseira compressa. Drug Development Research, 73(2), 82-89.

Milledge, J.J., Nielsen, B.V., & Bailey, D. (2016). High-value products from macroalgae: the potential uses of the invasive brown seaweed, Sargassum muticum. Reviews in Environmental Science and Bio/Technology, 15(1), 67-88.

Mineur, F., Arenas, F., Assis, J., Davies, A. J., Engelen, A. H., Fernandes, F., JanMalta, E., Thibaut, T., Nguyen, T., Vaz-Pinto, F., Vranken, S., Serrao, E., & De Clerck, O. (2015). European seaweeds under pressure: Consequences for communities and ecosystem functioning. *Journal of Sea Research*, *98*, 91-108.

Paoli, C., Vassallo, P., & Fabiano, M. (2008). An emergy approach for the assessment of sustainability of small marinas. *Ecological engineering*, *33*(2), 167-178.

Pereira, L. (2011). A review of the nutrient composition of selected edible seaweeds. *Seaweed: Ecology, nutrient composition and medicinal uses*, 15-47.

Perkol-Finkel, S., & Airoldi, L. (2010). Loss and recovery potential of marine habitats: an experimental study of factors maintaining resilience in subtidal algal forests at the Adriatic Sea. *PLoS one*, *5*(5), e10791.

Plaza, M., Cifuentes, A., & Ibáñez, E. (2008). In the search of new functional food ingredients from algae. *Trends in Food Science & Technology*, *19*(1), 31-39.

Rodríguez-Prieto, C., Ballesteros, E., Boisset, F., Afonso-Carrillo, J. (2013). Guía de las macroalgas y fanerógamas marinas del Mediterráneo occidental. Omega.

Sales, M., & Ballesteros, E. (2010). Long-term comparison of algal assemblages dominated by Cystoseira crinita (Fucales, Heterokontophyta) from Cap Corse (Corsica, North Western Mediterranean). *European Journal of Phycology*, *45*(4), 404-412.

Sales, M., Cebrian, E., Tomas, F., & Ballesteros, E. (2011). Pollution impacts and recovery potential in three species of the genus Cystoseira (Fucales, Heterokontophyta). *Estuarine, Coastal and Shelf Science*, *92*(3), 347-357.

Stein, J.R., & Borden, C.A. (1984). Causative and beneficial algae in human disease conditions: a review. *Phycologia*, *23*(4), 485-501.

Steneck, R.S., Graham, M.H., Bourque, B.J., Corbett, D., Erlandson, J.M., Estes, J.A., & Tegner, M.J. (2002). Kelp forest ecosystems: biodiversity, stability, resilience and future. *Environmental conservation*, *29*(4), 436-459.

TEEB, 2010. The Economics of Ecosystems and Biodiversity: Mainstreaming the Economics of Nature: a Synthesis of the Approach, Conclusions and Recom- mendations of TEEB.

Tenorio-Rodriguez, P.A., Murillo-Álvarez, J.I., Campa-Cordova, Á.I., & Angulo, C. (2017). Antioxidant screening and phenolic content of ethanol extracts of selected Baja California Peninsula macroalgae. Journal of food science and technology, 54(2), 422-429.

Thompson, R.C., Wilson, B.J., Tobin, M.L., Hill, A.S., & Hawkins, S.J. (1996). Biologically generated habitat provision and diversity of rocky shore organisms at a hierarchy of spatial scales. Journal of Experimental Marine Biology and Ecology, 202(1), 73-84.

Tilley, D.R., & Brown, M.T. (2006). Dynamic emergy accounting for assessing the environmental benefits of subtropical wetland stormwater management systems. *Ecological Modelling*, *192*(3), 327-361.

Vassallo, P., Bastianoni, S., Beiso, I., Ridolfi, R., & Fabiano, M. (2007). Emergy analysis for the environmental sustainability of an inshore fish farming system. *Ecological Indicators*, *7*(2), 290-298.

Vassallo, P., Paoli, C., Rovere, A., Montefalcone, M., Morri, C., & Bianchi, C.N. (2013). The value of the seagrass Posidonia oceanica: A natural capital assessment. *Marine Pollution Bulletin*, *75*(1), 157-167.

Vergés, A., Steinberg, P.D., Hay, M.E., Poore, A.G., Campbell, A.H., Ballesteros, E., Heck, K.L., Booth, D.J., Coleman, M.A., Feary, D.A., Figueira, W., Langlois, T., Marzinelli, E.M., Mizerek, T., Mumby, P.J., Nakamura, Y., Roughan, M., Van Sebille, E., Sen Gupta, A., Smale, D.A., Tomas, F., Wernberg, T., Wilson, S.K. (2014, August). The tropicalization of temperate marine ecosystems: climate-mediated changes in herbivory and community phase shifts. In *Proc. R. Soc. B* (Vol. 281, No. 1789, p. 20140846). The Royal Society.

Villegas, M. J., Laudien, J., Sielfeld, W., & Arntz, W E. (2008). Macrocystis integrifolia and Lessonia trabeculata (Laminariales; Phaeophyceae) kelp habitat structures and associated macrobenthic community off northern Chile. Helgoland Marine Research, 62(1), 33.

Yang, L.E., Lu, Q.Q., & Brodie, J. (2017). A review of the bladed Bangiales (Rhodophyta) in China: history, culture and taxonomy. *European Journal of Phycology*, 1-13.

Zhao, Z., Zhao, F., Yao, J., Lu, J., Ang, P.O., & Duan, D. (2008). Early development of germlings of Sargassum thunbergii (Fucales, Phaeophyta) under laboratory conditions. *Journal of applied phycology*, *20*(5), 925.

Zubia, M., Fabre, M.S., Kerjean, V., Le Lann, K., Stiger-Pouvreau, V., Fauchon, M., & Deslandes, E. (2009). Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chemistry, 116(3), 693-701.

Zuo, P., Wan, S.W., Qin, P., Du, J., & Wang, H. (2004). A comparison of the sustainability of original and constructed wetlands in Yancheng Biosphere Reserve, China: implications from emergy evaluation. *Environmental Science & Policy*, *7*(4), 329-343.

Thesis objectives and structure

The present PhD Thesis is addressed to study the interactions between human activities and coastal ecosystem, using as case study, the intertidal *Cystoseira* populations present along the Ligurian coast in the North-Western Mediterranean, taking into account the status of the *Cystoseira* habitat, its protection and restoration, the bioactive properties of these algae (*Cystoseira* and *Sargassum*) as potential natural sources, directly ad indirectly providing benefits to human well-being. Finally, the relevant importance of the natural capital provided by the *Cystoseira* habitat has been accounted in terms of ecosystems functions and services (*e.g*. water quality regulation, commercial species provisioning, ecotourism).

In order to tackle the points mentioned above, the thesis has been structured in the following four chapters:

Chapter 1 is focused on i) assessing the Environmental Status of the Ligurian coastal water bodies over a decade, according to the CARLIT Index. In order to establish a good long-term monitoring programme to be carried out by environmental regional agencies, ii) assessing the effects of intra-seasonal variability within the period of implementation (spring) and sampling operator effect of the CARLIT Index in order to comply with the requirements of marine environment directives, correctly classifying water bodies ES with an appropriate level of confidence.
Given the relevance of the structuring *Cystoseira* species, as the most sensible species by CARLIT Index, the abundances and distribution of *Cystoseira amentacea* and *Cystoseira compressa* communities have been studied. iii) The implementation of this Index, over a decade, allowed to observe changes on macroalgal communities of the coastal environment studied providing a benchmark, useful to detect changes along time on these *Cystoseira* populations with the aim of to conserve, manage or, potentially, restore. iv) In addition, herbaria records of *Cystoseira* species from the late 19th century in the Ligurian coast have contributed to the study of their presence and distribution until today, allowing to assess the loss of the most valuable intertidal species caused by anthropogenic pressures and to provide the potential areas to protect, conserve and/or restore.

Regarding restoration, in **Chapter 2** a protocol of a laboratory cultivation for *Cystoseira amentacea* was defined. Light intensity, temperature of sea water and appropriate substrates allowing the best attachment of *Cystoseira amentacea* zygotes were identified as optimal culture conditions. This protocol establishes the first step necessary to set up a restoration activity of *Cystoseira amentacea*. This species is particularly relevant for the Ligurian region because it is abundant along most of the rocky coast with the exception of the coast along the Marine Protected Area of Cinque Terre, providing clues to develop restoration actions, on basis of the information obtained in the previous study regarding the historical distribution of *Cystoseira amentacea* along the Ligurian coast.

Chapter 3 addresses the potential antioxidant and antimicrobial capacities of six Fucales species (belonging to *Cystoseira* and *Sargassum* genera) from the Mediterranean Sea and the Atlantic Ocean. For antioxidant

screening, the total phenolic content (TPC), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, the Oxygen Radical Absorbent Capacity (ORAC) and the Ferric reducing antioxidant power (FRAP) were assessed for each species.
The antimicrobial activity was measured by following the growth of the fungus *Candida albicans*, the gram-positive bacteria *Staphylococcus aureus* and the gram-negative bacterium *Pseudomonas aeruginosa*. The natural antioxidant and antimicrobial potential of the brown algae were successfully studied and evaluated.

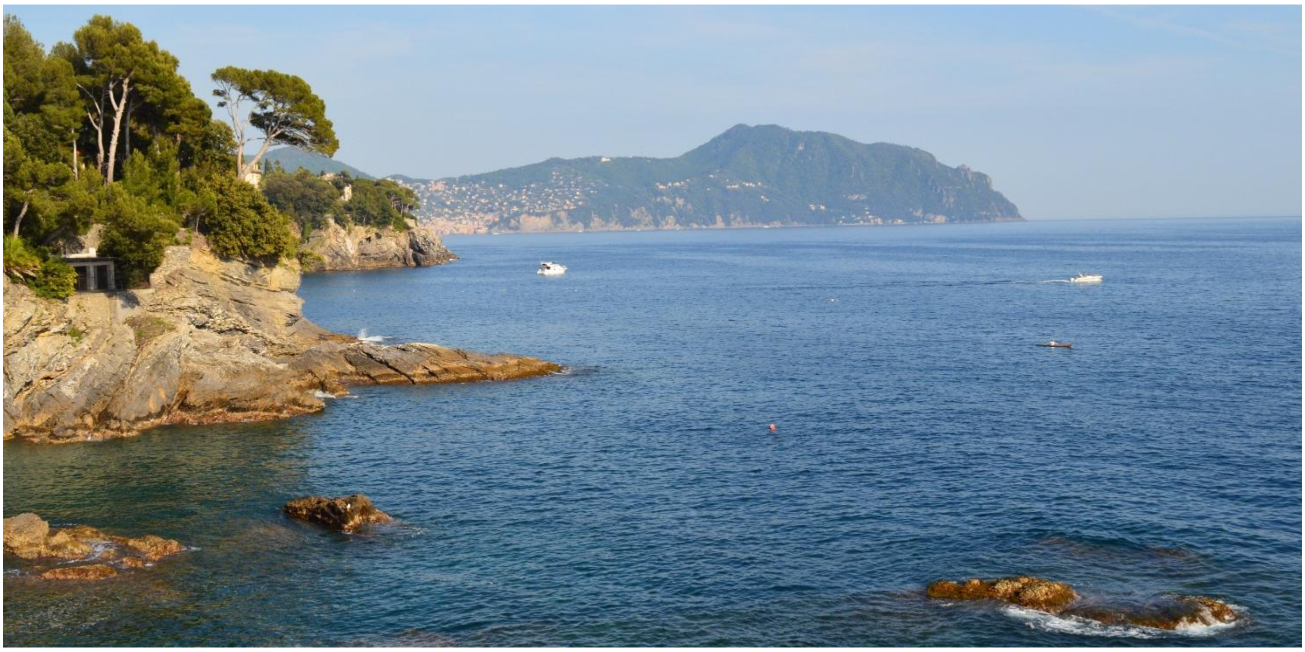
Finally, in **Chapter 4**, the effect of *Cystoseira* canopy-forming species on the midlittoral habitat value in the Ligurian coast was assessed through the emergy analysis. Emergy analysis allowed obtaining the functions and services and the natural capital of the middlittoral habitat present above the *Cystoseira* canopies. The variability of the natural capital of the midlittoral habitat, expressed in monetary terms, was influenced by the relative presence and the continuous or discontinuous features of the belt dominated by the two dominant species, *Cystoseira amentacea* and *Cystoseira compressa* fringe, which affected the structure of the of the midlittoral habitat in terms of infauna and macroalgal composition.

Chapter 1

Ecological status improvement over a decade along the Ligurian coast according to a macroalgae based index (CARLIT)

Authors: Gina De La Fuente, Mariachiara Chiantore, Federico Gaino, Valentina Asnaghi

Accepted in: Plos One



Abstract

According to the Water Framework Directive, within 2015 European Union countries must reach and maintain the "good" Ecological Status (ES), quantified through indices based on key biological elements as indicators. Along the Ligurian shallow rocky coasts (NW Italy), a macroalgae based index (CARtography of LITtoral and upper-sublittoral benthic communities, CARLIT), calibrated according to national characteristics and management needs, has been applied by the Regional Environmental Agency over the last ten years. In 2015, the "good" ES was achieved in all Ligurian water bodies except one, located in the Eastern Ligurian coastline, characterized by the present lack of the most sensitive species, *Cystoseira amentacea var. stricta*. A general ES improvement has been observed along the Ligurian coastline, also in comparison with other quality indices (macroinvertebrates and fecal bacteria), and in particular in the Genoa water body, as proved by a relevant increase of *C. amentacea* abundance, probably as a consequence of enhancement in wastewater treatments. In the present study, the reliability of the observed improvement of the ES over a decade has been assessed, teasing apart intra-seasonal and operator-related variability.

1. Introduction

The Water Framework Directive (WFD, 2000/60/EC) adopted by the European Community in 2000 with the goal of maintaining and improving the quality of aquatic environments, in its first management cycle, requires that member states achieve and maintain a "good" Ecological Status (ES) of all water bodies by 2015, postponed to 2021 and 2027 for the second and third management cycles. The ES has to be quantified applying indices based on appropriate key biological elements as indicators. A ratio between observed values and reference ones (EQR, Ecological Quality Ratio) of the indicator allows to rank water bodies according to different ES classes: bad, poor, moderate, good and high.

The CARLIT Index, CARtography of LITtoral and upper-sublittoral benthic communities (Ballesteros et al., 2007), assesses coastal water quality in the Mediterranean Sea using intertidal macroalgal assemblages as key biological elements: the EQR is calculated dividing the Ecological Quality Value, attributed following the dominant macroalgal community, by the reference value, according to the geomorphological relevant situation (Ballesteros et al., 2007). The selection of a proper reference site is fundamental for the CARLIT Index calculation (Mangialajo et al., 2007). This index is widely used in the EU Mediterranean countries: Spain (Catalan coast, Ballesteros et al., 2007; Alboran Sea, Bermejo et al., 2013); France (Southern coast and Corsica, Blanfuné et al., 2017); Italy (Rende et al., 2011; Ligurian Sea, Mangialajo et al., 2007; Asnaghi et al., 2009; Gulf of Naples, Buia et al., 2007; Northwestern Adriatic Sea, Sfriso & Facca, 2011; Sardinia, Ferrigno et al., 2014; Tyrrhenian islands, Lasinio et al., 2017); Croatia (Northeastern Adriatic Sea, Nikolić et al., 2013); Malta (Blanfuné et al., 2011) and in two non-EU country, Albania (Blanfuné et al., 2016) and Tunisia (Omrane et al., 2010).

The rationale of CARLIT Index is the different sensitivity to environmental stresses displayed by macroalgal assemblages along rocky shores (Litter & Murray, 1975; Panayotidis et al., 1999; Orfanidis et al., 2001; Krause-Jensen et al., 2007; Pinedo et al., 2007; Ling & Fong, 2008; Díez et al., 2009; Akcali & Kucuksezgin, 2011; Chakraborty et al., 2014). The lowest levels of sensitivity correspond to the opportunistic species (mainly Ulvales order: Díez et al., 1999; Airoldi & Bulleri, 2011), the intermediate values correspond to the stress-tolerant species (mainly Corallinales order: Arevalo et al., 2007; Mangialajo et al., 2008a) and the highest sensitivity levels refer to the species belonging to the *Cystoseira* genus (Fucales order) (Salas et al., 2006; Arevalo et al., 2007; Juanes et al., 2008). *Cystoseira* species display a large size and complex structure with an arborescent thallus: where well-developed, these species can form forests, which play an important role as ecosystem engineers, supporting a highly structured and diversified macroalgal assemblage and providing shelter (refuge) and food for marine invertebrates and for juvenile fish (Bulleri et al., 2002; Cheminée et al., 2013).

At present, the CARLIT Index is regularly implemented in Italy, where it has been calibrated according to national characteristics, as reported in the ISPRA (Italian National Institute for Environment Protection and Research) methodological report (Mangialajo et al., 2008; http://www.isprambiente.gov.it/it/archivio/notizie-e-novita-normative/notizie-ispra/anno-2009/pubblicazione-amb-mar). The first implementation of CARLIT Index was performed in Liguria, in 2005-2007 (Mangialajo et al., 2007; Asnaghi et al., 2009): in the following years, CARLIT monitoring has been carried out by the Regional Environmental Agency (ARPAL) as part of the institutional monitoring programme (Environmental and Sea Defense Ministry – Monitoring Programme, ex L. 979/82). According to ISPRA methodological report, the CARLIT Index can be calculated uninterruptedly along the entire rocky coast or, following a hierarchical design, encompassing large and representative stretches of coast for each water body, as reported by Asnaghi et al. (2009). In the Ligurian Sea, the CARLIT Index is calculated by ARPAL following a hierarchical design, encompassing 3 sites of at least 20 sectors (50 m) for each water body (24). Each WB is visited once every three years.

The use of the CARLIT Index as a monitoring tool for the Regional Agencies has several advantages, because of non-destructive sampling, reduced costs and easy-to-acquire taxonomic expertise (Ballesteros et al., 2007). Yet, the methodology has the disadvantage that needs to be implemented in a specific and limited time-frame, due to the strongly seasonal development of macroalgal assemblages. In fact, the CARLIT monitoring must be performed in late spring (April - June), when macroalgae reach their growth peak. Since the application of the CARLIT Index along the entire regional coast may be quite long due to unstable sea conditions in spring time, the seasonality of macroalgal growth may be considered a potential bias in the comparison of sites sampled at different times within the spring season. Particularly, *Cystoseira* spp. (the most sensitive species), show strong variability in their thalli length, that can potentially confound the attribution of the correct community category, affecting the CARLIT Index calculation. Additionally, a long term implementation of the index necessarily implies collection of data by different operators, that could represent an additional source of uncertainty, particularly in the attribution of intermediate categories.

The present research focuses on the assessment of the reliability of CARLIT Index results for the Ligurian coastline over a decade, teasing apart the effects of intra-seasonal variability within the period of implementation (spring) and sampling operator effect, in order to verify if the correct attribution of the ES class may be affected by such potential sources of variability.

2. Material and Methods

2.1 Study site

The Ligurian region is located in the NW of Italy (from 4° 58' W to 2° 22' W and from 43° 45' N to 44° 40' N) and encompasses more than 300 km of coast. The main superficial current along the Ligurian coast is formed by the convergence of the Tyrrhenian current and the West-Coast Corsica current, moving along the shore from East to West. Three of the largest Italian harbors are located in the region: Savona Vado (SV), Genova (GE) and La Spezia (SP), which carry out commercial, industrial and recreational activities. These harbors are located in the three Ligurian largest urbanized areas, particularly Genoa, with approximately 700.000 inhabitants. The Ligurian coast represents the ideal site for this validation study, because it has been the pilot area for the definition of the Italian CARLIT methodology and, being the first place where this index was applied, provides the longest temporal dataset.

2.2 Sampling strategies

In the present study, two sampling approaches were implemented in order to assess i) the ecological status of Ligurian region water bodies over a decade, considering operator-associated variability and ii) the potential intra-seasonal variability of the Index.

2.2.1 Ecological status over a decade

Since 2006, 24 water bodies (WB, as defined by the Ligurian Region Environmental Agency ARPAL; Fig. 1) are sampled along the Ligurian rocky coast.

To the aim of this study, samplings performed during spring season, applying the CARLIT methodology (Ballesteros et al., 2007), from 2006 to 2015 in seven WBs were considered: Capo Mortola (Mo), Laigueglia-Albenga (Ga), Genova-Camogli (Ge), Portofino (PF), Punta Mesco (PM), Cinque Terre (CT) and Portovenere (PV). These WBs have been selected in order to be representative of the entire Ligurian coastline. Each WB was divided into three areas: West, Central and East, and each area was composed by 20-30 sectors (50 meters each), depending on the length of the rocky shore in each WB.

The rocky coast was covered with a small boat and the dominant macroalgal community and geomorphological features of each sector were recorded on a cartographic support using the Quantum Geographical Information System (QGIS).

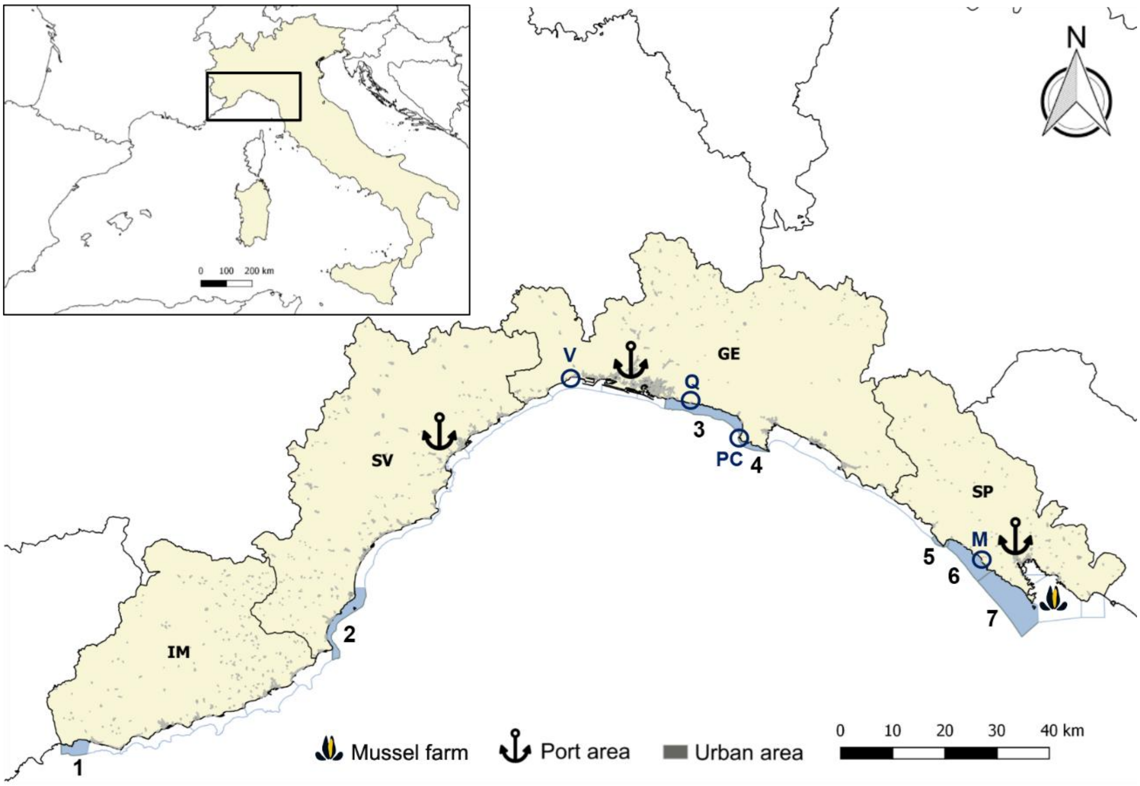


Fig 1. Geographical distribution of the different sites along the Ligurian coast (NW Med). The CARLIT Index was calculated in the WBs highlighted in pale blue: **1**- Capo Mortola (Mo); **2**- Laigueglia-Albenga (Ga); **3**- Genova-Camogli (Ge); **4**- Portofino (PF); **5**- Punta Mesco (PM); **6**- Cinque Terre (CT); **7**- Portovenere (PV); the intra-seasonal variability study has been performed in Sites marked with circles: **V**- Vesima; **Q**- Quarto dei Mille; **PC**- Punta Chiappa; **M**- Manarola. IM (Imperia), SV (Savona), GE (Genova) and SP (La Spezia) are the four Ligurian provinces. Main harbors and La Spezia mussel mariculture are reported with symbols.

2.2.2 Intra-seasonal variability

The intra-seasonal study was carried out in order to verify if the morphological features and percent cover of *Cystoseira* species, the most sensitive ones in the CARLIT assessment, affect the attribution of the correct community category, and thus the assessment of the correct ES, within the time-frame of index implementation. Samplings was performed during spring 2015 at four out of the seven sites: Vesima (V), Quarto dei Mille (Q), Punta Chiappa (PC) and Manarola (M).

Table 1. Summarized description and sensitivity of the main community categories as reported in the methodological contribution published by ISPRA (modified from Mangialajo et al. 2008b).

Category	**Description**	**SL**
Trottoir[a]	Large organogenic build-ups of *Lithophyllum byssoides*, *Lithophyllum trochanter*, *Dendropoma*[b]	**20**
Cystoseira brachycarpa / *crinita* / *elegans*	Community dominated by *Cystoseira brachycarpa* / *crinita* / *elegans*	**20**
Cystoseira sheltered	Community dominated by *Cystoseira foeniculacea* / *barbata* / *humilis* / *spinosa*	**20**
Cystoseira amentacea / *mediterranea* 5	Continuous belt of *Cystoseira amentacea* / *mediterranea*	**20**
Cystoseira amentacea / *mediterranea* 4	Almost continuous belt of *Cystoseira amentacea* / *mediterranea*	**19**
Cystoseira amentacea / *mediterranea* 3	Abundant patches of dense stands of *Cystoseira amentacea* / *mediterranea*	**15**
Cystoseira amentacea / *mediterranea* 2	Abundant scattered plants of *Cystoseira amentacea* / *mediterranea*	**12**
Cystoseira compressa	Community dominated by *Cystoseira compressa*	**12**
Cystoseira amentacea / *mediterranea* 1	Rare scattered plants of *Cystoseira amentacea* / *mediterranea*	**10**
Dictyotales / Stypocaulaceae	Community dominated by *Padina* / *Dictyota* / *Dictyopteris* / *Taonia* / *Stypocaulon*	**10**
Corallina	Community dominated by *Corallina* spp. (including *Ellisolandia elongata*)	**8**
Encrusting corallinales	Community dominated by *Lithophyllum incrustans*, *Neogoniolithon brassica-florida* and other encrusting corallines	**6**
Mussels	Community dominated by *Mytilus galloprovincialis*	**6**
Pterocladiella / Ulva / Schizymenia	Community dominated by *Pterocladiella / Ulva / Schizymenia*	**6**
Green algae	Community dominated by *Ulva* and / or *Cladophora*	**3**
Blue greens	Community dominated by *Cyanobacteria* and *Derbesia tenuissima*	**1**
Posidonia reef	Barrier and fringing reefs of *Posidonia oceanica*	**20**
Cymodocea nodosa	Superficial *Cymodocea nodosa* meadows	**20**
Zostera noltii	Superficial *Zostera noltii* meadows	**20**

[a] Except for the category Trottoir, which is generally found in the mediolittoral zone, all the other categories only have be taken into account when present in the infralittoral fringe zone.

[b] *Dendropoma* forms organogenic build-ups typical of Sicily and other South Italian regions.

[c] In the case of rare scattered plants of *Cystoseira amentacea* / *mediterranea*, the dominant community also has to be noted down. (Sensitivity level - SL: average value).

The sites were chosen for different reasons: 1- they display different ES scores, 2- are spread along the coast in order to encompass, as much as possible, the extension (and associated potential variability) of the Ligurian rocky coast, 3- are different in terms of urbanization impact (Vesima and Quarto dei Mille are differently affected by the Genoa urban center; Punta Chiappa and Manarola are located inside Marine Protected Areas).

Sampling was performed monthly from March to June, in order to encompass the period of maximum macroalgal development. At each site, three 50 meters long sectors (a stretch of coast of 150 m) were sampled according to the CARLIT methodology, identifying the appropriate community category in each one (Table 1; according to Mangialajo et al., 2008). According Ballesteros et al. (2007), the EQR for each site was obtained as the ratio between the value corresponding to the community category and the reference value characteristic for the corresponding geomorphological relevant situation (Table 2).

Table 2. Ecological quality values (EQi) for the six geomorphological relevant situations (GRS) in reference conditions.

GRS(i)	Coastal morphology	N/A	EQi
1	Decimetric blocks	Natural	12.2
2	Low coast	Natural	16.6
3	High coast	Natural	15.3
4	Decimetric blocks	Artificial	12.1
5	Low coast	Artificial	11.9
6	High coast	Artificial	8.0

2.3 Data analysis

2.3.1 CARLIT Index calculation

The CARLIT Index was calculated obtaining the ecological quality ratio (EQRs) values using the CARLIT package (Pecorino et al., 2015) with the free software R (R Development Core Team 2014, Version 3.1.0), which provides averaged values for each water body. The CARLIT package requires three datasets. The first one (containing actual data collected in the field) includes data columns in the following order: Site, Morphology (Decimetric blocks, Low coast, High coast), NatArt (natural or artificial substrate), Length (sector length) and Community (Categories of macroalgal communities). The second one is the Sensitive Level (SL) dataset, reporting the sensitivity of each macroalgal community category, as reported in the methodological contribution published by ISPRA (see Table 1). The third one is the reference dataset, reporting reference values for each geomorphological relevant situation (according Ballesteros et al., 2007). The benefit provided by the CARLIT package in R is that the two latter datasets may be easily adapted to the characteristics of each region/basin, according to the occurrence and the abundance of the commonest upper-infralittoral macroalgal communities or to regional/national management requirements.

The EQR values range from 0 to 1 and, according to the WFD, water bodies have been classified into five ecological status (ES) classes. The rating scale of EQR values was defined by Ballesteros et al. (2007): 0–0.25 (bad), >0.25–0.40 (poor), > 0.40–0.60 (moderate), >0.60–0.75 (good) and >0.75–1 (high).

2.3.2 Ecological status over a decade

Longitudinal data, such as repeated measurements across time (correlation structure), are better analyzed as a linear mixed effects model allowing to include random effects and use time as a continuous variable. For this reason, we choose the linear mixed model approach to study the variability in the EQRs calculated by the CARLIT Index due to different factors, as detailed below.

A dataset reporting EQR values obtained in each WB during four different years between 2006 and 2015 was created, also including information about the operator who performed the sampling (surveyor), who could be an important source of variability, particularly in the attribution of intermediate categories (*e.g. Cystoseira amentacea* 3 and 4, see Table 1). In the mixed model, EQR values are used as a response variable and Years, WB and Surveyor as predictors. WBs, by definition, should differ in terms of their ES, but they are included as factors in the model because of their relevance as variance components. A random slope and intercept model was fitted in R with lme4 package (Bates et al., 2005) by REML. The model allows to partition variability associated to the different factors, according to the following R code:

```
lmer (EQR ~ ( 1 | Surveyor ) + ( 1 | WB ) + ( 1 | Year ) + ( 0 + WB | Year ), data = Carlit, REML=T )
```

The total variance (σ^2_T) and variance components associated with each factor (σ^2_x) were estimated and then the percentage of variance explained by each factor (P_{samp}) was calculated, following Clarke et al. (2006):

$$P_{samp} = 100\sigma^2_x / \sigma^2_T$$

where,

$$\sigma^2_T = \sigma^2_{Year} + \sigma^2_{WB} + \sigma^2_{Surveyor} + \sigma^2_{WBx|Year} + \sigma^2_R$$

The model was validated by plotting the residuals against the fitted EQR values. No data transformation was required. The model was chosen after validation by AIC (Akaike Information Criteria).

In order to visualize interannual changes in the dominance of the different CARLIT communities within each water body, radar charts have been produced.

2.3.3 Intra-seasonal variability

In order to evaluate variability within the spring season, EQR values from March to June 2015 were calculated in the four selected sites and, consequently, the corresponding ES values were obtained and plotted in a barplot.

3. Results

3.1 Ecological status along ten years

The ES of the Ligurian rocky shores shows overall quite positive results (Fig.2): 6 out of the 7 WBs are classified as "good" or "high" in 2015. In particular, over the investigated time-frame, a shift from "moderate" to "good" and from "good" to "high" has been observed for two water bodies (respectively Cinque Terre, CT, and Genova-Camogli, Ge).

The two lowest ecological status classes, "bad" and "poor", have never been recorded along the Ligurian coast in the framework of the present study. The highest EQR scores were recorded in Portofino (PF), Capo Mortola (Mo) and Laigueglia-Albenga (Ga), slightly changing along the considered years, but remaining within the "high" ecological status class. On the contrary, Portovenere (PV) presented the lowest EQR scores and the ecological status was always maintained as "moderate" over the considered time-frame. In Punta Mesco (PM), the EQR scores fell in the "good" ecological status class, with the exception of 2009, when the ecological status decreased from "good" to "moderate". Instead, in Cinque Terre (CT) the ecological status improved from "moderate" (2006-2009) to "good" (2012-2015). The most relevant change occurred in Genova-Camogli (Ge), where in 2006 a "good" EQR score was obtained, although close to the upper-limit of "moderate": in the following years, since 2009, a constant increase in EQR scores was observed, showing values within the "high" class.

Linear mixed effects model results (Table 3) show that the highest percentage of variance (47%) was explained by WB, as expected (see Materials and methods section, paragraph 2.3.2). Factors Year and Surveyor, instead, did not show any associated variability (0%). Among WBs, Genova-Camogli explained the highest percentage of the inter-annual variability of the EQR values (20%). The low residual variability (7%) provides evidences that almost all variance is explained by the model.

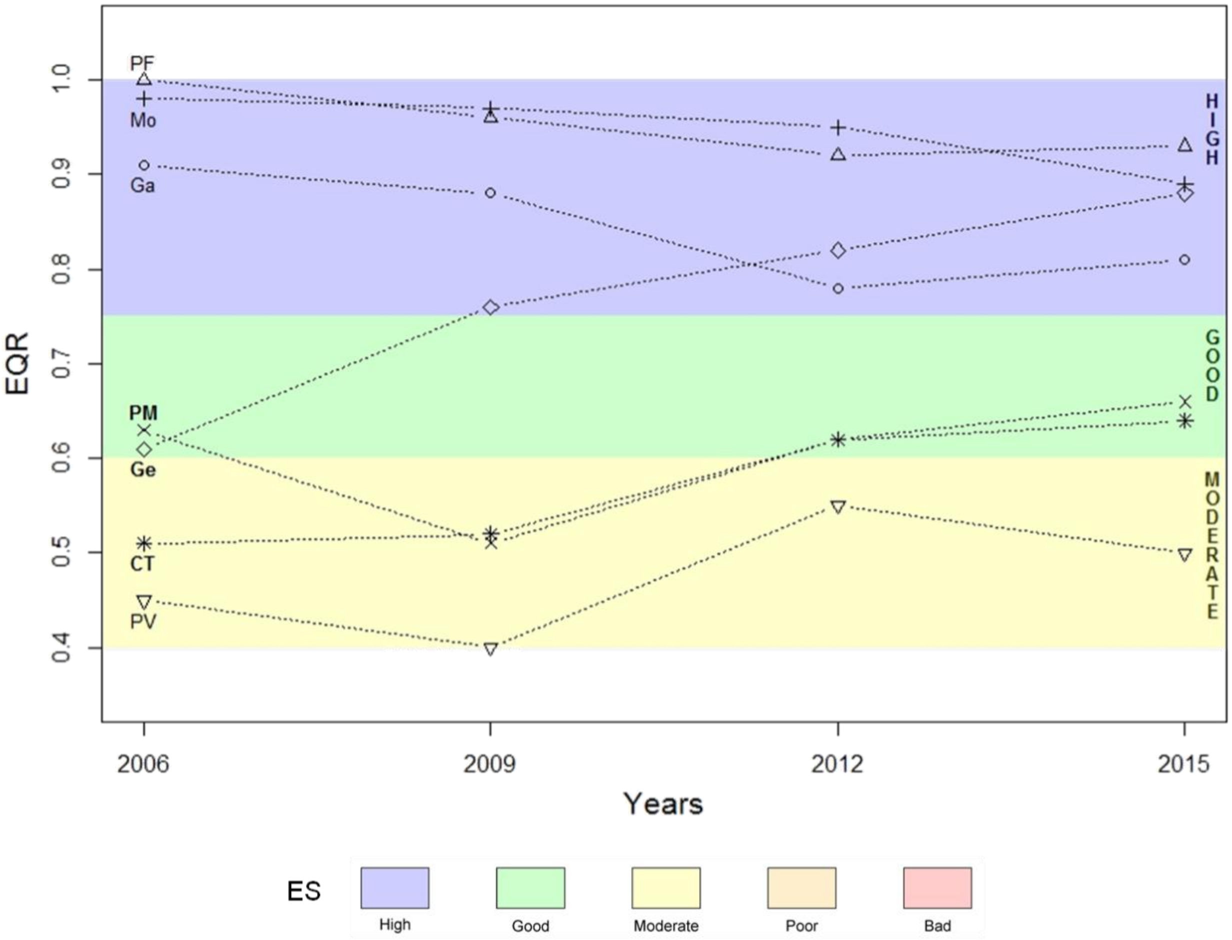


Fig 2. Ecological status (ES) in different years over 10 years in seven water bodies of the Ligurian coast: Capo Mortola (Mo); Laigueglia-Albenga (Ga); Genova-Camogli (Ge); Portofino (PF); Punta Mesco (PM); Cinque Terre (CT); Portovenere (PV).

Table 3. Results of linear mixed effects model fit by restricted maximum likelihood (REML). Untransformed EQR scores analyzed as a function of four random effects. WB- Water Body; P_{samp}- the proportion of total variance explained by each factor.

Groups	Levels	Type	Std.dev.	Variance	P_{samp} (%)
WB	7	Crossed	0.17800	0.031683	**47**
Year	4	Crossed	0.00000	0.000000	0
Surveyor	2	Crossed	0.00000	0.000000	0
Year:					
Capo Mortola			0.04112	0.001691	2
Laigueglia-Albenga			0.05730	0.003284	5
Genova-Camogli			0.11552	0.013276	**20**
Portofino			0.03608	0.001302	2
Punta Mesco			0.05869	0.003444	5
Cinque Terre			0.06932	0.004810	7
Portovenere			0.05869	0.003429	5
Residual			0.06637	0.004404	7

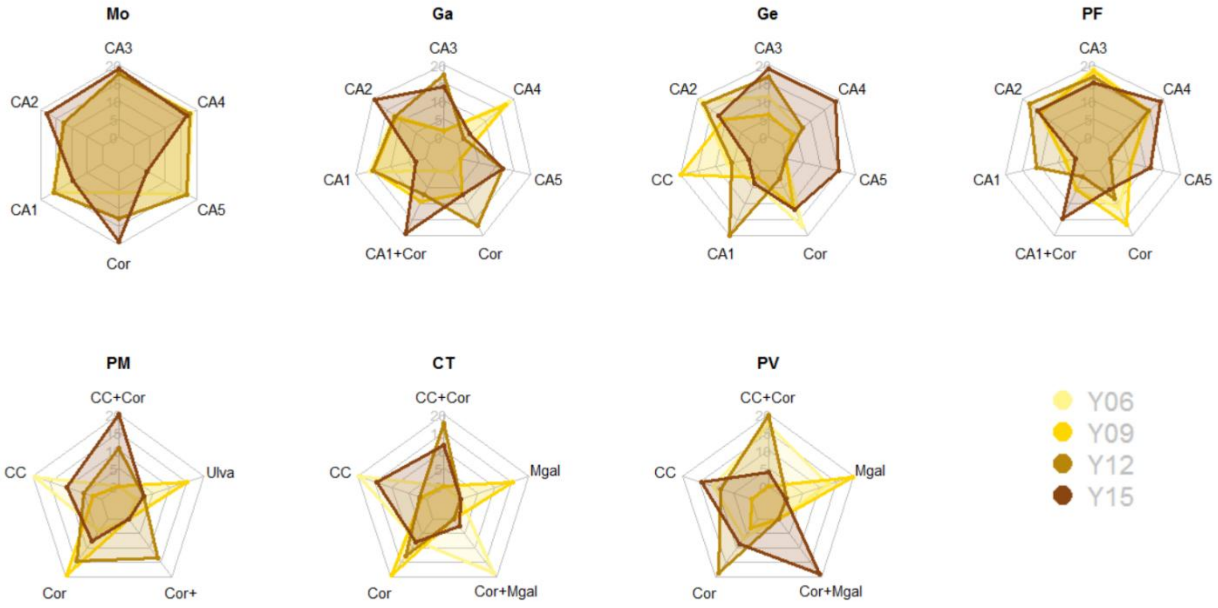


Fig 3. Radar charts reporting interannual changes in dominance of the different CARLIT community categories (CA1-2-3-4-5: *Cystoseira amentacea* 1-2-3-4-5; CC: *Cystoseira compressa*; Cor: *Corallina* spp.; Mgal: *Mytilus galloprovincialis*; Ulva: *Ulva* spp.) in 2006 (Y06), 2009 (Y09), 2012 (Y12), 2015 (Y15) within each water body (Mo: Capo Mortola; Ga: Laigueglia-Albenga; Ge: Genova-Camogli; PF: Portofino; PM: Punta Mesco; CT: Cinque Terre; PV: Portovenere).

Interannual changes in dominance of the different CARLIT community categories (defined in Table 1) are reported in Fig 3. In Capo Mortola the coast was commonly dominated by *Cystoseira amentacea*, homogenously distributed in the different categories, from scattered plants to continuous belts (CA1- CA5). In 2015 a decrease of *C. amentacea* dense stands (CA3), non continuous (CA4) or continuous belts (CA5) was observed, in favor of *Corallina* spp. (Cor). In Gallinara and Portofino a similar community dominance was observed, but higher percentages of stretches of coast covered by continuous belt of *C. amentacea* (CA5) were observed in 2015 (and also 2012 for Gallinara), with a concurrent increase in dominance of *Corallina* spp., associated to scattered *C. amentacea* thalli (CA1+Cor). In the Genova-Camogli water body, in 2006 and 2009 the community was generally dominated by *Corallina* spp., *Cystoseira compressa* (CC) and scattered *C. amentacea* thalli (CA1-2) and progressively shifted towards stretches of coast dominated by *C. amentacea* dense stands or belts (CA3-4-5) in 2012 and 2015, mirrored by a substantial increase in the CARLIT Index (Fig 2). In the easternmost side of the Ligurian coast, the only *Cystoseira* species present is *C. compressa*, dominant along most of the coast in Punta Mesco and Cinque Terre in 2006. This community (CC) shifted to a mixed one with *Corallina* spp. in the last years (CC+Cor). A concurrent decrease of *Ulva* sp. dominance in Punta Mesco and of *M. galloprovincialis* (Mgal)in Cinque Terre was observed. In Portovenere a constant massive presence of *M. galloprovincialis* was observed, with a relevant decrease in 2012 and a shift to a co-dominated community with *Corallina* spp. (Cor+Mgal) in 2015.

3.2 Intra-seasonal variability

The EQR scores obtained in each site from March to June, shown in the bar plots (Fig 4), fell in the same ecological status class in the different months within each site, with the exception of Quarto dei Mille. In this site, the values showed a different ecological status in March (“good”) with respect to April, May and June (“high”).

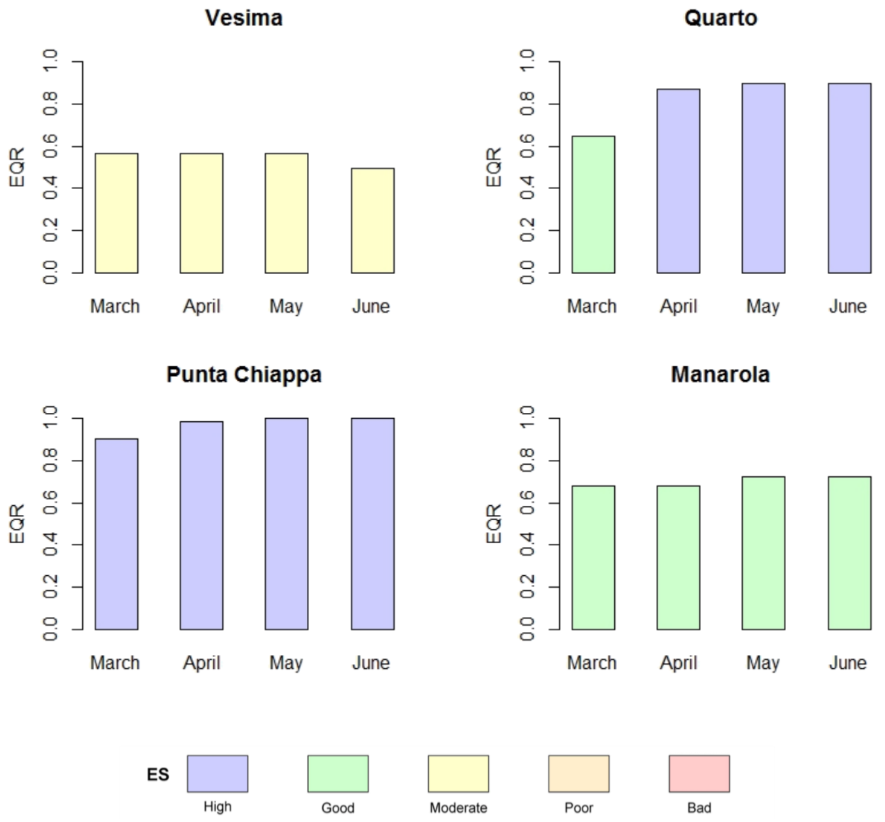


Fig 4. Ecological status of the four selected sites along the Ligurian coast, for each month during the spring season 2015, for the intra-seasonal variability assessment.

4. Discussion

As required by the Water Framework Directive (WFD, 2000/60/EC), all the Ligurian water bodies (with only one exception) reached the “good” ES by 2015. In addition to the achievement of the WFD’s goal, an encouraging general improvement of the ES along the Ligurian coastline has been observed in the considered decade (Fig. 2 and Fig. 3).

A general improvement of the ecological quality, based on CARLIT index, can be also observed at a larger scale in the North-Western Mediterranean basin according to a recent study by Blanfuné et al. (2017), who additionally underline the importance of considering a combination of different indices, as required by the WFD, in order to account for the ecological quality of a water body as a whole, the status of the ecosystems and natural and human-induced pressures.

The general increase in ES observed in time in the study area is actually mirrored by an improvement of coastal water quality, as proved also by other indices based on different key biological elements (*e.g.* AMBI Index on macroinvertebrates, Fig. 1S Supplementary Material) and bathing water quality (*i.e.* quantification of *Enterococcus* spp. and *Escherichia coli*, Fig. 2S and 3S Supplementary Material).

During the first CARLIT assessment performed along the Ligurian coast, scores below the “good-moderate” threshold were recorded in the Genoa urban area and in the sites at the easternmost side of the region (Mangialajo et al. 2007; Asnaghi et al. 2009), probably because of the low abundances or lack of the most sensitive macroalga considered by the Index, *Cystoseira amentacea* (Table 1).

In the present study we highlighted a particularly remarkable trend in Genova-Camogli WB (Ge), where the ES recorded in 2006 was close to the lowermost limit of the “good” class (EQR=0.61), while in 2015 it was classified in the uppermost boundary of the “high” class (EQR=0.88). Since no relevant artificial modification in coastal morphology (*e.g.* ports, breakwater, etc) and human pressure occurred in the area (both reported by Torras et al., 2015 as possible sources of change in ES), the improvement in ES should be ascribed to an actual amelioration in water quality, mirrored by an increase of the abundance of macroalgae sensitive species. At the beginning of the study the communities were generally dominated by intermediate sensitive species (*i.e. Corallina* spp., *Cystoseira compressa*) and scattered *C. amentacea* thalli, progressively shifting during the considered decade towards dense stands or belts of *C. amentacea* (Fig. 3). This amelioration could be related to the water treatment enhancement occurred in the last years (*i.e.* intervention on drainage pipe following two relevant flood events occurred in the area in 2011 and 2014 and improvement of sewage treatment plant in the last years performed by the competent company IRETI S.p.A.).

C. amentacea is still completely missing in the easternmost side of the Ligurian coastline starting from Punta Manara, around 20 km North-West from Cinque Terre (authors personal observation). This species has been recorded until the end of the 19th century (authors personal observation through herbaria records, Fig. 4S Supplementary Material) and its disappearance in the area can be explained by the habitat fragmentation due to coastal developments and the high sediment loads caused by intense excavating activities for extracting construction material (mostly for building railway and highway in the area) that occurred in the first half of the 20th century. Even if such disturbing activities have largely been reduced in the last decades, with significant changes in the riverine basin and sediment load to the sea, and a Marine Protected Area has been established, this sensitive species is still completely absent along these stretches of coast, probably as a consequence of its low dispersal capacity (< 1m, Johnson and Brawley, 1998; Gaylord et al., 2002; Susini, 2006; Mangialajo et al., 2012) that hampers the natural recovery of this species, additionally affected by other local factors such as mussel farming (competition for space with the settled mussel juveniles).

Despite *C. amentacea* absence, ES values belonging to the "good" class have been recorded also in this area, because of the increased percentage in the last years of stretches of coast dominated by species with intermediate sensitivity levels (*i.e. C. compressa*, and *Corallina* spp.), alongside with a decreasing trend of low value species (*Ulva* spp. in Punta Mesco and *Mytilus galloprovincialis* in Cinque Terre), with the exception of Portovenere which still remains in the "moderate" ES class, not fulfilling WFD's requirements. Low ES values may be ascribed to the proximity to a mussel mariculture and present activities carried out in the Port of La Spezia, such as shipbuilding, shipping, yachting and tourism, whose wastes are accumulated and driven along shore by the superficial current of the Gulf of La Spezia and the main current of the Ligurian Sea (Gasparini et al., 2009). A similar effect of the port area and current regime on ES values is observed on the Wester side of Genoa Port (Vesima, "moderate" class Fig. 4).

Though the present study, the reliability of the observed general improvement of the ES over a decade along Ligurian coasts has been addressed, teasing apart potential sources of variability associated to CARLIT index implementation.

Several factors are considered as sources of error in the different quality indices, which use marine macrophytes as Biological Quality Elements (Asnaghi et al., 2009; Bennet et al., 2011; Mascaró et al., 2013; Torras et al., 2015; Cavallo et al., 2016; Blanfuné et al., 2017): spatial variability (*e.g.* sites, depth), temporal variability, inter-annual variability and human associated error.

The spatial variability, due to high horizontal and depth-related heterogeneity, may be erroneously incremented by incomplete or confused sampling designs and, for this reason, in several cases, such variability has been identified as one of the most important causes of misclassification of ES. This possible confounding element leads to the strong recommendation to carry out monitoring at large spatial scales (Cavallo et al., 2016). Specifically, the CARLIT Index in the Ligurian Sea has been implemented following a hierarchical design, encompassing three different large (more than 1000 m) and representative stretches of coast in each water body, as performed in the ARPAL implementation of the index, following ISPRA methodological report indications (Mangialajo et al., 2008). This assures that the among water bodies variability observed and reported in the present study is reliable, unaffected by spatial variability and strictly related to actual ES differences.

The temporal variability, in terms of natural inter-annual variability, has been addressed in several previous studies using different indices, which reported that it may be considered very low, ranging between 0% and 15% (Mascaró et al., 2013). Specifically, for the CARLIT Index, two studies already supported low inter-annual variability. Asnaghi et al. (2009) did not find inter-annual differences comparing ES measured along the Ligurian rocky coast in two consecutive years, and Blanfuné et al. (2017) obtained similar results along French coasts, performing an assessment on two different periods (encompassing three years each) on a longer time scale. The outcomes of these studies support the effectiveness of performing CARLIT monitoring at a lower temporal resolution, *e.g.* once every three years for the whole water body (as implemented by the Ligurian Environmental Agency). On a longer time-frame, data collected every three years, instead, show trends that may be related to actual change in ES of individual water bodies (Fig. 2 and 3).

In the present study, temporal variability has been addressed also in terms of intra-seasonal variability. Although the recommended period to perform the CARLIT Index assessment in the NW Mediterranean is spring (April – June; Ballesteros et al., 2007), peculiar seasonal environmental conditions that trigger macroalgal development, such as light intensity and temperature, change along the season and trend may be inter-annually different, affecting the growth of macroalgal species, potentially affecting the ES attribution. Additionally, unfavourable meteo-marine conditions may force to delay samplings along the season, although within the defined spring time-frame. Over such time-frames, the abundances and the morphology of the macroalgal species of the rocky shores, particularly for the canopy forming *Cystoseira* spp., could vary largely, possibly causing a misclassification of the dominant macroalgal assemblages, affecting the EQR calculation.

In the present study, no variability in the classification into ES classes during the whole April-June time-frame emerged for all the investigated water bodies, supporting the appropriateness of the whole recommended time-frame for the implementation of the CARLIT Index (Fig. 4), since intra-seasonal variability does not hamper the attribution of a consistent community category.

In a long term comparison perspective, an additional factor to be taken into account is the possible bias related to the operator (surveyor) subjectivity in assessing community category. Such effect was tested in the present study, for the first time for the CARLIT Index, showing that operator-associated variability did not affect the uncertainty of the EQR calculation and consequently the classification of water bodies into the different ecological status classes along years. The same result has been obtained also for other macrophyte-based quality indices (Bennet et al., 2011; Mascaró et al., 2013).

Given all the above, the variations observed in the study area in time, showing a general increase in ES, may be considered as robust trends of the coastal water quality improvement of the Ligurian region.

5. Conclusions

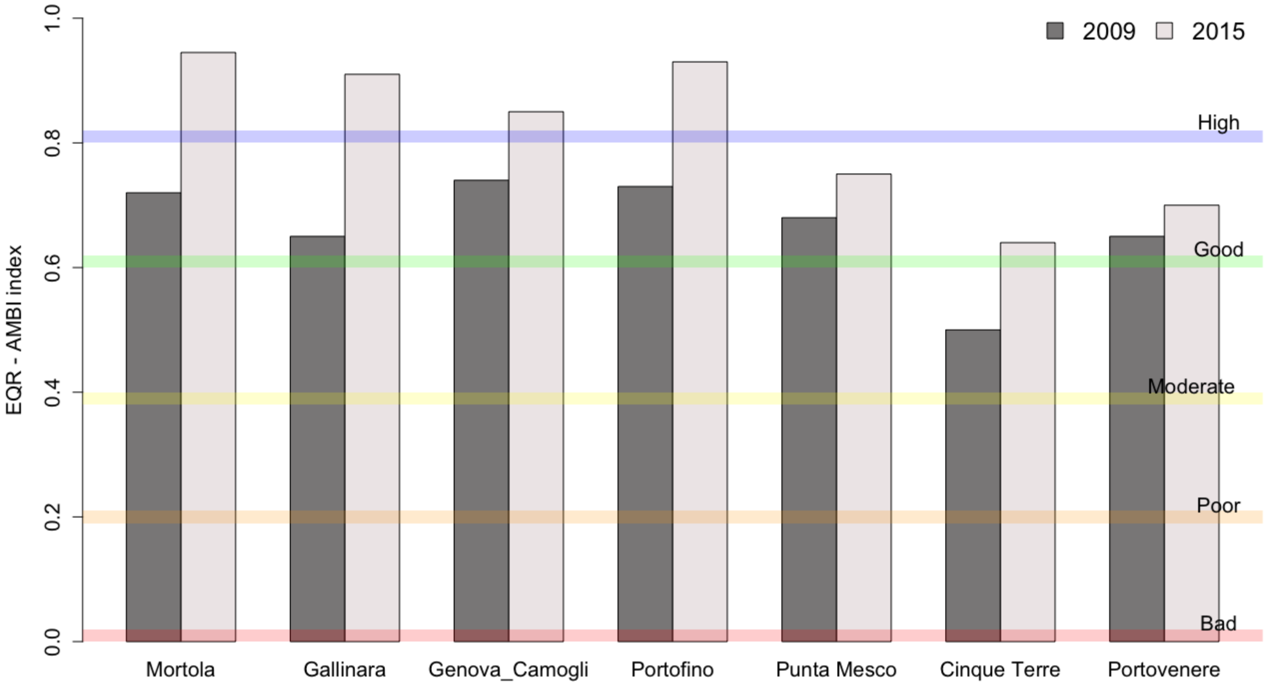
The reliability of quality indices is essential to comply with the requirements of marine environment directives, in order to correctly classify water bodies ES with an appropriate level of confidence. Hence, teasing apart any natural annual variability, spatial variability or human related one due to the operator is fundamental in order to establish a good long-term monitoring programme to be carried out by environmental regional agencies.

The present study allowed to elucidate different aspects linked to the variability of the CARLIT Index, in order to validate procedures carried out by monitoring agencies, and provided an overview of the ecological status of the Ligurian coasts. Our main findings are: i) an increase of *Cystoseira* spp. detected along the Ligurian coast in the last decade, in particular along Genova-Camogli stretch of coast, probably linked to a higher attention in waste-water treatment; ii) an improvement of the ES of almost all of the WB studied (six out of seven) in the Ligurian coast; iii) only one WB, Portovenere (PV), did not yet fulfill the WFD requirements for 2015, being classified as “moderate”, most likely because of present and past heavy anthropogenic impacts and limited dispersal of the most sensitive species, preventing self-recruitment; iv) the lack of intra-seasonal and operator effects in the estimation of the ES, supporting the reliability of the CARLIT Index implementation carried out by the Ligurian Environmental Agency.

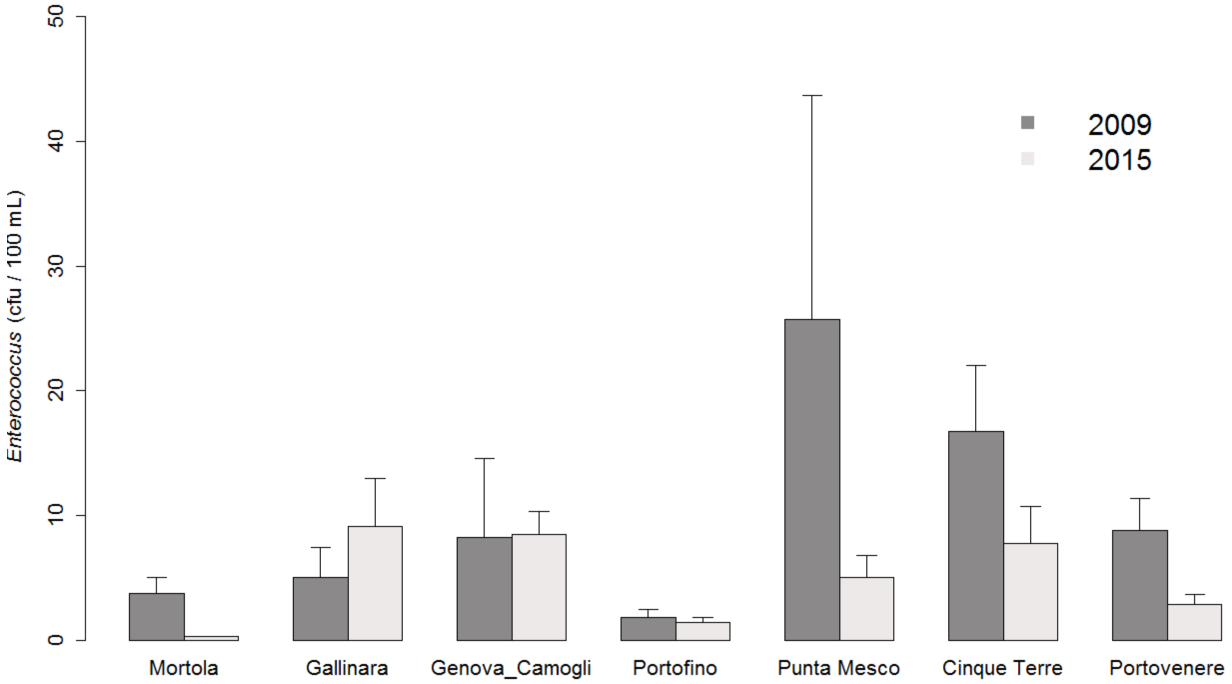
These results support the reliability of monitoring procedures carried out though the CARLIT Index and highlight the need and the effectiveness of reduction measures for anthropogenic impacts in order to achieve the ES required by European directives. The understanding of mechanisms that affect *Cystoseira* spp. distribution is a relevant issue in the light of monitoring and assessing potential changes in the Ecological Status assessment of Mediterranean rocky shores. Moreover, the implementation of CARLIT will provide a benchmark for *Cystoseira* species distribution and abundance. Its application along years will build a long-term dataset collected using a standardized methodology, which will be useful to assess the progression or regression of *Cystoseira* species with the aim of conservation (Nikolić et al., 2013), management and eventually restoration.

Supporting information

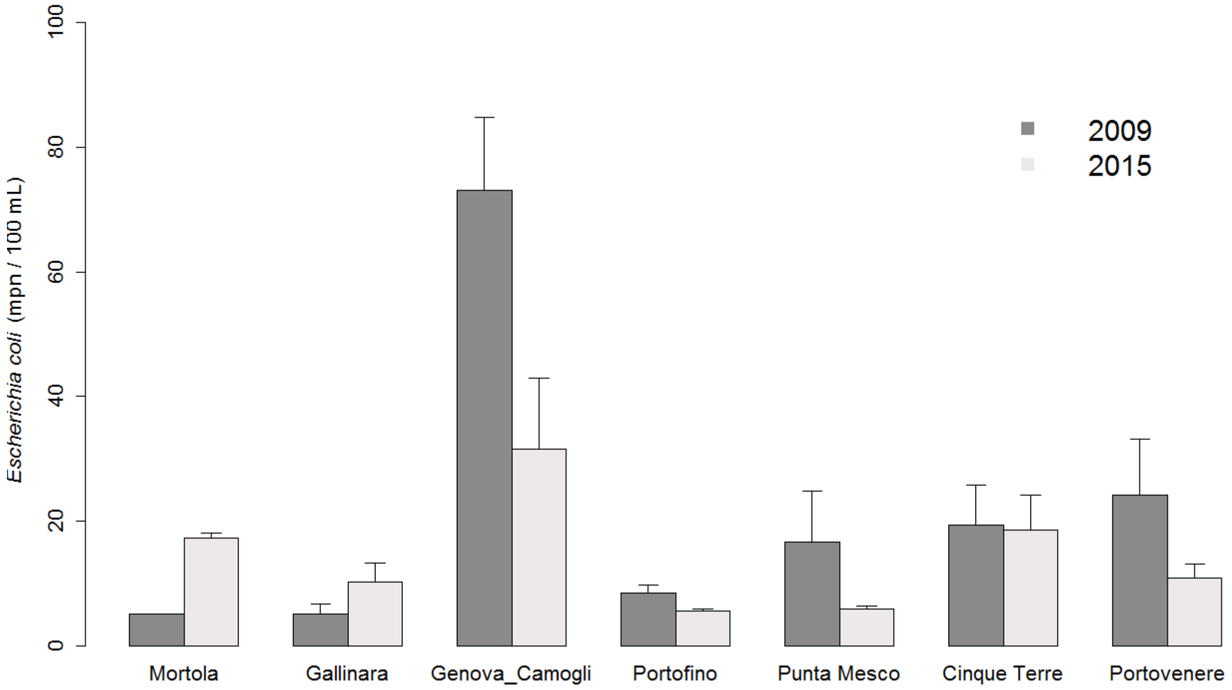
S1 Figure. AMBI index values for the investigated Sites in 2009 and 2015 (data available on ARPAL website: www.ambienteinliguria.it).



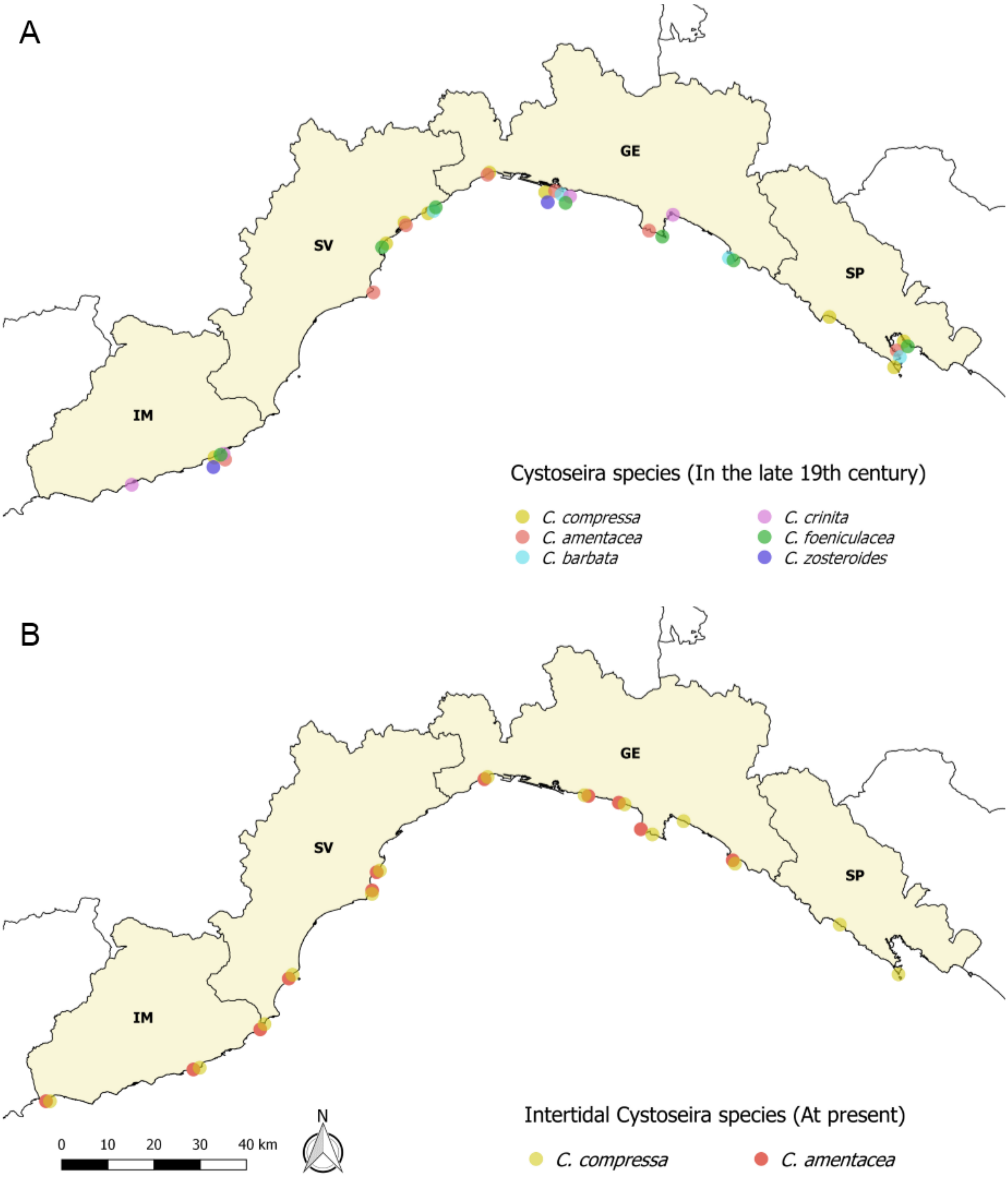
S2 Figure. Abundances of *Enterococcus* spp. (mean value + standard error) for the investigated Sites in 2009 and 2015 (data available on ARPAL website: [www.ambienteinliguria.it)](http://www.ambienteinliguria.it).



S3 Figure. Abundances of *Escherichia coli* (mean value + standard error) for the investigated Sites in 2009 and 2015 (data available on ARPAL website: [www.ambienteinliguria.it)](http://www.ambienteinliguria.it).



S4 Figure. Occurrence of *Cystoseira amentacea* along the Ligurian coast from herbaria records at the end of 19th century (A) and nowadays (B).



References

Airoldi, L., & Bulleri, F. (2011). Anthropogenic disturbance can determine the magnitude of opportunistic species responses on marine urban infrastructures.
PLoS One, 6(8), e22985, http://dx.doi.org/10.1371/journal.pone.0022985.

Arévalo, R., Pinedo, S., & Ballesteros, E. (2007). Changes in the composition and structure of Mediterranean rocky-shore communities following a gradient of nutrient enrichment: descriptive study and test of proposed methods to assess water quality regarding macroalgae. *Marine Pollution Bulletin*, *55*(1), 104-113, http://dx.doi.org/10.1016/j.marpolbul.2006.08.023.

Asnaghi, V., Chiantore, M., Bertolotto, R. M., Parravicini, V., Cattaneo-Vietti, R., Gaino, F., Moretto, P., Privitera, D. and Mangialajo, L. (2009), Implementation of the European Water Framework Directive: Natural variability associated with the CARLIT method on the rocky shores of the Ligurian Sea (Italy). *Marine Ecology*, http://dx.doi.org/10.1111/j.1439-0485.2009.00346.x.

Akcali, I., & Kucuksezgin, F. (2011). A biomonitoring study: heavy metals in macroalgae from eastern Aegean coastal areas. *Marine pollution bulletin*, 62(3), 637-645, http://dx.doi.org/10.1016/j.marpolbul.2010.12.021.

Ballesteros, E., Torras, X., Pinedo, S., García, M., Mangialajo, L., & De Torres, M. (2007). A new methodology based on littoral community cartography dominated by macroalgae for the implementation of the European Water Framework Directive. *Marine pollution bulletin*, 55(1), 172-180, http://dx.doi.org/10.1016/j.marpolbul.2006.08.038.

Bates, D. (2005). Fitting linear mixed models in R. *R news*, *5*(1), 27-30.

Bennett, S., Roca, G., Romero, J., & Alcoverro, T. (2011). Ecological status of seagrass ecosystems: an uncertainty analysis of the meadow classification based on the Posidonia oceanica multivariate index (POMI). *Marine pollution bulletin*, *62*(8), 1616-1621, http://dx.doi.org/10.1016/j.marpolbul.2011.06.016.

Bermejo, R., de la Fuente, G., Vergara, J. J., & Hernández, I. (2013). Application of the CARLIT index along a biogeographical gradient in the Alboran Sea (European Coast). *Marine pollution bulletin*, *72*(1), 107-118, http://dx.doi.org/10.1016/j.marpolbul.2013.04.011.

Blanfuné, A., Markovic, L., & Thibaut, T. (2011). Assessment of the CARLIT methodology in the Mediterranean rocky water bodies. *Journal of Phycology*, *46*, 173-173.

Blanfuné, A., Boudouresque, C. F., Verlaque, M., Beqiraj, S., Kashta, L., Nasto, I., Ruci, S., & Thibaut, T. (2016). Response of rocky shore communities to anthropogenic pressures in Albania (Mediterranean Sea): Ecological status assessment through the CARLIT method. *Marine pollution bulletin*, *109*(1), 409-418, https://doi.org/10.1016/j.marpolbul.2016.05.041.

Blanfuné, A., Thibaut, T., Boudouresque, C. F., Mačić, V., Markovic, L., Palomba, L., Verlaque, M., & Boissery, P. (2017). The CARLIT method for the assessment of the ecological quality of European Mediterranean waters: Relevance, robustness and possible improvements. *Ecological indicators*, *72*, 249-259, http://dx.doi.org/10.1016/j.ecolind.2016.07.049.

Buia, M. C., Silvestre, F., & Flagella, S. (2007, March). The application of the "CARLIT method" to assess the ecological status of the coastal waters in the gulf of Naples. In *Proceedings of the Third Mediterranean Symposium on Marine Vegetation. CAR/ASP Publ., Tunis* (pp. 253-254).

Bulleri, F., Benedetti-Cecchi, L., Acunto, S., Cinelli, F., & Hawkins, S. J. (2002). The influence of canopy algae on vertical patterns of distribution of low-shore assemblages on rocky coasts in the northwest Mediterranean. *Journal of experimental marine biology and ecology*, *267*(1), 89-106, http://dx.doi.org/10.1016/S0022-0981(01)00361-6.

Cavallo, M., Torras, X., Mascaró, O., & Ballesteros, E. (2016). Effect of temporal and spatial variability on the classification of the Ecological Quality Status using the CARLIT Index. *Marine pollution bulletin*, *102*(1), 122-127, http://dx.doi.org/10.1016/j.marpolbul.2015.11.047.

Chakraborty, S., Bhattacharya, T., Singh, G., & Maity, J. P. (2014). Benthic macroalgae as biological indicators of heavy metal pollution in the marine environments: A biomonitoring approach for pollution assessment. *Ecotoxicology and environmental safety*, 100, 61-68, http://dx.doi.org/10.1016/j.ecoenv.2013.12.003.

Cheminée, A., Sala, E., Pastor, J., Bodilis, P., Thiriet, P., Mangialajo, L., Cottalorda, J.M., & Francour, P. (2013). Nursery value of Cystoseira forests for Mediterranean rocky reef fishes. *Journal of experimental marine biology and ecology*, *442*, 70-79, http://dx.doi.org/10.1016/j.jembe.2013.02.003.

Clarke, R. T., Davy-Bowker, J., Sandin, L., Friberg, N., Johnson, R. K., & Bis, B. (2006). Estimates and comparisons of the effects of sampling variation using 'national'macroinvertebrate sampling protocols on the precision of metrics used to assess ecological status. In *The Ecological Status of European Rivers: Evaluation and Intercalibration of Assessment Methods* (pp. 477-503). Springer Netherlands, http://dx.doi.org/10.1007/s10750-006-0076-5.

Díez, I., Secilla, A., Santolaria, A., & Gorostiaga, J. M. (1999). Phytobenthic intertidal community structure along an environmental pollution gradient. *Marine Pollution Bulletin*, 38(6), 463-472, http://dx.doi.org/10.1016/S0025-326X(98)90161-8.

Díez, I., Santolaria, A., Secilla, A., & Gorostiaga, J. M. (2009). Recovery stages over long-term monitoring of the intertidal vegetation in the 'Abra de Bilbao' area and on the adjacent coast (N. Spain). *European Journal of Phycology*, *44*(1), 1-14, http://dx.doi.org/10.1080/09670260802158642.

Ferrigno, F., Boi, S., Cinti, M., Paliaga, B., & Guala, I. (2014). Spatial and temporal distribution of shallow algal communities in the marine protected area of Capo Carbonara (Sardinia). *Biol. Mar. Mediterr*, *21*(1), 257-258.

Gasparini, G. P., Abbate, M., Bordone, A., Cerrati, G., Galli, C., Lazzoni, E., & Negri, A. (2009). Circulation and biomass distribution during warm season in the Gulf of La Spezia (north-western Mediterranean). *Journal of Marine Systems*, *78*, S48-S62, http://dx.doi.org/10.1016/j.jmarsys.2009.01.010.

Gaylord, B., Reed, D. C., Raimondi, P. T., Washburn, L., & McLean, S. R. (2002). A PHYSICALLY BASED MODEL OF MACROALGAL SPORE DISPERSAL IN THE WAVE AND CURRENT-DOMINATED NEARSHORE.
Ecology, *83*(5), 1239-1251, http://dx.doi.org/10.1890/0012-9658(2002)083[1239:APBMOM]2.0.CO;2.

Johnson, L. E., & Brawley, S. H. (1998). Dispersal and recruitment of a canopy-forming intertidal alga: the relative roles of propagule availability and post-settlement processes. *Oecologia*, *117*(4), 517-526, http://dx.doi.org/10.1007/s004420050688.

Juanes, J. A., Guinda, X., Puente, A., & Revilla, J. A. (2008). Macroalgae, a suitable indicator of the ecological status of coastal rocky communities in the NE Atlantic. *Ecological indicators*, *8*(4), 351-359, http://dx.doi.org/10.1016/j.ecolind.2007.04.005.

Krause-Jensen, D., Carstensen, J., & Dahl, K. (2007). Total and opportunistic algal cover in relation to environmental variables. *Marine Pollution Bulletin*, 55(1), 114-125, http://dx.doi.org/10.1016/j.marpolbul.2006.08.019.

Lasinio, G. J., Tullio, M. A., Ventura, D., Ardizzone, G., & Abdelahad, N. (2017). Statistical analysis of the distribution of infralittoral Cystoseira populations on pristine coasts of four Tyrrhenian islands: Proposed adjustment to the CARLIT index. *Ecological Indicators*, *73*, 293-301, https://doi.org/10.1016/j.ecolind.2016.09.038.

Lin, D. T., & Fong, P. (2008). Macroalgal bioindicators (growth, tissue N, δ 15 N) detect nutrient enrichment from shrimp farm effluent entering Opunohu Bay, Moorea, French Polynesia. *Marine Pollution Bulletin*, 56(2), 245-249, http://dx.doi.org/10.1016/j.marpolbul.2007.09.031.

Littler, M. M., & Murray, S. N. (1975). Impact of sewage on the distribution, abundance and community structure of rocky intertidal macro-organisms. *Marine Biology*, *30*(4), 277-291, http://doi.org/10.1007/BF00390633.

Mangialajo, L., Ruggieri, N., Asnaghi, V., Chiantore, M., Povero, P., & Cattaneo-Vietti, R. (2007). Ecological status in the Ligurian Sea: the effect of coastline urbanisation and the importance of proper reference sites. *Marine Pollution Bulletin*, *55*(1), 30-41, http://dx.doi.org/10.1016/j.marpolbul.2006.08.022.

Mangialajo, L., Sartoni, G., Giovanardi, F. (2008a). *Quaderno Metodologico sull'elemento biologico MACROALGHE e sul calcolo dello stato ecologico secondo la metodologia CARLIT*. ISPRA – Istituto Superiore per la Protezione e la Ricerca Ambientale, Rome: 105 pp.

Mangialajo, L., Chiantore, M., Susini, M. L., Meinesz, A., Cattaneo-Vietti, R., & Thibaut, T. (2012). Zonation patterns and interspecific relationships of fucoids in microtidal environments. *Journal of experimental marine biology and ecology*, *412*, 72-80, http://dx.doi.org/10.1016/j.jembe.2011.10.031.

Mascaró, O., Alcoverro, T., Dencheva, K., Díez, I., Gorostiaga, J. M., Krause-Jensen, D., Balsby, T.J.S., Marbà, N., Muxika, I., Neto, J.M., Nikolić, V., Orfanidis, S., Pedersen, A., Pérez, M., Romero, J. (2013). Exploring the robustness of macrophyte-based classification methods to assess the ecological status of coastal and transitional ecosystems under the Water Framework Directive. *Hydrobiologia*, *704*(1), 279-291, http://dx.doi.org/10.1007/s10750-012-1426-0.

Nikolić, V., Žuljević, A., Mangialajo, L., Antolić, B., Kušpilić, G., & Ballesteros, E. (2013). Cartography of littoral rocky-shore communities (CARLIT) as a tool for ecological quality assessment of coastal waters in the Eastern Adriatic Sea. *Ecological indicators*, *34*, 87-93, http://dx.doi.org/10.1016/j.ecolind.2013.04.021.

Omrane, A., Guellouiz, S., Zarrouk, A., & Romdhane, M. S. (2010, December). Assessment of the ecological status of the Galite island coastal waters(northern of Tunisia) using the" CARLIT" method. In *Proceedings of the 4th Mediterranean Symposium on Marine Vegetation (Yasmine-Hammamet, 2–4 December 2010)* (pp. 204-206). RAC/SPA publ., Tunis.

Orfanidis, S., Panayotidis, P., & Stamatis, N. (2001). Ecological evaluation of transitional and coastal waters: a marine benthic macrophytes-based model. *Mediterranean Marine Science*, *2*(2), 45-66, http://dx.doi.org/10.12681/mms.266.

Panayotidis, P., Feretopoulou, J., & Montesanto, B. (1999). Benthic vegetation as an ecological quality descriptor in an Eastern Mediterranean coastal area (Kalloni Bay, Aegean Sea, Greece). *Estuarine, Coastal and Shelf Science*, 48(2), 205-214, http://dx.doi.org/10.1006/ecss.1998.0411.

Pecorino D, De la Fuente G, Torras X. (2015) CARLIT: Ecological Quality Ratios Calculation and Plot. R package version 1.0. http://CRAN.R-project.org/package=CARLIT.

Pinedo, S., García, M., Satta, M. P., De Torres, M., & Ballesteros, E. (2007). Rocky-shore communities as indicators of water quality: a case study in the Northwestern Mediterranean. *Marine Pollution Bulletin*, 55(1), 126-135, http://dx.doi.org/10.1016/j.marpolbul.2006.08.044.

Rende, F., Bacci, T., Gennaro, P., Penna, M., Trabucco, B., Austoni, M., Giovanardi, F., & Cicero, A. M. (2011, October) Study of Macrophytes in WFD 2000/60/EC: the response of the adopted indices to anthropogenic pressure along the Italian coast. In *XXI Congresso della Società Italiana di Ecologia*. (Palermo, 3-6 October 2011).

Sala, E., Ballesteros, E., Dendrinos, P., Di Franco, A., Ferretti, F., Foley, D., Fraschetti, F., Friedlander, F., Garrabou, J., Güçlüsoy, H., Guidetti, P., Benjamin S. Halpern, B.S., Hereu, B., Karamanlidis, A.A., Kizilkaya, Z., Macpherson, E., Mangialajo, L., Mariani, S., Micheli, F., Pais, A., Riser., K., Rosenberg, A.A., Sales, M., Selkoe, K.A., Starr, R., Tomas, F., Zabala, M. (2012). The structure of Mediterranean rocky reef ecosystems across environmental and human gradients, and conservation implications. *PloS one*, *7*(2), e32742, http://dx.doi.org/10.1371/journal.pone.0032742.

Salas, F., Marcos, C., Neto, J. M., Patrício, J., Pérez-Ruzafa, A., & Marques, J. C. (2006). User-friendly guide for using benthic ecological indicators in coastal and marine quality assessment. *Ocean & Coastal Management*, *49*(5), 308-331, http://dx.doi.org/10.1016/j.ocecoaman.2006.03.001.

Sfriso, A., & Facca, C. (2011). Macrophytes in the anthropic constructions of the Venice littorals and their ecological assessment by an integration of the "CARLIT" index. *Ecological Indicators*, *11*(3), 772-781, http://dx.doi.org/10.1016/j.ecolind.2010.10.002.

Susini, M. L. (2006). *Statut et biologie de Cystoseira amentacea var. stricta* (Doctoral dissertation, Thèse de Doctorat, Université de Nice-Sophia Antipolis).

Torras, X., Pinedo, S., García, M., Weitzmann, B., & Ballesteros, E. (2015). Environmental quality of Catalan coastal waters based on macroalgae: The interannual variability of CARLIT index and its ability to detect changes in anthropogenic Pressures over time. In *Experiences from Ground, Coastal and Transitional Water Quality Monitoring* (pp. 183-199). Springer International Publishing, http://dx.doi.org/10.1007/698_2015_370.

Chapter 2

Ex-situ cultivation protocol for *Cystoseira amentacea* var. *stricta* (Fucales, Phaeophyceae) from a restoration perspective

Authors: Annalisa Falace, Sara Kaleb, Gina De La Fuente, Valentina Asnaghi, Mariachiara Chiantore.

Published in: Plos One 13(2), e0193011

Abstract

Cystoseira forests are experiencing a significant decline due to multiple impacts, affecting the ecosystem services they provide. Although the implementation of conservation efforts, there is an urgent need to develop best practices and large-scale restoration strategies. Until now, only limited trials with juvenile or adult transplants have been conducted. The outplanting consisting in producing new recruits from fertile material in hatcheries represents a sustainable option for restoring without depleting natural populations.

In order to implement restoration actions, we developed an *ex-situ* protocol for *C. amentacea* var. *stricta* cultivation, aimed to reduce the time for laboratory culture, avoiding prolonged maintenance and minimizing costs. Specifically, we tested the effects of temperature, light and substratum on settlement and growth of early-life stages using a factorial experiment. Temperature (24 and 20°C) and photoperiod (15L:9D) were selected to reflect conditions experienced in the field during the reproductive period. Light intensities (250 and 125 μmol photons $m^{-2}s^{-1}$) were selected to mimic the condition experienced in the absence of canopy (i.e. barren - higher light intensity) or in the understory (lower light intensity) during gamete release. The substrata tested were flat polished pebbles and rough clay tiles. Gametes release and the successive embryo and germlings survival and development were followed for two weeks. Regardless culture conditions, rougher tiles showed higher zygote settlement, although the substrata did not affect the successive development. Zygote mortality after one week was 50% on average. At the end of the second week the embryonic survival was higher under lower light and temperature conditions, determining also the growth of larger embryos.

Introduction

The genus *Cystoseira* C. Agardh, brown algae belonging to the order Fucales, is distributed along the Mediterranean and Atlantic coasts from the surface to the lower sublittoral zone. This genus is ecologically relevant as an "ecosystem engineer" (Jones et al., 1994), with a key functional role in controlling habitat spatial heterogeneity, productivity, and nutrient cycling in temperate rocky reefs. In particular, *Cystoseira* forests provide refuge and food for many invertebrates and fishes and modulate the structure of the associated benthic community (Mineur et al., 2015).

At present, some *Cystoseira* populations (depending on species and location) are experiencing a decline/loss throughout the Mediterranean (Boudouresque, 2004; Thibaut et al., 2005, 2014; Micheli et al., 2013; Blanfune et al., 2016), largely due to multiple human impacts such as urbanization, overfishing and climate change. Consequently, many systems have shifted from complex and productive assemblages to simpler, less productive habitats such as barrens, turf-forming algae and other ephemeral opportunistic seaweeds, impacting the ecosystem services provided (Munda, 1993; Sala et al., 1998; Boudouresque, 2004; Falace et al., 2010; Fraschetti et al., 2011; Giakoumi et al., 2012; Marzinelli et al., 2014; Templado et al., 2014). *Cystoseira* spp. are listed as species of community interest according to the Habitat Directive (92/43/EEC), protected in Annex I of the Bern Convention and by the Barcelona Convention, considered vulnerable by several international organizations (IUCN, RAC/SPA, and MedPan). These species are environmental quality indicators according to the Water Framework Directive (2000/60/EC) and are used as benthic quality elements (BQEs) for index calculation in Mediterranean coastal waters (i.e., EEI- Orfanidis et al., 2003 and CARLIT- Ballesteros et al., 2007, Mangialajo et al., 2007).

The threat of losing *Cystoseira* is magnified by its low dispersal capacity due to rapid egg fertilization and zygote sinking (Guern, 1962; Clayton, 1990; Johnson & Brawley, 1998; Gaylord et al., 2002), which hampers natural recovery in the absence of adults.

Despite the implementation of significant conservation efforts, most degraded systems have not recovered, emphasizing the urgency to develop an active intervention to restore endangered habitats (Marzinelli et al., 2014). Interest in habitat restoration is increasing according to the Biodiversity Strategy to 2020 (Target 2; European Commission, 2011), which recommends the reintroduction of relevant species into areas where their historical presence has been recorded and the factors that led to their loss have been removed.

Small scale *Cystoseira* transplants have been attempted utilizing several techniques on short temporal scales (Falace & Bressan, 2006; Falace et al., 2006; Susini et al., 2007; Sales et al., 2011). The most frequently tested method is the transplantation of juveniles or adult thalli with fertile receptacles: the only major challenge of this approach is the appropriate fixing of individuals or installation in the target area.

Outplanting, which consists of producing recruits from fertile material in hatcheries to be placed in the sea, has been explored to a lesser degree for the genus *Cystoseira* (Falace et al., 2006; Susini et al., 2007; Sales et al., 2011; Verdura et al., 2015). In contrast, many studies have been performed using other large fucoid seaweeds (Vasquez & Tala, 1995; Stekoll & Deysher, 1996; Dudgeon & Petraitis, 2005; Hwang et al., 2006; Hays, 2007; Lamote & Johnson, 2008; Pang et al., 2009; Yatsuya, 2010; Yu et al., 2012), focusing in particular on the long-term maintenance of seedlings in culture (Zhao et al., 2008; Sun et al., 2009; Yu et al., 2012; Yan & Zhang, 2014; Yoon et al., 2014; Kerrison & Le, 2016; Liu et al., 2016).

Considering the high potential of *Cystoseira* to generate gametes and zygotes under optimal conditions, the cultivation of germlings starting from fertile receptacles represents a sustainable option for restoring endangered species without depleting natural populations. From this perspective, the development of effective cultivation protocols, tailored to the eco-physiological needs of different species, is a compulsory milestone.

Usually, the need for large amounts of germlings for outplanting represents a bottleneck in the design of large-scale restoration actions. Therefore, it is particularly challenging to plan an efficient, effortless and cost-effective seedling production system that fits the breeding needs of a specific species.

The aim of this study was to develop an in-laboratory protocol for *Cystoseira amentacea* var*. stricta* Montagne, a caespitose intertidal Mediterranean sensitive species, whose reduction/loss has primarily been recorded in several locations in the NW Mediterranean (Mangialajo et al., 2007; Thibaut et al., 2014).

The protocol aimed to maximize zygote settlement, minimize embryo development time and generate a dense coverage of healthy germlings for outplanting. With these objectives, we tested the effects of easily adjustable variables (temperature, light and substratum) on the settlement and growth of early-life stages to develop best practices for the restoration of this sensitive species.

Materials and methods

In June 2016, during the reproductive period of *C. amentacea* var. *stricta*, healthy apices ca. 3 cm in length holding mature receptacles were collected from a donor population at Bogliasco, Genoa (NW Italy) (Fig 1). This site is considered moderately urbanized, where *C. amentacea var. stricta* forms a continuous belt in the intertidal zone. In particular, the site is characterized by a tide in the range of 30 cm (the barometric tide may dominate the water level) and an average spring temperature of 20°C. The local macroalgal community in Bogliasco is dominated by *C. amentacea var. stricta*, followed by *Cystoseira compressa* and articulate corallinales.

After sampling, apices wrapped with seawater-wetted towels were delivered within 24h under dark, cold and humid conditions to the laboratory in Trieste (NE Italy) (Fig 1) for culture in controlled rooms.

The temperature and photoperiod were selected to reflect typical seasonal conditions at the sampling site during the reproductive phase of *C. amentacea* var*. stricta* (from late spring to summer). The photoperiod was set to a 15:9 h light : dark cycle. Light intensities were chosen to mimic two possible scenarios occurring in nature during the gamete release, fertilization and early-life growth stages of *Cystoseira*: in the absence of canopy, as on barren ground (higher light intensity) or in the understory (lower light intensity). Light was provided by LED lamps (AM366 Sicce USA Inc., Knoxville, USA), and irradiance was measured with a LI-COR LI-190/R Photometer (LICOR-Biosciences, Lincoln, NE, USA). Light irradiance (L) was set at 250 μmol photons $m^{-2}s^{-1}$ (L^+) or at 125 μmol photons $m^{-2}s^{-1}$ (L^-), while temperature(T) was set at 24°C (T^+) or at 20°C (T^-). The medium used in the experiments was Stosch's enriched seawater (VSE) (von Stosch, 1963; as modified by Guiry and Cunningham, 1984). The seawater was filtered and autoclaved prior to VSE addition. Aquaria were filled with 4 L of culture medium, renewed every 4 days to minimize any possible effects of nutrient limitation and continuously aerated by air pumps. Two substrata with differing natures and roughness were tested: flat polished pebbles (Pe) and rough clay tiles (Ti).

A factorial laboratory experiment was performed combining irradiance, temperature and substratum. Four combinations of culturing conditions consisting of two crossed levels for each environmental condition (L^+T^+, L^+T^-, L^-T^+, L^-T^-) and two substrata (Pe and Ti) were tested in a two-way crossed design.

Fertile apices were gently cleaned with a brush and rinsed with sterile seawater to remove adherent biofouling and surface detritus. Then, they were placed in individual aquaria: 3 apices with mature receptacles on each substratum in separate aquaria per condition (in triplicate).

Three additional replicates were placed on glass slides under each condition to observe zygote development with an inverted microscope (Leica, DM IL LED) and obtain photographs with a Canon Powershot G9, avoiding stress on the treatment replicates.

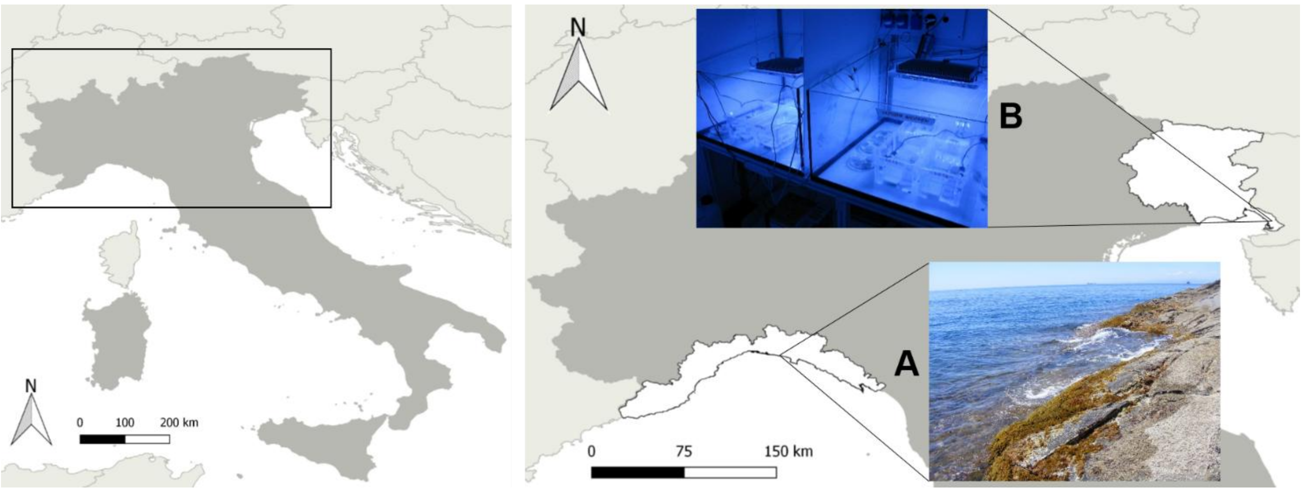


Fig 1. Map showing the geographical location of the collection site (A) and the laboratory site (B), North Italy.

Data analysis

Egg release and settlement

After 2h, gametes were released on all substrata and under all conditions. Next, the receptacles were removed, and their fresh weight (FW) was measured. Due to the high density of eggs released on each substratum and to reduce manipulation stress as much as possible, counts were performed by processing photographic data. Eggs were counted in 5 randomly selected 1x1 cm quadrants for each substratum in photos obtained from a Leica MZ6stereo microscope with a Nikon Coolpix 4500 camera.

The number of eggs per unit of receptacle FW was analyzed as a response variable to compare settlement on different substrata under different conditions. Two-way crossed ANOVA was performed using both factors and their interaction as fixed factors. The data were transformed by the square root to satisfy normality and homoscedasticity assumptions.

Embryo development

At the end of the first week (w1), replicate embryos in all four growth stages were visible on glass slides and were counted: I-embryos with 4 primary rhizoids, II-embryos with 8 rhizoids, III-embryos with short apical hair/s, and IV-embryos with long apical hair/s (Fig 2). To analyze the differences among conditions, a PERMANOVA analysis was applied using the percentage of individuals at each stage as a response variable and conditions as factors. Pairwise comparisons were performed on significant terms.

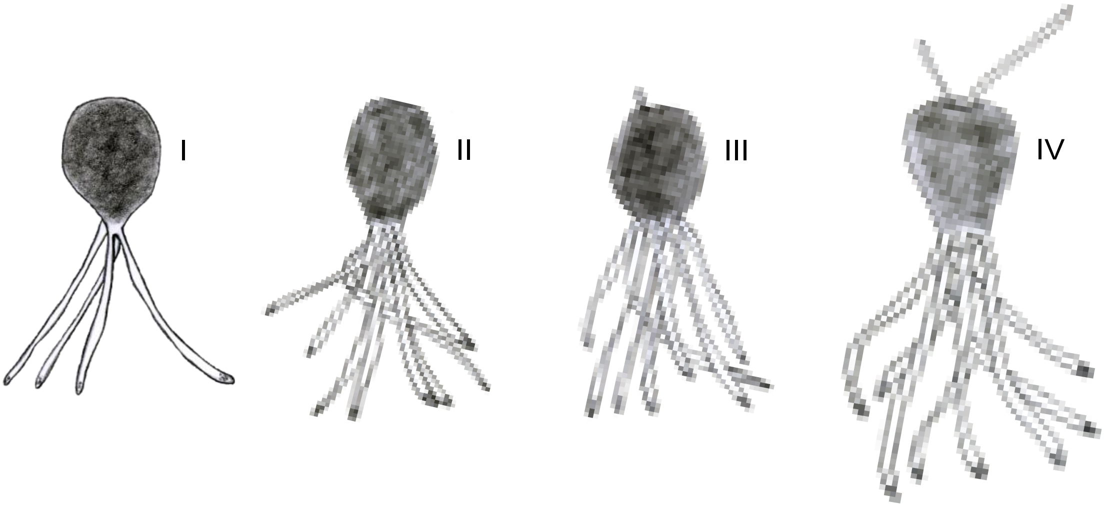


Fig 2. Early stages of *C. amentacea* var*. stricta* at week1: I-embryos with 4 primary rhizoids, II-embryos with 8 rhizoids, III-embryos with short apical hair/s, and IV-embryos with long apical hair/s.

Embryo survival

After 24h (T0), zygote numbers were counted on each substratum (5 1x1 cm quadrants) by processing photographic data. Counts were then repeated at w1 and w2 to calculate the germling survival rate.

We applied an ANCOVA analysis for both w1 and w2 with unequal slopes for survival rate as a response variable and density (i.e., number of fertilized eggs) as a covariate. Substrata, conditions and their interaction were used as fixed factors. Assumptions were validated after applying the arcsine square root transformation (suitable for proportional data). Post hoc SNK tests were performed on significant interaction terms.

Subsequent germling growth

At w2, three subsequent developmental stages were identifiable: I-round-shaped, II-elongated, and III-elongated with branch (Fig 3).

The area of ten randomly chosen individuals per shape was measured in each replicate substratum and used as a response variable. The area was quantified by processing photographic data using ImageJ software (Schneider et al., 2012). Conditions and substrata were used as crossed fixed factors in a PERMANOVA analysis. Pairwise comparisons were performed on significant terms.

Fig 3. *C. amentacea* var*. stricta* germling stages at week2: I-round-shaped, II-elongated, and III- elongated with branch.

3. Results

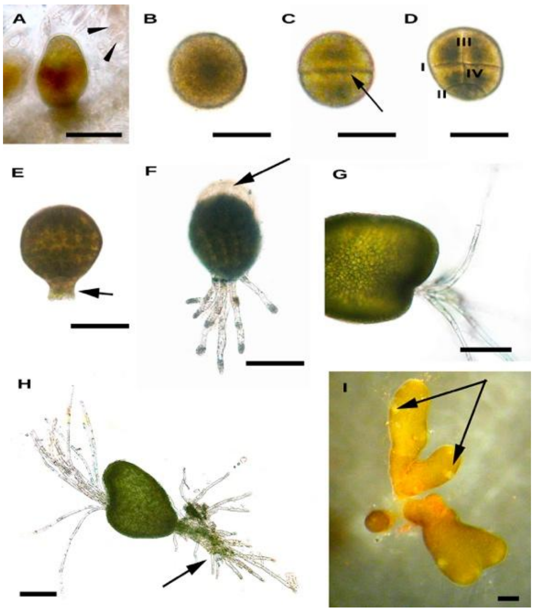
Morphogenesis

C. amentacea var*. stricta* is a monoic species, with female and male gametes produced in the same conceptacle (Figure 4A). In our trials, gamete release started soon after the receptacles were placed in the aquaria. The mean diameter of the eggs was 122±3µm (n=20). Fertilization occurred externally, and the development of a fecundation membrane around the zygote facilitated its adhesion to a substratum (Figure 4B). The zygote cytoplasm, which was initially homogeneous, became metabolically differentiated (polarization) with the establishment of a vertical growth axis (connecting the rhizoid and thallus pole). Twelve hours after fertilization (AF), the first division perpendicular to the growth axis was observed, leading to the formation of two equally sized cells (Figure 4C). The second division, which was parallel to the first, occurred in the lower cell 20-22 h AF (Figure 4D), while the third division, perpendicular to the first, appeared in the upper cell (Figure 4D). Within 32-34h AF, many divisions occurred without an increase in embryo volume.

Within the first week, the rhizoids developed as follows: the rhizoid mother cell, using perpendicular divisions, gave rise to four cells that differentiated into four primary rhizoids (Figure 4E and Fig 2) that grew further, forming long filaments (ca. 150-200 µm long). After detachment of the fecundation membrane, the length of the embryo increased through subsequent cell divisions, and secondary rhizoids were formed (Figure 4F). Thus, the embryo assumed an erect position, and an invagination with hyaline hairs appeared in the apical region (Figure 4H,4G). At w1 AF, the more developed embryos were 353±26 µm long and 259±37 µm wide (n=20). At w2 AF, germlings with numerous rhizoids grew further [466±26 µm long and 275±28 µm wide (n=20)] Small lateral branches with some cryptostomata began to appear (Fig 3). At w3 AF, numerous cryptostomata were observed (Figure 4I), and iridescence, which is typical of adult plants, was visible on the thallus surface (Fig. 5). At this time point, the germlings were 1386±130 µm long and 466±61 µm wide (n=20).

A few tiles were transported back to the donor population (Bogliasco - Genoa). Juveniles were 4737±543 µm long and 811±92 wide after 1 month in the field. They grew up to 9 cm in 9 months (April 2017).

Figure 4. Early development of *Cystoseira amentacea* var. *stricta*



A. Detail of a conceptacle with an oogonium and antheridia (arrowhead).

B. Zygote with a central large nucleus.

C. First zygote division (arrow).

D. Second zygote division (II), parallel to the first (I); third (III) and fourth divisions (IV), perpendicular to the first one.

E. Embryo with rhizoidal buds (arrow).

F. Embryo with secondary rhizoids. Note the detachment of the fecundation membrane (arrow) during embryo elongation.

G. Hyaline hairs growing from the invagination in the apical region of the embryo.

H. Embryo with long apical hairs and numerous rhizoids (arrow).

I. Germling with cryptostomata (arrows). Bar=200 µm.

Egg release and settlement

The number of settled eggs was higher on Ti (avg +/- er stand) than on Pe (avg +/- er stand), highlighting a significant effect of substratum roughness ($p<0.0001$; Table 1 Supp Mat). Conversely, no significant differences were detected between conditions or within the interaction term.

Early embryo development

The percentage of individuals in each of the four stages observed for the glass slide replicates (Fig 2) was calculated. An MDS ordination plot (Fig 5) showed three different groups: L^+T^-, L^+T^+ and one group comprising L^-conditions (L^-T^- and L^-T^+). PERMANOVA analysis confirmed significant differences among these groups ($p<0.001$; Table 2 Suppl Mat.).

Furthermore, a bar plot (Fig. 6) revealed a higher percentage of embryos in stage IV (embryos with long apical hair/s) under L^-conditions.

Table 2. Suppl Mat. One-way PERMANOVA performed at week1 based on the percent composition of three embryonic developmental stages under the different conditions. In bold significant effects.

	df	SS	MS	F	R^2	P
Condition	3	2.80	0.93	34.23	0.65	**0.001**[a]
Residual	56	1.52	0.03		0.35	
Total	59	4.32			1.00	

[a] Pairwise comparisons among Conditions: $L^+T^- \neq L^+T^+ \neq L^-T^- = L^-T^+$

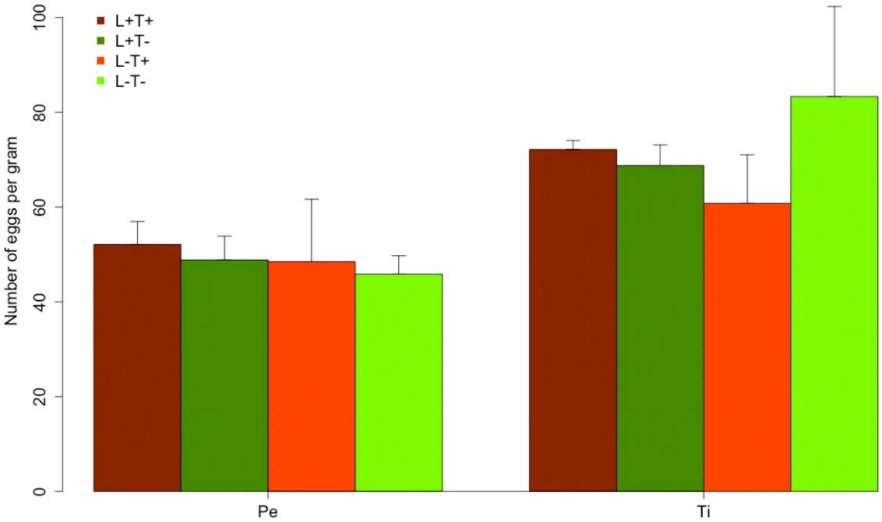


Fig 5. MDS ordination plot of the percent composition of embryonic developmental stages at week1 under the different conditions.

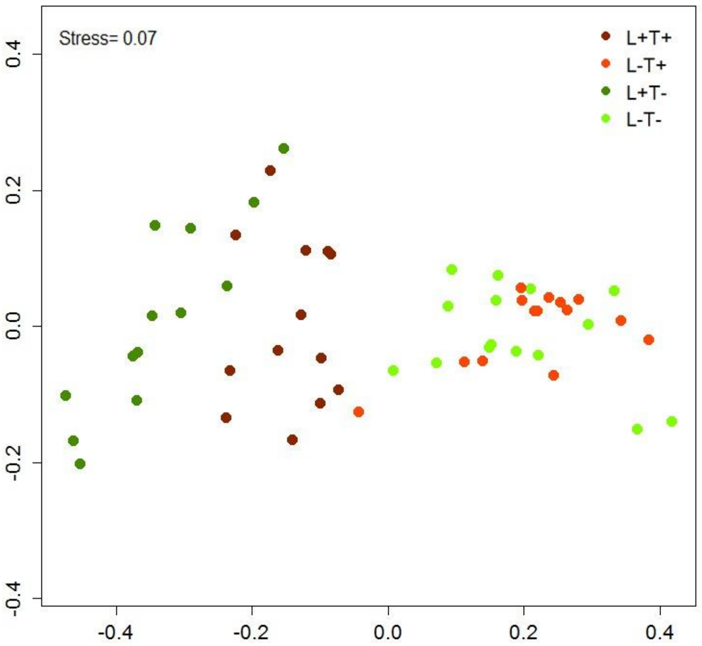


Fig 6. Bar plot of the percent composition of embryonic developmental stages at week1 under the different conditions.

Embryo survival

The results of an ANCOVA analysis indicated strong significant differences among conditions and small differences between the two substrata at w1 ($p<0.0001$ and $p<0.03$, respectively; Table 3 Suppl Mat).

At w2 the interaction term (condition X substratum) was significant ($p<0.001$; Table 4 Suppl Mat): on both substrata, extreme conditions did not differ ($L^+T^+=L^-T^-$) but differed significantly from the other two conditions (L^+T^-; L^-T^+). On Ti, L^+T^- and L^-T^+ did not significantly differ, although they differed on Pe. Additionally, ANCOVA indicated that L^+T^- was significantly different between substrata, while survival was slightly higher on Pe.

As shown in boxplots (Fig 7), the survival rate at w1 was higher under extreme conditions (L^+T^+; L^-T^-) compared to the other two conditions (L^+T^-; L^-T^+). The survival rate from w1 to w2 showed a conspicuous decrease (between 50 and 95%) for the L^+T^+, L^+T^- and L^-T^+ conditions, while the survival rate under L^-T^- remained more stable with a mortality below 30%.

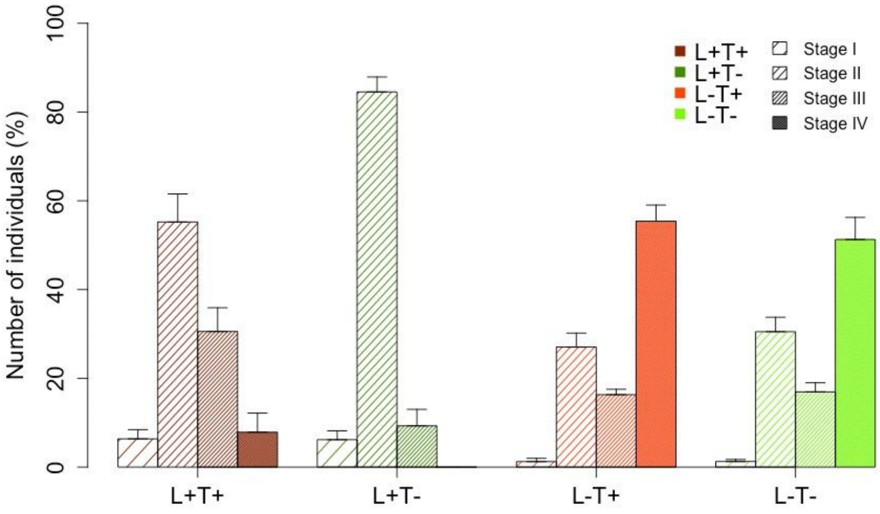


Fig 7. Boxplot of the survival rates at week1 (A) and week2 (B) among conditions and substrata.

Subsequent germling growth

PERMANOVA analysis performed on the germling area at different stages (Fig 3) at w2 showed significant differences among conditions (p = 0.001), with L$^-$T$^-$condition different from all the others (Table 5 Suppl. Mat.; Fig 8).

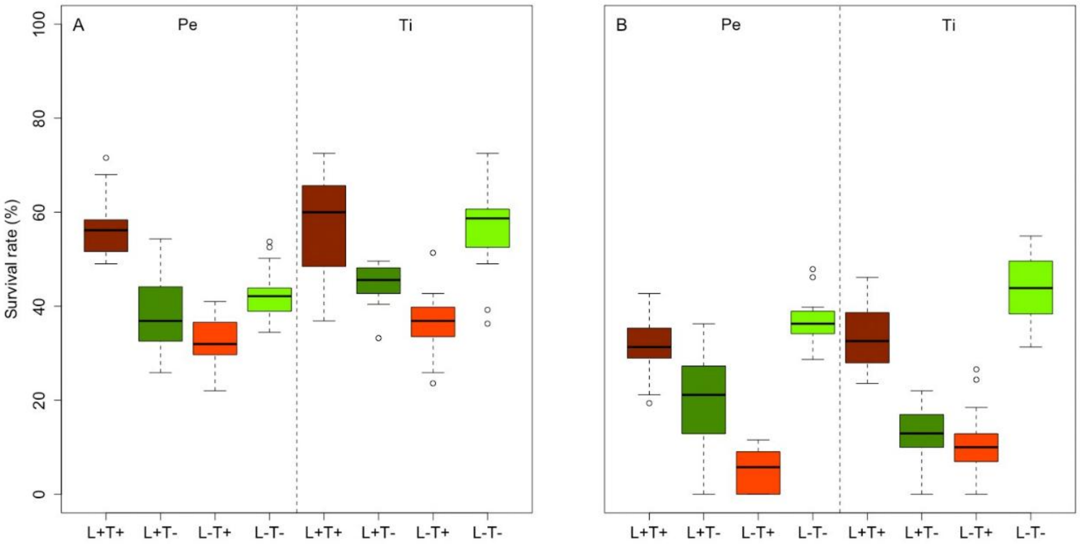


Fig 8. Boxplots of the area of the different stages (A: stage I; B: stage II; C: stage III) at week 2 among conditions for both substrata.

Discussion

Given the worldwide concern regarding the loss of key habitat-forming organisms, such as the large brown macroalgae of the order Fucales, and the downstream cascade effects of the services such organisms provide, there is an urgent need to develop best practices and restoration strategies. Studies that provide sound information on how best to undertake habitat restoration are crucial for managing coastal ecosystems.

Outplanting appears to be an ecologically sustainable approach that consists of two main steps: culturing germlings in the laboratory and transferring them into the field (Dudgein & Petraitis, 2005; Hays, 2007; Yu et al., 2012; Yoon et al., 2014). For *Cystoseira*, outplanting appears a feasible management option, providing a large number of healthy specimens for re-introduction to the environment without impacting natural populations (Falace et al., 2006; Sales et al., 2015; Verdura et al., 2015).

Herein, we focused on the first step in the outplanting process: developing an effective protocol to cultivate the early stages of *C. amentacea* var. *stricta*.

This approach is challenging because most eco-physiological studies of *Cystoseira*, both in the field and in the laboratory, have focused on the adult stages. Nevertheless, the single/few-celled stages are characterized by simplicity and sensitivity, and any environmental variable will exert greater effects on germling mortality and growth rate (Yakovleva et al., 1998; Wiencke et al., 2000) compared to adults. Thus, the needs of these ontogenetic stages must be understood because findings obtained from the macrothallus stages cannot be extrapolated to the microscopic stages (Altamirano et al., 2009). In this regard, the development of species-specific best practices for the cultivation of germlings is necessary to implement a successful restoration strategy at a large scale.

First, we tested whether it was possible to collect samples far away from the breeding facility (ca. 600 km) without damaging the reproductive materials, excluding possible negative effects related to the distance of the target site from the hatchery. Transporting under dark and cold conditions allowed immediate gamete release bypassing this specific step (thermal and light shock) in the laboratory. Indeed, receptacles that were placed in aquaria soon after their arrival in the laboratory immediately released gametes.

Nutrient limitation affects many processes, such as photosynthetic capacity (Pérez-Lloréns et al., 1996), protein content (Vergara et al., 1995; Martínez & Rico, 2002), photoprotection mechanisms (Korbee-Peinado et al., 2004; Korbee et al., 2005; Huovinen et al., 2006), behavior and settlement of eggs, embryonic development and growth rate (Chapman et al., 1978; Amsler & Neushul, 1990; Reed et al., 1999; Kinlan et al., 2003; Flavin et al., 2013; Morelisen et al., 2013). Therefore, the culture medium was enriched to allow the germlings to invest their photosynthetic energy in growth processes (Celis-Plà et al., 2014a, 2014b). Better growth of *C. amentacea* var. *stricta* germlings with nutrient enrichment was also observed by (Susini, 2006). Together with culture conditions, the choice of substrata must also favor the adhesion of gametes and zygotes and their successive development. Regardless of the culture conditions, rougher tiles showed higher zygote settlement than smoother pebbles, although the substrata did not affect successive germling growth or survival under any of the tested conditions.

Embryonic mortality after w1 was elevated under all conditions (50% on average), as expected given the *Cystoseira* very high stochastic gamete and zygote mortality in the natural environment.

At the end of w1, embryonic survival was positively affected by two of the tested conditions: L^+T^+ and L^-T^-. In the longer term (at w2), survival was still higher under the L^-T^- condition but significantly decreased under L^+T^+

treatment. The other two combined conditions demonstrated the lowest embryonic survival throughout the entire experiment. Low light and low temperature conditions also favor higher embryonic survival rate in *Sargassum vachellianum* Greville, 1848 cultivations (Yan & Zhang, 2014).

Lower light intensity also reduced the time required for embryo development, allowing a greater number of individuals to reach developmental stage IV (larger embryos) within w1, regardless of temperature. After w2 at low irradiance, lower temperature also strongly determined the growth of larger embryos.

Our findings corroborate that environmental conditions (specifically light and temperature) may interact and exert synergistic or antagonistic effects on the physiological responses in unpredictable ways, differently according to developmental stage. The distribution of *C. amentacea* var. *stricta* is restricted to the upper littoral zone, and therefore, this species is naturally exposed to high irradiance levels that potentially exceed its light energy requirements, as reported for other species that live close to the water surface (Delgado et al., 1995; Hader & Figueroa, 1997; Hanelt & Figueroa, 2012; Celis-Plà et al., 2014a). Generally, sun-adapted species (*sensu* Ramus, 1981) develop efficient photoprotection mechanisms to tolerate light stress in addition to dynamic photoinhibition (Mattoo et al., 1984; Ohad et al., 1984; Guenther & Melis, 1990; Critchley & Russel, 1994; Hader & Figueroa, 1997; Figueroa & Gomez, 2001; Franklin et al., 2003; Abdala-Diaz et al., 2006; Wilheim & Selmar, 2011; Hanelt & Figueroa, 2012; Celis-Plà et al., 2015). Our study highlighted the light-shade adaptation of *C. amentacea* var. *stricta* germlings, which showed enhanced growth at lower irradiance (125 μmol photons m^{-2} s^{-1}). Other *Cystoseira* species have been cultivated under different conditions, primarily depending on laboratory facilities: *C. susanensis* (16±1°C | 40 μmol photons m^{-2} s^{-1}; (Alongi et al., 1999) and *C. barbata* (16-17°C | 120 μmol photons m^{-2} s^{-1}; Falace et al., 2006). Susini (2006) described the morphological development of *C. amentacea* var. *stricta* embryos cultivated at 18±1°C and an average light intensity of 70 μmol photons $m^{-2}s^{-1}$ but did not report quantitative data.

Studies examining adult thalli of *Cystoseira* have demonstrated the absence of photosynthetic inhibition, even with very high irradiance (Cudret & Jupin, 1985). In *Cystoseira barbata* (Stackhouse) C. Agardh f. *aurantia* (Kuetzing) Giaccone, photoinhibition occurs only at irradiances higher than 1500 μmol photons $m^{-2}s^{-1}$ (Baghdadli et al., 1990), while photosynthesis in *C. mediterranea* is not saturated at an irradiance of 1600 μmol photons $m^{-2}s^{-1}$ (Delgado et al., 1995). Notably, the light requirements of adults should not be extrapolated to the microscopic stages because the presence of non-photosynthetic tissues in complex thalli increases the need for light energy (Hanelt & Figueroa, 2012). Moreover, in nature, the irradiance reaching the embryos is restricted by adult fronds (Van Tamelen et al., 1997; Dudgeon & Petraitis, 2005; Zhao et al., 2008). *Cystoseira* zygotes and germlings settle under adult plants, where they find a good protective screen against high irradiance and other stress. In nature, such community self-protection is particularly important during spring-summer, when *C. amentacea* var. *stricta* produces new recruits in the study area. Conversely, the lower irradiance requirements of germlings permit high-density cultures because self-shading is not a restricting factor.

At higher temperatures (24°C), the proliferation of biofouling was enhanced, particularly at lower light intensity, progressively affecting the development of *C. amentacea* var. *stricta* embryos.

Based on these results, we determined the best hatchery conditions to accelerate the development of high numbers of healthy, larger embryos.

Further studies are required to improve the second step in the outplanting process in order to increase the number of juveniles that are able to reach the adult stage once reintroduced into the field. Grazing pressure, timing and density dependent effects need to be taken into account to achieve the best restoration performance.

Supporting information

Table 1 Suppl Mat. Two-way crossed ANOVA performed on the number of eggs per gram. In bold significant effects.

	df	SS	MS	F	P
Substratum (S)	1	3020.75	3020.75	33.31	**2.87e-05**
Condition (C)	3	334.16	111.39	1.23	0.33
S:C	3	511.07	170.36	1.88	0.17
Residual	16	1451.29	90.71		

Table 2 Suppl Mat. One-way PERMANOVA performed at week1 based on the percent composition of three embryonic developmental stages under the different conditions. In bold significant effects.

	df	SS	MS	F	R^2	P
Condition	3	2.80	0.93	34.23	0.65	**0.001**[a]
Residual	56	1.52	0.03		0.35	
Total	59	4.32			1.00	

[a] Pairwise comparisons among Conditions: $L^+T^- \neq L^+T^+ \neq L^-T^- = L^- T^+$

Table 3 Suppl Mat. ANCOVA performed at week1 using survival, substratum and condition as factors and density as a covariate. In bold significant effects.

Week 1	df	SS	MS	F	P
Density	1	1702.2	1702.17	29.62	**3.18e^{-07}**
Substratum (S)	1	274.9	274.91	4.78	**0.03**
Condition (C)	3	8404.3	2801.43	48.74	**2.2e^{-16}**[a]
S:C	3	367.0	122.34	2.13	0.10
Residuals	111	6379.9	57.48		

[a] SNK test among Conditions: $L^+T^+ \neq L^-T^- \neq L^+T^- \neq L^-T$

Table 4. Suppl Mat. ANCOVA performed at week2 using survival as response variable, Substratum and Condition as factors and density as covariate. In bold significant effects.

Week 2	df	SS	MS	F	P
Density	1	2321.5	2321.5	48.11	**2.83e^{-10}**
Substratum (S)	1	192.7	192.7	3.99	0.05
Condition (C)	3	17125.8	5708.6	118.29	2.2e^{-16}
S:C	3	898.5	299.5	6.21	**0.0006**[ab]
Residuals	111	5356.8	48.3		

[a] SNK test among Substrata within Condition: Cond. L^+T^-, T≠ S; all other Cond. T=S

[b] SNK test among Conditions within Substratum: Sub. S, (L^+T^+ = L^-T^-) ≠ L^+T^- ≠ L^-T^+; Sub.T, (L^+T^+ = L^-T^-) ≠ (L^+T^- = L^-T^+)

Table 5. Suppl. Mat. Two-way PERMANOVA performed on the germling area at different stages at week2. Condition and Substratum are crossed fixed factors. In bold significant effects.

	df	SS	MS	F	R^2	P
Substratum (S)	1	0.01	0.002	0.51	0.01	0.47
Condition (C)	3	0.16	0.05	12.30	0.71	**0.001**[a]
S:C	2	0.01	0.004	0.98	0.04	0.40
Residual	13	0.06	0.004		0.25	
Total	19	0.22			1.00	

[a] Pairwise comparisons among Conditions: L^-T^- ≠ L^+T^+=L^-T^+=L^+T^-

References

Abdala-Dıaz RT, Cabello-Pasini A, Pérez-Rodrìguez E, Conde Alvarez RM, Figueroa FL. Daily and seasonal variations of optimum quantum yield and phenolic compounds in *Cystoseira tamariscifolia* (Phaeophyta). Mar Biol. 2006. 148: 459-465. doi: 10.1007/s00227-005-0102-6

Alongi G, Catra M, Cormaci M. Observations sur *Cystoseira susanensis* (Cystoseiraceae, Phaeophyta): une espèce méditerranéenne rare et peu connue. Cryptogam, Algol. 1999; 20(1): 25-33. https://doi.org/10.1016/S0181-1568(99)80004-X

Altamirano M, Flores-Moya A. Figueroa FL. Effects of UV radiation and temperature on growth of germlings of three species of *Fucus* (Phaeophyceae). Aquat Bot. 2009; 75: 9-20.

Amsler CD, Neushul M. Nutrient stimulation of spore settlement in the kelps *Pterygophora californica* and *Macrocystis pyrifera*. Mar Biol. 1990; 107: 297-304.

Baghdadli D, Tremblin G, Pellegrini M, Coudret A. Effects of environmental parameters on net photosynthesis of a free-living brown seaweed *Cystoseira barbata* forma *repens*: determination of optimal photosynthetic culture conditions. J Appl Phycol. 1990; 2: 281-287. doi: 10.1007/BF02179786

Ballesteros E, Torras X, Pinedo S, García M, Mangialajo L, and De Torres M. A new methodology based on littoral community cartography dominated by macroalgae for the implementation of the European Water Framework Directive. Mar Pollut Bull. 2007; 55(1): 172-180. http://dx.doi.org/10.1016/j.marpolbul.2006.08.038

Blanfune A, Boudouresque CF, VerlaqueM, Thibaut T. The fate of *Cystoseira crinita*, a forest-forming Fucales (Phaeophyceae, Stramenopiles), in France (North Western Mediterranean Sea). Estuar Coast Shelf Sci. 2016; 181: 196-208. https://doi.org/10.1016/j.ecss.2016.08.049

Boudouresque CF. Marine biodiversity in the Mediterranean: status of species, populations and communities. Sci Rep Port-Cros Natl Park, Fr. 2004; 20: 97-146.

Celis-Plá PSM, Korbee N, Gómez-Garreta A, Figueroa FL. Seasonal photoacclimation patterns in the intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta). Sci. Mar. 2014a; 78(3): 377-388. doi: http://dx.doi.org/10.3989/scimar.04053.05A

Celis-Plá PSM., Martínez B, Quintano E, García-Sánchez M, Pedersen A, Navarro NP, Copertino MS., Mangaiyarkarasi N, Mariath R, Figueroa FL, Korbee N. Short-term ecophysiological and biochemical responses of *Cystoseira tamariscifolia* and *Ellisolandia elongata* to environmental changes. Aquat Biol. 2014b; 22: 227-243. doi: 10.3354/ab00573

Celis-Plá PSM, Hall-Spencer JM, Horta PA, Milazzo M, Korbee N, Cornwall CE, Figueroa FL. Macroalgal responses to ocean acidification depend on nutrient and light levels. Front Mar Sci. 2015. 2:26. doi: 10.3389/fmars.2015.00026

Chapman ARO, Markham JW, Lüning K. Effects of nitrate concentration on growth and physiology of *Laminaria saccharina* (Phaeophyta) in culture. J Phycol. 1978; 14(2): 195-198. doi: 10.1111/j.1529-8817.1978.tb02448.x

Clayton MN. The adaptive significance of life history characters in selected orders of marine brown macroalgae. Austral Ecol. 1990; 15(4): 439-452. doi: 10.1111/j.1442-9993.1990.tb01469.x

Coudret A, Jupin H. Action de énergie lumineuse surle métabolisme de *Cystoseira elegans* Sauvageau. C.R. Acad. Sci. Paris. 1985; 301(19): 827-832.

Critchley C, Russel AW. Photoinhibition of photosynthesis in vivo: The role of protein turnover in photosystem II. Physio. Plant. 1994; 92(1): 188-196. doi: 10.1111/j.1399-3054.1994.tb06670.x

Delgado O, Rodriguez-Prieto C, Frigola L, Ballesteros E. Drought tolerance and light requirements on high and low sublittoral species of Mediterranean macroalgae of the genus *Cystoseira* C. Agardh (Fucales, Phaeophyceae). Bot Mar. 1995. 38: 127-132. https://doi.org/10.1515/botm.1995.38.1-6.127

Dudgeon S, Petraitis PS. First year demography of the foundation species, *Ascophyllum nodosum*, and its implications. Oikos. 2005; 109(2): 405-415. doi: 10.1111/j.0030-1299.2005.13782.x

EC, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal L 327, 22/12/2000 p.0001-0073.

EEC, 1992. Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. Official Journal L 206, 22/07/1992 p.0007-0050.

Falace A, Bressan G. Seasonal variations of *Cystoseira barbata* (Stackhouse) C. Agardh frond architecture. Hydrobiologia. 2006. 555:193–206.doi:10.1007/s10750-005-1116-2

Falace A, Zanelli E, Bressan G. Algal transplantation as a potential tool for artificial reef management and environmental mitigation. Bull Mar Sci. 2006; 8: 161-166.

Falace A, Alongi G, Cormaci M, Furnari G, Curiel D, Cecere E, Petrocelli A. Changes in the benthic algae along the Adriatic Sea in the last three decades. Chemistry and ecology 2010; 26(S1): 77-90. http://dx.doi.org/10.1080/02757541003689837Figueroa FL, Gómez I. Photosynthetic acclimation to solar UV radiation of marine red algae from the warm temperate coast of southern Spain. A review. J Appl Phycol. 2001. 13(3), 233-245. doi:10.1023/A:1011126007656

Flavin K, Flavin N, Flahive B. Kelp farming manual. A guide to the processes, techniques and equipment for farming kelp in New England Waters. Ocean Approved, Portland; 2013.

Franklin LA, Osmond CB, Larkum AWD. Photoinhibition, UV-B and algal photosynthesis. In: Larkum AWD, Douglas SE, Raven JA. *Photosynthesis in Algae. Advances in Photosynthesis and Respiration* Vol. 14. Kluwer, Dordrecht; 2003. pp 351-384. doi: 10.1007/978-94-007-1038-2_16

Fraschetti S, Guarnieri G, Bevilacqua S, Terlizzi A, Claudet J, Russo GF, Boero F. Conservation of Mediterranean habitats and biodiversity countdowns: what information do we really need? Aquat Conserv. 2011; 21(3): 299-306. doi: 10.1002/aqc.1185

Gaylord B, Reed DC, Raimondi PT, Washburn L, McLean SR. A physically based model of macroalgal spore dispersal in the wave and current dominated nearshore. Ecology. 2002; 83(5): 1239-1251. doi: 10.1890/0012-9658(2002)083[1239:APBMOM]2.0.CO;2

Giakoumi S, Cebrian E, Kokkoris GD, Ballesteros E, Sala E. Relationships between fish, sea urchins and macroalgae: The structure of shallow rocky sublittoral communities in the Cyclades, Eastern Mediterranean. Estuar Coast Shelf Sci. 2012; 109: 1-10. https://doi.org/10.1016/j.ecss.2011.06.004

Guenther JE, Melis A. The physiological significance of Photosystem II heterogeneity in chloroplasts. Photosynth Res. 1990; 23(1): 105-109. doi: 10.1007/BF00030070

Guern M. Embryologie de quelques espèces du genre *Cystoseira* Agardh 1821 (Fucales). Vie Milieu. 1962; 13: 649-679.

Guiry, M. D., & Cunningham, E. M. (1984). Photoperiodic and temperature responses in the reproduction of north-eastern Atlantic Gigartina acicularis (Rhodophyta: Gigartinales). Phycologia, 23(3), 357-367.

Hader DP, Figueroa FL. Photo-ecophysiology of marine macroalgae. Photochem Photobiol. 1997; 66(1): 1-14. doi: 10.1111/j.1751-1097.1997.tb03132.x

Hanelt D, Figueroa FL 2012. Physiological and photomorphogenic effects of light on marine macrophytes, in: Wiencke C et al. editors. Seaweed biology: Novel insights into ecophysiology, ecology and utilization. Ecological Studies; 219. pp. 3-23.

Hays CG. Adaptive phenotypic differentiation across the intertidal gradient in the alga *Silvetia compressa*. Ecology. 2007; 88(1): 149-157. doi: 10.1890/0012-9658(2007)88[149:APDATI]2.0.CO;2

Huovinen P, Matos J, Pinto IS, Figueroa FL. The role of ammonium in photoprotection against high irradiance in the red alga *Grateloupia lanceola.* Aquat Bot. 2006; 84: 308-316. doi:10.1016/j.aquabot.2005.12.002

Hwang E, Park C, Baek J. Artificial seed production and cultivation of the ediblebrown alga, *Sargassum fulvellum* (Turner) C. Agardh: Developing a new species for seaweed cultivation in Korea. J Appl Phycol. 2006; 18: 251-257. doi: 10.1007/978-1-4020-5670-3_4

Johnson LE, Brawley SH. Dispersal and recruitment of a canopy-forming intertidal alga: the relative roles of propagule availability and post-settlement processes. Oecologia. 1998; 117(4): 517-526. doi: 10.1007/s004420050688

Jones CG, Lawton JH, Shachak M. Organisms as ecosystem engineers. Oikos. 1994; 69(3): 373-386. doi: 10.2307/3545850

Kerrison P, Le HN. Environmental factors on egg liberation and germling production of *Sargassum muticum*. J Appl Phycol. 2016; 28(1): 481-489. doi: 10.1007/s10811-015-0580-y

Kinlan BP, Graham MH, Sala E, Dayton PK. Arrested development of giant kelp (Macrocystis pyrifera, Phaeophyceae) embryonic sporophytes: a mechanism for delayed recruitment in perennial kelps. J Phycol. 2003; 39:47-57. doi: 10.1046/j.1529-8817.2003.02087.x

Korbee-Peinado N, Abdala-Díaz RT, Figueroa FL, Helbling EW. Ammonium and UV radiation simulate the accumulation of mycosporine-like amino acids in *Porphyra columbina* (Rhodophyta) from Patagonia, Argentina. J Phycol. 2004; 40: 248-259. doi: 10.1046/j.1529-8817.2004.03013.x

Korbee N, Figueroa FL, Aguilera J. Effect of light quality on the accumulation of photosynthetic pigments, proteins and mycosporine-like amino acids in the red alga *Porphyra leucosticta* (Bangiales, Rhodophyta). J Photochem Photobiol. 2005; 80: 71-78. doi:10.1016/j.jphotobiol.2005.03.002

Lamote M, Johnson LE. Temporal and spatial variation in the early recruitment of fucoid algae: the role of microhabitats and temporal scales. Mar Ecol Prog Ser. 2008; 368: 93-102.doi: 10.3354/meps07592

Liu W, Wu H, Liu M, Duan D. Improvement of the zygote utilization and reduction of the seedling loss in the early stage of seedling production of *Sargassum thunbergii* (Fucales, Phaeophyta). 2016; Ocean. Limnol. 34(3): 492-497. doi:10.1007/s00343-016-5041-1

Mangialajo L, Ruggieri N, Asnaghi V, Chiantore M, Povero P, Cattaneo-Vietti R. Ecological status in the Ligurian Sea: the effect of coastline urbanisation and the importance of proper reference sites. Mar Pollut Bull. 2007; 55(1): 30-41. doi:10.1016/j.marpolbul.2006.08.022

Mangialajo L, Chiantore M, Susini ML, Meinesz A, Cattaneo-Vietti R Thibaut T. Zonation patterns and interspecific relationships of fucoids in microtidal environments. J Exp Mar Bio Ecol. 2012; 412: 72-8. doi:10.1016/j.jembe.2011.10.031

Martínez B, Rico JM. Seasonal variation of P content and major N pools in *Palmaria palmata* (Rhodophyta). J Phycol. 2002; 38: 1082-1089. doi: 10.1046/j.1529-8817.2002.01217.x

Marzinelli EM, Campbell AH, Vergés A, Coleman MA, Kelaher BP, Steinberg PD. Restoring seaweeds: does the declining fucoid *Phyllospora comosa* support different biodiversity than other habitats? J Appl Phycol. 2014; 26(2): 1089-1096. doi 10.1007/s10811-013-0158-5

Mattoo AK, Hoffman-Falk H, Marder JB, Edelman M. Regulation of protein metabolism: coupling of photosynthetic electron transport to in vivo degradation of rapidly metabolized 32-kilodalton protein of the chloroplast membranes. Proc Nat. Aca. Sci. 1984; 81: 1380-1384.

Micheli F, Levin N, Giakoumi S, Katsanevakis S, Abdulla A, Coll M, et al. Setting priorities for regional conservation planning in the Mediterranean Sea. PLoS ONE. 2013; 8(4): e59038.8. https://doi.org/10.1371/journal.pone.0059038

Mineur F, Arenas F, Assis J, Davies A J, Engelen AH, Fernandes F et al. European seaweeds under pressure: Consequences for communities and ecosystem functioning. J Sea Res. 2015; 98: 91-108.

Morelisen B, Dudley BD, Geange SW, Phillips NE. Gametophyte reproduction and development of *Undaria pinnatifida* under varied nutrient and irradiance conditions. J Exp Mar Biol Ecol. 2013; 448: 197-206. https://doi.org/10.1016/j.jembe.2013.07.009

Munda IM. Changes and degradation of seaweed stands in the Northern Adriatic. Hydrobiologia. 1993; 260-239. doi:10.1007/BF00049025

Ohad I, Kyle DJ, Artntzen CJ. Membrane protein damage and repair: Removal and replacement of inactivated 32-kilodalton polypeptides in chloroplast. J Cell Biol. 1984; 99: 481-485.

Orfanidis S, Panayotidis P, Stamatis N. An insight to the ecological evaluation index (EEI). Ecol Indic. 2003; 3(1): 27-33. https://doi.org/10.1016/S1470-160X(03)00008-6

Pang S, Liu F, Shann T, Gao S, Zhang Z. Cultivation of the brown alga *Sargassum horneri*: Sexual reproduction and seedling production in tank culture under reduced solar irradiance in ambient temperature. J App Phycol. 2009; 21: 413-422. doi:10.1007/s10811-008-9386-5

Pérez-Lloréns JL, Vergara JJ, Pino RR, Hernandez I, Peralta G, Niell FX The effect of photoacclimation on the photosynthetic physiology of *Ulva curvata* and *Ulva rotundata* (Ulvales, Chlorophyta). Eur J Phycol. 1996; 31: 349-359. doi:10.1080/09670269600 651581

Reed DC, Brzezinski MA, Coury DA, Graham WM, Petty RL. Neutral lipids in macroalgal spores and their role in swimming. Mar Biol. 1999; 133:737-744. doi:10.1007/s002270050515

Sala E, Boudouresque CF, Harmelin-Vivien M. Fishing, trophic cascades, and the structure of algal assemblages: evaluation of an old but untested paradigm. Oikos. 1998; 82(3): 425-439. doi: 10.2307/3546364

Sales M, Cebrian E, Tomas F, Ballesteros E. Pollution impacts and recovery potential in three species of the genus *Cystoseira* (Fucales, Heterokontophyta). Estuar Coast Shelf Sci. 2011; 92(3): 347-357. https://doi.org/10.1016/j.ecss.2011.01.008

Sales M, Ballesteros E, Vidal E, Tomas F, Moranta J, Cebrian E. New method for restoring degraded *Cystoseira* forests. Eur J Phycol. 2015. 50(sup1): 108-109.

Schneider CA, Rasband WS., Eliceiri KW. NIH Image to ImageJ*: 25 years of image analysis.* Nature Methods. 2012; 9:671-675. doi:10.1038/nmeth.2089

Stekoll MS, Deysher L. Recolonization and restoration of upper intertidal *Fucus gardneri* (Fucales, Phaeophyta) following the Exxon Valdez oil spill. In: Lindstrom SC, Chapman DJ editors. Fifteenth International Seaweed Symposium. Developments in Hydrobiology. Springer Netherlands. 1996. 116: pp. 311-316. doi: 10.1007/978-94-009-1659-3_44

Sun JZ, Zhuang DG, Sun QH, Pang SJ. Artificial cultivation trials of *Sargassum horneri* at Nanji islands of China. South China Fish Sci. 2009; 5(6): 41-46.

Susini ML. Statut et biologie de *Cystoseira amentacea*var. *stricta*. PhD Thesis, Université Nice-Sophia Antipolis. 2006. Available from: ftp://nephi.unice.fr/users/ecomers/2006/2006%20Susini%20Th%C3%A8se.pdf

Susini ML, Mangialajo L, Thibaut T, Meinesz A. Development of a transplantation technique of *Cystoseira amentacea* var. *stricta* and *Cystoseira compressa*. Hydrobiologia. 2007; 580(1): 241-244. doi: 10.1007/s10750-006-0449-9

Templado J. Future trends of Mediterranean biodiversity. In*:* Goffredo S, Dubinsky Z editors. The Mediterranean Sea. Springer, Dordrecht Netherlands; 2014. pp. 479-498. doi: 10.1007/978-94-007-6704-1_28

Thibaut T, Pinedo S, Torras X, Ballesteros E. Long-term decline of the populations of Fucales (*Cystoseira* spp. and *Sargassum* spp.) in the Alberes coast (France, North-western Mediterranean). Mar Pollut Bull. 2005; 50(12): 1472-1489. https://doi.org/10.1016/j.marpolbul.2005.06.014

Thibaut T, Blanfuné A, Markovic L, Verlaque M, Boudouresque CF, Perret-Boudouresque M, Maćic V, Botti L. Unexpected abundance and long-term relative stability of the brown alga *C. amentacea* var. *stricta*, hitherto regarded as a threatened species, in the north-western Mediterranean Sea. Mar Pollut Bull. 2014; 89(1): 305-323. doi: 10.1016/j.marpolbul.2014.09.043

Van Tamelen PG, Stekoll MS, Deysher L. Recovery. Processes of the brown alga *Fucus gardneri* following the 'Exxon Valdez' oil spill: settlement and recruitment. Mar Ecol Prog Ser, 1997; 160: 265-277.

Vasquez JA, Tala F. Repopulation of intertidal areas with *Lessonia nigrescens* in northern Chile. J Appl Phycol. 1995; 7: 347-349. doi:10.1007/BF00003791

Verdura J, Cefali ME, Orfila E, Verges A, Sanchez M, Cebrian E. Optimal environmental conditions in *Cystoseira* sp early life stages. Eur J Phycol. 2015. 50(sup1): 214-214.

Vergara JJ, Bird KT, Niell FX. Nitrogen assimilation following NH4+ pulses in the red alga *Gracilariopsis lemaneiformis*: effect on C metabolism. Mar Ecol Prog Ser. 1995; 122(1-3): 253-263. doi: 10.3354/meps122253

Wiencke C, Gómez I, Pakker H, Flores-Moya A, Altamirano M, Hanelt D, Bischof K, Figueroa FL. Impact of UV-radiation on viability, photosynthetic characteristics and DNA of brown algal zoospores:implications for depth zonation. Mar Ecol Prog Ser. 2000; 197: 217-229.

Wilhelm C, Selmar D. Energy dissipation is an essential mechanism to sustain the viability of plants: the physiological limits of improved photosynthesis. J. Plant Physiol. 2011; 168: 79-87. doi:10.1016/j.jplph.2010.07.012

Yan XH, Zhang J. Embryology of zygote and development of germling in *Sargassum vachellianum* Greville (Fucales, Phaeophyta). J Appl Phycol. 2014; 26: 577-585. doi: 10.1007/s10811-013-0102-8

Yakovleva IM, Dring M, Titlyanov EA. Tolerance of North Sea algae to UV and visible radiation. Rus. J. Plant. Physiol. 1998; 45(1): 45-54.

Yatsuya K. Techniques for the restoration of *Sargassum* beds on barren grounds. Bull Fish Res Agen. 2010; 32: 69-73.

Yoon JT, Sun SM, Chung G. *Sargassum* bed restoration by transplantation of germlings grown under protective mesh cage. J Appl Phycol. 2014; 26(1): 505-509. doi:10.1007/s10811-013-0058-8

Yu YQ, Zhang QS, Tang YZ, Zhang SB, Lu ZC, Chu SH, Tang XX. Establishment of intertidal seaweed beds of *Sargassum thunbergii* through habitat creation and germling seeding. Ecological Engineering. 2012; 44: 10-17. http://dx.doi.org/10.1016/j.ecoleng.2012.03.016

Zhao Z, Zhao F, Yao J, Lu J, Ang PO, Duan D. Early development of germlings of Sargassum thunbergii (Fucales, Phaeophyta) under laboratory conditions. In: Borowitzka MA, Critchley AT, Kraan S, Peters A, Sjøtun K, Notoya M. editors. Nineteenth International Seaweed Symposium. Developments in Applied Phycology, vol 2. Springer, Dordrecht; 2008. pp 475-481. doi: 10.1007/978-1-4020-9619-8_57

Chapter 3

Bioactive potential of six brown algae (Fucales: Sargassaceae) from the Mediterranean Sea and the Atlantic Ocean

Authors: Gina De La Fuente, Susete Pinteus, Joana Silva, Celso Alves, Rui Pedrosa

In preparation

Abstract

In recent years, research on the bioactive properties of macroalgae has increased, due to the great interest in exploring new products that can contribute to improve human health and well-being. In the present study, the antioxidant and antimicrobial potential of six different brown algae of the Fucales order was evaluated. Five species belonging to the *Cystoseira* genus and *Sargassum vulgare* were collected along the Mediterranean and Atlantic coasts. The antioxidant capacity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, the Oxygen Radical Absorbent Capacity (ORAC) and the Ferric reducing antioxidant power (FRAP), and related to the total phenolic content (TPC) quantified on each Fucales species. The antimicrobial activity was evaluated measuring the growth inhibition of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. The highest antioxidant capacity was obtained for *Cystoseira tamariscifolia* alongside to its high value of TPC. This species also reported the highest antimicrobial capacity, but only on *Staphylococcus aureus*. Overall, the highest bioactivities were reported for *Cystoseira tamariscifolia*, *Cystoseira usneoides* and *Cystoseira amentacea*. Thus, *Cystoseira* species can be considered a good potential source of bioactive compounds, especially, antioxidants.

1. Introduction

The economic, cultural and scientific development of our society has given rise to important changes in our food habits and life-style. Diets in developed countries are highly caloric, rich in saturated fats and sugars, while the consumption of complex carbohydrates and dietetic fiber is low. This fact, together with a decrease in physical activity, has given rise to an increase of obesity problems, and along with it, a raise in the incidence of heart diseases, diabetes and hypertension in the population (WHO, 2017). Therefore, the interest, among consumers and food industry, on products that can promote health and well-being has increased appreciably during the last decades and these products are called functional food (Sloan, 1999; Menrad, 2003; Plaza et al., 2008; Siro et al., 2008).

Marine macroalgae are a rich source of minerals, fibers and vitamins, while being low in calories and fat and, they have been used during centuries as food and feed, particularly in Southern-east Asian countries, where the interaction human-macroalgae has a long tradition. Besides nutritional and domestic uses, different macroalgal applications started to appear in more recent years in the industry field, e.g. gels, fertilizers, (Delaney et al., 2016) and, more recently in the biotechnology field as source of natural bioactive compounds (Holdt and Kraan, 2011). Studies on antimicrobial (Pinteus et al., 2015), antitumoral (Alves et al., 2016), anti-inflammatory (Smit, 2004; da Matta et al., 2011) and antioxidant activities (Hefferman et al., 2015; Pinteus et al., 2017) provide examples of the bioactive properties of red, green and brown macroalgae. For these reasons, macroalgae can be considered one of the potential future “functional food” (Plaza et al., 2008; Mohamed et al., 2012).

The antimicrobial resistance among key microbial pathogen is increasing at a great rate worldwide, consequently, the research on new antimicrobial agents has become one of the greatest challenges facing humanity. Today, the Gram-positive bacteria *Staphylococcus aureus* and *Enterococci*, the Gram-negative bacteria *Escherichia coli* and the fungus species of *Candida* genus are among the most resistant microbes. Several studies on the antimicrobial capacity of macroalgae to these and other microbial species have been performed (Val et al., 2001; Etahiri et al., 2003; Genovese et al., 2009; Taskin et al., 2010; Pinteus et al., 2015; Belattmania et al., 2016).

As far as antioxidant properties, a large search for antioxidants from natural sources has increased because of the huge interest among consumers and food industry for natural products that can promote human health and well-being (Sloan, 1999; Lobo et al., 2010). In fact, artificial antioxidants as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been widely used in food, cosmetics, and therapeutic industries. Nowadays, the use of synthetic food additives is under strict legislation because of the toxic effects observed on different species (Branen, 1975; Kahl, 1984; Bjelakovic et al., 2004; Stanner et al., 2004).

In particular, the highest antioxidant capacity is reported for brown algae, probably in relation to the higher content in phenolic compounds of these species (Al-Amoudi et al., 2009; Costa et al., 2010; Cox, Abu-Ghannam, & Gupta, 2010; Kang et al., 2004; Kindleysides, Quek, & Miller, 2012; Pinteus et al., 2017).

The present work aims to study the antioxidant and antimicrobial activities of six brown algae belonging to *Cystoseira* and *Sargassum* genus (Fucales), collected in the Mediterranean and Atlantic coasts, in order to assess their bioactive potential as natural marine sources.

The antioxidant screening was addressed to measure the total phenolic content (TPC), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, the Oxygen Radical Absorbent Capacity (ORAC) and the

Ferric reducing antioxidant power (FRAP). The antimicrobial screening was performed measuring the growth inhibition of the following species: *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*.

2. Material and Methods

2.1 Chemicals and reagents

Dymetil sulphoxide (DMSO), Folin-Ciocalteu reagent, gallic acid; 2,2- diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescin (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), ferric tripyridyl triazine (TPTZ), ferric chloride ($FeCl_3$) and ferrous sulfate ($FeSO_4$) were purchased from Sigma-Aldrich (Madrid, Spain). Methanol and dichlorometane were purchased from Fisher Chemical.

2.2 Collection and extraction of algae samples

Six brown algae species, belonging the order Fucales, were gathered in summer along the intertidal zone of Italy (Genoa; 44°22'31.46"N - 9°4'35.14"E) and Portugal (Peniche; 39°22'1.60"N - 9°22'25.98"W) as shown in Figure 1. *Cystoseira amentacea* var. *stricta*, *Cystoseria compressa* were collected in Genoa and *Cystoseria tamariscifolia*, *Cystoseira usenoides*, *Cystoseira baccata* in Peniche. *Sargassum vulgare* was collected in both sites.

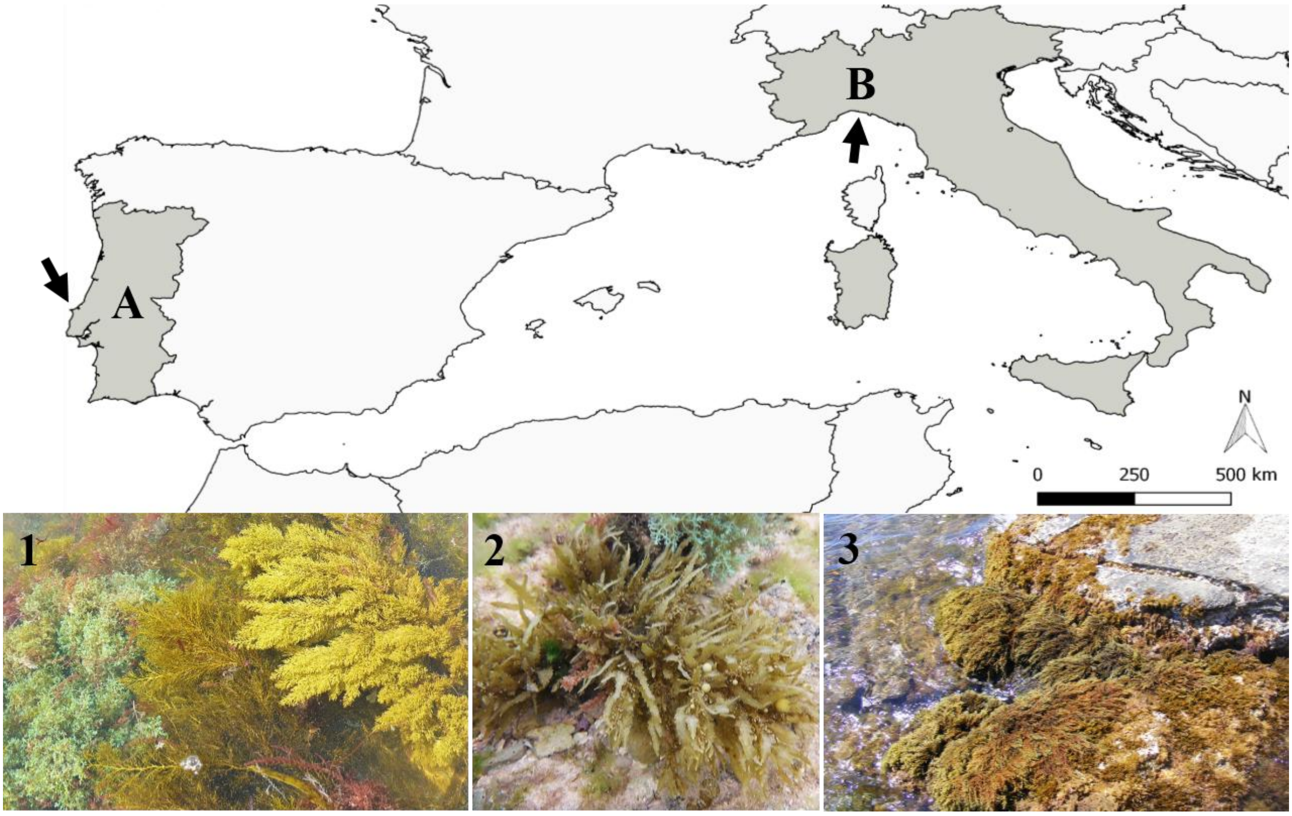


Figure 1. Map of the sampling sites. **A**- Atlantic site, Peniche, Portugal; **B**- Mediterranean site, Genoa, Italy. **1**- *C. tamariscifolia*, *C. baccata* and *C. usneoides*; **2**- *S. vulgare*; **3**- *C. amentacea* and *C. compressa*.

In the laboratory, the samples were cleaned accurately to remove detritus and epiphytes and then frozen at -80 ºC. In order to obtain a powder, the samples were lyophilized and ground. Each sample was extracted in

1:40 (w/v) biomass/solvent ratio with methanol and dichloromethane (50:50 - v/v) at constant stirring during 12h. The solvents were evaporated in a rotary evaporator (Laborota 4000, Heidolph, Schwabach, Germany) at 40 ºC and the extracts were solubilized in DMSO and stored at -20 ºC until further use.

2.3 Quantification of total phenolic contents (TPC)

The TPC of sample extracts were determined using Folin-Ciocalteu method adapted to microscale (Singleton & Rossi, 1965). Briefly, 2 µl of sample were added to 158 µl of distilled water, 10 µl of Folin-Ciocalteu reagent and 30 µl of 20% sodium carbonate. After one hour of reaction in the dark, the absorbance was measured at 755 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA) and used to calculate the phenolic content using gallic acid as the standard.

2.4 DPPH radical scavenging activity

The DPPH free radical scavenging method was performed according to Brand-Williams, Cuvelier, and Berset (1995) adapted to microscale with slight modifications. DPPH radical was dissolved in ethanol (0.1 mM). Various concentrations of 2 µL of sample solution were added to 198 µL of the DPPH radical solution. The mixture was vortexed for 1 min and allowed to stand at room temperature in the dark for 30 min, at which time the decrease in absorbance at 517 nm was measured (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments). The radical solution was freshly prepared each day. The ability to scavenge the DPPH radical was calculated using the follow equation:

$$\text{Scavenging effect (\%)} = \left[1 - \left(\frac{A\_sample - A\_sampleblank}{A\_control}\right)\right] \times 100$$

where,

A_control is the absorbance of the control (DPPH solution with DMSO)
A_sample is the absorbance of the test sample (DPPH solution plus test sample)
A_sampleblank is the absorbance of the sample in ethanol (sample without DPPH solution)

The results were expressed as antioxidant activity (%) relative to a control containing DMSO and as half maximal inhibitory concentration (IC_{50}) expressed in µg/mL.

2.5 Oxygen radical absorbent capacity (ORAC)

The ORAC assay was performed as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004) as follows: The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 µl. Sample (20 µl and fluorescein (120 µl; 70 nM, final concentration) were placed in the well of the microplate. The mixture was pre-incubated for 15 min at 37ºC. AAPH solution (60 µl; 12 mM, final concentration) was added rapidly using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence recorded every minute for 240 min. The microplate was automatically shaken prior each reading. A blank using phosphate buffer instead of the fluorescein and eight calibration solutions using Trolox (1-8 µM, final concentration) as antioxidant were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample.

Antioxidant curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor $\text{fluorescence}_{\text{blank,t=0}}/\text{fluorescence}_{\text{sample,t=0}}$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = 1 + \sum_{i=1}^{i-80} fi/f0$$

where f0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i.* The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples. ORAC values were expressed as Trolox equivalents by using the standard curve calculated for each assay. Final results were in µmol of Trolox equivalents (TE)/g of extract.

2.6 Ferric reducing antioxidant power (FRAP)

The ability to reduce ferric ions was measured using the modified method described by Benzie and Strain (1999). The FRAP reagent was freshly prepared from 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) made up in 20 mM $FeCl_3$. The mixture was incubated at 37 °C. Briefly, 2 µl of sample were added to 198 µl of FRAP reagent. After 4 min of reaction in the dark, the absorbances were read using a microplate reader at 593 nm. The FRAP value was calculated and expressed as µMl of $FeSO_4$ equivalents/g extract based on a calibration curve constructed using $FeSO_4$ as standard.

2.7 Antimicrobial activity

The antimicrobial activity was tested measuring the capacity to inhibit the Gram+ bacteria *Staphylococcus aureus* (ATCC 25923), the Gram- bacteria *Pseudomonas aeruginosa* (ATCC 27853) and the fungus *Candida albicans* (ATCC 10231) growth. Bacteria were cultured in Luria broth (LB) medium, and *C. albicans* in yeast extract peptone dextrose (YPD) medium. Tests were performed in 96-well plates at 37 °C. The growth was quantified by OD measurement at 600 nm after 6 h growth (exponential phase growth). The results of the antimicrobial activity were expressed as IC_{50}, defined as the concentration causing 50% reduction of microorganism growth.

2.8 Data analysis

The IC_{50} concentration was calculated from nonlinear regression analysis using the GraphPad Prism software with the equation: $Y = 100/ [1 + 10^{(X - LogIC50)}]$.

In order to assess differences of antioxidant activity among species, a PERMANOVA was performed using the values obtained from the different tests as response variables and the species as factor. Then, a pairwise PERMANOVA was performed, using the “RVAideMemoire” package. Additionally, a Principal Components

Analysis (PCA) was performed in order to visualize the relation between the antioxidant capacity and the total phenolic content, as well as to point out the differences among the Fucales species on their antioxidant capacity.

3. Results and discussion

3.1 Antioxidant capacity

The total phenolic content of the methanol and dichloromethane (50:50 - v/v) extract of six Fucales species, evaluated by Folin-Ciocalteu method is shown in Table 1. In this study*, Cystoseira baccata*, *Cystoseria usneoides* and *Cystoseira amentacea* obtained higher values (45-65 mg GAE / g extract) and, the highest value was obtained for *Cystoseira tamariscifolia* (~120 mg GAE / g extract). The lowest values were obtained for *Cystoseira compressa* (<30 mg GAE / g extract) and, particularly, for *Sargassum vulgare* (<7 mg GAE / g extract).

Table 1. Total phenolic content (TPC, mg GAE / g extract) of organic extracts of the different Fucales species. Values are mean of three independent experiments ± standard error of the mean (SEM).

Species	mg GAE / g extract	SEM
Cystoseira amentacea	45.95	5.88
Cystoseira baccata	52.27	4.63
Cystoseira compressa	27.84	2.87
Cystoseria tamariscifolia	121.52	27.43
Cystoseira usneoides	62.07	5.10
Sargassum vulgare (Mediterranean)	6.60	0.65
Sargassum vulgare (Atlantic)	5.48	0.99

The total phenolic content has been widely related with the antioxidant capacity. This correlation particularly stands for brown algae, where a higher antioxidant capacity is generally reported compared to green and red algae (Balboa et al., 2013), because brown algae are able to produce particular phenolic compounds, the phlorotannins that confer this property. These compounds are involved in chemical defenses against herbivores, provide structural functions in cell walls and are involved in growth and reproduction processes (Kubanek e al., 2004).

In the present study, the PCA (Figure 2) supports the correlation among TPC and the antioxidant activities (DPPH, ORAC and FRAP). The variability across the 18 extracts of Fucales species is accounted mainly by PC1 (87.7%), which is highly correlated with FRAP, separating *C. tamariscifolia* of the other species.

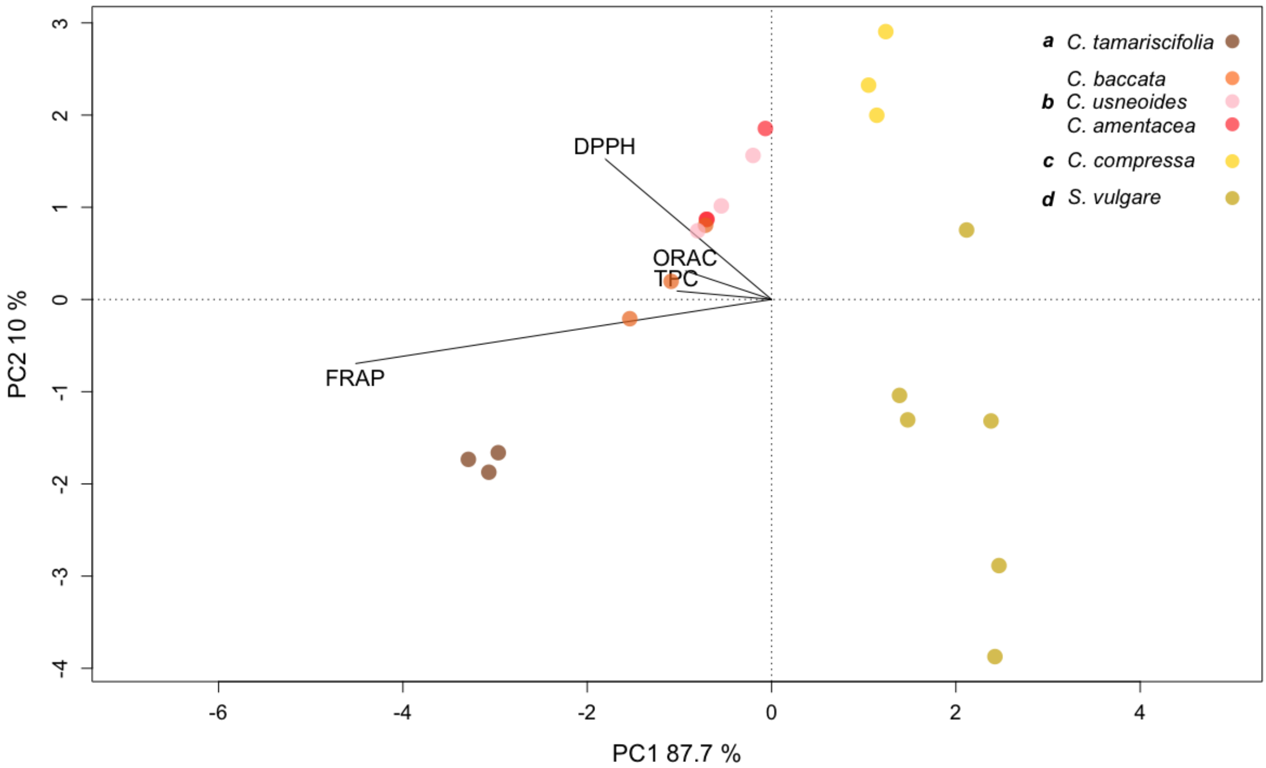


Figure 2. Principal components analysis of total phenolic content (TPC) and antioxidant activities (DPPH, ORAC and FRAP) of organic extracts (methanol:dichloromethane; 50:50 - v/v) of the six Fucales species from Mediterranean and Atlantic coasts.

On the other hand, the PERMANOVA results and the post-hoc analyses showed significant differences among all species (p-value< 0.01), except *C. amentacea*, *C. baccata* and *C. usneoides,* which were not significantly different from each other. The highest antioxidant capacity for *C*. *tamariscifolia*, which obtained the highest values in each antioxidant test performed, specifically in the FRAP test (1760 mg FeE / g extract, Table 4) is observed in the PCA. The group 2 with similar results among the species in the different antioxidant tests is also evident. Instead, *C*. *compressa* and *S*. *vulgare* obtained lower values (Tables 2-4), which were positioned at the opposite site (at right of the PC1) of the antioxidant capacity tests and the total phenolic content.

Table 2. The IC_{50} of 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity expressed as µg per millimeter (µg/ml). Values are expressed as means with 95% confidence intervals.

Species	**IC_{50} (µg/ml)**	**95% confidence interval (µg/ml)**
Cystoseira amentacea	87.29	73.81 - 103.20
Cystoseira compressa	142.5	122.20 - 166.20
Sargassum vulgare (Mediterranean)	695.5	548.30 - 882.30
Cystoseira tamariscifolia	27.02	22.33 - 32.69
Cystoseira baccata	60.64	53.19 - 69.14
Cystoseira usneoides	108.2	85.84 - 136.40
Sargassum vulgare (Atlantic)	619.3	519.80 - 737.70

Table 3. The values of the oxygen radical absorbance capacity (ORAC) are expressed as µmol of Trolox equivalents per gram of extract (µmol TE / g extract). Values are mean of three independent experiments ± standard error of the mean (SEM).

Species	**µmol TE / g extract**	**SEM**
Cystoseira amentacea	149.55	12.11
Cystoseira baccata	94.67	6.30
Cystoseira compressa	58.40	12.09
Cystoseira tamariscifolia	138.92	4.40
Cystoseira usneoides	148.24	10.77
Sargassum vulgare (Mediterranean)	25.45	1.87
Sargassum vulgare (Atlantic)	30.44	0.67

Table 4. The values of the ferric reducing antioxidant power (FRAP) are expressed as micromolar of $FeSO_4$ equivalents per gram of extract (µM FeE / g extract). Values are mean of three independent experiments ± standard error of the mean (SEM).

Species	µM **FeE / g extract**	**SEM**
Cystoseira amentacea	353.82	47.97
Cystoseira baccata	463.92	35.97
Cystoseira compressa	76.37	5.55
Cystoseira tamariscifolia	1761.19	19.89
Cystoseira usneoides	692.56	58.24
Sargassum vulgare (Mediterranean)	17.36	3.34
Sargassum vulgare (Atlantic)	86.99	20.06

The total phenolic content obtained for *C. compressa* differs from other studies (48.3 mg GAE / g extract; Chkhikvishvili & Ramazanov, 2000; ~61 mg GAE / g extract, Maldhebi et al., 2013). The low antioxidant capacity of *S. vulgare* observed in the present study is not in accordance with other studies performed on *Sargassum* species (Lim et al., 2002; Yangthong et al., 2009; Pinteus et al., 2017). Additionally, different results for *Sargassum vulgare* was obtained in Plaza et al., 2010, where the values obtained were much higher than our results, ranging from 26.9 to 70.9 mg GAE / g extract (SW-100 ºC; SW-200 ºC).

Other *Sargassum* species have shown very varied values of total phenolic content: *Sargassum furcatum* and *Sargassum desfrontrainessi* (29.7 and 16.8 mg GAE / g extract; Chkhikvishvili & Ramazanov, 2000); *Sargassum horridium* (~0.1 mg GAE / g extract; Tenorio-Rodriguez et al., 2017); *Sargassum muticum* (~10 mg GAE / g extract, Plaza et al., 2010; ~38 mg GAE / g extract, Lee and Kim, 2015; 24-71 mg GAE / g extract; Pinteus et al., 2017).

Regarding *Cystoseira tamariscifolia*, in other studies were reported similar results, as in the work of Zubia et al., 2009 to the brown algae species from Brittany coast, where *Cystoseira tamariscifolia* extract obtained also the highest value (109 mg GAE / g extract), and in Vizetto-Duarte et al., 2016 (~165 mg GAE / g DW). Contrary, in Pinteus et al., 2017, although the Fucales species obtained the highest values of phenolic content, *Cystoseira* *tamariscifolia* obtained a lower value than in this study (~57 mg GAE / g extract), instead, *Cystoseira usneoides* obtained a similar value (~86 mg GAE / g extract).

Overall, the highest antioxidant capacity performing TPC, ORAC and mainly DPPH is reported for Fucales genera, particularly in *Cystoseira* and *Fucus* (Zubia et al., 2009; Andrade et al., 2013; Vizetto-Duarte et al., 2016; Pinteus et al., 2017; Tenorio-Rodriguez et al., 2017). In the studies on polar extracts of *Cystoseira* species high values of phenolic content were reported but, as find for *Sargassum* species, with a noticeable variability among them: *Cystoseira sedoides* (50.3 mg GAE / g extract; Mhadhebi et al., 2013), *Cystoseira crinita* (56.5 mg GAE / g extract; Mhadhebi et al., 2013), *Cystoseira abies-marina* (6.81 and 48.09 mg GAE / g extract; Plaza et al., 2010) or *Cystoseira hakodatensis* (31.3 mg GAE / g extract).

Interestingly, the lowest antioxidant capacity was observed in the more resilient species, *C. compressa* and *S. vulgare,* while the highest antioxidant activity was observed for the threatened and protected species, such as *C. tamariscifolia* and *C. amentacea*. This fact may be due to the amount of phenolic content that is produced by these species in response to different stress conditions (Amsler and Fairhead, 2006; Stiger-Pouvreau et al., 2014): a lower production of these secondary metabolites is performed in those species which are more tolerant to moderately polluted habitats. Recent studies (Mannino et al., 2014, 2017) have elucidated variation of the TPC from temperate brown algae (Fucales and Dictyotales) due to different factors such as morphology, exposition, solar radiation and bathymetric level that can explain the disparity in antioxidant activity results among the studies. Additionally, season of collection and preparation of the extract are other important factors affecting the results of the different antioxidant tests (Tenorio-Rodriguez et al., 2017).

3.2 Antimicrobial activity

Antimicrobial activity of the extracts of six Fucales species was tested against *Staphylococcus aur*eus, *Pseudomonas aerug*inosa and *Candida albicans* growth. The Figure 3 shows antimicrobial capacity only in *S. aureus*, where the macroalgae extracts caused more than 50% growth inhibition at the maximum concentration tested (1mg / ml). In fact, several studies reveal the Gram+, as *S. aureus,* are more sensitive to macroalgal extracts than the more resistant Gram- bacteria, especially, *P. aeruginosa* (Pesando and Caram, 1984; Mhadhebi et al., 2011; Alghazeer et al., 2013; Pinteus et al., 2015). The higher resistance in Gram- bacteria is related to a complex envelope with an outer and an inner membrane delimiting a periplasmic space, forming various protein channels involved in the transport, uptake or efflux, of a large variety of compounds, therefore representing a barrier to the penetration of antibiotic (Pagès et al., 2008; Nikaido, 2009).

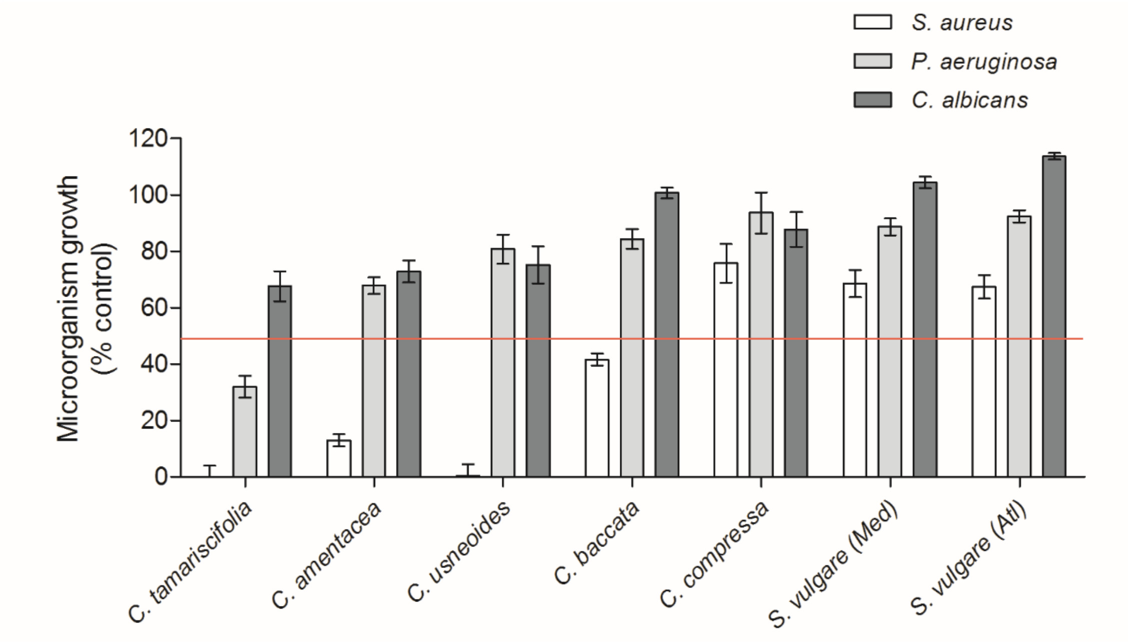


Figure 3. Antimicrobial activity of the extracts tested against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans* growth (1 mg/ml). Values in each column are the mean of two or three independent experiments ± standard error of the mean (SEM). Values below the red line indicate the capacity of the macroalage extracts to cause more than 50% growth inhibition.

In the present study, a higher capacity to inhibit *S. aureus* was observed for *C. tamariscifolia*, with an IC_{50} of 267.9 µg/mL, while a lower capacity was observed on *C. amentacea* and *C. usneoides* with similar values (IC_{50} ~400-425 µg/ml. The dose response analysis on *C. baccata* extracts revealed an IC_{50} >1000 µg/mL (Table 5).

Table 5. IC_{50} values of the growth inhibition of *Staphylococcus aureus* expressed as µg per millimeter (µg/ml). Values are expressed as means with 95% confidence intervals. The results are the mean of two or three independent experiments.

Species	**IC_{50} (µg/ml)**	**95% confidence interval (µg/ml)**
Cystoseira tamariscifolia	267.9	212.3 - 338.2
Cystoseira usneoides	396.7	288.2 – 546.1
Cystoseira amentacea	426.9	344.2 – 529.4
Cystoseira baccata	>1000	

For *P. aeruginosa* and *C. albicans* all macroalgal extracts presented an IC_{50} >1000 µg/mL. The absence of antimicrobial activity for *Cystoseira compressa* and *Sargassum vulgare* are in agreement with other previous studies (Ibtissam et al., 2009; Mhadhebi et al., 2011). On the contrary, in Etahiri et al. (2003) *Sargassum vulgare* displayed high antibacterial activity against *S. aureus*, whose growth was also inhibited in presence of *Cystoseira tamariscifolia* and *Cystoseira humilis*. In addition to these latter species, also *C. mediterranea* and *C. usneoides* show a high antibacterial activity for Gram+ bacteria (*S. aureus*) but also for Gram- bacteria (Ibtissam et al., 2009). In the present study, we observed high antibacterial activity for Gram+ bacteria, but no activity against Gram- bacteria (*P. aeruginosa*) for all *Cystoseira* extracts.

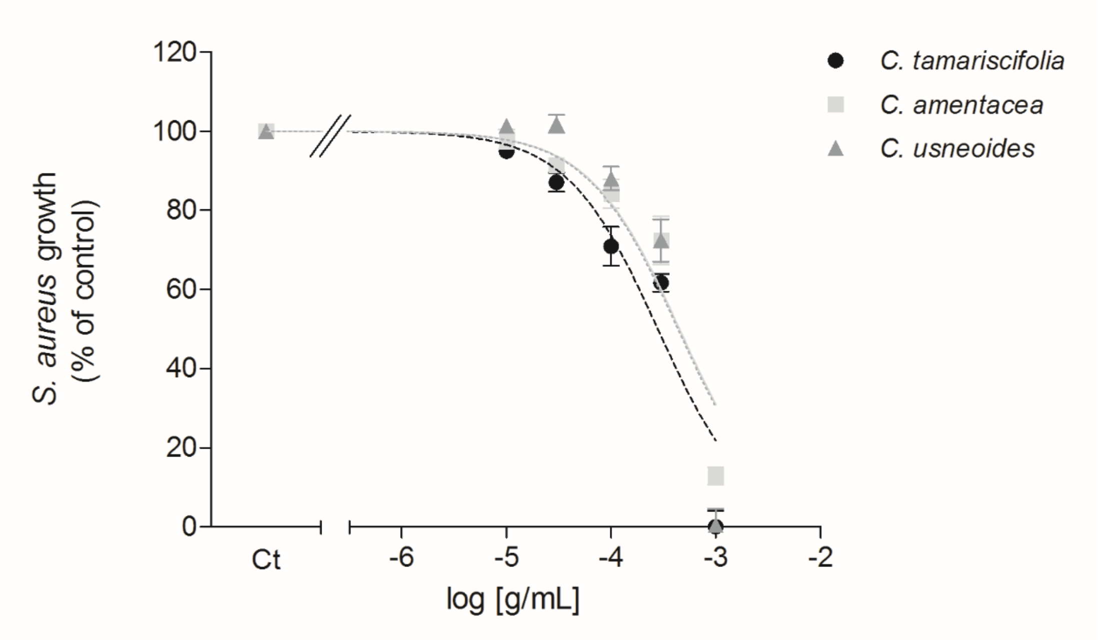


Figure 4. *Staphylococcus aureus* growth (6 hours) in the presence of different concentrations of polar extracts (10–1,000 µg/ml). The results are the mean of two or three independent experiments. ± standard error of the mean (SEM).

As far as antifungal activity against *Candida* species, in the present study, no activity was recorded for any of the investigated species, contrarily to the findings in *C. sedoides* and *C. crinita* (Mhadhebi et al., 2011).

Conclusions

The higher antioxidant activity of brown algae compared to green and red algae is clearly confirmed by the consistent results reported in literature. Instead the antibacterial activity is still controversial, reporting highest antibacterial activity in brown algae (Etahiri et al., 2003; Rizve and Shameel, 2004; Mhadhebi et al., 2011; Alghazeer et al., 2013) or in red algae (Mahasneh et al., 1995; Val et al., 2001; Kaaria et al., 2015; Pinteus et al., 2015), alternatively.

In this work, the antioxidant and antibacterial activities were highlighted for the polar extracts of several *Cystoseira* species. The highest bioactivities were obtained for *Cystoseira tamariscifolia* followed by *Cystoseira usneoides* and *Cystoseira amentacea*. Conversely, the *Sargassum vulgare* displayed limited antioxidant activity and no antimicrobial one. Therefore, *Cystoseira* species can be considered as good potential source of bioactive properties and a promising source of health products.

Aknowledgements

The Erasmus+ Programme / KA1 A.Y. 2017-18; *Student Mobility for traineeship*, has facilitated the collaboration of G. De La Fuente in the research activities of the present study at IPLeiria – Cetemares.

References

Al-Amoudi OA, Mutawie HH, Patel AV, Blunden G. Chemical composition and antioxidant activities of Jeddah corniche algae. Saudi Arabia. Saudi J Biol Sci. 2009; 16(1): 23-29.

Alghazeer R, Whida F, Abduelrhman E, Gammoudi F, Azwai S. Screening of antibacterial activity in marine green, red and brown macroalgae from the western coast of Libya. Nat Sci. 2013; 5(1): 7.

Alves C, Pinteus S, Horta A, Pedrosa R. High cytotoxicity and anti-proliferative activity of algae extracts on an *in vitro* model of human hepatocellular carcinoma. Springerplus. 2016; *5*(1): 1339.

Amsler CD, Fairhead VA. Defensive and sensory chemical ecology of brown algae. Adv. Bot. Res. 2006; 43: 1-91.

Andrade PB, Barbosa M, Matos RP, Lopes G, Vinholes J, Mouga T, Valentão P. Valuable compounds in macroalgae extracts. Food chem. 2013; 138(2): 1819-1828.

Balboa EM, Conde E, Moure A, Falqué E, Domínguez H. *In vitro* antioxidant properties of crude extracts and compounds from brown algae. Food chem. 2013; 138(2): 1764-1785.

Belattmania Z, Reani A, Barakate M, Zrid R, Elatouani S, Hassouani M, Eddaoui A, Bentiss F, Sabour B. Antimicrobial, antioxidant and alginate potentials of *Dictyopteris polypodioides* (Dictyotales, Phaeophyceae) from the Moroccan Atlantic coast. Pharma Chem. 2016; 8: 216-226.

Benzie IF, Strain JJ. [2] Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Method enzymol. 1999; 299: 15-27.

Brand-Williams W, Cuvelier ME, Berset CLWT. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci Technol. 1995; 28(1): 25-30.

Branen AL. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. J Am Oil Chem Soc. 1975; 52(2): 59-63.

Bjelakovic G, Nikolova D, Simonetti RG, Gluud C. Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. The Lancet. 2004; 364(9441): 1219-1228.

Brito da Matta CB, De Souza ÉT, De Queiroz AC, De Lira DP, De Araújo MV, Cavalcante-Silva LHA, De Miranda GEC, De Araújo-Junior JX, Barbosa-Filho JM, De Oliveiras Santos BV, Alexandre-Moreira MS. Antinociceptive and anti-inflammatory activity from algae of the genus *Caulerpa*. Mar drugs. 2011; 9(3): 307-318.

Chkhikvishvili ID, Ramazanov ZM. Phenolic substances of brown algae and their antioxidant activity. Applied Biochemistry and Microbiology. 2000; 36(3): 289-291.

Costa LS, Fidelis GP, Cordeiro SL, Oliveira RM, Sabry DA, Câmara RBG, Nobre LTDB, Costa MSSP, Almeida-Lima J, Farias EHC, Leite EL, Rocha HAO. Biological activities of sulfated polysaccharides from tropical seaweeds. Biomedicine & Pharmacotherapy. 2010; 64(1): 21-28.

Cox S, Abu-Ghannam N, Gupta S. An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. International Food Research Journal. 2010; 17: 205-220.

Dávalos A, Gómez-Cordovés C, Bartolomé B. Extending applicability of the oxygen radical absorbance capacity (ORAC− fluorescein) assay. Journal of agricultural and food chemistry. 2004; 52(1): 48-54.

Delaney A, Frangoudes K, Ii SA. Society and seaweed: understanding the past and present. In: Fleurence, J. & Levine, I, editors. Seaweed in Health and Disease Prevention. London: Elsevier Academic Press; 2016. p. 7-40.

Etahiri S, Bultel-Poncé V, Elkouri AE, Assobhei O, Zaoui D, Guyot M. Antibacterial activities of marine algae from the Atlantic coast of Morocco. Mar Life. 2003; 13: 3-9.

Genovese G, Tedone L, Hamann MT, Morabito M. The Mediterranean red alga *Asparagopsis*: a source of compounds against Leishmania. Marine drugs. 2009; 7(3): 361-366.

Holdt SL, Kraan S. Bioactive compounds in seaweed: functional food applications and legislation. Journal of Applied Phycology. 2011; 23(3): 543-597.

Ibtissam C, Hassane R, Jose M, Francisco DSJ, Antonio GVJ, Hassan B, Mohamed K. Screening of antibacterial activity in marine green and brown macroalgae from the coast of Morocco. African Journal of Biotechnology. 2009; 8(7).

Kaaria P, Wakibia J, Matiru V, Ndung'u M, Bii C. Antimicrobial Screening of Marine Endophytes and Epiphytes Isolated from Marine Algae of Kenyan Indian Ocean. Journal of Applied & Environmental Microbiology. 2015; 3(3): 70-74.

Kahl R. Synthetic antioxidants: biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. Toxicology. 1984; 33(3-4): 185-228.

Kang HS, Chung HY, Kim JY, Son BW, Jung HA, Choi JS. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. Archives of pharmacal research. 2004; 27(2): 194-198.

Kindleysides S, Quek SY, Miller MR. Inhibition of fish oil oxidation and the radical scavenging activity of New Zealand seaweed extracts. Food Chemistry. 2012; 133(4): 1624-1631.

Plaza M, Amigo-Benavent M, Del Castillo MD, Ibáñez E, Herrero M. Facts about the formation of new antioxidants in natural samples after subcritical water extraction. Food Research International. 2010; 43(10): 2341-2348.

Kubanek J, Lester SE, Fenical W, Hay ME. Ambiguous role of phlorotannins as chemical defenses in the brown alga *Fucus vesiculosus*. Marine Ecology Progress Series. 2004; 277: 79-93.

Lee JH, Kim GH. Evaluation of antioxidant activity of marine algae-extracts from Korea. Journal of Aquatic Food Product Technology. 2015; 24(3): 227-240.

Lim SN, Cheung PCK, Ooi VEC, Ang PO. Evaluation of antioxidative activity of extracts from a brown seaweed, *Sargassum siliquastrum*. Journal of Agricultural and Food Chemistry. 2002; 50(13): 3862-3866.

Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy reviews. 2010; 4(8): 118.

Mahasneh IHSAN, Jamal M, Kashashneh M, Zibdeh M. Antibiotic activity of marine algae against multi-antibiotic resistant bacteria. Microbios. 1995; 83(334): 23-26.

Mhadhebi L, Chaieb K, Bouraoui A. Evaluation of antimicrobial activity of organic fractions of six marine algae from Tunisian Mediterranean coasts. Int. J. Pharm. Pharm. Sci. 2012; 4(1): 534-537.

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*. Iranian journal of pharmaceutical research: IJPR. 2014; 13(1): 207.

Mannino AM, Vaglica V, Oddo E. Seasonal variation in total phenolic content of *Dictyopteris polypodioides* (Dictyotaceae) and *Cystoseira amentacea* (Sargassaceae) from the Sicilian coast. Fl Medit. 2014; 24: 39-50.

Mannino AM, Vaglica V, Oddo E. Interspecific variation in total phenolic content in temperate brown algae. Journal of Biological Research-Bollettino della Società Italiana di Biologia Sperimentale. 2017; 90(1).

Menrad K. Market and marketing of functional food in Europe. Journal of food engineering. 2003; 56(2): 181-188.

Mohamed S, Hashim SN, Rahman HA. Seaweeds: a sustainable functional food for complementary and alternative therapy. Trends in Food Science & Technology. 2012; 23(2): 83-96.

Nikaido H. Multidrug resistance in bacteria. Annual review of biochemistry. 2009; 78: 119-146.

Ozdemir G, Horzum Z, Sukatar A, Karabay-Yavasoglu NU. Antimicrobial activities of volatile components and various extracts of *Dictyopteris membranaceae* and *Cystoseira barbata* from the coast of Izmir, Turkey. Pharmaceutical biology. 2006; 44(3): 183-188.

Pagès JM, James CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nature Reviews Microbiology. 2008; 6(12): 893-903.

Pesando D, Caram B. Screening of marine algae from French Mediterranean coast for antibacterial and antifungal activity. Bot. Mar. 1984; 27: 381-386.

Pinteus S, Alves C, Monteiro H, Araújo E, Horta A, Pedrosa R. *Asparagopsis armata* and *Sphaerococcus coronopifolius* as a natural source of antimicrobial compounds. World Journal of Microbiology and Biotechnology. 2015; 31(3): 445-451.

Pinteus S, Silva J, Alves C, Horta A, Fino N, Rodrigues AI, Mendes S, Pedrosa R. Cytoprotective effect of seaweeds with high antioxidant activity from the Peniche coast (Portugal). Food chemistry. 2017; 218: 591-599.

Plaza M, Cifuentes A, Ibáñez E. In the search of new functional food ingredients from algae. Trends in Food Science & Technology. 2008; 19(1): 31-39.

Plaza M, Amigo-Benavent M, Del Castillo MD, Ibáñez E, Herrero M. Facts about the formation of new antioxidants in natural samples after subcritical water extraction. Food Research International. 2010; 43(10): 2341-2348.

Rizvi MA, Shameel M. Biological activity and elementology of benthic algae from Karachi coast. Pakistan Journal of Botany. 2004; 35(5; SPI): 717-730.

Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American journal of Enology and Viticulture. 1965; 16(3): 144-158.

Siro I, Kápolna E, Kápolna B, Lugasi A. Functional food. Product development, marketing and consumer acceptance—A review. Appetite. 2008; 51(3): 456-467.

Sloan AE. The new market: foods for the not-so-healthy. Food technology. 1999; 53(2): 54-60.

Smit AJ. Medicinal and pharmaceutical uses of seaweed natural products: a review. Journal of applied phycology. 2004;16(4): 245-262.

Stanner SA, Hughes J, Kelly CNM, Buttriss J. A review of the epidemiological evidence for the ‘antioxidant hypothesis’. Public health nutrition. 2004; 7(3): 407-422.

Stiger-Pouvreau V, Jégou C, Cérantola S, Guérard F, Le Lann K. Phlorotannins in Sargassaceae species from Brittany (France): Interesting molecules for ecophysiological and valorisation purposes. Adv. Bot. Res. 2014; 71: 379-412.

Taskin E, Caki Z, Ozturk M. Assessment of *in vitro* antitumoral and antimicrobial activities of marine algae harvested from the eastern Mediterranean Sea. African Journal of Biotechnology. 2010; 9(27): 4272-4277.

Tenorio-Rodriguez PA, Murillo-Álvarez JI, Campa-Cordova ÁI, Angulo C. Antioxidant screening and phenolic content of ethanol extracts of selected Baja California Peninsula macroalgae. Journal of food science and technology. 2017; 54(2): 422-429

Val A, Platas G, Basilio A, Cabello A, Gorrochategui J, Suay I, Vicente F, Portillo E, Río M, Reina G, Peláez F. Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain). International Microbiology. 2001; 4(1): 35-40.

Vizetto-Duarte C, Custódio L, Acosta G, Lago JH, Morais TR, de Sousa CB, Gangadhar KN, Rodrigues MJ, Pereira H, Lima RT, Vasconcelos MH, Barreira L, Rauter AP, Albericio F, Varela J. Can macroalgae provide promising anti-tumoral compounds? A closer look at *Cystoseira tamariscifolia* as a source for antioxidant and anti-hepatocarcinoma compounds. PeerJ. 2016; 4: e1704.

World Health Organization. Reviewed February 2018. Obesity and Overweight Fact Sheet no. 311. Available: www.who.int/mediacentre/factsheets/fs311/en/.

Yangthong M, Hutadilok-Towatana N, Phromkunthong W. Antioxidant activities of four edible seaweeds from the southern coast of Thailand. Plant foods for human nutrition. 2009; 64(3): 218-223.

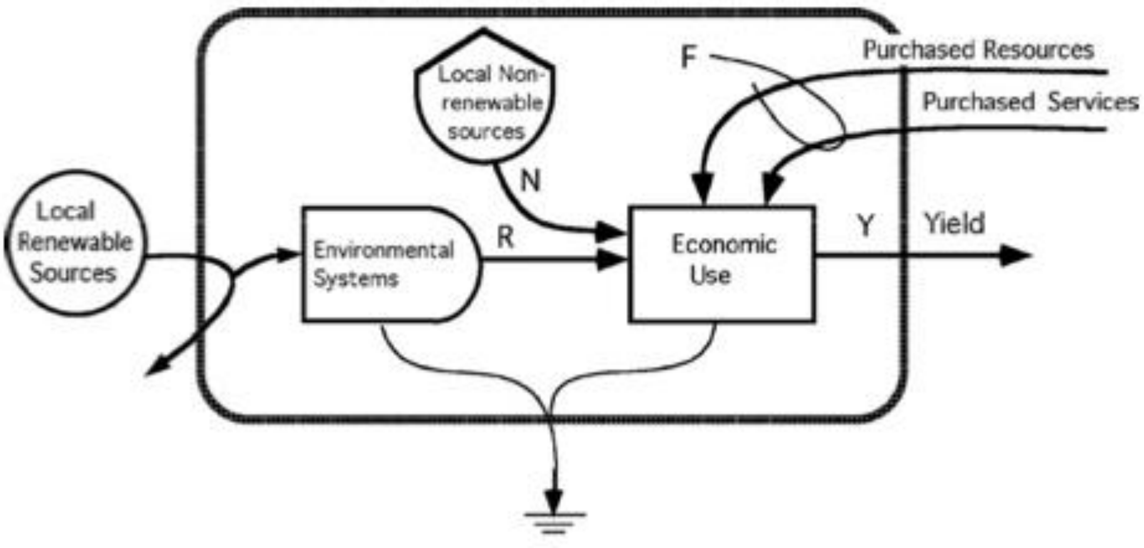
Zubia M, Fabre MS, Kerjean V, Le Lann K, Stiger-Pouvreau V, Fauchon M, Deslandes E. Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chemistry. 2009; 116(3): 693-701.

Chapter 4

The effect of *Cystoseira* canopy on the midlittoral habitat value in NW Mediterranean, an emergy assessment

Authors: Gina De La Fuente, Valentina Asnaghi, Mariachiara Chiantore, Paolo Povero, Paolo Vassallo, Chiara Paoli.

In preparation



Abstract

Along Mediterranean shallow rocky reefs, brown macroalgae belonging to the Cystoseira genus (e.g. Cystoseira amentacea and C. compressa) are ecosystem engineer, locally creating a continuous fringe in the midlittoral zone, supporting high biodiversity and productivity and providing relevant ecosystem services. A growing interest in the quantification of the economic value of ecosystems arose in the last decades aiming at raising awareness of the general public and supporting policy-makers in the process of creating new legal instruments towards the preservation of biodiversity. In this framework, a methodology for ecological and monetary evaluation of natural capital, based on biophysical accounting and on emergy theory is applied to the upper midlittoral habitat influenced by the continuous and non-continuous Cystoseira fringe. The proposed approach is a quantitative measure able to analyse the overall functioning of the system and its efficiency in exploiting available resources. The mean economic value of the midlittoral habitat assessed through the present study is 1.04 em€/m2, consistent with the average values calculated for other benthic habitats. Higher values and larger variability were observed in presence of continuous Cystoseira compressa fringe, which is present in sites with different levels of pollution. Instead, in presence of a continuous Cystoseira amentacea fringe, a noticeable sensitivity species, lower variability was recorded showing a deterministic homogeneity effect on the above midlittoral habitat. From this study, emergy analysis is confirmed to represent an effective and operative tool to provide a synthetic monetary assessment of ecosystem services making a complex information easily accessible to different stakeholders, from general public to territorial managers.

1. Introduction

Reefs are rocky marine habitats or biological concretions that rise from the seabed. Reefs may support a zonation of benthic communities of algae and animal species as well as concretions (corallogenic or not). They are generally subtidal but may extend as an unbroken transition into the intertidal zone, where they are exposed to the air at low tide. Reefs are very variable in form and in the communities that they support, which are characterised by communities of attached algae and invertebrates, usually associated with a range of mobile animals, including invertebrates and fish. The different tipologies of reef, which are characterised by a specific benthic community, are protected under the EU Habitats Directive (Council Directive 92/43/EEC), within the Natura 2000 ecological network of protected areas. In the Mediterranean Sea *Cystoseira*/*Sargassum* beds are the most relevant species within this habitat, supporting a mixture of other red algae (*e.g.* Gelidiales, Ceramiales), brown algae (*e.g.* Dictyotales) and green algae (*e.g.* Siphonales, Siphonacladales). Additionally, this habitat is characterised by the presence of biogenic reef forming animals, such as polychaetes (*e.g. Sabellaria alveolata*) and bivalve molluscs, and non-reef forming animals, as barnacles, hydroids, bryozoans, ascidians, sponges, gorgonians and polychaetes as well as diverse mobile species of crustaceans and fish.

Benthic organisms (macrophytes and macrofauna) have been identified as ecological quality status indicators for this habitat in the Water Framework Directive (WFD 2000/60/EC) of the European Community, according to their different sensitivity levels to environmental stress (Borja et al., 2000; Krause-Jensen et al., 2005; Pinedo et al., 2007; Romero et al., 2007; Orfanidis et al., 2011; Díez et al., 2012).

Rocky midlittoral, i.e. the zone between the high and low tides along coast and the interface between terrestrial and marine realms, is the most impacted by anthropization, in terms of coastal artificialization, human collection and trampling, floating pollutants, particularly affecting the first few meters and compromising superficial benthic communities (Arnoux and Bellan-Santini, 1972; Belsher and Boudouresque, 1974; Verlaque and Tine, 1979; Soltan et al., 2001), with consequent habitat impoverishment.

The midlittoral zone displays macroalgal assemblages mainly characterised by algal turfs (composed of a matrix of small macroalgae mainly belonging to the order of Corallinales, Ceramiales and other green and red filamentous algae). In the uppermost zone (supralittoral) the fleshy red algae, *Rissoella verruculosa* and *Nemalion helminthoides*, encrusting brown and red algae, colonial cyanobacteria and lichens (*Verrucaria* spp.) are present. Sessile animals thriving in the midlittoral zone are mostly mussels (in the lower midlittoral), limpets, topshells, littorinids and barnacles (in the upper midlittoral).

Along shallow rocky reefs, canopy-forming brown macroalgae belonging to the *Cystoseira* genus are listed among the most sensitive species for the ecological quality assessment (Ballesteros et al., 2007; Pinedo et al., 2007). *Cystoseira* spp. display a large size and complex structure with an arborescent thallus: where well-developed, these species can form forests, which play an important role as ecosystem engineers, supporting a highly structured tri-dimensional and diversified macroalgal assemblage with high biodiversity and productivity and providing shelter (refuge) and food for marine invertebrates and for juvenile fish (Bulleri et al., 2002; Cheminée et al., 2013). *Cystoseira amentacea* var. *stricta* and *Cystoseria compressa* typically thrive in the lower level of the midlittoral zone, being *C. amentacea* one of the most sensitivity species classified as protected (Habitat Directive (92/43/EEC); Bern and Barcelona Convention) and *C*. *compressa* the most resilient species of this genus.

The so-called *Cystoseira* fringe (specially *C*. *amentacea*), when present in a continuous belt in the lower midlittoral zone, displays relevant ecosystem functioning that can affect the above habitat. Notwithstanding the relatively small size of the *Cystoseira* species compared to large oceanic brown algae, given the narrow amplitude of the tide in the Mediterranean Sea, ranging in a decimetre scale (30-90 cm; NOAA, 2015), these species can play a similar role as kelps or fucoids, such as sheltering and water oxygenation (Schdmit et al., 2011; Smale et al., 2013).

A general regression of *Cystoseira* species is reported along the Mediterranean coasts, due to different factors: sedimentation (Perkol-Finkel and Airoldi, 2010), low water quality (Sales et al., 2011; Zavodnik et al., 2002) and coastal artificialization (Sales and Ballesteros, 2010; Thibaut et al., 2005). It is already well known that the regression of *Cystoseira* species, and more generally of canopy-forming species (Dayton, 1975; Dayton, 1985; Chapman, 1990; Benedetti-Cecchi et al., 2001, Smale and Wernberg, 2013), reduces the three-dimensionality, the biodiversity and the functioning of their habitat, affecting the structure of the understorey assemblages and the flattening in vertical distribution of habitats (Bulleri et al., 2002). Differently, the effect of the canopy on the functioning of the above located "upper midlittoral assemblages" has so far been poorly investigated.

Additionally, little studies have been performed on the vagile species community composition associated to algal assemblages, both within the *Cystoseira* fringe and in the above midlittoral zone (Thrush et al., 2011). Previous studies have demonstrated that algal assemblages can have strong effects on the distribution of macrofaunal organisms that live in the turf matrix (Abbiati et al., 1987; 1991; Giangrande, 1988; Sardà, 1991; Kelaher et al., 2001). In fact, algal turfs and canopies can provide protection from dislocation due to waves, provide shade and restrict the variability in humidity and temperature on the shore (Kelaher et al. 2001, Bulleri and Benedetti-Cecchi, 2008), filter swimming larvae or drifting adults, provide a refuge from predation by large mobile predators. These habitat-mediated effects imply that changes in algal habitat structure should influence the diversity of niches available to rocky shore infauna.

The focus of this study is to quantify the effect of the *Cystoseira* fringe on the upper midlittoral ecosystem in the Mediterranean Sea.

At this purpose the value of the natural capital stocked in habitats located in the midlittoral zone and above a continuous and discontinuous *Cystoseira* belt has been calculated.

The natural capital of the midlittoral habitat was estimated for both the autotroph (macroalgae) and heterotroph (sessile and vagile macroinvertebrates) components of the community through the application of emergy analysis.

Emergy approach is able to attribute a system value to a habitat through a biophysical accounting procedure. In particular, the value corresponds to the amount of resources used to create and maintain the habitat.

Emergy accounting is then a quantitative method able to assess the work of biosphere in terms of direct and indirect solar energy converging to support the production of goods and services (Odum, 1996). According to this method, the more work of the biosphere is embodied in generating the exploited natural resources, the greater is their value. Emergy units can be converted into currency equivalents through a conversion factor named "Emergy to money ratio" (EMR) to better convey results to administrative stakeholders and general public.

Recently this approach has been applied to natural capital evaluation (Brown and Ulgiati, 1999; Campbell and Brown, 2012; Campbell and Tilley, 2014; Franzese et al., 2015; Franzese et al., 2017; Picone et al., 2017; Lu et al., 2007; Pulselli et al., 2011; Vassallo et al., 2013; Vassallo et al. 2017; Paoli et al., 2018).

A growing interest in the quantification of the monetary value of ecosystems arose in the last decades aiming at raising awareness of the general public towards the preservation of biodiversity and at supporting the policy makers in the process of creating new legal instruments (Beaumont et al., 2008; Braüer et al., 2006; Brown et al., 2001; Costanza et al., 1997; Odum, 2000). Efforts were addressed not only to the monetisation of ecosystems but also to understand the link between ecosystems and human well-being.

Costanza and Daly (1992) introduced the concept of natural capital, combined with human capital and manufactured capital. Natural capital includes land, air, water, sea and ecosystems themselves; human capital comprises all individuals' capacities for work, while manufactured capital encompasses all material goods generated through economic activity and technological change. Interactions between the three capital types generate well-being and a tight link exists between ecosystem services provision and natural capital: only if natural capital is preserved the supply of services in the future and at the actual level can be guaranteed (De Groot et al., 2012). From natural capital, ecosystem functions arise: these latter represent the potential to generate services that arise from natural capital stocks; functions originate directly from the ecological structures and processes of ecosystems and their mutual interactions (De Groot et al., 2010). If functions depend on natural capital, ecosystem services (ES) arise from functions. ES represent the useful things that ecosystems directly and indirectly generate for the mankind. The main difference between functions and services resides in the fact that, while the value and the perception of services by humans change according to cultural, social and economic features, functions exist and bring their potential independently from humans (De Groot et al., 2010). Since ES generate well-being it is important to know and to evaluate each step or component of this so-called "pathway from nature to well-being" (De Groot et al., 2010; Paoli et al., 2017).

Emergy, in this context, i) being an input-state-output method (Pulselli et al., 2011) with a system approach can help understanding relationships leading from natural capital to well-being ii) is able to attribute a value, also expressed in monetary equivalents, to natural capital, allowing to easily monitor changes in studied communities.

2. Materials and Methods

2.1 Sites and sampling

Survey covered 10 sites located along the eastern Ligurian Riviera, Italy, extending over ~80 km of coastline (Figure 1). Sampling was conducted in July 2008. Each site consisted of a 150 m transect located in the rocky midlittoral zone (immediately above the mean low water level). All sites were chosen consistently in terms of slope and aspect to open water.

The start point of each transect was located in an unbiased, haphazard fashion and samples were collected at this point and every 10 m (measured with a tape measure), resulting in a sample size of 15 sampling points for each site. Each sampling point was also logged with a GPS (accuracy <10 m, RMS Garmin eTrex H).

At each sampling point, we visually censused a 20 × 20 cm quadrat to identify the large organisms (invertebrates and seaweeds) that define the algal turf habitat and assess quadrat species density (number of species per quadrat). To provide percent cover estimates, the quadrat was divided into 25 equal squares, each representing 4% of coverage. Each taxon observed was given an abundance rank (1 to 4) within each square (1⁄4 of the square = 1%) and the total percent substrate cover was obtained by adding up scores for all squares where the taxon was present. Organisms filling less than 1/4 of a square (<1%) were given a percent cover value of 0.5 (following Dethier et al. 1993). Visible, solitary organisms were also counted in each quadrat.

At each sampling point we also collected two cores, by pushing a stainless steel corer (5 cm internal diameter) firmly into the turf and encrusting fauna: the top of the core was then plugged while a flexible metal blade was slid against the rock to free the encrusting turf and organisms contained within the corer. At each sampling point, one of the cores was used to sample the turf infauna: the sample was placed into a plastic jar, preserved with 70% isopropyl alcohol and stained with Rose Bengal. Specimens were then sorted and enumerated at the lowest taxonomic resolution possible. The second core sample was collected for the analysis of turf organic matter and sediment content. This core was stored in a plastic bag, kept cool and in the dark until frozen on return to the laboratory. Samples for turf total organic and sediment content were defrosted and air dried. Then algae and large organisms with calcified shells (e.g. mussels and barnacles) were removed from the sample. Samples were then dried at 60°C for 48 h, weighed, then combusted at 400°C for 6 h, and reweighed to measure total organic and inorganic sediment content respectively.

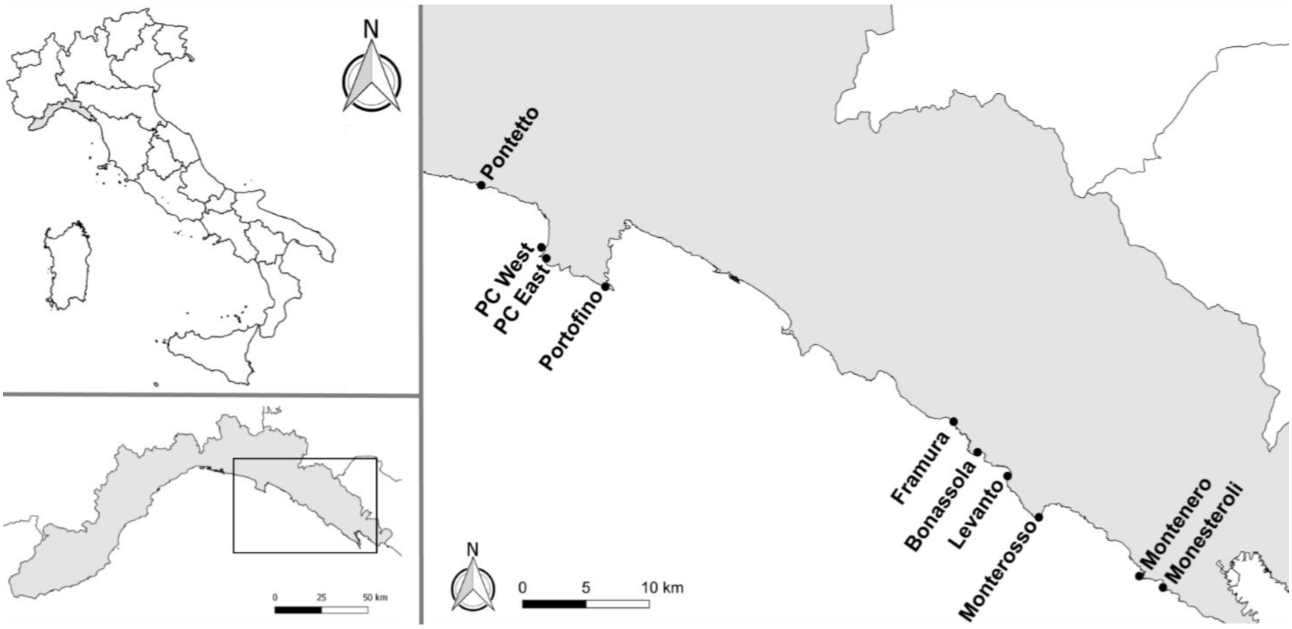


Figure 1. Sampling Sites along the eastern Ligurian Riviera, Italy.

2.2 Emergy analysis: theory and principles

Emergy analysis is a thermodynamic based methodology introduced during the ’80s by Howard Odum. It is an approach of quantitative analysis that standardizes the values of non-monied and monied resources, services and commodities in a single common unit (Brown and Herenden, 1996). This makes emergy a very versatile technique that can be applied to whatever natural or human system or to a mix of the two.

Emergy theory is defined by two key concepts: solar emergy itself and solar transformity. Solar emergy is identified by the quantity of solar energy required, directly or not, to provide a given flow or storage of energy or matter (Odum, 1996). Emergy is expressed in solar emergy Joule (seJ) and is usually calculated on an annual scale. Transformity measures the input of emergy per unit output and is calculated as the ratio of the emergy necessary to produce a flow or a storage to the actual energy of that flow or storage (Ulgiati and Brown, 2002). The transformity is expressed in solar emergy Joule per Joule of output flow (sej·J^{-1}). For certain products or flows, easily quantifiable in units of mass or money, a conversion value expressed in sej·g^{-1} or sej·$€^{-1}$ can be used (Paoli et al., 2008 a,b). These coefficients are respectively named specific emergy and emergy per unit money; transformity, specific emergy and emergy per unit money can be gathered under the locution Unit Emergy Value (UEV hereinafter, Paoli et al., 2013).

The first step of the methodology consists in the drawing of a system diagram where the system is represented as a box, containing the components and surrounded by all the inputs on left and upper boundaries. The outputs are located outside the right boundary while the heat losses are represented below. From the diagram an emergy table is built where in the first column all input to the ecosystem are listed, the second column has the corresponding unit of measure, the third contains UEVs and the fourth the emergy values obtained from the multiplication of input flows and UEV.

The sum of emergy values provides an estimate of total resources amount required by the ecosystem that can be considered a proxy of natural capital value as cost invested by nature.

When an emergy amount is ascribed to the natural capital the latter step consists in the calculation of the corresponding monetary value. In order to express the solar emergy as units of money, the standard practice (Odum, 1996) is to divide the solar emergy (sej) by the mean solar emergy-to-currency ratio (sej/€ in our case) of the economy that encompasses the flow of solar emergy (Campbell and Tilley, 2014). This ratio is named Emergy Money Ratio (EMR hereinafter) and it is calculated as ratio between the emergy flow used by a nation and its Gross Domestic Product. The ratio represents the emergy buying power of money in a system since it values how much emergy corresponds, on average, to one unit of money produced by the considered national economy (Odum, 1996; Campbell and Lu, 2009). As a consequence, given an emergy amount expressed in sej, it can be translated in whatever currency simply by dividing this amount by the chosen EMR. Here the 9.60E+11 sej/€ ratio is employed (Pereira et al., 2013). Monetary equivalents of biophysical values, such as the natural capital value of the midlittoral zone, are expressed in emergy-euros (em€). Currently, emergy scientists are debating about the conversion of the emergy value in monetary units (Campbell and Tilley, 2014). In emergy evaluations the monetary equivalents of solar emergy joules amounts are used mainly with communication purposes: the translation from emergy units to money units does not change the biophysical feature of the accounting method but, instead, it represents a tool to include nature's value in socio-economic and policy contexts (Franzese et al., 2017; Vassallo et al., 2017; Paoli et al., 2018).

2.2 Natural capital value assessment

Emergy has been recently employed for the study of ecosystems (Brown and Ulgiati, 1999; Odum and Odum, 2000; Pulselli et al., 2011; Franseze et al., 2015). Practical applications are mainly addressed to the study of terrestrial forests (Campbell and Tilley, 2014; Turcato et al., 2015), or marine systems, in particular Marine Protected Areas (MPAs; Vassallo et al., 2013, Vassallo et al., 2017: Franzese et al., 2017; Picone et al., 2017; Paoli et al., 2018).

The applied methodology is based on the scheme proposed by Vassallo et al. (2017) and then is based on three main steps:

1. Trophodynamic assessment: an estimate of the primary productivity exploited to generate and to maintain the benthic biodiversity of the study area
2. Supporting units' calculation: estimation of the space and time required to yields primary productivity calculated in the previous phase.

3. Ecologic and monetary equivalents valuation: an estimate, through Emergy, of the biophysical value ascribable to the productivity calculated in the trophodynamic assessment, then translated in monetary units,

Here below each phase is described in more detail:

2.2.1 Tropho-dynamic assessment

The main trophic and taxonomic groups composing the ecosystem were identified and described in Operational Taxonomic Units (OTUs) for both macroalgae and infauna. Then a database of the biomass per unit area associated with each trophic group was created.

As far as macroalgae are concerned, coverage values (from the visual quadrats) were converted into biomass using conversions factors (WCF) from Ballesteros (1985), except for the *Cystoseira* species, where conversion factors were calculated *ad hoc* on harvested samples (Table 1).

Dry weights of the considered algal species have been transformed in carbon weights (g/m^2) by means of the conversion factors (CCF) from Brey (2016). The sum of the carbon weights of the different species composes the total carbon weight of the primary producers OTUs.

Table 1. Macroalgal species and used conversion factors: from coverage values to biomass dry weight (WCF) and from dry weight to carbon content (CCF)

Acronym	**Species/OTUs**	**WCF**	**CCF**
CYS-CA	*Cystoseira amentacea* var. *stricta*	0.900	0.35
CYS-CC	*Cystoseira compressa*	0.900	0.35
ACA-C	Articulated calcareous *Corallina* spp.	0.030	0.15
ACA-J	Articulated calcareous *Jania*	0.020	0.27
CCA	Corallinales Crustose	0.175	0.27
NCA	NON-Corallinales Crustose	0.020	0.27
CFA	Corticated Foliose	0.050	0.29
CFA-C	Corticated Foliose-Calcified	0.050	0.29
FA	Filamentous	0.003	0.28
CM	Corticated	0.010	0.29

The midlittoral benthic fauna was grouped according to their systematics (phyla: Mollusca, Crustacea, Annelida, Echinodermata) and their feeding behaviour (grazers, deposit feeders, suspensions feeders and predators), for a total of eleven different OTUs (Table 2).

Table 2. OTUs of turf infauna benthic invertebrates.

Feeding behaviour category	**Systematic category**	**OTU**
GRAZERS	Mollusca	Mollusca gra
	Crustacea	Crustacea gra
DEPOSIT FEEDERS	Annelida	Annelida_dep
	Crustacea	Crustacea_dep
	Echinodermata	Echinod_dep

SUSPENSIONS FEEDERS	Mollusca	Mollusca_susp
	Annelida	Annelida_susp
	Crustacea	Crustacea_susp
PREDATORS	Annelida	Annelida_pred
	Crustacea	Crustacea_pred
	Pycnogonid	Pycnogonid_pred

The abundance values of each OTU from the individual samples were converted into biomass using individual weight conversion factors from Goren (1980) and Brey (1990) (Paoli et al., 2016; Paoli et al., 2018).
The individual weights ascribed to each considered species are reported in Table 3, classified according to the corresponding OTU.

Table 3. Species of turf fauna considered and individual weights

OTU	Species	Individual weight (gC)
Mollusca_susp	*Mytilus galloprovincialis*	3.02E-03
Mollusca_susp	*Mytilaster minimus*	3.02E-03
Mollusca_susp	*Musculus costulatus*	6.61E-04
Mollusca_susp	*Hyatella arctica*	3.02E-03
Mollusca_gra	*Patellidae indet.*	2.33E-02
Mollusca_gra	*Acanthochitona fascicularis*	2.11E-02
Echinod_dep	*Amphipholis squamata*	3.53E-02
Annelida_pred	*Nereididae indet.*	4.31E-04
Annelida_pred	*Nereis jacksoni*	5.48E-04
Annelida_pred	*Perinereis cultrifera*	4.31E-04
Annelida_pred	*Nereis perivisceralis*	5.48E-04
Annelida_pred	*Platynereis dumerilii*	1.97E-04
Annelida_pred	*Syllidae indet.*	6.71E-05
Annelida_pred	*Syllis gracilis*	8.08E-05
Annelida_pred	*Syllis prolifera*	8.08E-05
Annelida_pred	*Exogoninae indet.*	6.71E-05
Annelida_dep	*Tubificidae indet.*	4.65E-04
Annelida_dep	*Polyophthalmus pictus*	7.71E-04
Annelida_susp	*Sabellidae indet.*	2.80E-03
Annelida_susp	*Serpulidae indet.*	4.66E-05
Crustacea_pred	*Caprella hirsuta*	1.22E-04
Crustacea_gra	*Ampithoe spuria*	1.59E-04
Crustacea_gra	*Elasmopus pocillimanus*	1.12E-04
Crustacea_gra	*Jassa marmorata*	1.22E-04
Crustacea_gra	*Hyale grimaldi*	1.22E-04
Crustacea_pred	*Stenothoe tergestina*	1.20E-04
Crustacea_pred	*Podocerus variegatus*	1.22E-04
Crustacea_susp	*Tanais dulongii*	1.89E-04
Crustacea_gra	*Sphaeromidae sp.*	8.30E-03
Crustacea_pred	*Gnathiidae sp.*	8.30E-03

Crustacea_dep	*Anthuridae sp.*	8.30E-03
Crustacea_susp	*Balanomorpha spp.*	2.22E-03
Pycnogonid_dep	*Anoplodactylus petiolatus*	9.29E-05
Pycnogonid_dep	*Rhynchothorax voxorinus*	9.29E-05
Crustacea_pred	*Eriphia verrucosa*	3.43E-03
Crustacea_pred	*Decapoda indet.*	3.43E-03

The assessment of the biomass stocked in the trophic groups is the basic information for the calculation of the primary productivity required, in space and time, to generate the natural capital stocked within the habitat.

The total productivity associated to the natural capital stock of the ecosystem (B or primary biomass) was obtained as sum of autotrophic primary biomass (B_a) and heterotrophic one (B_e). B_a is given by the sum of the different primary producers' biomasses.

B_e is a function of each heterotrophic OTU biomass and its trophic level. Pauly and Christensen (1995) proposed a calculation for the evaluation of the primary productivity supporting fisheries based on the assumption that in nature a fixed rate of energy (trophic efficiency rate) is transferred from a trophic level to the following. According to Christensen and Pauly (1993), the transfer efficiency between trophic levels is assumed equal to 15% in coastal systems. The supporting primary productivity for heterotrophic biomass (B_e) stock is then calculated as:

$$B_e=\sum_{i=1}^{n} \text{heterotrophic OTU}_\text{i} \text{ biomass} * 7^{(\text{TL}_i-1)} \qquad \text{equation 1}$$

where TL_i is the trophic level of each heterotrophic OTU (Lindeman, 1942)

The biomasses and the obtained productivities were expressed in carbon grams per squared meters. The corresponding quantity of nitrogen and phosphorus fixed were calculated by adopting the classical mass ratio of C:N:P=41:7:1 (Redfield et al., 1963). The trophic levels were assessed by means of the simulations of the trophic web through Matlab ® software (Paoli et al., 2016).

2.2.3 Supporting units' calculation for natural capital

Once primary productivity B was calculated, the number of years during which this productivity has been generated has to be accounted (supporting time hereinafter). This calculation is required in order to apply the emergy procedure, based on the estimate of annual natural fluxes allowing the transformation of nutrients into organic matter.

Supporting time was calculated as ratio between required primary production and an average productivity rate. Here two rates were considered: for the biomass of macrophytes a specific productivity rate equal to 375 g/m^2 has been adopted (Charpy-Roubaud and Sournia, 1990), while for the productivity that supported the storage of heterotrophic biomass a rate of 650 g/m^2, considering macroalgae, seagrass, microphytobentonic and phytoplanktonic average productivity, has been taken into account. Supporting time multiplied by the considered surface (in our case a squared meter) provided the so called "supporting surface" (Table S1). Supporting surface gave an estimate of the total area required to potentially generate, in a single year, the primary productivity that allowed the storage of present natural capital through time.

2.3 Ecological valuation and estimate of monetary equivalents

Emergy associated to each sampling site was assessed starting from data of nutrients and natural fluxes calculated following the described approach.

From B calculation, inputs to emergy table, listed in Table 4, were obtained. Inputs are represented by the quantity of nutrients used to generate the required primary productivity and the amounts of natural resources necessary to convey and then transform them in organic matter.

Table 4. Emergy table formulas for the midlittoral habitat above the *Cystoseira* fringe; coefficients used in formulas are detailed in Supplementary materials (Table S1).

Items	Formula	UM	References
Carbon	B (see paragraph 2.2.1)	g	This work
Nitrogen	Carbon/41*7	g	This work
Phosphorus	Carbon/41	g	This work
Sun	Annual solar radiation per unit area*Supporting surface*(1-albedo)	J	Odum, 1996
Rain	Annual rainfall* Gibbs free energy* Water density* supporting surface	J	Odum, 1996
Wind	Air density*drag coeff.*(geostrophic wind velocity)^3*supporting surface*seconds per year	J	Campbell et al., 2005
Currents	½*water mass*velocity^2*supporting surface	J	Campbell et al., 2005
Geothermal heat	Geothermal flux*supporting surface	J	Odum, 2000; Cataldi et al., 1995
Tides	½*tides per year*(height)^2*density*supporting surface*gravity	J	Odum, 1996

The content of carbon, nitrogen and phosphorus of each site were translated in emergy terms by means of appropriate UEVs (Table 5) together with natural fluxes impacting on each station/site (namely sun, rain, geothermal heat, current, tides) for the supporting time are accounted. The UEVs used in this study were updated to the 1.52E+25 sej yr^{-1} biosphere emergy baseline calculated by Brown and Ulgiati (2010).

The sum of inputs' emergy values provided an estimate of the resources' amount required by the site and represents the biophysical assessment of the cost made by nature to generate all the resources exploited (Vassallo et al., 2017). The emergy values of different inputs were summed according to emergy algebra rules. As a consequence, items can be classified as: i) "splits" that are flows of the same kind, with different emergy but the same UEV (for example, a water stream that divides into two); ii) co-products that are outflows of a different kind and different UEV (for example, flows of wool and mutton produced by the sheep agricultural system are co-products). Co-products have different UEVs because they emerge from different stages in the series of transformations during the same process. According to algebra rules, only the maximum among co-products was chosen and included in the sum to avoid double counting. In particular, the nutrients were considered as a different set of co-products and then compared with sun, rain, wind and current (representing another co-product set), being these two groups generated on a different spatial and temporal scale. Tides and geothermal heat were considered as splits according to previous researches and being generated by different processes at global scale (Brown et al. 1991; Campbell et al., 2004).

Table 5. UEVs used in the present study

ITEM	UEV (seJ/unit)	Reference
C	1.02E+08	Campbell et al., 2014
N	7.40E+09	Odum, 1996
P	2.86E+10	Odum, 1996
Sun	1.00E+00	By definition
Rain	2.93E+04	Odum, 1996
Wind	2.41E+03	Odum, 1996
Geothermal heat	2.00E+04	Brown and Ulgiati, 2010
Tides	7.20E+04	Brown and Ulgiati, 2010
Currents	3.80E+04	Odum, 1996

The sum of 1) the greatest among nutrients emergy contributions, 2) the maximum among natural inputs and geothermal heat, 3) currents and 4) tides composed then the total emergy requirement for the midlittoral habitat in each site.

Once this emergy amount was calculated the corresponding economic value was obtained applying EMR. Here the 9.06 E11 sej/€ EMR was employed (Pereira et al. 2013).

The potential role of the different *Cystoseira* species and of their continuous or discontinuous fringe on the economic value of the above midlittoral community was evaluated by assessing, for each sampling point, the main condition: presence of a continuous belt of *C. amentacea,* presence of a discontinuous belt of *C. amentacea,* presence of a continuous belt of *C. compressa,* presence of a discontinuous belt of *C. compressa.* Data were then plotted and differences tested by ANOVA using Matlab ® software.

3. Results

3.1. System diagram

Figure 2 shows the system diagram of the midlittoral habitat above the *Cystoseira* fringe. The diagram summarizes the general functioning of the system and how *Cystoseira* influences it. Inputs are depicted on the left side of the diagram. Main box indicates habitat boundaries, including the habitat itself and the generated functions box. Ecosystem services are listed as outputs to humans on the right side of the diagram.

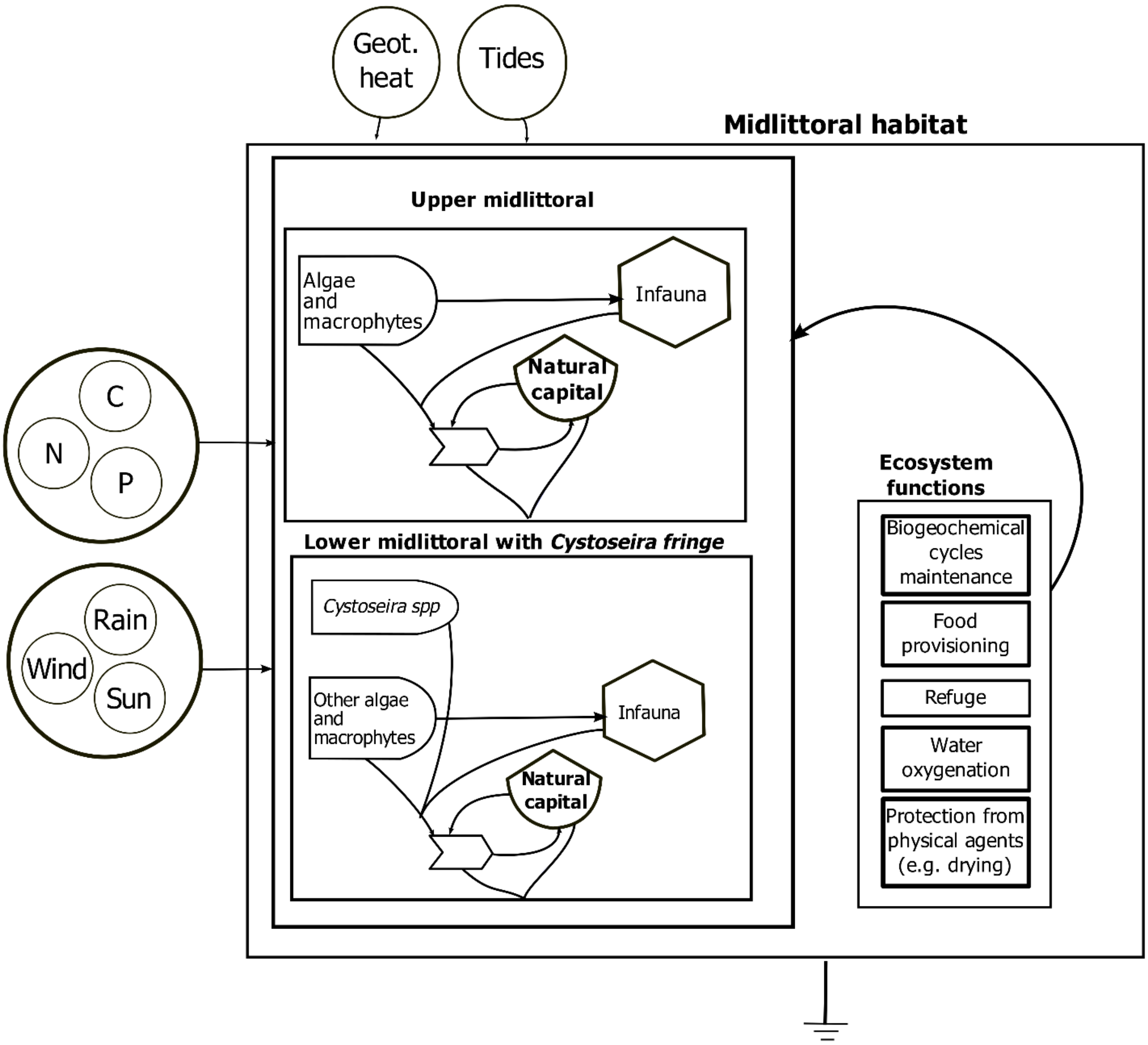


Figure 2. Emergy diagram of the midlittoral habitat above the *Cystoseira* fringe.

3.2 Biomass assessment

Figure 3 shows the proportional density of each OTU in the different sampling sites. Overall, the OTUs Mollusca_Susp, Crustacea_Graz and Crustacea_Dep have the highest values of density. In the Framura Site we observed the greatest density, which is due mainly by two OTUs: Mollusca_Susp, Crustacea_Graz, and, particularly the Mollusca_Susp presents the highest values of density.

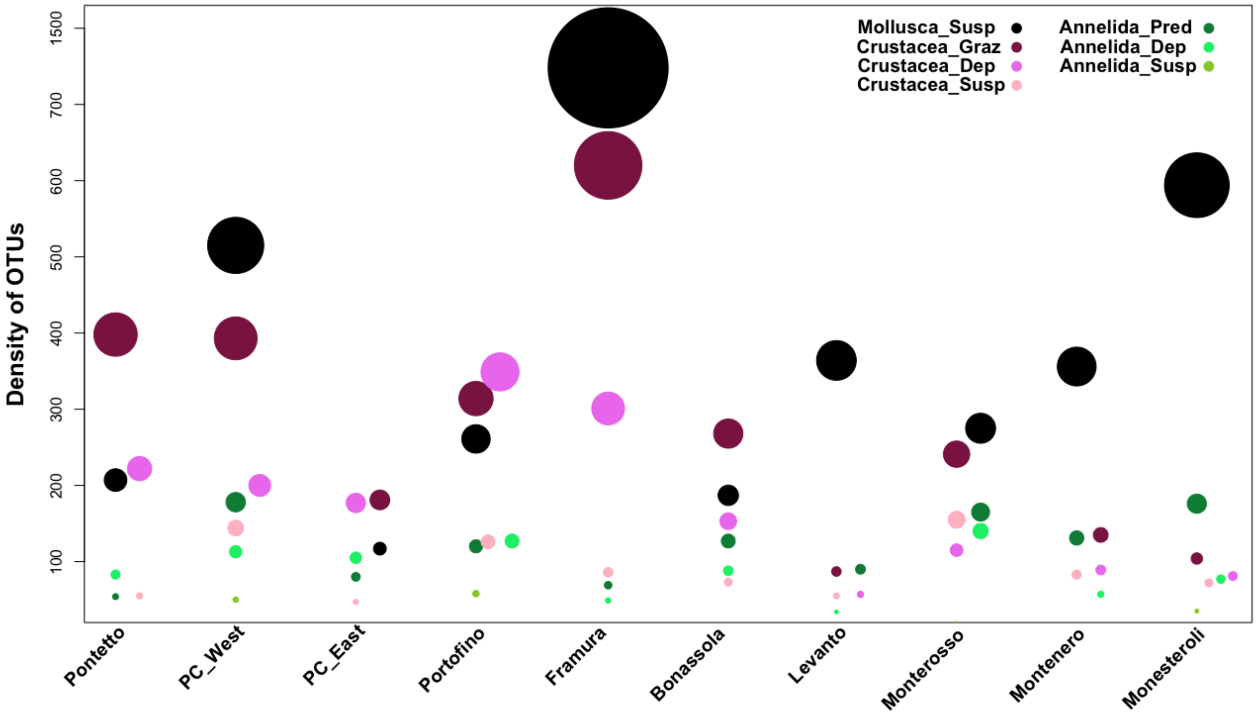


Figure 3. Proportional density of each infauna OTU for the different sites.

Values of biomass for the 10 sites, converted from density values of the 15 sampling points per site, are reported in Table 6. Autotrophic and heterotrophic components contribute almost equally to total biomass in half of the sites, while the heterotrophic component results significantly larger (over the 60%) than the autotrophic one only in a single site (Framura). In all the remaining sites, the heterotrophs' contribution is lower than 35%.

Table 6. Biomass in carbon grams in the considered sites

Biomasses (gC/m^2)	**Pontetto**	**PC West**	**PC East**	**Portofino**	**Framura**	**Bonassola**	**Levanto**	**Monterosso**	**Montenero**	**Monesteroli**
Autotrophs	1.17E+02	5.93E+01	6.79E+01	3.78E+01	9.71E+01	6.78E+01	8.09E+01	4.89E+01	6.72E+01	6.39E+01
Mollusca susp.	1.94E+01	4.66E+01	1.07E+01	2.29E+01	1.31E+02	1.90E+01	3.02E+01	2.66E+01	3.22E+01	5.93E+01
Mollusca graz.	3.58E+00	2.15E+00	2.15E+00	5.88E+00	5.73E+00	1.70E+01	2.86E+00	9.38E+00	5.73E+00	5.73E+00
Echinodermata det.	0.00E+00	1.20E+00	1.20E+00	0.00E+00	4.79E+00	3.59E+00	0.00E+00	0.00E+00	3.59E+00	1.20E+00
Annelida susp.	1.91E-01	4.56E+00	5.71E-01	4.48E+00	9.49E-02	0.00E+00	1.34E+00	1.43E+00	5.71E-01	3.14E+00
Annelida det.	4.86E-01	4.46E-01	7.36E-01	6.22E-01	3.43E-01	3.39E-01	1.90E-01	6.75E-01	3.22E-01	3.05E-01
Annelida pred.	9.65E-01	2.77E+00	1.32E+00	2.11E+00	1.09E+00	2.25E+00	1.38E+00	2.40E+00	2.27E+00	2.16E+00
Crustacea susp.	3.93E+00	3.68E+00	3.88E+00	5.01E+00	3.51E+00	4.46E+00	2.69E+00	2.99E+00	4.39E+00	3.22E+00
Crustacea det.	9.13E-01	8.22E-01	7.26E-01	1.43E+00	1.24E+00	6.29E-01	2.34E-01	4.73E-01	3.66E-01	3.33E-01
Crustacea graz.	2.35E+00	1.68E+00	7.94E-01	2.18E+00	7.62E+00	3.16E+00	1.77E+00	1.55E+00	5.79E-01	1.87E+00
Crustacea pred.	2.82E-01	5.82E-01	7.96E-01	1.28E+00	1.16E-01	1.26E+00	9.62E-01	3.35E+00	7.96E-01	6.80E-01
Chelicherata pred.	6.31E-03	4.10E-02	6.31E-03	8.52E-02	9.15E-02	3.15E-03	9.46E-03	5.05E-02	1.26E-02	1.58E-02
Heterotrophs	3.21E+01	6.46E+01	2.29E+01	4.60E+01	1.56E+02	5.17E+01	4.16E+01	4.89E+01	5.08E+01	7.79E+01
Total	1.49E+02	1.24E+02	9.08E+01	8.38E+01	2.53E+02	1.20E+02	1.23E+02	9.78E+01	1.18E+02	1.42E+02

3.3 Natural capital evaluation

Primary biomass (B) that supported in space and time the stored biomass was calculated together with supporting units for each site (Figure 4).

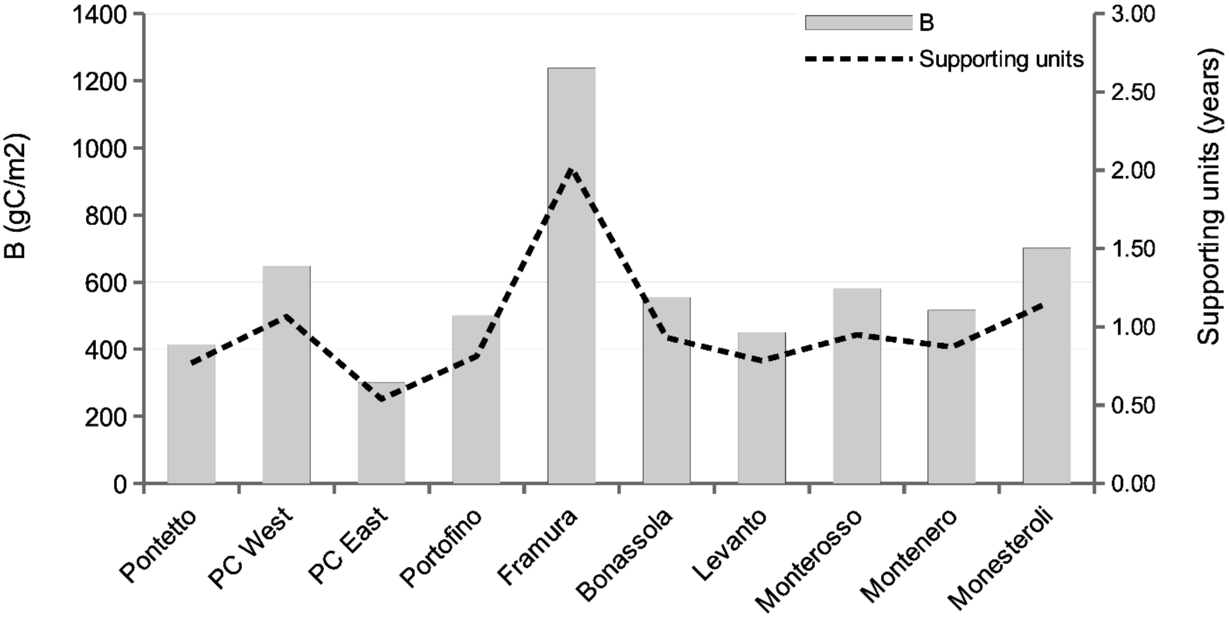


Figure 4: Primary biomass B of the midlittoral habitat above the *Cystoseira* fringe in the considered sites

Emergy tables were obtained from B and supporting units' values (Table 7).

The average emergy value of *Cystoseira* intertidal habitat is 1.00E+12 seJ/m^2, corresponding to 1.04 eM€/m^2, with a minimum value of 0.53 eM€/m^2 and a maximum one of 2.21 eM€/m^2. Coefficient of variation (=0.43) is rather high, because of the large variability among the considered sites. The emergy value is mainly due to the heterotrophic component, which contributes with a percentage from 68 to 91% of the total emergy amount.

Table 7. Emergy values of different inputs in the different sites of the midlittoral habitat above the *Cystoseira* fringe, total values in emergy terms and monetary equivalents are reported.

	Emergy values (seJ)	**Pontetto**	**PC West**	**PC East**	**Portofino**	**Framura**	**Bonassola**	**Levanto**	**Monterosso**	**Montenero**	**Monesteroli**
a	Carbon	4.23E+10	6.63E+10	3.07E+10	5.11E+10	1.27E+11	5.68E+10	4.60E+10	5.94E+10	5.29E+10	7.17E+10
b	Nitrogen	5.22E+11	8.19E+11	3.80E+11	6.32E+11	1.56E+12	7.02E+11	5.69E+11	7.34E+11	6.53E+11	8.86E+11
c	Phosphorous	2.89E+11	4.53E+11	2.10E+11	3.49E+11	8.65E+11	3.88E+11	3.14E+11	4.06E+11	3.61E+11	4.90E+11
d	Sun	3.47E+09	4.80E+09	2.43E+09	3.66E+09	8.98E+09	4.15E+09	3.55E+09	4.21E+09	3.85E+09	5.09E+09
e	Rain (chemical potential)	9.40E+10	1.30E+11	6.60E+10	9.94E+10	2.46E+11	1.14E+11	9.59E+10	1.16E+11	1.07E+11	1.41E+11
f	Wind	7.47E+10	1.03E+11	5.24E+10	7.89E+10	1.96E+11	9.05E+10	7.62E+10	9.22E+10	8.46E+10	1.12E+11
g	Currents	6.12E+08	8.48E+08	4.29E+08	6.46E+08	1.60E+09	7.41E+08	6.24E+08	7.56E+08	6.93E+08	9.17E+08
h	Geothermal heat	8.71E+10	1.21E+11	6.11E+10	9.21E+10	2.99E+11	1.38E+11	1.16E+11	1.41E+11	9.87E+10	1.31E+11
i	Tides	4.72E+09	6.54E+09	3.31E+09	4.99E+09	1.24E+10	5.72E+09	4.81E+09	5.83E+09	5.35E+09	7.07E+09
	Total=max(a,b,c)+ max(d,e,f,g)+h+i	**7.08E+11**	**1.08E+12**	**5.10E+11**	**8.28E+11**	**2.12E+12**	**9.59E+11**	**7.86E+11**	**9.97E+11**	**8.64E+11**	**1.16E+12**
	Monetary equivalents (eM€)= total/EMR	**7.38E-01**	**1.12E+00**	**5.31E-01**	**8.63E-01**	**2.21E+00**	**9.99E-01**	**8.19E-01**	**1.04E+00**	**9.00E-01**	**1.21E+00**
	Heterotrophic component percentage	**68.41%**	**89.50%**	**74.58%**	**91.29%**	**90.84%**	**85.85%**	**79.39%**	**90.18%**	**85.15%**	**89.53%**

The large variability of the natural capital for the individual site was investigated taking into account the type and condition of the underlying *Cystoseira* fringe. Data were grouped across sites according to the four classes (continuous and discontinuous fringe of *C. amentacea* or *C. compressa*; Figure 5).

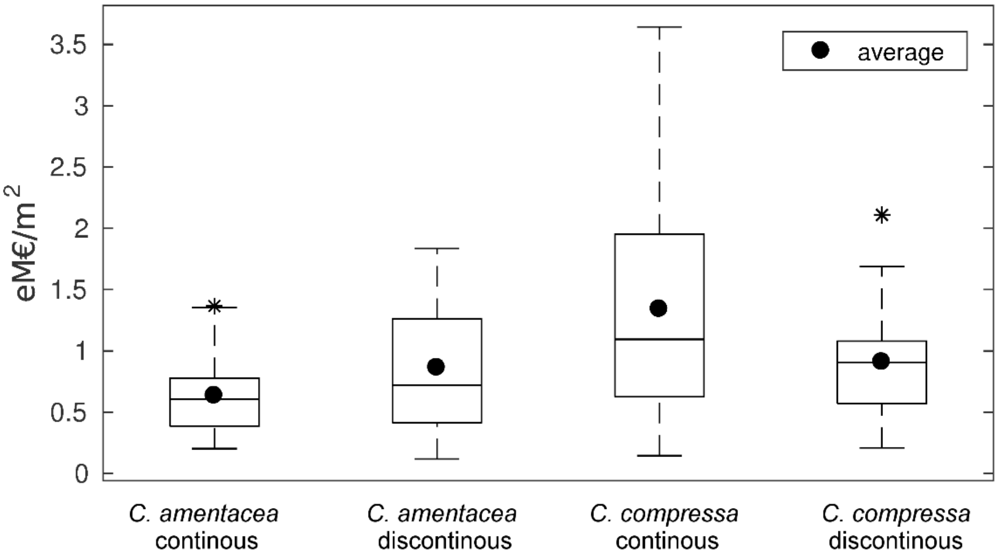


Figure 5: Variability of natural capital value according to species type and condition of the underlying *Cystoseira* belt

In order to understand if the natural capital value is influenced by the condition of the underlying *Cystoseira* belt, a one-way ANOVA test was performed: results are shown in Table 8 and prove the four groups to be significantly different.

Table 8. One-way ANOVA results regarding effects of type/condition of the *Cystoseria* fringe on the natural capital

Source	SS	df	MS	F	Prob>F
Cystoseira fringe	11.33	3	3.77672	8.22	4.6890e-05
Residual	60.16	131	0.45925		
Total	71.49	134			

Results from multiple comparison performed on pair of groups' means identified significative differences between *C. amentacea* and *C. compressa* continuous fringes (p-value < 0.001) and between continuous and discontinuous fringes of *C. compressa* (p-value=0.023).

4. Discussion

There is a strong scientific consensus that coastal marine ecosystems, along with the goods and services they provide, are threatened by anthropogenic global climate change (Hays et al., 2005; Hsieh et al., 2005; Harley et al., 2006; Helmuth et al., 2006; Parmesan, 2006; Harvey, 2007). In particular, midlittoral ecosystems are seriously threatened by the increasing anthropogenic pressure on coastal areas, due to high population densities and rapid economic development (Sekovski et al., 2012; Guarnieri et al., 2016). Additionally, they are particularly sensitive to the potential effects of changing climate, because of they are exposed to a wide range of environmental conditions at the extreme edge of both marine and terrestrial environment (Harley et al., 2006; Helmuth et al., 2006; Blanchette et al., 2008). Midlittoral habitats are highly dynamic environments that provide a range of ecosystem functions supporting high biological productivity (McLusky et al., 1992; Ysebaert et al., 2003; Vassallo et al., 2012), contributing to flood defence (Dixon et al., 1998) and providing important habitats for marine and terrestrial organisms (Elliott et al., 2007; Nagelkerken et al., 2008; Ramos et al.,2012; Yates et al., 2014; Atkinson et al., 2004; Mander et al., 2007; Rog et al., 2017).

In the Mediterranean, the midlittoral zone is widely spread by species of the *Cystoseira* genus that often dominating complex and diverse benthic assemblages (Giaccone et al., 1994; Ballesteros, 1998; Hereu et al., 2008), playing an important role as habitat-forming species in the ecosystem functioning and structure. For these reasons, it is overwhelming important to provide metrics to assess the health status of these environments in order to set up effective management measures.

This study provides an emergy assessment of the value of the natural capital of the midlittoral habitat associated with the underlying *Cystoseira* fringe.

In the proposed emergy diagram (Figure 2) the main ecosystem functions of midlittoral habitat associated with the underlying *Cystoseira* fringe were identified (i.e., refuge, biogeochemical cycles maintenance, food provisioning, water oxygenation and protection from physical agents), from which arise related ecosystem services of this habitat: water quality regulation, commercial species provisioning and ecotourism.

The provision of these functions and services at the present level relies on the maintenance of the natural capital intact, and therefore the value of the midlittoral habitat natural capital was assessed.

Obtained values of the natural capital can be compared with those recently calculated with the same approach by Franzese et al. (2017), Picone et al. (2017) and Paoli et al. (2018) (Table 9) in some Italian marine protected areas (MPAs).

The value of the midlittoral habitat natural capital, on average equal to 1.04 em€/m^2, is consistent with the average values calculated for the benthic habitats of the MPAs varying from 0.29 to 2.81. A certain variability can be observed among the MPAs’ habitats, nonetheless the natural capital of the midlittoral habitat seems to be significantly lower than those recorded for photophilic and sciaphilic hard bottoms, especially in the comparison with data obtained in the same geographical area (Paoli et al., 2018).

Yet, values from previous studies presented in Table 9 were all obtained on infralittoral habitats and within MPA areas, where the habitats value benefits of a protection regime partially or totally limiting the stress imposed by human activities on benthic communities.

Conversely, the upper midlittoral habitat is subjected to a huge variability of environmental conditions (e.g. wetting degree, intensity of wave action, temperature, salinity, light). This variability can affect fundamental biotic and abiotic processes such as recruitment, predation and competition, availability of resources, trophic relations and it is mirrored by strong differences in macro-benthic communities found in the habitat (Terlizzi et al., 2002). The trophic network is furthermore limited in both complexity and biomass storage by physical condition. The value of the habitat can then be strongly variable and significantly reduced according to the difficult existing conditions and the ability of organisms to cope with them.

Table 9: habitats' natural capital values from previous researches

		Paoli et al. (2018)	Paoli et al. (2018)	Franzese et al. (2017)	Picone et al. (2018)
		Northen Italy Western Liguria	Northen Italy Eastern Liguria	Central Italy	Southern Italy
		em€/m^2			
Photophilous algae	Photophilic hard bottom	6.02	5.74	0.77	2.56
Sciaphilous infralittoral algae	Sciaphilic hard bottom	5.73	4.11	2.97	2.48
Sciaphilous infralittoral algae		-	5.03		
Coralligenous		15.00	10.92		
Cymodocea nodosa	*Posidonia oceanica* seagrass bed	2.29	-	0.62	2.45
Cymodocea nodosa on matte		2.69	-		
P. oceanica dead matte		2.40	1.68		
P. oceanica and dead matte		3.64	3.62		
P. oceanica		7.22	6.35		
P. oceanica on rocks		6.42	6.73		
Coastal detritic	Soft bottom	3.14	1.46	0.11	1.03
Muddy detritic		-	0.83		
Muds		0.26	0.35		
Sands		0.64	0.44		
Stones and pebbles		1.10	0.73		
Average		0.68	2.81	0.29	2.16

Concerning the *Cystoseira* fringe underlying the upper midlittoral habitat, highest and most variable values were observed in presence of *C. compressa* continuous fringe. Here the average emergy value of the natural capital is 1.29E+12 seJ/m^2 corresponding to 1.34 eM€/m^2. Moreover *C. compressa* continuous fringe displays the overall highest value (3.64 eM€/m^2). Where the fringe is not continuous (partially replaced by turf assemblages), the above midlittoral community shows very similar average values (0.91 eM€/m^2 for *C. compressa* discontinuous and 0.86 eM€/m^2 for *C. amentacea* discontinuous).

In the *C. amentacea* continuous fringe condition, the average value is lower than the unit (0.63 eM€/m^2). Nonetheless, in presence of a *C. amentacea* continuous fringe, the natural capital values show the lowest scatter (*C. amentacea* continuous: st. dev. = 0.35; *C. compressa* continuous: st. dev. = 0.88; *C. amentacea* discontinuous: st. dev. = 0.55; *C. compressa* discontinuous: st. dev. = 0.48), suggesting its role in habitat stabilization.

Since the important role of *C. amentacea* in shallow ecosystems is widely recognised, being a key species in maintaining habitat complexity and species diversity in its understorey community compared to *C. compressa* (Mangialajo et al., 2008), which has shorter branches and less tridimensional structure, it can be surprising to find a low value of the midlittoral community above a continuous fringe of *C. amentacea*. A possible interpretation of this finding may be that midlittoral organisms tend to concentrate in the understorey of this species rather than above because of its higher engineering function, and consequently the economic value obtained for the above community in presence of *C. amentacea* fringe is lower. As far as the lower variability in the economic value recorded for *C. amentacea* compared to *C. compressa* may instead be explained by the higher resilience of the latter, which can thrive in both moderate polluted and unpolluted zones, therefore in a larger range of environmental conditions.

5. Conclusions

The natural capital value can represent a valid baseline to monitor if the conditions of the considered environment change along time as a consequence of existing or new pressures. The economic value, expressed in currency units, can be conveyed not only to scientists but also to managers and general public.

The findings obtained in this work, referred to the upper midlittoral zone, encourage further studies in order to evaluate the overall natural capital of the midlittoral zone in the Mediterranean Sea. For this reason, it is necessary to encompass also the *Cystoseira* habitat, evaluating the ecosystem functioning and services that it provides as foundation species. The estimation of the natural value of the *Cystoseira* habitat will allow to raise awareness of the public towards the preservation of this habitat-forming species in order to protect biodiversity, support the policy makers in the process of creating new legal and management instruments in terms of protection, conservation and restoration actions if required, as strongly supported by European funding programmes (e.g. LIFE Programme).

Acknowledgments

The present work is a contribution to the ROC-POP Life project (Promoting biodiversity enhancement by Restoration Of *Cystoseira* POPulations), co-funded by the European Commission's LIFE Programme under Agreement N° LIFE16 NAT/IT/000816.

Supplementary materials

Table S1 Values employed for the inputs' calculation formulas.

	Pontetto	PC_West	PC_east	Portofino	Framura	Bonassola	Levanto	Monterosso	Montenero	Monesteroli	U.M.	Reference
Annual solar radiation per unit area	4.86E+09	4.85E+09			4.80E+09		4.87E+09	4.77E+09	4.76E+09		J/m^2	http://re.jrc.ec.europa.eu/pvgis/apps4/pvest.php
Albedo on the sea	7.00										%	Payne, 1972
Annual rainfall	8.46E-01										m	http://www.istat.it/it/archivio/5679
Gibbs free energy	4.94E+00										J/g	Odum, 1996
Water density	1.00E+06										g/m^3	
Air density	1.30E+00										kg/m^3	
Drag coefficient	3.00E-03											La Rosa et al., 2008
Geostrophic coefficient	1.67E+00											La Rosa et al., 2008
Wind velocity	4.13										m/s	http://atlanteeolico.rse-web.it/
Seconds per year	3.15E+07										sec	
Current velocity	2.50E-01										m/s	Doglioli et al., 2004
Photic zone height	3.50E+01										m	
Sea water density	1.03E+03										Kg/m^3	
Geothermal heat flux	1.89E+06										J/m^2	http://geothopica.igg.cnr.it/
Tides' height	2.48E-01										m	
Tides per year	7.30E+02										num	
Gravity	9.80E+00										m/s^2	

References to Table S1:

La Rosa, A.D., Siracusa, G., Cavallaro, R., 2008. Emergy evaluation of Sicilian red orange production. A comparison between organic and conventional farming. Journal of Cleaner Production, 16, 1907-1914.

Odum, H.T., 1996. Environmental Accounting: Emergy and Environmental Decision Making. John Wiley and Sons. New York, 370 pp.

Payne, R., 1972. Albedo of the Sea Surface. Journal of the Atmospheric Sciences, 29, 959–970

References

Abbiati, M., Bianchi, C. N., Castelli, A. (1987). Polychaete vertical zonation along a littoral cliff in the Western Mediterranean. Marine Ecology, 8(1), 33-48.

Abbiati, M., Bianchi, C. N., Castelli, A., Giangrande, A., Lardicci, C. (1991). Distribution of polychaetes on hard substrates of the midlittoral–infralittoral transition zone, western Mediterranean. Ophelia, 5(Suppl), 421-432.

Arnoux, A., Bellan-Santini, D. (1972). Relations entre la pollution du secteur de Cortiou par les détergents anioniques et les modifications des peuplements de Cystoseira stricta. Tethys, 4(3), 583-586.

Atkinson, P. W., Crooks, S., Drewitt, A., Grant, A., Rehfisch, M. M., Sharpe, J., Tyas, C. J. (2004). Managed realignment in the UK–the first 5 years of colonization by birds. Ibis, 146(s1), 101-110.

Ballesteros, E. (1985). Els vegetáis i la zonado litoral: especies, comunitats ifactors que influeixens en la seva distribució. Tesis doctoral. Universidad de Barcelona.

Ballesteros, E., Sala, E., Garrabou, J., Zabala, M. (1998). Community structure and frond size distribution of a deep water stand of Cystoseira spinosa (Phaeophyta) in the Northwestern Mediterranean. European journal of phycology, 33(2), 121-128.

Ballesteros, E., Torras, X., Pinedo, S., García, M., Mangialajo, L., de Torres, M. (2007). A new methodology based on litoral community cartography dominated by macroalgae for the implementation of the European Water Framework Directive. Marine Pollution Bulletin 55: 172-180.

Beaumont, N.J., Austen, M.C., Mangi, S. C., Townsend, M. (2008). Economic valuation for the conservation of marine biodiversity. Marine pollution bulletin, 56(3), 386-396.

Belsher, T., Boudouresque C.F., 2004. L'impact de la pollution sur la fraction algale des peuplements benthiques de Mditerranée. Atti Tavola Rotonda Internazionale: La Biologia Marina Per La Difesa E Per La Prolduttivit-8 Del Mare. Livorno: 215-260, 1974.

Benedetti-Cecchi, L., Pannacciulli, F., Bulleri, F., Moschella, P. S., Airoldi, L., Relini, G., Cinelli, F. (2001). Predicting the consequences of anthropogenic disturbance: large-scale effects of loss of canopy algae on rocky shores. Marine Ecology Progress Series, 214, 137-150.

Blanchette, C. A., Melissa Miner, C., Raimondi, P. T., Lohse, D., Heady, K. E., Broitman, B. R. (2008). Biogeographical patterns of rocky intertidal communities along the Pacific coast of North America. Journal of Biogeography, 35(9), 1593-1607.

Borja, A., Franco, J., Pérez, V. (2000). A marine biotic index to establish the ecological quality of soft-bottom benthos within European estuarine and coastal environments. Marine Pollution Bulletin, 40(12), 1100-1114.

Bräuer, I., Müssner, R., Marsden, K., Oosterhuis, F., Rayment, M., Miller, C., Dodoková, A. (2006). The use of market incentives to preserve biodiversity. ECOLOGIC, 51p

Brey, T. (1990). Estimating productivity of macrobenthic invertebrates from biomass and mean individual weight. MEERESFORSCHUNG/REP. MAR. RES., 32(4), 329-343.

Brey, T. (2016). Population dynamics in benthic invertebrates. A Virtual Handbook (2016). http://www.thomas-brey.de/science/virtualhandbook/

Brown, M. T., Tennenbaum, S., Odum, H. T. (1991). Emergy Analysis and Policy Perspectives for the Sea of Cortez, Mexico. University of Florida, Center for Wetlands and Water Resources.

Brown, M. T., Ulgiati, S. (1999). Emergy evaluation of the biosphere and natural capital. Ambio, 486-493.

Brown, M. T., Herendeen, R. A. (1996). Embodied energy analysis and EMERGY analysis: a comparative view. Ecological Economics, 19(3), 219-235.

Brown, K., Adger, W. N., Tompkins, E., Bacon, P., Shim, D., Young, K. (2001). Trade-off analysis for marine protected area management. Ecological Economics, 37(3), 417-434.

Brown, M. T., Ulgiati, S. (2010). Updated evaluation of exergy and emergy driving the geobiosphere: a review and refinement of the emergy baseline. Ecological Modelling, 221(20), 2501-2508.

Bulleri, F., Benedetti-Cecchi, L., Acunto, S., Cinelli, F., Hawkins, S. J. (2002). The influence of canopy algae on vertical patterns of distribution of low-shore assemblages on rocky coasts in the northwest Mediterranean. Journal of experimental marine biology and ecology, 267(1), 89-106.

Bulleri, F., Benedetti-Cecchi, L. (2008). Facilitation of the introduced green alga Caulerpa racemosa by resident algal turfs: experimental evaluation of underlying mechanisms. Marine Ecology Progress Series, 364, 77-86.

Campbell, D. E., Brandt-Williams, S. L., Meisch, M. E. (2005). Environmental accounting using emergy: Evaluation of the state of West Virginia (p. 116). US Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Atlantic Ecology Division.

Campbell, E. T., Brown, M. T. (2012). Environmental accounting of natural capital and ecosystem services for the US National Forest System. Environment, development and sustainability, 14(5), 691-724.

Campbell, E. T., Tilley, D. R. (2014). Valuing ecosystem services from Maryland forests using environmental accounting. Ecosystem Services, 7, 141-151.

Cataldi, R., Mongelli, F., Squarci, P., Taffi, L., Zito, G., Calore, C. (1995). Geothermal ranking of Italian territory. Geothermics, 24(1), 115-129.

Chapman, A. R. O. (1990). Effects of grazing, canopy cover and substratum type on the abundances of common species of seaweeds inhabiting littoral fringe tide pools. Botanica Marina, 33(4), 319-326.

Charpy-Roubaud, C., Sournia, A. (1990). The comparative estimation of phytoplanktonic, microphytobenthic and macrophytobenthic primary production in the oceans. Marine Microbial Food Webs, 4(1), 31-57.

Cheminée, A., Sala, E., Pastor, J., Bodilis, P., Thiriet, P., Mangialajo, L., Cottalorda, J.-M. Francour, P. (2013). Nursery value of Cystoseira forests for Mediterranean rocky reef fishes. Journal of experimental marine biology and ecology, 442, 70-79.

Christensen, V., Pauly, D. (1993). Trophic models of aquatic ecosystems. ICLARM Conf. Proc. 26, 390 p.

Costanza, R., Daly, H. E. (1992). Natural capital and sustainable development. Conservation biology, 6(1), 37-46.

Costanza, R., d’Arge, R., de Groot, R., Farber, S., Grasso, M., Limburg, K., Naeem, S., O’Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 253–260

Dayton, P. K. (1975). Experimental evaluation of ecological dominance in a rocky intertidal algal community. Ecological monographs, 45(2), 137-159.

Dayton, P. K. (1985). The structure and regulation of some South American kelp communities. Ecological Monographs, 55(4), 447-468.

De Gaetano, P., Doglioli, A. M., Magaldi, M. G., Vassallo, P., Fabiano, M. (2008). FOAM, a new simple benthic degradative module for the LAMP3D model: an application to a Mediterranean fish farm. Aquaculture research, 39(11), 1229-1242.

De Groot R., Fisher B., Christie M., Aronson J., Braat L., Haines-Young R., Gowdy J., Maltby E., Neuville A., Polasky S., Portela R., Ring I. (2010). Integrating the ecological and economic dimensions in biodiversity and ecosystem service valuation in Kumar P. (Ed.), The Economics of Ecosystems and Biodiversity: Ecological and Economic Foundations, Earthscan, London

De Groot, R., Brander, L., Van Der Ploeg, S., Costanza, R., Bernard, F., Braat, L., Christie, M., Crossman, M., Ghermandi, A., Hein, L., Hussain, S., Kumar, P., McVittie, A., Portela, S., Rodriguez, L. C., Ten Brink, P., Van Beukering, P. (2012). Global estimates of the value of ecosystems and their services in monetary units. Ecosystem services, 1(1), 50-61.

Dethier, M. N., Graham, E. S., Cohen, S., Tear, L. M. (1993). Visual versus random-point percent cover estimations:'objective'is not always better. Marine ecology progress series, 93-100.

Díez, I., Bustamante, M., Santolaria, A., Tajadura, J., Muguerza, N., Borja, A., Muxica, I., Saiz-Salinas, J.I. Gorostiaga, J. M. (2012). Development of a tool for assessing the ecological quality status of intertidal coastal rocky assemblages, within Atlantic Iberian coasts. Ecological indicators, 12(1), 58-71.

Elliott, M., Burdon, D., Hemingway, K. L., Apitz, S. E. (2007). Estuarine, coastal and marine ecosystem restoration: confusing management and science–a revision of concepts. Estuarine, Coastal and Shelf Science, 74(3), 349-366.

Franzese, P. P., Buonocore, E., Paoli, C., Massa, F., Donati, S., Miccio, A., Mollica, M., Navone, A., Russo, G.F., Povero, P., Vassallo, P. (2015). Environmental accounting in marine protected areas: the EAMPA Project. Journal of Environmental Accounting and Management, 3(4), 324-332.

Franzese, P. P., Buonocore, E., Donnarumma, L., Russo, G. F. (2017). Natural capital accounting in marine protected areas: The case of the Islands of Ventotene and S. Stefano (Central Italy). Ecological Modelling, 360, 290-299.

Giaccone, G., Alongi, G., Pizzuto, F., Cossu, A. V. L. (1994). La Vegetazione marina bentonica fotofila del Mediterraneo: 2.: Infralitorale e Circalitorale: proposte di aggiornamento. Bollettino dell'Accademia Gioenia di scienze naturali, 27(346), 111-157.

Giangrande, A. (1988). Polychaete zonation and its relation to algal distribution down a vertical cliff in the western Mediterranean (Italy): a structural analysis. Journal of Experimental Marine Biology and Ecology, 120(3), 263-276.

Goren, M. (1980). Development of benthic community on artificial substratum at Ashdod (Eastern Mediterranean). Oceanologica Acta, 3(3), 275-283.

Guarnieri, G., Bevilacqua, S., De Leo, F., Farella, G., Maffia, A., Terlizzi, A., Fraschetti, S. (2016). The challenge of planning conservation strategies in threatened seascapes: understanding the role of fine scale assessments of community response to cumulative human pressures. PloS one, 11(2), e0149253.

Hays, G. C., Richardson, A. J., Robinson, C. (2005). Climate change and marine plankton. Trends in ecology & evolution, 20(6), 337-344.

Harley, C. D., Randall Hughes, A., Hultgren, K. M., Miner, B. G., Sorte, C. J., Thornber, C. S., Rodriguez L.F., Tomanek L., Williams, S. L. (2006). The impacts of climate change in coastal marine systems. Ecology letters, 9(2), 228-241.

Harvey, A. L. (2007). Natural products as a screening resource. Current opinion in chemical biology, 11(5), 480-484.

Helmuth, B., Mieszkowska, N., Moore, P., Hawkins, S. J. (2006). Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change. Annu. Rev. Ecol. Evol. Syst., 37, 373-404.

Hereu, B., Mangialajo, L., Ballesteros, E., Thibaut, T. (2008). On the occurrence, structure and distribution of deep-water Cystoseira (Phaeophyceae) populations in the Port-Cros National Park (north-western Mediterranean). European Journal of phycology, 43(3), 263-273.

Hsieh, C. H., Glaser, S. M., Lucas, A. J., Sugihara, G. (2005). Distinguishing random environmental fluctuations from ecological catastrophes for the North Pacific Ocean. Nature, 435(7040), 336.

Kelaher, B. P., Chapman, M. G., Underwood, A. J. (2001). Spatial patterns of diverse macrofaunal assemblages in coralline turf and their associations with environmental variables. Journal of the Marine Biological Association of the United Kingdom, 81(6), 917-930.

Krause-Jensen, D., Greve, T. M., Nielsen, K. (2005). Eelgrass as a bioindicator under the European Water Framework Directive. Water Resources Management, 19(1), 63-75.

Lindeman, R. L. (1942). The trophic-dynamic aspect of ecology. Ecology, 23(4), 399-417.

Lu, H., Campbell, D., Chen, J., Qin, P., Ren, H. (2007). Conservation and economic viability of nature reserves: an emergy evaluation of the Yancheng Biosphere Reserve. Biological Conservation, 139(3), 415-438

Mander, L., Cutts, N. D., Allen, J., Mazik, K. (2007). Assessing the development of newly created habitat for wintering estuarine birds. Estuarine, Coastal and Shelf Science, 75(1-2), 163-174.

Mangialajo, L., Bertolotto, R., Cattaneo-Vietti, R., Chiantore, M., Grillo, C., Lemee, R., Melchiorre N., Moretto P., Povero P., Ruggieri, N. (2008). The toxic benthic dinoflagellate Ostreopsis ovata: Quantification of proliferation along the coastline of Genoa, Italy. Marine pollution bulletin, 56(6), 1209-1214.

McLusky, D. S., Bryant, D. M., Elliott, M. (1992). The impact of land-claim on macrobenthos, fish and shorebirds on the forth estuary, eastern Scotland. Aquatic conservation: marine and freshwater ecosystems, 2(3), 211-222.

Nagelkerken, I. S. J. M., Blaber, S. J. M., Bouillon, S., Green, P., Haywood, M., Kirton, L. G., Meynecke, J.-O., Pawlik, J., Penrose, H.M., Sasekumar, A., Somerfield, P. J. (2008). The habitat function of mangroves for terrestrial and marine fauna: a review. Aquatic Botany, 89(2), 155-185.

Odum, H. T. (1996). Environmental accounting: emergy and environmental decision making. John Wiley and Sons, New York.

Odum, H.T. (2000). Handbook of Emergy Evaluation Folio #2: Emergy of Global Processes. Center for Environmental Policy, University of Florida, Gainesville. 30p.

Orfanidis, S., Panayotidis, P., Ugland, K. (2011). Ecological Evaluation Index continuous formula (EEI-c) application: a step forward for functional groups, the formula and reference condition values. Mediterranean marine science, 12(1), 199-232.

Paoli, C., Vassallo, P., Fabiano, M. (2008a). Solar power: an approach to transformity evaluation. Ecological engineering, 34(3), 191-206.

Paoli, C., Vassallo, P., Fabiano, M. (2008b). An emergy approach for the assessment of sustainability of small marinas. Ecological engineering, 33(2), 167-178.

Paoli, C., Gastaudo, I., Vassallo, P. (2013). The environmental cost to restore beach ecoservices. Ecological engineering, 52, 182-190.

Paoli, C., Morten, A., Bianchi, C.N., Morri C., Fabiano M., Vassallo P., 2016b. Capturing ecological complexity: OCI, a novel combination of ecological indices as applied to benthic marine habitats. Ecological Indicators 66: 86-102.

Paoli C., Vassallo P., Dapueto G., Fanciulli G., Massa F., Venturini S., Povero P., 2017. The economic revenues and the emergy costs of cruise tourism. Journal of Cleaner Production 166, 1462-1478.

Paoli, C., Povero, P., Burgos, E., Dapueto, G., Fanciulli, G., Massa, F., Scarpellini, P., Vassallo, P. (2018). Natural capital and environmental flows assessment in marine protected areas: The case study of Liguria region (NW Mediterranean Sea). Ecological Modelling, 368, 121-135.

Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. Annu. Rev. Ecol. Evol. Syst., 37, 637-669.

Pauly, D., Christensen, V. (1995). Primary production required to sustain global fisheries. Nature, 374(6519), 255-257.

Pereira, L., Zucaro, A., Ortega, E., Ulgiati, S. (2013). Wealth, trade and the environment: carrying capacity, economic performance and wellbeing in Brazil and Italy. Journal of Environmental Accounting and Management, 1(2), 159-188.

Perkol-Finkel, S., Airoldi, L. (2010). Loss and recovery potential of marine habitats: an experimental study of factors maintaining resilience in subtidal algal forests at the Adriatic Sea. PLoS one, 5(5), e10791.

Picone, F., Buonocore, E., D'Agostaro, R., Donati, S., Chemello, R., Franzese, P. P. (2017). Integrating natural capital assessment and marine spatial planning: A case study in the Mediterranean Sea. Ecological Modelling, 361, 1-13.

Pinedo S, García M, Satta MP, de Torres M, Ballesteros E (2007). Rocky-shore communities as indicators of water quality: A case study in Northwestern Mediterranean. Marine Pollution Bulletin 55: 126-135.

Pulselli, F. M., Patrizi, N., Focardi, S. (2011). Calculation of the unit emergy value of water in an Italian watershed. Ecological modelling, 222(16), 2929-2938.

Redfield, A. C. (1963). The influence of organisms on the composition of seawater. The sea, 2, Wiley, London, 27-77.

Rog, S. M., Clarke, R. H., Cook, C. N. (2017). More than marine: revealing the critical importance of mangrove ecosystems for terrestrial vertebrates. Diversity and Distributions, 23(2), 221-230.

Romero, J., Martínez-Crego, B., Alcoverro, T., Pérez, M. (2007). A multivariate index based on the seagrass Posidonia oceanica (POMI) to assess ecological status of coastal waters under the water framework directive (WFD). Marine Pollution Bulletin, 55(1-6), 196-204.

Sales, M., Ballesteros, E. (2010). Long-term comparison of algal assemblages dominated by Cystoseira crinita (Fucales, Heterokontophyta) from Cap Corse (Corsica, North Western Mediterranean). European Journal of Phycology, 45(4), 404-412.

Sales, M., Cebrian, E., Tomas, F., & Ballesteros, E. (2011). Pollution impacts and recovery potential in three species of the genus Cystoseira (Fucales, Heterokontophyta). Estuarine, Coastal and Shelf Science, 92(3), 347-357.

Sardà, R. (1991). Polychaete communities related to plant covering in the mediolittoral and infralittoral zones of the Balearic Islands (Western Mediterranean). Marine Ecology, 12(4), 341-360.

Schmidt, A. L., Coll, M., Romanuk, T. N., Lotze, H. K. (2011). Ecosystem structure and services in eelgrass Zostera marina and rockweed Ascophyllum nodosum habitats. Marine Ecology Progress Series, 437, 51-68.

Sekovski, I., Newton, A., Dennison, W. C. (2012). Megacities in the coastal zone: Using a driver-pressure-state-impact-response framework to address complex environmental problems. Estuarine, Coastal and Shelf Science, 96, 48-59.

Smale, D. A., Weinberg, T. (2013). Extreme climatic event drives range contraction of a habitat-forming species. In Proc. R. Soc. B (Vol. 280, No. 1754, p. 20122829). The Royal Society.

Smale, D. A., Burrows, M. T., Moore, P., O'Connor, N., Hawkins, S. J. (2013). Threats and knowledge gaps for ecosystem services provided by kelp forests: a northeast Atlantic perspective. Ecology and evolution, 3(11), 4016-4038.

Soltan, D., Verlaque, M., Boudouresque, C. F., Francour, P. (2001). Changes in macroalgal communities in the vicinity of a Mediterranean sewage outfall after the setting up of a treatment plant. Marine Pollution Bulletin, 42(1), 59-70.

Terlizzi A., Fraschetti S., Guidetti P., Boero F., 2002. The effect of sewage discharge on shallow hard substrate sessile assemblages. Marine Pollution Bulletin, 44 (2002), 544-550

Thibaut, T., Pinedo, S., Torras, X., Ballesteros, E. (2005). Long-term decline of the populations of Fucales (Cystoseira spp. and Sargassum spp.) in the Alberes coast (France, North-western Mediterranean). Marine pollution bulletin, 50(12), 1472-1489.

Thibaut, T., Blanfuné, A., Markovic, L., Verlaque, M., Boudouresque, C. F., Perret-Boudouresque, M., Maćic, V., Bottin, L. (2014). Unexpected abundance and long-term relative stability of the brown alga Cystoseira amentacea, hitherto regarded as a threatened species, in the north-western Mediterranean Sea. Marine pollution bulletin, 89(1), 305-323.

Thrush, S. F., Chiantore, M., Asnaghi, V., Hewitt, J., Fiorentino, D., Cattaneo-Vietti, R. (2011). Habitat–diversity relationships in rocky shore algal turf infaunal communities. Marine Ecology Progress Series, 424, 119-132.

Turcato, C., Paoli, C., Scopesi, C., Montagnani, C., Mariotti, M. G., Vassallo, P. (2015). Matsucoccus bast scale in Pinus pinaster forests: a comparison of two systems by means of emergy analysis. Journal of Cleaner Production, 96, 539-548.

Ulgiati, S., Brown, M. T. (2002). Quantifying the environmental support for dilution and abatement of process emissions: the case of electricity production. Journal of Cleaner Production, 10(4), 335-348.

Vassallo, P., Paoli, C., Fabiano, M. (2012). Ecosystem level analysis of sandy beaches using thermodynamic and network analyses: a study case in the NW Mediterranean Sea. Ecological indicators, 15(1), 10-17.

Vassallo, P., Paoli, C., Rovere, A., Montefalcone, M., Morri, C., Bianchi, C. N. (2013). The value of the seagrass Posidonia oceanica: A natural capital assessment. Marine Pollution Bulletin, 75(1), 157-167.

Vassallo, P., Paoli, C., Buonocore, E., Franzese, P. P., Russo, G. F., Povero, P. (2017). Assessing the value of natural capital in marine protected areas: A biophysical and trophodynamic environmental accounting model. Ecological Modelling, 355, 12-17.

Verlaque, M., Tine, J. (1979). Végétation marine de Toulon (Var-France): grande rade et rade-abri. Marine nationale| Phytobenthos marin et pollution.

Yates, K. K., Rogers, C. S., Herlan, J. J., Brooks, G. R., Smiley, N. A., Larson, R. A. (2014). Diverse coral communities in mangrove habitats suggest a novel refuge from climate change. Biogeosciences, 4321.

Ysebaert, T., Herman, P. M. J., Meire, P., Craeymeersch, J., Verbeek, H., Heip, C. H. R. (2003). Large-scale spatial patterns in estuaries: estuarine macrobenthic communities in the Schelde estuary, NW Europe. Estuarine, coastal and shelf Science, 57(1), 335-355.

Zavodnik, N., Ivesa, L., Travizi, A. (2002). Note on recolonisation by fucoid algae Cystoseira spp.and Fucus virsoides in the North Adriatic Sea, Acta. Adriat. 43: 25-32.

General conclusions

In the present PhD thesis, interactions between coastal environments and human well-being have been addressed in order to tackle the objectives required by the recent marine policies.
Overall, the findings reported in the **Chapter 1,** through the implementation of the CARLIT Index, show that the Ligurian rocky coast meets requirements of the Water Framework Directive (WFD), which aimed to reach the "good" Ecological Status (ES) of the EU's coastal water bodies within 2015. In the Ligurian region, the CARLIT Index monitoring, performed over a decade, has also allowed to i) elucidate the ES improvement in some water bodies that have even reached the maximum ES ("high"), ii) identify the relevant improvement of waste treatment performed in the zone, iii) compare the results with other indicators (AMBI, bathing quality water), and iv) observe the increase in abundance of the founding species *Cystoseira amentacea*.
All of these results are also consistent with the Marine Strategy Framework Directive (MSFD) that aims to achieve Good Environmental Status (GES) of the EU's marine waters by 2020, protecting more effectively the marine environment relating their resources with economic and social activities. Eleven qualitative descriptors have been listed in the MSFD in order to determine GES of the marine waters and five of them (1, 4, 5, 8, and 10) can be linked to the main findings obtained in **Chapter 1**. Among these five, three (5, 8, 10) are related to marine pollution in terms of eutrophication, concentration of contaminants (*e.g.* pesticides, anti-foulants, heavy metals) and marine litter resulting from different sources (*e.g*. untreated municipal sewerage, fishing industry, shipping), which are in part directly related to WFD. Instead, the 1 and 4 descriptors address to the maintenance of biodiversity, ensuring the long-term quality and occurrence of the habitats and abundance of species of marine ecosystems. The application of the CARLIT Index assesses indirectly the abundances of the protected *Cystoseira* species and habitat (1170, "reef"), which is in accordance with these latter descriptors of the MSFD. In the line of encompassing different aspects that affect the protection and maintenance of the biodiversity of marine ecosystems and, therefore, to the economic and social activities of the human population, other descriptors, as for instance Descriptor 2 (non-indigenous species), can be taken into account in further studies.

Besides protection and conservation, restoration actions on marine habitats are also encouraged by the MSFD and by the Biodiversity Strategy to 2020. In the Mediterranean, several studies have reported about the past and the present distribution and abundance of different canopy-forming species belonging to the *Cystoseria* genus (Sales et al., 2010; Thibaut et al., 2014), detecting regression or loss of some of them caused by different factors (Thibaut et al., 2005; Perkol-Finkel & Airoldi, 2010; Sales & Ballesteros, 2010).
In **Chapter 1**, the presence and absence of intertidal *Cystoseira* species along the Ligurian coast (S4 Figure) show the loss of several species since 19th century. The species that are still present are *C. compressa* and *C. amentacea*, although the lack of this latter one is recorded in Cinque Terre MPA (Marine Protected Area). The causes that could have affected the disappearance of this species in the past (habitat fragmentation and high sediment loads caused by coastal developments occurred in the area in the first half of the 20th century), discussed in the first chapter, are no longer present and the adequate protection, given by the MPA itself, is assured. In the other hand, large abundances of *C. amentacea* are steadily present in Portofino MPA. For that reasons, the Ligurian Region is an ideal study case for a restoration action of *C. amentacea*, since its coast can

provide both donor and receiving sites. In addition, the implementation of restoration actions, coupled with ecosystem-based management in Marine Protected Areas (MPAs), is considered a successful strategy to preserve of *Cystoseira* stands in the Mediterranean Sea (Mangialajo et al., 2012; Gianni et al., 2013).

These observations are taken into account under the *Promoting biodiversity enhancement by Restoration Of Cystoseira POPulations* project (ROC-POP-LIFE), since one of its goals is to enhance marine biodiversity of the Ligurian rocky shores, by restoring *C. amentacea* in Cinque Terre MPA. Consequently, in **Chapter 2**, a protocol of *Cystoseira amentacea* culture in lab conditions is described to be applied, as first step, in outplanting activities, in order to produce, efficiently, a high number of healthy and large embryos. The second step is the transfer of embryos/juveniles into the field on appropriate substrates, which presents various difficulties caused by different environmental factors that may not ensure the survival of embryos/juveniles transferred up to the fertile adult stage. In addition, in the Fucales species, the patterns of algal stand structure are largely determined in the microscopic early life stages, which is when the highest mortality occurs (Schiel and Foster, 2006). For this reasons, the study of the factors affecting early stages transferred (*e.g.* attachment, site selection, timing and grazing) is essential, in order to achieve a good restoration performance.

The attachment of the substrates on which embryos are settled onto the rocky shores can be realized either through epoxy resin or drilling. The latter one can be a good choice that allows a strong attachment of the substrates, but also any easy removal, if necessary, without permanent remains in the environment.

In the intertidal zone, where direct exposure to light irradiance and associated temperature stress can affect the healthy embryos transferred, attaching the substrates in zones with presence of a canopy or an algal turf can be a good decision (Harris et al., 1984; McConnico and Foster, 2005), because of the protection action that they can play on embryos. In addition, during summer season, also the nutrient competition becomes a relevant factor that affect the early life stages, because fast growth at high temperatures requires higher nutrient demands (Steen and Scrosati, 2004). Another threat is grazing, especially, acting on the highly vulnerable early life stages (Bertness et al., 2002), which are prey to a wide range of herbivores (e.g. small crustaceans, snails, sea urchins) that can increase their activity during hot seasons when macroalgal biomass is increased. A delay in development can occur in the early stages of Fucales species when seasonal conditions are unfavorable (DeWreede, 1986, Worm et al., 1999), ensuring their survival while waiting for better conditions in order to increase their growth. For this reason, one option is to transfer early stages into the rocky shores at the very beginning of spring or even in cold season (autumn/winter), in order to decrease early stages mortality rate caused by the above mentioned factors.

In Europe, along both Atlantic and Mediterranean coasts, there is a growing demand of marine resources in the biotech sector, for different applications as nutraceutical, pharmaceutical, medicine or additive food. For instance, *Cystoseira barbata* and *Cystoseira crinita* are used as food additives in medical treatment of oncological patients in Ukraine and Bulgaria countries, and the brown algae *Dictyota spiralis* and *Halopteris scoparia* are used in pharmaceutical applications (Pereira, 2016). In green algae, some species have been reported as a potential source for medical use, for example *Bryopsis plumosa* (favors cardiovascular and nervous activity, Milchakova, 2011) or *Ulva intestinalis*, which, besides being widely used as food, is also used in different medical treatments as aphthae, back pain or goiter (Oh, 1990). Regarding red ones, *Gracilaria*, *Bangia* and *Porphyra* genera are some examples, particularly the latter one, which is worldwide well-known as alga Nori used as food, being one of the most important commercial macroalgal species. Interestingly, its antioxidant capacity has also demonstrated (Souza et al., 2007; Zubia et al., 2009). Other examples of red species, *Asparagopsis armata* and *Sphaeroccocus coronopifolius*, show high potential as antimicrobial activity (Pinteus et al., 2015).

Clearly, green, red and brown macroalgae are marine resources with many potential uses for human health and well-being. Further studies on the characterization of the compounds responsible of each bioactivity in the different species are essential to encourage positioning of the European industries on macroalgal market in terms of functional food and natural compounds, as well as, to promote an increase of the macroalgal farming in Europe.

Results reported in **Chapter 3** reinforce the potential bioactive properties of Fucales species, in particular, the antioxidant activity in *Cystoseira* genus, which is related with their phenolic content. Polyphenols in brown algae are mostly phlorotannins, which have been recognized as the most significant group of biologically active substances determining the pharmacological value of algae (Kim and Himaya, 2011). Phlorotannins have been identified from several brown algal families such as Alariaceae, Fucaceae and Sargassaceae (Wang et al., 2009; Holdt and Kraan, 2011), therefore, also in the species belonging *Cystoseira* genus (Montero et al., 2014). The antioxidant activity of polyphenols extracted from brown algae has been widely demonstrated by in vitro assays (Shibata et al., 2008; Wijesekara et al., 2010; Balboa et al., 2013). However, due to the complex chemical structure of these bioactive polyphenols in brown algae and the multiple roles of phlorotannins, it is still necessary to identify which types of phlorotannins are responsible for the different potential bioactivities.

Finally, in **Chapter 4**, the results show the influence of canopy-forming *Cystoseira* on the associated middlitoral community. The economic values estimated, through emergy analysis, for middlitoral fringe associated to *Cystoseira amentacea* continuous belt obtained lower values than those associated with *C. compressa* or non-continuous belt. Despite of that, the variability of the values was lower, showing a homogeneous deterministic effect on the infauna and macroalgae associated to the *C. amentacea* continuous belt. These findings stimulate further research about assessing the natural capital of the valuable habitats provided by *Cystoseria* species. To estimate the economic value of *Cystoseira* habitat, it is necessary biomass data of the given canopy, and of its understory, in order to evaluate the ecosystem functioning and services that it provides. Given the ecological relevance of the canopy-forming *Cystoseira*, the sampling methodology to collect this data could compromise its preservation. This data could be also obtained from the many studies carried out on *Cystoseira* population in the Mediterranean Sea, therefore, their availability by the authors is highly encouraged.

On the same line, the economic evaluation of the protection against restoration costs in marine and coastal environments is very important to decision-makers, planners, and researchers. The decision about to protect or restore these habitats could depend on the relative costs of the two options, taking into account the efficiency and feasibility of restoration actions (Suding 2011, La Peyre et al. 2014). Nevertheless, optimal conservation results require both protection and restoration (Possingham et al. 2015) depending on their relative costs, the rate at which habitat is being lost, and the time required between restoring habitat and the recovery of its ecosystem services. Monitoring activities to identify the most cost-efficient restoration techniques and avoid project failure are particularly needed (Bayraktarov et al., 2016).

For these reasons, the *Cystoseira* restoration in Cinque Terre MPA, under the ROC-POP-LIFE project, will be a good opportunity to assess relative costs, paying attention the objective of the marine directives that aim to provide benefits to environment, human health and economy.

References

Balboa, E. M., Conde, E., Moure, A., Falqué, E., & Domínguez, H. (2013). In vitro antioxidant properties of crude extracts and compounds from brown algae. Food chemistry, 138(2), 1764-1785.

Bayraktarov, E., Saunders, M. I., Abdullah, S., Mills, M., Beher, J., Possingham, H. P., ... & Lovelock, C. E. (2016). The cost and feasibility of marine coastal restoration. Ecological Applications, 26(4), 1055-1074.

Bertness, M. D., Trussell, G. C., Ewanchuk, P. J., & Silliman, B. R. (2002). Do alternate stable community states exist in the Gulf of Maine rocky intertidal zone?. Ecology, 83(12), 3434-3448.

De Wreede, R. E. (1986). Demographic characteristics of Pterygophora californica (Laminariales, Phaeophyta). Phycologia, 25(1), 11-17.

Gianni, F., Bartolini, F., Airoldi, L., Ballesteros, E., Francour, P., Guidetti, P., ... & Mangialajo, L. (2013). Conservation and restoration of marine forests in the Mediterranean Sea and the potential role of Marine Protected Areas. Advances in oceanography and limnology, 4(2), 83-101.

Harris, L. G., Ebeling, A. W., Laur, D. R., & Rowley, R. J. (1984). Community recovery after storm damage: a case of facilitation in primary succession. Science, 224, 1336-1339.

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. Journal of Applied Phycology, 23(3), 543-597.

Kim, S. K., & Himaya, S. W. A. (2011). Medicinal effects of phlorotannins from marine brown algae. Advances in food and nutrition research, 64, 97-109.

La Peyre, M., Furlong, J., Brown, L. A., Piazza, B. P., & Brown, K. (2014). Oyster reef restoration in the northern Gulf of Mexico: Extent, methods and outcomes. Ocean & Coastal Management, 89, 20-28.

Mangialajo, L., Chiantore, M., Susini, M. L., Meinesz, A., Cattaneo-Vietti, R., & Thibaut, T. (2012). Zonation patterns and interspecific relationships of fucoids in microtidal environments. Journal of experimental marine biology and ecology, 412, 72-80.

McConnico, L. A., & Foster, M. S. (2005). Population biology of the intertidal kelp, Alaria marginata Postels and Ruprecht: a non-fugitive annual. Journal of Experimental Marine Biology and Ecology, 324(1), 61-75.

Milchakova, N. A. (2011). Marine Plants of the Black Sea: An Illustrated Field Guide. DigitPrint Press.

Montero, L., Herrero, M., Ibáñez, E., & Cifuentes, A. (2014). Separation and characterization of phlorotannins from brown algae Cystoseira abies-marina by comprehensive two-dimensional liquid chromatography. Electrophoresis, 35(11), 1644-1651.

Oh, Y. S., Lee, I. K., & Boo, S. M. (1990). An annotated account of Korean economic seaweeds for food, medical and industrial uses. Korean J. Phycol, 5, 57-71.

Perkol-Finkel, S., & Airoldi, L. (2010). Loss and recovery potential of marine habitats: an experimental study of factors maintaining resilience in subtidal algal forests at the Adriatic Sea. PLoS one, 5(5), e10791.

Pereira, L. (2016). Edible seaweeds of the world. CRC Press.

Pinteus, S., Alves, C., Monteiro, H., Araújo, E., Horta, A., & Pedrosa, R. (2015). Asparagopsis armata and Sphaerococcus coronopifolius as a natural source of antimicrobial compounds. World Journal of Microbiology and Biotechnology, 31(3), 445-451.

Possingham, H. P., Bode, M., & Klein, C. J. (2015). Optimal conservation outcomes require both restoration and protection. PLoS biology, 13(1), e1002052.

Sales, M., & Ballesteros, E. (2010). Long-term comparison of algal assemblages dominated by Cystoseira crinita (Fucales, Heterokontophyta) from Cap Corse (Corsica, North Western Mediterranean). European Journal of Phycology, 45(4), 404-412.

Schiel, D. R., & Foster, M. S. (2006). The population biology of large brown seaweeds: ecological consequences of multiphase life histories in dynamic coastal environments. Annu. Rev. Ecol. Evol. Syst., 37, 343-372.

Shibata, T., Ishimaru, K., Kawaguchi, S., Yoshikawa, H., & Hama, Y. (2008). Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae. Journal of Applied Phycology, 20(5), 705.

de Souza, M. C. R., Marques, C. T., Dore, C. M. G., da Silva, F. R. F., Rocha, H. A. O., & Leite, E. L. (2007). Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. Journal of applied phycology, 19(2), 153-160.

Steen, H., & Scrosati, R. (2004). Intraspecific competition in Fucus serratus and F. evanescens (Phaeophyceae: Fucales) germlings: effects of settlement density, nutrient concentration, and temperature. Marine Biology, 144(1), 61-70.

Suding, K. N. (2011). Toward an era of restoration in ecology: successes, failures, and opportunities ahead. Annual Review of Ecology, Evolution, and Systematics, 42.

Thibaut, T., Pinedo, S., Torras, X., & Ballesteros, E. (2005). Long-term decline of the populations of Fucales (Cystoseira spp. and Sargassum spp.) in the Alberes coast (France, North-western Mediterranean). Marine pollution bulletin, 50(12), 1472-1489.

Thibaut, T., Blanfuné, A., Markovic, L., Verlaque, M., Boudouresque, C. F., Perret-Boudouresque, M., ... & Bottin, L. (2014). Unexpected abundance and long-term relative stability of the brown alga Cystoseira amentacea, hitherto regarded as a threatened species, in the north-western Mediterranean Sea. Marine pollution bulletin, 89(1), 305-323.

Wang, T., Jonsdottir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food chemistry, 116(1), 240-248.

Wijesekara, I., Yoon, N. Y., & Kim, S. K. (2010). Phlorotannins from Ecklonia cava (Phaeophyceae): biological activities and potential health benefits. Biofactors, 36(6), 408-414.

Worm, B., Lotze, H. K., Boström, C., Engkvist, R., Labanauskas, V., & Sommer, U. (1999). Marine diversity shift linked to interactions among grazers, nutrients and propagule banks. Marine Ecology Progress Series, 309-314.

Zubia, M., Fabre, M. S., Kerjean, V., Le Lann, K., Stiger-Pouvreau, V., Fauchon, M., & Deslandes, E. (2009). Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chemistry, 116(3), 693-701.