ACCLIMATION AND ADAPTATION OF INVASIVE SEAWEEDS
- a case study with the brown alga *Sargassum muticum*

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Declaro ser a autora deste trabalho, que e original é inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Abstract

Non-indigenous seaweeds impact natural communities worldwide, affecting biodiversity, ecosystem functioning, and ecosystem services, resulting in significant economic and social consequences. Among major impacts are the displacement of native species, a threat to endangered species, and effects on ecological and evolutionary processes within the invaded communities. While critical to predict the fate of introduced species, understanding the mechanisms of acclimation and adaptation following introduction represents a great challenge in seaweeds. In this thesis, we investigated some acclimation processes, with an emphasis on the role of associated microbiota, and examined their effects with *Sargassum muticum* as a model species. This brown seaweed native to Asia, is an emblematic invader among seaweeds, with a distribution now ranging from Mexico to Alaska in America and from Morocco to Norway in Europe. We first reviewed the competitive advantages and traits that may contribute to its invasiveness. In addition, we provided an overview of putative underlying mechanisms of acclimation and adaptation and highlighted their role in seaweed invasions. We then examined the effects of ocean acidification on the microbiome of *S. muticum* and revealed that it does not have significant effects on the seaweed-associated microbiota despite certain changes in the microbial community. We further investigated the seasonal changes in the seaweed-associated microbiota and revealed significant differences between seasons and geographic locations. Finally, we applied ecological niche modelling, but innovatively accounting for phenology, to project the distribution of *S. muticum* under two future climate change scenarios. According to our projections, by 2100 the distribution of *S. muticum* is expected to shift northwards along its European, North American and Asian distributions with a partial retreat from the currently occupied areas.

Resumo

As algas não indígenas afetam comunidades naturais em todo o mundo, afetando a biodiversidade, o funcionamento dos ecossistemas e os seus serviços, com consequências económicas e sociais significativas. Entre os principais impactos estão deslocamento de espécies nativas e espécies ameaçadas e efeitos sobre processos ecológicos e evolutivos nas comunidades invadidas. A compreensão dos mecanismos de aclimatação e adaptação após a introdução representa um grande desafio nas algas marinhas. Nesta tese investigamos alguns processos de aclimatação, com ênfase no papel dos microorganismos associados e examinamos os seus efeitos usando *Sargassum muticum* como espécie modelo. Esta alga marinha nativa da Ásia é uma importante invasora, com uma distribuição atual do México ao Alasca na América e de Marrocos à Noruega na Europa. Primeiro analisamos as vantagens e características competitivas que podem contribuir para sua invasibilidade. Além disso, fornecemos uma visão geral dos mecanismos de aclimatação e adaptação subjacentes e destacamos seu papel nas invasões de algas marinhas. Em seguida, examinamos os efeitos da acidificação dos oceanos no microbioma de *S. muticum* e revelamos que não tem efeitos significativos sobre a comunidade microbiana das algas marinhas apesar de causar algumas mudanças na comunidade. No estudo das mudanças sazonais na microbiota associada às algas marinhas revelamos diferenças significativas entre as estações e os locais geográficos. Finalmente, realizamos modelação de nicho ecológico de forma inovadora para a fenologia, para projetar a distribuição de *S. muticum* em dois cenários futuros de mudança climática. De acordo com nossas projeções, até 2100, a distribuição de *S. muticum* deverá expandir-se para norte ao longo das suas distribuições européia, norte-americana e asiática, com retração parcial das áreas atualmente ocupadas.

Resume

Les algues non-indigènes ont des impacts sur les communautés naturelles à une échelle mondiales: elles affectent la biodiversité, le fonctionnement des écosystèmes et les services écosystémiques, avec des conséquences économiques et sociales. Parmi les conséquences majeures de ces introductions sont le déplacement d'espèces indigènes, des menaces sur des espèces en danger et des effets sur les processus écologiques et évolutifs dans les communautés envahies. Bien que crucial pour anticiper le devenir des espèces non-indigènes, comprendre les mécanismes d'acclimatation et d'adaptation agissant suite à leur introduction reste un challenge chez les algues. Au cours de cette thèse, nous avons étudié certains processus d'acclimatation, en particulier le rôle des bactéries associées, et leurs effets, en utilisant comme modèle d'étude *Sargassum muticum*. Cette algue brune originaire d'Asie, est une espèce emblématique parmi les algues introduites avec, de nos jours, une présence du Mexique à l'Alaska en Amérique et du Maroc à la Norvège en Europe. Nous avons réalisé une revue bibliographique des traits et avantages compétitifs qui pourraient contribuer à son succès invasif. Nous avons aussi réalisé une revue des mécanismes d'acclimatation et d'adaptation qui pourraient jouer un rôle dans les processus d'invasions chez les algues. Ensuite, nous avons étudié les effets attendus de l'acidification des océans sur le microbiome de *S.muticum* et montré que l'acidification n'a pas d'effets significatifs sur les bacteries associées, bien que certains changements de la communauté microbienne aient été observés. Nous avons poursuivi notre étude en examinant les changements saisonniers du microbiome : des différences significatives sont observées entre saisons et localités. Enfin, nous avons utilisé un modèle de niche écologique, original car prenant en compte la phénologie de l'algue, pour prédire la distribution de *S. muticum* sous deux scénarios de changement climatique. Selon nos résultats, d'ici 2100, la distribution de *S. muticum* devrait se déplacer vers le nord en Europe, en Amérique du Nord et en Asie, s'accompagnant de disparitions dans certaines des zones actuellement occupées par cette algue.

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Chapter 1

General Introduction

1. General Introduction

1.1 Background

The continuous growth of the number of alien species introductions observed in the aquatic and terrestrial environments worldwide raises significant concerns about its ecological, economic and social implications. Introductions of non-indigenous species are known to produce inevitable impacts on natural communities, as well as affect biodiversity and ecosystem functioning (Molnar et al. 2008, Grosholz 2002). Introduced species have been demonstrated to threaten endangered species and cause declines in native species populations (Gurevitch & Padilla 2004), while the processes of disturbance themselves have been shown to facilitate further introductions and make communities even more vulnerable to invasions (Hobbs & Huenneke 1992). Through the facilitation of each other′s invasion or increase of each other′s impact, non-indigenous species can cause more significant consequences for native communities. According to the estimates of the Global Invasive Species Programme (GISP), the impacts of invasive species constitute at least US$ 1.4 trillion annually, while the impacts on public health result in infectious diseases with severe implications.

Alike in terrestrial environments, the rate of establishment of non-native invasive species in aquatic environments appears to be increasing (Johnson & Chapman 2007, Seebens et al. 2017). Europe is a hot-spot for such aquatic introductions with 1071 species in the marine environment and 481 species in the freshwater environment recorded to have ecological and economic impacts (DAISIE project estimates cited in Vila et al. 2009). Among the major vectors of marine species introductions are: the transport with ballast water, hull fouling, via waterways (like the Suez canal), use as packing materials (i.e. algae), as well as accidental and intentional releases related to fisheries, aquaculture and aquarium trade (Carlton 1999, Ruiz et al. 1997). Among all aquatic non-indigenous species recorded in Europe, macroalgae represent one of the largest groups and constitute between 20 and 29% of all marine introduced species (Williams & Smith 2007).

The issue of introductions of non-indigenous macroalgae is particularly relevant for the European region, where a number of opportunistic species have been shown to displace native algae (Johnson & Chapman 2007), resulting in the disappearance of characteristic and increased abundance of tolerant algal species (Schaffelke & Hewitt 2007). For instance, the introduced green macroalga *Caulerpa racemosa* has reduced diversity and abundance of native seaweed species on the coast of Tuscany, Italy (Piazzi et al. 2001). The introduction of *Caulerpa taxifolia* into the

French Mediterranean coast has also resulted in significant ecological impacts, comprising decreased biomass and diversity of native species of algae and invertebrates (Boudouresque et al. 1992). Expansion of the invasive brown seaweed *Sargassum muticum* resulted in the displacement of native species in various parts of the world (Engelen et al. 2015). As seaweeds represent particular importance for coastal ecosystems by forming complex habitats and supporting high biodiversity, extensive displacement of dominant native algae with introduced species may significantly impact coastal productivity, food web structure, and ecosystem services (Schaffelke & Hewitt 2007). The extent of such impacts is expected to be even more significant in combination with climate change, as changes in temperature, CO_2 levels, pH, coastal salinity, nutrient runoff, wave heights, sea-level, etc. will introduce new environmental conditions for native species to cope with (Harley et al. 2012). While there is a great need for estimating the potential for future spread and evolution of invasiveness, understanding the mechanisms and estimating the speed of acclimation and adaptation of non-indigenous seaweeds into their new range represents a great challenge. Among the numerous processes involved in the sustainable settlement of non-native seaweeds and evolution of invasiveness, seaweed-associated microbial communities play an important role. Rapid changes in the seaweed microbiota occurring in response to changing environmental conditions can affect seaweed fitness and contribute to adaptation of invasive seaweeds in the novel range (Saha et al. 2016).

The main objective of the research is to investigate some of the acclimation processes and examine their effects on the success of invasive seaweeds with *Sargassum muticum* as model species. The major issue to be addressed is assessing the role of associated bacterial communities in adaptation and evolution.

1.2 Significance of Research

In the last decades, the larger part of studies on invasive macroalgae focused on the mechanisms of invasion, opportunistic life history traits, and the effects of abiotic stress on algal growth (Hunt & Denny 2008) and metabolism (Davidson & Pearson 1996). However, the knowledge of these has not been sufficient to explain the success of seaweed invasions. The given research summarizes the current knowledge on the seaweed invasions and identifies required approaches for future research.

Up to now, many insights on invasive species acclimation (short-term epigenetic changes) and adaptation (long-term genetic changes) have been provided by the evidence from terrestrial systems, including plants (Buswell et al. 2011, Lavergne & Molofsky 2007, Ellstrand & Schierenbeck 2000), while the research on rapid evolution of invasive macroalgae has been quite limited. Unlike existing publications, the review presented in this thesis focuses on the differences between the processes of acclimation and adaptation with an emphasis on invasive seaweeds. It summarizes the tools and mechanisms available to invasive seaweeds upon introduction into the novel area.

One of the major threats to aquatic ecosystems associated with ongoing climate change is the ocean acidification. Recent studies demonstrate that it has a significant impact on the abundance and composition of the host-associated microbiota though alteration of services required by/provided for their hosts (Stiling et al. 2003). Increased CO2 levels and subsequent decrease in pH have been shown to result in substantial changes in the seaweed-associated microbial communities, leading to further changes in the gutmicrobiomes of grazers and adaptation of their digestive systems. Given research allows a better understanding of the role of microbiota in the adaptation of invasive seaweeds, as well as the direct and cascading effect of ocean acidification on the gutmicrobiome of mesoherbivores.

Although various studies examined the role of microbiota in increasing fitness of macroalgae (Head & Carpenter 1975, Croft et al. 2005, Boyd et al. 1999) and its effects on morphogenesis (Matsuo et al. 2003, Marchall et al. 2006, Wichard 2015), more case studies characterizing associated bacterial communities across space and time in different (invasive) seaweed species are needed. The given research reveals the shifts in bacterial community abundance and composition between the seasons and geographic locations with potential consequences for *S. muticum*. It provides new insights regarding the impact of season on the seaweed-associated microbiota, as well as seasonal changes among different types of algal tissues.

Finally, this thesis provides projections of future invasion risk of *Sargassum muticum* for two different future climate scenarios (the most optimistic by 2050 under RCP 2.6 and the most pessimistic by 2100 under RCP 8.5) assuming niche and phenological constraints conservatism over time. To provide more reliable forecasts of future seaweed distribution, an innovative approach has been applied incorporating phenology into niche modelling. Future projections demonstrate that by 2100, the distribution of *S. muticum* is expected to shift northwards with a substantial retreat from currently occupied southern habitats. This may have significant ecological consequences for sensitive subarctic ecosystems.

1.3 Thesis Structure

Chapter 2 on the Circumglobal Invasion by the Brown Seaweed *Sargassum muticum* represents a review. It summarizes the current state of the art of the invasions of this species, identifies research gaps, and assesses to which extend this species can function as a model species for marine invasions in general. The scientific review paper was published in 2015 in the *Oceanography and Marine Biology Annual Reviews* 53: 81-126 (Impact Factor 7).

Chapter 3 on Acclimation vs. Adaptation of Invaders also represents a review. It aims to elucidate evolutionary aspects of invasive seaweeds, by providing an integrated overview of the underlying mechanisms of acclimation and adaptation, and highlighting the role of these mechanisms in seaweed invasions. The review focuses on the differences between the processes of acclimation and adaptation with an emphasis on invasive seaweeds and intends to contribute to the understanding of eco-evolutionary mechanisms following their introduction and the factors that increase their invasiveness. One important aspect of this review paper is to highlight the role of the bacterial community associated with invasive algae as a potential key mechanism facilitating acclimation process.

Chapter 4 examines the effects of ocean acidification and grazing on the microbiome of the invasive brown seaweed *Sargassum muticum* and a native isopod consumer *Synisoma nadejda*. It focuses on the changes in associated microbiota of the hosts after three weeks of exposure to the ambient CO2 conditions (380ppm, pH 8.16) and an acidification treatment (1000ppm, pH 7.86).

Chapter 5 on the Seasonal Variation of Bacterial Communities Associated with *S. muticum* is focused on examining the seasonal variation of bacterial communities associated with *S. muticum* (identifying temporal shifts in bacterial community abundance and composition across different tissues) in northern and southern Portugal across different times of the year.

Chapter 6 is concerned with Accounting for Phenology in Marine Invasion Predictions under Climate Change Scenarios. In this study, future invasion risk of *S. muticum* is predicted, assuming niche and phenological constraints conservatism over time. Ecological niche modelling is applied to forecast the alien's distribution under different future climate scenarios but innovatively with consideration of phenology affecting the species' reproductive period. The reproductive windows for sea surface temperature and day length along the latitudinal gradient of its distribution are used for improving predictions of *S. muticum* invasions. This is the first time that phenological data are applied to improved predictions of invasive seaweeds distribution under climate change.

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Chapter 2

Circumglobal Invasion by the Brown Seaweed *Sargassum muticum*

Oceanography and Marine Biology – An Annual Review 53: 81-126.

2. Circumglobal Invasion by the Brown Seaweed *Sargassum muticum*

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Oceanography and Marine Biology – An Annual Review 53: 81-126.

2.1 Abstract

Hundreds of macroalgal species have been spread outside their natural range by human activities, and many of these introductions are occurring at a worldwide scale. This review considers one of the best-studied and most widespread invasive macroalgae, *Sargassum muticum*, to determine the traits and processes important in marine invasions and to identify important lines of future research. Particular emphasis is placed on the ecology of *S. muticum* in its native range and on the four stages of invasion—transport, colonization, establishment, and spread—integrating taxonomy, invasion history, dispersal, impact, invasiveness and invasibility, and general ecology. Although *S. muticum* has received a lot of scientific attention, with more than 650 papers on this species, key information on its taxonomy, invasive biology, and evolutionary potential is still lacking. Most previous studies have been local or descriptive or provide circumstantial evidence, and too few have been hypothesis driven. Only by local-scale research conducted in different geographical regions, especially including the native range, and developed in an eco-evolutionary framework, will it be possible to greatly improve our understanding of the complex of factors, traits, and processes involved in macroalgal invasions.

Keywords: seaweed, interactions, taxonomy, non-indigenous species, invasive species, invasion, ecology, impact, *Sargassum muticum*

Introduction

Human-mediated transport of species significantly alters their distribution at a global scale. Since the seminal work by Elton (1958), non-indigenous species (NIS) introductions and invasions have increasingly attracted scientific attention for addressing basic issues in ecology and evolution, for instance, the traits favouring the establishment of a species in a new environment (e.g., plasticity; Richards et al. 2006); the mechanisms that facilitate rapid adaptive processes (e.g., Rius & Darling 2014, Rius et al. 2014); and the likelihood of change in climatic niche between different geographic areas (e.g., Guisan et al. 2014). In addition, NIS are a recognized major threat to natural ecosystems, with a range of impacts at population, community, and ecosystem levels (Carlton 2000, Taylor & Hastings 2005, Molnar et al. 2008, Simberloff et al. 2013). As such, the study of NIS is also an important field of applied research for supporting management strategies for conserving and utilizing natural ecosystems. The European programme DAISIE (Delivering Alien Invasive Species Inventories for Europe) recognized 12,122 non-native species present in Europe, with around 1000 species reported so far in marine environments. However, for several reasons, including problems of access to marine environments and taxonomic uncertainties about many marine taxa, this number is likely to be a significan't underestimate (Carlton 2009).

Marine systems are particularly prone to invasions, with fewer than 20% of marine ecoregions having no reported introductions, although some of them have not been surveyed/examined thoroughly (Molnar et al. 2008). Numerous vectors have been described, but maritime trafic and aqua- culture are the most important ones (Carlton 2001, Molnar et al. 2008). Unsurprisingly, increasing worldwide trade led to increasing rates of biological invasions in marine systems since the beginning of the twentieth century (Reise et al. 1999, Goulletquer et al. 2002, Inderjit et al. 2006). Macroalgae are no exception to this phenomenon.

At a global scale, Williams & Smith (2007) reported 277 introduced marine macroalgae, some of them having been introduced in several regions (e.g., *Undaria pinnatifida*, Voisin et al. 2005 and references therein; *Asparagopsis* sp., Dijoux et al. 2014). Williams & Smith (2007) also pointed out that NIS are found in all major taxonomic groups of macroalgae, but some families have more NIS than expected by chance, such as the Alariaceae, Chordariaceae, and Fucaceae. At a global scale, introduction vectors of macroalgae fall broadly into four categories (Mineur et al. 2015, Williams & Smith 2007): 1) transportation by ships either on hulls as a fouling organism or within ballast tanks; 2) accidental introduction with oysters or other farmed shellfish; 3) accidental release from aquaria (e.g., *Caulerpa taxifolia*; Jousson et al. 1998); and 4) intentional introductions for cultivation (e.g., the Asian edible kelp *Undaria pinnatifida*; Voisin et al. 2005, Wallentinus 2007). The relative importance of these vectors is likely to be different compared to animal NIS, with, for example, only a few cases of macroalgae being introduced via ballast water. In addition, some vectors are regionally specific; for instance, the opening of the Suez Canal has promoted 'Lessepsian' or 'Erythrean' introductions (i.e., species native to the Red Sea expanding their distribution to the Mediterranean Sea) (Por 1971, Galil 2006).

Both ecological and economic impacts have been documented following the establishment of non-indigenous macroalgae; the effects are numerous but still poorly investigated as compared to animal species (Schaffelke & Hewitt 2007, Williams & Smith 2007). As pointed out recently by Simberloff et al. (2013), invasion biology is a cross-disciplinary field requiring an integrative approach to investigate and, more importantly, to quantify the impacts, as well as to provide a better understanding of the mechanisms involved in the successful establishment and spread of NIS. Numerous conceptual frameworks, empirical approaches, and methodologies have been developed for the study of patterns and processes in the introduction of non-native species (for recent reviews, see Facon et al. 2006, Blackburn et al. 2011, Gurevitch et al. 2011). Among others, three important research themes are 1) 'eco-evolutionary' studies (see details in Facon et al. 2006), which aim to investigate how ecological and evolutionary forces interact and change during the introduction process (e.g., how migration may influence adaptation of the NIS to its new environment), leading to the successful establishment of an NIS; 2) building up a relevant framework for practical solutions to invasions in support of decision makers in charge of conservation and management of coastal areas, integrating prevention, detection, early response, and control; and 3) examining and predicting the fate of NIS and indigenous species affected by them, as well as their interactions in light of global climate change. All of these research themes are challenging considering the amount of data and knowledge required for their investigation.

Benefiting from the wide range of studies carried out on the brown alga *Sargassum muticum*, the aim of the present review is to illustrate the potential of integrative approaches in developing a better understanding of the evolutionary and ecological changes associated with trans- and interoceanic invasions mediated by human activities. *Sargassum muticum* constitutes an outstanding model to gain insights into patterns and processes involved in the success of an introduced species: it is distributed circumglobally but was introduced at different time periods across its present range, providing a basis for comparing introduction events under different conditions and populations established for different lengths of time. Patterns of establishment and spread, life-history traits, impact on native communities, and biotic resistance to invasion are discussed in turn, and directions for future work are provided.

***Sargassum muticum* in its native region**

Most studies of invasive species have, for obvious reasons, been conducted in introduced regions (i.e., outside their natural range). Yet, there is a lot of evidence that invaders often differ in key aspects between their native and introduced range(s). A species may, for example, become more abundant (Fowler et al. 1996, Williamson & Fitter 1996); attain higher growth rates (Blossey & Nötzold 1995, Fowler et al. 1996); grow larger (Pritchard 1960, Blossey & Nötzold 1995); or be more fecund (Noble 1989, Rees & Paynter 1997) in their introduced range. To identify the key traits relevant to species invasiveness, studies of invasive species in their native range are essential.

Taxonomic status

The taxonomic history and status of *Sargassum muticum*, like many species of the speciose genus *Sargassum*, is confusing due to variable morphological characters (like ‘leaf’ length and width and vesicle shape). *Sargassum muticum* (Yendo) Fensholt was first described as *S. kjellmanianum* f. *muticus* by Yendo (1907). Okamura (1924) described two growth forms of *S. kjellmanianum* based on differences in leaf size. The form with larger leaves resembled *S. kjellmanianum* f. *muticus* of Yendo and was common on the eastern (Pacific) and western (Sea of Japan) coasts of Japan, whereas the smaller-leaf form was more common in the warmer seas further south, on the Pacific side of the main island (Honshu). In places where both forms are found, the larger-leaf form is found in deeper water. However, a third form of *S. kjellmanianum* (*S. kjellmanianum* f. *longifolium*) was described from China (Tseng & Chang 1954). Fensholt (1955) elevated *S. kjellmanianum* f. *muticum* to spe- cies level as *S. muticum*, with a sterile specimen from Itsumo, Kii Province (present-day Wakayama Prefecture), Honshu, collected in April 1902 as the lectotype (Yoshida 1978, 1980). Based on the examination of the reproductive organs of a single population from Misaki, Kanagawa Prefecture, Inoh (1930) concluded that *S. muticum* is monoecious. In a detailed study on Japanese *Sargassum* belonging to the subgenus *Bactrophycus*, Yoshida (1983) believed that *Sargassum kjellmanianum* f. *longifolium* should be synonymized with *S. muticum*, but Tseng & Lu (2000) still kept it under *S. muticum* f. *longifolium*.

The typical form of *Sargassum kjellmanianum* was synonymized as *S. miyabei* Yendo by Yoshida (1978). Based on the description of Okamura (1924), the typical form of *S. kjellmanianum*

(= *S. miyabei*) differs from *S. kjellmanianum* f. *muticum* (= *S. muticum*) in having shorter leaves, 1.5 cm long, 1–2 mm wide compared to the leaves 2–3 cm long and 3–4 mm wide of *S. muticum* (Yoshida 1983, 1998). However, Tseng & Lu (2000) described the leaves of *S. muticum* f. *longifolium* as 5–6 cm long and 3–6 mm wide. In addition, *S. miyabei* also has elliptical to fusiform apiculate vesicles, whereas the typical vesicles of *S. muticum* are blunt. Apiculate vesicles, however, have been seen in *S. muticum* specimens from China (Tseng & Lu 2000). The main clear difference between *S. muticum* and *S. miyabei* is that the latter is dioecious and has a discoid holdfast when young that eventually develops fibrous or filamentous outgrowth (Yoshida 1983), whereas the former is assumed monoecious (Inoh 1930) with a holdfast that remains discoid throughout its life. Molecular sequencing of the nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) indicated that *S. muticum* and *S. miyabei* are the most closely related and similar of all *Sargassum* species under the subgenus *Bactrophycus* (Oak et al. 2002, Stiger et al. 2003).

Fronds without reproductive structures and holdfasts can be extremely difficult to identify and can be confused with many more *Sargassum* species like *S. ammophilum* (Yoshida 1983). Cheang et al. (2010b) revealed two recently diverged lineages in the native range of *S. muticum*. A dominant lineage, representing populations from central and western Japan, South Korea, and northern China, is identical to that in the entire introduced region. This shows that even though *S. muticum* is one of the most important globally invasive macroalgae, its taxonomic status remains uncertain, a situation encountered in other important invasive macroalgae.

In non-native marine species, DNA-based studies have recently been used to identify cryptic species (Stepien & Tumeo 2006) or small organisms (Patil et al. 2005) as well as to (in) validate previous morphological identification (McGlashan et al. 2008). In the case of introduced macro-algae, such tools have enabled the detection of various newly introduced species (e.g., Rueness 2005, Verlaque et al. 2009, Mineur et al. 2010b, 2012b), as well as revealing the introduced status of cryptogenic species (e.g., McIvor et al. 2001). Given the taxonomic complexity within *Sargassum*, with several morphologically similar Asian *Sargassum* species, these tools might help not only to resolve the possible taxonomic confusion within the genus *Sargassum*, but also to ascertain the taxonomic status of the introduced species at a worldwide scale.

Such a study based on phylogeography and phylogenetics was recently carried out successfully on two invasive red macroalgae, *Asparagopsis taxiformis* and *A. armata*; for both, new lineages and clades were discovered pointing out the existence of new subspecies and sister species in the *Asparagopsis* genus (Dijoux et al. 2014 and references therein). Some of the mitochondrial markers (e.g., trnW-I) developed for Laminariaceae (Voisin et al. 2005) were found to be conserved in *Fucus* sp. (Engel et al. 2008). Cheang et al. (2010a,b) also showed the usefulness of this marker in *Sargassum muticum*, *S. thunbergii*, and *S. hemiphyllum*. Even though the region was poorly polymorphic at the within-species level, it could be used for species comparison, suggesting its utility for further phylogenetic investigation. Concatenating the sequence obtained at trnW-I with sequences obtained for regions coding for cytochrome oxidase III (cox3) increased the within-species genetic resolution for *S. muticum* in Korea (Bae et al. 2013).

Ecology

In its native region, *Sargassum muticum* is a typical temperate species reported to be widely distributed in the north-western Pacific from Kuri Island Group and Sakhalin in Russia to Haifong in southern China (Figure 1; Yamada 1956, Kang 1966, Yoshida 1983, Tseng & Lu 2000, Lee & Kang 2002) in seawater temperatures of 2–15°C in winter to 21–28°C in summer. The actual range of distribution, however, may be much narrower than reported (see Cheang et al. 2010b for details). Yoshida (1998) doubted the presence of *S. muticum* as far north as the Kuril Islands and Sakhalin. Attempts to find *S. muticum* in Guandong Province in southern China, including Hong Kong, were also unsuccessful (Cheang et al. 2010b). The most likely range of distribution might therefore be from northern Honshu, Japan (41°N), to Dongshan, Fujian Province, south-eastern China (around 23°N). Surprisingly, *S. muticum* is not known to be a dominant species in its native region. It was not even mentioned by Taniguti (1962) in his series of papers on the phytosociological studies of Japanese marine algae. Populations are common in the Bohai and Yellow Seas (China), but become less common towards the south (Tseng & Lu 2000). In Japan, the species is most common in the Inland Sea (Region V, e.g., Seto Bay) (Tokuda et al. 1994).

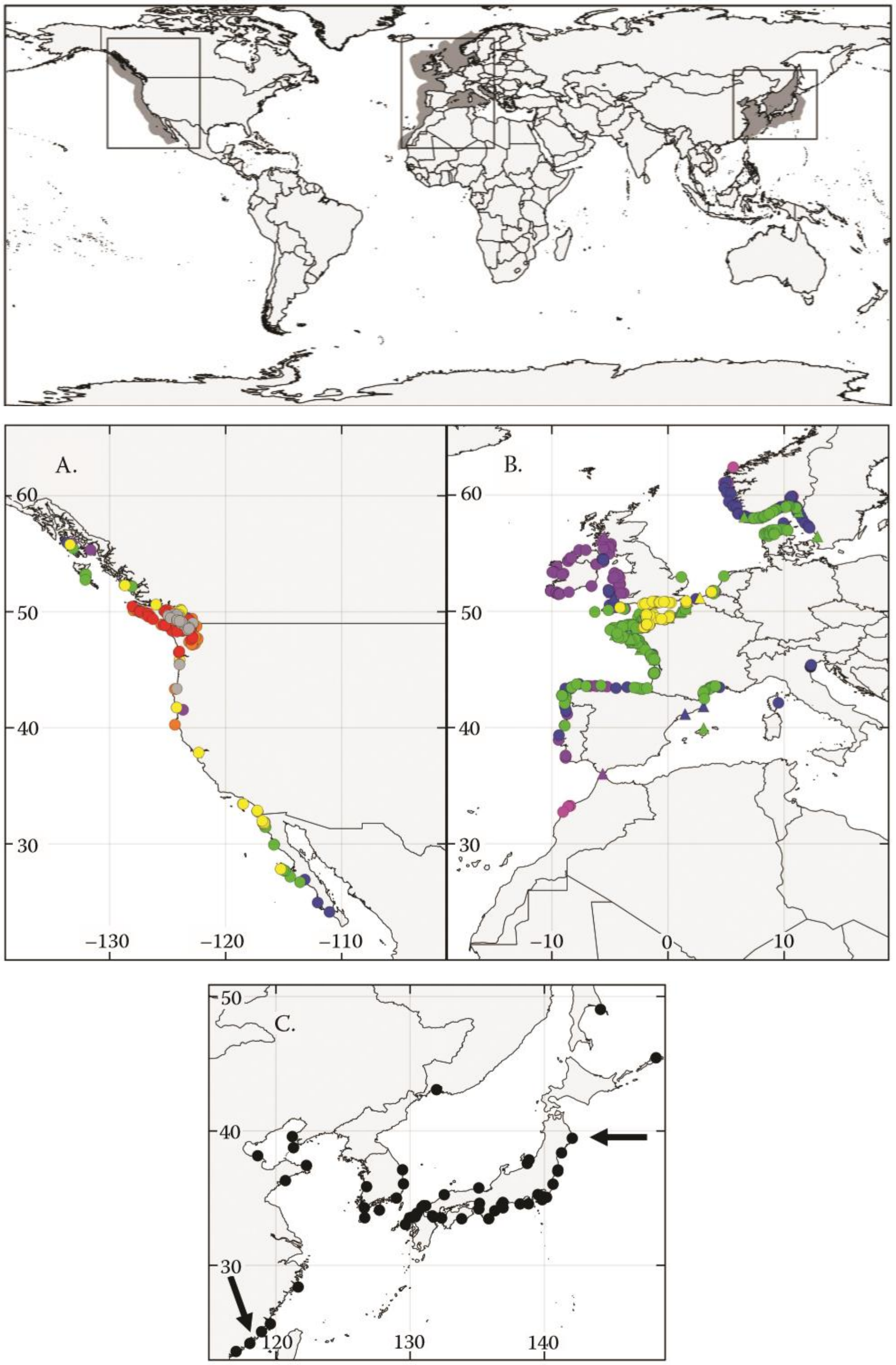


Figure 1 Geographical distribution of *Sargassum muticum* in the native and introduced regions by decade: ● 1940s; ● 1950s; ● 1960s; ● 1970s; ● 1980s; ● 1990s; ● 2000s; ● 2010s: A. north-eastern Pacific; B. north-eastern Atlantic; C. native range in the north-western Pacific. Triangles in Europe indicate drift specimens. Arrows in the native range indicate the most likely actual limits of distribution (see text for details).

The ecology and phenology of *S. muticum* in its native region is not well documented. It is found in the low intertidal to shallow subtidal down to 4 m below lower low tide (LLT) on shores that are more sheltered (Yoshida 1998, Tseng & Lu 2000), where it can be found on hard substrata and in seagrass or kelp beds together with other *Sargassum* species like *S. horneri* and *S. patens*. In the Inland Sea of Japan, it is more common in the intertidal to shallow subtidal than in deeper areas (P. Ang personal observation). The thalli can grow up to 1 m long in the intertidal and reach lengths of 2–3 m in subtidal environments, although lengths greater than 4 m are not exceptional (A.H. Engelen personal observation; see Figure 2). Individuals are reproductive in winter to early summer in Japan (Yoshida 1983, 1998) and spring in China (C.C. Cheang personal communication) and start to die back in late June (in Dalian, North China; R. Luan personal communication).

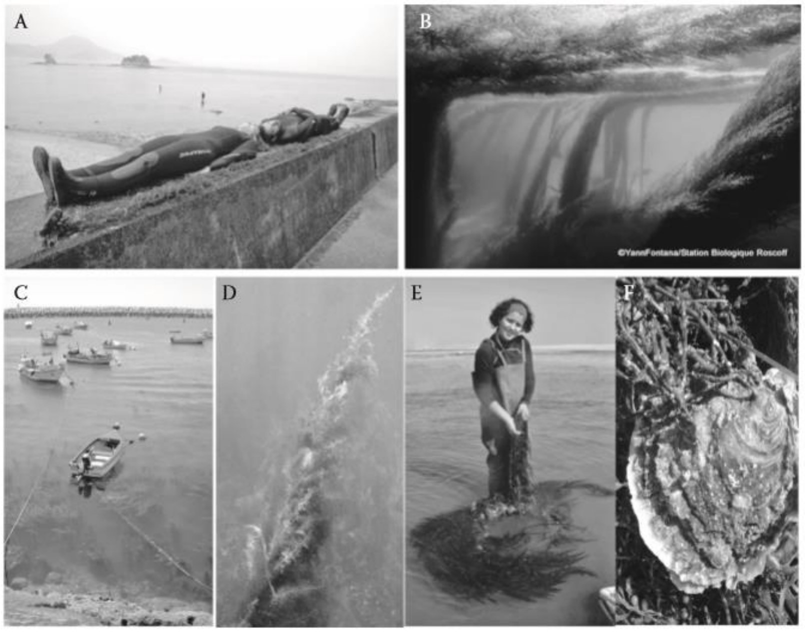


Figure 2 *Sargassum muticum* thallus length demonstrated (A) in the Seto Inlet Sea, Japan (summed length of the two people, 3.2 m; photo Rui Santos); (B) underwater during low tide in the shallow subtidal (2 m depth) of Morlaix Bay, Brittany, France (photo Yann Fontana); and (C) in a fishing harbour in Albufeira, southern Portugal (photo Aschwin Engelen). (D) *Sargassum muticum* with overgrowth of filamentous brown algae, shallow subtidal (depth ~1 m below the level of the lowest astronomical tide), Firth of Clyde, Scotland (photo Philip Smith). E. A clump of individuals within the Ria Formosa soft- bottom lagoon (photo Rui Santos). F. On Pacific oysters, *Crassostrea gigas*, at 1 m depth at low tide in the Ria Formosa, Portugal (photo Aschwin Engelen).

Sargassum muticum seems not to be an aggressive colonizer in its native region. As large extents of kelp or other macroalgal beds in Japan have been denuded by sea urchins (a condition called ‘isoyake’ in Japanese), massive efforts have been undertaken over the years to restore the macroalgal bed through the establishment of artificial reefs (known as ‘tsukiiso’, artificial reefs and their establishment). However, despite monitoring of macroalgal colonization, there are no records of *S. muticum* recruiting to these artificial reefs (Tokuda et al. 1994). For example, in Karatsu, north- ern Kyushu, where *S. muticum* has been collected before, several species of *Sargassum*, including *S. fulvellum*, *S. horneri*, *S. patens*, *S. piluliferum*, and *S. siliquastrum*, some of which co-occur with *S. muticum*, became dominant on artificial reefs, covering 50% to 70% of the rock surface, yet no *S. muticum* was recorded.

Spread history

Sargassum muticum was observed for the first time outside its native region in 1941, in the Strait of Georgia, British Columbia, Canada (Scagel 1956; Figure 1). Over the next 30 years, it was subsequently found in many other localities in British Columbia and adjacent areas in Washington State (Scagel 1956, University of British Columbia Herbarium n.d.), a region characterized by a semiprotected, spatially complex coastline (small islands, straits, fjords). Early in the eastern Pacific invasion (1947), *S. muticum* was found south of the initial introduction, in Coos Bay, Oregon (Fensholt 1955). It reached northern California in 1965 (Abbott & Hollenberg 1976) and Mexico, 100 km south of the border with the USA, in 1973 (Devinny 1978). The spread of *S. muticum* continued southwards along the western coast of Baja California during the 1980s (Espinoza 1990). At the northern edge of its eastern Pacific range, *S. muticum* has also been collected in south-eastern Alaska (1974; University of Alaska Southeast Herbarium n.d.) and was found in the Queen Charlotte Islands in 1981 (University of British Columbia Herbarium n.d.). In the eastern Pacific, its current distribution extends now from south-eastern Alaska to Baja California (Aguilar-Rosas et al. 1984, Aguilar-Rosas & Aguilar-Rosas 1985).

The second major introduction of *Sargassum muticum* occurred in Europe in the English Channel area (Figure 1). In 1972, a drift specimen was found on the Belgian coast, followed by several sightings in northern France (Coppejans et al. 1980). Established populations were first recorded in the Isle of Wight (United Kingdom) in 1973 (Jones & Farnham 1973). *Sargassum muticum* subsequently spread along both coasts of the English Channel (Gruet 1977, Critchley et al. 1983), populations became established in the Oosterschelde estuary in 1979 (Prud'Homme Van Reine & Nienhuis 1982), and it reached the Nissum Bredning in Denmark in 1984 (Christensen 1984) and Arcachon in 1983 (Gruet 1983). *Sargassum muticum* was eventually reported in the Mediterranean, where it was found in the Thau Lagoon, southern France in 1981 (Critchley et al. 1983, Knoepffler-Péguy et al. 1985) and Venice in 1992 (Curiel et al. 1995). Nowadays, *S. muticum* is present in suitable habitat (i.e., rocky shores and artificial hard substrata) on most European Atlantic coasts, and it is still spreading, especially on the Iberian Peninsula (southern coast; Bermejo et al. 2012) and in the British Isles. Recently, it also arrived on the coast of Morocco (Sabour et al. 2013).

With these new southern observations, it becomes questionable whether the species has reached its limit, including in the Mediterranean Sea, where it seemed to be contained in the Thau and Venice lagoons. Some drifting specimens were found on the Spanish Mediterranean coast and in the Balearic Islands, but the species apparently did not become established there (Barceló Martí et al. 2001). It is likely that, in the light of global warming, the species is still spreading northwards.

Transport: primary vector of introduction

Although *Sargassum* species have high drifting capabilities, the first appearance of *S. muticum* on the western coast of North America was not until the twentieth century, supporting the hypothesis of human-mediated introductions. The hypothesis of specimens drifting with the clockwise surface currents of the North Pacific cannot be completely ruled out, as this mode of transport was demon- strated by the arrival of debris from Japan along the Pacific coasts of North America after the 2011 tsunami (Eriksen 2012, Ward 2012). From the late nineteenth century, Pacific oysters (*Crassostrea gigas*) were translocated from Japan to British Columbia and the neighbouring area of Puget Sound (USA) (Scagel 1956, Druehl 1973). Therefore, the first sightings of *Sargassum muticum* precisely in those areas (recorded from 1941) is circumstantial evidence of an introduction associated with oyster imports.

Similarly, the first records of *Sargassum muticum* in Europe followed the importation of *Crassostrea gigas* spat from Japan, which began in France in the 1960s (Mineur et al. 2014b). Between 1971 and 1977, there were also large-scale importations of adult *C. gigas* from British Columbia (Gruet et al. 1976, Grizel & Héral 1991). Yoshida (1983) examined some European specimens of *Sargassum muticum* from Zeeland, the Netherlands, and found them to be similar to samples from Seto Bay in having long leaves (5 cm) arising sparsely from the axis. *Sargassum muticum* f. *longifolium* of Tseng & Chang (1954) also resembled those from Seto Bay and the Netherlands (Yoshida 1983).

Three putative source areas have been identified in the literature, but recent molecular evidence suggests that the most likely source for introduction of *Sargassum muticum* to the eastern Pacific and later to the eastern Atlantic was oyster spat for aquaculture sourced from Hiroshima, inside the Seto Inland Sea (Cheang et al. 2010b and references therein). However, as noted, oysters have also been imported to Europe directly from British Columbia, and this possibility has not been well investigated. Thus, although oyster imports are the likely vector for the European invasion, it is unclear whether primary (i.e., from the native range) or secondary introduction (i.e., from another non-native area) from British Columbia spread *S. muticum* to Europe. Experimental evidence shows that shells of shipped oysters, even if visibly clean, can host a wide range of macroalgal species in microscopic life-history stages (spores, gametes, or germlings), including *S. muticum* (Mineur et al. 2007a). Moreover, adult thalli have been observed in some oyster shipments transferred between European regions (Critchley & Dijkema 1984).

Vectors associated with shipping, especially hull fouling, are considered an important dispersal vector for macroalgae (e.g., Ribera-Siguan 2002, Hewitt et al. 2004), but this was prob- ably not the introduction vector of *Sargassum muticum*, as macroalgae found on the hulls of ships and barges are typically opportunistic cosmopolitan taxa, usually filamentous or foliose (such as Ulvales, Ectocarpales, and Ceramiales) (Godwin 2003, Mineur et al. 2007b), even though some larger macrophytes, such as the invasive kelp *Undaria pinnatifida* do occur on vessel hulls (Hay 1990). *Sargassum muticum* occasionally develops on artificial structures in ports and marinas, like floating pontoons, which may facilitate its settlement on vessel hulls. Another potential vector associated with shipping is ballast water. However, there is no evidence that this vector can transport *S. muticum*, and it seems unlikely that *S. muticum* germlings could survive the conditions encountered in a ballast tank. Moreover, it is improbable that *S. muticum* thalli drifting at the surface would be drawn into subsurface ballast water intakes.

Even though oyster importation from Japan is probably the main source of the introduction of *S. muticum* to North America and then to Europe, the possibility of cryptic introductions (recurrent, unrecognized introductions) with other vectors and from other regions cannot be completely ruled out. Indeed, thanks to molecular markers, such events have been detected in a wide range of marine taxa, including invertebrates (e.g., *Cyclope neritea*; Simon-Bouhet et al. 2006) and macroalgae (e.g., *Undaria pinnatifida*; Voisin et al. 2005). A molecular approach would help in tracing the introduction pathways (i.e., origin and vectors for the introduction) as well as in evaluating patterns of expansion within the introduced range, such as gradual (stepwise) versus jump (long-distance and stochastic) dispersal (e.g., Wilson et al. 1999, Holland 2001, Dupont et al. 2003, Viard et al. 2006, Grulois et al. 2011). Invasion genetics in marine organisms is still in its infancy, but recent studies have shown promise for elucidating invasion processes (Rius et al.

2014). This approach should be facilitated with the development of highly polymorphic markers, like microsatellites or RAD-seq markers, which is now much easier, even in non-model organisms. Such DNA-based studies would allow very fine-scale processes to be studied; recruitment could, for instance, be investigated in *Sargassum muticum* populations as a complementary approach to eld surveys and population-dynamics studies (see 'Colonization' and 'Establishment' sections below).

Regional spread

Although the chronological spread maps in Figure 1 show the overall spatial pattern of spread, they do not provide quantitative information about spread dynamics. Several studies have estimated the rate of spread of *Sargassum muticum* in different regions. In the north-eastern Pacific, rate estimates are quite consistent: 43 km yr^{-1} (Shanks et al. 2003), 68 km yr^{-1} (F. Mineur unpublished), and 89 km yr^{-1} (Lyons & Scheibling 2009). Recent spread rate estimates in the north-eastern Atlantic fall in a similar range: less than 90 km yr^{-1} (Shanks et al. 2003), 52 km yr^{-1} (Lyons & Scheibling 2009), and 69 km yr^{-1} (Mineur et al. 2010a). However, the European dataset shows clearly that the spread rate was higher before 1990 (105 km yr^{-1}; F. Mineur et al. unpublished).

One reason for the high rate of spread and larger spatial occupation in the north-eastern Atlantic (Mineur et al. 2010a) may be the greater 'macrocomplexity' of European coasts, compared to the linear coast of western North America. North-eastern Atlantic currents may also be more complex (mesoscale gyres, eddies, tidal currents, etc.) than the large-scale along-shore currents in the north-eastern Pacific. Consequently, thalli drifting off shore in Europe may have a better chance of encountering a shoreline and successfully releasing propagules within an appropriate habitat. This complexity may also multiply the number of potential pathways for vectors, such as shellfish transfers and recreational yachting, and increase the opportunities for secondary introductions. Another possible explanation is that extensive sandy beaches along the Oregon and California coastlines could both slow the rate of spread and reduce the potential maximum spatial occupation. However, in North America, the distribution of *S. muticum* in Oregon and California is still poorly known relative to other regions (e.g., Washington, British Columbia), and this informational gap could influence the results. It is likely that new portions of coastline already are or will be invaded. Moreover, it is probable that the range of suitable habitats (including northern and southern limits) will be subject to modifications, especially given the potential for future changes in water temperatures and ocean circulation caused by climate change.

Mechanisms of secondary dispersal

Secondary dispersal in the introduced region is mainly a combination of the natural spread capacity of the species and anthropogenic dispersal vectors. For *Sargassum muticum*, it is assumed that the wide and quick spread in introduced regions has been strongly facilitated by detached, drifting adult plants (Deysher & Norton 1982). Although most detached individuals probably are cast ashore, some may stay afloat for months (Norton 1976), even become fertile while afloat (Deysher & Norton 1982, Norton & Deysher 1989), (self-) fertilize, and shed their propagules in the meantime. Drifting adults with gas vesicles have the potential to disperse long distances; however, no study has quantified long-distance dispersal. The ability of drift material to grow and become or remain reproductively viable has been shown for *Sargassum* species from Sao Paulo, Brazil, in culture (Paula 1984). Drift material of *Sargassum* spp. is common throughout their geographical range and can form drifting 'islands' in the open sea with their own community. Individual species such as *S. kjellmanianum*, a species that may be conspecific with *S. muticum*, has been found oating in the Pacific Ocean almost 600 km from the coast of Japan (Vozzhinskaja 1964), and tagged specimens of other *Sargassum* species have been shown to drift 900 km at an average speed of 18 km d^{-1} (Segawa et al. 1962).

The annual shedding of floating branches carrying reproductive units coupled with the high reproductive potential gives *Sargassum muticum* long-distance spreading capability, which has been instrumental in the species' rapid expansion after its introduction on the North American Pacific and European Atlantic coasts (Scagel 1956, Farnham et al. 1973, North 1973, Critchley 1983a, Steen 2004). Dissemination of reproductive units from fertile drifting branches may also play an integral part in the species' local expansion (Critchley 1983a,b, Critchley & Dijkema 1984), as offspring released from attached specimens have a limited dispersal range (Kendrick & Walker 1995). It is assumed that the colonization of California from the north must have taken place through drift material spreading south for 1100 km along the entire coast of California (Deysher & Norton 1982).

The spread of *Sargassum muticum* in the north-eastern Pacific and north-eastern Atlantic has almost certainly been enhanced by secondary introductions associated with oyster transfers. In Europe, translocations between regions are a routine element of cultivation methods, and they potentially occur between all regions, but unfortunately quantities are not well documented (Mineur et al. 2007a). Transfers among regions also seem to occur regularly along the western coast of the USA (Wasson et al. 2001). Several sites where *S. muticum* has been secondarily introduced, such as the Oosterschelde estuary, the Nissum Bredning in Denmark, Arcachon Bay (western France), Strangford Lough in Northern Ireland, the Thau Lagoon and the Venice Lagoon in Europe, and Willapay Bay (Washington) and Coos Bay (Oregon) in the USA, are relatively confined embayments. These sites were also relatively distant from existing populations of *S. muticum*, and all are oyster cultivation areas. It is likely that 'hitchhiking' on oysters was the vector for the *S. muticum* introductions in these bays.

Vectors associated with shipping, especially hull fouling, are considered important for dispersal of microalgae (e.g., Ribera-Siguan 2002, Hewitt et al. 2004). For large macroalgae such as *Sargassum muticum*, however, there is no evidence that hull fouling plays an important role as a vector as this species is in general not found on ship hulls (F. Mineur and K. Britton-Simmons personal observations). In Europe and North America, however, *S. muticum* is often observed on floating structures such as pontoons (docks). For example, *S. muticum* was found on a floating dry dock, which was moored for a period in San Diego and subsequently towed from California to Hawaii (Godwin 2003), but *S. muticum* does not appear to have become established in Hawaii (Abbott & Huisman 2003). So, such floating structures (including aquaculture cages and oil platforms) can present a risk because they usually do not receive the same amount of servicing (i.e., application of antifouling coating) as ships (Mineur et al. 2012a).

Besides hull fouling, vectors associated with recreational yachting may be important for the regional spread of *S. muticum*. Indeed, in invaded areas such as the west of France, thalli of *S. muticum* are occasionally found attached to floating pontoons (F. Viard personal observation) and are regularly found entangled around the propellers of sailing boats (Mineur et al. 2008). Because the engine of a recreational sailing vessel is used only intermittently, thalli could potentially be transported long distances and then subsequently released when the propeller is engaged.

Aside from the introduction in Mediterranean lagoons, however, it is difficult to completely rule out natural spread, such as drifting of detached mature thalli. Estimating the natural spread of invasive species such as *Sargassum muticum* is problematic as the observed spread is likely to have been enhanced by anthropogenic vectors. It is impossible to retrospectively assess the proportion of spread that is attributable to natural versus human-mediated mechanisms. Moreover, even anthropogenic vectors can be viewed as part of the environment, and currently any species' dispersal ability will depend on its predisposition for exploiting human-mediated vectors.

In the invaded range, *Sargassum muticum* is found in rocky habitats in the shallow subtidal zone, with some populations extending into the lower intertidal zone on emergent rocks or as high as the midintertidal in tide pools. Subtidally, it is most abundant between approximately 1 and 3 m below mean lower low water (MLLW), diminishing in abundance with increasing depth below that zone. In California, USA, *S. muticum* is abundant subtidally to depths of 18 m, with some plants found as deep as 30 m, which is probably due to the higher light availability in clear waters (Foster & Schiel 1992).

Colonization

After dispersing (by natural or non-natural means) into a new area, the colonization success of *Sargassum muticum* first and foremost depends on the match between the recipient environmental regime and the species' physiological tolerances. The introduced individuals must be able not only to survive the environmental conditions in the invaded area but also to grow, reproduce, and recruit. The success of this depends on the environmental tolerance and acclimatization capacity of the introduced individuals. This section critically evaluates the life-history traits considered to contribute to the invasive character of the species.

Abiotic tolerances

Temperature and salinity are often considered two of the most important environmental abiotic drivers of the geographical distribution of marine algae, and tolerance to these factors often varies between different life stages (Lüning 1990). Experiments have shown that adult *Sargassum muticum* tolerate temperatures between −1°C and 30°C (comparable with the seasonal range in the native range) and survive in salinities below 10‰ (Norton 1977, Hales & Fletcher 1989). Adult *S. muticum* have a wide physiological tolerance to several environmental variables, similar to other *Sargassum* species with wide latitudinal distributions, such as *S. lipendula*, a species living at temperatures ranging from 4.5°C to 32°C (Earle 1969). However, other life-cycle stages have lower tolerance. Multifactorial experiments have shown that fertilization and post-fertilization development in *S. muticum* are reduced at low temperatures and salinities (Norton 1977, Hales & Fletcher 1989, Steen & Rueness 2004). Fertilization in *S. muticum* is not possible at salinities of 15‰ or less, and growth of germlings is strongly reduced at temperatures below 10°C (Norton 1977, Hales & Fletcher 1989, Steen 2004). In spite of these lower tolerances, the species' life cycle may still be completed in areas with such conditions, providing that temperatures are greater than

8°C for at least 4 months of the year. Accordingly, *S. muticum* will be able to complete development in wide geographical areas of the world.

Temperature and salinity tolerances are only indicators of potential distributional ranges, and laboratory conditions with constant temperature and salinity do not represent natural, variable conditions. Thus, inferring potential distributional ranges from physiological experiments under controlled conditions should be done with caution. However, this broad tolerance to temperature is in line with the present-day introduced range of *S. muticum*, spanning from Alaska to Baja California in America and from Norway to Morocco and the Mediterranean Sea in Europe (Figure 1).

Reproductive Traits

Reproductive output

Sargassum muticum is a pseudoperennial species with strong seasonality in its life cycle. In Europe, the fertile season peaks in spring/summer, but small fertile individuals are also present in autumn, especially in southern populations. Maturation is gradual, within both individuals (lateral branches have an acropetal maturation pattern in which lateral mature branches coexist with immature branches) and populations (larger individuals become fertile earlier in the season). This gradual maturation hinders the estimation of reproductive allocation (RA) of the species, usually calculated as the percentage of dry biomass allocated to fertile structures (receptacles; Figure 3). Norton & Deysher (1989) found that receptacles represented 50% of biomass in fertile individuals. Arenas & Fernández (1998) estimated mean values of reproductive biomass around 20–24%, with a maximum RA of 55% in a single individual. These values are not higher than the RA in native north-eastern Atlantic fucoids: *Ascophyllum nodosum*: 48–70% (Cousens 1986), 54% (maximum 92%; Åberg 1996); *Fucus spiralis*: 28–78% (Mathieson & Guo 1992); *F. vesiculosus*: 62% (Mathieson & Guo 1992); *F. distichus*: 50–62% (Mathieson & Guo 1992); *F. serratus*: 38.6–50.5% (Brenchley et al. 1996). Nevertheless, the number of recruits released by an individual *Sargassum muticum* may be high due to the size it may attain and its long fertile period. Each receptacle releases around 300 embryos, and the number of receptacles is estimated to be around 100 g^{-1} (fresh weight, FW) of vegetative tissue (Norton & Deysher 1989). These authors estimated that a small individual of 15 g FW might release up to 500,000 propagules. Dense populations of *S. muticum* with fresh biomass density of 0.6–12 kg m^{-2}, like those frequently found in invaded areas (Givernaud et al. 1991), would be able to release up to several million propagules per square metre.

This high fertility is a common characteristic of large brown macroalgae. In fact, the production of new recruits in high numbers seems to be crucial for fucoids like *S. muticum* because of the low probability of successful settle- ment and high post-settlement mortality. In a recent study, Vaz-Pinto et al. (2012) found that high availability of propagules together with adequate substratum availability was crucial for the estab- lishment of *S. muticum* populations.

Self-fertilization

Sargassum muticum is monoecious, bearing both male and female reproductive organs (called conceptacles) within each reproductive structure (called receptacles), which enables self-fertilization (Figure 3). Numerous studies have shown that isolated reproductive structures give rise to healthy new recruits in the laboratory, so successful recruitment can take place through self-fertilization (e.g., Fletcher 1980). Other *Sargassum* species are also monoecious, such as most of the *Sargassum* species at wave-exposed sites on the coast of the state of São Paulo (Brazil), and are capable of self- fertilization (Paula & Eston 1987). On the other hand, *Sargassum* species from sheltered sites (the dominant habitat of *S. muticum*) on the same Brazilian coast are predominantly dioecious (Paula & Eston 1987). It remains unknown to what extent self-fertilization is important in natural populations of *S. muticum* because no published studies based on population genetic markers with sufficient resolution are currently available.

Gamete release

For fertilization to be successful, synchrony of gamete release may be important and may contribute to generating the densities of propagules required for the establishment of populations. *Sargassum muticum* releases gametes intermittently at intervals of 14 days following a semilunar periodicity (Fletcher 1980, Norton 1981, May & Clayton 1991, Engelen et al. 2008) throughout the reproductive season. Although this synchronization might be affected by the onset of senescence at the end of the reproductive season (Monteiro et al. 2009), this greatly increases the chances of at least one release coinciding with favourable conditions for fertilization/settlement (Deysher & Norton 1982). In general, *Sargassum* species display a periodicity of egg release that is synchronized around the new moon (May & Clayton 1991 for *Sargassum vestitum*; Inoh 1930 for 12 *Sargassum* species in Japan) or both new and full moon (Tahara 1909, 1913). To what extent this contributes to the invasive character of *S. muticum* remains uncertain.

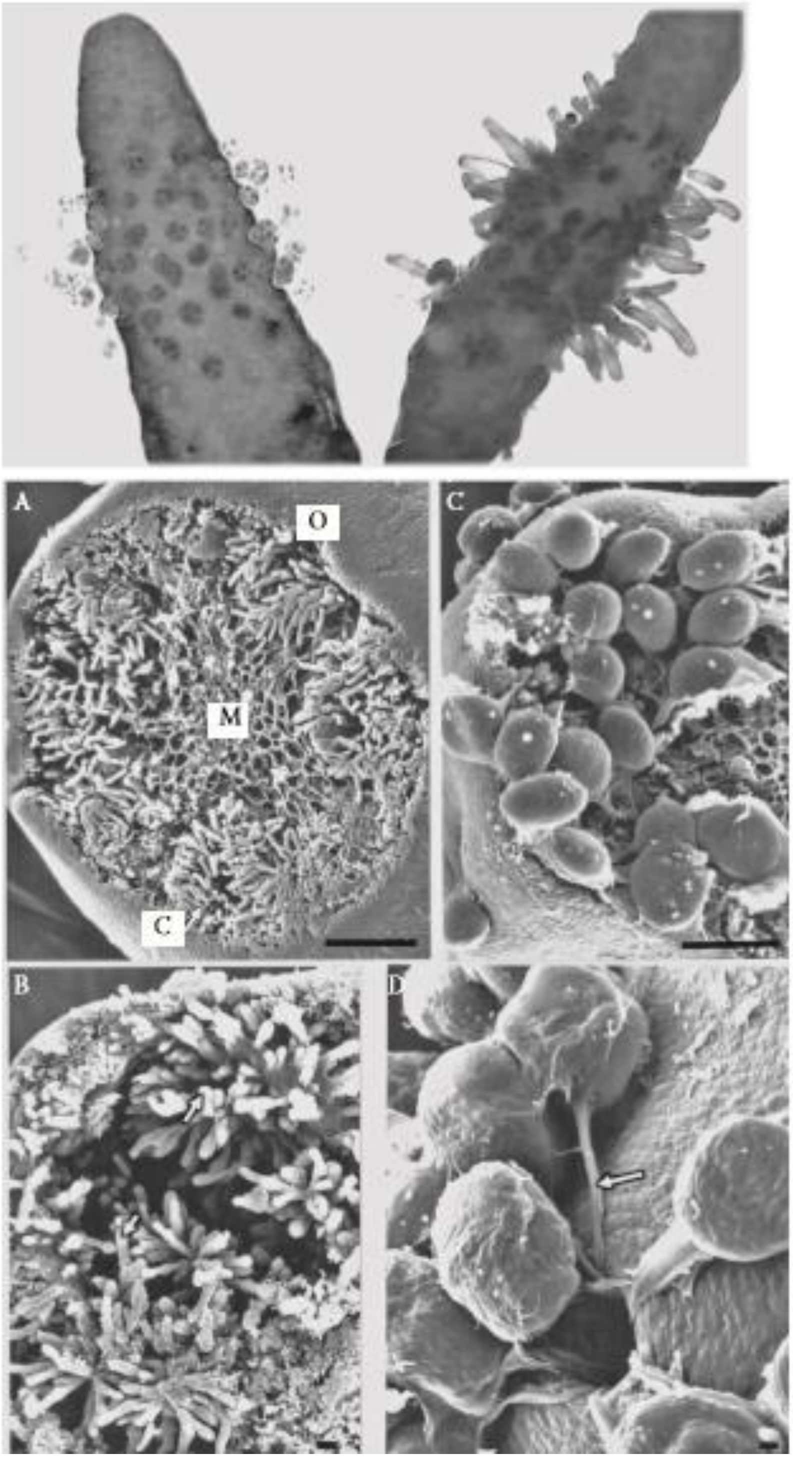


Figure 3 *Sargassum muticum* receptacles with attached eggs (top left) and multicellular germlings (top right). Scanning electron micrographs of *S. muticum* receptacles: (A) Transverse section of receptacle with medulla (M), conceptacle (C), and ostiole (O); scale bar represents 100 µm. (B) Conceptacle with antheridia (arrow); scale bar represents 10 µm. (C) Conceptacle with oogonia; scale bar represents 100 µm. (D) Eggs retained on the outside of the receptacle after expulsion, still connected to the inside of the conceptacle; scale bar represents 10 µm. (Photos by Francisco Arenas)

Propagule retainment

Another feature that likely enhances the post-invasion success of S*argassum muticum* is the ability to retain germlings on the parental tissue after fertilization (Fletcher 1980; Figure 3). As in other *Sargassum* and *Cystoseira* species, *Sargassum muticum* has a form of parental care in which propa- gules remain on the reproductive structures for up to a few days after fertilization (Norton 1976, Fletcher 1980, Schiel 1985, Paula & Eston 1987, Engelen et al. 2008, Monteiro et al. 2009, 2012). Most germlings are released from the receptacle surface 2–3 days after fertilization (Fletcher 1980, Engelen et al. 2008, Monteiro et al. 2009, 2012) but apparently may remain attached for as long as 18 days (Norton 1980, 1981). However, propagules retained beyond the normal incubation time develop normally but seem unable to liberate or attach to the substratum when released (Deysher & Norton 1982; Figure 3). The normal retainment of a few days implies that the most vulnerable early post-fertilization life stage is passed while the young germlings are anchored and somewhat protected on the receptacle surface, which is thought to increase their chances of survival and subsequent settlement on the sea floor. After detachment, propagules sink much faster (>0.5 mm s^{-1} in stationary water) than other algal propagules investigated (Coon et al. 1972, Okuda & Neushul 1981), but probably similar to other *Sargassum* (Paula & Eston 1987) and *Cystoseira* species, and are capable of quick attachment on contact with a surface (Norton & Fetter 1981, Deysher & Norton 1982). Large multicellular propagules coupled with rapid germling growth may give *Sargassum muticum* a competitive advantage during the early post-settlement stages.

Embryo dispersal

Like many fucoid macroalgae, *Sargassum muticum* can disperse as embryos and as adults. When attached to the substratum, *S. muticum* drops the vast majority of its embryos within a few metres of the parent plant, although a few settle up to 30 m away (Deysher & Norton 1982). Embryos have been found in the plankton up to 1.3 km from the nearest reproductive adult (Deysher & Norton 1982). Physical modelling of *S. muticum* embryo dispersal by Gaylord et al. (2002) confirmed that the majority of embryos will settle within a few metres at a current speed of 2 cm s^{-1} and wave height of 0.5 m, but that at current speeds of decimetres per second and wave height of 1 m or higher, dispersal will be on the order of kilometres. Embryos kept in suspension for up to 40 days maintained the ability to attach (Deysher & Norton 1982).

Substratum requirements

Sargassum muticum has less-specific substratum requirements than many other perennial macrophytes, and specimens have been observed growing on a variety of substrata, including floating pontoons, bottles, rope structures, empty shells (Figure 3), and small stones and pebbles, often with little competition from other algal species. Low substratum specificity may increase the species' chances of establishment, especially in areas where resistance to species invasions is otherwise high. If the introduced species exploits previously unoccupied niches, this may lead to increased marine production and diversity (Russell & Balazs 1994, Fletcher & Farrell 1998, Occhipinti- Ambrogi & Savini 2003, Buschbaum et al. 2006).

Establishment

If the physiological requirements of *Sargassum muticum* are met and the species successfully colonizes a new area, its establishment will depend on interactions with resident species, which may be negative (competition, 'biotic filters'), positive (facilitation), or both (Sánchez et al. 2005). The competitive ability of *S. muticum* is related to its high rate of biomass accumulation and rapid thallus elongation (Critchley 1983a, Wernberg et al. 2001, Pedersen et al. 2005). A multispecies comparison made in Danish waters showed that *S. muticum* had a higher relative growth rate (expressed in biomass) than other perennial macroalgae in this system (Pedersen et al. 2005). Another cross-species comparison of early post-settlement development in Scandinavian waters revealed that germlings of *S. muticum* were capable of growing several times faster than germlings of potential competitor fucoid species (Steen & Rueness 2004). However, the outcome of competitive interactions between *S. muticum* and native species will likely depend on environmental conditions, and *S. muticum* germlings are more negatively affected by reductions in salinity and temperature than germlings of several potential competitor fucoid species in European waters (Serrão et al. 1996, Malm et al. 2001, Wikström et al. 2002, Steen 2004, Steen & Rueness 2004). Reduced growth and competitive ability at low temperatures and salinities may thus decrease the invasiveness and ecological impact of *S. muticum* in cold-temperate and brackish waters, respectively. However, given optimal environmental conditions, *S. muticum* may grow as fast as 4 cm d^{-1} (Jephson & Gray 1977, Nicholson et al. 1981) and attain lengths of up to 10 m (Abbott & Hollenberg 1976, Belsher & Boyen 1983), which makes the species a strong competitor that may have negative effects on indigenous floral components (den Hartog 1997, Viejo 1997, Cosson 1999, Stæhr et al. 2000, Wernberg et al. 2001, Britton-Simmons 2004, Sánchez et al. 2005). On the other hand, Baer & Stengel (2010) showed that *S. muticum* was heavily colonized by the epiphytic brown alga *Pylaiella littoralis* in a sheltered site in Ireland, a situation that may impede growth of *S. muticum*. In the Firth of Clyde, Scotland, *S. muticum* becomes overgrown by a similar brown alga in mid- to late summer (Figure 2D; I.P. Smith personal communication).

Phenotypic plasticity

Sargassum muticum has a modular growth form with a structural complexity resembling that of terrestrial plants. Individual *S. muticum* are attached to the substratum by a perennial holdfast with a single stem that yearly sprouts several apically growing main branches. These primary branches will bear lower-order branches, also with apical growing meristems. This structural complexity gives *S. muticum* the ability to modify the production of modules (especially main branches) and to vary branch elongation, reducing interference among neighbours and allowing plants to grow in locally dense populations (Arenas et al. 2002). It also serves as a mechanism to compensate for eventual canopy losses, promoting the persistence of local populations and preventing invasion by other species. Baer & Stengel (2010) showed that morphological and developmental variation between populations in Ireland corresponded to degree of wave exposure (open shore vs. sheltered bays). In view of the recent colonization of these sites (since 2001), Baer & Stengel (2010) suggested that phenotypic plasticity was the most likely explanation for such variations.

Latitudinal variation in phenology

Sargassum muticum has an annual cycle of growth, reproduction, and quiescence, which was well described by Wernberg-Møller et al. (1998). The new primary laterals start growing in late winter or spring, depending on seawater temperature, and are represented by short branches with large basal leaves, which increase the photosynthetic surface area. As the lateral growth progresses, gas vesicles and secondary branches begin to develop. Multiple vesicles keep the thallus upright in the water column towards the sunlight, while the colour of the thallus becomes lighter. In the middle of the season, basal leaves considerably reduce in number. Laterals of lower orders develop, and their number of vesicles is high. Receptacles start to develop, and the thallus is yellowish. In late summer, fertile receptacles prevail, giving the plant a dark brown colour. There are almost no vesicles, and the plant loses buoyancy. At the end of the season, new primary branches with vesicles detach from older perennial parts and float away, carrying fertile propagules. The holdfast with some of the old primary laterals remains and overwinters. Such an adjustment of morphology, along with the physiological slowdown during the (late) summer and the subsequent period of dormancy (Strong 2003), demonstrates a highly specialized adaptation of *S. muticum* to life in a seasonal environment (Wernberg-Møller et al. 1998).

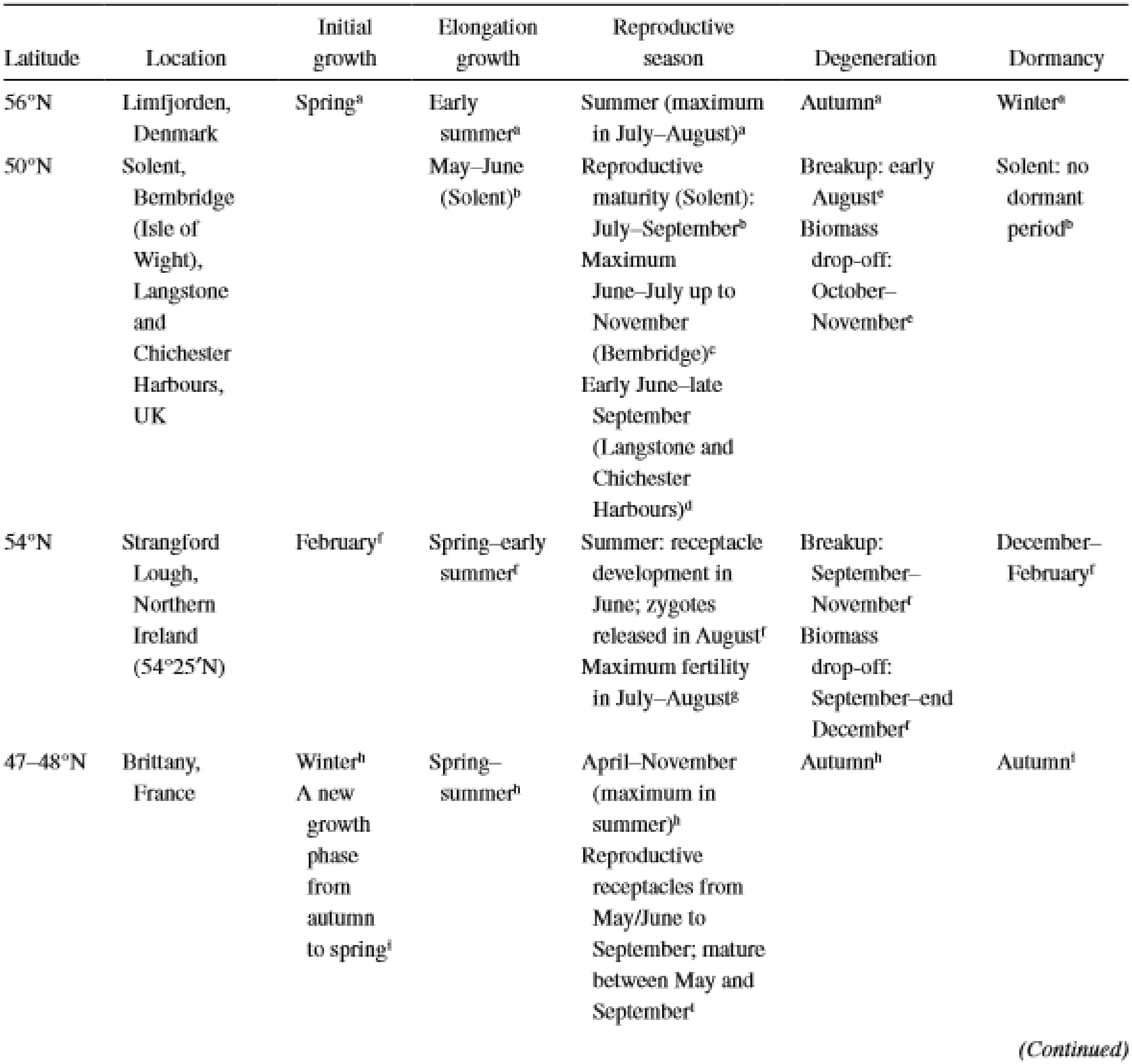
The timing of the different phases of the annual life cycle varies geographically (Table 1). In the native area (Japan, 34–39°N), reproductive individuals of *Sargassum muticum* are found from winter to early summer (Yoshida 1983, 1998). After reproduction, individuals enter a dormant phase, which is characterized by dieback to the perennial holdfast. Growth then recommences in late autumn (Yendo 1907, Rueness 1989). In China, fertile individuals are recorded in spring (C.C. Cheang personal observation), with degeneration in late June (in Dalian, North China, 39°N; R. Luan personal communication).

In invaded regions (Figure 1), mature individuals of *Sargassum muticum* can be observed in early spring, summer, and early autumn. Moving southwards along the western coast of North America (49–26°N), the seawater temperature increases, and the occurrence and duration of dormancy appear to decline (Jephson & Gray 1977). In autumn, new branches start to develop, and throughout the late winter–spring period (February–May), the plants undergo rapid vegetative growth. The reproductive period follows in June to September. However, in more southerly locations, the reproductive period tends to begin earlier in the year and to last longer (Norton & Deysher 1989). In Southern California, USA (33°N), fertile individuals can be found throughout the year (Nicholson et al. 1981). A similar situation is observed on the north-western coast of Baja California, Mexico (32°N), where mature individuals are found all year round, with the highest percentage from May to July (spring–summer) and the lowest from December to March (winter) (Aguilar-Rosas & Machado-Galindo 1990). After the end of the reproductive period, the growth rate declines rapidly and the laterals start to degenerate.

In southern Europe (37–45°N), the winter period of dormancy is not always evident. The alga undergoes its highest vegetative growth from winter to early summer with laterals reaching over 4.5 m in length (Sfriso & Facca 2013). Rapid growth is induced by the increasing water tempera- ture and availability of light. During this period, as reproductive receptacles develop, vegetative growth reduces. Numerous gas vesicles keep the thalli afloat close to the water surface. On the northern coast of Portugal (42°N), the reproductive period can last from April to August, while in southern Portugal (37°N), it occurs between January and September, depending on the location (Engelen et al. 2008). In the Lagoon of Venice, Italy (45°N), the growth rate of individual

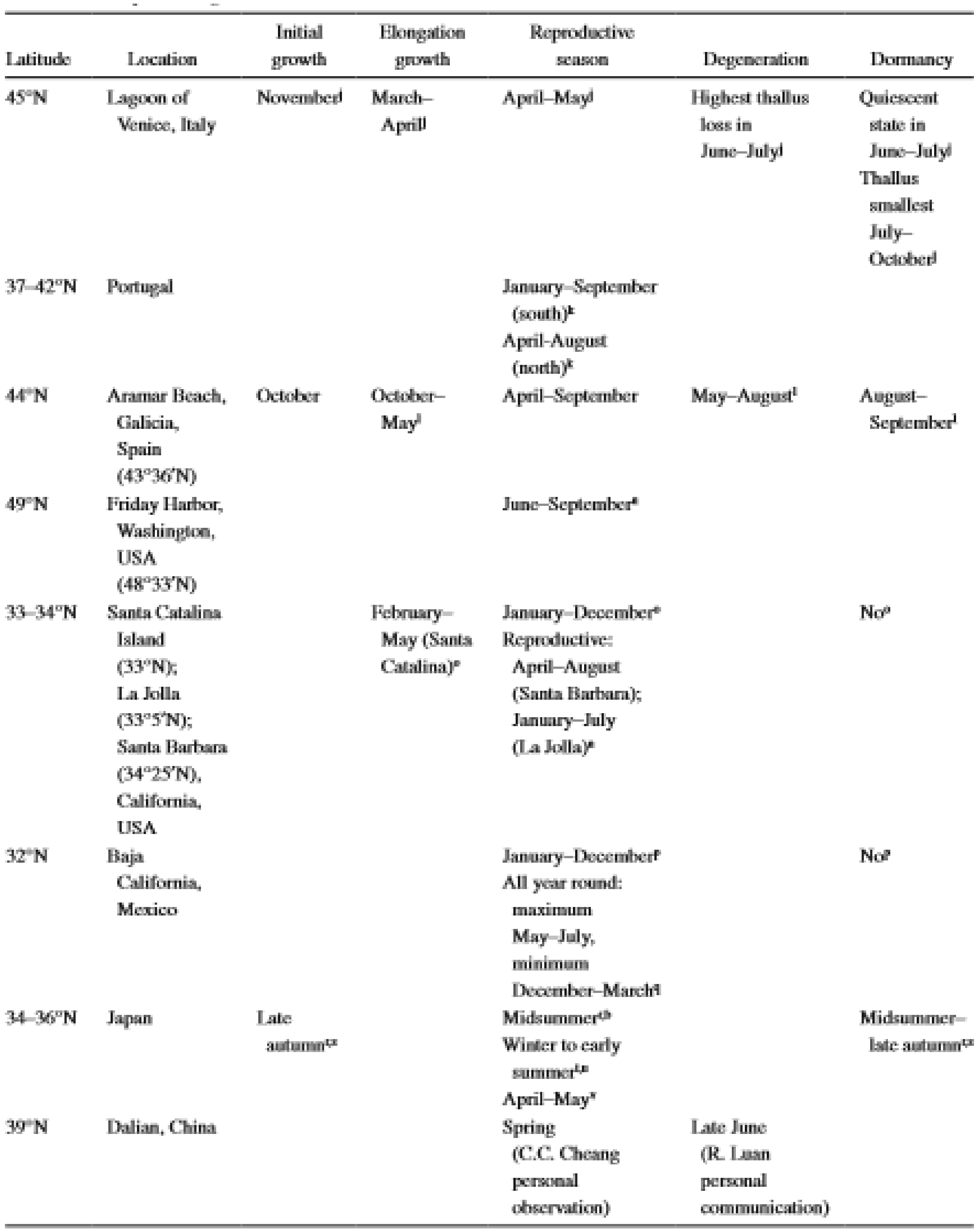
Sargassum muticum increases in March, with maximum length reached in April (Sfriso & Facca 2013). In June–July, when water temperature exceeds 22–25°C, *S. muticum* ceases growth. Plants begin losing their primary branches, keeping only the short axis. As a result, the thallus reduces or disappears completely, with minimal length recorded from July to October. After surviving the warm season, *S. muticum* begins growing again in November, when the temperature falls to 15°C. Above the optimal temperature range (15–18°C or maximum 20°C), growth rates are negative due to the loss of primary laterals (Sfriso & Facca 2013).

Table 1 Regional variations in the annual cycle of growth, reproduction, and dormancy of *Sargassum muticum*



Latitude	Location	Initial growth	Elongation growth	Reproductive season	Degeneration	Dormancy
56°N	Limfjorden, Denmark	Spring[a]	Early summer[a]	Summer (maximum in July–August)[a]	Autumn[a]	Winter[a]
50°N	Solent, Bembridge (Isle of Wight), Langstone and Chichester Harbours, UK		May–June (Solent)[b]	Reproductive maturity (Solent): July–September[b] Maximum June–July up to November (Bembridge)[c] Early June–late September (Langstone and Chichester Harbours)[d]	Breakup: early August[e] Biomass drop-off: October–November[e]	Solent: no dormant period[b]
54°N	Strangford Lough, Northern Ireland (54°25′N)	February[f]	Spring–early summer[f]	Summer: receptacle development in June; zygotes released in August[f] Maximum fertility in July–August[g]	Breakup: September–November[f] Biomass drop-off: September–end December[f]	December–February[f]
47–48°N	Brittany, France	Winter[h] A new growth phase from autumn to spring[i]	Spring–summer[h]	April–November (maximum in summer)[h] Reproductive receptacles from May/June to September; mature between May and September[i]	Autumn[h]	Autumn[i]

(Continued)



Latitude	Location	Initial growth	Elongation growth	Reproductive season	Degeneration	Dormancy
45°N	Lagoon of Venice, Italy	November[j]	March–April[j]	April–May[j]	Highest thallus loss in June–July[j]	Quiescent state in June–July[j] Thallus smallest July–October[j]
37–42°N	Portugal			January–September (south)[k] April-August (north)[k]		
44°N	Aramar Beach, Galicia, Spain (43°36′N)	October	October–May[l]	April–September	May–August[l]	August–September[l]
49°N	Friday Harbor, Washington, USA (48°33′N)			June–September[n]		
33–34°N	Santa Catalina Island (33°N); La Jolla (33°5′N); Santa Barbara (34°25′N), California, USA		February–May (Santa Catalina)[o]	January–December[o] Reproductive: April–August (Santa Barbara); January–July (La Jolla)[v]		No[o]
32°N	Baja California, Mexico			January–December[p] All year round: maximum May–July, minimum December–March[q]		No[p]
34–36°N	Japan	Late autumn[t,u]		Midsummer[r,s] Winter to early summer[t,u] April–May[v]		Midsummer–late autumn[t,u]
39°N	Dalian, China			Spring (C.C. Cheang personal observation)	Late June (R. Luan personal communication)	

[a] Wernberg-Møller et al. 1998; [b]Jephson & Gray 1977; [c]Fletcher & Fletcher 1975; [d]Critchley 1983a; [e]Critchley 1983c; [f]Strong 2003; [g]Davison 1999; [h]Le Lann et al. 2012; [i]Plouguerné et al. 2006; [j]Sfriso & Facca 2013; [k]Engelen et al. 2008; [l]Fernández 1999; [m]Arenas & Fernández 1998; [n]Norton & Deysher 1989; [o]Nicholson et al. 1981; [p]Espinoza 1990; [q]Aguilar-Rosas & Machado-Galindo 1990; [r]Rueness 1989; [s]Yendo 1907; [t]Yoshida 1983; [u]Yoshida 1998; [v]Deysher 1984.

In northern Europe, the annual life cycle of *Sargassum muticum* is quite different and constitutes the following phases: initial growth (spring), elongation growth (early summer), reproductive growth (summer), and degeneration (autumn) (Wernberg-Møller et al. 1998). The primary laterals start growing rapidly during the spring and summer months, with notable prevalence of larger individuals in summer. During late spring and early summer, the growth rate is estimated to be 2 cm d^{-1} (in Northern Ireland, 54°N; Strong 2003) and can reach up to 4 cm d^{-1} between May and June (southern England, 49°N; Jephson & Gray 1977). In Denmark (56°N; Wernberg-Møller et al. 1998) and at a wave-exposed site on the south-western coast of Ireland, individuals reach their maximum length around July (53°N; Baer & Stengel 2010). In Strangford Lough, Northern Ireland (54°N), maximum length is achieved in August (Strong 2003). The maximum plant biomass also occurs in this period. The reproductive season is short and is restricted to summer. In southern England, *S. muticum* reaches its reproductive maturity between July and September (Jephson & Gray 1977); receptacle development typically begins in June, and zygotes are released during August. In Denmark, the number of mature individuals is maximal in July and August, while on the south-western coast of Ireland, individuals are not fertile until August (Baer & Stengel 2010). On the western coast of Brittany, France (48°N), mature individuals are observed until autumn, when fragmentation begins (Le Lann et al. 2012), and there is a rapid decrease in length in October (Plouguerné et al. 2006). In Northern Ireland, receptacle development typically starts in June, and zygotes are released during August (Strong 2003). By November, the number of primary laterals is significantly decreased. The individual biomass reaches its lowest value by the end of December, and in February, the laterals do not exceed 0.1 m in length (Strong 2003). While the period of dormancy usually lasts from December to February, in certain locations (e.g., southern England), no dormant period has been recorded as the growth of the next generation of laterals begins before the previous year's branches have decayed (Jephson & Gray 1977).

Published results from various locations suggest a latitudinal dependency in the phenology of *Sargassum muticum*. In more southerly locations, the reproductive period of this alga tends to begin much earlier in the year and to last longer. However, such differences in reproductive phenology are generally interpreted as caused by environmental factors rather than physiological differences in plants found at different latitudes. Common garden (transplantation) experiments might reveal the relative contributions of tolerance and acclimatization associated with these observations. While higher water temperatures generally lead to more rapid development and early reproduction of the alga (Norton & Deysher 1989), this does not apply to every population of *S. muticum*. For instance, Aguila-Ross & Machado-Galindo (1990) found that the peak of the reproductive period in Baja California occurs much later than expected, even though reproductively mature individuals are present throughout the year. They attributed the lateness of the reproduction period to an effect of coldwater upwelling along the coast (Aguilar-Rosas & Machado-Galindo

1990). In addition to the impacts of water temperature, day length is important in the reproductive timing of *S. muticum*. Delayed reproductive maturity with increasing latitude can also be explained by the response to photoperiod (Strong 2003). The beginning of growth of *S. muticum* in winter matches the finding of Hwang & Dring (2002), who showed under controlled conditions that the development of erect thalli is a photoperiodic response. In addition, main-branch elongation is promoted by short days and suppressed under long-day regimes (Uchida et al. 1991).

Although numerous publications have provided information on the phenology of the species, these all relate to local studies, none of which has properly monitored environmental parameters like temperature along with *Sargassum muticum* phenology. This makes it practically impossible to integrate their individual findings. Phenological events in terrestrial plants (e.g., leaf unfolding, flowering, fruiting, etc.) are tightly linked to photoperiod and temperature (e.g., Campoy et al. 2011) and as such are sensitive to climate change. Further progress in understanding the phenology of *S. muticum* would greatly benefit from a latitudinal approach in which the species and its environmental conditions (especially photoperiod, salinity, and temperature) are monitored and integrated in temperature accumulation models (Chuine et al. 2003). For scientific interest, this should be combined with the use of molecular techniques to provide important insights into the molecular basis and control of phenology.

Demography

Invasion biology must ultimately address patterns at the level of population dynamics because it is at this level that an invasion either fails or succeeds. By elucidating patterns of variation in population growth rates or demographic rates, such as fecundity and survival, it should be possible to test theoretical ideas about invasiveness (the traits that enable a species to invade), invasibility (the susceptibility of recipient communities to the establishment of new species), and rates of spread (Mack 1985). Standard deterministic approaches in demography provide a framework for modelling structured populations in which growth, death, and birth rates depend on age, size, or developmental stage. Demographic insights can provide an essential connection between quantitative field data and invasion theory. Unfortunately, demographic approaches are rare in invasion biology and have rarely been applied in macroalgal invasions.

Following a stochastic matrix model approach, Engelen & Santos (2009) identified the demographic traits of *Sargassum muticum* that are most important for local population growth along the Portuguese coast. They investigated tide pool invasions by following the fate of hundreds of *S.*

muticum individuals monthly over 2 years, and their demographic analyses did not show any shift in the importance of any life-history strategy or traits with the progress of invasions. Elasticity analysis indicated that persistence of non-reproductive adults contributed most to the population growth rate during both phases of invasion, although survival of microrecruits was important in one of the years during colonization. During colonization, growth rate and growth rate sensitivity (expressed as elasticities) were more variable between years than in relation to when the invader was established. Persistence of adults was high, with 70% to 80% of individuals remaining after 1 year. These findings showed that locally an invader can rely on persistence of adults, and that there is not always a need for changes in life-history strategy as invasion progresses.

Although life-history traits can be considered the ecological basis of invasiveness, apparently invaders do not necessarily have different traits from non-invasive species, as the demographic characteristics of *S. muticum* do not differ markedly from other canopy-forming, non-invasive brown macroalgae, such as *S. polyceratium* (Engelen 2004, Engelen et al. 2005) and *Ascophyllum nodosum* (Åberg 1992a,b). Demographic data collected close to Oviedo (Asturian coast of Spain, 43°N) and Plymouth (south-western England, 50°N) suggest that at these higher latitudes, persistence of individuals might be considerably lower than in southern Portugal (37°N; C. Fernández & F. Arenas unpublished). Consequently, new recruitment might be more important for sustaining populations.

It is evident that there is a great need for demographic approaches that elucidate differences in population dynamics between invaders and natives and between invasive species in their native and introduced regions, as well as the interactions between an invading population and its host community.

Biotic filters

Herbivory

Palatability of Sargassum muticum *to herbivores*

To our knowledge, only a few studies have investigated the palatability of *Sargassum muticum* to herbivores. Since *S. muticum* is primarily found in temperate seas, mesoherbivores are probably the most important grazers of this species. *Sargassum muticum* sometimes appears as free-floating algal clumps that are inhabited by a variety of crustacean herbivores (Vandendriessche et al. 2006). *Idotea baltica* did not prefer *Sargassum muticum* as a habitat (the highest percentage of isopods was found on *Fucus vesiculosus* and *Enteromorpha* sp.). However, it did prefer to feed on

Sargassum muticum, *Ascophyllum nodosum*, and *Enteromorpha* sp. over *Fucus vesiculosus* and *Himanthalia elongata*. *Gammarus crinicornis*, on the other hand, preferred to reside on *Sargassum muticum*, but fed little on all the algal species tested; that is, no clear difference in feeding preference could be detected (Vandendriessche et al. 2006).

The enemy release hypothesis (ERH; Elton 1958) states that introduced plant species will have a competitive advantage over native plants due to reduced control by natural enemies (e.g., pathogens, herbivores, etc.; Colautti et al. 2004). For instance, native specialist herbivores will not recognize them as food. Some investigations have tested (aspects of) the ERH in feeding preference experiments using *Sargassum muticum* and co-occurring native macroalgae. Engelen et al. (2011) found that *S. muticum* was the least-preferred species (compared to six native macroalgal species) in multiple-choice feeding experiments with two native herbivores (the herbivorous snail *Hydrobia ulvae* and the amphipod *Gammarus insensibilis*). Grazers affected the growth rate of all macroalgae, but *Sargassum muticum* had the highest biomass growth rates with and without grazers. Similar results were obtained by Cacabelos et al. (2010), who concluded that in general native meso- and macrograzers (*Littorina littorea*, *Aplysia punctata*, and *Paracentrotus lividus*) preferred native macroalgae to *Sargassum muticum*. From this, they suggested, perhaps prematurely, that *S. muticum* is not under substantial pressure from grazers on the Galician coast.

The feeding preferences of herbivorous snails (*Littorina littorea*) and crustacean herbivores (*Idotea* spp.) for *Sargassum muticum* and two co-occurring native fucoid macroalgae (*Fucus serratus* and *Halidrys siliquosa*) were tested in a series of two-choice feeding preference experiments (Hill 2006). *Littorina littorea* preferred to feed on *Sargassum muticum* rather than both native macroalgae, but no preference was detected for *Idotea* spp. Hill therefore concluded that the introduced macroalga was not released from enemies, and the ERH was rejected. In Strangford Lough, Northern Ireland, Strong et al. (2009) came to the same conclusion based on their finding that the most abundant herbivorous amphipod on *Sargassum muticum*, *Dexamine spinosa*, removed more tissue from *Sargassum muticum* than from three native macroalgae (*Saccharina latissima*, *Halidrys siliquosa* and *Fucus serratus*) when cultured together.

In Washington State, USA, the common gastropod *Lacuna vincta* preferred to feed on *Sargassum muticum* in choice feeding trials with fresh tissue and artificial foods (Britton-Simmons et al. 2011). Available data even indicated that the gastropod acquired this preference more than 30 years after the initial invasion (Britton-Simmons et al. 2011).

It is difficult to draw general conclusions from these studies with regard to the ERH; their findings are inconsistent. This could very well be because of the different species of grazer interacting with *Sargassum muticum* in different geographical areas. On the other hand, the ERH was first postulated for terrestrial habitats where specialist herbivores are common, whereas specialist herbivores are rare in the marine environment. Hence, macroalga-herbivore interactions are commonly dominated by generalist herbivores and so the ERH may not apply to marine habitats. To test effectively for the release of natural enemies as the cause of invasiveness, it is essential to incorporate studies in the native region, such as that by Vermeij et al. (2009).

Chemical defences against herbivores

Macroalgae have evolved adaptations to herbivory, for example, by escaping in time and space, tolerating biomass losses through rapid growth, or resisting herbivory through low nutritional quality, morphological or chemical defences, or a combination of these (Duffy & Hay 1990). Brown macroalgae, including species of *Sargassum*, contain a class of polyphenolic secondary metabolites called phlorotannins. Phlorotannins are polymers of 1,3,5-trihydroxy-benzene and are usually measured by colorimetric methods (e.g., Folin-Denis or Folin-Ciocalteus) that quantify the total phenolic content of the macroalgal tissues (e.g., Amsler & Fairhead 2006). Phlorotannins deter grazing by gastropod herbivores in the fucoids *Fucus vesiculosus* and *Ascophyllum nodosum* (Gieselman & McConnell 1981, Pavia & Toth 2000).

Van Alstyne & Paul (1990) indirectly tested the deterrent effect of *Sargassum muticum* phlorotannins on herbivores by offering tropical herbivorous fish in Guam (western Pacific) palatable local species of algae coated with methanol extracts of *S. muticum* collected in California, USA. Fish consumed significantly less treated algae than untreated controls, indicating deterrence (Van Alstyne & Paul 1990). Methanol extracts not only contain phlorotannins but also may contain other unidentifed compounds; therefore, the herbivore-deterrent activity of the extracts cannot unequivocally be attributed to phlorotannins. However, to our knowledge, this is the only study that has investigated the herbivore-deterrent ability of extracted secondary metabolites (phlorotannins or other compounds) from *S. muticum*.

The tissue phlorotannin content in brown macroalgae varies on several spatial and temporal scales (Amsler & Fairhead 2006). Connan et al. (2006) investigated the variation in phlorotannin content between *Sargassum muticum* and three co-occurring brown algae (*Ascophyllum nodosum*, *Laminaria digitata*, and *L. hyperborea*) collected in Brittany, France. On average, *Sargassum muticum* contained similar phlorotannin levels to *Ascophyllum nodosum* (4.9 ± 2.9% and 4.8 ± 0.5%

of dry weight, mean ± standard deviation [SD], respectively) and high levels compared to the two *Laminaria* species (0.2 ± 0.1% and 2.6 ± 1.5% dry weight, mean ± SD, in *L. digitata* and *L. hyperborea*, respectively). Steinberg (1986) reported somewhat lower whole-plant levels of phlorotannins (3.77 ± 0.39% dry weight, mean ± standard error [SE]) in *Sargassum muticum* collected in California, USA. Furthermore, although no statistically significant difference in phlorotannin content was detected between different parts of *S. muticum* individuals, the apical parts appeared to have lower tissue phlorotannin content (1.6 ± 0.1% dry weight, mean ± SD) than the basal (7.0 ± 1.1% dry weight, mean ± SD) and median (6.2 ± 1.6% dry weight, mean ± SD) parts (Connan et al. 2006). The phlorotannin content in Swedish *S. muticum* was 3.10 ± 0.75% dry weight (mean ± SE) in apical parts and 5.55 ± 0.20% dry weight (mean ± SE) in holdfasts (K.E. Hill unpublished). Taken together, the results of these local studies indicate that the phlorotannin levels in *S. muticum* vary on several spatial scales, from populations separated by thousands of kilometres to parts within a macroalgal thallus. Recently, Tanniou et al. (2013) assessed this spatial variability in phenolic content across latitudes in Europe, from Norway (60°N) to Portugal (37°N) and not only confirmed the spatial variation patterns of the foregoing studies but also detected higher phenolic content and the greatest activity (antireactive oxygen species) in the most southerly populations investigated.

To our knowledge, no compounds apart from phlorotannins have been implicated in herbivore-deterrent effects in *Sargassum muticum*. However, other metabolites have been isolated from *S. muticum*; for example, Zang et al. (1991) isolated phytohormones (cytokinins) that were hypothesized to function in different growth processes within the algae, although this hypothesis was not tested experimentally. Furthermore, alkylphenols that may have been generated from alkylated phlorotannin-type compounds (Van Heemst et al. 1996), apo-9′-fucoxanthinone (Yang et al. 2013), and formaldehyde (Yang et al. 1998) were found in *S. muticum*, but no hypothesis or experimental test of their ecological function was proposed. However, the lack of identified bioactive metabolites in *S. muticum* compared to other species (e.g., Paul et al. 2001) may be due to a lack of relevant experiments (i.e., bioassay-guided fractionation of extracts using ecologically relevant herbivores) rather than a true deficiency of herbivore-deterrent secondary metabolites in this species.

Induced herbivore resistance

Chemical defences in macroalgae are either produced and are present continuously within a plant (e.g., Paul et al. 2001) or may be induced in response to different environmental cues (e.g., direct grazing damage or damage-related chemical cues; Pavia & Toth 2000, Toth & Pavia 2000). The presence of induced resistance in *Sargassum muticum* was tested by exposing individuals either to direct grazing by the isopod *Idotea granulosa* or to waterborne chemical cues from macroalgae that were being grazed by the isopods in a 1-week laboratory induction experiment (Toth 2007). After termination of the induction experiment, the individual algae were freeze-dried, homogenized, and incorporated into artificial diets. Induced resistance was evaluated in two-choice feeding experiments, where naive isopods were allowed to choose between artificial diets containing either induced or control individuals previously kept without herbivores or cues. Artificial diets, rather than fresh algal tissues, were used to maximize the chance of detecting induced resistance by avoiding induction in control algal pieces during the bioassays. *Idotea granulosa* preferred to feed on an artificial diet prepared from control algae rather than the grazed-algae diet, indicating that direct grazing by the isopods induces resistance to further grazing in *Sargassum muticum*. However, there was no evidence of induced resistance in the macroalgae exposed to waterborne cues (Toth 2007).

Using a similar approach with fresh and constitutive food assays, Yun et al. (2012) found no consistent evidence for induced defences in *Sargassum muticum* (in contrast to native brown macro-algae). However, chemical defence was induced in native macroalgae when exposed to seawater in which *S. muticum* had been grazed. This strongly indicated that grazing on *S. muticum* can induce defences in nearby native brown macroalgae through waterborne cues, analogous to ‘eavesdropping’ in terrestrial plants.

Biotic resistance to invasion by macroalgae

Interactions with native species can strongly constrain the success of invading species. One of the oldest ideas about biotic resistance to invasion is the diversity-invasibility hypothesis, which states that the invasibility of native communities will be inversely related to their species diversity (Elton 1958). The diversity-invasibility hypothesis has been widely tested in terrestrial systems, but studies in the marine realm are scarce (but see Stachowicz et al. 1999). Observational studies in invaded regions, like those by Mineur et al. (2008b), have found positive relationships between native and invader macroalgal diversity in the introduced range at three different scales (from sampling plot to site and region), suggesting that processes promoting native diversity also do so with non-native diversity. Similar results were found by Klein et al. (2005), but in that study, relationships were only significant at the site scale but not at the sampling-plot scale (the regional scale was not examined).

In the case of invasion by *Sargassum muticum*, Cacabelos et al. (2013) found that the relationship between invasion success of *S. muticum* and species diversity was negative at small scales (10^{-2}–10^{0} m) but positive at large scales (10^{5} m). These results are in agreement with the "invasion paradox" proposed by Fridley et al. (2007), which suggests that at local scales, native species richness can contribute to invasion resistance by means of neighbourhood interactions (competition and other negative species interactions), while at larger scales, conditions promoting high native species richness also promote invasions. However, observational studies were unable to establish causality between invasion success and diversity in macroalgal assemblages (Arenas et al. 2006). In fact, several experimental studies have explicitly tested the diversity-invasibility hypothesis in the context of the *S. muticum* invasion (Britton-Simmons 2006, White & Shurin 2007, Vaz-Pinto et al. 2012). Whether native algal communities facilitate or inhibit invasion was determined by the net outcome of individual species or functional group effects integrated over the entire life cycle of *S. muticum* (Britton-Simmons 2006).

Numerous studies have found that the *Sargassum* invasion is disturbance dependent (Deysher & Norton 1982, DeWreede 1983, Britton-Simmons 2006, Sánchez & Fernández 2006), indicating that established assemblages of native algae inhibit invasion by *S. muticum*. Native algae appear to moderate invasion by *S. muticum* by preempting key resources, mainly space and light. For example, *S. muticum* recruitment is a space-limited process (Deysher & Norton 1982, DeWreede 1983, Britton-Simmons 2006, but see Vaz-Pinto et al. 2012), and native species that occupy primary space effectively preclude recruitment by *S. muticum* (Deysher & Norton 1982, Britton-Simmons 2006). In addition, some native canopy species may act as a barrier that reduces the probability of *S. muticum* propagules encountering the substratum (Sánchez & Fernández 2006). Vaz-Pinto et al. (2012) found that availability of light in the recipient assemblages is a key factor explaining the invasion success of *S. muticum*. Once *S. muticum* has successfully recruited, larger native algae are important competitors for light (Foster & Schiel 1992, Britton-Simmons 2006). Moreover, detailed studies of biotic resistance to invasion by *S. muticum* highlighted the influence of particular functional groups or species on invasion. Britton-Simmons (2006) found that the effects of native functional groups on the biotic resistance of the community can differ between the pre- and post-settlement invader phases depending on their resource-use strategies. Functional groups that utilize primary space obstruct the recruitment of *S. muticum* propagules, while canopy-forming functional groups that shade the substratum inhibit post-settlement growth. Similarly, F.

Arenas (unpublished) found that the establishment of *S. muticum* in tide pools was strongly inhibited by native algae, with the exception of encrusting species, which promoted invasion by providing a suitable substratum for the recruits.

Collectively these studies suggest that species or functional group identity and resource availability are key to understanding biotic resistance to *Sargassum muticum* by (assemblages of) native algae. The role of particular functional groups is context dependent and can be complex, usually depending on the stage of the invasion. Canopy algae compete for light with *S. muticum* in the subtidal zone, but on intertidal substrata they can simultaneously reduce *S. muticum* recruitment by shading and sweeping and increase their survivorship by preventing desiccation (Sánchez & Fernández 2006). Similarly, White & Shurin (2007) found that in experimental communities (25 × 25 cm), local native diversity facilitated the recruitment of *S. muticum* but subsequently reduced the growth and survivorship of the invader.

Impact on recipient species and communities

The widespread invasion of *Sargassum muticum* along shorelines of the north-western United States of America and Europe over the last four to five decades led several authors to predict serious impacts on local algal communities (e.g., Fletcher & Fletcher 1975, Rueness 1989, Walker & Kendrick 1998). In this section, studies of the impact of *S. muticum* on native species and communities are reviewed to assess whether the predicted impacts have materialized. The effects of *S. muticum* on recipient species and communities have been studied in a wide range of habitats in many regions in North America and Europe.

Effects on native macrophytes

Not surprisingly, algae have been the focus of many studies because of the obvious potential for competitive interactions between *Sargassum muticum* and native macroalgae. The earliest work was done at Santa Catalina Island in Southern California, where Ambrose & Nelson (1982) observed the establishment of *S. muticum* at a study site following a large-scale die back of giant kelp (*Macrocystis pyrifera*) during an El Niño event. Subsequent experiments in this subtidal community demonstrated that *M. pyrifera* recruitment increased when *Sargassum muticum* was removed, even when the latter's holdfast was left attached to the substratum. Although Ambrose & Nelson (1982) did not investigate the mechanism involved, their results suggest that shading by *S. muticum* prevented establishment of *Macrocystis pyrifera* (Ambrose & Nelson 1982). However, a concurrent but longer-term observational study also carried out at Santa Catalina showed that *M. pyrifera* had largely recovered 4 years after the El Niño, indicating that the stress-mediated transition to a '*Sargassum muticum* state' was reversible (Foster & Schiel 1992).

Only a few studies have investigated the impact of *Sargassum muticum* on subtidal communities in Europe. The first was from the coast of Lower Normandy (France), where Cosson (1999) reported a substantial decline in *Laminaria digitata* from several sites between 1983 and 1997, a loss that was coincident with the invasion of *Sargassum muticum* in the area. The second study was from Limfjorden (Denmark), where *S. muticum* was first detected in 1984. By 1997, it had become the most dominant alga, and the invasion has been followed by a significant decline of leathery, canopy-forming algae (e.g., *Saccharina latissima* [= *Laminaria saccharina*], *Halidrys siliquosa*, *F. serratus* and *Fucus* spp.) and a less-pronounced decline of many understory algae (e.g., *Codium fragile*, *Chondrus crispus*, and *Dictyota dichotoma*) (Stæhr et al. 2000). In subsequent years, the invasion of Limfjorden by *Sargassum muticum* continued, and while it appeared to have stabilized by 2005, abundance of many native algal species continues to decrease (M.F. Pedersen unpublished data).

Using a block-designed field experiment, Lang & Buschbaum (2010) found that the abundance of native macroalgae, such as *Polysiphonia nigrescens*, *Antithamnion plumula*, and *Elachista fuciola* decreased with increasing densities of *Sargassum muticum* on soft-bottom Pacific oyster (*Crassostrea gigas*) beds in the shallow subtidal of the German Wadden Sea. The latest evidence was provided by the work of Strong & Dring (2011) in Strangford Lough (Northern Ireland). Manipulating densities in pure and mixed stands of *Saccharina latissima* and *Sargassum muticum*, the authors concluded that *S. muticum* will not affect stands of adult *Saccharina latissima*. Experimental work in subtidal habitats of the San Juan Islands of Washington State showed that *Sargassum muticum* reduces the abundance of native algae, including kelps, *Desmarestia* spp., and understory red algae (Britton-Simmons 2004). Moreover, shading by *Sargassum muticum* reduced kelp growth and had an indirect effect on native algae by causing a shift in the relative abundance of native species, with the more shade-tolerant kelp *Agarum* sp. increasing in abundance relative to *Laminaria* sp. However, also in this case, the transition to domination by *Sargassum muticum* was reversible—native species recovered within approximately 1 year following the experimental removal of *S. muticum*.

The effects of *Sargassum muticum* on intertidal algae have been investigated in both Europe and North America. Studies of emergent rocky substrata have been carried out in Canada and Spain.

In the Strait of Georgia (British Columbia, Canada), *S. muticum* prevented the re-establishment of native red algae once it had invaded following experimental disturbance (DeWreede 1983, DeWreede & Vandermeulen 1988). In northern Spain, Sánchez et al. (2005) sampled three low-intertidal sites prior to invasion by *S. muticum* in 1977 and then twice following the invasion (1996 and 2002). They found significant changes in the structure of the algal assemblage following the invasion of *S. muti- cum*, including a significant increase in canopy-forming *Bifurcaria bifurcata* and a marked decline of another canopy-forming species, *Gelidium spinosum*. Algal species richness increased with time, probably because epiphytic algae were included in the species richness metric (a large number of epiphytic algae grow on *Sargassum muticum*). Sánchez et al. (2005) suggested that competition for light, especially with the previously dominant *Gelidium spinosum*, might have been important. In a second study in the same area, Sánchez & Fernández (2005) conducted a removal experiment over 2 years. The removal of *Sargassum muticum* from experimental plots had no detectable effects on the abundance, species richness, or species diversity of the native algal assemblage. Sánchez & Fernández (2005) suggested that the density of *S. muticum* (ca. 24 plants m^{-2}) in the intertidal zone of the experimental area might have been too low to significantly affect the algal assemblage. In north-western Spain, Olabarria et al. (2009) conducted a similar removal experiment and reached the same conclusions: a weak effect on the recipient assemblage, with a reduction in the number of species and in the cover of two understory functional groups (filamentous and foliose algae).

Unlike the results from lower rocky shores, studies of the effects of *Sargassum muticum* on rock pools have led to similar conclusions: they generally produce little evidence for effects of *S. muticum* on rock pool algal assemblages. The earliest rock pool work was conducted by Viejo (1997) on the northern coast of Spain, where *S. muticum* removal experiments were carried out at three sites for a period of 2 years. Total algal cover and cover of thick leathery algae (especially *Bifurcaria bifurcata*) only increased slightly when *Sargassum muticum* was removed, suggesting weak effects of *S. muticum* on native algae in this system. In similar experiments in northern Spain, Sánchez (2005) showed that *S. muticum* had a modest negative effect on *Bifurcaria bifurcata*, but that other taxa were unaffected, and there was no evidence of an effect on algal richness or diversity. The same pattern continues even 12 years after the start of the experiment (C. Fernández unpublished data). Finally, Wilson (2001) coupled *Sargassum* removal experiments with surveys over a large geographical region to examine the impact of *S. muticum* on tide pool communities in California (USA) and Baja California (Mexico). She found no evidence for effects of *S. muticum* on rock pool algal assemblages.

Interspecific competition among macroalgae is most often for light (through shading), major nutrients (often nitrogen or phosphate), or space (through domination of the substratum). *Sargassum muticum* is likely to be a strong competitor for light because it has a tall, positively buoyant thallus, extending as high as 4 m into the water column in subtidal habitats, although it tends to be much shorter in the intertidal zone (up to 2 m). Indeed, many authors have speculated that shading by *S. muticum* was the mechanism underlying effects on native algae (e.g., Ambrose & Nelson 1982, Sánchez et al. 2005). However, few studies have quantified resource availability in experiments to evaluate which resources are involved in interactions between *S. muticum* and native algae. Critchley et al. (1990) and Strong et al. (2006) found that surface photosynthetically active radiation (PAR) was reduced by 97% in the uppermost 0.1 m within a dense *S. muticum* meadow where the fronds extended to the water surface. Manipulative field experiments on the western coast of Vancouver Island, Canada, demonstrated density-dependent effects of *S. muticum* on macroalgal richness through light competition by shading smaller, understory macroalgae (White & Shurin 2011). Likewise, Britton-Simmons (2004) showed that removal of *S. muticum* increased light transmission to the substratum by 30–75%. The shading effect varies seasonally, depending on the development of *S. muticum* fronds, with the most intense shading in late spring (southern Europe) to midsummer (northern Europe) according to the period in which the species reaches the annual largest size (Fernández 1999, Stæhr et al. 2000). If this period coincides with the period of growth of native macroalgae, such as *Gelidium spinosum* in northern Spain (Sánchez et al. 2005), the effect may be severe. Because the holdfast of *Sargassum muticum* is relatively small (approximately 2 cm in diameter in large specimens), it seems unlikely that *S. muticum* would be a strong competitor for primary substratum, except where it occurs at very high densities. Britton-Simmons (2004) found no evidence that *S. muticum* altered nutrient availability, sedimentation, or water flow. Strong et al. (2006) obtained similar results, finding no difference in sedimentation or reduction in water flow due to *S. muticum* compared with a native species (*Saccharina latissima*). Although the measurements of these variables were limited in space and time, they are the only data currently available for these factors. Thus, existing evidence suggests that any negative effects of *Sargassum muticum* on native algae are mainly due to competition for light, but with a possible role for allelopathy.

Most of the research on the effects of *Sargassum muticum* has focused on its interactions with native algae and to a lesser extent with native seagrasses. However, there was some concern from early in the invasion that this alga might compete with the native seagrass *Zostera marina* in the eastern Pacific (Druehl 1973) and the Netherlands (den Hartog 1997). These two species have different habitat requirements: *Sargassum muticum* needs hard substratum for holdfast attachment, and *Zostera marina* only grows in soft sediment (North 1973). Consequently, the only habitats where they co-occur are those in which the substratum is mixed (i.e., mud or sand mixed with cobbles, shells, or other hard substrata) (DeWreede 1978, den Hartog 1997) or where clams inhabit seagrass meadows (White & Lindsay 2011). These clams can be either native or introduced, potentially facilitating colonization by *Sargassum muticum* (Simberloff & Von Holle 1999). Hard substratum availability is limiting and sedimentation load is high in these habitats, and as a result *S. muticum* does not reach high densities (K.H. Britton-Simmons personal observation). Although some competition (especially for light) between *S. muticum* and *Zostera marina* may occur under these limited circumstances, this possibility has not been well investigated. Until recently, it was considered unlikely that *Sargassum muticum* is having or ever will have a strong effect on the distribution and abundance of *Zostera marina* in the eastern Pacific (DeWreede 1978). However, a study by Tweedley et al. (2008) at Salcombe, south-western England, showed that the majority of *Sargassum muticum* plants within *Zostera marina* beds were attached to hard objects buried within the soft sediment; outside the seagrass bed, though, this was much less common. Therefore, Tweedley et al. (2008) suggested that the presence of *Z. marina* may actually enhance *Sargassum muticum* colonization of soft sediments by trapping drifting fragments and allowing viable algae to settle on the seagrass matrix in an otherwise-unfavourable environment. So, the consequences for the invasion of seagrass beds by *S. muticum* may therefore be more severe than previously thought.

Effects on native fauna

Effects on benthic animals

One might expect the effects of *Sargassum muticum* on light and native algae to have consequences for benthic invertebrates, particularly herbivores. However, the available data suggest that *S. muticum* invasions have no effect on most benthic invertebrates (Wilson 2001, Britton-Simmons 2004), with one notable exception. In Southern California, Wilson (2001) found evidence that the sea urchin *Strongylocentrotus purpuratus* was more abundant in tide pools where *Sargassum muticum* was absent. Likewise, in Washington State, the urchin *Strongylocentrotus droebachiensis* was significantly more abundant in experimental plots from which *Sargassum muticum* had been removed (Britton-Simmons 2004). *Sargassum muticum* is distasteful to

Strongylocentrotus droe- bachiensis (Britton-Simmons 2004), and field data suggest that *Sargassum muticum* has a negative, indirect effect on *Strongylocentrotus droebachiensis* by reducing the abundance of *Saccharina* (= *Laminaria*) *bongardiana* on which it prefers to feed (Britton-Simmons 2004). In contrast, DeWreede (1983) reported seeing *Strongylocentrotus droebachiensis* feed on intertidal *Sargassum muticum* in British Columbia. This last observation notwithstanding, available evidence does suggest that sea urchins in the genus *Strongylocentrotus* are negatively affected by the *Sargassum muticum* invasion across a broad geographical area (Britton-Simmons et al. 2009). However, it is possible that there are regional differences in how sea urchins respond to *S. muticum* or that the interaction between these species has changed over time.

Effects on fish

Perhaps the least-studied aspect of the *Sargassum muticum* invasion is how this species interacts with native fishes. Since *S. muticum* often forms large canopies and is inhabited by a diverse epifaunal invertebrate community (Viejo 1999, Engelen et al. 2013), it provides potentially good habitat for fish. Wilson (2001) found no effect of *S. muticum* on abundance of tide pool sculpins (Cottoidea), and in Washington State at least four species of fish (mostly gunnels, Pholidae) have been docu- mented using *S. muticum* as habitat (Norton & Benson 1983, Giver 1999).

Although these studies provide some information about demersal fishes, we have no information about more mobile fishes that are unlikely to be captured in epifaunal collections or tide pool samples. For example, in the San Juan Islands, striped sea perch (*Embiotoca lateralis*) and tubesnout (*Aulorhynchus avidus*) are routinely observed in *Sargassum muticum* canopies, and *Aulorhynchus avidus* frequently deposits eggs on them (K.H. Britton-Simmons personal observation). Moreover, sea perch and other fishes are known to prey on the snail *Lacuna vincta* and other herbivores that feed on *Sargassum muticum*. In the northern Wadden Sea, the native pipe fish *Entelurus aequoreus* stock is promoted by *Sargassum muticum* due to provision of habitat and food (harpacticoid copepods) (Polte & Buschbaum 2008). Recently, Salvaterra et al. (2013) showed that generalist fish, like *Gobius paganellus*, *Symphodus melops*, and *Pomatoschistus pictus*, were numerically dominant in experimental macroalgal assemblages incorporating *Sargassum muticum* in south-western Ireland. Salvaterra et al. (2013) linked these changes to changes in the physical environment and a decrease in primary consumers in the presence of *S. muticum*. The ecological interactions of fish with *S. muticum* and its herbivores are potentially important and deserve further study.

Effects on macroalgae-associated fauna

Marine algae and seagrasses harbour diverse assemblages of small, mobile invertebrates dominated by peracarid crustaceans, gastropod molluscs, and polychaete worms (Edgar 1983, Dean & Connell 1987). Besides forming the first link between primary producers and higher trophic levels, these small invertebrates play an important role in the structuring of macroalgal communities (Duffy & Hay 2000). The interaction between macroalgae and epifauna also acts the other way around. Macroalgae can affect epibiotic assemblages by providing microhabitat and by modifying physical factors, such as water flow (Duggins et al. 1990) and light (Clark et al. 2004).

Since the arrival of *Sargassum muticum* in Europe in the early 1970s, there has been interest in the associated fauna of *S. muticum* and possible impacts on native fauna, as the species was reported to displace native macrophytes (Druehl 1973, Withers et al. 1975). The first casual inspections of the epifauna associated with *S. muticum* indicated that a rich fauna was associated with the alga, and that it seemed unlikely that the introduction of the species would result in a significant change in local epibiotic communities (Withers et al. 1975). Since then, various studies have been conducted in different ways and across different habitats and locations.

Invasion by *Sargassum muticum* of areas without native canopy-forming algae typically results in additions to the local fauna (Viejo 1999, Buschbaum et al. 2006). *Sargassum muticum* can also be the host for other NIS, such as the colonial and invasive tunicate *Perophora japonica* in northern Brittany (F. Viard personal observation). In most cases, however, *Sargassum muticum* competes directly with and possibly replaces native primary producers (mostly macroalgae but sometimes seagrasses). In these situations, the associated fauna found on *S. muticum* resemble the fauna of the native competitor(s) when these are morphologically similar, such as *Cystoseira nodicaulis* (Viejo 1999), *Halidrys siliquosa* (Wernberg et al. 2004, Buschbaum et al. 2006, R. Svenson unpublished), and *Cystoseira humilis* (Engelen et al. 2013). However, faunal compositions can differ slightly due to small differences in the density of many faunal species in species-rich areas like southern Portugal (Engelen et al. 2013) or due to large differences in the density of a few faunal species, such as *Caprella laeviuscula*, which is strongly attracted to *Sargassum muticum* (Norton & Benson 1983). Native algal competitors with a simpler morphology in general support a different and less-diverse assemblage of associated fauna. This has most clearly been shown in the San Juan Islands, where the community supported by *S. muticum* is more abundant and species rich compared to that of the displaced native *Saccharina latissima* (Giver 1999), but also applies to species such as *Chorda lum* in Sweden (R. Svenson unpublished) and *Dictyota dichotoma* in

Scotland (Harries et al. 2007). Differences between *Sargassum muticum* and native macroalgae with an intermediate morphology like *Fucus vesiculosus* are less clear. For example, on the German island Sylt, *F. vesiculosus* supported a different and less-diverse assemblage of associated fauna (Buschbaum et al. 2006), whereas in northern Spain, differences were small (Viejo 1999).

Predicting the impact of *Sargassum muticum* on macroalgae-associated fauna is complicated by the fact that fauna can differ between habitats (littoral vs. sublittoral, low-shore tide pools vs. high-shore tide pools, exposed vs. sheltered, and rocky vs. sandy shore) and locations (Viejo 1999, Buschbaum et al. 2006, Engelen et al. 2013). Differences in associated fauna among macroalgal species need not necessarily be caused directly by the host species but can be related to the epiphytic algal community or silt associated with the host (Norton & Benson 1983).

Macroalgae-associated fauna in the introduced range of *Sargassum muticum* in general are opportunistic organisms with a relative low host specificity, which probably enables them to survive the spatial and temporal variability of macroalgal composition and abundance. Only when faunal species are host specific and the morphological or chemical characteristics of *S. muticum* are very different from the native host species can marked differences in associated fauna be expected.

Even though the invasion impact of *Sargassum muticum* on macroalgae-associated fauna seems to be generally small, several authors have pointed out that, in contrast to the pseudoperennial life history of *S. muticum*, most native competitors are perennial species. These native perennials, such as *Halidrys siliquosa*, provide a more seasonally constant biomass, which results in a temporally constant faunal biomass compared to *Sargassum muticum*. Species richness and abundance of asso- ciated fauna on both host species, however, exhibit clear seasonal variation (Wernberg et al. 2004).

Most information about the associated fauna of *Sargassum muticum* has originated from descriptive studies. Few experimental studies have been conducted that manipulated *S. muticum* abundance to assess the potential effects of the invader on epifauna. The only experimental study that used this approach on native tide pool communities (Wilson 2001) concurred with the descriptive studies: *Sargassum muticum* had little or no effect on the communities studied.

Effects of *Sargassum muticum* on infaunal assemblages have rarely been studied. Strong et al. (2006) investigated modification of infaunal assemblages, but unfortunately, they did not compare possible effects of the invader and native macrophytes. At both study sites in Strangford Lough, Northern Ireland, community analyses revealed significant differences between the assemblages under *S. muticum* canopies and those in adjacent unvegetated areas, although communities differed between study sites. In northern Spain, placement of *S. muticum* wrack on intertidal sand flats had only a small effect on the organic matter content of intertidal marine sediments and benthic micro-algae (Olabarria et al. 2010).

Although drifting *Sargassum muticum* is assumed to be partly responsible for the wide distribution of the species, almost nothing is known about the fauna associated with drift material of this species, let alone the potential role of drifting *S. muticum* as a disperser of fauna. Vandendriessche et al. (2006) found that the macrofauna associated with detached floating macroalgae differed between drifting masses composed primarily of native species (*Ascophyllum nodosum* and *Fucus vesiculosus*) and those composed primarily of *Sargassum muticum.* The dissimilarity in faunal composition between the native macroalgae and *S. muticum* was mainly caused by the abundance of *Gammarus crinicornis* and *Idotea baltica* in the native macroalgae and *Liocarcinus holsatus* (by far the most important contributor) and *Stenothoe marina* in the invader. Faunal densities were higher in drifting masses dominated by *Sargassum muticum*, but diversity and evenness were lower compared to samples dominated by the native macroalgae. Described faunal differences may relate to the source of the drift material, how long it had been drifting, and under what conditions it was drifting.

Ecosystem effects

Although the dominance of *Sargassum muticum* in invaded habitats would be expected to have impacts at the ecosystem level, few studies have reported this. On sandy beaches of northern Spain, which do not represent ‘invaded habitats’ as such, detached *S. muticum* was an important food resource for the amphipod *Talitrus saltator* and to a lesser extent for the isopod *Tylos europaeus* (Rossi et al. 2010). The input of organic material from *Sargassum muticum* was not important, however, during spring (March and May), and Rossi et al. (2010) attributed this to the high availability of the native macrophyte *Cystoseira baccata*. *Sargassum muticum* was one of the few species available as beach wrack during autumn and as such might sustain the population abundance of *Talitrus saltator* in a season when it normally declines (Rossi et al. 2010). Rossi et al. (2011) found that addition of *Sargassum muticum* wrack to beach sand resulted in higher ^{15}N mobilization to sediments and macrofauna. However, as mixtures of *S. muticum* with *Fucus vesiculosus* and *Ulva* sp. were used, the contribution of *Sargassum muticum* is uncertain (Rossi et al. 2011).

Experimental manipulations in field mesocosms have shown that the presence of *Sargassum* *muticum* can increase food web complexity (measured as connectance) as well as the proportion of species of intermediate trophic level and decrease the proportion of top predators (Salvaterra et al. 2013). Increased food web complexity was caused by the arrival of new generalist species and a redistribution of existing species to higher trophic levels (Salvaterra et al. 2013). In addition, using artificial macroalgal assemblages, Vaz-Pinto et al. (2014) demonstrated that, under controlled conditions, *S. muticum* increased respiration and production of the assemblages due to its high biomass. Overall, these studies suggest that effects of *Sargassum muticum* extend to the ecosystem level, but this area of research is at an early stage.

Effects on human activities

In contrast to biological impacts, scientific information about impacts of *Sargassum muticum* on human activities is limited. This topic received some attention in the 1980s, but not much after that. Large, dense stands of *S. muticum* may cause loss in amenity and recreational use of water areas; for example, swimming, water skiing, surfing, dinghy sailing, and fishing can be inhibited (Eno et al. 1997). This can be especially the case when thalli become detached and form large floating mats (Farnham 1980), like the mats of up to 50 m^2 that were common on calm days in June and July 1976 in the eastern Solent (Gray & Jones 1977). However, fixed thalli can also be a nuisance, as they have been in the tidal outdoor swimming pools of Praia Norte in Viana do Castelo, northern Portugal.

In addition, *Sargassum muticum* can also affect commercial activities. In the form of fouling, it can obstruct industrial seawater intakes, and detached material can clog water inlets of ships and industrial installations (Critchley et al. 1986). Floating mats of *S. muticum* foul commercial fishing lines and nets and concentrate floating debris, creating an eyesore (Critchley et al. 1986). Large beach casts can cause problems when rotting, for example, by producing offensive smells on resort beaches. Dense growths of *S. muticum* on oyster beds in British Columbia interfered with oyster cultivation, and it was feared that the buoyant fronds of *S. muticum* attached to the oyster shells could carry them out of the culture area (Scagel 1956, Franklin 1979, Critchley et al. 1986), although quantitative data on this subject are lacking.

In general, there is a strong focus on biological impacts in marine biological invasion science, in marked contrast to research on terrestrial biological invasions, which has shown that invasions can cause significant damage to economic interests, such as agriculture and forestry. Since the impact of *Sargassum muticum* on human activities is understudied, the absence of evidence of impact should not be taken to indicate lack of impact, but rather it should be a stimulus for research on this topic.

Climate change and *Sargassum muticum* invasion

The ecological literature shows that invasive species often have physiological or life-history traits that could be advantageous under climate change, promoting the success of these species under future environmental scenarios (Dukes & Mooney 1999). While some research has been done on the physiological tolerances of *Sargassum muticum*, for example, to salinity and particularly temperature (see ‘Abiotic tolerances’ section), future climate change will involve several physical parameters changing simultaneously. Thus, any research addressing the interplay between climate changes and invasions should include multiple physical variables. In fact, research on the biological impacts of climate change in the marine realm has focused primarily on two physical factors—sea-water temperature and pH—as the main drivers of climate-related biological changes in the oceans (Hale et al. 2011, Diaz-Pulido et al. 2012).

To date, two published studies have addressed the consequence of multiple stressors for the outcome of *Sargassum muticum* invasions. Using experimental mesocosms where temperature and seawater pH were altered to levels as predicted in the worst-case scenarios for the future ocean (air pCO_2 of 1000 ppm, resulting in a seawater pH of 7.68 ± 0.02 to 7.91 ± 0.05 depending on temperature; and +5°C in seawater temperature), Olabarria et al. (2013) found evidence that assemblages from rock pools invaded by *S. muticum* might be more resilient to climate change than those dominated by the native canopy-forming species *Cystoseira tamariscifolia*. Similarly, in a recent experiment using a similar approach, Vaz-Pinto et al. (2013) found that the effects of those environmental factors on the species were dependent on the life-cycle stage. Thus, survivorship of *Sargassum muticum* was enhanced at high CO_2 (1000 ppm) and ambient temperature (15°C) after 3 days and reduced at ambient CO_2 and high temperature after 10 days. After 6 months in the field, residual effects of laboratory treatments remained, with *S. muticum* reaching higher cover in most assemblages previously subjected to ambient pCO_2. These seminal studies on multiple stressors showed our deficiencies when predicting feedbacks and interactions between stressors and their consequences for the outcome of the invasion of *S. muticum.* Much more research on this key subject is urgently needed.

Management strategies for invasions

In the past, there have been various attempts to contain invasions of *Sargassum muticum*. Chemical methods using herbicide, such as diquat, Stomp®, copper sulphate, sodium hypoclorite, K-lox®, and Nortron®, failed due to lack of selectivity, the large doses needed, the incubation period required, as well as application problems (Lewey & Jones 1977). Studies of biocontrol showed molluscs and amphipods can consume small germlings, but this had no restrictive effect on the local spread of *S. muticum* (Critchley et al. 1986). Removing *S. muticum* by hand turned out to be extremely time consuming and needed to be repeated, probably indefinitely (Farnham 1980). In addition, removal by trawling, cutting, and suction has also been tried. Trials in the mid-1970s using tractors equipped with harrows, cultivators, and fore-end loaders were abandoned due to problems with containment of collected material and physical damage inflicted on the shore (Critchley et al. 1986).

Despite great efforts (more than 30 tonnes of wet *S. muticum* were removed during summer harvests from Portmouth Harbour and Bembridge, southern England, in 1973–1976), the spread could not be contained (Lewey 1976). It was estimated that in 1976 a total of 450 tonnes of wet *S. muticum* was collected in England in an attempt to eradicate the species (Gray & Jones 1977). Whatever method was used, the alga always quickly regrew, and effective methods for its permanent removal have not been found, although cutting and suction have been the preferred method applied (Farnham et al. 1981, Critchley et al. 1986). As another complicating factor, individuals surviving in cleared areas became fertile sooner, and clearance allowed regeneration of increased density of recruits (Lewey 1976).

However, it must be mentioned that these clearances took place in the reproductive season of the invader. In addition, sites were difficult to access, and collected material was difficult to contain and dispose of. The main concern associated with mechanical removal techniques has been regrowth from very small and numerous stages that are not removed or from holdfast remnants. In addition, small recruits of the target species are unlikely to be removed due to difficulties in finding, identifying, and actually removing them (Critchley et al. 1986).

Demographic matrix models simulating several harvesting regimes revealed that the most effective time of year to reduce population growth rates would be before the onset of reproduction (Engelen & Santos 2009). At that time, the density of recruits will be minimal, and there will also be little chance of additional spread caused by transportation of embryo-carrying thalli normally involved in harvesting. This is a realistic hazard since spring tides provide the most time between high tides to carry out removals; this is also the tidal phase at which *Sargassum muticum* releases its eggs and embryos (Engelen et al. 2008). During a removal campaign before the onset of reproduction, all large individuals should be removed as completely as possible since the possibility exists that fragments of a single holdfast left behind may reproduce vegetatively and thereby diminish the effectiveness of the removal campaign.

Although simulations provide great insight into possible management strategies and their effectiveness, the simulations of harvesting regime mentioned were mainly hypothetical, and real harvests are needed to provide information on how well individual *Sargassum muticum* are removed by people participating in these campaigns. Single harvests will not be enough to eradicate the species locally. This is expected to be true especially when other populations are nearby. In March 2007, the knowledge was applied to a trial removal of *S. muticum* from the Ria Formosa lagoon in southern Portugal, where a soft-bottom habitat was dominated by large *S. muticum* individuals attached to shells, pebbles, and bivalves. About 5 tonnes were removed manually, and this was apparently successful in eradicating the invader from the lagoon, as since then no *S. muticum* has been observed (A.H. Engelen & R.O. Santos unpublished). The mobile nature of substratum in soft-sediment habitats may have contributed significantly to the success of the removal campaign. This case highlights that there are opportunities to eradicate the species in specific situations.

The recent rapid increase in the number of studies testing chemical compounds obtained from *Sargassum muticum* for biotechnological applications (e.g., Bazes et al. 2009, Plouguerné et al. 2010, Piao et al. 2011, González-López et al. 2012, Tanniou et al. 2013) suggests that, in the near future, industrial interest in biomass of the species might facilitate management activities. Eradication actions could indeed provide biomass to the seaweed industry, but a guaranteed quantity and quality of supply would require commercial crops that would act as a major source for local and regional recruitment.

Conclusions

Sargassum muticum is the most successful macroalgal invader worldwide, as judged by the extent of its non-native geographical distribution, from Alaska to Baja California in the eastern Pacific and from Norway to Morocco in the eastern Atlantic, including the Mediterranean, which is much larger than its native distribution range, from southern Russia to southern China in the western Pacific. An obvious realization is that the species has the capacity to establish populations across a wide range of environmental regimes, including cold- and warm-temperate zones, through adjustment of its physiological, developmental, and phenological processes. But, is this enough to explain the success of this invader and to grasp the fundamental attributes required for an introduced species to successfully establish and spread?

After the initial transport phase, which in the case of *Sargassum muticum* appears tightly linked to the trade of oysters for aquaculture, as well as for many other species (Wolff & Reise 2002), the environmental regime/physiological tolerance filter determines the species that colonize a new site. The available data reviewed here show that *S. muticum* is able to colonize a broad range of habitats and endure a wide range of environmental conditions. The life-cycle stages most likely to start colonization are the early stages or holdfasts that travel attached to oyster shells. Even though the growth of the initial germling phases of the life cycle is strongly reduced at temperatures below 10°C, which occur in its cold-temperate geographical distribution range, the life cycle may still be completed providing the water temperature is above 8°C for at least 4 months per year. Once the adult stage is achieved, *S. muticum* can tolerate wide ranges of environmental conditions, such as temperature (−1°C to 30°C) and salinities (down to 10‰).

Following the colonization phase, an invading species must compete successfully with other species and survive herbivore pressure to establish self-sustaining, expanding populations. Evidence indicates that the competitive performance of *Sargassum muticum* is intrinsically linked to the size that plants may attain, lengths of up to 10 m. As *S. muticum* has a pseudoperennial life cycle, over-wintering in the form of small holdfasts with reduced branches, maximum size is attained within a few months. Shading of competitors is a strong competitive trait of *S. muticum*, which is a species that is not a good competitor under low light, as it only develops dense, canopy-forming populations in shallow areas. The high morphological plasticity of *S. muticum*, with the potential to be profusely branched, is a trait that may contribute to its competitive ability with other canopy-forming species, particularly leathery laminarians and fucoids. *Sargassum muticum* can modify the production and elongation of branches, reducing interference among neighbours and allowing plants to grow in dense populations. It is not surprising, then, that demographic modelling revealed that the vital rate that contributes most to the population growth of *S. muticum* is precisely the survival of adults. Interestingly, growth rates are more variable during the initial colonization phase than when the species has become established.

The large size of *Sargassum muticum*, the fact that a high percentage of the branch biomass becomes reproductive, and a long fertile season result in a high reproductive output, a trait that is critical for invasiveness. High propagule pressure and the low substratum specificity shown by the species are probably fundamental to achieving high recruitment. Other reproductive traits of the species, such as the possibility of self-fertilization and the parental care of embryos retained on receptacles, developing a multicellular organization before sinking to attach on the substratum, probably also constitute competitive advantages. However, the recruitment success rates of *S. muticum* need to be compared with other competitive macroalgae to reveal if indeed those reproductive traits are critical for invasiveness.

In theory, introduced species will have competitive advantage over natives because they are released from native specialist herbivores that will not recognize them as food (ERH). This hypothesis is not clearly supported for *Sargassum muticum*, probably because specialist herbivores are rare in the marine environment. On the other hand, invasive species may evolve increased competitive ability by shifting resources from defences to growth in response to chronically lower losses to herbivores (evolution of increased competitive ability). There is no scientific evidence of a deficiency of herbivore-deterrent secondary metabolites in this species, and there is a scarcity of relevant experiments to assess if antigrazing adaptations contribute to the invasiveness of the species. In any case, *S. muticum* has been shown to have higher growth rates (both in length and biomass) than native competitors, with or without grazers.

For an established species to become invasive, it needs to spread successfully and thus to have effective mechanisms for long-distance dispersal. The spread rate of *Sargassum muticum* is similar in the north-eastern Pacific and the north-eastern Atlantic, and it is among the highest reported for macroalgae. The species relies on a high reproductive potential coupled with annual shedding of oating fertile branches. Drifting fragments may stay afloat for months and even become fertile during drift. The ability to self-fertilize then allows colonization of remote locations by drifting thalli from a single individual, thus increasing the spread of the species over short periods.

This review shows that *Sargassum muticum* has many attributes that may explain its invasive behaviour. From a devil’s advocate perspective, we are left with an unanswered question: of the more than 40 species of *Sargassum* found in the native region of *S. muticum*, why has only this spe- cies been introduced to and spread in North America and Western Europe? This is puzzling as most of the invasiveness traits of *S. muticum* are also common to other *Sargassum* species. In fact, Paula & Eston (1987) studied the traits of Brazilian *Sargassum* species and concluded that many of them have most of the traits mentioned for *S. muticum*, yet are not invasive.

The reason other *Sargassum* species have not spread could be that they do not survive long-distance transport or that the early stages of their life cycles may have narrower physiological tolerances, preventing their initial introduction. However, considering the close similarities in traits among *Sargassum* species, doubts are cast on these explanations. Recent introduction and fast local spread of the Asian species *S. horneri* (Aguilar-Rosas et al. 2011) and *S. licinum* (Aguilar-Rosas et al. 2007, Riosmena-Rodríguez et al. 2012) in the Californias suggest that *Sargassum* species other than *S. muticum* can invade under some circumstances. Furthermore, there is no single trait that explains invasiveness, but rather a set of traits, as in the case of *S. muticum*. Spatially explicit demographic models must be developed to test the sensitivity of population growth rate to species traits, so that the most critical traits for invasiveness may be assessed.

Other research fields also need to be developed, in particular DNA-based studies. For a long time, most of the work and much of the literature on invasion has dealt with ecology and population dynamics. In the last two to three decades, the evolutionary impact and the genetics of alien species have been emphasized (for reviews, see, for instance, Roman & Darling 2007, Estoup & Guillemaud 2010, Geller et al. 2010, Dormontt et al. 2011, Lawson Handley et al. 2011, Fitzpatrick et al. 2012, Rius & Darling 2014, Rius et al. 2014). It is noteworthy that major findings for biological invasion studies came from algal invasion genetics. For instance, focusing on the geographical origin(s) of *Codium* spp., Goff et al. (1992) provided among the first invasion genetic studies to show the usefulness of genetic approaches to investigate introduction pathways. The study of Voisin et al. (2005) on the Japanese kelp *Undaria pinnatifida* was among the first to show that introduction processes can, on a worldwide scale, distribute the genetic diversity existing within and among populations in the native range, with various consequences. For instance, if a non-native area receives introductions from several genetically different populations in the native range, genetic diversity in the introduced population may be higher than in any one of the source populations. Such genetic admixture may further enhance invasiveness if it leads to new genotypes (by recombination) that confer an advantage in the new environment. More specifically for marine invasion studies, using *Codium fragile*, Provan et al. (2005) performed a pioneer study demonstrating the usefulness of DNA obtained from historical (herbarium) specimens to trace introduction processes.

DNA-based studies of marine invaders have proliferated in the last decade, including studies of macroalgae. Yet, despite its status as a major worldwide invader, only Cheang et al. (2010b) and Bae et al. (2013) have studied introduced populations of *Sargassum muticum* by means of molecular tools. The studies by Cheang et al. (2010b) and Bae et al. (2013) examined the polymorphism of sequence data in native and introduced populations. Although providing support for the hypothesis of a secondary introduction from North America to Europe (Cheang et al. 2010b) and documenting the distribution of mitochondrial genetic diversity in the Korean part of the native range (Bae et al. 2013), the low polymorphism of the marker used in both studies prevented investigation of fine-scale processes (e.g., founder events, self-fertilization rates in introduced populations, spreading routes, extent of genetic admixture).

Such approaches are nevertheless pivotal: the use of genetic approaches is important not only for retracing the introduction processes but also for investigating eco-evolutionary scenarios behind the introduction (Facon et al. 2006). As time series are essential to elucidate the long-term effect of invaders, a promising approach would be to compare populations of *Sargassum muticum* in the eastern Pacific, introduced more than 60 years ago, with the more recently introduced European populations and include herbarium specimens preserved in museums. A further step for future research is to determine whether there is more than a broad tolerance underlying the success of the species. Did acclimatization and adaptation of *S. muticum* occur following its introduction into new environments, and if so, what were the mechanisms (e.g., selection from standing genetic variation, enhanced phenotypic plasticity, etc.)? The use of genetic and genome-wide approaches is particularly promising in this field, for instance, to examine the role of natural selection in shaping the new genetic architecture of introduced populations or the relative importance of phenotypic plasticity and genetic changes in this successful invasive macroalga. The recent publishing of the mitochondrial genome of *S. muticum* might offer new molecular tools for this type of research (Liu & Pang 2014).

In total, over 650 papers have been published on the single species of *Sargassum muticum*, and as such, it is probably the most studied invasive macroalga around the globe. Most published studies have been local and descriptive or provide circumstantial evidence rather than being hypothesis driven. Only by hypothesis-driven research, beyond local scales, conducted in different geographical regions, especially including the native region, and developing this research in an eco-evolutionary framework will it be possible to greatly improve our scientific knowledge of the web of factors, traits, and processes involved in macroalgal invasions. As climate change is likely to increase biological invasions further (Stachowicz et al. 2002), studies of the effects of global warming and ocean acidification should be incorporated into future research.

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Chapter 3

Acclimation vs. Adaptation of Invasive Seaweeds.

3. Acclimation vs. Adaptation of Invasive Seaweeds.

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Abstract

Rapid adaptive changes after introduction play a crucial role in invasive seaweeds establishment and persistence. Plastic and genetic variation represent two major ways of adapting to new conditions, as through phenotypic and/or genetic changes driven by the environment, introduced seaweeds may rapidly evolve throughout time. However, it is not yet entirely understood whether phenotypic plasticity or genetic changes contributes most to the evolution of invasiveness. In this review, we elucidate the evolutionary aspects of invasive seaweeds, provide an integrated overview of the underlying mechanisms of acclimation and adaptation acting on non-indigenous macroalgae following the introduction, highlight the role of these mechanisms in seaweed invasions, and provide new insights regarding factors that may increase invasiveness.

Keywords: invasive seaweeds, acclimation, adaptation

Introduction

Introductions of non-indigenous seaweeds are known to produce significant ecological and evolutionary impacts on natural communities, affecting biodiversity and ecosystem functioning, as well as resulting in significant economic and social implications (Grosholz 2002, Chapman et al. 2006, Schaffelke & Hewitt 2007, Williams & Smith 2007, Molnar et al. 2008, Blackburn et al. 2014, Davidson et al. 2015). Declines in and displacement of native species (Johnson & Chapman 2007), threatening of endangered species (Gurevitch & Padilla 2004), and increases in abundance of tolerant introduced species (Schaffelke & Hewitt 2007) are among the major ecological threats inevitably leading to changes in native community composition, habitat alteration and changes in ecosystem processes (Davidson et al. 2015). As seaweeds represent a particular importance for coastal ecosystems by forming complex habitats and supporting high biodiversity, extensive displacement of dominant native seaweeds with introduced species may adversely impact coastal productivity, food web structure, and ecosystem services (Schaffelke & Hewitt 2007, Mineur et al. 2015). The extent of such impacts is expected to be even more significant in combination with climate change (Harley et al. 2012, Vye et al. 2014, Samperio-Ramos et al. 2015) and habitats degradation due to the anthropogenic activities in coastal areas (Bulleri et al. 2016), as shifts in environmental factors are predicted to favor invasive seaweeds distribution and abundance offering new niches for colonization (Verbuggen et al. 2013, Marcelino & Verbuggen 2015). While there is an urgent need to assess the potential for future spread and evolution of invasiveness, understanding the mechanisms and estimating the speed of acclimation and adaptation of non-indigenous seaweeds into their new range represents a great challenge.

It is accepted that rapid adaptive changes after introduction play a crucial role in invasive populations' establishment and persistence (Lee 2002, Facon et al. 2006, Roman & Darling 2007, Prentis et al. 2008). Plastic and genetic variation represent two major ways of adapting to new conditions (Meyers & Bull 2002). However, there is no consensus regarding whether phenotypic plasticity or genetic changes contributes most to the evolution of invasiveness. Because the evolutionary rate and outcome depend on the intensity of selection, as well as the nature and extent of heritable variation, different forms and levels of genetic variance within invasive populations can indicate the evolutionary potential in invaders' traits and affect the possibility of adaptive evolution during the various stages of invasion (Meyers & Bull 2002, Prentis et al., 2008). Understanding how the source of variation (genetic or epigenetic) affects adaptation is crucial. It may reveal the factors maintaining genetic variation in invasive populations and allow assessing the speed of adaptation in the novel range (Barett & Schluter 2008).

The term acclimatization corresponds to a ‘‘within life time phenotypic adaptation’’, which results in increased fitness and is subsiding when the stress is relieved (Horowitz 2001). Acclimatization (in the field) or acclimation (experimentally induced) both are forms of phenotypic plasticity, which provide organisms with a fitness advantage over individuals that lack the possibility to acclimate to the particular environment (Leroi et al. 1994). Properly acclimatized organisms might be able to better sustain environmental change (Harley et al. 2012). In contrast to acclimatization, adaptation stands for a genetically-fixed organism’s response to environmental conditions (Lee 2002). It refers to genetic changes (in physiological, morphological or behavioral traits) in response to natural selection, which results in increased performance of an organism (i.e. increased survival, growth or reproduction) with respect to some environmental feature and improved suitability of an organism to the surrounding environment (Lee 2002, Fitzpatrick 2012).

The present review aims to elucidate evolutionary aspects of invasive seaweeds, by providing an integrated overview of the underlying mechanisms of acclimation and adaptation, and highlighting the role of these mechanisms in seaweed invasions. The review focuses on the differences between the processes of acclimation (short-term epigenetic changes or changes in the relative expression of already expressed genes) and adaptation (long-term genetic changes) with an emphasis on invasive seaweeds and intends to contribute to the understanding of eco-evolutionary mechanisms following their introduction and the factors that increase their invasiveness.

Seaweed Specificities

While studying the processes of acclimation and adaptation of invasive seaweeds, certain seaweeds specificities should be taken into consideration, as functional traits are likely to impact these processes through their effect on genetic composition. Among the life-history traits that affect genetic processes within introduced populations, breeding systems are particularly important as they define gene transmission to the next generation, alter genetic variance and, as such, can affect local adaptation (Leimu & Fischer 2008, Glemin & Galtier). One of such systems is clonality. Clonality affects genetic composition, as the next generation is genetically identical to parents unless a mutation occurs (Glemin & Galtier 2012). Clonal reproduction is usually associated with limited genetic diversity within populations that can represent significant constraints to natural selection (Geng et al. 2007). While clonal growth in seaweeds is expected to result in genetically uniform ramets (Santelices 2004), marine clonal macrophytes populations often exhibit high levels of genetic variation (McLellan et al. 1997, Van der Strate et al. 2002), which may be generated by intraclonal variation obtained from genetic exchanges (Poore & Fagerstrom 2000). This refers to thalli that are nonsexually reproductive and don’t undergo meiosis or sexual recombination (Santelices 2004). Selfing can also affect genetic composition. Although self-fertilization allows establishment of very small founder populations (a single individual can initiate an invasion), it can limit or entirely prevent genetic admixture (Frankham 2005). Since mating occurs within the same lineage, alleles are not able to spread beyond their lineage of origin; recombination is not effective, and selfers tend to be less diverse and more structured (Hamrick & Godt 1996, Glemin et al. 2006).

The life-cycle stage of the seaweed is also able to affect acclimation ability, as tolerance to environmental variables (i.e. temperature, salinity) varies between different life stages (Lüning 1990). Fertilization, post-fertilization development, and growth depend on environmental factors, which determine whether the life-cycle will be completed. Individuals at the initial germling phases are more sensitive to environmental changes and stages other than adulthood may be impossible at low salinities. Germling growth may also be significantly reduced at lower temperatures and, if the temperature is below optimal, the life cycle may not be completed (Norton 1977, Hales & Fletcher 1989, Steen 2004). Once the adult stage is achieved, seaweeds are able to tolerate a wider range of temperatures and survive at lower salinities (Norton 1977, Hales & Fletcher 1989), resulting in a broader abiotic tolerance range over which acclimation can occur. Ecological differences between the haploid and diploid stages of the seaweed life-cycle particularly matter when considering the processes of acclimation, as haploid individuals (gametophytes) and diploid individuals (sporophytes) tend to interact with the environment differently. Because diploids have two copies of every gene and can better survive the effects of deleterious mutations, they are thought to have a fitness advantage over haploids (Van der Strate et al. 2002). However, in haploids, deleterious mutations will be faster eliminated by selection and advantageous mutations will provide higher fitness. Species with an ability to shift between these states (i.e. biphasic life cycles) may exploit a broader range of environmental conditions (Willson 1981).

Reproductive traits can also assist acclimation. High fertility, longer reproduction period, rapid germling growth, and embryo dispersal can be crucial for post-settlement survival of seaweeds populations (Vaz-Pinto et al. 2012). Synchrony of gamete release may be important for successful fertilization, as it can increase the probability of at least one release coinciding with conditions favorable for fertilization and settlement (Deysher & Norton 1982). The ability to retain germlings on the parental tissue after fertilization may increase the probability of their survival and settlement (Fletcher 1980). Protected on the receptacle surface, large multicellular zygotes are then capable of faster sinking (Coon et al. 1972, Okuda & Neushul 1981) and quicker attachment to the substratum (Norton & Fetter 1981).

Dispersal capacity is another major regulator of gene movement within and among seaweeds populations (Valero et al. 2001). This particularly refers to the fragmentation and dispersal of thalli with reproductive structures, as some broken thalli have the ability to re-establish from small fragments by reattaching to the substrate and forming new independent individuals (Collado-Vides 2002a, Andreakis 2009). Fragmented thalli also can carry reproductive structures that may have different levels of genetic diversity compared to the mother thallus (Andreakis 2009).

Among the numerous processes that play a role in sustainable settlement of non-indigenous seaweeds and evolution of invasiveness, acclimation and adaptation mechanisms are thought to be crucial through: genetic adaptation by selection on standing genetic variation (Barett & Schluter 2008), adaptive introgression (Currat et al. 2008, Prentis et al. 2008), and phenotypic plasticity (Richards et al. 2006). Through phenotypic and/or genetic changes driven by the environment, introduced seaweeds may rapidly evolve throughout time (Buswell et al. 2011).

1. Mechanisms available to non-indigenous seaweeds upon invasion

1.1 Phenotypic plasticity

Non-indigenous species often arrive in novel areas with low initial genetic diversity. A high degree of plasticity represents the first way to which they may respond to new conditions (Hanfling & Kolmann 2002, Keller & Taylor 2008**.** Plasticity allows organisms to express different phenotypes in response to changing abiotic and biotic factors (Richards et al. 2006). However, because plasticity is a property of certain traits in specific environmental conditions, a given genotype in the same environment may be plastic for one, but not for another trait (Richards et al. 2006).

Plasticity can provide a fitness advantage to populations where local adaptation has not yet developed or cannot develop due to the shortage of genetic variation (Richards et al. 2006). It can enhance ecological niche breadth and, through expressing advantageous phenotypes, allow occupation of a wide range of habitats (Richards et al. 2006). Phenotypic plasticity can affect the invaders' ability to become established and even lead to outcompeting native species (Davidson et al. 2011). As a result of phenotypic plasticity, many introduced species might be able to spread successfully in the absence of developing new adaptations (Keller & Taylor 2008). For instance, an invasive Mediterranean strain of *Asparagopsis taxiformis* (lineage 2) was shown to have great levels of phenotypic plasticity in response to temperature, which enables this species to cope better with thermal stress and colonize deeper habitats (Zanolla et al. 2015). Photosynthetic responsiveness to different temperatures indicates adaptive advantages that likely contribute to the invasiveness of this lineage (Zanolla et al. 2015). The invasive brown *Sargassum muticum* showed lower rates of elongation at exposed sites in response to wave action, compared to sheltered areas (Viejo et al. 1995, Baer & Stengel 2010), and longer and fewer branches in response to crowded conditions (i.e. self-thinning, Arenas & Fernandez 2000, Arenas et al. 2002) that could also be a result of plasticity.

Phenotypic plasticity is particularly important in clonal seaweeds, representing their primary strategy of adjusting to novel conditions (Geng et al. 2004). Although accumulation of deleterious mutations is more likely to contribute to clonal species extinction due to restricted gene flow (Glemin & Galtier 2012), clonality is able to increase the likelihood of local adaptation if clonal growth allows phenotypic plasticity (Leimu & Fischer 2008). In clonal morphology, plasticity can serve as a resource-acquisition strategy through the expression of an increased branching intensity in conditions of higher resource supply (de Kroons & Hutchings 1995, Collado-Vides 2002a). Morphological plasticity enables effective exploitation of resources in patchy environments, as it allows individual ramets to continuously search for favorable habitats while escaping unfavorable ones (de Kroons & Hutchings 1995). For instance, the clonal green siphonous seaweed *Caulerpa prolifera* develops dense and thick branches with small laminas in sunny reef patches, and thin and long laminas, allowing acquisition of light, in shady patches (Collado-Vides 2002b). Such morphological plasticity allows *C. prolifera* to acclimate to different light conditions (Collado-Vides 2002b). Clonal seaweeds, however, do not undergo self-thinning in crowded conditions, compared to non-clonal (Scorati & De Weede 1997).

Often, the phenotypic response to environmental stressors is conditioned by the previous experience of organisms with such stressors (Ellers & Stuefer 2010). In these cases, the adaptive potential is determined by the levels of phenotypic variance within introduced populations (Fierst 2011). A history of phenotypic plasticity can define the evolution of genetic architecture and decrease the time necessary for phenotypic variance to arise from mutations and recombination (Fierst 2011). Pre-existing variation for plasticity may thus contribute to phenotypic adaptation providing introduced populations with a better ability to adapt (Lande 2009, Fierst 2011).

Like other traits, phenotypic plasticity can evolve through natural selection (Pigliucci 2005, 2006, Richards et al. 2006). If genetic variation for plasticity is available, and genotypes with higher plasticity have a fitness advantage in new conditions, this will result in the evolution of increased plasticity (Pigliucci 2005, 2006, Richards et al. 2006). Environmentally induced traits can pass through selection processes and genetic accommodation enhanced by standing genetic variation or by genetic recombination (West-Eberhard 2005). If adaptive plasticity is a subject to selection, genotypes from the introduced area can be more plastic than native ones (Hanfling & Kolmann 2002). Whether acting on the level of plasticity or the environmentally induced traits, selection on plasticity will result in increased plasticity (Crispo 2007). Phenotypic plasticity may evolve rapidly in invasive species and play an important role in its successful establishment and spread (Richards et al. 2006). Frequent environmental changes will favor selection on phenotypic and developmental plasticity (Meyers et al. 2005), while infrequent environmental changes will favor local adaptation (Bock et al. 2015). Adaptation to extreme fluctuations of the environment can also assist to the rapid increase of plasticity (Lande 2009, 2015). The evolution of plasticity can lead to increased mutational variance and standing genetic variation allowing populations quicker respond to selection (Draghi & Whitlock 2012). For instance, the genus *Asparagopsis* expresses significant morphological plasticity in response to light availability, as its branches are shorter and denser at patches exposed to light, compared to those in a shadow. Such competition for light or space may select for plasticity, maintaining these capacities (Monro & Poore 2009). Multiple selections may eventually shape more compact phenotypes in high-light patches while maintaining elongated phenotypes in low-light patches (Monro & Poore 2009). As a result of selection, shade-induced plasticity in *Asparagopsis armata* may evolve adaptively (Monro & Poore 2009).

Phenotypic plasticity can eventually result in significant ecological consequences, as generated phenotypes may lead to additional impacts on direct interactions of organisms with its environment or on indirect interactions in which multiple species are involved (Miner et al. 2005). It can particularly affect competition (Relyea 2000), trophic interactions (Trussel et al. 2002), population dynamics (Karban & Baldwin 1997) and population stability (Miner et al. 2005). For instance, increased toughness of thalli tissue in *Fucus vesiculosus*, in response to wave exposure, resulted in feeding preferences changes of its main consumer the snail *Littorina obtusata* (Molis et al. 2015). Plasticity can also result in a niche construction (Donohue 2005). In particular, through elongation of stem and branches in response to shading by competitors and overtopping the neighbors, plants can increase the amount of available light (Smith et al. 1990). This may determine the type of selection, affect phenotypic expression, and influence the expression of genetic variation (Donohue 2005). Through the effect on indirect interactions, phenotypic plasticity can alter biodiversity (Miner et al. 2005) and profoundly impact ecosystem functioning (Hooper et al. 2005).

1.2 Epigenetic processes and non-genetic inheritance

While an ability to produce a plastic response reflects a property of a particular genotype, the DNA sequence alone is not sufficient to determine the phenotype of an organism (Bender 2002, Chinnusamy & Zhu 2009). Epigenetic processes play an important role in this context, as they can generate changes in phenotype (without underlying changes in DNA sequence) through gene expression and destabilization of the genome (Bender 2002, Aubin-Horth & Renn 2009, Hoffman & Togham 2010). Epigenetic changes are based on a set of regulatory molecular mechanisms, which may directly impact gene expression through activation, reduction or disabling the activity of particular genes (Bossdorf et al. 2008). Gene expression is known to be affected by DNA methylation, histone modifications, chromatin remodelling, and RNA interference (Gupta et al. 2012). These mechanisms activate the signals involved in development programs and generate new phenotypes (Gupta et al. 2012). Epigenetic systems function as transmitting chains from detecting environmental change to an adjustment in gene expression, and their? Ability to act immediately and reversibly underlines the flexibility of organisms' responses to the abiotic and biotic stressors

(Grant-Downton & Dickinson 2006). Epigenetic processes are at the basis of various types of phenotypic plasticity, including the environmental tolerance in seaweeds (Pearson et al. 2001, Roeder et al. 2005, Sung et al. 2011). Gene expression processes are thought to be responsible for desiccation tolerance (Pearson et al. 2001) and herbivore-induced defense in *Fucus vesiculosus* (Flothe et al. 2014), response to oxidative stress in *Laminaria digitata* (Roeder et al. 2005), and physiological tolerance in *Undaria pinnatifida* (Henkel & Hoffman 2008b). While research in this area is in its infancy, studies propose that epigenetic processes play an important role in natural variation and microevolution and are crucial for understanding invasiveness (Bossdorf et al. 2008; Ardura et al. 2017).

DNA methylation currently represents the best-understood epigenetic mechanism in plants (Zhang et al. 2013). Environmental stress can generate changes in gene expression through hypomethylation or hyperethylation of DNA (Chinnusamy & Zhu 2009). Because transposons, comprising a significant part of plant genomes, are maintained in a repressed state by DNA methylation, environmental factors can activate them through DNA demethylation (Chinnusamy & Zhu 2009). Such a responsiveness of DNA methylation (i.e. control by frequency and distribution of DNA methylation) to environmental change is thought to affect plants morphology and development, and therefore, is considered to be a long-term mediator of plasticity at the phenotypic level (Bender 2002, Zhang et al. 2007). In seaweeds, research on DNA methylation is still in its early stage. For instance, acclimation of *Gracilaria dura* to heavy metal stress caused changes in DNA methylation (Kumar et al. 2012). Cadmium exposure slowed algal growth rate while causing demethylation of DNA, which could serve as a defensive mechanism for regulating gene expression (Kumar et al. 2012). The frequency and distribution of DNA methylation was also suggested to regulate morphogenesis in *Ulva reticulata* (Gupta et al. 2012).

Gene expression driven by environmental stress also often depends on nucleosome histone post-translational modifications (Chinnusamy & Zhu 2009). Some histone modifications enhance, while others repress transcription (Sridhar et al. 2007, Zhang et al. 2007). Overall, environmental signals can repress the target genes by a reduction in histone acetylation levels (Chinnusamy & Zhu 2009). While DNA methylation and histone modifications are thought to play a major role in gene expression and plant development under stress (Chinnusamy & Zhu 2009), none of these mechanisms are thought to be working in brown seaweeds as the genes responsible for these processes are not present in the *Ectocarpus* genome (Cock et al. 2010, Maumus et al. 2011). The transcriptomic study of this brown alga demonstrated that unlike terrestrial plants, *Ectocarpus* *siliculosus* experienced extensive reprogramming of its transcriptome during the acclimation to abiotic stress (Dittami et al. 2009, 2011). Certain classical stress responses were absent, while some novel reactions unique to *E. siliculosus* were observed (Dittami et al. 2009). In the brown seaweed *Laminaria digitata,* defense responses against pathogens and grazers were similar to those in terrestrial plants, but also included new responses involving "tightly regulated iodine metabolism" (Cosse et al. 2009). Nevertheless, recent research on the brown seaweed *Sargassum horneri* demonstrated that changes in DNA methylation induced adventitious calli and buds in this species, with younger plants exhibiting "higher capacity for forming calli and buds than in the older explants or regenerated plantlets" (Uji et al. 2015).

RNA-mediated silencing represents another gene regulatory mechanism (Cerutti et al. 2011). Although its key components have been shown to be widely distributed among algal lineages, they seem to be entirely lost in some species with quite small nuclear genomes. "Complex sets of endogenous small RNAs" (i.e. candidate microRNAs and small interfering RNAs) have been revealed in green, red, and brown seaweeds (Cerutti et al. 2011). Although the role of small RNAs in algae is not yet well understood, it is suggested that they may act in "defense mechanisms against transposon mobilization, in responses to nutrient deprivation and, possibly, in the regulation of recently evolved developmental processes" (Cerutti et al. 2011).

While many stress-induced modifications represent transitional changes and are reset once the stress is relieved, some modifications may become inherited (Bond & Finnegan 2007). Transfer of epigenetic information between generations can provide a memory of environmental stress experienced in previous generations (Molinier et al. 2006). DNA methylation and histone modifications are thought to retain stress memory for longer periods of time (Chinnusamy & Zhu 2009). For example, experimentally altered DNA methylation in flax (*Linum usitatissimum*) had a significant effect on phenotypes in at least four generations (Fields & Amyot 1999). Such abiotic stress-induced epigenetic changes (epigenetic stress memory) have an adaptive advantage and may help plants to cope with environmental stress (Chinnusamy & Zhu 2009). Transgenerational plasticity is thought to be increasingly involved in plants adaptation (Sultan et al. 2009, Holeski et al. 2012).

Epigenetic modifiers do not regulate gene activity independently, but rather in a complex, playing a crucial role in genome organization, stability and gene expression control (Grant-Downton & Dickinson 2005). As epigenetic processes play a role in plant acclimation under stress, they are also likely to play a role in acclimation of seaweeds. Because epigenetic variance is induced directly by ecological interactions, it may provide an additional, quick pathway for evolutionary change of non-indigenous seaweeds in their novel range (Bossdorf et al. 2008).

1.3 Adaptation processes (genetically-determined)

Upon invasion, non-indigenous seaweeds become placed in the environment with no history of previous adaptation that generates strong selection pressures on both invasive and native species (Facon et al. 2006). As such, the success of invaders will depend on their ability to respond to natural selection, in addition to physiological tolerance and plasticity (Lee 2002). An effective response to selection may be crucial for the establishment of self-sustaining populations and range expansion (Keller & Taylor 2008). Selection of beneficial traits can occur rapidly, resulting in a competitive advantage of introduced over native species within a few generations (Reznick & Ghalambor 2001, Prentis et al. 2008). Life history traits, including growth rate, reproductive capacity, and phenotypic plasticity, all are subjected to selection pressures (Lee 2002).

Studies on successful marine invasions often document the availability of high genetic variance within introduced populations that may be related to selective processes (Rius et al. 2014). As introduced populations face high selection pressures, selection acting on the genetic background of introduced individuals appears to be crucial (Rius et al. 2014). Multiple introductions, outcrossing, and hybridization with native species may lead to high genetic variation (Barett & Richardson 1986) on which natural selection could act. In contrast, fewer introductions, inbreeding, and clonal reproduction can result in lower genetic variation and pose significant constraints to selection (Conner & Hartl 2004). Thus, availability of genetic variation for a trait, along with selection processes, defines whether trait selection will produce an evolutionary response. Under natural selection, non-indigenous seaweeds can become adapted to novel environmental conditions (Keller & Taylor 2008). For instance, the invasive Mediterranean strains of *Asparagopsis taxiformis* are characterized by high genetic diversity within its populations in contrast to the native strains (Andreakis et al. 2009).

1.3.1. Selection on standing genetic variation

At the time of introduction, a significant part of the adaptive material is already present within populations or, is taken from the standing genetic variation (Hermisson & Pennings 2005). Beneficial alleles are instantly available for selection to act upon (Anderson 2012). They come at initially high frequencies that reduce the fixation time (Hermisson & Pennings 2005, Anderson

2012), and may have already been tested by selection in previous environments or in other species with which genes were exchanged (Rieseberg et al. 2003). Also, alleles with neutral or deleterious effects in the native area may become advantageous in the introduced area (Prentis et al. 2008). In most cases, alleles from standing genetic variation dominate when adaptation occurs over a short time frame (Barett & Schluter 2008).

If a major part of adaptive material comes from standing genetic variation, adaptation to new conditions will depend on the quality and amount of genetic variation available within introduced populations (Hermison & Pennings 2005). Therefore, sufficient amount of heritable genetic variation should be present, for evolution by natural selection to occur (West-Eberhard 2003). High levels of genetic variation within introduced populations can increase their potential to cope with and adapt to new biotic and abiotic conditions (Jump et al. 2009). Standing variation may thus represent the first source of adaptive genetic variation within introduced populations and lead to their rapid evolution in the novel range (Hermisson & Pennings 2005, Barett & Schluter 2008, Prentis et al. 2008, Hedrick 2011). For instance, the invasion of Caribbean islands and Central America by Brazilian water hyacinth *Eichhornia paniculata* is thought to be due to the adaptation from standing genetic variation (Fenster & Barett 1994, Barett et al. 2008). Rapid adaptation from standing variation seems to be particularly relevant in clonal species (Prentis et al. 2008), compared to selfing, which is expected to reduce polymorphism (Glemin & Ronfort 2013). Due to the ability to regenerate from fragments, non-native clonal seaweeds (i.e. *Caulerpa, Codium, Bryopsis*) can sustain intense grazing by native herbivores (Williams & Smith 2007). In the genus *Caulerpa, resistance (or high tolerance) to herbivory* due to the production of toxic metabolites (Boudouresque et al. 1996, Bulleri & Malquori 2015) indicated that this genus accumulated herbivores throughout time and that strains capable of efficient vegetative reproduction (Renoncourt and Meinesz 2002) were rapidly selected.

1.3.2. Genetic admixture

Genetic admixture represents another mechanism that can contribute to the adaptive potential of non-indigenous seaweeds in the novel range (Verhoeven et al. 2011, Facon et al. 2008). Many successful seaweed invasions are associated with multiple introductions from different locations in the native range and subsequent mixing between populations that do not normally come in contact in its native range (Dlugosch & Parker 2008, Verhoeven et al. 2011, Andreakis et al. 2016). Although a single introduction may not necessarily cause species invasive ability, repeated introductions can result in segregation and recombination between genotypes from various source populations and formation of new genotypes with novel combinations of traits that can be advantageous to invaders (Ellstrand & Schierenbeck 2000, Lavergne & Molofdky 2007). The major long-term benefit of admixture is an increased genetic variation, which can increase the probability of adaptation and the likelihood of successful establishment of colonizing genotypes (Hufbauer et al. 2013). Admixture can also mask deleterious mutations while heterosis or hybrid vigour (Facon et al. 2008), resulting in the phenotypic superiority of hybrids compared to their parents (Lippman & Zamir 2007), can raise inbreeding depression and decrease the impact of genetic bottlenecks (Verhoeven et al. 2011). Multiple introductions from genetically different source populations can often restore lost genetic diversity, and result in even higher diversity than in the native range, contributing to invasion success (Dlugosch & Parker 2008, Keller & Taylor 2010, Verhoeven et al. 2011). For instance, high genetic diversity within the populations of the invasive red seaweed *Asparagopsis taxiformis* in the Mediterranean Sea, indicated multiple introductions or a single introduction by a genetically diverse and large group of founders (Andreakis et al. 2009). Similarly, increased genetic variation in the introduced populations of the invasive brown alga *Undaria pinnatifida* (compared to the native Asian populations), was associated with multiple introduction events from highly differentiated sources (Voisin 2005). The study on the highly invasive green alga *C. fragile ssp. tomentosoides* suggested that despite the low genetic variance and genetic bottlenecks within its populations, there have been at least two separate introductions from its native range in Japan to the North Pacific (Provan et al. 2005). The invasive red alga *Polysiphonia harveyi* is thought to be introduced into the North Atlantic from Japan at least twice from two independent native lineages: the cold-temperate island Hokkaido and the warm-temperate island Honshu? (McIvor et al. 2001).

1.3.3 Increased phenotypic plasticity (genetically determined)

Adaptation of invasive seaweeds in the novel range can also occur through conversion of non-heritable environmentally induced variation (adaptive phenotypic plasticity) to adaptive heritable variation (Baldwin effect or genetic assimilation; Ghalambor et al. 2007). Traits initially induced by the environment, through the process of natural selection, can become genetically determined (assimilated) and canalized (a loss of plasticity) so that the environmental stimulus previously required to produce the trait is no longer required (Ghalambor et al. 2007, Richards et al. 2006). If the new phenotype has an increased fitness, it will be selected for. The continuous process of adjustment of a phenotype (by selection) to new conditions, with no exposure to the original environment, may lead to a stable expression of the new phenotype and gradual reduction of plasticity for a trait (Richards et al. 2006). Over time, individuals with novel phenotypes will survive, while originally induced phenotypic responses may be lost as they become less beneficial (Richards et al. 2006). Genetic assimilation is particularly relevant for invasions, as exposure to new conditions can result in the generation of new phenotypes and can occur for example when invaders are released from the herbivore pressure in the novel range (Keane & Crawley 2002, Mitchell & Power 2003).

Genetic assimilation represents a second step in the adaptation to new environments via adaptive plasticity and can play an important role in the establishment of self-sustaining populations. Through this process, populations can adapt genetically to novel environments resulting in new evolutionary paths (Richards et al. 2006). For instance, “depth- and nutrient-dependent selection on chemical defenses” in the brown seaweed *Fucus vesiculosus*, may maintain high genetic diversity required for rapid evolution of this species (Jormalainen & Honkanen 2004). A similar potential for evolutionary change was revealed in the red seaweed *Asparagopsis armata*, which opportunistic life-history traits may be selected for fast growth and high fecundity (Monro & Poore 2009).

2. Acclimation and adaptation mechanisms across the phases of invasion process

From the moment of introduction, the non-indigenous seaweed becomes involved in the interactions with the new environment and ecosystem and has to overcome various obstacles to become successful (Kolar & Lodge 2001, Blackburn et al. 2011). These interactions, whether positive or negative (as failure may occur at any given time), will determine whether species will become established in the novel range and expand their range (Kolar & Lodge 2001). Genetic drift and reduction in population size resulting from founder events, during the introduction and colonization stages, may lead to the reduction of overall genetic diversity and affect invasion success (Sakai et al. 2001). Founder effects may also be a reason of post-establishment lag phase, and introduced species become invasive after evolutionary adjustments have occurred (Richards et al. 2006, Roman & Darling 2007, Dlugosh & Parker 2008). Although gene flow can have a positive effect through an increase in genetic diversity, it may also have an adverse impact via the introduction of undesirable genes.

2.1 Primary introduction

Dispersal mechanisms have a direct impact on the genetic composition and structure of invasive species populations (Hamrick et al. 1993). Gene dispersal, occurring through human-mediated transport or natural processes, can affect genetic quality and diversity of introduced populations and thereby impact their ability to adapt to novel environmental conditions (Roman & Darling 2007). Propagule pressure is particularly important for the invasion success of a species, as well as for determining genetic diversity of introduced populations, unless the species are selfing (Roman & Darling 2007). Increased propagule pressure, resulting from individual introductions with large propagule pools (introductions of a high number of founders from a single diverse source) or multiple introductions (introductions from multiple sources) is likely to result in increased genetic diversity (Simberloff 2009, Bock et al. 2015). Increased propagule pressure can raise the probability of introducing genotypes with high fitness in the recipient environment (i.e. pre-adapted genotypes, Barett & Schluter 2008, Rius et al. 2015). Genetic admixture, arising from multiple introductions, can then further contribute to the success of introduced population through formation of new genetic combinations (Rius & Darling 2014). A transport of a large propagule pools is particularly effective via the ballast water (MacIsaac et al. 2002, Uwai et al. 2006), especially if individuals possess life-history traits favoring the long-distance transport (Nyberg & Wallentinus 2005, 2009). The ability to survive in conditions of darkness, extreme temperatures, dehydration and starvation, gametophytes can disperse via shipping in the ballast water (Flagella 2003, Nyberg & Wallentinus 2009). For instance, the initial introduction of *Undaria pinnatifida* from Asia to New Zealand is thought to occur via the transport of alive gametophytes (and fertile sporophytes) attached to ship hulls or in the ballast water tanks (Uwai et al. 2006). Due to the tolerance to high temperatures, the gametophytes of *U. pinnatifida* could survive transportation through the tropics (Uwai et al. 2006). Oyster transportation (i.e. fouling on oyster shells) also has the capacity for large propagule pool delivering and is likely to result in increased genetic diversity (Fofonoff et al. 2003). For instance, the most invasive seaweed in the world the green algae *Codium fragile* ssp. *tomentosoides* is thought to be transported around the world with culture shellfish (Trowbridge 1998, Nyberg and Wallentinus 2005). In contrast to transportation vectors described, aquarium releases introduce much smaller amounts of propagules meaning that multiple introductions are necessary to create diverse populations (Roman & Darling 2007). For instance, the aquarium strain of *Caulerpa taxifolia* in the Mediterranean is characterized by significantly reduced diversity (Jousson et al. 1998).

Natural spread capacity, including the long-distance dispersal via propagules and drift thalli (i.e. *Sargassum muticum, Undaria pinnatifida,* etc.) or capacity to spread via fragmentation (i.e. *Caulerpa taxifolia*), acts in combination with anthropogenic dispersal vectors. Clonality represents one of the strategies for long-distance dispersal through transplantation of fragments (Van der Merwe 2010). Fast colonization ensured by fragmentation may contribute to invasive success, as segments separated due to the effects of biotic or abiotic factors are able to reattach to the substrate and form new independent individuals. Re-attachment after clonal fragmentation was observed in *Acanthophora spicifera* (Mshigeni 1978). Fragmentation also played an important role in the spread of *Caulerpa taxifolia* in the Mediterranean (Meinesz et al. 1995). Fragmentation is crucial for seaweeds dispersal as fragmented units are more likely to grow faster and spread more rapidly than spores (Meneses & Santelices 1999). Moreover, due to somatic mutations dispersed and replicated by growth, genetic differentiation of strains may occur (Meinesz et al. 1995). At the stage of dispersal, natural selection may operate directly on dispersal mechanisms (Holt et al. 2005). Since the introduction pathway may favor different characteristics, the invader may be selected for different features at different stages of the life cycle.

2.2 Colonization

If invaders survive the long-distance transport, the success of colonization will depend on whether their physiological tolerance to abiotic factors and life history match the new environment sufficiently for survival and reproduction (Wikstrom 2004, Cebrian & Rodriguez-Prieto 2012). At this stage, the breadth of the native range can serve as an indicator of invasive potential, ensuring that at least one genotype would be suited to the new range (Bock et al. 2015). Reproductive capacity and growth strategies become particularly important at this phase, as the availability of pre-adapted traits (i.e. broad environmental tolerance, asexual reproduction, high fecundity, rapid growth rates, phenotypic plasticity) can contribute to colonization success (Nyberg & Wallentinus 2005, Hu & Lopez-Bautista 2013, Bock et al. 2015). Phenotypic plasticity is likely to represent the first response of introduced genotypes to their new environment (Richards et al. 2006, Prentis et al. 2008). Expression of advantageous phenotypes may result in better fitness (i.e. increased survival and higher population growth; Sexton et al. 2002, West Eberhard 2003) in conditions, in which individuals would otherwise be maladapted (Crispo 2007). In the absence of plastic responses, during the first generations of colonization, populations' growth may be low and extinction more probable. For instance, the ability of *Caulerpa racemosa* to adapt to various light conditions (i.e.

photosynthetic plasticity) may have contributed to its invasive success in the Coastal Mediterranean (Raniello et al. 2004). Polyploidy is particularly relevant at this stage. Polyploid organisms were shown to have high levels of plasticity, allowing increases in fitness and competitiveness (Hahn et al. 2012), and they are thought to be better colonizers than diploids (Soltis & Soltis 2000, Brochmann et al. 2004, Pandit et al. 2011). Among invasive macroalgae, the relationship between polyploidy and invasiveness has been revealed in invasive red seaweed *Asparagopsis taxiformis,* lineage 2 (Andreakis 2007) and *Undaria pinnatifida* (Uwai et al. 2006).

As the ability to generate a plastic response is determined by genetic variation available within introduced populations, pre-existing genetic variation represents the first source of adaptive genetic variation (Prentis et al. 2008). During the colonization process, the genetic changes allowing non-indigenous seaweeds to adapt to new environmental conditions during the first generations are most likely to be due to alleles from standing genetic variation (Barett & Schlutter 2008, Prentis et al. 2008). If introduction events repeat, this may lead to genetic admixture, which results in increased genetic variance and formation of new allelic combinations (Lavergne & Molofsky 2007, Rius & Darling 2014). Admixture, in turn, can generate an increase in fitness and enhance a species response to local selection pressures, which may be crucial in conditions of low environmental matching (Lavergne & Molofsky 2007). Recent research on the ragweed *Ambrosia artemisiifolia* demonstrated that admixture can shape phenotypic variation in traits important for colonization success (Chun et al. 2011). Admixture is also prevalent in species with low dispersal abilities and extensive spread (Rius & Darling 2014).

2.3 Establishment

If physiological requirements of non-indigenous seaweeds are fulfilled, their establishment will depend on interactions with native species (Sanchez et al. 2005, Scheibling et al. 2008, Strong et al. 2009). This implies successful competition and surviving biotic pressures for the establishment of self-sustaining and growing populations (Sumi & Scheibling 2005, Gollan & Wright 2006). For instance, the successful establishment of *Codium fragile ssp. tomentosoides* in Nova Scotia is thought to be due to the release from grazing by the sea urchins *Strongylocentrotus droebachiensis* compared to its native range in England (Sumi & Scheibling 2005, Lyons & Scheibling 2008).

Studies comparing the plasticity of invaders and native species support the ideas that a) on average, invasive species may be more plastic than non-invasive or native ones, and b) invasive species may have evolved higher plasticity in their introduced range than in their native range (Richards et al. 2006). Invasive populations often exhibit increased performance in competitive ability (i.e. growth rate, size, fecundity) in the recipient range compared to the native area (Cano et al. 2008, Parker et al. 2013). For instance, invasive populations of *S. muticum* have larger thallus, higher population abundance and increased reproduction levels, compared to native populations (Crawley 1987). This could be a result of phenotypic plasticity in response to the favorable environmental conditions (Crawley 1987) or the absence of natural enemies leading to more rapid growth and reproduction (Keane and Crawley 2002, Mitchell & Power 2003).

Invaders frequently have a broad ecological tolerance and genetic variation for plasticity (Richards et al. 2006). Although individuals introduced from an area with a wider range of environmental conditions are already better prepared in the introduced range, an increased plasticity of invaders vs. natives could be evolved through selection on the presence of high levels of disturbance, plasticity of physiological or morphological traits (Cano et al. 2008). For instance, the capacity of *Asparagopsis taxiformis'* Mediterranean lineage 2, to turn plastic physiological responses into adaptive benefits, is likely to support its invasive success in its introduction range (Zanolla et al. 2015).

Because plasticity provides a fitness advantage that allows enlarging the breadth of environments in which individuals can survive and reproduce, it has been proposed to contribute to the development of invasiveness (Richards et al. 2005, Davidson et al. 2011). It may enable a larger increase in fitness in more favorable conditions ("master-of-some" scenario), expressing an opportunistic behavior and rapidly taking advantage of available resources (Richards et al. 2006, Davidson et al. 2011). Alternatively, it can rather maintain fitness in and express robustness towards the unfavorable environmental conditions ("Jack-of-all trades" scenario; Richards et al. 2006, Davidson et al. 2011). Alternatively, both elements of opportunism and robustness can be combined so that the phenotypic plasticity in response to more favorable environmental conditions increases fitness, while plasticity in response to stressful conditions allows a smaller fitness decline ("Jack-and-master" scenario; Richards et al. 2006). However, for biological invasions, the role of plasticity represents particular interest when it maintains positive fitness over a wide range of environments (Richards et al. 2006, Davidson et al. 2011).

Genetic admixture can further contribute to post-colonization population success (Verhoeven et al. 2011). If it results in increased fitness, it may lead to bigger and more productive populations, characterized by a higher probability of successful establishment and probability of range expansion, as well as a lower probability of extinction (Rius & Darling 2014). Still, the most invasive seaweeds often have common reasons why they become established: they are highly tolerant and opportunistic (Nyberg & Wallentinus 2005).

2.4 Range expansion

Range expansion of introduced seaweeds significantly depends on their long-distance dispersal capacity and is limited if the drifting capacity and ability to reproduce during drift are absent. For instance, wide and rapid expansion of *S. muticum* is closely related to its high capacity for long-distance dispersal, as its detached thallus, may stay afloat for months (Norton 1976) while being fertile during drift (Deysher & Norton 1982, Norton & Deysher 1989). Reproductive units may then disseminate even further when detached from fertile drifting branches (Deysher & Norton 1982). Ability to stay alive for significant periods of time allows detached thalli of *S. muticum* to survive long-distance transport while shedding fertile propagules (Engelen et al. 2015). Fragmentation and substratum requirements play a major role in range expansion. Species with low substratum specificity can attach to various long-distance drifting objects and as such, spread on significant distances (Engelen et al. 2015). For instance, a significant dispersal potential of the red seaweed *Asparagopsis armata* is attributed to the fragmentation and attachment to floating structures, in addition to a free-floating tetrasporophyte in its life-cycle (Wiencke & Bischof 2012). Long-distance dispersal also seems to play a role in the spread of *Asparagopsis taxiformis* (Andreakis 2009), *Gracilaria vermiculophylla* (Nyberg & Wallentinus 2005, 2009, Hu et al. 2014), and *Acanthophora spicifera* (O´Doherty & Sherwood 2007).

If sufficient amount of genetic variation is available within introduced populations, adaptation from standing genetic variation will dominate rapid evolution during the range expansion process (Prentis et al. 2008). This mechanism can be particularly important for invasive species with ecogeographic variation. Clines were observed in the introduced environments of invasive flowering plants (Keller et al. 2009, Colautti et al. 2010). Still, adaptation from standing genetic variation in invasive species can be limited if genetic constraints prevent natural selection from enhancing certain combinations of traits. If this takes place, introduced populations in marginal habitats can experience a reduction in population growth. For instance, evidence of adaptation from standing genetic variation was observed during the northward expansion of *Lythrum salicaria* in North America (Colautti et al. 2010). Its adaptation was however constrained at the northern limits of the introduced range due to the lack of genetic variation for a particular combination of traits (Colautti et al. 2010).

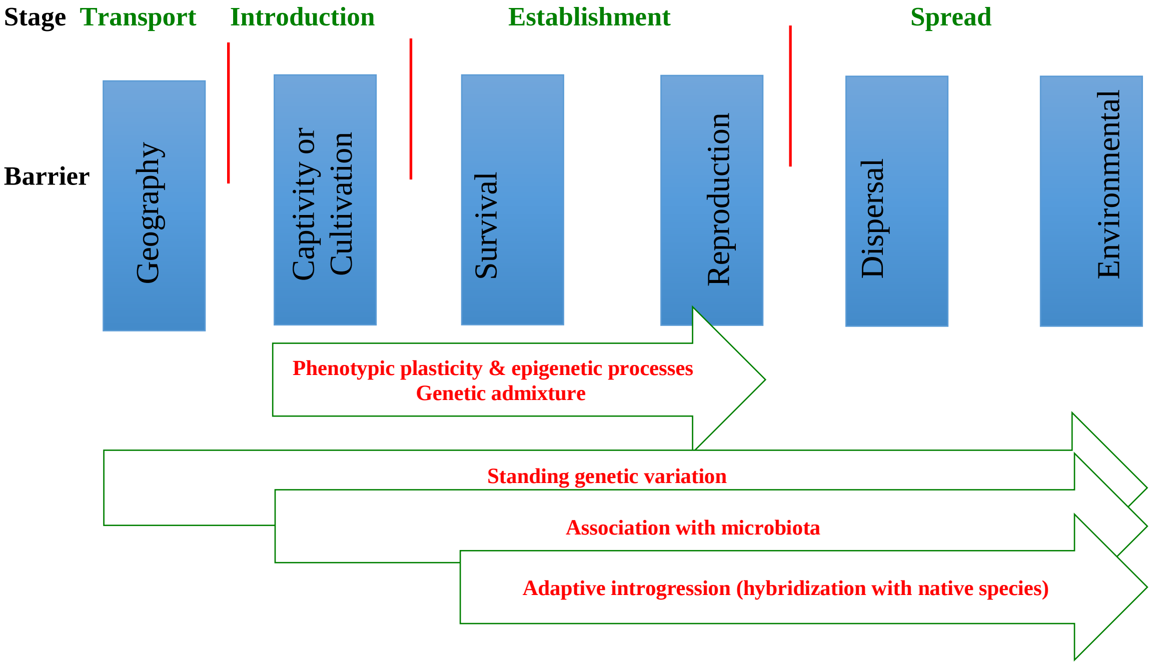

Figure 3.1 Mechanisms of acclimation and adaptation acting throughout the stages of seaweeds invasion process based on the unified framework for biological invasions proposed by Blackburn et al. 2011.

3. Species are not alone

3.1 The role of associated bacterial communities in adaptation and evolution

Recent studies reveal that adaptive capacity of non-indigenous seaweeds depends not only on their gene pools but also on association with their microbiota (Aires et al., 2013, 2015, Saha et al. 2016). These provide the seaweed holobiont (i.e. the host and its associated organisms, Fig. 3.2) with a significant amount of genetic information, often considerably exceeding that of its host (Zilber-Rosenberg & Rosenberg 2008, Bosch & McFall-Ngai 2011).

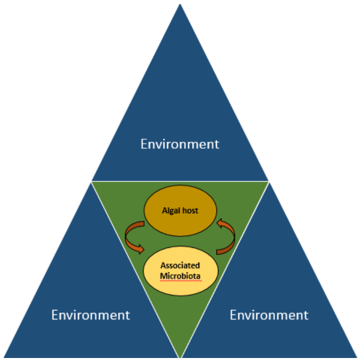

Figure 3.2 The seaweed holobiont (comprising the host and its associated microbiota) as a unit of selection by environmental factors.

Microorganisms play a pivoting role in expanding seaweeds' physiological capacities, broadening their environmental tolerance, and providing the capacity to occupy new ecological niches (Goecke et al. 2010, Egan et al. 2013, Hollants et al. 2013). For instance, specific bacteria are linked to low salinity tolerance in *Ectocarpus* cultures facilitating acclimation to environmental change (Dittami et al. 2016). Seaweed-associated bacteria were also shown to have growth-promoting and nutritional effects (Head & Carpenter 1975, Dimitrieva et al. 2006), as well as providing vitamins to their hosts (Croft et al. 2005, Wichard et al. 2015). For instance, *Azotobacter* bacteria, associated with *Codium fragile*, were shown to be involved in nitrogen fixation, and are thought to supply combined nitrogen compounds stimulating algal growth, which could be associated with rapid expansion of this seaweed (Head & Carpenter 1975).

Seaweed microbiota is also involved in the production of biologically active and defensive compounds, protecting algal surfaces from pathogens, herbivores and fouling (Burgess et al. 1999, Rao et al. 2007, Penesyan et al. 2009, Egan et al. 2014, Saha et al. 2014). Through antibiotic activity or production of metabolites causing negative effects on fouling organisms, bacteria control the microbial population on seaweed surfaces (Boyd et al. 1999, Egan et al. 2000, Joint et al. 2007, Kanagasabhapathy et al. 2008, Romero et al. 2011). For instance, bacteria associated with the green seaweed *Ulva reticulata* were shown to inhibit settlement of the polychaete *Hydroides elegans* (Dobretsov & Qian, 2002). In *Laminaria saccharina*, 50% of isolated bacterial strains demonstrated antimicrobial activity, inhibiting the growth of at least one Gram-negative and Gram-positive bacterium (Wiese et al. 2009). In *Splachnidium rugosum*, 36% of bacterial isolates exhibited antimicrobial activity against one or more strains tested (Albakosh et al. 2016). Extracts from invasive *Grateloupia turuturu* and *Sargassum muticum* also exhibited anti-microfouling activities and microbial inhibition (Plouguerne et al. 2008). Seaweed-associated microbiota also supports the normal morphology of their hosts (Matsuo et al. 2003, Wichard et al. 2015). *Ulvales* green algae are known for the loss of their typical morphological development in the absence of specific bacterial strains or biologically active morphogenetic compounds isolated from those bacterial strains (Wichard et al. 2015). Bacteria-induced morphogenesis was observed in *Ulva lactuca* (Provasoli & Pinter 1980), *Ulva linza* (Marshal et al. 2006), *Ulva pertusa* (Nakanishi et al. 1996), and *Ulva mutabilis* (Spoerner et al. 2012), among other species.

Disturbances and stress, resulting from changing environmental conditions, are known to affect the seaweed-associated microbial composition (directly or via its physiological responses) and cause its structural, functional or behavioral changes (Pruzzo et al. 2008, Saha et al. 2016).

Taking into consideration the significant amount of genetic information contained in the diverse microbiota, shifts in its diversity and abundance occurring rapidly in response to fluctuating environmental factors (Dinsdale et al. 2008), may affect invaders´ fitness and, as such, represent an important tool for adaptation to new environmental conditions (Zilber-Rosenberg & Rosenberg 2008, Tonon et al. 2011, Dittami et al. 2014). For instance, the invasion success of the red seaweed *Gracilaria vermiculophylla* is thought to be enhanced by the rapid defense adaptation ensured by bacterial epibionts from the invaded range (Saha et al. 2016). Poor defense of invasive populations of *G. vermiculophylla* by microbial epibionts from the native range, along with the weak defense of native populations against bacterial epibionts from the invaded range, indicate an adaptation of their defense capacity during the invasion process and loss of capacity to cope with old epibionts (Saha et al. 2016). *Sargassum muticum* showed spatial variability in phenolic contents and antibacterial activities along its European distribution range with greatest quantities in Norway and Portugal, suggesting that defense could be induced in response to stress in extreme conditions (i.e. UV radiation, seawater temperature, Tanniou et al. 2014). Specific bacterial strains associated with invasive *Caulerpa racemosa* and their absent in the native range, are also thought to play a role in its stress tolerance (Aires et al. 2013, 2015).

Acquisition of new symbionts from the environment and multiple contacts with various microorganisms during the lifetime can also result in changes in the seaweed-associated microbiota (Gachon et al. 2010, Goecke et al. 2010, Bosch & McFall-Ngai 2011). Under favorable conditions, acquired microorganisms may increase in abundance and affect the phenotype of the alga (Harvell et al. 2009, Campbell et al. 2011, Burge et al. 2013). For instance, environmentally-acquired opportunistic pathogenic bacteria caused bleaching disease in *Delisea pulchra* (Kumar et al. 2016). Alternatively, acquiring new symbionts can also be beneficial as it may introduce entirely new genes into the seaweed holobiont (Bosch & McFall-Ngai 2011, Saha et al. 2016). The proximity and high density of bacteria may also accelerate the rate of gene transfer between different microbial species (Bosch & McFall-Ngai 2011). Through horizontal gene transfer genetic information between pathogens and symbionts might be exchanged (Hacker et al. 2005).

Changes in microbial diversity generated by amplification, acquisition from the environment, and horizontal gene transfer can result in increased genetic information of the seaweed holobiont and, as such, can affect organism's fitness (Zilber-Rosenberg & Rosenberg 2008, Bosch & McFall-Ngai 2011). Physical proximity and increasing contact can accelerate the evolutionary changes in the genomes of symbiotic partners, and thereby, association with microbial communities is considered as an important mechanism of driving evolution (Guerrero et al. 2013, Dittami et al. 2014). Association with microorganisms may allow non-indigenous seaweeds to adapt to the conditions of the novel environment, as based solely on their genomes, they would evolve slowly (Bosch & McFall-Ngai 2011). The diverse microbiota can, therefore, assist the seaweed holobiont to survive, reproduce and gain the time necessary for the seaweed genome to evolve (Zilber-Rosenberg & Rosenberg 2008). Because the combination of host and microbiota genomes acts as a unit of natural selection by environmental factors, association with microbial communities can be crucial for acclimatization of alien seaweeds during the colonization and range expansion processes.

3.2 Adaptive introgression

Adaptive introgression represents another potential source of adaptive genetic variation and can take place during seaweed invasions if adaptive variation transmits to introduced species by interbreeding or introgression with native species (Currat et al. 2008, Hendrick 2013). For such genetic exchange between local and introduced populations to occur, adaptive introgressive variants have to be introduced through the process of hybridization (Arnold & Martin 2009, Hendrick 2013). Hybridization results in genomic changes (chromosomal rearrangements, genome expansion, gene expression and gene silencing), which may eventually lead to the generation of new beneficial phenotypes (Baack & Rieseberg 2007). Compared to adaptation from standing genetic variation, adaptive variation from introgression usually has a lower initial frequency, and the rate of adaptive change is thought to be slower (Hendrick 2013). Species of hybrid origin, characterized by new adaptive genetic combinations, tend to have more extreme trait values compared to their parental species and are thought to colonize novel environments through the expression of transgressed traits (Baack & Rieseberg 2007). First generation hybrids are often characterized by increased size, growth rate and yield (i.e. hybrid vigour or heterosis; Lippman & Zamir 2007). Among invasive seaweeds, hybridization with native species contributed to continuing expansion of the European rockweed *Fucus serratus* along the coastline of eastern Canada (Johnson et al. 2012). Hybridization and introgression in *Fucus evanescens* (introduced from the North Pacific), and the native *Fucus serratus*, resulted in “incomplete reproductive isolation and widespread hybridization” throughout the North Atlantic (Coyer et al. 2007). Hybridization can play an important role in the adaptation of non-indigenous seaweeds to new environmental conditions (Kim et al. 2008) and is crucial during the phases of establishment and range expansion (Prentis et al. 2008).

Conclusions and future directions

Multiple studies on the role of rapid adaptive evolution, conducted in recent decades, have demonstrated the potential of introduced species to adapt rapidly to the conditions in the novel range (Lee 2002, Richards et al. 2006, Barett & Schluter 2008, Currat et al. 2008, Prentis et al. 2008, Buswell et al. 2011, Saha et al. 2016). Despite this, many insights in this area come from the research on terrestrial plants, which can provide valuable evidence for understanding the acclimation and adaptation mechanisms in seaweeds, as many physiological and biochemical processes are similar to those in vascular plants (Rudiger & Lopez-Figueroa 1992, Stirk et al. 2003, Bradley 1991, Evans & Trewvas 1991). To allow a better understanding of mechanisms underlying rapid adaptive changes in seaweeds, further research characterizing the source of variation contributing to adaptive evolution is required. Regarding invasion genetics, application of more advanced genetic tools and analytical approaches is required (Viard et al. 2016). Examining the museum and herbarium specimens is particularly important for identifying genes involved in invasiveness and tracing back the genetic invasion history, while early detection and identification of cryptic invasive seaweeds is crucial for conservation and undertaking timely preventive measures (Le Cam et al. 2015, Andreakis et al. 2016). Future research should include seaweed epigenetics, as limited evidence in this area still leaves space for further exploration (Pearson et al. 2001; Roeder et al. 2005, Molinier et al. 2006, Sung et al. 2011). This particularly concerns the brown seaweeds, for which not all molecular mechanisms underlying acclimation to environmental stress are known. Assessing the role of genetic versus epigenetic variation represents an important direction in the research, as epigenetic mechanisms in invaders with minimal genetic differentiation may already be sufficient for the invasion success.

Another non-genetic mechanism that has received increasing attention in recent years represents the association of seaweeds with their microbiota (Tujula et al., 2010, Burke et al. 2011, Lachnit et al. 2011, Saha et al. 2016). Although it has already been demonstrated that adaptive capacity of invasive seaweeds in the novel range can be strengthened through association with their microbiomes (Aires et al., 2016, Saha et al. 2016), temporal and spatial variation of seaweed-associated microbial communities is still not entirely understood (Bengtsson et al. 2010, Burke et al. 2011, Campbell et al. 2015, Mancuso et al. 2016). Limited studies on seasonal differences in seaweed-associated microbiota currently do not allow making clear conclusions regarding which bacteria are characteristic for each season (Bengtsson et al., 2010; Lachnit et al., 2011; Mancuso et al., 2016). Moreover, detailed studies on seasonal differences among different algal tissues are required (Mancuso et al., 2016; Staufenberger et al., 2008). Research on seasonal aspects would also benefit from analyses of similarities between the seaweed and seawater microbiomes over time (Bengtsoon et al., 2010; Burke et al., 2011; Lachnit et al., 2009; Mancuso et al., 2016; Taylor et al., 2004), providing insights on future bacterial shifts and possible changes in behavior of invaders as a consequence of ongoing climate change. Although some studies have already explored the effects of acidification on the coral-associated microbiota (Meron et al. 2012, Webster et al. 2013), the research on seaweeds has been quite limited. Further studies, exploring whether increased CO2 and reduced pH generate shifts in seaweed-associated microbiota towards bacteria associated with stress and disease (as it was documented in corals Meron et al., 2012, Vega Thurber et al., 2009, Webster et al., 2013) are necessary. Understanding the responses of seaweed-associated microbiota to environmental stressors is crucial as it has a potential to affect seaweeds adaptation capacity (Aires et al., 2016). Moreover, seaweeds were shown to act as vectors of coral disease (Nuges et al., 2004, Morrow et al. 2017), and if shifts in seaweed microbiomes will be analogous to those in corals, organisms that are in contact or proximity with them will be threatened. As acidification can further affect the seaweed chemistry and nutritional quality of algal tissue, indirectly impacting grazers consumption rates (Urabe et al. 2003, Van de Waal et al. 2009), further research on the impacts of multiple stressors and species interactions would be of great value.

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Glossary

Acclimation: a physiological, biochemical or morphological response of an organism resulting from continuous exposure to new experimentally induced environmental conditions. It refers to the rapid and reversible response of an organism, or the response occurring slowly during the process of organism development. (Woods & Harrison 2002)

Acclimatization: a physiological, biochemical or morphological response of an organism, resulting from continuous exposure to new environmental factors (Woods & Harrison 2002).

Adaptation: genetic change in response to natural selection, resulting in improved performance of an organism in relation to some environmental feature. (Fitzpatrick 2012)

Adaptive plasticity: is an ability of a genotype to express a phenotype that enhances its fitness in response to environmental induction. (Fitzpatrick 2012)

Admixture: the occurrence of individuals (in the same population) from multiple genetically distinct sources (geographically separated native populations, temporally distinct samples from the same geographic population, or populations of closely related species). (Roman & Darling 2007)

Introgression (introgressive hybridization): incorporation of alleles from one species into the gene pool of another species usually via hybridization or backcrossing (Anderson 1949).

Colonization: is a phase of the invasion process, during which abiotic filters determine whether propagules survive in the new environmental conditions and affect growth rates.

Epigenetic: a factor, changing the phenotype of an organism, which is not associated with changes in its DNA sequence, but rather in gene expression (Prentis et al 2008).

Epigenetic inheritance: an inheritance of epigenetic marks throughout multiple generations (Schlihting and Wund 2014).

Establishment: is a phase of the invasion process, characterized by the overcoming (by individuals in an introduced population) the barriers to successful survival and reproduction in a novel environment, resulting in a positive long-term population growth and formation of self-sustained populations (Blackburn et al 2011).

Genetic assimilation: a process by which a character state, initially produced by the environmentally induced plastic response, becomes fixed via selection that leads to the evolutionary reduction and eventual loss of plasticity. (Schlihting and Wund 2014)

Hybridization: the breeding of individuals from genetically different populations (within or between species), resulting in genotypes with new combinations of alleles. (Roman & Darling 2007)

Invasive species: an introduced species, established outside of its native range, with the potential to cause ecological or economic harm. (Roman & Darling 2007)

Lag phase: the time frame between the phases of introduction and establishment of a non native species. (Roman & Darling 2007)

Phenotypic plasticity (=plasticity): the ability of an individual genotype to express different phenotypes in response to changing environmental conditions. (Fitzpatrick 2012)

Standing genetic variation: genetic variation already available within the population at the moment of environmental change (Barett & Schluter 2008)

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Chapter 4

Acidification increases abundances of *Oceanospirillales*, *Vibrionales* and *Planctomycetia* associated to a seaweed (*S. muticum*)-grazer (*S. nadejda*) system: potential consequences for disease and prey digestion efficiency.

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4. Acidification increases abundances of *Oceanospirillales*, *Vibrionales* and *Planctomycetia* associated to a seaweed (*S. muticum*)-grazer (*S. nadejda*) system: potential consequences for disease and prey digestion efficiency.

Alexandra Serebryakova, Tania Aires, Frederique Viard, Ester A. Serrão, Aschwin H. Engelen

4.1 Abstract

Ocean acidification significantly affects marine organisms in several ways, with complex interactions. Seaweeds might benefit from raising CO2 through increased photosynthesis and carbon acquisition, with subsequent higher growth rates. However, changes in plant leaf chemistry due to increased CO2 may result in reduced nutritional quality of tissue for grazers. In addition, organisms live in close association with diverse microbiota, which can also be influenced by environmental changes, with feedback effects. As gutmicrobiomes are often linked to diet, changes in seaweed characteristics and associated microbiome can affect the gut microbiome of the grazer with possible fitness consequences. In this study, we experimentally investigated the effects of acidification and grazing on the microbiome of the invasive brown seaweed *Sargassum muticum* and a native isopod consumer *Synisoma nadejda*. Both were exposed to ambient CO2 conditions (380ppm, pH 8.16) and an acidification treatment (1000ppm, pH 7.86) for three weeks. The microbiome diversity and composition was determined using hight-throughput sequencing of the variable regions V5-7 of the 16S rDNA. We anticipated that as a result of acidification, the seaweed-associated microbial community would change, leading to further changes in the gut microbiome of grazers. While no significant effects of CO2 on the overall microbial community structure and composition were revealed in the seaweed, substantial changes were observed in the grazer gut. Some specific bacterial groups were affected. *Oceanospirillales*, *Vibrionales* and *Planctomycetia* showed increased abundance in elevated CO2 treatments of the seaweed and in the grazer gut. As these bacteria are linked to disease, this could mean an increased potential for seaweeds to act as reservoirs for pathogens under acidified conditions. In addition, an increased *Planctomycetes* abundance could provide the isopod with an elevated algal digestion and availability of inorganic compounds to compensate the shifted C/N ratio under acidification in their food. So, although acidification may have limited effects on general host associated bacterial composition and diversity, the specific changes, potential have considerable negative (pathogenic) and positive (digestive) functional consequences.

Keywords: invasive seaweeds, ocean acidification, seaweed microbiomes, grazer microbiomes, metabarcoding, *Sargassum muticum, Synisoma nadejda*.

4.2 Introduction

Ocean acidification significantly affects marine organisms in diverse ways (Fabry et al., 2008; Kroeker et al., 2013). In the case of species interactions (e.g. prey-predators), the outcome of such effects can be difficult to predict as antagonistic or synergistic effects may be observed (Asnaghi et al. 2013; Branch et al. 2013; Poore et al. 2013). This is particularly true for non-calcifying seaweeds, which in contrast to most other organisms can benefit from raising CO2 through increased photosynthesis and carbon acquisition, and subsequently acquire higher growth rates (Porzio, Buia & Hall-Spencer, 2011; Harley et al., 2012; Koch et al., 2013; Olischlager et al., 2013). However, changes in plant leaf chemistry in response to elevated carbon supply are expected to result in higher C:N and C:P ratios and, as such, reduced the nutritional quality of tissue for grazers (Urabe, Togare, & Elser, 2003; van de Waal, 2009). Variations in the palatability of seaweeds may lead to changes in consumption rates by herbivores, which will have to absorb nutrients more efficiently or consume more to compensate low concentrations of essential nutrients (Gutow et al., 2014). Thus, ocean acidification could have positive effects on seaweeds growth rate but at the same time induce behavioral changes of the herbivores and increased grazing rates. Therefore, it is critical to understand the effects of multiple stressors (e.g. ocean acidification and predation).

Seaweeds and marine organisms feeding on them live in a close association with diverse and abundant microbial communities (King et al., 2012; Hollants et al., 2013; Egan et al., 2013; Dudek et al., 2014). Interactions among host and their associated bacterial communities affect the holobionts' physiology and health (Hollants et al., 2013; Egan et al., 2013), and play an important role in the functioning of the hosts (Singh et al. 2011; Singh & Reddy 2014). Seaweeds comprise dynamic species-specific bacterial communities (Aires et al. 2015; Aires, Serrao, & Engelen 2016; Vieira et al. 2016). The communities are recognized to have growth-promoting and nutritional effects (Head & Carpenter, 1975; Dimitrieva, Crawford, & Yuksel, 2006), and to be involved in the production of biologically active (Chojnacka et al., 2012) and defensive (Burgess et al., 1999) compounds. Symbiotic bacteria inhabiting the guts of marine herbivores are also known to complement the genomes of their hosts by supporting important physiological functions (Hacquard et al., 2015). This includes the mediation of the digestion of food components by producing critical digestive enzymes for breaking down nutrients with complex molecular structures (McBee & McBee 1982; Mackie et al., 2004). However, the host-associated microbiota is affected by environmental changes (Banin et al., 2000; Ben-Haim, Zicherman-Keren, & Rosenberg, 2003;

Sunagawa et al., 2009), and alterations in its structure may influence the abundance and composition of seaweed and grazer associated microbial communities (Weimer et al., 1999; Russell & Rychlik, 2001; Koren & Rosenberg, 2006).

In addition to digestive functions, grazers depend on seaweed-associated microbiota for nutrients found in the algal biofilm (i.e. proteins, polysaccharides, lipids, etc.; Tietjen, 2014). As such, diet represents an important factor in shaping microbial diversity in the intestinal systems of grazers, and any seaweed microbial alterations may change the grazer's bacterial composition (Tietjen, 2014). The altered microbial composition of the seaweed host may result in diet-induced changes in the gut microbiota of grazers that may eventually affect the metabolism of the host, as well as its fitness and biology (Tietjen, 2014, Mattila et al., 2014). Because carbon acquisition is expected to be facilitated for seaweeds at elevated CO2 levels, higher nutrient uptake is anticipated to help obtain other nutrients in the right balance with carbon. Part of these nutrients might be obtained through the microbiome, and therefore, the microbiome demand might putatively rise. Because ocean acidification is expected to affect the interactions between marine herbivores and seaweeds through increased consumption of carbon enriched algal tissue (Gutow et al., 2014), the microbiomes of grazers might help with nutrient acquisition. While better understanding of the diversity and functions of associated symbiotic bacteria is needed, few studies have addressed the diversity and composition of gut microbiota of marine grazers (but see Hong et al., 2011; Devine, Pelletreau, & Rumpho, 2012; Davis et al., 2013; Dudek et al., 2014).

To predict the responses of aquatic organisms to ocean acidification, it is necessary to understand responses of the host-associated microbiota to increasing CO2 and reduced pH. Little is known about the responses of associated microbiota to changes in pCO2, including microbial metabolic capabilities or the ability to rapidly shift the host range (Morrow et al., 2015). Also, there is no consensus regarding whether a decrease in pH causes increase (Kerfahi et al., 2014), decrease (Taylor et al., 2014) or no changes (Hassenruck et al. 2016) in microbial richness and prevalence of dominant microbial taxa under acidification conditions. Furthermore, the current knowledge of acidification effects on the host-associated microbial communities is mostly based on the results of experiments conducted on corals. These experiments demonstrated that reduced pH initiates shifts in the coral microbiota towards microorganisms associated with stress and disease (Meron et al., 2011, Vega Thurber et al., 2009, Webster et al., 2013). Moreover, seaweeds have been demonstrated to function as vectors of coral disease and, if seaweed microbiomes shift analogously to coral microbiomes, this may have consequences for other organisms, which are in direct contact, close proximity or feeding on it (Nugues et al., 2004). Therefore, there is a need for relevant studies on seaweeds, with a particular interest in species interactions as species may not respond similarly to ocean acidification and as effects can act synergistically. This response is particularly relevant to be examined in marine introduced seaweeds, because they may benefit from future ocean acidification conditions, and thus increase their invasiveness.

In this study, we experimentally investigated the effects of acidification on the microbiomes of an emblematic invasive seaweed, the brown alga *Sargassum muticum*, and the gut microbiome of a native isopod consumer, *Synisoma nadejda*, following a three-week exposure to elevated pCO2, and compared the bacterial community (hereafter microbiome) responses in these two hosts. We anticipated that as a result of acidification, the seaweed-associated microbial community would change, leading to further changes in the gutmicrobiome of grazers and adaptation of its digestive systems. Because the composition of associated microbial communities is linked to host health, we predicted that seaweed and grazer gut-associated microbial communities under acidification treatment would be distinct from those under ambient conditions with the potential loss of acidification-sensitive bacteria and increased disease and stress-related bacteria. An increase in disease-associated bacteria would indicate an increased potential for seaweeds to act as reservoirs and vectors for pathogens under acidified conditions. Furthermore, we expected that if part of the microbiome assists the seaweed obtaining nutrients and rare elements under elevated pCO2 conditions, this need would be increased and consequently this part of the microbiome should increase. An increased abundance of bacteria assisting in digestion could provide the isopod with an elevated algal digestion and availability of inorganic compounds to compensate the shifted C/N ratio under acidification in their food.

4.3 Methodology

Experimental set-up

The experiment was performed at CCMAR's Ramalhete field station during the spring of 2014. Ambient (380 ppm CO2, and pH 8.16) and elevated pCO$_2$ (1000 ppm CO2, and pH 7.86) conditions were controlled by two separate CO$_2$ sensors systems. For each condition, CO$_2$ was injected in seawater deposits that provided seawater for the experimental units. In both systems salinity was 36, alkalinity 2550 μmol kg^{-1} and seawater temperature 15 ºC. Experimental units consisted of 3 L flowthrough mesocosms receiving each 30 L seawater per hour. Experimental units were placed in one square meter tanks with 15 cm of the overflown seawater of the experimental units to stabilize temperature conditions in the units. A preliminary test showed that a wet weight of 1 g seaweed did not affect the pH conditions in experimental units at the used volume and flow. Each CO2 condition contained four replicates for each treatment combination of the seaweed *Sargassum muticum* with or without the presence of three *Syniosoma nadejda* isopods. In total, 16 experimental items were used to obtain 24 samples: 16 seaweed samples corresponding to two CO2 conditions X four replicates for seaweed in the presence and absence of grazer, and 8 grazer gut samples (dissected; see below) corresponding to the two CO2 conditions with four replicates each. The experiment ran for three weeks, during this period the units were cleaned, twice a week, to avoid epiphyte overgrowth on the experimental unit walls. In each experimental unit, seawater pH and the calibration of the automated CO2 injection system was manually checked daily to make sure the pH was stable. At the end of the three weeks, the isopods and seaweeds were flash frozen, transported to the laboratory in liquid nitrogen and stored there at -80ºC until further processing.

Hight-throughput sequencing of the microbiome

DNA was extracted from four replicate tissue samples of each host x treatment combination using the Quick–gDNA kit (Zymo Research™) according to the manufacturer protocol. Before extraction, isopods where dissected by removal of both ends and pulling out the intestinal tract. The total 16S rRNA was amplified using the universal primers 27F and 1492r with the changes to the original protocol (Lane 1991) as in Aires et al. 2016: an initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 90 s, with a final extension was at 72 °C for 3 min. The 25 µl reaction mixture contained 250 µM dNTPs, 0.6 µM of each primer, 1 × 2 PCR buffer mix, 2 µl of template DNA (with a final concentration of about 10 ng µl−1) and 0.3 µl of Taq polymerase (Advantage R2 Clontech). PCR products were cleaned using ExoFastAP enzyme following the Thermo Scientific™ protocol. Amplified DNA was sent to Molecular Research (MR DNA), Shallowater, Texas where a nested–PCR was performed prior to sequencing. The modified 8 bp key–tagged primer 799F along with the reverse primer 1193R (fragment ~ 400 bp) were used to avoid chloroplast cross amplification (Bodenhausen et al., 2013). PCR conditions were as follow: 95 °C for 3 min, 10 cycles of 95 °C for 20 s, 50 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 3 min. Samples were pooled together in equal proportions based on their molecular weight and DNA concentrations and purified using calibrated Ampure XP beads. DNA libraries were prepared by following Illumina TruSeq

DNA library preparation protocol and paired–end (2 x 250 bp) sequencing performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

Sequence analysis and bioinformatics

The microbial community analyses were performed using the Quantitative Insights into Microbial Ecology (QIIME version 1.8.0) software (Caporaso et al., 2010). Sequences were screened and filtered for a minimum read length of 350 bp and less than 2 or more undetermined nucleotides. The filtered dataset, containing only high-quality sequences, was submitted to a conservative chimera detection filter using the ChimeraSlayer method (Haas et al., 2011). Selected high-quality chimera-free sequences were clustered into Operational Taxonomic Units (OTUs) within reads using UCLUST (Edgar, 2010) using a pairwise identity threshold of 0.97. Representative sequences for each OTU were picked using the "most-abundant" method, and OTU sequence alignment was performed with Pynast (Caporaso et al., 2010). RDP classifier (Wang et al., 2007) was used for taxonomic assignment with a 95 % confidence threshold. Each OTU was assigned to the closest matching described taxon in the Greengenes reference database (version 13_5) (McDonald et al., 2012) with a maximum e-value to record an assignment of 0.001. Eukaryotes (i.e., chloroplasts and mitochondria) matching sequences were excluded from the OTU table in downstream analyses as well as rare OTUs (singletons and doubletons) and unassigned sequences.

The filtered rarefied Operational Taxonomic Unit (OTU) table was applied to calculate alpha diversity statistics using the QIIME software, including the Chao I richness estimates (Chao, 1984), the observed number of species, and the Shannon index. Permutational multivariate analyses of variance (PERMANOVA) were performed to compare the microbiome of *S. muticum* vs. the microbiome of grazer gut samples. A PERMDISP procedure was used to test for the homogeneity of bacterial community structure among these factors (i.e. seaweed vs. grazer). To visualize differences and to assess dissimilarity between samples, Canonical Analysis of Principal coordinates (CAP) plots were constructed using the PRIMER 7 software (Clarke & Warwick, 1994; Clarke & Gorley, 2006). Similarities and dissimilarities in microbial communities between acidification treatments were explored using SIMPER analyses with PRIMER-E (Clarke & Gorley, 2006). For bacterial groups, which exhibited notable differences in abundance between the grazer gut and the seaweed and, which responded to elevated CO2 levels, two-way analyses of variance (ANOVA) were performed (with the preliminary tests for normality and homogeneity of variances being implemented). Species (*S. muticum* and isopod) and acidification (CO2 ambient and elevated) were tested as factors affecting the structure /composition bacterial communities. For microbial species for which significant interaction was detected, a post-hoc t-test was implemented (P(T<=t) two tail < 0.05).

4.4 Results

Bacterial communities associated with S. muticum *and the gut of* S. nadejda

In total, 3,204,094 partial 16S rRNA gene sequences were acquired from 24 samples (i.e. two CO2 conditions X four replicates for seaweed in the presence and the absence of grazer and four grazer gut samples with two CO2 conditions). Quality filtering resulted in 2,877,493 high-quality sequences, with an average of 119,896 ± 46,294 reads per sample, which were clustered into unique operational taxonomic units (OTUs). The OTU table was rarefied to the minimum number of sequences (66,831). As a result, a total of 41,040 unique sequences were observed.

Overall, 563 bacterial genera from 76 classes distributed across 30 phyla (including unidentified bacteria) were detected. Among them, 551 bacterial genera distributed across 29 phyla (including unidentified bacteria) were associated with *S. muticum* (over all samples and treatments), compared to 450 genera distributed across 24 phyla (including unidentified bacteria) for gutbiome. The diversity of seaweed-associated bacterial communities was 1.2 to 1.4 times higher in *S. muticum* than in the grazer gut for Shannon index (2-way ANOVA, F=2.507, P=0.139), OTU richness (2-way ANOVA, F=3.361, P=0.092) and Chao 1 (2-way ANOVA, F=6.226, P=0.028), respectively.

Acidification did not affect microbial diversity at OTU level as estimated by diversity indexes: Chao 1 (2-way ANOVA, F=0.003, P=0.960), unique OTU richness (2-way ANOVA, F=0.178, P=0.681) and Shannon index (2-way ANOVA, F=0.048, P=0.831). The addition of grazers did not result in significant changes in microbial community structure (PERMANOVA, p=0.459) and composition (PERMANOVA, p=0.372) of *S. muticum*. Bacterial phyla-specific to hosts and specific conditions are presented in Table S1 of the Supplementary Material. Interestingly, at elevated CO2, *Vibrionales* were significantly more abundant within *S. muticum* in the absence of grazers than in their presence (Two-tailed t-test, P=0.018).

As no significant effect of grazers on *S. muticum* was revealed, further analyses focusing on the comparison of *S. muticum* vs grazer gut, implemented only for *S. muticum* in the presence of grazers. *S. muticum* associated bacterial communities in the presence of grazers were dominated by

Bacteroidetes (75.4%; *Flavobacteria* 75.2%) and *Proteobacteria* (16.2%; *Alphaproteobacteria* 12.2%), while the isopod gut communities were dominated by *Proteobacteria* (47.2%; *Alphaproteobacteria* 43.5%), *Planctomycetes* (32.4%; *Planctomycetia* 32.1%), and *Bacteroidetes* (17.7%; *Flavobacteria* 16.8%); Fig. 4.1). *Flavobacteriales* (73.3%) was the most common bacterial order associated with *S. muticum*, while *Rickettsiales* (38.4%), *Pirellulales* (30.9%) and *Flavobacteriales* (16.8%) were the most abundant grazer gut-associated orders. Altogether, bacterial communities associated to *S. muticum* differed significantly from those in the gut of *S. nadejda* (PERMANOVA p=0.001; Fig. 4.2). The main differences were due to a higher abundance of *Bacteroidetes* (73.7%) associated to *S. muticum* (contributing 15.11% to the dissimilarity; SIMPER) and to more *Proteobacteria* (47.2%) and *Planctomycetes* (32.4%) in the gut of *S. nadejda*, contributing 11.76 and 14.98% to the dissimilarity, respectively (Fig. 4.3).

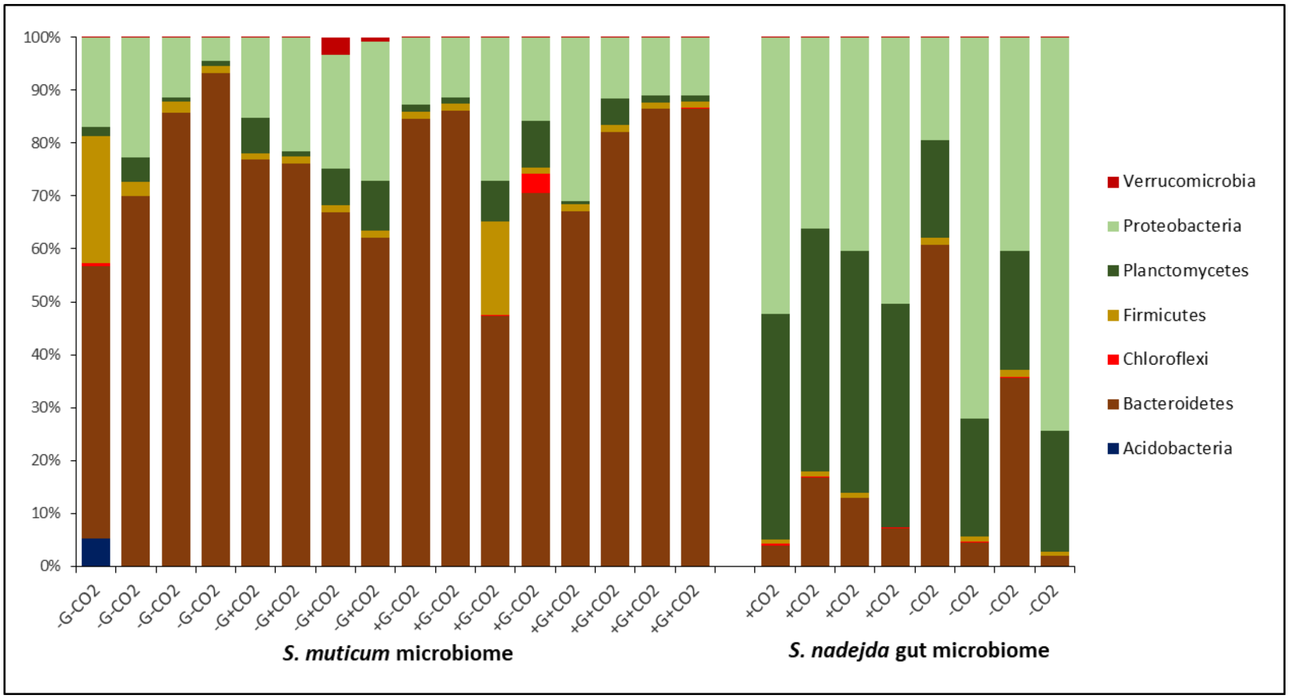


Figure 4.1 Relative distribution of the bacteria phyla associated to the brown seaweed *Sargassum muticum*, without (-G) and with (+G) *Synisoma nadejda* isopods, and to the gut of the isopod after three weeks on a *Sargassum muticum* diet, under ambient (380ppm; -CO2) and elevated (1000ppm; +CO2) CO2 conditions.

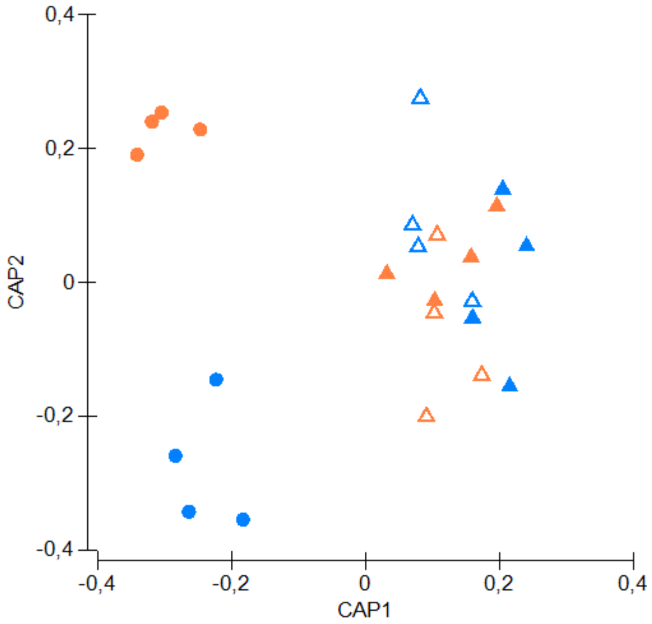


Figure 4.2 Canonical Analysis of Principal coordinates (CAP) of the bacterial communities associated to the brown seaweed *Sargassum muticum*, without (filled triangles) and with *Synisoma nadejda* isopods (empty triangles), and the gut microbiome of the isopod on a *Sargassum muticum* diet, under ambient (380ppm; blue symbol) and elevated (1000ppm; orange symbol) CO2 conditions for three weeks.

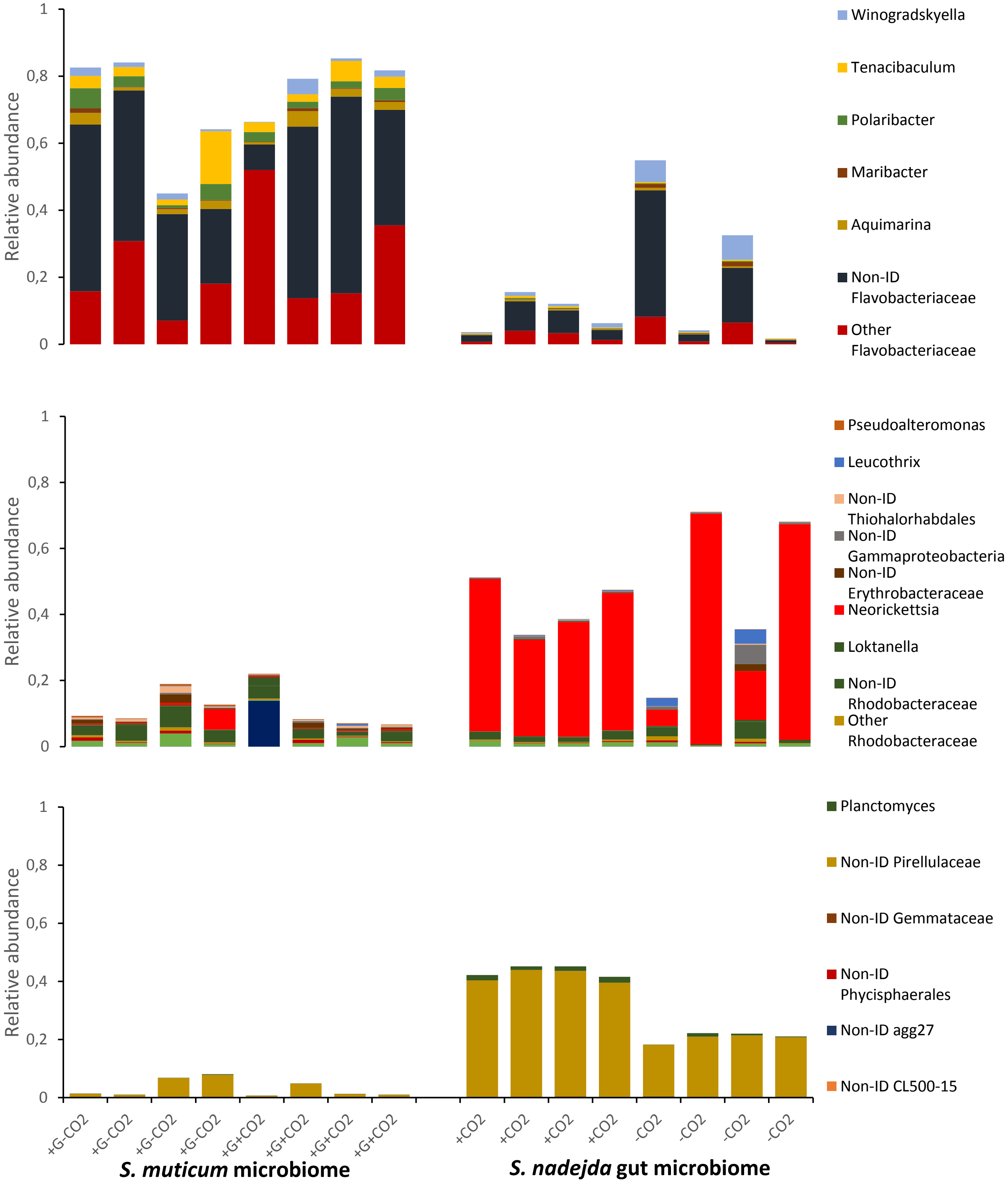


Figure 4.3 Relative abundance of the bacterial phyla *Bacteroidetes* (top), *Proteobacteria* (middle), and *Planctomycetes* (bottom) associated to the brown seaweed *Sargassum muticum* with (+G) *Synisoma nadejda* isopods present (left side), and the gut microbiome of the isopod on a *Sargassum muticum* diet (right side), after three weeks under ambient (380ppm; -CO2) and elevated (1000ppm; +CO2) CO2 conditions.

Several bacterial groups were significantly more abundant within *S. muticum* in the presence of grazers than in the isopod gut: *Flavobacteriales* from the phylum *Bacteroidetes* (2-way ANOVA, F=49.124, P<0.001); *Bdellovibrionales* from the class *Deltaproteobacteria* (2-way ANOVA, F=13.221, P=0.003); as well as *Acidithiobacillales* (2-way ANOVA, F=22.589, P<0.001), *Alteromonadales* (2-way ANOVA, F=15.285, P=0.002), and *Oceanospirillales* (2-way ANOVA, F=6.407, P=0.026) from the class *Gammaproteobacteria* (Fig. 4.4). In contrast, the gut microbiome had higher abundances of *Rickettsiales* from the class *Alphaproteobacteria* (2-way ANOVA, F=18.574, P=0.001); the low abundance bacteria from the phylum *TM6* – class *SJA-4* (2-way ANOVA, F=12.491, P=0.004); and the low abundance order *AKAU3564-Phycisphaerae* from the phylum *Planctomycetes* (2-way ANOVA, F=7.325, P=0.019) than within *S. muticum* in the presence of grazer (Fig. 4.4).

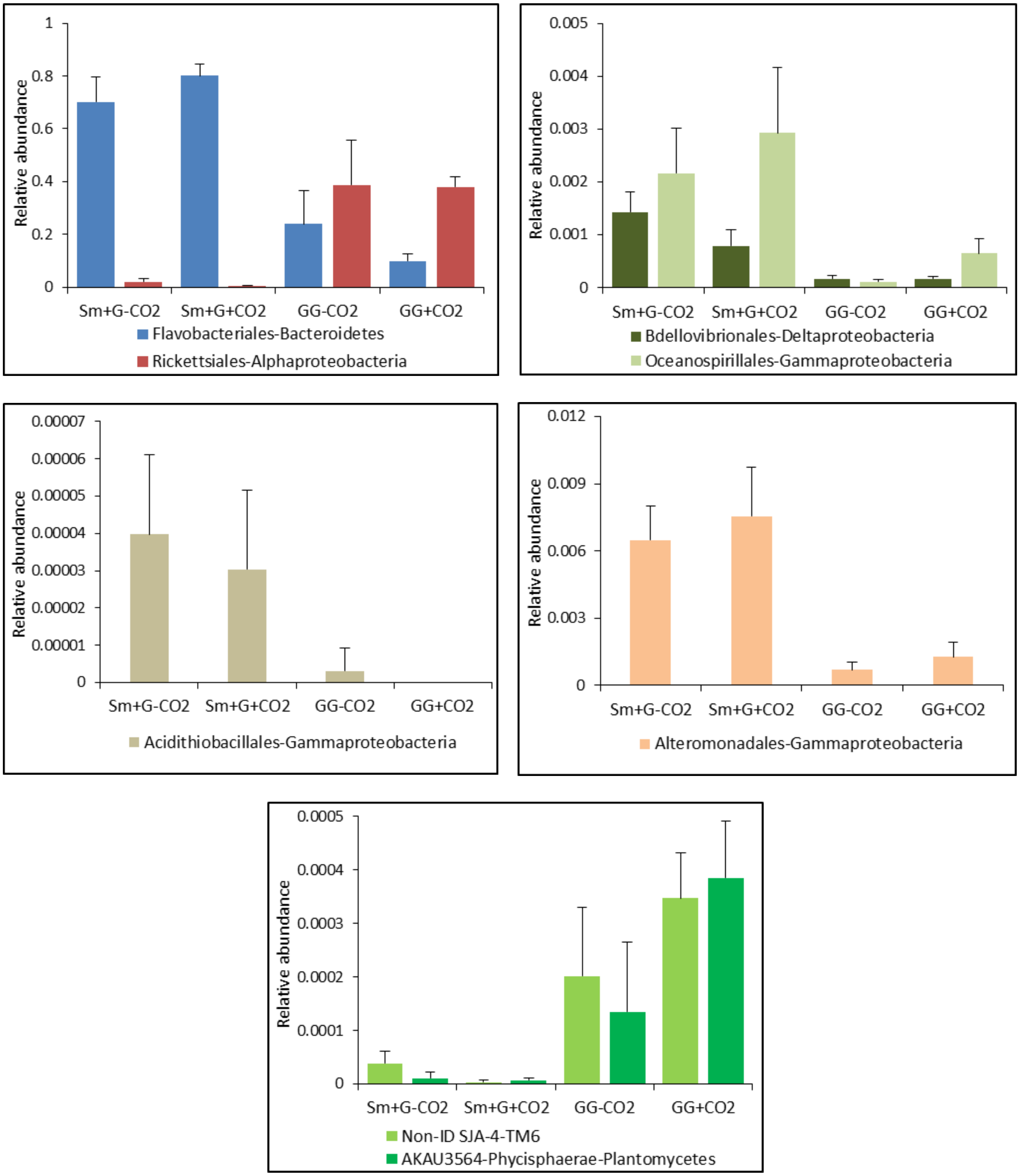


Figure 4.4 Mean relative abundances of bacterial classes, and respective orders, significantly more abundant in either *Sargassum muticum* (Sm) or the gut of *Synisoma nadejda* (GG), after three weeks under ambient (380ppm; -CO2) and elevated (1000ppm; +CO2) CO2 conditions. Alpha = 0.05, error bars show standard error per treatment (n=4).

Bacterial diversity, structure and composition under acidification conditions

Acidification treatment did not affect overall microbial structure (PERMANOVA, p=0.711) and composition (PERMANOVA, p=0.584) associated with *S. muticum*, but significantly affected the structure (PERMANOVA, p=0.030) and composition (PERMANOVA, p=0.022) associated with the gut of *S. nadejda*. Under acidified conditions (and in the presence of grazers), the number of phyla in the seaweed-associated bacterial community dropped by 9 (from 26 to 17 (including one unidentified)), compared to the ambient conditions (One-way ANOVA, F=7.228, P=0.009). In contrast, the number of phyla in the grazer gut-associated bacterial community under acidification treatment increased by 5 (from 18 to 23 (including one unidentified)), compared to the ambient conditions (One-way ANOVA, F=1.923, P=0.171). The phyla specific to the microbiomes of *S. muticum* and gut of *S. nadejda* are presented in Table 1 of the Supplementary Material.

Some bacterial groups were unique to its hosts under specific conditions (Fig. 4.5). The seaweed, in the presence of grazer and under the ambient conditions, had the highest number of unique bacterial genera (n=28, 5%), while the grazer gut in the ambient conditions had the lowest number of unique bacterial genera (n=2). *S. muticum* in the presence of grazer and under the ambient conditions contained a large part of genera belonging to *Planctomycetes* (n=3, 24.8%), *Proteobacteria* (n=9, 19.8%) and *Bacteroidetes* (n=9, 19.5%). *S. muticum* in the presence of grazer, but under acidification treatment, contained 13 unique bacterial genera, most of which belonged to *Proteobacteria* (n=7, 74.9%). *Sphingobacterium* (*Bacteroidetes*) and *Caldicoprobacter* (*Firmicutes*) were unique to the gut of *S. nadejda* in the ambient conditions, compared to 23 genera unique to the grazer gut in the acidified conditions, a significant part of which belonged to *Planctomycetes* (n=2, 50.7%) and *Proteobacteria* (n=8, 15.8%).

A core microbial community (present in at least 75% of samples) was composed of 181 bacterial genera (32.1%; including unidentified) (Fig. 4.5). Within this core bacterial community, the highest number of genera belonged to *Bacteroidetes* (n=29; 55.3%), *Proteobacteria* (n=90; 26.7%) and *Planctomycetes* (n=7; 13.2%). Shared microbial communities within *S. muticum* in the presence of grazer between the ambient and acidified conditions (n=29; 5.2%) had the highest number of bacterial genera belonging to *Proteobacteria* (n=14; 47.2%), *Firmicutes* (n=8, 19.9%), and *Bacteroidetes* (n=5; 18.5%). Shared bacterial communities in the gut of *S. nadejda* between the ambient and acidified conditions (n=9; 1.6%), belonged mostly to *Planctomycetes* (n=3; 54.8%) and *Proteobacteria* (n=4, 32.3%) (Fig. 4.5).

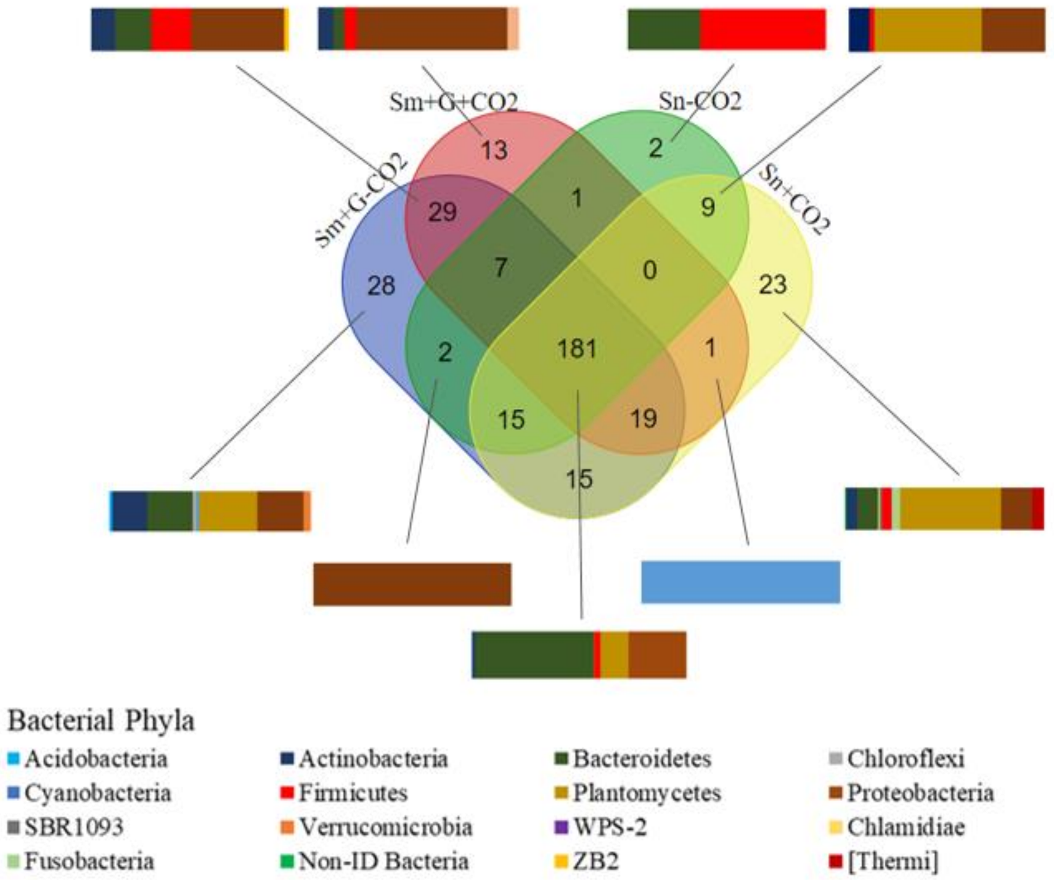
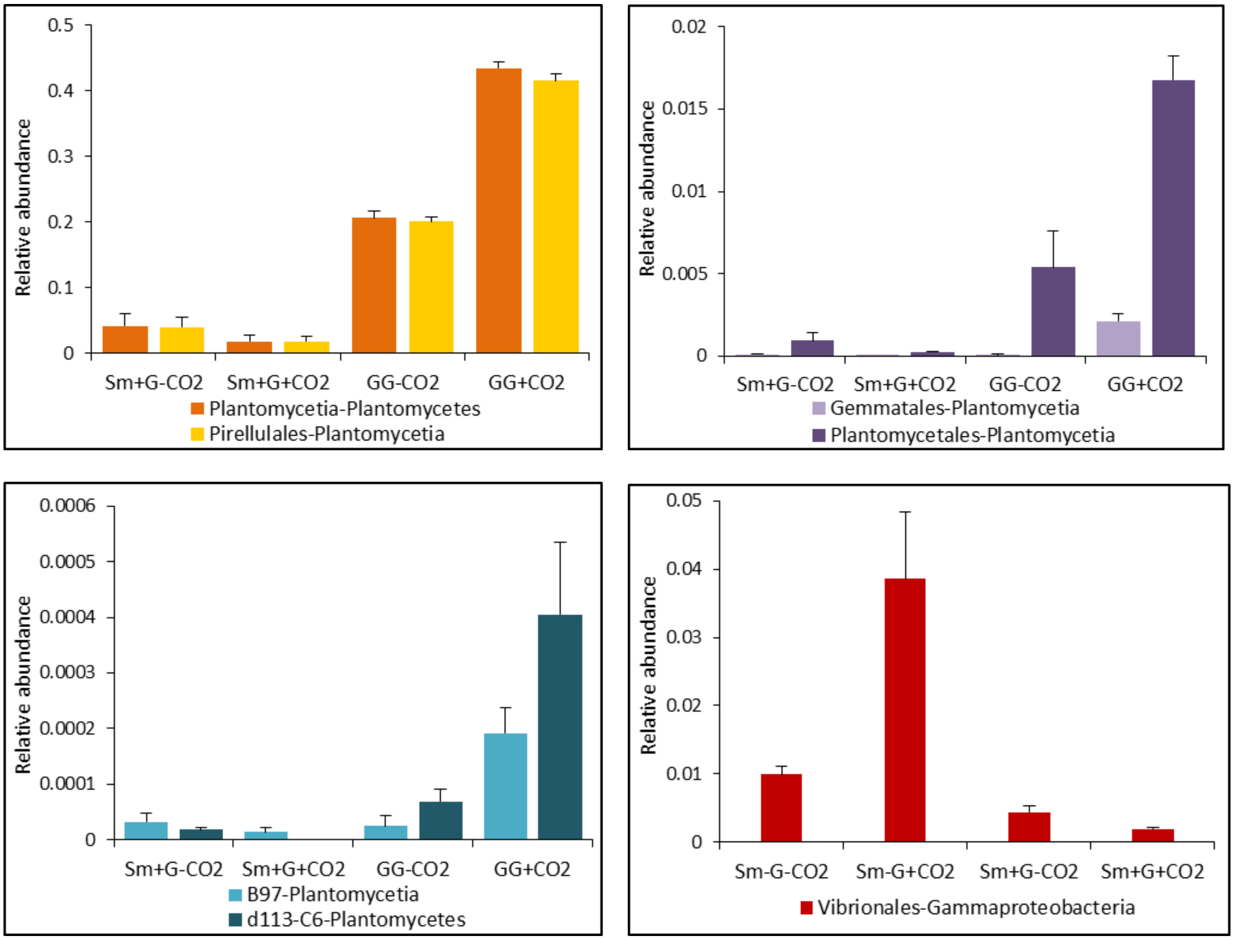


Figure 4.5 Venn diagram representing the number of bacterial genera (present in at least 75% of samples) shared between the different CO2 treatments and associated to *Sargassum muticum (Sm)* with (+G) *Synisoma nadejda* isopods, and the gut microbiome of the isopod on a *Sargassum muticum* diet. The bar graphs show the distribution of Phyla of selected intersections.

Under elevated CO2 treatment relative abundances of *Gammaproteobacteria* from the orders *Oceanospirillales* and *Vibrionales*, particularly *Pseudoalteromonas,* increased on *S. muticum* but only significantly in the absence of grazers (P=0.037 and P=0.047, respectively; Fig. 4.6).

The class *Planctomycetia* at elevated CO2 showed a significantly increased abundance (Fig. 4.6). Under the acidification treatment, the abundance of *Planctomycetia* associated with the grazer gut was significantly higher than at the ambient CO2 levels (Two-tailed t-test, P<0.001), as was their abundance (at elevated CO2) in the grazer gut than on *S. muticum* (Two-tailed t-test, P<0.001). Deeper analyses of bacterial abundance within *Planctomycetia* revealed that all bacterial orders detected within this class responded to acidification treatment (Fig. 4.6). A significant interaction was confirmed within the grazer gut under different CO2 conditions and between the seaweed and the grazer gut under increased CO2. At elevated CO2 levels, the abundance of *B97* (Two-tailed t-test, P=0.031), *Gemmatales* (Two-tailed t-test, P=0.009), *Pirellulales* (Two-tailed t-test, P<0.001) and *Planctomycetales* (Two-tailed t-test, P=0.008) were increased in the grazer gut. Alongside, under the acidification treatment, the abundance of the bacterial orders *B97* (Two-tailed t-test, P=0.010), *Gemmatales* (Two-tailed t-test, P=0.008), *Pirellulales* (Two-tailed t-test, P<0.001) and *Planctomycetales* (Two-tailed t-test, P<0.001) was significantly higher in the grazer gut than in/on the seaweed in the presence of grazers. The class *C6-Planctomycetes* also exhibited a significant increase in abundance under elevated CO2 conditions, but only within the grazer gut (Two-tailed t-test, P=0.039) (Fig. 4.6). This was mostly due to the significant increase of bacteria from the order *d113* (Two-tailed t-test, P=0.039) (Fig. 4.6). The abundance of *d113* under acidified conditions was significantly higher in the grazer gut than within *S. muticum* (Two-tailed t-test, P=0.018) (Fig. 4.6). Overall, under acidification treatment, the abundance of *Planctomycetes* in the grazer gut increased from 20.9% to 43.8% (1-way ANOVA, F=306.663, P<0.001).



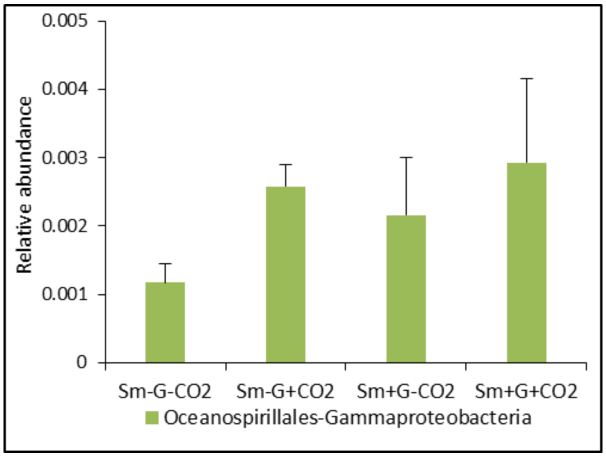


Figure 4.6 Mean relative abundances of bacterial orders associated with *Sargassum muticum* (Sm) and the gut of *Synisoma nadejda* (GG) under ambient (380ppm; -CO2) and elevated (1000ppm; +CO2) CO2 conditions that responded to acidification. Alpha = 0.05, error bars show standard error per treatment (n=4).

4.5 Discussion

The results presented in this study demonstrated that acidification affected specific bacterial groups but hardly influenced the overall microbiome of the invasive brown seaweed *S. muticum*, while causing substantial changes in the gutmicrobiome of a native isopod consumer *S. nadejda* after three weeks of exposure to elevated pCO2 treatment. Interestingly, acidification increased abundances of *Planctomycetia* in the gut of *S. nadejda* and of *Oceanospirillales* and *Vibrionales* associated to *S. muticum*, raising hypotheses about their functional role in these conditions.

The guts of isopods have been shown to be populated by symbiotic bacteria (e.g. Eberl, 2010; Fraune & Zimmer, 2008; Wang et al., 2007; Zimmer & Bartholmé, 2003) that assist in utilization of food by the host (Zimmer 2002; Zimmer et al., 2002; Fraune & Zimmer 2008; Zimmer & Bartholmé, 2003). Because the diversity of gut bacterial communities is shaped by host diet (Titjen, 2014), changes in seaweed characteristics and its microbiome, as a result of ocean acidification, can affect the gut microbiome of the grazer with possible fitness consequences. Here, we documented that the most striking change resulting from acidification was a significantly increased abundance of *Planctomycetes* in the gut of *S. nadejda*, more specifically due to an increase of Non-ID *Pirellulaceae.* Although the role of *Pirellulaceae* is hardly known, its presence was documented in the gastrointestinal tract of fish (Parris et al., 2016) and the microbiomes of marine copepods (Moisander et al., 2015). *Planctomycetes* constituted the second most prevalent phylum (after *Proteobacteria*) in the gut of *S. nadejda*. They are known to colonize aquatic and terrestrial ecosystems (Lage & Bondoso, 2014), and are common in anoxic zones of the ocean (Kirkpatrick et al., 2006, Kuypers et al., 2003, Woebken et al., 2008). *Planctomycetes* are frequently reported in marine taxa (Singh & Reddy, 2014) and widespread in the associated bacterial communities of macroalgae (Lage & Bondoso, 2014), among which is *Caulerpa taxifolia* (Meusnier et al., 2001) and the brown kelp seaweed *Laminaria hyperborea* (Bengtsson & Ovreas 2010). Here, it was the third most abundant bacterial phylum associated with *S. muticum*. Bacteria from the phylum *Planctomycetes* are suggested to have potential benefits for its hosts, thanks to their ability to mineralize organic molecules into inorganic compounds that match the nutritional requirements of macroalgae (Lage & Bondoso, 2014). *Planctomycetes* are also proposed to function as "slow-acting decomposers of organic matter" (i.e. algal polymer degradation; Bodelier & Dedysh, 2013) and important contributors to the global nitrogen cycle (i.e. anammox *Planctomycetes*, Fuerst & Sagulenko 2011). In this study, acidification increased the abundance of *Planctomycetes* strongly in the bacterial gut communities of *S. nadejda* compared to their food source (*S. muticum*). An increased *Planctomycetes* abundance could provide the isopod with an elevated algal digestion and inorganic compounds availability to compensate the shifted C/N ratio under acidification in the food.

Another important change resulting from acidification was a significant increase in *Oceanospirillales* (Non-ID *Oceanospirillaceae*) and *Vibrionales* (*Pseudoalteromonas*) associated with *S. muticum* in the absence of grazers. They all belong to the Class of *Gammaproteobacteria* which have been previously found in association with various seaweed species including the red macroalgae *Amphiroa anceps* and *Corallina officinalis* (Huggett et al., 2006), and the green alga *Enteromorpha sp.* (Patel et al., 2003). Members of the class *Gammaproteobacteria* are known to produce biologically active metabolites that mediate antifungal (Barbieri et al., 2001), antifouling (i.e. *Alteromonas*, *Pseudomonas*; Holmström, Rittschof, & Kjelleberg, 1992; Holmström & Kjelleberg, 1999; Maki et al., 1988; Mary et al., 1993) and antibacterial activities (Hentschel et al., 2001). There are no studies documenting the effect of acidification on these bacteria in seaweeds. Moreover, the function of these bacteria was examined only in invertebrates and their effects on seaweeds remains to be investigated. Concerning specifically *Oceanospirillaceae* and *Vibrionaceae*, their increase has often been observed associated with stressed and diseased marine invertebrates; they are also known as coral pathogens (Bourne et al., 2008; Bourne & Munn, 2005; Sunagawa et al., 2009; Meron et al., 2011). Bacteria from the order *Oceanospirillales* are known to be heterotrophic and capable of degrading complex organic compounds (Garrity et al., 2005, Goffredi et al., 2007). They are also associated with carbon fixation and sulfur oxidation in corals (reviewed in Lawler et al. 2016). The abundance of *Oceanospirillales* was also associated with the collagenase activity in the deep-sea siboglinid polychaetes *Osedax (Goffredi et al., 2007).*

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Vibrionales are known as nitrogen fixers and were isolated from healthy and diseased corals (reviewed in Lawler et al. 2016). *Vibrionales* were also responsible for a number of infections in humans and animals (Vezzulli et al., 2016), and identified as potential pathogens of sablefish larvae (Schulze et al., 2006) and bivalve mollusks (Asplund et al., 2014). Interestingly, it has been shown that under low pH conditions, the coral-associated pathogen *Vibrio sp.* increased in abundance (Meron et al., 2011), while the blue mussel pathogen *Vibrio tubiashii* became more infectious (Asplund et al., 2014). In this study, among *Vibrionales* that experienced a significant increase were predominantly *Pseudoalteromonas.* While certain members of the genus *Pseudoalteromonas* were reported to have antibacterial activity in corals, providing it with defense against potential pathogens (Shnit-Orland et al., 2012), multiple studies identify *Pseudoalteromonas* as opportunistically pathogenic to marine organisms (Liu et al., 2010, Wang et al., 2013, Song et al., 2012). Therefore, ocean acidification could potentially result in shifts from healthy-associated microbial communities within seaweeds towards a high prevalence of pathogenic bacteria and increased vulnerability to disease. Still, the vulnerability of seaweeds facing these bacteria remains unknown.

Among bacteria that were significantly more abundant within *S. muticum* than in the isopod gut were *Flavobacteriales* from the *Bacteroidetes* phylum. *Bacteroidetes* are known to widely colonize marine and freshwater environments (Thomas et al., 2011), populating a wide variety of surfaces, including macroalgae (Beleneva & Zhukova, 2005; Staufenberger et al., 2008) and marine sediments (Devine et al., 2012). *Bacteroidetes* were isolated from *Caulerpa taxifolia* (Meusnier et al., 2001), *Ulva australis* and the red alga *Delisea pulchra* (Longford et al., 2007), suggesting that these typical marine bacteria are common seaweed associates (Tujula et al., 2010). *Bacteroidetes* represent some of the most abundant marine bacteria (Glöckner, Fuchs, & Amann, 1999; Simon, Glockner, & Amann, 1999; Cottrell & Kirchman, 2000) that serve as degraders of complex organic matter (Church, 2008). *Flavobacteria*, the most prevalent class detected within the seaweed, are known as producers of enzymes for polymer degradation (Fenchel, 2012). Most *Flavobacteria* are able to degrade cellulose, chitin, proteins, and nucleic acids (Kirchman, 2002; Fenchel, 2012). However, *Bacteroidetes* are also related to stress conditions (such as pH) and were often found in diseased corals (Vega Thurber et al., 2009; Barneah et al., 2007). For instance, “exposure of the coral *Porites compressa* to high pCO2 (and so low pH) resulted in an increase of disease associated bacteria within the class *Flavobacteria*” (Vega Thurber et al., 2009).

Flavobacteriales, the most widely represented *Flavobacteria* order within *S. muticum*, are mostly associated with degradation of complex particle biomacromolecules, as well as algal debris (Kirchman, 2002). *Flavobacteriaceae* are known for degrading “proteins, agars, xylan, fucoidan, cellulose, and chitin” (Devine et al., 2012). Among the frequently occurred members (after Other and non-ID *Flavobacteriaceae*) were bacteria from the genera *Aquimarina* and *Tenacibaculum*, known for their presence as free-living bacteria in marine environments (Nedashkovskaya et al., 2006) and fixation to the surfaces of marine organisms (Suzuki et al., 2001). Bacteria from the genus *Tenacibaculum* are thought to induce morphogenesis in algae (Hanzawa et al., 1998; Matsuo et al., 2003) and enhanced seaweed growth could be related to these bacteria. In this study, acidification resulted in insignificant decrease of seaweed-associated *Flavobacteriales* (just in the absence of grazer) which contrasts with results of the study conducted on the natural biofilms from the Great Barrier Reef, which reported an increase in the relative abundance of *Flavobacteriales* (*Flavobacteriaceae*), with *Flavobacteriaeae* increasing with decreasing pH (Witt et al., 2011). As algae can function as vectors of coral pathogens (Nugues et al., 2004) increased acidification on reefs could potentially facilitate the shift to algal dominated reefs.

Because *Bacteroidetes* are associated with seaweeds, they are also found in the guts of seaweed grazers (Hong et al., 2011), where they have been shown to degrade the high molecular weight compounds (Tremaroli & Backhed, 2012). *Bacteroidetes* were also found to dominate the gut of the gastropod snail *Achatina fulica* (Cardoso et al., 2012), and to be present in the intestinal tracts of the limpet *Patella pellucida* (particularly *Flavobacteria*; Dudek et al., 2014) and terrestrial isopod *Armadillidium vulgare* (Moisander et al. 2015). *Bacteroidetes* (which are so abundant in the food) consumed by grazers putatively contribute to the degradation of brown algal polysaccharides in the gastrointestinal tract (Dudek et al., 2014). Despite being the most abundant in association with *S. muticum*, *Bacteroidetes* constituted only the third most abundant bacterial phylum in the intestinal tract of *S. nadejda*. This was quite unexpected considering that the diet regularly shapes the gut microbiota. Because *Bacteroidetes* prevailed in/on the seaweed, we would expect it to be the most abundant in the grazer gut as well, but this was not the case. In contrast, there was a large difference in the microbiome of the seaweed and the gut (which is usually not the case) and the 17% *Bacteroidetes* in the gut could be a remnant from the food. The lower abundance of *Bacteroides* in the grazer gut could potentially be driven by lesser need to hydrolyze algal polysaccharides. It could also be because the seaweed is a non-native; however, to our knowledge, such observation had never been made in non-native seaweeds or animals.

In contrast to *Bacteroidetes* and *Gammaproteobacteria* prevailing within *S. muticum*, *Alphaproteobacteria* (*Rickettsiales*), as well as bacteria from the phylum *TM6* (the class *SJA-4*) were more abundant in the grazer gut microbiome. *Proteobacteria* dominated (>80%) the gut microbiomes of molluscs *Haliotis cracherodii* and *H. sorenseni* (King et al., 2012), the limpet *Patella pellucida* (Dudek et al., 2014), and the sea slug *Elysia chlorotica* (Devine et al., 2012). *Alphaproteobacteria* were one of the associates significantly more abundant in marine copepods (Moisander et al., 2016). Bacteria from the order *Rickettsiales*, dominating the gut of *S. nadejda*, are known as pathogenic to humans and animals (Perlman, Hunter, & Zchori-Fein 2006), and were found in the intestinal tract of infected isopod *Armadillidium vulgare* (Dittmer et al., 2016). *Neorickettsia* is known as a parasite to which the invertebrates usually serve as vectors (Greiman et al. 2014, 2015). While the role of bacteria from the class *SJA-4* is unclear, it was reported to be a part of the microbiomes of sponges (Hardoim & Costa, 2014; Alex & Antunes 2015).

The results of this study demonstrated that addition of grazers did not result in significant changes in *S. muticum* microbial community structure and composition. Nevertheless, under acidified conditions, there was a lower abundance of seaweed-associated *Vibrionales* in the presence of grazer than in its absence. Although the simultaneous addition of CO2 and grazers resulted in the significant loss of the bacterial phyla, these changes were not significant in abundance, as they were observed among the less abundant members of the seaweed-associated bacterial community.

A word of caution concerning the results obtained in this study, is that controlled experiments are not able to fully reconstruct the dynamic conditions of nature. The aquarium conditions may contribute to stress and disturbance of the microbial communities, as was documented for a coral species, which had different associated microbial communities in laboratory compared to the field (Kooperman et al., 2007, Meron et al. 2012). Other effects that are not representative of nature may be present in the design and time scale of the experiments. First of all, pH and CO2 conditions used in the various studies are not always realistic and, in some cases, are less than those expected in the next 100 years (Meron et al. 2011, Vega Thurber et al., 2009). Second, while the effects of ocean acidification on marine organisms occur over long time scales, many laboratory studies are short-term and last only days or weeks (O'Brien et al. 2016). Third, the absence of data for comparison regarding the unfed/starving grazers limits our conclusions and interpretation of results.

The results of this study demonstrate that microbial communities associated with "aquarium-acclimated" seaweed and the grazer gut are dynamic and responsive to changes in CO2 and pH. The observed changes in the associated bacterial communities of the seaweed and the grazer gut might not be a negative consequence of stressful conditions but rather acclimation variations that facilitate tolerance and survival, as suggested by Meron et al. (2012). The capacity of the host to accommodate a highly diverse microbiota, accompanied by the development of a distinct combination of microbial communities, may influence the fitness and success of the host and enhance its ability to survive changing environmental conditions (Morrow et al., 2015). Further research is required to understand better the processes and conditions under which different symbiotic bacteria can increase tolerance of the host organisms to various disturbances. The sensitivity of associated microbial communities can serve as an early indicator of the host and ecosystem responses to ocean acidification (Webster et al., 2013).

4.6 Conclusion

The responses of bacteria associated with *S. muticum* revealed in this study suggest that worst case acidification scenarios, apparently, do not greatly affect bacterial community composition and diversity. This seems to have less significant impact on seaweed microbial ecology than other environmental stressors, such as increased temperature (Mensch et al. 2016). Acidification hardly influenced the overall microbiome of *S. muticum* but did increase the abundance of seaweed-associated *Oceanospirillales* and *Vibrionales,* as well as *Planctomycetia* in the gut of *S. nadejda*. The addition of a grazer did not affect microbial structure and composition of the seaweed microbiome. *Bacteroidetes*, *Proteobacteria,* and *Planctomycetes,* previously isolated from other seaweed species (Huggett et al., 2006; Lachnit et al., 2011; Burke et al., 2011), were the most dominant, suggesting that they account for core roles in the metabolisms of *S. muticum* and *S. nadejda*. The seaweed associated bacterial communities were dominated by *Flavobacteria* and *Alphaproteobacteria*, while the isopod gut communities were dominated by a parasite *Neorickettsia (Alphaproteobacteria)*, for which invertebrates are usually the vectors, as well as *Pirellulaceae (Planctomycetes),* which play a role in polymer degradation. *Pirellulaceae* was one of the bacterial groups that responded to CO2 enrichment, as their abundance strongly increased in the grazer gut. An increased *Planctomycetes* abundance could provide the isopod with an elevated algal digestion and inorganic compounds availability to compensate the shifted C/N ratio as seaweed becomes less nutritional under acidification. *Oceanosirilllales and Vibrionales,* which abundance significantly increased within *S. muticum,* may have negative consequences for the seaweed and other organisms as they can be considered pathogenic. *Bacteroidetes,* despite being important degraders of organic matter (dominant within the seaweed and strongly reduced in the grazer gut), were also related to stress conditions (such as pH) and often found in diseased corals (Vega Thurber et al., 2009; Barneah et al., 2007). As algae can function as vectors of coral pathogens (Nugues et al., 2004) increased acidification on reefs could potentially facilitate the shift to algal dominated reefs. Nevertheless, the abundances of these bacteria were not increased in the grazer gut and, as such, the grazer cannot function as a transport vector for these bacteria.

4.7 Acknowledgements

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4.9 Supplementary Material

Table S1 Bacterial phyla specific to hosts or / and specific conditions apart from those present in all treatments

Microbiome	**Seaweed**				**Isopod gut**	
	No grazer		**With grazer**			
	CO2-	**CO2+**	**CO2-**	**CO2+**	**CO2-**	**CO2+**
Gemmatimonadetes	+	+	+	+	-	+
Nitrospirae	+	+	+	+	-	+
Fusobacteria	+	+	+	-	+	+
Tenericutes	+	+	+	-	-	+
BRC1	+	+	+	-	-	-
Chlamydiae	-	+	+	-	+	+
OP3	+	-	+	-	-	+
Non-classified *BH180-139*	+	+	-	-	-	-
Caldithrix	+	-	+	-	-	-
Fibrobacteres	+	-	+	-	-	-
Non-classified *NKB19*	+	-	-	-	-	-
SAR406	+	-	-	-	-	-
GNO2	-	-	-	-	-	+
WS3	-	-	+	-	-	+
Other bacteria	+	-	+	+	+	-
Non-ID bacteria	+	-	+	-	+	+

Chapter 5

Seasonal variation of bacterial communities associated with invasive brown seaweed *Sargassum muticum*.

Submitted to PLOS ONE

5. Seasonal variation of bacterial communities associated with invasive brown seaweed *Sargassum muticum.*

Alexandra Serebryakova, Tania Aires, Aschwin H. Engelen, Frederique Viard, Ester A. Serrão

5.1 Abstract

Seaweed-associated microbiota is known to experience spatial and temporal shifts in response to changing environmental conditions and seaweed physiology. In particular, seasonal shifts are thought to be characterized by specific winter and summer bacterial communities. Shifts in microbial abundance and composition, in response to changing environmental parameters, may result in structural, functional and behavioral changes within the host with potential consequences for its fitness, as well as represent an important tool for adaptation to changing environmental conditions. Despite this, the current knowledge of seasonal variation of seaweed-associated microbiota is still quite limited. In this study, we explored the seasonal variation of bacterial communities associated with the invasive brown seaweed *S. muticum* (across different tissue types) in northern and southern Portugal, in September, March and July-August (summer). The microbiome diversity and composition were determined using next generation sequencing of the variable regions V5-7 of the 16S rDNA. We anticipated that seaweed-associated microbial communities would be more similar at the same season across different geographic locations within Portugal than at different seasons in the same location. We also expected to observe a clear successional pattern in the seaweed-associated bacterial communities over time with gradual addition of pathogenic bacteria towards the end of the annual life cycle. Interestingly, the study revealed notable shifts in *S. muticum* microbiome between the seasons and geographic locations with significant abundance of Non-ID *Rhodobacteraceae* and *Loktanella* in September-March and significant prevalence of Non-ID *Pirellulales* in July-August. In the North of Portugal and on basal parts, bacterial diversity was higher than in the South and on apical parts. At both locations, seasonal differences were most pronounced in the apical tips and sediments, but the bacteria contributing to these differences differed between locations. Small and large scale spatial differences in associated bacterial communities were most pronounced in summer and apical tissues. Holdfasts were characterized by the highest bacterial diversity among all algal tissues examined, after the sediments. All these changes have the potential to affect seaweed ecology, fitness and adaptation capacity to environmental changes.

Keywords: associated bacterial communities, seasonal variation, *Sargassum muticum*

5.2 Introduction

Seaweeds live in association with abundant and diverse microbiota, which plays an important role in the life of its hosts and is dynamic in nature [1, 2, 3]. Seaweeds rely on associated microbial communities for diverse functions including morphological development [4, 5, 6, 7, 8], consumption of organic matter and nitrogen source [9], defense [10, 11, 12, 13, 14, 15, 16], or provision of vitamins [17]. Microbial host-specificity has been reported (e.g. in fucoid seaweed *Phyllospora comosa* [18]) but most of the studies document important changes of the microbial communities associated to seaweeds in response to various factors and conditions [11, 18].

Structure and composition of the seaweed-associated microbiota are known to change with the host conditions, as well as in space and time [11, 18, 19, 20, 21, 22, 23, 24, 25]. Evidence exists that seaweed-associated microbiota can change depending on host condition [11, 26, 27]. For instance, healthy vs. stressed *Ecklonia radiata* exhibit different microbial communities [28]. This suggests that host traits may be critical in determining the community structure of associated microbiota. Other studies documented changes across locations [29]. For instance, high within-species variability was observed between microbial communities associated with specimens of *Ulva australis* from different rock pools [30]. However, most studies document shifts in microbial communities over different time scales, varying from short-term (i.e. less than a day) to long-term (i.e. inter-annual) with seasonal variability (even with several years' time interval) pointed out as the strongest variability [21, 31]. Some bacterial taxa seem more representative of a given season than others [19, 21, 22, 25]. For instance, summer bacterial communities of *Fucus vesiculosus* were dominated by *Alphaproteobacteria*, *Bacteroidetes*, *Verrucomicrobia* and *Cyanobacteria*, while winter bacterial communities were dominated by *Gammaproteobacteria* with an entire absence of *Cyanobacteria* [21]. Persistent seasonal variation of bacterial communities associated with *Fucus vesiculosus* was revealed over two consecutive years at the phyla level [21]. Recent research conducted on the Mediterranean seaweed *Cystoseira compressa* also revealed its associated microbiota to be temporally variable [22]. Bacterial communities associated with thalli of *C. compressa* displayed a clear successional pattern over time as well as an increase in abundance of pathogenic bacteria, associated with natural degradation of thalli at the end of the annual life cycle [22].

Seasonal variations are thought to be related to the combined effect of biotic (i.e. seaweed growth cycle, the age of the algal tissue) and abiotic factors (i.e. seawater temperature) [19, 22, 25]. For instance, *Planctomycetes*, *Verrucomicrobia* and *Alphaproteobacteria* were detected among the early colonizers of young thalli in *Laminaria hyperborea* [19]. As the algal tissue aged and biofilms matured, communities associated with *L. hyperborea* were complemented by *Gammaproteobacteria, Bacteroidetes*, and *Cyanobacteria* [19]. Other biotic factors include interactions (including competition) among bacterial taxa (i.e. both internal and from the surrounding water; [19]), consequences of biological interactions with organisms from other trophic levels (i.e. grazing, cross-feeding) as well as the loss of certain functions by bacteria (which results in the dependence on services provided by other microorganisms, [32]). Concerning abiotic factors, seasonal shifts in microbial community composition generally mirror seasonal environmental changes [31]. In particular, the summer increase of water temperature is likely to modify bacterial community composition [19, 22]. During summer, microbial communities on *L. hyperborea* were for instance characterized by a high diversity and such conditions were thought to be beneficial for seaweed-associated bacteria populating algal exudates [19]. Some bacteria, such as *Betaproteobacteria* and *Verrucomicrobia* associated with *L. hyperborea,* were observed only in specific months when the seawater temperature was below 10°C, suggesting a preference for colder seawater temperatures [19]. Climate change and ocean acidification are also expected to intensify these changes [33, 34, 35].

Apart from the seasonality, host-associated microbiota is documented to be tissue-specific [24]. While microbial tissue-specificity was studied previously on for example corals [36], the number of studies conducted on seaweeds is very limited, but suggest that different seaweed tissues are populated by specific bacteria [24]. For instance, *Laminaria saccharina* had specific bacteria within its young and undisturbed tissues regardless of seasonality or geographic location [24]. Among other marine organisms, bacterial communities associated with different compartments of the coral *Porites lutea* (i.e. mucus, tissue and skeleton) showed seasonal variation, with dissolved oxygen and rainfall thought to be the most influential environmental factors [36]. It still remains to be explored to what extent tissue differentiation is affected seasonally. In addition, although many studies explored the seasonal differences of seaweed-associated microbiota and surrounding water column [19, 21, 22, 30, 37], no studies looked at similarities between the seaweed microbiome and surrounding water over time and, more importantly, at tissue specificity.

Microbiota can play an important role in increasing fitness and resilience of its hosts [2, 38]. It is able to respond faster to external stressors compared to its host. Consequently, seaweed-bacterial interactions and changes in seaweed-associated microbiota are particularly relevant to investigate in invasive seaweeds: microbiota might play a key role in the acclimatization of non-native seaweeds to environmental stressors and novel conditions, and finally enhance host invasiveness [39, 40, 41, 42, 43]. The present study was conducted to address the above mentioned limitations using the invasive brown seaweed *Sargassum muticum* as a case study. This brown alga is one of the most invasive macroalgae in the northern hemisphere, but its invasive success is not yet entirely understood [44]. Next-generation sequencing of the variable regions V5-V7 of bacterial 16S rDNA genes was applied to characterize the diversity of associated microbiota and describe changes in bacterial composition.

We hypothesized that bacterial communities associated to *Sargassum muticum* vary seasonally, but the extent of such variation depends on location and tissue. As detailed before, several studies indeed showed that bacterial seasonal variations are driven by environmental factors, notably seawater temperature. Seawater temperature data from Southern to Northern Portugal clearly demonstrate that there is a large variation in temperature between seasons in the north as compared to the south. We thus hypothesized that composition of microbial communities associated to *S. muticum* (including the water and sediment samples) would be more similar between samples taken in the same season but at different geographic locations within Portugal than between samples taken in different seasons at the same location. We also expected that associated bacteria vary between tissues and that such differences in microbiomes among different algal tissues would change depending on season. If sediments show more seasonal variation, we would expect that the lower tissues of *S. muticum* would also be more variable if they are strongly influenced by sedimental bacteria. Finally, we hypothesized that even though microbial communities associated with the seaweed would be different from those in the surrounding water column, there would be similarities between the seaweed and seawater microbiomes over time. In particular, we explored whether seasonal changes in seawater and sediment are similar to the changes across the tissues of *S. muticum*.

5.3 Methodology

Study area and samples collection

The samples were collected in Praia Norte (Viana do Castelo) and Praia do Queimado (Porto Covo) in September 2013, as well as in March, July (Porto Covo) & August (Viana do Castelo) 2014. The tissues collected constituted: the tip, basal blades and holdfast, as well as reproductive structures (receptacles) collected in July in Porto Covo and in September & August in Viana do Castelo. Each tissue was sampled in triplicate at each location and month. In addition, sediments and water samples were collected. The sediments contained three replicates in each month apart from the September in Viana do Castelo, which contained only 2 replicates. The seawater samples also generally had three replicates, apart from September (in both locations), which had 2 replicates, and August in Viana do Castelo, which had one replicate only. In the field, seawater microbiome was sampled by filtering 0.5 L seawater over a 0.2 μm filter. All samples, seaweed and environmental, were preserved in the field directly up on collection in Xpedition lysis buffer (ZymeResearch, California, USA). Overall, 94 samples were collected across both locations: 47 from Porto Covo and 47 from Viana do Castelo.

Hight-throughput sequencing of the microbiome

Epiphytic and endophytic bacteria were extracted from differentiated structures (holdfast, basal blades, tip blades & reproductive structures) of *S. muticum* using MoBio PowerSoil® DNA Isolation Kit in accordance with the manufacturer protocol. Variable regions V5-V7 of bacterial 16S rDNA genes were PCR-amplified using the primer pair 799F& 1193R which avoid chloroplast cross amplification [45]. Following the initial denaturation at 95 ºC for 2min, conditions constituted 35 cycles of denaturation at 95 ºC for 20s, annealing at 55 ºC for 20s, and extension at 72 ºC for 90s. The final extension was at 72 ºC for 3min. The 25 µl reaction mixture contained 250 µM dNTPs, 0.6 µM of each primer, 1 × 2PCR buffer mix, 2 µl of template DNA (with a final concentration of about 10 ng µl–1), and 0.3 µl of Taq polymerase (Advantage R2 Clontech). PCR products were cleaned using the ExoFastAP enzyme according to the manufacturer protocol (Thermo Scientific™) and amplified DNA was applied to the Molecular Research (MR DNA), Shallowater, Texas where a nested–PCR was implemented before the sequencing. Modified 8 bp key–tagged primer 799F along with the reverse primer 1193R (fragment ~ 400 bp), which avoid chloroplast cross amplification [46], were applied and PCR conditions constituted: 95 °C for 3 min, 10 cycles of 95 °C for 20 s, 50 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 3 min. Samples were pooled together in equal proportions based on their molecular weight and DNA concentrations and purified using calibrated Ampure XP beads. DNA libraries were prepared applying the Illumina TruSeq DNA library preparation protocol and paired–end (2 x 250 bp) sequencing performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines.

Sequence analysis and bioinformatics

The microbial community analysis was implemented using the Quantitative Insights into Microbial Ecology (QIIME version 1.8.0) program [47]. Sequences were screened and filtered for a minimum read length of 350 bp and less than 2 undetermined nucleotides. The filtered dataset, comprising only high quality sequences, was applied to a conservative chimera detection filter using the ChimeraSlayer method [48]. Selected high quality chimera-free sequences were clustered into Operational Taxonomic Units (OTUs) within reads using the UCLUST algorithm [49] with a pairwise identity threshold equaling 0.97. Representative sequences for each OTU were selected using the "most-abundant" method and OTU sequence alignment was implemented with the Pynast tool [47]. The Ribosomal Database Project (RDP) classifier [50] was applied for taxonomic assignment with a 95 % confidence threshold. To assign each OTU to the closest matching described taxon, the search was performed against the Greengenes reference database (version 12_10) [51] with a maximum e-value to record an assignment of 0.001. Sequences with the best match for eukaryotes (i.e. chloroplasts & mitochondria), rare OTUs (i.e. singletons & doubletons), and unassigned sequences were removed from the OTU table in the downstream analysis. The filtered rarefied OTU table was applied to calculate alpha diversity statistics, including the Chao I richness estimates [52], the observed number of species, and the Shannon index, using the QUIIME software. Permutational multivariate analysis of variance (PERMANOVA) was conducted to compare the microbiomes of *S. muticum* across seasons and tissues. To visualize dissimilarity between samples, Canonical Analysis of Principal coordinates (CAP) plots were constructed using the PRIMER 6 software [53, 54]. To determine the contributions bacteria to the similarity and dissimilarity between microbial communities in different months and tissues, SIMPER analysis was implemented using the PRIMER-E [54]. For bacterial groups, which showed notable differences in abundance between seasons, two-way analyses of variance (ANOVA) were implemented (with the preliminary tests for normality and homogeneity of variances being implemented).

5.4 Results

In the end, 2719 high quality sequences were used for each sample. Overall, the diversity was 1.2 times higher in Northern compared to Southern Portugal when expressed as Chao1 (PERMANOVA, F=11.875, P=0.001) and OTU Richness (PERMANOVA, F=13.935, P=0.001), except for Chao1 in March and all months for the Shannon index (Fig. 5.1). Significant differences between tissues were revealed for Chao1 (PERMANOVA, F=14.540, P=0.001), OTU Richness (PERMANOVA, F=17.965, P=0.001), and Shannon index (PERMANOVA, F=3.339, P=0.009) (Fig. 5.1). However, a significant interaction was observed between the month and tissue for OTU Richness (PERMANOVA, F=2.139, P=0.035). Overall, holdfasts were characterized by the highest bacterial diversity among all algal tissues examined, after the sediments (Fig. 5.1). Bacterial diversity tended to decrease towards the apical seaweed tissues with the lowest diversity associated to the tips (Fig 5.1).

Overall, 794 bacterial genera were distributed across 137 classes from 52 phyla. In Porto Covo, 623 genera were distributed across 104 classes from 40 phyla, while in Viana do Castelo 682 genera were distributed across 124 classes from 52 phyla. September-March bacterial communities associated to *S. muticum* were dominated by *Proteobacteria* (60.5%; *Alpha* 36%, *Gamma* 17.9% & *Delta* 4.9%), *Bacteroidetes* (29.9%; *Flavobacteriia* 21.1% & *Saprospirae* 4.7%), and *Actinobacteria* (7.3%; *Acidimicrobiia* 7.2%) (Fig. 5.2). In contrast, summer (July-August) bacterial communities were dominated by *Proteobacteria* (40%; *Alpha* 18.3% & *Gamma* 17.5%), *Bacteroidetes* (31.4%; *Flavobacteriia* 15.5% & *Saprospirae* 11.7%), *Planctomycetes* (14.5%; *Plantomycetia* 11.4%) and *Spirochaetes* (6.5%; *Spirochaetes* 6.5%) (Fig. 5.2).

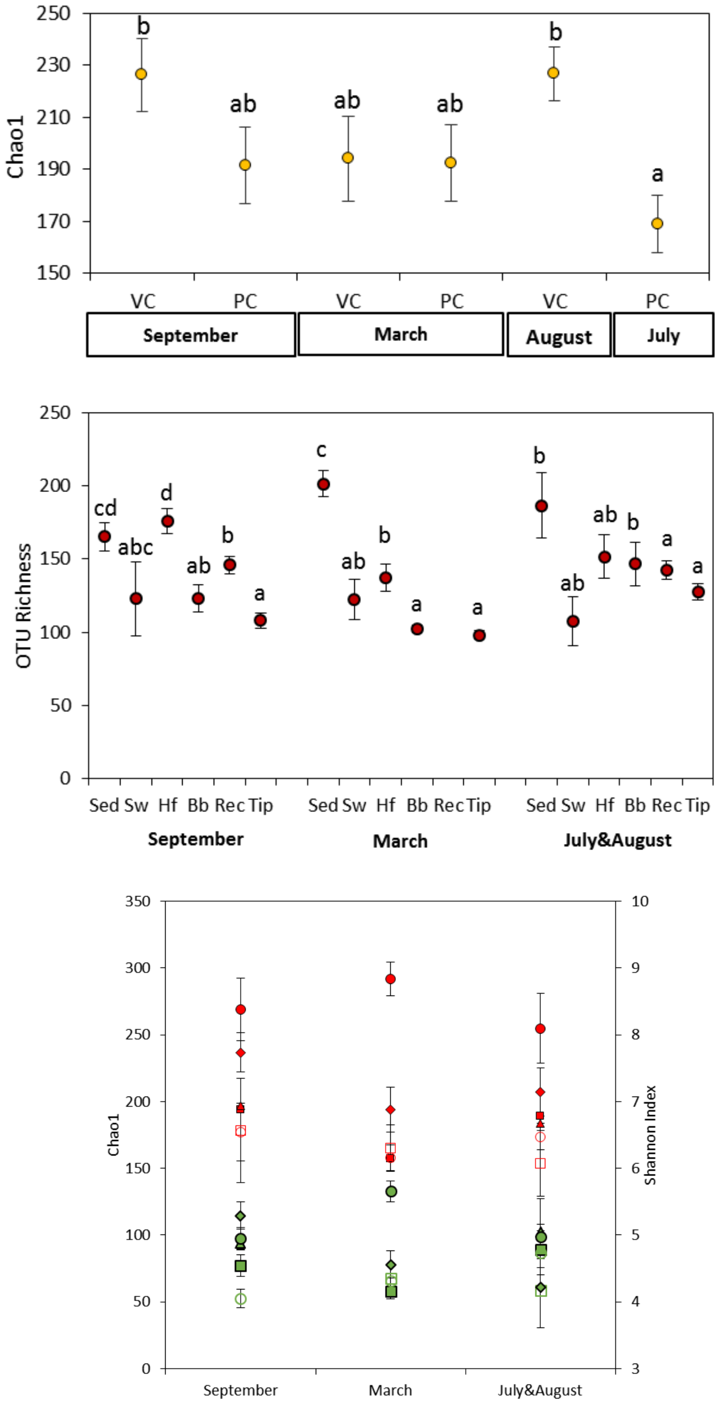


Figure 5.1 Chao 1 (top and bottom) and OTU richness (middle) alpha diversity measures of bacterial communities associated to *S. muticum* across seasons at its northern (Viana do Castelo) and southern distribution in Portugal (Porto Covo). Differentiated are sediments (color circle), seawater (empty square), holdfast (rombe), basal blades (color square), receptacles (color triangle), and tip (empty circle). Values are means ± standard error (n=3).

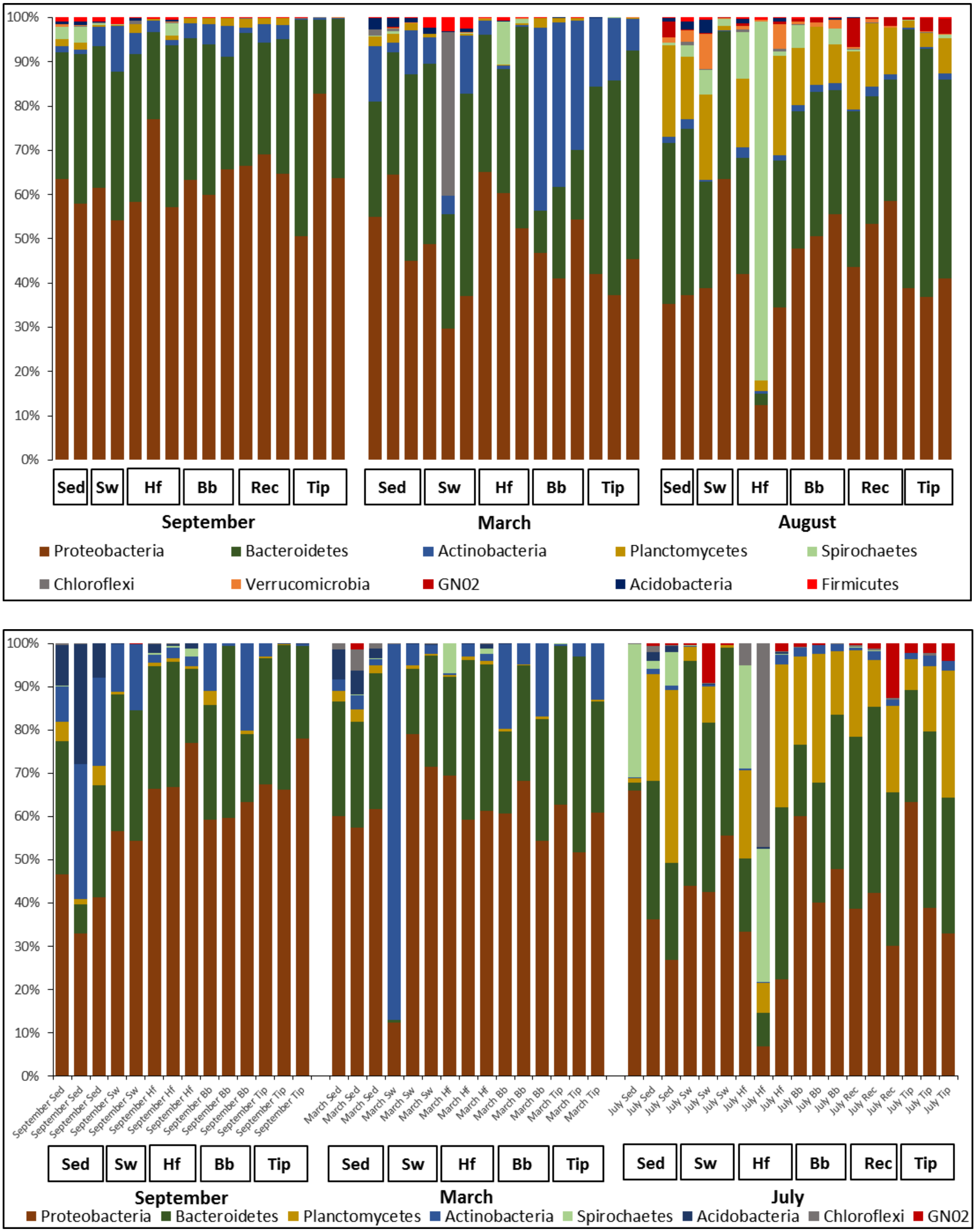


Figure 5.2 Relative distribution of the bacteria phyla associated to the brown seaweed *Sargassum muticum*, sediments and surrounding seawater in Viana do Castelo (top) and Porto Covo (bottom).

The PERMANOVA analyses revealed significant structural differences in bacterial communities associated to *S. muticum*, sediments and seawater between September-March and July-August (PERMANOVA, p=0.006) (Fig. 5.3).

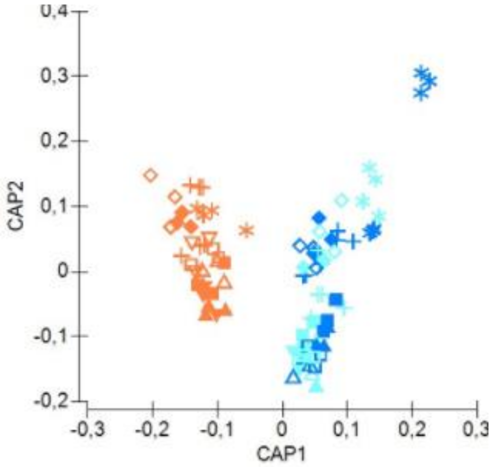


Figure 5.3 Canonical Analysis of Principal coordinates of the bacterial communities associated to *Sargassum muticum*, sediments and seawater samples collected in Porto Covo and Viana do Castelo in September (light green), March (blue) and July-August (orange) (PERMANOVA, p=0.006).

The most significant changes in summer compared to March and September constituted a decrease in *Proteobacteria* by 20.5% and *Actinobacteria* by 7.4%, as well as an increase in *Planctomycetes* by 13.8% (Fig. 5.2). The decrease in *Proteobacteria* in summer, compared to September, occurred mostly due to the significant decline in abundance of Non-ID *Rhodobacteraceae* (within the tip, receptacles & basal blades) and *Loktanella* (within the tip, receptacles, basal blades and holdfast) both belonging to the order *Rhodobacterales* (Table 5.1, Fig. 5.4). Significantly increased abundance of *Planctomycetes* in summer, compared to September, occurred mostly due to an increase of Non-ID *Pirellulaceae* within the receptacles, basal blades and holdfast (Table 5.1; Fig. 5.4).

Table 5.1 Bacteria that exhibited significant differences (Two-way ANOVA) in abundance between September and summer within *S. muticum* tissues.

Bacteria	**Tissue**	**F**	**P**
Non-ID Rhodobacteraceae	Basal blades	18.845	0.002
	Receptacles	15.441	0.017
	Tip	13.337	0.006
Loktanella	Holdfast	14.780	0.005
	Basal blades	42.563	< 0.001
	Receptacles	23.663	0.008
	Tip	108.995	< 0.001
Non-ID Pirellulaceae	Holdfast	10.591	0.012
	Basal blades	43.952	< 0.001
	Receptacles	46.287	0.002

Overall, in September-March, the most abundant bacteria associated with *S. muticum* constituted Non-ID *Rhodobacteraceae* (9.9%), *Loktanella* (8.6%), Non-ID *Flavobacteriaceae* (8.5%) and Non-ID *Hyphomonadaceae* (5.4%). In contrast, in July-August, the most abundant bacteria constituted Non-ID *Saprospiraceae* (10.2%), Non-ID *Pirellulaceae* (10%), Non-ID *Gammaproteobacteria* (9.4%), Non-ID *Flavobacteriaceae* (7%), *Spirochaeta* (6.4%), and Non-ID *Rhodobacteraceae* (5.5%).

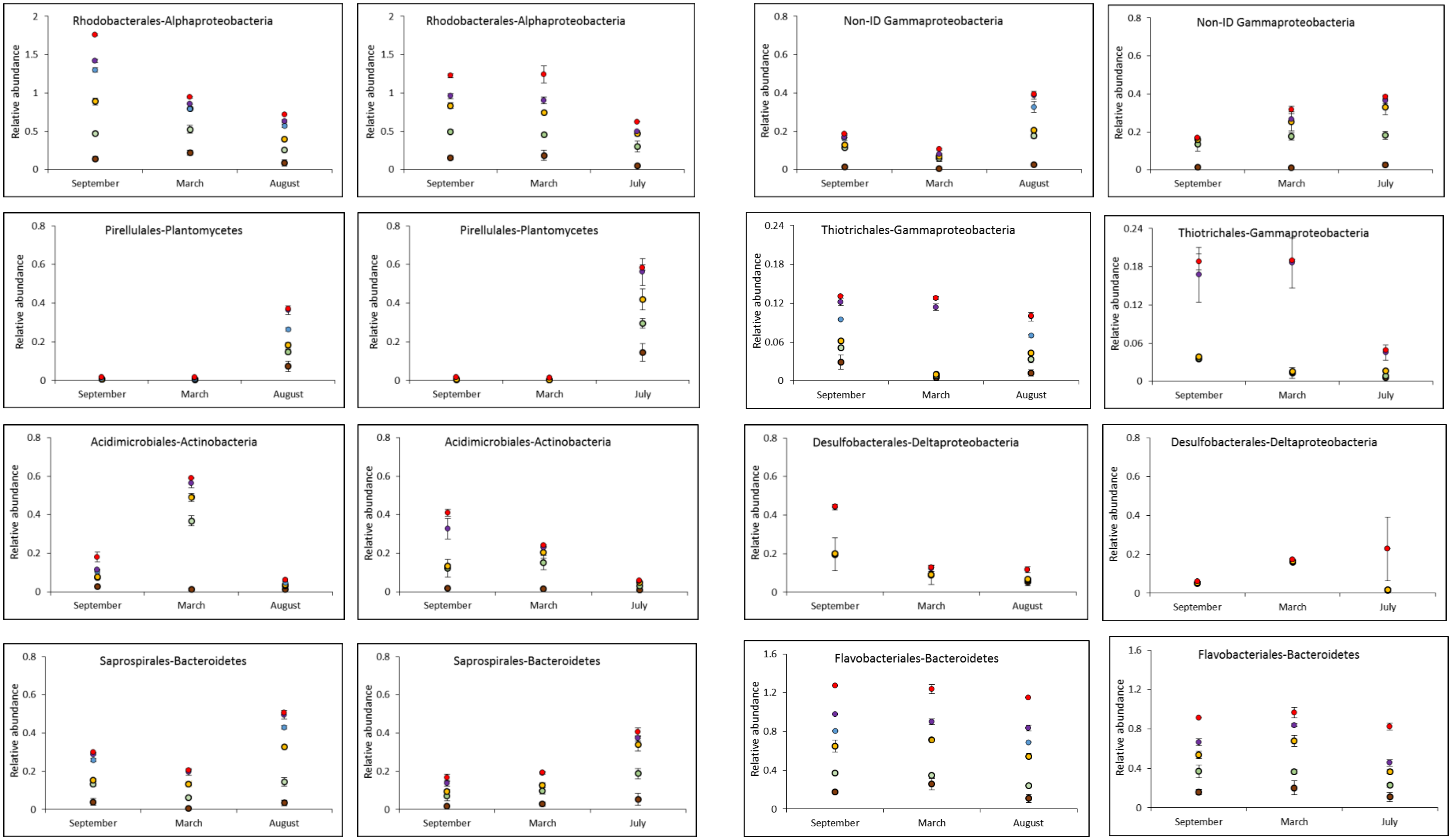


Figure 5.4 Mean relative abundances of bacterial orders associated to *Sargasum muticum* that exhibited significant differences between the seasons and geographic locations within the sediments (violet), seawater (red), holdfast (brown), basal blades (green), receptacles (blue), and tip (yellow) at Viana do Castelo (left) and Porto Covo (right). Alpha = 0.05, error bars show standard error (n=3).

Northern (Viana do Castelo) vs. Southern (Porto Covo) Portugal

The main differences between the Southern and Northern Portuguese location were due to the significant prevalence of Non-ID *Pirellulaceae (Pirellulales)* and Non-ID *Gammaproteobacteria* in Porto Covo (contributing 1.71% and 1.67% to dissimilarity respectively; SIMPER), and significantly higher abundance of *Loktanella (Rhodobacterales)* and Non-ID *Saprospiraceae (Saprospirales)* in Viana do Castelo (contributing 1.65% and 1.62% to the dissimilarity respectively; SIMPER) (Fig. 5.4). There was a significant difference in the sediment-associated bacterial communities in September between two locations (Pair-wise t-test, P=0.037).

The core microbiomes of *S. muticum* (bacteria present in 100% of samples in all tissues at all times) constituted 18 and 19 bacterial genera in Porto Covo and Viana do Castelo, respectively. The cores constituted *Alphaproteobacteria*, *Gammaproteobacteria*, *Flavobacteria*, *Saprospirae* and *Acidimicrobia* at both locations (Fig. 5.5). The core communities were very similar between the locations with the exception of summer. While *Proteobacteria* and *Bacteroidetes* constituted the seasonally-independent bacterial phyla observed in all months sampled, *Planctomycetes* were unique to *S. muticum* only in July and August (Table 5.2).

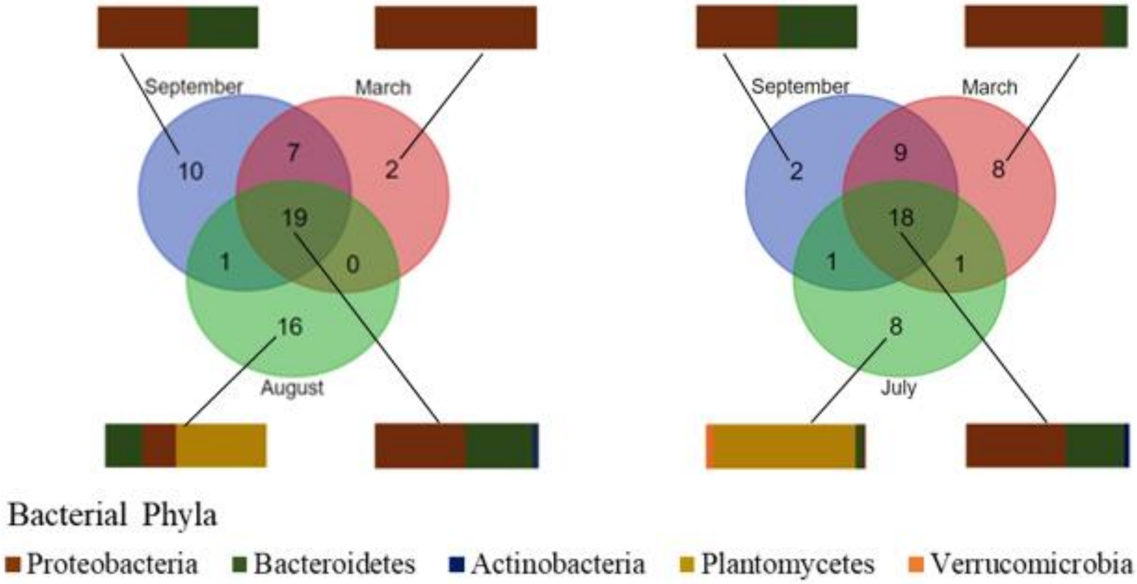


Figure 5.5 Venn diagram representing the number of bacterial genera (present in all tissues) shared between bacterial communities associated with *S. muticum* in Viana do Castelo (left) and Porto Covo (right) in different months.

Table 5.2 Bacterial genera unique to *Sargassum muticum* at each sampling season.

Sampling time	Viana do Castelo	Porto Covo
September	*Ulvibacter & Tenacibaculum-Flavobacteria;* *Anaerospora-Alphaproteobacteria;* *Non-ID OM27-Deltaproteobacteria;* *Glaciecola, Congregibacter, Thiothrix & Other-Gammaproteobacteria;* *Lewinella-Saprospirae;* *Non-ID NS1112-Sphingobacteria.*	*Psychroserpens-Flavobacteria;* *Octadecabacter-Alphaproteobacteria.*
March	*Other Rhizobiales & Phaeobacter -Alphaproteobacteria;*	*Crocinitomix-Flavobacteria;* *Non-ID Flammeovirgaceae-Cytophagia;* *Non-ID Rhodospirillales, Non-ID BD7-3, Phaeobacter, & Other Rhizobiales-Alphaproteobactria;* *Non-ID HTCC2089 & Congregibacter-Gammaproteobacteria;*
July & August	*Algibacter, Winogradskyella & Psychroserpens-Flavobacteria;* *Non-ID SB-1-Bacteroidia;* *Non-ID Flammeovirgaceae-Cytophagia;* *Pseudoruegeria-Alphaproteobacteria;* *Non-ID Comamonadaceae-Betaproteobacteria;* *Psychromonas & Marinicella-Gammaproteobacteria;* *Non-ID Bacteriovoracaceae-Deltaproteobacteria;* *Non-ID Helicobacteraceae-Epsilonproteobacteria;* *Non-ID Saprospirales;* *Non-ID agg27, Non-ID CL500-15, Plantomyces & Non-ID Pirellulaceae- Planctomycetes.*	*Lewinella & Non-ID BME43-Bacteroidetes;* *Non-ID Spirobacillales-Deltaproteobacteria;* *Non-ID agg 27, Plantomyces, Non-ID Pirellulaceae- Planctomycetes;* *Verrucomicrobium & non-ID Verrucomicrobiaceae-Verrucomicrobia.*

Seasonal differences within seaweed tissues

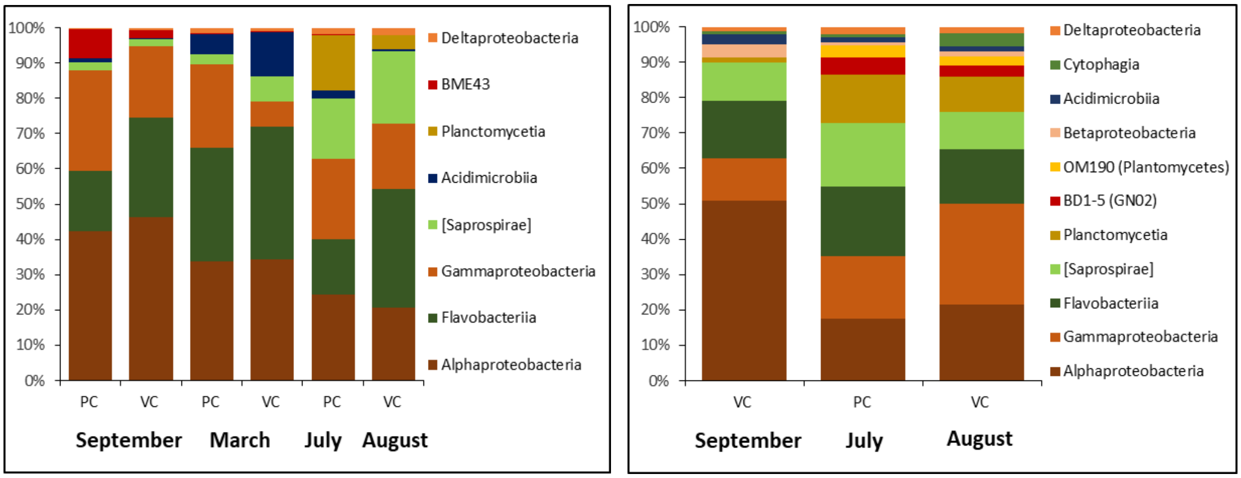
While the comparisons among different seaweed tissues were not the goal of this study, it focused on the seasonal differences within seaweed tissues. Tip associated communities changed over all sampled seasons, but differently so between the northern and southern *S. muticum* populations (see Supplementary Material). Between March and Summer, Non-ID *Acidimicrobiales (Acidimicrobia)* and *Tenacibaculum (Flavobacteria)* contributed most to the dissimilarity (4.77% and 3.81% respectively) in Viana do Castelo (47.74% average dissimilarity, SIMPER), while Non-ID *Pirellulaceae (Planctomycetia)* contributed most (4.58%) to the dissimilarity in Porto Covo (52.10%) (see Fig. 5.6 for their abundances). Between Summer and September, *Loktanella (Alphaproteobacteria)* and Non-ID *Saprospiraceae (Saprospirae)* contributed most to the dissimilarity (4.47% and 4.22% respectively) in Viana do Castelo (45.64% average dissimilarity, SIMPER), while *Glaciecola (Gammaproteobacteria)* and Non-ID *Pirellulaceae (Planctomycetia)* contributed most to the dissimilarity (4.34% and 4.22% respectively) in Porto Covo (55.64% average dissimilarity, SIMPER) (Fig. 5.6). Between September and March, Non-ID *Acidimicrobiales (Acidimicrobiia)* and *Loktanella (Alphaproteobacteria)* contributed most (5.68% and 4.6% respectively) to the dissimilarity in Viana do Castelo (43.04% average dissimilarity, SIMPER), while Non-ID *Rhodobacteraceae (Alphaproteobacteria)* contributed most (3.66%) to the dissimilarity in Porto Covo (40.98% average dissimilarity, SIMPER) (Fig. 5.6).

Receptacles were only found present during August (Viana do Castelo) and September (both locations). At Viana do Castelo, significant differences were detected between summer and September (average dissimilarity 39.65%, SIMPER) with Non-ID *Pirellulaceae (Planctomycetia)* and *Loktanella (Alphaproteobacteria)* contributing most to the dissimilarity, 3.48% and 3.41% respectively (Fig. 5.6).

Bacterial communities associated with basal blades showed differences between months only at the northern location (Viana do Castelo, Supplementary Material). Between Summer and September (average dissimilarity 39.45%, SIMPER) Non-ID *Pirellulaceae (Planctomycetia)* contributed most (3.45%) to the dissimilarity, while between March and Summer (average dissimilarity 54.01%, SIMPER) most contribution to the dissimilarity was done by Non-ID *Acidimicrobiales* (4.38%) and non-ID *JdFBGBact* (3.06%) both belonging to the class *Acidimicrobiia* (Fig. 5.6). Between September and March (average dissimilarity 42.59%, SIMPER), Non-ID *Acidimicrobiales* contributed most (5.12%) to dissimilarity (Fig. 5.6). No significant seasonal differences in bacterial structure were revealed within the holdfast tissues.

Sedimental communities differed in structure between March and summer at both locations (Supplementary Material), but whereas at Viana do Castelo (average dissimilarity 51.69%, SIMPER) Non-ID *Pirellulaceae* contributed most (2.09%), in Porto Covo (average dissimilarity 58.44%, SIMPER) *Spirochaetales* (3.53%) and Non-ID *Desulfobacterales* (3.27%)

contributed most to the dissimilarities. No significant seasonal differences in bacterial structure were revealed in the seawater.


A) Tip B) Receptacles

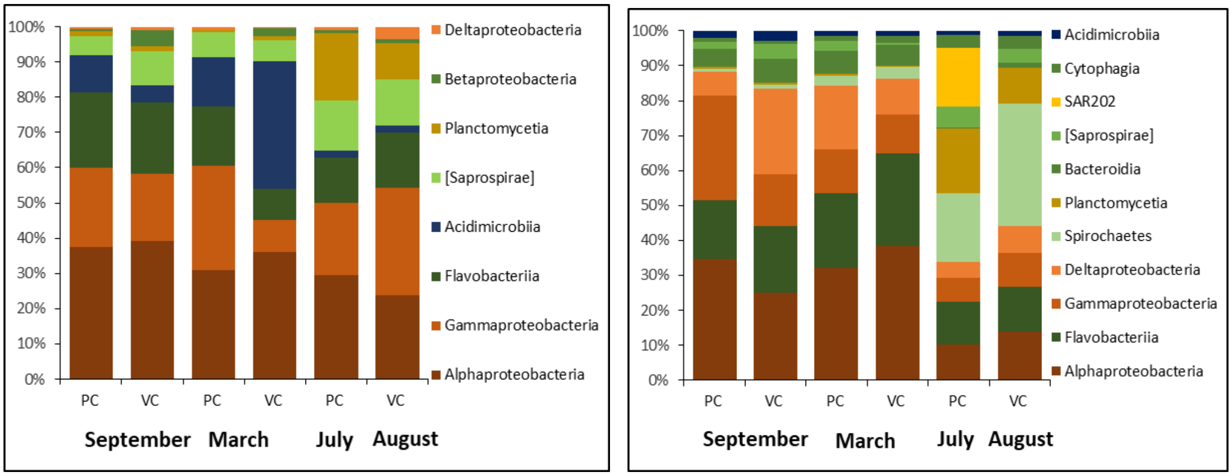

C) Basal Blades D) Holdfast

Fig 5.6 The most abundant bacterial classes associated with the different types of *S. muticum* tissues across seasons: A) Tip; B) Receptacles; C) Basal blades; D) Holdfast.

5.5 Discussion

Variation of bacterial communities associated with S. muticum between different months

In this study, we demonstrated a clear shift in the structure and composition of bacterial community associated with *S. muticum* among different times of the year (March, summer and September). The most significant changes were the significantly decreased abundance of *Proteobacteria* (by 20.5%) and *Actinobacteria* (by 7.4%) and increased abundance of

Planctomycetes (by 13.8%) in summer, compared to September and March. The summer decrease in *Proteobacteria* occurred mostly due to the significant decline in abundance of Non-ID *Rhodobacteraceae* (within the tip, receptacles & basal blades) and *Loktanella* (within the tip, receptacles, basal blades and holdfast). *Rhodobacterales* have been previously isolated from the seagrasses *Thalassia hemprichii* [55] and *Zostera marina* [56] and are primary colonizers of in marine surfaces with a known ability to fix nitrogen [57, 58]. Although not all members of the *Rhodobacteraceae* are considered pathogens, certain members are known to cause infections and disease (with the potential of getting more severe at increased temperatures) in *Fucus vesiculosus* [59] and *Delisea pulchra* [60]. *Loktanella* are known from various macroalgal species [2, 38, 61], including *Fucus vesiculosus* [21, 59], *Ulva australis* [30] and *Cystoseira compressa* [22]. Bacteria from this genus are highly adaptive and known for their capacity to utilize and metabolize rapidly organic carbon sources from seaweed exudates [20, 62, 63]. A significant increase of these bacteria was associated with the natural degradation of aged thalli in *Cystoseira compressa* during annual shedding in October [22]. Decrease in abundance of *Rhodobacteraceae* in response to elevated temperature levels was also observed in association with *Fucus vesiculosus* forma *mytili* [64].

Another important change was the summer increase of *Planctomycetes,* which occurred mostly due to the increase of Non-ID *Pirellulaceae* from the order *Pirellulales* (within the holdfast, basal blades & receptacles). *Planctomycetes* are frequent associates of macroalgae [2, 9, 19, 30, 65]. They were reported in association with *Laminaria hyperborea* [66, 67], *Macrocystis pyrifera* [68], *Porphyra umbilicalis* [61], *Ulva australis* and *Delisea pulchra* [29], *Fucus vesiculosus, Gracilaria vermicuphylla* and *Ulva intestinalis* [21]. *Planctomycetes* are known for their ability to mineralize organic into inorganic compounds matching nutritional requirements of macroalgae [65]. *Planctomycetes* are also proposed to degrade algal polymers and important contributors to the global nitrogen cycle [69]. Because *Planctomycetes* contain a high number of sulfatases genes, they could participate in the degradation of sulfated polysaccharides produced by *S. muticum* [70, 71]. Although *Planctomycetes* are known to be abundant on macroalgae [72, 65], their relative abundances vary substantially between seasons and seaweed species [21]. The summer increase of *Planctomycetes* was in accordance with the study on *Laminaria hyperborea*, where the abundance of this bacterial phyla was minimal in September and maximal in July [19]. It has been proposed that without the seaweed chemical defense, *Planctomycetes* could lose their competitiveness over other bacteria, resulting in the low abundance observed in September [66]. A high abundance of *Planctomycetes* is not unique to macroalgae as in seawater during a diatom bloom, *Planctomycetales* (i.e. *Pirellula*), attached to diatom cells, were among the prevalent bacteria [73].

Seasonally Independent Bacterial Communities

Overall, *Proteobacteria* and *Bacteroidetes* constituted the most abundant bacterial phyla associated with *S. muticum* that was consistent with previous studies [19, 21, 22, 24, 25, 30]. *Proteobacteria* (*Alpha*, *Gamma & Delta*), *Bacteroidetes* and *Actinobacteria* were prevalent in association with Baltic and North Sea *Fucus vesiculosus* [59, 64, 74], *Cystoseira compressa* [22] and *Macrocystis pyrifera* [68]. Prevalence of *Proteobacteria*, *Bacteroidets* and *Actinobacteria* over the year indicates of their seasonal independence (consistent with results of [21]) and temporal adaptation, as well as an important role towards *S. muticum* and functioning of its associated bacterial community.

Differences between North and South

Loktanella (*Alphaproteobacteria*) and Non-ID *Saprospiraceae* (*Bacteroidetes*) were more prevalent at the northern, while Non-ID *Pirellulaceae* (*Planctomycetes*) and Non-ID *Gammaproteobacteria* were more abundant at the southern location. Between September-March and summer, *Loktanella* and Non-ID *Gammaproteobacteria* exhibited significant decrease in abundance, while Non-ID *Saprospiraceae* and Non-ID *Pirellulaceae* showed significant increase. Overall, the effect of season on *S. muticum* associated microbiota was more significant than the effect of geographic location. A possible explanation for these results might be that despite the temperature gradient between the two locations throughout the year, the difference in seawater temperature might not be sufficient to significantly re-organize and re-structure associated microbial communities.

Differences by Tissue between Different Months

The most notable differences in *S. muticum* microbiota were detected within the tip tissues as they were observed between all months sampled. This could be due to the fact that tips represent new tissues and as such recently colonized, compared to older holdfast tissues. Another major change among all months sampled were the significant differences within the basal blades, observed in Viana do Castelo, but not in Porto Covo. This could be due to the higher gradient in water temperature in the North of Portugal compared to the South. Higher gradient in seawater temperature in the North could also explain significant difference in associated microbiota within receptacles, which was observed between September and summer in Viana do Castelo. As reproductive activity of *S. muticum* in Northern Portugal occurs between April and August, by September it is already finished and re-organization of associated microbiota is likely to have taken place.

Sources of Bacteria

Variation of bacterial communities associated with *S. muticum* could be due to the abundance of specific bacteria in the environment from where they could be acquired, at the particular season [75]. The differences in composition between bacterial communities associated with *S. muticum* and seawater maybe due to the influence of the chemistry of the seaweed surface tissue (i.e. effect of seaweed metabolites on bacterial growth and attachment), which selectively attracts specific bacteria forming microbial composition driven by seaweed exudates [38, 68]. Pre-existing bacterial communities on the seaweed surface may affect the ability of settling bacteria to attach [75]. Changes in *S. muticum* microbiota are likely to be caused by increased water temperature, as shown in the significant shifts in restructuring of associated microbiota of *Fucus vesiculosus* [59, 64].

In this study, the abundance of Non-ID *Rhodobacteraceae* was generally the highest in the seawater (followed by *S. muticum*) and the lowest in the sediments, indicating that these bacteria could be acquired by the seaweed from the surrounding water. Similarity in patterns between Non-ID *Rhodobacteraceae* observed within *S. muticum* and in the seawater suggest that these bacteria could follow the seasonal pattern of its availability in the water column in response to variations in temperature, light availability and other factors. In contrast, *Loktanella* was more abundant in association with *S. muticum* than in the seawater across all seasons and locations, and least abundant in the sediments. An increased abundance of *Loktanella* during September-March could be linked to the fact that these bacteria could be attracted by seasonal changes in algal exudates at these months. The abundance of Non-ID *Pirellulaceae* was higher in the sediments than within *S. muticum* and the lowest in the seawater across all seasons and locations, indicating that these bacteria could also be acquired by the seaweed from the sediments.

Considerations

While temperature has been previously demonstrated to be a key factor in altering the seaweed-associated bacterial communities [59], salinity and irradiance represent other substantial factors as shown in the study on *F. vesiculosus* forma *mytili* [76]. Furthermore, seasonal changes in other physical and chemical environmental factors (i.e. day-length, land runoff, seasonal winds, currents), also affect microbial composition [31]. Ongoing climate change may also generate changes in abundance and composition of bacteria sensitive to temperature [59, 64], CO2- [59, 64] and pH [77] with effects on seaweed fitness with the potential for invaders to spread further. Nevertheless, understanding the effect of season on seaweed-associated microbiota is necessary for assessing the seaweed response and adaptation to future climate conditions [78]. When analyzing the seasonal changes in seaweed-associated microbiota, it is important to consider that they have “an annual repeating pattern” and as such, “data need to be collected over several consecutive years” to assure that the pattern is actually seasonal and not just changing over months [31].

In this study, we demonstrated that bacterial communities associated with *S. muticum* experience significant temporal shifts as well as variation between geographic locations. The temporal effect was reflected in significant abundance of *Non-ID Rhodobacterales* and *Laktonella* in September-March and substantial prevalence of Non-ID *Pirellulales* in summer. Such changes within *S. muticum* microbiota could be related to the seaweed productivity as temporally changing algal exudates attract different bacteria, which degrade algal polysaccharides and cell walls among other functions [66]. The changes between months occurred mostly within the tip tissues and less within the basal blades and, possibly, receptacles. This could be related to the tips being younger compared to the other seaweed tissues and possibly under more direct colonization by bacteria from the surrounding environment. Significant re-organization and re-structuring of microbiota associated to *S. muticum* may have potential consequences for the seaweed fitness and adaptation to environmental changes resulting in increased invasiveness [78].

5.6 Acknowledgments

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5.8 Supplementary Material

Table S1 PERMANOVA results: Structure of bacterial communities associated to *S. muticum*, seawater and sediments (ti) at two locations (lo) and three seasons (mo).

Source	df	SS	MS	Pseudo-F	P(perm)	perms
lo	1	3282.1	3282.1	4.0008	0.001	999
mo	2	16513	8256.5	10.065	0.001	999
ti	5	33975	6795.1	8.2831	0.001	999
loxmo	2	3594	1797	2.1905	0.001	996
loxti	5	6939	1387.8	1.6917	0.001	996
moxti**	9	6681	1853.4	2.2593	0.001	995
loxmoxti**	8	9016.7	1127.1	1.3739	0.006	998
Res	61	50042	820.35			
Total	93	1.4764E5				

Table S2 PERMANOVA Pair-wise tests of locations by month and tissue of the interaction term 'location x month x tissue' of bacterial communities structure associated to *S. muticum*, seawater and sediments at two locations and three seasons.

VC=Viana do Castelo; PC=Porto Covo; rec=receptacles; hf=holdfast; sed=sediment; sw= seawater. Significant comparisons: p<0.05.

Month	Tissue	Groups	t	P(perm)	P(MC)
March	sed	PC. VC	1.5153	0.117	0.119
March	blade	PC. VC	1.6701	0.097	0.067
March	hf	PC. VC	0.79017	0.825	0.619
March	tip	PC. VC	1.6571	0.094	0.076
March	sw	PC. VC	1.3595	0.102	0.174
Summer	sed	PC. VC	1.1091	0.309	0.34
Summer	rec	PC. VC	1.3685	0.093	0.154
Summer	blade	PC. VC	1.4804	0.089	0.125
Summer	hf	PC. VC	0.99608	0.52	0.438
Summer	sw	PC. VC	1.1779	0.224	0.347
Summer	tip	PC. VC	1.866	0.081	0.043
September	sed	PC. VC	2.2183	0.109	0.037
September	blade	PC. VC	1.3552	0.11	0.164
September	hf	PC. VC	1.0558	0.491	0.381
September	sw	PC. VC	1.1224	0.322	0.372
September	tip	PC. VC	1.3171	0.098	0.17

Table S3: PERMANOVA Pair-wise tests of months by location and tissue of the interaction term 'location x month x tissue' of bacterial communities structure associated to *S. muticum*, seawater and sediments at two locations and three seasons.

VC=Viana do Castelo; PC=Porto Covo; rec=receptacles; hf=holdfast; sed=sediment; sw= seawater. Significant comparisons: $p<0.05$.

Location	Tissue	Groups	t	P(perm)	P(MC)
PC	sed	March. Summer	1.5848	0.081	0.104
		March. September	1.789	0.089	0.063
		Summer. September	1.6571	0.106	0.088
PC	blade	March. Summer	1.7339	0.11	0.063
		March. September	1.1085	0.297	0.329
		Summer. September	1.679	0.114	0.057
PC	hf	March. Summer	1.457	0.105	0.133
		March. September	0.87437	0.801	0.574
		Summer. September	1.7711	0.095	0.058
PC	sw	March. Summer	1.4429	0.097	0.154
		March. September	0.93501	0.532	0.484
		Summer. September	1.3253	0.104	0.199
PC	tip	March. Summer	2.2234	0.11	0.022
		March. September	1.1941	0.2	0.269
		Summer. September	2.1224	0.094	0.029
VC	sed	March. Summer	2.0869	0.098	0.028
		March. September	1.871	0.105	0.07
		Summer. September	1.7677	0.104	0.088
VC	rec	Summer. September	2.1926	0.115	0.019
VC	blade	March. Summer	2.698	0.099	0.012
		March. September	2.4515	0.117	0.013
		Summer. Sept	2.0846	0.08	0.019
VC	hf	March. Summer	1.7134	0.097	0.052
		March. September	1.2519	0.106	0.223
		Summer. September	1.529	0.095	0.121
VC	sw	March. Summer	1.7391	0.269	0.134
		March. September	1.5115	0.096	0.139
		Summer. September	1.5679	0.333	0.295
VC	tip	March. Summer	2.8434	0.086	0.011
		March. September	2.0498	0.111	0.031
		Summer. September	2.154	0.105	0.017

Table S4: PERMANOVA results: Composition of bacterial communities associated to *S. muticum*, seawater and sediments (ti) at two locations (lo) and three seasons (mo).

Source	df	SS	MS	Pseudo-F	P(perm)	perms
lo	1	3423.3	3423.3	4.6357	0.001	996
mo	2	12236	6118	8.2847	0.001	998
ti	5	21050	4210	5.701	0.001	997
loxmo	2	2564.2	1282.1	1.7362	0.003	997
loxti	5	5320.7	1064.1	1.441	0.003	993
moxti**	9	10532	1170.2	1.5847	0.001	996
loxmoxti**	8	7091.8	886.48	1.2004	0.032	995
Res	61	45047	738.47			
Total	93	1.1267E5				

Table S5 PERMANOVA Pair-wise tests of locations by month and tissue of the interaction term 'location x month x tissue' of bacterial communities composition associated to *S. muticum*, seawater and sediments at two locations and three seasons.

VC=Viana do Castelo; PC=Porto Covo; rec=receptacles; hf=holdfast; sed=sediment; sw= seawater.

Month	Tissue	Groups	t	P(perm)	P(MC)
March	sed	PC. VC	1.4206	0.1	0.122
	blade	PC. VC	1.3299	0.094	0.157
	hf	PC. VC	0.93704	0.521	0.494
	sw	PC. VC	1.2363	0.11	0.244
	tip	PC. VC	1.4189	0.097	0.13
Summer	sed	PC. VC	1.2031	0.093	0.25
	rec	PC. VC	1.3002	0.097	0.198
	blade	PC. VC	1.3488	0.097	0.18
	hf	PC. VC	1.28	0.119	0.209
	sw	PC. VC	0.90852	1	0.511
	tip	PC. VC	1.4834	0.108	0.111
September	sed	PC. VC	1.5573	0.102	0.111
	blade	PC. VC	1.539	0.099	0.11
	hf	PC. VC	1.0061	0.505	0.43
	sw	PC. VC	1.1999	0.325	0.336
	tip	PC. VC	1.3185	0.098	0.203

Table S6 PERMANOVA Pair-wise tests of months by location and tissue of the interaction term 'location x month x tissue' of bacterial communities composition associated to *S. muticum*, seawater and sediments at two locations and three seasons

VC=Viana do Castelo; PC=Porto Covo; rec=receptacles; hf=holdfast; sed=sediment; sw= seawater. Significant comparisons: $p<0.05$.

Location	Tissue	Groups	t	P(perm)	P(MC)
PC	sed	March. Summer	1.4175	0.102	0.166
		March. February	1.5574	0.108	0.093
		Summer. February	1.4426	0.106	0.155
PC	blade	March. Summer	1.4121	0.101	0.130
		March. February	1.1602	0.202	0.284
		Summer. February	1.5018	0.107	0.087
PC	hf	March. Summer	1.4132	0.097	0.133
		March. February	0.85208	0.9	0.577
		Summer. February	1.6431	0.081	0.072
PC	sw	March. Summer	1.3411	0.113	0.181
		March. February	0.88123	0.813	0.553
		Summer. February	1.2726	0.09	0.236
PC	tip	March. Summer	1.7394	0.099	0.041
		March. February	1.162	0.171	0.294
		Summer. February	1.5211	0.117	0.08
VC	sed	March. Summer	2.0208	0.102	0.026
		March. February	1.3997	0.093	0.178
		Summer. February	1.7371	0.097	0.102
VC	rec	Summer. February	1.6227	0.095	0.084
VC	blade	March. Summer	2.0963	0.101	0.026
		March. February	1.6506	0.083	0.09
		Summer. February	1.579	0.095	0.072
VC	hf	March. Summer	1.6953	0.092	0.061
		March. February	1.2843	0.111	0.212
		Summer. February	1.4438	0.102	0.109
VC	sw	March. Summer	1 .4843	0.276	0.218
		March. February	1.1644	0.105	0.306
		Summer. February	1.1937	0.338	0.413
VC	tip	March. Summer	2.1613	0.097	0.016
		March. February	1.5928	0.116	0.095
		Summer. February	1.7939	0.089	0.046

Chapter 6

Integration of reproductive phenology in marine invasion predictions under climate change scenarios.

6. Integration of reproductive phenology in marine invasion predictions under climate change scenarios.

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6.1 Abstract

The phenology and demography of aquatic and terrestrial macrophytes have a latitudinal dependency, with reproductive periods beginning earlier in the year and more prolonged at lower latitudes. Temperature and day length are suggested to be strong environmental drivers of the reproductive timing of species. This makes phenological events sensitive to climate change and suitable to improve predictions of species´ distributions. In this study, we predicted future invasion risk of the alien seaweed *Sargassum muticum* assuming niche and phenological constraints conservatism over time. We used ecological niche modelling to forecast the alien's distribution under two different future climate scenarios. Our results indicate how the distribution of *S. muticum* may change under diferent climate change scenarios and the restrictions on northward shifts imposed by the reproductive window. Under the most drastic climate change scenario, the predicted northward expansions may have significant ecological consequences for subarctic coastal ecosystems whereas in lower latitudes, habitats currently occupied by *S. muticum* will no longer be suitable, creating opportunities for significant community changes.

Keywords: niche modelling, *Sargassum muticum*, phenology, climate change

6.2 Introduction

New environmental conditions resulting from climate changes are expected to result in shifts of (invasive) species distribution and abundance (Bates et al. 2014). By altering abiotic filters, climate change may benefit non-native species adapted to the changed conditions and promote the success of warm water-tolerant species in aquatic environments (Rahel 2002). It may also result in increased abundance and invaded range expansions (Rahel & Olden 2008, Hellmann et al. 2008), leading to impacts on native ecosystems´ functioning and evolutionary processes (Edwards & Richardson 2004). Among the physical and biological factors shaping the species’ distribution boundaries (i.e. abiotic, biotic, dispersal strategies and capacity to adapt to new conditions; Soberon and Peterson 2005), temperature is believed to be the most important (Hoek 1982, Luning et al. 1990, Eggert 2012) as it affects performance (i.e. photosynthesis, growth, and reproduction) and determines tolerance or survival limits (Breeman 1990, Eggert 2012). Northern boundaries are established “by low lethal winter temperatures, or by summer temperatures too low for growth and/or reproduction”, while southern boundaries are established “by high lethal summer temperatures, or by winter temperatures too high for induction of a crucial step in the life cycle” (Breeman 1990). Seaweeds are particularly sensitive to changing temperatures and will be directly affected as a result of climate change. The long-term temperature increase may affect phenotypically plastic species and, as such, all temperature sensitive phenotypically plastic traits will shift (Visser 2005). This may eventually favor opportunistic and tolerant seaweeds increasing their competitive ability, result in replacement of less tolerant species (Dukes 2007), and lead to the downwards shift of the upper limit of the intertidal and subtidal seaweeds (Harley & Paine 2009; Harley 2011). Because temperature represents a key factor for the geographic distribution of seaweed species (Hoek 1982a,b; Breeman 1988), the effects of changing seawater temperature, as a result of climate change, can be predicted (Breeman 1990).

One of the tools that became increasingly important in conservation biology and climate change research is ecological niche modelling (i.e. species distribution modelling / habitat suitability modelling). These models are based on the statistical relationship between species occurrence or abundance data with environmental factors (i.e. abiotic habitat features) and/or spatial characteristics (Elith & Leathwick 2009, Guisan & Zimmermann 2000, Guisan & Thuiller 2005, Richards et al. 2007, Schroeder 2008). Through characterization of niche aspects that determine species´ distribution (i.e. the set of biotic and abiotic conditions under which species can survive, persist and reproduce, Warren 2012), maps of habitat suitability and the probability of occurrence can be produced (Kearney & Porter 2009, Marcelino & Verbuggen 2015). Ecological niche models allow predictions of species distribution across terrestrial and aquatic landscapes at various spatial and temporal scales and provide useful ecological and evolutionary insights (Elith & Leathwick 2009, Guinan et al. 2009, Pauly et al. 2011, Carlos-Junior et al. 2015).

For seaweeds, ecological niche modelling can be applied in invasion biology to assess the likelihood of introductions´ success and establishment in the novel area, identify critical routes and arrival points, and forecast range expansions under climate change (Peterson 2003, Elith & Leathwick 2009, Marcelino &Verbruggen 2015, Verbruggen et al. 2009, 2013). Changes in environmental conditions generated by climate change may result in increased invasions to areas in which alien species could not survive before, and reduced invasions in areas that become environmentally unfavorable (Stachowicz et al. 2002, Walther et al. 2009). Therefore, ecological niche modelling allows to predict the seaweeds invasive potential under future climate scenarios. In fact, a number of studies with application of niche models for investigating algal niche features or distributions have been conducted (Neiva et al. 2014, Pauly et al. 2011, Raybaud et al. 2013, Tyberghein et al. 2012, Verbuggen et al.. 2009, 2013). Although "the lack of knowledge regarding seaweeds physiological responses to the synergistic action of changing environmental factors" is still an uncertainty, this limitation can be overcome by increased accessibility of "data on seaweeds physiology and environmental data sets for future climate" (Marcelino & Verbruggen 2015).

While developing the niche models for predicting the risk of invasions, species phenology should be taken into consideration. This is important because for invasive species earlier and longer warm seasons may lead to a prolonged period of reproduction and wider physiological tolerance in the invaded ranges, with more resilient species demonstrating larger phenological shifts (Jones and Cresswell 2010, Rahel & Olden 2008). Shifts in reproduction phenology (i.e. variations in timing and length of reproductive seasons) are among the primary organisms´ responses to climate change (Parmesan & Yohe 2003; Menzel et al. 2006; Edwards & Richardson 2004, Nemani et al. 2003, Dunn 2004). Through an extended reproductive season and resulting increased fecundity (Edwards & Richardson 2004, Nemani et al. 2003), climate change-induced shifts in reproductive phenology may affect species´ population dynamics (Miller-Rushing et al. 2010), species composition and competition within communities (Waser & Real 1979; Post et al. 2001) and trophic interactions through altered food-web structures (Harrington et al. 1999; Edwards & Richardson 2004, Thackeray et al. 2010). Considering the physiological response of organisms to temperature and its importance in phenological studies, sea surface temperature can be used as an indicator of climate change in the marine environment (Eswards & Richardson 2004). Day length is also an important factor in seaweeds´ phenology and distribution as it affects reproductive timing (Breeman 1993, Cunningham et al. 1993, Voskoboinikov et al. 1996). Reproductive maturity, for example, is delayed with increasing latitude and short day lengths (Strong 2003). Despite this, maturation of species seems a cumulative and combined response to temperature, day length, and irradiance, as each of these factors affects the initiation of fertility (Hales & Fletcher 1990). Increased temperatures promote the beginning of fertility under long-day conditions (Hales & Fletcher 1990).

To better predict past, present and future seaweed distributions, further progress should made to add physiology and phenology in predictions produced with correlative niche models. Integrating phenological trends in the prediction of aquatic invasive species and vulnerable native species is particularly important and would facilitate the assessment of species' sensitivity to climate change (Diez et al. 2012). Development of species-specific phenological models would improve the prediction of distributional shifts at local and regional scales in response to climate change (Cleland et al. 2007, Chuine et al. 2003). Finally, accurate forecasting of species´ reproductive phenology could increase the accuracy of ecosystems productivity predictions under future climate scenarios and allow further understanding of population dynamics (Cleand et al. 2007). Nevertheless, despite the increasing importance of phenological predictions, there is a lack of research on responses of reproductive phenology to climate change (Price & Waser 1998; Dunne, Harte, & Taylor 2003). The majority of studies aimed to understand the seasonal phenological shifts in relation to climate were conducted on terrestrial species (Walther et al. 2002, Parmesan & Yohe 2003), with a limited number on aquatic species (Thackeray et al. 2010, Burrows et al. 2011) and phenology of invasive species (Wolkovich & Cleland 2011).

In this study, we use ecological niche modelling to predict the invasion risk of *Sargassum muticum* under current and future conditions but innovatively with accounting for reproductive phenology. Under the assumption of niche and phenological constraints conservatism over time, we use reproductive windows associated to combined sea surface temperature and day length along the latitudinal gradient of the distribution to produce more reliable forecasts. This is the first time that phenological data is used to improve predictions of seaweed´ invasions under climate change.

6.3 Methodology

Biological data and study area

As study area, the entire distribution range of *S. muticum* in the Northern Hemisphere was considered with an emphasis on the European region. *S. muticum* presence records were derived from the literature and online databases, including the Global Biodiversity Information Facility (GBIF; http://www.gbif.org/), Ocean Biogeographic Information System (OBIS; www.iobis.org), and *S. muticum* distribution records in Europe of the Ghent University Phycology Lab (http://www.phycology.ugent.be/research/invasives/map.html; Engelen et al. 2015).

The occurrences were reviewed to correct or exclude those with referencing errors. From the determined geographic extent, cells ranging from 0 to 30 m depth were selected, as the maximum depth at which attached *S. muticum* is found along the coastline. Delimiting a depth threshold was implemented using the 30 arc-seconds resolution bathymetric data derived from the General Bathymetric Chart of the Oceans (GEBCO) website (http://www.gebco.net/). For georeferencing of *S. muticum* occurrence data and environmental variables, a resolution of 5 arc minutes (~ 9.2 km) was applied. In total, 2587 *S. muticum* occurrence records were obtained, which corresponded to 1115 presence cells.

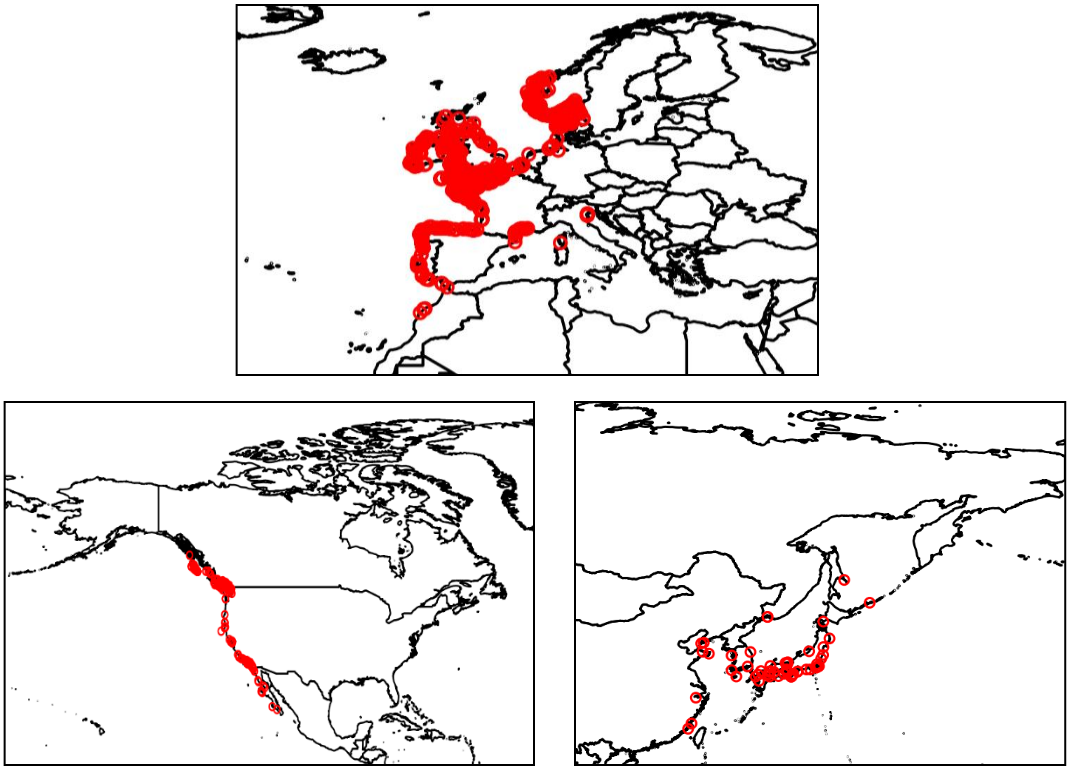

Figure 6.1 Current distribution of *Sargassum muticum* in the Northern Hemisphere with its native Asian and invasive European and North American ranges.

Environmental data

Environmental variables included the monthly means of: the sea surface temperature (SST max., mean, min., and range), surface air temperature (SAT max., mean, min. and range), and mean salinity for current conditions relevant to the study region. The data were derived from the Bio-ORACLE dataset (Tyberghein *et al*. 2012) and tested by Pearson's correlation coefficient < |0.7| in our study area. Multi-model ensembles were calculated based on five Ocean General Circulation Models (CNRM-CM5, HadGEM2-ES, IPSL-CM5A-LR, MPI-ESM-MR, & NASA/GISS) belonging to the Coupled Model Intercomparison Project Phase 5 (CMIP5; http://cmip-pcmdi.llnl.gov/cmip5/). To project the future distribution of *S. muticum*, ensembles were generated for two extreme future conditions by 2050 (from 2030 to 2050) and 2100 (from 2080 to 2100) with RCP (Representative Concentration Pathways) 2.6 representing the low emissions scenario and RCP 8.5 as the most pessimistic emissions scenario according to the Intergovernmental Panel on Climate Change - IPCC (Moss et al. 2010; van Vuuren et al. 2011).

Current and future SDMs

To obtain the current and future climate scenarios' predictions for *S. muticum*, an ensemble approach was applied. The *"biomod2"* package (Thuiller et al. 2014) was used to perform six presence–absence algorithms: flexible discriminant analysis (FDA), generalized additive model (GAM), generalized boosting model (GBM), generalized linear model (GLM), multiple adaptive regression splines (MARS), and randomForest (RF). We used an equal number of pseudo-absences as presence cells extracted at random. All models were implemented using the biomod2 package in R (Thuiller et al. 2014). We run 5 iterations for each modelling technique, thus, 30 models for each time and scenario were generated to produce the corresponding ensembles. The data was split into a calibration (70%) and a validation set (30%) in each of the 5 iterations performed for each model. The models' performance was assessed using the true skill statistic (TSS; Allouche et al. 2006), the receiver operating characteristic (ROC) curve (AUC), as well as ROC sensitivity and specificity (Fielding & Bell 1997). The threshold chosen optimized the ROC and TSS scores (Thuiller et al. 2014).

The final prediction under current conditions represented an ensemble calculated through the average of binary predictions (committee averaging ensemble), which was previously demonstrated to have the best performance in predicting coastal species (Chefaoui et al. 2016).

To produce the ensemble, only the models which obtained TSS > 0.8 were used. To project future distribution of *S. muticum,* ensembles were projected to the RCP 2.6 and RCP 8.5 scenarios. To assess the uncertainty of future projections, a clamping mask was generated. This allowed assessment of uncertainty areas through comparison of different values of each variable between the present and projected distributions. The importance of each variable was assessed using a procedure similar to "randomForest" (Liaw and Wiener 2002). A relative importance value from 0 to 1 (with one being of highest importance) was obtained for each variable by the correlation between the full model and a model rearranged without the examined variable using three iterations (Thuiller et al. 2014).

Hybrid model

To define the reproductive window of *S. muticum*, we collected observational data on SST and daylength available for each occurrence in the literature. Overall, 34 publications related to the reproductive phenology of *S. muticum* were revewed. We used the minimum and maximum values of monthly mean SST of the warmest and coldest months within *S. muticum* reproductive window when provided with the studies (in this case Deysher 1984; Norton & Deysher 1989; Espinoza 1990; Arenas & Fernandez 1998: Fernandez 1999). The minimum and maximum values of monthly mean day length during the reproductive window at required coordinates in the northern hemisphere were estimated using the NOAA Solar Calculator (https://www.esrl.noaa.gov/gmd/grad/solcalc/). After obtaining the correlative SDMs, a filter to the predictions concerning the reproductive phenology of the species was applied.

6.4 Results

Species distribution models

Following the Pearson correlation analysis, three variables were used in the modelling: maximum SST, minimum SST and salinity. Overall, the validation scores for the models of *S. muticum* were high (Table S1, Supplementary Material). The generalized boosting model (GBM) and RandomForest (RF) were the most accurate with the highest validation scores in the ensemble model TSS=0.859 (Table S1, Suplementary Material). The lowest validation scores were produced by the generalized additive model (GAM) with TSS=0.828 (Table S1, Supplementary Material). Maximum sea surface temperature was the variable with the highest relative importance (SST max. = 0.77) for the ensemble model, compared to minimal surface temperature (SST min. = 0.45) and salinity (S = 0.21). This suggests that the maximum SST may act as a limiting factor of the current distribution of *S. muticum* in the southern limit. Salinity seems to have the least effect in the distribution of the species.

For the future, even the least changing scenario (low emissions 2.6 RCP by 2050) led to predictions of a northward shift in the distribution of *S. muticum* compared to the current state. In the native range, the highest probability for the future *S. muticum* distribution was northwards from Vladivostok in Russia, Central and Southern Sakhalin, Hokkaido and the entire chain of the Kuril Islands up to Komchatka. Along the West coast of North America, substancial northwards shift is projected with a complete retreat of habitats from Baja California. The highest probability for the species coincided with its current presence along the coast of the US Washington state and British Columbia, and the lower probability of occurrence along the coast of Alaska (the Gulf of Alaska) (Figure S1, Supplementary Material). However, significant colonization was projected along the US East Coast, including from the Chesapeake Bay northwards along the coast of New England to Nova Scotia and Newfoundland, and up to the southern coast of the Hudson Bay (Figure S1, Supplementary Material). In Europe, the most probable future distribution of *S. muticum* also coincided with its current distribution in the South-Western fiords of Norway, down to UK, Danish, Dutch, Belgian, French, Spanish and Portugueses coastlines (Figure S1, Supplementary Material). However, significant colonization was also projected in Azores, Mediterranean (along the Southern French, Northern Italian and Croatian coastlines) and the Black Sea. Overall, the relative area increase by 2050 under RCP 2.6, compared to the current distribution, is expected to constitute 59% (Figure S1, Supplementary Material).

In the most extreme future scenario (8.5 RCP by 2100), *S. muticum* demonstrated an even more significant shift northwards. In the native region, the distribution of *S. muticum* is predicted to shift from Chinese and Korean shorelines northwards to Russian coast in the Japanese Sea, the Sakhalin Island, Kamchatka and the Kuril Islands (Fig. 6.3). Future distribution along the coast of North America was projected to spread extensively along the Gulf of Alaska (with the lower presence probability in the Islands of Alaska in the Bering Sea). Our model projected also appropriate coasts along the East Coast of US and Canada (extending up to Newfoundland on the East and the Southern Coast of the Hudson Bay on the North), in case the species could arrive there (Fig. 6.3). In Europe, future projections indicate that *S. muticum* will be distributed from the Azores to the Faroe Islands, along the Mediterranean Sea up to the Croatian coast and the Black Sea (with higher presence probability in the Azov Sea) (Fig. 6.3). There is a lower presence probability of *S. muticum* spreading in Southeastern Iceland, North-Western fjords of Norway and along the Russian coastline in the Barents and Kara Seas. The total area increase by 2100 under RCP 8.5 is expected to constitute 88%, compared to the current distribution (Fig. 6.2).

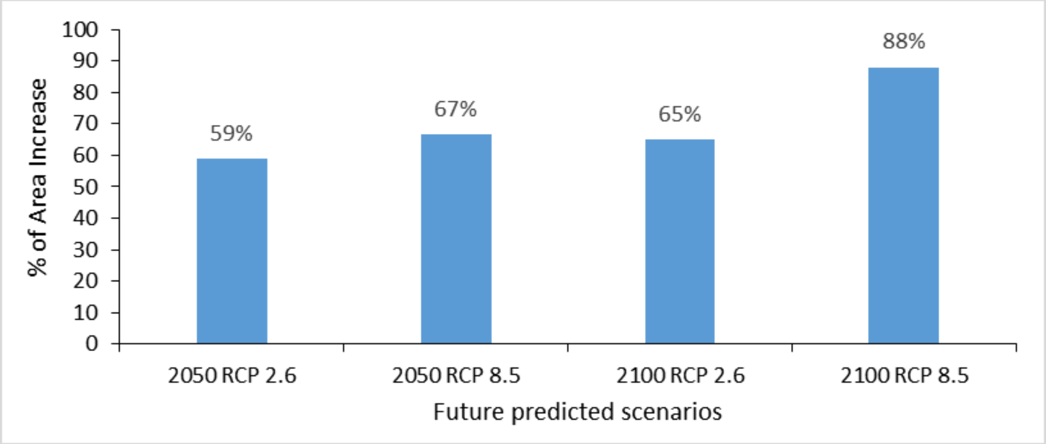

Figure 6.2 Relative area increase of invasion risk of *Sargassum muticum* between the present and future scenarios.

Overall, the area of *S. muticum* persistence between the present and 2100 under RCP 8.5 calculated using the TTS threshold constituted 15%, while the congruence between the area with high probability of occurrence (estimated arbitrarily as a probability of occurrence higher than 80%) found for the two scenarios constituted 59%.

The uncertainty in future predictions for both scenarios according to the clamping masks is presented in Figure S2 of the Supplementary Material. The areas with a higher uncertainty due to extreme values outside the range of the current conditions constituted the eastern part of the Canadian Archipelago located north from the Hudson Bay (characteristic for both the most optimistic and the most pessimistic scenarios), as well as the areas along the Barents and Kara Seas (characteristic for the worst case scenario). However, these uncertainty areas do not affect the predicted habitats with high probability of occurrence.

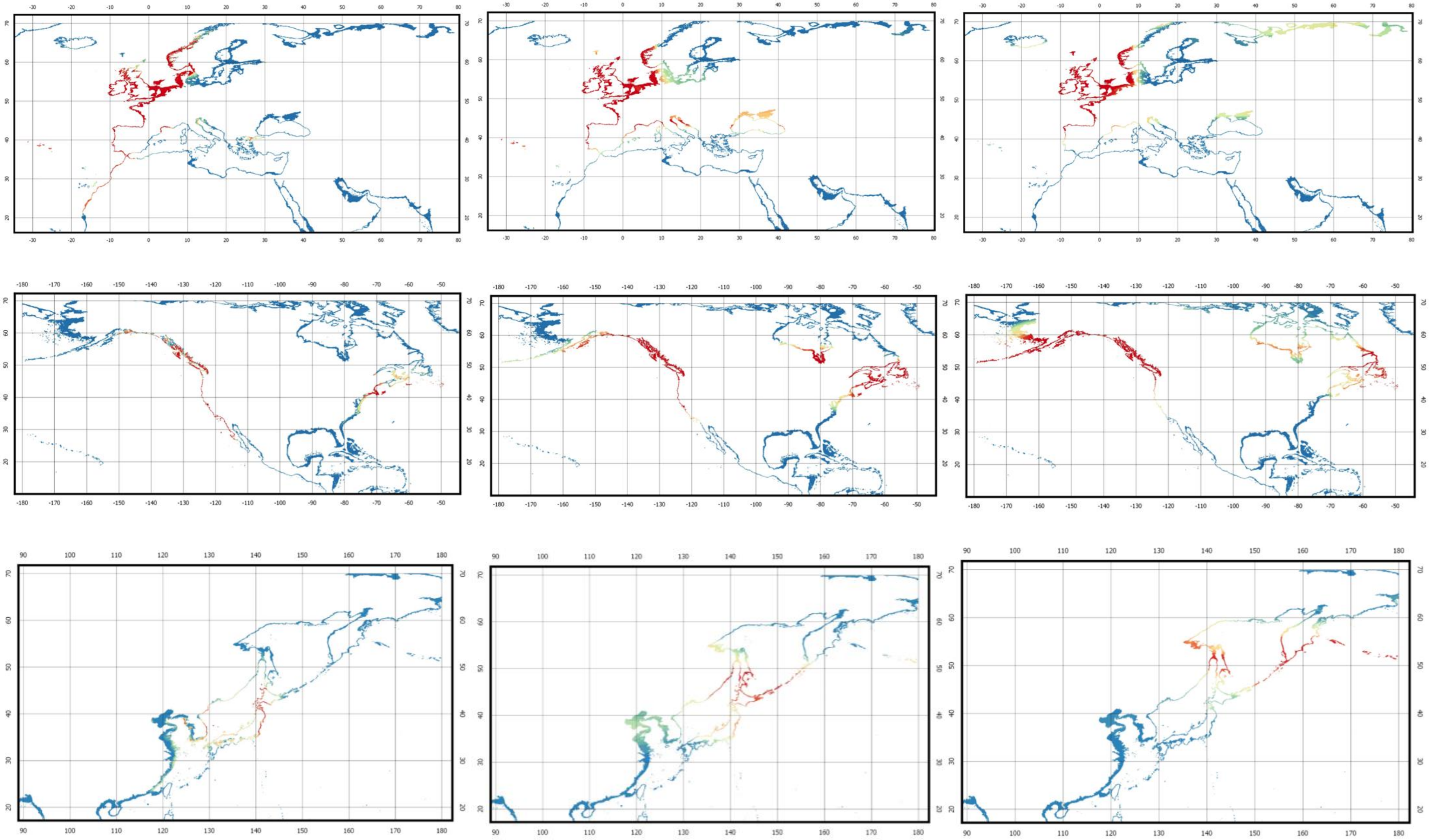

Figure 6.3 Estimated probability of occurrence for *S. muticum* in Europe (top), North America (middle), & Asia (bottom): present (left), projected by 2100 at RCP 2.6 (middle), & projected by 2100 at RCP 8.5 (right). Occurrence probability shown from high (red) to low (blue).

Hybrid model

Based on the data collected from the literature, the reproductive SST window varied from 10ºC to 27ºC (Deysher 1984; Norton & Deysher 1989; Espinoza 1990; Arenas & Fernandez 1998: Fernandez 1999). These data were used to filter predictions. As such, an analysis of projected distributions depending on the sea surface temperature threshold for the reproductive window (10-27°C) was implemented. In addition, the days of the year with light according to the thresholds (day length window from 10h48min to 15h54min) for the European range of distribution (latitude 30-70) were calculated. For latitude 30, those days were from 34 to 314, while for latitude 70, the days were from 70 to 104 and from 243 to 277. According to these results, the day length does not seem to be a limitation for the species persistence.

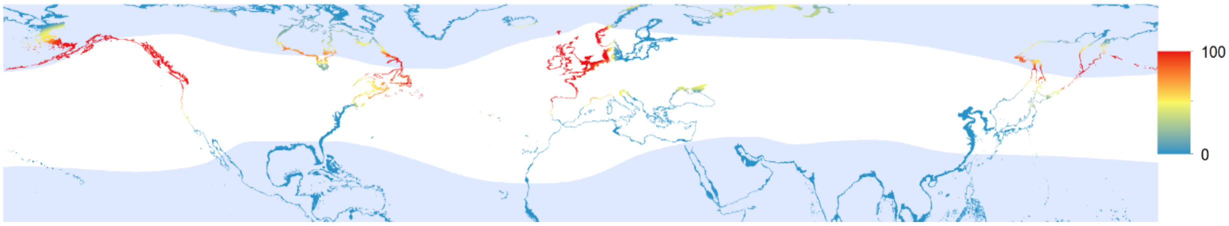

Figure 6.4 Hybrid model for *Sargassum muticum* composed by the prediction by 2100 under RCP 8.5 scenario filtered afterwards with the SST found to delimit the reproductive window of *S. muticum* under the same future scenario. The regions outside the limits of the SST reproductive window are in light blue colour. In the future, wide regions of North America and Asia would not be available for the reproduction of *S. muticum* despite showing a high probability of occurrence according to the correlative SDMs.

6.5 Discussion

In this study, we used ecological niche modelling to predict the invasion risk of *Sargassum muticum* under current and future conditions while accounting for phenological observations affecting the species' reproductive period. According to the prediction scenario by 2100 under RCP 8.5, a significant northward shift of possible occurrence of *S. muticum* was projected with substancial retreat from the current habitats. In the native region, the current distribution ranges of *S. muticum* in China, Korea, Japan, and Russia´s Vladivostok Region are projected to shift nothwards to the Central and Northern Sakhalin, the western part of the Sea of Okhotsk, Kiril islands, and Kamchatka. A substancial northward shift in *S. muticum* distribution was also projected in the European region towards the North-Western fjords of Norway, South-

Eastern coast of Iceland, Faroe Islands and along the coasts of Barents and Kara Seas (particularly the Gulf of Ob). A risk of *S. muticum* spread with a slight probability of occurrence was also projected in the Mediterranean along the Italian coastline, as well as in the Northern Adriatic and Azov Seas. The areas of current distribution of *S muticum* in Morocco and Southern Portugal are projected to retreat entirely. Along the coast of North America, the current habitats in Baja California and California in the United States are also projected to retreat. In contrast, the habitats in Alaska (along the Gulf of Alaska and Alaska Peninsula) are projected to expand significantly northwards up to the Bering Sea and Eastwards to Aleutian Islands. Significant risk of spread was also projected along the US East coast from New England to Newfoundland and along the Sourthern coast of the Hudson Bay. Notable increase in distribution area of *S. muticum* was revealed under each of the four scenarios ranging from 59% by 2050 under RCP 2.6 (the best case scenario) to 88% by 2100 under RCP 2.8 (the worst case scenario).

The above-described northward shift projected for *S. muticum* is in accordance with projections made for kelp forests (Assis et al. 2017a) and other fucoid algae: *Fucus serratus, F. vesiculosus* and *Ascophyllum nodosum* in the North-Atlantic (Jueterbock et al. 2013; Assis et al. 2014). These studies projected that by 2100, these temperate seaweed species will shift northwards to Arctic shores with particularly suitable habitats being found in Spitsbergen, Greenland and Canada, while currently suitable habitats below 45 degrees North by 2200 will become unsuitable. Significant northward shifts are projected in multi-species seaweed studies (e.g. Muller et al. 2009, Assis et al. 2017a), which forecasted extensions of cold-temperate seaweeds distribution from the Northern Atlantic to the High Arctic with a notable retreat of seaweed distribution along the northeastern Atlantic.

The northward shift in *S. muticum* distribution, along with the retreat from the currently occupied habitats, demonstrate that this invasive seaweed species may thrive well in those areas. Expansion into newly favoured habitats requires the capacity to disperse there (Assis et al. 2017b). Effective colonization of the North Atlantic and subarctic coastal habitats may be facilitied by the advantagous life-history traits and high dispersal capabilities of *S. muticum* (Engelen et al. 2015), although this may be strongly dependent on oceanographic currents (Buonomo et al. 2017). This may endanger sensitive Arctic ecosystems, which are already in the state of shift as a result of extensive pressures from multiple anthropogenic stressors associated with the warming climate. Raising sea water temperature may compromise the distribution of the indigenous seweed species making them more vulnerable to invasions and, if acting in combination with changes in other environmental parametes, habitats may become unsuitable and result in local species extinction. This may lead to the redistribution of species in the subarctic territories with potential evolutionary consequences, as well as generate cascading and irreversible impacts on local biodiversity and ecosystems.

While ecological niche models have been previously developed for various seaweed species (i.e. *Halimeda*, Verbuggen et al. 2009; *Caulerpa cylindracea*, Verbuggen et al. 2013), this study for the first time integrated phenology into seaweed distribution predictions. The study clearly demonstrates that including important phenological characteristics such as reproduction can affect predictions tremendously. Nevertheless, while the temperature and the day length were successfully incorporated to improve the predictions of future *S. muticum* distribution, fluctuations of other environmental parameters as a result of changing climate, such as salinity, concentration of nutrients or grazers could also be important to be integrated into future niche models (Engelen et al. 2015). In addition, incorporation of impacts from local anthropogenic stressors would also be beneficial, even though these data are not readily available.

Up to now, projections were only made to forecast the changes in horizontal distribution and abundance of *S. muticum* but future research would benefit from a modelling approach that would incorporate vertical shifts, so relevant to intertidal and subtidal seaweeds. Because the upper and lower limits of seaweed distribution may be controlled by temperature, light, salinity, and grazing (Hurd et al. 2014), variations in these parameters as a result of climate change may result in further distributional shifts (Harley 2011). As a result of long-term increases in temperature and intertidal thermal and desiccation stress (Harley 2003), the upper limits of intertidal seaweeds may shift downwards (Harley & Paine 2009), as shown for other fucoids (Pearson et al. 2009). As *Sargassum muticum* is a phenotypically plastic species, new conditions generated by changing climate (i.e. increasing temperature and CO2 levels, decreasing pH) may favor this species as it might be able to increase its competitive ability (Dukes 2007). This may affect the entry pathways of these species, as well as its colonization, establishment, and future spread (Capdevila-Argüelles & Zilletti, 2008). Deeper understanding of the genetic and epigenetic factors that contribute to the invasive success of *S. muticum* may further improve predictions of its future spread and identify potential impacts on native ecosystems.

Under the assumption of niche and phenological constraints conservatism over time, we used reproductive windows found for sea surface temperature and day length along the latitudinal gradient of its distribution to produce more reliable forecasts. We demonstrated how the distribution of *S. muticum* may change under diferent climate change scenarios and the restrictions on northward shifts imposed by the species reproductive window. By 2050 under RCP 2.6 the environmental changes would result in moderate distributional shifts. In contrast, by 2100 under RCP 8.5 the distribution of *S. muticum* is projected to significantly shift northwards with a partial retreat from the currently occupied habitats. Such a shift in the distribution of *S. muticum* in response to climate change may have significant ecological econsequences for subarctic coastal ecosystems. As this species is highly tolerant and opportunistic, it may potentially displace native species populations in the regions where its distribution will be expanding. In contrast, some habitats currently occupied by *S. muticum* will no longer be suitable and may be occupied by other seaweed species that may benefit from newly available habitats. This may result in further impacts and cascading efects on local ecosystems (Mineur et al. 2015).

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6.8 Supplementary Material

Table S1 Average validation scores of models obtained for each algorithm: Generalized linear model (GLM), generalized boosting model (GMB), generalized additive model (GAM), flexible discriminant analysis (FDA), multiple adaptive regression splines (MARS), and RandomForest (RF).

Model	**AUC(±SD)**	**TSS (±SD)**	**Sensitivity(±SD)**	**Specificity(±SD)**
GLM	0.962(±0.005)	0.831(±0.018)	94.328(±3.364)	88.557(±2.319)
GBM	0.969(±0.005)	0.859(±0.020)	96.194(±1.275)	90.075(±2.045)
GAM	0.960(±0.006)	0.828(±0.012)	93.731(±3.403)	88.806(±2.763)
FDA	0.964(±0.006)	0.831(±0.021)	94.179(±1.055)	90.896(±1.056)
MARS	0.963(±0.007)	0.832(±0.015)	94.627(±2.254)	87.861(±3.005)
RF	0.970(±0.006)	0.859(±0.021)	94.328(±0.000)	91.045(±0.000)

Table S2 Validation scores for the Ensemble. Accuracy of the Ensemble for the Present Conditions

	Testing data	**Cut-off**	**Sensitivity**	**Specificity**
KAPPA	0.000	1000	86.726	93.835
Mean TSS	0.843	449.5	96.143	88.184
ROC	0.960	450.0	96.143	88.184

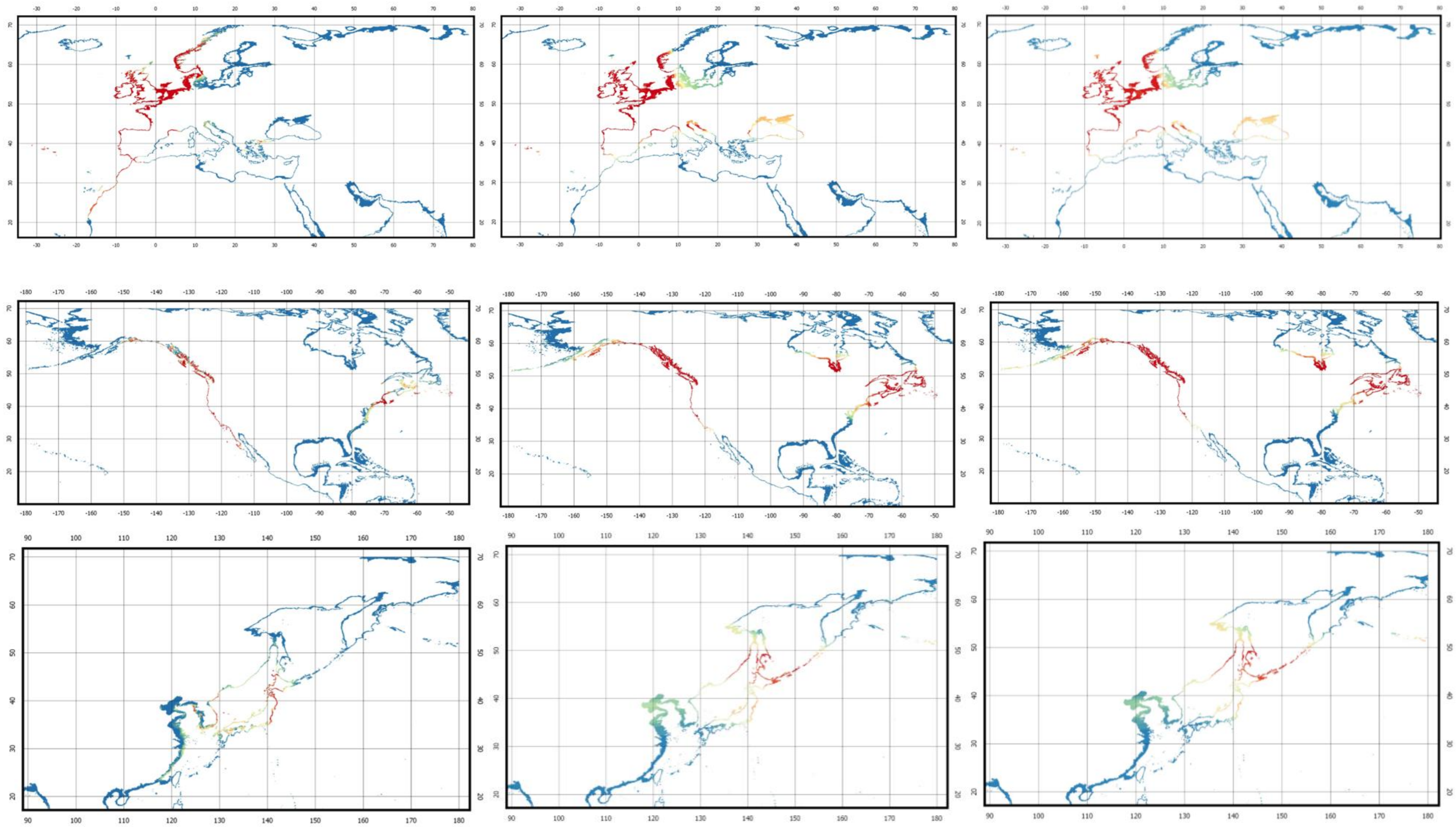

Figure S1. Estimated probability of occurrence for *S. muticum* in Europe (top), North America (middle) & Asia (bottom): present (left), projected by 2050 at RCP 2.6 (middle) and projected by 2050 at RCP 8.5 (right). The highest probability is presented in red, while the lowest probability is presented in blue color.

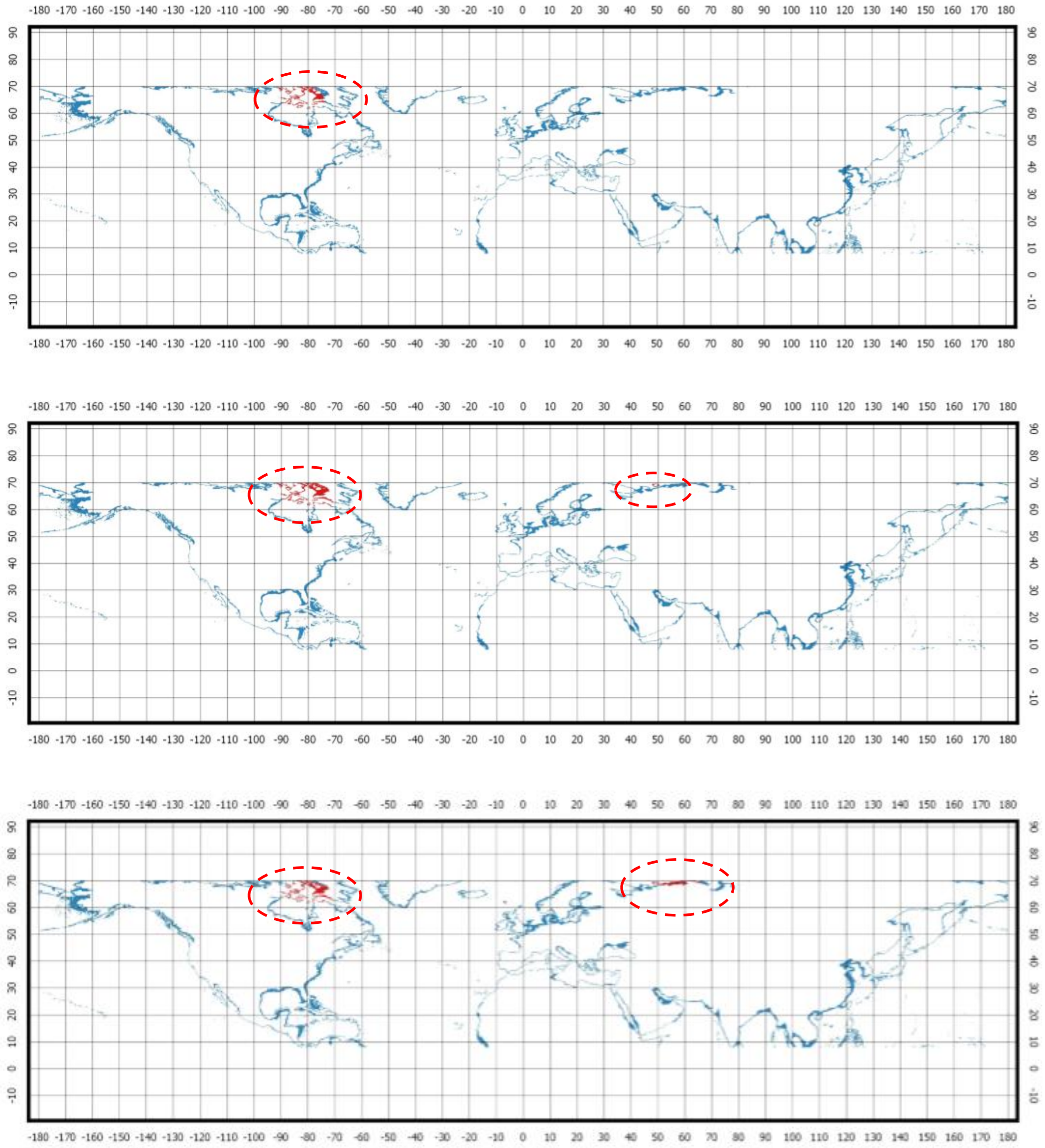

Figure S2. The areas with a higher uncertainty of *S. muticum* estimated probability of occurrence projected by 2050 at RCP 2.6 (top), 2050 at RCP 8.5 (middle), & 2100 at RCP 8.5 (bottom). There was no the area of uncertainty revealed under scenario by 2100 at RCP 2.6.

Chapter 7

General Discussion

7. General Discussion

Invasive species represent a global issue with important ecological and evolutionary consequences. Among all known invasive seaweeds, *Sargassum muticum* is a global invader, which represents an interesting case study, as its invasiveness and potential for future distribution are not yet entirely understood. However, invasions are very complex and need to be studied in an integrative framework. In the thesis, we combined reviews of the bibliography (Chapters 2 & 3), experimental studies (Chapters 4 & 5) and a modelling approach (Chapter 6) to better understand the patterns and processes associated with the success of *Sargassum muticum*.

Sargassum muticum is a successful introduced seaweed, which has a number of competitive advantages that may contribute to its invasiveness. This species is highly tolerant, opportunistic and has a high physiological tolerance allowing to adapt to a wide range of environmental conditions by adjusting its physiology, morphology, and phenology. The review provided in the Second Chapter of the thesis shows most of these characteristics: the high propagule pressure, low substratum specificity, fast growth, high morphological plasticity, shading of competitors, advantageous reproductive traits (i.e. high fecundity, self-fertilization, propagules retainment), and effective mechanisms for long-distance dispersal (i.e. high dispersal rate, reproduction during drift) (Engelen et al. 2015). However, most of these traits are also inherent to non-invasive *Sargassum* species. In addition, conversely to many marine introduced species, *S. muticum* is genetically depauperate.

The reasons for the success of this species is unclear and represent an important knowledge gap, particularly regarding the role that is played by the seaweed-associated bacteria. Associated microbiota has been shown to be potentially important in enhancing seaweed adaptability (Arnaud-Haond et al. 2017), and it represents a key issue addressed in this thesis. The mechanisms and processes playing a role in invasions are identified in Chapter 3 on Acclimation vs. Adaptation of Invasive Seaweeds. The review clearly demonstrates that knowledge regarding terrestrial plants is much further advanced and there is a lack of molecular studies on emerged marine invaders. Despite the fact that molecular ecology studies on *S. muticum* and invasive seaweeds are rare, they can provide some useful insights in regard to this issues.

Another knowledge gap lies in the understanding of how success of *S. muticum* may interact with climate change. This is a second issue addressed in the thesis through experimental (bacterial response to acidification) and a modelling approaches. We anticipated that bacterial communities are not stable and that they may have a role in the adaptation to different environmental conditions and invasion process. The given research demonstrated that bacterial communities associated with *S. muticum* experience significant shifts between the seasons and geographic locations. In the North of Portugal and on basal parts bacterial diversity was higher than in the South and on apical parts. Bacterial community composition in summer was very distinct from September and March independent of the location of the study site, mainly due to a strong decrease in *Proteobacteria* and *Actinobacteria*, as well as an increase in *Planctomycetes*. At both locations, seasonal differences were most pronounced in the apical tips and sediments, but the bacteria contributing to these differences differed between locations. Core bacteriomes were highly similar between North and South Portugal in September and March, but not so in summer. Small and large scale spatial differences in associated bacterial communities were most pronounced in summer and apical tissues. These differences have the potential to affect seaweed ecology, fitness and adaptation capacity to environmental changes.

There is a link between the invasion and climate change **(**Dukes & Mooney 1999, Hellmann et al. 2008, Bellard et al. 2013**)**, as climate change is expected to further affect the distribution of *S. muticum* in response to increased temperature and acidification processes. This issue is addressed in the thesis with two contrasting approaches: the role played by bacteria in response to acidification and through the niche modelling of *S. muticum* distribution under different climate change scenarios. The responses of bacteria associated with *S. muticum* indicated that acidification hardly influenced the overall microbiome of *S. muticum* and did not seem to change the relationship between the seaweed and its microbiota significantly. It did however increase the abundance of *Oceanospirillales* and *Vibrionales* that might have consequences for the seaweed. The niche model shows that by 2100, invasion of *S. muticum* is expected to spread further North rather that South. A large northward distribution shift can be expected away from its current southern range. However, the innovative approach considering the reproductive phenology, highlights that part of the future northern distribution of *S. muticum* will have to rely entirely on yearly recruitment from outside as reproduction will not be able to take place in those territories and as the species can't resettle drifting material. In the future, the inclusion of reproduction predicts a much smaller distribution of the species. It is probable that inclusion of this variable would do the same for all organisms used in niche models.

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