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# Marine forests loss : causes and effects in the framework of their conservation and restoration

Margalida Monserrat Barcelo

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$$e^{i\pi} + 1 = 0$$

# THÈSE DE DOCTORAT

## Régression des forêts marines : Causes et effets dans le cadre de leur conservation et restauration

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Présentée en vue de l'obtention du grade de docteur en  
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DOCTORAL THESIS

**REGRESSION DES FORETS MARINES : CAUSES ET EFFETS DANS LE CADRE DE  
LEUR CONSERVATION ET RESTAURATION**

-

**MARINE FORESTS LOSS: CAUSES AND EFFECTS IN THE FRAMEWORK OF  
THEIR CONSERVATION AND RESTORATION**

Margalida Monserrat Barcelo

Presented for the obtention of the degree of Doctor of Environmental Sciences from the  
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*A ses meves padrines Margalides,*

*a n'es padrí Joan i Francisco*

*i a sa Madrina.*

*Als meus pares i a n'en Miquel.*





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**RESUME**

Les grandes algues brunes sont des espèces clés dans les écosystèmes marins des latitudes tempérées où elles forment les forêts marines, parmi les écosystèmes les plus productifs et diversifiés au monde. En Méditerranée, elles sont représentées par les espèces du genre *Cystoseira sensu lato*, pour la plupart endémiques et caractérisées par leur longue durée de vie et faible dispersion. Cependant, les impacts anthropogéniques causent leur régression, entraînant des changements abruptes vers des communautés moins complexes (communautés gazonnantes et déserts marins), rendant l'écosystème potentiellement plus vulnérable aux phénomènes émergents, tels que les efflorescences de microalgues benthiques nuisibles. Les efflorescences de dinoflagellés benthiques du genre *Ostreopsis*, se développant sur les communautés macroalgales ont augmenté au cours des dernières décennies dans les régions tempérées, y compris la Méditerranée. Elles sont connues pour leurs effets néfastes sur la santé publique, les écosystèmes et l'économie qui en dépend.

Les objectifs de cette thèse sont d'évaluer (i) les causes abiotiques (température et acidification) et biotiques (herbivorie, facilitation écologique) potentiellement à l'origine de la régression des forêts marines Méditerranéennes, et (ii) les éventuelles conséquences de cette régression dans la facilitation des efflorescences d'*Ostreopsis* spp. Les deux premiers chapitres de cette thèse sont focalisés sur les causes de la régression de *Cystoseira s.l.* Dans le Chapitre 1, les effets du changement climatique et de la facilitation écologique sur le recrutement de *Cystoseira compressa* ont été étudiées par des expériences en laboratoire. Un effet interactif du réchauffement et de l'acidification de l'océan, qui affecte négativement les recrues de *C. compressa*, a été observé, ainsi qu'un effet négatif de la présence d'algues corallines incrustantes. Dans le Chapitre 2, la pression herbivore de différents invertébrés sur les recrues de *C. compressa* a été étudiée par des expériences sur le terrain et en laboratoire. Les résultats montrent une forte pression herbivore de plusieurs espèces d'invertébrés (mollusques, décapodes et isopodes) sur les recrues de *C. compressa*, qui pourraient représenter une menace pour les populations à long terme et une cause d'insuccès des actions de restauration.

Les chapitres 3 et 4 se focalisent sur le lien entre la perte de forêts marines et les efflorescences d'*Ostreopsis* spp. Dans le Chapitre 3, une revue bibliographique s'intéresse au rôle de l'habitat dans la facilitation/régulation des efflorescences. Malgré un évident manque d'informations à l'échelle globale sur les méso- et macrohabitat plus propices aux efflorescences, les connaissances actuelles démontrent que les substrats les plus échantillonnés pour étudier ces espèces sont des macroalgues formant des communautés peu complexes, suggérant que ces communautés hébergent les efflorescences les plus importantes. Dans le Chapitre 4, la relation entre les efflorescences d'*Ostreopsis* et les communautés macroalgales a été étudiée par des expériences sur le terrain en



## RESUME

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Italie et en France. Des différences significatives ont été observées sur un des deux sites d'étude tandis que dans le deuxième on estime que la forte variabilité puisse avoir caché les éventuelles préférences d'*Ostreopsis* spp. Mais, des études à plus grande échelle seraient nécessaires pour conforter ces résultats.

Les résultats de cette thèse représentent d'importantes avancées sur les causes et les effets de la régression des forêts de *Cystoseira s.l.*, confortant l'importance de leur conservation et (où nécessaire) restauration, en contribuant à la conception de stratégies de gestion, non seulement pour préserver un des écosystèmes les plus productifs en Méditerranée, mais aussi dans le but de limiter d'éventuelles conséquences inattendues, telles que les efflorescences d'*Ostreopsis* spp.

**Mots clé :** forêts marines, *Cystoseira compressa*, communautés de macroalgues, réchauffement des océans, acidification des océans, changement climatique, changements de régime, efflorescences algales nuisibles, dinoflagellés benthiques, *Ostreopsis*

**ABSTRACT**

Large brown forest-forming macroalgae are dominant foundation species, ecosystem engineers of marine macroalgal forests. In the Mediterranean Sea, they are mainly represented by *Cystoseira sensu lato* spp. most of which are endemic. They are also characterized for being long-lived species with short dispersal of the zygotes. Marine forests are one of the most productive and diverse ecosystems on earth. However, anthropogenic impacts are pushing them to the edge, causing regime shifts towards less complex communities such as shrubs, turfs, or even barren grounds. Marine forest loss affects the whole ecosystem, eventually making it more vulnerable to emergent phenomena such as benthic harmful algal blooms (BHABs). BHAB of the genus *Ostreopsis* spp. have been expanding in recent decades through temperate regions such as the Mediterranean Sea, where they have important public health, ecological and economic consequences. Major blooms are generally observed on macroalgal turfs and shrubs, suggesting that less structurally complex macroalgal communities could have an active role in promoting the proliferation of blooms.

The main objectives of this thesis are (i) to assess some abiotic (climate change) and biotic (herbivory) causes of marine forests loss in the Mediterranean Sea and (ii) the potential consequences this loss can have in the context of BHABs proliferation. In the first two chapters, the causes of *Cystoseira s.l.* spp. loss were assessed. In Chapter 1, the effects of climate change and species facilitation on the recruitment of *Cystoseira compressa* were studied in controlled laboratory experiments. The major results from this chapter show that the interactive effects of ocean warming and acidification negatively affect *C. compressa* recruits, which are also negatively affected by the presence of crustose coralline algae. In Chapter 2 the grazing pressure and the effects of different invertebrates on recruits of *C. compressa* were assessed through field surveys and both field and laboratory-based experiments. The results obtained show a high grazing rate of several common invertebrate species (molluscs, decapods et isopods) on recruits of *C. compressa*, representing a threat to natural populations in the long term, but also affecting the success of restoration actions.

In chapters 3 and 4 the consequences of forest loss and in particular, the facilitation of *Ostreopsis* spp. blooms was approached by a literature review and field experiments. The review, reported in Chapter 3, focussed on the role of habitat in the facilitation of *Ostreopsis* spp. blooms. Despite an evident lack of information at the global scale on the meso- and macrohabitat fostering *Ostreopsis* spp. blooms, the present knowledge suggests a relationship between the abundance of *Ostreopsis* spp. and the complexity of the macroalgal communities. In Chapter 4 *Ostreopsis* spp. blooms have been studied in relation to macroalgal communities in field experiments in Italy and in France. A high variability on *Ostreopsis* spp. abundances was observed in the different macroalgal species

## ABSTRACT

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and communities sampled, in some cases likely hiding other potential patterns of *Ostreopsis* spp. preferences. Larger scale studies would be needed to confirm these results.

These findings provide important insights into the causes and effects of *Cystoseira s.l.* spp. loss and are of major interest for the conservation of Mediterranean marine forests, contributing to the development of effective management measures. The results presented support the importance of marine forests restoration in the Mediterranean Sea, as recommended by the 2030 European Biodiversity Strategy, the United Nations Decade on Ecosystem Restoration and the 2030 Agenda for Sustainable Development objectives. Such restoration actions will not only increase the productivity and biodiversity of coastal ecosystems but could potentially mitigate the public health, ecological and economic consequences of *Ostreopsis* spp. blooms.

**Keywords:** marine forests, *Cystoseira compressa*, macroalgal communities, ocean warming, ocean acidification, climate change, herbivory, regime shifts, HABS, benthic dinoflagellates, *Ostreopsis*

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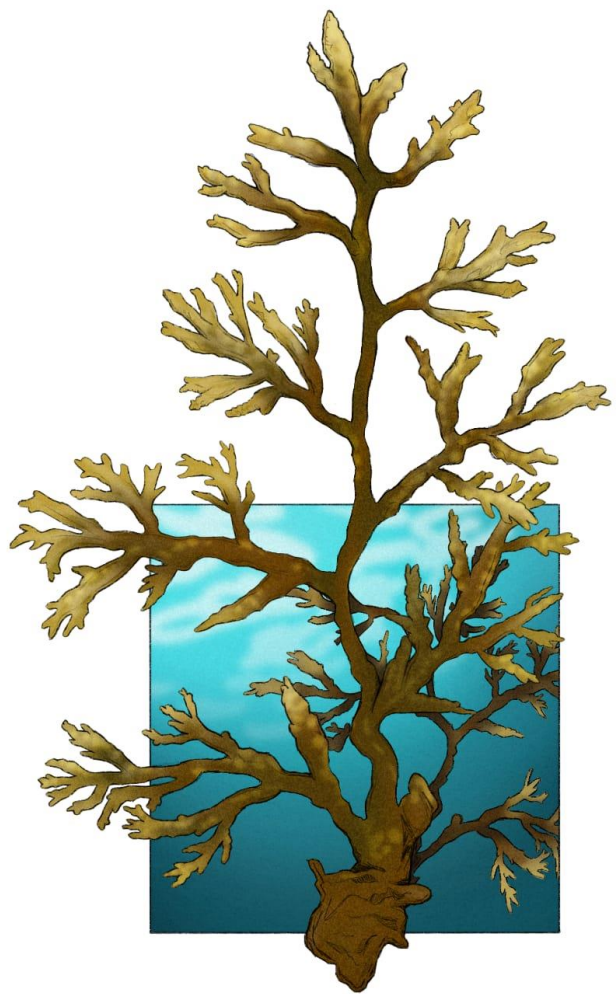
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# GENERAL INTRODUCTION AND OBJECTIVES



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## **THE MARINE ENVIRONMENT**

### ***A threatened biodiversity***

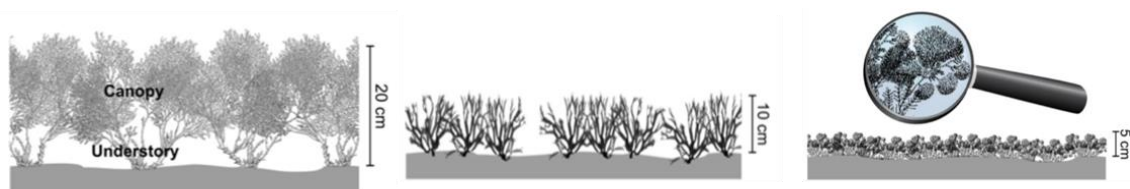
Oceans constitute the largest volume of life on the planet covering 70.8 % of the Earth's surface (Boeuf, 2011). They host an important number of species but also play an important role in climate regulation and provide major ecosystem services for humans (Mcleod et al., 2011). However, the increasing number of human populations (reaching 8 billion in November 2022; United Nations Department of Economic and Social Affairs, Population Division, 2022) is changing global biodiversity at unprecedented rates (Zalasiewicz et al., 2011). A large and rising proportion of the population lives close to the coast (three times more people than the global average is living less than 100 km from the sea; Small and Nicholls, 2003; Todd et al., 2019), negatively impacting the coastal environment, which constitutes an important zone with a lot of spatial heterogeneity influencing the temperature, salinity, and primary production of coastal ecosystems.

Different types of anthropogenic stressors can impact marine ecosystems, some at a global scale, such as global warming and ocean acidification; and others at a regional or local scale, such as urbanisation, sedimentation, overfishing, invasive species and water pollution. As a consequence of these impacts and their interactions, reductions in habitat structure, biodiversity and trophic complexity are expected (Doney et al., 2012; Duarte, 2014; Hall-Spencer and Harvey, 2019). Among the anthropogenic pressures, climate change is expected to be the strongest force of biodiversity change at the global scale (Bellard et al., 2012). In the present, climate change is already producing changes in demographic rates and forcing the redistribution, adaptation and acclimation of species (Mooney et al., 2009; Doney et al., 2012, 2020; Bernhardt and Leslie, 2013). All these impacts will probably influence the communities composition and interactions among species (Bakker et al., 2016; Pagès et al., 2018), producing cascade effects at the ecosystem level, with ecological and socioeconomic consequences (Hoegh-Guldberg and Bruno, 2010; Pecl et al., 2017; Doney et al., 2020). At present there is not a single marine ecosystem unthreatened by anthropogenic stressors (Halpern et al., 2008b, 2011, 2018), but the hard-bottom coastal areas and rocky reefs are the highest impacted (Halpern et al., 2007, 2019). To assess the ongoing impacts of climate change, the Intergovernmental Panel on Climate Change (IPCC) prepares Assessment Reports about the state of scientific, technical and socio-economic knowledge on climate change using the Shared Socioeconomic Pathways (SSPs). The SSPs are scenarios of projected socioeconomic global changes up to 2100 used to derive greenhouse gas emissions scenarios from different climate policies (IPCC; 2022). The last IPCC report is the Sixth Assessment Report (AR6 Climate Change 2022: Mitigation of Climate Change — IPCC).



### *The key role of macroalgae dominated communities*

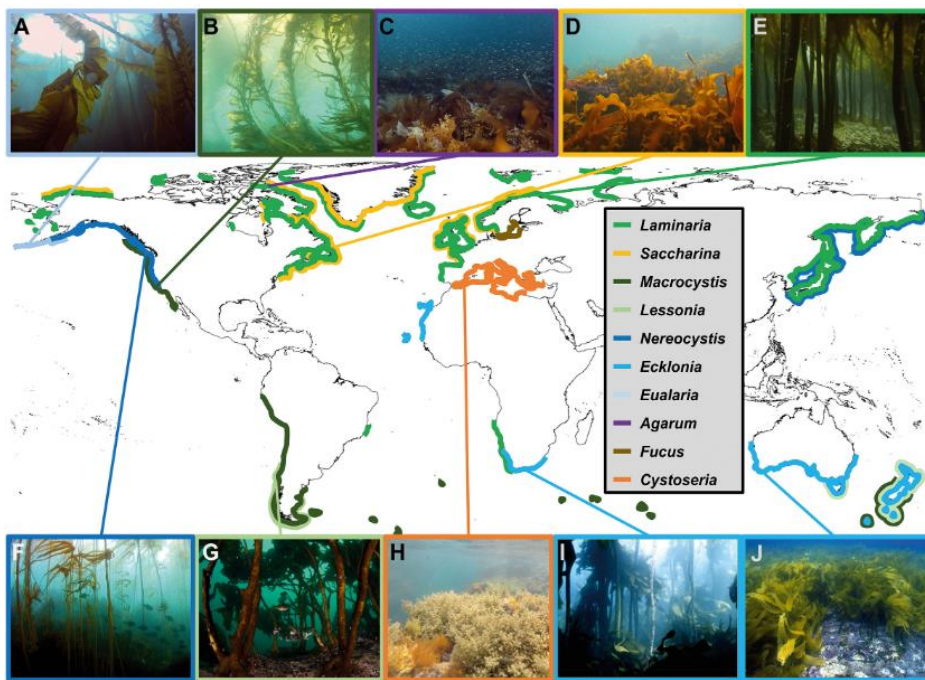
Macroalgae or seaweeds are key organisms in temperate coastal ecosystems functioning around the globe, largely contributing to benthic primary production (Steneck et al., 2002; Cheminée et al., 2013; Teagle et al., 2017; Piazzini et al., 2018). Macroalgae also participate in carbon sequestration by capturing, storing, and potentially sequestering CO<sub>2</sub> in the ocean through transportation to deep marine sediments (Krause-Jensen and Duarte, 2016; Krause-Jensen et al., 2018; Filbee-Dexter and Wernberg, 2020; Filbee-Dexter et al., 2022; Wright et al., 2022). Furthermore, they provide other invaluable ecosystem services such as supporting fisheries and mariculture, nutrient cycling and protecting the shoreline (Bennett et al., 2015a; Blamey and Bolton, 2018; Eger et al., 2021a). Consequently, changes in the macroalgal composition may sway coastal ecosystems (Sala et al., 1998; Shears and Ross, 2010; Mineur et al., 2015; Smale et al., 2022). The diverse macroalgal species thriving on rocky reefs can be characterised by their sizes, shapes, structure and architecture of their thallus. Based on these factors, we can differentiate turf, erect and forest-forming or canopy-forming macroalgae. Turf-forming macroalgae are represented by species with tightly packed fronds and filamentous thin axes, usually forming dense and compact mats (Stewart, 1983; Sala et al., 2012; Connell et al., 2014; Thiriet et al., 2016; Mauffrey et al., 2020). Erect macroalgae are described as foliose laminar, ribbon-like, massive or fan-like thallus and erect arborescent tufts, which do not form a canopy but a shrub (Sala et al., 2012; Thiriet et al., 2016; Bertolini, 2019). Forest-forming macroalgae include the most structurally complex macroalgae with cylindrical axes, branched and tree-like ramifications (Sala et al., 2012; Thiriet et al., 2014; Bertolini, 2019; Shelamoff et al., 2019; Assis et al., 2020). The macroalgae from this group are considered habitat-forming species able to create three-dimensional habitats with a canopy and understory, which supports distinct communities of fish, invertebrates and other plants (Cheminée et al., 2013, 2017; Thiriet et al., 2016; Shelamoff et al., 2019; Wernberg and Filbee-Dexter, 2019). Analogically to terrestrial environments, the physical traits of the dominant species are used to classify macroalgal communities into turfs, shrubs and forests, indicating the functional similarities between habitats on land and in the sea (Figure 1; Wernberg and Filbee-Dexter, 2019).



**Figure 1:** Different types of marine macroalgal habitats in function of the structural characteristics of the dominant species: marine forest, shrub and turf. Modified from Thiriet (2014).

**MARINE FORESTS**

Macroalgal marine forests are constituted by large brown forest-forming macroalgae of the orders Laminariales, Tylopteridales, Desmarestiales and Fucales (Wernberg and Filbee-Dexter, 2019) and are considered among the most productive and biodiversity-rich ecosystems on Earth (Wernberg and Filbee-Dexter, 2019; Pessarrodona et al., 2022). They form the tallest biotic component of the seascape, altering the physical and biological environment in the understory (Thiriet et al., 2014; Veiga et al., 2014). These foundation species dominate intertidal and subtidal rocky shores in temperate and polar regions worldwide (Figure 2; Feldmann, 1934; Boudouresque et al., 2016; Wernberg and Filbee-Dexter, 2019). They provide functions very similar to terrestrial forests such as: (i) habitat, food and shelter to a multitude of species at different life stages, (ii) supporting a high biodiversity of primary and secondary producers and decomposers, and (iii) playing an important role in the functioning and structure of the ecosystem maintaining food-webs and enhancing the secondary production (Ballesteros et al., 2009; Smale et al., 2013; Teagle et al., 2017; Wernberg et al., 2019a; Fragkopoulou et al., 2022).



**Figure 2:** Distribution of marine forests of large brown macroalgae for different regions. Figure from Wernberg and Filbee-Dexter (2019).

The structure and productivity of marine forests are influenced by many environmental factors that drive the growth, survival, reproduction and metabolism of forest-forming macroalgae, which in turn affect the whole habitat or ecosystem (Irving et al., 2009; Cardona et al., 2013; Pessarrodona et al., 2022; Smale et al., 2022; Smith et al., 2022). A variety of anthropogenic impacts are altering the environmental parameters that drive the functioning of ecosystems (Krumhansl et al., 2016; Pessarrodona et al., 2019), and as a result negatively affect marine forests that at the present are in

regression worldwide (Filbee-Dexter and Wernberg, 2018; Pessarrodona et al., 2021). Several local and global impacts are involved in the regression of marine forests such as urbanisation, marine farming, local pollution and herbivory (Steneck et al., 2002; Krumhansl et al., 2016; Orfanidis et al., 2021), but also ocean warming (OW) and potentially ocean acidification (OA; Wernberg et al., 2011; Connell et al., 2013; Friedlander et al., 2020; Smale, 2020). These stressors are putting marine forests to the limit, confining them to refuge locations characterized by less unfavourable conditions (Straub et al., 2019; Verdura et al., 2021); and making them less resilient against future impacts (Capdevila et al., 2019; Straub et al., 2019).

Global change effects on marine forests can vary according to the location, the population characteristics and the species (Krumhansl et al., 2016; Hollarsmith et al., 2020; Verdura et al., 2021). There is evidence that early-life stages of these species are more vulnerable than adults and this, together with a very high natural mortality rate during early-life stages (Ang, 1991; Vadas et al., 1992; Capdevila et al., 2015), could lead, in the long term, to their loss (Coelho et al., 2000; Schiel and Foster, 2006; de Caralt et al., 2020).

### **MEDITERRANEAN MARINE FORESTS**

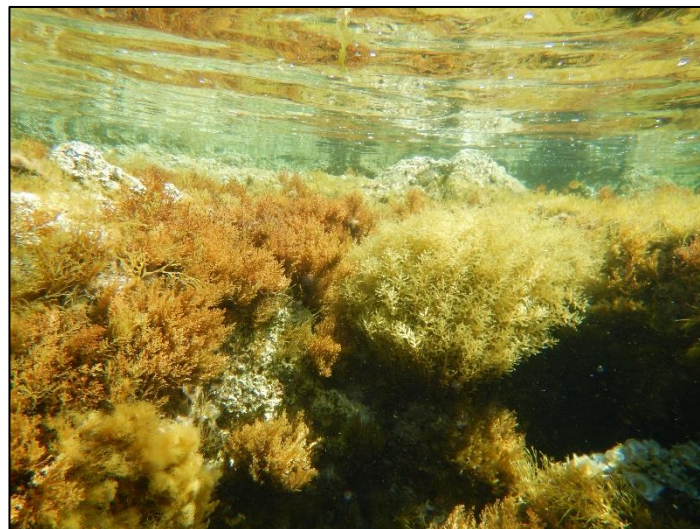
The Mediterranean Sea is the largest (more than 2 500 000 km<sup>2</sup>) and deepest (average and maximum depth of 1 500 m and 5 267 m, respectively) enclosed sea on Earth (Coll et al., 2010). Nowadays the Mediterranean coasts support a high density of inhabitants and represent one of the first touristic destinations in the world, with the consequent environmental cost that it entails (Segreto et al., 2009; Coll et al., 2010). The Mediterranean Sea, characterized by small tides, oligotrophic waters and high salinity and mean water temperature (Ros et al., 1985), is one of the major hotspots of biodiversity, especially on coastal areas (Coll et al., 2010). Some of these unique communities are the meadows of the endemic phanerogam *Posidonia oceanica*, the coralligenous community built up by crustose coralline algae, the vermetid platforms, the *Litophyllum brissoides* algal reef and the Fucales forests (Ros et al., 1985; Boudouresque, 2004).

Macroalgal marine forests in the Mediterranean Sea are represented mainly by species belonging to the orders Fucales; in particular to the genera *Cystoseira*, *Gongolaria* and *Ericaria*, from now referred as *Cystoseira sensu lato* species (Box 1; Wernberg and Filbee-Dexter, 2019; Molinari - Novoa and Guiry, 2020). There are more than thirty different species, most of them endemic to the Mediterranean Sea (Molinari - Novoa and Guiry, 2020), they are the dominant forest-forming species in intertidal and sublittoral rocky bottoms and despite showing smaller average size have functional traits comparable to those of larger kelps (Wernberg and Filbee-Dexter, 2019). Only two species of Laminariales are found in the Mediterranean Sea forming marine forests: *Laminaria ochroeluca*, in the immediacies of the Strait of Gibraltar, western coast of Africa and the Strait of

Messina (Drew, 1974; Flores-Moya, 2012); and the critically endangered and endemic deep-water *Laminaria rodriguezii* (Feldmann, 1934; Bo et al., 2011).

**BOX 1: *Cystoseira sensu lato* species**

*Cystoseira s.l.* spp. have a high morphological plasticity that makes their identification complex using morphological traits only (Rožić et al., 2012; Orellana et al., 2019). Analysis of DNA demonstrated the multiple phylogenetic origin of the Mediterranean genus *Cystoseira* (Draisma et al., 2010) leading to its division in three clades (Bruno de Sousa et al., 2019). A few years ago, Orellana et al. (2019) assigned three different genera to the clades based on their morphological characteristics: *Cystoseira* C. Agardh, 1820; *Treptacanta* Kützing, 1843; and *Carpodesmia* Greville, 1830. More recently, Molinar-Novoa and Guiry (2020) revised this proposition concluding that *Gongolaria* Boehmer, 1760 and *Ericaria* Stackhouse, 1890 have priority over the ones proposed by Orellana et al. (2019), and now *Cystoseira*, *Ericaria* and *Gongolaria* are the accepted names for the three clades (Molinari - Novoa and Guiry, 2020).



Shallow *Cystoseira s.l.* forest in Saint Honorat Island (Lérins Islands, Cannes, France) composed by *Ericaria amentacea*, *Ericaria brachycarpa* and *Cystoseira compressa*.

*Cystoseira s.l.* spp. form canopies up to 1 m height in exposed or sheltered locations from the upper littoral to the circalittoral zone, down to 50 m depth, and represent the highest level of Mediterranean macroalgae complexity (Giaccone, 1973; Ballesteros, 1990a, 1990b; Sala et al., 2012). *Cystoseira s.l.* spp. are considered habitat-forming as they generate a three-dimensional structure from which it is possible to differentiate different strata: an arboreal layer, on the upper part of the canopy made by the forest-forming species (i.e. *Cystoseira s.l.*) and the epiphytes; a middle shrubby layer on the middle canopy, formed by erect shrub-forming macroalgae; a turf layer, formed by turf-forming calcareous and sciaphilic macroalgae; and a basal layer, mainly formed by crustose calcifying algae (Ros et al., 1985). *Cystoseira s.l.* forests are one of the most productive habitats in the Mediterranean Sea (Figure 3; Ballesteros, 1989a; Sales and Ballesteros,



2012; Duarte et al., 2022; Pessarrodona et al., 2022) and provide habitat to a large number of associated species, supporting trophic networks (Ballesteros, 1992; Ballesteros et al., 2009; Thiriet et al., 2016; Piazzini et al., 2018).

In function of their distribution and vertical zonation (that is influenced by many factors such as depth, light intensity, hydrodynamics, resistance to breaking, temperature, nutrient availability and herbivory pressure; Feldmann, 1934; Vergés et al., 2009; Sant and Ballesteros, 2020a, 2021), the forests are dominated by different *Cystoseira s.l.* spp. Some species are widely distributed along the Mediterranean Sea and others restricted to very specific locations, being most of the species found on shallow sheltered or exposed locations (Ribera et al., 1992).

### ***Threats and status of Cystoseira sensu lato forests***

Many anthropogenic impacts are pushing Mediterranean marine ecosystems to the edge (Coll et al., 2010). The overexploitation and habitat loss (e.g. urbanisation) have been affecting the ecosystems for centuries and at present, together with fishing impacts, pollution, eutrophication and climate change, represent the most important threats to almost all species, including *Cystoseira s.l.* forests (Mangialajo et al., 2008b; Coll et al., 2010; Giakoumi et al., 2012; Orfanidis et al., 2021). The Mediterranean Sea is very sensitive to climate change (Belkin, 2009; Lejeune et al., 2010; Tuel and Eltahir, 2020) and the seawater temperature is already increasing (Lionello and Scarascia, 2018) as well as the intensity and frequency of marine heatwaves (Oliver et al., 2018, 2019). In addition, global biodiversity scenarios also predict that invasive species will be an increasing problem in the Mediterranean Sea, which seems to be more threatened than other ecosystems (Sala et al., 2000). Therefore the introduction and the establishment of alien species of herbivores, macroalgae and microalgae can represent an additional stressor for marine forest (Vergés et al., 2014a, 2014b; Marampouti et al., 2020; Iveša et al., 2021).

As many other foundation species, *Cystoseira s.l.* spp. are very sensitive to both local and large scale variations on environmental conditions (Irving et al., 2009; Cardona et al., 2013; Mancuso et al., 2019; de Caralt et al., 2020) and, in some cases, the unfavourable conditions are restricting *Cystoseira s.l.* populations to refuge locations where requisites for their survival are ensured (Verdura et al., 2021). Because of the high sensitivity to human disturbances (Mangialajo et al., 2008b; Sales and Ballesteros, 2009) these species are used in many protocols with the objective of characterising coastal habitats (De La Fuente et al., 2018; Bahbah et al., 2020). Since decades, declines of *Cystoseira s.l.* forests have been reported all along the Mediterranean Sea (Cormaci and Furnari, 1999; Thibaut et al., 2005, 2015; Bianchi et al., 2014; Buonomo et al., 2018; Mariani et al., 2019). Consequently, most of the *Cystoseira s.l.* spp. are included in the Annex II of the Barcelona Convention (UNEP/MAP, 2013) and considered threatened or endangered and in need

of protection measures (Verlaque et al., 2019). The only *Cystoseira s.l.* spp. not included in the annex is the shallow widely distributed *Cystoseira compressa* (Verlaque et al., 2019).

Declines in marine forests are attributed mainly to anthropogenic factors, local stressors seem to have a strong direct and indirect impact (Russell et al., 2009; Leal et al., 2018; de Caralt et al., 2020; Gissi et al., 2021), but global stressors such as OW and OA are also affecting these ecosystems (Celis-Plá et al., 2015; Fernández et al., 2020; Smale, 2020; Verdura et al., 2021). Locally, the proliferation of herbivores is believed to be one of the most important biological factors controlling the mortality of early-life stages of *Cystoseira s.l.* spp. and responsible for the degradation and maintenance of post-regime shifts communities, such as turfs or barren grounds (Giakoumi et al., 2012; Vergés et al., 2014a; Gianni et al., 2017). Alongside, eutrophication and pollution affect the biomass, growth and photosynthetic yield of *Cystoseira s.l.* spp. and have been responsible for the regression of the populations in many locations (Thibaut et al., 2005; Pinedo et al., 2013; Blanfuné et al., 2019; de Caralt et al., 2020). Globally, OW has already been signalled as a threat for the survival of both adults and recruits of *Cystoseira s.l.* spp., affecting their phenology, recruitment and growth (Celis-Plá et al., 2017a; Bevilacqua et al., 2019; Falace et al., 2021; Verdura et al., 2021; Bennett et al., 2022; Orlando-Bonaca et al., 2022). Instead, the effects of OA are expected to favour the growth of *Cystoseira s.l.* spp. when other environmental conditions, such as temperature and nutrients, are optimal (Celis-Plá et al., 2015, 2017a). However, the interactive effects of local and global stressors, need to be further investigated to better understand the causes of such declines (Hepburn et al., 2011; Connell et al., 2013; Celis-Plá et al., 2017a; Leal et al., 2017).

The substitution of marine forests by structurally less complex macroalgal communities such as shrubs and turfs, and even barren grounds or deserts, has been observed and studied in the Mediterranean Sea (Chemello et al., 2018; Benedetti-Cecchi et al., 2019; Álvarez-Losada et al., 2020) and the miniaturisation of the habitats is a common event on temperate areas worldwide (Filbee-Dexter and Wernberg, 2018; Pessarrodona et al., 2021). The loss of these habitat forming species involves a series of cascade effects with profound changes on the ecosystem function together with the loss of ecosystem services (Airoldi et al., 2008; Hall-Spencer and Harvey, 2019; Pessarrodona et al., 2019; Smale et al., 2022).

The characteristics of *Cystoseria s.l.* spp., long-lived species with a short dispersal capacity (because of the large size of their zygotes, 100 – 120 µm), make very difficult the natural recovery of the forests, especially considering that populations are in many cases fragmented (Clayton, 1990; Capdevila et al., 2018; Riquet et al., 2021). Even if the improvement of environmental conditions has been attained in some locations (e.g. improvement in water quality) allowing the regeneration of some populations (Thibaut et al., 2005, 200; Perkol-Finkel and Airoldi, 2010; Blanfuné et al., 2019), natural recovery is rare (Iveša et al., 2016). In most cases, the only solution for restoring

locally extinct or highly endangered populations is the active restoration (Gianni et al., 2013; Eger et al., 2022). Recently, great efforts have been done to explore innovative restoration techniques for recovering or improving the status of endangered populations (Falace et al., 2018; De La Fuente et al., 2019; Cebrian et al., 2021). So far, successful restoration actions of *Cystoseira s.l.* forests in the Mediterranean Sea are scarce (Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019; Savonitto et al., 2021). The next main issues that need to be addressed and further studied for the accomplishment of restoration actions are herbivory and climate change (Gianni et al., 2013; Tamburello et al., 2019; Medrano et al., 2020).

### **BENTHIC HARMFUL ALGAL BLOOMS PROLIFERATION**

The same factors that are driving *Cystoseira s.l.* forests to the edge (e.g. degradation of natural habitats due to pollution, urbanisation, overfishing and climate change) and are promoting regime shifts could also make ecosystems more vulnerable to new biological threats. Recently, an increase in harmful algal blooms (HABs) has been recorded in temperate locations around the globe including the Mediterranean Sea (Anderson et al., 2019). Anthropogenic activities and climate change are considered the main contributors of alien invasions and main enablers of HAB events (Marampouti et al., 2020). In the global change context, ocean warming is expected to promote the expansion and growth of tropical and sub-tropical harmful algal dinoflagellates, including the genera *Ostreopsis*, *Gambierdiscus* and *Fukoya* (Tester et al., 2020). A significant proportion of studies concerning benthic HABs are focused on tropical benthic dinoflagellate ecology, primarily as a result of the incidence of ciguatera fish poisoning (CFP) in tropical and subtropical areas (Litaker et al., 2009) and the expansion of toxic species in temperate waters (Rhodes, 2011). To the present, no CFP episodes have been reported in the Mediterranean Sea, but the recent findings of species of *Gambierdiscus* in Crete (Aligizaki and Nikolaidis, 2008) and in Balearic Islands (Laza-Martínez et al., 2016; Tudó et al., 2018, 2020), are rising the alert. Still, *Ostreopsis* spp. (Box 2) are already widely distributed in the Mediterranean Sea, where their blooms are frequent and important in magnitude (Mangialajo et al., 2011; Accoroni et al., 2012; Accoroni and Totti, 2016; Açaçaf et al., 2020). The increasing rate of *Ostreopsis* spp. blooms and the hazardous impacts that they have on the environment, economy, and human health make it important to have accurate knowledge about its development (Berdalet et al., 2016, 2022). The increased seawater temperatures in the Mediterranean Sea, predicted to become the norm in the mid-21<sup>st</sup> century, should stimulate more intense *Ostreopsis* spp. blooms in the future (Tester et al., 2020) and both *Gambierdiscus* and *Ostreopsis* populations are expected to increase, especially in warm locations (Açaçaf et al., 2020; Tester et al., 2020).

*Proliferation of Ostreopsis species***BOX 2: *Ostreopsis* Johns.Schmidt, 1901**

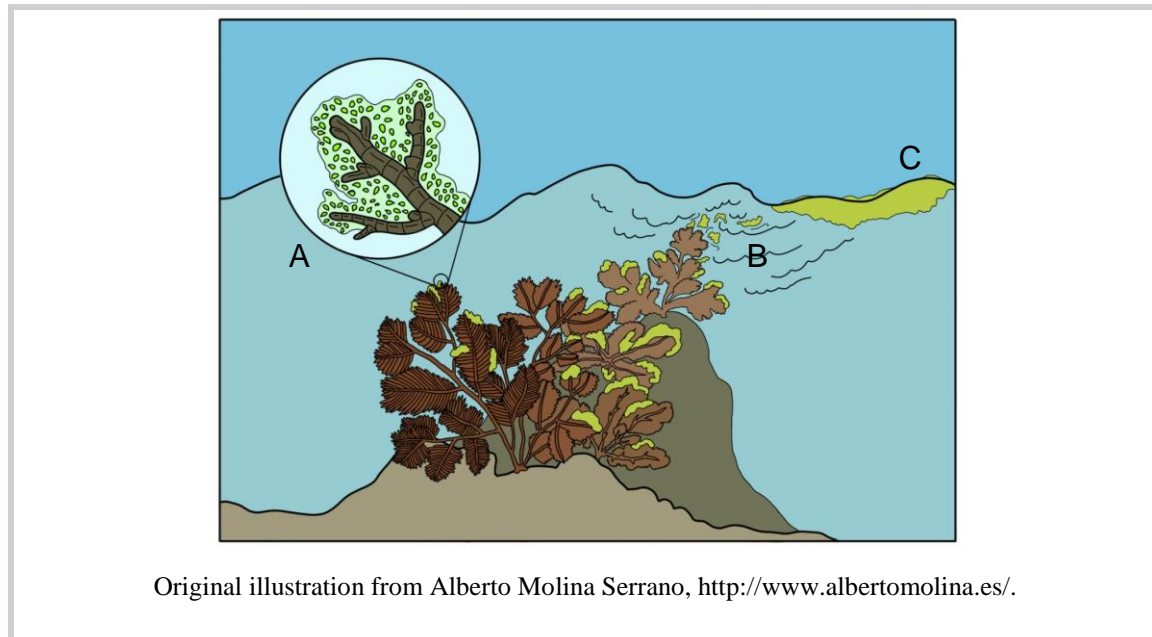
The genus *Ostreopsis*, belonging to the family of Ostreopsidaceae (Gonyaulacales, Dinophyceae, Dinzoa) includes several species largely distributed from tropical to temperate marine coastal areas worldwide.

*Ostreopsis* is a benthic dinoflagellate that develop in close relationship with a biotic or abiotic substrate (Totti et al., 2010). *Ostreopsis* spp. are usually epiphytic on macrophytes (Rhodes, 2011), but can also be found on dead corals, sediments or rocks, constituting the stock of cells (A; Bomber et al., 1989; Vila et al., 2001; Shears and Ross, 2009; Totti et al., 2010). Also, due to a combination of physical and biological processes *Ostreopsis* spp. cells can be found in the water column (B) or forming aggregates on the water surface (C; Mangialajo et al., 2011; Pavaux et al., 2021; Berdalet et al., 2022).

*Ostreopsis* spp. have been reported for a long time in tropical ciguatera endemic areas and, since some decades, have become common in temperate areas as well (Litaker et al., 2009; Rhodes, 2011). Recurrent *Ostreopsis* spp. blooms have been recorded throughout the globe (Chang et al., 2000; Rhodes et al., 2000; Lenoir et al., 2004; Shears and Ross, 2009; Nascimento et al., 2012b; Yamaguchi et al., 2012b; Gomaa et al., 2018; Tibirica et al., 2019; Solino et al., 2020; Zou et al., 2020) and in the Mediterranean Sea (Vila et al., 2001; Penna et al., 2005; Chiantore et al., 2008; Ismael and Halim, 2012; Açaçaf et al., 2020; Gémin et al., 2020).

Within the eleven species of *Ostreopsis* identified (Fukuyo, 1981; Norris et al., 1985; Quod, 1994; Faust and Morton, 1995; Faust, 1999), at least five produce palytoxin-like compounds, known for being among the most toxic marine compounds (Usami et al., 1995; Ukena et al., 2001; Ciminiello et al., 2010; Suzuki et al., 2012; Varela et al., 2021). Humans can be affected by *Ostreopsis* spp. blooms by the inhalation of marine aerosols which can lead to respiratory disease (Tichadou et al., 2010; Tubaro et al., 2011; Vila et al., 2016). The most important human intoxications in temperate locations were recorded in the Mediterranean Sea, in Italy in 2001 and 2005 (Brescianini et al., 2006) and in Spain in 2004 and 2006 (Barroso García et al., 2008), affecting hundreds of locals swimmers and beach goers, mainly affected by respiratory intoxications caused by the inhalation of seawater aerosols containing toxins (Mangialajo et al., 2011). Recurrent events of symptoms on humans due to *Ostreopsis* spp. blooms have been observed in several parts in the Mediterranean Sea (Tubaro et al., 2011; Illoul et al., 2012) and in 2021 an important event was recorded for first time in the Atlantic French coast (in Biarritz; Drouet et al., 2021; Chomérat et al., 2022). The whole ecosystem is also affected by *Ostreopsis* spp. blooms (Turner et al., 2021), and potentially macroalgae (Iveša et al., 2021). Most intense blooms of *Ostreopsis* spp. can result in mass mortalities of invertebrates such as bivalves, gastropods, crustaceans, and echinoderms (Shears and Ross, 2009; Ramos and Vasconcelos, 2010; Guidi-Guilvard et al., 2012; Parsons et al., 2012; Accoroni and Totti, 2016; Migliaccio et al., 2016; Neves et al., 2018).





Surface seawater temperature is thought to be one of the cardinal factors affecting the development of *Ostreopsis* spp. blooms, while the role played by other abiotic factors such as hydrodynamics, salinity and nutrients is still unclear (Accoroni and Totti, 2016). This lack of knowledge highlights that, as reported in Pavaux et al. (2020), only a small part of the studies on *Ostreopsis* spp. focus on its ecological aspects. Nevertheless, it seems that the synergic effect of more than one of these factors could influence the development of blooms (Accoroni and Totti, 2016). On one hand, most studies relate larger abundances of *Ostreopsis* spp. to sheltered or low hydrodynamic conditions (e.g. Chiantore et al., 2008; Shears and Ross, 2009; Richlen and Lobel, 2011; Mohammad-Noor et al., 2016; Asnaghi et al., 2017; Boisnoir et al., 2018; Hachani et al., 2018), while others suggest larger abundances of *Ostreopsis* spp. in higher or slightly higher levels of water motion (Vila et al., 2001; Selina et al., 2014). The relationship between blooms of *Ostreopsis* spp. and hydrodynamics could be related to the dense mucilaginous aggregations formed by cells of *Ostreopsis* spp. covering different substrates (e.g. macrophytes), that can be released due to wave action and suspended in the water column or on the sea surface (Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Chiantore et al., 2008; Shears and Ross, 2009; Totti et al., 2010; Tester et al., 2020).

On the other hand, the inshore occurrence of *Ostreopsis* spp. has raised the question concerning their association with nutrient enrichments due to human activities (Faust et al., 1996), but there are mixed conclusions on how nutrient concentrations influence population dynamics of *Ostreopsis* spp. (Ungano et al., 2010; Cohu et al., 2011b; Accoroni et al., 2012). In some studies dinoflagellate abundances were positively correlated with several nutrient parameters, such as nitrates, nitrites, phosphates and silicates (Delgado et al., 2006; Parsons and Preskitt, 2007; Skinner et al., 2013; Accoroni et al., 2020), while other studies found no significant relationship (Okolodkov et al., 2007; Asnaghi et al., 2012; Nascimento et al., 2012a). Nevertheless, abundances of *Ostreopsis* spp.

decrease with depth (Richlen and Lobel, 2011; Cohu and Lemee, 2012), maybe because of the light availability, even if, again, there are controversial results between light intensity and the growth of *Ostreopsis* spp. in experimental conditions (Accoroni and Totti, 2016).

### ***Preferences for Ostreopsis species proliferation***

Due to the benthic nature of *Ostreopsis* spp. is expected that the substrate plays a major role in the development of blooms. However, substrate preferences for *Ostreopsis* spp. are still ambiguous sometimes giving discordant patterns (Monti et al., 2007; Totti et al., 2010; Cohu et al., 2013; Blanfuné et al., 2015; Moncer et al., 2017; Meroni et al., 2018; Gémin et al., 2020; Ternon et al., 2020). The abundances of *Ostreopsis* on different substrates are highly variable and linked to several assessment limitations that make comparisons difficult. The comparisons usually depend on the type and characteristics of the substrate (e.g. surface for rocks and fresh weight for macrophytes), ideally not allowing direct comparisons (Mangialajo et al., 2017; Tester et al., 2022). In order to avoid the technical issues linked to the measurements different techniques have been proposed such as the use of artificial substrates (Tester et al., 2014; Jauzein et al., 2016; Yong et al., 2018; Fernandez-Zabala et al., 2019; Carreira-Flores et al., 2020), the syringe method (Abbate et al., 2012) and the benthic dinoflagellate integrator device (BEDI; Mangialajo et al., 2017) to obtain comparable estimates of number of cells at multiple sites. However, these techniques are not yet widely used. The high variability on abundances of *Ostreopsis* spp. among substrates and the discordant patterns, could be due to several biotic and abiotic factors acting at a larger scale than the substrate itself, such as the morphology, palatability, and microbial community associated to the substrate and the herbivory and allelopathic interactions due to the production of secondary metabolites (Cruz-Rivera and Villareal, 2006; Totti et al., 2010; Accoroni et al., 2015; Pavaux et al., 2020; Ternon et al., 2020).

Recent studies focusing on CFP are suggesting that the sea bottom complexity and heterogeneity could affect the development of benthic harmful dinoflagellates (Meroni et al., 2018; Yong et al., 2018; Boisnoir et al., 2019; Bravo et al., 2020; Lee et al., 2020). Some studies suggest that *Ostreopsis* spp. prefer habitats with high coral cover (Yong et al., 2018) while others associate *Gambierdiscus* and *Ostreopsis* spp. with turf algal communities, suggesting an effect of the architecture of the thallus of the macrophyte on hosting benthic dinoflagellates (Totti et al., 2010; Catania, 2017; Mustapa et al., 2019; Bravo et al., 2020; Lee et al., 2020). Furthermore, lower abundances of *Ostreopsis* spp. could be related to *Cystoseira s.l.* forests (Catania, 2017; Meroni et al., 2018). These results are consistent with observations of higher abundances of *Ostreopsis* spp. at sites heavily impacted by humans where healthy macroalgal communities are replaced by opportunistic and fast-growing turf macroalgae (Rhodes, 2011; Fraga et al., 2012; Meroni et al., 2018; Roselli et al., 2022). According to some authors, the continued and extensive destruction of

natural habitats and large metropolitan areas could promote *Ostreopsis* spp. and *Gambierdiscus* spp. blooms (Rhodes, 2011; Fraga et al., 2012; Meroni et al., 2018; Mustapa et al., 2019; Tester et al., 2020; Roselli et al., 2022). The artificialisation of the coast and the miniaturisation and degradation of macroalgal communities due to regime shifts could, therefore, favour the expansion of *Ostreopsis* spp. (Fraga et al., 2012; Mangialajo et al., 2017; Meroni et al., 2018; Lee et al., 2020; Tester et al., 2020; Roselli et al., 2022).

### **OBJECTIVES**

The main objective of this thesis is to assess the causes and some potential effects of marine forests loss in the Mediterranean Sea. As causes of loss we assessed the effects of abiotic (climate change) and biotic (species facilitation and herbivory) factors. As effects of loss, we investigated for the first time the relationship between the present regression of marine forests and the proliferation of benthic harmful algal blooms.

This thesis focuses on *Cystoseira s.l.* forests from shallow coastal areas, mainly inhabiting rockpools and shallow sheltered locations, usually very impacted by human activities (e.g. urbanisation, recreational activities, climate change) which could be magnified due to the low turnover rate conditions of these locations. In view of the new restoration techniques developed for Mediterranean *Cystoseira s.l.* forests (see Cebrian et al., 2021 for a review), we consider necessary to further study the causes that could negatively impact *Cystoseira s.l.* spp. in the future (e.g. climate change and species interactions) and compromise the success of such restoration actions. In addition, despite the efforts done in understanding the mechanisms compromising *Cystoseira s.l.* forests in the Mediterranean still little is known on how the interactive effects of climate change (i.e. ocean warming and acidification) affect this species, especially its early-life stages. We have selected *Cystoseira compressa* (Box 3) as a target species for several reasons. First, this macroalgal species is considered the most resistant *Cystoseira s.l.* spp. and, still at the present time, can be found in both cosmopolitan and pristine locations in many coastal areas (including the French Riviera; Thibaut et al., 2015; Verlaque et al., 2019). Second, there are currently no specific studies on the effect of climate change on adults and recruits of *C. compressa*, nor results of restoration attempts for this species. Thus, the first objective is to study the causes of loss, assessing how climate change and species interactions affect the recruits of *C. compressa*.

**BOX 3: *Cystoseira compressa* (Esper) Gerloff & Nizamuddin 1975**

Caespitose macroalgae, light brown colour, very variable in size, from several centimetres to 80 cm in maximum height according to the degree of wave action. Some specimens are perennial but often present an annual development. Cylindrical or flattened axes of several centimetres high (2-3 cm), almost inexistent in juveniles (A). Apices of the axes are smooth (B). Tophules are absent. Primary branches totally flattened, at least at their base. No spiny appendages. Aerocysts usually associated with the receptacles. Receptacles in the apices of the terminal branches, simple or more frequently branched, sometimes located on an aerocyst. Male and female gametangia in conceptacles grouped in receptacles (C and D). They present a monophasic diploid life cycle (Garreta, 2000; Rodriguez-Prieto et al., 2013).

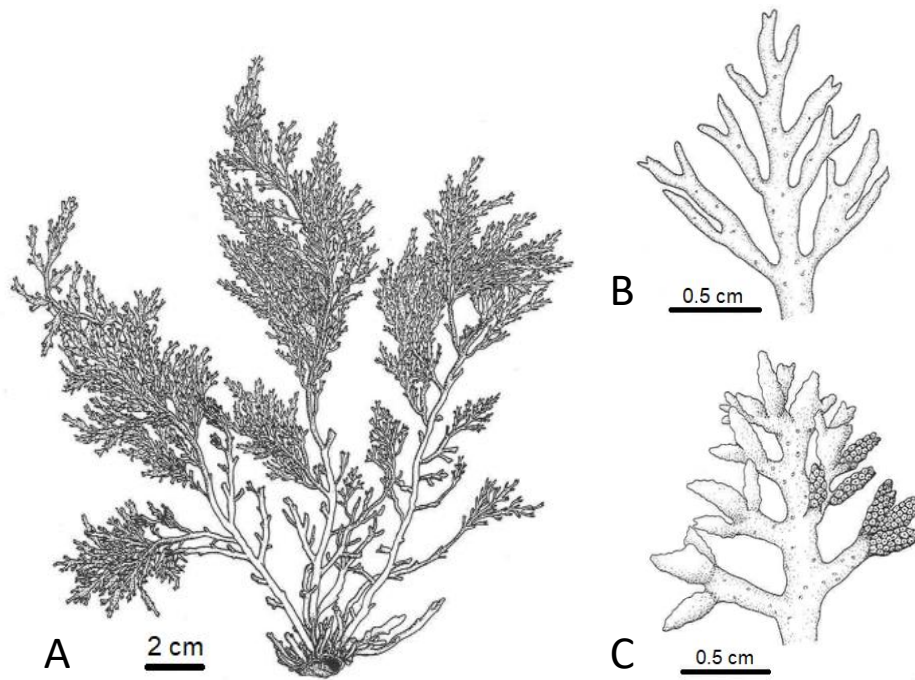
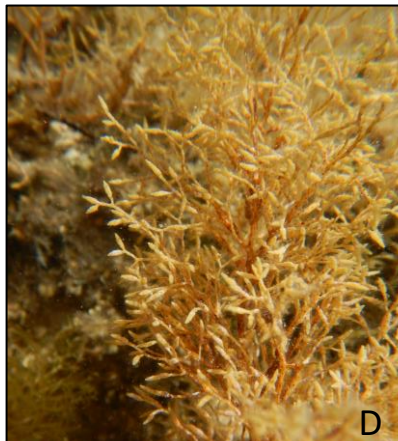


Image modified from Garreta (2000).



The disappearance of Mediterranean marine forests and their substitution by less structurally complex macroalgal communities could lead to profound changes on ecosystems, facilitating emergent phenomena such as harmful algal blooms. Thus, as an effect of Mediterranean marine forests loss, we selected the emergent phenomena of benthic harmful algal blooms proliferation of the genus *Ostreopsis*. Benthic harmful algal blooms are causing growing concerns in the Mediterranean Sea given their catastrophic impacts on coastal ecosystems and humans, and their economic consequences (tourism, fishing, etc.; Berdalet et al., 2022). While *Ostreopsis* spp. are well studied, not much is known about their ecology (Pavaux et al., 2020) and since they settle on benthic surfaces, a better understanding of their relationship with marine vegetation could improve the risk assessment and management of toxic blooms. Therefore, the second objective of this thesis is to study the proliferation of toxic benthic dinoflagellates from the genus *Ostreopsis* as potential consequence of *Cystoseira s.l.* forests loss.

The chapters presented hereafter combine field surveys, experiments in the field, controlled experiments in the laboratory and a systematic review. The specific objectives of each chapter are detailed below:

- **Chapter 1.** *Climate change and species facilitation affect the recruitment of macroalgal marine forests.* In this chapter we assess the effects of ocean warming, acidification and species facilitation on the recruitment of *C. compressa* in controlled experiments in the laboratory. We determine how future climate change scenarios could affect recruitment and growth of *C. compressa* and how the presence of crustose coralline algae can facilitate the recruitment under the different treatments of temperature and pH.
- **Chapter 2.** *Grazing effects on recruits of the forest-forming furoid *Cystoseira compressa*.* Here we examine the effects of different herbivorous species on recruits of *C. compressa* through (i) field surveys, to characterise the herbivores present in our experimental locations; (ii) experiments in the field, to study the effect of the *in-situ* herbivory on recruits obtained using different restoration techniques; and (iii) in the laboratory, specifically testing the potential of some selected species to graze on recruits of *C. compressa*.
- **Chapter 3.** *The role of habitat in the facilitation of *Ostreopsis* spp. blooms.* This chapter is the first step to collect information and better understand the relationship between blooms of *Ostreopsis* spp. and marine vegetation. In this literature review, we collect information on *Ostreopsis* spp. blooms at the substrate, habitat, and ecosystem scale, in order to assess eventual preferences and trends for the development of blooms of *Ostreopsis* spp.
- **Chapter 4.** **Ostreopsis* spp. blooms in relation to macroalgal communities.* In this chapter we collect information on abundances of *Ostreopsis* spp. at the substrate, habitat and

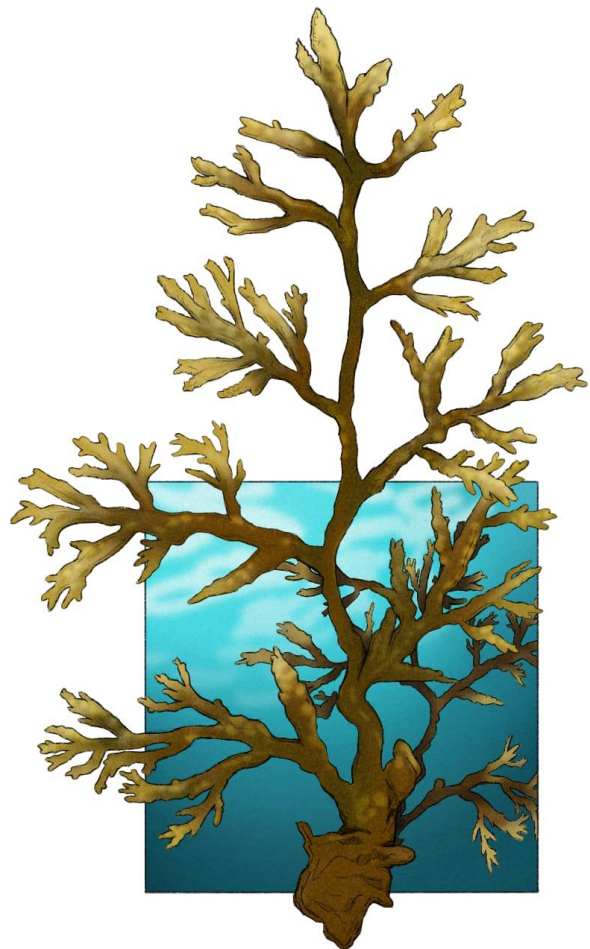
ecosystem scale during an annual bloom in Rochambeau (Villefranche-sur-Mer, France) and Vernazzola (Genova, Italy), to assess the preferences and trends of *Ostreopsis* spp. on different types of substrates (macroalgal and artificial substrates) at different spatial scales.





# CHAPTER 1: CLIMATE CHANGE AND SPECIES FACILITATION AFFECT THE RECRUITMENT OF MACROALGAL MARINE FORESTS

Monserrat, M., Comeau, S., Verdura, J., Alliouane, S., Spennato, G., Priouzeau, F., et al. (2022). Climate change and species facilitation affect the recruitment of macroalgal marine forests. *Sci Rep* 12, 1–12. doi: [10.1038/s41598-022-22845-2](https://doi.org/10.1038/s41598-022-22845-2).







## ABSTRACT

Marine forests are shrinking globally due to several anthropogenic impacts including climate change. Forest-forming macroalgae, such as *Cystoseira s.l. spp.*, can be particularly sensitive to environmental conditions (e.g. temperature increase, pollution or sedimentation), especially during early-life stages. However, not much is known about their response to the interactive effects of ocean warming (OW) and acidification (OA). These drivers can also affect the performance and survival of crustose coralline algae, which are associated understory species likely playing a role in the recruitment of later successional species such as forest-forming macroalgae. We tested the interactive effects of elevated temperature, low pH and species facilitation on the recruitment of *Cystoseira compressa*. We demonstrate that the interactive effects of OW and OA negatively affect the recruitment of *C. compressa* and its associated coralline algae *Neogoniolithon brassica-florida*. The density of recruits was lower under the combinations OW and OA, while the size was negatively affected by the temperature increase but positively affected by the low pH. The results from this study show that the interactive effects of climate change and the presence of crustose coralline algae can have a negative impact on the recruitment of *Cystoseira s.l. spp.* While new restoration techniques recently opened the door to marine forest restoration, our results show that the interactions of multiple drivers and species interactions have to be considered to achieve long-term population sustainability.

**Keywords:** Macroalgal forests, Coralline algae, Climate change, Ocean acidification, Ocean warming, Species facilitation, *Neogoniolithon brassica-florida*, *Cystoseira compressa*

## 1. INTRODUCTION

The ocean plays an important role in the regulation of climate and offers numerous ecosystem services for humans. However, the ocean is affected by multiple anthropogenic impacts including climate change (IPCC, 2022). Ocean warming (OW) and ocean acidification (OA) are expected to affect most marine ecosystems with consequences to humans (Doney et al., 2012; Gattuso et al., 2015; Hall-Spencer and Harvey, 2019). Ecosystems all around the globe are expected to experience reductions in habitat structure, biodiversity and trophic complexity as sea temperature rises (Straub et al., 2019) and oceanic pH decreases (Kroeker et al., 2011; Connell et al., 2013). At the same time, an expansion of opportunistic and turf-forming species is foreseen, with the consequent loss of ecosystem services (Wernberg et al., 2016; Sunday et al., 2017; Harvey et al., 2021).

Large brown forest-forming macroalgae (which include the orders Laminariales, Tylopteridales, Desmarestiales, and Fucales) are dominant foundation species on intertidal and subtidal rocky shores in temperate and cold regions (Steneck et al., 2002). They form what is known as marine forests, which (Schiel and Foster, 2006; Wernberg and Filbee-Dexter, 2019) provide important ecosystem functions (Cheminée et al., 2013; Smale et al., 2013; Carbajal et al., 2022). However,

marine forests are shrinking globally (Filbee-Dexter and Wernberg, 2018; Pessarrodona et al., 2021) due to several impacts such as urbanisation, marine farming, local pollution and herbivory (Steneck et al., 2002; Krumhansl et al., 2016; Orfanidis et al., 2021); making these habitats more sensitive to global change (Capdevila et al., 2019; Straub et al., 2019). The structure and productivity of marine forests are influenced by many environmental factors that drive the growth, survival, reproduction and metabolism of the organisms, which in turn affect the whole habitat or ecosystem (Irving et al., 2009; Smale et al., 2022; Smith et al., 2022). Global change effects on marine forests can vary according to the location, the population characteristics and the species (Krumhansl et al., 2016; Hollarsmith et al., 2020; Verdura et al., 2021). As a result, in several cases, marine forests are constrained to locations with the most favourable conditions which could act as a refuge (Mariani et al., 2019; Smale, 2020; Verdura et al., 2021). There is evidence that early-life stages of these species are more vulnerable than adults which could lead, in the long term, to the loss of marine forests (Coelho et al., 2000; Schiel and Foster, 2006; de Caralt et al., 2020). A high mortality rate is naturally observed during the early stages and the resilience of a population to future impacts can be largely dependent on efficient recruitment and development of juveniles (Vadas et al., 1992; Capdevila et al., 2015).

The shift in carbonate chemistry associated with OA causes an increase in dissolved  $\text{CO}_2$  that could favour photosynthesis and then the growth of photosynthetic organisms (Connell et al., 2013; Koch et al., 2013). The increase in  $\text{CO}_2$  modifies the dissolved  $\text{CO}_2$  to  $\text{O}_2$  ratio at the RuBisCO active site, i.e., the key enzyme in carbon fixation metabolism. Because the latter emerged in an oxygen poor environment, it is characterised by a higher affinity for  $\text{O}_2$  than  $\text{CO}_2$  (Shih et al., 2016). Therefore, the current increase in dissolved  $\text{CO}_2$  to  $\text{O}_2$  ratio favours RuBisCO carbon fixation efficiency and then can favour the growth of photosynthetic organisms. To increase the RuBisCO carbon fixation efficiency, many algae also developed carbon concentration mechanisms (CCM), that increase the  $\text{CO}_2$  to  $\text{O}_2$  ratio in front of the RuBisCO fixation site (Connell et al., 2013; Cornwall et al., 2017a). Still, despite having or not having CCM it is not clear if most algae respond positively to an increase of  $\text{CO}_2$  (Hepburn et al., 2011; Connell et al., 2013; Cornwall et al., 2017a). OA might, therefore, have beneficial effects for some species like large brown forest-forming macroalgae, that are thought to thrive at high  $\text{CO}_2$  concentrations (Hepburn et al., 2011; Porzio et al., 2011; Connell et al., 2013). However, calcifying organisms (e.g. foundation species like corals and coralline algae) are expected to be particularly affected by OA (Kroeker et al., 2013a). In particular, crustose coralline algae, which are important components of the understory of marine forests, are among the organisms potentially the most susceptible to OA (Kroeker et al., 2010; Rindi et al., 2019). This species can be directly and indirectly impacted by OA due to reduced calcification rates and increasing competition with algae which benefit from elevated  $\text{CO}_2$  (James et al., 2014; Comeau and Cornwall, 2016; Cornwall et al., 2017a). Crustose coralline algae are among the first colonizers

of bare rock on euphotic marine habitats and are quickly overgrown by later successional species such as the more structurally complex large brown forest-forming macroalgae (Airoldi, 2000; Asnaghi et al., 2015). Some authors (Bulleri et al., 2002b) report that crustose coralline algae could help in the maintenance of alternative habitat states by preventing the recruitment of later colonizers (e.g. large brown macroalgae), even if this could be species-specific (Villas Bôas and Figueiredo, 2004). While other studies suggest that crustose coralline algae could, in contrast, enhance biodiversity by facilitating the settlement of later colonists, including invertebrates (Bulleri et al., 2002a; Maggi et al., 2011; Asnaghi et al., 2015) and by creating a positive association with the forest-forming macroalgae (Melville and Connell, 2001; Irving et al., 2004, 2005). Thus, a reduction of crustose coralline algae cover, because of climate change, may affect the recruitment of forest-forming macroalgae and therefore the maintenance of marine forest habitats (Breitburg, 1984; Bulleri et al., 2016) and their resistance against climate change (Comeau and Cornwall, 2016; van der Heide et al., 2021).

In Mediterranean rocky bottoms, *Cystoseira sensu lato* species (including the genera *Cystoseira*, *Ericaria* and *Gongolaria*, hereafter referred to as *Cystoseira*) are the main representatives of marine forests (Wernberg and Filbee-Dexter, 2019; Molinari - Novoa and Guiry, 2020). However, only a few studies have investigated the effect of climate change on this taxon and even fewer have focused on their early stages (Celis-Plá et al., 2017a; Hernández et al., 2018; Capdevila et al., 2019; Falace et al., 2021). Most studies show a negative impact of OW for both recruits and adults of *Cystoseira* on their survival (Falace et al., 2018, 2021; Verdura et al., 2021), resilience (Capdevila et al., 2019) and phenology (Bevilacqua et al., 2019; Savonitto et al., 2021). In contrast, decreasing pH increased the productivity, antioxidant activity and production of photoprotective compounds of adult *Cystoseira* (Celis-Plá et al., 2017a; Hernández et al., 2018). Despite that, some species of *Cystoseira* (including *Cystoseira compressa* and *Cystoseira foeniculacea*) are considered CCM species whose CCM does not downregulate due to additional CO<sub>2</sub> and, thus, could not benefit from increasing CO<sub>2</sub> (Cornwall et al., 2017a). To our knowledge, there are no studies on the combined effects of OW and OA on early-life stages or recruits of *Cystoseira*, and only one study (Celis-Plá et al., 2017a) has investigated the effects of both drivers on adults.

Here, we tested the interactive effects of temperature, pH and species facilitation on the recruitment of *Cystoseira compressa*. This species is a common forest-forming macroalgae that can create dense populations on shallow and sheltered rocky shores around the Mediterranean Sea (Mangialajo et al., 2012). It is considered one of the most resistant *Cystoseira* species and it is the only one that is not protected under the Barcelona Convention (Annex II; United Nations Environment Programme/Mediterranean Action Plan-UNEP/MAP; Verlaque et al., 2019). We designed two separate experiments to test the effects of elevated temperature, low pH and the presence of crustose coralline algae on the early-life stages of *C. compressa*. The first experiment focused on the effects

of the temperature on the recruits of *C. compressa*. Based on the results of the first experiment, we ran a second complementary experiment to assess the role of the interactive effects of temperature, pH and species facilitation (crustose coralline algae) on the recruitment of *C. compressa*. Because coralline algae are sensitive to OA and are a potentially favourable substrate for the recruitment of *Cystoseira*, we assessed the recruitment of *C. compressa* on living and dead *Neogoniolithon brassica-florida*, one of the most common species in association with shallow *Cystoseira* forests (Benedetti-Cecchi and Cinelli, 1992; Asnaghi et al., 2015). We also compared the recruitment of *Cystoseira* on abiotic artificial clay substrates that have been proposed as an efficient substrate for restoration (Falace et al., 2018; De La Fuente et al., 2019; Orlando-Bonaca et al., 2021b). The main hypothesis of this study is that climate change will negatively affect the recruitment of *C. compressa*. Our hypotheses are that OW may have a direct negative impact on the recruits of *C. compressa* while OA may increase their growth and productivity. We also hypothesize that the settlement and survival of *C. compressa* might be indirectly affected by the effects of climate change on its associated understory species (crustose coralline algae) that act as a substrate.

## 2. MATERIALS AND METHODS

### 2.1. *Experiment 1: Effects of ocean warming on the recruitment of Cystoseira compressa*

#### 2.1.1. Collection and obtention of recruits

Apical fertile branches of *Cystoseira compressa* were hand-collected on the 13th of July 2020 from a donor population situated in a rockpool (between the surface and 1m depth) in Sainte Marguerite Island (Lérins Islands, France). This site is a Nature 2000 site situated in front of the coast of Cannes and it is one of the last locations with healthy *Cystoseira* populations in the French Riviera (Thibaut et al., 2015). After visually checking that the receptacles contained fertile conceptacles, about 140 gram fresh weight (FW) of apical fertile branches were manually collected and transported in cool and dark conditions in plastic bags to the laboratory. The sampling was non-destructive, as only apical branches (roughly 5 cm long) were collected. A temperature data logger (HOBO Pendant MX Temp, ONSET), that took measurements every hour, was installed in the rock pool to monitor the temperature in the donor population site. The receptacles were conserved at 4°C in the dark overnight before placing them in experimental tanks filled with filtered seawater (20 µm) and marble substrates that acted as settlement substrates (Falace et al., 2018; Verdura et al., 2018; Orlando-Bonaca et al., 2021b). In each tank, 15g FW of receptacles were placed in a net on the surface allowing the zygotes to fall on the substrates. The receptacles were kept in the tanks for 72 hours without water circulation to facilitate the settlement of the zygotes. The temperature was maintained during the releasing and the settlement of the zygotes at the target temperature treatment. After opening the water system, the receptacles were removed and the recruits were kept

in tanks for 96 days. Submersible water pumps (NEWA) provided water motion in each experimental tank.

### 2.1.2. Experimental set-up and treatments

Three independent 5 L tanks ( $n=3$ ) were set up for each temperature (24, 28 and 32°C; Table 1), for a total of 9 experimental tanks, with three square marble substrates of about 25 cm<sup>2</sup> placed inside each tank. Filtered seawater (20 µm) pumped from Villefranche Bay at 3 m depth was continuously delivered into the experimental tanks at a rate of 7 L h<sup>-1</sup>. The experimental tanks were placed inside a thermoregulated bath to maintain the temperature at the targeted value. The temperature was controlled in two thermoregulated baths per treatment with a temperature controller (T CONTROLLER TWIN AQUA MEDIC). Light was provided by 37 W LED light bars (PRO<sup>2</sup> LED, Aquaristik) and the irradiance gradually increased from 0 µmol photons m<sup>-2</sup> s<sup>-1</sup> at 06:30 to a maximum of 110 µmol photons m<sup>-2</sup> s<sup>-1</sup> between 12:00 and 14:00, and gradually decreased to 0 µmol photons m<sup>-2</sup> s<sup>-1</sup> at 21:00 (LI-185B with an LI-190SB quantum sensor, LI-COR Biosciences, Lincoln, USA). The three temperature treatments were selected according to the temperatures registered in the donor population during the reproductive season of *C. compressa* and the expected increase in temperature due to global warming.

**Table 1:** Measured (regular characters) and expected (bold characters) seawater physico-chemical parameters (temperature in °C,  $pH_T$  in total scale, calculated  $pCO_2$  in µatm, and total alkalinity in µmol Kg<sup>-1</sup> with mean ± SD) according to different treatments.

Treatment	Temperature (°C)	$pH_T$ n = 15	$pCO_2$ (µatm) n = 15	Total alkalinity (µmol kg <sup>-1</sup> ) n = 8
<b>24°C</b>	24.15 ± 0.43 n = 23	-	-	-
<b>28°C</b>	28.3±0.41 n = 23	-	-	-
<b>32°C</b>	31.47 ± 0.60 n = 23	-	-	-
<b>28°C pH 8.07</b>	28.22 ± 0.71 n = 15	8.04 ± 0.04	434.23 ± 42.63	2564.32 ± 5.80
<b>28°C pH 7.8</b>	28.37 ± 0.74 n= 15	7.83 ± 0.12	795.89 ± 170.97	2568.73 ± 22.51
<b>32°C pH 8.07</b>	31.56 ± 0.47 n = 15	8.01 ± 0.03	468.05 ± 45.53	2564.96 ± 6.23
<b>32°C pH 7.8</b>	31.61 ± 0.70 n = 15	7.83 ± 0.063	788.17 ± 135.69	2563.26 ± 5.50

### 2.1.3. Measurements

The density and size of *C. compressa* recruits were selected as response variables. The density of recruits was calculated by taking pictures every 10 or 15 days and counting from the picture the total number of recruits on a 3x3 cm area in the middle of each substrate using the software ImageJ

(ImageJ, NIH US Department of Health and Human Services). The size of recruits ( $\mu\text{m}$ ) was determined monthly on 5 recruits that were removed from each substrate ( $n = 45$ ). Their total length was measured using a microscope equipped with a graduated eyepiece.

### ***2.2. Experiment 2: Effects of climate change and species facilitation on the recruitment of *Cystoseira compressa****

#### **2.2.1. Collection and obtention of recruits**

Apical fertile branches of *C. compressa* were collected on the 4<sup>th</sup> of August 2021 from the same donor population as mentioned above and following the same protocol (see section 2.1.1.). For this experiment, about 500 g FW of apical fertile branches were manually collected and transported in cool and dark conditions in plastic bags to the laboratory. The receptacles were conserved at 4°C in the dark overnight before placing them under the experimental conditions, in the experimental tanks, to obtain recruits on the different substrates and under the different conditions of temperature and acidification. In each tank, 10 g FW of receptacles placed inside a net were kept on the surface for 72 hours. During this period, the temperature and pH were maintained at the target treatment conditions. Afterwards, the receptacles were removed and the recruits were kept in tanks for 75 days.

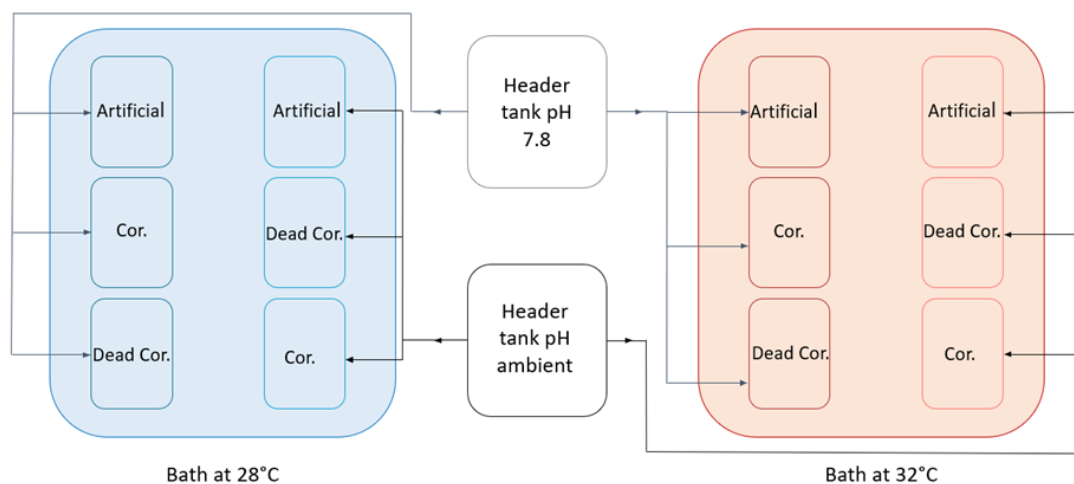
#### **2.2.2. Experimental set-up and treatments**

The recruitment of *Cystoseira compressa* was assessed in two different conditions of temperature, 28 and 32°C, and two pH levels, ambient ( $\text{pH}_T = 8.07$ ) and low pH ( $\text{pH}_T = 7.8$ ). In order to investigate the potential facilitation effect of *Neogoniolithon brassica-florida*, three different substrates were used for the settlement of recruits: 1) living and 2) dead *N. brassica-florida* (respectively factors “coralline” and “dead coralline”) and 3) artificial clay substrates (factor “artificial substrate”). The temperatures were selected according to the results obtained during the first experiment (experiment 1). The low pH condition ( $\text{pH}_T = 7.8$ ) corresponds to the pH value expected by the end of the century under the SSP2 - 4.5 CO<sub>2</sub> emissions scenario (Celis-Plá et al., 2015; Kwiatkowski et al., 2020). The surface of the three substrates was about 10 cm<sup>2</sup> (3 cm diameter and 1 cm height for the artificial substrates). The coralline algae were collected in April 2021 from Anse des Fossés (Saint-Jean-Cap-Ferrat, France) between 0.5 and 1 m depth. Samples that were the most homogeneous and the least colonized by other organisms were selected. Then, they were cleaned and epiphytes were removed using brushes and tweezers. Dead coralline substrates were obtained by putting the *N. brassica-florida* substrates in freshwater with bleach (1:50) for 24 hours. They were then rinsed several times with freshwater and dried, before placing them in the tanks. All the substrates were placed in the experimental tanks at ambient seawater

temperature and pH and gradually brought to the experimental temperatures and pH levels over 3 months before the start of the experiment.

The experimental set-up consisted of four independent 1.8 L tanks (n=4) for each of the 12 conditions (Substrate type  $\times$  pH  $\times$  Temperature) for a total of 48 experimental tanks. Each tank contained five replicates of a substrate (either artificial, coralline or dead coralline substrates). One substrate from each experimental tank was used as a control and was not seeded with zygotes of *C. compressa*. Seawater from the Bay of Villefranche was continuously delivered into eight 20 L header tanks that then gravity fed six 1.8 L independent experimental tanks each with a water rate of 3 L h<sup>-1</sup> (Figure 1). Seawater pH was manipulated inside 4 20 L header tanks. pH was maintained at the target value using pH controllers (APEX, Neptune Systems) that controlled the bubbling of pure CO<sub>2</sub> in the header tanks. The experimental tanks were placed inside thermoregulated baths (four per temperature) connected to the same control system (APEX, Neptune Systems; Figure 6) to maintain the temperature at 28 and 32°C. Submersible water pumps (NEWA) provided water motion in each experimental tank.

Light was provided by 89 W LED light bars (Aqualumix, Aquaristik) and irradiance gradually increased from 0  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at 06:30 to a maximum of 175  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  between 12:00 and 14:00, and gradually decreased to 0  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  at 21:00 (LI-185B with an LI-190SB quantum sensor, LI-COR Biosciences, Lincoln, USA; Verdura et al., 2021).



**Figure 1:** Experimental set-up used to test the effects of temperature (28 and 32°C), pH (ambient 8.07, and low 7.8) and species facilitation (artificial, coralline and dead coralline substrates) on the recruits of *C. compressa*. The experimental set-up was repeated 4 times, resulting in 8 header tanks, and 48 experimental tanks in which the different types of substrates were randomly assigned.



### 2.2.3. Carbonate chemistry

pH in the header and experimental tanks was measured weekly using a handheld pH-meter (826 pH mobile, Metrohm) calibrated with TRIS buffer (batch #T33 provided by A. Dickson, Scripps Institution of Oceanography). Total alkalinity was measured weekly in eight randomly selected tanks and was determined by potentiometric titration using a Metrohm 888 Titrand following the method of Dickson et al. (Dickson et al., 2007), the samples were measured three times and the mean value was used. Certified reference material (Batch #186) provided by A. Dickson was used to assess the accuracy of the measurements and was within  $7.73 \mu\text{mol kg}^{-1}$ . The seawater from the Bay of Villefranche was  $2565.41 \pm 12.1 \mu\text{mol kg}^{-1}$  (mean  $\pm$  SD; Table 1; Appendix A: Chapter 1 Supplementary material S1).

### 2.2.4. Measurements

The density and size of recruits of *C. compressa* and calcification rate of the living coralline algae substrates were selected as response variables. The density of recruits was assessed by counting directly in the tanks the total number of recruits on the substrates using a magnifying table lamp. The total number of recruits was normalized by the surface of the substrate. The size of the recruits was determined by measuring the length of ten individuals randomly picked from each substrate ( $n = 160$ ). When ten or fewer individuals were present on the substrate, all of them were measured. The measurements were done using graph paper under a magnifying table lamp.

Total calcification rate of *N. brassica-florida* was assessed using the buoyant weight technique (Davies, 1989). Weighing was done before obtaining *C. compressa* recruits and at the end of the experiment, 64 days later. Changes in wet weight were converted to dry weight using the following equation:

$$\text{Dry weight} = \frac{\text{Wet weight}}{\left(1 - \frac{\text{Water density}}{\text{Calcite density}}\right)}$$

with a calcite density of  $2.73 \text{g cm}^{-3}$ . Calcification rate was determined as the change in dry weight normalized by the surface of coralline algae at the moment of the weighting and the number of days (64 days). Surfaces of coralline substrates were determined on photographs using the software ImageJ (ImageJ, NIH US Department of Health and Human Services).

All experiments were carried out according to relevant regulations and guidelines concerning *Cystoseira compressa* and *Neogoniolithon brassica-florida* sampling. The latter were collected under prefectural order No. 277, delivered by the Interregional Directorate of the Mediterranean Sea, Regulatory/Control Service, authorizing the ECOSEAS Laboratory to sample fauna and flora for scientific purposes only.

## 2.3 Data analysis

Experiment 1: A Generalized Linear Mixed-Effects Model (GLMM), with a Poisson link log distribution function was used to test the effect of the temperature on the density of recruits, with temperature (three levels) and time (seven levels) as fixed factors, and substrate nested within tank as random. A two-way ANOVA was used to test the effect of temperature on the size of the recruits, with temperature (three levels) and time (three levels) as fixed factors. The assumptions of normality and equality of variance were evaluated through graphical analyses of residuals using QQ plot functions. An alpha of 0.01 was used when the assumption of equality of variance was not achieved.

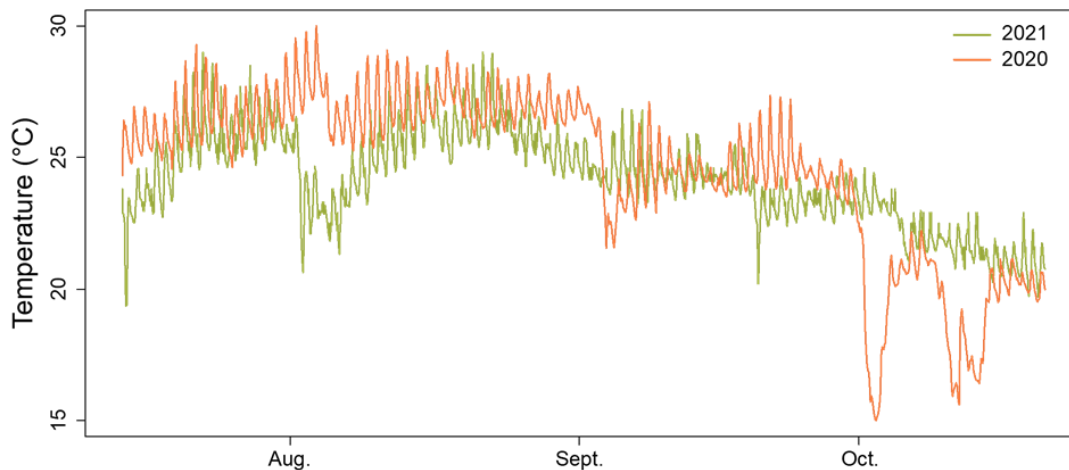
Experiment 2: A GLMM with a Poisson distribution was used to test the effect of temperature, pH and substrate type on the density of recruits, with temperature (two levels), pH (two levels), substrate type (three levels) and time (five levels) as fixed factors and substrate nested within tanks as random, to account for the lack of independence between observations (repeated measures over time). The response of the variable size to the treatments was analysed using a GLMM with a Gamma error distribution function and the logit link function ‘inverse’, with temperature (two levels), pH (two levels), substrate type (three levels) and time (three levels) as fixed factors and tank as random. The total calcification rate of the coralline algae was analysed with a Linear Model (LM), with temperature (two levels) and pH (two levels) as fixed factors.

GLMM and LM models were fitted to analyse the effect of the variables and the AICs likelihood minimum was used to select the best model among the possible combinations. The different models were fitted using the functions “glmer” and “lm” from the package lme4 (Bates et al., 2015) in the statistical environment R (R: The R Project for Statistical Computing). P-values were obtained by means of a Wald  $\chi^2$  test using the “ANOVA” function from the CAR package (Fox and Weisberg, 2018). Finally, the function “emmeans” from the package emmeans (Lenth et al., 2022) was used to perform the post-hoc analysis of the LM and GLMM models while the test “snk” (Student-Newman-Keuls) was used to perform the post-hoc analysis for the two-way ANOVA.

## 3. RESULTS

### 3.1 In-situ temperatures at the donor population location

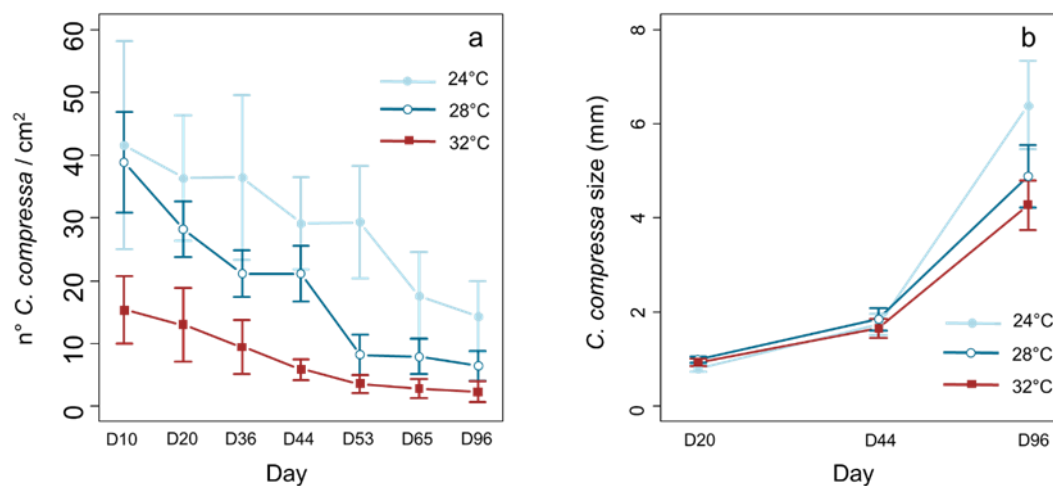
The temperatures at the *Cystoseira compressa* donor population site during the periods that cover the first (14<sup>th</sup> July-13<sup>th</sup> October 2020) and second experiment (10<sup>th</sup> August – 20<sup>th</sup> October 2021) varied between minimums and maximum values of 15.2°C (27<sup>th</sup> October) and 29.5°C (2<sup>nd</sup> August) in 2020 and between 19.4°C (14<sup>th</sup> July) and 29.0°C (22<sup>nd</sup> July) in 2021 (Figure 2).



**Figure 2:** *In-situ* mean seawater temperature at the donor population site.

### 3.2 Experiment 1: Effects of ocean warming on the recruitment of *Cystoseira compressa*

The temperature negatively affected the density of recruits since the beginning of the experiment (GLMM,  $P$ -value  $< 0.001$ ; Appendix A: Chapter 1 Supplementary material S2). Recruit density was significantly lower at 32°C than at 28°C and 24°C since the first sampling dates (day 10; Figure 3a), while from day 36 densities at 24°C remained higher than the ones at 28°C and 32°C (Figure 3a). The temperature also affected the size of the recruits at the end of the experiment, recruits at 24°C being significantly larger than the ones grown at warmer temperatures (ANOVA,  $P$ -value  $< 0.001$ ; Appendix A: Chapter 1 Supplementary material S2 and Figure 3b).

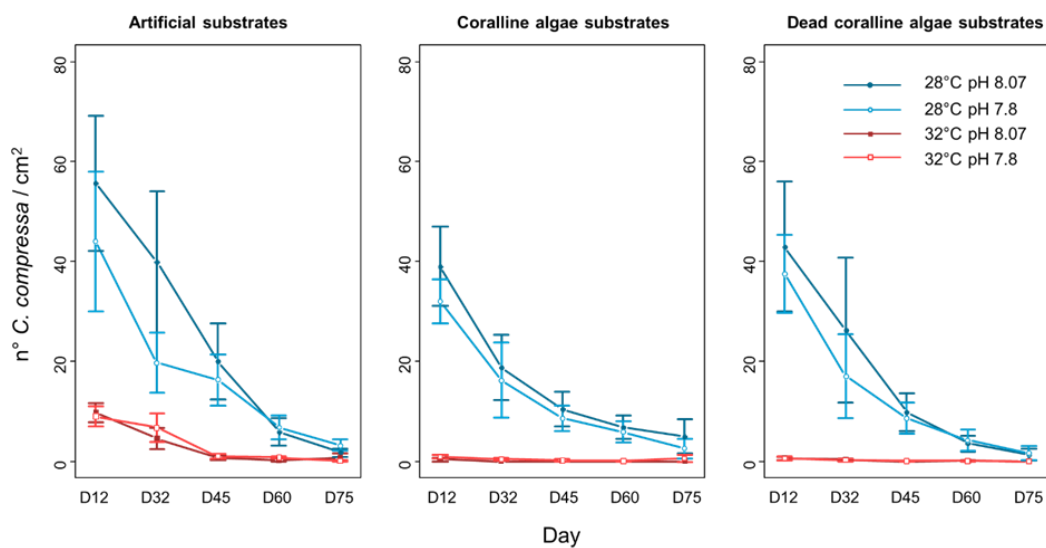


**Figure 3:** Density (a) and size (b) of recruits of *C. compressa* as a function of temperature during the first experiment (96 days). The errors bars show the confidence intervals.

### 3.3 Experiment 2: Effects of climate change and species facilitation on the recruitment of *Cystoseira compressa*

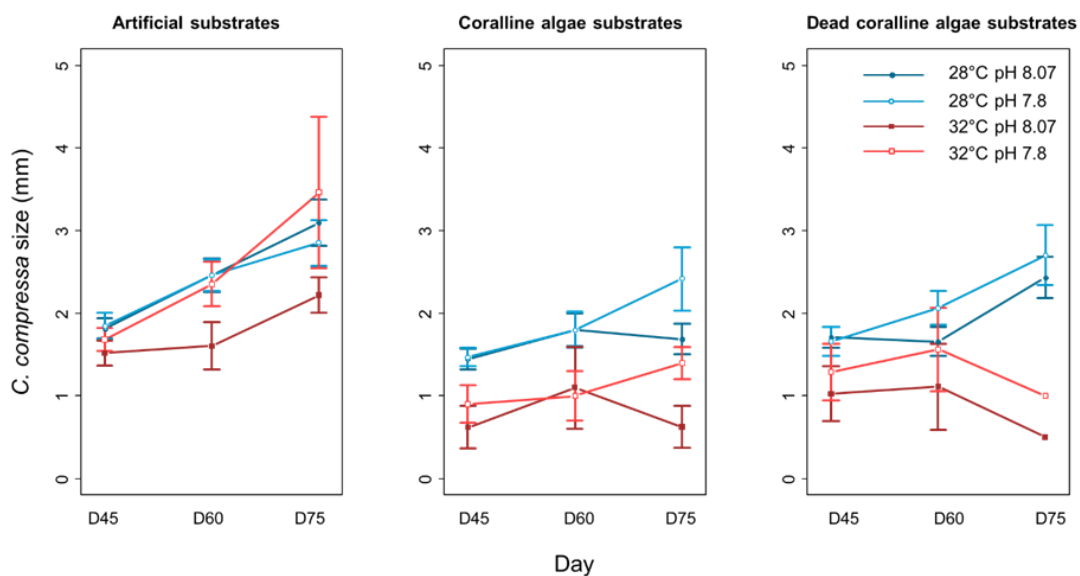
#### 3.3.1. Recruits of *Cystoseira compressa*

The temperature and pH, separately and together with substrate type and time, had an interactive effect on the density of recruits (GLMM, P-value < 0.001; Appendix A: Chapter 1 Supplementary material S3). There was a strong negative effect of the temperature on the density of recruits, especially in presence of *Neogolithon brassica-florida* (Figure 4). Low pH negatively affected the density of recruits in absence of *N. brassica-florida*, but no differences in density between pH levels were detected in presence of the coralline algae (Appendix A: Chapter 1 Supplementary material S3 and S4). The presence of the living and dead coralline algae negatively affected the density of recruits of *C. compressa*, compared to the higher densities found on artificial substrates. The interaction among temperature, pH and time (Temperature  $\times$  pH  $\times$  Time) also affected the density of recruits (GLMM, P-value < 0.001; Appendix A: Chapter 1 Supplementary material S3). Higher densities of recruits were found at low temperature-ambient pH while no differences between pH levels were detected at elevated temperature (Appendix A: Chapter 1 Supplementary material S3). In general, higher densities of recruits were found at low temperature, ambient pH and in absence of *N. brassica-florida*. The density of recruits decreased with time which led to a homogenization of results for most of the considered factors at the end of the experiment (significant differences in pH and temperature were found only in absence of the coralline algae and at low temperature, Appendix A: Chapter 1 Supplementary material S4). This is because only a few recruits survived until the end of the experiment (Figure 4).



**Figure 4:** Densities of recruits on living and dead *Neogoniolithon brassica-florida* and on artificial substrates, as a function of temperature and pH under the different treatments. The errors bars show the confidence intervals.

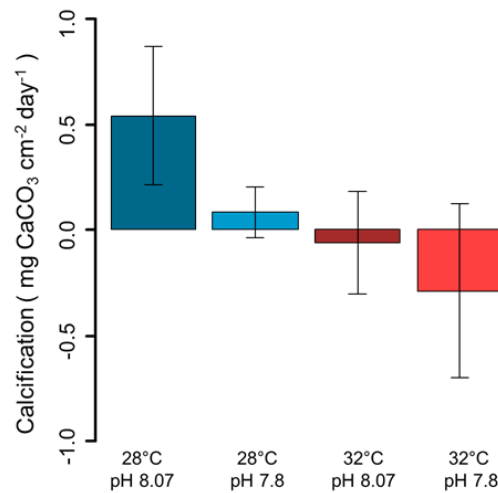
Several interactive effects involving temperature, pH, presence of coralline algae and time affected the size of recruits (GLMM, P-value < 0.001 for the interactions pH  $\times$  Substrate Type  $\times$  Time, Temperature  $\times$  pH and Temperature  $\times$  Substrate type, Appendix A: Chapter 1 Supplementary material S3). The size of recruits was larger at low temperature and low pH. The size of recruits was also larger in absence of *N. brassica-florida* in all the treatment combinations (Figure 5). Differences in size were observed between recruits on living and dead *N. brassica-florida*, but only at low temperature. By the end of the experiment, recruits grown in association with the living coralline algae were smaller, while there were no differences in size between recruits grown on dead *N. brassica florida* and in clay substrates (Figure 5; Appendix A: Chapter 1 Supplementary material S5).



**Figure 5:** Size of recruits on living and dead *Neogoniolithon brassica-florida* and on artificial substrates as a function of temperature and pH under the different treatments. The errors bars show the confidence intervals.

### 3.3.2. Calcification of *Neogoniolithon brassica-florida*

The temperature and pH negatively affected the net calcification of living *Neogoniolithon brassica-florida* (LM, P-value < 0.01 for Temperature and P-value < 0.05 for pH; Appendix A: Chapter 1 Supplementary material S3). Calcification rates were statistically significantly higher at 28°C than at 32°C and ambient pH than at low pH (Figure 6).



**Figure 6:** Calcification rate of *Neogoniolithon brassica-florida* after 64 days under different treatments. Error bars show the confidence intervals.

#### 4. DISCUSSION

Marine forests are in regression in many locations worldwide (Filbee-Dexter and Wernberg, 2018; Christie et al., 2019; Pessarrodona et al., 2021) and particularly in the Mediterranean Sea (Thibaut et al., 2005, 2015; Orlando-Bonaca et al., 2021a). Despite this, the response of the different life stages to climate change remains poorly known, efforts have been done in recent years to evaluate the single or combined effect of climate change on forest-forming species together with other local stressors such as pollution, highlighting and strong negative effect of OW (Leal et al., 2018; Capdevila et al., 2019; Fernández et al., 2021). Our results showed that the temperature, pH and the presence of potentially facilitating species had substantial effects on the density of recruits of *Cystoseira compressa*. The warmer temperature had, in agreement with our initial hypotheses, the largest negative effect on both the density and the size of recruits, as already observed on other forest-forming species, both on recruits (Leal et al., 2018; Capdevila et al., 2019; Verdura et al., 2021) and adults (Lind and Konar, 2017; Fernández et al., 2020; Smale, 2020; Falace et al., 2021). Warmer temperatures not only affect the survival and growth of *Cystoseira* but also affect their metabolism (Celis-Plá et al., 2015, 2017a; Mancuso et al., 2019). A study on *C. compressa* reported that the maximum quantum yield ( $F_v/F_m$ ) of the macroalgae started decreasing from 28°C, while the total phenolic content increased with seawater temperature (Mancuso et al., 2019). Globally, the increase in seawater temperature is a direct threat to marine forests, isolating forest-forming macroalgae to refuge locations with more suitable conditions, while consequent local extinctions can be expected in the northern limits of distribution of some species (e.g. the northern limit of the Mediterranean Sea; Mariani et al., 2019; Verdura et al., 2021). Moreover, indirect temperature-driven effects on marine forests are not negligible, because they contribute to the tropicalization of

habitats and the range expansion of warm water species that can re-shape algal communities and trophic cascades (Vergés et al., 2014a, 2014b).

Seawater pH affected both the density and size of recruits in opposite ways, as density was negatively affected by the low pH, whereas size was positively affected. Low pH has been shown to negatively influence the settlement and early-life stages of other key species such as the giant kelp (Gaitán-Espitia et al., 2014; Hollarsmith et al., 2020), corals and molluscs (Kroeker et al., 2013a) which has led to the paradigm that early-life stages could be more sensitive to global change and therefore could constitute a bottleneck (Capdevila et al., 2019; Falace et al., 2021). However, in our experiment, lower pH levels positively affected the size of *C. compressa*, potentially showing a better performance as reported in some studies for the giant kelp (Roleda et al., 2011; Leal et al., 2017). Non-calcifying macroalgae are generally considered as not particularly sensitive to OA as they can benefit from increasing dissolved CO<sub>2</sub>, particularly the carbon-limited species that do not possess a CCM (Hepburn et al., 2011; Connell et al., 2013). The increase in dissolved CO<sub>2</sub> with OA is therefore expected to positively affect carbon-limited species and neutrally or positively affect non-carbon-limited species with CCM (Hepburn et al., 2011). Most large brown macroalgal species have attributes suggesting the presence of CCM, and yet they benefit from increased CO<sub>2</sub> (Celis-Plá et al., 2015, 2017a; Zhang et al., 2020). The same seems to happen here with the beneficial effects of high CO<sub>2</sub> on the size of the recruits. *C. compressa*, possessing a high affinity CCM for dissolved inorganic carbon (DIC), would not change the activity of the CCM due to OA and, thus, would not specially benefit under elevated CO<sub>2</sub>. As a result, they would potentially end up being less competitive than other species that will benefit from elevated CO<sub>2</sub> (Cornwall et al., 2017a). It is important to note that in some of our experimental conditions (i.e. 32°C and low pH), the biggest sizes corresponded to the lowest densities, which does not allow us to exclude the effect of density-dependent processes in addition to the effect of CO<sub>2</sub>. OA could also have indirect effects on marine forests, by favouring the increased performance of turfs that are generally carbon-limited, especially in the presence of nutrients (e.g. local nutrient pollution; Connell et al., 2013; Falkenberg et al., 2013). Turf-forming species are fast-growing and therefore are great space competitors that could limit the recruitment of long-life species such as forest-forming species. As a result, turf-forming species could expand and replace foundation species (e.g. *Cystoseira s.l.* spp.) that are already affected by global warming and other anthropogenic impacts (Nagelkerken et al., 2016; Filbee-Dexter and Wernberg, 2018). This indirect effect of OA on marine forests might decrease the structural complexity of marine forests, compromising their functioning and promoting regime shifts (Connell and Russell, 2010; Connell et al., 2013; Wernberg et al., 2016).

Our results are in agreement with studies suggesting that OA might have a lesser direct effect on forest-forming macroalgae than warming (Kroeker et al., 2013a): the effect of the pH on the density of recruits was likely masked by the strong effect of the temperature. Nevertheless, the highest



densities were recorded under low temperature (28°C) and ambient pH (8.07), while the size was negatively affected by the high temperature (32°C), but positively affected by a decrease in pH (7.8). Complex interactions of abiotic and biotic factors are well known in natural systems. In our study we initially hypothesized that OW and OA would have affected the early-colonizer coralline algae *Neogoniolithon brassica-florida*, decreasing its potential facilitative effect on *C. compressa* recruitment. The calcification rate of the coralline algae was strongly affected by the temperature and pH, probably explaining the stronger effects of the temperature and pH on recruits growing in association with the coralline algae. However, the presence of both living and dead coralline algae had a negative effect on both the density and the size of the recruits, with the artificial clay substrate being the most favourable. Contrary to what is reported in the literature for other species (Asnaghi et al., 2015), the very common coralline alga *N. brassica florida* did not have a facilitating role in the recruitment of the later successional species *C. compressa* in our experiment. Since the very beginning of the experiment fewer *C. compressa* were observed in association with the coralline algae, living and dead. In the case of the living coralline algae substrates, the physiological state of *N. brassica-florida* likely does not explain this result as they exhibited calcification rates consistent with those reported in other species of coralline algae (Martin and Gattuso, 2009; Cornwall et al., 2017b). The lower recruitment on living coralline could be due to an inhibition of the settlement and development of recruits of *C. compressa* caused by changes in pH or other chemical parameters in the boundary layer formed on the surface of the coralline algae (Cornwall et al., 2014, 2017b). Furthermore, crustose coralline algae have biotic interactions linked to their microbiome (Gefen-Treves et al., 2021) and to their physical and chemical anti-fouling mechanisms to control epiphytes (Johnson and Mann, 1986; Keats et al., 1997; Villas Bôas and Figueiredo, 2004). These characteristics of the surface of coralline algae could not be optimal for the recruitment of *C. compressa* and hence have reduced the settlement in our experiment. From an applicative point of view, it is worth noting that clay substrate, which is already used in many restoration actions in the Mediterranean Sea (Falace et al., 2018; De La Fuente et al., 2019; Orlando-Bonaca et al., 2021b), was an adequate substrate for the settlement of *C. compressa*. Our results support that this substrate is of particular interest because it favours settlement and offers many technical practicalities (e.g. they are cheap, biodegradable, easy to produce and can be formed into any shape; Falace et al., 2018; De La Fuente et al., 2019; Orlando-Bonaca et al., 2021b).

In general, our results show that OW and OA additively, affect both the recruitment of *C. compressa* and the calcification rate of *N. brassica-florida*. Interestingly the interactive effect of OW and OA are likely exacerbated in presence of the coralline *N. brassica-florida*. This could be 1) because of the lower recruitment in the presence of coralline algae or 2) because the effects of OW and OA on coralline algae exacerbate, in turn, its inhibiting effect on *C. compressa* (Maggi et al., 2011; Bulleri et al., 2016). Our experiment shows that complex interactions of biotic and abiotic factors could



affect the key species that shape marine forest communities, with an ultimate effect at the ecosystem level (Smale et al., 2022). As most experimental studies performed in controlled conditions, some limitations have to be highlighted. First of all, our experiment did not allow to separate the effects of the different drivers on the settlement process and the survival of recruits. Secondly, epiphytes (turf algae) progressively appeared in our experimental tanks and their proliferation was enhanced at higher temperatures and low pH (author's personal observation), which in turn eventually affected the performance of *C. compressa*. But this phenomenon is likely to be observed also in natural conditions, where turf-forming species are expected to proliferate under OW and OA (Filbee-Dexter and Wernberg, 2018). Many studies on recruits and adults of *Cystoseira* report elevated mortality under experimental conditions, which confirm the difficulty of maintaining *Cystoseira* in tanks (Falace et al., 2018; De La Fuente et al., 2019; de Caralt et al., 2020) and of replicating the conditions of natural habitats in the laboratory. This could be due to different variability in light, temperature and flow conditions in the aquarium facilities (Irving et al., 2009). Different associated organisms (e.g. microbiome, epiphytic algae, invertebrates) could also explain the different performances observed when culturing these algae (Mancuso et al., 2016). Our experiments were stopped when recruits showed signs of degradation, but the duration of our experiments is consistent with other studies on *Cystoseira* (de Caralt et al., 2020; Falace et al., 2021; Verdura et al., 2021).

The densities obtained in our experiments are extremely high, reaching an average of  $94\ 100.07 \pm 89\ 324.78$  *C. compressa* ind.  $m^{-2}$  (mean  $\pm$  SD,  $n = 28$ ) after two months in the first experiment and  $14\ 213.20 \pm 17.67$  *C. compressa* ind.  $m^{-2}$  (mean  $\pm$  SD,  $n = 197$ ) after 2 months in the second experiment (min.: 1000 ind.  $m^{-2}$ ; max.: 320 000 ind.  $m^{-2}$  for the first experiment and min.: 10 000 ind.  $m^{-2}$  and max.: 210 000 ind.  $m^{-2}$  for the second). Interestingly, a parallel study performed by our team at the same time as the first experiment, seedling natural stones with the same technique presented in this study directly in the field, produced lower densities after 2 months. On the contrary, the recruits growing in the field under natural conditions were bigger ( $11.33 \pm 3.27$  mm) than in the first ( $5.18 \pm 2.70$  mm) and second experiment ( $2.51 \pm 1.61$  mm) after 2 months, showing that the conditions in tanks are not optimal for the growth of *C. compressa* and/or that density-dependent factors can result in high density and smaller size of recruits in the laboratory. Natural densities observed in the donor population reach only  $76.36 \pm 0.72$  of *C. compressa* ind.  $m^{-2}$  (mean  $\pm$  SD,  $n = 22$ ; maximum: 128 ind.  $m^{-2}$  and minimum: 16 ind.  $m^{-2}$ ; author's personal observations, article in prep.). Even if the comparison with natural conditions often highlight some limitation for the studies in tanks, it is important to continue with this approach as it is the only one that allows testing multiple factors under controlled conditions (i.e. temperature and pH).

The results from this study demonstrate that the interactive effects of climate change have a pronounced negative impact on shallow marine forests. This result is especially striking for forest-

forming macroalgae thriving in rock pools, which are expected to be more acclimated to local factors variation (i.e. temperature and pH). Marine forests are facing several other stressors than global change (e.g. water pollution, urbanization, trampling, herbivory; Orfanidis et al., 2021) that already put them on the edge. Some of these stressors have been addressed by management measures and are now mitigated (e.g. water quality; Blanfuné et al., 2019) allowing, in a few cases, the natural recovery of the forest or the feasibility of planning restoration actions (Gorman and Connell, 2009; Gianni et al., 2013; Cebrian et al., 2021; Riquet et al., 2021). Recently, new restoration techniques opened the door to the possibility of restoring these ecosystems, still, marine forests will be increasingly impacted by global change (IPCC, 2022) and it has to be considered to achieve long-term population sustainability and/or successful restoration actions. The next step for the protection of these key ecosystems is to understand how climate change and other drivers acting at the local scale can interact, eventually providing additive or synergetic effects (Halpern et al., 2008a), likely causing the restructure and redistribution of marine forests and affecting their ability to resist and recover under extreme conditions (Smale, 2020; Verdura et al., 2021; Smale et al., 2022).

## **DATA AVAILABILITY**

Data Availability Statement: Data Availability Statement: The datasets generated and analysed during the current study are available in the Knowledge Network for Biocomplexity repository, <https://knb.ecoinformatics.org/view/urn:uuid:daa9cc97-47eb-48a3-addc-ed0047f0f3c4>

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## **AUTHOR'S CONTRIBUTION**

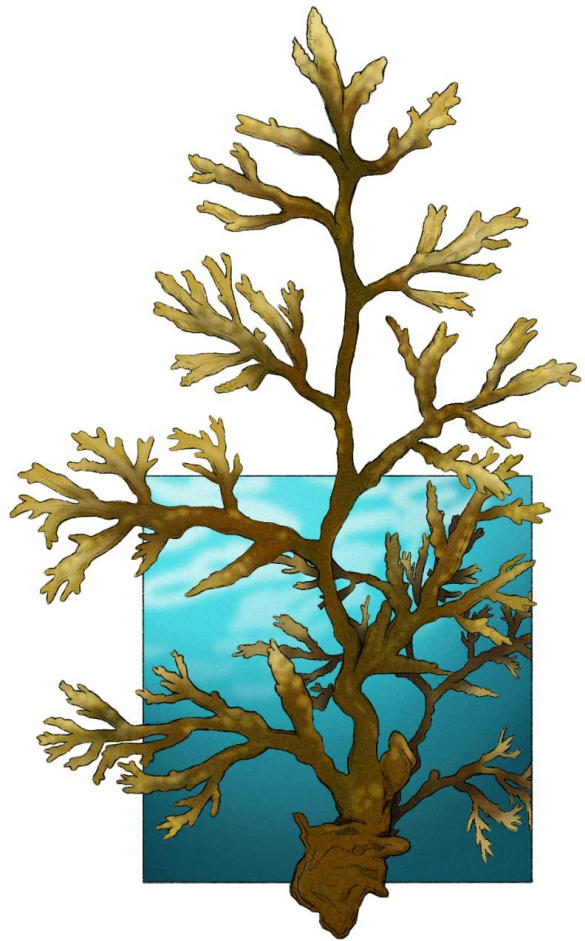
Author Contribution Statement: L.M. got the funding. L.M., S.C. and M.M. designed the study. L.M., J.V. and M.M. were involved with fieldwork. L.M., S.C., J.V. and M.M. performed the experiments. G.S. and G.R. helped in counting and measures from the first experiment in 2020. M.M., G.R. and F.P. did the clay substrates. S.A. contributed to the maintenance and measures of

## CHAPTER 1

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carbonate chemistry. S.C. and M.M. analysed the data. M.M. wrote the first draft of the manuscript which was then finalized by all co-authors.

**CHAPTER 2: GRAZING EFFECTS ON  
RECRUITS OF THE FOREST-FORMING FUCOID  
*Cystoseira compressa***





## ABSTRACT

Grazing is one of the most important biological factors controlling the mortality of early-life stages of furoids and one of the major problems when restoring marine forests. Benthic macro-invertebrates (e.g. sea urchins) and fish shape and regulate benthic macroalgal communities from temperate to tropical regions, being responsible for the formation of turfs or even barren grounds. However, other smaller invertebrates associated with marine forests could significantly participate in the grazing, especially on early-life stages. Knowing which invertebrates could participate in the grazing of recruits of forest-forming macroalgae could be crucial for their conservation. We performed several experiments in the field and in mesocosms in order to investigate the herbivory pressure and the effects of different grazers on recruits of *Cystoseira compressa*. The results highlight that non-strict herbivorous species, such as *Clibanarius erythropus*, *Cerithium vulgatum* and *Idotea balthica*, can potentially graze on recruits of *Cystoseira s.l.* spp. probably affecting the success of marine restoration actions. From the different invertebrates studied in the mesocosms, *I. balthica* had the higher consumption rates of recruits of *C. compressa*. We conclude that biotic factors such as herbivory need to be well understood when planning restoration actions and that anti-herbivory devices adapted to the local diversity of potential herbivores should be included when restoring marine forests.

**Key words:** *Cystoseira compressa*, Herbivory, Plant-herbivore interactions, Grazing, Restoration, *Clibanarius erythropus*, *Cerithium vulgatum*, *Idotea balthica*, *Gammarus*, sea urchins, *Sarpa salpa*

## 1. INTRODUCTION

Marine forests of large brown macroalgae represented by the species from the orders Laminariales, Tylopteridales, Desmarestiales and Fucales form structurally complex habitats in cold and temperate regions around the world (Wernberg and Filbee-Dexter, 2019). These forests, commonly located on intertidal and subtidal rocky bottoms, provide important ecosystem functions, such as habitat, food and shelter to multiple species and are one of the most productive ecosystems in the world participating in the carbon sink (Boudouresque et al., 2016; Wernberg and Filbee-Dexter, 2019; Filbee-Dexter et al., 2022; Pessarrodona et al., 2022). Despite the important ecological functions marine forests provide, multiple anthropogenic stressors are pushing them to the edge (Filbee-Dexter and Wernberg, 2018; Carnell and Keough, 2019; Wernberg et al., 2019b). A worldwide decline of marine forests is occurring, mainly driven by the destruction of habitats, reduced water quality, global change and proliferation of herbivores (Wernberg et al., 2019b; Orfanidis et al., 2021; Pessarrodona et al., 2021). In particular, overgrazing by sea urchins and herbivorous fish species is in some locations responsible for such declines (Foster and Schiel, 2010; Gianni et al., 2017; Sharma et al., 2021; Barrientos et al., 2022), shaping and regulating benthic macroalgal communities in a variety of natural rocky reef systems from temperate to tropical

regions (Scheibling et al., 1999; Vanderklift et al., 2009; Vergés et al., 2009). Although local stressors and regional variations can dominate marine forest dynamics (Smith et al., 2017, 22; Smale, 2020; Verdura et al., 2021), many studies have shown how sea urchins, and native and invasive herbivorous fish, and even omnivorous fish, are a potential threat to macroalgal communities and are responsible in the formation of turfs or barren ground extensions (Tegner et al., 1995; Vergés et al., 2014b, 2016; Papadakis et al., 2021). Other smaller invertebrates associated to marine forests such as decapods, gastropods, amphipods and isopods can significantly participate in the grazing of different life stages of forest-forming species (Arrontes et al., 2004; Jonne et al., 2006; Gunnarsson and Berglund, 2012; Hong et al., 2021; Navarro-Barranco et al., 2022; Molis et al., 2010) but little is known about the magnitude of their possible effects.

In the Mediterranean Sea, density and abundance of macroalgae are also controlled by herbivorous (Vergés et al., 2009). The two species of sea urchin *Paracentrotus lividus* and *Arbacia lixula* are the most common benthic macro-herbivores of the sublittoral rocky bottoms (Bulleri et al., 1999; Agnetta et al., 2015). Both species of sea urchin have been responsible alongside anthropogenic stressors (e.g. date mussel harvesting), for the degradation and regime shifts of forest-forming and shrub-forming macroalgal assemblages to barren grounds, favouring the maintenance of post-regime shifts stable states (e.g. bare rock) in many areas (Sala et al., 1998; Bulleri et al., 1999; Guidetti and Dulčić, 2007; Guidetti, 2011; Giakoumi et al., 2012).

However, sea urchins are not the only macro-herbivores that play a role in the structure of macroalgal assemblages of the Mediterranean Sea. Salema fish, (*Sarpa salpa*), the only true native herbivorous fish in the Mediterranean Sea, is also important in structuring macroalgal communities, and can also be responsible for the depletion of *Cystoseira s.l.* forests (Gianni et al., 2017). More recently, the range-expansion of tropical herbivorous fishes such as the rabbitfish (*Siganus* spp.) has also been signalled for severely reducing the biomass and diversity of macroalgal species in the Mediterranean Sea, driving regime shifts from macroalgal dominated communities to barrens (Vergés et al., 2014b, 2016; Zarco-Perello et al., 2020). Herbivorous range-shifting fishes have also been reported for preferring to graze on turf, constituting also a menace for forest-forming species as they could graze on the microscopic life-stages present in the turf matrix (Barrientos et al., 2021). *Cystoseira s.l.* spp. in the Mediterranean Sea form prominent and dense canopies that are analogous to kelp forests of other temperate rocky coasts (Giaccone, 1973; Ballesteros et al., 1998), and are amongst the most productive and complex habitats of the Mediterranean Sea (Ballesteros, 1989a, 1990a; Clayton, 1990). The vertical zonation of *Cystoseira s.l.* spp. depends on multiple depth-dependent physical factors such as light, hydrodynamics, temperature, and availability of nutrients (Ballesteros, 1989b), but also on biotic factors such as herbivory (Ruitton et al., 2000; Vergés et al., 2009; Sala et al., 2011).

Because of the important ecosystem services marine forests provide, important efforts have recently been made to promote the conservation and the viability of their restoration (Eger et al., 2021b). Different restoration techniques have been used to restore marine forests from transplantation of adults to *ex-situ* and *in-situ* recruitment enhancement techniques (Verdura et al., 2018; Cebrian et al., 2021; Falace et al., 2018; De La Fuente et al., 2019; Savonitto et al., 2021). However, still, some problems need to be overcome (e.g. herbivory and climate change) to ensure the success of conservation and restoration actions (Gianni et al., 2013; Cebrian et al., 2021; Savonitto et al., 2021). Because grazing is believed to be one of the most important biological factors controlling the survival of early-life stages of furoids (Chapman, 1995), it constitutes one of the major problems when planning the restoration of marine forests (Gianni et al., 2013, 2018; Tamburello et al., 2019; Savonitto et al., 2021). Knowing which species graze on different life stages of forest-forming species could be crucial for successful conservation and restoration actions. It is of special interest to study early-life stages as they are more vulnerable than adults and high mortality rates are naturally observed (Aberg, 1992; Coelho et al., 2000; Schiel and Foster, 2006), and because the resilience of the whole population to future impacts can be largely dependent on efficient recruitment and growth of the recruits (Ang, 1991; Capdevila et al., 2015).

The main objective of this study was to investigate the herbivory pressure in the field and the effect of different species (sea urchins, fishes, decapods, gastropods, amphipods and isopods) on recruits of *Cystoseira compressa*. In order to assess species-specific herbivory pressure on the survival and growth of recruits of *C. compressa* we performed experiments in the field and in mesocosms. More specifically, in the field we performed herbivory exclusion experiments on recruits of *C. compressa* obtained from both *in-situ* and *ex-situ* recruitment enhancement techniques. In laboratory, we assessed for the first time the potential grazing impact of non-strict herbivorous species that have not been considered before as potential herbivores (decapods, gastropods, amphipods and isopods) on recruits of *C. compressa*.

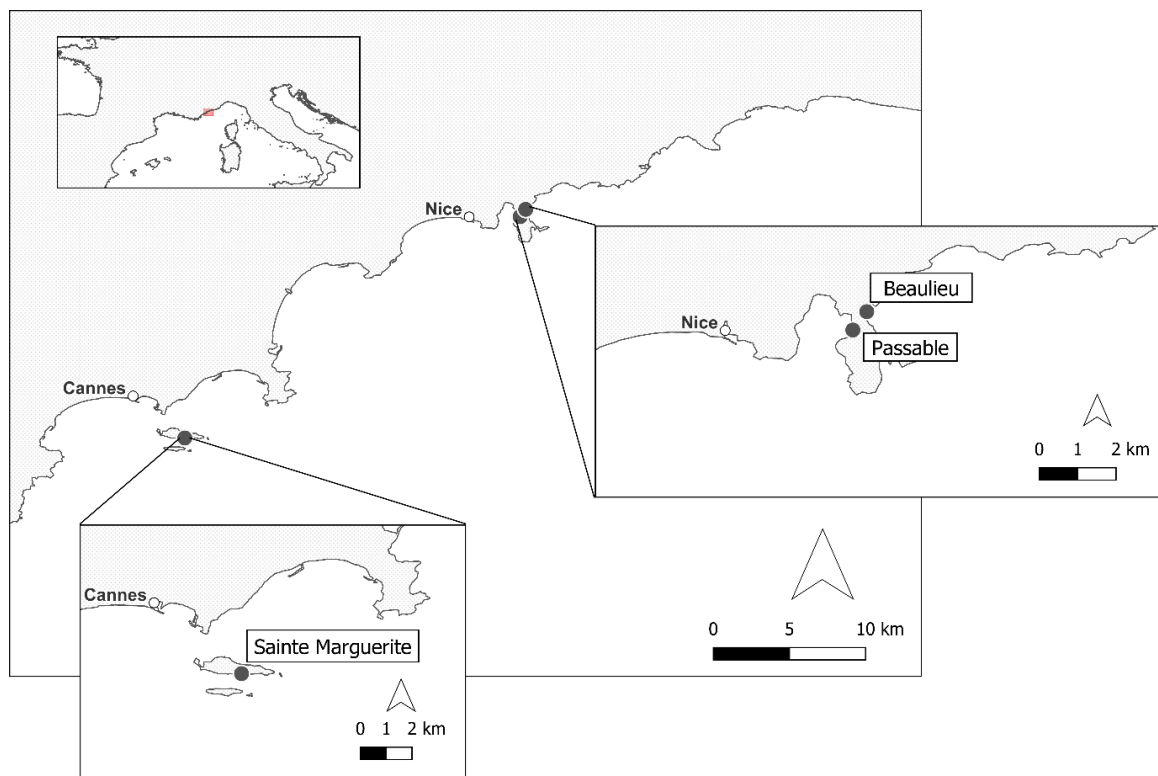
## 2. MATERIAL AND METHODS

### 2.1. Study sites and target species

*Cystoseira compressa* was the selected species because it is considered one of the most resistant species to manipulation and the only *Cystoseira s.l.* spp. not protected under Barcelona Convention Annex II and United Nations Environment Programme/Mediterranean Action Plan-UNEP/MAP (Mangialajo et al., 2012; Verlaque et al., 2019). This species, that could also have a role in facilitating the settlement of other *Cystoseira s.l.* spp. (Mangialajo et al., 2012), is still present in the French Riviera, where patches can be found along exposed rocky shores but dense populations are only found on rockpools in Lérins Islands (author's personal information; Thibaut et al., 2015).



Three sites were selected to study the herbivory pressure on recruits of *C. compressa*: one site on a *Cystoseira s.l.* forest (Sainte Marguerite Island) and two sites where *C. compressa* was reported in the past but where at present is lost (Beaulieu-sur-Mer and Passable; Thibaut et al., 2015). The forest site corresponds to a rockpool system located in Sainte Marguerite Island (from now Sainte Marguerite; WN-Mediterranean Sea - Lérins Islands, Cannes, France), a protected Natura 2000 site at 1.3 km from the coast of Cannes where there is one of the last well-conserved shallow marine forests in the Côte d'Azur (Figure 1). The rockpool system (between 0.2 and 1.0 m depth) is composed mainly of *C. compressa*, *Gongolaria barbata*, *Ericaria crinita* and *Ericaria brachycarpa*. The two sites deprived of *Cystoseira s.l.* forests are located on the continental open coast in (i) Fourmis' Bay in Beaulieu-sur-Mer (from now Beaulieu), a semi-exposed site between 0.5 and 1 m depth, and (ii) Passable in Saint – Jean - Cap – Ferrat (from now Passable), a sheltered site around 0.5 m depth (Figure 1).



**Figure 1:** Location of the three experimental sites: the rockpool system in Sainte Marguerite Island in Lérins Islands ( $43^{\circ}30'57.6''\text{N}$ ,  $7^{\circ}3'14.4''\text{E}$ ), and the two open coast locations Beaulieu ( $43^{\circ}42'3.6''\text{N}$ ,  $7^{\circ}19'48''\text{E}$ ) and Passable ( $43^{\circ}41'42''\text{N}$ ,  $7^{\circ}16'33.6''\text{E}$ ).

## 2.2. Herbivores survey

Before the start of the experiments, the presence and density of known and potential herbivorous species, including sea urchins, decapods, gastropods and *Sarpa salpa*, were characterized at the three locations. The density of decapods and gastropod was determined using 20\*20 cm quadrates in the rockpool system in Sainte Marguerite ( $n = 40$ ) and in the open coast in Beaulieu ( $n = 20$ ) and

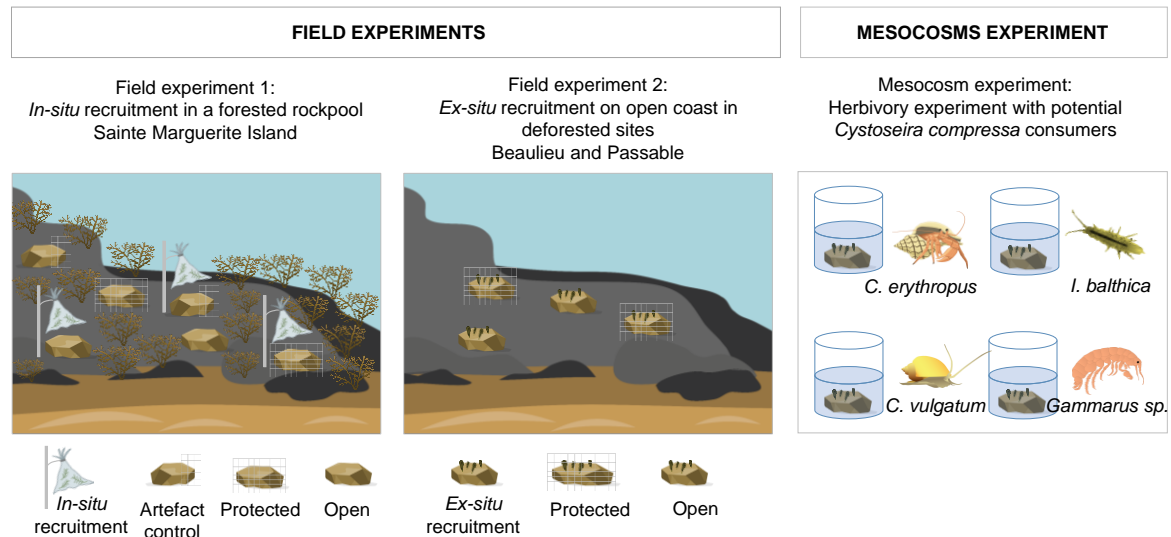
Passable ( $n = 20$ ); while the density of sea urchins was assessed using 50\*50 cm quadrates ( $n = 20$  per site). The surveys were performed from summer to autumn 2020 and 2021 on five different days in Sainte Marguerite and in 2021 on three different days in Beaulieu and Passable. Herbivorous fish, mainly *Sarpa salpa* larger or equal to 5 cm length, were visually quantified in Sainte Marguerite, while in Beaulieu and Passable, herbivorous fish densities were quantified by recording stationary videos at random points and counting number of *S. salpa* individuals in a radius of 5 m during 5 min ( $n = 10$ ; Sala and Ballesteros, 1997).

### **2.3. Field experiment 1: Herbivory on *in-situ* recruitment substrates in a rockpool**

In order to study the effectiveness of the *in-situ* recruitment enhancement and the herbivory pressure on recruits in the field, an herbivory exclusion experiment was performed in the rockpool system in Sainte Marguerite (Figure 1). 18 substrates were randomly fixed using Epoxy to the bottom of the rockpool system, to provide free substrate. In order to test the effect of recruitment enhancement, half of these substrates were seeded using the non-destructive *in-situ* recruitment enhancement technique (Verdura et al., 2018). To do so, on July 2020, after having observed mature conceptacles under the microscope, apical fertile branches (ca. 3 cm in length) were manually collected and transported in cold and dark conditions in plastic bags to the laboratory. The receptacles were conserved at 4°C in the dark overnight before placing them on the selected substrates *in-situ*. On each substrate, a net bag containing 5 g fresh weight (FW) of receptacles was placed floating above the substrate for three days. In order to assess the herbivory impact on the recruits, again half of the substrates were protected (factor Protection, three levels) to avoid herbivory (mainly from fishes and sea urchins, factor level protected), artefact controls and substrates with no protection (levels artefact control and open, respectively) were also included (Figure 2; Recruitment enhancement  $\times$  Herbivory Protection,  $n = 3$ ). The density (as number of recruits per surface) and size (cm) as length of the longest axis of each *C. compressa* individual growing on the substrates were monitored at 2, 9 and 12 months and used as variables. The size of the recruits was calculated by measuring 10 individuals from each substrate, in the case of substrates with less than 10 individuals, all the individuals were measured. The natural density of the *C. compressa* individuals present in the rockpool of Sainte Marguerite was monthly monitored from May to October 2020 using 20\*20 cm quadrats ( $n = 25$ ) and the global mean value was used as a reference to assess the evolution during our experiments.

The density of recruits of *C. compressa* on 0.04 m<sup>2</sup> was used as response variable. A Generalized Linear Model (GLM), with a Quasipoisson link log distribution function, was used to test the effect of the herbivory protection and recruitment enhancement on recruits of *C. compressa* after 2 months, with herbivory protection (three levels) and recruitment enhancement (two levels) as fixed factors. After testing the effect of the recruitment enhancement, this factor was no longer considered

for further analysis. Then, a Generalized Linear Mixed-Effects Model (GLMM) with a Poisson link log distribution function was used to test the effect of the factor protection over time with the factors protection and time (both three levels) as fixed factors and the factor substrate as random factor, as GLMM can cope with repeated measures over time (Pinheiro and Bates, 2000). A GLMM with an inverse gaussian link log distribution was used to test the effects of the herbivory protection on the size of the recruits (cm), with the factors herbivory protection and time (both three levels) as fixed factors and the substrate as a random factor.



**Figure 2:** Representation of the different experiments performed. The two herbivory experiments in the field, to assess the herbivory pressure on recruits of *C. compressa*; and the mesocosm experiment, to assess the potential herbivory on recruits of *C. compressa* of the decapod *Clibanarius erythropus*, the gastropod *Cerithium vulgatum*, the amphipod *Gammarus* sp. and the isopod *Idotea balthica*.

#### 2.4. Field experiment 2: Herbivory on *ex-situ* recruitment substrates on the open coast

In order to study the herbivory pressure in the field and the success of the *ex-situ* recruitment enhancement technique in absence of a *Cystoseira s.l.* spp. forest, an herbivory exclusion experiment was performed in the two locations situated in the open coast: Beaulieu and Passable (Figure 1). In August 2021, after having observed mature conceptacles under the microscope, about 180 g FW apical branches of *C. compressa* containing fertile receptacles, were collected from the same *C. compressa* donor population located in the rockpool system in Sainte Marguerite, following the same non-destructive technique described before (see section 2.3; Falace et al., 2018; Verdura et al., 2018; De La Fuente et al., 2019). The apical branches were transported in cold and dark conditions and once in the laboratory they were gently cleaned with tweezers. To stimulate the release of gametes, the apical branches were maintained at 4°C in the dark overnight before placing them on the surface of three 30 L closed system tanks (60 g FW of fertile receptacles per tanks) to obtain recruits on substrates placed on the bottom of the tanks. The receptacles were kept for four

days on the surface of the tanks while releasing the zygotes. The recruits of *C. compressa* were kept for 1 month in the aquarium facilities, with air pumps and under natural light conditions, before the transplant to the two coastal locations was done. The seawater (filtered using a 200  $\mu\text{m}$  mesh) of the tanks was changed every two days and no culture medium was provided to the cultures. After 2.5 months in the tanks, 24 substrates with similar densities of *C. compressa* recruits were transplanted and fixed using Epoxy at the sea bottom of the two open coast locations in October 2021. Some substrates with recruits were maintained in the aquarium facilities and used as controls ( $n = 6$ ). As for experiment 1, half of the transplanted substrates were protected to avoid herbivory (mainly fishes and sea urchins), while the others were not ( $n=6$ ; Figure 2).

The density of recruits of *C. compressa* on 0.04  $\text{m}^2$  was used as response variable. A Generalized Linear Mixed-Effect Model (GLMM) with a Poisson link log distribution function, was used to test the effect of the herbivory protection on recruits of *C. compressa*, with the herbivory protection (two levels) and time (two levels) as fixed factors, and the site (two levels) and substrate as random to cope with repeated measures over time (Pinheiro and Bates, 2000).

### **2.5. Mesocosm experiment: Potential herbivory of different species**

Four experiments were done in a mesocosm in the laboratory to study the potential herbivory role of some invertebrates on recruits of *C. compressa*. The selected species consisted of a decapod (*Clibanarius erythropus*), a gastropod (*Cerithium vulgatum*), an amphipod (*Gammarus* sp.) and an isopod (*Idotea balthica*). The species and their densities were selected in function of the observations in the field and in function of the information reported in the literature for other herbivory experiments performed on furoid species (Engkvist et al., 2000; Gunnarsson and Berglund, 2012; Suzuki et al., 2020; Hong et al., 2021; Navarro-Barranco et al., 2022).

*C. erythropus* and *C. vulgatum* individuals were obtained in the Sainte Marguerite, within the marine forest, while *I. balthica* and *Gammarus* sp. were obtained from Anse des Fossés (Saint-Jean-Cap-Ferrat, France) within leaves of *Posidonia oceanica* detritus. Different numbers of individuals of each species were selected and placed in separated close system experimental tanks (2.5 L). In each experimental tank, there was one substrate with similar biomass of recruits of *Cystoseira compressa* (between 2-3 months old). Recruits were obtained in the laboratory following the same *ex-situ* cultivation technique as in section 2.4 and kept in 30 L tanks until the beginning of the mesocosm experiment. The substrates with *C. compressa* were cleaned from epiphytes and/or growing biofilm with tweezers and a brush before the start of the experiment. The selected densities of individuals for *C. erythropus* were 1, 2 and 3 individuals per tank ( $n = 5$ ); for *C. vulgatum* were 1, 3 and 5 individuals per tank ( $n = 5$ ); and for *Gammarus* sp. and *I. balthica* 1 individual per tank ( $n = 7$ ). Controls with 0 herbivorous were included ( $n = 5$ ). As the experiments with *C. erythropus* and *C. vulgatum* were performed simultaneously, the same controls ( $n = 5$ ) were used for both

species. The experiments with *Gammarus* sp. and *I. balthica* were performed later and, as were again performed simultaneously, both species share the control experimental tanks with 0 individuals ( $n = 5$ ). This experiment consisted in a total of 50 experimental tanks. All the species were kept without food for 48h before the start of the experiment. Every two days the temperature was measured and the filtered seawater (200  $\mu\text{m}$  mesh) of the tanks was changed, faeces were cleaned every day. *C. erythropus* and *C. vulgatum* were maintained in the experimental tanks 23 days and *Gammarus* sp. and *I. balthica* 4 days, until they consumed all or almost all of the available biomass of *C. compressa*. *C. erythropus* and *C. vulgatum* were only weighted once at the end of the experiment (mg FW) after extracting them from their shells. *Gammarus* sp. and *I. balthica* individuals were weighted (mg FW) every 24h. The mean biomass of *Gammarus* sp. individuals was  $0.08 \pm 0.02$  g and the mean biomass of *I. balthica* individuals was  $0.07 \pm 0.01$  g (mean  $\pm$  SD) at the beginning of the experiment. The consumption of the different species was assessed as the consumed biomass (mg FW) of recruits and calculated from the number of *C. compressa* recruits. At the start of the experiment the mean biomass of *C. compressa* on each tank was  $5.02 \pm 0.53$  mg FW for *C. erythropus* and *C. vulgatum* and  $6.05 \pm 0.91$  mg FW (mean  $\pm$  SD) for *Gammarus* sp. and *I. balthica* (Appendix B: Chapter 2 Supplementary material). The biomass of recruits was evaluated every 24h the first week and then twice a week.

The biomass (mg FW) of the *C. compressa* recruits was extrapolated from a linear regression [Volume ( $\text{mm}^3$ )  $\sim$  biomass (mg FW)]. To do so, the volume of each recruit was calculated approximating its shape to a cone and semi sphere (Appendix B: Chapter 2 Supplementary material). The maximum length and maximum width of each recruit was measured under a stereomicroscope. Then this volume was transformed to g FW, using the previously adjusted volume ( $\text{mm}^3$ )  $\sim$  biomass (mg FW) linear regression using other recruits ( $R^2 = 0.994$ ). To obtain the linear regression, the volume of 38 recruits was calculated by water displacement (mL). The volume of each recruit was measured six times and the mean of the measurements was used. Then each recruit was dried with absorbent paper before being weighed three times in mg (0.001), for obtaining the FW. Correlating the volume and the biomass of each recruit we adjusted the linear regression (Appendix B: Chapter 2 Supplementary material).

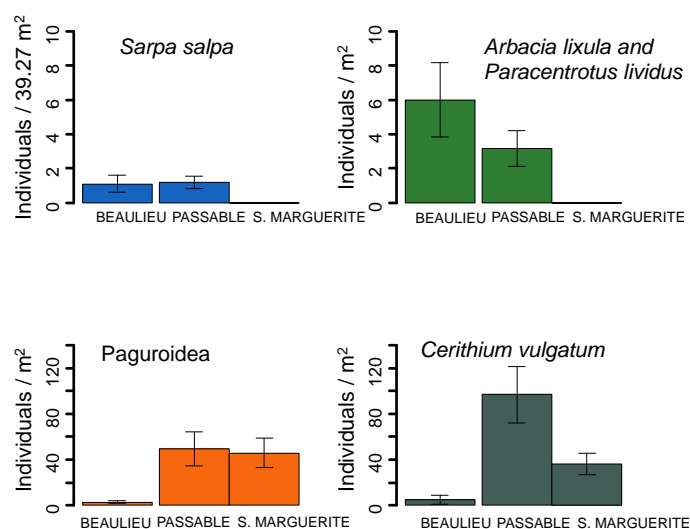
A Linear Mixed-Effects Model (LMER) was used to test the cumulative biomass consumption of recruits of *C. compressa* by *C. erythropus*, *C. vulgatum* and *I. balthica*, with the density of herbivorous (four levels for *C. erythropus*, *C. vulgatum* and two levels for *I. balthica*) as a fixed factor, and time and the aquarium as a random factor as the LMER models can cope with repeated measures over time. Finally, a Linear Model (LM) was used to test the consumption rate (mg *C. compressa*  $\text{mg herbivor}^{-1} \text{ day}^{-1}$ ) of the different species, with the species (three levels) as fixed factor. *Gammarus* sp. was excluded as no consumption was detected.

All the GLMM and LM models were fitted to analyse the effect of the variables and the AICs likelihood minimum was used to select the best model among the possible combinations. All the different models were fitted using the functions “glmer” and “lm” from the package lme4 (Bates et al., 2015) in the statistical environment R (R Core Team, 2019). For all the models, the assumptions of normality and equality of variance were evaluated through graphical analyses of residuals using QQ plot functions. P-values were obtained by means of a Wald  $\chi^2$  test using the ‘ANOVA’ function from the car package (Fox and Weisberg, 2018). Finally, the function ‘emmeans’ from the package emmeans (Lenth et al., 2022) was used to perform the post-hoc analysis.

### 3. RESULTS

#### 3.1. Herbivores survey

In the open coast sites mean densities of *Sarpa salpa* of  $1.1 \pm 1.59$  ind.  $39.27 \text{ m}^{-2}$  and of  $1.2 \pm 0.84$  ind.  $39.27 \text{ m}^{-2}$  (mean  $\pm$  SD, hereafter) were respectively found in Beaulieu and Passable. Sea urchins (*Paracentrotus lividus* and *Arbacia lixula*) were present at densities of  $6.00 \pm 6.86$  ind.  $\text{m}^{-2}$  and of  $3.14 \pm 3.89$  ind.  $\text{m}^{-2}$ ; Paguroidea decapods were present at densities of  $2.91 \pm 6.32$  ind.  $\text{m}^{-2}$  and of  $49.45 \pm 69.29$  ind.  $\text{m}^{-2}$ ; and *Cerithium vulgatum* was present at densities of  $5.09 \pm 18.07$  ind.  $\text{m}^{-2}$  and of  $96.73 \pm 116.27$  ind.  $\text{m}^{-2}$ , respectively for Beaulieu and Passable (Figure 3). In the rockpool system in Sainte Marguerite, neither *Sarpa salpa* (>5 cm) or sea urchins, *Paracentrotus lividus* or *Arbacia lixula*, were observed during the experiment. In this location mean density of Paguroidea decapods was  $45.90 \pm 102.18$  ind.  $\text{m}^{-2}$  and of *Cerithium vulgatum* was  $36.46 \pm 73.73$  ind.  $\text{m}^{-2}$  (mean  $\pm$  SD). Paguroidea decapods in the three locations consisted mainly in *Clibanarius erythropus* but individuals of *Diogenes pugilator* and few of *Calcinus tubularis* were also observed. In general, higher densities of herbivores were observed in Passable (Figure 3).

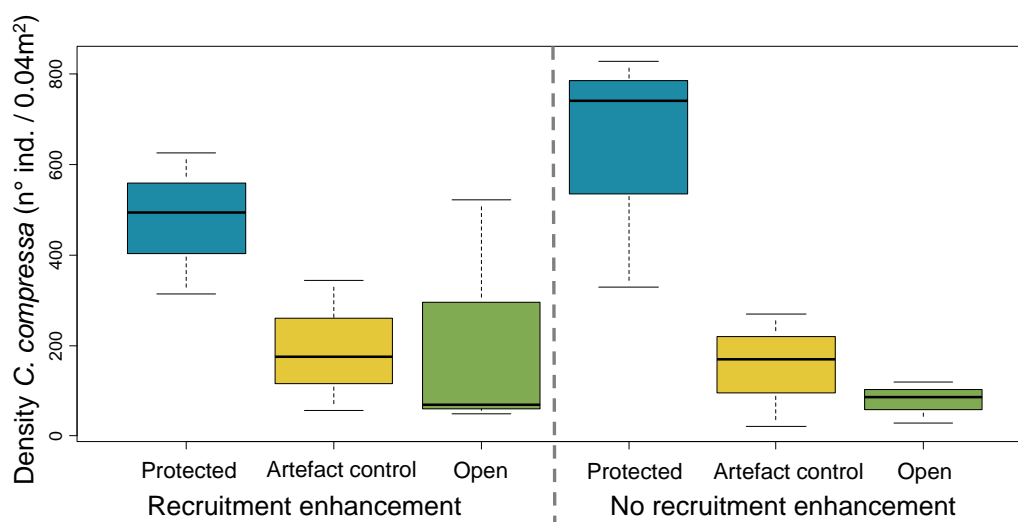


**Figure 3:** Density of herbivores in the three studied locations. The errors bars show the standard error.



### 3.2. Field experiment 1: Herbivory on in-situ recruitment substrates in a rockpool

The density of recruits (ind.  $0.04 \text{ m}^{-2}$ ) on the substrates in Sainte Marguerite, were affected at two months only by the herbivory protection, and not the recruitment enhancement (GLMM, P-value < 0.001; Figure 4 and Table 1). At this time, the post-hoc analysis found higher densities of recruits on protected substrates than on open or artefact control substrates. Due to the non-significance of the factor recruitment enhancement on the density of recruits at two months, this factor was no longer considered and the data was pooled together in the next analysis.



**Figure 4:** Boxplot of the density of recruits of *C. compressa* per  $0.04 \text{ m}^2$  of natural substrates in the rockpool in Sainte Marguerite 2 months after the starting of the experiment. The bold horizontal lines indicate the median value (Q2), the box marks the interquartile distances (Q1 and Q3), the whiskers mark the values that are less than  $Q3 + 1.5 * \text{IQR}$  but greater than  $Q1 - 1.5 * \text{IQR}$ .

When considering the three sampling dates, the herbivory protection and the time exerted an interactive effect on the density of recruits (GLMM, P-value < 0.001 for the interaction Herbivory protection  $\times$  Time; Figure 5 and Table 1). The post-hoc analysis detected differences in densities of *C. compressa* between the levels of herbivory protection for all sampling days. At month 2 and 12 densities of *C. compressa* were higher on protected substrates, while at month 7 there were no differences between protected and open substrates. For all the herbivory protection levels, the densities of *C. compressa* decreased over time, but at month 12 they were still higher than the ones observed in the natural population ( $3.05 \pm 0.14$  ind. *C. compressa*  $0.04 \text{ m}^{-2}$  on natural populations; Figure 4).

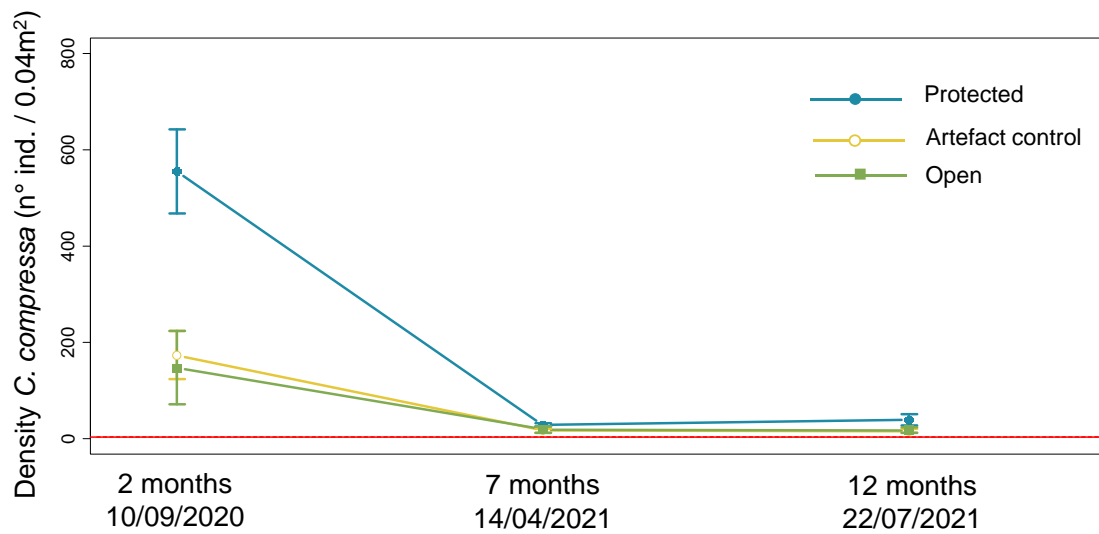
**Table 1:** Results from the statistical analyses.

Experiment	Model	Factor levels	DF	Chi sq	P-value
Experiment Lérins	<b>glm (Density (ind 0.04 m<sup>-2</sup>) 2 months ~ Protection * Recruitment enhancement, quasipoisson(link = "log"))</b>	Protection (Protected, Artefact control and Open)	2	16.3084	< 0.001 ***
		Recruitment enhancement (Yes/No)	1	0.006	0.940
		Protection * Recruitment enhancement	2	2.238	0.327
	<b>glmer (Density (ind 0.04 m<sup>-2</sup>) ~ Protection * Time + (1   Substrate), poisson (link = "log"))</b>	Protection (Protected, Artefact control and Open)	2	19.872	< 0.001 ***
		Time (4 levels)	2	3736.725	< 0.001 ***
		Herbivory protection * Time	4	53.427	< 0.001 ***
Experiment Beaulieu and Passable	<b>glmer (Density (ind 0.04 m<sup>-2</sup>) ~ Protection * Time + (1   Site) + (1   Substrate), poisson (link = "log"))</b>	Herbivory Protection (Protected and Open)	1	3.793	0.051
		Time (2 levels)	2	2115.406	< 0.001 ***
		Herbivory protection * Time	2	123.771	< 0.001 ***
Experiment C. <i>erythropus</i>	<b>lmer (Biomass consumption (mg FW) ~ n° herbivores + (1   Time) + (1   Tank))</b>	N° herbivores (0, 1, 2 and 3)	3	45.129	< 0.001 ***
Experiment C. <i>vulgatum</i>	<b>lmer (Biomass consumption (mg FW) ~ n° herbivores + (1   Time) + (1   Tank))</b>	N° herbivores (0, 1, 3 and 5)	3	42.651	< 0.001 ***
Experiment I. <i>baltica</i>	<b>lmer (Biomass consumption (mg FW) ~ n° herbivores + (1   Time) + (1   Tank))</b>	N° herbivores (0 and 1)	1	45.562	< 0.001 ***
All herbivores	<b>lm (Consumption Rate mg FW C. <i>compressa</i> + mg FW herbivores<sup>-1</sup> * Day<sup>-1</sup>) ~ Species of herbivores)</b>	Species of herbivores (4 levels)	2	27.666	< 0.001 ***

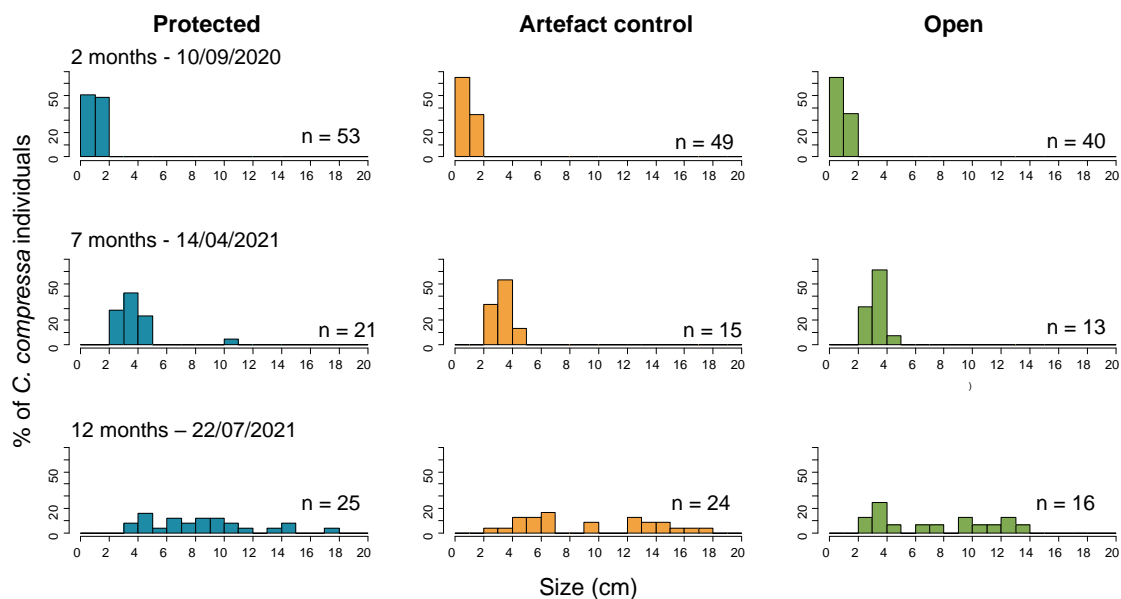
Only time, and not the herbivory protection had a significant effect on the size of the recruits (GLMM, P-value < 0.001 for the factor Time; Figure 6 and Table 1). The size was increasing during the sampling dates with a maximum at month 12 (July 2021), with a mean size of  $9 \pm 4.18$  cm (mean  $\pm$  SD), and maximum and minimum of 18 and 3 cm, respectively (Figure 6 and Table 1). The natural population density of *Cystoseira compressa* in Sainte Marguerite was  $3.05 \pm 0.14$  ind.



0.04 m<sup>-2</sup> ( $76.36 \pm 0.72$  individuals m<sup>-2</sup>; mean  $\pm$  SD), with maximum densities of 5 and a minimum of 1 individual 0.04 m<sup>-2</sup>.



**Figure 5:** Density of recruits of *C. compressa* per 0.04 m<sup>2</sup> of substrates in the rockpool in Sainte Marguerite. The natural population densities of *C. compressa* in Sainte Marguerite are represented in red considering the mean and the standard error ( $3.05 \pm 0.14$  individuals 0.04 m<sup>-2</sup>, mean  $\pm$  SD). The errors bars show the standard error.

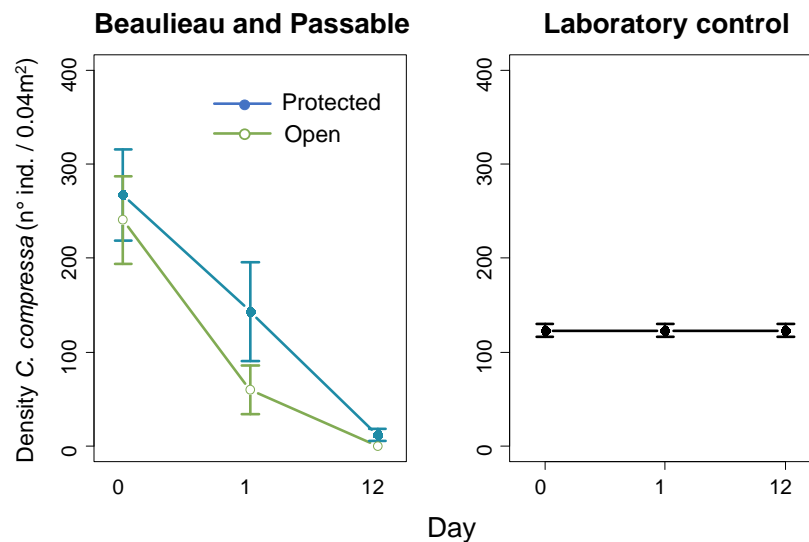


**Figure 6:** Size class distribution of the *C. compressa* recruits on Sainte Marguerite's substrates over time for each treatment. The X-axis represent the size classes in 1 cm intervals and the Y-axis is the proportion of each size class per treatment.

### 3.3. Field experiment 2: Herbivory on ex-situ recruitment substrates on the open coast

The herbivory protection and time had an interactive effect on the density of recruits of *C. compressa* on the open coast (GLMM, P-value < 0.001 for the interaction Herbivory protection  $\times$

Time; Figure 7 and Table 1). The post-hoc analysis found higher densities of recruits of *C. compressa* on protected substrates after 1 and 12 days. However most of the recruits (> 95%), even on protected substrates, disappeared after 24h, while densities of the recruits that remained in the tanks in the laboratory (substrates that were not transplanted to the field) remained stable after 12 days (Figure 7).

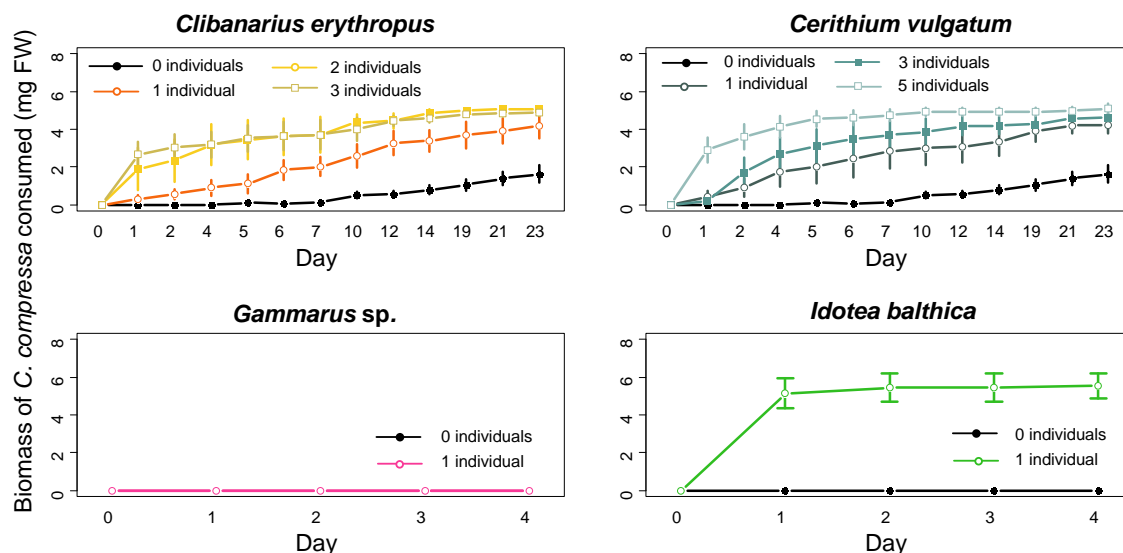


**Figure 7:** Average evolution of the density of recruits of *C. compressa* (individuals per 0.04 m<sup>2</sup>) on the two open coast sites and in the laboratory controls. The density on the laboratory control substrates was lower as the most recruited substrates were selected to be transplanted to the field. The errors bars show the standard error.

### 3.4. Mesocosm experiment: Potential herbivory of different species

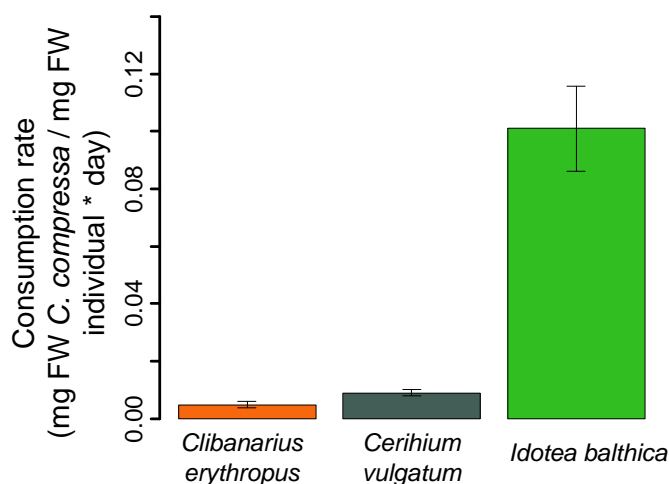
All the studied herbivorous species, except *Gammarus* sp., consumed recruits of *C. compressa*. For *Clibanarius erythropus*, *Cerithium vulgatum* and *Idotea balthica* the post-hoc analysis found significant differences in the consumption of *C. compressa* biomass when compared to the controls (factor number of individuals; GLMM, P-value < 0.001; Figure 8 and Table 1). No consumption and neither significant mortality of *C. compressa* was found in the tanks with *Gammarus* sp. (Figure 8).

For *C. erythropus*, the only differences were between the control and the presence of the herbivores, whatever the density was (without significant differences according to the number of individuals). While for *C. vulgatum* there were significant differences between the highest and the lowest number of individuals (5 individuals versus 1 individual; Figure 8 and Table 1). 24h after the start of the experiment, *I. balthica* individuals consumed 100% of the biomass of recruits available in four of the seven experimental tanks, and more than the 85% in two of the seven experimental tanks (Figure 8).



**Figure 8:** Cumulative consumption of *C. compressa* biomass (mg FW) by *Clibanarius erythropus*, *Cerithium vulgatum*, *Gammarus* sp. and *I. balthica*, and control substrates. Not that the biomass loss in the controls (0 individuals) it is not due to herbivory. The errors bars show the standard error.

Regarding the results of the LM used to test the consumption rate of *C. compressa* by *C. erythropus*, *C. vulgatum* and *I. balthica*, the last one was the herbivore consuming more *C. compressa*, with no difference between the consumption rate of *C. erythropus* and *C. vulgatum* (Figure 9 and Table 1).



**Figure 9:** Consumption rate (mg *C. compressa* mg herbivore<sup>-1</sup> day<sup>-1</sup>) for the three herbivores for the whole duration of each experiment. *Gammarus* sp. is not represented as we could not observe consumption of *C. compressa* by this species. The error bars show the standard error.

#### 4. DISCUSSION

Our study is a first step to elucidate the herbivory pressure in the field and direct effects of different species on the grazing of recruits in the framework of shallow *Cystoseira s.l.* restoration, as grazing have been observed and quantified on transplanted adult individuals and recruits (Susini et al., 2007;

Mangialajo et al., 2012; Tamburello et al., 2019; Savonitto et al., 2021). During the experiments *Cerithium vulgatum* and decapods (mainly *Clibanarius erythropus*) were the most abundant herbivorous observed on all our sampling sites in exception of Beaulieu, and higher densities were observed on patches of bare rock just next to the macroalgal communities (author's personal observation). In the rockpool system in Sainte Marguerite the density of *Cystoseira compressa* recruits changed in function of herbivory protection, showing how important it is to control herbivory on early-life stages for increasing restoration success. The first assessment of density and sizes of recruits on this location was performed 2 months after the substrates provision and recruitment enhancement because of the difficulty of identifying the early-life stages. Even if in this location the densities of recruits drastically decreased on month 7, on month 12 there were still significantly more recruits on the protected substrates than on the artefact control and open substrates. The variations on the density of recruits could be because of seasonal changes or also due to density-dependant effects: the density decreases as the size increases (Chapman, 1995). Nevertheless, on all the substrates from the three treatments (protected, artefact control, and open) the density of recruits of *C. compressa* after 12 months was still higher than the density observed in the surrounding natural population. On the experiment in the field we did not observe an effect of the factor recruitment enhancement, probably due to the presence of fertile *C. compressa* adults in the rockpool, which could have recruit on all our substrates. Also, the fact of not observing an effect of the recruitment enhancement factor could be because the recruitment enhancement performed on half of our substrates had a larger scale effect, affecting all the substrates and not only the substrate just underneath. In addition, there were no differences on the size of the recruits independently of the herbivory protection as the size of the recruits increased over time in all the substrates. At month 12, a slightly higher number of recruits was reported than at month 7, those new recruits were probably settled later during the season.

In the two open coast locations, Beaulieu and Passable, the dramatic decrease in density of the transplanted recruits after only 24 h could be due to several reasons. Unlike in Sainte Marguerite, where the conditions for the growth of *Cystoseira s.l.* spp. were ensured (presence of a healthy forest), the environmental conditions for the survival of recruits of *C. compressa* in the two open coast locations might have not been adequate (e.g. water quality, hydrodynamics, irradiation, absence of a canopy... Irving et al., 2009). Another reason for this *ex-situ* technique to fail in these locations could be the methodology used for obtaining recruits: the recruits that were adapted to the stable conditions in the tanks for 2.5 months might have been sensitive to different environmental parameters such as hydrodynamics, variations in temperature, higher irradiance, different water chemistry etc. This problem could be solved by transplanting the recruits sooner to the sea, as suggested in other studies (Falace et al., 2018; De La Fuente et al., 2019; Savonitto et al., 2021). Replicating natural conditions in experimental tanks is difficult, and during the 2.5 month that the

recruits were in the laboratory we could observe that they grew less sizes than the recruits in Sainte Marguerite, obtained from the *in-situ* recruitment enhancement technique (author's personal observation). Even if it is not considered a major stressor for the recruits (Falace et al., 2018; De La Fuente et al., 2019), the transport of the recruits from the laboratory facilities to the experimental locations on the coast could have affected the recruits. However, the fact of not observing mortality of recruits on the substrates that were kept in the laboratory (substrates that were not transplanted in Beaulieu and Passable and were kept as laboratory controls), could be due to the lower densities of recruits they had in comparison to the transplanted substrates in the field. As said before, substrates with higher densities of recruits could have experienced mortality due to density-dependant effects, known as self-thinning (Chapman, 1995).

The recruits for the two field experiments were obtained using different techniques in function of the characteristic of each location (Cebrian et al., 2021) which prevent from comparing the results obtained between Sainte Marguerite, and Beaulieu and Passable. In the rockpool system in Sainte Marguerite, the *in-situ* technique with substrate provision and natural and artificial seedling was used, as it is the one preferred for sheltered locations with low herbivory pressure (Cebrian et al., 2021). While in the open coast in Beaulieu and Passable, the *ex-situ* technique was used as it is the one recommended for deforested locations (Cebrian et al., 2021). However, herbivory pressure change in function of the physical environmental conditions and the structure of the benthic communities (Lubchenco, 1986). One study (Gianni et al., 2018) performed on the open infralittoral fringe of the French Riviera (N-W Mediterranean Sea), where sea urchins were not present, concluded that *S. salpa* was the most efficient grazer on adult *Cystoseira amentacea* and able to limit the success of restoration actions. However, Ferrario et al. (2016) observed how individuals from the same species (fishes and decapods) exerted a stronger herbivory pressure on artificial habitats than on natural ones, and the same could have happened during our experiments in Beaulieu and Passable that are located in urbanized areas. The recruits obtained in the rockpool within a *Cystoseira s.l.* forest (Sainte Marguerite) could have been less affected by herbivores because of the larger abundance and variety of other more palatable species of macroalgae present in the site that were not present in the open coast location (Beaulieu and Passable), possibly explaining the rapid decrease in recruits observed in the urban open coast locations.

The mesocosm experiment performed in this study is a first approach for elucidating which other species, apart from the most studied ones (mainly urchins and herbivorous fish), can have a role in the grazing of recruits of *Cystoseira s.l.* spp. Ferrario et al. (2016) already highlighted that most species consuming or interacting with *Cystoseira s.l.* spp. are usually classified as omnivorous rather than herbivorous. During our experiment, the species that had the highest and fastest consumption of recruits of *C. compressa* was *Idotea balthica*, followed by *Clibanarius erythropus* and *Cerithium vulgatum*. Even if in our experiment the amphipod (*Gammarus* sp.) did not graze on

recruits of *C. compressa*, it is reported that amphipods heavily feed on brown macroalgae (Duffy and Hay, 2000) including Fucales (Jonne et al., 2006), but they preferences could be species specific. Some studies have already shown how molluscs, and decapods can graze or interact (i.e. clipping and cutting the thalli) with recruits and adults of some forest-forming macroalgae including *C. compressa* (Lubchenco, 1983; Perkol-Finkel et al., 2012; Ferrario et al., 2016; Gianni et al., 2017).

*I. balthica* is the most widespread Idoteidae in European Seas (Guarino et al., 1993) that is frequently used as model species to study the interactions between macroalgae and herbivores (Gutow et al., 2014; Lavaut et al., 2022). This species can be found living in macroalgal communities and marine forests (Guarino et al., 1993; Lavaut et al., 2022) and it is an important consumer of forest-forming brown macroalgae such as the shallow *Fucus vesiculosus* in the Atlantic (Kotta et al., 2000; Jonne et al., 2006; Vesakoski et al., 2008; Molis et al., 2010; Schaal et al., 2016). *I. balthica* has an important grazing effect on structuring *F. vesiculosus* populations in the Baltic Sea, preferring younger tissue over older (Engkvist et al., 2000; Boström and Mattila, 2005). It could also play an important role in grazing recruits of shallow *Cystoseira s.l.* spp. in the Mediterranean Sea according to our results. Even if macroalgae species, including *Cystoseira s.l.* spp., contain metabolites such as phenolic compounds, that deter feeding in macroalgae and especially on young tissue, this chemical defence may not be enough to prevent grazing by some herbivorous species. For example, in another mesocosm experiment (Vergés et al., 2007) *C. vulgatum* was the only invertebrate unaffected by the chemical deterrence extracts from the phanerogam *Posidonia oceanica*, and it was one of the species with the highest densities in our study location. It is however important to note that the lack of other potentially more palatable species of macroalgae or the limited amount of biomass of *C. compressa* in each tank could have impacted the consumptions rates of *C. compressa* reported here during the mesocosm experiment.

We highlight that non-strict herbivorous species can potentially graze on recruits of *Cystoseira s.l.* spp. affecting the success of marine forests conservation and restoration actions. The protected substrates in the studied locations had significantly more densities of recruits of *C. compressa*, showing the negative effect of herbivory on early-life stages obtained from different recruitment techniques. However, we cannot exclude that others characteristics of the locations had an effect on the density of recruits. Despite adapting the restoration techniques to the particularities of each locations, the degree of success of the restoration is not assured. We conclude that, in addition to making sure that abiotic conditions (e.g. water quality, seawater temperature, anthropogenic pressures) are favourable for the restoration of marine forest, biotic factors such as herbivory should be well understood as a great variety of species could impact the recruits (e.g. in function of the size of the recruits). In locations with high herbivory pressure, anti-herbivory devices should be included when restoring marine forests (Cebrian et al., 2021) and these devices should be adequate

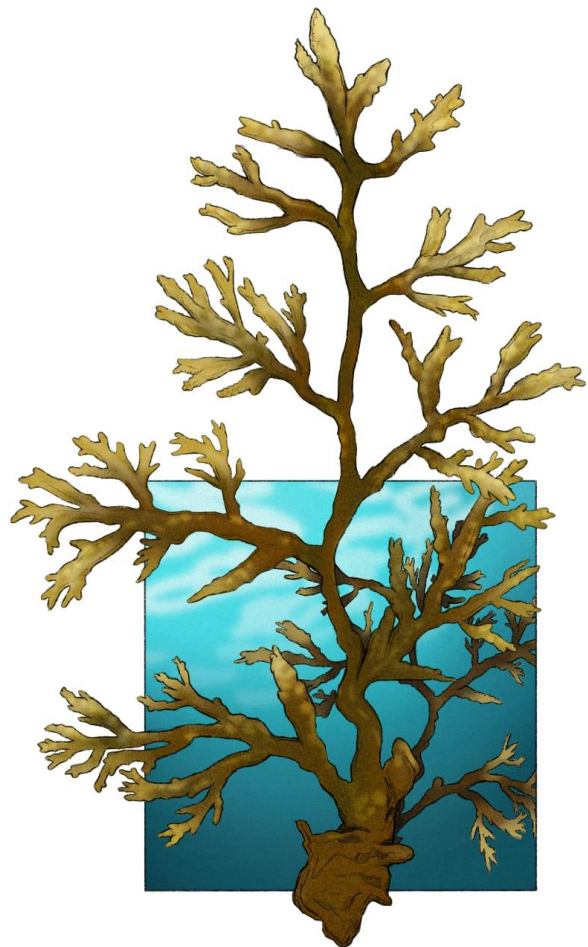
to the diversity of potential herbivorous species present in the site. More studies are needed to predict how the feeding behaviour and preferences of different native and invasive species could change under climate change to evaluate the threat they pose for marine forests conservation in the future (Tomas et al., 2011; Asnaghi et al., 2013; Gutow et al., 2014; Vergés et al., 2014b; Mitterwallner et al., 2021).

### **ACKNOWLEDGEMENTS**

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# CHAPTER 3: THE ROLE OF HABITAT IN THE FACILITATION OF *Ostreopsis* spp. BLOOMS

Monserrat, M., Catania, D., Asnaghi, V., Chiantore, M., Lemée, R., and Mangialajo, L. (2022). The role of habitat in the facilitation of *Ostreopsis* spp. blooms. *Harmful Algae* 113, 102199. doi: [10.1016/j.hal.2022.102199](https://doi.org/10.1016/j.hal.2022.102199).







## ABSTRACT

In recent decades, recurrent *Ostreopsis* spp. blooms have been recorded throughout the globe, causing public health issues and mass mortalities of invertebrates. *Ostreopsis* spp. are benthic and develop in shallow waters in close relation with a substrate, but possible substrate preferences are still ambiguous. Bloom develops on both living and dead substrates and several interacting biotic and abiotic factors acting at different spatial scales can potentially foster or regulate *Ostreopsis* spp. development. The objective of this review is to collect and summarize information on *Ostreopsis* spp. blooms related to the habitat at different spatial scales, in order to assess preferences and trends. References including *Ostreopsis* spp. samplings in the field were analysed in this review, as potentially including information about the micro- (substrate), meso- (community) and macrohabitat (ecosystem) related to *Ostreopsis* spp. blooms. The sampled substrate and the ecosystem where *Ostreopsis* spp. were collected were generally reported and described in the studies, while the description of the mesohabitat was rarely reported. *Ostreopsis* spp. were generally described as attached to biotic substrates and in particular, macroalgae, even in studies conducted in coral reefs, where macroalgae are generally not dominant (but they can be in case of coral reef degradation). In both temperate and tropical areas, *Ostreopsis* spp. were mostly sampled on algal species usually forming medium or low complexity communities (erect or turf-forming algae), often characteristic of post-regime shift scenarios, and rarely on canopy-forming species (such as fucoids and kelps). This literature review highlights the need of collecting more information about the mesohabitat where important *Ostreopsis* spp. blooms develop, as much as of the underlying mechanisms driving eventual differences on *Ostreopsis* spp. abundances. This knowledge would allow a better risk assessment of *Ostreopsis* spp. blooms, identifying areas at high risk on the base of the benthic habitats.

**Keywords:** benthic HABs, *Ostreopsis*, substrate, community, habitat, ecosystem, algae

## 1. INTRODUCTION

Over the past decades, coastal areas throughout the world experienced an accelerating trend of harmful algal blooms (HABs) events (Anderson et al., 2019) including the ones due to benthic species (Parsons et al., 2012). A significant proportion of studies concerning benthic HABs is focused on tropical benthic dinoflagellate ecology, mostly because of the incidence of ciguatera fish poisoning (CFP) in tropical and subtropical areas (Litaker et al., 2009) and the geographic expansion of some toxic species in temperate waters (Rhodes, 2011). Among them, recurrent *Ostreopsis* spp. blooms have been recorded throughout the globe: in the Mediterranean Sea (Vila et al., 2001; Penna et al., 2005; Turki, 2005; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008a; Totti et al., 2010; Cohu et al., 2011a; Ismael and Halim, 2012; Manca et al., 2015; Açaçaf et al., 2020; Gémin et al., 2020), the East Atlantic Ocean (Solino et al., 2020), the West Atlantic Ocean

(Nascimento et al., 2012b; Tibirica et al., 2019), the South-West Pacific (Chang et al., 2000; Rhodes et al., 2000; Shears and Ross, 2009), the North-West Pacific (Yamaguchi et al., 2012a; Zou et al., 2020), the South-West Indian Ocean (Lenoir et al., 2004) and the Red Sea (Gomaa et al., 2018). At least five out of the eleven identified species (Fukuyo, 1981; Norris et al., 1985; Quod, 1994; Faust and Morton, 1995; Faust, 1999) produce palytoxin-like compounds, one of the most toxic marine compounds (Usami et al., 1995; Ukena et al., 2001; Ciminiello et al., 2010; Suzuki et al., 2012; Varela et al., 2021), causing respiratory disease after inhalation of marine aerosols (Tichadou et al., 2010; Tubaro et al., 2011; Vila et al., 2016). The whole ecosystem is affected by *Ostreopsis* spp. blooms (Turner et al., 2021), due to mass mortalities of invertebrates such as bivalves, gastropods, crustaceans, and echinoderms (Shears and Ross, 2009; Ramos and Vasconcelos, 2010; Guidi-Guilvard et al., 2012; Parsons et al., 2012; Accoroni and Totti, 2016; Migliaccio et al., 2016; Neves et al., 2018). In order to effectively manage and mitigate *Ostreopsis* spp. blooms, a thorough understanding of the bloom dynamics of these species is needed. Samplings have been worldwide restricted mostly to shallow waters as it has been demonstrated in both tropical and temperate areas that *Ostreopsis* spp. cell abundances are negatively correlated with depth (Richlen and Lobel, 2011; Cohu and Lemee, 2012). The mechanisms that affect *Ostreopsis* spp. populations are unclear, but most studies relate larger abundances of *Ostreopsis* spp. to sheltered zones or in low hydrodynamic conditions (Di Turi et al., 2003; Chiantore et al., 2008; Shears and Ross, 2009; Battocchi et al., 2010; Cabrini et al., 2010; Richlen and Lobel, 2011; Accoroni and Totti, 2016; Mohammad-Noor et al., 2016; Boisnoir et al., 2018; Hachani et al., 2018; Meroni et al., 2018), while others suggest larger abundances of *Ostreopsis* spp. in higher or slightly higher levels of water motion (Vila et al., 2001; Selina et al., 2014). The inshore occurrence of *Ostreopsis* spp. has also raised the question concerning their association with nutrient enrichments due to human activities (Faust et al., 1996), but there are mixed conclusions on how nutrient concentrations influence population dynamics of *Ostreopsis* spp. (Ungaro et al., 2010; Cohu et al., 2011a; Accoroni et al., 2012; Asnaghi et al., 2012). A concise summary of the effects of surface seawater temperature, salinity and nutrient concentrations on *Ostreopsis* spp. populations in temperate areas can be found in Accoroni and Totti's (2016) and Tester's et al. (2020) reviews. Results from the above-mentioned studies are sometimes contradictory, likely due to the fact that *Ostreopsis* spp. blooms are a global phenomenon controlled by several factors in very different contexts and, as a consequence, blooms are very variable in space and time, making difficult defining general trends (Mangialajo et al., 2011).

Due to their benthic nature, *Ostreopsis* spp. develop in close relation with a substrate. *Ostreopsis* spp. are often described as epiphytic on macroalgae and seagrasses (Rhodes, 2011), but can also be found on dead corals, sediments, rocks, and in the water column (Bomber et al., 1989; Vila et al., 2001; Shears and Ross, 2010, 2010; Totti et al., 2010). However, the substrate preferences of

*Ostreopsis* spp. are still ambiguous (Vila et al., 2001; Cohu et al., 2013; Sparrow et al., 2017; Ternon et al., 2020; Tester et al., 2020), although some studies reported larger abundances on some substrates compared to others (Vila et al., 2001, 2012; Mohammad-Noor et al., 2007; Widiarti, 2008; Cabrini et al., 2010; Totti et al., 2010; Accoroni et al., 2011, 2012; Mangialajo et al., 2011; Accoroni and Totti, 2016; Yong et al., 2018; Boisnoir et al., 2019; Bravo et al., 2020; Lee et al., 2020). Such comparisons are difficult because of the limits of cell quantification in function of the type of substrate (i.e. surface for rocks, fresh weight for macrophytes), but also because the measures depend often on some characteristics of the substrate (i.e. the specific weight of sampled species). As an example, some macroalgae species seem to host larger abundance of *Ostreopsis* spp. than most other species, e.g. *Corallina* spp. (Simoni et al., 2004; Aligizaki and Nikolaidis, 2006; Monti et al., 2007; Chiantore et al., 2008) and *Padina* spp. (Aligizaki and Nikolaidis, 2006; Cabrini et al., 2010; Hachani et al., 2018; Gémin et al., 2020). Nonetheless, other species provide discordant patterns such as: *Ulva* spp. (Aligizaki and Nikolaidis, 2006; Monti et al., 2007; Okolodkov et al., 2007; Totti et al., 2010; Ismael and Halim, 2012), *Dictyota* spp. (Cohu et al., 2013; Blanfuné et al., 2015; González et al., 2019; Gémin et al., 2020; Ternon et al., 2020), and *Cystoseira sensu lato* spp. (Aligizaki and Nikolaidis, 2006, 2008; Monti et al., 2007; Blanfuné et al., 2015; Catania, 2017; Moncer et al., 2017; Meroni et al., 2018). *Ostreopsis* abundances on different substrates are highly variable and, despite the technical issues linked to the measurement cited above, several biotic factors acting at different spatial scales can be responsible for such variability: morphology and palatability of macrophytes, herbivory and allelopathic interactions due to the production of secondary metabolites by living substrates, among others (Cruz-Rivera and Villareal, 2006; Totti et al., 2010; Accoroni et al., 2015, 2016; Ternon et al., 2020).

Benthic ecosystems are dynamic systems and, especially due to human impacts, can experience regime shifts, resulting in profound changes in the structure and composition of communities (Hughes, 1994; Benedetti-Cecchi et al., 2001; Chemello et al., 2018; Filbee-Dexter and Wernberg, 2018; Johns et al., 2018; Melis et al., 2019; Pessarrodona et al., 2021). In both temperate and tropical reefs, regime shifts cause a change from healthy communities dominated by foundation species to communities dominated by less structurally complex species (Filbee-Dexter and Scheibling, 2014; Jouffray et al., 2015; Wernberg et al., 2016; O'Brien and Scheibling, 2018; Pessarrodona et al., 2021). In temperate areas forest-forming macroalgae are lost and replaced by less complex communities (Strain et al., 2014; Vergés et al., 2014a; Bulleri et al., 2016); several studies relate major densities of *Ostreopsis* spp. on algal turfs (Bravo et al., 2020) and in particular on highly urbanised coasts that are usually characterized by post-regime shift algal communities (Mangialajo et al., 2008a; Widiarti, 2008; Cohu et al., 2013), compared to healthier habitats dominated by forest-forming brown algae (Meroni et al., 2018). In tropical areas, ciguatera fish poisoning episodes recurrently follow disturbances to coral reefs by natural and artificial events

such as heat waves, hurricanes, dredging, and shipwrecks, among others, where regime shifts from coral to algae are observed (de Sylva, 1994; Jouffray et al., 2015; Rains and Parsons, 2015; Johns et al., 2018). In this case, higher benthic dinoflagellate abundances are recorded, both on turf-forming (Yong et al., 2018; Lee et al., 2020) and forest-forming species, such as *Sargassum* spp. (Chinain et al., 2020).

To our best knowledge, only few studies assessed the variability of *Ostreopsis* spp. at a level higher than the substrate (Cohu et al., 2013; Meroni et al., 2018; Yong et al., 2018; Bravo et al., 2020; Lee et al., 2020) with the aim of comparing different habitats to help understanding *Ostreopsis* spp. variability. To ascertain how the habitat positively or negatively affects *Ostreopsis* spp. blooms, a literature review was conducted in order to describe trends at different spatial scales. In this review we define: (i) the microhabitat (substrate, spatial scale of cm<sup>2</sup>), as the biotic or abiotic object sampled to quantify/collect *Ostreopsis* spp. cells (including living or dead organisms and abiotic substrates), (ii) the mesohabitat, as the community or the abiotic material patch where the substrate is sampled (spatial scale of few to a dozen of m<sup>2</sup>, e.g. the macroalgal turf where a certain macroalgal species is sampled as a substrate or the sandy bottom where a pebble is sampled as a substrate; Meroni et al., 2018; Bravo et al., 2020; Lee et al., 2020), and (iii) the macrohabitat, as the ecosystem or the seascape (spatial scales of hundreds/thousands of m<sup>2</sup>) where the mesohabitat is found (i.e. coral reefs, macroalgal-dominated rocky shores; Yong et al., 2018).

## 2. MATERIALS AND METHODS

A bibliographic review assessment was performed using three databases: Aquatic Science and Fisheries Abstracts (ASFA), Web of Science (WoS) and SCOPUS (last update at 1<sup>st</sup> February 2021). Search parameters were kept as wide as possible to ensure that all the relevant publications on the topic were found in the database search. The keyword used for the search was “*Ostreopsis*” and the parameters of the search were to find the word “*Ostreopsis*” ‘everywhere’ in the body or text of the publications, between anytime and February 1<sup>st</sup>, 2021.

To quantify and compare the relationship between *Ostreopsis* spp. and the micro- meso and macrohabitats, only articles involving field collection of *Ostreopsis* spp. were included in this review. The key information extracted from each article included, when available: microhabitat (e.g. sampled substrates such as macrophytes, rocks, shells, artificial substrates, others), mesohabitat (community/abiotic patch type), macrohabitat (ecosystem/seascape), ocean and sea of study, country, temperate or tropical area (tropical areas were defined as areas between -35° and 35°, including both tropical and subtropical climate), geographic coordinates (if not available, they were obtained from Google Earth, whenever possible) of the sampling location where *Ostreopsis* spp. were sampled, maximum recorded abundance in the study and sampling dates. The maximum abundances of *Ostreopsis* spp. recorded in the studies were classified in three categories: No bloom

(< 200 × 10<sup>3</sup> cells per gram fresh weight (FW) of macrophyte), Bloom (> 200 × 10<sup>3</sup> cells per gram FW of macrophyte) and Major bloom (> 1000 × 10<sup>3</sup> cells per gram FW of macrophyte) as in Mangialajo et al. (2017). Several studies (132) were conducted in two or more separate regions around the globe, seas, countries, locations and/or involving different substrates. In this case, a different line was inserted in the table for each different score, therefore it is possible that one article was accounted more than once. All the graphs have been produced using R Project version 3.6.2. (R: The R Project for Statistical Computing).

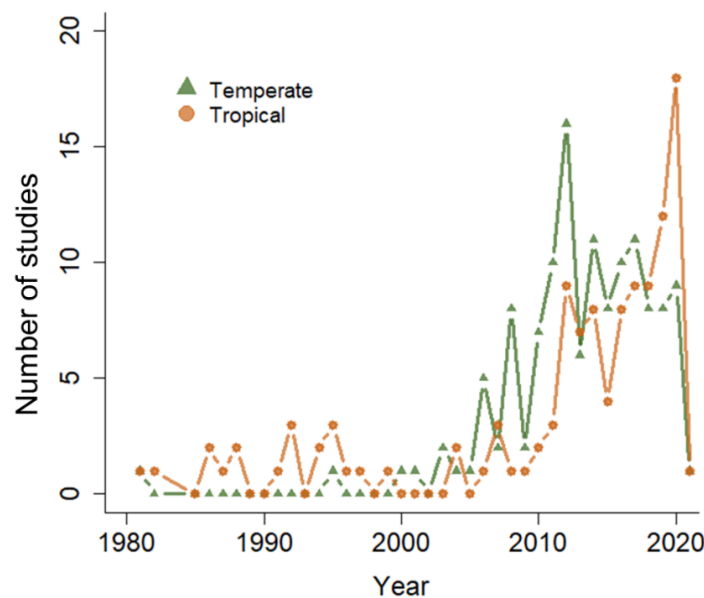
Macroalgal species are characterised by different sizes and shapes, and the communities dominated by different species can be more or less structurally complex, comparable to continental forests, shrubs and turfs, terms often used in the scientific literature (see below). Because abundances of *Ostreopsis* spp. on macroalgae are subjected to differences in the structure, surface, and fresh weight, the different algal species sampled as a substrate in the reviewed studies were grouped in the following three categories according to their structural morphology:

- Turf-forming algae: algae with tightly packed fronds and filamentous thin cylindrical axes, with a horizontal coverage several times higher than their height forming a dense and compact mat (Stewart, 1983; Tittley and Neto, 1995; Sales and Ballesteros, 2010; Sala et al., 2012; Connell et al., 2014; Thiriet et al., 2016; Mauffrey et al., 2020).
- Shrub-forming algae: foliose laminar, ribbon-like, massive or fan-like thallus and erect arborescent tufts. Algae usually having a smaller size and forming less complex communities than forest-forming species. At the same time, in their region, they generally have a bigger size and form communities characterised by higher complexity than turf forming algae (Sala et al., 2012; Thiriet et al., 2016; Bertolini, 2019).
- Forest-forming algae: cylindrical axes, branched and tree-like characterised by a complex tri-dimensional structure, generating a canopy. Algae having a large size compared to other algae growing in the same region. This term is generally used for Laminariales, Fucales and some Tilopteridales (Sala et al., 2012; Strain et al., 2014; Thiriet et al., 2014, 2016; Bertolini, 2019; Shelamoff et al., 2019; Assis et al., 2020).

### 3. RESULTS

The bibliometric search resulted in 1157 publications including the word “*Ostreopsis*” in the text from 1973 to 2021. Only 249 studies, published from 1981 to 2021, were based on field sampling of *Ostreopsis* spp. cells and were therefore considered as relevant for the review (Appendix C: Chapter 3 Supplementary material). The 249 considered studies comprise 957 sampling locations, involving a total of 1644 different substrates sampled between 1972 and 2019. 90% of the papers from this review (224) focused on *Ostreopsis cf. ovata*.

The number of studies per year reveals a positive trend of ecological studies involving sample collection in the field (Figure 1) in both temperate and tropical areas. The first studies involving field sampling were performed in the tropics, but, since year 2000, studies performed in temperate areas have exceeded in number the tropical ones. Since 2018 tropical studies are again more numerous than in temperate areas, but this trend has to be verified in the next years. Nearly half of the 957 different locations investigated between 1972 and 2019 are in temperate areas (471), the other half (486) in tropical ones. The exact geographical coordinates were available for 595 (62.2%) sampling locations and it was possible to position 132 (13.8%) additional ones on the basis of the description of the location in the text. A distribution map of the 727 locations, out of the 957 sampled in the 249 relevant studies, is reported in Figure 2.



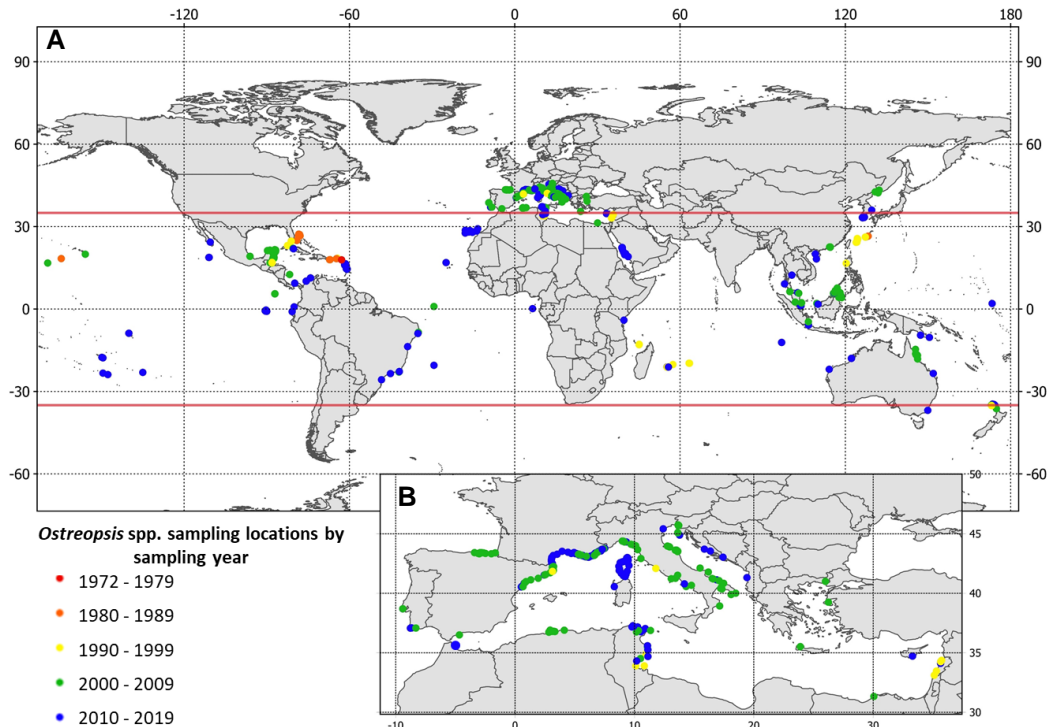
**Figure 1:** Trend of the number of peer-reviewed studies involving field sampling of *Ostreopsis* spp. from 1981 to 2021.

### 3.1 Spatial scales

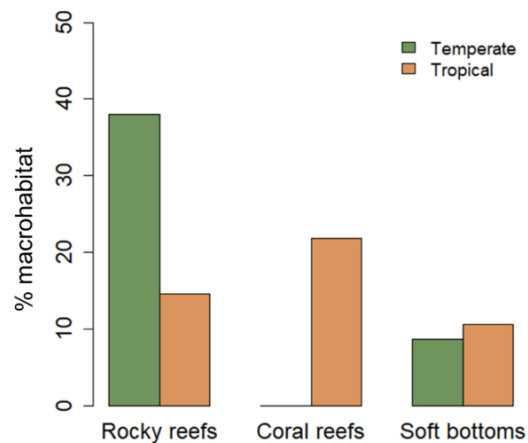
#### 3.1.1 Macrohabitat

The macrohabitat was detailed in a total of 197 out of the 249 papers (79.1%). In 12 of them (6.1% studies) at least two different ecosystems/seascapes were sampled. From the 957 sampled locations, 897 (93.7%) included or allowed extrapolation of the information at the large scale (ecosystem/seascape; Figure 3). In temperate areas, *Ostreopsis* spp. is mostly sampled in rocky reefs (usually dominated by macroalgal communities), while in tropical areas *Ostreopsis* spp. is generally sampled in coral reefs. Soft bottoms are also regularly sampled, especially in tropical areas.





**Figure 2:** Map of the sampling locations. The colour corresponds to the year it was first sampled. The two red lines ( $35^{\circ}\text{N}$  and  $35^{\circ}\text{S}$ ) separate the tropical/subtropical from the temperate areas; (A) global distribution; (B) detailed map for the Mediterranean Sea.



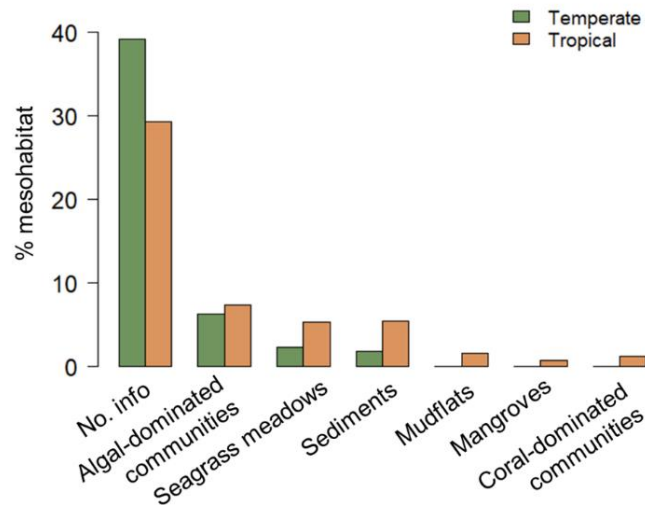
**Figure 3:** Macrohabitats where *Ostreopsis* spp. have been sampled in temperate and tropical areas.

### 3.1.2 Mesohabitat

The mesohabitat was detailed in a total of 87 out of the 249 papers (34.9%). In 30 of them (12% studies) at least two different mesohabitats were sampled. From the 957 sampled locations, 301 (31.4%) included or allowed extrapolation of the information at the intermediate scale (Figure 4). In temperate and in tropical areas, *Ostreopsis* spp. is mostly sampled in algal communities (6.2%



and 7.3% respectively). Seagrass meadows are also regularly sampled, especially in tropical areas (5.2%).



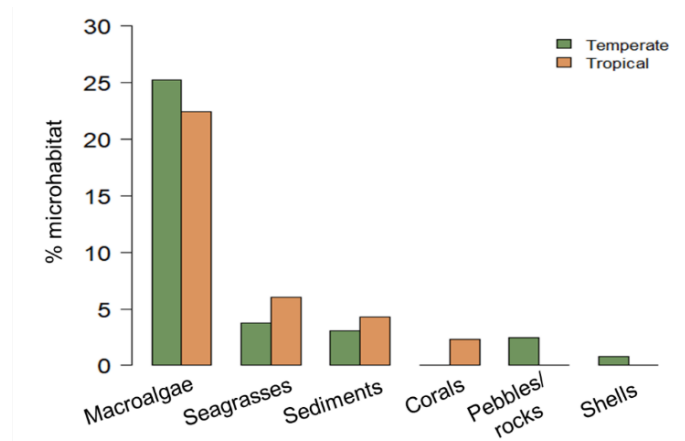
**Figure 4:** Mesohabitats where *Ostreopsis* spp. have been sampled in tropical and temperate areas.

### 3.1.3 Microhabitat

The microhabitat was detailed in a total of 243 out of the 249 (97.6%) studies considered. In almost half of the studies (113 studies, 45.4%) at least two different substrates were sampled. From the 957 sampled locations, 950 (99.3%) included information about the sampled substrate. On 527 (55.1%) sampled locations out of the 957, two or more substrates were sampled, resulting in a total of 1644 different sampled substrates. The frequency of the total sampled substrates is reported in Figure 5.

Three additional substrates were sampled and are not accounted for in the present study: a Bryozoan (Di Pippo and Congestri, 2017), the gut contents of the herbivorous fish *Sarpa salpa* (Bellassoued et al., 2013) and floating plastic debris (Masó et al., 2003; Casabianca et al., 2019; Tibirica et al., 2019, 2019).

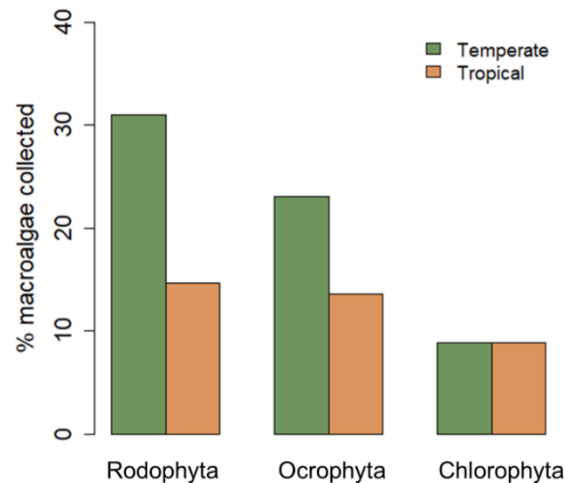
Artificial substrates, that allow an easier standardisation of *Ostreopsis* spp. abundances (Jauzein et al., 2018; Yong et al., 2018; Fernandez-Zabala et al., 2019; Lee et al., 2020); were used in 24 out of the 249 studies (3.6% in temperate areas and 6% in tropical), and in 136 out of the 1644 sampled substrates (2.3% in temperate areas and a 6% in tropical). Seawater is also commonly sampled and, in some countries, thresholds alerts are based on cell concentrations in seawater at dozen of centimetres above the sea bottom (Funari et al., 2015). Water was sampled in 100 out of the 249 studies (32.5% in temperate and 7.6% in tropical areas) and represent 332 out of the 1644 sampled substrates (15.7% in temperate and 4.5% in tropical areas).



**Figure 5:** Microhabitats (substrates) where *Ostreopsis* spp. have been sampled in temperate and tropical areas.

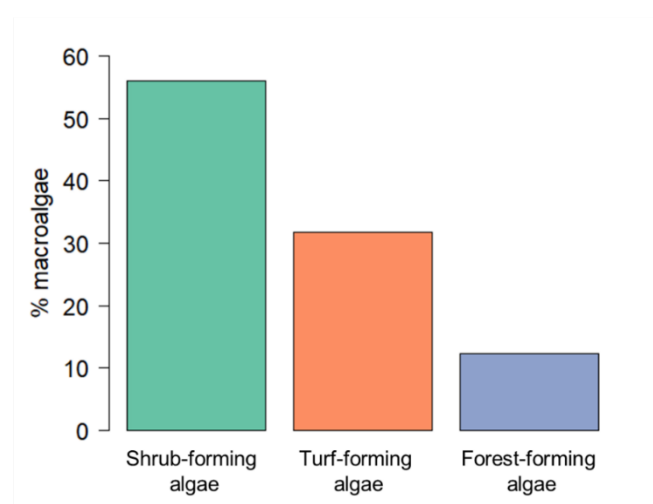
Without considering seawater, macroalgae are by far the preferred substrate for sampling *Ostreopsis* spp. Out of the 249 studies, 179 (71.9 %) included at least a macroalgal sample. A total of 792 macroalgae specimens were sampled in tropical and temperate areas. Macroalgae are commonly sampled both in temperate (where they dominate rocky reefs habitats) and in tropical areas (where, on the contrary, habitats are usually dominated by corals). The second preferred substrate is represented by seagrasses, followed by sand and sediments, coral fragments, pebbles/rocks and dead mollusc shells. Most studies (56.8%) sampling macrophytes identified the sample at the species level, while 20.1% out of them identified the macrophyte at genus level. Some studies (2.9%) identified the species sampled only at a level higher than genus or using non-taxonomic classification (i.e. turf). The remaining fraction (20.2%) did not provide any taxonomic information. It is worth noting that quantification of *Ostreopsis* spp. cell abundances on invertebrates (corals or molluscs) is generally performed on dead coral fragments or shells (Faust et al., 1996; Faust, 1999; Mohammad-Noor et al., 2007; Okolodkov et al., 2007; Xu et al., 2014).

Macroalgae samples, that involved the collection of 792 different specimens, represent nearly half of the sampled substrates (47.7%) and are a polyphyletic and extremely diversified group of organisms. The different macroalgal phyla, Rhodophyta, Ochrophyta and Chlorophyta (Ruggiero et al., 2015) were sampled in different proportions, in particular in temperate areas where Rhodophyta (49.2%) seem to be sampled preferentially, followed by Ochrophyta (36.7%) and Chlorophyta (14%). In tropical areas, the sampling frequency of each phylum is similar (39.4%, 36.7% and 23.8%, respectively; Figure 6).



**Figure 6:** Macroalgae sampled in function of the phylum.

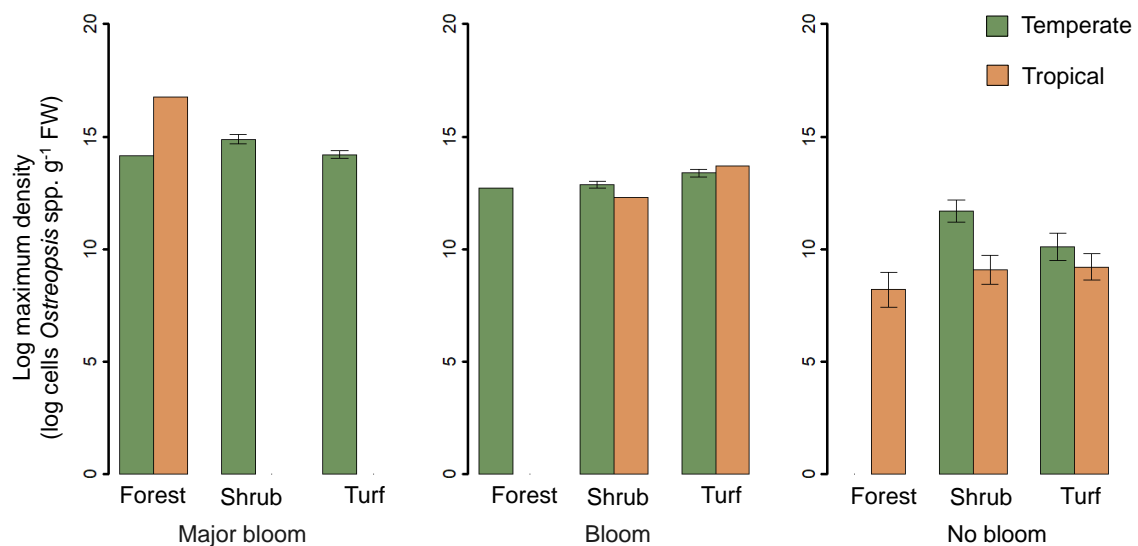
One of the major limitations and controversies for studying macrophyte preferences is the difficulty of standardising cell densities. In order to compare and assess the role of each macroalgae species in structuring the community, we classified the macroalgae sampled as a substrate in three different groups according to their physical structure. The algae sampled in the considered studies were classified, based to our knowledge about their mean size and their appearance in “forest-forming”, “shrub-forming” and “turf-forming”, as defined in the “Materials and methods” section. Following this classification, most of the macroalgae sampled in both temperate and tropical areas correspond to the category shrub-forming (55.9%), followed by the turf-forming (31.7%) and the forest-forming (12.3%; Figure 7).



**Figure 7:** Macroalgae sampled in function of the type of community they can form.

Of the 249 studies, 82 (32.9%) reported abundances of *Ostreopsis* spp. cells per gram FW of macrophyte. Such quantification is dependent on the specific weight and morphology of the different species (Mangialajo et al., 2017) and would not, ideally, allow a direct comparison of

abundances on different macroalgal species. At present, the few alternative methods allowing a standardised quantification (Tester et al., 2014; Jauzein et al., 2016; Mangialajo et al., 2017) are only sporadically applied and large-scale comparisons are performed on the measures of cells per gram FW. In order to have a global vision of the *Ostreopsis* spp. abundances, the maximal abundance reported on each study indicating the host macrophyte has been reported, when available, in Table 1 (expressed as cells/g of macroalga FW). From the data reported on Table 1, the maximum cell densities of *Ostreopsis* spp. (cells g<sup>-1</sup> FW macroalgae) are grouped according to the type of mesohabitat the macroalgae sampled can form and the intensity of the bloom (N = 54; Figure 8; Mangialajo et al., 2017). In the case of Bloom and No bloom scenarios (according to Mangialajo et al. (2017)), the abundance of *Ostreopsis* spp. cells seems to be higher on shrub and turf-forming species. For the Major bloom scenario (see classification in Mangialajo et al. (2017)), the abundance of *Ostreopsis* spp. seems to be higher on shrub-forming species in temperate areas (followed by high values in forest-forming) and on forest-forming species in tropical areas.



**Figure 8:** Maximum cell densities of *Ostreopsis* spp. (cells g<sup>-1</sup> FW macroalgae) for each study grouped according to the morphological structure of the macroalgae.

**Table 1:** Maximum cell densities of *Ostreopsis* spp. (cells g<sup>-1</sup> FW macrophyte) for each study on different microhabitats.

Macrophyte	Maximum abundance (cells <i>Ostreopsis</i> spp. g <sup>-1</sup> FW)	Turf, shrub or forest-forming	Country	Tropical/temperate	Reference
<b>Rhodophyta</b>					
<i>Acanthophora spicifera</i>	1500	Turf	Belize	Tropical	Faust (2009)
<i>Acanthophora spicifera</i>	1500	Turf	Belize	Tropical	Morton and Faust (1997)
<i>Asparagopsis taxiformis</i>	230000	Shrub	Italy	Temperate	Carella et al. (2015)
<i>Asparagopsis taxiformis</i>	236276	Shrub	France	Temperate	Bire et al. (2015)
<i>Corallina</i> sp.	79000	Turf	Algeria	Temperate	Illoul et al. (2012)
<i>Hypnea musciformis</i>	1700000	Turf	Italy	Temperate	Totti et al. (2010)
<i>Ellisolandia elongata</i>	760000	Turf	Spain	Temperate	Casabianca et al. (2013)
<i>Ellisolandia elongata</i>	28000	Turf	Lebanon	Tropical	Accoroni et al. (2016)
<i>Ellisolandia elongata</i> or <i>Jania rubens</i>	1000000	Turf	Spain	Temperate	Vila et al. (2016)
<i>Ellisolandia elongata</i> or <i>Jania rubens</i>	1480000	Turf	Spain	Temperate	Carnicer et al. (2015)
<i>Ellisolandia elongata</i>	880694	Turf	Lebanon	Tropical	Açaf et al. (2020)
<i>Galaxaura elongata</i>	186	Shrub	Philippines	Tropical	Pocsidio and Dimaano (2004)
<i>Galaxaura</i> sp.	10000	Shrub	Mascarene Islands	Tropical	Quod (1994)
<i>Jania rubens</i>	11000	Turf	France	Temperate	Cohu and Lemée (2012)
<i>Laurencia complex</i>	1040000	Turf	Monaco	Temperate	Fricke et al. (2018)
<i>Laurencia</i> sp.	99000	Turf	Brazil	Tropical	Nascimento et al. (2012b)
<i>Pterocliadiella capillacea</i>	18194	Turf	Hawaii Island (USA)	Tropical	Parsons and Preskitt (2007)

<i>Pterocладиella capillacea</i>	545000	Turf	Italy	Temperate	Ciminiello et al. (2014)
<i>Spyridia filamentosa</i>	2640000	Turf	Croatia	Temperate	Gladan et al. (2019)
<b>Ochrophyta</b>					
<i>Carpophyllum maschalocarpum</i>	1095	Forest	New Zealand	Tropical	Chang et al. (2000)
<i>Carpophyllum plumosum</i>	1406000	Forest	New Zealand	Temperate	Shears and Ross (2009)
<i>Dictyopteris polypodioides</i>	1300000	Shrub	Italy	Temperate	Accoroni et al. (2012)
<i>Dictyopteris sp.</i>	33405	Shrub	Galapagos (Ecuador)	Tropical	Carnicer et al. (2020)
<i>Dictyota dichotoma</i>	330000	Shrub	Italy	Temperate	Cabrini et al. (2010)
<i>Dictyota sp</i>	79000	Shrub	Cuba	Tropical	Moreira et al. (2012)
<i>Dictyota sp.</i>	57000	Shrub	Virgin Islands	Tropical	Kohler and Kohler (1992)
<i>Dictyota sp.</i>	53231	Shrub	Mexico	Tropical	Irola-Sansores et al. (2018)
<i>Dictyota sp.</i>	44000	Shrub	Puerto Rico	Tropical	Ballantine et al. (1988)
<i>Dictyota sp.</i>	220079	Shrub	Cape Verde	Tropical	Fernandez-Zabala et al. (2019)
<i>Dictyota spp.</i>	8540000	Shrub	France	Temperate	Cohu et al. (2013)
<i>Dictyota spp.</i>	24939	Shrub	Guadeloupe (France)	Tropical	Boisnoir et al. (2019)
<i>Dictyota spp.</i>	830000	Shrub	France	Temperate	Gémin et al. (2020)
<i>Ericaria crinita</i>	334306	Forest	Croatia	Temperate	Pfannkuchen et al. (2012)
<i>Halopteris scoparia</i>	658448	Shrub	Italy	Temperate	Guidi-Guilvard et al., (2012)
<i>Halopteris scoparia</i>	311552	Shrub	France	Temperate	Blanfune et al. (2012)
<i>Halopteris scoparia</i>	330000	Shrub	France	Temperate	Bire et al. (2013)

## CHAPTER 3

<i>Halopteris scoparia</i>	2289000	Shrub	Italy	Temperate	Jauzein et al. (2018)
<i>Halopteris scoparia</i>	2890528	Shrub	Italy	Temperate	Meroni et al. (2018)
<i>Halopteris scoparia</i>	2289100	Shrub	Italy	Temperate	Giussani et al. (2017)
<i>Halopteris scoparia</i>	195152	Shrub	France	Temperate	Blanfune et al. (2015)
<i>Halopteris scoparia</i>	2900000	Shrub	Italy	Temperate	Vassalli et al. (2018)
<i>Halopteris scoparia</i>	3700000	Shrub	France	Temperate	Brissard et al. (2014)
<i>Halopteris scoparia</i>	596000	Shrub	Spain	Temperate	Vila et al., (2001)
<i>Halopteris sp.</i>	359900	Shrub	France	Temperate	Mangialajo et al. (2017)
<i>Padina pavonica</i>	1900	Shrub	Egypt	Tropical	Ismael and Halim (2012)
<i>Sargassum sp.</i>	2860	Forest	Indonesia	Tropical	Skinner et al. (2011)
<i>Sargassum sp.</i>	15000	Forest	Saint Martin Island, Lesser Antilles	Tropical	Boisnoir et al. (2020)
<i>Sargassum sp.</i>	19000000	Forest	French Polynesia	Tropical	Chinain et al. (2020)
<i>Turbinaria decurrens</i>	143	Shrub	Saudi Arabia	Tropical	Catania et al. (2017)

### Chlorophyta

<i>Cladophora sp.</i>	16000	Turf	Italy	Temperate	Battocchi, et al. (2010)
<i>Cladophora wrightiana</i>	102	Turf	Korea	Tropical	Shah et al. (2013)
<i>Derbesia sp.</i>	8660	Turf	Korea	Tropical	Kim et al. (2011)
<i>Halimeda sp.</i>	596	Shrub	Kiribati	Tropical	Xu et al. (2014)
<i>Ulva rigida</i>	74000	Shrub	Italy	Temperate	Perini et al. (2011)

### Seagrasses

<i>Cymodocea nodosa</i>	1940	Tunisia	Tropical	Ben Gharbia et al. (2019)
<i>Halophila stipulacea</i>	1669	Guadeloupe (France)	Tropical	Boisnoir et al. (2018b)
<i>Posidonia oceanica</i>	360000	Tunisia	Tropical	Turki (2005)
<i>Posidonia oceanica</i>	2000	Tunisia	Tropical	Moncer et al. (2017)
<i>Thalassia testudinum</i>	3318	Colombia	Tropical	Arbelaez et al. (2017)

#### 4. DISCUSSION

The first indexed papers involving *Ostreopsis* spp. sampling have been published in tropical areas. The bibliometric study shows a rise in scientific publications presumably linked to the increasing HABs incidences in recent decades (Anderson et al., 2019), and in particular since *Ostreopsis* spp. blooms have spread in temperate areas (Shears and Ross, 2009). The search performed in this study did not take into account non-indexed papers or grey literature likely providing a low estimation of old papers, in particular in developing countries. Since the 90s *Ostreopsis* spp. are reported in temperate areas; a rapid increase in publications in temperate areas after 2006 is likely due to the first recognised mass intoxication in Genoa (Italy) in 2005 (Brescianini et al., 2006) that encouraged several European research groups to focus on this emergent phenomenon. An increase in publications in tropical areas is registered after 2010, often in synergy with research performed in temperate areas. It is worth noting that the peak of *Ostreopsis* spp. publications in temperate areas registered in 2012 is due to the publication of 18 articles in a special issue (*Cryptogamie Algologie*) resulting from the proceedings of the International Congress on *Ostreopsis* Development (ICOD conference, 2011; Lemee et al., 2012).

Benthic dinoflagellate development is affected by interacting biotic and abiotic factors linked to the habitat and acting at different scales (i.e. substrate, benthic community and seascape). Interestingly the present study highlighted that the sampled substrate (herein the microhabitat, e.g. coral fragment, macroalgal species) and the ecosystem (herein the macrohabitat, e.g. coral reefs, macroalgal-dominated rocky shores, sandy or muddy bottoms) where *Ostreopsis* spp. are sampled are generally reported and described. In 98% and 79% of the studies the sampled substrate and the seascape are respectively described or, alternatively, the text allows undoubtful extrapolation of this information. Concerning the microhabitat, and in particular organic substrates, most studies detail the species (56.8%), or at least the genus (20.1%) of the organisms sampled. Yet, even if the coral fragment or shell is identified at the species level, it is not clearly reported if 1) it is a living organism and 2) it is the direct *Ostreopsis* spp. host. Some studies (Widiarti, 2008; Yong et al.,



2018) suggested that small filamentous macroalgae developing on dead or damaged coral surfaces could foster *Ostreopsis* spp. development, showing the role of macroalgae as a favourable substrate. The same considerations could be valid for shells, pebbles, and stones, but there is no specific literature on these particular cases to our knowledge. On the contrary, the description of the mesohabitat (e.g. macroalgal turfs, macroalgal forests, coral-dominated communities, seagrasses, or sandy patches) is rarely reported (35% of the considered studies). As highlighted in Pavaux et al. (2020) it is undeniable that only a small part of the research on *Ostreopsis* spp. is focused on ecological aspects. Interestingly, recent studies, mainly focused on tropical species related to ciguatera, suggest that habitat heterogeneity and complexity affect benthic dinoflagellate communities (Rains and Parsons, 2015; Meroni et al., 2018; Yong et al., 2018; Boisnoir et al., 2019; Bravo et al., 2020; Lee et al., 2020). In particular Yong et al. (2018) found higher *Gambierdiscus*, *Prorocentrum* and *Amphidinium* cell abundances on reefs with high turf algal cover and coral rubble, while *Ostreopsis* preferred mesohabitats with high coral cover. Lee et al. (2020) found that the mesohabitat influence the distribution of benthic harmful algal blooms and that *Ostreopsis* and *Gambierdiscus* are both associated with turf algal communities and hard coral. Bravo et al. (2020) observed an influence of the macrophyte's thallus architecture on dinoflagellates abundances and also reported higher abundances of *Ostreopsis* on turf Rhodophyta, while *Gambierdiscus* and *Sinophysis* seem to be more abundant on Rhodophyta and Ochrophyta. More information at the global scale on how habitat affects benthic dinoflagellate blooms would allow to better disentangle the different factors playing a role in *Ostreopsis* spp. blooms.

Results from this literature review highlight that most data available on benthic substrates are from studies focusing on *Ostreopsis* spp. attached to biotic substrates and in particular, macroalgae. Interestingly, independently of the macrohabitat sampled, most of the studies in both temperate and tropical areas chose macroalgae (mainly visually obvious macroalgae species) as substrate. Even in studies conducted in coral reefs (where macroalgae are usually not dominant), macroalgae, rather than living corals or dead coral fragments, are selected as sampled substrate, meaning that i) macroalgae could be very abundant in this particular site and/or ii) scientists believe there are larger abundances of *Ostreopsis* spp. on macroalgae rather than on other substrates. However, choosing macroalgae over corals as substrate to be sampled could be for ethical reasons (coral species may be protected under international or national environmental law) or other practicalities of the sampling. Concerning the different species, Park et al. (2020) report higher *Ostreopsis* spp. abundances on red (*Spyridia filamentosa* and *Laurencia* complex) and brown macroalgae (i.e. *Dictyota* spp., *Halopteris scoparia*, *Dictyopteris polypodioides* and *Carpophyllum plumosum*) that are also the most sampled macroalgal groups while lower densities are reported in Chlorophyta, and in particular *Ulva* spp. Conversely, some of these results contrast with the results from studies on the effect of metabolites from macroalgae on the growth and settlement of *Ostreopsis cf. ovata*

(Accoroni et al., 2015; Ternon et al., 2020) that suggest a strong negative effect of some Dictyotales on *O. cf. ovata*. However, Pavaux et al. (2020) and Ternon et al. (2020) suggest that macroalgae surface chemistry may be not enough to explain *Ostreopsis* spp. settlement preferences and that other factors such as other organisms of the epiphytic community (bacteria, viruses, parasites, microalgae, and fauna) and the whole surrounding community are likely to modulate the growth of *Ostreopsis* spp.

With respect to the mesohabitat, the information in the literature is scarce. The most sampled macroalgae are species forming medium to low complexity communities, such as shrubs formed by *Padina* spp., *Dictyota* spp., *Halopteris* spp. (Cabrini et al., 2010; Widiarti and Anggraini, 2012; Cohu et al., 2013; Blanfuné et al., 2015; Boisnoir et al., 2019) and turfs formed by *Corallina* spp., *Jania* spp., *Laurencia* spp., *Hypnea* spp. (Simoni et al., 2004; Monti et al., 2007; Totti et al., 2010; Kim et al., 2011; Abbate et al., 2012; Ismael and Halim, 2012; Blanfuné et al., 2015; Yong et al., 2018). The sampling is often performed on the most abundant or predominant macroalgal species (Mangialajo et al., 2008a, 2011; Accoroni et al., 2011; Lemee et al., 2012; Rhodes et al., 2017; Jauzein et al., 2018; Boisnoir et al., 2019; Chinain et al., 2020; Gémin et al., 2020) and it can therefore be deduced that the representative mesohabitats in the sites where scientists study *Ostreopsis* spp. blooms are potentially characterized by algal shrubs and turfs. This is in agreement with the results of the few ecological studies considering the mesohabitat scale, where larger *Ostreopsis* spp. densities are found on low-complexity macroalgal communities (shrubs or turfs) characteristic of post-regime shift scenarios in highly impacted locations (Mangialajo et al., 2008a; Meroni et al., 2018; Bravo et al., 2020; Lee et al., 2020). This is particularly true in temperate areas, where macroalgal communities dominate rocky reefs, and suggest that lower abundances of *Ostreopsis* spp. could be found on large brown algae forests of fucoids and kelps, e.g. *Cystoseira sensu lato* spp., *Sargassum* spp., etc; (Mangialajo et al., 2008b; Catania, 2017, 2; Meroni et al., 2018). Nevertheless, blooms are observed on *Ericaria crinita* in the Adriatic Sea (Pfannkuchen et al., 2012) and on *Carpophyllum plumosum* populations in New Zealand (Shears and Ross, 2009). In tropical areas important blooms of *Ostreopsis* spp. can be observed on forest-forming macroalgal species (*Sargassum* spp., Chinain et al., 2020), where regime shifts from coral to algae (often with a dominance of large size species; Payri and Naïm, 1983; Stiger and Payri, 1999) and subsequent coral reef degradation, are usually associated to human impacts (Stiger and Payri, 1999). But such results, based on the maximum *Ostreopsis* spp. abundances occurring at a study site have to be considered with care, because of the potential bias linked to the classic sampling method, that quantify cells/gram of macroalga, providing an estimate that is species dependent (i.e. specific weight, Mangialajo et al., 2017).

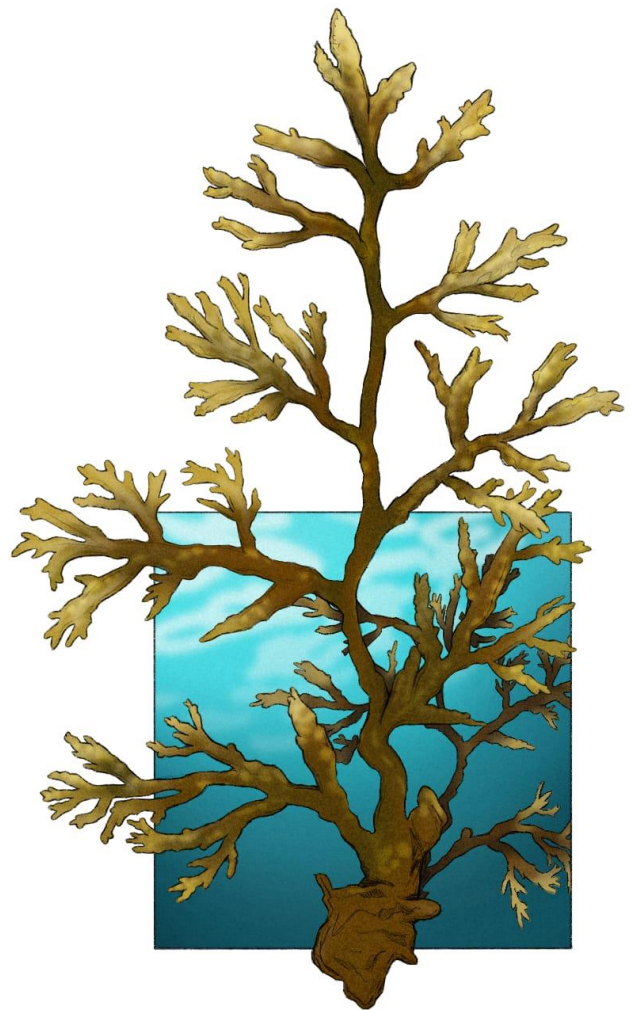
The present review reveals that most studies do not provide detailed descriptions of the mesohabitats (or benthic communities) where the studied blooms occur and how the mesohabitat

could play a role on *Ostreopsis* spp. bloom dynamics. Benthic dinoflagellate preferences at different habitat scales (substrate, community, and ecosystem) and biotic interactions present untapped fields of research with great potential which still need to be addressed in the future. Our results seem to predict a larger risk exposure to toxic effects for humans in post-regime shifts communities such as urban or degraded areas, where marine forests of large brown algae and/or reef building corals are often lost and replaced by less complex species that could host large densities of *Ostreopsis* spp. (Hughes, 1994; Benedetti-Cecchi et al., 2001; Mangialajo et al., 2008a; Catania, 2017; Filbee-Dexter and Wernberg, 2018; Johns et al., 2018; Meroni et al., 2018). Understanding the underlying mechanisms on how the habitat can affect *Ostreopsis* spp. blooms, would allow a better risk assessment of the phenomenon, identifying areas at risk simply on the base of the benthic habitats. These findings underline the need of a better understanding of *Ostreopsis* spp. ecology to prevent socio-economic damage, reduce human health risks in coastal regions, and reduce ecological impacts to marine coastal ecosystems.

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# CHAPTER 4: *Ostreopsis* spp. BLOOMS IN RELATION TO MACROALGAL COMMUNITIES





## ABSTRACT

There has been an increasing occurrence of benthic harmful algal blooms (BHAB) in temperate locations during the last decades. Moreover, the expansion and growth rates of benthic harmful dinoflagellates of the genus *Ostreopsis* could be favoured under future ocean warming scenarios. *Ostreopsis* spp. are known to develop on the surface of macroalgae in both temperate and tropical areas. There is evidence that the morphology of the macrophytes could play a role in the abundance of dinoflagellates, being the filamentous morphotypes suggested to host higher abundances of the genus *Ostreopsis*. The fact that *Ostreopsis* spp. could be associated with structurally less complex macroalgal communities is of special interest, due to the generalized phenomenon of habitat miniaturization as the result of regime shifts in temperate ecosystems. In the present study samples for *Ostreopsis* spp. were taken at different spatial scales and on different macroalgal species and communities, using different sampling techniques and methodologies to quantify abundances. The objective was to understand the role that different macroalgal species (microhabitat) and communities (mesohabitat) can play in controlling the distribution and abundance of *Ostreopsis* spp. In general, the results obtained highlight the high variability and wide distribution of *Ostreopsis* spp. among macroalgal species and communities during a bloom, and the suitability of less complex macroalgal communities in hosting *Ostreopsis* spp. The high variability of *Ostreopsis* spp. abundances on bloom locations could be due to the stronger effects at the large scale, that in turn, could influence the dominance of specific macroalgal communities in the location. Larger scale studies would be needed to confirm these results.

**Keywords:** *Ostreopsis*, Benthic HABs, macroalgae, community, habitat, substrate

## 1. INTRODUCTION

Harmful algal bloom (HABs) events have been increasing in coastal areas around the globe in the last decades (Anderson et al., 2019). Such increase is referred to ocean warming resulting from global change that would favour the expansion and growth rates of tropical and sub-tropical benthic harmful dinoflagellates including the genera *Ostreopsis*, *Gambierdiscus* and *Fukoya* in areas where habitat requirements are satisfied (Tester et al., 2020). The expansion and faster growth rates of toxic benthic dinoflagellates could multiply the number and intensity of benthic harmful algal blooms (BHABs) events in temperate areas, which could have negative repercussions on human and marine ecosystems health (Tester et al., 2020).

The genus *Ostreopsis*, belonging to the family of Ostreopsidaceae (Gonyaulacales, Dinophyceae, Dinozoa) includes several species largely distributed from tropical to temperate marine coastal areas worldwide. *Ostreopsis* is a benthic dinoflagellate that has been reported for a long time in tropical ciguatera endemic areas and, for some decades has become common in temperate areas as well (Litaker et al., 2009; Rhodes, 2011; Zingone et al., 2020). In temperate areas, its blooms are

often associated with toxic effects on humans and benthic marine ecosystems (Accoroni and Totti, 2016). Some species of *Ostreopsis* produce palytoxin-like compounds, one of the most toxic marine compounds (Ukena et al., 2001; Suzuki et al., 2012; Varela et al., 2021), causing respiratory disease after inhalation of marine aerosols in humans (Vila et al., 2016) and mass mortalities of invertebrates in marine ecosystems (Parsons et al., 2012; Accoroni and Totti, 2016). With the aim of better understanding and modelling blooms of *Ostreopsis* spp. an important research effort has been done for assessing the role of the effects of environmental factors (e.g. nutrients, salinity and seawater temperature) on the occurrence and dynamics of HABs. The results of such studies are often contrasting, showing a highly variable response of *Ostreopsis* spp. to these factors (reviewed in Parsons et al., 2012; Accoroni and Totti, 2016).

*Ostreopsis* spp. blooms develop in close relationship with the substrate and different interactions at different spatial scales (i.e. microhabitat, mesohabitat and macrohabitat) could regulate *Ostreopsis* spp. development (Cohu et al., 2013; Monserrat et al., 2022). Even if an antagonistic relationship between macroalgae and microalgae has been reported in both natural and experimental conditions (Lee and Olsen, 1985; Fong et al., 1993), *Ostreopsis* spp. are known to develop on the surface of macroalgae forming a biofilm (Totti et al., 2010; Accoroni et al., 2015). In both temperate and tropical areas *Ostreopsis* spp. are mainly sampled on macroalgal species that form low or medium complex communities (turf and shrub-like macroalgal communities), typical of post-regime shift scenarios and degraded habitats (Mangialajo et al., 2008a; Sales and Ballesteros, 2010; Meroni et al., 2018; Yong et al., 2018; Bravo et al., 2020; Lee et al., 2020). Recent studies highlighted the importance of the macrophyte morphology on the abundance of dinoflagellates: filamentous morphotypes and macroalgae forming entangled groups (turf-forming macroalgae) are suggested to host higher abundances, especially from the genus *Ostreopsis* (Totti et al., 2010; Mustapa et al., 2019; Bravo et al., 2020). The fact that BHABs could be associated with structurally less complex macroalgal communities (turf and shrubs) is of special interest, due to the generalized phenomenon of habitat miniaturization result of regime shifts in temperate ecosystems (Pessarrodona et al., 2021). Mainly due to anthropogenic pressures (e.g. local pollution, habitat destruction, climate change), structurally complex macroalgal communities, such as marine forests, are being substituted by less complex macroalgal communities, such as turfs, with the consequent loss of ecosystem functions and perturbations at the ecosystem level that could favour BHAB (Parsons et al., 2012; Filbee-Dexter and Wernberg, 2018; Benedetti-Cecchi et al., 2019; Orfanidis et al., 2021; Smale et al., 2022). Furthermore, it is thought that the metabolites produced by some macroalgal species could also influence the settlement and growth of *Ostreopsis* spp. (Accoroni et al., 2015; Ternon et al., 2020). Some macroalgal species such as brown macroalgae are known to produce secondary metabolites with an important chemical effect against marine herbivores (Schnitzler et al., 2001; Wiesemeier et al., 2007; Jormalainen and Ramsay, 2009). Some studies even suggest the



use of macroalgae in the prevention, control and mitigation of BHABs as some macroalgal species have been proven to exert negative effects against harmful benthic dinoflagellates, including *Ostreopsis* cf. *ovata* (Accoroni et al., 2015; Ternon et al., 2020). Nevertheless, the substrate and habitat preferences of *Ostreopsis* spp. are still unclear, and possibly linked to other biotic and abiotic factors including other components of the epiphytic community (Pavaux et al., 2020; Ternon et al., 2020; Roselli et al., 2022). Because of that, the existence of direct and indirect effects at smaller (e.g. epiphytic community) and larger (e.g. the macroalgal community) spatial scales than the substrate that affect *Ostreopsis* spp. proliferation are expected.

Yet, direct comparisons among different macroalgae, the most common sampled substrate (Monserrat et al., 2022), are hindered by the quantification procedure that, most of the time takes into account the number of microalgal species as cells per gram of fresh weight (FW) of the macroalgae. Such quantification is strongly affected by the specific weight and morphology of the macroalgae, therefore not allowing appropriate comparisons between macroalgal species (Totti et al., 2010; Mangialajo et al., 2017; Tester et al., 2022). Even if efforts have been performed using alternative methodologies independent from the specific weight of the substrate allowing a standardized quantification, they are not yet applied at a large scale (Tester et al., 2014; Jauzein et al., 2016; Mangialajo et al., 2017), hindering finding trends for *Ostreopsis* spp. preferences.

There is currently a need for a better understanding of the ecology of *Ostreopsis* spp. and to reduce human health and ecological risks in coastal regions, preventing indirect socioeconomic damage (Berdalet et al., 2016). Understanding the mechanisms affecting *Ostreopsis* spp. blooms, would allow a better risk assessment of BHABs, and eventually the identification of areas at risk in function of their benthic characteristics. The objective of this study is to assess the effect of different macroalgal species (small scale or microhabitat) and macroalgal communities (medium scale or mesohabitat) on the abundances of *Ostreopsis* spp. We hypothesise that (i) individual forest-forming macroalgae and marine forests, the most structurally complex macroalgal communities (dominated by large brown forest-forming macroalgae) will host lower abundances of *Ostreopsis* spp.; while (ii) medium and low structurally complex macroalgal species and related macroalgal communities (dominated by fast-growing macroalgal species with a larger number of filaments and entangled clumps, (i.e. shrub and turf-forming macroalgae) characteristic in post-regime shift scenarios and degraded habitats; will foster the highest abundances of *Ostreopsis* spp.

As *Ostreopsis* spp. abundances on the biofilm associated to macroalgae can vary in function of different factors at the microhabitat (macroalgae) scale (i.e. morphology, surface, allelopathy) and at the mesohabitat (macroalgal community) scale (i.e. resistance to wave energy, presence of herbivores), in each location we sampled: (i) different macroalgal species according to their structural morphology (i.e. turf-forming, shrub-forming and forest-forming); (ii) artificial



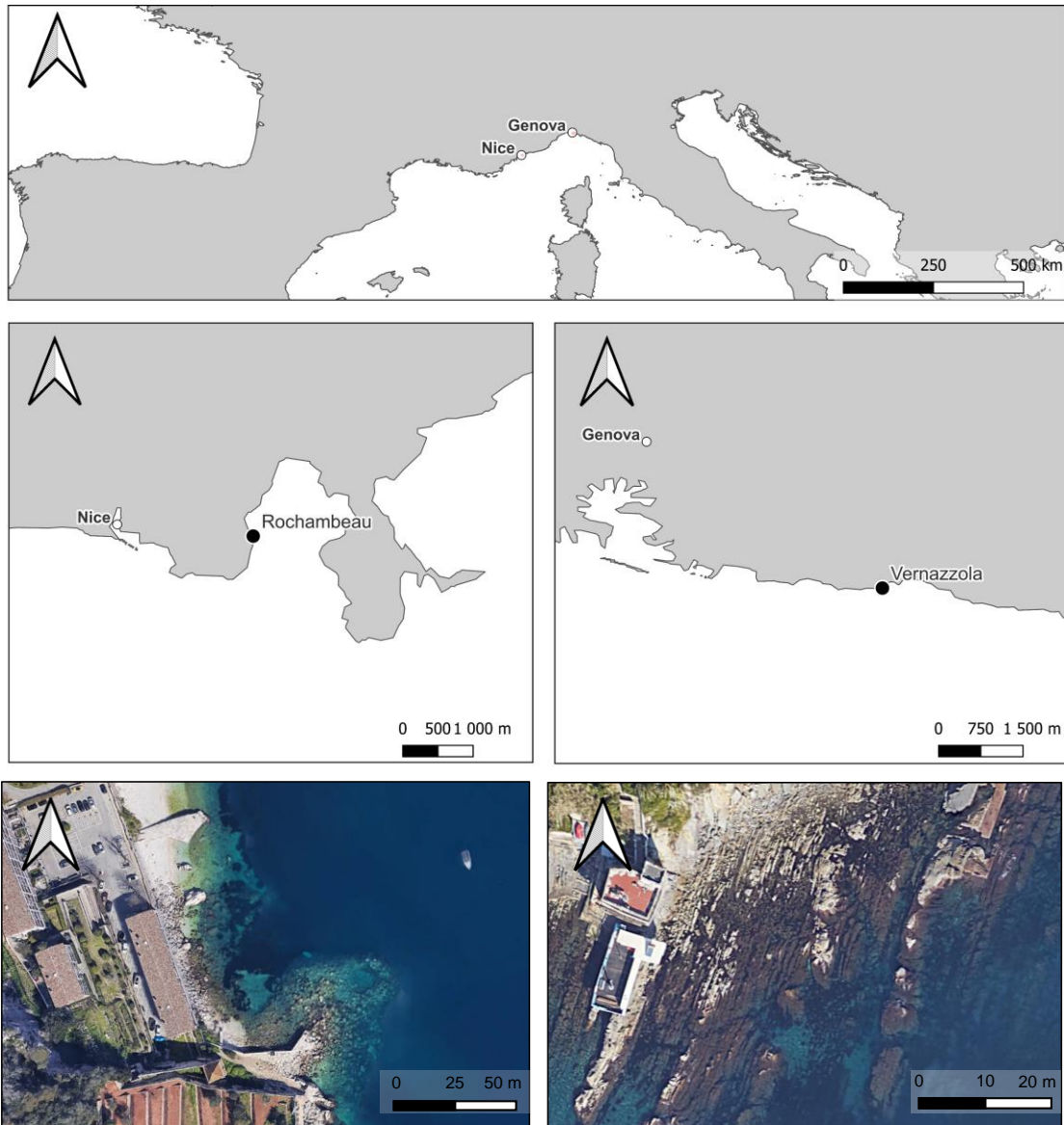
macroalgae also according to their structural morphology, dismissing any chemical or allelopathic effect; and (iii) different macroalgal communities, characterized by the same dominant species sampled for the microhabitat scale preference assessment.

## 2.1. MATERIAL AND METHODS

### 2.1. *Sampling sites*

Two locations with known annual summer blooms of *Ostreopsis* spp. were selected for the study. The two sampling sites, Rochambeau and Vernazzola, are situated on the continental N-W Mediterranean coast more than 150 km apart. The sampling site Rochambeau (Villefranche-sur-Mer, France; 43°41'34.83" N and 7°18'31.66" E), consists in a small creek of the Bay of Villefranche-sur-Mer characterized by a sheltered rocky coast. The sampling site Vernazzola (Genova, Italy; 44°23'24.23" N and 8°58'40.13" E), consists in a shallow exposed rocky coast next to the centre of Genova (Figure 1). The marine vegetation in both locations is characterized by turf and shrub-forming macroalgae, mainly *Halopteris scoparia* and *Padina pavonica* and to a lesser extent *Dictyota* spp., *Ulva* spp., *Caulerpa cylindracea* and *Jania rubens*. In each sampling site, we sampled different types of natural and artificial macroalgae species (for the small scale or microhabitat scale) and macroalgal communities (for the medium or mesohabitat scale) according to their structural morphology: turf, shrub and forest-forming macroalgae species were sampled. However, not all the different types of macroalgae species were present in the sampling locations, in this case, we transplanted individuals and stones colonized with the missing macroalgal species one week before starting the experiment.

For the microhabitat preference assessment, in each sampling location five species (or groups of species) of individual natural macroalgae were sampled ( $n = 4$ ; Coahu et al., 2013; Jauzein et al., 2018). The sampled macroalgal species are reported in Table 1; as much as possible the same species were selected in the two sampling locations. Artificial macroalgae made of latex with the same surface area but different sizes and number of ramifications were also sampled for the microhabitat preference assessment. The artificial macroalgae were attached to a plastic lid and fixed to the sea bottom with cable ties as in Fricke et al. (2018) in both sampling locations ( $n = 4$ ; see Appendix D Chapter 4, Supplementary material S1). Four different types of artificial macroalgae were used and classified according to their similarity to natural macroalgae, from a smaller and higher number of ramifications to larger and fewer number of ramifications: artificial Turf, *Dictyota*, *Halopteris* and *Cystoseira* like, and an empty lid was used as a control treatment (Appendix D Chapter 4 Supplementary material S1).



**Figure 1:** The two sampling locations: Rochambeau in the Bay of Villefranche-sur-Mer (France) and Vernazzola in Genova (Italy).

**Table 1:** Macroalgae species (microhabitat) and communities (mesohabitat scale) sampled in Rochambeau and Vernazzola.

MACROALGAL SPECIES AND COMMUNITIES	SAMPLING SITE	BIOMASS (g FW / 0.04 m <sup>2</sup> , mean ± SD)	COMPOSITION
<b>TURF – DICTYOTA SPP. TURF - FORMING</b>	Rochambeau		<i>Jania rubens</i> (80%), <i>Dictyota</i> spp. (20%)
<b>TURF TURF - FORMING</b>	Rochambeau	55.34 ± 16.45	Small <i>Padina pavonica</i> and <i>Halopteris scoparia</i> (80%), <i>J. rubens</i> (10%), crustose coralline (10%), <i>Acetabularia acetabulum</i> and <i>Cladophora</i> sp. (1%)
<b>TURF TURF - FORMING</b>	Vernazzola	133.50 ± 26.16	<i>Polysiphonia</i> sp., <i>Chondracantus acicularis</i> and other filamentous macroalgae (50%), <i>Dictyota</i> spp. (20%), <i>Ellisolandia elongata</i> (20%), <i>Caulerpa cylindracea</i> (5%), <i>Hypnea</i> sp. (5%) and <i>Ulva</i> sp. (2%)
<b>DICTYOTA SPP. SHRUB -FORMING</b>	Rochambeau	75.65 ± 22.30	<i>Dictyota</i> spp. (80%), <i>J. rubens</i> (10%) and crustose coralline (10%)
<b>DICTYOTA SPP. SHRUB -FORMING</b>	Vernazzola	87.00 ± 25.45	<i>Dictyota</i> spp. (80%) and crustose coralline (20%)
<b>HALOPTERIS SCOPARIA SHRUB -FORMING</b>	Rochambeau	135.98 ± 34.36	<i>H. scoparia</i> (90%), <i>P. pavonica</i> (10%), <i>A. acetabulum</i> and <i>Haliptilon virgatum</i> (2%), <i>Caulerpa cylindracea</i> , <i>Peyssonnelia</i> sp., <i>Sphaerococcus coronopifolius</i> and <i>E. elongata</i> (1%)
<b>HALOPTERIS SCOPARIA SHRUB -FORMING</b>	Vernazzola	120.00 ± 45.25	<i>H. scoparia</i> (70%), <i>Dictyota</i> spp. (20%), <i>Caulerpa cylindracea</i> (5%), <i>E. elongata</i> (5%)
<b>CYTOSEIRA COMPRESSA FOREST-FORMING</b>	Rochambeau	150.75 ± 59.44	<i>Cystoseira compressa</i> (90%), crustose coralline (10%) *
<b>CYTOSEIRA COMPRESSA FOREST-FORMING</b>	Vernazzola	241.50 ± 34.65	<i>C. compressa</i> (85%), <i>Sargassum vulgare</i> (10%), crustose coralline (5%)
<b>GONGOLARIA BARBATA FOREST-FORMING</b>	Rochambeau	53.79 ± 1.77	<i>Gongolaria barbata</i> (90%), crustose coralline (10%) *
<b>SARGASSUM VULGARE FOREST-FORMING</b>	Vernazzola	96.50 ± 21.92	<i>S. vulgare</i> (90%), <i>C. compressa</i> (5%), crustose coralline (5%)

\* By the end of the experiment the two transplanted macroalgal communities in Rochambeau corresponding to *Cystoseira compressa* and *Gongolaria barbata*, experienced a reduction in the macroalgal biomass.

The sampled macroalgal communities (mesohabitat scale) were dominated by the same species of macroalgae selected for the microhabitat assessment (Table 1). However, because of the lack of forest-forming species in Rochambeau, stones colonized by *Cystoseira compressa* and *Gongolaria barbata* were transplanted to Rochambeau for the duration of the experiment from Sainte Marguerite Island (Lérins Islands, Cannes, France). Sainte Marguerite Island is one of the last locations with healthy *Cystoseira sensu lato* forests in the French Riviera (Thibaut et al., 2015). In Rochambeau, a modified BEDI device (Benthic Dinoflagellate Integrator; Mangialajo et al., 2017) was used in order to sample the selected communities (see section 2.2. Collection and processing of samples). In both sites, 0.04 m<sup>2</sup> scrapings (n=3) of the selected communities were performed in

order to extrapolate the abundances of *Ostreopsis* spp. from the communities, expressed as cells per surface of seabottom (see section 2.2. Collection and processing of samples).

For each site, also abundances of *Ostreopsis* spp. cells on the surrounding water were sampled ( $n = 4$  in Rochambeau and  $n = 2$  in Vernazzola). The temperature was monitored every hour during the duration of the experiment at both sampling sites at 0.5 m depth using a temperature data logger (HOBO Pendant MX Temp, ONSET).

## **2.2. Collection and processing of samples**

*Ostreopsis* spp. cells on the surrounding water were determined from seawater samples collected in 250 mL plastic flasks 20 cm above the natural macroalgal substrate, in particular turf (Table 1), as described in Cohu et al. (2013). Samples of individual natural macroalgae (microhabitat scale samples) were carefully collected into a 250 mL flask with the surrounding water, as described by Cohu et al. (2013). Abundances of *Ostreopsis* spp. on artificial macroalgae, with the surrounding water, were sampled in 1 L flasks coiled to the lid at the base of the artificial macroalgae, in a similar way as in Fricke et al. (2018), closing the flask underwater. Then, once the flask was hermetically closed, the lid with the macroalgae and the flask were detached from the base cutting the cable tides and pulled out of the water. Afterwards the flask was shaken to detach the cells of *Ostreopsis* spp. from the artificial macroalgae and the lid. Finally, the 1 L flask with the sample was closed using another lid and the lid with the artificial macroalgae was fixed again at the same point on the sea bottom with cable tides.

The cells at the mesohabitat scale were assessed in two ways: (i) using the BEDI device and (ii) extrapolated from the total biomass of macroalgae obtained from the scraping. In the first assessment, a modified adapted BEDI device (Mangialajo et al., 2017) consisting of a hollow plastic cylinder open at both ends was used. In order to avoid cell loss, the bottom part was provided of a fixed rubber seal and the upper part was kept out of the water. The adapted BEDI device used in this study covered an area of 314.16 cm<sup>2</sup> of the sea bottom and had a total volume of 9.42 L. To homogenize cells of *Ostreopsis* spp. in the area and volume covered, a propeller was installed on the inside upper part of the BEDI. Once the device was placed on the macroalgal communities, the sea water level inside the BEDI was noted and the propeller was rotated 10 times from the outside of the BEDI thanks to a crank. Then a 60 mL sample was extracted from the inside of the adapted BEDI device using a syringe. For the second assessment, the total biomass of macroalgae for each macroalgal community was calculated from the total biomass of macroalgae obtained from the scrapings. In both sites, 0.04 m<sup>2</sup> of all the considered communities were sampled ( $n = 3$ ). Once in the laboratory, the dominant species were centrifugated for 30 seconds and weighted (FW). In both cases (BEDI sampling and extrapolation from scrapings) the results can be expressed as the number

of cells per seabottom surface area (i.e. 0,04 m<sup>2</sup>) independently of the specific weight of the substrate (Mangialajo et al., 2017).

All the samples were fixed with acidic Lugol (2% v/v). For the seawater samples the Utermöhl method (Utermöhl, 1958) was used to estimate abundances (in number of cells / L). The fixation of the natural macroalgae samples was followed by vigorous shaking and filtration of the sample through a 500 µm mesh to separate the macroalgae from the seawater containing *Ostreopsis* spp. cells and the macroalgae were weighed ( $\pm$  0.01 g; Jauzein et al., 2018). The abundances of *Ostreopsis* spp. cells on natural macroalgae, artificial macroalgae and macroalgal communities were estimated under a microscope (Axio Observer D1, Zeiss) using a Sedgewick Rafter counting chamber. The abundance of cells at the microhabitat scale were expressed as number of cells per gram of fresh weight of macroalgae (cells / g FW). The density of cells of *Ostreopsis* spp. on artificial macroalgae was expressed as cells / cm<sup>2</sup> of surface of artificial macroalgae (Appendix D Chapter 4 Supplementary material S1). At the mesohabitat scale abundances were expressed as cells / 0.04 m<sup>2</sup>.

Our study was conducted during the different phases of the bloom (exponential, peak and decreasing phase) to assess eventual changes in *Ostreopsis* spp. preferences in function of the dynamic of the bloom. In order to define the magnitude of the bloom, the sampled abundances of *Ostreopsis* spp. were classified in Major bloom ( $>1000 \times 10^3$  cells / g FW), Bloom ( $>200 \times 10^3$  cells / g FW), Alert ( $>30 \times 10^3$  cells / L), and No bloom ( $<200 \times 10^3$  cells / g FW) according to the definition from Funari et al. (2015) and Mangialajo et al. (2017).

### **2.3. Data analysis**

Abundances of *Ostreopsis* spp. on natural macroalgae (microhabitat scale): A Generalized Linear Mixed-Effects Model (GLMM), with a Gamma link log distribution function was used to test the effect of the macroalgal species on the abundance of cells of *Ostreopsis* spp. The variable tested was cells per gram of macroalgal FW in Rochambeau and Vernazzola, with (i) macroalgal species (five levels) as a fixed factor, and (ii) the sampling day (5 levels in Rochambeau and 6 in Vernazzola) as random.

Abundances of *Ostreopsis* spp. on artificial macroalgae (microhabitat scale): A GLMM with a Gamma link log distribution function was used to test the effect of the macroalgal morphology on the abundance of cells of *Ostreopsis* spp. The variable was cells per surface (cm<sup>2</sup>) in Rochambeau and Vernazzola with (i) macroalgal species (five levels) as a fixed factor, and (ii) the sampling day (5 levels) and the replicate as random to account for repeated measures over time.

Abundances of *Ostreopsis* spp. on macroalgal communities (mesohabitat scale): A GLMM with a Gamma link log distribution function was used to test the effect of the benthic macroalgal

community on the abundance of cells of *Ostreopsis* spp. The tested variables were cells in 0.04 m<sup>2</sup> of sea bottom using the adapted BEDI device in Rochambeau and cells in 0.04 m<sup>2</sup> of sea bottom extrapolated from the biomass of macroalgae obtained from the scrapings in Rochambeau and Vernazzola, with (i) the macroalgal community type (five levels) as a fixed factor, and (ii) the sampling day (6 levels) and the replicate as random to account for repeated measures over time.

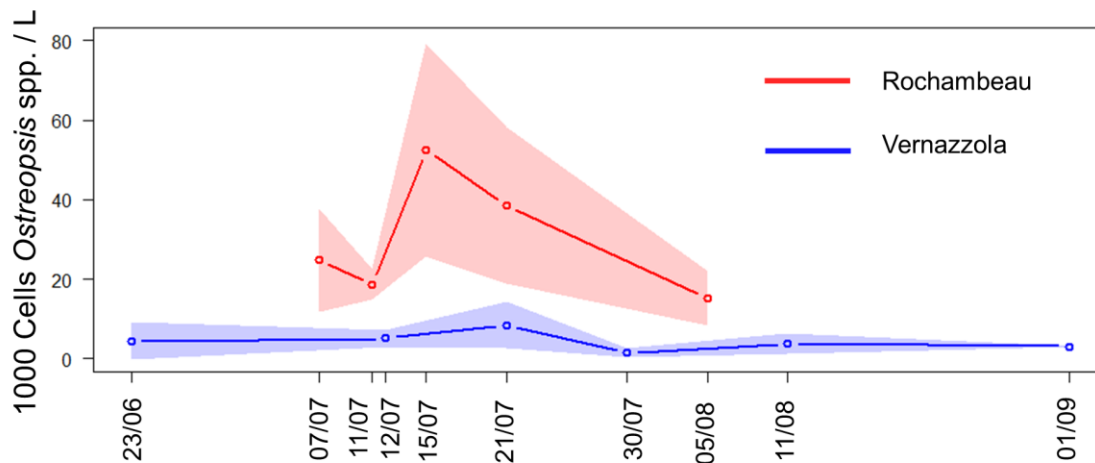
All GLMM were fitted to analyse the effect of the variables and the AICs likelihood minimum was used to select the best model among the possible combinations. The different models were fitted using the functions “glmer” and “lm” from the package lme4 (Bates et al., 2015) in the statistical environment R in the statistical environment R (R: The R Project for Statistical Computing). P-values were obtained by means of a Wald  $\chi^2$  test using the “ANOVA” function from the CAR package (Fox and Weisberg, 2018). Finally, the function “emmeans” from the package emmeans (Lenth et al., 2022) was used to perform the post-hoc analysis of the GLMM models.

## RESULTS

The mean seawater temperature was  $24.99 \pm 1.25^\circ\text{C}$  and  $26.77 \pm 1.68^\circ\text{C}$  (mean  $\pm$  SD), respectively in Rochambeau and Vernazzola, during the duration of the experiment, with maximum and minimum temperatures of  $28.27^\circ\text{C}$  (on the 30<sup>th</sup> of July 2021) and  $21.49^\circ\text{C}$  (on the 2<sup>nd</sup> July 2021) in Rochambeau and of  $30.54^\circ\text{C}$  (on the 15<sup>th</sup> of August 2021) and  $23.59^\circ\text{C}$  (on the 28<sup>th</sup> of June 2021) in Vernazzola.

### 3.1. *Ostreopsis* spp. concentrations in seawater

Concentrations of *Ostreopsis* spp. in the water in Rochambeau (n = 4) situate the peak of the bloom on 15/07 (day 3, mean cell abundance of  $5.25 \times 10^4$  *Ostreopsis* spp. cells / L), reaching a maximum concentration of  $7.90 \times 10^4$  *Ostreopsis* spp. cells / L (the maximum cell concentration in seawater measured in this study; Figure 2A). In Vernazzola, the seawater samples of *Ostreopsis* spp. (n = 2) show the peak of the bloom on day 3 (21/07, mean cell abundance of  $8.47 \times 10^3$  *Ostreopsis* spp. cells / L), reaching a maximum concentration of  $1.26 \times 10^4$  *Ostreopsis* spp. cells / L (Figure 2B). According to the definition of bloom (Funari et al., 2015; Mangialajo et al., 2017), the seawater samples from Rochambeau reached concentrations corresponding to a state of alert ( $>30 \times 10^3$  cells / L) the sampling days 1 (07/07), 3 (15/07) and 4 (21/07), while seawater abundances of *Ostreopsis* spp. in Vernazzola never surpassed the values of routine monitoring.

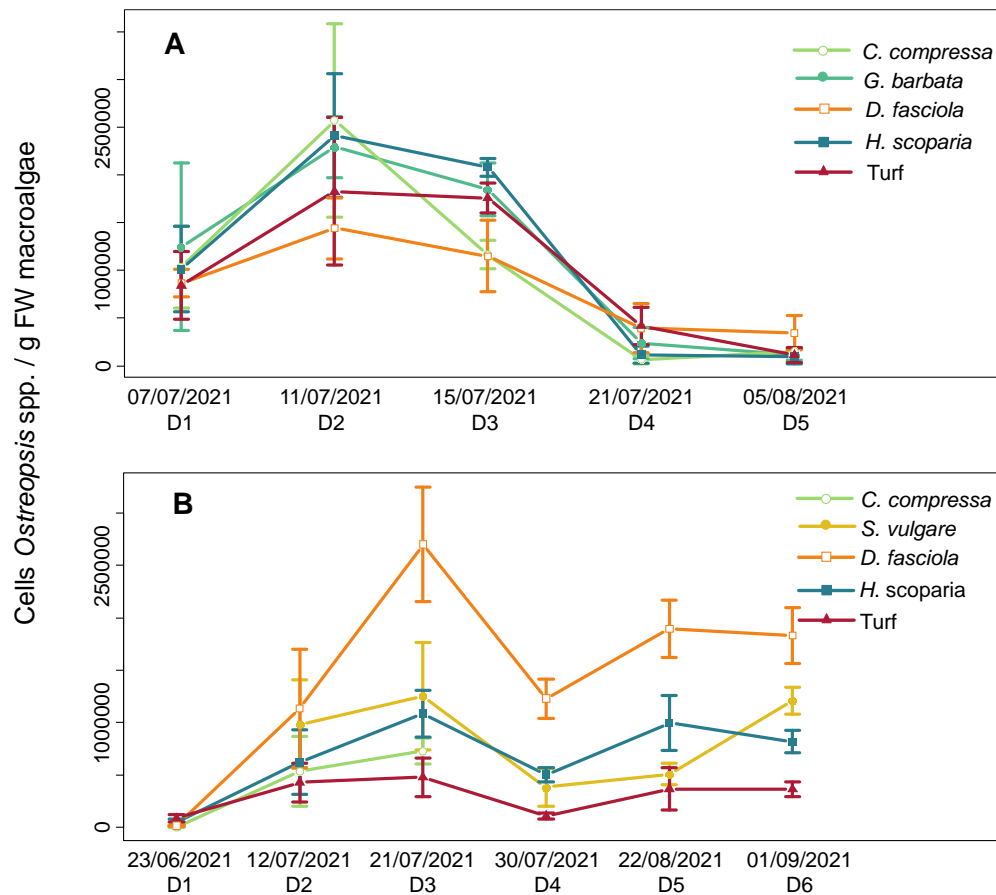


**Figure 2:** Abundances of *Ostreopsis* spp. in seawater (1000 Cells *Ostreopsis* spp. / L) for Rochambeau (n = 4) and Vernazzola (n = 2) for the duration of the experiment. The confidence interval represents the standard error.

### 3.2. Abundances of *Ostreopsis* spp. on natural macroalgae (microhabitat scale)

The ANOVA from the GLMM model did not find differences in abundances of *Ostreopsis* spp. among the different macroalgal species sampled in Rochambeau (Table 2). For this sampling site, the peak of the benthic bloom on natural macroalgae was on day 2 (11/07, mean cell abundance of  $2.06 \times 10^6$  cells / g FW), with maximum benthic abundances of *Ostreopsis* spp. reaching  $4.00 \times 10^6$  cells / g FW (the maximum benthonic cell abundances measured in this study) on turf (day 2, 11/07; Figure 3A). Major bloom abundances were reached on days 1 (07/07), 2 (11/07) and 3 (15/07; on 35 out of 83 total samples), and bloom abundances on days 1 (07/07), 2 (11/07), and 5 (05/08; on 29 out of 83 total samples). Abundances of no bloom on macroalgae were recorded on days 4 (21/07) and 5 (05/08; on 19 out of 83 total samples).





**Figure 3:** Abundances of *Ostreopsis* spp. on natural macroalgae (cells *Ostreopsis* spp. / g FW macroalgae) in Rochambeau (A) and Vernazzola (B). The error bars show the standard error.

In Vernazzola, significant differences on abundances of *Ostreopsis* spp. were found among macroalgal species (Figure 3B and Table 2). Significantly higher abundances of *Ostreopsis* spp. were found on *Dictyota fasciola* than in *Sargassum vulgare*, *Cystoseira compressa* and turf. *Halopteris scoparia* hosted significantly higher abundances of *Ostreopsis* spp. than *C. compressa* (Figure 3B). For this sampling site, the peak of the benthic bloom on natural macroalgae was on day 3 (21/07, mean abundance of  $1.03 \times 10^6$  cells / g FW), and the maximum abundances were observed on *D. fasciola*. ( $3.76 \times 10^6$  cells / g FW; Figure 3B). In Vernazzola major bloom and bloom abundances (Funari et al., 2015; Mangialajo et al., 2017) were reached on days 2 (12/07), 3 (21/07), 4 (30/07), 5 (11/08) and 6 (01/09; on 28 and 49 out of 97 total samples for the major bloom and the bloom scenarios respectively), and no bloom abundances on days 1 (23/06), 4 (30/07) and 5 (11/08; on 20 out of 97 total samples).

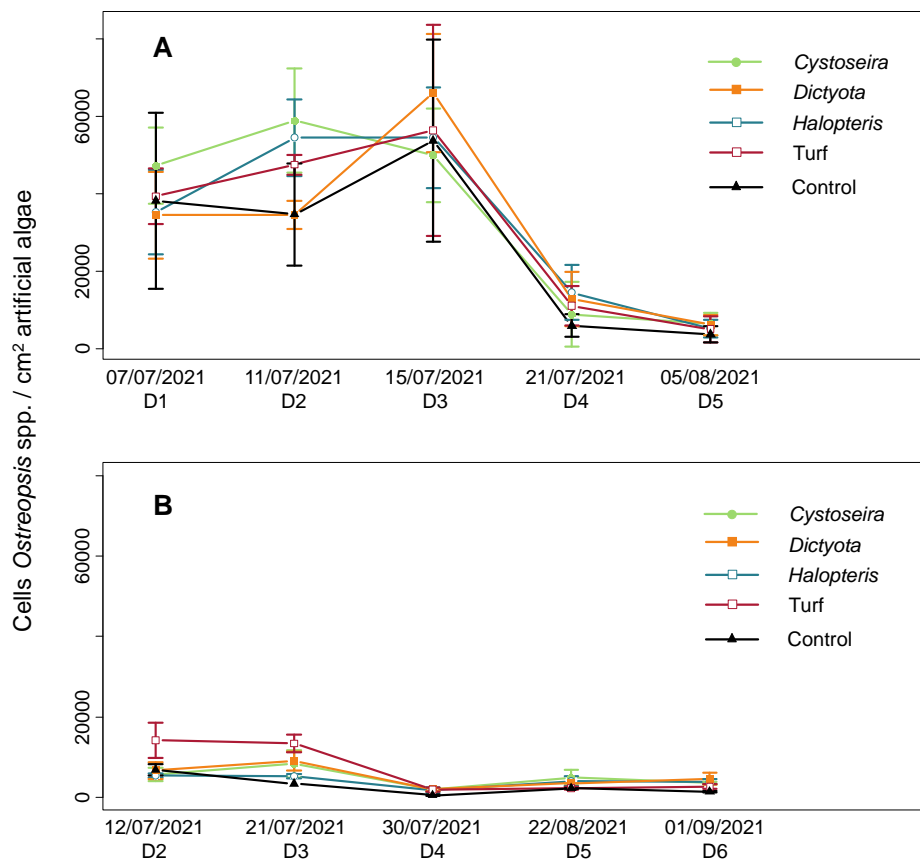


**Table 2:** Results from the statistical analysis.

Experiment	Model	Factor levels	DF	Chi sq	P-value	Site	
<b>Cells <i>Ostreopsis</i> spp. / FW macroalgae</b>	Cells <i>Ostreopsis</i> spp. / g FW macroalgae ~ Algal species + (1 Sampling day), Gamma (link = "log")	Algal species (5 levels)	4	0.567	0.967	Rochambeau	
			4	40.404	<0.001	***	Vernazzola
<b>Cells <i>Ostreopsis</i> spp. / cm<sup>2</sup> artificial macroalgae</b>	Cells <i>Ostreopsis</i> spp. / cm <sup>2</sup> ~ Algal types + (1 Sampling day) + (1 Replicates), Gamma (link = "log")	Algal species (5 levels)	4	1.800	0.772	Rochambeau	
			4	21.656	<0.001	***	Vernazzola
<b>Cells <i>Ostreopsis</i> spp. / 0.04 m<sup>2</sup> macroalgal community - BEDI</b>	Cells <i>Ostreopsis</i> spp. / 0.04 m <sup>2</sup> of sea bottom~ Macroalgal communities + (1 Sampling day) + (1 Replicates), Gamma (link = "log")	Communities (6 levels)	5	2.902	0.715	Rochambeau	
<b>Cells <i>Ostreopsis</i> spp. / 0.04 m<sup>2</sup> macroalgal community – From scrapings</b>	Cells <i>Ostreopsis</i> spp. / 0.04 m <sup>2</sup> sea bottom~ Macroalgal communities + (1 Sampling day) + (1 Replicates), Gamma (link = "log")	Communities (5 levels)	4	12.578	<0.05	**	Rochambeau
			4	13.948	<0.005	***	Vernazzola

### 3.3. Abundances of *Ostreopsis* spp. on artificial macroalgae (microhabitat scale)

The ANOVA from the GLMM model did not find differences in abundances of *Ostreopsis* spp. among the different types of artificial macroalgae, neither with the control in Rochambeau (Figure 5A and Table 2). In Rochambeau, abundances of *Ostreopsis* spp. on artificial macroalgae remained high until day 3 (15/07) when they started decreasing (Figure 4A). The highest abundances on artificial macroalgae reached  $1.38 \times 10^5$  cells / cm<sup>2</sup> on turf the day 3 (15/07), mean abundances on this sampling day were also the highest (mean abundance of  $5.57 \times 10^4$  cells / cm<sup>2</sup>).



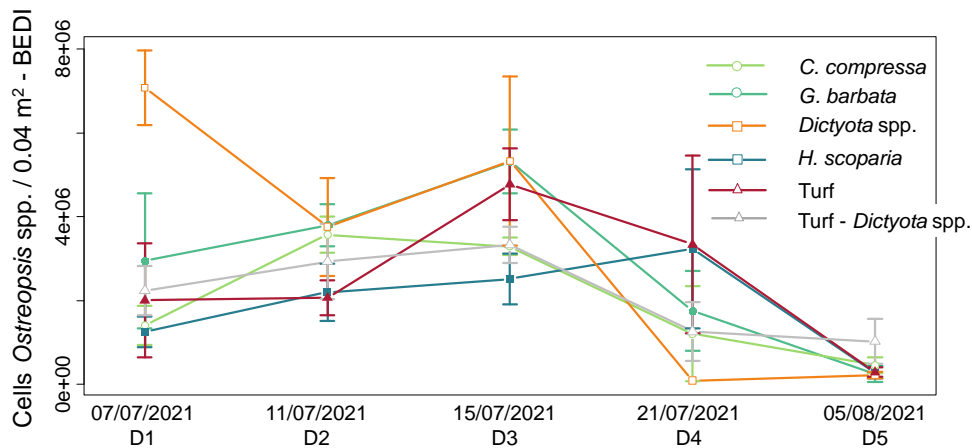
**Figure 4:** Abundances of *Ostreopsis* spp. on artificial macroalgae (cells *Ostreopsis* spp. / cm<sup>2</sup> artificial macroalgae) in Rochambeau (A) and Vernazzola (B). The error bars show the standard error.

However, significant differences among types of artificial macroalgae were found in Vernazzola, where the control hosted lower abundances of *Ostreopsis* spp. than the artificial algal types *Cystoseira*, *Dictyota* and Turf (Figure 5B and Table 2). In this sampling location, abundances of *Ostreopsis* spp. on artificial macroalgae remain high on the two first sampling days (12/07 and 21/07) when they start decreasing (Figure 5B). Maximum abundances on artificial macroalgae in Vernazzola were lower than in Rochambeau, reaching only  $2.33 \times 10^4$  cells / cm<sup>2</sup> on turf on day 1 (12/07), the highest mean abundances were recorded on day 2 (mean abundance of  $32/07$ ;  $8.54 \times 10^3$  cells / cm<sup>2</sup>).

### 3.4. Abundances of *Ostreopsis* spp. on macroalgal communities (mesohabitat scale)

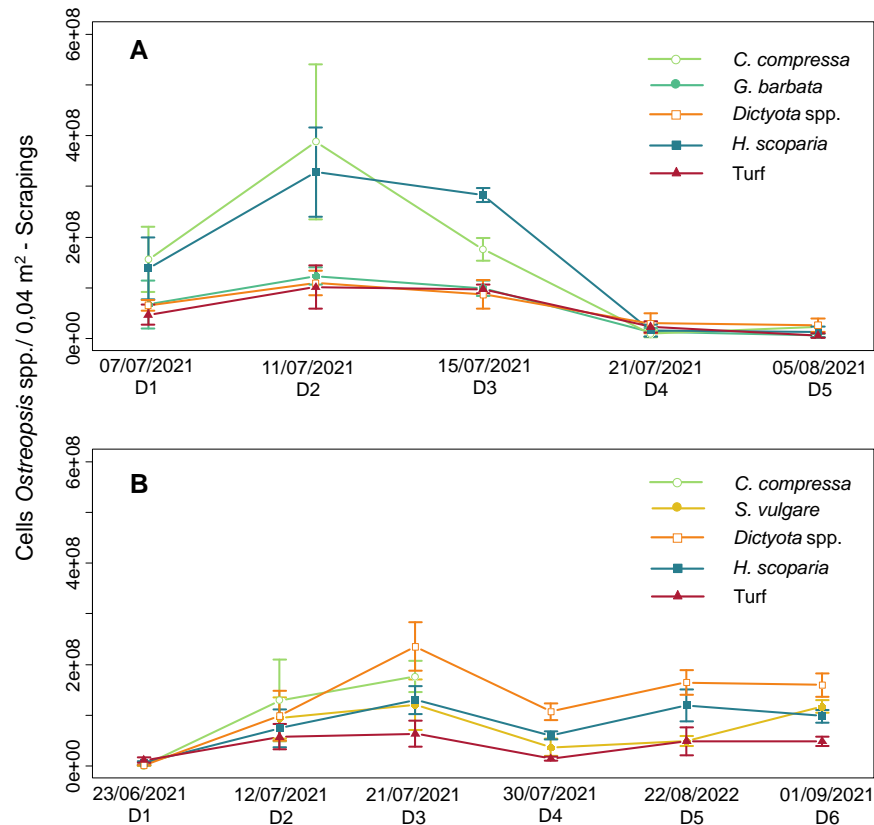
The ANOVA from the GLMM model did not find significant differences in abundances of *Ostreopsis* spp. among the different macroalgal communities sampled with the adapted BEDI device in Rochambeau (Table 2). The peak of the bloom on natural communities (mesohabitat scale) in Rochambeau was day 3 (15/07, mean abundance of  $3.98 \times 10^6$  cells / 0.04 m<sup>2</sup>), and after this day the abundances started decreasing (Figure 5). The maximum abundances measured reached  $8.95 \times 10^6$  cells / 0.04 m<sup>2</sup> on the turf community on day 4 (21/07). *Ostreopsis* spp. abundances on

natural communities in Rochambeau reached values corresponding to a major bloom ( $> 1319$  cells /  $\text{cm}^2$ ) on the sampling days 1 (07/07), 2 (11/07), 3 (15/07) and 4 (21/07), globally on 76 out of 100 total samples. Bloom values ( $> 279$  cells/  $\text{cm}^2$ ) were reached on days 4 (21/07) and 5 (05/08), and globally on 13 out of 100 total samples (for major bloom and bloom definition, see Mangialajo et al., 2017).



**Figure 5:** Abundances of *Ostreopsis* spp. on natural macroalgal communities (cells *Ostreopsis* spp. / 0.04 m<sup>2</sup> macroalgal communities) sampled in Rochambeau with the adapted BEDI device. The error bars show the standard error.

Regarding the cells of *Ostreopsis* spp. on 0.04 m<sup>2</sup> of sea bottom (mesohabitat scale), extrapolated from the total biomass of macroalgae obtained from the scrapings, significant differences were found among the different communities sampled in both Rochambeau and Vernazzola (Table 2). In Rochambeau, the abundances of cells was significantly higher on *Halopteris scoparia* than on *Gongolaria barbata* dominated communities, without differences among the other species (Figure 6A). The highest mean abundances of cells were recorded the day 2 (11/07; mean abundance of  $1.90 \times 10^8$  cells / 0.04 m<sup>2</sup>). In Vernazzola significantly higher abundances were found on *Dictyota* spp. dominated communities than on all the other communities (Figure 6B). The highest mean abundances were recorded the day 3 (21/07; mean abundance of  $1.45 \times 10^8$  cells / 0.04 m<sup>2</sup>).



**Figure 6:** Concentration of *Ostreopsis* spp. on natural macroalgal communities (cells *Ostreopsis* spp. / 0.04 m<sup>2</sup>) in Rochambeau (A) and Vernazzola (B). The error bars show the standard error.

## DISCUSSION

The objective of this study was to expand our understanding of the role that macroalgal species (microhabitat) and/or communities (mesohabitat) can play facilitating blooms of *Ostreopsis* spp. To do so, samples of *Ostreopsis* spp. were taken at different spatial scales and on different macroalgal species and communities, using different sampling techniques and methodologies to quantify abundances of *Ostreopsis* spp.

The maximum concentrations of cells of *Ostreopsis* spp. in the seawater observed in this study are comparable with the ones reported in Rochambeau ( $10.4 \times 10^4$  cells / L), and the ones reported in several sites in Genova, including Vernazzola ( $8.4 \times 10^4$  cells / L; Mangialajo et al., 2008a, 2011). On both sampling sites, the number of cells of *Ostreopsis* spp. on natural macroalgae (cells / g FW) reached abundances characteristic of a major bloom (Funari et al., 2015; Mangialajo et al., 2017) and are comparable with the maximum abundances reported in or near the same locations. In Rochambeau in July 2008 one of the maximal epiphytic abundances reached  $8.54 \times 10^6$  cells / g FW on *Dictyota* spp. (Cohu et al., 2013). In Genova-Quarto on July 2017 Meroni et al. (2018b) reported abundances reaching  $2.89 \times 10^6$  cells / g FW on *Halopteris scoparia*.

The present study highlights the high variability of *Ostreopsis* spp. preferences when using different sampling techniques to assess the abundances of cells. At the microhabitat scale, in Rochambeau abundances of cells of *Ostreopsis* spp. per gram FW of macroalgae were not significantly different for the sampled species. Contrarily, significant patterns were observed in Vernazzola, where the cells of *Ostreopsis* spp. per gram FW of macroalgae were higher on *Dictyota fasciola* and *Halopteris scoparia* than on most of the other sampled macroalgal species. Such results are in agreement with our hypotheses that higher abundances are found on shrub-forming macroalgae (*D. fasciola* and *H. scoparia*) than on forest-forming (*Cystoseira compressa* and *Sargassum vulgare*; Meroni et al., 2018; Yong et al., 2018; Bravo et al., 2020; Lee et al., 2020).

Some recent studies already highlighted the importance of the macrophyte morphology on the abundance of dinoflagellates (Vila et al., 2001; Parsons and Preskitt, 2007; Totti et al., 2010), and the need for standardized methods allowing direct comparisons, as some of the ones used in the present study (i.e. cells per surface of artificial macroalgae or cells per surface of sea bottom). Our study did not highlight significant differences among the different artificial macroalgae. In Vernazzola the control was hosting a significantly lower number of cells in 3 out of the 4 treatments (except *Halopteris*), while in Rochambeau even the control was not significantly different from the other treatments. Therefore, highlighting that the considered different morphologies of artificial macroalgae are not influencing *Ostreopsis* spp. abundances (no differences among treatments); that allelopathic interactions may be at the origin of the patterns observed; and that the small scale variability can be very high (no significant differences with the control in Rochambeau). The maximum abundances of *Ostreopsis* spp. recorded on the artificial macroalgae were found in Rochambeau, and were of the same order of magnitude of the abundances observed on natural macroalgae (Totti et al., 2010) in the Adriatic Sea and higher than the abundances reported on other artificial substrates in the N-W Mediterranean Sea (Fricke et al., 2018).

At the mesohabitat scale, no differences in *Ostreopsis* spp. per cm<sup>2</sup> of sea bottom were found among the sampled macroalgal communities using the adapted BEDI device in Rochambeau. This result can be explained by the high variability in cell abundances observed in this location. When extrapolating the abundances of cells of *Ostreopsis* spp. per surface of sea bottom using the total biomass of macroalgae obtained from the scrapings, significant differences at the mesohabitat scale among communities were found both in Rochambeau (*Halopteris scoparia* higher than *Gongolaria barbata*) and in Vernazzola (*Dictyota* spp. higher than all the other communities). Yet, while no differences among communities were highlighted by the cell's abundances obtained with the BEDI device, the extrapolation of the same variable using the total biomass of macroalgae obtained from the scrapings allowed to observe higher abundances on a shrub-forming species (*H. scoparia*) than on a forest-forming (*G. barbata*) in agreement with our hypothesis. Nevertheless, such results have to be taken with care as the abundances on the other considered communities were not significantly

different. We cannot exclude that such results could be due to a high variability associated to the BEDI sampling, that can be difficult to apply on irregular sea bottoms and that integrates the cells of *Ostreopsis* spp. on the macroalgal community and the cells present in the water column, potentially confusing the absolute values of the cells in the macroalgal biofilm, especially in the situations of “bloom” or “major bloom”. Nevertheless, our calculations suggest that the mean cells of *Ostreopsis* spp. sampled in seawater, represent less than the 10 % of the mean cells in the macroalgal biofilm. Concerning the transplanted communities, we observed that the *C. compressa* and *G. barbata* dominated communities moved to Rochambeau from Sainte Marguerite Island lost about a 30 % of their biomass by the end of the experiment, possibly reducing the facilitating or deterrent effects of these communities on *Ostreopsis* spp. proliferation. The loss of biomass of the transplanted forest-forming communities was partially due to fish grazing, but sign of degradation were also observed, potentially due to unfavourable environmental conditions in Rochambeau or to the effects of the bloom, that have been suggested to negatively impact other *Cystoseira s.l.* spp. (Jauzein et al., 2017; Iveša et al., 2021).

In Vernazzola, patterns were more clear, observing higher abundances of cells at the mesohabitat scale on *Dictyota* spp. dominated communities, in agreement with the samplings at the microhabitat scale (cells / g FW macroalgae) and with other studies on other locations in the Mediterranean Sea (Blanfuné et al., 2015; González et al., 2019; Gémin et al., 2020; Ternon et al., 2020). It is worth noting that one of the maximal epiphytic abundance ever reported for *Ostreopsis* cf. *ovata* in the N-W Mediterranean was found on species belonging to this genus (Cohu et al., 2013). Nonetheless, studies in mesocosm found that *Dictyota* spp. could inhibit the growth of *Ostreopsis* cf. *ovata* (Accoroni et al., 2015; Ternon et al., 2020). These differences among studies in the field and in laboratory could be due to the changes in macroalgal metabolites when exposed to culture conditions or because of the lack of other effects at larger scale that could modify the preferences of *Ostreopsis* spp. (Mancuso et al., 2016). Moreover, older identifications of *Dictyota* spp. as substrate for the development of *Ostreopsis* spp. should be reviewed as this genus has suffered taxonomic changes (Tronholm et al., 2010).

Overall, the results obtained extrapolating the *Ostreopsis* spp. cells in 0.04 m<sup>2</sup> of sea bottom from the cells per gram FW of macroalgae are in agreement with the results from other studies reporting higher abundances of cells of harmful benthic dinoflagellates in shrubs and turfs (Meroni et al., 2018b; Yong et al., 2018; Bravo et al., 2020) and from a recent review (Monserrat et al., 2022). Some studies (Yong et al., 2018; Lee et al., 2020) highlight the importance of the mesohabitat for the proliferation of BHAB and developed methodologies (artificial substrates) to allow the comparison between different macroalgal communities. Unfortunately, studies using either artificial substrates or quantification of cells per surface of sea bottom (using the BEDI device or

extrapolating from macroalgal biomass) are scarce and do not allow us to compare the results obtained in this study.

The comparison of cell abundances between different macroalgal species from the literature is controversial, especially due to differences on macrophyte morphologies and surfaces (Totti et al., 2010; Mangialajo et al., 2017). Our results, as the ones reported in other studies (Totti et al., 2010; Blanfuné et al., 2015; Meroni et al., 2018b; Bravo et al., 2020) show an important variability in the abundance of *Ostreopsis* spp. in function of the sampled macroalgal species and communities, sometimes not allowing to find clear patterns of preference for *Ostreopsis* spp. (Berdalet et al., 2017; Jauzein et al., 2018; Tester et al., 2022) as in the case of Rochambeau. The abundance of cells per area of sea bottom could be a measure better estimating the public health risk of blooms, as it represents the total number of cells in a determinate area (Mangialajo et al., 2017). The methodologies and standardisations better representing the reality of the ecosystem (e.g. real macroalgal biomass and area available for the settlement of *Ostreopsis* spp. per sea bottom surface) should be further studied and, if possible, preferred (Tester et al., 2022).

In general, the results obtained in this study highlight the wide and highly variable distribution of *Ostreopsis* spp. on all macroalgal species and communities sampled. Nevertheless, the fact of not having found in some cases clear preferences on cell abundances among natural or artificial macroalgae could be due to the high abundances of *Ostreopsis* spp. during the bloom that could homogeneously colonize all the available substrates. This could be the case of Rochambeau, the sampling location where higher abundances of *Ostreopsis* spp. were observed in this study, and where the preferences of *Ostreopsis* spp. were less clear. In this site, the variability on *Ostreopsis* spp. abundances could also be due to the characteristic morphology of the coast, consisting in a small bay with different expositions affecting in different ways important environmental factors such as hydrodynamics and light. On the opposite, in Vernazzola the coast is more homogenous and the whole sampling location is equally exposed, possibly leading to smaller variability in cell abundances (Figure 1). Furthermore, *Ostreopsis* spp. abundance variability in Rochambeau could be spatial and temporal, as the exposition of the coast can condition the sooner or later development of the bloom (Chang et al., 2000; Shears and Ross, 2009b; Accoroni and Totti, 2016; Fricke et al., 2018).

Our results highlight that preferences of *Ostreopsis* spp. can be species-, site- and period of the bloom-specific. Effects at a larger scale (i.e. at the macrohabitat scale, not assessed in this study) could in turn directly influence *Ostreopsis* spp. abundances or have indirect effects influencing the dominance of specific macroalgal communities that could facilitate or regulate *Ostreopsis* spp. blooms (Mangialajo et al., 2008b). Results from this study show how variable *Ostreopsis* spp. cell assessment can be in function of the scale (micro and mesohabitat) and of the quantification method

used, opening new research topics such as the study of blooms at a bigger scale (macrohabitat) and testing different methods for the standardization of the cells independently on the substrate. Some patterns of substrate's preferences for *Ostreopsis* spp. proliferation have been observed, indicating the role that benthic communities can potentially play in the frequency and magnitude of blooms. The effect of the dominant macroalgal communities has to be further investigated in order to: (i) mitigate BHABs on current and future regime shift scenarios, where the most complex forest-forming macroalgal communities, possibly hosting fewer abundances of benthic toxic dinoflagellates, are disappearing (Rindi et al., 2020; Pessarrodona et al., 2021); (ii) better assess locations with high public health risk of BHABs and (iii) estimate priority locations for possible actions of mitigation (remediation and/or restoration).

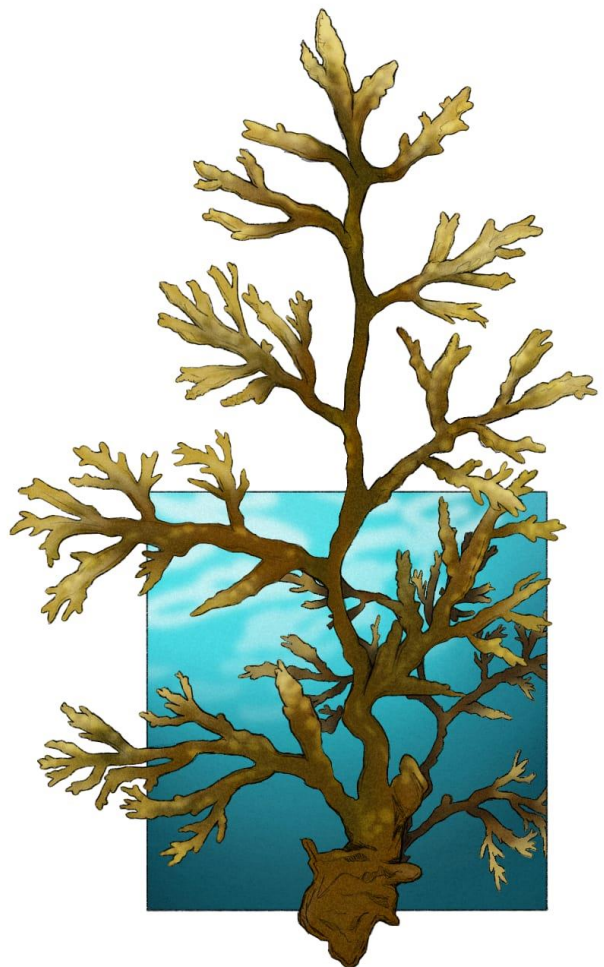
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# GENERAL DISCUSSION AND CONCLUSIONS





The aim of this dissertation is to unravel some of the effects of global change on shallow *Cystoseira s.l.* forests, using a cosmopolitan habitat-forming species, *Cystoseira compressa*, as a model species and to assess if the proliferation of blooms of *Ostreopsis* spp. could be a consequence of *Cystoseira s.l.* forests loss. Considering that in each chapter a specific discussion was addressed, we present here the main findings of the experiments focussing on some causes involved in the loss of *Cystoseira s.l.* forests and the consequences, such as the proliferation of benthic harmful algal blooms (BHAB) of *Ostreopsis* spp. In this section, we provide a discussion on the implications of the loss of coastal Mediterranean marine forests and the expansion of *Ostreopsis* spp. in the conservation of shallow coastal habitats, as well as a view of the application and contribution of the thesis to coastal conservation, management and restoration.

### **THE DECLINE OF MARINE FORESTS AND REGIME SHIFTS**

Forest-forming brown macroalgae, covering ~28% of the coastline worldwide have been valued at over 10<sup>6</sup> USD km<sup>-1</sup> year<sup>-1</sup> (Filbee-Dexter and Wernberg, 2018) and their conservation and restoration is clearly justified by the importance of these species in hosting high biodiversity, providing several ecosystem services and being highly productive (Filbee-Dexter and Wernberg, 2018; Wernberg et al., 2019a; Pessarrodona et al., 2022). It is then critical to understand the factors promoting the decline of forest-forming brown macroalgae and its consequences for ecosystem functioning, such as fostering harmful algal blooms. Therefore, we investigated different ecological interactions, such as species facilitation (Chapters 1, 3 and 4), and herbivory (Chapter 2), which are key factors in structuring communities and ecosystems (Stachowicz, 2001; Kordas et al., 2011; Kroeker et al., 2013b).

Microalgae and macroalgae are the base of marine trophic nets and any perturbation in their populations can have an enormous effect on the whole ecosystem (Cardona et al., 2013; Smale et al., 2022). Human impacts on forest-forming macroalgae are expected to continue or increase in the future (Halpern et al., 2007, 2019), potentially leading to changes in the macroalgal composition and its structural complexity (i.e. regime shifts). The substitution of long-lived large brown forest-forming species, including *Cystoseira s.l.* spp., by fast-growing opportunistic turf-forming macroalgae and even barren grounds (Chemello et al., 2018; Filbee-Dexter and Wernberg, 2018; O'Brien and Scheibling, 2018) have already been reported along hundreds of kilometres along Canadian, European, and Australian coasts (Filbee-Dexter and Wernberg, 2018). Regime shifts have also been observed for *Cystoseira s.l.* spp. in several areas of the Mediterranean Sea. It is expected that only the most tolerant populations or species will be able to resist under future global change scenarios (Thibaut et al., 2005, 2015; Blanfuné et al., 2016; Rindi et al., 2020; Orlando-Bonaca et al., 2021a). For example, in the Mediterranean Sea, Thibaut et al. (2005) and (2015) report the high regression and fragmentation of *Cystoseira s.l.* populations in the French Riviera

and Alberes Coast (North Western Mediterranean), where only five of the fifteen historically present species were still thriving and only *C. compressa* presented no signs of regression. The causes responsible for regime shifts and miniaturisation of habitats are multiple and ultimately related to anthropogenic activities (Benedetti-Cecchi et al., 2001; Mangialajo et al., 2008b; Sala et al., 2012; Chemello et al., 2018). Some of the abiotic and biotic stressors related to marine forests decline have been addressed in this thesis, using the Mediterranean widely distributed *Cystoseira compressa* as a model for forest-forming macroalgae. *C. compressa*, independently of being the only non-protected *Cystoseira s.l.* spp. (Verlaque et al., 2019), is considered very tolerant to manipulation and interesting in the restoration of Mediterranean marine forests due to its potential facilitation role in the recruitment of other forest-forming species (Mangialajo et al., 2012).

### **ABIOTIC INTERACTIONS ON MARINE FORESTS: CLIMATE CHANGE**

Abiotic factors such as habitat destruction, local pollution, eutrophication and overfishing contribute to the loss of marine forests (Connell et al., 2013; de Caralt et al., 2020; Smale, 2020; Verdura et al., 2021), but climate change is expected to be the ultimate impact that all species around the world will need to overcome, especially macroalgae (Doney et al., 2012; Harvey et al., 2013; Smale, 2020).

#### ***Ocean warming***

Ocean warming (OW) is expected to affect forest-forming macroalgae around the globe, including *Cystoseira s.l.* spp. (Wernberg et al., 2016; Capdevila et al., 2019; Smale, 2020). Several studies in the Mediterranean Sea already linked the increase in seawater temperatures with (i) perturbations in the phenology of *Cystoseira s.l.* (Bevilacqua et al., 2019), (ii) a decrease in the resilience of adults (Capdevila et al., 2019), and, in agreement with the results presented in this thesis, (iii) a higher mortality of early-life stages in the laboratory and in the field (Falace et al., 2018, 2021; Capdevila et al., 2019; Verdura et al., 2021). Because early-life stages are more sensitive than adults to any change in environmental conditions (Coelho et al., 2000; de Caralt et al., 2020; Verdura et al., 2021), and present high mortality rates (Chapman, 1995), they are considered as a bottleneck for the continuity of the populations. Therefore, further studies on the recruits of these species are needed for the conservation and restoration of marine forests of *Cystoseira s.l.* spp.

In our experiment, the warmest temperature (32°C) had a negative effect on the density of recruits since the start of the experiment, while differences in the density of recruits at 28°C and 24°C were only observed after one month and a half. However, no differences in the size of recruits relative to the different temperature treatments were observed until the third month, potentially indicating that the temperature was not affecting the growth of the surviving individuals (Celis-Plá et al., 2015, 2017a; Mancuso et al., 2019). These results on *C. compressa* show how recruits of this species could be more tolerant and resistant to climate change, and thus more widely distributed, than early-

life stages of other *Cystoseira s.l.* spp. (Bennett et al., 2022). For example, *Ericaria crinita*, drastically decreased in density after 5 days in experimental tanks at 28°C (Verdura et al., 2021) or *Ericaria giacconei*, which zygotes did not survived at 28°C in the laboratory (Falace et al., 2021). The contrasting responses against OW of different *Cystoseira s.l.* spp. manifest that not all the *Cystoseira s.l.* spp. are ecological analogues (Bruno de Sousa et al., 2019; Orellana et al., 2019). In general, the negative effects of OW have already been observed on other forest-forming species, both on recruits (Leal et al., 2018; Capdevila et al., 2019; Verdura et al., 2021) and adults (Lind and Konar, 2017; Fernández et al., 2020; Smale, 2020; Falace et al., 2021).

Specific studies on the effect of OW on adults of *C. compressa* in the Mediterranean Sea have also been performed. In a mesocosm experiment, Bennett et al. (2022) found the maximum gross primary production of *C. compressa* from Cyprus and Mallorca (Balearic Islands) at  $28 \pm 4$  °C, and a thermal limit where the net production become negative at  $23 \pm 0.5$  °C. The results reported by Bennett et al. (2022) on adult *C. compressa*, presumably less sensitive than recruits, are comparable with the mortality we observed in the tanks at similar temperatures. Our results in the treatments at warmer temperatures (28 °C and 32°C) are also in concordance with the results reported by Mancuso et al. (2019) who observed a decrease in the photosynthetic activity of *C. compressa* from 28°C. Moreover, *C. compressa* shows a reduction in net production (Bennett et al., 2022) and an increase in phenolic contents (Mancuso et al., 2019) with the increment in temperature. The impacts of OW and extreme climatic events such as marine heat waves have also been claimed as responsible for the decline and local extinction of *Cystoseira s.l.* populations in the Mediterranean Sea (Mariani et al., 2019; Verdura et al., 2021). In fact, OW can, in some cases, limit populations to specific refuge locations with more favourable conditions (Smale, 2020; Verdura et al., 2021). Finally, indirect temperature-driven effects, such as the range expansion of warm water, fast-growing or opportunistic species, on marine forests are not negligible, because they contribute to the tropicalization of habitats and can re-shape macroalgal communities (Vergés et al., 2014a, 2014b).

### ***Ocean acidification***

Ocean acidification (OA) due to increasing anthropogenically-derived atmospheric CO<sub>2</sub> levels is leading to an increase in dissolved inorganic carbon (DIC), a decrease in pH, CO<sub>3</sub><sup>2-</sup> and carbonate saturation state, having adverse consequences for marine calcifiers, such as crustose coralline algae, and probably affecting the organisms' cellular homeostasis (Hurd et al., 2020). Nevertheless, the increase in CO<sub>2</sub> and DIC levels can be beneficial for some non-calcifying macroalgae (Connell et al., 2013; Cornwall et al., 2017a). For instance, OA could stimulate photosynthesis (Connell et al., 2013; Fernández et al., 2015; Rautenberger et al., 2015; Zhang et al., 2020), thus favouring carbon-limited macroalgae and macroalgae with the ability to down-regulate their carbon concentration

mechanisms (CCM; Cornwall et al., 2017a; Cornwall and Hurd, 2019), altering the dominant species and re-shaping macroalgal communities (Hepburn et al., 2011; Connell et al., 2013; Koch et al., 2013; Sunday et al., 2017).

Besides the evident impact of climate change on forest-forming species around the world, studies on the interactive effects of OW and OA have focused on Laminariales rather than on Fucales (Poore et al., 2013; Gaitán-Espitia et al., 2014; Leal et al., 2018; Hollarsmith et al., 2020; Fernández et al., 2021) and to my knowledge, this study represents the first quantification of the effects of OA on the recruitment of *Cystoseira s.l.* spp. In the first chapter of this thesis, and as reported in other studies, OA seems to have a less pronounced effect on recruits of *C. compressa.* than OW. Experiments on the effects of OA on adults of *Cystoseira s.l.* spp. (Celis-Plá et al., 2017b, 2017a) showed that OA could be beneficial when there are enough light and nutrients and in moderate temperature ranges. In agreement with our results, the few studies investigating the interactive effects of OW and OA on adult *Cystoseira s.l.* spp., performed on *Ericaria selaginoides* by Celis-Plá et al. (2017b, 2017a), found that the negative effects were due to the increase in temperature. The same authors showed that low pH increased biomass, maximal electron transport rate ( $ETR_{max}$ ), polyphenol content and antioxidant activity ( $EC_{50}$ ) of *E. selaginoides* in ambient temperature (20°C). They also concluded that ongoing OW and OA can increase photoprotection and photosynthesis of shallow forests of *Cystoseira s.l.* spp.

In our experiment, lower pH levels positively affected the size of *C. compressa*, potentially showing a better performance under elevated CO<sub>2</sub>. Indeed, OA seems to favour the size of *Cystoseira s.l.* spp. (Celis-Plá et al., 2017b, 2017a), including *C. compressa*, even if they are considered CCM species that cannot down regulate their CCM and thus are not expected to be as favoured by OA as other carbon-limited macroalgae or macroalgae that can down regulate their CCM (Cornwall et al., 2017a). In the Mediterranean Sea, surveys of coastal CO<sub>2</sub> seeps have repeatedly shown how brown macroalgae, such as *Cystoseira s.l.* spp., *Dictyota* spp., *Sargassum vulgare* and *Padina pavonica*, proliferate as CO<sub>2</sub> levels rise (Porzio et al., 2011; Baggini et al., 2014). Moreover, one specific study performed in CO<sub>2</sub> vents investigating the effects of OA on *C. compressa*, reports that in elevated CO<sub>2</sub> and shaded conditions, *C. compressa* had higher carbon content and antioxidant activity, independently of nutrients concentrations (Celis-Plá et al., 2015).

However, future responses of *Cystoseira s.l.* spp. to OA may depend on multiple factors, such as temperature, light and nutrient availability (Celis-Plá et al., 2014, 2015, 2017b, 2017a). For example, Britton et al. (2016) reported that *Eklonia radiata*, a CCM-enabled species, relies on diffusive CO<sub>2</sub> as a carbon source for photosynthesis when exposed to lower daytime pH, but did not show an increase in growth rates, as expected when CCMs are down-regulated. Some studies on the giant kelp *Macrocystis pyrifera* (Roleda et al., 2011; Leal et al., 2017) reported better

performance and increased growth rates of the macroalgae under OA treatments, while others did not report any benefit of elevated CO<sub>2</sub> on the photosynthesis and growth of the same species (Gaitán-Espitia et al., 2014; Fernández et al., 2021). This suggests that the benefits of OA on non-calcifying macroalgae depend on multiple drivers or combined effects of multiple environmental factors, like temperature and nutrient availability, (Fernández et al., 2015) and also on the possible effects of the seawater carbonate system on physiological processes (Hurd et al., 2020).

In the first chapter, the effects of the low pH on the density of recruits of *C. compressa* seemed to be negatively stronger during the first experimental days. Similarly, OA showed a negative influence on the settlement and early-life stages of other forest-forming macroalgae such as giant kelp (Gaitán-Espitia et al., 2014; Hollarsmith et al., 2020), and other key species such as corals and molluscs (Kroeker et al., 2013a). These results together with the magnified effects of OW on recruits of *Cystoseira s.l.* spp. (Capdevila et al., 2019; Falace et al., 2021; Verdura et al., 2021) has led to the paradigm that early-life stages could be more sensitive to global change and therefore could constitute a bottleneck (Chapman, 1995; Coelho et al., 2000).

Overall, OA could act both as a stressor and as a resource (Connell et al., 2013), favouring the replacement of some species by others more resistant and with different functional roles, compromising ecosystem function and stability (Kroeker et al., 2013b; Teixidó et al., 2018). Changes in the biomass of macroalgal species can directly affect their consumers (Ghedini and Connell, 2016; Nagelkerken and Connell, 2022), leading to bottom-up driven alterations to the food web (Nagelkerken et al., 2020; Smale et al., 2022). Under climate change, key species could be replaced by opportunistic species able to survive in disturbed environments (O'Brien and Scheibling, 2018; Álvarez-Losada et al., 2020) and physiologically less sensitive to the effects of OA (Connell et al., 2013; Wittmann and Pörtner, 2013; Sunday et al., 2014).

Our results on OW and OA are striking for *C. compressa* thriving in rock pools, which are expected to be more acclimated to large variability in environmental factors (i.e. temperature, pH, salinity). Rockpools can experience strong fluctuations of biotic and abiotic factors due to biological activity, irradiance variation, tides, weather-driven changes in water level, etc (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983; Olabarria et al., 2013). *C. compressa* can express a high plasticity in function of the origin location (Bennett et al., 2022), and our recruits, obtained from a donor population situated in a rockpool, are expected to be adapted to a high range of temperatures, pH and salinity. Populations usually exposed to larger extremes in temperatures, such as our population, could have higher thermal tolerances than populations of the same species not acclimated to such environmental variations, however, the effects of pH variability are less clear to provide a better adaptation to future OA (Rivest et al., 2017; Cornwall et al., 2018; Legrand et al., 2018). It has already been demonstrated how intraspecific variability of other *Cystoseira s.l.* spp.



to certain environmental parameters such as irradiance allows these species to be better adapted to them (Sant and Ballesteros, 2020b, 2021). Nonetheless, in habitats where organisms are near, or at their physiological limits, environmental variability could exacerbate even more the future effects of climate change (Lawson et al., 2015; Pansch and Hiebenthal, 2019; Kroeker et al., 2020). Therefore, it is very important to know the variability species are dealing with at present as this could affect their responses under climate change scenarios or ongoing marine heat waves.

Assessing the interactive effects of OW and OA on marine forests is, therefore, essential for their conservation and restoration. Next steps to evaluate the responses of Mediterranean marine forests under climate change could be: (i) testing the interactive effects of OW and OA on recruits of other *Cystoseira s.l.* spp., including deep-sea species, as some species could be more sensitive than others (Capdevila et al., 2019; Falace et al., 2021; Verdura et al., 2021); (ii) studying the effects of climate change on recruits of the same species from different locations, e.g. from the open coast and from rockpools, as the effects of their environmental story could make them more resistant to variations in environmental factors (Fernández et al., 2021; Sant and Ballesteros, 2021; Verdura, 2021; Bennett et al., 2022); (iii) elucidating the interactive effects of climate change on the development and settlement of the recruits (Leal et al., 2017; Savonitto et al., 2019); and (iv) assessing how the photosynthetic activity and metabolome of the recruits change under future climate change scenarios (Fernández et al., 2015; Celis-Plá et al., 2017a; Mancuso et al., 2019).

## **BIOTIC INTERACTIONS ON MARINE FORESTS: SPECIES FACILITATION AND HERBIVORY**

### ***Species facilitation***

Marine forests are complex and rich ecosystems interacting (i.e. predation, competition, facilitation, allelopathy) with other species of macroalgae, microalgae, vertebrates, and invertebrates. Therefore, in the first chapter we considered it important to evaluate the direct and indirect effects of climate change in presence of other species. Several studies have already investigated the role the canopy has on the maintenance of the understory crustose coralline algae (Melville and Connell, 2001; Connell, 2003; Irving et al., 2004, 2005; Irving and Connell, 2006), however, the role of understory crustose coralline algae on the latter successional forest-forming species is less well understood (Benedetti-Cecchi and Cinelli, 1992; Asnaghi et al., 2015). We evaluated the role of one of the most common crustose coralline algae in shallow Mediterranean forests, *Neogoniolithon brassica-florida* (Benedetti-Cecchi and Cinelli, 1992; Braga et al., 2009), on the recruitment of *C. compressa* under different OW and OA treatments. Our results show strong effects of OW and OA on the calcification rate of *N. brassica-florida* which could have cascading effects on later successional species such as *C. compressa*. From the start of our experiment, *C. compressa* showed lower densities when associated with the crustose coralline algae, either living or dead. Studies at

CO<sub>2</sub> seeps (Porzio et al., 2011; Baggini et al., 2014) have reported a decrease in coralline algae abundance with decreasing pH and CO<sub>3</sub><sup>2-</sup>, while brown algae, such as *Cystoseira s.l. spp.*, proliferated. This suggests that the disappearance of crustose coralline algae does not affect the maintenance of *Cystoseira s.l. spp.* Even if some studies (Asnaghi et al., 2015) found a facilitation effect of *N. brassica-florida* on *Cystoseria s.l. spp.*, our experiment showed lower densities of recruits of *C. compressa* in association with this crustose coralline algae, independently of the treatment. This result potentially relates to the anti-fouling mechanisms of crustose coralline algae (Johnson and Mann, 1986; Keats et al., 1997; Villas Bôas and Figueiredo, 2004) and is of major importance for future restoration actions, because it suggest that natural biotic substrate are not necessarily the best option. Supporting the suitability of the bare substrate, including the widely used artificial clay substrate in the Mediterranean Sea (Falace et al., 2018; De La Fuente et al., 2019) for the settlement of *Cystoseira s.l. spp.*

### **Forest-forming macroalgae as refuge from ocean acidification**

Forest-forming macroalgae can metabolically induce diel pH fluctuations through photosynthesis and respiration (higher pH during the daytime, lower at night) modifying local seawater chemistry and therefore, potentially acting as a refuge from OA in the vicinity of these habitats for sensitive species such as calcifying organisms (Middelboe and Hansen, 2007; Wootton et al., 2008; Krause-Jensen et al., 2015, 2016). Nevertheless, it is not established if these refuges from OA could still be effective under future OA scenarios (Britton et al., 2016; Murie and Bourdeau, 2020), especially considering the above mentioned negative effects of climate change on marine forests. *Fucus vesiculosus* can induce diurnal pH fluctuations of about one pH unit (Middelboe and Hansen, 2007; Saderne and Wahl, 2013), but the magnitude of the fluctuations would be inversely proportional to the spatial scale considered, being more important in locations with little water turnover rates such as rockpools (Wahl et al., 2015, 2018). Moreover, pH fluctuations are not temporally and spatially uniform at the community and organismal scales (Britton et al., 2016). For example, Houlihan et al. (2020) show that an increase in irradiance and thus, photosynthesis, and low hydrodynamics, increase pH and the thickness of the diffusion boundary layer (Hurd et al., 2011; Cornwall et al., 2015) of crustose coralline algae, affecting an habitat at the dozen of microns to millimetres scale, in which sea urchin larvae, and other species of invertebrates and algae can settle. However, this increase in pH would not perdure during the night due to strong variations in the pH at such a small scale because of respiration processes (Hurd et al., 2011; Cornwall et al., 2015). In fact, Cornwall et al. (2013) report pH fluctuations of 0.94 units within a shallow *Macrocystis pyrifera* forest demonstrating that diurnal variability in pH was as important as a mean decrease on pH due to OA in controlling the growth rates of understory coralline algae.

### ***Herbivory***

Biotic factors such as the proliferation of herbivorous species can be responsible for driving complex systems toward range shifts (Sala et al., 1998, 1999; Chemello et al., 2018). In tropical areas, the impact of benthic herbivores such as sea urchins is well documented (Lewis and Wainwright, 1985; Foster, 1987; Coyer et al., 1993; Burkepile and Hay, 2008) although omnivorous and herbivorous fishes exert the greatest control on macroalgal abundance and distribution (Hiatt and Strasburg, 1960; Cronin et al., 1997; Vergés et al., 2016). In contrast, in temperate areas, herbivory by benthic invertebrates, especially sea urchins and gastropods seems to be predominant (Hagen, 1983; Harrold and Reed, 1985; Norderhaug and Christie, 2009; Filbee-Dexter and Scheibling, 2014). However, while there is a considerable amount of information showing an inverse correlation between the abundance of macroalgae and sea urchin densities (Giakoumi et al., 2012; Sala et al., 2012), the relationship between herbivorous fish and other invertebrates (decapods, gastropods, amphipods, isopods) and the conservational status of *Cystoseira s.l.* forests has not been assessed. Recently, the proliferation of herbivorous fish such as *Sarpa salpa*, and also omnivorous species such as *Diplodus vulgaris*, *Coris julis* or *Thalassoma pavo*, in the Mediterranean Sea has been related to the formation of shrubs and turfs, where forest-forming species are substituted by erect and/or turf-forming macroalgae (Vergés et al., 2009; Gianni et al., 2017; Papadakis et al., 2021). These post regime shift scenarios (i.e. shrubs, turfs or barren grounds), either created by the proliferation of sea urchins, herbivorous fish or other herbivores, are considered stable states because of the difficulty to reverse the ecosystem to its original state of healthy marine forests, mainly due to the demographic characteristics of the forest-forming macroalgae (i.e. long lived and short dispersal; Chapman, 1995; Capdevila et al., 2018; Riquet et al., 2021). The effects of gastropods, decapods, amphipods and isopods have also been investigated on forest-forming species, revealing a variety of behaviours of the herbivores towards the macroalgae such as physical damage or clipping, facilitation effects by removing sediments or herbivory (Pavia and Toth, 2000; Perkol-Finkel et al., 2012; Gutow et al., 2014; Suzuki et al., 2020). However, the herbivory pressure is more studied on adults of *Cystoseira s.l.* spp. (Vergés et al., 2009, 2014b; Gianni et al., 2017; Papadakis et al., 2021) than on recruits. Since some studies found that a variety of invertebrates could graze on early-life stages of forest-forming macroalgae (Lubchenco, 1983; Korpinen and Jormalainen, 2008; Alestra and Schiel, 2014; Suzuki et al., 2020; Barrientos et al., 2021; Savonitto et al., 2021) we wanted to study the potential herbivory effect of species usually present within shallow *Cystoseira s.l.* forests on recruits of these species.

In the Mediterranean Sea, the grazing on *Cystoseira s.l.* spp. has been principally studied on adults and for sea urchins and *Sarpa salpa* (Vergés et al., 2009; Agnetta et al., 2015; Gianni et al., 2017; Medrano et al., 2020; Miller et al., 2022). But as herbivory is believed to contribute to the high mortality observed during early-life stages (Chapman, 1995), in the second chapter we performed

a series of experiments in the field and in the laboratory to better understand the herbivory pressure on *Cystoseira s.l.* recruits and for the first time, we selected small size non-strict herbivorous species (*Clibanarius erythropus*, *Cerithium vulgatum*, *Idotea balthica* and *Gammarus* sp.). The experiment in the field allowed us to elucidate the herbivory pressure in locations with different characteristics (e.g. presence or past presence of a *Cystoseira s.l.* forest). We observed a high mortality rate of the early-life stages, possibly linked to density-dependant processes, but also a significant effect of the herbivory protection. However, the effects of herbivory on recruits in the field should be reviewed and monitored during larger periods of time, as *Cystoseira s.l.* spp. are long-lived species and macroalgal populations that need decades to become functional and self-sustaining.

The experiments in the laboratory reported in the second chapter of this thesis also demonstrate that a variety of other non-strict herbivorous species usually found in shallow macroalgal communities (*Clibanarius erythropus*, *Cerithium vulgatum* and *Idotea balthica*) can graze on recruits of *C. compressa*, potentially contributing to the mortality of recruits observed in the field. These experiments represent a first approach to evaluate the role of potential herbivores and to elucidate which species must be considered to reduce or avoid grazing on recruits of *Cystoseira s.l.* spp. The results observed during our experiment are in agreement with the results performed with other species of fucoids and other macrophytes, reporting the important role *Clibanarius erythropus*, *Cerithium vulgatum* and *Idotea balthica* exert in structuring macroalgal assemblages (Jonne et al., 2006; Vergés et al., 2007; Gunnarsson and Berglund, 2012; Suzuki et al., 2020). Our results showed that fish, sea urchins, decapods, gastropods and isopods can exert a top-down control potentially limiting the recruitment of shallow *Cystoseira s.l.* forests by consuming early-life stages of these species.

Contrarily to the effects of OW and OA on marine forests, the effects of the proliferation of herbivores are usually local and, therefore, could be addressed to prevent the loss of key species with effective management of affected adult or young restored populations (Gianni et al., 2018, 2020; Suzuki et al., 2020; Tamburello et al., 2021). To achieve this, recruits and mature adults of threatened populations should be protected against herbivory by using deterrent devices to ensure the continuity of the population (Gianni et al., 2020; Savonitto et al., 2021). In the case of smaller invertebrates the herbivory deterrents may not be sufficient and when considering restoration actions the use of more expensive *ex-situ* cultivation techniques could be evaluated, as larger recruits could be less susceptible to the herbivory effects of smaller invertebrates (Cebrian et al., 2021). Despite that, these measures will not address the underlying cause of the proliferation of herbivores and are unlikely to provide a long-term solution to conserve and restore forests and ecosystem function on its own (Miller et al., 2022). Due to the growing number of restoration projects the topic of herbivory is more up to date than ever for being considered as one of the main causes for unsuccessful restoration actions of marine forests (Gianni et al., 2013; Tamburello et al., 2019;

Savonitto et al., 2021). The regulation of populations of herbivores could also be considered as tested by Medrano et al. (2020) and Miller et al. (2022). Therefore, when considering or performing restoration actions it is critical to include herbivory management actions to limit its effects on the survival of recruits and adults transplanted (Cebrian et al., 2021). As an example, the proliferation of sea urchins has been largely related with the creation of barren grounds or marine deserts (Giakoumi et al., 2012; Sala et al., 2012; Azzarello et al., 2014; Agnetta et al., 2015) and their removal is considered a tool for restoring macroalgal forests (Miller et al., 2022). Assessing the herbivory pressure in marine forests is essential for their conservation and restoration and for the conception of efficient herbivory deterrents. Further studies could be: (i) better identifying species grazing on *Cystoseira s.l.* populations in function of the life stage, (ii) performing feeding preference experiments to elucidate the real impact that potential herbivores have in locations where marine forest populations are present, and (iii) to elucidate if the feeding preferences and behaviours of these species can change under global change (Barrientos et al., 2021).

In particular, climate change can affect herbivores either by altering their densities, changing their feeding behaviour, their food preferences or their consumption rates (Gutow et al., 2014; Barrientos et al., 2021; Mitterwallner et al., 2021). On one hand, sea urchins have been reported to decrease in abundance near natural CO<sub>2</sub> seeps, possibly decreasing its herbivory rate and being less competitive under OA (Porzio et al., 2011; Asnaghi et al., 2013, 2020; Baggini et al., 2014). On the other hand, the proliferation of non-indigenous warm water herbivorous fish, such as *Siganus* spp., is an indirect effect of OW on macroalgal communities, potentially leading to higher herbivory pressure and regime shifts (Vergés et al., 2014a, 2014b; Bennett et al., 2015b; Barrientos et al., 2021). It is suggested that range-shifting herbivorous fish can also affect recruits by consuming microscopic stages present in the turf matrix (Bennett et al., 2015b; Barrientos et al., 2021). In addition, metabolic rates of herbivores could increase due to OW and OA increasing also their consumption rates (Rich et al., 2018). For example, according to Barrientos et al. (2021) the feeding rates on early-life stages of *Eklonia radiata* were higher under most tropicalized scenarios. Also, the carbon content of macroalgae could increase under OA altering, in some cases, the consumption rates by herbivores (Gutow et al., 2014; Leung et al., 2018).

### **MARINE FORESTS LOSS AND FACILITATION OF BLOOMS OF TOXIC BENTHIC DINOFLAGELLATES**

The degradation and losses that marine forests are experiencing and will experience under global change, will have profound consequences for the ecosystems (Álvarez-Losada et al., 2020; Smale et al., 2022). We have already discussed some causes responsible for marine forests loss: the abiotic interactive effect of OW and OA and the biotic effects of species facilitation and herbivory on recruits of *C. compressa*. In the framework of interspecific interactions, in the two last chapters of

this thesis, we investigated a possible effect of marine forests loss, in particular the blooms of *Ostreopsis* spp. The results from the third chapter of this thesis, in agreement with the results of the latest bibliographic reviews on the ecology of toxic benthic dinoflagellates (Berdalet and Tester, 2018; Tester et al., 2020), clearly demonstrate the current proliferation of blooms of *Ostreopsis* spp. in temperate zones, including the Mediterranean Sea. The observed increase in harmful algal blooms in the last decades relates to more effective detection through improved observation and monitoring capacities (Hallegraeff et al., 2021), but also to the increasing anthropogenic impacts and global climate change effects (Berdalet et al., 2017; Tester et al., 2020).

It is expected that the ongoing OW can facilitate the expansion and proliferation of blooms of the genus *Ostreopsis*, as their seasonal variation is often linked to temperature fluctuations, especially in the warm season (Ingarao and Pagliani, 2013; Accoroni and Totti, 2016). The effects of OA on *Ostreopsis* spp. still require further investigations, but Di Cioccio et al. (2014) found bloom abundances of *Ostreopsis* cf. *ovata* in CO<sub>2</sub> seeps in Ischia Island (Mediterranean Sea) where pH values are similar to those predicted for the end of the century. Furthermore, an indirect effect of OW and OA on the proliferation of blooms in tropical areas could be the substitution of corals for macroalgae, that are a preferred substrate for the development of *Ostreopsis* spp. (Berdalet et al., 2017). Our results, predicting larger abundances of *Ostreopsis* spp. on shrub and turf-forming macroalgal communities; also foresee climate change to indirectly promote the development of blooms of *Ostreopsis* spp. through regime shifts, either from corals to macroalgae (Jouffray et al., 2015; Rains and Parsons, 2015, 2015; Johns et al., 2018) or from complex macroalgal communities to less complex ones (Bulleri et al., 2016; Filbee-Dexter and Scheibling, 2017; Pessarrodona et al., 2021), that can trigger this species development (Fraga et al., 2012). Despite that, our bibliographic review makes patent that the ecological aspects involved in the proliferation of blooms of *Ostreopsis* spp. are still largely understudied and that most of the studies do not focus on the ecology of these species (Pavaux et al., 2020) and in particular on the description of the meso- and macrohabitat of the sampled locations.

Even if the growth, abundance, and distribution of benthic dinoflagellates are controlled by chemical (nutrients and salinity) and physical (temperature, irradiation and hydrodynamics) factors, biological (competition, predation, facilitation) factors are likely to influence *Ostreopsis* spp. (Pavaux et al., 2020; Tester et al., 2020). It has been recently suggested that the characteristics of the macroalgal substrate, such as the thallus architecture and the production of allelopathic compounds, could facilitate or regulate the development of epibenthic dinoflagellates (Totti et al., 2010; Accoroni et al., 2015; Ternon et al., 2018; Pavaux et al., 2020; Tester et al., 2022). Allelochemicals produced by many macrophytes can inhibit or stimulate epibionts (Accoroni et al., 2015; Ternon et al., 2018, 2020) and consequently affect the epibenthic community. Following the trend highlighted in the third chapter, where certain macroalgal groups are more commonly sampled



in *Ostreopsis* spp. studies, we performed a preference experiment in the field. In the last chapter, we tested the effects of the micro- and mesohabitat on the facilitation of *Ostreopsis* spp. proliferation in the field, during an annual bloom. The effects at large scale were, in some cases, more important than the effects at small scale for the proliferation of *Ostreopsis* spp. The high variability on cell abundances and the contradictory results among macroalgal species and habitats in function of the sampling and methodology used for the quantification of the cells, has already been reported in other studies (Berdalet et al., 2017; Jauzein et al., 2018; Tester et al., 2022). At the small scale, the variability in the abundance of cells can be high, and linked to local habitat characteristics such as hydrodynamics, light exposure, predation, nutrients, available surface, etc. (Berdalet et al., 2017; Tester et al., 2020). However, our results indicate that the physical structure of macroalgae alone does not explain the preferences of *Ostreopsis* spp. Overall, according to our results, *Dictyota* spp. seems to be a good substrate for the development of *Ostreopsis* spp. (this thesis Chapter 4; Park et al., 2020; Ibgghi et al., 2022), contrarily to the results from studies on allelopathic interactions (Accoroni et al., 2015; Ternon et al., 2020) that suggest a strong negative effect of some Dictyotales on *Ostreopsis* cf. *ovata*. The preferences could be due to physical characteristics of the macroalgae, to their allelochemical proprieties, to the presence of other organisms from the epiphytic community or to environmental factors acting at larger scale (Ternon et al., 2018; Pavaux et al., 2020).

As reported, in the last two chapters, abundances of *Ostreopsis* spp. can change in function of the methodology used in the sampling and quantification of the cells (Mangialajo et al., 2017; Jauzein et al., 2018; Vassalli et al., 2018). Therefore, the highly used quantification of cells of *Ostreopsis* spp. per gram FW of macroalgae may not be representative of what is really happening in the macroalgal community, as it is dependent on the specific weight of the macroalgae. The methodologies and quantifications that are best representing the reality of the ecosystem should be further investigated and better implemented in future studies. Finding the ideal sampling procedure for *Ostreopsis* spp. will not be an easy task, and should reflect the real risk of bloom for the sampled location. Also, the method should consider the structural complexity and characteristics of the benthic habitats sampled, the diversity of available substrates and the spatial and temporal scales of variability (Berdalet et al., 2017). The high variability on preferences of *Ostreopsis* spp. at the small scale (micro- and mesohabitat) could be influenced by conditions acting at the large scale, at the ecosystem level (abundance of patches of favourable mesohabitat, hydrodynamics, water quality temperature...), and for this reason so far has not been possible to reach clear conclusions (Accoroni and Totti, 2016; Fricke et al., 2018).

Macroalgal species can affect benthic dinoflagellates abundances, but epiphytic dinoflagellates in turn can also affect the macroalgal substrates (Iveša et al., 2021). For example, *O.* cf. *ovata* cells show a high affinity for ammonia (Berdalet et al., 2017; Jauzein et al., 2017) which can lead to

thallus necrosis in *Cystoseira s.l.* populations due to the ammonium concentrations present on the mucilaginous aggregates of the microalgae during blooms (Iveša et al., 2021). Further studies are needed to investigate the effects of *Ostreopsis* spp. biofilm on macroalgae, therefore we cannot exclude that recurrent blooms of benthic dinoflagellates could constitute another factor favouring the local loss of fucoids (Iveša et al., 2021). To better understand the link between the disappearance of marine forests and the proliferation of blooms of *Ostreopsis* spp., we should collect information about the deterioration of the environmental parameters in locations with annual bloom of *Ostreopsis* spp., and historical and present data about marine forest presence.

Managing harmful algal blooms is extremely important, and with the increasing number of coastal urban areas, mitigating the impacts of blooms of *Ostreopsis* spp. on human health is becoming of major interest (Berdalet et al., 2016, 2022). The contact with contaminated seawater or the inhalation of aerosols can result in human intoxications and with ecological (mass mortalities of invertebrates) and socio-economic consequences in coastal touristic locations, such as the Mediterranean coast (Berdalet et al., 2016, 2017, 2022). It is estimated that in the Provence-Alpes-Côte-d'Azur region, N-W Mediterranean Sea, an increase in *Ostreopsis* spp. outbreaks could have an economic cost from several hundred thousand to several million euros from loss tourism (Lemee et al., 2012). The results obtained in this thesis highlight the high variability of *Ostreopsis* spp. abundances on macroalgal species and communities. However, the comparisons of cell abundances of *Ostreopsis* spp. on macroalgal species is controversial and makes it difficult to find clear patterns for the development of blooms of *Ostreopsis* spp. Next steps to better elucidate the relationship between *Ostreopsis* spp. blooms and macroalgal communities should involve: (i) setting similar studies at large scale, avoiding transplantation of missing macroalgal communities (Bravo et al., 2012, 2020; Accoroni et al., 2014; Yong et al., 2018; Lee et al., 2020), (ii) performing allelopathic experiments on different species of *Ostreopsis* and macroalgae (Accoroni et al., 2015; Ternon et al., 2020), and (iii) extending these experiments to other species of toxic benthic dinoflagellates (Mustapa et al., 2019; Bravo et al., 2020).

## GENERAL CONCLUSIONS

Globally, the results of this thesis allowed a step forward in the understanding of the causes and effects of *Cystoseira s.l.* forests loss in the Mediterranean Sea in the framework of BHABs proliferation. The findings provide important insights into the causes and effects of *Cystoseira s.l.* spp. loss and are of major interest in the conservation of Mediterranean marine forests, contributing to the development of effective management measures. The early-life stages of *Cystoseira compressa* are negatively affected by ocean warming and acidification, and by the presence of *Neogoniolithon brassica-florida*. Recruits of *C. compressa* can also be affected by the herbivory pressure of several non-strict herbivorous species. Blooms of *Ostreopsis* spp. seem to prefer shrub



## GENERAL DISCUSSION AND CONCLUSIONS

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and turf-forming macroalgae, such as *Dictyota* spp. and *Halopteris scoparia*, even if, in some locations, the high variability of cells of *Ostreopsis* spp. among substrats and communities could hide other potential patterns of *Ostreopsis* spp. preferences. Larger scale studies would be needed to confirm these results.

The restoration of ecosystems is essential in slowing biodiversity and ecosystem services loss. Marine forests, due to their ecosystem services, offering nursery, habitat, protection and food for many invertebrates and fishes, have to be considered when restoring coastal locations (Wernberg and Filbee-Dexter, 2019). It is important to know the causes driving marine forest loss and prevent the effects of their decline, such as BHABs. It is not always possible to remove environmental causes of marine forests loss (e.g. herbivory), or even virtually impossible (e.g. OW and OA). Future restoration actions could limit the effects of marine forests loss, especially through joint efforts, selecting the most adequate techniques prioritizing resistant species and/or populations and the less impacted areas for restoration. Such restoration actions will not only increase the productivity and biodiversity of coastal ecosystems but could potentially mitigate the public health, ecological and economic consequences of *Ostreopsis* spp. blooms.

The results presented in this thesis follow the European Union Biodiversity Strategy for 2030, United Nations Decade on Ecosystem Restoration and the 2030 Agenda for Sustainable Development objectives, widening the view of the effects of global change (i.e. OW, OA and herbivores proliferation) on the disappearance of marine forests, potentially triggering the development of *Ostreopsis* spp. blooms in the Mediterranean Sea. Overall, the insights reported in this thesis can be useful across species and could help us (i) in the conservation of *Cystoseira s.l.* forests, (ii) in the conception of future restoration actions and guidelines in a changing sea, and (iii) in better assessing and eventually regulating *Ostreopsis* spp. blooms.

## Key highlights by chapter

### Chapter 1

- High temperatures (32°C) and low pH (7.8) negatively affect the density of recruits of *Cystoseira compressa* and the calcification rate of its associate understory crustose coralline algae *Neogoniolithon brassica-florida*.
- *C. compressa* recruit size is larger under lower temperatures (24°C and 28°C) and low pH (7.8).
- The presence of *N. brassica-florida* negatively affect the recruitment of *C. compressa*, independently of the temperature and pH level.
- OW and OA are expected to have a negative impact on *C. compressa* populations.
- Clay substrates are a good support for the recruitment of *C. compressa* and recommended for restoration actions.

### Chapter 2

- Herbivory control has to be considered for the protection of *Cystoseira s.l.* forests and the success of restoration actions.
- Herbivory pressure on recruits of *C. compressa* can differ among locations.
- Different herbivorous and omnivorous species can graze on recruits of *Cystoseira compressa* compromising the population recruitment and future restoration actions.

### Chapter 3

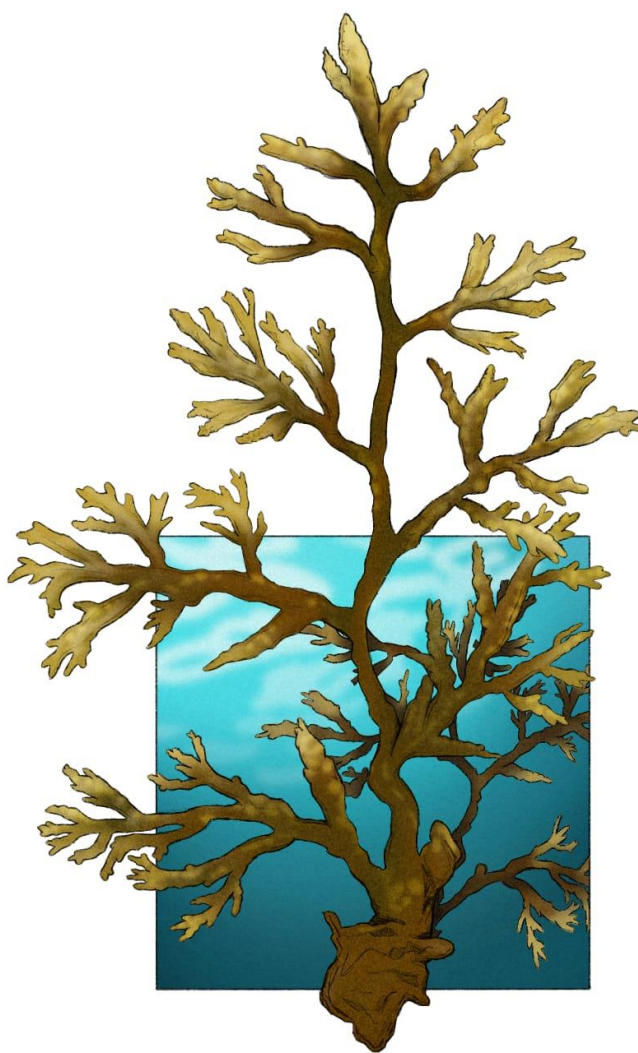
- Studies involving field sampling of *Ostreopsis* spp. increased in the last decade in both temperate and tropical areas.
- In both temperate and tropical areas, erect and turf-forming macroalgae are the most sampled substrate for *Ostreopsis* spp. sampling, independently of the mesohabitat or macrohabitat dominant in the sampling location.
- *Ostreopsis* spp. are often sampled on macroalgal communities characteristic of post-regime shift scenarios.
- The description of the mesohabitat where *Ostreopsis* spp. are sampled is rarely reported.
- There is a need to collect more information at the mesohabitat scale to better understand the preferences of *Ostreopsis* spp.

### Chapter 4

- *Ostreopsis* spp. seem to prefer shrub and turf-forming macroalgae even if, in Rochambeau, the high variability of cells among substrats and communities could hide other potential patterns of *Ostreopsis* spp. preferences. Larger scale studies would be needed to confirm these results.
- *Dictyota* spp. are potentially a good substrate for *Ostreopsis* spp. proliferation in Vernazzola.
- The morphology of the macroalgae alone, excluding allelopathic interactions, is likely not enough to explain *Ostreopsis* spp. preferences.
- The macroalgal preferences of *Ostreopsis* spp. can vary in function of the counting methodology and the type of quantification used for the sampling.



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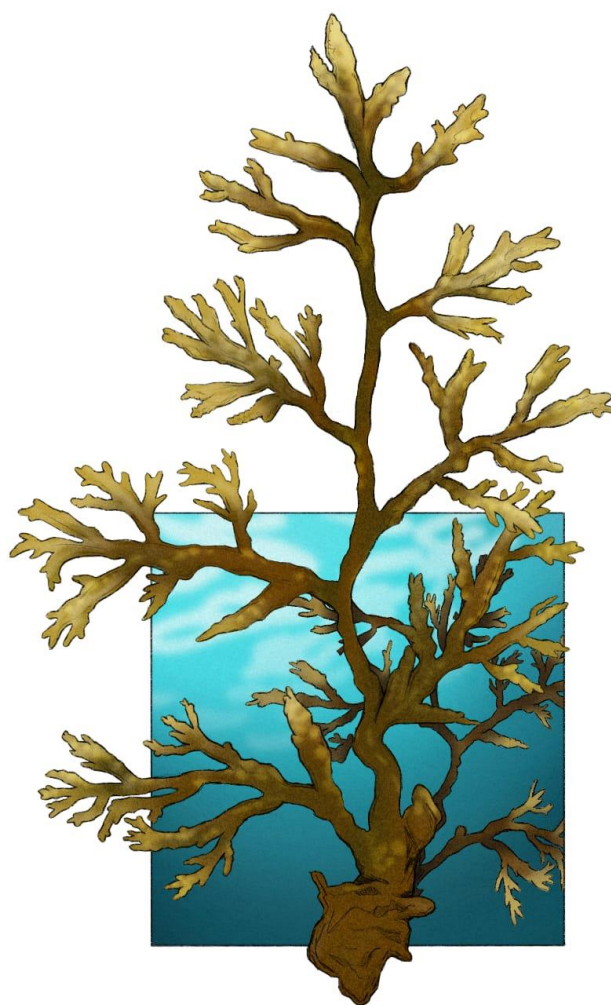
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# APPENDICES







**APPENDIX A: CHAPTER 1 SUPPLEMENTARY MATERIAL**

**Supplementary material S1:** Measured (regular characters) and expected (bold characters) seawater physico-chemical parameters (temperature in °C, *p*H in total scale, calculated *p*CO<sub>2</sub> in  $\mu$ atm, and total alkalinity in  $\mu$ mol Kg<sup>-1</sup> with mean and SD) according to different treatments and substrate types.

Treatment	Substrate type	Temperature (°C)		<i>p</i> H		<i>p</i> CO <sub>2</sub> ( $\mu$ atm)		Total alkalinity ( $\mu$ mol kg <sup>-1</sup> )	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b>28°C pH 8.07</b>	<b>Artificial</b>	28,25	0,79	8,05	0,05	429,42	57,51	2562,06	5,79
	<b>Coralline</b>	28,19	0,66	8,04	0,04	439,46	50,41	2563,77	3,60
	<b>Dead Coralline</b>	28,21	0,69	8,04	0,04	435,52	50,13	2566,66	7,20
<b>28°C pH 7.8</b>	<b>Artificial</b>	28,29	0,75	7,83	0,14	807,15	192,03	2564,20	3,52
	<b>Coralline</b>	28,38	0,73	7,84	0,07	784,99	154,70	2564,79	15,41
	<b>Dead Coralline</b>	28,43	0,73	7,84	0,13	793,41	166,83	2561,56	3,59
<b>32°C pH 8.07</b>	<b>Artificial</b>	31,57	0,44	8,01	0,04	468,46	47,74	2566,97	5,36
	<b>Coralline</b>	31,58	0,48	8,00	0,03	477,15	39,59	2565,73	6,77
	<b>Dead Coralline</b>	31,56	0,48	8,02	0,04	459,05	47,13	2562,03	6,51
<b>32°C pH 7.8</b>	<b>Artificial</b>	31,61	0,76	7,82	0,07	805,88	155,87	2561,92	4,89
	<b>Coralline</b>	31,54	0,70	7,83	0,06	783,92	135,27	2562,58	6,06
	<b>Dead Coralline</b>	31,67	0,64	7,84	0,05	774,72	112,88	2565,43	5,90



Supplementary material S2: Statistic results from the experiment 1.

Variable	Model	Factor levels	DF	Chi sq	P-value
<i>C. compressa</i> /cm2	glmer( <i>C.compressa</i> /cm2 ~ Temperature*Day + (1 Tank/Substrate), family="poisson", na.action = na.omit)	Temperature (28°C and 32°C)	2	296.56	<0.01
		Day (7 days)	6	581.09	<0.01
		Temperature*Day	12	42.937	<0.01
<i>Size C. compressa</i> (mm)	Anova ( <i>Size C. compressa</i> ~ Temperature*Day)	Temperature (28°C and 32°C)	2	768590551	316.643
		Day (3 days)	2	18087101	7.451
		Temperature*Day	4	21189426	8.730
		Residuals	441	2427311	

Experiment 1

Supplementary material S3: Statistic results from the experiment 2.

Variable	Model	Factor levels	DF	Chi sq	P-value	
<b>C. compressa /cm2</b>	glmer (C.compressa /cm <sup>2</sup> ~ Temperature*pH*Substrate Type*Day + (1 Tank/Substrate), family="poisson", na.action = na.omit)	Temperature (28°C and 32°C)	6	628.25	<0.01	
		pH (ambient and 7.8)	1	134.99	<0.01	
		Substrate type (artificial, Corallinales and dead Corallinales)	7	145.70	<0.01	
		Day (6 days)	9	4362.03	<0.01	
		Temperature*pH	1	36.81	<0.01	
		Temperature*Substrate type	5	129.45	<0.01	
		pH*Substrate type	2	10.38	<0.01	
		Temperature*Day	7	107.99	<0.01	
		pH*Day	4	40.74	<0.01	
		Substrate type * Day	11	157.09	<0.01	
		Temperature*pH*Substrate type	2	3.94	>0.05	
		Temperature*pH*Day	4	41.87	<0.01	
		Temperature*Substrate type*Day	8	30.97	<0.01	
		pH*Substrate type*Day	8	45.99	<0.01	
		Temperature*pH*Substrate type*Day	8	1.28	>0.05	
<b>Size C. compressa (mm)</b>	glmer (C.compressa size ~ Temperature*pH*Substrate type*Day + (1 Tank), Gamma(link = "inverse"), na.action = na.omit=subs)	Temperature (28°C and 32°C)	1	0.59	>0.05	
		pH (ambient and 7.8)	1	7.82	<0.01	
		Substrate type (artificial, dead Corallinales and Corallinales)	2	193.46	<0.01	
		Day	2	277.65	<0.01	
		Temperature*pH	1	16.31	<0.01	
		Temperature*Substrate type	2	66.18	<0.01	
		pH*Substrate type	2	17.92	<0.01	
		Temperature*Day	2	2.20	>0.05	
		pH*Day	2	4.40	>0.05	
		Substrate type*Day	4	8.80	>0.05	
		Temperature*pH*Substrate type	2	1.83	>0.05	
		Temperature*pH*Day	2	1.96	>0.05	
		Temperature*Substrate type*Day	4	3.26	>0.05	
		pH*Substrate type*Day	4	19.12	<0.01	
		Temperature*pH*Substrate type*Day	4	5.08	>0.05	
<b>Calcification rate ( mg CaCO3 cm-2 day-1 )</b>	lm (Calcification rate ~ Temperature*pH, na.action = na.omit)	Temperature (28°C and 32°C)	1	7.38	8.6446	<0.01
		pH (ambient and 7.8)	1	3.96	4.6321	<0.05
		Temperature*pH	1	0.15	0.1789	>0.05
		Residuals	118	100.80		

Experiment 2

# APPENDICES

**Supplementary material S4:** Results from the post-hoc analysis of the experiment 2 on the density of *C. compressa* for the significant interactions pH × Substrate Type × Day, Temperature × Substrate Type × Day and Temperature × pH × Day. AS is artificial substrates, CS coralline algae substrates and DCS dead coralline algae substrates. \* means significant differences and = means no significant differences.

(*) significant differences (=) no significant differences	pH ambient														pH 7.8																
	AS D12	AS D32	AS D45	AS D60	AS D75	DCS D12	DCS D32	DCS D45	DCS D60	DCS D75	CS D12	CS D32	CS D45	CS D60	CS D75	AS D12	AS D32	AS D45	AS D60	AS D75	DCS D12	DCS D32	DCS D45	DCS D60	DCS D75	CS D12	CS D32	CS D45	CS D60	CS D75	
AS D12	*															*															
AS D32	*	*														*	*														
AS D45	*	*	*													*	*	*													
AS D60	*	*	*	*												*	*	*	*												
AS D75	*	*	*	*	*											*	*	*	*	*											
DCS D12	*					*										*						*									
DCS D32	*					*	*									*						*	*								
DCS D45	*					*	*	*								*						*	*	*							
DCS D60	*					*	*	*	*							*						*	*	*	*						
DCS D75	*					*	*	*	*	*						*						*	*	*	*	*					
CS D12	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D32	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D45	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D60	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D75	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
AS D12	*															*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AS D32	*	*	*	*	*											*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AS D45	*	*	*	*	*											*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AS D60	*	*	*	*	*											*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AS D75	*	*	*	*	*											*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
DCS D12	*					*										*						*									
DCS D32	*					*	*									*						*	*								
DCS D45	*					*	*	*								*						*	*	*							
DCS D60	*					*	*	*	*							*						*	*	*	*						
DCS D75	*					*	*	*	*	*						*						*	*	*	*	*					
CS D12	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D32	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D45	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D60	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*
CS D75	*					*	*	*	*	*	*	*	*	*	*	*						*	*	*	*	*	*	*	*	*	*



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	pH ambient 28°C					pH 7.8 28°C					pH ambient 32°C					pH 7.8 32°C					
	D12	D32	D45	D60	D75	D12	D32	D45	D60	D75	D12	D32	D45	D60	D75	D12	D32	D45	D60	D75	
pH ambient 28°C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
pH 7.8 28°C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
pH ambient 32°C	*	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=	=
pH 7.8 32°C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

**Supplementary material S5:** Results of the post-hoc analysis of the experiment 2 on the size of *C. compressa* for the significant interactions pH × Substrate Type × Day, Temperature × Substrate Type and Temperature × pH. AS is artificial substrates, CS coralline algae substrates and DCS dead coralline algae substrates. \* means significant differences and = means no significant differences.

(*) significant differences (=) no significant differences	pH ambient									pH 7,8								
	AS D45	AS D60	AS D75	DCS D45	DCS D60	DCS D75	CS D45	CS D60	CS D75	AS D45	AS D60	AS D75	DCS D45	DCS D60	DCS D75	CS D45	CS D60	CS D75
AS D45	=	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
AS D60	=	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
AS D75	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
DCS D45	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
DCS D60	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
DCS D75	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
CS D45	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
CS D60	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
CS D75	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
AS D45	=	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
AS D60	=	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
AS D75	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
DCS D45	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
DCS D60	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
DCS D75	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
CS D45	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
CS D60	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*
CS D75	*	*	*	*	*	*	*	*	*	=	*	*	*	*	*	*	*	*

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		28°C			32°C		
		AS	DCS	CS	AS	DCS	CS
28°C	AS		*	*	=		
	DCS	*		*		*	
	CS	*	*				*
32°C	AS	=				*	*
	DCS		*		*		=
	CS			*	*	=	

		pH ambient		pH 7.8	
		28°C	32°C	28°C	32°C
pH ambient	28°C		*	*	
	32°C	*			*
pH low	28°C	*			*
	32°C		*	*	

## APPENDIX B: CHAPTER 2 SUPPLEMENTARY MATERIAL

## Supplementary material S1

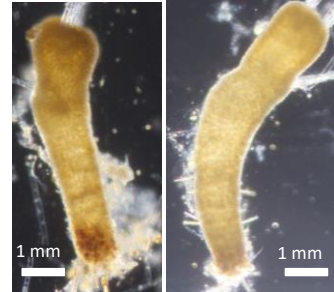
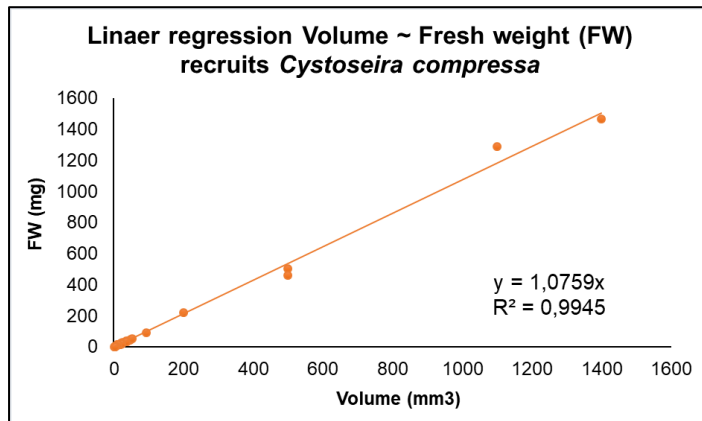


Image of the substrates used in the mesocosm experiment recruited with 2 – 3 months old *Cystoseira compressa* ( $5.02 \pm 0.53$  mg FW *C. compressa*; mean  $\pm$  SD).





**APPENDIX C: CHAPTER 3 SUPPLEMENTARY MATERIAL****Studies considered for the review:**

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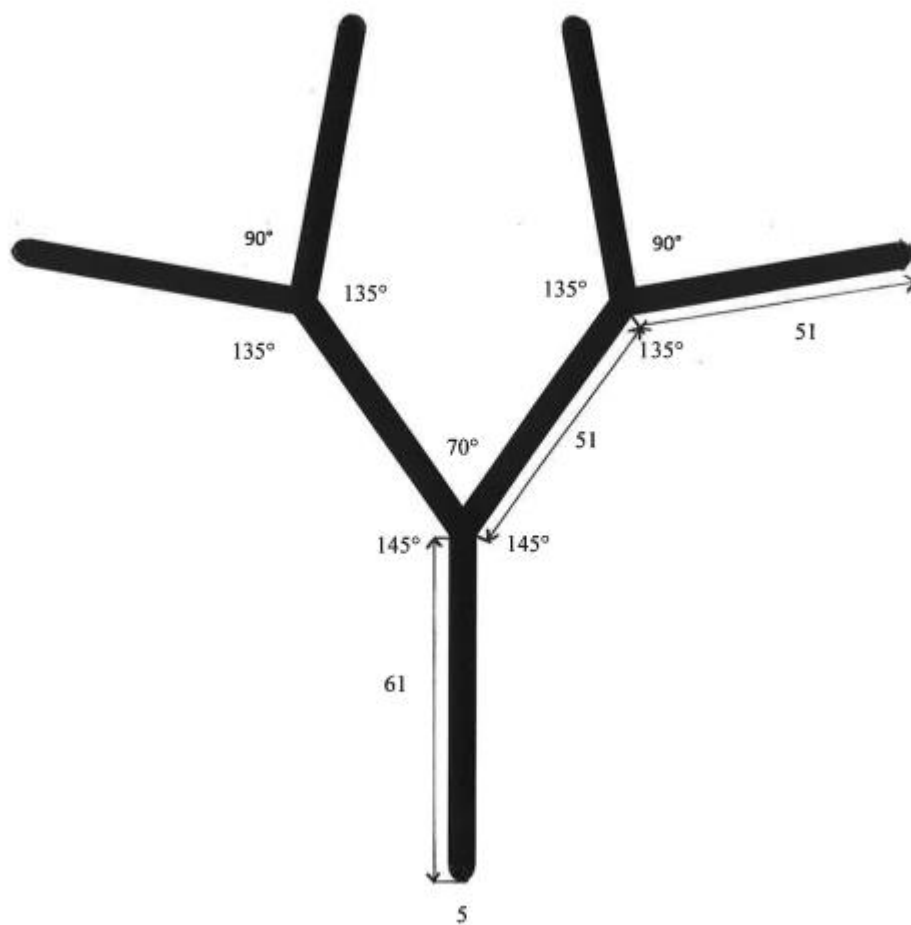
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## APPENDIX D: CHAPTER 4 SUPPLEMENTARY MATERIAL

## Supplementary material S1: Artificial macroalgae design

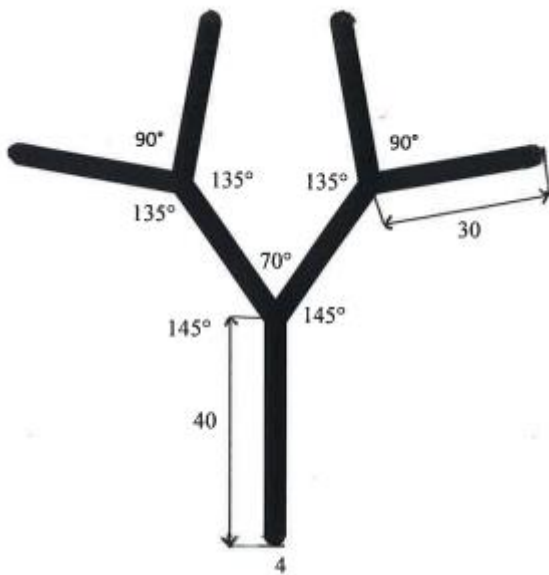
ARTIFICIAL MACROALGAE TYPE	NUMBER OF ALGAL UNITS	DIAMETER (CM)	THICKNESS (CM)	TOTAL SURFACE (CM <sup>2</sup> )	FIGURES OF THE UNITS	PHOTOS OF ARTIFICIAL MACROALGAE
<i>CYTOSEIRA</i>	2	0.5	0.2	99.99	1	1
<i>DICTYOTA</i>	4	1.1	0.2	100.85	2	2
<i>HALOPTERIS</i>	2	0.4	0.2	100.98	3	3
TURF	16	0.2	0.2	99.77	4	4
CONTROL	0	-	-	0 (78.5 lid's surface)	-	5



**Figure 1:** Real size artificial macroalgae unit *Cystoseira* type. The measures are in mm.



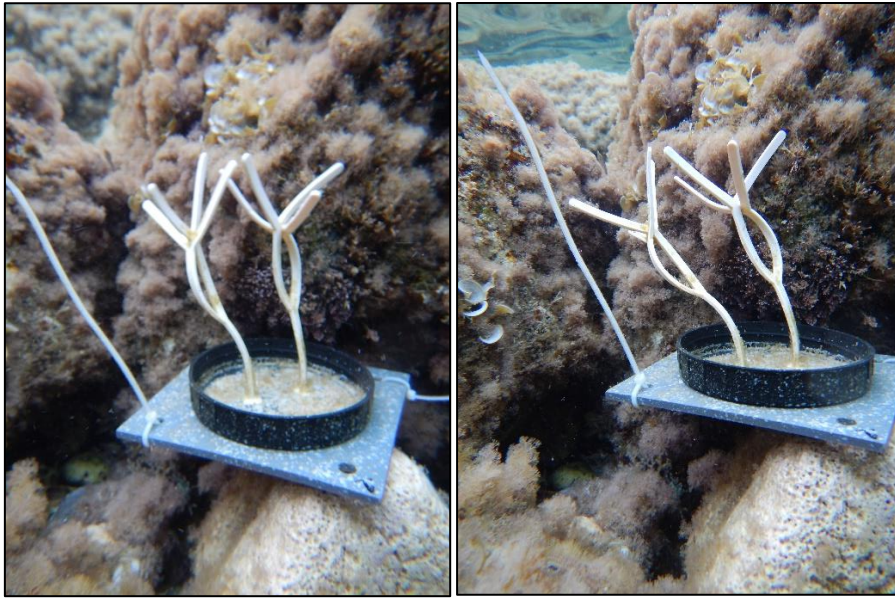
**Figure 2:** Real size artificial macroalgae unit *Dictyota* type. The measures are in mm.



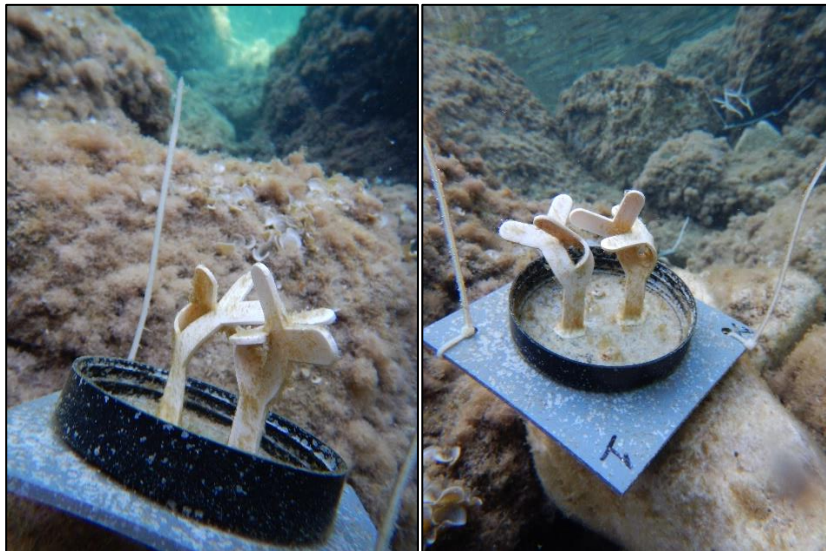
**Figure 3:** Real size artificial macroalgae unit *Halopteris* type. The measures are in mm.



**Figure 4:** Real size artificial macroalgae unit turf type. The measures are in mm.

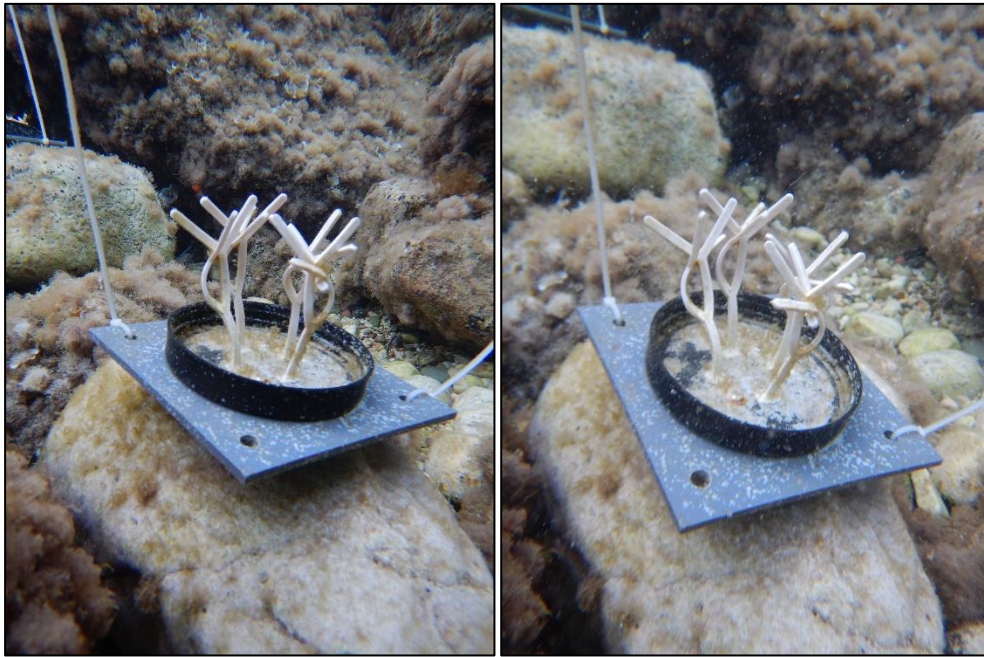


**Photo 1:** Artificial macroalgae *Cystoseira* type.

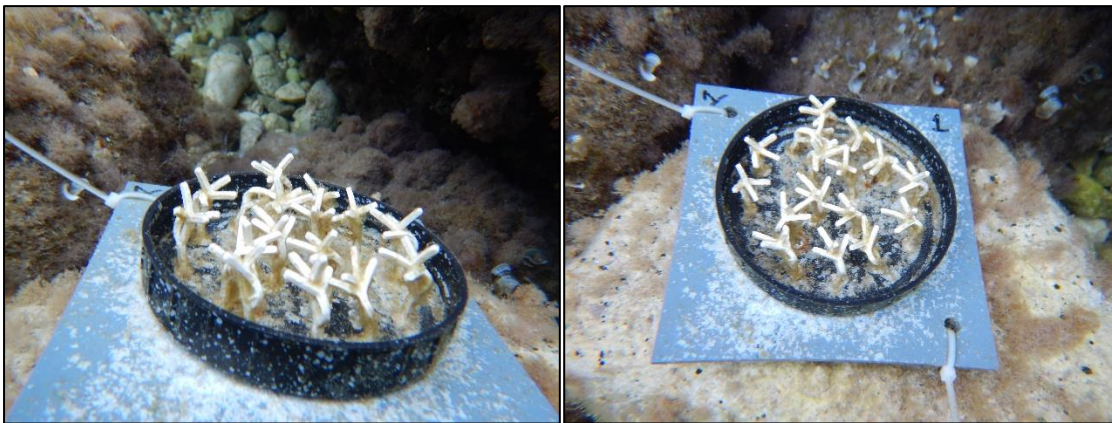


**Photo 2:** Artificial macroalgae *Dictyota* type.





**Photo 3:** Artificial macroalgae *Halopteris* type.



**Photo 4:** Artificial macroalgae turf type.



**Photo 5:** Control for the surface of the artificial macroalgae (the lid).

