ULVA GROWTH, DEVELOPMENT AND APPLICATIONS

by

Fatemeh Ghaderiardakani

A thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

School of Biosciences
The University of Birmingham
August 2019

Abstract

The green marine macroalgae *Ulva* (Ulvophyceae) are common algae distributed worldwide, which play a key role in aquatic ecosystems. *Ulva* species are a potentially valuable resource for food, feed, fertiliser and fuel but can also cause massive nuisance blooms if they grow unchecked.

For correct growth and development, *Ulva* requires the presence of a combination of regulatory morphogenetic compounds released by associated epiphytic bacteria in addition to nutritional parameters. The first results chapter examines the extent of specificity or generality of bacteria-induced morphogenesis in *Ulva*, by cross-testing bacteria isolated from several *Ulva* species on *Ulva* mutabilis and *Ulva* intestinalis. We show that pairs of bacterial strains isolated from *Ulva* species can fully rescue *U. mutabilis* or *U. intestinalis* morphology.

In the second results chapter, activity of algal growth- and morphogenesis-promoting factors (AGPFs) derived from bacteria were estimated in a land-based integrated multitrophic aquaculture system (IMTA) of fish and macroalgae (located at the coastal lagoon Ria de Aveiro, Portugal), using a standardised bioassay with axenic cultures of *Ulva*. Nutrient availability was also assessed in this IMTA system. The study thus informs aspects of the various potential aquaculture-environment interactions. It was observed that both the water from the lagoon (external to the farm system) and the water from the fish pond (input for algae cultures) could completely restore the normal growth and morphology of the macroalga under axenic conditions. The results highlight the presence of a sufficient chemical cocktail of AGPFs in this IMTA system required for growth and morphogenesis of *Ulva*. In addition, the water of fish farming increases the nutrient availability (nitrate and ammonium) needed for macroalgae production. The conclusion of this chapter is that *Ulva´*s sustainable growth and development can benefit from

multitrophic aquaculture systems and shallow water systems, due to the naturally enriched AGPFs and their *in-situ* production by bacteria in intensive algal aquacultures.

In the final results chapter, the effects of *U. intestinalis* extracts on germination and root development in the model land plant *Arabidopsis thaliana* were examined. *Ulva* extract concentrations above 0.1% inhibited *Arabidopsis* germination and root growth. *Ulva* extract <0.1% stimulated root growth. All concentrations of *Ulva* extract inhibited lateral root formation. An abscisic-acid insensitive mutant showed altered sensitivity to germination- and root growth-inhibition. Ethylene- and cytokinin-insensitive mutants were partly insensitive to germination-inhibition. This suggests that different mechanisms mediate each effect of *Ulva* extract on early *Arabidopsis* development and that multiple hormones contribute to germination-inhibition.

Taken together, the results of this thesis highlight: (i) Specific *Ulva*-associated bacterial functions (promoting cell division, or cell differentiation) that cannot be assigned to a specific genus/taxonomic group of bacteria, (ii) an IMTA system ensuring an adequate supply of nutrients and a sufficient chemical mixture of AGPFs for reliable *Ulva* cultivation and (iii) the first-characterised mechanisms to date by which *Ulva* extract can impact germination and growth in *Arabidopsis*.

This thesis and all my academic achievements are dedicated to my mother and father. Without their endless support and encouragement, I would never have been able to complete my graduate studies.

Acknowledgements

I would like to take this opportunity to express my heartfelt gratitude and sincere thanks to all those who helped me to complete my thesis.

Dr. Juliet Coates, my fantastic supervisor, I would like to thank you for agreeing to take me as PhD student, for your regular advice, immense support, thoughtful guidance, critical comments, correction of my papers and thesis and eventually for boosting my confidence throughout the entire PhD journey. I feel so lucky to have you as my supervisor.

Dr. Thomas Wichard, I thank you from the bottom of my heart for accepting me as visiting researcher in your lab and mentoring my experiments at the Friedrich Schiller University Jena. You have been a constant source of motivation, encouragement and inspirational guidance. You introduced me a sense of discipline and attention to details in my work. Thanks for the time you spent to answer my questions and all discussions.

Xulyu Cao, Clare Clayton and Alexandros Phokas, I would like to thank you as my fantastic friends for sharing your knowledge and all cheerful time we spent together. I would also like to thank my lab mates in Jena, Ralf Kessler, Anne Weiss, Jan Frieder Mohr and Gianmaria Califano for your help and advice.

Dr. Daniel Gibbs, I am grateful to you and your wonderful group for supporting me. Thanks to Dr. Helena Abreu and all amazing members of ALGAplus company for your assistance, support and advices to perform my investigation in Portugal and for that particular dream you helped to come true.

Finally, a big thank you to my sister and brothers, for those lots of support you all gave me during my long educational journey, to my mother-in-law and father-in-law for your understanding and sacrifices, to my sisters-in-law and brothers-in-low, you have been a source of strength and motivation during moments of distress and discouragement and to

Reza, for putting up with me at home throughout my PhD and for all seaweed collecting journeys.

I would like to acknowledge the Islamic Development Bank for funding my PhD studentship, networking support by the COST Action ‘Phycomorph’ FA1406 through Short Term Scientific Missions, the British Phycological Society, International Phycological Society, Estuarine and Coastal Sciences Association and Society for Experimental Biology for travel grants that enabled me to present my research in the UK, Denmark, Poland and Italy.

Contents

List of figures

List of tables

CHAPTER 1:
INTRODUCTION

1.1 Phylogeny of macroalgae

Seaweeds or marine macroalgae are sessile multicellular photosynthetic eukaryotes. They form a diverse and ubiquitous group of oxygenic photosynthetic organisms that commonly occupy a wide range of habitats (aquatic and some terrestrial habitats), including salt and freshwater, wet soil and hot springs (Egan et al., 2013). The importance of seaweed's role in both terrestrial and marine ecological investigations has been extensively studied. Algae have been established as model organisms in variety of research (e.g. *Ulva mutabilis* (De Clerck et al., (2018), *Ectocarpus siliculosus*, Cock et al., (2010), *Chondrus crispus*, Collen et al., (2013), *Pyropia yezoensis*, Nakamura et al., (2013)). The high diversity and complexity of their life histories raises a great deal of fundamental questions about the science of evolutionary biology (Bhattacharya and Medlin, 1998).

Macroalgae are differentiated from plants due to the absence of true specialised tissues (e.g. root system, internal vascular structures, seeds or flowers) (Graham and Wilcox, 2000). There are approximately 9,000 species of macroalgae commonly divided into three evolutionarily diverse groups based on their pigmentation: brown algae (Phaeophyta); green algae (Chlorophyta) and red algae (Rhodophyta) (Khan et al., 2009).

Algae are the plant taxa (Figure 1.1) that preceded embryophytes (informally called land plants) in the phylogeny (Bennici, 2008). Based on the findings of plant evolutionary developmental biology, land plants arose from a green algal ancestor: this was a pivotal event in the history of life and has directed substantial changes in the global environment and defined the evolutionary origin of the whole terrestrial ecosystem during the early Paleozoic (Kenrick and Crane, 1997, Leliaert et al., 2012). Some lines of evidence indicate that the establishment of an endosymbiotic relationship between cyanobacteria and a heterotrophic eukaryotic host cell formed the first identifiable photosynthesising

eukaryote. In fact, this cyanobacterium later became firmly integrated and eventually turned into a plastid (Archibald, 2009, Keeling, 2010, Leliaert et al., 2012).

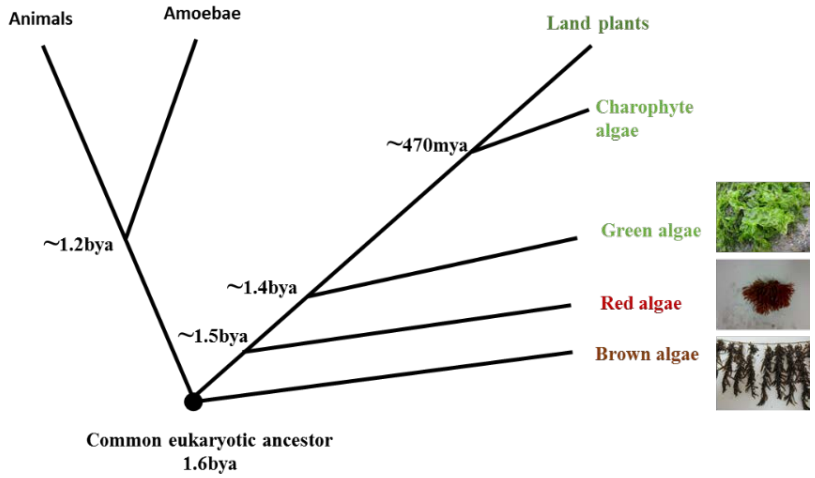

Figure 1.1 Eukaryotic phylogenetic tree demonstrating the position of green, red and brown seaweeds. All evolved from a common ancient ancestor. Brown alga are a separate lineage to red/ green algae and land plants. Figure adopted from (Coates and Charrier, 2015) and modified.

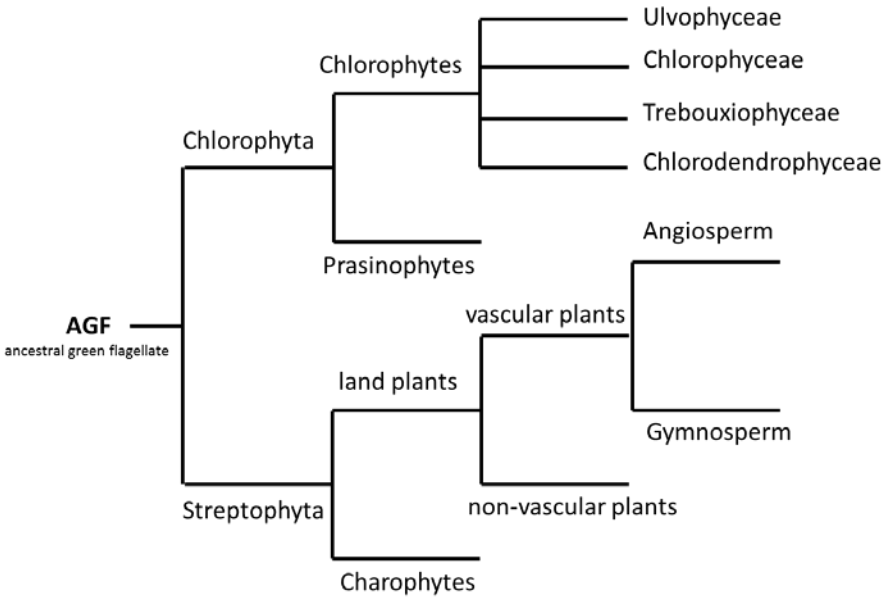

Figure 1.2 Summary phylogeny of the green lineage. Phylogenetic tree summarising relationships among main lineages of green plants (Viridiplantae). The Viridiplantae consists of two major phyla: Chlorophytes and Streptophytes and green algae including Ulvophyceae diverged/evolved from Chlorophytes.

1.2 The functions and benefits of macroalgae

1.2.1 The ecological importance of macroalgae

Macroscopic marine algae (seaweeds) are significant primary producers in the oceans, which cover about 71% of Earth's surface. Seaweeds are known as 'ecosystem engineers' due to their critical roles in marine environments, where they modulate the supply of resources to other species and alter the physical state of the surrounding environment, including sediments and water flow (Jones et al., 1994). Seaweeds make a major contribution to maintaining local biodiversity (Schiel and Lilley, 2007), create a protective environment for numerous invertebrate species (Wilson et al., 1990, Bulleri et al., 2002) and provide an essential habitat for a range of epibionts (organisms that live on the surface of other organisms' bodies) from microscopic organisms to macroinvertebrates (Fraschetti et al., 2006, Burke et al., 2011b).

It has been reported by Murphy et al. (2000) that a larger variety of associated marine species (such as bay pipefish, gunnels, perch and juveniles of commercial species including salmonids, rockfish, gadids and flatfish) have been supported by macroalgal populations compare to habitats where macroalgae are not exist. This support is suggested to be in the forms of providing food and/or covering from predators (Simenstad 1994).

Solid negative effects on the local environmental conditions and benthic (lowest-level of the water) community were reported following the complete removal of the fucoid alga *Hormosira banksia* from the mid-intertidal areas of moderately exposed rocky coasts in southern New Zealand over two years (Lilley and Schiel, 2006). Naturally, these habitat-dominant algal species form a dense protective canopy layer on the mid regions of the shore. After removal of this algal canopy, the abundance of about half of the species in *H. banksii* beds was lost or significantly reduced compared to controls (Lilley and Schiel,

2006). These species were either epifauna associated with *H. banksii* or understory benthic species. It seems that understory species are able to survive in the mid-shore areas only by relying on a protective and established canopy-forming alga. It has suggested that during low tide and periods of emersion (desubmergence) the algal canopy can decrease the heat- and temperature stress for these delicate species including other fucoid alga (e.g. *Cystophora* spp., *Dictyota* spp. and *Ulva* spp.) (Lilley and Schiel; 2006; Schiel and Lilley, 2007).

In parallel, besides the significant role of marine macroalgae in ecosystems as well as the bio-based economy, some species are responsible for important environmental hazards. Due to eutrophication and habitat alterations caused by human activity, seaweeds are considered a significant causative factor of massive green, golden or red "tides" and also toxic and noxious blooms that have harmed global aquatic ecosystems, causing decreases in biological diversity due to oxygen deficiency, chemical pollution, nutrient imbalances and ocean acidification (COST, 2014). In addition, they can cause economic issues for the tourism industry, aquaculture facilities and traditional fisheries (Smetacek and Zingone, 2013). For instance, upon the algal blooming phenomenon if, the biomass is not collected quickly, seaweeds can decompose and become foul-smelling. The rotten biomass can generate toxic hydrogen sulphide gas (Schramm, 1996), which leads to unfavourable and harmful influences on the affected shores (Smetacek and Zingone, 2013). Moreover, the consumption of dissolved oxygen through the decay process leads to "hypoxic" (low oxygen) conditions which have deleterious consequences on fish and invertebrates as well (Buapet et al., 2008).

Typically, only a few genera of macroalgae are known as responsible for the massive algal tides. The role of two specific genera is apparent in the majority of algal blooms: floating species of *Ulva* and *Sargassum* are the main causative species in "green tides" and "golden

tides" respectively (Smetacek and Zingone, 2013, Blomster et al., 2002, Laffoley et al., 2011).

Making a step-change in the knowledge of basic algal biology is needed to meet actual and future demands in this field (COST, 2014). In order to manage the algal blooms that happen as a result of a complex contingency of situations such as anthropogenic eutrophication, environmental elements (i.e. geographical locations or hydrodynamic conditions etc) and the principle biological characterisations of algae species, it is necessary to gain an in-depth and comprehensive knowledge about the growth dynamics involved in tidal events as well as the influences of chemical, physical and biological factors and their interactions on coastal ecosystems. This new knowledge could be counted as a requirement for establishing and emerging cost-effective mitigation approaches to deal with the problems between human and nature. Additionally, such knowledge provides useful information that is necessary to find ways for using algal biomass as an alternative valuable resource e.g. conversion to biofuel, biogas or making algal extract (Smetacek and Zingone, 2013, Liu et al., 2013, Milledge and Harvey, 2016).

1.2.2 Nutritional and biomedical values of macroalgae

In a commercial context, there is increasing interest in the use of marine biomass worldwide with multiple traditional and novel applications in food, feed, fuel, high-value chemical and pharmaceutical industries and also in aquaculture, which is one of the promising market sectors (Kraan, 2013).

According to the United Nations report (2015) "World Population Prospects: The 2015 Revision", the world population is estimated to reach 9.6 billion by 2050 (DESA, 2015). To meet this huge future challenge for feeding our planet, as well as decreasing the

pressure on natural resources to keep them safe for next generations, all agriculture sectors including crops, livestock, fisheries, aquaculture and forestry make a huge contribution.

Seaweed has been known a source of nutrition and health for human for thousands of years (since between 14,220 and 13,980 years ago) according to the archaeological evidence found in Chile (Dillehay et al., 2008). About nine seaweed species were recovered from this archaeological site, and interestingly, four of these seaweed species, *Durvillaea antarctica*, *Porphyra* sp., *Gracilaria* sp., and *Sargassum* sp., are edible and presented some important medicinal effects (Dillehay et al., 2008).

Out of 221 species of algae that are used today, about 145 species (66%) are consumed directly as food including 79 species of red algae, 38 species of brown algae and 28 species of green algae. 101 species are utilised in phycocolloid industry and 24 species in traditional medicinal uses (Pereira, 2011).

In 2012, about 9 million tonnes of the 23.8 million tonnes of cultivated seaweeds (‘aquatic plants’) worldwide were consumed as human food. The equivalent figure reached to over 30 million tonnes in 2016 (FAO, 2018). In these studies, only forms recognisable as seaweed were counted, appliction of phycocolloids (e.g., agars, alginates, carrageenans) to foods was not included (FAO, 2014). While the incorporation of seaweeds into Japanese diets is ranging from 9.6 (in 2014) to 11.0 (in 2010) g per day (Wells et al., 2017, MHLW, 2014), the main application of macroalgae in the western countries is using as hydrocolloids, thickening or gelling agents and stabilizers, in foods and beverages. Therefore, seaweeds remain unexploited as food resources in the West (Pereira, 2011).

Algal species contain a highly variable nutritional composition, which consequently will affect their dietary value. Some parameters such as the species, geographical area and harvest season, in addition to external factors such as water, temperature, irradiance, light

quality, salinity and nutrient concentration in the water are involved in the variability in nutritional composition of algae (Makkar et al., 2015). For example, the concentration of amino acids is highest in red algae followed by green and brown algae, respectively. Amino acid contents of seaweeds collected during the autumn is lower compared to the spring (Gillard et al., 2018).

According to Wells et al., (2017), some aspects of the health benefits of food products with algal origin are not investigated yet. These aspects include:

(i) Comprehensively assessing the biological availability of different fractions of algal food products to humans and detecting which parameters impact how nutritional components get released from the food matrix. These parameters are highly diverse, ranging from processing and preparation methods, the effect of saliva and different enzymes in the stomach and small intestine involved in digestion process, to genetic variation of microbial flora in the gut.

(ii) elucidating the interaction between algal nutritional and functional elements and complex human metabolic systems and intermediary pathways involved (Reviewed in Wells et al., 2017).

There is extensive evidence for the health values of seaweed-based food products. Beneficial and possible negative influences of these products are still largely qualitative. For example, algal proteins and derived peptides, in addition to carbohydrates and minerals, may benefit humans by balancing the intestinal mucosal barrier function (Cian et al., 2015). By showing prebiotics property algal-derived food can modulate intestinal epithelial cell, macrophage and lymphocyte proliferation and differentiation (Cian et al., 2015). Moreover, indirectly by promotion of bacterial responses, seaweed-based foods can play a role in regulating immune system responses (Cian et al., 2015). Even though

quantifying the potential “nutritional or functional food value” of seaweed-based products and their possible beneficial or harmful impacts on human health is quite challenging, it needs to be investigated substantially using case-by-case basis research and new experimental and collaborative experiments (Wells et al., 2017).

Marine macroalgae are dietary sources with rich iodine contents. Consuming seaweed is beneficial to health and can reduce the risk of iodine deficiency disorders include goitre (swelling of the thyroid gland) (Yeh et al., 2014). The recommended amount of iodine intake for adults is 150 µg per day in United States (Yeh et al., 2014). The average of iodine contents in dried *Porphyra* sp., *Undaria* sp., and *Laminaria* sp., were measured as 36.9 mg/kg, 139.7 mg/kg, and 2523.3 mg/kg, respectively (Yeh et al., 2014). So obviously *Laminaria* species could be excellent potential biosources for iodine. *Laminaria* species traditionally have been consumed extensively as a health supplement for medical purposes such as myxoedema and treatment of hyperactive thyroid gland and goitre (Müssig, 2009; reviewed in Holdt et al., 2011).

The inhibitory effect of iodine on breast tumour progress based on experimental results is supported by the relatively low occurrence of breast cancer in Japanese women who have a nutritional diet containing seaweeds with high amounts of iodine (Smyth, 2003). Regarding the link between iodine content of seaweeds and cancer, it has been reported that an aqueous extract of *Undaria pinnatifida* (mekabu) suppressed tumourigenesis in rats with carcinogen-induced mammary tumours (Funahashi et al., 2001). Although the mechanism of mekabu extract was not defined clearly, less occurrence of mammary tumours and significantly smaller tumours observed in rats treated with mekabu extract. Moreover, i*n vitro* results showed that apoptosis was induced significantly in three types of human breast cancer cell lines. No significant adverse impact (apoptosis induction) was detected in human normal mammary cells during this experiment (Funahashi et al., 2001).

Considering the reported apoptosis induction effects of mekabu treated groups, the authors suggested that iodine might cause increasing activity of superoxide dismutase (one of very important cellular antioxidants against reactive oxygen species (Younus, 2018, Funahashi et al., 2001).

It is important to note that, foods with a large proportion of iodine could also lead to serious concerns such as iodine-induced toxic effects. Crawford et al., (2010) reported iodine toxicity caused thyroid dysfunction in adults who used soy milks which were fermented in seaweed (*Laminaria* sp.) to improve the flavour in Australia. Following a series of reports of hypothyroidism and clinical events, these products were recalled in Australia and New Zealand in 2009.

Algal-derived food products are considered as “functional food” or “nutraceuticals”. Apparently, there is no official definition for functional food, but this term is being used widely in marketplace (Wells, et al., 2017). According to Madhusudan et al., (2011) a functional food is a food given potential positive effects in physiological functions, improves optimal health and well-being and/or helps to eliminate the risk of disease. The main reason behind the widespread interest in using seaweeds for their functional food potential is that in addition to basic nutritional values (e.g. variety of vitamins and mineral elements), they are rich sources of various compounds with biological activities (Reviewed in Wells, et al., 2017; Reviewed in Mariya and Ravindran, 2013; Holdt and Kraan, 2011). Dietary fibre (a group of edible plant polysaccharides that cannot be hydrolysed by human digestive enzymes), long-chain polyunsaturated fatty acids, sulphated polysaccharides and halogenated compounds are in the list of algal bioactive metabolites (Mariya and Ravindran, 2013, Holdt and Kraan, 2011). So, they may benefit human health by delivering several physiological functions such as anti-hypertensive, anti-oxidant or anti-inflammatory effects along with antibacterial, antiviral, antitumor and antioxidant

activities (Goldberg and Hasler, 1996, Madhusudan et al., 2011; reviewed in Mariya and Ravindran, 2013).

1.2.3 Seaweed aquaculture

Based on available data collected by the Food and Agriculture Organisation of the United Nations (FAO), the significant role of the fisheries and aquaculture at different economic levels (national, regional and global) in promoting health, the contribution that small-scale aquaculture makes to poverty elimination and food security cannot be neglected. This role especially is quite important in developing countries (FAO, 2010). Interestingly from developing countries, such as China, Thailand and Vietnam, net exports of fish and fishery products are higher than net exports of several other agricultural commodities, like rice, meat, sugar, coffee and tobacco (FAO, 2010).

It should be considered that according to the data recorded and presented by the FAO reports, aquaculture involves not only “food fish” but also aquatic algae (mostly seaweeds) and non-food products (e.g. pearls and seashells for ornamental and decorative uses). Food fish includes finfishes, crustaceans, molluscs, amphibians, freshwater turtles and other aquatic animals such as sea cucumbers, sea urchins, sea squirts and edible jellyfish, which are produced for the projected use as food for human consumption (FAO, 2014).

In the past decade, aquaculture has become one of the fastest-growing food producing sectors (human and animal food) in the world (Skladany et al., 2007). The production of aquatic plants in China almost doubled between 2000 and 2012. During the same time, world production of farmed algae also increased twofold and reached 23.5 million tonnes (US$ 6.4 billion) (FAO, 2014).

Numerous advantages have been offered by only this part of aquaculture (seaweed production). Seaweeds do not compete with terrestrial food and biofuel crops for arable

land, fertiliser and freshwater resources. Macroalgae can grow very fast (which may reach several meters in length through one year), in a wide range of temperatures (from tropical to polar climates), using only sunlight, atmospheric carbon and nutrients naturally present in sea waters. Furthermore, several seaweeds produce more biomass per square metre than land plants (e.g. *Saccharina latissimi* with 26 tonnes dry weight per hectare per year, compared to 2.1, 4.1 and 5.1 for soybean, wheat and maize respectively; (Broch and Slagstad, 2012)).

However, the aquaculture sector, which provides at least 50 percent of animal protein to millions of people in low-income countries – is already under multiple pressures, such as overfishing, habitat loss and water pollution (McGuire, 2015). Achieving the long-term goal of economic, social and environmental sustainability in this sector depends on formulation and implementation of well-informed policies, proper strategies and plans by governments. So, regarding food demands, aquaculture must now expand, intensify and diversify.

Talking about this specific part of aquaculture, seaweed production, to translate these (mentioned above) goals into practical action, using applied research is one of promising approaches (FAO, 2010). So obviously a deep understanding of the biology and physiology of algae, in particular their development, morphogenesis, life cycle regulation and life-history strategies are fundamental to us being able to engineer new and useful biological systems. For example, better control of the different developmental steps in the seaweed lifecycle will lead to better seaweed management in an algal cultivation system and to eradicate ecological harm and environmental pests. The development of comprehensive sequenced genomes for algal model organisms such as *Ectocarpus siliculosus* (brown algae) (Cock et al., 2010), *Chondrus crispus* (Collén et al., 2013),

Pyropia yezoensis (Nakamura et al., 2013) will facilitate further progression to a functional knowledge rather than a descriptive understanding.

It is also becoming clear that different species of seaweeds naturally harbour a rich diversity of epiphytic bacteria with functions related to host growth and morphological development (Provasoli and Pintner, 1980, Marshall et al., 2006, Wichard, 2015a, Ghaderiardakani et al., 2017). So, using filtered seawater (or axenic medium) in scientific investigations and even in commercial aquaculture (e.g. land-based aquaculture operations) may directly affect the success of biomass production due to the morphogenetic effect of bacteria on the growth and development of seaweeds (e.g. green macroalgae like *Ulva* spp.) (Grueneberg et al., 2016).

Therefore, beside the necessity of translation of this investigative research into practical outcomes, the challenges lie in applying more emerging sustainable, cost-effective and lucrative techniques to grow, harvest, transport and process algae in large-scale aquaculture settings (Kraan, 2013).

The following case study is described to further emphasise the importance of applying basic knowledge to address practical challenges in the real world. Gao (2016) highlighted the damaging effects of periodic reproduction leading to growth and productivity fluctuations, as one of the main technical problems restricting the extent and scope of seaweed (*Ulva rigida*) cultivation. To tackle this issue, three "innovative techniques" were suggested: (i) Optimising the growth conditions of *Ulva*. This combination of growth parameters has been recommended: "a light intensity of 80 μmol photons m^{-2} s^{-1}; 150 μM N and 7.5 μM P; 12^{o}C, and an aeration rate of 100 L $minute^{-1}$ for 16 hour day^{-1}". (ii) Applying a multiple-harvest approach to collect low-productive thallus tissue (including potentially reproductive parts before spore formation) and decaying thalli fragments.

Consequently, the multiple harvest regime helped to keep a high growth rate of *Ulva rigida* during long-term farming. (iii) Developing a "sterile strain" or a mutant strain of *U. rigida* that could demonstrate high growth rate and low reproduction levels over long-term cultivation, using a combination of different doses of UV radiation and chemical treatments (Gao, 2016).

1.3 The genus *Ulva*

Ulva is a cosmopolitan macroalgal genus, the main multicellular branch of the Chlorophyte algae, and the most abundant Ulvophyceae representative (Hayden et al., 2003, Guiry and Guiry, 2014). The genus *Ulva* was one of the first taxonomic descriptions used by Linnaeus (1753) for algal classification. In the first place, it involved a range of algae we now know are unrelated to *Ulva* such as *Fucus* (a brown alga), *Conferva* (now the brown alga *Ectocarpus*) and *Chara* (a freshwater Charophyte alga). Later, after about 100 years, *Ulva* was reclassified into distinct genera, one of which was *Enteromorpha* (Link, 1820) that included the green tubular seaweeds (Hayden et al., 2003). The *Ulva* genus was retained for green seaweeds with distromatic (i.e. two-cell thick) thallus, and monostromic (i.e. one-cell thick) blades in the form of a hollow tube were documented as the *Enteromorpha* Link (Hayden et al., 2003). Investigating phylogenetic relationships based on the Ribulose-1,5-bisphosphatecarboxylase/oxygenase large subunit (*rbc*L) gene and nuclear ribosomal DNA internal transcribed spacer (ITS) sequences analyses, together with former molecular and culture data, determined that *Ulva*, *Enteromorpha* and *Chloropelta* are not separate evolutionary entities. So, they should not be known as distinct genera. Since *Ulva* was the oldest genus, species in *Enteromorpha* and *Chloropelta* genera were reclassified as *Ulva* (Hayden et al., 2003).

1.4 Identification of *Ulva*

Ulvophyceae form a group of multicellular algae (Figure 1.2) with some typical properties of multicellular land plants e.g. they contain pigments including chlorophyll that trap light from the sun and they are covered by a variety of different types of extracellular matrix consisting of various polysaccharide and proteoglycan components such as Cellulose, β-mannans, β-xylans, sulfated polysaccharides (Domozych et al., 2012)), although they are morphologically more simple than land plants. These are opportunistic organisms, having a potential for rapid and proliferous growth (Blomster et al., 1998). Although *Ulva* species are primarily marine taxa distributed widely in marine and brackish environments around the world (brackish water is water that is saltier than fresh water but not as much as seawater, normally in intertidal and estuarine habitats), they can also proliferate in freshwater habitats.

One of the essential concepts for comparison of all biological sub-categories is that of a species, which is apparently a controversial issue not just for its precise definition but also for distinguishing the boundaries and numbers of species (De Queiroz, 2007). Twenty-four different theoretical concepts for the species have been documented by Mayden (1997). According to biological concept, the property of species is "interbreeding (natural reproduction resulting in viable and fertile offspring)" (Mayr, 1999, Wright, 1940, Dobzhansky, 1950; reviewed by De Queiroz, 2007).

In terms of *Ulva*, the issue of species concept itself has been added to a problem relating to species identification, because of high phenotypic plasticity of many members of Ulvales which resulting in a "historical misinterpretation" in species level of *Ulva* (Steinhagen et al., 2019b).

Identification of *Ulva* species from morphology is quite challenging due to extensive variability of physiological and morphological traits and also a high degree of intraspecific variation and phenotypic plasticity presented by many members (Lobban and Harrison, 1995) Moreover, few reliable characteristics are available for differentiating taxa and morphological differences between species are small and difficult to detect. In fact, the morphological and cytological features which are used in classification are profoundly affected by environmental conditions (such as season, wave energy, latitude and geographical location), age of the thallus and life style even within an individual population at a certain time (Heesch et al., 2009, Wolf et al., 2012). For instance, the appearance or nonappearance of branching was the most convenient character distinguishing two *Ulva* species, *U. intestinalis* and *U. compressa*, but there was an element of vagueness and uncertainty because branching in *U. intestinalis* could be induced by low salinity or salinity shock (Blomster et al., 1998).

Because of the problems with identification of members of this genus, many species names have been misapplied (Silva et al., 1996) and this has caused the combination of species under a single name or the allocation of a single species to two or more *Ulva* species. Due to all these limitations with morphological identification only, the application of molecular methods along with detailed anatomical data has been supposed to pave the way for more reliable outcomes to resolve the taxonomic problems.

To show the plasticity of *Ulva* species' morphogenesis which might result from environmental drivers or symbiont-dependent development, we can refer to morphological plasticity of *U. compressa* in northern Germany, North Sea and Baltic Sea besides misidentification of *U. compressa* and *U. mutabilis* (Steinhagen et al., 2019a, Steinhagen et al., 2019b).

Typically, the thallus's unique tubular shape and its branching pattern were considered a distinguishing factor for the taxonomic identification of *U. compressa*, but this is now deemed controversial (see references in Steinhagen et al., 2019b). As it has been noted earlier, many researchers believed that branching patterns cannot be a solid parameter for taxonomic classifications as branching can be affected by external factors such as salinity (Blomster et al., 2002, Steinhagen et al., 2019b).

By using plastid-encoded genetic marker *tuf*A as a genetic approach taken together with morphological observations, Steinhagen et al., (2019) identified both attached and branched tubular form of *U. compressa* in the North Sea, whereas in the Baltic Sea, *U. compressa* specimens were observed with a very distinct morphology of unattached and sheet-like thalli.

When for the first-time blooms of unattached *U. compressa* U. Linnaeus 1753 with an atypical sheet-like morphotype were observed in the German Baltic, Steinhagen et al., (2019a) reported that this nuisance alga is conspecific with the type strain of *U. mutabilis* Föyn 1958 from Faro in Portugal. The results from comparisons on vegetative and reproductive features of *U. mutabilis* and German *U. compressa* as well as interbreeding experiments of gametes of these two species highly support the monophyly of *U. compressa* and *U. mutabilis*, which can be treated as the same species (Kostamo et al., 2008, Steinhagen et al., 2019a).

1.5 *Ulva*'s life cycle

In order to understand seaweed biology as well as the biology of land plants, we need to establish cultures under laboratory conditions (controlled light intensity, light quality, photoperiod, temperature, inorganic and organic nutrients, osmotic stress, pH of the medium). These studies rely on tractable model systems with robust methodological

procedures, but this is challenging in seaweeds for several reasons. The type of nutrient supply influences of laboratory conditions on the uptake and growth responses of seaweeds, and propagation under controlled conditions are some of the major prerequisites for successful exploitation of seaweeds in the laboratory.

A detailed description of *Ulva*'s life cycle (Figure 1.3) was an additional crucial step in order to establish cultures under laboratory conditions. An isomorphic alternation of generations occurs, with gametophytic and sporophytic multicellular generations with the same general morphologies during life cycle progression in all *Ulva* species, according to historic research on *U. mutabilis, U. rigida* and *U. lactuca* (Føyn, 1958, Phillips, 1990, Wichard and Oertel, 2010). The haploid gametophytic plants produce biflagellate haploid gametes through mitosis, and diploid sporophytes release haploid zoospores with four anterior flagella (at a rate of 105-106 per plant per day) through meiosis (Maggs and Callow, 2003). In fact, *Ulva* gametophytes and sporophytes can be distinguished by the type of released zoid. There is an identical pattern for the development of both gametophyte and sporophyte (Løvlie and Bryhni, 1978). Diploid sporophytes initiate from the mating of a plus- and a minus mating-type gamete. Haploid gametophytes can reproduce either from meiotically-formed haploid zoids of sporophytes, by germination of unfused gametes or from diploid parthenosporophytes arising from unmated gametes by spontaneous diploidization, which usually occurs at later growth stages. Their zoids are only from one mating type and may later develop into new haploid gametophytes (Føyn, 1958, Hoxmark, 1975, Løvlie and Bryhni, 1978, Phillips, 1990). Reproductive activities mainly happen along the margins of *Ulva* fronds or at damaged parts of the thalli (Stratmann et al., 1996).

Figure 1.3 *Ulva* isomorphic life cycle.

All *Ulva* species exhibit two isomorphic phases: gametophyte (n) and sporophyte (2n). Mature gametophytes release both male and female haploid gametes (or sexual cells) with two flagella and noticeable eyespots. Fusion of opposite mating type gametes leads to form a zygote. Two arrows show the parthenogenetic development of gametophytes resulted from unfused gametes. Diploid sporophytes develop from the zygotes. The sporophyte phase releases swimming haploid spores after meiosis division. Following discharging the spores, they grow and give rise to gametophytes (n) again. The haploid (n) stages are indicated by blue and diploid (2n) stages by green colours.

1.6 Features of the *Ulva mutabilis* life cycle

More recently the *U. mutabilis* 'slender' mutant is often used for experimental investigations (Løvlie and Bryhni, 1978) mainly because it displays a much shorter developmental cycle and is amenable to laboratory culture. Following the analysis of the vegetative cell cycle by radioactive labelling with ^{14}C-uracil, Stratmann et al., (1996) demonstrated that the transformation of vegetative thalli into fertile gametangia/sporangia is controlled by two regulatory factors or sporulation inhibitors (SIs): a high molecular weight cell wall glycoprotein (SI-1) and a low molecular weight factor (SI-2) in space between two cell layers of the thallus. Apparently, SI-2 keeps the more basal sections of blades in the vegetative state ensuring that differentiation only happen in the blade's apical parts, through forming an internal vertical concentration gradient between the two-cell layers of blade (Stratmann et al., 1996, Wichard and Oertel, 2010).

Differentiation of vegetative blades initiates only if both SI-1 and SI-2 levels descend below inhibitory levels or they are not perceived by the alga anymore. One of the advantageous features of *U. mutabilis* slender is that the induction of gametogenesis can be artificially induced in the laboratory by removal of the both sporulation inhibitors: this can be induced by cutting the thallus into single-layer fragments and subsequently washing and transferring them into fresh medium (Stratmann et al., 1996, Wichard and Oertel, 2010). Gametogenesis induction in a laboratory environment was also successfully shown in *U. intestinalis* (Ghaderiardakani et al., 2017, Vesty et al., 2015). So this regulatory mechanism might have significant role in the dynamics of green tide blooms as fragmentation reduces the levels of the inhibitors, which leads to subsequent propagation of *Ulva* and this has been documented by observation of *Ulva prolifera* propagation during algal blooms (Gao et al., 2010).

Wichard and Oertel (2010) provided a detailed description of the time course of gametogenesis using scanning electron microscopy (SEM) in *U. mutabilis*. Katsaros et al., (2017) also explained the mechanisms involved in gametogenesis by investigating the fine structural transformations occurring throughout developing gametangia specifically focused on cell wall differentiation and the microtubule cytoskeleton organisation. The length of cell cycles in vegetative blade cells is precisely one day and these cycles are perfectly tailored to the natural day-night cycles. The gametangium differentiation is controlled by circadian rhythms as well. In the defined procedure (Stratmann et al., 1996) induction preferably is performed at noon, in the middle of the G1 cell-cycle phase. The very first assessable response is an arrested S-phase in the evening, which is followed by releasing a substance (swarming inhibitor (SWI)) into the medium throughout the whole differentiation process. Generally, SWI prevents gamete (or spore) release once gametes (or spores) have been formed three days later. Around second day's noon after induction (removal of sporulation inhibitors through mincing and washing of mature thalli) when the thallus cells are irreversibly devoted to the differentiation, the chloroplasts became reoriented, and conical cell projections or papillae appeared towards the exterior surface of the blade cells. During the following night the papillae transformed to "capped" pores, whereas all progametes matured into sixteen entirely differentiated biflagellate gametes. On the third morning after induction, the plug-shaped caps of the conical cell wall projections were continually removed and the open exit pores became visible. Due to the presence of SWI in the surrounding media, the mature gametes remained stacked inside the cells despite open pores and exposing to light. They were motionless, connected together by thin cytoplasmic bridges. Upon removal of the SWI and receiving light signals, the gametangia were discharged quickly and the mating possibility was increased (Stratmann et al., 1996, Wichard and Oertel, 2010, Katsaros et al., 2017).

1.7 The ecological and economic importance of *Ulva* species

1.7.1 Ecological and economic benefits

Economically speaking, species within *Ulva* genus are considered as a novel food with many nutritional values, and also for their diverse uses in industry and medicine for different aims (Kraan, 2013). Accordingly, a brief explanation of two *Ulva* spp. potential applications, is as follows.

1.7.1.1 Ulvan, a unique polysaccharide found in *Ulva* genus with potential values in biotechnology and industry

With today's growing attention on new renewable and valuable sources (especially marine resources) of chemicals and polymers, *Ulva*'s underexplored (but massive in bloom events) biomass is an essentially untapped source waiting to be discovered. Among the polymers synthesized by members of Ulvales, many current studies focused on ulvan, a water-soluble polysaccharide mainly extracted from cell wall (Lahaye and Robic, 2007).

The name ulvan came from "ulvin" and "ulvacin", the terms defined by Kylin (1946) to refer to different fractions of water-soluble sulfated polysaccharides of *U. lactuca* extract (Lahaye and Robic, 2007). The main repeating disaccharide unit identified in ulvan structure is β-D-glucuronosyluronic acid (1→4) L-rhamnose 3-sulphate, named as aldobiouronic acid (Paradossi et al., 1999). Based on different extraction and purification methods, the ulvan extraction yield varies from 8% to 29% of dry weight of the seaweed biomass (Lahaye and Robic, 2007).

Due to ulvan's structural diversity, functional characteristics and special physicochemical, rheological and biological properties, this polysaccharide has been introduced as a

promising potential option for a range of innovative applications: in the chemical industry, to supply rare sugars to produce fine chemicals; in the pharmaceutical industry, to provide iduronic acid that is a prerequisite to the synthesis of heparinoid anticoagulant (to prevent blood-clots formation) substances; in the food industry, to be utilised in functional foods and enrich human nutrition; in the agricultural industry, to be used as elicitors (Lahaye and Ray, 1996, Lahaye and Robic, 2007).

1.7.1.1.1 Elicitor activity of ulvan

Growing concerns about the environment and public health, plus increasing durability of disease-resistance genes and consequently the rising risk of disease-resistance breakdown in commercial varieties of crop plants, have been resulted in research interest to develop an efficient, eco-friendly approach for controlling plant diseases and a potential substitute to the current economically costly and environmentally undesirable chemical disease control methods (Thakur and Sohal, 2013).

Elicitors are compounds involved in activating chemical defence responses during plant-pathogen interactions (Cluzet et al., 2004; Thakur and Sohal, 2013). *Ulva* extract has been considered as a potential elicitor: an alternative option for disease control in agronomic crops (Cluzet et al., 2004; Thakur and Sohal, 2013). Cluzet et al., (2004) used microarray analyses for the first time to describe the elicitor activity of *Ulva* extract on the model legume, *Medicago truncatula*. According to the results of this research, when *Ulva* extract inserted into plant tissues or sprayed on the plant's leaves, the extract acted as an effective elicitor of an extensive range of defence responses *in M. truncatula*. Additionally, this research showed that pre-treatment of *M. truncatula* with the *Ulva* extract led to an almost complete protection against the pathogenic fungus *Colletotrichum trifolii*, well-known as the main causal agent of anthracnose disease (Cluzet et al., 2004).

Through later studies it has been suggested that ulvan (known the main component of *Ulva* extract (Berri et al., 2017) is able to act as a plant protectant in *Arabidopsis thaliana* against *Alternaria brassicicola* infection via a mechanism that is dependent to NADPH oxidase but independent to hypersensitive reaction (de Freitas and Stadnik, 2015). de Freitas and Stadnik (2015) showed that although the mycedial growth of *Alternaria brassicicola* (a very common plant pathogenic fungus causes black spot disease on most *Brassica* species) on *A. thaliana* was not completely inhibited by foliar spraying of ulvan, the "severity" of infection was declined by 90% in wild type as well as mutant, *AtrbohF*, plants. The severity of disease was analysed by measuring the percentage of necrotic area on an infected leaf. Moreover, it has been reported that spraying of ulvan resulted in an elevated-NADPH-oxidase activity as well as enhanced-hydrogen-peroxide levels (de Freitas and Stadnik, 2015).

The potential use of ulvan as elicitor to induce the plant defence response was previously shown for treated bean plants against rust disease caused by the fungus *Uromyces appendiculatus* (Borsato et al., 2010) and anthracnose disease caused by the fungus *Colletotrichum lindemuthianum* (de Freitas and Stadnik, 2015). Ulvan enhanced defence-related enzyme's activities, namely the activity of glucanase and then peroxidase in bean plants after infection (de Freitas and Stadnik, 2015).

The plant protectant activity of ulvan was also shown to be identical with the transcriptomic signature that was observed in methyl jasmonate-tre*ated Medicago trunc*atula plants (Jaulneau et al., 2010). Based on the results of different tests including the "protease inhibitory activity" assessment and "hormonal profiling", it has been shown that jasmonic acid contributes to ulvan response (Jaulneau et al., 2010). Together with the results of expression of defence genes in response to ulvan treatment in *A. thaliana* wild

type and its hormone mutants, the authors outlined that ulvan-induced defence in plants happened through the jasmonic acid signalling pathway (Jaulneau et al., 2010).

1.7.1.2 *Ulva* as a co-culture species in integrated multi-trophic aquaculture (IMTA)

The fast development and sustainable expansion of fish farming has resulted in growing attention and concern on the more societal aspects of this industry. The current concerns about the amount of waste emitted from fish rearing demonstrates that this will be a decisive factor in the sustainability of aquaculture industry. Nitrogen (N) and phosphorous (P), the main end-products released by fish, are the source of most dissolved N and P waste resulting from intensive aquaculture systems. The excess of these two elements in the effluents of aquaculture operations causes eutrophication and subsequent variations in the aquatic environment if their supply exceeds the assimilation capacity of the ecosystem (Jahan et al., 2003, Lazzari and Baldisserotto, 2008).

Normally nutrients generated by each farmed fish include soluble inorganic components (such as phosphate and ammonia) and particulate organic compounds resulting from metabolic excretion besides residuals of uneaten feed and soluble organic nutrients resulted from particulate segments resuspension (Olsen et al., 2008). Using the mass balance calculated for salmon cage aquaculture with a feed conversion efficiency factor equal to 1.16 kg dry feed/ kg produced fish, it has been evaluated that slightly more than a third of N and P contents of fish feed are consumed by fish and the rest is released into the ecosystem as waste (Olsen et al., 2008). For the worldwide environmental concerns resulting from the sustainable expansion of the aquaculture industry, effective and reasonable treatment of fish farm effluents is crucial (Naylor et al., 2000, Neori et al., 2003).

Use of live macroalgae for the treatment of aquaculture effluents (e.g. shrimp and finfish) or in order to bioremediate civil waste water before discharging into the ecosystem has been drawing growing interest from scientists, stakeholders and legislators as a promising approach (Chopin et al., 2013, Nobre et al., 2010). It has been suggested as one of the efficient and cost-effective strategies to reduce nutrient loads from the aquatic environment as well as preventing the loss of phosphates and nitrates from run-off due to the high uptake rate of these nutrients by macroalgae (Bolton et al., 2009, Ridler et al., 2007). Such integrated systems, referred to IMTA (IMTA; e.g. shellfish and/or finfish with seaweeds) not only can eliminate large quantities of inorganic components from the system, they are also able to collect heavy metals and other water trace contaminants (Suzuki et al., 2005).

IMTA systems have been designed to take ecological and socio-economic advantage of balancing ecosystem functions by co-culture/farming aquaculture species inhabiting different trophic positions (referring to the various nutritional levels of species in a food chain) close to each other and in a unique system (Chopin et al., 2013). Through this approach, excess nutrients including uneaten feed and by-products of higher trophic-level species would be input (i.e. fertilizer, food, and energy source) for lower trophic-level crops (Chopin et al., 2013).

Counterbalance between all physical, chemical parameters including light, dissolved oxygen, acidity, temperature, CO_2 contents and water flow rate, as well as biological factors within a working IMTA unit is strictly important. In case of using seaweed in an IMTA system, some of the biological parameters are including “interplant variability” which is dependent on selecting suitable target species and their proportions. In fact, an appropriate combination of different functional roles of various algal species in the ecosystem is the first critical phase toward implementing an IMTA operation. “Type of tissue”, “nutrient prehistory”, “life history stages/age” and “control of parameters

triggering reproduction stages" are some of the other important biological factors (Chopin et al., 2001a, Soto, 2009). Typically, within members of the same species, the nutrient uptake rate during earliest phases of their life history is higher compared to mature phases.

Troell and Berg, (1997) and Chopin et al., (2001a) documented the results of co-cultivating seaweed, *Gracilaria chilensis*, with salmon cages in southern Chile. They reported that considering seaweed's double function as a "nutrient scrubber" and value-added crop, the risk for eutrophication of the environment would be significantly reduced and economic profits significantly increased. About 5% of dissolved inorganic nitrogen and 27% of dissolved phosphorous discharged from the salmon-farming sea cages in southern Chile was removed by growing 1 ha of seaweed nearby the fish cages (Troell and Berg, 1997, Chopin et al., 2001a). The co-cultured algal species in biofilter systems can be used for producing economically important by-products such as agar, or consumed as a dietary ingredient for high-value marine species such as fish (e.g. gilthead seabream (Shpigel et al., 2017)), abalone and sea urchins (Shpigel et al., 2005), and even non-marine animals such as ruminants (Arieli et al., 1993). Nobre et al., (2010) in a case study in South Africa, demonstrated the technical and economic feasibility and direct environmental advantages of including seaweed in abalone farming systems compare to abalone monocultures as follows:

(i) A reduction in "negative environmental externalities". The discharge of nitrogen to the sea reduced by 3.7 to 5.0 tons per year depends of different experimental schemes. (ii) A decline in collecting of kelp as feed for abalone from wild seaweed beds (by 2.2 to 6.6 ha per year) and (iii) a reduction of greenhouse gases measured as carbon dioxide equivalent emissions (by 290 to 350 tons CO_2 equivalent per year) also reported (Nobre et al., 2010).

Ulva is broadly used in IMTA approaches (Bolton et al., 2009), commonly interplanted with *Gracilaria* species (Guttman et al., 2018; reviewed in Soto et al., 2009). The main reasons behind the extensive use of *Ulva* are that (i) they are economically valuable, (ii) many members of this genus naturally adopt unattached morphotypes, which fits more suitably to the commercial aquaculture units and (iii) *Ulva* species represent special affinities for growing in waters with high levels of nitrogen such as waste water from fish farms (Bolton et al., 2009).

Ammonium and phosphate biofiltering efficiency was reported by interplanting *Ulva rotundata*, *Ulva intestinalis* and *Gracilaria gracilis* using the waste water from seabass (*Dicentrarchus labrax*) cultivation units (Hernández et al., 2002, Martínez-Aragón et al., 2002). In order to find other application for seaweeds grown in IMTA units, Valent et al., (2006) investigated the effects of *Ulva rigida* and two *Gracilaria* species as dietary additives on the "growth performance", "nutrient utilisation" and "body composition" of seabass juveniles. According to the outcomes of this research, a combination of *Gracilaria bursa-pastori*s and *Ulva rigid*a could replace up to 10% of fish diet, while *Gracilaria corne*a could replace up to 5% of this diet (Valente et al., 2006).

In fact, IMTA systems mimic the normal functioning of marine ecosystems, where algae take up available dissolved inorganic nutrients (ammonia, nitrate, phosphate and CO_2) present in water discharged from aquatic animal production (Abreu et al., 2011). With the massive worldwide demand for food, sustainably farmed fish and macroalgae will play a crucial role in tackling this problem. As mentioned before, *Ulva* species are currently used for food, nutraceuticals, feed functional additives, plant care and are investigated for novel biomaterials (e.g. bioplastics). Farmed biomass has the advantage of quality control and traceability, essential traits for its use in food and/or health products (Abreu et al., 2011, Abreu et al., 2014).

1.7.2 Ecological and economic disadvantages of *Ulva*

Ulva species are known as major fouling green algae on manmade infrastructures mostly because of the robust adhesive nature of their zoospores after settlement (Callow et al., 1997). The prevention of colonisation and growth of *Ulva* is the major target for antifouling studies (Finlay et al., 2008, Callow et al., 1997, Wendt et al., 2013). In contrast, increasing settlement and growth of this genus is the main objective of aquaculture sector in order to obtain higher biomass yield and also for bioremediation of nitrate and phosphate from aquatic waste water bodies (Sode et al., 2013).

In addition to *Ulva* spp being identified as prominent fouling green algae (Finlay et al., 2008, Callow et al., 1997), they were also introduced as a 'pollution indicator' due to their massive biomass accumulation in eutrophic aquatic environments (Morand et al., 1991), a great bio-monitor for both metal pollution and nutrient enrichment (Lee and Wang, 2001) and the causative macroalgae of vast green algal mats, a phenomenon known as "green tides" observed in many coastal regions along the world including the Yellow Sea in China (Gao et al., 2010), Tokyo Bay, Japan (Yabe et al., 2009) and the central Philippines (Largo et al., 2004). As noted earlier, these massive blooms of unattached and free-floating *Ulva* species have the potential to cause severe ecological and economic damage so they became one of rising global concerns (Smetacek and Zingone, 2013). For instance, the costs of clean up and emergency responses through the massive green tide in the summer of 2008 in Beijing, China were estimated about 30.8 million US dollars, not including losses to the aquaculture and tourism industries (Liu et al., 2013).

So, settlement and attachment could be considered as an important phenomenon distinguishing the advantages and disadvantages of *Ulva* in ecological and economic studies.

1.7.2.1 *Ulva* settlement and its regulators

Settlement is defined as the first step towards three stages of "colonisation" of *Ulva*. Settlement initiated by detecting a suitable substratum by swarmers, either biflagellate gametes or quadriflagellate spores. The second step is called "adhesion". Through adhesion, the flagella will be disappeared, and an adhesive will be secreted which forms a plaque. Adhesives are macromolecular polymers released from lots of vesicles which they exist in the anterior region of settled swarmers. The third step called "establishment". Through this phase, expansion and development of new cell wall, germination following by the growth of the germling and additional exploitation of the substratum are typical occurrences (Marshall, 2004, Callow and Callow, 2011).

Several "settlement cues" are incorporated into *Ulva* spore's attraction and then attachment process to a specific surface. In addition to being negatively phototactic, which directs swimming spores to dark areas, chemical stimuli such as fatty acids or the presence of bacteria may initiate a chemotactic reaction and lead spores towards a suitable location and consequently to higher levels of spore attachment and settlement (Callow and Callow, 2002, Marshall, 2004). Wheeler et al., (2006) demonstrated the role of Acyl -homoserine lactones (AHLs) in the regulation of *U. intestinalis* zoospore's settlement rate and detected a novel chemokinetic mechanism by which *Ulva* spores respond to quorum sensing signals. This research showed that the swimming speed of *Ulva* zoospores was quickly reduced in the presence of AHLs (Wheeler et al., 2006). Settlement of *Ulva* spores is also regulated by the surface's physical characteristics such as hydrophobicity and microtopography and other factors such as temperature (Callow and Callow, 2011). A higher number of spore settlements have been observed by increasing the temperature in *U. intestinalis* and *U. compressa* (Callow et al., 1997). Gao et al., 2017a also showed that

higher temperature (18 °C) significantly boosted the settlement of *U. rigida* gametes (Gao et al., 2017a).

1.8 Model organisms

Through the investigations about the growth, development and evolution of land plants, newly emerging model systems were developed and broadly recruited by researchers as an effective and strong tool to integrate genetic and genomic methods. The wealth of information provided by model organisms could be exploited for either fundamental researches, e.g. to identify specifics variations exist between different species or to address actual "applied" issues (Cock et al., 2006, Peters et al., 2004, Irish and Benfey, 2004). Studies on model organisms mainly focus on "conserved processes". In fact, comparative studies and genomics approaches in model systems provide the opportunity to facilitate the translation of understanding of fundamental biological processes in model plants and to reveal evolutionary and mechanistic insights in other plant species, particularly valuable crop plants (Cock et al., 2006).

Arabidopsis for instance is a well-defined and broadly studied model for dicots and flowering plants (Arabidopsis_Genome_Initiative, 2000) to investigate and uncover principles and pathways involved in different developmental and physiological traits and functions (Irish and Benfey, 2004). The possibility of using the vast wealth of data given by *Arabidopsis thaliana* complete genome sequence and available functional genomics tools, allow scientists working on economically important crop plant to find solution for real-tangible- "applied" problems (Cock et al., 2006).

1.8.1 *Arabidopsis thaliana* a well-defined model organism

The model plant *Arabidopsis* has been used effectively to understand processes that are conserved in crop plants. This dicotyledonous angiosperm belonging to the *Brassicaceae*

(cabbage) family is undoubtedly the most thoroughly studied green plant studied (Koornneef and Meinke, 2010). The small size of the *Arabidopsis* genome was a distinct advantage for molecular studies (Meyerowitz and Pruitt, 1985). *Arabidopsis* was the first plant with a complete sequenced genome (The *Arabidopsis* genome initiative, 2000) and development of a variety of phenotypic screens in *Arabidopsis* resulted in extensive mutant collections which are now available for use, including mutants in hormone signalling and perception, and mutants involved in nutrient uptake and processing (Azpiroz-Leehan and Feldmann, 1997, Tissier et al., 1999, Parinov and Sundaresan, 2000, Sussman et al., 2000, Alonso et al., 2003). Through comprehensive studies of plant growth and development on *A. thaliana*, most biosynthetic pathways of plant hormones have been previously determined, which has yielded a broad understanding of plant responses to many different stimuli (Hasegawa et al., 2000, Qin et al., 2011, Wani et al., 2016). Also, this reference plant has a very short generation time compared to many other plant species (6–8 weeks) and is amenable to most known culture techniques. Employing *Arabidopsis* as a model organism has enabled translation of the understanding of basic biological principles of plant growth and development to crop plants and agricultural practices which implies how indispensable a better understanding of their functioning is (Hayashi and Nishimura, 2006, Hochholdinger and Zimmermann, 2008, Sah et al., 2016). For instance, oilseed rape seeds that fall to the ground during dehiscence cause a huge loss in harvest for the farmer. To solve this problem, the mechanisms and genes related to silique opening were studied in detail in *Arabidopsis thaliana* (Job, 2002), presenting that research with *A. thaliana* is directly related to agricultural applications.

1.8.2 Algal model systems

Macroalgae as sessile multicellular photosynthetic eukaryotes that in the absence of specialised tissues (e.g. root system and vascular structures) are distinguished from land

plants (Graham and Wilcox, 2000), are suggested as representative examples or model systems in biology. The study of plants relies on a detailed understanding of model systems and exploring and clarifying the key processes that make them what they are also will enable researchers to apply these conclusions in future investigations in similar fields.

Cock et al., (2006) suggested *Ectocarpus siliculosus*, a small filamentous seaweed, as a genetic and genomic model for brown algae. *Ectocarpus* is one of the well-described brown algae mainly for following reasons: being highly fertile, the whole life cycle can be completed under laboratory conditions and quite rapidly (about three months), towards the sexual reproduction by fusing different mating type of gametes sexual crosses can be happened and existence of small evolutionary distance between members of Ectocarpales and Laminariales which are known as commercially important seaweeds (Cock et al., 2006). To establish the genetic and genomic tools of *Ectocarpus*, the entire and relatively small sized 214 Mbp genome has been sequenced completely (Cock et al., 2006).

Delisea pulchra, a red macroalga, has been introduced as an ideal marine model organism to investigate different factors incorporated into the occurrence of bleaching disease. These factors include host stress and its chemical defensive approaches (e.g. halogenated furanones), climate change, the interactions between opportunistic bacterial pathogens (such as *Nautella italica* R11 and *Phaeobacter* sp. LSS9 belonging to Roseobacter clade), the natural symbiotic bacteria and the algal host (Kumar et al., 2016, Fernandes et al., 2012, Case et al., 2011). Typically, upon bleaching disease, loss of photosynthetic pigments happens in restricted sections, mid-thallus, of infected algae, which can be followed by tissue necrosis and death and possibly mass mortality and population extinction (Campbell et al., 2014, Kumar et al., 2016).

In aquatic environments, the abundance of green algae provides testable systems that could be used as suitable representative model organisms (Wichard et al., 2015. *Ulva* species have been extensively used for decades as an ideal model system for studies on different aspects of *Ulva* spores colonisation, behaviour of spores, understanding the cellular mechanisms involved in adhesion system, regulation of these processes by a range of biological, physical and chemical interactions with submerged surfaces (Marshal 2004, Callow et al., 2006; Callow et al., 2011). These investigations have crucially important contribution to shed light on the practical consequences of this natural phenomenon which is "marine biofouling" and the management of applied problems caused by biofouling in the real world (Callow et al., 2006; Callow et al., 2011). Nonetheless, utilising of green macroalga as model systems remains vastly under-exploited and largely understudied in comparison to land plants. (Wichard et al., 2015; Vesty et al., 2015).

Recently, the green seaweed *Ulva mutabilis* (sea lettuce; green nori) has been introduced as an emerging experimentally tractable non-land plant model organism for studies of macroalgal development, growth, morphogenesis (Wichard, 2015b).

The unique properties that make *Ulva* species especially attractive as model systems are:

- The small genome: de Clerck et al., (2018) reported 98.5 Mbp haploid genome, about 12924 protein coding genes of the established model system *Ulva mutabilis*.

- Simple organisation of the thallus consisting of just three differentiated cell types: blade, stem and rhizoid cells

- Cultivation in the laboratory (Spoerner et al., 2012)

- Symbiotic growth with bacterial epiphytes: Cock et al., (2006) argued that in marine biology, understanding the interaction between organisms and their functions are more important than the basic biological knowledge about them. In case of *Ulva*, the complete

morphology and development is largely driven by morphogenetic compounds or "morphogens" (reviewed in Wichard, 2015). Pure thallusin, a chemical cue, showed a significant morphogenesis- and differentiation-inducing activity in *Monostroma oxyspermum*, suggesting complex symbiotic and chemically-mediated communications between seaweed and their associated bacterial communities in the marine ecosystem (Matsuo et al., 2005).

- A wide variety of naturally-occurring developmental mutants (e.g. *Ulva mutabilis*), and the ability to generate stable transgenic lines (Fjeld and Lovlie, 1976, Oertel et al., 2015, Wichard et al., 2015b).

1.9 Aims of this research

i. *The extension and development of* Ulva intestinalis *as an experimental model system*

Although the suitability of *Ulva intestinalis* as a tractable model system is already established (Kostamo et al., 2008), one aspect of this study has been the development and establishment of a reproducible culture method to provide conditions for completion of the *U. intestinalis* life cycle in the laboratory environment. Moreover, due to availability of *U. intestinalis* along most UK coastlines and therefore its easy accessibility, it might be more suitable alternative to employ as a UK model *Ulva* species rather than *U. mutabilis* (Chapter2/Section 2.3.2 for more details).

ii. *Further understanding of green macroalgae morphogenesis and development*

For several species of green algae, it has been demonstrated that the epiphytic bacterial communities are essential for correct development and typical morphogenesis (Matsuo et al., 2005, Marshall et al., 2006, Spoerner et al., 2012, Wichard et al., 2015a). However, the mechanism(s) by which this regulation occurs are still poorly understood. For elucidating the complexity of interactions between green algae and associated bacteria, these are

reduced to a tripartite symbiosis in the case of *U. mutabilis* (Spoerner et al., 2012). This tripartite community of *U. mutabilis*, *Roseovarius* sp. strain MS2 and *Maribacter* sp. strain MS6 has been introduced as a novel algal model system that is ideal for characterising microbial communities associated with algal hosts (Spoerner et al., 2012). Now a key research question is the extent of specificity of epiphytic bacteria involved in the *Ulva*–bacterial interaction. So, another facet of this study has been to determine the specificity of bacteria-induced morphogenesis of *Ulva* by cross-testing of two very important *Ulva* species, *U. mutabilis* and *U. intestinalis*, and their epiphytic bacteria.

Furthermore, another study revealed that *Ulva* can benefit from morphogenetic compounds with similar activity to the compounds released by the *Roseovarius* sp. and *Maribacter* sp. (or equivalents of these strains), as waterborne morphogens (Grueneberg et al., 2016). Therefore, another aspect of this research has been evaluating the impacts of sterile-filtered natural water samples collected from a fish farm on *Ulva* morphogenesis and development.

iii. *Comparative studies at a mechanistic level to further understand how seaweed fertilisers can affect land plant growth and development*

Seaweed fertilisers have been used in agricultural programmes to improve soil management, disease management, nutritional strategies, water efficiency and drought tolerance (Arioli et al., 2015). The mechanism by which seaweed fertilisers affect plant growth, development and yield is currently unknown. My thesis aims to tackle this key point by establishing a “standardised” laboratory-based system (employing a common UK *Ulva* species) to help determine the molecular mechanisms by which seaweeds can affect land plant productivity, using model organisms. This will be done by using two experimentally tractable organisms which will deepen our understanding of the plant mechanisms and signalling pathways that can be triggered by utilising algal extract as a biostimulant.

1.10 Concluding remarks

Our dependence on plants for food, medicine, energy and their critical ecological role highlights how essential a better understanding of plant- and seaweed functions and processes is. Investigation into seaweeds is in its infancy compared to that of terrestrial plants. Today, expert studies conclude that the better understanding of the physiological, molecular and genetic mechanisms that control seaweed morphogenesis, growth and development, besides further identification of the critical factors of seaweed aquaculture and environmental challenges such as algal bloom management, will be key points to enabling a sustainable future. This research will address some questions by developing robust assays for analysing *Ulva*-bacteria interactions and the waterborne morphogenetic activities as well as explorative surveys for assaying the effect of *Ulva* extract on plants growth and development. These data will expand our knowledge of essential processes such as the morphogenesis and development of seaweeds.

CHAPTER 2:
MORPHOGENESIS AND DEVELOPMENT INDUCED BY BACTERIA IN *ULVA* SPECIES

2.1 The symbiosis between marine macroalgae and bacterial communities

2.1.1 Microbial abundance and diversity

Mutualistic relationships between organisms are reciprocally beneficial in nature (Herre et al., 1999). In the marine ecosystem, surface colonisation is ubiquitous and (healthy) macroalgal surfaces are consistently subjected to new/fresh colonisation by surrounding bacteria and subsequently biofilm formation (Egan et al., 2013). It has been assessed that more than two million different bacterial taxa live in the oceans and a single litre of surface seawater, on average, contains more than 10^9 bacterial cells (Curtis et al., 2002). Bacteria that inhabit marine environments represent a diverse and abundant population performing a range of complex ecological functions (Friedrich, 2012).

Shiba et al., (1980) documented 10^4 - 10^6 culturable heterotrophic bacteria per cm^2 on *Monostroma nitidum* and *Ulva linza* (syn. *Enteromorpha linza*). Among this bacterial population, most of pigmented bacteria were identified as belonging to the *Flavobacterium-Cytophaga* group. But because only a small fraction (as low as <1%) of the total diversity of bacteria which exists in a given environment can be grown in the laboratory on growth media (Yamamoto, 2000), these numbers are probably an underestimate of the bacterial densities. Using a substitute technique of direct counts from scanning electron micrographs, 2.6 x 10^6 bacteria per cm^2 on the surface of *U. reticulata* were reported (Dobretsov and Qian, 2002). Most common bacteria retrieved from macroalgal surfaces belong to the phyla Proteobacteria (Gram-negative) and Actinobacteria and Firmicutes (Gram-positive) (Tait et al., 2009, Salta et al., 2013), whereas the free-living isolates include members of the alphaproteobacterial SAR11 and gammaproteobacterial SAR86 clades (Morris et al., 2002, Dupont et al., 2012).

2.1.2 Importance and function of algal-bacteria interaction

A review of recent research points to the rapid expansion and diverse range of knowledge focusing on many individual aspects of interactions between marine algae and their associated microbiota, including: the structuring of microbial communities on the surface of the algal host (Burke et al., 2011b), bacterial diversity (Lachnit et al., 2009, Lachnit et al., 2011, Barott et al., 2011), stimulation release and settlement of algal spores by epiphytic bacteria (Joint et al., 2000, Thomas and Allsopp, 1983), inhibitory activity of associated bacteria against common biofouling organisms (Dobretsov et al., 2006, Egan et al., 2008) and algal pathogens and diseases (Case et al., 2011, Gachon et al., 2017). A great deal of substantial laboratory-based evidence confirmed that macroalgal health, performance and resilience are functionally controlled and assisted partially by associated bacterial isolates (reviewed in (Egan et al., 2013).

Despite the role of biological and physical properties of the macroalgal surface in shaping the associated bacteria and their metabolic activity both qualitatively and quantitatively (Salaün et al., 2012, Egan et al., 2013, Bengtsson et al., 2011), several studies have revealed that chemically-mediated algal-bacterial cooperative interactions through the exchange of nutrients, minerals and signal molecules, is the basis of this cross-talk (Thomas et al., 2008, Goecke et al., 2010, Hollants et al., 2013).

Marine macroalgae are able to make organic compounds (carbon-rich constituents of macroalgal cell walls e.g. agar, carrageenan, alginate, fucan, laminarin, cellulose, and pectin) and oxygen, that can be exploited by their associated bacterial communities (Goecke et al., 2010, Ramanan et al., 2016). Associated bacteria, instead, may cater for their algal host by providing CO_2 (which is absolutely necessary for algal photoauthrophy), fixed nitrogen, vitamins, minerals (especially for those grown in oligotrophic environments or with the lack of nitrogen fixation ability), antibiotics, phytohormones and

morphogenic factors that play crucial roles in algal growth and development (reviewed in (Egan et al., 2013). As an example, Fries (1975) described that *Enteromorpha linza*- and *Enteromorpha compressa*-associated bacterial isolates had the ability to transform tryptophan to indole-3-acetic acid (IAA) (Fries, 1975). Later on, it was shown by Singh et al., (2011) that *Exiguobacterium homiense* and *Bacillus* spp. isolated from red macroalga *Gracilaria dura* can make IAA that is included in defining the number of buds and regeneration of this algae (Singh et al., 2011).

Other than nutrients, some of these symbiotic bacteria adopt various strategies which allow them to inhibit biofouling and pathogen invasion or extend the defence mechanisms of their hosts. “Competitive colonisation” is one of these supportive strategies. The presence of symbiotic bacteria on the surface of algae may provide a ‘protective’ role from unfavourable or even harmful colonisers and prevent subsequent biofouling by other organisms more likely via releasing of deterrent or inhibitory chemicals (Armstrong et al., 2001). On *Gracilaria vermiculophylla* and *Delisea pulchra*, epiphytic bacteria such as the *Rhizobiales*, *Actinobacter* and *Roseobacter*, which are well known as predominant bacteria on the algal surfaces, can facilitate their colonisation by their effective antibacterial properties (Longford et al., 2007, Rao et al., 2007, Spoerner et al., 2012). Generally speaking, macroalgal–bacterial interaction malfunctions can lead to algal diseases [reviewed in (Goecke et al., 2010)]. Moreover, these microorganisms primarily attracted a great scientific and economic interest as potential sources of novel bioactive metabolites (Romanenko et al., 2008, Zhang et al., 2009).

2.2 Uniqueness and variability of the epiphytic bacterial community on macroalgae

2.2.1 Is the epibacterial community on macroalgae host-specific?

Selected aspects of macroalgal-bacterial interactions have been reviewed in many studies. A number of studies have shown that not only are these macroalgal-associated bacteria distinct from the surrounding seawater communities but also different species of seaweeds (specially growing in the same ecosystem) are associated with species-specific bacterial strains (Lachnit et al., 2009, Lachnit et al., 2011, Barott et al., 2011), leading to the hypothesis that the association between microorganisms and algae is host-specific. Host-specificity implies that it would be possible to identify or isolate the specific communities of associated bacteria on particular macroalgal species that are not present on other algal species (or only found in low numbers) or even only present on certain parts of the algal body (Egan et al., 2013). Although the mechanisms of this specificity have not yet been well understood, ecological roles have been elucidated for some of the associations (Goecke et al., 2010).

This assumption is supported by observations that a significantly different phylum composition of bacteria was associated with each of three co-existing algae sampled at regular intervals over two years (Lachnit et al., 2011). Moreover, the same species of seaweeds growing in different ecological habitats can associate with similar bacterial species (Lachnit et al., 2009). Although it has been suggested that the bacterial-algal association is determined by the algal host (Longford et al., 2007), bacterial isolates from seaweeds can vary with season and host life-cycle stage (Lachnit et al., 2011). It was also reported by Cray et al., (2013) that the pre-eminence of some phyla e.g. Proteobacteria and Firmicutes is the result of their ability to compete with other phyla due to (i) high

resistance to various stress factors (ii) existence of different pathways for generating energy (Cray et al., 2013).

2.2.2 The lottery hypothesis

In contrast, based on a large-scale sequencing analysis, Burke et al., (2011) suggested "the competitive lottery model" for algal-associated bacteria, originally developed by Sale (1976) for explaining the coexistence of reef fish species in the same niche (Sale, 1976). Different bacterial species were isolated from different *Ulva australis* samples in the same niche space and at different times in the year. The model states that the structuring of microbial communities on the surface of host algae is controlled by the presence of particular microbial functional genes rather than microbial taxonomic entities (Burke et al., 2011b). It is estimated that these functions are related to the ecophysiological roles of alga-associated microbial communities in general, i.e. detecting and moving towards the host, followed by attaching to the host and forming a biofilm, then responding to host environmental factors (Burke et al., 2011b, Friedrich, 2012). This functional assistance would result in formation of a holobiont, an entity composed of an alga with its associated functionally important bacteria (Egan et al., 2013).

2.2.3 The sea lettuce *Ulva* only adopts a typical morphotype with the right bacteria

The first attempt to address the important questions about the bacteria-induced morphogenesis in *Ulva lactuca* was made by Provasoli in 1985 through fundamental experiments to test whether bacteria-free cultures of *Ulva* would grow on mineral media or whether they would need organic factors. Provasoli and Pintner (1980) showed that *Ulva* cultures lost the typical foliose morphology and adopted an atypical pincushion-like morphotype when grown axenically (Provasoli and Pintner, 1980).

To study microbial-algae interactions in the lab, axenic cultures of macroalgae pave the way for comparative research. Gamete purification takes into account that using antibiotics might affect the growth of samples (Andersen, 2005), the often low effect of antibiotics in seawater and the high antibiotic resistance of marine bacteria. Interestingly it has been shown that several different species of *Ulva* can be stably cultivated under laboratory conditions starting with axenic germ cells purified via their phototactic movement towards light, without applying antibiotics, over a very similar time frame (Spoerner et al., 2012, Wichard, 2015, Vesty et al., 2015, Ghaderiardakani et al., 2017).

2.3 A symbiotic tripartite community as a bioassay-driven approach

A requirement for at least two bacteria, *Roseovarius* sp. strain MS2 (GenBank EU359909) and *Maribacter* sp. strain MS6 (GenBank EU359911), for proper morphogenesis in *Ulva mutabilis* (originally collected by B. Føyn at the southern Atlantic coast of Portugal (Føyn, 1958)) has been demonstrated by Spoerner et al., (2012), who also partially purified some of the regulatory factors that are needed for normal morphogenesis. It was demonstrated that bacterially-derived substances govern rhizoid and blade development (Spoerner et al., 2016). The established tripartite community can be considered an ideal model system with which to have controlled, repeatable conditions for further investigation of the interaction between a macroalga and its associated bacteria (Wichard et al., 2015b, Grueneberg et al., 2016). Strictly sterile (axenic) *U. mutabilis* cultures derived from purified phototactic gametes have an atypical “pincushion” morphotype, in which a lack of holdfast and exterior cell wall distortions are the main characteristics. Co-cultivation experiments using axenic gametes and *Roseovarius sp.* MS2 revealed that this bacterium promotes cell division and algal blade cell growth, analogous to cytokinin function in land plants. A similar experiment using *Maribacter sp.* MS6 showed that MS6 induces formation of a proper cell wall and a primary rhizoid (Wichard et al., 2015b, Grueneberg et al., 2016).

2.3.1 Cross-testing experiment

Marshall et al., (2006) assessed the effects of 38 unique bacterial strains, isolated from three species of *Ulva*, on the growth rate and morphological development of *U. linza* axenic plantlets (treated with antibiotics) for 28 days. A subset of 20 isolates was selected from this collection for more detailed investigations and identified by 16S rDNA sequencing. Plant morphology assessment relied on a semi-quantitative scale based on the number and state of extension of tubules from the central callus of each plant. Bacteria that led to plants with more than fifty well-developed tubular extensions from the central callus were assigned a score of 3 (the biggest difference compared to the controls); between 30 and 50 tubular extensions resulted in a score of 2 and 10–30 extensions by a score of 1. In comparison to axenic controls, growth of only 0–10 tubular extensions were assigned a score of 0 (Marshall et al., 2006). Five isolates significantly increased the algal growth rate and induced marked morphological development with more than 30 well-developed tubular extensions (Marshall at al., 2006). However, no single bacterium was able to completely restore normal morphology to axenic *U. linza* (Vesty et al., 2015), in contrast to a recent observation in *U. mutabilis* applying bacteria isolated from *U. rigida* (Grueneberg et al., 2016). Grueneberg et al., (2016) also showed the bioactive bacterial factors do not need to be close to *Ulva* plants to have their effect and indicated that morphogenesis of *Ulva* can also benefit from bacterial sources other than its own epiphytic bacteria. This raises the question of specificity of the morphogen-producing bacteria and also discredits the species-specificity hypothesis as algae could pick up signals from their neighbours. Previous studies did not explore whether bacteria from certain species of *Ulva* can function in cross-species “swap” experiments, and only very few studies have systematically addressed the still unanswered research question of the species-specificity

of epiphytic bacteria involved in the *Ulva*–bacterial interaction (Vesty et al., 2015, Grueneberg et al., 2016, Weiss et al., 2017, Ghaderiardakani et al., 2017).

Establishing an additional standardised tripartite system with more than one species of *Ulva* presents an ideal possibility for elucidating the complexity and specificity of algal-bacterial interactions. The following cross-testing and comparative investigations defined pairs of bacterial strains isolated from several *Ulva* species that can completely recover the normal growth of either *U. mutabilis* or *U. intestinalis* axenic cultures, demonstrating that different compositions of microbial communities with similar functional characteristics can enable complete algal morphogenesis and development into mature thalli (Ghaderiardakani et al., 2017).

2.3.2 A new tripartite system more suited to the UK

The majority of recent *Ulva* lab-based basic research is conducted on the fast-growing tubular mutant of *U. mutabilis* called 'slender' (Løvlie and Braten, 1978) which presents a much shorter life cycle and is amenable to laboratory culture. *U. mutabilis* wild-type is found mostly off the Portugal shoreline in Europe. So, it might be difficult to access and collect samples from outside of borders particularly for researchers in UK. Indeed, keeping the mutants alive and growing them under laboratory conditions would be a little bit tricky.

U. intestinalis is a new system providing a key point of comparison to study morphogenesis, morphogenetics and mutualistic interactions. Phylogenetic analysis has suggested a very close relationship between *U. intestinalis* and *U. compressa* (Blomster et al., 1998) and also, in spite of the variation in morphologies and life cycles, between *U. mutabilis* and *U. compressa* (Hayden et al., 2003, Lövlie, 1963, Tan et al., 1999). *U. intestinalis* has a widespread distribution along UK coastal area (Figure 2.1) and easy accessibility, so it would be an ideal candidate to be exploited as a UK model *Ulva*

species. Trying to use domestic species in this kind of investigation also provides opportunities for development of other comparative *Ulva* model systems that may offer additional information.

I attended a specific training programme at the Institute for Inorganic and Analytical Chemistry at the Friedrich Schiller University of Jena which gave me this opportunity to employ known procedures to a new Ulvophyte system and made possible cross-testing of epibacterial species and comparison of their morphogenesis and development (Ghaderiardakani et al., 2017).

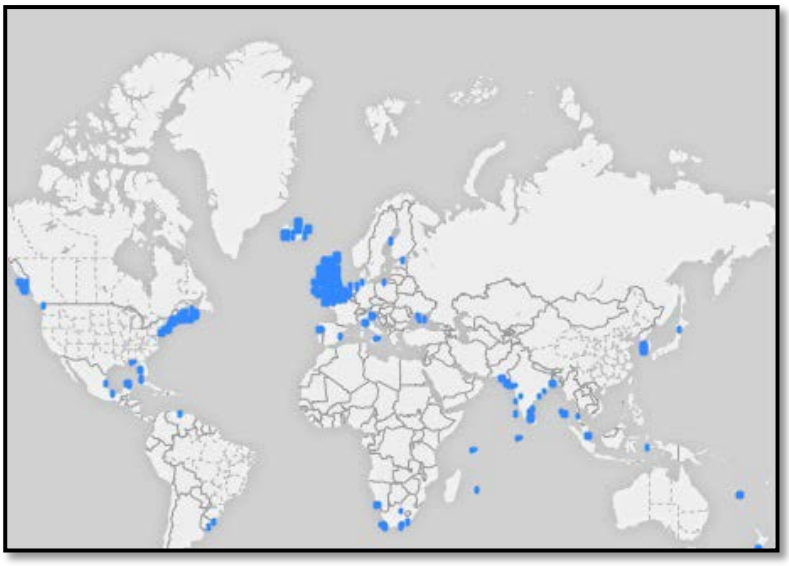

Figure 2.1 Distribution of *U. intestinalis* across the world. The data supplied by the Ocean Biogeographic Information System. https://www.marlin.ac.uk/species/detail/1469

Figure 2.2 Sample collection location. Llantwit major, South Wales (51°23'46.5"N 3°30'06.0"W). This figure illustrates the *U. intestinalis* samples predominantly distributed on the upper-mid region of the shore. The beach has a range of *Ulva* spp. and other seaweed samples including both red and brown algae.

2.4 Chapter aims

The general objective of this part of my project is to determine whether there is a core group of associated bacterial species, which makes contribution to host normal morphogenesis and development, or if there is a kind of specificity of the morphogen-producing bacteria involved in this mutual interaction. A series of bioassays will be performed to tackle the key points outlined below.

- Confirmation that the *Ulva* at the collection site at Llantwit Major (Figure 2.2) is *U. intestinalis*

- The extension and development of a new tripartite system by *U. intestinalis*. To establish a detailed and standardised tripartite system, as a first step we need to determine if it is possible to induce *U. intestinalis* to form gametes and axenic cultures.

- Analysing the effect of bacterial isolates isolated from *U. mutabilis* individually and in combination, on *U. intestinalis* axenic gametes.

- Analysing the effect of bacterial isolates selected from currently available culture collections isolated from several *Ulva* species individually and in combination, on *U. mutabilis* axenic gametes.

2.5 Materials and methods

2.5.1 Algal samples

(i) Vegetative and fertile *Ulva intestinalis* (Figure 2.3) blades were collected three times between March 2015 and April 2016 from Llantwit Major beach, South Wales (51°23'46.5"N 3°30'06.0"W) (Figure 2.2). A freshwater stream probably containing agricultural run-off enters the top of the beach from the North East. The seaweed *U. intestinalis* is more frequently found in the inter tidal zone, upper-mid region of the beach.

Indeed, another *Ulva* species with flat bi-layer morphology was collected from the lowest point of the tidal region. These flat-shape samples were found individually or in small bunches attached to the hard substratum via rhizoids.

Excess water and epiphytic species were removed at the site by blotting the sample's surface before storage on ice for transport back to the laboratory. This species cannot be reliably identified solely using morphological characteristics, so plastid-encoded *rbc*L (RuBisCo large subunit) markers and the plastid-coding elongation factor gene (*tuf*A), as a typical barcode of green algae which has recently been established (Saunders, 2010), were used for molecular identification.

(ii) Developmental and fast-growing mutant of *U. mutabilis* "slender" (sl-G(mt+)) (Føyn, 1958, Løvlie, 1964) was gratefully received from Dr Thomas Wichard's group and cultured in liquid medium. Haploid gametophytes of *U. mutabilis* "slender" used for all cross-testings and comparative investigations with *Ulva intestinalis*. Typical lab cultures of *U. mutabilis* (A) and *U. intestinalis* (B) are shown in Figure 2.3.

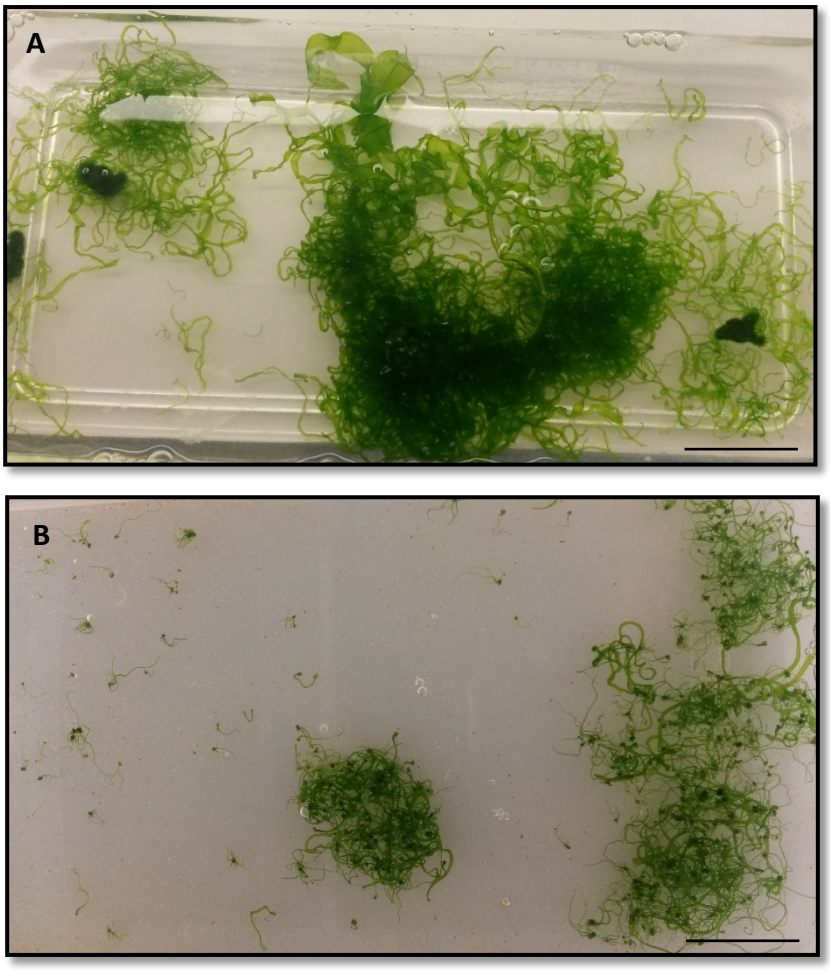

Figure 2.3 Typical cultures of (A) *U. mutabilis* Føyn mutant (slender), (B) *U. intestinalis*. The cultures are 4 weeks old. Scale bar = 2cm.

2.5.2 Preparation of *Ulva* culture medium (UCM)

UCM was made by adding 10ml of solutions II, III and IV to 1 litre of solution I then adding 2ml of filter sterilised vitamin solution V and 1 ml HCL 0.5M (Table 2.1). Solutions II, III and IV were autoclaved to sterilise and kept in UV resistant version of amber stained laboratory glass bottles (Duran®) at 4°C to reduce the particle formation and degradation. Solution V was sterilised by filter into 50ml Falcon™ tubes (Fisher Scientific) and stored at -20°C.

2.5.3 Cultivation conditions

The mutant slender (sl-G(mt+)) strains of *U. mutabilis* and *U. intestinalis* gametophytes were propagated from unmated gametes or zooids derived from gametophytes or sporophytes in sterile culture flasks with gas-permeable screw caps (Nunc Int., Denmark) containing 100 mL UCM under the standard growth conditions including a 17:7 h light/dark regime at 18 °C with an illumination about 60 µmol photons m^{-2} s^{-1} provided by 50 % GroLux, 50% day-light fluorescent tubes (Stratmann et al., 1996). Additional information about the materials demanded, the culture conditions and the preparation of the seed stock can be found in (Wichard and Oertel, 2010).

Solution I	
Chemical	Quantity
NaCl	19.14g
Na_2SO_4	3.21g
$MgCl_2$	8.39g
$CaCl_2$	0.83g
$NaNO_3$	8.5g
$(NH_4)_2SO_4$	1.1g
distilled H_2O	1 litre

Solution II (100x stock)	
Chemical	Quantity
NaH_2PO_4	0.7g
$NaHCO_3$	8.8g
$C_4H_{11}NO_3$	10g
distilled H_2O	1 litre

Solution III	
Chemical	Quantity
KBr	7.84g
KCl	54.2g
$SrCl_2$	1.95g
distilled H_2O	1 litre

Solution IV	
Chemical	Quantity
$C_{10}H_{16}N_2O_8$ (50x)	20ml
H_3BO_3 (100x)	10ml
$FeSO_4$ (1000x)	1ml
$CuSO_4$ (10,000x)	100µl
$NaMoO_4$ (10,000x)	100µl
$MnCl_2$ (1000x)	1ml
$ZnSO_4$ (1000x)	1ml
$CoCl_2$ (10,000x)	100µl
NH_4VO_3 (10,000x)	100µl
KI (10,000x)	100µl
Na_2SeO_3 (5000x)	50µl
Na_2WO_4 (20,000x)	50µl
distilled H_2O	1 litre

Solution V (Vitamin solution)	
Chemical	Quantity
Vitamin B_{12} (2.3mM)	1.6µl
Thiamin (16.62mM)	4ml
Nicotinic acid (4.06mM)	20ml
Pantothenic acid (11.4mM)	4ml
Pyridoxine (59.11mM)	400µl
ρ-aminobenzoic acid 18.23mM)	400µl
Biotin (1.02mM)	2ml
Thymine (15.86mM)	40ml
Inositol (55.51mM)	10ml
Orotic acid (41.64mM)	4ml
Folic acid (45.34mM)	1µl
Putrescine (113.44mM)	400µl
Riboflavin (13.29mM)	100µl
Pyridoxamine (14.86mM)	800µl
Choline (345.6mM)	1ml
distilled H_2O	To 100ml

Table 2.1 Components of solutions I, II, III, V and V for assembly of *Ulva* Culture Medium (UCM).

2.5.4 Artificial induction of gametogenesis and preparation of axenic cultures

Intact mature thalli of *Ulva mutabilis* and *Ulva intestinalis* were artificially induced to form gametangia and produce gametes were washed for 15 min with distilled water at noon followed by chopping (Zyliss® herb chopper) individual blades into small pieces approximately 3-5 mm. Tissue was washed three times by leaving in 100ml UCM for 15 minutes, filtering through gauze and placing in fresh UCM. This process removes/dilutes the naturally occurring sporulation inhibitors, allowing induction to happen. After three repeats pieces were distributed in IKEA containers containing about 75ml UCM and placed in growth chamber at 18°C, 7h light and 17h dark for three days.

2.5.4.1 Purification of gametes

Afterwards, on the third morning in daylight, gametes were released from the gametangia by removing/diluting the swarming inhibitor (SWI). This was done by an additional medium change and filtering *Ulva* pieces, washing them with UCM and returning to containers containing fresh media.

Then, freshly-released gametes were purified from their accompanying bacteria by taking advantage of the gametes fast movement towards light through a narrow horizontal capillary under strictly sterile conditions in a laminar flow hood (Figure 2.4 A,B,C). In fact, biflagellate gametes can swim quicker than any flagellated cells, bacteria or diatoms that might be contamination reason of the culture, toward the light. In case there is spores instead of gametes, because spores are negatively phototactic and they swim to the farthest point away from the light source of Pasteur pipette (Figure 2.4 D).

This method was repeated at least three times to obtain bacteria-free gametes. Upon collecting in high enough densities of gametes they were transferred to microcentrifuge tubes and placed in front of light to help further concentration and purification (Figure 2.4 C). As final step, concentrated gametes collected from the top of the Pasteur pipette and bacterial contamination was checked by plating a drop of the ‘gamete solution’ on Marine Agar plates (Roth, Karlsruhe, Germany, supplemented with 1 % agar) and by PCR amplifications of the 16S DNA (Spoerner et al., 2012; Wichard, 2015).

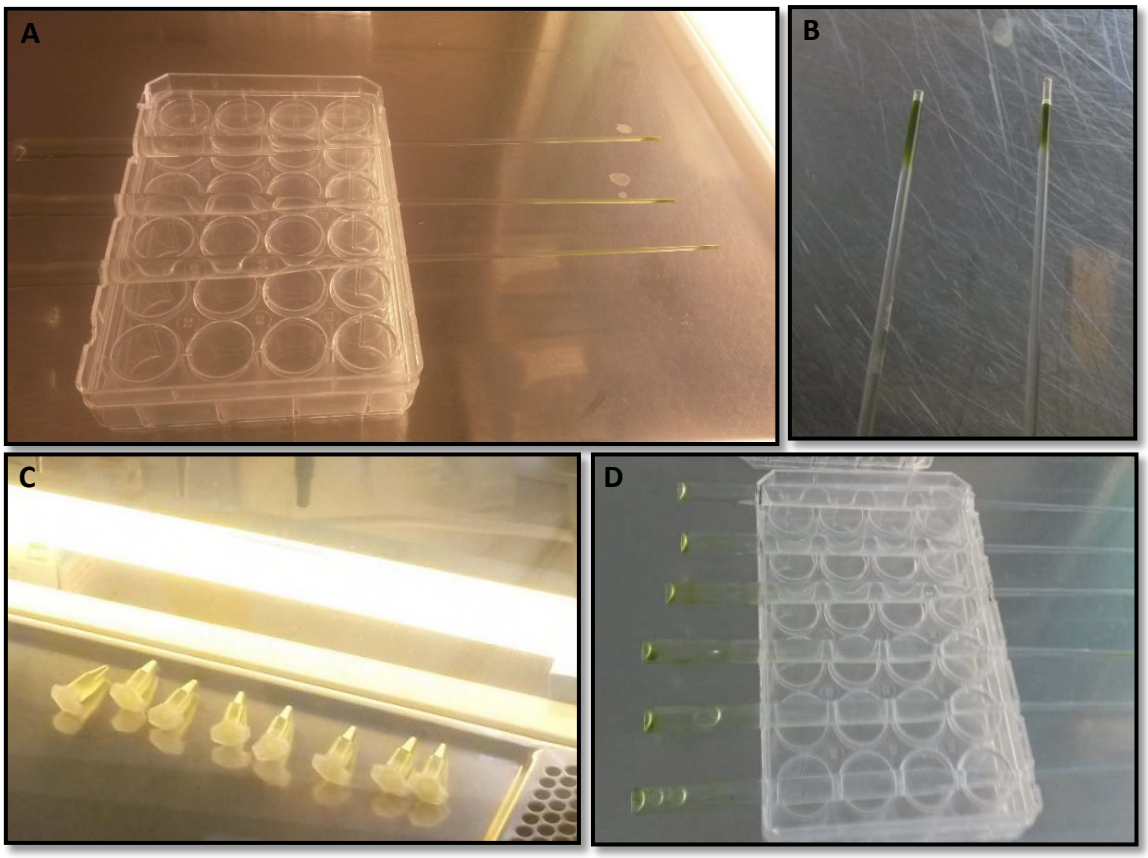

Figure 2.4 Gamete purification by positively phototactic movement along a capillary pipette. (A) Freshly released gametes swam towards light at narrow end of the Pasteur pipette **(B)** and collected at the tip. **(C)** The purified gametes collected and transferred to microcentrifuge tubes and placed back in front of bright light to make them more concentrated **(D)** Quadriflagellate spores shows negative phototactic movement and remained at wide end of pipette.

2.5.5 Phylogenetic characterisation of *Ulva*

2.5.5.1 Genomic DNA extraction

Genomic DNA was extracted from 30 mg seaweed samples using an ISOLATE II Genomic DNA Kit (Bioline, London, UK) according to the manufacturer's recommendations. Briefly, homogenised tissue was lysed and filtered through a column. Binding conditions were then adjusted with binding buffer and DNA bound to a column membrane. Membrane was washed three times with provided washing buffers before

adding warmed (65°C) elution buffer, incubating at 65°C and centrifuging to elute DNA. By using NanoDrop spectrophotometer (Thermo Scientific), samples were quantified and stored at -20°C before applying to PCR. For long term storage samples were stored at -80°C.

2.5.5.2 Primer sequences and PCR conditions

DNA fragments of the *rbc*L gene were amplified by PCR using 30 ng DNA and 1 µl VELOCITY DNA Polymerase (2 units/µl) (Bioline Ltd, UK) in a final volume of 50 µl per reaction according to the manufacturer's protocol. Two primer pairs were used:

Forward-*rbc*LStart 5'-ATGGCTCCAAAAACTGAAAC-3'

Reverse-750 5'-GCTGTTGCATTTAAGTAATG-3'

Forward-650 5'- GAAAACGTAAACTCACAACC-3'

Reverse-*rbc*LEnd 5'-TTCTTTCCAAACTTCACA-3'

The primers tested for *tuf*A marker were:

*tuf*A: F 5'-GGNGCNGCNCAAATGGAYGG-3'

*tuf*A R 5'-CCTTCNCGAATMGCRAAWCGC-3' (Famà et al., 2002).

The PCR conditions were as follows: an initial denaturation step at 98°C for 2 min, 32 cycles of 98°C for 30 s, 58°C annealing for 30 s and 72°C extension for 1 min 30 s. The cycles were succeeded by a final elongation step at 72°C for 5 min. PCR products were cleaned using the Thermo Fisher Scientific GeneJET™ PCR Purification Kit and sequenced on a capillary sequencer (ABI 3730, Applied Biosystems, USA) at the Functional Genomics Laboratory of the University of Birmingham.

2.5.5.3 Cloning the *tuf*A sequences

The following detail explains the mechanism by which the targeted gene, *tuf*A sequences, that used for identification and didn't work very well were selected more accurately. For ligation of inserts into target vectors, PCR products were extracted, purified (mentioned above) and ligated into the PCR® Blunt vector using the Zero Blunt™ PCR Cloning Kit and subsequently transformed into DH5α competent cells, following the manufacturer's instructions (Invitrogen). Transformation mixtures were plated out onto LB agar petri dishes containing 50µg/ml ampicillin for selection of transformants and incubated overnight at 37°C. Five colonies which grew on plates were selected and used to inoculate individual 10 ml LB+50µg/ml Kanamycin liquid cultures, which were subsequently incubated in an orbital incubator overnight at 37^{o}C. Plasmid DNA was then isolated and screened for correct integration of the PCR product by digesting the resulting plasmids using *EcoRI* (sites present at either side of the Zero Blunt™ PCR vector Multiple Cloning site). Inserts were then sequenced from both ends using the universal primers M13F and M13R.

2.5.5.4 Agarose gel electrophoresis

0.8% agarose was made using 1xTBE (90mM Tris, 90mM boric acid, 2.5mM EDTA) and poured into gel trays containing 5µl/100ml Gel Red (10000X). DNA samples were mixed with 6xDNA loading buffer (New England BioLabs® Inc.) prior to loading into wells and running alongside a 1kb marker ladder (Invitrogen/NEB). Expression was visualised with Molecular Imager® Gel Doc™ XR+ system with Image Lab™ software (Bio-Rad).

2.5.5.5 Sequence analysis of *rbc*L and *tuf*A genes

The two primer pairs amplified two PCR products from the *rbc*L gene, 1–750 and 650–1430 (the 3' end) that overlapped, meaning a sequence for almost the entire gene could be

obtained by sequencing and aligning the PCR products. PCR products were fully sequenced from both ends using the primers used to amplify them. The resulting sequences were aligned manually (there were no mismatches in the double reads for each PCR product) using the overlapping central 100 bp (650–750) to generate a consensus *rbc*L sequence. A single PCR product was generated for *tuf*A, which was sequenced from both ends. Alignment of the forward and reverse *tuf*A sequences demonstrated that they were identical. The consensus sequences enabled the *Ulva* sample to be identified to species level by comparing the acquired sequence data with already available sequence data in GenBank by using a Basic Local Alignment Search Tool (BLASTN; Johnson et al., 2008).

2.5.5.6 Phylogenetic analysis

The sequences were aligned with reference strains obtained from GenBank using SeaView Version 4 through an interface with CLUSTALO programme (Gouy et al., 2009). For taxonomic robustness, an unrooted tree was constructed with high quality *rbc*L and *tuf*A gene sequences. The consensus trees cluster the *Ulva* samples into groups where the closest affiliations lie. The trees were observed in Fig Tree v1.4.3 and transferred to a windows metafile.

2.5.6 Bacterial strain selection

By using axenic gametes in a standardised bioassay, it is possible to determine which microbes induce the algal morphogenesis through morphogenetically active substances (morphogens). A large collection of *Ulva*-associated bacteria was available, isolated by the Callow laboratory (Marshall, 2004, Marshall et al., 2006). These bacterial strains isolated from multiple *Ulva* species (including *U. linza, U. lactuca, U. compressa* and *Enteromorpha* sp.) have been maintained at –80◦C in glycerol as source cultures since collection: not all have been previously assigned a genus [(Marshall, 2004, Marshall et al.,

2006), J. Callow unpublished; Table 2.2]. First, UL19, EC19, UL16, EC34, E1, UL2 were selected, which caused a wide range of degrees of growth of axenic *Ulva* plantlets (based on Marshall et al., 2006 or our preliminary tests; Tables 2.2). In the second round of this experiment, another set of 9 species plus EC19 as an MS6-equivalent (control) were investigated (UL1, UL4, UL9a, UL10, UL12, UL13, UL15, EC19, UL23, UL27). These bacteria were all isolated from *U. linza* only, covered a diverse range of genera and were subsequently used in the bioassay against the axenic gametes. The procedure in this part was the same as the methods used for initial bioassay.

Isolate ID	**Original Genbank accession number**	**Algal host**[a]	**Morphology score**[b]	**Closest matching strain in GenBank after collection**	**% Sequence similarity**
UL19	AM180742	*Enteromorpha compressa*	2	*Shewanella gaetbuli*	96%
EC19	Not previously identified	*Enteromorpha compressa*	-	Not previously identified	-
E34	Not previously identified	*Enteromorpha sp.*	-	Not previously identified	-
E1	Not previously identified	*Enteromorpha sp.*	-	Not previously identified	-
UL16	AM180741	*Ulva linza*	3	*Cellulophaga* sp.	92%
UL2	AM180737	*Ulva linza*	1-2	*Bacteroidetes bacterium*	94%

Table 2.2 Previous data for bacterial strains chosen for this study.

Note: [a] Callow JA, unpublished data. [b] "Morphology after 28 days assessed on a semi-quantitative scale. 0: Little tubular growth (G10) from central callus of *Ulva linza*; 1: 10–

30 tubular extensions; 2:30–50 tubules; 3: 950 well-developed tubules” (Marshall et al., 2006).

2.5.6.1 Phylogenetic characterisation of bacteria

2.5.6.1.1 Preparation of bacterial isolates

The most typical way to detect taxonomic groups at the genus level in bacteria is known as comparison of 16S rRNA genes which contains adequate information for trustable phylogenetic characterisation and is universally disseminated (Amann et al., 1995). To reassess the phylogenetic characterisation (based on new database information) of the six bacterial isolates originally isolated from various *Ulva* species (Marshall et al., 2006), partial 16S rDNA sequences (approx. 1500 bp) were amplified from these strains and sequenced. Ten µL of each of bacterial isolate was cultivated in 10 mL Marine Broth (MB; Roth, Karlsruhe, Germany) and then directly streaked onto Marine Agar plates to obtain single colonies. The plates were incubated at 20 °C for 5 days, then distinct colonies were picked off and transferred with a sterile loop into new bottles containing 10 mL MB.

2.5.6.1.2 DNA extraction, primer sequences and PCR conditions

Bacterial DNA was extracted according manufacturer's instructions using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). To identify, or re-classify, the identity of the six bacterial strains using to up to date classifications, Partial 16S rDNA from single isolates was amplified by PCR amplification and using the primer pair 27f (ggg ttt gat cct ggc tca g) and 1390r (acg ggc ggt gtg trc aa) (Spoerner et al., 2012, Grueneberg et al., 2016). The reaction master mix contained: 2.5 µL of PCR buffer 10% (100 mmol L−1 Tris/HCl pH 8.3, 500 mmol L−1 KCl, 15 mmol L−1 MgCl2), 1.25 µL of BSA (20 mg/ml), 1µL each of forward and reverse primer (20 mM), 0.5 µL dNTPs 100 mM (dATP, dCTP, dGTP, dTTP), 0.15 µL Taq polymerase (5 units / µl), ~100 ng of template DNA. The PCR protocol included a 5-min initial denaturation at 95°C, followed by 31 cycles at 95°C for 30 s, 58 °C for 30 s, 72 °C for 90 s, finally 1 cycle of 7 min at 72 °C and storage at 4 °C.

PCR products then were subjected to forward primer sequencing using the chain termination method (GATC, Göttingen Germany).

2.5.6.1.3 Taxonomic classification

The purified product from Section 2.6.7.1.2 (previous section) was sequenced from both ends using the primers used to amplify them. This was undertaken by the Genomics Laboratory, School of Biosciences, University of Birmingham. The consensus sequences enabled the bacteria isolates to be identified to species level by comparing the acquired sequence data with previously identified and registered sequences in GenBank by using a Basic Local Alignment Search Tool (BLASTN; Johnson et al., 2008). Alignment of the forward and reverse partial 16S rDNA sequences demonstrated that they were identical. The sequences which had the most percentage of match to previously submitted isolates have been considered, and a final consensus sequence of about 1500 bp was submitted to Genbank.

2.5.7 The *Ulva* bioassay array

2.5.7.1 Gamete distribution

To survey the potential activity of potentially morphogenesis-inducing bacteria, the '*Ulva* bioassay array' based on a multiwell plate format was used (Grueneberg et al., 2016). This standardised bioassay was designed and developed for large screening based on a previous study with the tripartite community of *U. mutabilis*, *Roseovarius* sp. MS2 and *Maribacter* sp. MS6 (Grueneberg et al., 2016). To avoid any bias and thus minimise variation between several experimental set ups that would make the results ambiguous, positive and negative controls were run on identically prepared 96-well plates at the same time. As positive controls, *U. mutabilis* axenic gametes were incubated with MS2 alone, MS6 alone and MS2+MS6 (Figure 2.5); in addition, axenic gametes of *Ulva intestinalis* were incubated

with MS2 alone, MS6 alone and MS2+MS6 (Figure 2.5). As a negative (axenic) control, 14 wells in one row were left without any bacterial inoculation in each plate (Figure 2.5). For further comparison and evaluation, *U. intestinalis* was growing in flasks with the normal complement of *U. intestinalis*-associated bacteria by using non-purified gametes. Three biological replicates were conducted in parallel for each experiment.

The stock solution of freshly prepared axenic gametes was diluted with *Ulva* Culture Medium (UCM; Stratmann et al., 1996) to obtain the optimum concentration of gametes (about 300 gametes / mL). The density of gametes in the axenic stock solution was measured by flow cytometry (BD Accuri® C6). The gamete solution was distributed in 96-well micro array plates, 100 µL in each well. After incubation of plates overnight at room temperature in darkness, gametes randomly settled down to the bottom of plates.

To observe the morphogenetic effects of *Ulva*-associated bacteria, *U. intestinalis* and *U. mutabilis* (*slender* G^+) axenic gametes were inoculated with the bacteria isolated from three different *Ulva* species and *U. mutabilis*, individually and in combinations.

2.5.7.2 Bacteria inoculation

Bacterial strains were firstly washed and then re-suspended in sterile UCM. The final optical density of bacteria in each well was adjusted to 10^{-5} by making serial dilution of the stock solution harvested at OD = 1.0. Each number in experimental pattern correspond to one individual bacterial isolate. To avoid any contamination, plates were covered with gas permeable sealing film (Breathe-Easy, Diversified Biotech, MA, USA) and transferred to growth chamber under standard conditions (Wichard and Oertel, 2010). Over the next three weeks, plantlets were observed under the inverted microscope and qualitative features recorded.

2.5.7.3 Microscopy and quantification of parameters

Differentiation of plantlets was observed through inverted microscope (DM IL LED, Leica, Wetzlar, Germany). Images were captured using a Nikon Digital Sight DS-Fil camera on a Nikon SMZ 1000 stereomicroscope. The qualitative features considered under microscopic observation included the presence of unusual cell wall protrusions ('bubble-like' structures), thallus length, and differentiated rhizoid cells (Spoerner et al., 2012). Quantification of the average blade cell number and the percentage of thalli with entirely normal cell walls was carried out.

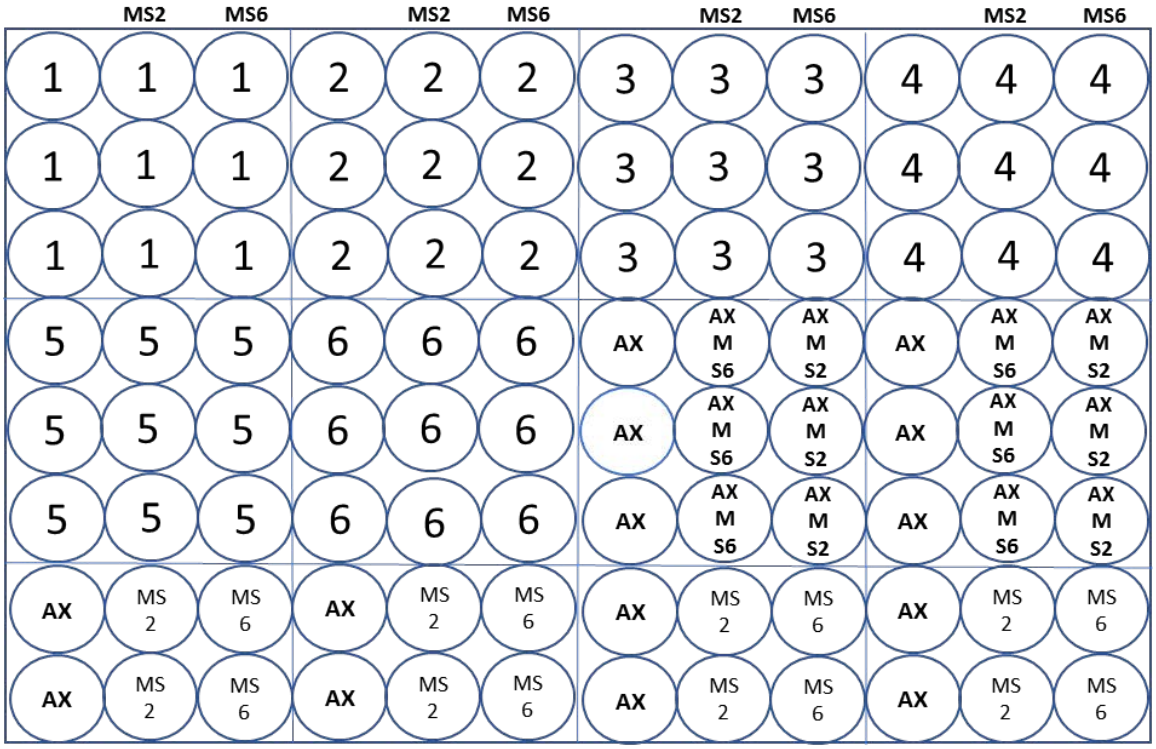

Figure 2.5 Experimental pattern. Numbers 1-6 correspond to six bacteria isolates, AX: Axenic gametes, AX+MS2: Axenic gametes + *Roseovarius* sp. MS2, AX+MS6: Axenic gametes + *Maribacter* sp. MS6.

2.6 Results

2.6.1 Identification of *Ulva* samples

2.6.1.1 Tubular-shape samples

The resulting sequence of *rbc*L gene (1334 bp) is deposited in GenBank under accession number MF038885. Also, a final consensus sequence of 772 bp (*tuf*A gene) was submitted to Genbank (MF162336) (Ghaderiardakani et al., 2017). Our sequences each had 100% match to only *U. intestinalis* samples. The sequence data were then used in further phylogenetic analysis. Figures 2.6 and 2.7 shows the neighbour joining tree of the species from this study compared to already submitted *Ulva* species in the GenBank database for both *rbc*L and *tuf*A genes. The morphotype of tubular-shape samples are shown in Figure 2.8.

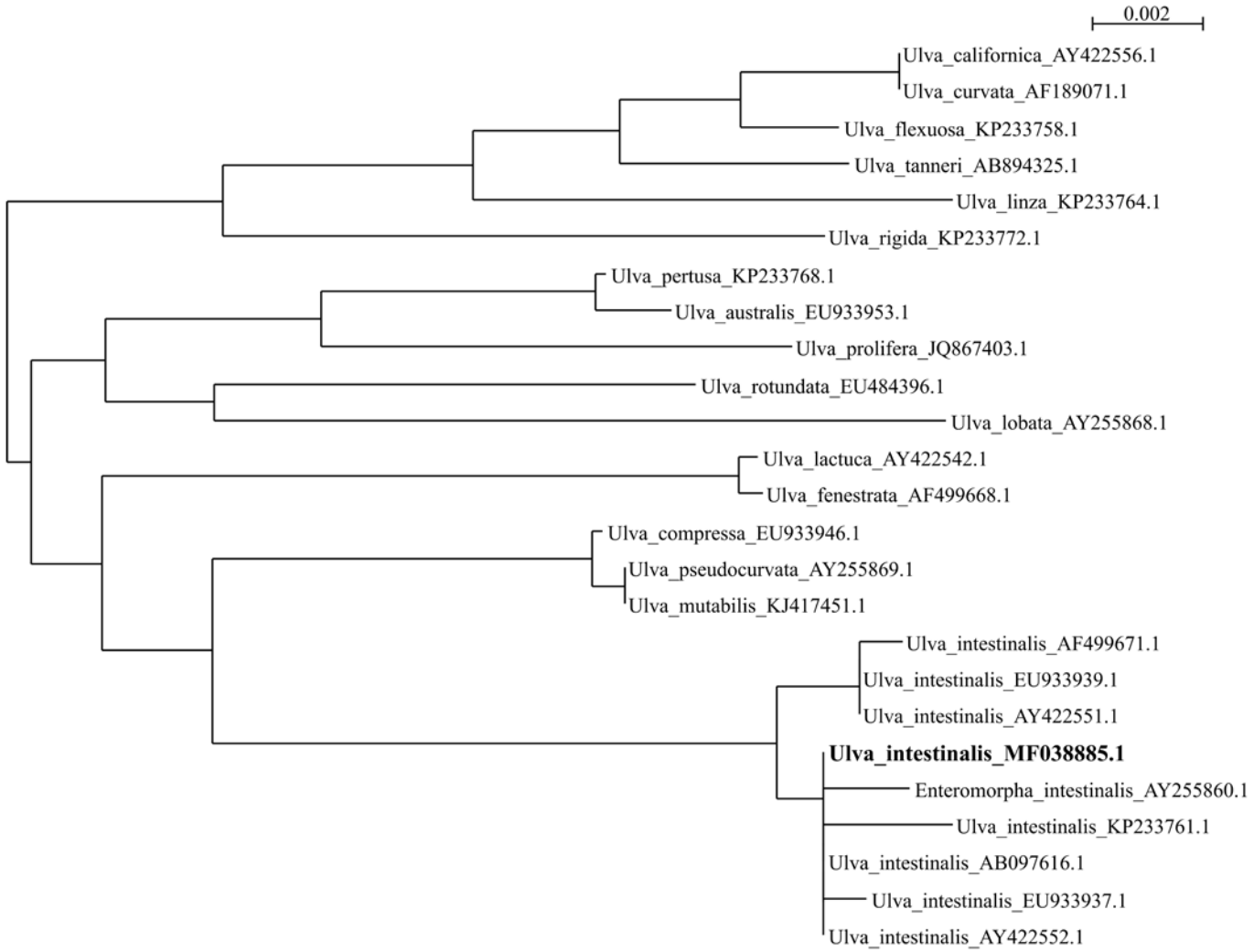

Figure 2.6 Phylogenetic analysis of plastid-encoded *rbc*L (RuBisCo large subunit) markers in *U. intestinalis*. The phylogenetic tree was constructed by BIONJ algorithm (an improved version of the neighbor-joining algorithm) using SeaView Version 4 programme.

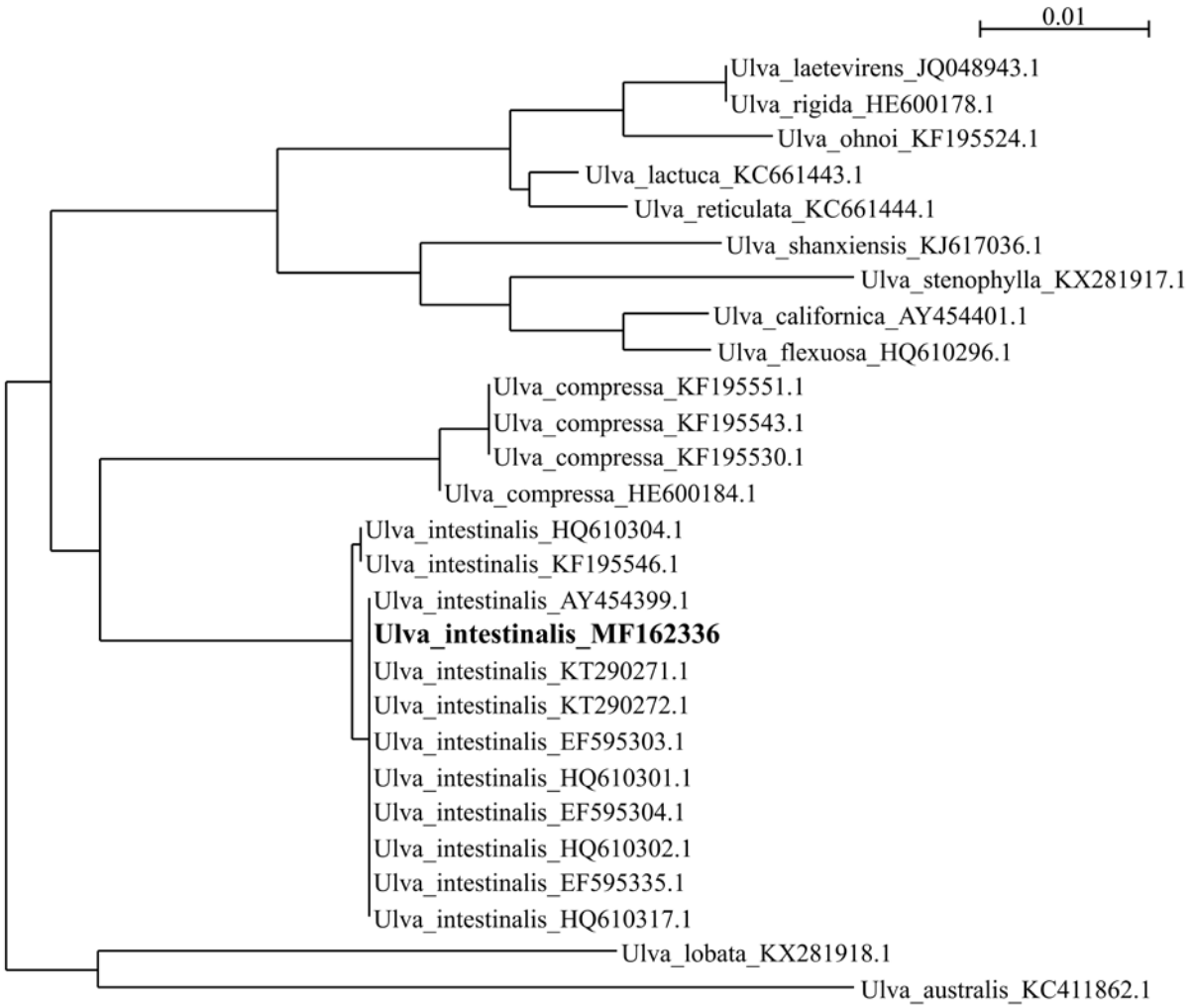

Figure 2.7 Phylogenetic analysis of plastid-coding elongation factor gene (*tuf*A) in *U. intestinalis*. The phylogenetic tree was constructed by BIONJ algorithm (an improved version of the neighbor-joining algorithm) using SeaView Version 4 programme.

2.6.1.2 Flat-shape samples

After trimming the ambiguous nucleotides, the alignment showed that the two sequences were identical at 731 aligned sites. Molecular phylogenetic data for *tuf*A gene placed the specimen from Llantwit Major in a well-supported clade along with published sequences of *U. laetevirens* (JQ048942.1 and JQ048943.1), which have identical sequences, and slightly more divergent to a mixture of GenBank accessions given as *U. rigida*, *U. reticulata*, *U. ohnoi* and *U. fasciata* (Figures 2.8, 9 and 10).

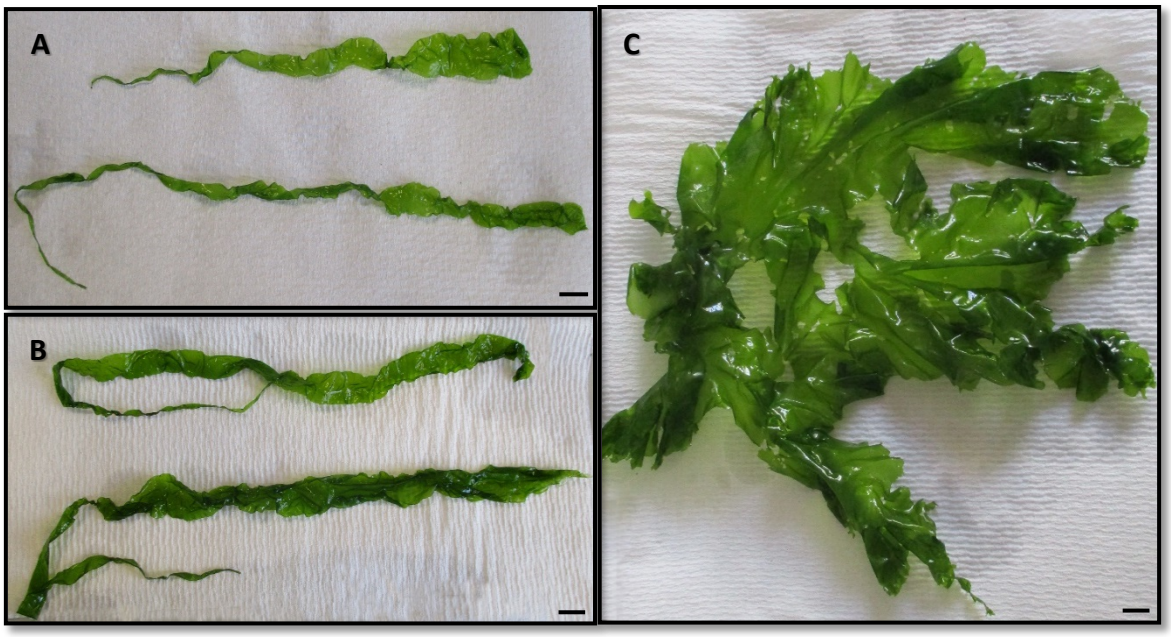

Figure 2.8 Different morphologies of *Ulva* samples collected from Llantwit Major. A) and **B)** *U. intestinalis* tubular morphology, **C)** *U. laetevirens* flat sheet with fluted margin. Scale bar = 1 cm.

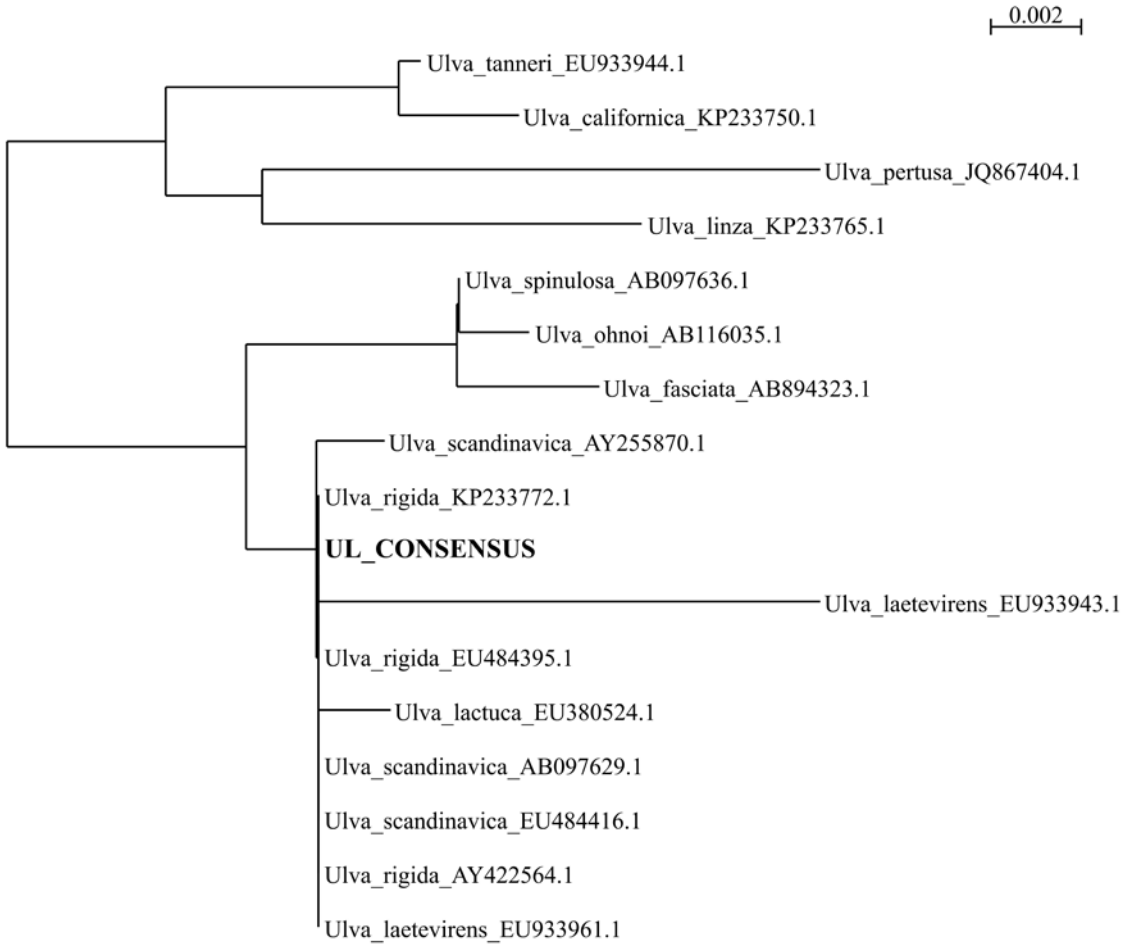

Figure 2.9 Phylogenetic analysis of plastid-encoded *rbc*L (RuBisCo large subunit) markers in *U. laetevirens*. The phylogenetic tree was constructed by BIONJ algorithm (an improved version of the neighbor-joining algorithm) using SeaView Version 4 programme.

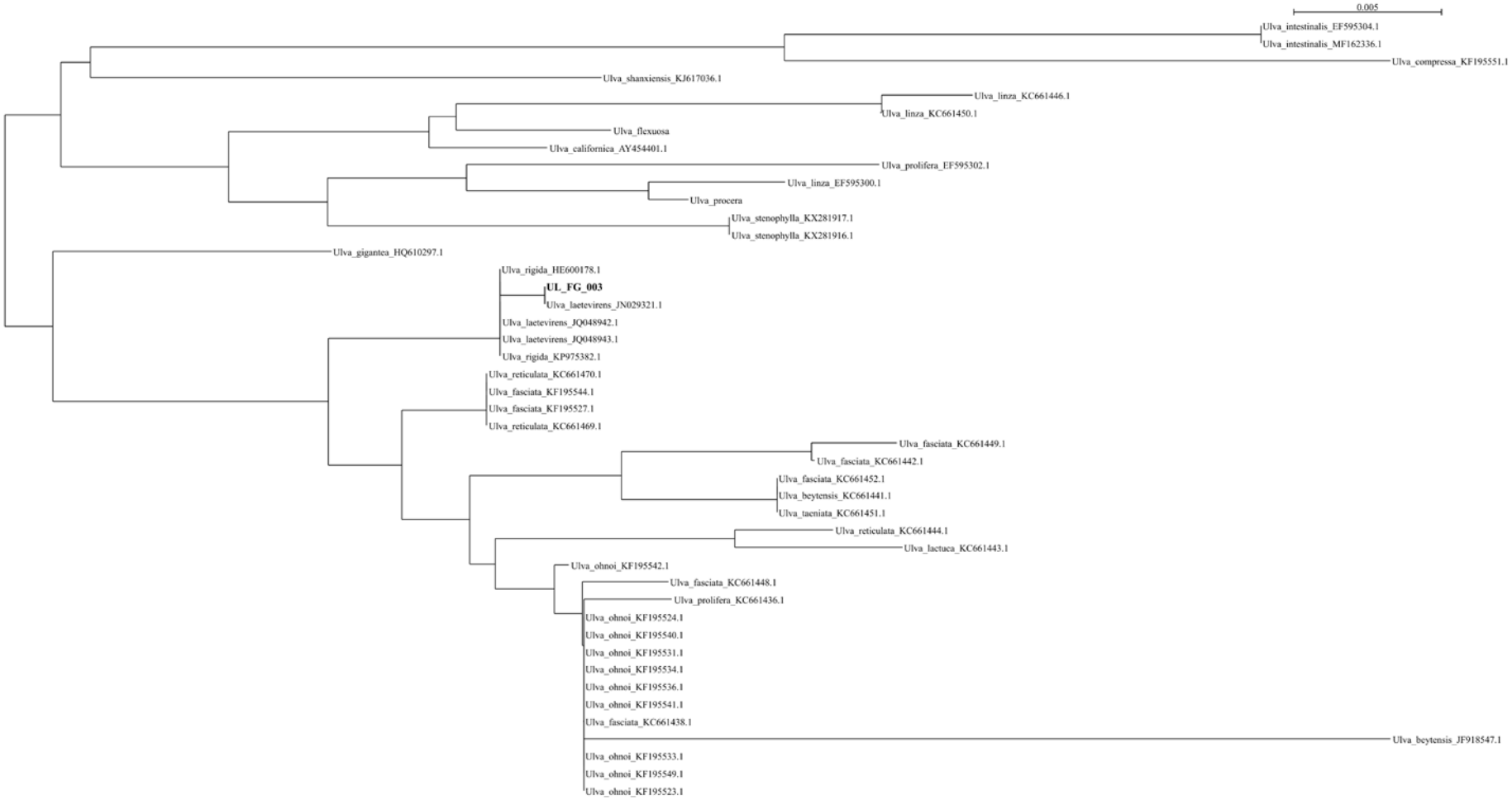

Figure 2.10 Phylogenetic analysis of plastid-coding elongation factor gene (*tuf*A) in *U. laetevirens*. The phylogenetic tree was constructed by BIONJ algorithm (an improved version of the neighbour-joining algorithm) using SeaView Version 4 programme.

2.6.2 Identification of bacteria

2.6.2.1 Initial screening of morphologically active *Ulva* spp. surface-associated bacterial strains

According to Marshall investigation (2004) the diversity of strains isolated from the surface of the three-different species of *Ulva* (*linza*, *compressa* and *lactuca*) was wide and 32 of 38 individual bacterial strains initiated some level of morphological change (Marshall, 2004). The first step of the present assay was set up by using six strains that significantly increased relative growth rates of *U. linza* axenic plantlets (Marshall, 2004). The sequences were run in a BLASTN search (Johnson et al., 2008) and compared with those already published. The closest homologous sequences in the GenBank database were recorded in Table 2.3. Two isolates belonged to the phylum Proteobacteria (Alphaproteobacteria class), two to the phylum Actinobacteria, one to the phylum

Isolate ID	Phenocopy of?	Closest matching strain in GenBank	Phylum	New Genbank Accession No#	% Sequence similarity

Bacteroidetes and one belonged to the phylum Firmicutes (Table 2.3).

UL19	Axenic	*Microbacterium* sp.	Actinobacteria	KY827088.1	100%
EC19	MS6	*Microbacterium* sp.	Actinobacteria	KY827089	99%
E34	MS2	*Paracoccus* sp.	Alpha-proteobacteria	KY827090	99%
E1	Axenic	*Planococcus* sp.	Firmicutes	KY827091	99%
UL16	MS2	*Cellulophaga* sp.	Bacteroidetes	KY827092	99%
UL2	MS2	*Paracoccus* sp.	Alpha-proteobacteria	KY827093	99%

Table 2.3 Current classification of bacteria isolated from three *Ulva* spp plus summary of new data in this chapter. This data now supersedes the classification of Marshall (2004).

2.6.2.2 Assay of nine further *U. linza* surface-attached bacteria and their influence on morphogenesis

The aim of this experiment was to test how a larger variety of bacterial strains, isolated from the periphytic microflora of *Ulva linza* by Marshall (2004) and characterised again (Table 2.4), influence the growth and morphology of *U. intestinalis* to find out better potential alternatives for new tripartite system. However, to this author's knowledge, the *Ulva* species mentioned by Marshall (2004) have not been identified by molecular methods. Therefore, there is not reliable evidence to prove that the samples have been called *U. linza* were actually *U. linza*. The second step of this study was set up by selecting nine (out of 21 species and after removing the replicates) individual bacteria from different phyla, labeled by Marshall as *U. linza* isolated periphytic bacteria strains for further assessment on *Ulva* samples collected from Llantwit Major and assumed to be *U. linza* (by morphological identification). The sequence data were then used in further phylogenetic analysis afterwards and demonstrated that they are different species and identifies as *U. intestinalis* (Section 2.7.1.1).

The closest homologous sequences of these bacteria in the GenBank database were recorded in Table 2.4. The *Ulva* bioassay array was repeated (in Birmingham University) to investigate the effects of individual and combinations of more bacterial strains upon the growth and morphology of the axenic plants. The changes in morphology of the plants were observed and some pictures have been taken by Zeiss inverted microscope (Invertoskop ID02, Carl Zeiss Microscopy).

Isolate ID	Phenocopy of?	Closest matching strain in GenBank	Phylum	% Sequence similarity
UL1	Axenic	*Frigoribacterium* sp.	Actinobacteria	100%
UL4	MS2	*Psychrobacter* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL5	----	*Bacillus* sp.	Firmicutes	99%
UL6b	----	*Psychrobacter* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL7	----	*Frigoribacterium* sp.	Actinobacteria	100%
UL9a	MS2	*Cobetia marina.*	Proteobacteria (Gamma-proteobacteria)	99%
UL10	Axenic	*Lysinibacillus chungkukjangi*	Firmicutes	100%
UL11	----	*Cobetia* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL12	MS6	*Maribacter* sp.	Bacteroidetes	100%
UL13	MS2	*Psychrobacter* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL15	Axenic	*Bacillus* sp.	Firmicutes	100%
UL18	----	*Salinibacterium* sp.	Actinobacteria	99%
UL20	----	*Cobetia* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL21	----	*Cobetia* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL23	MS2	*Celeribacter* sp.	Proteobacteria (Alpha-proteobacteria)	100%
UL24	----	*Psychrobacter* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL25	----	*Cobetia* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL27	Axenic	*Zobellia* sp.	Bacteroidetes	100%
UL28	----	*Cobetia* sp.	Proteobacteria (Gamma-proteobacteria)	100%
UL29	----	*Psychrobacter* sp.	Proteobacteria (Gamma-proteobacteria)	99%
UL62	----	*Bacillus* sp.	Firmicutes	100%

Table 2.4 Current classification of bacteria isolated from *Ulva linza* plus summary of resulted data in this chapter. This sequence-match data and associated accession numbers now supersedes the classification of Marshall (2004). The nine strains that their influence on morphogenesis have been assayed in second experiment are shown in bold.

2.6.3 Induction and purification of *U. intestinalis* gametes

Artificial induction and purification based on positive phototactic movement of gametes were fundamentals of the protocol developed originally for use with *U. mutabilis* slender (Wichard and Oertel, 2010). The aim was to identify if these techniques could be successfully applied to an alternative Ulvophyte system and a common wild-type species such as *U. intestinalis* in order to establish a new maintainable culture of bacteria-free plantlets**.** Ghaderiardakani et al., (2017) demonstrated that *U. intestinalis* gametophyte is inducible for gametogenesis and in fact *U. intestinalis* controls the gametogenesis in much the similar way as *U. mutabilis*. Biological changes of blade cells during gametogenesis have been shown in Figure 2.11.

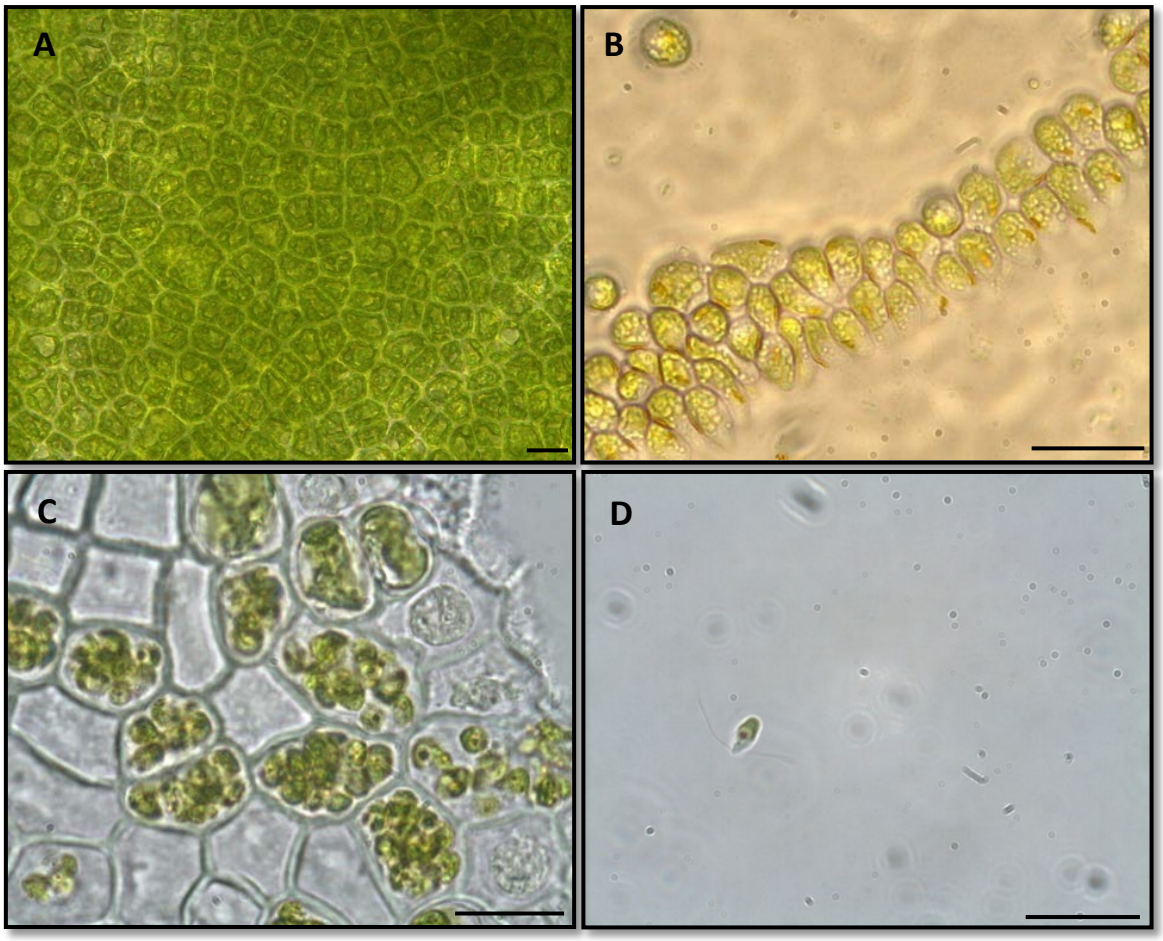

Figure 2.11 Artificial induction of gametogenesis in *U. intestinalis*. (A) 48 h after induction multiple granular bodies were visible, **(B)** 72h after induction all progametes matured into 16 fully differentiated biflagellate gametes which tightly packed within the cells, **(C)** after changing the medium on third day and gametes are ready to be released **(D)** A biflagellate gamete. Scale bar A and B= 20µm. Scale bar C and D= 80µm.

2.6.4 Bioassay-guided classification of the bacteria-induced morphogenesis of *Ulva mutabilis*

As demonstrated by Spoerner et al., (2012), axenic *U. mutabilis* plants develop a characteristic morphology with a lack of holdfast and distortions of the exterior cell wall (Figure 2.12). The effect of six individual bacterial species isolated from *Ulva* species were assessed for their ability to "rescue" the morphology of axenic *U. mutabilis* gametes back towards the complete non-axenic state (Figure 2.12). A range of different morphotypes were stimulated by the individual bacterial strains, but none of them could

solely elicit complete algal morphogenesis and normal development of *U. mutabilis* (Figure 2.12).

Various *Ulva* bacterial isolates were able to promote marked morphological changes in *U. mutabilis*. Three out of these four isolates, *Paracoccus* sp., strains E34 and UL2, as well as *Cellulophaga lytica* UL16 caused cell divisions, similarly to the reference strain *Roseovarius* sp. MS2 (Figure 2.12). As previously observed, the release of the MS2-like factor was not genus-dependent (Gruenberg 2016). Although in previous studies the MS2-like factor was frequently assigned to genera from the Alphaproteobacteria, this experiment shows that the specific morphogenetic activity of blade induction can also be carried out by *Cellulophaga* sp. (Figure 2.12).

As the MS2-like factor does not drive normal cell wall development and protrusions remained visible (Figure 2.12), further bacteria are necessary to complement these functional traits and to complete *Ulva´*s morphogenesis. The *Microbacterium* sp. EC19 possesses this activity and can induce both cell differentiation and cell wall formation, but failed to induce a proper blade, which is analogous to the activity of the reference strain MS6 (Figure 2.12). The two other tested bacteria *Microbacterium* sp. UL19, and *Planococcus* sp. E1, had no distinct effect on the growth and morphology of *U. mutabilis* and at the end of the experiment, algae cultured with these bacteria resembled axenic controls (Figure 2.12). In addition, the strain E1 seems to negatively interfere with MS6, as the typical morphogenetic activities of MS6 are not visible in the presence of E1 (Figures 2.12 and 2.13). Overall, this shows that the morphogenetic activity of bacteria towards *U. mutabilis* is bacterial strain-specific rather than correlating with bacterial genus.

2.6.5 Bioassay-guided classification of the bacteria-induced morphogenesis of *Ulva intestinalis*

To address the question of how *Ulva* species-specific the morphogenetic activities of bacteria are, axenic cultures of *U. intestinalis* were prepared through application of the methods originally developed for *U. mutabilis*. In the absence of epiphytic bacteria, *U. intestinalis* plantlets reverted to an undifferentiated callus of cells (Figure 2.13, controls), similar to axenic plantlets of *U. mutabilis* (Spoerner et al., 2012, Vesty et al., 2015) with unusual colourless protrusions from the exterior cell wall instead of the normal tubular morphology (Figure 2.13, controls).

2.6.5.1 Initial screening of morphologically active *Ulva* spp. surface-associated bacterial strains

In an original experiment using 6 morphologically active strains, as observed for *U. mutabilis*, the mode of action of *Paracoccus* sp. E34, *Cellulophaga* sp. UL16 and *Paracoccus* sp. UL2 on *U. intestinalis* plantlets was indistinguishable from the activities of the control reference strain MS2 (compare Figures 2.12 and 2.13). The same was true for the respective activity of *Microbacterium* sp. EC19. Under the influence of EC19 axenic gametes of the "slender" mutant develop into minute short rows of degenerated blade cells with normal cell walls and rhizoid formation. EC19 thus revealed similarity to the activity of the MS6-like factor with *U. intestinalis* in addition to its activity with *U. mutabilis* (Figure 2.13, compare with the MS6-control). The strong effect on rhizoid formation was prominent, forming multiple secondary rhizoids (Figure 2.13).

2.6.5.2 Assay of nine further *U. linza* surface-attached bacteria and their influence on morphogenesis

There were difficulties throughout this investigation. The most important one was finding and collecting wildtype gametophytes in the beach. Although Marshall (2004) suggested

that vegetative and fertile plants were recognisable by the presence of whitish tips, it was still quite challenging to collect gametophytes and not sporophytes. It seems that the population of gametophytes and sporophytes in nature is determined by variety of factors. The effect of external parameters on the balance between *Ulva* "haploid gamete-producing" and "diploid zoospore producing" generations was investigated by Løvlie and Bryhni, (1978). The occurrence of two reproduction methods, sexual and parthenogenetic, could be affected by temperature, density of gametes in the sea water (which itself is dependent on population density), size of mature plants, gametes releasing synchrony. Low temperatures, suboptimal for gamete fusion, would increase "abortive mating" and subsequent parthenogenetic reproduction. Obviously, low population densities would lead to the same results (Løvlie and Bryhni, 1978). This might explain the observation that *U. lactuca* sporophytes are relatively rare on the western shore line of Sweden (Lovlie 1978), where the gamete-producing *Ulva* plants develop directly by parthenogenetic reproduction (Bliding, 1968, Kapraun, 1970), in contrast to the collecting area of present study, where sporophytes are common and algal density is high (personal observations).

Inoculation of wells with the new selection of bacteria was carried out for one replicate, due to lack of gametes. Additionally, plant morphology assessment based on the semi-quantitative scale (the number and state of extension of tubules from the central callus of each plant) could not be done mainly because of the low quality of images (Figure 2.14). The results used for second column (Phenocopy of?) within Table 2.4 were from the only successful replicate of second assay for 9 strains - UL1, UL4, UL9a, UL10, UL12, UL13, UL15, UL23, UL27 plus EC19 from first experiment (as MS6 equivalent for control) – out of 21 strains identified and listed in Table 2.4.

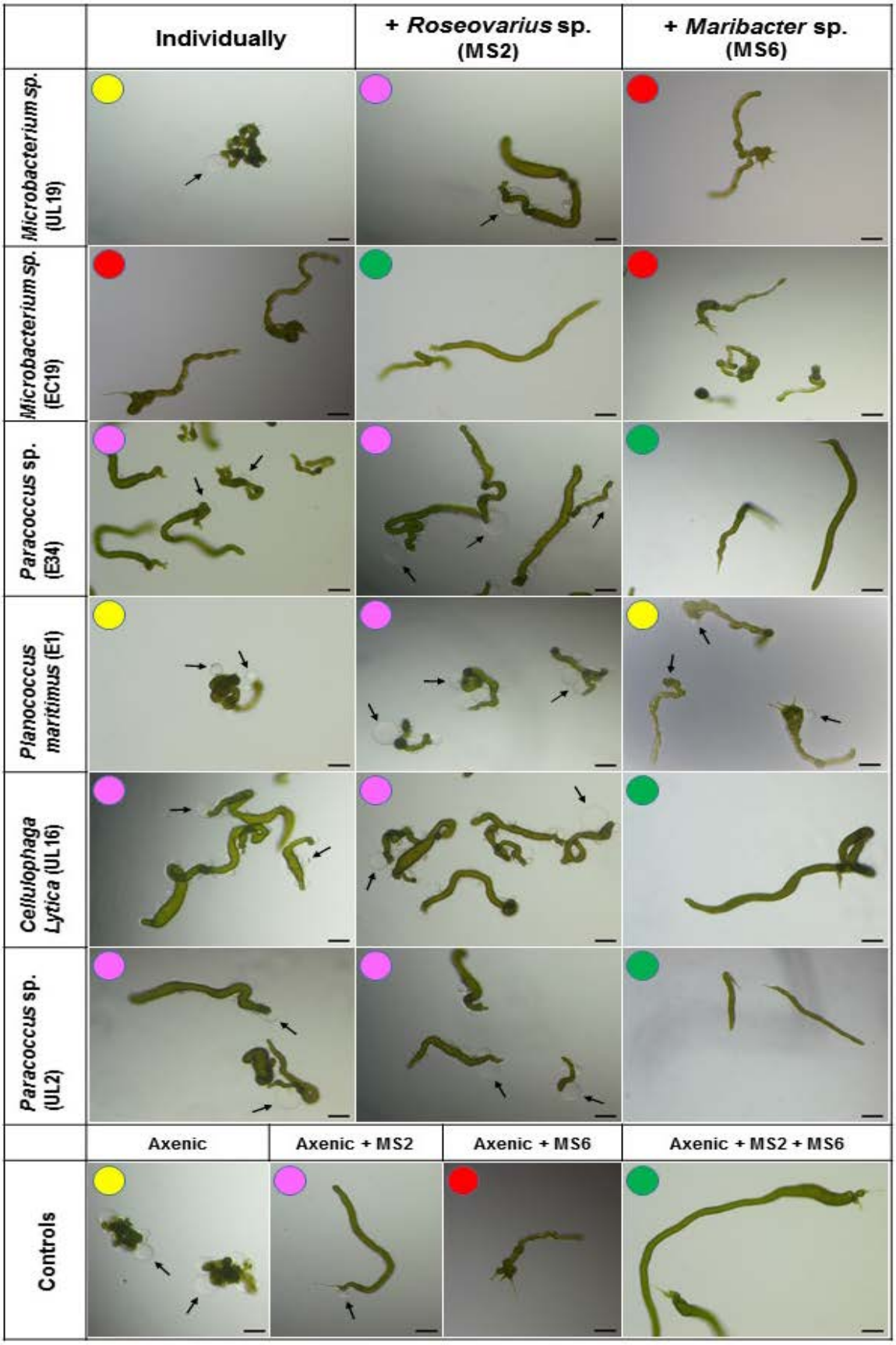

Figure 2.12 Morphogenesis assessment of *U. mutabilis* using the '*Ulva* bioassay array'. The multiwell-based testing system of morphogenetic activity using axenic gametes of *U.*

mutabilis allows the fast determination of the different morphotypes induced by bacteria isolated from various *Ulva* species, singly and in pairwise combination with the bacteria *Roseovarius* sp. MS2 and *Maribacter* sp. MS6.

Representative morphotypes are categorised by a colour code: Yellow circle (axenic morphotype): calluslike cultures with typical colourless cell wall protrusions. Magenta circle (morphotype induced by the MS2-like factor)**:** germlings with normal cell division towards one direction but still covered by protrusions and differentiated rhizoid cells are missing. Red circle (morphotype induced by the MS6-like factor)**:** plantlets show a proper cell wall and rhizoid formation, but the blade does not develop. Green circle (completely recovered morphotype)**:** characteristic usual morphotype with normal blade and rhizoid formation. Propagules are 3 weeks old. Controls are shown in the bottom row. Arrows indicate the typical colourless protrusions from the exterior cell walls of axenic cultures. Scale bars = 100 μm.

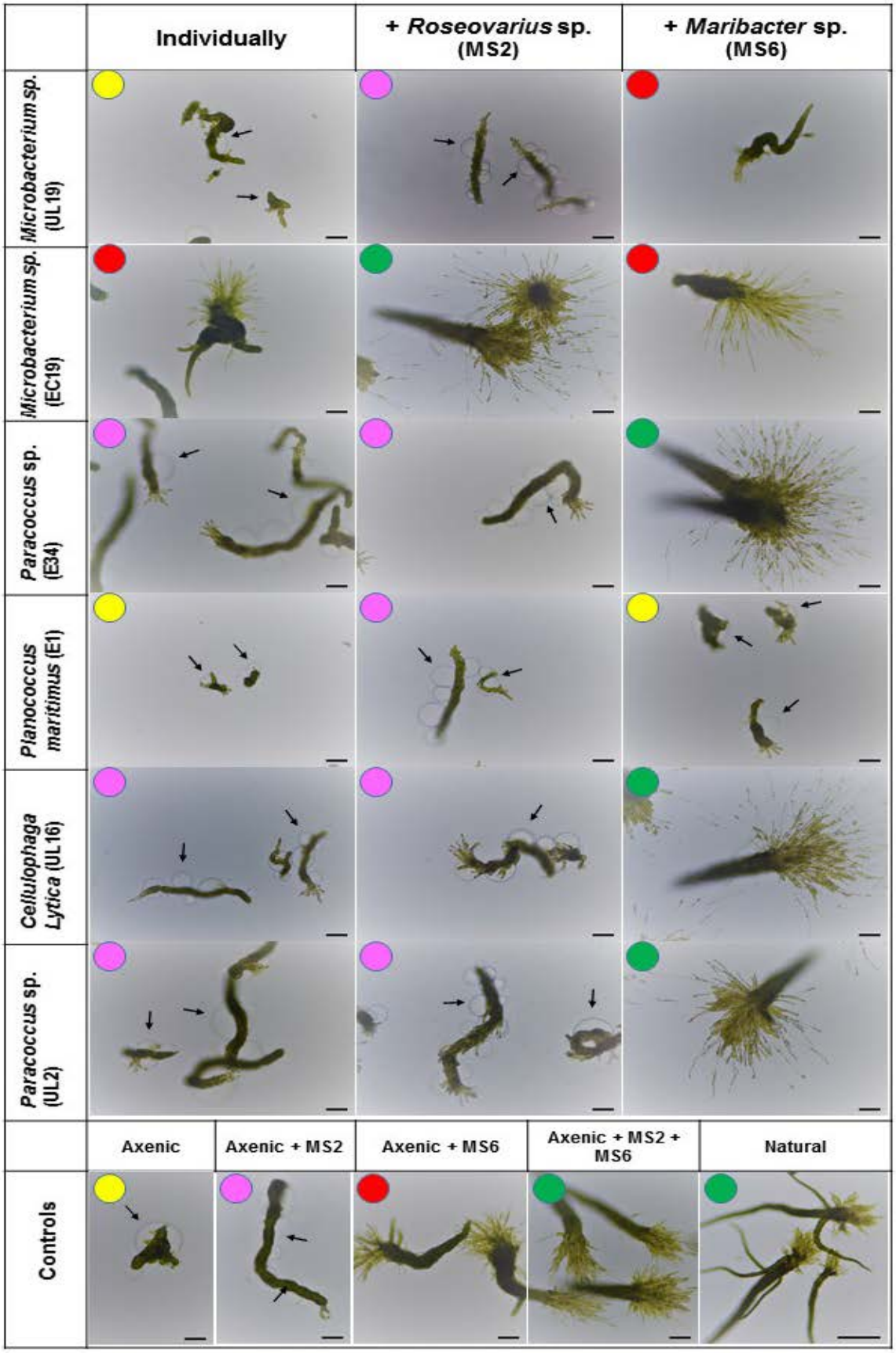

Figure 2.13 Morphogenesis assessment of *U. intestinalis* using the '*Ulva* bioassay array'. Different morphotypes of *U. intestinalis* induced by bacteria isolated from various *Ulva*

species singly and in pairwise combination with the bacteria *Roseovarius* sp. MS2 and *Maribacter* sp. MS6. Arrows indicate the typical colourless protrusions from the exterior cell walls of axenic cultures. Representative morphotypes are categorised by the same colour code as described in Figure 2.12. Propagules are 3 weeks old. Controls are shown in the bottom row. There were no significant differences in growth and morphology between propagules grown in the presence of the strains MS2 and MS6 compared to those grown in the presence of the natural associated bacteria ('Natural'). Scale bars = 100 µm.

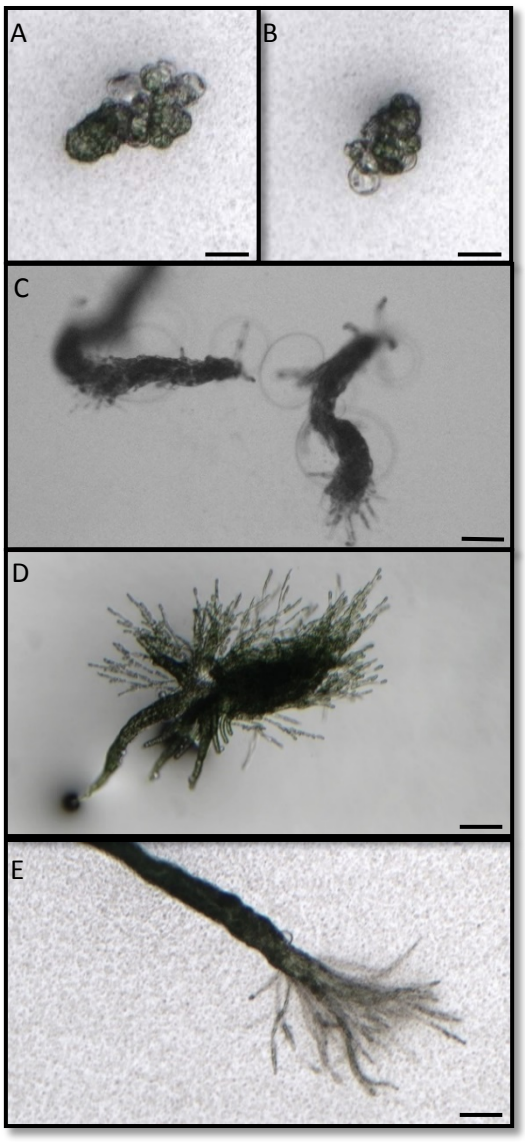

Figure 2.14 Morphogenesis assessment of *U. intestinalis* using the '*Ulva* bioassay array' through second experiment. (A) Axenic culture control, (B) Axenic morphotype induced by UL27, *Zobellia* sp., (C) MS2-like morphotype induced by UL13, *Psychrobacter* sp., (D) MS6-like morphotype induced by UL12, *Maribacter* sp., and (E) normal and complete morphotype in presence of UL9a, *Cobetia marina*, (M2-eauivalent) and EC19, *Microbacterium* sp., (MS6-equivalent). Propagules are 3 weeks old. Scale bars = 100 µm.

2.6.6 Semi-quantification of the morphogenesis inducing activity of bacteria

For further evaluation, a more detailed quantitative analysis of morphogenetic rescue was conducted. The number of cells produced by developing *Ulva* plantlets (Figure 2.15 A,B) and the degree of formation of cell wall protrusions as a result of a lack of MS6-morphogens was determined (Figure 2.15 C,D). Upon the inoculation of axenic gametes of *U. mutabilis* with the strains E34, UL16 or UL2 the average cell numbers increased four-fold (Figure 2.15 A; $p < 0.05$) within two weeks: these strains were therefore as active as the reference strain MS2. There was no significant difference between the activity of MS2 and the MS2-like bacteria E34, UL2 and UL16 on *U. mutabilis*: all bacteria can rescue the cell division to the same degree (Figure 2.15). However, two-way ANOVA revealed that the morphogenetic-activity of the bacteria E34, UL16 and UL2 was significantly lower on *U. intestinalis* (Figure 2.15 B; $p < 0.05$) than on *U. mutabilis* (Figure 2.15 A; $p < 0.05$) within the two-week bioassay. Overall, we conclude that differences in growth of both algae are due to slower growth rates of *U. intestinalis* compared to *U. mutabilis* rather than the mode of action of the factors released by the respective bacteria.

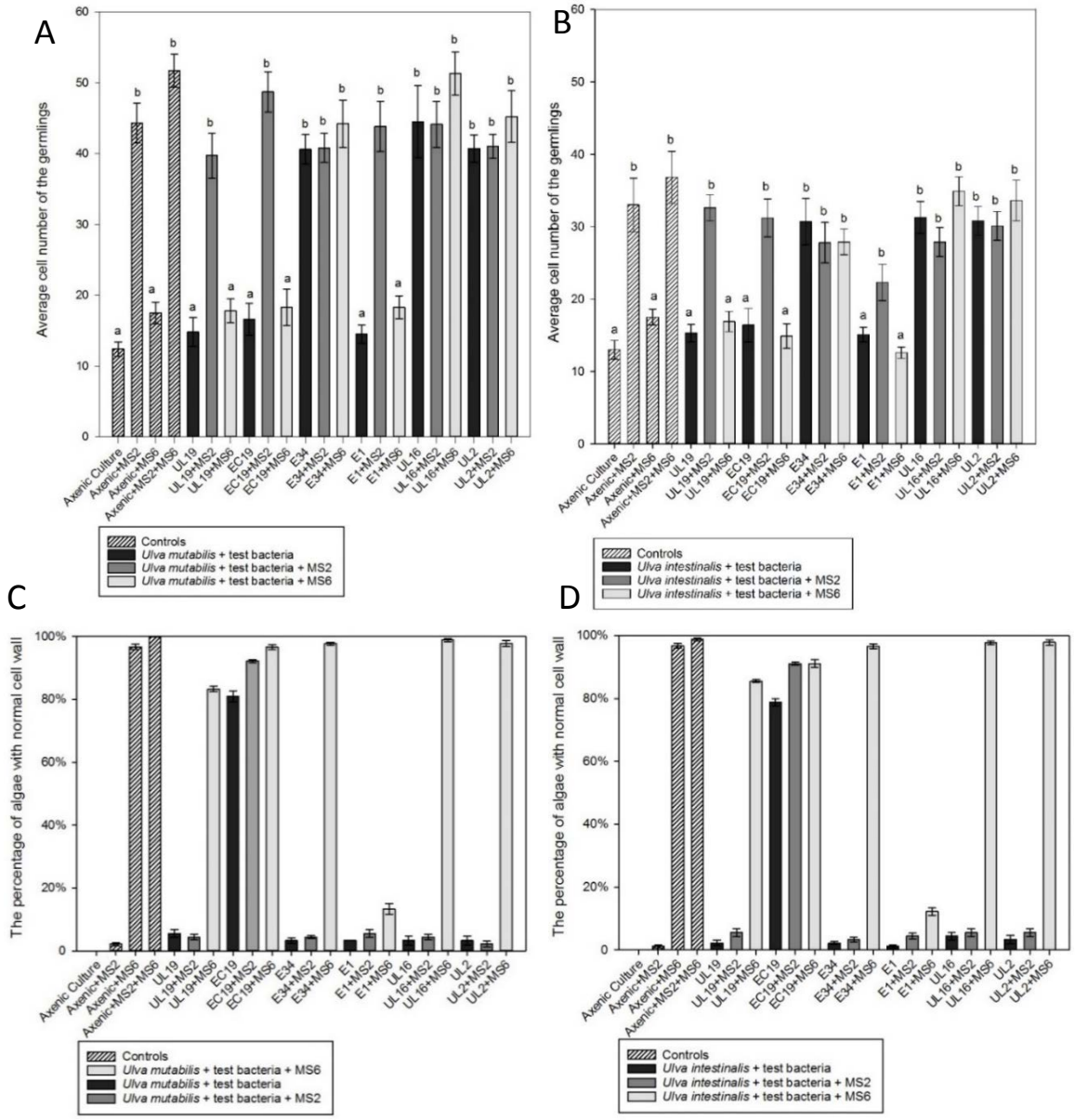

Figure 2.15 Semi-quantitative data of bacteria-induced growth and morphogenesis derived from the '*Ulva* bioassay array' with axenic *U. mutabilis* (A,C) and *U. intestinalis* (B,D) gametophytes. (A and B) To estimate the activity of the MS2-like factor, the total cell numbers in thalli of *U. mutabilis* (A) and *U. intestinalis* (B) plantlets were counted 10 days after inoculation with *Microbacterium* sp. EC19, *Microbacterium* sp. UL19, *Planococcus* sp. E1, *Paracoccus* sp. E34, *Cellulophaga* sp. UL16 or *Paracoccus* sp. UL2. Controls show the morphogenetic activity on gametes without bacteria, with the bacterial strain MS2, with the bacterial strain MS6 and with both MS2 and MS6 bacterial strains. (C and D) To determine the activity of the MS6-like factor, the proportion of thalli of *U. mutabilis* (C) and *U. intestinalis* (D) with normal cell wall development was evaluated as a percentage of total thalli 10 days after inoculation with

bacteria listed above. A one-way ANOVA was performed to reveal statistically significant differences, followed by a Dunn's multiple comparison test to determine which groups differ ($P < 0.05$), indicated by the letters a and b. Error bars represent (A,B) confidence intervals ($P = 0.95$; $n > 30$ individual algae) or (C,D) standard deviations ($n > 30$ individual algae).

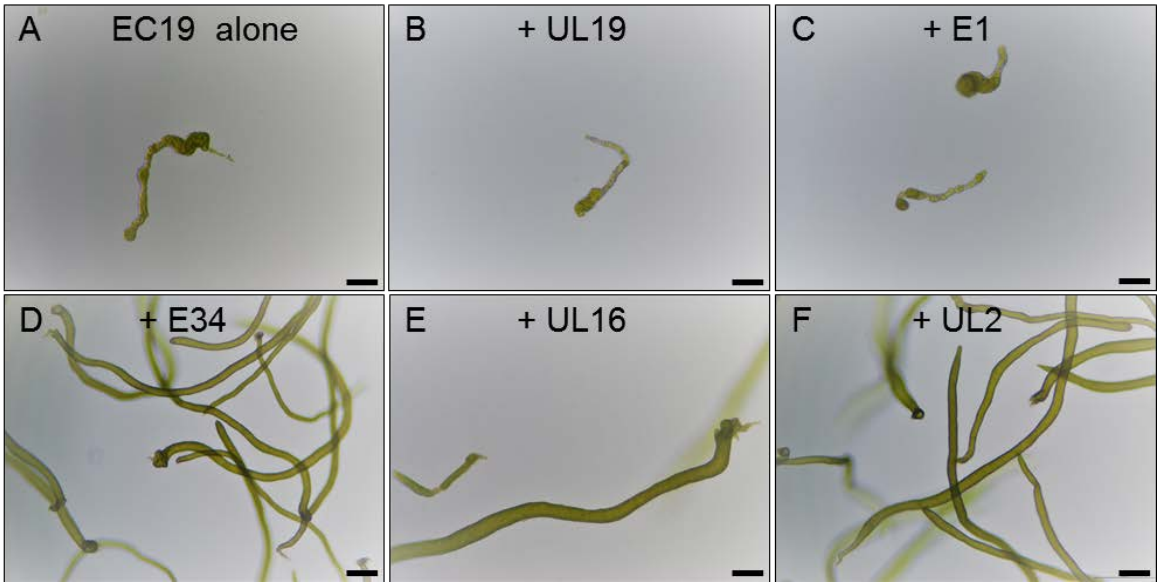

Figure 2.16 Establishment of a tripartite community of *U. mutabilis* with novel bacteria. Three-week-old *U. mutabilis* gametophytes are shown inoculated with bacteria isolated from different *Ulva* species in pairwise combination. Axenic gametes of *U. mutabilis* were inoculated with (**A**) *Microbacterium* sp. EC19 only, and together with (**B**) *Microbacterium* sp. UL19, (**C**) *Planococcus* sp. E1, (**D**) *Paracoccus* sp. E34, (**E**) *Cellulophaga* sp. UL16 or (**F**) *Paracoccus* sp. UL2. (**D-F**) Due to the complementary functional traits of the bacteria, the tripartite community can completely recover the morphogenesis of *U. mutabilis*, whereas the bacterial isolates UL19 and E1 do not contribute to the algal development. The bioassay system was scaled up using sterile culture flasks. Scale bars = 100 µm.

2.6.7 A new tripartite system established with *U. intestinalis* and *U. mutabilis*

The applied strains have been tested in previous studies with *U. linza* and bacterial activities were classified according to morphological scores by Marshall et al., (2006) (Table 2.2), but different functional traits for growth and morphogenesis were not

determined at that time. Therefore, in this study, bacterial strains were selected according to their two main functional traits in order to define new tripartite communities with *U. mutabilis* (Figures 2.16) or *U. intestinalis* (Figures 2.17). Importantly, there was no species-specificity between *U. intestinalis* and *U. mutabilis*, because a range of bacteria can perform their eco-physiological functions similarly in both species (Figures 2.16 and 2.17).

The morphogenesis of *U. intestinalis* and *U. mutabilis* axenic germlings completely recovered in co-cultivation experiments with *Microbacterium* sp. EC19, the only selected strain that could phenocopy the *Maribacter sp*. MS6, in combination with any one of E34, UL16 or UL2, which phenocopy the *Roseovarius* sp. MS2 (Figures 2.16 and 2.17). Upon inoculations, bacteria grew and formed a cluster around the rhizoid of *U. intestinalis* (Figure 18 A) resembling the tripartite *U. mutabilis-Roseovarius-Maribacter* system (Spoerner et al., 2012). It is not clear whether a single, or both, bacterial species are present at the rhizoid or how they achieve this, as only some species of *Microbacterium* sp. EC19 and *Paracoccus* sp. E34 are motile (Kelly et al., 2006). Starting with this biofilm, *U. intestinalis* continues growing in the presence of any of the pairs of specifically selected bacterial species (Figure. 2.17 G-I). In summary, a newly standardised *U. intestinalis* tripartite system has been established with various pairs of bacterial symbionts isolated from multiple *Ulva* species (Figure 2.18).

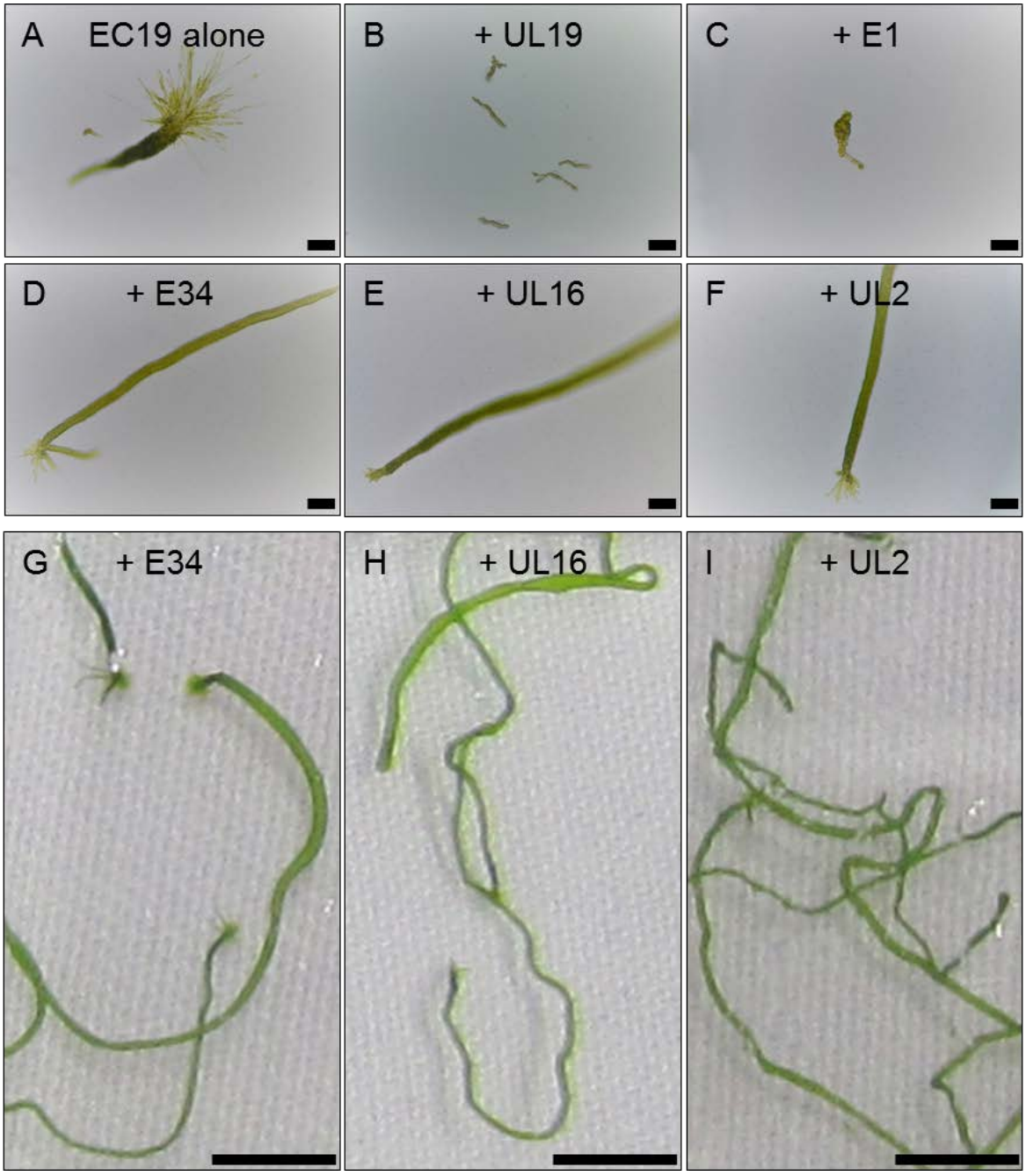

Figure 2.17 Establishment of a tripartite community of *U. intestinalis*. Three-week-old *U. intestinalis* gametophytes are shown inoculated with bacteria isolated from different *Ulva* species in pairwise combination. Axenic gametes of *U. mutabilis* were inoculated with (**A**) *Microbacterium* sp. EC19 only, and together with (**B**) *Microbacterium* sp. UL19, (**C**) *Planococcus* sp. E1, (**D**) *Paracoccus* sp. E34, (**E**) *Cellulophaga* sp. UL16 or (**F**) *Paracoccus* sp. UL2. (**D-F**) Due to the complementary functional traits of the bacteria, the tripartite community can completely recover the morphogenesis of *U. intestinalis*. (**G–I**) The thallus of *U. intestinalis* continues growing under these conditions and increases

significantly in size within one more week. (**A–F**) Scale bars = 100 µm and (**G–I**) scale bars = 1 cm.

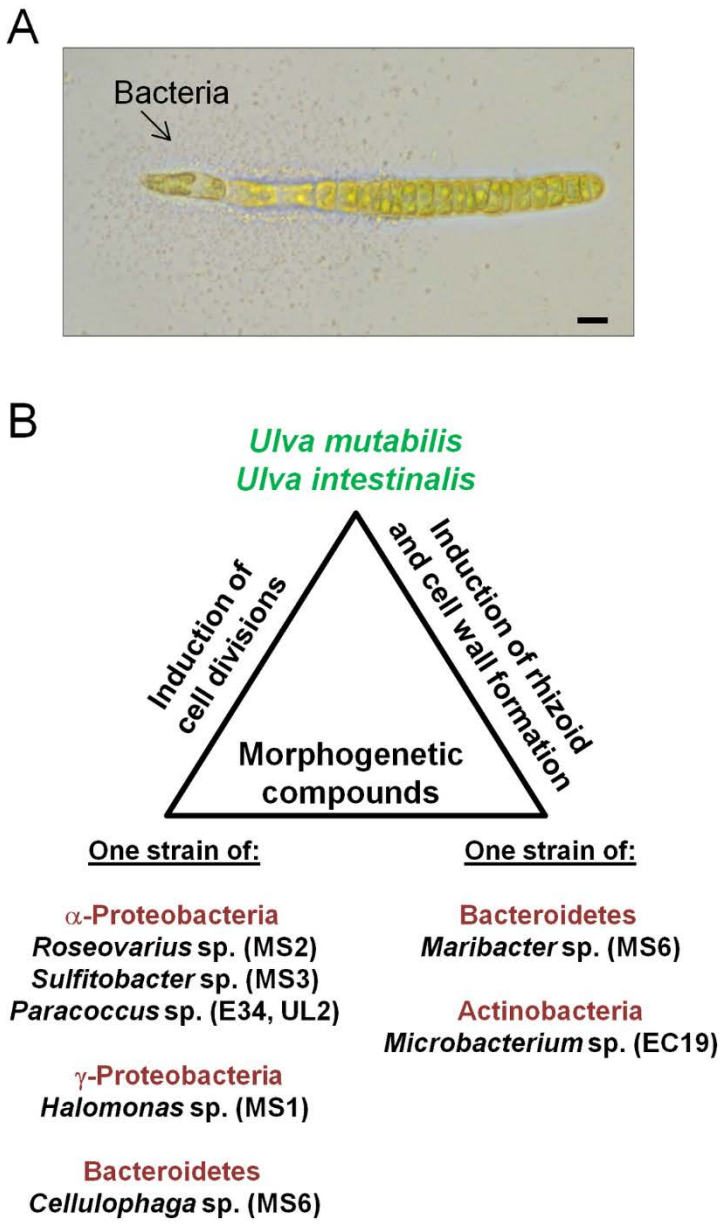

Figure 2.18 Model systems for bacteria–macroalgae interactions. (**A**) Bacterial biofilm formation upon inoculation of *U. intestinalis* axenic gametes with *Microbacterium* sp. EC19 and *Paracoccus* sp. E34 for 5 days. Bacteria concentrate around the rhizoid. Scale bars=10 µm. (**B**) Effects of a defined bacterial community can be reliably tested using tripartite systems of *U. mutabilis* or *U. intestinalis* and multiple combinations of algal morphogenesis-inducing bacteria. Figure was adapted and changed from Grueneberg et al., (2016). Names of bacterial strains, which were tested in this study for the first time, are printed in black.

2.7 Discussion

2.7.1 Development of a sustainable method for creating axenic *Ulva* cultures

One of the main objectives of this project was to produce axenic cultures and generate a consistent, reliable and repeatable assay so that the effect of naturally occurring periphytic bacteria on the morphology and growth could be evaluated. It is not easy to confirm that there is complete removal of bacteria from algal gametes. The maintenance of these cultures in an axenic condition, without any bacteria contamination is equally difficult. Undoubtedly, approaches used for culturing land plants axenically are inappropriate for algae mainly because of the involvement of toxic chemicals such as hypochlorite, which cannot easily penetrate the cuticle of the seed coat. Due to absence of such a defensive barrier in *Ulva*, substitute methods must be considered to remove the bacteria from gametes. Antibiotics were the obvious approach and they were shown to eliminate the bacteria numbers. However, the reduction (atelicity) was not kept all over the experiment and bacteria numbers started to increase between 2 and 7 days (Marshall, 2004). Additionally, they can affect the *Ulva* organelles (e.g. Chloramphenicol, despite killing bacteria by inhibiting protein synthesis, can disturb chloroplasts as well. So, they are potentially challenging for use in treatment of *Ulva* (Marshall, 2004).

This study, started with axenic cultures, where axenicity was defined as a bacterial load that is undetectable by culturing on plates and by a lack of PCR-amplification of any bacterial genomic DNA from the supernatant in which the purified gametes were resuspended prior to inoculation of the cultures (Ghaderiardakani et al., 2019b). Furthermore, the subsequent bioassays undertaken demonstrate that these cultures of *Ulva* develop only callus-morphology germlings and that only addition of specific bacteria recovered growth and morphogenesis to that resembling wild-type *Ulva* species (Figures

2.12 and 2.13). We cannot rule out the possibility that this method of gamete purification results in gametes associated with a small number of uncultivatable bacteria whose genomic DNA is undetectable by PCR in the supernatant from the purification procedure. The results of the present study have shown that phylogenetically distinct bacteria isolated from *Ulva* species other than *Ulva mutabilis* possess morphogenetic activity and can be used in combination to set up a tripartite system in an established model and phenocopy the reference strains MS2 and MS6. We have also shown that that the economically important *U. intestinalis* can function similarly in a tripartite system. We have defined new 'minimal' bacterial communities that promote growth, development and morphogenesis in *U. mutabilis* and *U. intestinalis*. The morphogenetic activity of all positively-tested bacterial strains was comparable with the activity found in sterile-filtered natural water samples collected from the lagoon Ria Formosa (Portugal) using the same standardised bioassay (Grueneberg et al., 2016).

2.7.2 Morphogenetic activity of bacteria is not limited to specific phyla: the first report of MS2-like activity from Bacteroidetes and MS6-like activity from Actinobacteria

This is the first report demonstrating the activity of an MS2-like factor within the phylum Bacteroidetes. Although experiments with boiling extracts of the *Maribacter* sp. MS6 revealed that this strain produces an MS2-like factor as well, the morphogenetic compound is not released into the environment (Spoerner et al., 2012). Our data contrasts with Grueneberg et al., (2016), who reported two isolates, *Algoriphagus* sp. and *Polaribacter* sp. that could each singly rescue complete morphology in *U. mutabilis*. This experiment reveals again that strains of the same genus, UL19 and EC19, can harbour different functional traits.

Until now, only very few Actinobacteria have been tested on *Ulva* species for their effect on algal morphogenesis (Marshall et al., 2006) and *Microbacterium* sp. EC19 is the first representative of this phylum with a defined activity to *U. mutabilis* and *U. intestinalis*. Interestingly, the phylum Actinobacteria was also one of the major beneficial bacterial phyla detected on *Gracilaria vermiculophylla* from the North Sea (Lachnit et al., 2011) and associated with *Laminaria* populations (Wiese et al., 2009, Salaün et al., 2010).

2.7.3 Host specificity of epiphytic bacteria on *Ulva* species, or lottery theory?

This study tested whether a consistent core community is necessary to drive complete morphogenesis of *Ulva* species or whether a range of bacterial isolates can phenocopy the algal phenotypes induced by the strains MS2 (*Roseovarius*) and MS6 (*Maribacter*).

Large-scale 16S rRNA gene sequencing of the bacterial populations present on various individual of *U. australis* demonstrated that a consistent core microbiota could not be detected, and a large number of bacterial individuals are able to colonise the algal surfaces (Burke et al., 2011a). The temporal and spatial comparisons carried out by Tujula et al., (2010) have revealed that the microbiota on *U. australis* varies considerably among the individuals collected from both the same, and three different, tidal pools and also over different seasons. Despite these considerable shifts, it also has been demonstrated that a set of bacterial epiphytes belonging to Alphaproteobacteria and Bacteroidetes remained stable over space and time, implying their possible significant role in function of this bacterial community (Tujula et al., 2010). However, bacteria belonging to the less-abundant phylum Actinobacteria on *Ulva´*s surface (Friedrich, 2012), can harbour strong (morphogenetic) effects on algal growth as demonstrated in our study.

Bioassays testing bacteria-induced morphogenesis, starting with axenic cultures, provide a unique approach to assess the specificity of bacterial functional traits within bacteria-

macroalga-interactions. Some evidence suggested that the activities of the strain MS6, promoting rhizoid growth and normal cell wall development, were rare, in contrast to the activity of strain MS2, which promotes growth and blade development. Therefore, the MS6-like factor was considered to be a genus-specific functional trait, also due to the fact that those marine bacteria are hard-to-culture (Wichard, 2015b, Grueneberg et al., 2016). With the findings of the current study, we show for the first time that both functional traits can be delivered by more than one bacterial phylum. The tripartite community of *Ulva* and bacteria can be established as long as certain bacteria release compounds with cytokinin-like activity, whereas others provide an auxin-like activity (Fig. 6). Overall, our data support the competitive lottery hypothesis (Sale, 1976, Burke et al., 2011a), which implies that colonizing of a bacterial community on a specific niche (algal surface) is random and based on the presence of functional genes and functional characteristics rather than on a requirement for bacteria to belong to particular taxonomic groups. Our study shows that in the lab, two species of green algae can use combinations of compounds derived from multiple species of bacteria to drive their correct morphogenesis, and we hypothesise that similar situations may arise in their natural environment, where algae are exposed to multiple bacteria and waterborne compounds.

2.8 Conclusions

Designed bacterial communities were used to test the algal morphogenesis-inducing traits of bacteria in both the standard test strain *U. mutabilis* and a new algal species, *U. intestinalis*. Elaborated bioassays revealed that more than one *Ulva* species can respond to the same range of bacteria that affect algal growth, development and morphology *via* microbial morphogens. There is specificity in the bacterial signals regulating algal development, e.g. with some bacteria inducing rhizoid formation, but the influences of bacteria cannot be assigned to a specific genus taxonomic group. This study supports

Grueneberg et al., (2016) who show that the presence of specific (epiphytic) bacteria does not directly matter as long as *U. mutabilis* perceives sufficient diffusible morphogenetic compounds even from bacteria in the vicinity of other *Ulva* species within a shared habitat. Establishing an additional standardised tripartite community (model system) with more than one species of *Ulva* presents an ideal possibility for elucidating the complexity of algal-bacterial partnership. The combined use of the tripartite communities will help to increase understanding of algal growth and development, to shed light on the underlying mechanisms involved in the cross-kingdom cross-talk of algae and bacteria. As *U. intestinalis* is a widespread alga with biofouling properties, our research presents a new way of understanding and controlling the life cycle of an economically important alga.

CHAPTER 3:

THE EFFECT OF WATERBORNE COMPOUNDS ON *ULVA* MORPHOGENESIS AND REMOVAL OF PHOSPHATE AND NITRATE BY THE GREEN SEAWEED *ULVA RIGIDA* IN AN IMTA SYSTEM

3.1 How fragile are marine ecosystems?

The word eutrophication is defined by Nixon (1995) as “an increase in the rate of supply of organic matter to an ecosystem”. He also emphasised that eutrophication is a process not a trophic status (a classification based on organic carbon supply like oligotrophic or mesotrophic etc.). A wide range and diversity of sources and materials contribute to the most common eutrophication in marine ecosystems, which is nutrient enrichment (Nixon, 1995). Anthropogenic eutrophication (organic loading from urban sewage, manufacturing wastes, agricultural, and aquacultural discharges) caused by increased human population, socio-economic development, enhanced urbanisation and use of the coastal areas, in addition to expanding usage of fertilisers, has led to accumulation of nutrients into marine ecosystems (Carpenter et al., 1998, Smith, 2002). According to Desmit et al., (2018) marine eutrophication directly depends on N and P enrichment at the river discharges and, to a lower degree, on atmospheric N deposition and upwellings (ocean-margin exchanges) in the North-East Atlantic.

Eutrophication is harshly threatening coastal ecosystems in many ways (Bricker et al., 2008) of which one is the promotion of green tide events. The number of records on the occurrence of large-scale green tides around the world has increased in recent years (Smetacek and Zingone, 2013, Kwon et al., 2017). Beside the world’s largest macroalgal (*Ulva* sp.) bloom in the Yellow Sea in China (Liu et al., 2013), the huge green tides have also been reported from many other locations such as southern California estuary (Boyle et al., 2004) or along the coasts of Brittany in France (Ménesguen and Piriou, 1995, Charlier et al., 2006) and Hiroshima Bay in Japan (Yoshida et al., 2015). Visually unpleasant, noxious-smelling masses of algae can adversely impact local fisheries and tourism industries and may persist for years and can pervasively and fundamentally alter the structure and function of aquatic ecosystems (Valiela et al., 1997). But conversely, some

areas use this excess biomass for biofuels, feed or soil improvements. Green tide-forming seaweeds especially *Ulva* spp represented as a possible source of renewable energy (Ceylan and Goldfarb, 2015), animal feeds (Bolton et al., 2009) and soil additive as compost (Cole et al., 2016). So far it has been even suggested that Utilising of seaweed biomass from green tides might be the best approach to control green tides in the Yellow Sea (Zhang et al., 2016).

3.2 Clean-up of the aqueous environment by seaweeds

In contrast to seaweeds being potential hazards to the environment, many recent studies have associated a reduction in dissolved nutrients' loads from aquaculture effluents or waste streams by seaweeds, which at the same time provide biomass for various industries such as bioenergy or production of green chemicals (as feedstock for the acetone, butanol and ethanol fermentation) and biofertilisers (Kraan, 2013, van der Wal et al., 2013). As mentioned before the world is facing crucial environmental issues such as polluted waters with a variety of contaminants from various human activities and developmental efforts. Re-thinking about waste water is an urgent need in order to manage natural resources properly (Clark and Deswarte, 2015). Furthermore, there is an increased pressure on the food production industry and agriculture due to the growing global population and food demand (FAO, 2016) which leads to use of more fertilisers and pesticides. Amongst agricultural fertilisers, the key elements for plants to grow are P (phosphorus) and N (nitrogen) and their run-off, from land washes into natural water ecosystems along with effluent from finfish and shellfish farms, from urban sewage and from industry, are raising too many environmental concerns (EPA, 2003). In fact, a considerable amount of valuable nutrient resources from terrestrial soil washes out with most agricultural practices faster than its recovery by natural processes. For example, by transporting to permanent sediments, freshwater P can be removed from circulation. (Carpenter and Bennett, 2011,

Compton et al., 2011). These nutrient loads can cause negative impacts on downstream biological communities (Lawton et al., 2013) and ultimately, these discharged nutrients into natural water systems to be deposited into deep ocean, that is one of these serious environmental concerns (Kraan, 2013). P and N flow from land and freshwater to the sea plays a key role in driving expansion of local oxygen depletion and deoxygenation (anoxia) in oceans (Diaz and Rosenberg, 2008, Rabalais et al., 2009).

3.3 Recycling natural resources by macroalgae

Undoubtedly, the industry of food production intensively depends on availability of phosphate-bearing rocks (mostly from marine and freshwater sediments) which is a non-renewable fertiliser ore and it has been predicted about half the world's remaining phosphate reserves will be depleted by 2030, considering the growing world population and for the sake of agricultural purposes (von Horn and Sartorius, 2009). Almost 30 countries around the world are currently extracting phosphate rock and the USA alone produces 33% of the total global output (Smil, 2000).

By adding manure or fertilisers containing organic and inorganic phosphate compounds, P initially is quite soluble and available. However, when it comes in contact with soil, several reactions begin occurring that make the phosphate less soluble and less available to plants. Some factors such as pH, clay content and type of clay, moisture content, temperature, and the P minerals already existing in the soil impact on the rate and products of these reactions. Adsorbing onto soil particles (mainly affected by texture of soil) and chemically combining with soil elements (e.g. calcium, aluminium, and iron) and forming new solid complexes are two common ways that phosphate ions react with soil. This phenomenon results in unavailability of phosphate to plants (Zhang et al., 2002).

Although nitrogen (mostly in form of free N_2 in the atmosphere) is not considered as such a restricted resource, reactive N or "biologically available" N, which is the result of "nitrogen fixation", accounts for less than 2% of the nitrogen on Earth (Galloway, 1998). Conversion of N_2 to a bioaccessible form of nitrogen is possible in three different ways: (1) through geochemical processes such as electrical discharges in the atmosphere, (2) biologically through the action of the nitrogenase enzyme to catalyse the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3) (discovered by (Beijerinck, 1901)). This enzyme is found only in a particular group of prokaryotes. These microorganisms are categorised into (i) non-symbiotic nitrogen fixing forms such as aquatic organisms (e.g. cyanobacteria), free-living soil bacteria (e.g. *Azotobacter*) and also bacteria that form associative relationships with plants, e.g. *Azospirillum* (ii) symbiotic N_2-fixing bacteria, such as *Rhizobium* and *Bradyrhizobium*, that form symbioses with leguminous plants (Postgate, 1982), (3) industrially through the Haber–Bosch process. Chemically, the conversion of free atmospheric nitrogen to ammonia as nitrogenous fertiliser (by the Harber-Bosch method), is an energy demanding process because of the strong triple covalent bonds between the two nitrogen atoms in N_2, which make the molecule highly inert and nonreactive. Approximately 1% of annual energy supply of the world is needed for preparing the necessary high temperature and pressure to break the N-N triple bond (Smith, 2002).

Considering these limited resources, a great deal of effort is required to retain and recycle N and P in order to postpone their global shortages (more specifically a P shortage and the energy or fossil fuels associated with producing a biologically useable form of N) in addition to declining the eutrophication of the aquatic ecosystems (Carpenter and Bennett, 2011).

The dissolved inorganic nutrients from the water can be assimilated by macroalgae, acting as a biofilter and removing the dissolved nutrient loads (e.g. excess N and P) and at the same time transforming into valuable biomass, adding to the income and the diversity of a fish farm (Cohen and Neori, 1991; Neori et al., 2003; Hernandez et al., 2006). In fact, this is the concept of an ecologically engineered ecosystem management approach, integrated multi-trophic aquaculture (IMTA), which involves farming finfish along with either algae or shellfish. Through this practice by-products (waste nutrients) from the finfish are recycled to become inputs for co-culture species (algae or shellfish etc.) (Abreu et al., 2011).

Normally, 60–70% of fish farm effluents consist of ingested nitrogen such as ammonia-N ($NH_3 + NH_4^+$), dissolved organic nitrogen which can quickly turned into ammonia-N and all these inorganic features of N (NH_4^+, NO_3^-, NO_2) are then accessible for assimilation by seaweeds (Porter et al., 1987; Krom et al., 1995; Cowey, 1995; Neori, 1996; Harrison and Hurd, 2001).

Fish culture effluents also contain phosphorus in forms of phosphate (PO_4^{3-}), dissolved organic phosphate and particulate organic P, and up to half of the P excreted by fish might be available for algal production (Dosdat et al., 1995, Neori et al., 1996). These commercial biofilters are providing more sustainable technologies and more ecologically sound practices. In fact, seaweed production by this approach should be considered as a "mixed blessing" strategy. Excess nutrients can be trapped by seaweeds and transformed into biomass and possibly into valuable products, such as carrageenan. Meanwhile through this process the negative ecological effects of excess nutrient loads will also be reduced (Ellner et al., 1996, Neori et al., 2004, Holdt et al., 2011).

Apart from challenges such as area needs and making a balance between high production expenses with high value consumption of the algae biomass, it is obvious that the algal biomass that has been used for bioremediation or as biofilter are supposed to absorb toxic burden of any waste water. Water environmental pollution due to toxic heavy metals raises concerns about potential but serious environmental and public health problems. Geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources are some of main reported sources of heavy metal contaminations in the environment (Tchounwou et al., 2012).

Thus, seaweeds used as biofilters might also contain high/toxic levels of heavy metals. The key question now is how these rich biomasses can be dumped? Conversion of these biomasses, in case the C/N ratio is high, to different types of biofuel (liquid, gaseous and solid) might be part of this solution (Nikolaisen et al., 2011). On the contrary, alleviating assimilation of nitrogen by algae will result in producing biomass with a low C/N ratio. Consequently, the biomass will be disqualified for bioenergy production, but still biomass could be Utilised for production of higher value products: feed, protein or soil improvement fertilisers (Sode et al., 2013). Surely the heavy metal ratio should not be too high.

3.3.1 Case study: impact of global warming on eutrophication and expanding green tides

Given the ecological and socio-economic effects of *Ulva* green tides, Gao et al., (2017) studied the interactive influences of ocean warming, acidification, and eutrophication on a range of attributes such as gamete settlement, germination, growth, and reproduction and main biochemical features of *U. rigida*, as one of the main green tide-causing species (Fletcher, 1996). This experiment indicated that the higher temperature accelerated germination rate as well as growth of young *Ulva* plants (*Ulva* reproduction), which

increased further in combination with elevated pCO_2 and nitrate. Based on the obtained results, climate change may lead to more severe green tides, especially when eutrophication cannot be mitigated efficiently. However, the study suggested that controlling the level of nitrate could be a part of the solution to tackle the green tide problem, since *Ulva* gametes are very sensitive to low nitrate. So simply, if nitrate can be restricted, it would possibly prevent germling growth (Gao et al., 2017).

3.4 Characteristics of the ideal candidate for bioremediation

To achieve substantial reduction of excess dissolved nutrients through the application of algal bioremediation, choosing a suitable algal sample is the key point. A variety of parameters are involved in selecting the best species for bioremediation purposes (Lawton et al., 2013, Chopin et al., 2012). *Ulva* species can be considered as an appropriate alternative owing to their efficient nutrient uptake and fast growth (Sode et al., 2013). Growing through a wide range of conditions is another vital characteristic, which satisfies the need for having year-round production in open culture systems (de Paula Silva et al., 2012). Furthermore, for eliminating the risk of invasion of target species into the natural environment and affecting local biodiversity, target species should be part of the native ecosystem/be a native species while possibly having a wide distribution potential (so that systems can be set up anywhere in the world). Perhaps the most important characteristic for selecting the appropriate target species among all candidates is to find a species that has suitable attributes for bioremediation, including high growth rate and nutrient uptake (Lawton et al., 2013).

Many studies have emphasised the importance of selecting the suitable species as the first critical step in algal bioremediation process. Lawton et al., (2013) discussed that *Ulva* species are “ideal candidates” as they have suitable traits for this purpose. In several ways,

different species within this genus have the most compelling features for using biomass throughout their global distribution. Fast growing, high stocking densities and high tolerance against epiphytes and a wide range of environmental conditions, contribute to ecological success of this genus (Callow et al., 1997). Thus, bioremediation of waste water with macroalgae such as *Ulva* might contribute to making a solution for both tackling the growing needs for recovery of nutrients from waste streams as well as the requests for sustainable production of protein and fertiliser in the future.

3.5 Determination of nitrate and phosphate in seawater

Detecting nitrate and phosphate in seawater is challenging. The concentrations of nitrate and phosphate are below the "limit of detection" of conventional methods of measurement (typically at nanomolar concentrations) over much of the world's oceanic surface waters (about 40% of the world's oceans). This is mainly due to depletion of these nutrients by active biological uptake in these waters (Patey et al., 2008). However, remineralisation of sinking particulate matter gives back the dissolved nutrients to the water column and subsequently the concentration of nitrate and phosphate enhances to micromolar in deeper areas of the oceans. Due to the important role of nitrate and phosphate in primary productivity and carbon sequestration in surface waters, a number of analytic methods have been established to deal with this issue (Patey et al., 2008). In order to measure the concentration of nitrate and phosphate in seawater, a variety of techniques have been performed traditionally, which Patery et al., (2008) have classified into three broad groups:

"(1) manual methods, where each sample is treated individually;

(2) automated methods, which are usually based on flow analysis; and,

(3) sensors, which, upon contact with the seawater, monitor a signal that is indicative of the analyte concentration." (Patey et al., 2008).

3.5.1 Nitrate analysis

The reduction method (nitrate to nitrite) using a copperised cadmium column has been accepted as the standard procedure in the routine analysis of nitrate (Wood et al., 1967). Then nitrite can be readily detected by spectrophotometer (at 540 nm) upon formation of a highly coloured dye by the diazotisation method originally proposed by Griess (1879) (Griess, 1879). This technique has been described as a very sensitive and robust one, which is not affected by the presence of other naturally occurring elements in seawater (Hansen et al., 1999).

3.5.2 Phosphate analysis

The typical method described for phosphate determination is using the colour reaction between orthophosphate and acidified ammonium molybdate to form 12-molybdophosphate, a yellow-coloured complex which based on the reaction condition can be detected by spectrophotometer (Murphy and Riley, 1962).

3.5.3 Nanomolar analytic methods for nitrate and phosphate

Patery et al., (2008) introduced a system as an ideal approach for recording nitrate and phosphate concentration through routine field studies, including liquid waveguide capillary cells connected to a conventional segmented-flow autoanalyser and using miniaturised spectrophotometers. Employing this approach, they managed to achieve “limit of detection” of 0.8 nM phosphate and 1.5 nM nitrate in the surface waters of North-East Atlantic Ocean. In this investigation, the nitrate concentrations in surface waters recorded in the range <1.5–165 nM and phosphate concentrations in the range 10–90 nM. The higher concentrations were supposed to be caused by upwelling of nutrient-rich waters and horizontal advection of nutrient-rich waters (Patery et al., 2008).

3.6 Importance and function of waterborne compounds for macroalgae morphogenesis

Besides the fundamental mechanisms and the metabolites involved in algal-bacteria cross-talk, it is particularly interesting to understand whether this process is only dependent on the growth-promoting morphogenetic compounds released from bacteria in the chemosphere of the symbionts or whether the direct physical cell contact is essential.

Contrary to some studies which showed that bacteria-free extract can partially resume the aberrant morphology of axenic *Monostroma* species (Provasoli and Pintner, 1964, Tatewaki et al., 1983) Nakanishi et al., (1996) reported the loss of morphology on removal of active morphogenesis-inducing bacteria in the culture chamber of *U. pertusa* axenic germlings, which indicated the requirement of actual presence of bacteria to promote algal development (Nakanishi et al., 1996), Interestingly, by adding synthesised thallusin to cell-free cultures of *Monostroma* it has been shown that in the absence of bacteria morphogenesis can be induced only in conditions where thallusin is provided continuously (Matsuo et al., 2005).

Similarly to plant growth-promoting bacteria (PGPB), which are microorganisms that naturally participate in complex ecological interactions (Liu et al., 2017), for example, in the rhizosphere with land plants (del Carmen Orozco-Mosqueda et al., 2018), bacteria also induce growth and morphogenesis in macroalgae (Singh and Reddy, 2014, Wichard, 2015b). A cocktail of compounds, harbouring different eco-physiological effects, is involved in macroalgal development (Spoerner et al., 2012).

Through subsequent studies Spoerner et al., (2012), by using a two-chamber system and a semi-permeable membrane, confirmed that the bacterial-algal interactions can happen even while bacteria and axenic gametes are not in direct contact with each other. By this

method, diffusible morphogenetic substances were secreted by bacteria in the medium and *U. mutabilis* perceived them without any direct contact (Spoerner et al., 2012).

Later, applying a bioassay-driven approach, Grueneberg et al., (2016) screened a collection of sterile-filtered natural water samples collected from 20 sampling sites and tidal pools along the lagoon Ria Formosa (Algarve, Portugal) to examine the ecological relevance of the waterborne bacterial morphogens by evaluating their morphogenetic impacts on the axenic gametes of *U. mutabilis*. This survey revealed that *Ulva* can benefit from morphogenetic compounds with similar activity to the compounds released by the *Roseovarius* sp. and *Maribacter* sp. (or equivalents of these strains), as waterborne morphogens were present, which were diffusible in the studied water body. Based on these findings, beyond the eco-physiological implications, the morphogenetic inductive potential of filtered seawater has to be taken into account particularly in the translation of this investigative research into practical outcomes. Using filtered seawater in scientific investigations and even in commercial aquaculture (e.g. land-based aquaculture operations) may directly affect the success of biomass production due to its morphogenetic effect on the growth and development of *Ulva* (Grueneberg et al., 2016).

A new abbreviation, AGPFs, for Algal Growth and morphogenesis-Promoting Factors derived from bacteria, is introduced by Ghaderiardakani et al., 2019b. In fact, these factors induce cell division, cell wall formation and holdfast development. AGPFs are under investigation in several studies and can induce various morphogenetic changes across the prokaryote-eukaryote boundary (Wichard and Beemelmanns, 2018).

In addition to the bacterial signals required for early *Ulva* development, mature blade cells of *U. mutabilis* excrete regulatory factors into their cell walls and the environment. These factors, a glycoprotein (Sporulation Inhibitor (SI)-1) and a low molecular weight factor

(SI-2) are essential for the maintenance of the vegetative state (Stratmann et al., 1996, Vesty et al., 2015) and are of great interest for algal aquaculture management (Charrier et al., 2017)

3.7 Chapter aims

It has been reported by previous investigators that *Ulva* spp. could be applied for removal of nutrients in land-based aquaculture (Bartoli et al., 2005, da Silva Copertino et al., 2009, Robertson-Andersson et al., 2008). The bioremediation efficiency of *Ulva* species has also been tested to take up nutrients from urban sewage and reject water from anaerobically digested waste water sludge (Tsagkamilis et al., 2010, Sode et al., 2013). Given the antibacterial effects of *Ulva* spp. on the waste water, reducing the health-related issues of the waste water is another advantage of such treatments (Lu et al., 2008).

The main aim of the present study is to determine the efficiency of the green macroalga, *U. rigida*, for bioremediation of nutrient-rich wastewaters from a land-based fish farm (Ilhavo, Portugal) to improve the quality of effluent water that is discharged from the fish farm to the environment, to reduce eutrophication risks and ultimately to provide basic guidelines for treatment, implementation and management. The nitrate and phosphate removal efficiency of an *Ulva*-based system receiving fish farm effluent waters assessed by:

- Determining the nitrate and phosphate level in water samples collected across the fish farm and seaweed cultivation system at different times during one day.
- To determine the efficiency of the green macroalga, *U. rigida*, for bioremediation of effluent water from a fish farm.

- To test the hypothesis that the lagoon Ria de Aveiro, linked to the IMTA, provides a reservoir of as-yet unidentified AGPFs for *Ulva´*s growth, while fish farming enriches the

water additionally with nutrients. The enrichment of potentially morphogen-releasing bacteria was determined by algae-bacteria bioassay analysis.

3.8 Materials and methods

3.8.1 Location and field site

The experiment was conducted at the ALGAplus company and a fish farm in the vicinity of this company in the coastal lagoon Ria de Aveiro, Portugal. The fish farm (2/3 seabass and 1/3 seabream in each pond) operates in a semi-intensive regime, at a low production density (up to 2 kg/m^3) and with artificial feeding counting only for 10-20% of the fish diet (natural food, as crabs, shrimps, algae accounts for the rest). Fish feeding normally occurs twice a day, early morning and late afternoon. Water flows in one direction only, entering the production system at each high tide and flowing back to the lagoon at low tides. The fish production units consist of an entrance water pond that feeds several fish ponds (4000-5000 m^2) individually controlled by gates; the fish effluents flow out to sedimentation ponds and then back to the lagoon. The macroalgae system, at the time of this study, was set to receive water that is pumped from two fish ponds (n3 and n5, Figures 3.2 and 3.3), mechanically filtered to remove particulate matter (drum filter with a mesh size >40 µm) and then continuously distributed across several seaweed tank. Each seaweed tank is individually controlled in terms of water exchange rates in order to minimize temperature variations and also nutrient availability. The algal species in cultivation is *U. rigida*, but some tanks are dedicated to the production of other green (*Codium tomentosum*) and red algae (*Porphyra and Gracilaria*). In this study only algae tanks A and B with *U. rigida* were investigated. The outflow from all production units (i.e. ponds and tanks), at the time of this study, is discharged to the sedimentation pond and thus mixed with the fish effluent before entering back to the lagoon (Figure 3.2). During this experiment, the water exchange rate at the sampled seaweed tanks was set in order to assure one renewal per day of the total tank volume. The main algae species in culture and the one considered for this experiment was *Ulva rigida* and, during this work, kept at a stocking density of 1kg/m^2.

3.8.2 First experiment: Measuring bioremediation capacity of *U. rigida*

Initially an outdoor experiment was designed to determine the nutrient uptake capacity of *U. rigida*. Therefore, a simple cultivation system was set-up using six experimental containers supplied with effluents from land-based fish commercial intensive aquaculture ponds as the nutrient source. During this investigation, *U. rigida* was cultivated for 28 days in cultivation tanks. Three tanks were set to receive a continuous independent flow of effluent from fish cultivation ponds for 24 hours (one renewal per day). A parallel batch experiment was designed in which the inlet water was closed during the day (closed from 9.00 until 18.00; ½ renewal per day) (Figure 3.1 A). The water flow in each container was set manually and the system was aerated constantly by air diffusers (L-shaped plastic pipes with holes set up on a plastic accessory attached to the end of the tub) that were placed about 1 cm above the container bottom (Figure 3.1 B). Aeration helped *Ulva* biomass to be exposed to the light and nutrients more efficiently (Abreu et al., 2011). The stocking density was 0.56 kg (wet weight) for each container (recommended by ALGAplus team). *Ulva* biomass for "seeding" was collected from *Ulva* cultivation ponds of ALGAplus company. Considering the main aim of this experiment, which was to examine the bioremediation capacity of *Ulva*, two electrodes, a Nitrate Ion-Selective Electrode and an Ammonium Ion-Selective Electrode (Vernier, Beaverton, USA) were bought and taken to Portugal for measurements. Table 3.1 summarises treatments and the water sampling times for each set of experiment. Electrodes were calibrated for each time-point measurement, but the collected values were not stable and did not look trustable. After 28 days, because of incorrect functions of the electrodes the experiment was stopped and a new aim pursued, to survey the waterborne morphogenetic compounds. Water samples collected from all over the IMTA area including the entrance ponds, fish ponds, algae cultivation

ponds and sediment pond at four different time points (9.00, 11.00, 14.00, 17.00) during the last day of research visit in Portugal (Section 3.8.3).

Figure 3.1 *Ulva* cultivation experimental system. A) Six containers and initial *Ulva* stock were prepared for experiment. **B**) The air diffuser to provide a constant movement in each system throughout the experiment.

Treatment	Time	Water samples
First treatment (1 renewal/day)	9:00 12:00 15:00 18:00	Inlet + Inside Inlet + Inside Inlet + Inside Inlet + Inside
Second treatment (½ renewal /day)	9:00 12:00 15:00 18:00	Inlet + Inside Inside Inside Inlet + Inside

Table 3.1 Experimental design.

3.8.3 Second experiment: Determination of growth and morphogenesis promoting factors and nutrients

In this study, the experiment area consists 22 sample points across the fish farm and seaweed cultivation system (Figure 3.2 and 3.3 – red spots). In February 2016, subsurface water was taken from sample points or at four time (9:00, 11:00, 14:00, 17:00) to survey the morphogenetic activity induced by waterborne compounds as well as evaluating the nitrate, ammonium, and orthophosphate. At each sample site, 20 mL water samples were collected with sterile screw cap polypropylene tubes (BD Falcon, Biosciences, Germany), transported immediately to the AlGAplus company's laboratory and filter-sterilised using 0.22 μm pore size syringe filters into sterile tubes under strictly sterile conditions within a laminar flow cabinet. The sterile-filtered water samples were frozen and stored at -80°C for the morphogenesis bioassays. Each sample (total number) was tested in the *Ulva* bioassay assayed separately for both morphogenetic activity and levels of nitrate and orthophosphate – and each bioassay was carried out at least 3 times for each sample. Originally the aim was to measure morphogenetic compounds at four time points over the day, but due to organisational issues (three concentrations, 22 sample points biological and technical repeats etc), I was only able to run the bioassay of morphogenetic activity for three time points (9:00, 11:00 and 17:00) (Figure 3.8).

Each sample was tested in the *Ulva* bioassay assayed separately for both morphogenetic activity and levels of nitrate and orthophosphate – and each bioassay was carried out at least 3 times for each sample. Ultimately, nitrate, orthophosphate and heavy metal data (Ghaderiardakani et al., 2019b) from replicates taken within each pond but also from ponds of the same type were combined to calculate the mean and to produce the results (i.e. ´Entrance ponds´ #1 and #2 (4 samples in total), ´Fish ponds´ #3 and #5 (8 samples in

total), ´Algal tanks´ A (T29) and B (T39) (6 samples in total) and the 3 discharge points of the algae system ´Sedimentation ponds´ (3 samples total) (Figure 3.3).

The heavy metal and ammonium (samples collected from seaweed tanks) analyses were performed in University of Jena by our collaborator Dr Thomas Wichard. In addition, to identify potential morphogen-producing bacteria, and determine composition and diversity of these bacteria in *Ulva´*s aquaculture, a next-generation DNA sequencing and subsequent metagenomic analysis was performed using water collected in the lagoon and algal tanks by Dr Wichard's lab (Ghaderiardakani et al., 2019b).

Figure 3.2 The study area at Lagoon Ria de Aveiro. The numbers correspond to the sampling points across the IMTA system.

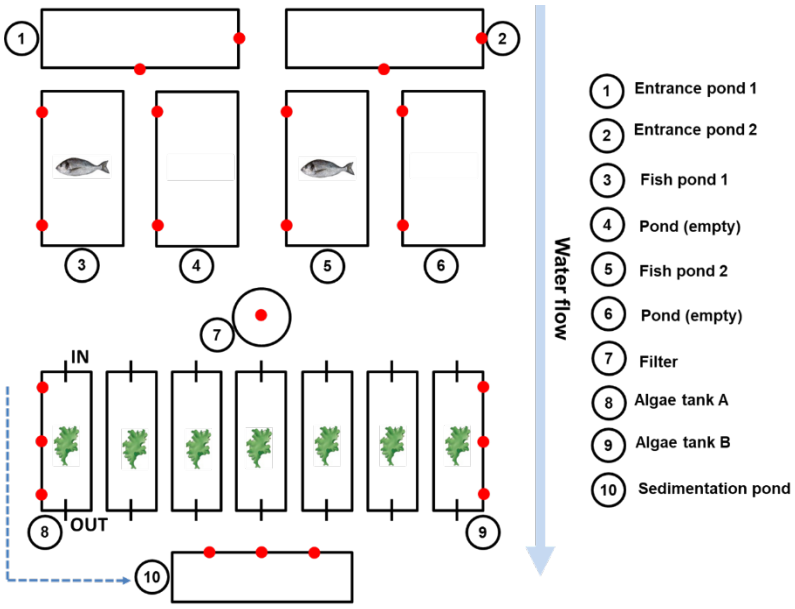

Figure 3.3 The scheme shows the sampling points (red dots) where the sterile-filtered water samples have been collected.

3.8.3.1 Determination of growth- and morphogenesis- promoting factors

A readily applicable *Ulva* bioassay array was used, the same as the previous experiments with the tripartite *U. mutabilis-Roseovirus-Maribacter* community (Section 2.3). The only

difference was for testing the morphogenetic influences of the aquaculture water samples on *Ulva* gametes, UCM was replaced with the filter-sterilised water samples. Since the bioassay array was run with a dilution series of water samples and to avoid any shortage of nutrients throughout the experiment, water dilutions were prepared in UCM. This '*Ulva* bioassay array' enables monitoring of growth promoting and morphogenetic activities in natural aquaculture water samples under controlled conditions in the laboratory with more biological replicates and different dilutions, because large numbers of *Ulva* gametes can be obtained from several individuals of the same culture batch or even from a single *Ulva* blade, and used for individual tests of germling formation.

Dilution hereby refers to the total volume [x / (x+y)] where x= parts of the collected aquaculture water and y= the parts of UCM. In this experiment three dilution series, 1:2 (one part of the fish farm effluents with 1 part of UCM), 1:10 (one part of the fish farm effluents with 9 parts of UCM), 1:100 (one part of the fish farm effluents with 99 parts of UCM), were tested to evaluate the potential morphogenetic activity on *U. mutabilis* axenic gametes (Figure 3.4). To minimise the number of differences between several experimental trials that would make the results ambiguous, positive and negative controls were applied on same tested 96-well plates each time. *U. mutabilis* axenic gametes (negative controls) were incubated with *Roseovarius* sp. alone, *Maribacter* sp. alone and *Roseovarius* sp.+ *Maribacter* sp., as positive controls (Figure 3.4). In each given experiment triplicates of each controls were performed.

After inoculation of axenic gametes with sterile aquaculture water, plates were covered with gas permeable sealing film (Breathe-Easy, Diversified Biotech, MA, USA) and transferred to growth chamber under standard growth conditions (Wichard and Oertel, 2010).

Thallus development and morphogenesis of about 45 - 60 germlings in total for each type of pond and tanks were examined in biological triplicates (15 - 20 germlings for each replicate) for each dilution step with (1:2, 1:10 and 1:100 dilution) and three time points (9:00, 11:00 and 17:00). An inverted microscope (DM IL LED, Leica, Wetzlar, Germany) equipped with a digital camera (Nikon, Düsseldorf, Germany) was used for inspection of the algal growth and morphogenesis. The qualitative features of inspected germlings in the tested population of *Ulva* included the presence of malformed cell walls (protrusions structures), cell divisions and blade formation and differentiated rhizoid cells (Spoerner et al., 2012). After ten days, upon the first appearance of malformed cell walls in the negative (axenic) control, quantification of the average cell number of the growing germlings and the percentage of thalli with entirely normal cell walls were carried out. Averaged values were calculated from samples of the same pond- or tank-type of each dilution.

For statistical analysis of differences between measurements for AGPFs and metals, a Kruskal-Wallis one-way analysis of variance followed by a Dunn's multiple comparisons test was performed at the $p < 0.05$ level. Here, a Gaussian distribution could not be assumed and thus the mean rank of each data set was compared with the mean rank of each other data set using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA). The graphs generated by SigmaPlot 13 software (Systat Software, San Jose, CA).

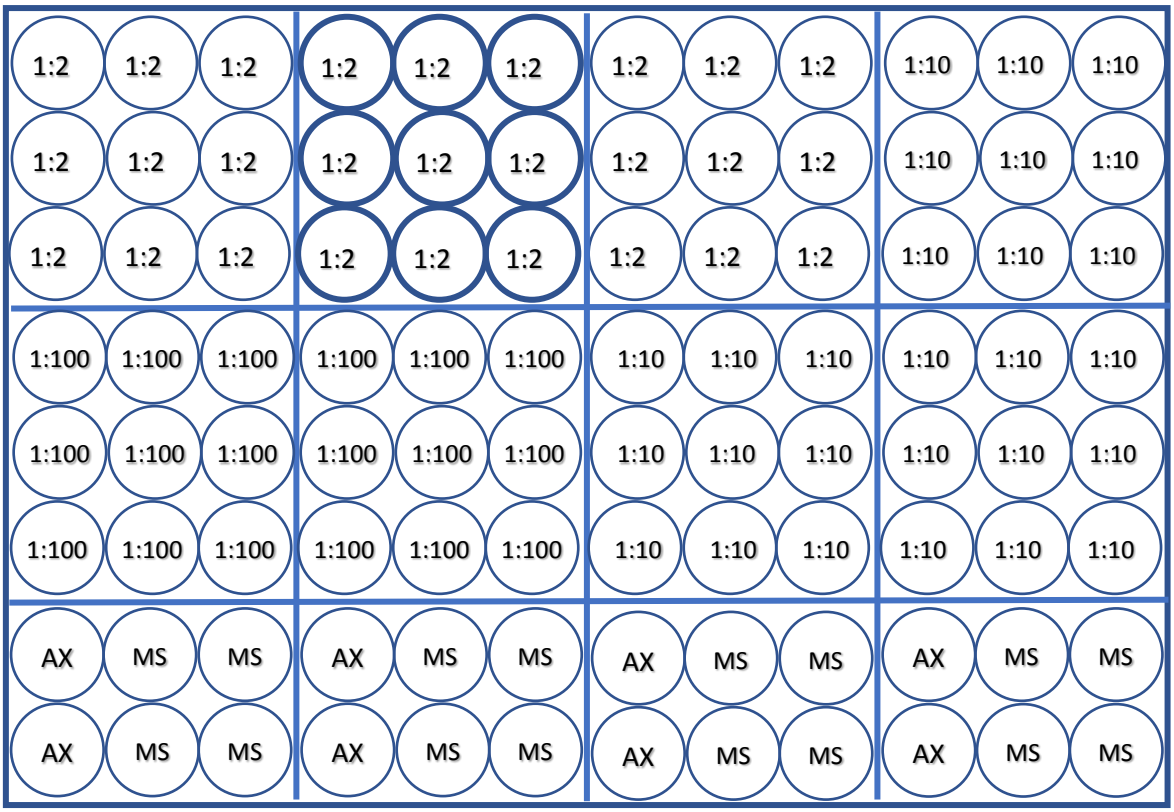

Figure 3.4 Experimental pattern. 1:2, 1:10 and 1:100 are different dilutions tested in this experiment to estimate the potential morphogenetic impact of fish farm sterile water., AX: Axenic gametes, MS2: Axenic gametes + *Roseovarius* sp. MS2, MS6: Axenic gametes + *Maribacter* sp. MS6 as controls.

3.8.3.2 Nitrate and orthophosphate measurement in the water from the aquaculture site

Concentrations of nitrate were determined using the colorimetric test kit VISOCOLOR® (MACHEREY-NAGEL, Dueren, Germany) and concentrations of orthohosphate by NANOCOLOR® O-phosphate kit (MACHEREY-NAGEL, Dueren, Germany), following the manufacture instruction. Comparison of the colours has done by spectrophotometer (Thermo Fisher, Germany). A control solution was set up using 20 ml H_2O with 1 ml reagent 1 and 1ml reagent 2 for nitrate measurement was used. Similarly, control solution of 20 ml distilled water with 1 ml reagent 1 and 1ml reagent 2 for orthophosphate measurement was prepared. Test and control samples were then transferred to 1.6 ml

Sarstedt polystyrene cuvettes and their absorbance read at 540 nm for nitrate and at 436 nm for orthophosphate against the control. Absorbance values for each sample were read in triplicate, and for each repeat the spectrophotometer was recalibrated against the control.

For statistical analysis of differences between different measurements for nitrate and orthophosphate a one-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA). The graphs generated by SigmaPlot 13 software (Systat Software, San Jose, CA).

The NO_3^- assay was firstly tested in water containing only NO_3^- stock in order to confirm the assay detected NO_3^- in the required manner. The calibration graph constructed by varying the NO^{3-} content from 0 to 36.5 mg/l produced the linear relationship displayed in Figure 3.5. As with the NO_3^- assay, a calibration graph for orthophosphate was obtained using 0-39 mg of KH_2PO_4 stock dissolved in distilled water. The obtained calibration graph displayed in Figure 3.6.

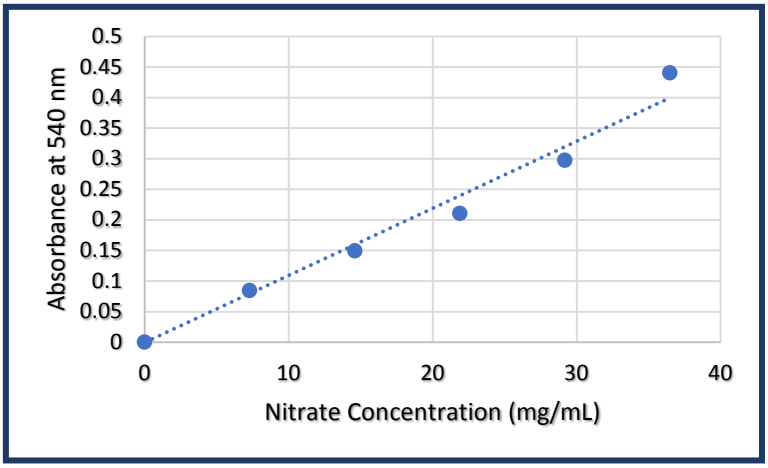

Figure 3.5 A calibration graph for measurement of nitrate displaying the change in absorbance with increasing nitrate concentration. R2 = 0.9743. Trend line and R2 value determined using Microsoft Excel linear regression.

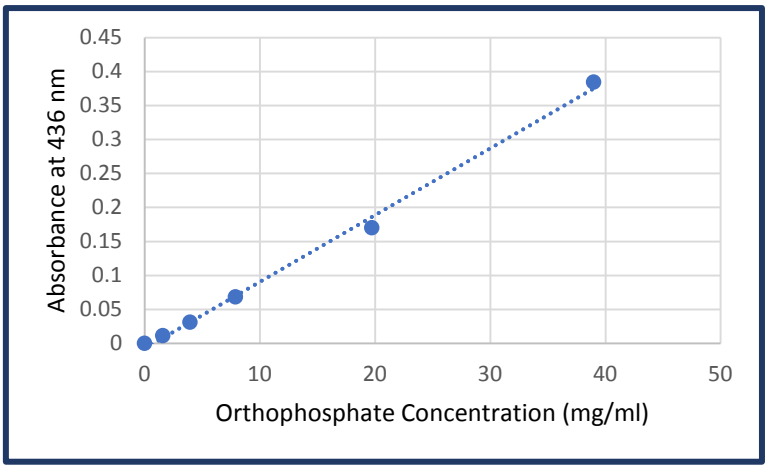

Figure 3.6 A calibration graph for measurement of orthophosphate displaying the change in absorbance with increasing orthophosphate concentration. R2 = 0.9962. Trend line and R2 value determined using Microsoft Excel linear regression.

3.9 Results

To determine the levels of algal growth-regulating substances in the integrated multitrophic aquaculture (IMTA) system, a "snapshot" was taken at various time points over a single day as *Ulva* growth yields into 3 kg fresh weight m^{-2} $month^{-1}$ with few variations over the year in this system. The approach allows us to determine many growth-promoting variables at the same time. The parameters measured were algal growth-promoting and morphogenetic factor activity, vital nutrients and metals (measured by Dr Wichard's lab).

3.9.1 Survey of algal growth and morphogenesis promoting factors (AGPFs)

Sterile-filtered water samples were tested to determine where and when morphogenesis-inducing compounds were present in the IMTA (Figures 3.3 and 3.4) using axenic cultures of *U. mutabilis* (Figure 3.7A). The activity of morphogens within the test samples was assessed via the standardised bioassay array with *U. mutabilis* (Figure 3.7) by applying a dilution series of the collected samples with UCM (Grueneberg et al., 2016). Mean cell numbers and percentage of *Ulva* germlings with normal cell walls increased upon

administration of seawater from the aquaculture units (Figure 3.8A and B) with a complete recovery of *Ulva´*s morphogenesis. Morphogenetic compounds could be detected in excess in each water sample, as 1:2 diluted sterile-filtered water samples revealed a complete recovery of morphogenesis. Even 1:10 diluted samples showed significant induction of cell division (Figure 3.8A) and the formation of normal cell wall (Figure 3.8B). The activities of both the *Roseovarius*-factor (cell division) and the *Maribacter*-factor were not apparent in a dilution of 1:100 (Figure 3.8A and B), when almost all gametes developed consistently into a callus-like morphotype, which is typical for an axenic culture (Figure 3.7A). The activity demonstrated both by the number of cells in individual germlings and the percentage of algae with normal cell wall formation did not significantly differ ($p > 0.05$) between the water samples at various collection points (Figure 3.4) or at different times of day in the IMTA (Figs. 3.8A and B). Importantly, the activities of the *Roseovarius*- and *Maribacter*-factors did not differ between the lagoon water (‘Entrance’) and after the Fish pond (‘After filtration’) (Figure 3.8A and B). In summary, these results show that morphogenetic compounds are present in the used lagoon water, throughout the IMTA system and throughout the day when the experiment was performed, in sufficient quantities to promote *Ulva´*s full morphogenesis.

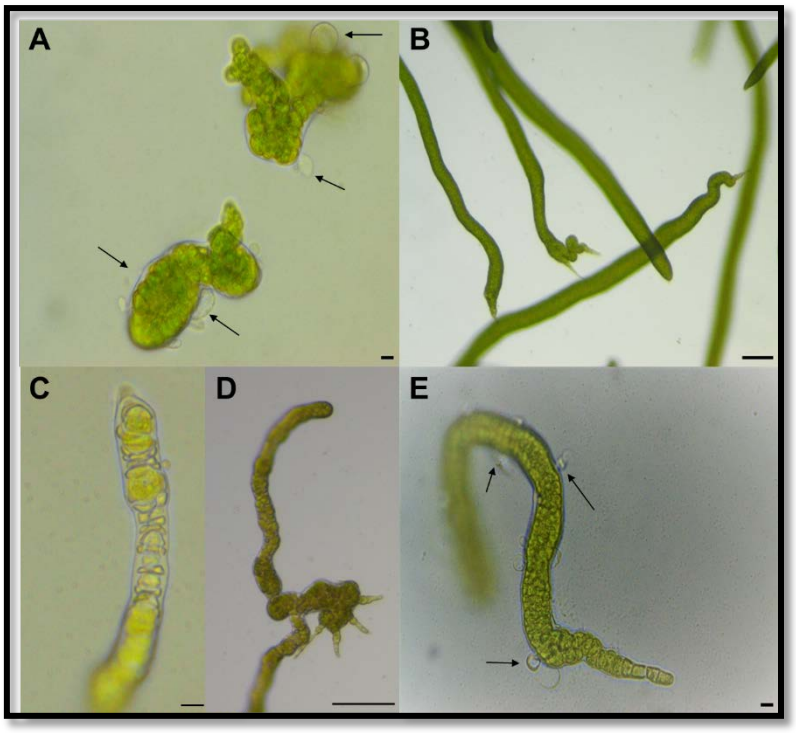

Figure 3.7 Representative morphotypes within the *Ulva* tripartite community. (**A-E**) The standardized '*Ulva* bioassay array' comprising axenic gametes of *U. mutabilis*, *Roseovarius* sp. MS2 and *Maribacter* sp. MS6 allows the fast determination of the different morphotypes induced by waterborne morphogenetic compounds in aquaculture water samples. (**A**) Axenic callus-like morphotypes of *Ulva* were compared with (**B**) axenic cultures inoculated with sterile-filtered water samples (see also Figure 3.8A and B) after three weeks. Controls are showing the morphotypes inoculated with *Maribacter* sp. only (**C, D**) or with *Roseovarius* sp. (**E**). Arrows (A, E) indicate the typical colourless protrusions from the exterior cell walls due to the lack of morphogens released by *Maribacter* sp. Scale bar (A, E) = 10 µm. Scale bars (B, C and D) = 100 µm.

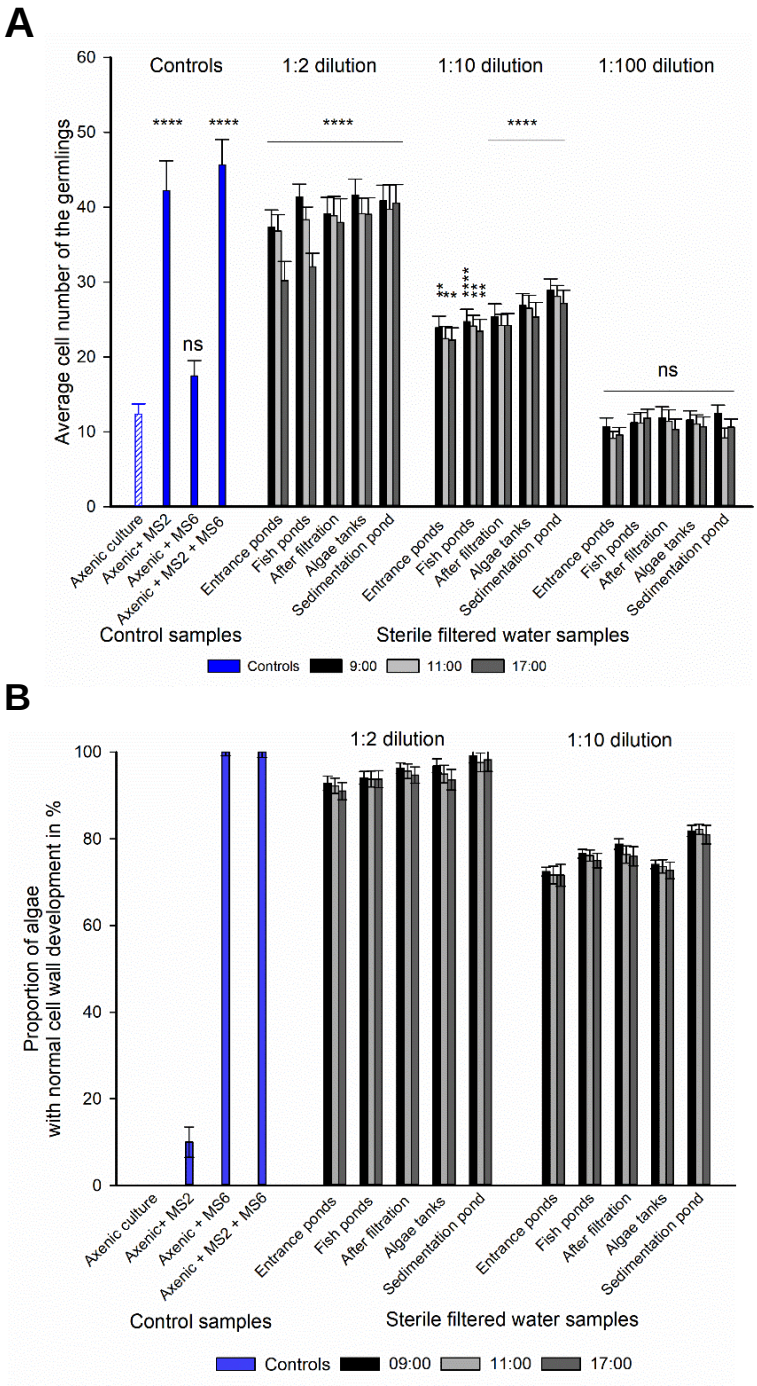

Figure 3.8 '*Ulva* bioassay array' of the morphogenetic activity of water samples from different locations on the aquaculture farm. (A) Semi-quantitative data derived from this bioassay showed the potential algal growth-promoting factors released by bacteria using dilution series of the ratios 1:2, 1:10 and 1:100 of doubly sterile-filtered water samples from each location as indicated on axenic *U. mutabilis* gametophytes. To estimate the activity of the *Roseovarius*-factor, the total cell numbers in thalli of *U. mutabilis* plantlets were counted 10 days after inoculation. Controls (blue bars) show the morphogenetic activity on gametes without bacteria (axenic, blue striped column), with

Roseovarius sp., with the bacterial strain *Maribacter* sp., and in combination with both bacterial strains. Data were analysed using a Kruskal-Wallis test followed by a multiple comparisons test with Dunn correction for "Axenic culture" control versus treated groups. Significant differences were indicated by asterisks. ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ and ****: $p \leq 0.0001$. (B) To determine the activity of the *Maribacter*-factor, the proportion of thalli of *U. mutabilis* with normal cell wall development was evaluated as a percentage of total thalli 10 days after inoculation with bacteria listed above as controls, with 1:2 dilution and 1:10 dilution. The effect of the *Maribacter*-factor completely vanished at 1:100 dilutions (i.e. 0 %, not shown). For A and B, error bars represent confidence intervals (P = 0.95; n = 45-60 individual algae).

3.9.2 The circulating system in the fish farm provides >=4mg/L nitrate and >=2mg/L orthophosphate throughout the day

In the entrance ponds, where water comes in from the river to supply the fish and algal tanks, nitrate levels peak at 14.00 (3.4 mg/L), with the lowest concentrations (2.3 mg/L) at 09.00. This is lower than the subsequent locations, reflecting the fact that water is entering from the river and has not been exposed to the fish or feed in the farm. Similarly to nitrate, the orthophosphate content of the entrance ponds peaks at 14.00 (0.16 mg/L) and is lowest (the lowest across all sample-sites) at 09.00 (0.14 mg/L) (Figure 3.9A and 3.10A).

In each fish pond, nitrate peaked at 11.00 (average 5.68 mg/L) and orthophosphate at 14.00 (0.4 mg/L): this reflects fish being fed at 07.00-08.00 and digestion/subsequent excretion of nitrate or orthophosphate occurring. The lowest amount of nitrate detected is at 17.00 (4.3 mg/L) and for orthophosphate at 09.00 (0.26 mg/L) (Figure 3.9B and 3.10B).

After filtration of the water from the fish ponds, the maximum amount of nitrate was recorded at 11.00 (5.4 mg/L) and orthophosphate at 11.00 and 14.00 (0.4 mg/L), at other times the nitrate and orthophosphate levels were similar to those at to 09.00 (Figure 3.9C and 3.10C). In both seaweed tanks at all sample points, the nitrate concentration, similarly to orthophosphate concentration, increased until 14.00 ((5.5 mg/L) and (0.3 mg/L)

respectively), and then decreased again at 17.00 (Figure 3.9D and 3.10D). In the sediment pond, overall levels of nitrate are higher at each corresponding time point ($p<0.01$) than in other locations (6 mg/L) and peak at 14.00 (Figure 3.9E and 3.10E). The higher levels of orthophosphate recorded in water samples collected after filtration (0.38 mg/L) which compared to in the algal tanks (compare Figure 3.10C and D) suggest the absorption of phosphate by seaweeds (6.9% decrease at 09.00, 18.4% at 11.00, 13.1% at 14.00 and 12.1% at 17.00).

Taken together, this data shows that there is variation in nitrate and orthophosphate levels in the fish farm during the day but that in the algal tanks the level of nitrate ranges from 2.3-6mg/L and phosphate from 1.4-0.4 mg/L). The levels of nitrate and orthophosphate in the entrance ponds are lower than in the other tanks, suggesting that a substantial proportion of the nitrate and phosphate in the algal tanks comes from fish metabolism. Moreover, it appears that the seaweed is able to deplete the water of orthophosphate while nitrate levels remain in abundance.

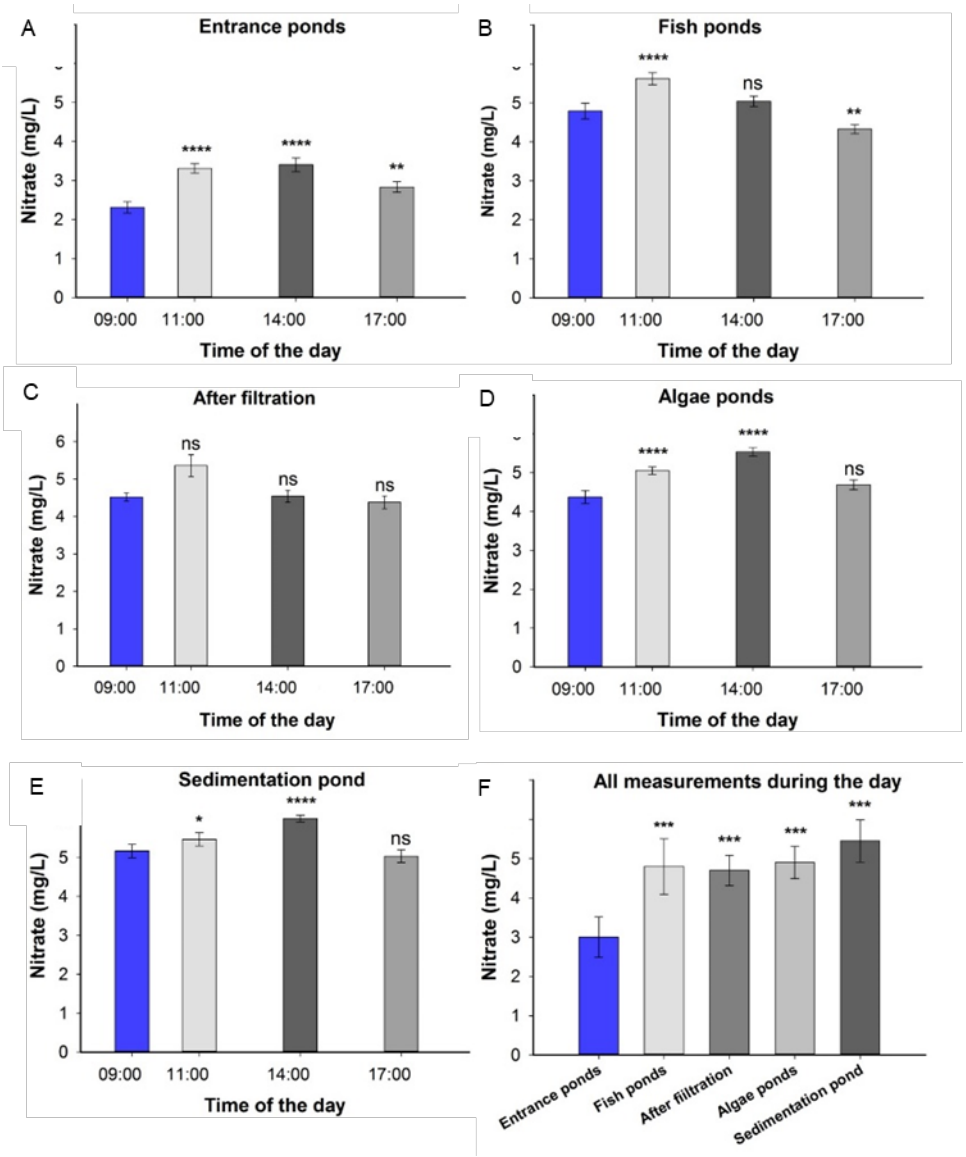

Figure 3.9 Comparison the concentration of nitrate (mg/L) along the sampling site. Values display the average of nitrate concentrations in (**A**) Entrance ponds 1 and 2, (**B**) Fish ponds 1, 2, 3 and 4, (**C**) After filtration, (**D**) Algal tanks (T29 and T39), **E**) Sediment pond and **F**) Mean nitrate concentrations measured over the day of all sample were compared with the water from the Entrance pond. A one-way ANOVA was performed to reveal statistically significant differences, followed by Dunnett's post-hoc comparisons tests to determine which groups differ significantly from 9:00 within a single pond. Error bars represent confidence intervals ($p = 0.95$). Significant differences were indicated by asterisks. ns: $p > 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ and ****: $p \leq 0.0001$.

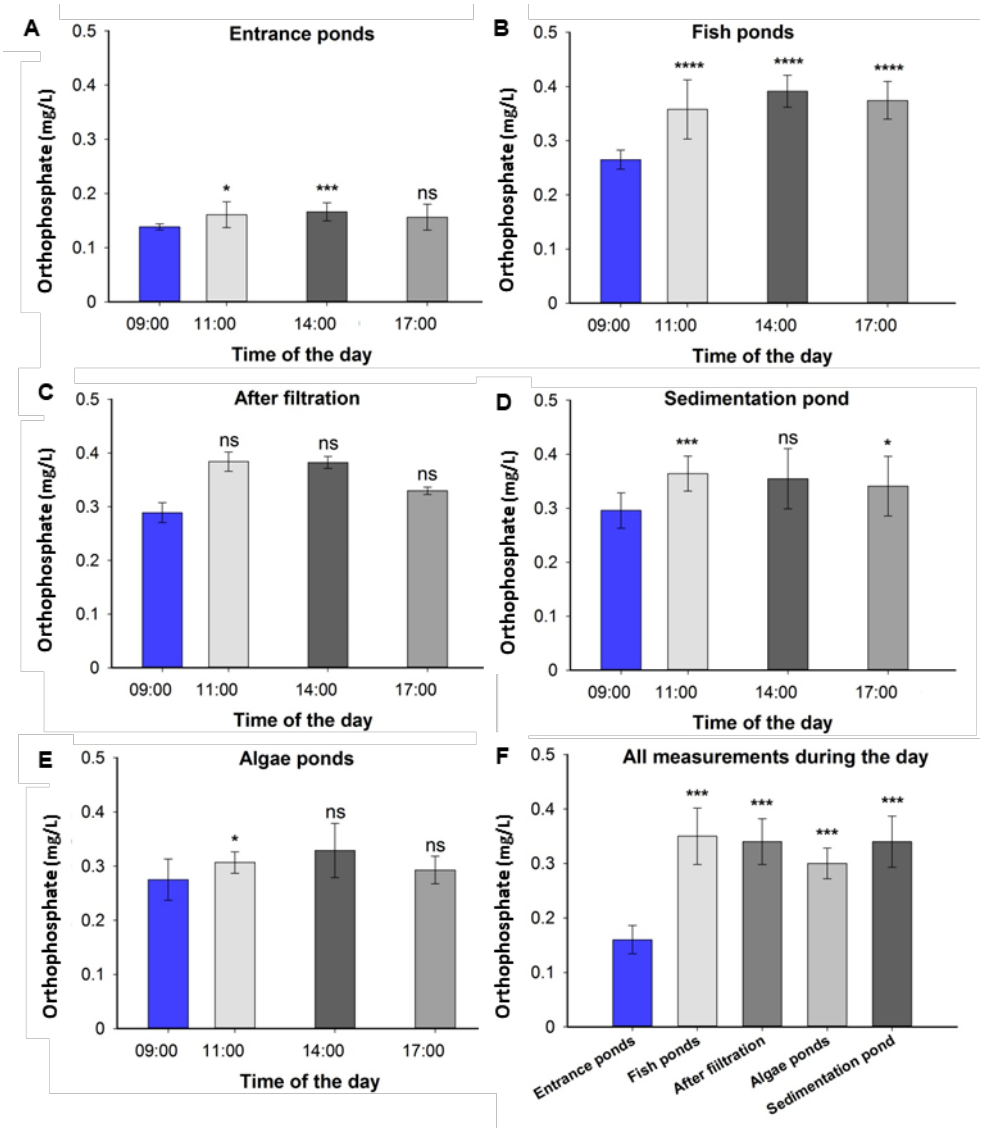

Figure 3.10 Comparison the concentration of orthophosphate (mg/L) along the sampling site. Values display the average of phosphate concentrations in (**A**) Entrance ponds 1 and 2, (**B**) Fish ponds 1, 2, 3 and 4, (**C**) After filtration, (**D**) Algal tanks (T29, T39) and **E**) Sediment pond and **F**) Mean orthophosphate concentrations measured over the day of all sample were compared with the water from the Entrance pond. A one-way ANOVA was performed to reveal statistically significant differences, followed by Dunnett's post-hoc comparisons tests to determine which groups differ significantly from 9:00 within the same pond. Error bars represent confidence intervals (p = 0.95). Significant differences were indicated by asterisks. ns: $p > 0.05$, *: $p \leq 0.05$, ***: $p \leq 0.001$ and ****: $p \leq 0.0001$.

3.9.3 N requirements for *Ulva intestinalis* growth in a lab-based system

Additionally, our preliminary data from an experiment to test the differential growth response of *Ulva intestinalis* to different sources of nitrogen (NH_4^+; NO_3^-) in Provasoli medium (Bold and Wynne, 1985) showed that in presence (or probably after depletion) of nitrate, *Ulva* pieces break down and generate spores/gametes. The *Ulva* pieces colour in medium enriched with $NaNO_3$ is more yellow-green. While the ammonium-enriched medium induced a higher increase in surface area expansion than the nitrate-enriched medium. The colour of the blade pieces enriched with NH_4Cl is steadily greener than pieces treated with nitrate which may be a result of the accumulation of chlorophyll in the cells (Figure 3.11).

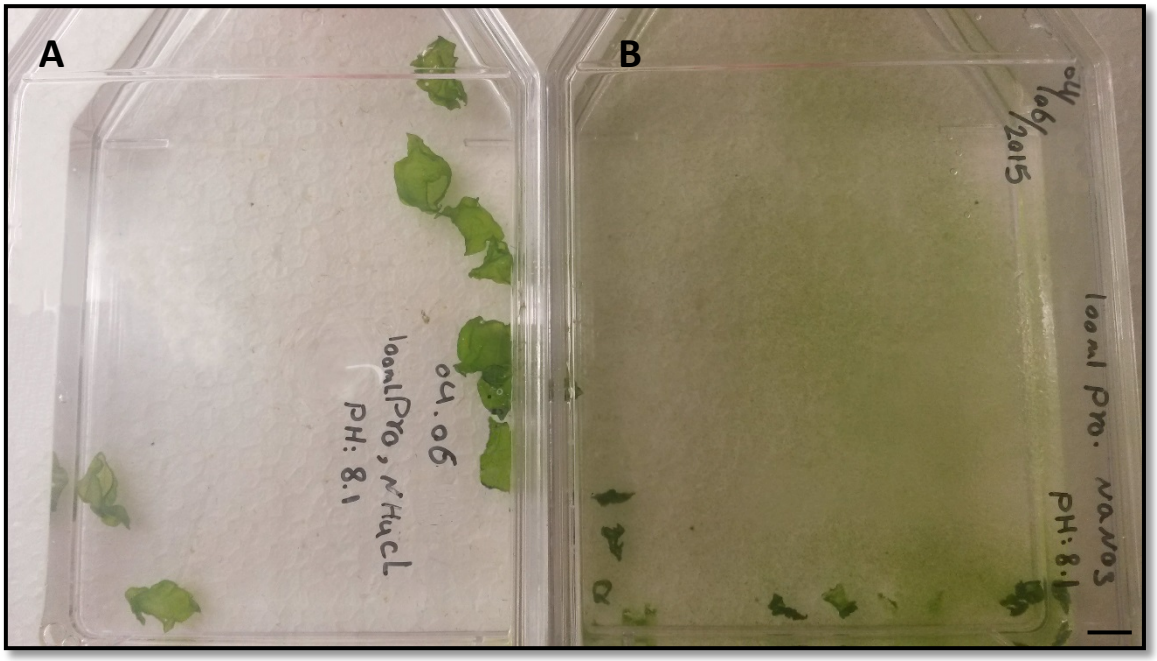

Figure 3.11 The growth difference of the surface area in 14-day cultures of *U. intestinalis* blade pieces in Provasoli medium enriched with different (A) ammonium and (B) nitrate nutrients. Scale bar = 1cm.

3.10 Discussion

Seaweed aquaculture, alone, or integrated into an animal production, requires a fundamental understanding of the underlying biological mechanisms controlling macroalgal life cycles from the production of germ cells to the growth and fertility of the adult organisms (Charrier et al., 2017). In this study, the input water to the farm from the surrounding lagoon (Ria de Aveiro), and several points within the IMTA system itself, were analysed for the activity of bacterial signals (not yet structure-elucidated) required for *Ulva´*s development and morphogenesis in addition to nutrients and metals. The current study presents a one-day snapshot of relevant AGPFs, nutrients and metals for land-based IMTA.

3.10.1 Water from the lagoon and aquaculture system promotes complete *Ulva´*s morphogenesis

Sterile-filtered aquaculture water samples affected the growth, development and morphology of axenic *U. mutabilis* gametes in a positive way (Figure 3.7). This demonstrates the presence of the activity of morphogenetic compounds that have also been determined in the lagoon water of the Ria Formosa (Portugal) (Grueneberg et al., 2016) and are likely to originate from bacteria (Wichard and Beemelmanns, 2018). Our semi-quantitative data indicates that the aquaculture water can initiate growth and morphogenesis of axenic germlings through released morphogenetic compounds present in excess, but the identity of those compounds originated from bacteria must still be determined (Wichard and Beemelmanns, 2018). Activity of potential morphogenetic compounds has already been found in tidal pools and lagoons where *Ulva* is abundant, and the shallow water body is separated from a more substantial body of water by barriers (Grueneberg et al., 2016). In this context, Matsuo et al., (2005) reported the potentially important role of thallusin for the normal development of *Monostroma* and this has also

been suggested for *Ulva* (Wichard and Beemelmanns, (2018); unpublished results). Even diluted samples provided the morphogenetic activity for the recovery of morphogenesis. These results also support earlier investigations that a direct cell-cell-contact with bacteria is not essential for triggering the morphogenesis in *Ulva* (Matsuo et al., 2003, Marshall et al., 2006, Spoerner et al., 2012).

Marshall (2004) reported that the necessity for bacterial attachment was not clear-cut and there were bacterial individuals such as (UL16, *Cellulophaga* sp.), where a low bacterial number was observed by microscopy, still can stimulate obvious morphology and growth changes in *U. linza*. Marshall believed in such cases, the bacterial isolates may not require direct contact and could be producing extracellular constituents that promote the morphological change.

The prevalence of AGPFs phenocopied the activities of the *Roseovarius*- and *Maribacter*-factors (Spoerner et al., 2012, Weiss et al., 2017) in water samples of the studied IMTA. The AGPFs can thus sustainably support the development of *Ulva* plantlets. Interestingly, the dilution series influences the effects of the AGPFs to varying degrees (Figure 3.8 A and B), which indicates two different molecular mechanisms for the two AGPFs. When the concentration of the *Maribacter*-factor was decreased by dilution (Figure 3.8B), the phenotype of some individuals indicated that the *Roseovarius*-factor solely controlled them, as protrusions could be observed again (similarly to Figure 3.7E).

Overall, there was no significant difference in the activity of each of these potential AGPFs between the entrance pond and fish ponds, indicating that fish farming does not jeopardise the positive effect of the lagoon water by removing or blocking the activity of beneficial morphogens. The lagoon can thus provide a reservoir of those compounds; this might explain in part the success in producing *Ulva* on this site. Therefore, AGPFs from

the lagoon water might be considered a naturally occurring boosting parameter of *Ulva´*s growth in land-based aquacultures and commercial-sized intensive systems leading to increased yield of algal biomass or conversely having the potential to initiate green tide formation in marine ecosystems. In other words, the presence or absence and even the amount of waterborne morphogenetic substances in water (which supply the water requirement for these aquacultures) might play a very important role in dealing with a land-based *Ulva* aquaculture (Grueneberg et al., 2016). Moreover, keeping the conditions stable and reproducible with regards to the waterborne morphogenetic compounds that change the growth conditions in IMTA could be challenging due to interfering features such as tides, temperature, salinity differences, marine currents, fish age, numbers and fish food ingredients.

As *Ulva* is supposed to "garden" its microbiome (Kessler et al., 2018), essential AGPF-producing bacteria may be selected on *Ulva´*s surface. Indeed, a bacteria enrichment analysis in the algae tanks supports this assumption, as the abundance of key genera such as *Maribacter* and members of the '*Roseobacter* clade' were significantly increased according to Ghaderiardakani et al., (2019b). In fact, the authors concluded that morphogens can be provided by *in situ* bacterial production in the algae tanks (Ghaderiardakani et al., 2019b).

3.10.2 Nutrients can be provided constantly for algae production by fish farming within an IMTA system

Ulva species were identified as ideal candidates for biofiltering fish pond effluents two decades ago (Cohen and Neori, 1991). IMTA-produced seaweed has generally shown higher productivity levels compared to seaweed from the natural environment, due to the continuous steady supply of nutrients (Shpigel et al., 2017). Ammonium, excreted by the fishes, is the preferential N source compared to nitrate for many algae since ammonium

can be directly assimilated into amino acids, and in contrast to oxidized N forms of nitrate and nitrite, it does not need to be reduced prior to amino acid synthesis (Barsanti and Gualtieri, 2006, Ale et al., 2011). Ammonium is also the most common N-form in fish-farms, especially in intensive systems. In contrast to ammonium, which decreases by ~50-60% across the algae tanks, from the inlet to outlet (Ghaderiardakani et al., 2019b), the variations seen in nitrate and orthophosphate before and after the algae tanks are not significant. The result is suggesting although the nitrate is in excess, but ammonium is taken up preferentially.

From an algal aquaculture point of view, the important point is that the nitrate concentration in this system does not fall below 2.1 mg/L at any time during the day through the algae tanks. The rise in nitrate and orthophosphate during the day in the entrance ponds is likely due to a combination of tidal regime and upwelling of nutrient-rich waters and horizontal currents of nutrient-rich waters in the lagoon. In this system, the fish are fed in the early morning (approx 07.00) and late afternoon (15.00-16.00) so the variations in N seen is in agreement with (Echevarréa et al., 1993), where dissolved inorganic nutrient (NH_4 and NO_3) peaks in the water are expected between 2h and 6h after feeding time. Although there is a reduction in orthophosphate in the seaweed tanks compared to the filtered water, suggesting some uptake of phosphate by *Ulva* (in accordance with (Tsagkamilis et al., 2010)), the level of nitrate remains relatively constant suggesting that it is present in excess due to its abundance in the lagoon and the fish effluent.

Interestingly, in periods of fish absence, the yield and quality of the seaweed biomass drops, with a negative impact in the seaweed farm profitability (unpublished observations on the site of AlGAplus). Therefore, we suggest that the IMTA arrangement is necessary

for sustainable algal aquaculture as it might reduce fluctuations in nutrients. At the same time, fish farming does not endanger the morphogenetic factors provided by lagoon water.

The measurements of N and P in the present study are in line with those found in other marine environments. Mean levels of nitrate and phosphate concentrations along the drainage network of the North-East Atlantic (NEA) were measured (Desmit et al., 2018). Nitrate concentration values fluctuate in a range from 0.45 to 12.9 mg N l^{-1} (32 to 921 μmol l^{-1}) in some rivers of the NEA, particularly in Brittany (in the north of France) and Belgium. Regarding orthophosphate concentration, values recorded around 0.16 mg P l^{-1} (5 μmol l^{-1}) in most rivers across the NEA excluding some Portuguese rivers and the Scheldt and Thames estuaries where values increase to 0.65 mg P l^{-1} (21 μmol l^{-1}) (Desmit et al., 2018).

3.10.3 Reproductive phases might be triggered by depletion of nitrate

Using a closed system under standardised conditions and controlled bacteria, Alsufyani et al., (2017) suggest that depletion of nitrate and the absence of sporulation inhibitors corresponded to the change of reproductive phases in *U. mutabilis* (Alsufyani et al., 2017). However, it is still unclear whether this was due to nitrogen availability or due to the absence of nitrate as other nitrogen sources were not monitored.

In the present study, the non-limiting resource of nitrate provided from both entrance (lagoon water) and fish ponds (efflux) (Figure 3.8) might be one of the key factors, along with the presence of the sporulation inhibitors (Alsufyani et al., 2017, Stratmann et al., 1996), keeping algae in the vegetative phase and preventing them from breaking down as correlated by Alsufyani et al., (2017), which results in the constant yield at this production site.

Periodic biomass losses resulting from reproduction has been suggested as a restrictive factor for expansion of land-based *Ulva* cultivation (Gao et al., 2017b). Gao et al., (2017b) reported that by mutating a wild type of *U. rigida* using ultraviolet radiation, they obtained mutant strains which (2.5 mm discs) remained in the green vegetative phase during the whole period of experiment (27 days), while the wild type discs switched to reproductive phase after 17 days (day 18). This observation is in contrast with previous findings as in this study the medium was changed every day to avoid nutrient limitation. Additionally, the growth rate of mutants was five times higher than the wild type. Accordingly, nitrate and phosphate uptake rates by mutant strains were 40.0% and 30.9% respectively higher than wild type. Gao et al., (2017b) argued that considering the boosted capacity of mutant strains for nutrient uptake, they potentially could be effective candidates for wastewater bioremediation or as biofilters. Considering all these investigations and preliminary results mentioned in Section 3.9.3, we suggest that possibly the type of nitrogen source might be contributing to the triggering of the reproductive phase.

This was also the case in an investigation conducted by Ale et al., (2011) that reported a favourable growth response to ammonium relative to other nitrogen sources through monitoring the growth kinetics of *U. lactuca*. In this study *Ulva* frond disks were exposed to different sources of nitrogen (NH_4^+; NO_3^-; and the combination NH_4NO_3) for 10 days (Ale et al., 2011). The NH_4Cl and $NaNO_3$ rich media (50 μM of N) both enhanced *U. lactuca* growth to a maximum specific growth rate of 16.4±0.18% day^{-1} and 9.4±0.72% day^{-1}, respectively. But ammonium boosted the biomass yield significantly ($P<0.05$) compare to the nitrate treatment (Ale et al., 2011). It has been reported that ammonium is the preferential N source compared to nitrate for many algae since ammonium can be directly assimilated into amino acids and in contrast to nitrate, it does not need to be reduced prior to amino acid synthesis (Barsanti and Gualtieri, 2006).

The ammonium measurements (Ghaderiardakani et al., 2019b) support previous work showing the importance of ammonium uptake in *Ulva*. Although the favourable growth response to ammonium as the nitrogen source was shown, Fort et al., (2019) have recently reported that nitrate accumulation and consumption was positively correlated with *Ulva´*s growth which is happening with higher growth rates during the night than during light periods (Fort et al., 2019).

3.10.4 Nutrient recycling using *Ulva* for bioremediation needs optimisation

The results of nutrient uptake (nitrate and orthophosphate) in present study do not suggest using *Ulva rigida* as a biofilter in the conditions (water flow velocity, initial stocking density, etc.) applied in ALGAplus company at the time of this study, simply because the nitrate is not depleted in the exit water of algal tanks although ammonium is significantly reduced. This assumption is supported by the previous experiments that concluded the water velocity influenced biomass yields and biofiltration performance of *U. lactuca* treated with low nutrient concentrations in laboratory investigations (Msuya and Neori, 2008).

However, Sode et al., (2013) tested successfully the reject water from anaerobically digested sewage sludge as a nutrient source for *Ulva lactuca* cultivation. In this study the growth dynamics and biochemistry of *Ulva* cultivated with reject water were not changed compared to *Ulva* cultivated with NH_4^+ (used as a tool for optimisation of any similar phycoremediation system). *Ulva* treated with reject water concentrations equivalent to 50 µM NH_4^+ showed the maximal growth performance and nutrient (N and P) uptake. Highest nutrient removal rates of 22.7 mg N g DW^{-1} d^{-1} and 2.7 mg P g DW^{-1} d^{-1} were achieved at reject water concentrations equivalent to 80 and 89 µM NH_4^+, respectively. The biomass

produced at these nutrient concentrations was rich in protein, and the concentration of all measured metals ranged between the common concentrations allowed for use for animal feed or soil improvement (Sode et al., 2013).

Emphasising the critical role of bioremediation conditions, a study conducted by Tsagkamilis et al., (2009) demonstrates how it is crucial to launch the optimum combination of initial algal mass density, effluent concentration and flow rate that resulted in a reasonable removal of phosphate. Tsagkamilis et al., (2009) designed a continuous-flow system with a 1/4 volume per hour water turnover, in a mixture of 60% sewage effluent: 40% sea water and 30g L^{-1} initial biomass of algae (renewed every 10 days. One green alga *Ulva lactuca* and two brown algae *Halopteris scoparia* and *Cystoseira compressa* were investigated for their capacity to remove phosphate, improve the water quality and reduce eutrophication hazards. Due to better growth and survival in low salinity waters, *U. lactuca* was chosen eventually. 50% of phosphate content of the effluent was taken by *U. lactuca* under these conditions and during this period of time (Tsagkamilis et al., 2010).

3.11 Conclusion

The work presented in this chapter explored the presence and effects of the algal growth promoting factors, which are diffusible compounds that can fully induce the morphogenesis of *Ulva* in water of the lagoon Ria de Aveiro, Portugal. This study was only a one-day snapshot (a preliminary investigation) and future work could be undertaken in a longer time period and could be more comprehensive. For example, future studies should include a time course of measurements over a year and should include sporulation inhibitor measurements. Based on the results of the current study, fish farming has no adverse effect on the effect of these substances and provides nutrients (mainly ammonium,

Ghaderiardakani et al., 2019b) for seaweed production. Essential dissolved nutrients such as nitrate and orthophosphate were not depleted albeit Ghaderiardakani et al., (2019b) showed that ammonium was significantly reduced in the algal tanks. The authors also concluded that although trace metals are available in water samples across the field, potentially toxic elements are far below the recommended limits (Ghaderiardakani et al., 2019b).

The permanent circulation of water ensures thus an adequate supply of nutrients. We argue that the presence of morphogens (probably sporulation inhibitors (Spoerner et al., 2012)) contributes to the sustainable cultivation of *Ulva* in aquacultures supplied by water from the lagoon and fish ponds. Future studies need to show how these morphogens contribute to *Ulva* development during the various life cycle phases. Despite the fact that morphogens might be taken up or metabolised by the organisms present in this ecosystem, the bacteria generating growth-promoting morphogens were enriched in *Ulva´*s aquaculture system (Ghaderiardakani et al., 2019b). This observation (the results of the bacterial investigation) corresponded well to the excess of morphogenetic activity in the water measured. Further studies are needed to determine fluctuations in AGPF (e.g. morphogens including sporulation inhibitors) over the whole growth from seeding until harvesting during the year. The extra identification and characterisation of morphogens would be worthwhile to enable continuous *Ulva* growth without breakdown to reproduction or for further possibilities to control biomass production in aquacultures.

CHAPTER 4:

EFFECTS OF A GREEN SEAWEED, *ULVA INTESTINALIS*, EXTRACT ON *ARABIDOPSIS THALIANA* GERMINATION AND EARLY DEVELOPMENT

4.1 The importance of abiotic stresses on agricultural yield

Drought (water deficit), excessive water (flooding), extreme temperatures (cold, frost and heat), salinity and mineral (metal and metalloid) toxicity are the most common abiotic stresses (environmental factors) negatively affecting growth, development, reproduction (e.g. seed quality) or yield of crops and other plants – these stresses thus generate a threat to sustainable agriculture (Suzuki et al., 2014). This has become an important issue due to concerns about the increasing frequency of these environmental stresses and their influence on plant resources, biodiversity and global food scarcity (Slama et al., 2015). Current global food challenges and the substantial pressure on the food production industry are due at least in part to the exponentially growing human population in the context of a world with increasing soil- and water issues compounding the pressure induced by anthropogenic climate change (Atkinson and Urwin, 2012).

Complicating the above and adding to a persistent decrease in agricultural productivity, land quality declining (or accelerating land degradation) is also making negative impacts on agriculture. Owing to a shortage of additional adequate arable land to expand agriculture, to meet new food demands, 90% of improved yield must come from increases in agricultural yield of existing farmland and only 10% from the use of additional land (FAO, 2012). Moreover, the consequences of intensive farming practices are also contributing to widespread occurrence of unfavourable conditions for crop growth, development and survival (Battacharyya et al., 2015).

As mentioned above, abiotic stresses are the primary causes of considerable crop losses worldwide (Challinor et al., 2010). These include stresses imposed by the changing climate. The current global warming poses a major and growing threat to worldwide food security (FAO, 2016). According to The World Bank report (2012) 0.8°C warming above pre-industrial levels has already had adverse impacts on different sectors including

agricultural yields and food security. Significant crop yield losses have already been observed at 0.8°C warming, and as temperatures rise to 2°C and beyond, the intensity and severity of impacts on agriculture will increase (Kit, 2014). For instance, more intense and more frequent droughts in Africa, southern South America and Southern Europe and increased flooding in temperate regions will result in potential decline in future crop yields (Schellnhuber et al., 2012, Shepherd et al., 2013, Kit, 2014).

Without adopting early but strong actions such as CO_2 fertilisation, effective adaptation, and genetic improvement and in the absence of substantial policy action, the global yields of major crops are estimated to reduce. For example, the yields of wheat, rice, maize, and soybean would be decreased on average by 6.0%, 3.2%, 7.4% and 3.1% respectively, per each degree-Celsius rise in global mean temperature (Zhao et al., 2017).

In another study (more focused on particular areas), some instances has been listed of projected effects in potential crop yields for around 2°C warming, without adapting further actions, as 30% to 70% reduction in soybeans yield and up to 50% decrease for wheat in Brazil, in Central America and the Caribbean about 50% decline in wheat yield, and 10% to 50% reduction for wheat in Tunisia (Kit, 2014).

Another scenario resulting from warming would be land desertification, which would put further pressure on water resources and agriculture. Land desertification can trigger and cause other stresses such as loss of vegetation coverage, soil erosion, dust storms, salinization, and a decrease in soil productivity. All these stresses pose severe threats and lead to decreasing crop yields per area or agricultural yields and loss of biodiversity (Kit, 2014). It also should be considered that natural environment normally involves the simultaneous exposure of plants to multiple abiotic stresses and/or even biotic factors. Plants' exposure to such unfavourable environmental stresses can cause dynamic and

complex responses in plant systems and these responses might be elastic (reversible) or plastic (irreversible) (Cramer et al., 2011).

To reduce the substantial risks resulting from abiotic stresses on agricultural productivity and to enhance crop production within the limited and endangered water, land and energy resources, it is absolutely necessary to improve crop gain in the presence of unfavourable environmental constraints (Santaniello et al., 2017).

Two main types of strategies have been introduced to improve sustainable agriculture: (i) a conventional breeding and/or biotechnological approach (Medrano et al., 2009) and (ii) development of agronomic practices (Tilman et al., 2011), such as employing specific products aimed at promoting stress tolerance traits in crops and other plants. To manipulate the synthesis of health-promoting chemicals of crops and thereby improve their nutritional values, numerous efforts have been undertaken through genetic engineering such as, increasing iron (Fe) content in rice (Goto et al., 1999) and enrichment of tomato fruit with health-promoting anthocyanins (Butelli et al., 2008). However, international argument about the application of genetic manipulation to crop improvement has decelerated the adoption rate of GM (genetically modified) crops as well as in European countries (Khush, 2012). So, there is an urgent need for proper science-based, cost-effective and time-effective regulatory strategies. As one of these strategies recently, special attention is being directed towards applying biostimulants and biofertilisers, usually based on natural extracts, in agricultural practices to enhance crop yields, resistance to stress and disease, without adverse environmental effects (Jayaraj et al., 2008, Khan et al., 2009, Santaniello et al., 2017).

4.2 Application of seaweed extract in agriculture: past, present and future

4.2.1 The importance of seaweed extracts

Seaweeds, referring to several species of macroscopic, multicellular, marine algae, one of the important marine living resources, could be termed as the futuristically promising plants. Applying seaweed-based biostimulants and biofertiliser is only one of the many possible approaches that farmers who are willing to alleviate adverse consequences of abiotic stresses on crops can use. The earliest written reference about using seaweed as a fertiliser dates back to Roman times (Henderson, 2004). Being continuously bathed with nutrient-rich seawater results in absorbance of high levels of nutrients. It might be the reason why seaweed products have been used for centuries by humans, predominantly along the coastal areas where seaweed is found in abundance, for multifarious purposes including food, fuel, aquaculture, cosmetic products, colouring dyes and, particularly, therapeutic and botanical applications (Dhargalkar and Pereira, 2005, Kelly and Dworjanyn, 2008, Notoya, 2010, Battacharyya et al., 2015).

The interest in employing seaweed extracts in organic and sustainable agriculture continues to grow (Craigie, 2011). This is especially the case due to the fact that seaweed extracts, unlike chemical fertilisers, are biodegradable, non-toxic, non-polluting and non-hazardous to humans, animals and birds (Dhargalkar and Pereira, 2005). More recently, numerous studies have revealed the beneficial effects of applying seaweeds and seaweed extracts in modern agricultural practices, reviewed in (Khan et al., 2009, Craigie, 2011, Arioli et al., 2015). With the inclusive use of seaweed extracts in agriculture, many positive impacts were reported including increased seed germination rates, improved plant development such as flowering, leaf quality and root system architecture (Hernández-Herrera et al., 2014), elevated defence against pathogens and pests (Zhang et al., 2003,

Jayaraj et al., 2008) and protection against nutrient deficiency and environmental stresses including salinity (Nabati et al., 1994), cold or drought (Santaniello et al., 2017, Zhang and Schmidt, 2000). de Carvalho et al., (2019) reported the results of an experiment investigating the impacts of seaweed extracts (from *Ascophyllum nodosum*, *Hypnea musciformis*, *Lithothamnium* sp., and *Sargassum vulgare*) on a commercial field of *Niagara Rosada* vines. In the most experiments, treatments with seaweed extract enhanced photosynthesis, stomatal conductance and water use efficiency. Plant production and yield estimates were specifically improved by foliar applications with *A. nodosum* (de Carvalho et al., 2019).

The well-documented uses of seaweed as organic and mainstream fertilisers highlight the potential importance of seaweeds to agricultural productivity, in terms of boosting crop yield and conferring resistance to stress and disease. Seaweed fertilisers have been also used in agricultural programmes to improve soil structure, disease management, nutritional strategies, water efficiency and climatic stresses tolerance (Arioli et al., 2015).

4.2.2 Commercial seaweed extract industry

A variety of manufacturing practices are used to liquefy seaweed biomass. These include alkaline or acid hydrolysis, cellular disruption under pressure, or fermentation (Milton, 1952, Craigie, 2011, Arioli et al., 2015). These seaweed extracts are marketed as liquid biofertilisers or biostimulants containing a wide variety of potentially plant growth-promoting components. But the point is here can we get farmers to adopt these methods, or will yields and profitability be compromised? There are some field-based studies that have demonstrated the benefits of successful application of seaweed extracts in the field rather than just in the laboratory. Field studies carried out by Burchett et al. (1988) showed that spraying a seaweed extract (Maxicrop) on winter barley improves winter hardiness and

increases the frost tolerance (Burchett et al., 1998). Application of *Ascophyllum nodosum* commercial extract (3-10 L/ha) once a month resulted in an increased concentration of phenolic compounds and antioxidant composition in cabbage, potato and onion (Lola-Luz et al., 2013, Lola-Luz et al., 2014). The effect of commercial *A. nodosum* extract has been evaluated for six years on coffee plant development and production. Fernandes et al., (2019) reported that application of the commercial biostimulant *Ascopyllum*-based extract increased the productivity of the coffee trees grown under closed conditions up to 43%. So, collecting more evidence from field-based assessments is likely to be essential for more application and adaptation.

4.3 Mode of action of growth-regulatory substances in algal extracts

Components identified in seaweed/algal extracts include plant growth regulators (phytohormones), minerals and trace elements, quaternary ammonium molecules such as betaines and proline, polyuronides including alginates/fucoidans and lipid-based molecules such as sterols (Arioli et al., 2015). Seaweed products suitable for use in agriculture are available as liquid extracts or in a soluble powder form.

The mechanism by which seaweed fertilisers affect plant growth, development and yield is as-yet unexplored. One hypothesis is that the effects of seaweed fertilisers are either due directly or indirectly to phytohormones or changes in nutrient availability. In other words, seaweed extracts may themselves contain beneficial phytohormones or other nutrients or may contain substances that trigger land plant signalling pathways that usually respond to hormones or nutrients. Which, if either, of these scenarios actually occurs is not clear.

4.3.1 First scenario: Key plant regulators' biostimulatory activity in algal extract

Phytohormones play an important role throughout the growth and development regulation of higher plants (Wani et al., 2016). Crop plants treated with algal extracts showed similar physiological responses to those treated with plant growth-regulatory substances. The diverse growth responses promoted by algal extracts have led to the speculation that probably more than one group of phytohormones or growth-stimulating factors are involved [reviewed in (Khan et al., 2009)] (Tay et al., 1985, Crouch and Van Staden, 1993b). Phytohormones detected in algal extracts are auxins, cytokinins, gibberellins, abscisic acid and brassinosteroids (Crouch and Van Staden, 1993a, Hussain and Boney, 1973, Stirk et al., 2004) which may explain the wide range of growth responses induced by seaweed extracts. However, despite the growing evidence for the phytohormone-like activity of seaweed extracts, their function remains elusive due to detection of a number of chemical components (other than phytohormones themselves) which elicit physiological effects reminiscent of plant hormones (Wally et al., 2013). The following sections introduce these hormones and summarise some of their roles in plants.

4.3.1.1 Auxin

Auxins, a class of essential plant hormones, were the first plant regulators to be discovered and are synthesised by many species including all land plant lineages and some multicellular Charophycean algae (Ross and Reid, 2010). This group of regulators orchestrates just about every feature of plant growth and development as well as responses to the environment (Woodward and Bartel, 2005, Vanneste and Friml, 2009, Kazan, 2013). IAA (indole-3-acetic acid) is the most commonly detected natural auxin in higher plants. It occurs predominantly in young leaves, floral organs and developing fruits and seeds, with the ability to impact at several aspects of different processes throughout plant development such as the differentiation of vascular tissues, elongation growth, apical dominance, lateral root initiation, fruit setting and ripening (Macdonald, 1997, Woodward and Bartel, 2005).

Moreover, it is well-documented that IAA is involved in regulating plant growth under stress conditions. As an example, it has been reported that the root and shoot growth of plants growing under salinity or heavy metal stresses has been alleviated in presence of IAA (Sheng and Xia, 2006, Egamberdieva, 2009). Interestingly, it has been illustrated that auxins and auxin-like compounds are abundant in marine algae (Crouch and Van Staden, 1993b). For instance, each gram of *Ascophyllum nodosum* dry extract contains as high as 50 mg IAA. Two different methods, by ethyl acetate and methanol, were used for extraction and purification of crude aqueous solutions of *A. nodosum* in this experiment (Kingman and Moore, 1982). More examples are reviewed in Khan (2009).

4.3.1.2 Abscisic acid (ABA)

ABA is an isoprenoid plant hormone and its existence has been reported widely throughout all kingdoms of life excluding the Archea (Hauser et al., 2011). The name of abscisic acid refers to its function in abscission of plant leaves, but this phytohormone is more usually studied in terms of assisting the plants in adaptive responses to and tolerance of biotic and abiotic stresses (Wani et al., 2016). ABA also plays a key role in regulating many aspects of plant physiological and developmental processes, such as seed dormancy and germination, controlling the stomata, embryo morphogenesis, germination and fruit ripening and synthesis of storage proteins and lipids (Sreenivasulu et al., 2010, Wasilewska et al., 2008).

A noticeable inhibition of hypocotyl growth has been shown upon using aqueous growth inhibitors extracted from *Laminaria digitata* and *Ascophyllum nodosum* on lettuce (Hussain and Boney, 1973) (Hussain and Boney 1973). Through investigation by bioassay, thin-layer, and gas-liquid chromatography analysis it has been discovered that one of these compounds resembled ABA. Later, in addition to this ABA-like activity of algal extracts,

ABA has been detected in green algae e.g. *Dunaliella parva*, *Chlamydomonas reinhardii*, *Ulva lactuca*, *Draparnaldia mutabilis* and *Chara foetida* using gas chromatography either with packed glass columns or fused silica capillary columns by Tietz et al., (1989) and in *Ascophyllum nodosum* by Kingman and Moore (1982). The content of ABA in *Ulva* was estimated to be about 0.44 ng/g of fresh weight (Tietz et al., 1989). Extraction of algal material in this experiment was performed with 96% ethanol and 8% methanol.

4.3.1.2.1 ABA regulatory genes in *Arabidopsis*

ABA is one of a group of important plant hormones, which are key mediators coordinating plant responses to environmental signals. ABA is particularly important during exposure to abiotic stresses (Wani et al., 2016). ABA levels are key factors in regulating the formation and accumulation of seed storage compounds, and ABA action prevents seed germination, enables the transition of seeds to dormancy and maintains seeds in the dormancy phase (Kulaeva and Prokoptseva, 2004, Wani et al., 2016). Mutants with deficiency in ABA biosynthesis, plus ABA-insensitive mutants, have been instrumental in elucidating the pathway by which ABA synthesis and signalling is regulated in plants. Since the pathway and the corresponding genes are considered as generally conserved in angiosperms, therefore the data acquired from *Arabidopsis* research is potentially valid for other plant species (Xiong and Zhu, 2003). The ABA-signalling pathway is activated by abiotic stresses (e.g. drought and salt stress) possibly by triggering a "Ca^{2+}-dependent phosphorelay cascade" and through this process the expression of genes which are involved in ABA biosynthesis will be stimulated (reviewed in Zhu, 2016, Xiong and Zhu, 2003).

The ABA biosynthesis pathway initiates in the chloroplast with hydroxylation of β-carotene to zeaxanthin and then zeaxanthin is converted to antheraxanthin. These two forms of xanthin are converted to violaxanthin by zeaxanthin-epoxidase (ZEP).

Violaxanthin is transformed to xanthoxin by 9-cis-epoxycarotenoid-dioxygenase NCED family, which includes five identified members in *Arabidopsis*. The rest of process happens in cytosol by exporting xanthoxin products to cytosol and oxidising to abscisic aldehyde which is converted to abscisic acid by aldehyde oxidase (AAO). The last step of catalysis is activated by the molybdenum cofactor (MoCo) sulfurase (MCSU) (Xiong and Zhu, 2003, Bauer et al., 2013). According to Xiong and Zhu, (2003) studies in *Arabidopsis* have shown that that genes encoding *ZEP*, *AAO3*, and *MCSU* are all up-regulated by ABA in addition to their regulation by stress. It means that exogenous ABA can stimulate and increase the expression of these genes noticeably, in contrast to the NCED gene, which cannot be induced by exogenous ABA (Xiong and Zhu, 2003).

4.3.1.3 Cytokinins

Cytokinins are modified forms of adenine that stimulate cytokinesis, with an influential impact in mediating many growth and developmental processes in plants including cell division, extension and differentiation, chloroplast and vascular tissue development, shoot growth, fruit and flower development, apical dominance and senescence (Auer, 1997). Evidence from the literature indicates that the complementary and cooperative actions of the two phytohormones auxin and cytokinin regulate a wide variety of plant responses and developmental processes (Chandler and Werr, 2015, Schaller et al., 2015, El-Showk et al., 2013, Bielach et al., 2017). Accordingly, the balance of auxin-cytokinin ratio is very carefully adjusted because any amendments can affect morphological and physiological events (Coenen and Lomax, 1997).

Cytokinins have been found in fresh samples of brown algae *Laminaria digitata* (Hussain and Boney, 1969) and in a commercial aqueous seaweed extract (Brain et al., 1973). Later, Stirk et al., (2003) analysed thirty-one seaweed species for cytokinin by HPLC-linked

mass spectrometry. Seaweed extract was prepared with 70% ethanol supplemented with deuterium labelled standards of all known isoprenoid cytokinins. They reported that regardless of seaweeds' taxonomy and growing area, cytokinin profiles were similar in all algal samples. Moreover, zeatin (Z) and isopentenyl (IP) derivatives of cytokinins are the predominant cytokinin types (Stirk et al., 2003).

4.3.1.4 Ethylene

Ethylene is a simple gaseous phytohormone that is associated with several phases of plant growth and development, notably fruit ripening, flower initiation and leaf and flower senescence, besides playing a decisive role as regulator of stress responses, particularly the submergence response (Zhu and Guo, 2008). It has been proposed that ethylene is involved in stimulating germination and breaking dormancy in seed plants alongside its other actions (Corbineau et al., 2014). Ethylene often acts through a network of interacting responses and in combination with other phytohormones such as auxin, ABA, jasmonates and salicylic acid. The presence of ethylene receptors has been reported in the charophyte *Klebsormidium flaccidum*, a freshwater alga (Gallie, 2015).

4.3.2 Second scenario: Chemical components biostimulatory activity in algal extract

On the other side, biochemical studies conducted to assess bioactivities observed in extract-treated plants have shown that seaweeds contain high macro- and micro-element levels which are proposed to boost nutrition or play a significant role in plant development along with a wide range of bioactive compounds such as sterols, phenols and fatty acids (Tang et al., 2002, Hong et al., 2007). Although seaweeds could potentially have a beneficial effect on plant growth due to their containing macronutrients, including nitrogen (N), phosphorus (P), ammonium (NH_4) and potassium (K), studies with a diverse range of

seaweeds have consistently shown that the beneficial effects of seaweed extracts are not just due to the presence of these macronutrients, particularly at the concentrations that the extracts are used in the field (Khan et al., 2009, Blunden et al., 1996). Very dilute seaweed extracts (1:1000 or below) still have biological activity but the compound(s) involved in their activity are unknown: the beneficial effects may involve several plant growth-promoters working synergistically together (Fornes et al., 2002, Vernieri et al., 2005, Tay et al., 1985, Crouch and Van Staden, 1993a), or it could be due to the presence of larger molecules (oligomers and polysaccharide elicitors). Enhanced protection against pathogens or insect damage resulting from defensive responses elicited by polymers are other important features of particular interest in agriculture (Craigie, 2011). These polymers which are synthesised by seaweeds are a range of unusual and complex polysaccharides not found in land plants such as agars, alginates, carrageenans, fucans, phlorotanins (Siegel and Siegel, 1973, PAINTER, 1983, Blunden et al., 1986, Duarte et al., 2001). For example, the brown seaweed *Ascophyllum nodosum* contains laminaran (a polysaccharide of glucose) with an ability to elicit endogenous β-D-(1,3) glucanases, which are known to be important regulators of plant defence and development (Patier et al., 1993). A reduction in bean root rot caused by soil fungi, *Fusarium solani f. phaseoli*, has been shown in soil samples treated with chitin and laminarin (Mitchell, 1963).

4.4 Standardised laboratory-based system: Investigation the molecular mechanisms of stimulatory factors in algal extracts

Although seaweed extracts are already bringing boosted agricultural productivity, a better knowledge of their biological modes of action will further improve productivity in the future. How can we understand at a mechanistic level how seaweed fertilisers can affect land plant growth and development? The concentration of an algal extract is a critical factor in its effectiveness (Finnie and Van Staden, 1985, Crouch and Van Staden, 1993b,

Rayorath et al., 2008, Hernández-Herrera et al., 2014). However, because of the range of plants, seaweeds and extraction methods used, “positive” concentrations of algal extract in previous studies ranged from 0.002% to 0.2% while inhibitory concentrations ranged from 0.1% to 1%. Previous studies have applied a diverse range of algal extracts from brown (*Ascophyllum*, *Sargassum*, *Padina*), green (*Ulva*) and red (*Gracilaria*, *Hypnea*) seaweeds to a heterogeneous range of crop plants (Finnie and Van Staden, 1985, Hernández-Herrera et al., 2014). Generally, lower concentrations of a particular algal extract have beneficial effects on root and shoot growth while higher concentrations have inhibitory effects, as shown with both *Ulva lactuca* and *Padina gymnospora* in tomato (Hernández-Herrera et al., 2014), with *Gracilaria* and *Hypnea* in tomato, chili and aubergine (Rao and Chatterjee, 2014), with *Sargassum* in green and black gram (Kalaivanan and Venkatesalu, 2012, Kumar et al., 2012) and with *Ulva reticulata* in black gram (Selvam and Sivakumar, 2013). Applying algal extract as a root drench is more effective than application as a foliar spray for increasing biomass (Hernández-Herrera et al., 2014). In tomato, *U. lactuca* and *P. gymnospora* extracts also show a biphasic effect (stimulatory at high concentration, inhibitory at low concentration) on germination (Hernández-Herrera et al., 2014). *Ascophyllum* extract promotes growth in *Amaranthus tricolor* and may protect against salt stress (Aziz et al., 2011). Foliar spray algal extracts can also increase fruit yield (*Ascophyllum* applied to orange (Fornes et al., 2002)) and (unspecified algal extract applied to olive (Chouliaras et al., 2009)).

My thesis aims will allow to establish a “standardised” laboratory-based system to help determine the molecular mechanisms by which seaweeds can affect land plant productivity, using model organisms.

4.4.1 The importance of model systems

As it is not reasonable to study every extant biological system, so biologists select key examples to pave the way for further comparative studies on a range of similar systems. Studying model systems has long contributed to fundamental biological knowledge and subsequently in recent years the potential of model-organism-based studies has been grown substantially. Basic biological knowledge of life cycles, processes and behaviour of a system as well as fundamental evolutionary perspectives from key plant lineages have been immeasurably advanced by using knowledge acquired from testing and manipulating of related model organisms (Coates, 2016). Model organisms are normally chosen based on their appropriateness to experimental conditions, their relevance and importance to that particular experiment and their ease of use, handling and maintenance.

Utilising model organisms and understanding of their biological mechanisms is the driving theme behind these present experiments and will enable us to systematically decipher the effect of seaweed extract on growth and development of plants. Applying model systems in this way will provide insights into a range of similar systems and provide this opportunity to productively interpret and apply them. There is still an enormous amount to uncover in this field, and approaches using model systems continue to be one of the most productive ones.

4.4.2 Regulation of early growth and development in *Arabidopsis*

Previous studies surveyed the effect of algal extract(s) on germination, root growth in green gram (Kumar et al., 2012), tomato (*Solanum lycopersicum*) (Hernández-Herrera et al., 2014), black gram (Selvam and Sivakumar, 2013) and seedling establishment of broccoli (*Brassica oleracea*) (Bakker et al., 2009). In the model plant *Arabidopsis thaliana*, these parameters can be easily assayed in the lab and different phenotypes can be identified, analysed and interpreted (Weigel and Glazebrook, 2002). Moreover, the

mechanisms controlling *Arabidopsis* early growth and development (the parameters) are well understood, including for example, the role of phytohormones such as auxin, ABA, cytokinin, gibberellins, jasmonates etc on early growth stages and in abiotic stress tolerance (Wani et al., 2016), or the regulatory effects of the nutrients on plant development and also crosstalk between nutritional and hormonal signalling (Krouk et al., 2011) or interaction between plant hormones together (Wani et al., 2016).

Analysis of *Arabidopsis* mutants which are impaired in responses to these hormones and nutrients due to disruption in genes required for hormone response or nutrient transport, respectively, can reveal important regulatory roles of these hormones and nutrients in plants growth and development. The importance of mutant screens in the developing of *Arabidopsis* as a model genetic system has been extensively reviewed in (Koornneef and Meinke, 2010). Mutants for almost every gene in the *Arabidopsis* genome are available from stock centres (http://signal.salk.edu/cgi-bin/tdnaexpres, https://www.arabidopsis.org/portals/mutants/stock centers.jsp). In the context of my research, it is possible to compare directly the germination and root-growth parameters of different *Arabidopsis* genotypes by exposing seeds or seedlings to different concentrations or composition of algal extract against an untreated control. Therefore, I decided to use selected mutants and a wild-type strain, Col-0, as a powerful lab-based approach to elucidate the mechanisms by which algal extract impacts the early growth and development in plants, in addition to discover the bioactive compounds in algal extract.

4.5 Chapter aims

This chapter aims to elucidate the first mechanistic insights into how algal extracts affect plant growth and development, via hormone signalling. The role of algal extract on plant growth and development was assessed by:

- Analysing the effects of different concentration of *Ulva* extract on *Arabidopsis* seed (wild-type and mutants) germination rate

- Analysing the effects of different concentration of *Ulva* extract on *Arabidopsis* (wild-type and mutants) primary- and lateral root growth.

- Identifying the changes that can be caused by *Ulva* extract in seeds by a gene expression analysis. In the context of this study, expression of the *ZEP*, *AAO3*, and *MCSU* genes, known to be induced by exogeneous ABA, were analysed, to test the hypothesis that algal extract contained ABA or affected *Arabidopsis*' ABA signalling.

The species of *Ulva* chosen for this study was *Ulva intestinalis*, a cosmopolitan intertidal alga found worldwide, which, in addition, can be grown in laboratory similarly to *Ulva mutabilis* (Spoerner et al., 2012, Ghaderiardakani et al., 2017). Our bioassay-driven approach enables us to compare directly the growth- or inhibition parameters of different concentrations of *Ulva intestinalis* extract versus a constant control, applied to both wild type and mutant *Arabidopsis* genotypes. By using two well-characterised model systems we have begun to shed light on the plant mechanisms and signalling pathways that can be triggered by applying algal extract as a biostimulant. As *Ulva* genetic manipulation becomes better-established (Oertel et al., 2015) this raises the possibility of future engineering of improved macroalgal fertiliser.

4.6 Materials and methods

4.6.1 Collection and identification of algal samples

Vegetative and fertile *U. intestinalis* blades were collected from the intertidal zone at low tide, three times between March 2015 and April 2016, from the coastal area of Llantwit Major beach, South Wales, UK (51°23'46.5"N 3°30'06.0"W). Excess water and epiphytic

species were removed at the site by blotting the sample's surface before storage on ice for transport back to the laboratory. Epiphyte-free samples were subjected to a molecular identification using plastid-encoded *rbc*L (large unit ribulose bisphosphate carboxylase) and *tuf*A (plastid elongation factor) markers as identification solely by morphological characteristics is not reliable (Section 1.4).

4.6.2 Preparation of water-soluble *Ulva* extract

Ulva samples were washed with tap water to remove surface salt, shade dried for 10 days, oven-dried for 48 h at 60 °C, and then ground to a fine powder using a coffee grinder (Crofton, China) to less than 0.50 mm. 10 g of this milled material was added to 100 mL of distilled water with constant stirring for 15 min followed heating for 45 minutes at 60°C in water bath (Selvam and Sivakumar, 2013). The contents then were filtered through 2 layers of muslin cloth. This *Ulva* extract was designated as 10% stock solution and added to MS solution to make up specific concentrations and autoclaved. 1% *Ulva* extract stock was subjected to pH measurement and elemental analysis. All measurements were performed in triplicate.

4.6.3 Germination bioassay

Arabidopsis thaliana wild-type Col-0 and mutant lines *abi1-1*, *tir1-1*, *axr1-3*, *cre1*, *etr1-3*, and *ein3-1*, *akt1*, *nhx1*, *nhx4*, *nrt*1.1, *nrt*2, *amt*1 and *amt*2 (Table 4.1) were obtained from the Nottingham Arabidopsis Stock Centre (Loughborough, UK). *Arabidopsis* seeds were sterilised in 20% Parozone^{TM} bleach on a turning wheel for 10 minutes and subsequently washed 2-3 times in sterile water. Seeds were vernalised in cold room for 48h and placed on 1% agar, containing MS medium and *Ulva* extract (different concentrations). Afterwards, plates were transferred to the growth room for 7-10 days and incubated at 22 ± 2 °C with a 16-h-light photoperiod and a light intensity of 120 µmol m^{-2} s^{-1}. Germination

was observed daily as in (Moody et al., 2016). A seed was scored as germinated when its radicle had emerged from within the seed coat. Germination percentage (GP) was calculated as follows: GP = (the number of germinated seeds/total number of seeds) ×100). Data from 3 independent biological repeats (n=30-90 seeds per genotype and treatment) was combined. To identify significant differences between treatments and genotypes, Kruskal-Wallis tests followed by a Dunn's post-hoc test were performed.

4.6.3.1 *Arabidopsis* mutant lines references

abi1-1: Koornneef et al., (1982) isolated abscisic acid deficient mutants by identifying and selecting mutants at the "abscisic acid insensitive 1" locus of *Arabidopsis* seedlings. These lines showed a declined sensitivity to the abscisic acid phytohormone (Koornneef et al., 1982). According to Koornneef et al., (1982), the physiological consequences are including "reduced seed dormancy, symptoms of withering, increased transpiration and a lowered ABA content in developing and ripe seeds and leaves". ABI1 turned out to be a protein phosphatase that interacts with the ABA receptors (Miyazono et al., 2009). The enzymatic activity of ABI1 in seeds and in vegetative tissues is necessary for an appropriate receptiveness of ABA and its signal transduction (Leung et al., 1997).

tir1-1: Following by *Arabidopsis* genetic analysis, the role of a number of genes that are involved in auxin responses has been clarified. To discover further genes required for auxin-mediated pathways, a survey was undertaken by Ruegger et al., (1997) through *Arabidopsis* mutants that are resistant to the auxin-transport inhibitors including *N*-1 -naphthylphthalamic acid (Katekar and Geissler, 1977) and 2-carboxyphenyl-3-phenylpropane-1,2-dione (Ruegger et al., 1997). The authors reported 16 independent mutants suggesting the involvement of seven genes, named Transport Inhibitor Response (*TIR*1-7) genes in auxin transport process. Indeed, the deficiency in response to an auxin

stimulus by transport inhibitor response 1 (*tir*1) mutants was shown at the same report. Further molecular description identified that the TIR1 protein is functioning as a member of a protein complex which is required for ubiquitin-mediated processes in response to auxin as same as AXR1 (Ruegger et al., 1998). Later, it has been shown that TIR1 is an auxin receptor that facilitates transcriptional responses to auxin by direct binding to auxin (Kepinski and Leyser, 2005; Dharmasiri et al., 2005).

axr1-3: The auxin-resistant1, *AXR*1, gene is essential for the growth mechanisms controlled by auxin in the most plant tissues (Lincoln et al., 1990). This gene encodes a protein (a subunit) of RUB1 (Related to UBiquitin)-activating enzyme or E1. E1 is the first enzyme in the ubiquitin-protein conjugation cascade (Leyser et al., 1993). This activation is happening by making a thiol-ester bond between c-terminal carboxyl group of ubiquitin and the cysteine residue of E1 (Ruegger et al., 1997, Haas and Rose, 1982).

cre1: Inoue et al., (2001) documented the identification of *cre*1 (cytokinin response 1) mutants in *Arabidopsis.* Mutagenesis in *CRE*1 gene led to a reduced sensitivity to cytokinin. The authors included evidences (using a yeast mutant) that CRE1 is a cytokinin receptor which can be activated by cytokinins to initiate phosphorelay signalling.

ein3-1: A group of *Arabidopsis* mutants including *etr1*, *etr2*, *ein2*, *ein3*, *ein4*, *ein5*, and *ein6* were identified, with lower sensitivity or insensitivity to high concentrations of ethylene due to defective ethylene perception and signalling (Guzman and Ecker, 1990; Sakai et al., 1998). Ethylene insensitive mutants (*ein*l and *ein*2) over-generate ethylene. The blocked autoregulation inhibitory system in ethylene-biosynthesis process in *Arabidopsis* seedlings may cause this increased ethylene production (Guzman and Ecker, 1990). EIN3 is known as a crucial transcription factor facilitating gene expression and a range of plant responses in presence of ethylene in *Arabidopsis* (Guo and Ecker, 2003).

etr1-3: Ethylene resistant, *etr*, mutants function also by blocking the autoinhibitory regulation system in etiolated *Arabidopsis* plantlets, resulting in an elevated generation of ethylene in vegetative tissues as same as *ein*2 mutants (Guzman and Ecker, 1990, Woeste et al., 1999). ETR1 is a member of ethylene-receptor family (including ETR1, ERS1, ETR2, ERS2, and EIN4 in *Arabidopsis* (Chang and Stadler, 2001). Histidine kinase activity of ETR1 has been confirmed by Gamble et al., (1998), however, the exact role of this activity was not clear in ethylene signalling. Later, regulatory role of ETR1 enzymatic activity in ethylene signalling has been demonstrated by Hall et al., (2012).

akt1: akt1 isolated and identified by Hirsch et al., (1998) as an *Arabidopsis thaliana* mutant with disruption in AKT1 channel gene and lack of measurable AKT1 channel activity in roots. Consequently, these mutants appeared to be defective in potassium uptake. A poor growth was exhibited by *akt1* mutants on media with a potassium concentration of 100 mM K^+ or lower in comparison to the wild type plants (Hirsch et al., 1998).

nhx1: Apse et al., (2003) identified a T-DNA insertional mutant of *At*NHX1 (*Arabidopsis thaliana* Na^+/H^+ exchanger). This gene encodes the most abundant Na^+/H^+ antiporter in vacuoles. These antiporters have very important functions such as: regulating of both K and Na ions transportation into the vacuole, keeping the pH balance in the cells, the facilitating of protein transferring and involving the salt tolerance mechanisms (Apse et al., 2003).

nhx4: *At*NHX4 is one of six vacuolar/endosomal Na^+/H^+ antiporters among the eight members of cation:proton antiporter-1 (CPA1) family in *Arabidopsis* (Yokoi et al., 2002). *At*NHX4 has a vital role in *Arabidopsis* responses to abiotic stresses particularly salt stress. Disruption of *At*NHX4 gene expression caused enhanced salt tolerance/sensitivity to salt in

*nhx*4 mutant compared with wild-type *Arabidopsis* plants (Li et al., 2009). It was suggested that *At*NHX4 contributes to "ions uptake and ion homeostasis" (Li et al., 2009). For present experiment *nhx1* and *nhx4* were selected because these mutants are impaired in potassium uptake (due to the role of *At*NHX1 in cytosolic and vacuolar distribution of potassium). Potassium is a vital

nutrient in plants that impacts most of the biochemical and physiological pathways and thus can highly influence metabolism and promote growth (Wang et al., 2013) so, it could be an active ingredient of algal fertilisers.

nrt1: Tsay et al., (1993) documented the isolation and description of the *Arabidopsis* CHL1 gene as an important nitrate regulatory gene and described the role of this gene in encoding an "electrogenic nitrate transporter". It has been suggested that this transporter induces the initial depolarisation of plasma membrane mainly in *Arabidopsis* roots in presence of nitrate (Tsay et al., 1993).

nrt2: Wang and Crawford (1996) demonstrated that two mutations in *NRT2* gene (*nrt2* mutants) led to a reduction in the "constitutive, high-affinity nitrate uptake system" in *Arabidopsis*. The authors argued that *NRT2* gene is critical for "constitutive, high-affinity nitrate uptake" system and it does not contribute to the "inducible, high-affinity nor constitutive, low-affinity" systems. In fact, the result of this research supported the hypothesise that specific separate transporters are responsible for different nitrate uptake kinetics instead of changed forms of an individual transporter (Wang and Crawford, 1996).

amt1: The role of four root -expressed ammonium transporter (*AMT1-1*, *AMT1-2*, *AMT1-3* and *AMT2-1*) genes involved in regulation of ammonium uptake in *Arabidopsis* roots was shown by (Gazzarrini et al., 1999) and (Sohlenkamp et al., 2002). Loque et al., (2006) introduced two T-DNA inserted lines *amt1* and *amt3* and defined the role of

AMT1-1 and AMT1-3 to "high affinity ammonium uptake" in *Arabidopsis* roots upon exposure to nitrogen (Loqué et al., 2006).

amt2: Sohlenkamp et al., (2000) identified a subfamily ammonium transporter in *Arabidopsis* which was described as "more closely related to bacterial ammonium transporters" compared to AMT1 family in plants in terms of various patterns of gene expression and specific biochemical features of protein. Transportation of ammonium by AMT2 needs energy that is supplied by membrane electrical potential (Sohlenkamp et al., 2000).

Stock code	Allele	Locus	AGI code	Description
Col-0	Wild-type	-	-	Wild-type
N3798	*tir1*-1	TIR1	At3g62980	Auxin transport inhibitor resistant
N3075	*axr1*-3	axr1	AT1G05180	Auxin resistant
N3070	*etr1*-3	etr1	At1g66340	Ethylene insensitive
N8052	*ein3*-1	ein3	At3g20770	Ethylene insensitive
N660854	*cre1*-12	cre1	At2g01830	Cytokinin insensitive
N22	*abi1*-1	abi1	At4g26080	Abscisic acid insensitive
N664533	-	AtNHX1	At3g06370	Potassium/cation transporter defective
N3762	*akt1*-1	akt1	At2g26650	Potassium transporter defective
N55504	-	AtNHX4	At3g06370	Potassium/cation transporter defective
N6384	*chl*1-5	nrt1.1	-	Nitrate transporter defective
N508253	-	nrt2	At1g08090	Nitrate transporter defective
N57021	-	amt1	At4g13510	Ammonium transporter defective
N619678	-	amt2	At2g38290	Ammonium transporter defective

Table 4.1 *Arabidopsis thaliana* seeds used in this experiment. Stock code (in Nottingham *Arabidopsis* Stock Centre (NASC)), name and mutation description are provided (Information taken from *Arabidopsis* info).

4.6.4 Root bioassay

Experiments were conducted using 10cm square agar plates for root assays. 20 seeds were placed individually on the agar following a line across the top of the plate. The plates were sealed with Micropore tape (3M), taped together and incubated vertically in standard growth conditions as in (Moody et al., 2016).

From day 7 to 14 the seedlings were photographed and primary root (PR) lengths were measured with ImageJ open-source software (http://rsb.info.nih.gov/ij). The number of visible emerged lateral roots (LR) on each primary root was also counted and the lateral root density was calculated by dividing the number of LRs present by the length of that root. To identify significant differences between treatments and controls in wild-type plants, two-tailed t-tests were performed using SigmaPlot 13 software (Systat Software, San Jose, CA), comparing the results of each *Ulva* extract concentration to the control (without *Ulva* extract). To identify significant differences between treatments and genotypes, Kruskal-Wallis tests followed by a Dunn's post-hoc test were performed. All experiments were repeated a minimum of two and a maximum of four times with similar trends observed in each repeat.

4.6.5 Seed treatment and RNA extraction

Arabidopsis wild-type Col-0 seeds were harvested and sterilised as defined in Section 4.6.3. Half of seeds vernalised by distil water and half of them incubated in *Ulva* extract 1% for 48 h at cold room. Seeds were grinded and homogenised using a pre-chilled mortar and pestle under ribonuclease (RNase)-free conditions, firstly in liquid nitrogen preceding by thawing in buffer provided by Bioline Isolate II RNA plant kit (Bioline Ltd, UK) according to manufacturer's instructions.

RNA quality and quantity were assessed with NanoDrop spectrophotometer (Thermo Scientific). RNA samples were stored at -20°C for short-term store and at -80°C for long-term.

4.6.6 Reverse-transcription PCR (RT-PCR)

Reverse transcription (to generate first-strand cDNA followed by PCR) was performed through two ways: Bioline MyTaq™ One-Step RT-PCR kit (Bioline Ltd, UK) and The Superscript™ II reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions.

4.6.6.1 Bioline MyTaq™ One-Step RT-PCR

MyTaq One-Step PCR mix (for 25µl) was prepared as following:

MyTaq™ One-Step mix	12.5µl
Reverse transcriptase	0.25µl
RiboSafe RNase inhibitor	0.5µl
Forward primer (10µM)	1µl
Reverse primer (10µM)	1µl
DEPC-H_2O	4.75µl
RNA template(4ng/µl)	5µl

Forward and reverse primers were used at a final concentration of 400nM (0.4µM) in each PCR reaction. The following thermal cycling conditions for PCR were applied as the most recommended one but depends on the images and results, some changes have been made along the experiment in cycles number, RNA template concentration, annealing temperature etc. Thermal cycling conditions included: Reverse transcription (one cycle) 45°C for 20m, polymerase activation (two cycles) 95°C for 2m, 40 cycles: denaturation (95°C for 10s), annealing (65°C for 10s) and extension (72°C for 30s) followed by a final extension for 5m at 72 °C.

4.6.6.2 The Superscript™ II reverse transcriptase

Up to 1 μg of RNA template was subjected to first strand cDNA synthesis with the aid of Superscript™ preamplification system and oligo (dT) primers (Invitrogen Life Technologies) and following the steps according to the manufacturer's instructions. Then cDNA was used as a template for amplification in PCR using VELOCITY DNA Polymerase (Bioline Ltd, UK). The PCR conditions were as follows: an initial denaturation step at 98°C for 2m, 32 cycles of 98°C for 30s, 58°C annealing for 30s and 72°C extension for 1.5m. The cycles were succeeded by a final elongation step at 72°C for 5 min.

The primers (Table 4.2) that have been subjected to this section, were designed by hand from sequence data of actin, AAO3, MCSU and ZEP genes.

Gene	Forward primer sequence	Reverse primer sequence
Actin	TCGTACAACCGGTATTGTGCT	TTACAATTTCCCGCTCTGCTG
AAO3	CGTTCTCTCCTCCAAAGCGT	GCATAGTCGCTGGAACAGGT
MCSU	GCGACACCGAATTCAAGAG	GCACCTGATGTCGAGCATC
ZEP	GTCTGTTGGCTACCGGGTTT	GTCGCATGCAACAAGTCGAG

Table 4.2 The list of primers for gene expression experiment.

4.7 Results

Our analysis on the effect (stimulatory or inhibitory) of different concentrations of *Ulva* extract on seed germination and root development of *A. thaliana* are resulted in following take home messages:

4.7.1 Concentrations of *Ulva* extract of 0.5% and above inhibit wild-type *Arabidopsis* seed germination

Previous experiments have shown conflicting effects of different concentrations of algal extract on seed germination, e.g. in tomato (Finnie and Van Staden, 1985, Hernández-Herrera et al., 2014). To investigate the effect of *Ulva intestinalis* extract on *Arabidopsis* germination, a range of concentrations of *Ulva* extract from 0 – 1.0% was tested (Figure 4.1A, 4.2, 4.5). All *Ulva* extract concentrations from 0.5% upwards delayed wild-type germination. The final germination percentage was reduced in 0.8% and 1.0% *Ulva* extract: only about half the seeds germinated in 1.0% *Ulva* extract after a week (Figure 4.1A, 4.2). Concentrations of 0.3% *Ulva* extract and below had no effect on seed germination and no stimulatory effect of *Ulva* extract on germination was observed at any concentration tested (Figure 4.1A).

4.7.2 The germination-inhibitory effect of *Ulva* extract is not apparent in an ABA-insensitive mutant

We next sought to determine whether ABA signalling could play a role in the effects of *Ulva* extract on *Arabidopsis* development to uncover the mechanism by which *Ulva* extract inhibits germination. *Arabidopsis* seeds from the ABA-insensitive mutant *abi1* (Meyer et al., 1994, Leung et al., 1994) were assayed for their response to *Ulva* extract. *abi1* seeds are unresponsive to the inhibitory effect of *Ulva* extract and behave similarly to untreated controls under all treatments (Figure 4.1B). This suggests that the inhibition of *Arabidopsis* seed germination by *Ulva* extract depends on a functional ABA signalling pathway in the seeds.

4.7.3 Cytokinin- and ethylene-signalling mutants show some insensitivity to inhibition of germination by *Ulva* extract

We also tested the germination behaviour of cytokinin receptor mutant *cre1* and the ethylene receptor mutant *etr1* on 0.1-1% *Ulva* extract. Both mutants' seeds showed some

insensitivity to germination-inhibition compared to wild type (Figure 4.1C, 4.1D, 4.2, 4.3), but were not as insensitive as the *abi1* mutant (Figure 4.1B). Both *cre1* and *etr1* also showed a higher final germination percentage in comparison to WT germination on 0.8% and 1% *Ulva* extract over the same period of time (Figure 4.1C, 4.1D, 4.2). This suggests that the inhibition of *Arabidopsis* seed germination by *Ulva* extract is influenced by the cytokinin- and ethylene signalling pathways in addition to the ABA signalling pathway.

4.7.4 *Arabidopsis* nutrient uptake mutants are inhibited in germination by *Ulva* extract

To determine whether the germination-inhibitory effect of *Ulva* extract could be due to phytohormones or non-phytohormone substances that trigger the inhibition pathway in *Arabidopsis,* a range of nutrient uptake mutants were tested. Almost all these potassium, nitrate and ammonium uptake mutants had delayed onset of germination and reduced rate of germination compared to the wild-type control, even in the absence of *Ulva* extract. In some cases, the mutants are also more sensitive than WT to the inhibitory effect of algal extract (Figure 4.4). As an example, *amt1*, *amt2* (ammonium transporter defective) and *nrt2* (nitrate transporter defective) mutant seeds at 1% concentration of *Ulva* extract did not show any germination (0%) while in WT at same treatment on day 8 showed 33.2% germination. Also in case of *Atnhx1*, *Atnhx4* (potassium/cation transporter defective) and *chl1*-5 (nitrate transporter defective) germination rate at 1% concentration of *Ulva* extract is lower than 10%. Similarly, in 0.8% treatment of *Ulva* extract on final day of experiment, the recorded germination rate for all of genotypes are lower than WT in same date and treatment (94.7%).

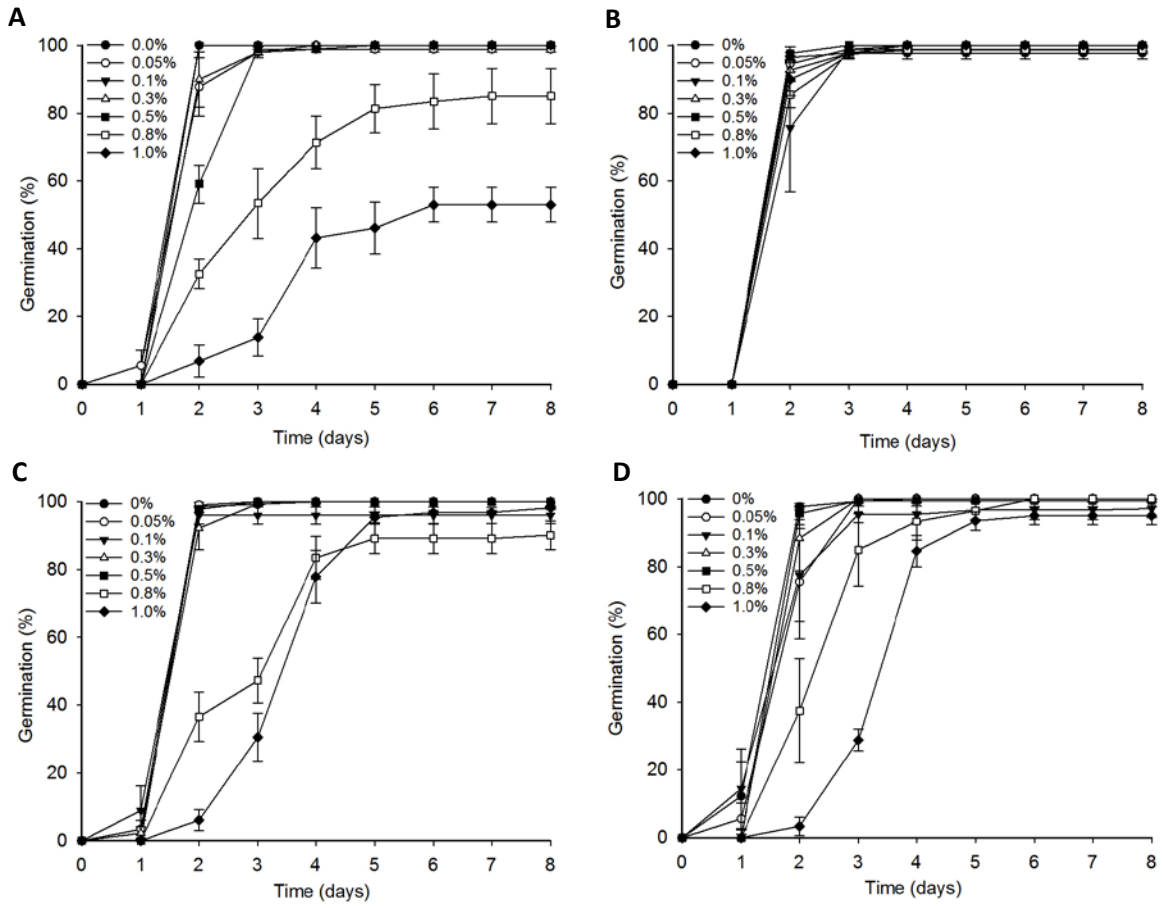

Figure 4.1 Effect of different concentrations (0.05-1%) Ulva extract on wild type and mutants Arabidopsis seed germination.

A) Wild type: On days 3 and 4, 0.8% *Ulva* extract was significantly different to the control, 0.1%, 0.3% treatments ($p<0.05$) and the 1% treatment was significantly from the control, 0.05%, 0.1%, 0.3% and 0.5% ($p\leq0.05$). On days 5 and 6 the control, 0.05%, 0.1%, 0.3%, 0.5% treatments were significantly different from 0.8% and 1% ($p\leq0.05$) but on day 7 only 1% demonstrates a significant difference from the control ($p\leq0.01$), 0.05% ($p\leq0.05$), 0.1% ($p\leq0.01$), 0.3% ($p\leq0.01$), 0.5% ($p\leq0.01$) treatments.

B) Germination of abi1 seeds on increasing concentrations of *Ulva* extract. There are no significant differences between treatments apart from on day 2 when 0.5% and 0.8% treatments are significantly different from one another ($p\leq0.05$).

C) Germination of cre1 mutant seed on varying concentrations of *Ulva* extract. On days 3 and 4, 1% *Ulva* extract is significantly different ($p<0.05$) from the control, 0.05%, 0.1%, 0.3% and 0.5%. On the rest of the days there is not any significant difference between treatments.

D) Germination of etr1 mutant seed on varying concentrations of *Ulva* extract. On days 3 and 4, 1% *Ulva* extract is significantly different ($p<0.05$) from the control, 0.05%, 0.1%, 0.3% and 0.5%. On the rest of the days there is not any significant difference between treatments.

Three biological repeats were performed, each with similar results – this data is a combination of the 3 biological repeats (each with n=30-90 seeds) with average percentage germination shown. Bars represent standard error of the mean. All germination data were analysed for significant differences between treatments and genotypes using a Kruskal-Wallis test followed by a Dunn's post-hoc test.

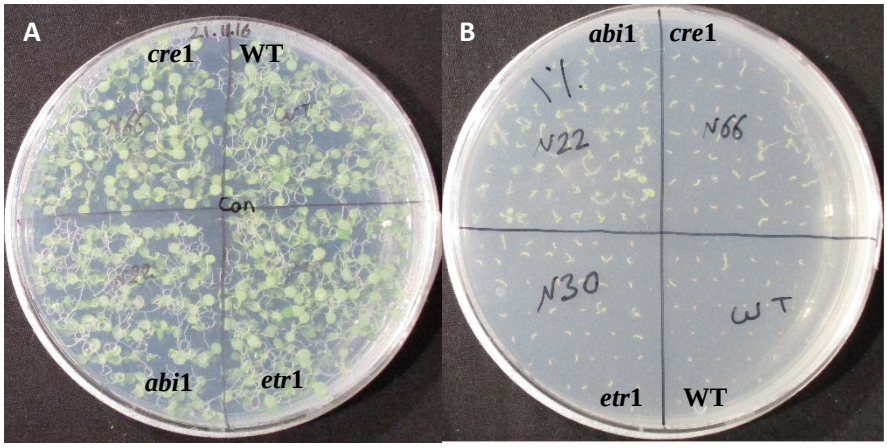

Figure 4.2 *Ulva* extract impact germination in *Arabidopsis thaliana* wild-type (WT), *cre1* (N66), *etr1* (N30) and *abi1* (N22) mutants. Seeds were germinated on concentrations of A) 0 and B) 1%. All genotypes germinate fully on no-extract control plates. On 1% concentrations only *abi1*, *cre1* and *etr1* mutant seeds showed some insensitivity to inhibition and germinated. Seedlings are 10 days old.

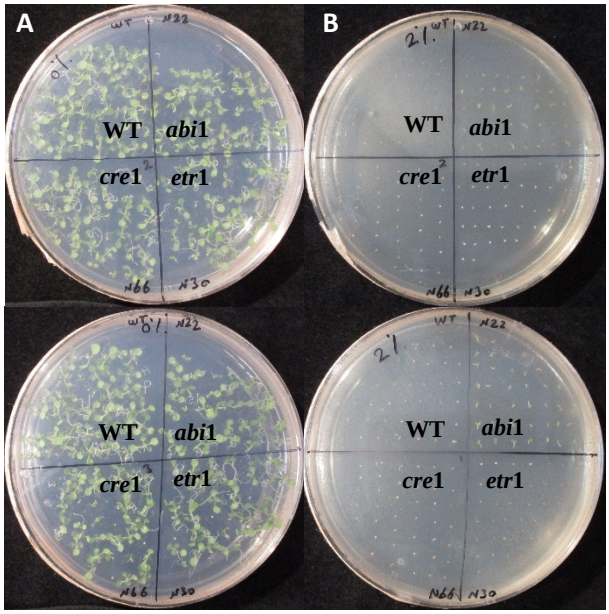

Figure 4.3 High concentration of *Ulva* extract impact germination in *Arabidopsis thaliana* wild-type and *abi1* mutant. Seeds were germinated on concentrations of **A)** 0 and **B)** 2%. On 2% concentrations only *abi1* mutant seeds showed some insensitivity to inhibition and germinated. Seedlings are 10 days old.

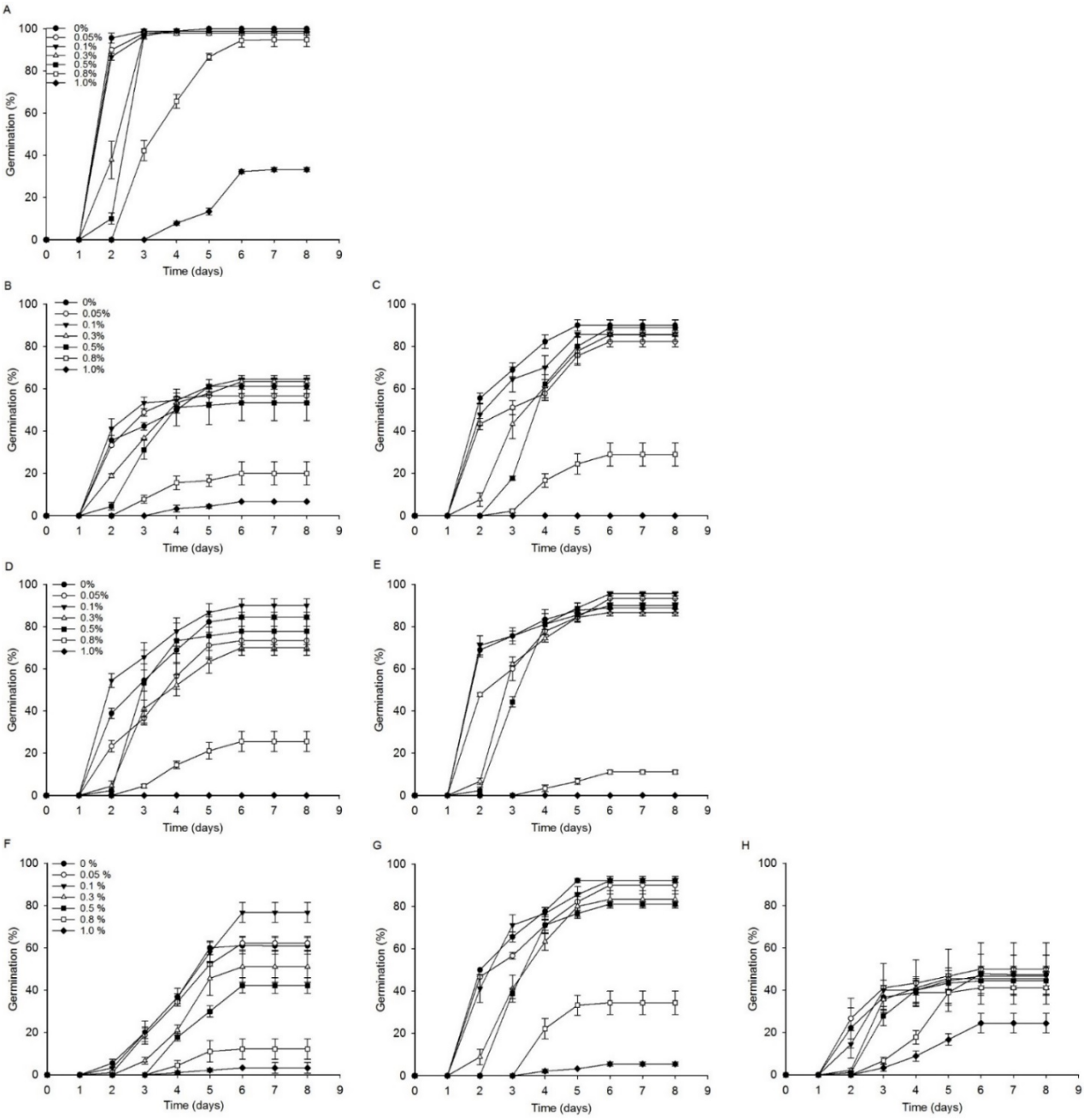

Figure 4.4 Effect of different concentrations (0.05-1%) *Ulva* extract on wild type and mutants *Arabidopsis* seed germination.

A) On day 2 the 0.8% *Ulva* extract was significantly different to the control and 0.3% ($p<0.05$) plus 0.1% ($p<0.01$) and 1% treatment was significantly from the control, 0.05%, 0.3% ($p<0.05$), and 0.1% ($p<0.01$).

On days 3, 4 and 5, 0.8% *Ulva* extract was significantly different to the control, 0.1% and 0.3%, on days 4 and 5 plus 0.5% treatments ($p<0.05$) and the 1% treatment these on days 3,4,5 and 7, was significantly from the control($p\leq0.05$), 0.1%, 0.3% and 0.5 % ($p<0.01$)

B) Germination of *nrt1* seeds on increasing concentrations of *Ulva* extract. On day 2, 0.5% treatment was significantly different with control ($p \leq 0.05$) and 0.05% and 0.1% ($p \leq 0.01$). 0.8% treatment with 0.1% ($p \leq 0.05$), and 1% treatment with control ($p \leq 0.05$), and 0.05% and 0.1% ($p \leq 0.01$).

On day3, 0.5% treatment was significantly different with 0.1% ($p \leq 0.05$), 0.8% with control, 0.05% and 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.05%, 0.1% and 0.3 ($p \leq 0.01$).

On next days, 0.5% treatment was significantly different with 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.05% and 0.1% ($p \leq 0.01$).

C) Germination of *nrt2* mutant seed on varying concentrations of *Ulva* extract. On days 2, 3 and 4, 0.8% treatment was significantly different with control, 0.05% and 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.05% and 0.1% ($p \leq 0.01$) and 0.3% ($p \leq 0.05$). On next days, 0.8% treatment was significantly different with control and 0.05% ($p \leq 0.05$) and 1% treatment with control, 0.05% ($p \leq 0.01$), 0.1% and 0.3% ($p \leq 0.05$).

D) Germination of *amt1* seeds on different concentrations of *Ulva* extract. On day 2, 0.5% treatment was significantly different with control ($p \leq 0.05$), 0.8% treatment with control, 0.05% and 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.05%, 0.1% ($p \leq 0.01$) and 0.3% ($p \leq 0.05$). On the next days, 0.8% treatment was significantly different with control and 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.1% ($p \leq 0.01$), 0.3% and 0.5% ($p \leq 0.05$).

E) Germination of *amt2* seeds on different concentrations of *Ulva* extract. On day 2, 0.8% treatment was significantly different with control, 0.05% and 0.1% ($p \leq 0.05$), 1% treatment with control, 0.05%, 0.1% and 0.3% ($p \leq 0.01$). On the next days, 0.8% treatment was significantly different with control, 0.1% and 0.3% ($p \leq 0.05$) and 1% treatment with control, 0.1%, 0.3% ($p \leq 0.01$) and 0.05%, ($p \leq 0.05$).

F) Germination of *Atnhx1* seeds on increasing concentrations of *Ulva* extract. On day 2, 0.8% treatment was significantly different with control, 0.05% and 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.05%, 0.1% ($p \leq 0.01$) and 0.3% ($p \leq 0.05$). On day 3, 0.8% treatment was significantly different with control, and 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.05%, 0.1% ($p \leq 0.01$) and 0.5% ($p \leq 0.05$). On the next days, 0.8% treatment was significantly different with control and 0.1% ($p \leq 0.05$) and 1% treatment with control, 0.05% and 0.1% ($p \leq 0.01$).

G) Germination of *Atnhx4* seeds on increasing concentrations of *Ulva* extract. On day 2, 0.5% and 0.8% treatments were significantly different with control and 0.05% ($p \leq 0.05$) and 1% treatment with control, 0.05% and 0.1% ($p \leq 0.01$). On the next days, 0.5% treatments were significantly different with 0.05% ($p \leq 0.05$), 0.8% treatment with control ($p \leq 0.05$) and 0.05% ($p \leq 0.01$) and 1% treatment with control, 0.05% ($p \leq 0.01$), 0.1% and 0.3% ($p \leq 0.05$).

H) Germination of *akt1* mutant seed on varying concentrations of *Ulva* extract. On day 2, 0.5%, 0.8% and 1% treatments were significantly different with control ($p \leq 0.01$), 0.05% and 0.1% ($p \leq 0.05$). On next days 0.5% and 0.8% with control ($p \leq 0.05$) and 1% treatment with control ($p \leq 0.01$), 0.05%, 0.1% and 0.3% ($p \leq 0.05$).

Three biological repeats were performed, each with similar results – this data is a combination of the 3 biological repeats (each with n=30-90 seeds) with average percentage germination shown. Bars represent standard error of the mean. All germination data were analysed for significant differences between treatments and genotypes using a Kruskal-Wallis test followed by a Dunn's post-hoc test.

4.7.5 *Ulva* extract stimulates *Arabidopsis* primary root growth at low concentrations and inhibits root growth at higher concentrations

Having demonstrated that seed germination is inhibited by *Ulva* extract, we sought to discover whether the next stage of development, primary root elongation, was also affected by *Ulva* extract.

Seeds were germinated, and seedlings grown, on standard growth medium containing a range of *Ulva* extract concentrations ranging from 0 to 2%. *Ulva* extract significantly stimulated *Arabidopsis* wild-type root growth at concentrations from 0.03-0.08% (~80% stimulation at 0.06%), while concentrations of 0.3% and above had an inhibitory effect on root growth (~68% inhibition at 2%) (Figure 4.5A).

In order to ascertain whether the inhibitory effect of *Ulva* extract concentrations ≥0.3% on root growth was simply a consequence of delayed germination (Figure 4.5A), we

conducted an experiment where seedlings were germinated on normal growth medium for 3 days before transferring to medium containing *Ulva* extract. Root growth was once again inhibited by *Ulva* extract, showing that higher concentrations of *Ulva* extract have an inhibitory effect on root growth, independent from any effect on germination (Figure 4.5B).

4.7.6 Hormone mutants, *axr1*, *tir1*, *etr1* and *cre1* behave similarly to wild-type *Arabidopsis* with respect to root growth

To clarify whether additional hormone signalling systems could be involved in primary root-inhibition by *Ulva* extract, mutants in auxin *axr1* perception (auxin resistant) and *tir1* (auxin transport inhibitor resistant) also mutants in ethylene- and cytokinin perception (the ethylene receptor mutant *etr1* and the cytokinin receptor mutant *cre1*, respectively) were tested. All these mutant's seedings showed inhibitory effect on primary root growth in concentration of 0.3% *Ulva* extract and above compared to the control (wild-type on 0% *Ulva* extract) (Figure 4.5C). At low concentrations 0, 0.05% and 0.1% a range of behaviours compared to control have been recorded. At 0 concentration, *axr1*, *etr1* and *cre1* did not have any significant difference with control but *tir1* is significantly ($p \leq 0.05$) lower than control. At 0.05% concentration *axr1* ($p \leq 0.05$), *tir1* ($p \leq 0.05$) and *etr1* ($p \leq 0.001$) are significantly lower than control while *cre1* is not showing any significant difference. At 0.1% concentration *axr1*, *tir1* and *cre1* are not significantly different but *etr1* is significantly different ($p \leq 0.05$) compared to control (Figure 4.5C).

4.7.7 The *abi1* mutant's root growth responds normally to low concentrations of *Ulva* extract and is slightly insensitive to higher concentrations of *Ulva* extract

Since the *abi1* mutant is impaired in its germination response to *Ulva* extract and since ABA is known to have a biphasic effect on root growth (Li et al., 2017), we tested the effect of *Ulva* extract on the root growth of the *abi1* mutant. The *abi1* mutant behaved similarly wild type plants at concentrations below 0.5% (0.1%-0.5%) of *Ulva* extract (Figure 4.5A,D), representing continuous inhibition. However, stimulatory effects were seen at concentrations below 0.1% similarly to wild type suggesting that the stimulatory effect of *Ulva* extract on root growth cannot be attributed to changes in ABA signalling in the plant (Ghaderiardakani et al., 2019a). At higher concentrations, 0.8% and 1%, of *Ulva* extract, the *abi1* mutant showed some insensitivity to inhibition of root growth (Figure 4.5A D), but this was of a much smaller magnitude than the *abi1* mutant's insensitivity during germination. This suggests that changes in ABA signalling in *Arabidopsis* may partially contribute to the inhibitory effect of *Ulva* extract on root growth.

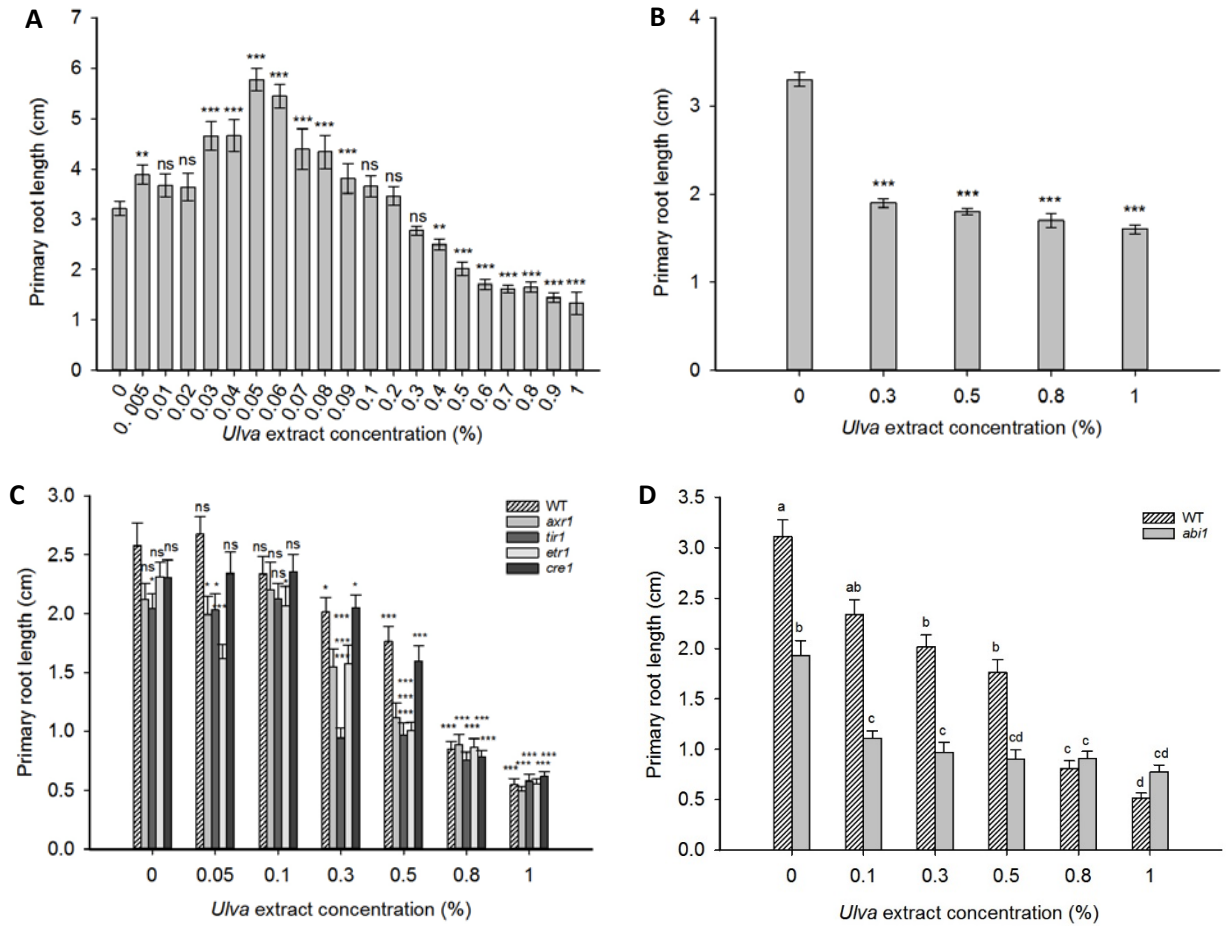

Figure 4.5 *Ulva* extract promotes root growth at low concentrations and inhibits root growth at higher concentrations.

A) Effect of *Ulva* extract on wild-type root growth in seedlings germinated on algal extract. Seeds were germinated, and seedlings grown on concentrations of 0 - 1% *Ulva* extract and the primary root length of every seedling was measured after 14 days. Pairwise t-tests were carried between each percentage of *Ulva* extract treatment and the control (0% *Ulva* extract). Asterisks denote significant differences, * P<0.05, ** P<0.01, *** P<0.001. ns denotes non-significant. n=60 seedlings per treatment; error bars represent standard error of the mean. Representative of 3 biological repeats.

B) Effect of inhibitory *Ulva* extract concentrations (0.3% and above) on wild-type root growth after transfer of 3-day old seedlings to *Ulva* extract followed by growth for 7 days. N=30-35 seedlings per treatment. Asterisks denote significant differences in pairwise t-tests against the 0% *Ulva* extract control, *** P<0.001. Bars represent standard error of the mean.

C) Effect of *Ulva* extract on wild-type and hormone-response mutants root growth in seedlings germinated on algal extract. Seeds were germinated, and seedlings grown on concentrations of 0 - 1% *Ulva* extract and the primary root length of every seedling was measured after 11 days. Pairwise t-tests were carried between each percentage of *Ulva* extract treatment and the control (wild-type on 0% *Ulva* extract). Asterisks denote significant differences, * P<0.05, ** P<0.01, *** P<0.001. ns denotes non-significant. n=45-50 seedlings per treatment; error bars represent standard error of the mean. Representative of 3 biological repeats.

D) Effect of *Ulva* extract on wild-type and *abi1* (abscisic acid insensitive) root growth in seedlings germinated on algal extract. Seeds were germinated, and seedlings grown on concentrations of 0 - 1% *Ulva* extract and the primary root length of every seedling was measured after 10 days. An analysis of variance followed by a Tukey's post-hoc test was carried out to determine significant differences between genotypes and treatments and the letters above the bars represent this. Wild type on 0% *Ulva* extract is significantly different to wild type on 0.3% (P<0.01%) and to wild type on 0.5%, 0.8% and 1% (P<0.001). Wild type on 0% is significantly different to *abi1* on 0 (P<0.05%), 0.1% -1% (P<0.001%). *abi1* on 0% *Ulva* extract is significantly different to *abi1* on 0.1% and 0.3% (p<0.01) and 0.5%-1% *Ulva* extract (p<0.001%). Thus, *Ulva* extract treatments significantly decrease root length in both wild type and *abi1*. Data is demonstrating some insensitivity from *abi1* to *Ulva* extract on higher concentrations with respect to root length inhibition in comparison with wild-type seedlings. n=60 seedlings per treatment; error bars represent standard error of the mean. Data is representative of 3 biological repeats.

4.7.8 *Ulva* extract inhibits *Arabidopsis* lateral root formation

Once the *Arabidopsis* primary root is established, it acquires branches, or lateral roots (LRs), as the seedling matures to secure anchorage and extract micro- and macronutrients from the soil (Lynch, 1995, Chang et al., 2013). Having ascertained that *Ulva* extract affects primary root growth, we went on to investigate the effect of *Ulva* extract on LR formation. Increasing concentrations of *Ulva* extract show a progressive inhibition in the density of LR branching from the primary root, even at concentrations that stimulate primary root growth (Figure 4.6).

In summary, *Ulva* extract inhibits germination, has a biphasic effect on primary root growth (stimulatory at low concentrations (Ghaderiardakani et al., 2019a); inhibitory at higher concentrations) and inhibits LR formation. Taken together, these observations are very reminiscent of the effect of the plant hormone, abscisic acid (ABA) on germination and early root development, as ABA is a negative regulator of germination (Li et al., 2010b), shows a biphasic effect on primary root growth (Li et al., 2017, WATTS et al., 1981, Xu et al., 2013), and inhibits LR growth at concentrations that stimulate primary root growth but do not affect germination (De Smet et al., 2003).

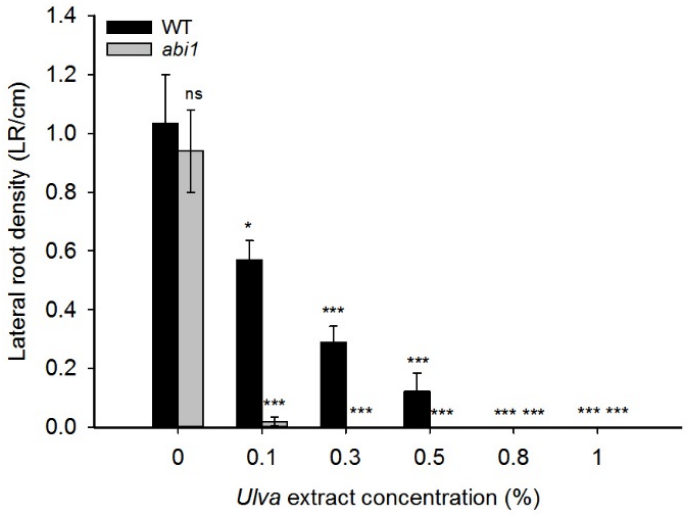

Figure 4.6 Effect of 0.1-1% *Ulva* extract on lateral root density of wild type and *abi1* seedlings. Asterisks denote significant differences compared to the 0% *Ulva* extract control in a t-test * P<0.05, ** P<0.01, *** P<0.001 N=30-35 seedlings per treatment. Representative of 3 biological repeats.

4.7.9 Could the germination-inhibitory effect of *Ulva* extract be due to elemental components?

Despite reports of ABA in *Ulva* (Tietz et al., 1989) and other seaweeds (Moore, 2004), because in the present experiments the *Ulva* extract was water-soluble, its effects on the *Arabidopsis* ABA signalling pathway are likely to be indirect: ABA is much more soluble in organic solvents than in water (Fu et al., 2011, Zhang, 2014). To understand further the mechanism by which *Ulva* extract may be affecting *Arabidopsis* development, elemental analysis of *U. intestinalis* biomass was performed by our collaborator Dr. Neil Graham at the University of Nottingham (dried samples were prepared at the University of Birmingham). The concentration of a panel of 31 water-soluble ions in the *Ulva intestinalis* samples was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as explained in Thomas et al., (2016) (Thomas et al., 2016) . With this measurement we aimed to find out whether significant differences exist between levels of elemental substances in *Ulva* tissue samples and those levels in a land plant standard

(tomato) or between elemental levels in *Ulva* tissues and those in our standard *Arabidopsis* growth medium. Thus would ultimately determine if the existence of any of the elements could explain the effects of *Ulva* extract on *Arabidopsis* seedling development. More details about elemental measurements can be found in Ghaderiardakani et al., (2019a).

The level of sodium in some algal extracts can raise the argument that the inhibitory effect of *Ulva* extract is most likely attributable to salt stress. However, the amount of sodium in the 1% *Ulva* extract (10.5mM; Table 4.3) is lower than the reported salt concentration that inhibited the germination and root development in *Arabidopsis* which reported as <150mM (Ghaderiardakani preliminary and unpublished data) (Gao et al., 2006). Therefore, the inhibition of root growth seen in the present experiments could not be due to salinity stress. Interestingly, this conclusion is in accordance with the fact that the *abi1* mutant is not wholly insensitive to the root growth inhibition (Figure 4.5 C,D) since salt stress responses are mediated by ABA signalling (Shi et al., 2003, West et al., 2004).

According to Ghaderiardakani et al., (2019a), two elements (Al^{3+} and Cu^{2+}) due to their high levels and in accordance with what previous literature (Yuan et al., 2013, Sun et al., 2009) showed in terms of their involvement in inhibitory effects on root growth, could be contributing to the inhibition in root growth that we see at concentrations of *Ulva* extract ≥0.3%.

Element	Seaweed composition (mg/kg)	Tomato control composition (mg/kg)	Significantly higher concentration in seaweed than tomato? (F-test)	Concentration of element in 0.05% *Ulva* extract - stimulatory for root growth	Concentration of element in 1% *Ulva* extract - inhibitory for germination and root growth	Concentration of element in Arabidopsis medium
B	**131.51±5.65**	31.18±0.25	p<0.05	6µM	121µM	100µM
Na	**61860.69±259.87**	116.79±4.96	p<0.001	530µM	**10.5mM**	**100µM**
Mg	**14291.19±1182.33**	10178.93±71.28	p<0.05	295µM	**5.9mM**	**1.5mM**
P	2511.15±76.31	2307.47±15.21	No	40µM	800µM	1.25mM
S	24810.4±872.48	9841.569±110.91	No	385µM	7.7mM	>1.5mM
K	16466.06±2383.77	27310.14±197.01	No	210µM	4.2mM	>19mM
Ca	37045.93±3114.25	46397.44±256.12	No	460µM	9.2mM	3mM
Ti	18.13±0.36	18.79±0.13	No	190nM	3.8µM	N/A
Li	2.88±0.19	0.5±0.02	No	150nM	3µM	N/A
Be	0.05±0.00	0.01±0.01	No	3nM	60nM	N/A
Al	**1397.88±177.11**	478±2.71	p<0.05	25.9µM	**518µM**	**N/A**
V	**2.85±0.22**	0.74±0.00	p<0.01	28nM	**560nM**	**N/A**
Cr	2.38±0.23	1.29±0.24	No	23nM	460nM	N/A
Mn	22.9±1.23	228.11±1.6	No	210nM	4.2µM	100µM
Fe	**795.33±95.13**	322.87±3.18	p<0.05	5µM	100µM	100µM
Co	0.34±0.04	0.47±0.00	No	3nM	60nM	100nM
Ni	1.68±0.13	1.4±0.1	No	154.5nM	290nM	N/A
Cu	**13.37±2.22**	1.71±0.05	p<0.05	105nM	**2.1µM**	**100nM**
Zn	26.03±0.87	25.77±0.18	No	200nM	4µM	30µM
As	**3.07±0.21**	0.13±0.00	p<0.01	20.5nM	**410nM**	**N/A**
Se	**0.13±0.01**	0.07±0.00	p<0.05	1nM	**20nM**	**N/A**
Rb	5.87±0.76	14.09±0.09	No	34.5nM	690nM	N/A
Sr	125.03±1.47	86.5±0.29	No	715nM	14.3 µM	N/A
Mo	0.34±0.04	0.44±0.02	No	2nM	40nM	1µM
Ag	0.09±0.00	0.00±0.00	No	415pM	8.3 nM	N/A
Cd	0.19±0.01	1.48±0.00	No	1nM	20nM	N/A
Cs	**0.28±0.033**	0.05±0.00	p<0.05	1nM	**20nM**	**N/A**
Ba	10.37±0.91	64.04±0.24	No	38nM	760nM	N/A
Tl	**0.09±0.01**	0.04±0.00	p<0.01	220pM	**4.4nM**	**N/A**
Pb	**1.79±0.12**	0.54±0.00	p<0.05	4.5nM	**90nM**	**N/A**
U	**0.08±0.01**	0.03±0.00	p<0.05	170pM	**3.4nM**	**N/A**

Table 4.3 Elemental analysis of *Ulva intestinalis* compared to land plant (tomato) control. Light grey represents elements that show a significant difference between their concentration in *Ulva* compared to tomato. The concentration of each element in 0.05% *Ulva* extract (stimulates root growth) and 1% *Ulva* extract (inhibitory to germination and root growth) is shown, compared to the concentration of the same element in our normal *Arabidopsis* growth medium (0.5x MS). Higher concentrations in 1% *Ulva* extract than in 0.5MS, has been shown in dark grey. The elemental analysis was conducted by Dr. Neil

Graham at the University of Nottingham and the data analysis was performed by JC. Samples were prepared in University of Birmingham by FG. Table has been adopted from Ghaderiardakani et al., (2019a).

4.7.10 RT-PCR analysis of AAO3, MCSU and ZEP genes

In order to check the presence of ABA or an ABA-like activity in *Ulva* extract, to explain the *abi1* insensitivity to *Ulva* extract and to test the hypothesis that *Ulva* extract could be able to regulate an inhibitory effect on germination by increasing biosynthesis of ABA in seeds, ABA biosynthesis gene expression was examined in treated *Arabidopsis* seeds and controls. RT-PCR analyses were performed using RNA harvested from seeds treated by *Ulva* extract and also seeds vernalised with water. By incubating these two groups of seeds for 48 hours in cold room we tried to re-shape the conditions as it was happened in germination experiment for the seeds cultivated on culture media without and with *Ulva* extract. Actin was chosen as control in this analysis. In three independent biological repeats, no difference in gene expression was seen between *Ulva* extract treatment and controls. (Figure 4.7).

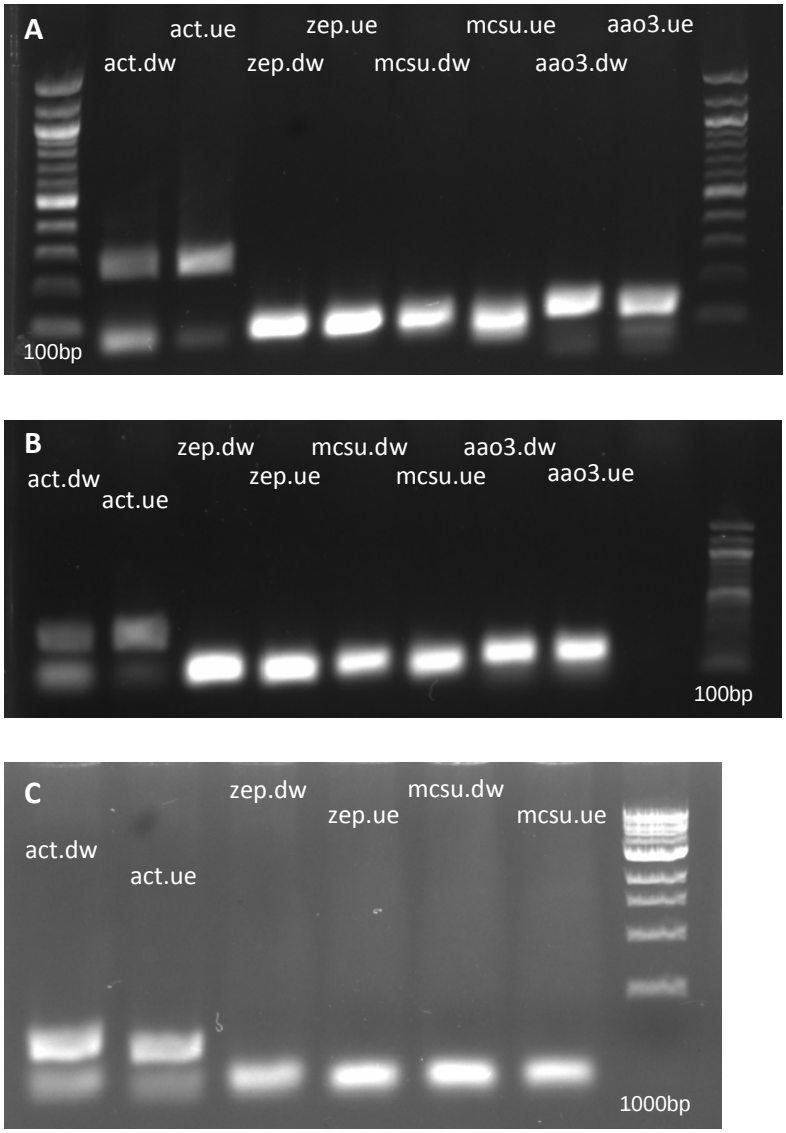

Figure 4.7 Expression of ABA biosynthesis genes is not changed in seeds by application of *Ulva* extract in a semi-quantitative experiment. Actin (act), ZEP gene (zep), MCSU gene (mcsu), AAO3 gene (aao3), dw (treated with distil water), ue (treated with *Ulva* extract). Panels A, B and C are three biological repeats. It seems that RNA has been extracted so not seeing any differences might be because of (i) there was not ABA in *Ulva* extract (ii) ABA was not the key regulator of inhibition in this experiment (iii) experimental issues e.g. could be too many PCR cycles to pick up subtle differences, etc.

4.8 Discussion

4.8.1 *Ulva* extract can inhibit *Arabidopsis* germination, root growth and lateral root formation

We have shown that *Ulva* extract at concentrations of 0.5%-1% inhibits wild-type *Arabidopsis* seed germination. Moreover, *Ulva* extract ≥0.3% reduces wild-type *Arabidopsis* primary root growth and the extract inhibits wild type LR formation even at concentrations below 0.1%, suggesting that LRs are more sensitive than the primary root to the inhibitory agent(s) in the *Ulva* extract.

Our results concur with those already in the literature, where seaweed extract at high concentrations had inhibitory effects on seed germination and seedling growth. An obvious reduction of the germination rate occurred in pepper seeds primed with a brown seaweed (*Ascophyllum*) extract at 1:250 (0.4%) and at higher concentrations (10%) of Maxicrop (a commercial seaweed extract) solution compared to control seeds primed with only water (Sivritepe, 2008). Treatments with higher concentrations (1.0%) of water-extracts from the brown seaweeds *Caulerpa sertularioides*, *Padina gymnospora* and *Sargassum liebmannii* had toxic effects on tomato germination parameters as well as seedlings and resulted in detrimental consequences such as radicle browning and disintegration of plumules (Hernández-Herrera et al., 2014). Application of 2%-10% aqueous extracts from *Sargassum johnstonii* led to similar detrimental effects on tomato (Kumari et al., 2011). Concentrations of kelp waste extracts (KWE) ranging from 10–100% inhibited germination of pakchoi (*Brassica chinensis L.*), with no seeds germinating on 100% KWE. However, this was attributed to high levels of NaCl, which are absent from our *Ulva* extract (Zheng et al., 2016). Arnon and Johnson (1940) reported similar detrimental effects on early tomato development as a result of higher pH in the growth medium. However, in

our experiments, the pH value was adjusted to be the same for all concentrations of *Ulva* extract concentrations so the effects we see are not due to altered pH.

4.8.2 *Ulva* extract stimulates *Arabidopsis* primary root growth but not germination or lateral root formation at concentrations below 0.1%

Our data shows that *Ulva* extract has a growth-stimulating effect on wild type *Arabidopsis* primary root elongation specifically at concentrations between 0.025-0.08%. Once again, this result is in accordance with work carried out in other species. Seaweed extract may improve water and nutrient uptake efficiency by root systems (Crouch et al., 1990) leading to enhanced general plant growth and vigour. Commercial extracts made from the brown seaweed *Ecklonia maxima* stimulated tomato root growth only at low concentrations (1:600; 0.17%) while higher concentrations (1:100; 1%) strongly inhibited root growth (Finnie and Van Staden, 1985). Rayorath et al., (2008) also reported root growth enhancement effects of *Arabidopsis thaliana* plants treated with aquaeous *Ascophyllum nodosum* extracts at very low concentrations (0.1 g L^{-1}; 0.01%), whereas plant height and number of leaves were affected positively at concentrations of 1 g L^{-1} (0.1%) (Rayorath et al., 2008). Hernández-Herrera et al., (2014) indicated that tomato seeds treated with lower concentrations (0.2 %) of extracts of both *Ulva lactuca* (green seaweed) and *P. gymnospora* (brown) were more effective at enhancing germination. However, we observed no boost in germination to *Arabidopsis* seeds with *Ulva intestinalis* extract under our growth conditions where we vernalise seeds at 4˚C for 48 hours to break dormancy before an assay so this may explain the discrepancy between the experiments.

A concentration of kelp waste extract (KWE) of 2% stimulated the germination of pakchoi seeds, with an 8.33% increase compared to the control (Zheng et al., 2016). Similar trends were obtained with other germination and seedling growth parameters, and this data is in-line with our observed root growth stimulation at low concentrations, followed by

inhibition at higher concentrations. The growth performance of pakchoi seedlings (plumule length, radicle length, fresh weight and dry weight) was improved by treatment with KWE at 2-5% (Zheng et al., 2016). The kelp waste extract was prepared differently (using cell wall digestion and centrifugation) to our *Ulva intestinalis* extract, which may explain why higher concentrations of KWE than *Ulva* extract give stimulatory effects, in addition to the fact that pakchoi is a larger plant than *Arabidopsis*.

4.8.3 Potential mechanisms of the inhibitory and stimulatory effects of *Ulva* extract

The stimulatory effect of low concentrations of KWE (2-5%) on germination of pakchoi seeds may be attributed to the combined effects induced by the presence of soluble sugars, amino acids and various mineral elements in KWE (Zheng et al., 2016). Better growth performance in pakchoi plants treated with higher concentrations of KWE (10-20%) was also attributed to the existence of these compounds. Sugars are immediate substrates for intermediary metabolism and as effective signalling molecules. So, accessibility of sugars is an influential factor in the regulation of plant growth and development (Smeekens et al., 2010).

Furthermore, the growth-enhancing potential of algal extract is related to the presence of diverse polysaccharides, including unusual and complex polysaccharides not present in land plants (Blunden et al., 1986, Craigie, 2011). However, other research suggests a role for macro- and microelements, vitamins and plant growth regulators (phytohormones) (Tay et al., 1985, Tay et al., 1987, Crouch and Van Staden, 1992, Stirk and Van Staden, 1997, Khan et al., 2009, Stirk et al., 2004).

Since our *Ulva* extracts are water-based, it is unlikely that they contain high quantities of plant hormones, which are soluble in organic solvents. Our *Arabidopsis* mutant analysis

demonstrates that inhibition of germination by *Ulva* extract is dependent on activation of the *Arabidopsis* ABA signalling pathway, with cytokinin- and ethylene-signalling also playing a role. This suggests that a substance or substances in the *Ulva* extract activates endogenous plant hormone signalling pathways to inhibit germination. *Ulva* extract-mediated inhibition of primary root growth is partly blocked in an ABA-insensitive mutant, while cytokinin-, auxin- and ethylene-signalling mutants all respond similarly to wild type with respect to root growth. This implies that although ABA signalling plays a role in primary root growth inhibition by *Ulva* extract, additional pathways also contribute to root growth inhibition. Furthermore, lateral root development is inhibited via a different mechanism to primary root growth, as the ABA-insensitive *abi1* mutant's LR development is inhibited by *Ulva* extract to a greater extent than wild-type (Figure 4.5D).

In order to further elucidate the mechanisms by which *Ulva* extract exerts its deleterious effects on *Arabidopsis* germination we also tested a range of nutrient uptake mutants to understand if a specific nutrient imbalance is involved in triggering the inhibition pathway or is somehow linked to hormone metabolic or signalling pathways. The effect of these substances as very important intermediates in plant metabolism has been well documented (Cao et al., 1993, Zhang and Forde, 2000, Linkohr et al., 2002, Li et al., 2010a).

For example, about the inhibitory role of NH_4^+, it has been reported that although with ammonium as the sole nitrogen source, the growth of many plants is strongly inhibited but a low concentration of nitrate or potassium can moderate these detrimental effects (Cao et al., 1993). Considering the known inhibitory role of some phytohormones on root growth, Cao et al., (1993) tested a range of *Arabidopsis* mutants (resistant to high levels of auxin and other hormones) and showed that these mutants are also resistant to ammonium inhibition because they developed roots in presence of high levels of ammonium and in the absence of potassium. They concluded that there is a link between mechanisms that

arbitrate the ammonium inhibition of root growth and hormone metabolism or signalling pathways (Cao et al., 1993).

Since *Ulva* extract-mediated inhibition of germination in the present study happened in all the nutrient uptake mutants tested, quite similarly to the wild-type response to *Ulva* extract, and also our preliminary results of root assay showed similar behaviour to *Ulva* extract as same as wild-type, these results led us to this conclusion that the inhibitory effect of *Ulva* extract is not be related to potassium, nitrate and ammonium.

The inhibitory effects of *Ulva* extract are being attributed to Al^{3+} based on elemental analysis of *Ulva* tissue reported by Ghaderiardakani et al., (2019a). According to these data, the aluminium cation is present in amounts known to inhibit *Arabidopsis* primary root growth (Sun et al., 2009, Yang et al., 2017). Sun et al., (2009) concluded the possibility cannot be ruled out that Al^{3+} is the only inhibitory substance present and a correlative role for auxin, ethylene and cytokinin in root responses to Al^{3+} stress has been reported (Sun et al., 2009). Root elongation in ethylene signalling and auxin polar transport mutants was less inhibited by Al^{3+} than that in wild-type plants. However, in our current research, a reduced inhibition of root elongation or seed germination was not apparent in the mutants compared to wild type from our mutant root assays (except for *abi1-1*). It has been argued that there may be other hormones involved in seed- and root responses to Al^{3+} stress: the effects of Al^{3+} on germination and lateral root development in *Arabidopsis* has not previously been studied. Furthermore, the relatively high levels of Mg^{2+} detected in the extract (In 1% *Ulva* extract, 4x that present in *Arabidopsis* growth medium), may rescue the detrimental effects of Al^{3+} in the *Ulva* extract (Ghaderiardakani et al., 2019a; Deng et al., 2006).

Al^{3+} stress has a range of physiological effects such as changing the function of the plasma membrane by interacting with the lipids, that could affect root growth and development. Therefore, membrane potentials could be affected by Al^{3+} stress and consequently ions transportation across membranes including Ca^{2+} could be disturbed. This can cause changes in cytoplasmic Ca^{2+} homeostasis. In fact, cytoplasmic Ca^{2+} is known to mediate many cell signalling, metabolism and cell growth processes including root development (due to direct or indirect contribution of aluminium-induced disruption of cytoplasmic Ca^{2+} homeostasis to the inhibition of the cell division or root elongation) (Panda et al., 2009).

Moreover due to Al^{3+} toxicity there are changes in the expression and activity of the plasma membrane H^{+}-ATPase which directly correlates with physiological processes by controlling cytosolic pH and membrane potentials (Zhang et al., 2017). Several reports have been described that seaweeds contain a high levels of certain cations: macroelements (Na, P, K, Ca) and microelements (Fe, B, Mn, Ca, Mo, Zn, Co) that have a critical role in plant development and growth (Hong et al., 2007, Rayirath et al., 2009).

In many vegetable crops, the accumulation of sodium ions restrains embryo or seedling development, leading to reduced germination, uneven morphogenesis and loss of crop production e.g. (Almodares et al., 2007). Ghaderiardakani et al., (2019a) also demonstrated the data suggesting that the only macroelement present at higher concentrations in *Ulva* extract than in plant tissues (or indeed plant growth medium) is Na^{+}, but Na^{+} was not present at high enough concentrations to explain the inhibition of germination, root growth and lateral root development that has been seen. *Ulva* is a species known to tolerate low salinity despite being a marine alga, and our *Ulva* sampling site is where a river meets the sea, and the salinity of the seawater is low (F. Ghaderiardakani, unpublished).

A reduction in germination rate and growth of tomato attributable to salt (and perhaps reduced imbibition of water by seeds) was suggested upon applying brown seaweed (*Caulerpa sertularioides* and *Sargassum liebmannii*) liquid extracts, but not with *U. lactuca* and *P. gymnospora* with a lower salt concentration (Hernández-Herrera et al., 2014). Interestingly, some seaweed extracts alleviate salt stress: the survival of Kentucky bluegrass (*Poa pratensis* L. cv. Plush) treated with a proprietary seaweed extract ($38Lha^{-1}$) increased significantly, under various levels of salinity, with improved growth and promotion of rooting of the grass at a soil salinity of $0.15Sm^{-1}$ (Nabati et al., 1994). Application of seaweed extract activated a mechanism reducing the accumulation of Na^{+} in plants; grass treated with seaweed extract had less sodium in the shoot tissue (Yan, 1993, Latef et al., 2017).

In comparison with the land plant control, the microelements B and Fe were present at higher concentrations in *Ulva* tissue, but at levels that are very similar to that found in *Arabidopsis* growth medium which has been used, so the observed stimulatory or inhibitory effects of *Ulva* extract cannot be attributed to them (Ghaderiardakani et al., 2019a). The content of minerals in the macroalgae used in that research was in general agreement with the typical values for *Ulva* ssp. have been reported previously such as *Ulva lactuca* (Hernández-Herrera et al., 2014) and *Ulva reticulata* (Selvam and Sivakumar, 2013, Hong et al., 2007).

4.8.4 Effects of *Ulva* extract in alleviating salinity stress in *Arabidopsis*

In order to decrease the toxic effects as a result of high salinity on plant growth several approaches have been suggested, such as plant genetic engineering (Wang et al., 2003), and more recently, applying plant growth-promoting bacteria (PGPB) (Dimkpa et al., 2009). Spray application of marine bioactive substances has been shown to enhance ion

uptake and water stress tolerance in potted *Vitis vinifera* plants (Mancuso et al., 2006). I designed an experiment for investigating the effect of *Ulva* extract applied at 0.1% concentration to *Arabidopsis* plants subjected to salinity stress. A range of NaCl concentrations (10 mM, 20 mM, 50 mM, 80 mM, 100 mM, and 120 mM) were used in this test. However, my preliminary data showed that *Ulva* extract, at least at this particular concentration (0.1%), cannot alleviate the inhibition of germination rate, primary root length and lateral root development by salt, but in fact exacerbates it, but further work would need to be carried out using lower concentrations of *Ulva* extract to fully explore potential beneficial effects against salt stress.

4.8.5 Implications for agriculture – fertiliser or weedkiller?

Using seaweed extracts as biofertilisers due to their direct or indirect stimulatory impacts on plant metabolism has been suggested as one of their key beneficial applications (Arioli et al., 2015). Taken together, our results and others' suggest that for plants to benefit optimally from algal extracts, only a small quantity should be used or even could be mixed with commercially available fertilisers (Sridhar and Rengasamy, 2010). Sridhar et al., (2010) reported the synergistic effect of chemical fertiliser and seaweed liquid fertilisers (SLFs) towards the yield of crop. For minimising chemical fertilisers' amounts (or costs) and improving yield of *Arachis hypogaea*, a combination of 50% of the recommended rate of chemical fertilisers plus 1.0% SLFs (*Sargassum wightii* and *Ulva lactuca*) was suggested. The maximum yield was found in plants that received a combination of chemical fertilisers with *S. wightii* extract, about 11% fresh weight more than plants that received 100% recommend rate of chemical fertilisers (Sridhar and Rengasamy, 2010).

Our data demonstrates that *Ulva* extract can inhibit *Arabidopsis* seed germination, early root growth and lateral root development, even at concentrations below 1%, by activating

endogenous plant hormone signalling pathways. Could this in itself be useful? One of the top priorities in organic agriculture is the eradication of weeds from the production area (Walz, 1999). Concerns about improvements in agriculture centre on diminishing their adverse effects on environment and improving the sustainable development of agricultural systems. New approaches are required to integrate biological and ecological processes into food production and minimise the use of practices that lead to the environmental harm (Pretty, 2008). Considering the observed biological inhibitory effects resulting from the action of seaweed extracts on crops' germination and early development particularly at high concentration, it might be worthwhile to employ seaweed extracts as organic herbicides. The evidence at hand establishes that there are benefits to be obtained from Utilising macroalgal products in agricultural systems.

4.9 Conclusion

This study shows that water-soluble algal extracts from *Ulva intestinalis* were effective at stimulating the primary root growth of *Arabidopsis thaliana* only when applied at low concentrations. High concentrations of *Ulva* extract inhibit germination and root development, perhaps in part due to Al^{3+} toxicity (Ghaderiardakani et al., 2019a), with endogenous plant ABA signalling playing a role in this inhibition. We have shown using a model plant that the effects of algal extracts on *Arabidopsis* development are likely mediated by a complex interplay of hormones. Future work targeting candidate genes in *Ulva* (Wichard et al., 2015b) may enable us to discover more about how *Ulva* extracts exerts their effects on plant hormone signalling. Although if used sparingly, seaweed extracts are potential candidates to produce effective biostimulants, perhaps they may be just as beneficial as organic herbicides by targeting plants' ABA signalling mechanisms.

Further translational studies are required to define the appropriate algal sources for commercial biostimulants (considering inherently different algal extracts and also the availability of seaweed biomass in a particular area), their application form and frequency, the timing of applications in relation to plant developmental stages and the optimal dosages needed to maximise both agricultural productivity and economic advantages. Cross-disciplinary research could help farmers to benefit optimally from the use of algal extracts in the future, particularly for cost-effective organic farming and an environmentally-friendly approach for sustainable agriculture.

CHAPTER 5:
GENERAL DISCUSSION/CONCLUSION

5.1 Aims and objectives

The aim of the research presented in this thesis was to investigate:

(i) the microbiology behind the development and morphogenesis processes of a group of marine green algae, *Ulva* spp., in the early stages of their life cycle in order to understand the extent of specificity of epiphytic bacteria involved in the *Ulva*–bacterial interaction. This was carried out using axenic gametes released from gametophytes of the macroalga *Ulva intestinalis* collected during the spring and summer months from Llantwit major, south Wales along with *Ulva mutabilis* gametes from laboratory-propagated cultures (Protocol was developed by Wichard et al., in University of Jena).

(ii) practical aspects of the seaweed cultivation industry. Acquiring a better knowledge of applying seaweed as biofertiliser or using seaweed biomass as phytoremediation to absorb excess nitrogen, phosphorus and heavy metals from nutrient-rich aquaculture water in an IMTA system.

5.1.1 The cross-kingdom cross-talk of algae and bacteria

Previous studies on *Ulva*-bacteria interactions have revealed that bacteria-free gametes of the *Ulva mutabilis* Føyn display abnormal development into slow-growing callus-like colonies with incomplete cell division resulting in an undifferentiated 'pin cushion' appearance (Spoerner et al., 2012). It was therefore of interest to investigate the details of events surrounding the previous findings in order to determine whether specificity of epiphytic bacteria is involved in the *Ulva*–bacterial interaction in more than one species.

Macroalgal surfaces as particular niches are regularly colonised by bacteria, more likely species with equivalent functionality that would let them to be part of a surface-associated community. If initial colonisation can be considered as "by chance" (a 'lottery') from a set

of functionally equivalent bacteria (a ‘guild’ of bacteria that all possess the necessary metabolic abilities to colonise), then although the combination of the final guild will have no recognisable taxonomic pattern (core community of species), it will still hold constantly all the traits that are required for an algal-associated community to function (Burke et al., 2011b).

In order to address these hypotheses, the following experimental objectives were set:

1. Develop a maintainable algal culture of *U. mutabilis* slender and collect *U. intestinalis* wild type gametophytes and identify these wild type samples by *rbc*L and *tuf*A gene sequences.

2. Artificial induction of gametogenesis in thallus cells and parthenogenetic propagation (preparation of axenic cultures) to employ as the basis of a reliable and repeatable assay to evaluate the influence of specific bacterial strains on the growth and morphology of the *Ulva*.

3. Identify epiphytic bacterial strains isolated from the surfaces of several *Ulva* species (by Marshall 2004) and characterise (phylotype) them by 16S rDNA sequencing.

4. Cross-testing of potentially morphogenesis-inducing bacteria, isolated from various *Ulva* species, between the model system *U. mutabilis* and the cosmopolitan UK species *U. intestinalis* to determine if more than one *Ulva* species can respond to the same range of bacteria that affect algal growth, development and morphology via microbial morphogens

5. Observe and record the effect of bacterial strains on the growth and morphology of the axenic *U. intestinalis* and *U. mutabilis* plantlets.

5.1.1.1 Novel outcomes of the research

The work explored in the first result chapter of this thesis investigated the effect of individual bacterial strains and combinations of bacteria (a designed bacterial community) on the morphology and growth of axenic *Ulva intestinalis* and *U. mutabilis* plants. To summarise the achievements of this research work, a maintainable axenic culture of *U. intestinalis* was developed for the first time through phototactic movements of gametes. It was also revealed that the economically important *U. intestinalis* can induce (to generate gametes) and function similarly to *U. mutabilis* in the tripartite system. Finding the gametophytes at the beach is rather challenging but feasible. However, finding the gametophytes is easier in the spring.

As part of the investigation, bacteria isolated from the microbial epiphytic communities on three species of *Ulva* (including *U. linza*, *U. lactuca*, *U. compressa* and *Enteromorpha* sp.) by Marshall (2004) were selected based on the wide range of degrees of growth of axenic *Ulva* plantlets (Marshall et al., 2006), and their identity and phylogeny was re-confirmed/re-defined using 16S rDNA. Afterwards, these bacteria were inoculated to the axenic gametes of both *Ulva* species within the tripartite bioassay system, either singly or in pairwise combination, or in pairs with the bacteria known to restore full development to *U. mutabilis*, *Roseovarius* sp. MS2 and *Maribacter* sp. MS6 (Spoerner et al., 2012).

Among all the bacteria tested in this project, only one bacterium, the *Actinobacterium Microbacterium* sp. EC19 initiated marked morphological changes similar to *Maribacter* sp. MS6 in both *U. intestinalis* and *U. mutabilis* plants, leading to the formation of a normal cell wall and rhizoid but failing to induce a proper blade. Three isolates also, *Paracoccus* sp., strains E34 and UL2, as well as *Cellulophaga lytica* UL16 induced cell divisions, like the reference strain *Roseovarius* sp. MS2. The combination of EC19 (the

MS6 equivalent strain) and one of MS2 equivalent strains recapitulated the full normal growth and morphology of *U. mutabilis* and *U. intestinalis* (Ghaderiardakani et al., 2017).

The findings of this experiment showed that:

(i) more than one *Ulva* species (*U. mutabilis* and *U. intestinalis* tested in this research) can have their normal morphogenetic programme promoted by the same range of bacteria that affect algal growth, development and morphology via microbial morphogens.

(ii) there is specificity in associated bacterial communities to their living hosts regarding the particular bacterial signals regulating algal development, e.g. for induction of rhizoid formation or induction of cell division. Therefore, epiphytic bacterial assemblages cannot be considered as a random association, particularly, when it is known that MS6 activity is present in far fewer bacterial species compare to MS2 activity (Grueneberg et al., 2016).

(iii) the variability of members of associated bacteria between different *Ulva* species implies that a kind of functional redundancy exists within this host-associated microbial community. In fact, data from this research suggested that the functions of bacteria (i.e. promoting cell division versus cell differentiation/cell wall formation) cannot be attributed to a specific genus taxonomic group. These are rather distributed throughout the various groups of bacteria which have the (required by algal-host) functional genes (Ghaderiardakani et al., 2017).

5.1.1.2 Future work

Marshall (2004) argued that the interactions of bacteria in combination are complex. By using an assay to evaluate the morphological effect of a combination of two bacteria

strains on *U. linza* plantlets, it was suggested that individual strains without effect, when added in combination, will suppress the other strains that stimulate changes (Marshall 2004). Coupled with the present study results, it may well be of interest to demonstrate if there is a competition and a particular order of distribution between different active bacteria (bacteria that can stimulate morphogenesis changes) including *Roseovarius* sp. MS2 and *Maribacter* sp. MS6 and the bacteria isolated from different *Ulva* species, *Roseovarius* sp., MS2 equivalents and *Maribacter* sp. MS6 equivalent or between active bacteria and non-active ones.

It would also be worthwhile to further investigate whether the bacteria isolated from different *Ulva* species or even different algae species can stimulate any kind of morphological changes on *Ulva* axenic gametes. The tripartite bioassay system, either *U. mutabilis* or *U. intestinalis* and their associated bacteria, which was used in this study, could potentially be used to record the morphological effects of any bacterial strains of interest. It also allows the plants growth to be studied and studied quantitatively (i.e. cell numbers and thallus length).

To clarify the relationship between the epiphytic bacteria and their algal host in more detail, a number of further experiments could be undertaken. It would be worth trying to develop a bioassay system similar to the tripartite system being used in this study, for other algae species including other green algae or red and brown algae. In some cases, considering the absence of gametophytes in nature (e.g. *Macrocystis* sp. (Kinlan et al., 2003)), spores could be substituted for the gametes or alternatively another way developed to create axenic algal culture such as axenic protoplasts (Gupta et al., 2012).

In nature, a single species of *Ulva* has been observed occurring with completely different morphologies, which correlate with different behaviours (e.g. attachment versus non-

attachment) that affect the seaweed's impact on the environment (e.g. beneficial versus nuisance (Smetacek and Zingone, 2013, Steinhagen et al., 2019b)). Under these circumstances it could therefore be relevant to explore whether the bacterial flora on each algae morphotype is identical or whether individual *Ulva* morphotypes are associated with different bacterial strains that have varying functions in terms of cell division and differentiation. Additionally, the parameters that determine the morphology, the attachment/unattachment and thereby also the tendency to form nuisance blooms in some *Ulva* species should be defined. Diversity of bacteria could be assessed through isolation and identification by both traditional microbiological techniques and molecular tools.

Community analyses could be an option, such as those undertaken by Tujula et al., (2010) where denaturing gradient gel electrophoresis (DGGE) fingerprinting was used to assess the whole bacterial communities on *U. australis* surfaces. However, this method is subject to some restrictions to report comprehensively the diversity, variability and uniqueness of the bacterial community on *Ulva* sp. surface (Muyzer et al., 1993, Burke et al., 2011b). Both (traditional microbiological) methods provide valuable data, but in the case of using bioassay techniques such as the tripartite system and because the bacteria will be subject to further experimental bioassays, the bacteria must be culturable.

As noted before when the concentration of the *Maribacter*-factor was decreased by dilution, the phenotype of some individuals indicated that the *Roseovarius*-factor solely controlled them (Section 3.8.3.1). Further studies are certainly needed to monitor and ideally to identify the underlying molecular structure of the AGPFs throughout different seasons, in various geographic locations, even by using different algal species. In other words, it is necessary to further define the extent of the specificity involved in bacteria-algae interactions between kingdoms using either symbiotic bacteria isolated from *Ulva* species from quite different geographic origins or symbiotic bacteria isolated from

brown/red algae. Furthermore, the assay could be repeated using filter-sterilised bacterial supernatants employing the 'tripartite' lab-based system as a bioassay method, (as in Spoerner et al., 2012) rather than the bacterial cultures themselves. This would demonstrate that morphological rescue of *Ulva* is dependent on diffusible signals from bacteria rather than direct contact with bacteria, as suggested by the filtered seawater experiments in Chapter 4 and the previous data of Spoerner et al., (2012). Bacterial cultures could also be autoclaved so that inoculation involved no live bacteria, although whether signals would remain present after this treatment is an open question.

There is a possibility that the lab-based growth medium used, UCM, could be affecting the performance of the *Ulva*-associated bacteria (in terms of morphological activity) compared to the medium (seawater) supporting bacteria-*Ulva* interactions in natural ecosystems. So, the bacterial-dependent morphological assays could be repeated using *Ulva* growing in reduced media such as artificial seawater.

The chemical structures of bacterial morphogens, their mode of action, their effective concentrations, their possible receptors in *Ulva*, their perception circumstances by *Ulva*, their mutual effects on each other and on *Ulva* are some examples of many further questions has been raised and hopefully will be considered for future investigations in this field. Fortunately, the *Ulva mutabilis* genome sequencing project has been completed which will provide a great resource to answer fundamental questions using this model system.

The complete genome sequence of *Ulva* can be also applied more widely for discovery of genes functions and as *Ulva* is a model organism and experimental tool, it would be an opportunity to shed light on different aspects of not only phycological/plant investigations but also environmental, energy-and food-related subjects. For instance, in the case of green

tide studies (regarding to both frequency and intensity of *Ulva* blooms), by performing large-scale (transcriptomic) gene expression analysis and comparing attached and unattached *Ulva* spp., the molecular mechanisms underpinning responses to various environmental conditions or to designed bacterial systems by using a range of associated known- and unknown- bacteria (in terms of producing morphogens) can be discovered. The mechanisms that are involved in modulating the reproduction, growth and development of *Ulva* spp which lead to bloom formation can thus be uncovered. Moreover, clarifying pathways incorporating into receiving and recognising morphogens and also the possible receptors for sporulation inhibitors would be exciting challenges for future cellular investigation. Considering this point that the sequenced genome makes mapping feasible and reasonably easier, informative mutant screens using naturally occurring mutants or other mutants generated in the lab can be performed to look for mutants that cannot respond the same as wild types to morphogens, sporulation inhibitors, environmental stresses etc.

Conducting research to detect and identify the bacterial or algal metabolites in each phase of *Ulva* growth and development (under controlled condition in the lab) would be also useful. To nail this purpose, labelled carbon sources can be used in bacteria or *Ulva* growth medium. By labelled nutritious units, the synthesis pathway and therefore the released metabolite in *Ulva*-bacteria chemosphere can be traceable. The labelled metabolites will distinguish between *Ulva* and bacterial substances which will be useful to understand better the cross-talk between *Ulva* and its associated bacteria and their specific roles in this community. In fact, more work can be directed to explore chemical ecology and resulting physiological changes of *Ulva* in different phases throughout its growth, with and without associated bacteria (in tripartite system), by surveying the whole metabolome and transcriptome via a combination of transcriptomics and metabolomics analysis. These

methods may also assist our understanding of how *Ulva* is responding under different conditions like environmental or nutritious stresses (with and without useful associated bacteria).

Global change is leading to increased risks and instability in environmental and ecological factors and ecosystem services, including climate change, ocean warming and acidification, coastal eutrophication, rising the sea-level (Kit, 2014). The consequences of these changes, for example whether the bacterial community would shift to be dominated by opportunistic pathogens, are unknown. Investigation of the traits that underlie the successful adaptation of *Ulva* species under different environmental circumstances, or stress adaptation regarding to their successful cosmopolitan distribution would be new opportunities to be taken by using genome data. By tracking metabolome- and gene expression data, generating profiles of stress-driven morphological, physiological and metabolic changes in *Ulva* would be possible (Gupta and Hemant et al., 2017). These approaches may also assist researchers to find an inclusive understanding about the role of beneficial associated bacteria to protect poorly-defended algal hosts and the impact of beneficial bacteria on algal diseases and fitness. It must be considered that under non-laboratory conditions, all or several of these factors (environmental, disease etc.) have interactions together, so the interactive effects of these elements on *Ulva* growth and development also need to be investigated.

5.1.2 Biotechnological applications of *Ulva*

To produce ever greater amounts of food to meet the demands of the expanding population globally by intensive agriculture and aquaculture, huge concerns have been raised about growing environmental contamination (Lazzari and Baldisserotto, 2008, Turcios and Papenbrock, 2014). This thesis tried to fill some of the gaps in our knowledge of

alleviating this environmental contamination via seaweed i.e. using *Ulva* as a biofilter and as an organic biostimulator.

5.1.2.1 Applications of seaweed extracts in agriculture

The necessity of enhancing crop productivity with more environmentally-friendly activities has generated new interest in novel biological approaches to exploit such as applying seaweed extracts as biofertiliser due to their potent plant growth-enhancing properties through metabolic benefits, activating disease response pathways and alleviating stress tolerance (Arioli et al., 2015). Therefore this research aimed at identifying the molecular mechanisms by which seaweeds can affect land plant productivity, using model organisms.

In order to achieve the second main aim of this thesis, a number of experimental objectives were set:

1. Developing a bioassay using two model organisms, *Ulva intestinalis* and *Arabidopsis thaliana*.

2. Preparing aqueous extract from *Ulva intestinalis* samples collected from south Wales.

3. Applying a range of *Ulva* extract to the culture medium of wild type *Arabidopsis* and a variety of mutants in hormone signalling and perception, and mutants involved in nutrient uptake and processing

4. Quantifying the effects of different concentration of *Ulva* extract on *Arabidopsis* seed (wild-type and mutants) germination rate.

5. Evaluating the effects of different concentration of *Ulva* extract on *Arabidopsis* (wild-type and mutants) primary and lateral root growth.

5.1.2.1.1 Achievements of the research

The convenience of model organisms (*Ulva intestinalis* and *Arabidopsis thaliana*) increases the detail with which we can demonstrate the cellular mechanisms and molecular processes involved in both positive and negative effects of *Ulva* extract on the growth and development of plants, or physiological responses of plants to algal extract, implying that the potential future traits and best possible outcome of application of seaweed extract as "plant biostimulants" may be discovered.

The work explored in this thesis examined the effect of different *Ulva* extract concentrations on seed germination, primary and lateral roots of *Arabidopsis thaliana* wild-type and a range of mutants. Data have shown that *Ulva* extracts at concentrations of 0.5%-1% inhibits wild-type *Arabidopsis* seed germination (Ghaderiardakani et al., 2019a). In these assays, it was also observed that *Ulva* extract ≥0.3% reduces wild-type *Arabidopsis* primary root growth and the extract inhibits wild type LR formation even at concentrations below 0.1%, suggesting that LRs are more sensitive than the primary root to the inhibitory agent(s) in the *Ulva* extract (Ghaderiardakani et al., 2019a). However, *Ulva* extract promotes *Arabidopsis* primary root growth but not germination or lateral root formation at concentrations below 0.1% (Ghaderiardakani et al., 2019a).

The mechanisms moderating growth changes caused by *Ulva* extract in wild-type *Arabidopsis* were investigated via observations and assays using *Arabidopsis* mutants treated by *Ulva* extract. While almost all mutants showed the same trend as wild-type (inhibited in germination, primary and lateral root growth by increasing the concentration of *Ulva* extract), *abi1* seeds were unresponsive to the inhibitory effect of *Ulva* extract and the *abi1* mutant's root growth behaved normally to low concentrations of *Ulva* extract and was slightly insensitive to higher concentrations of *Ulva* extract (Ghaderiardakani et al., 2019a).

The hypothesis that *Ulva* extract was affecting the *Arabidopsis* directly via ABA in the *Ulva* extract in this experiment was rejected because the extract being used was aqueous while ABA is more soluble in organic solvents than in water (Fu et al., 2011, Zhang, 2014). The ABA was not detected in *Ulva* samples (dried samples) when it was measured by our collaborator Dr. Wichard (University of Jena). Moreover, the hypothesis that *Ulva* extract functioned solely by affecting the ABA signalling pathway in *Arabidopsis* was rejected because the responses from *abi1* primary root assay showed slight insensitivity to higher concentrations of *Ulva* extract, but not complete insensitivity. Furthermore, based on the elemental analysis and the high amount of Al^{3+}, Ghaderiardakani et al., (2019a) argued that aluminium toxic effects could be the reason of inhibitory effects of *Ulva* extract particularly at higher concentrations of *Ulva* extract. This finding might be also extended to explain the results of other investigations that showed the inhibitory effects of higher concentration of algal extract on different crops (Hernández-Herrera et al., 2014, Rao and Chatterjee, 2014, Kalaivanan and Venkatesalu, 2012, Kumar et al., 2012).

5.1.2.1.2 Future work

A combined approach of chemical analysis and molecular biology or 'omics' tools will further explore the chemical combination, effective compounds, the cellular mechanisms moderating morphology or growth changes and ultimately the mode of action of mechanisms involved in the physiological responses in *Arabidopsis* or generally speaking in all plants (particularly important agricultural crops) to algal extract.

Considering the importance of the extraction method which results in extracts with different characteristics and consequently different effects (Craigie et al., 2008), it would be beneficial to investigate other methods of extraction of algal extract. Moving forward it would be useful to alter the components of extract for the sake of synergistic effects by

mixing extracts resulting from different ways or extracts from various algae species or even with artificial extracts. This is relevant in an applied sense to fertiliser production.

To get further at mechanisms, future work could look at *Arabidopsis* mutants impaired in aluminium response (Zhang et al., 2019).

Several questions have been raised that still need to be answered in terms of application of seaweed extract as biofertiliser to obtain the maximum benefits and desired outcome, such as: at what stage of the plant's growth should an extract be applied, how much extract should be applied each time, how frequently should an extract be applied, and which procedure should be used e.g. drainage or spraying, etc. Surveying genetic variation in plants responses when they are treated by algal extract can be another interesting subject for future research. For all these studies a series of high-resolution time-lapse images can be used in comparison to traditional method for measuring root or shoot growth. For instance, Mairhofer et al., (2017) revealed a protocol of X -ray compute (CT) for imagining and quantification of plant root architecture and tracking the effect of different factors (natural or non-natural) on roots in the soil (Mairhofer et al., 2017). This non-destructive, 3D phenotyping method would be an excellent way to quantify the root changes in presence or absence of *Ulva* (or any other seaweed) extract.

It would be worthwhile to test the hypothesis that hormones are involved in algal extract impact on growth and development of plants by adding hormones as controls to the experiment was designed at present study.

The subsequent detailed mechanisms and perception into how seed germination and root development respond to various seaweed extracts will be helpful in terms of handling crop development by using non-chemical biofertilisers.

5.1.2.2 Applications of seaweed in aquaculture (IMTA system)

The attention recently paid to the quantity of residue produced by fish farming installations implies that this will be a decisive feature in the sustainability of fish farming in the near future. Nitrogen (N) and phosphorus (P) are the main metabolic waste produced by intensive fish farming, and can affect not only the rearing water, but also the whole environment. Many endogenous and exogenous variables such as genetics, life stage, size, rearing system, and diet, control the output of N and P metabolic end-products deposited into the environment by aquaculture operations (Lazzari and Baldisserotto, 2008).

The aim of the present study was to assess the capability of *Ulva rigida* to biofilter nitrate and phosphate, the dissolved nitrogenous compounds excreted by the sea bass and also to show implications of accumulated algal growth factors (as a side project) in the effluents of an IMTA system. Applying such bioremediative approaches can be complex and require a detailed understanding of the physiology of the selected species (e.g. macroalgae species in present study) and compromising between conflicting ultimate goals (e.g. biomass production versus bioremediation efficiency (Chopin et al., 2001b)).

To tackle these key points, the following experimental objectives were set:

1. Selecting the points across the sampling area, including entrance ponds, rearing fish ponds, after filtration, seaweed growing ponds and sediment ponds.

2. Collecting samples in 4 different times along a day.

3. Filtering water samples to make them sterile.

3. Analysing the morphogenetic compounds, phosphate and nitrate amounts of each water sample.

By measuring the nitrate and phosphate in water samples in entrance ponds (before the samples became enriched by fish rearing operations) and within fish ponds, then in algae cultivation ponds, it was possible to determine the amount of nitrate and phosphate assimilation by seaweeds. Although the level of nitrate remains rather constant (comparing before and after seaweed tanks), some decrease in case of phosphate is being recorded. The results emphasise the importance of the point mentioned above about the ultimate long-term aim, which means that the conditions such as water flow rate or stocking density should be arranged according to the final purpose, biomass production or bioremediation. Other factors like sea currents and tide conditions are also important in such fish farms which water movement controls with tides.

5.1.2.2.1 Future work

Considering the possible future challenges in this field which could be requiring adequate land or area and balancing high production costs with high value application of the algal biomass (Sode et al., 2013), it would be worth local investigations into the quality of waste water which is going to be used as nutrient source (i.e. civil sewage, agricultural run-off or aquaculture effluents) and therefore, determining the bioremediation capacity of algae species over a range of nutrient concentrations by using the waste water only, or dilute waste water if it is massively concentrated.

Obviously, to fully benefit from the use of live macroalgae as a biofilter and to improve their effectivity, more scientific investigation, innovation and local field trials are required. It would be necessary to assess the risks of introducing a non-endemic species, of unwanted blooms of algal species which is used as candidate for bioremediation, and of whether using live seaweed can promote the subsequent bloom of diatoms or planktons in aquatic ecosystem by disturbance in water's nutrition (for instance silica ratio in water) or

cause occurrence of epiphytes, such as *Polysiphonia* sp. (Hurtado et al., 2006; Bindu and Levine, 2010).

With considering the high propagation rate during growth periods and a comparatively remarkable metal accumulation capacity, *Ulva* spp. may form a considerable biomass rich of heavy metals (Boubonari et al., 2008) so that care needs to be taken for utilising as food or animal feed. But it would not disqualify the biomass for any other substitute utilisation e.g. as biofuel feedstocks regarding concentrations of heavy metals.

In the future, studies with a greater temporal resolution will be necessary to monitor the dynamics in nitrogen consumption in aquaculture. In addition, elaborated studies are certainly required to verify the presence of swarming inhibitor and sporulation inhibitors (identify the glycoproteins), which cannot be tested using the bioassays established by Kessler et al., (2018) due to the species-specificity of the inhibitors (Stratmann et al., 1996, Kessler et al., 2018). Stratmann et al., (1996) argued that in natural ecosystems, preventing gametes release would be beneficial for *Ulva* to avoid releasing mature gametes when conditions are not appropriate (for example when algae is not submerged in water because of low tide). Accordingly, in aquaculture units, the existence and level of sporulation inhibitors and swarming inhibitors may play important roles to regulate reproduction, inhibit early reproduction and keep seaweed in the vegetative phase.

5.2 Final conclusion

- Bacteria can work across *Ulva* species and multiple bacteria can induce the same morphogenetic functions
- Using the model organism *Arabidopsis*, we have shown that *Ulva*-extract inhibition works in part via ABA signalling but other hormone pathways are involved too.

Inhibition may be in part due to heavy metals and positive (stimulation) effects are only apparent at low *Ulva* extract concentrations.

- *Ulva* can take up phosphate and grows well when nitrate is not limiting.

Going forward, these results can be beneficial to address questions of fundamental biology of green macroalgae, aquaculture and seaweed production, uses of seaweeds for remediation or fertilisation of land plants.

Chapter 6:
References

ABREU, M. H., PEREIRA, R. & SASSI, J.-F. 2014. Marine algae and the global food industry. *In:* PEREIRA, R. & NETO, J. (eds.) *Marine algae, biodiversity, taxonomy, environmental assessment, and biotechnology.* Bocca Raton, FL, USA: CRC Press.

ABREU, M. H., PEREIRA, R., YARISH, C., BUSCHMANN, A. H. & SOUSA-PINTO, I. 2011. IMTA with *Gracilaria vermiculophylla*: productivity and nutrient removal performance of the seaweed in a land-based pilot scale system. *Aquaculture,* 312**,** 77-87.

ALE, M. T., MIKKELSEN, J. D. & MEYER, A. S. 2011. Differential growth response of Ulva lactuca to ammonium and nitrate assimilation. *Journal of Applied Phycology,* 23**,** 345-351.

ALMODARES, A., HADI, M. & DOSTI, B. 2007. Effects of salt stress on germination percentage and seedling growth in sweet sorghum cultivars. *Journal of Biological Sciences,* 7**,** 1492-1495.

ALONSO, J. M., STEPANOVA, A. N., LEISSE, T. J., KIM, C. J., CHEN, H., SHINN, P., STEVENSON, D. K., ZIMMERMAN, J., BARAJAS, P. & CHEUK, R. 2003. Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science,* 301**,** 653-657.

ALSUFYANI, T., WEISS, A. & WICHARD, T. 2017. Time course exo-metabolomic profiling in the green marine macroalga *Ulva* (Chlorophyta) for identification of growth phase-dependent biomarkers. *Marine drugs,* 15**,** 14.

AMANN, R. I., LUDWIG, W. & SCHLEIFER, K.-H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews,* 59**,** 143-169.

ANDERSEN, R. A. 2005. *Algal culturing techniques*, Academic press.

APSE, M. P., SOTTOSANTO, J. B. & BLUMWALD, E. 2003. Vacuolar cation/H+ exchange, ion homeostasis, and leaf development are altered in a T -DNA insertio AtNHX1, the Arabidopsis vacuolar Na+/H+ antiporter. *The plant journal,* 36**,** 229-239.

ARABIDOPSIS_GENOME_INITIATIVE 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *nature,* 408**,** 796.

ARCHIBALD, J. M. 2009. The puzzle of plastid evolution. *Current Biology,* 19**,** R81-R88.

ARIELI, A., SKLAN, D. & KISSIL, G. 1993. A note on the nutritive value of Ulva lactuca for ruminants. *Animal Science,* 57**,** 329-331.

ARIOLI, T., MATTNER, S. W. & WINBERG, P. C. 2015. Applications of seaweed extracts in Australian agriculture: past, present and future. *Journal of applied phycology,* 27**,** 2007-2015.

ARMSTRONG, E., YAN, L., BOYD, K. G., WRIGHT, P. C. & BURGESS, J. G. 2001. The symbiotic role of marine microbes on living surfaces. *Hydrobiologia,* 461**,** 37-40.

ATKINSON, N. J. & URWIN, P. E. 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of experimental botany,* 63**,** 3523-3543.

AUER, C. A. 1997. Cytokinin conjugation: recent advances and patterns in plant evolution. *Plant Growth Regulation,* 23**,** 17-32.

AZIZ, N., MAHGOUB, M. & SIAM, Z. 2011. Growth, flowering and chemical constituents performance of Amaranthus tricolor plants as influenced by seaweed (Ascophyllum nodosum) extract application under salt stress conditions. *J Appl Sci Res,* 7**,** 1472-1484.

AZPIROZ-LEEHAN, R. & FELDMANN, K. A. 1997. T-DNA insertion mutagenesis in Arabidopsis: going back and forth. *Trends in Genetics,* 13**,** 152-156.

BAKKER, C., SWANTON, C. & MCKEOWN, A. 2009. Broccoli growth in response to increasing rates of pre-plant nitrogen. I. Yield and quality. *Canadian journal of plant science,* 89**,** 527-537.

BAROTT, K. L., RODRIGUEZ-BRITO, B., JANOUŠKOVEC, J., SMITH, J. E., KEELING, P. & ROHWER, F. L. 2011. Microbial diversity associated with four functional groups of benthic reef algae and the reef building coral *Montastraea annularis*. *Environmental microbiology,* 13**,** 1192-1204.

BARSANTI, L. & GUALTIERI, P. 2006. Algae Anatomy, Biochemistry, and Biotechnology Taylor and Francis Group LLC. Boca Raton London New York CRC Press.

BARTOLI, M., NIZZOLI, D., NALDI, M., VEZZULLI, L., PORRELLO, S., LENZI, M. & VIAROLI, P. 2005. Inorganic nitrogen control in wastewater treatment ponds from a fish farm (Orbetello, Italy): denitrification versus Ulva uptake. *Marine Pollution Bulletin,* 50**,** 1386-1397.

BATTACHARYYA, D., BABGOHARI, M. Z., RATHOR, P. & PRITHIVIRAJ, B. 2015. Seaweed extracts as biostimulants in horticulture. *Scientia Horticulturae,* 196**,** 39-48.

BAUER, H., ACHE, P., LAUTNER, S., FROMM, J., HARTUNG, W., AL-RASHEID, K. A., SONNEWALD, S., SONNEWALD, U., KNEITZ, S. & LACHMANN, N. 2013. The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. *Current Biology,* 23**,** 53-57.

BEIJERINCK, M. W. 1901. Über oligonitrophile Mikroben [On oligonitrophilic microbes]. *Centralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene.,* 7**,** 561–582.

BENGTSSON, M. M., SJØTUN, K., STORESUND, J. E. & ØVREÅS, L. 2011. Utilization of kelp-derived carbon sources by kelp surface-associated bacteria.

BERRI, M., OLIVIER, M., HOLBERT, S., DUPONT, J., DEMAIS, H., LE GOFF, M. & COLLEN, P. N. 2017. Ulvan from Ulva armoricana (Chlorophyta) activates the PI3K/Akt signalling pathway via TLR4 to induce intestinal cytokine production. *Algal research,* 28**,** 39-47.

BHATTACHARYA, D. & MEDLIN, L. 1998. Algal phylogeny and the origin of land plants. *Plant Physiol,* 116**,** 9-15.

BIELACH, A., HRTYAN, M. & TOGNETTI, V. B. 2017. Plants under stress: Involvement of auxin and cytokinin. *International journal of molecular sciences,* 18**,** 1427.

BINDU, M. S., & LEVINE, I. A. 2011. The commercial red seaweed Kappaphycus alvarezii—an overview on farming and environment. *Journal of Applied Phycology*, 23, 789-796.

BLIDING, C. 1968. A critical survey of European taxa Ulvales II. Ulva, Ulvaria, Monostroma, Kornmannia. *Bot. Not.,* 121**,** 535-629.

BLOMSTER, J., BÄCK, S., FEWER, D. P., KIIRIKKI, M., LEHVO, A., MAGGS, C. A. & STANHOPE, M. J. 2002. Novel morphology in Enteromorpha (Ulvophyceae) forming green tides. *American Journal of Botany,* 89**,** 1756-1763.

BLOMSTER, J., MAGGS, C. A. & STANHOPE, M. J. 1998. Molecular and morphological analysis of Enteromorpha intestinalis and E. compressa (Chlorophyta) in the British Isles. *Journal of Phycology,* 34**,** 319-340.

BLUNDEN, G., CRIPPS, A., GORDON, S., MASON, T. & TURNER, C. 1986. The characterisation and quantitative estimation of betaines in commercial seaweed extracts. *Botanica Marina,* 29**,** 155-160.

BLUNDEN, G., JENKINS, T. & LIU, Y.-W. 1996. Enhanced leaf chlorophyll levels in plants treated with seaweed extract. *Journal of applied phycology,* 8**,** 535-543.

BOLD, H. & WYNNE, M. 1985. introduction to the Algae. –720 pp. Prentice-Hall, inc., englewood Cliffs, New Jersey, USA.

BOLTON, J., ROBERTSON-ANDERSSON, D., SHUULUKA, D. & KANDJENGO, L. 2009. Growing *Ulva* (Chlorophyta) in integrated systems as a commercial crop for abalone feed in South Africa: a SWOT analysis. *Journal of Applied Phycology,* 21**,** 575-583.

BORSATO, L. C., DI PIERO, R. M. & STADNIK, M. J. 2010. Mechanisms of defense elicited by ulvan against uromyces appendiculatus in three bean cultivars. *Tropical Plant Pathology,* 35**,** 318-322.

BOUBONARI, T., MALEA, P. & KEVREKIDIS, T. 2008. The green seaweed Ulva rigida as a bioindicator of metals (Zn, Cu, Pb and Cd) in a low-salinity coastal environment. *Botanica Marina,* 51**,** 472-484.

BOYLE, K. A., KAMER, K. & FONG, P. 2004. Spatial and temporal patterns in sediment and water column nutrients in a eutrophic southern California estuary. *Estuaries,* 27**,** 378-388.

BRAIN, K., CHALOPIN, M., TURNER, T., BLUNDEN, G. & WILDGOOSE, P. 1973. Cytokinin activity of commercial aqueous seaweed extract. *Plant Science Letters,* 1**,** 241-245.

BROCH, O. J. & SLAGSTAD, D. 2012. Modelling seasonal growth and composition of the kelp Saccharina latissima. *Journal of applied phycology,* 24**,** 759-776.

BUAPET, P., HIRANPAN, R., RITCHIE, R. J. & PRATHEP, A. 2008. Effect of nutrient inputs on growth, chlorophyll, and tissue nutrient concentration of Ulva reticulata from a tropical habitat. *Science Asia,* 34**,** 245-252.

BULLERI, F., BENEDETTI-CECCHI, L., ACUNTO, S., CINELLI, F. & HAWKINS, S. J. 2002. The influence of canopy algae on vertical patterns of distribution of low-shore assemblages on rocky coasts in the northwest Mediterranean. *Journal of experimental marine biology and ecology,* 267**,** 89-106.

BURCHETT, S., FULLER, M. & JELLINGS, A. Application of seaweed extract improves winter hardiness of winter barley cv Igri. The Society for Experimental Biology, Annual Meeting, The York University, 1998.

BURKE, C., STEINBERG, P., RUSCH, D., KJELLEBERG, S. & THOMAS, T. 2011a. Bacterial community assembly based on functional genes rather than species. *Proceedings of the National Academy of Sciences,* 108**,** 14288-14293.

BURKE, C., THOMAS, T., LEWIS, M., STEINBERG, P. & KJELLEBERG, S. 2011b. Composition, uniqueness and variability of the epiphytic bacterial community of the green alga Ulva australis. *The ISME journal,* 5**,** 590.

BUTELLI, E., TITTA, L., GIORGIO, M., MOCK, H.-P., MATROS, A., PETEREK, S., SCHIJLEN, E. G., HALL, R. D., BOVY, A. G. & LUO, J. 2008. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature biotechnology,* 26**,** 1301-1308.

CALLOW, J. A. & CALLOW, M. E. 2011. Trends in the development of environmentally friendly fouling-resistant marine coatings. *Nature communications,* 2**,** 244.

CALLOW, M. E. & CALLOW, J. A. 2002. Marine biofouling: a sticky problem. *Biologist,* 49**,** 1-5.

CALLOW, M. E., CALLOW, J. A., PICKETT -HEAPS, J. D.
adhesion of Enteromorpha (Chlorophyta, Ulvales) propagules: quantitative settlement studies and video microscopy. *Journal of Phycology,* 33**,** 938-947.

CAMPBELL, A. H., VERGÉS, A. & STEINBERG, P. D. 2014. Demographic consequences of disease in a habitat -forming seaw
enemies. *Ecology,* 95**,** 142-152.

CAO, Y., GLASS, A. D. & CRAWFORD, N. M. 1993. Ammonium inhibition of Arabidopsis root growth can be reversed by potassium and by auxin resistance mutations aux1, axr1, and axr2. *Plant Physiology,* 102**,** 983-989.

CARPENTER, S. R. & BENNETT, E. M. 2011. Reconsideration of the planetary boundary for phosphorus. *Environmental Research Letters,* 6**,** 014009.

CARPENTER, S. R., CARACO, N. F., CORRELL, D. L., HOWARTH, R. W., SHARPLEY, A. N. & SMITH, V. H. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological applications,* 8**,** 559-568.

CASE, R. J., LONGFORD, S. R., CAMPBELL, A. H., LOW, A., TUJULA, N., STEINBERG, P. D. & KJELLEBERG, S. 2011. Temperature induced bacterial virulence and bleaching disease in a chemically defended marine macroalga. *Environmental Microbiology,* 13**,** 529-537.

CEYLAN, S. & GOLDFARB, J. L. 2015. Green tide to green fuels: TG–FTIR analysis and kinetic study of Ulva prolifera pyrolysis. *Energy Conversion and Management,* 101**,** 263-270.

CHALLINOR, A. J., SIMELTON, E. S., FRASER, E. D., HEMMING, D. & COLLINS, M. 2010. Increased crop failure due to climate change: assessing adaptation options using models and socio-economic data for wheat in China. *Environmental Research Letters,* 5**,** 034012.

CHANDLER, J. W. & WERR, W. 2015. Cytokinin–auxin crosstalk in cell type specification. *Trends in plant science,* 20**,** 291-300.

CHANG C, STADLER R (2001) Ethylene hormone receptor action in *Arabidopsis*. *Bioessays* 23, 619–627

CHANG, L., RAMIREDDY, E. & SCHMÜLLING, T. 2013. Lateral root formation and growth of Arabidopsis is redundantly regulated by cytokinin metabolism and signalling genes. *Journal of experimental botany,* 64**,** 5021-5032.

CHARLIER, R. H., MORAND, P., FINKL, C. W. & THYS, A. Green tides on the Brittany coasts. US/EU Baltic International Symposium, 2006 IEEE, 2006. IEEE, 1-13.

CHARRIER, B., COATES, J. C. & STAVRIDOU, I. 2017. Surfing amongst oil-tankers: connecting emerging research fields to the current international landscape. *Trends in plant science,* 22**,** 1-3.

CHOPIN, T., BUSCHMANN, A. H., HALLING, C., TROELL, M., KAUTSKY, N., NEORI, A., KRAEMER, G. P., ZERTUCHE -GONZÁLEZ,
2001a. Integrating seaweeds into marine aquaculture systems: a key toward sustainability. *Journal of Phycology,* 37**,** 975-986.

CHOPIN, T., COOPER, J. A., REID, G., CROSS, S. & MOORE, C. 2012. Open -water integrate
multi -trophic aquacu
fed aquaculture by extractive aquaculture. *Reviews in Aquaculture,* 4**,** 209-220.

CHOPIN, T., MACDONALD, B., ROBINSON, S., CROSS, S., PEARCE, C., KNOWLER, D., NOCE, A., REID, G., COOPER, A. & SPEARE, D. 2013. The Canadian Integrated

ecosystem responsible aquaculture. *Fisheries,* 38**,** 297-308.

CHOPIN, T., YARISH, C., NEEFUS, C., KRAEMER, G., BELYEA, E., CARMONA, R., SAUNDERS, G., BATES, C., PAGE, F. & DOWD, M. 2001b. Underutilized tools: seaweeds as bioremediation and diversification tools and bioindicators for integrated aquaculture and coastal management. *Journal of Phycology,* 37**,** 12.

CHOULIARAS, V., TASIOULA, M., CHATZISSAVVIDIS, C., THERIOS, I. & TSABOLATIDOU, E. 2009. The effects of a seaweed extract in addition to nitrogen and boron fertilization on productivity, fruit maturation, leaf nutritional status and oil quality of the olive (Olea europaea L.) cultivar Koroneiki. *Journal of the Science of Food and Agriculture,* 89**,** 984-988.

CIAN, R., DRAGO, S., DE MEDINA, F. & MARTÍNEZ-AUGUSTIN, O. 2015. Proteins and carbohydrates from red seaweeds: evidence for beneficial effects on gut function and microbiota. *Marine drugs,* 13**,** 5358-5383.

CLARK, J. H. & DESWARTE, F. 2015. *Introduction to chemicals from biomass*, John Wiley & Sons.

CLUZET, S., TORREGROSA, C., JACQUET, C., LAFITTE, C., FOURNIER, J., MERCIER, L., SALAMAGNE, S., BRIAND, X., ESQUERRÉ -TUGAYÉ, M.
Gene expression profiling and protection of Medicago truncatula against a fungal infection in response to an elicitor from green algae Ulva spp. *Plant, Cell & Environment,* 27**,** 917-928.

COATES, J. C. 2016. Model plants for understanding evolution. *eLS*.

COATES, J. C. & CHARRIER, B. 2015. Understanding "green" multicellularity: do seaweeds hold the key? *Frontiers in plant science,* 5**,** 737.

COCK, J. M., SCORNET, D., COELHO, S., CHARRIER, B., BOYEN, C. & PETERS, A. F. 2006. 5. MARINE GENOMICS AND THE EXPLORATION OF MARINE BIODIVERSITY. *The Exploration of Marine Biodiversity Scientific and Technological Challenges***,** 117.

COCK, J. M., STERCK, L., ROUZÉ, P., SCORNET, D., ALLEN, A. E., AMOUTZIAS, G., ANTHOUARD, V., ARTIGUENAVE, F., AURY, J.-M. & BADGER, J. H. 2010. The Ectocarpus genome and the independent evolution of multicellularity in brown algae. *Nature,* 465, 617.

COENEN, C. & LOMAX, T. L. 1997. Auxin—cytokinin interactions in higher plants: old problems and new tools. *Trends in plant science,* 2**,** 351-356.

COHEN, I. & NEORI, A. 1991. Ulva lactuca biofilters for marine fishpond effluents. I. Ammonia uptake kinetics and nitrogen content. *Botanica Marina,* 34**,** 475-482.

COLE, A. J., ROBERTS, D. A., GARSIDE, A. L., DE NYS, R. & PAUL, N. A. 2016. Seaweed compost for agricultural crop production. *Journal of applied phycology,* 28**,** 629-642.

COLLÉN, J., PORCEL, B., CARRÉ, W., BALL, S. G., CHAPARRO, C., TONON, T., BARBEYRON, T., MICHEL, G., NOEL, B. & VALENTIN, K. 2013. Genome structure and metabolic features in the red seaweed *Chondrus crispus* shed light on evolution of the Archaeplastida. *Proc Natl Acad Sci USA,* 110, 5247-5252.

COMPTON, J. E., HARRISON, J. A., DENNIS, R. L., GREAVER, T. L., HILL, B. H., JORDAN, S. J., WALKER, H. & CAMPBELL, H. V. 2011. Ecosystem services altered by human changes in the nitrogen cycle: a new perspective for US decision making. *Ecology Letters,* 14**,** 804-815.

CORBINEAU, F., XIA, Q., BAILLY, C. & EL-MAAROUF-BOUTEAU, H. 2014. Ethylene, a key factor in the regulation of seed dormancy. *Frontiers in plant Science,* 5**,** 539.

COST 2014. ADVANCING KNOWLEDGE ON SEAWEED GROWTH AND DEVELOPMENT European Cooperation in the field of Scientific and Technical Research.

CRAIGIE, J. S. 2011. Seaweed extract stimuli in plant science and agriculture. *Journal of Applied Phycology,* 23**,** 371-393.

CRAIGIE, J. S., MACKINNON, S. L. & WALTER, J. A. 2008. Liquid seaweed extracts identified using 1H NMR profiles. *Journal of applied phycology,* 20**,** 665-671.

CRAMER, G. R., URANO, K., DELROT, S., PEZZOTTI, M. & SHINOZAKI, K. 2011. Effects of abiotic stress on plants: a systems biology perspective. *BMC plant biology,* 11**,** 163.

CRAWFORD, B. A., COWELL, C. T., EMDER, P. J., LEAROYD, D. L., CHUA, E. L., SINN, J. & JACK, M. M. 2010. Iodine toxicity from soy milk and seaweed ingestion is associated with serious thyroid dysfunction. *The Medical Journal of Australia,* 193**,** 413-415.

CRAY, J. A., BELL, A. N., BHAGANNA, P., MSWAKA, A. Y., TIMSON, D. J. & HALLSWORTH, J. E. 2013. The biology of habitat dominance; can microbes behave as weeds? *Microbial biotechnology,* 6**,** 453-492.

CROUCH, I., BECKETT, R. & VAN STADEN, J. 1990. Effect of seaweed concentrate on the growth and mineral nutrition of nutrient-stressed lettuce. *Journal of Applied Phycology,* 2**,** 269-272.

CROUCH, I. & VAN STADEN, J. 1992. Effect of seaweed concentrate on the establishment and yield of greenhouse tomato plants. *Journal of Applied Phycology,* 4**,** 291-296.

CROUCH, I. J. & VAN STADEN, J. 1993a. Commercial seaweed products as biostimulants in horticulture. *Journal of Home & Consumer Horticulture,* 1**,** 19-76.

CROUCH, I. J. & VAN STADEN, J. 1993b. Evidence for the presence of plant growth regulators in commercial seaweed products. *Plant growth regulation,* 13**,** 21-29.

CURTIS, T. P., SLOAN, W. T. & SCANNELL, J. W. 2002. Estimating prokaryotic diversity and its limits. *Proceedings of the National Academy of Sciences,* 99**,** 10494-10499.

DA SILVA COPERTINO, M., TORMENA, T. & SEELIGER, U. 2009. Biofiltering efficiency, uptake and assimilation rates of Ulva clathrata (Roth) J. Agardh (Clorophyceae) cultivated in shrimp aquaculture waste water. *Journal of Applied Phycology,* 21**,** 31-45.

DE CARVALHO, R. P., PASQUAL, M., DE OLIVEIRA SILVEIRA, H. R., DE MELO, P. C., BISPO, D. F. A., LAREDO, R. R., & LIMA, L. D. A. S. (2019). "Niágara Rosada" table grape cultivated with seaweed extracts: physiological, nutritional, and yielding behavior. *Journal of Applied Phycology*, 1-12.

DE FREITAS, M. B. & STADNIK, M. J. 2015. Ulvan-induced resistance in Arabidopsis thaliana against Alternaria brassicicola requires reactive oxygen species derived from NADPH oxidase. *Physiological and Molecular Plant Pathology,* 90**,** 49-56.

DE PAULA SILVA, P. H., DE NYS, R. & PAUL, N. A. 2012. Seasonal growth dynamics and resilience of the green tide alga Cladophora coelothrix in high-nutrient tropical aquaculture. *Aquaculture Environment Interactions,* 2**,** 253-266.

DE QUEIROZ, K. 2007. Species concepts and species delimitation. *Systematic biology,* 56**,** 879-886.

DE SMET, I., SIGNORA, L., BEECKMAN, T., INZÉ, D., FOYER, C. H. & ZHANG, H. 2003. An abscisic acid [illegible] in Arabidopsis thaliana lateral root development. *Plant Journal,* 33**,** 543-555.

DEL CARMEN OROZCO-MOSQUEDA, M., DEL CARMEN ROCHA-GRANADOS, M., GLICK, B. R. & SANTOYO, G. 2018. Microbiome engineering to improve biocontrol and plant growth-promoting mechanisms. *Microbiological research,* 208**,** 25-31.

DENG, W., LUO, K., LI, D., ZHENG, X., WEI, X., SMITH, W., THAMMINA, C., LU, L., LI, Y. & PEI, Y. 2006. Overexpression of an Arabidopsis magnesium transport gene, AtMGT1, in Nicotiana benthamiana confers Al tolerance. *Journal of experimental botany,* 57**,** 4235-4243.

DESA, U. 2015. World population prospects: The 2015 revision, key findings and advance tables. *Working PaperNo*.

DESMIT, X., THIEU, V., BILLEN, G., CAMPUZANO, F., DULIÈRE, V., GARNIER, J., LASSALETTA, L., MÉNESGUEN, A., NEVES, R. & PINTO, L. 2018. Reducing marine eutrophication may require a paradigmatic change. *Science of The Total Environment,* 635**,** 1444-1466.

DHARGALKAR, V. & PEREIRA, N. 2005. Seaweed: promising plant of the millennium.

DIAZ, R. J. & ROSENBERG, R. 2008. Spreading dead zones and consequences for marine ecosystems. *science,* 321**,** 926-929.

DIMKPA, C., WEINAND, T. & ASCH, F. 2009. Plant–rhizobacteria interactions alleviate abiotic stress conditions. *Plant, cell & environment,* 32**,** 1682-1694.

DOBRETSOV, S., DAHMS, H.-U. & QIAN, P.-Y. 2006. Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling,* 22**,** 43-54.

DOBRETSOV, S. V. & QIAN, P.-Y. 2002. Effect of bacteria associated with the green alga Ulva reticulata on marine micro-and macrofouling. *Biofouling,* 18**,** 217-228.

DOBZHANSKY, T. 1950. Mendelian populations and their evolution. *The American Naturalist,* 84**,** 401-418.

DOMOZYCH, D., CIANCIA, M., FANGEL, J., MIKKELSEN, M., ULVSKOV, P. & WILLATS, W. 2012. The Cell Walls of Green Algae: A Journey through Evolution and Diversity. *Frontiers in Plant Science,* 3.

DOSDAT, A., GAUMET, F. & CHARTOIS, H. 1995. Marine aquaculture effluent monitoring: methodological approach to the evaluation of nitrogen and phosphorus excretion by fish. *Aquacultural Engineering,* 14**,** 59-84.

DUARTE, M. E., CARDOSO, M. A., NOSEDA, M. D. & CEREZO, A. S. 2001. Structural studies on fucoidans from the brown seaweed Sargassum stenophyllum. *Carbohydrate Research,* 333**,** 281-293.

DUPONT, C. L., RUSCH, D. B., YOOSEPH, S., LOMBARDO, M.-J., RICHTER, R. A., VALAS, R., NOVOTNY, M., YEE-GREENBAUM, J., SELENGUT, J. D. & HAFT, D. H. 2012. Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage. *The ISME journal,* 6**,** 1186.

ECHEVARRÉA, G., ZARAUZ, N. & LÓPEZ-RUIZ, J. 1993. Study of nitrogen excretion in the gilthead seabream (*Sparus aurata L.*): influence of nutritional state. *Comp Biochem Physiol A Physiol,* 105**,** 17-19.

EGAMBERDIEVA, D. 2009. Alleviation of salt stress by plant growth regulators and IAA producing bacteria in wheat. *Acta Physiologiae Plantarum,* 31**,** 861-864.

EGAN, S., HARDER, T., BURKE, C., STEINBERG, P., KJELLEBERG, S. & THOMAS, T. 2013. The seaweed holobiont: understanding seaweed–bacteria interactions. *FEMS Microbiology Reviews*, 37**,** 462-476.

EGAN, S., THOMAS, T. & KJELLEBERG, S. 2008. Unlocking the diversity and biotechnological potential of marine surface associated microbial communities. *Current opinion in microbiology,* 11**,** 219-225.

DHARMASIRI, N., DHARMASIRI, S., & ESTELLE, M. 2005. The F-box protein TIR1 is an auxin receptor. *Nature*, 435(7041), 441.

EL-SHOWK, S., RUONALA, R. & HELARIUTTA, Y. 2013. Crossing paths: cytokinin signalling and crosstalk. *Development,* 140**,** 1373-1383.

ELLNER, S., NEORI, A., KROM, M. D., TSAI, K. & EASTERLING, M. R. 1996. Simulation model of recirculating mariculture with seaweed biofilter: development and experimental tests of the model. *Aquaculture,* 143**,** 167-184.

EPA 2003. National Environmental Monitoring Programme for Transitional, Coastal and Marine Waters Environmental Protection Agency.

FAMÀ, P., WYSOR, B., KOOISTRA, W. H. & ZUCCARELLO, G. C. 2002. Molecular phylogeny of the genus Caulerpa (Caulerpales, Chlorophyta) inferred from chloroplast tufA gene1. *Journal of phycology,* 38**,** 1040-1050.

FAO 2010. Technical Paper. No. 500/1. Rome 105 pp.

FAO 2014. The State of World Fisheries and Aquaculture 2014. Rome. 223 pp.

FAO 2016. The State of Food and Agriculture–Climate Change, Agriculture and Food Security. Food and Agriculture Organization of the United Nations Rome.

FAO 2018. The state of world fisheries and aquaculture.

FERNANDES, N., STEINBERG, P., RUSCH, D., KJELLEBERG, S. & THOMAS, T. 2012. Community structure and functional gene profile of bacteria on healthy and diseased thalli of the red seaweed Delisea pulchra. *PloS one*, 7**,** e50854.

FERNANDES, A. L. T., SILVA, R. O., SALDANHA, L., BETTINI, M. O., BROETTO, F., 2019. Effect of seaweed extract formulation on coffee plants at different irrigation levels. *Asian Academic Research Journal of Multidisciplinary*, 5.

FINLAY, J. A., KRISHNAN, S., CALLOW, M. E., CALLOW, J. A., DONG, R., ASGILL, N., WONG, K., KRAMER, E. J. & OBER, C. K. 2008. Settlement of Ulva zoospores on patterned fluorinated and PEGylated monolayer surfaces. *Langmuir,* 24**,** 503-510.

FINNIE, J. & VAN STADEN, J. 1985. Effect of seaweed concentrate and applied hormones on in vitro cultured tomato roots. *Journal of Plant Physiology,* 120**,** 215-222.

FJELD, A. & LOVLIE, A. 1976. Genetics of multicellular marine algae. *Botanical monographs*.

FLETCHER, R. 1996. The occurrence of “green tides”—a review. *Marine benthic vegetation.* Berlin, Heidelberg, 7-43: Springer.

FORNES, F., SANCHEZ-PERALES, M. & GUARDIOLA, J. 2002. Effect of a seaweed extract on the productivity of'de Nules' clementine mandarin and navelina orange. *Botanica Marina,* 45**,** 486-489.

FORT, A., LEBRAULT, M., ALLAIRE, M., ESTEVES-FERREIRA, A. A., MCHALE, M., LOPEZ, F., FARINAS~FRANCO, J. M., ALSEEKH, S., FERNIE, A. R. & SULPICE, R.

2019. Extensive variations in diurnal growth patterns and metabolism amongst Ulva spp strains. *Plant Physiology***,** pp.01513.2018.

FØYN, B. 1958. Über die Sexualität und den Generationswechsel von Ulva mutabilis. *Arch. Protistenkd,* 102**,** 473-480.

FRASCHETTI, S., TERLIZZI, A., BEVILACQUA, S. & BOERO, F. 2006. The distribution of hydroids (Cnidaria, Hydrozoa) from micro-to macro-scale: spatial patterns on habitat-forming algae. *Journal of Experimental Marine Biology and Ecology,* 339**,** 148-158.

FRIEDRICH, M. W. 2012. Bacterial communities on macroalgae. *Seaweed biology.* Springer.

FRIES, L. 1975. Some observations on the morphology of Enteromorpha linza (L.) J. Ag. and Enteromorpha compressa (L.) Grev. in axenic culture. . *Bot Mar* 18**,** 251-253.

FU, J., SUN, X., WANG, J., CHU, J. & YAN, C. 2011. Progress in quantitative analysis of plant hormones. *Chinese Science Bulletin,* 56**,** 355-366.

FUNAHASHI, H., IMAI, T., MASE, T., SEKIYA, M., YOKOI, K., HAYASHI, H., SHIBATA, A., HAYASHI, T., NISHIKAWA, M. & SUDA, N. 2001. Seaweed prevents breast cancer? *Japanese journal of cancer research,* 92**,** 483-487.

GACHON, C. M., STRITTMATTER, M., BADIS, Y., FLETCHER, K. I., WEST, P. V. & MÜLLER, D. G. 2017. Pathogens of brown algae: culture studies of *Anisolpidium ectocarpii* and *A. rosenvingei* reveal that the Anisolpidiales are uniflagellated oomycetes. *European Journal of Phycology,* 52**,** 133-148.

GALLIE, D. R. 2015. Ethylene receptors in plants-why so much complexity? *F1000prime reports,* 7.

GALLOWAY, J. N. 1998. The global nitrogen cycle: changes and consequences. *Environmental pollution,* 102**,** 15-24.

GAMBLE, R. L., COONFIELD, M. L., & SCHALLER, G. E. 1998. Histidine kinase activity of the ETR1 ethylene receptor from Arabidopsis. *Proceedings of the National Academy of Sciences,* 95, 7825-7829.

GAO, G. 2016. Developing systems for the commercial culture of Ulva species in the UK.

GAO, G., CLARE, A. S., ROSE, C. & CALDWELL, G. S. 2017a. Eutrophication and warming-driven green tides (Ulva rigida) are predicted to increase under future climate change scenarios. *Marine pollution bulletin,* 114**,** 439-447.

GAO, G., CLARE, A. S., ROSE, C. & CALDWELL, G. S. 2017b. Reproductive sterility increases thecapacity to exploit the green seaweed *Ulva rigida* for commercial applications. *Algal Res,* 24, 64-71.

GAO, S., CHEN, X., YI, Q., WANG, G., PAN, G., LIN, A. & PENG, G. 2010. A strategy for the proliferation of Ulva prolifera, main causative species of green tides, with formation of sporangia by fragmentation. *PLoS One,* 5**,** e8571.

GAO, X., REN, F. & LU, Y.-T. 2006. The Arabidopsis mutant stg1 identifies a function for TBP-associated factor 10 in plant osmotic stress adaptation. *Plant and cell physiology,* 47**,** 1285-1294.

GAZZARRINI, S., LEJAY, L., GOJON, A., NINNEMANN, O., FROMMER, W. B. & VON WIRÉN, N. 1999. Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots. *The Plant Cell,* 11**,** 937-947.

GHADERIARDAKANI, F., COATES, J. C. & WICHARD, T. 2017. Bacteria-induced morphogenesis of Ulva intestinalis and Ulva mutabilis (Chlorophyta): a contribution to the lottery theory. *FEMS microbiology ecology,* 93.

GHADERIARDAKANI, F., COLLAS, E., DAMIANO, D. K., TAGG, K., COATES, J. C. 2019a. Effects of green seaweed extract on *Arabidopsis* early development suggest roles for hormone signalling in plant responses to algal fertilisers. *Scientific reports* 9(1), 1983.

GHADERIARDAKANI, F., Califano, G., Mohr, J. F., Abreu, M. H., Coates, J. C. and Wichard, T. 2019b. Analysis of algal growth-and morphogenesis-promoting factors in an integrated multi-trophic aquaculture system for farming Ulva spp. *Aquaculture Environment Interactions*, 11, 375-391.

GOECKE, F., LABES, A., WIESE, J. & IMHOFF, J. F. 2010. Chemical interactions between marine macroalgae and bacteria. *Marine Ecology Progress Series,* 409**,** 267-299.

GOLDBERG, I. & HASLER, C. 1996. Functional Foods--Designer Foods, Pharmafoods, Nutraceuticals. *American Journal of Clinical Nutrition,* 64**,** 255-255.

GOTO, F., YOSHIHARA, T., SHIGEMOTO, N., TOKI, S. & TAKAIWA, F. 1999. Iron fortification of rice seed by the soybean ferritin gene. *Nature biotechnology,* 17**,** 282-286.

GOUY, M., GUINDON, S. & GASCUEL, O. 2009. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular biology and evolution,* 27**,** 221-224.

GRAHAM, L. E. & WILCOX, L. W. 2000. Algae. Upper Saddler River. *NJ: Prentice Hall,* 25**,** 29.

GRIESS, P. 1879. Bemerkungen zu der abhandlung der hh. Weselsky und benedikt „ueber einige azoverbindungen" . *Berichte der deutschen chemischen Gesellschaft,* 12**,** 426-428.

GRUENEBERG, J., ENGELEN, A. H., COSTA, R. & WICHARD, T. 2016. Macroalgal morphogenesis induced by waterborne compounds and bacteria in coastal seawater. *PloS one,* 11**,** e0146307.

GUIRY, M. & GUIRY, G. 2014. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway.

GUO, H., & ECKER, J. R. 2003. Plant responses to ethylene gas are mediated by SCFEBF1/EBF2-dependent proteolysis of EIN3 transcription factor. *Cell,* 115, 667-677.

GUPTA, V., BIJO, A., KUMAR, M., REDDY, C. & JHA, B. 2012. Detection of epigenetic variations in the protoplast-derived germlings of Ulva reticulata using methylation sensitive amplification polymorphism (MSAP). *Marine biotechnology,* 14**,** 692-700.

GUPTA, V., & KUSHWAHA, H. R. 2017. Metabolic regulatory oscillations in intertidal green seaweed Ulva lactuca against tidal cycles. *Scientific reports,* 7, 16430.

GUTTMAN, L., BOXMAN, S. E., BARKAN, R., NEORI, A. & SHPIGEL, M. 2018. Combinations of Ulva and periphyton as biofilters for both ammonia and nitrate in mariculture fishpond effluents. *Algal Research-Biomass Biofuels and Bioproducts,* 34**,** 235-243.

GUZMAN, P. & ECKER, J. R. 1990. Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *The Plant Cell Online,* 2**,** 513-523.

HAAS, A. & ROSE, I. 1982. The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis. *Journal of Biological Chemistry,* 257**,** 10329-10337.

HALL, B. P., SHAKEEL, S. N., AMIR, M., HAQ, N. U., QU, X., & SCHALLER, G. E. 2012. Histidine kinase activity of the ethylene receptor ETR1 facilitates the ethylene response in

Arabidopsis. *Plant Physiology*, 159, 682-695.HANSEN, H., KOROLEFF, F., GRASSHOFF, K., KREMLING, K. & EHRHARDT, M. 1999. Methods of seawater analysis. *Grasshoff, K., Kremling, K., Ehrhardt, M. Wiley-VCH, Germany*.

HASEGAWA, P. M., BRESSAN, R. A., ZHU, J.-K. & BOHNERT, H. J. 2000. Plant cellular and molecular responses to high salinity. *Annual review of plant biology,* 51**,** 463-499.

HAUSER, F., WAADT, R. & SCHROEDER, J. I. 2011. Evolution of abscisic acid synthesis and signaling mechanisms. *Current Biology,* 21**,** R346-R355.

HAYASHI, M. & NISHIMURA, M. 2006. Arabidopsis thaliana—a model organism to study plant peroxisomes. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1763**,** 1382-1391.

HAYDEN, H. S., BLOMSTER, J., MAGGS, C. A., SILVA, P. C., STANHOPE, M. J. & WAALAND, J. R. 2003. Linnaeus was right all along: Ulva and Enteromorpha are not distinct genera. *European journal of phycology,* 38**,** 277-294.

HEESCH, S., BROOM, J. E., NEILL, K. F., FARR, T. J., DALEN, J. L. & NELSON, W. A. 2009. Ulva, Umbraulva and Gemina: genetic survey of New Zealand taxa reveals diversity and introduced species. *European Journal of Phycology,* 44**,** 143-154.

HENDERSON, J. 2004. *The Roman book of gardening,* Psychology Press.

HERNÁNDEZ-HERRERA, R. M., SANTACRUZ-RUVALCABA, F., RUIZ-LÓPEZ, M. A., NORRIE, J. & HERNÁNDEZ-CARMONA, G. 2014. Effect of liquid seaweed extracts on growth of tomato seedlings (Solanum lycopersicum L.). *Journal of applied phycology,* 26**,** 619-628.

HERNÁNDEZ, I., MARTÍNEZ-ARAGÓN, J., TOVAR, A., PÉREZ-LLORÉNS, J. & VERGARA, J. 2002. Biofiltering efficiency in removal of dissolved nutrients by three species of estuarine macroalgae cultivated with sea bass (Dicentrarchus labrax) waste waters 2. Ammonium. *Journal of Applied Phycology,* 14**,** 375-384.

HERRE, E. A., KNOWLTON, N., MUELLER, U. G. & REHNER, S. A. 1999. The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution,* 14**,** 49-53.

HIRSCH, R. E., LEWIS, B. D., SPALDING, E. P. & SUSSMAN, M. R. 1998. A role for the AKT1 potassium channel in plant nutrition. *Science,* 280**,** 918-921.

HOCHHOLDINGER, F. & ZIMMERMANN, R. 2008. Conserved and diverse mechanisms in root development. *Current opinion in plant biology,* 11**,** 70-74.

HOLDT, S., WERNERB, A., DRINGB, M., MØHLENBERGA, F. & PEDERSENC, P. 2011. Nutrient uptake and growth of Irish Moss (Chondrus crispus, Rhodophyta) in dilute fish effluent.

HOLDT, S. L. & KRAAN, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. *Journal of applied phycology*, 23**,** 543-597.

HOLLANTS, J., LELIAERT, F., CLERCK, O. & WILLEMS, A. 2013. What we can learn from sushi: a review on seaweed–bacterial associations. *FEMS microbiology ecology*, 83**,** 1-16.

HONG, D. D., HIEN, H. M. & SON, P. N. 2007. Seaweeds from Vietnam used for functional food, medicine and biofertilizer. *Journal of Applied Phycology*, 19**,** 817-826.

HOXMARK, R. 1975. Experimental analysis of the life cycle of Ulva mutabilis. *Botanica Marina,* 18**,** 123-129.

HURTADO, A. Q., CRITCHLEY, A. T., TRESPOEY, A., & LHONNEUR, G. B. 2006. Occurrence of Polysiphonia epiphytes in Kappaphycus farms at Calaguas Is., Camarines Norte, Phillippines. *Journal of Applied Phycology*, 18, 301-306.

HUSSAIN, A. & BONEY, A. 1969. Isolation of kinin-like substances from Laminaria digitata. *Nature,* 223**,** 504.

HUSSAIN, A. & BONEY, A. 1973. Hydrophilic growth inhibitors from Laminaria and Ascophyllum. *New Phytologist,* 72**,** 403-410.

IRISH, V. F. & BENFEY, P. N. 2004. Beyond Arabidopsis. Translational biology meets evolutionary developmental biology. *Plant physiology,* 135**,** 611-614.

JAHAN, P., WATANABE, T., KIRON, V. & SATOH, S. 2003. Improved carp diets based on plant protein sources reduceenvironmental phosphorus loading. *Fisheries Science,* 69**,** 219-225.

JAULNEAU, V., LAFITTE, C., JACQUET, C., FOURNIER, S., SALAMAGNE, S., BRIAND, X., ESQUERRÉ-TUGAYÉ, M.-T. & DUMAS, B. 2010. Ulvan, a sulfated polysaccharide from green algae, activates plant immunity through the jasmonic acid signaling pathway. *BioMed Research International,* 2010.

JAYARAJ, J., WAN, A., RAHMAN, M. & PUNJA, Z. 2008. Seaweed extract reduces foliar fungal diseases on carrot. *Crop Protection,* 27**,** 1360-1366.

JOB, D. 2002. Plant biotechnology in agriculture. *Biochimie,* 84**,** 1105-1110.

JOHNSON, M., ZARETSKAYA, I., RAYTSELIS, Y., MEREZHUK, Y., MCGINNIS, S., & MADDEN, T. L. (2008). NCBI BLAST: a better web interface. Nucleic acids research, 36(suppl_2), W5-W9.

JOINT, I., CALLOW, M. E., CALLOW, J. A. & CLARKE, K. R. 2000. The attachment of Enteromorpha zoospores to a bacterial biofilm assemblage.

KALAIVANAN, C. & VENKATESALU, V. 2012. Utilization of seaweed Sargassum myriocystum extracts as a stimulant of seedlings of Vigna mungo (L.) Hepper. *Spanish Journal of Agricultural Research,* 10**,** 466-470.

KAPRAUN, D. F. 1970. Field and cultural studies of Ulva and Enteromorpha in the vicinity of Port Aransas, Texas. *Contr. Mar. Sci.,* 15**,** 205-285.

KATEKAR, G. F., & GEISSLER, A. E. (1977). Auxin transport inhibitors. II. 2-(1-pyrenol) benzoic acid, a potent inhibitor of polar auxin transport. *Functional Plant Biology,* 4, 321-325.

KATSAROS, C., WEISS, A., LLANGOS, I., THEODOROU, I. & WICHARD, T. 2017. Cell structure and microtubule organisation during gametogenesis of Ulva mutabilis Føyn (Chlorophyta). *Botanica Marina,* 60**,** 123-135.

KAZAN, K. 2013. Auxin and the integration of environmental signals into plant root development. *Annals of botany,* 112**,** 1655-1665.

KEELING, P. J. 2010. The endosymbiotic origin, diversification and fate of plastids. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 365**,** 729-748.

KELLY, M. & DWORJANYN, S. 2008. The potential of marine biomass for anaerobic biogas production: a feasibility study with recommendations for further research. *Scotland: Scottish Association for Marine Science*.

KENRICK, P. & CRANE, P. R. 1997. The origin and early evolution of plants on land. *Nature,* 389**,** 33.

KEPINSKI, S., & LEYSER, O. 2005. The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature*, 435, 446.

KHAN, W., RAYIRATH, U. P., SUBRAMANIAN, S., JITHESH, M. N., RAYORATH, P., HODGES, D. M., CRITCHLEY, A. T., CRAIGIE, J. S., NORRIE, J. & PRITHIVIRAJ, B. 2009. Seaweed extracts as biostimulants of plant growth and development. *Journal of Plant Growth Regulation,* 28**,** 386-399.

KHUSH, G. S. 2012. Genetically modified crops: The fastest adopted crop technology in the history of modern agriculture. *Agriculture & Food Security,* 1**,** 14.

KINGMAN, A. & MOORE, J. 1982. Isolation, purification and quantitation of several growth regulating substances in Ascophyllum nodosum (Phaeophyta). *Botanica marina,* 25**,** 149-154.

KINLAN, B. P., GRAHAM, M. H., SALA, E. & DAYTON, P. K. 2003. ARRESTED DEVELOPMENT OF GIANT KELP (MACROCYSTIS PYRIFERA, PHAEOPHYCEAE) EMBRYONIC SPOROPHYTES: A MECHANISM FOR DELAYED RECRUITMENT IN PERENNIAL KELPS? 1. *Journal of Phycology,* 39**,** 47-57.

KIT, O. 2014. Turn down the heat: confronting the new climate normal.

KOORNNEEF, M., JORNA, M., BRINKHORST-VAN DER SWAN, D. & KARSSEN, C. 1982. The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of Arabidopsis thaliana (L.) Heynh. *Theoretical and Applied Genetics,* 61**,** 385-393.

KOORNNEEF, M. & MEINKE, D. 2010. The development of Arabidopsis as a model plant. *The Plant Journal,* 61**,** 909-921.

KOSTAMO, K., BLOMSTER, J., KORPELAINEN, H., KELLY, J., MAGGS, C. A. & MINEUR, F. 2008. New microsatellite markers for Ulva intestinalis (Chlorophyta) and the transferability of markers across species of Ulvaceae. *Phycologia,* 47**,** 580-587.

KRAAN, S. 2013. Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. *Mitigation and Adaptation Strategies for Global Change,* 18**,** 27-46.

KROUK, G., RUFFEL, S., GUTIÉRREZ, R. A., GOJON, A., CRAWFORD, N. M., CORUZZI, G. M. & LACOMBE, B. 2011. A framework integrating plant growth with hormones and nutrients. *Trends in plant science,* 16**,** 178-182.

KULAEVA, O. & PROKOPTSEVA, O. 2004. Recent advances in the study of mechanisms of action of phytohormones. *Biochemistry (Moscow),* 69**,** 233-247.

KUMAR, N. A., VANLALZARZOVA, B., SRIDHAR, S. & BALUSWAMI, M. 2012. Effect of liquid seaweed fertilizer of Sargassum wightii Grev. on the growth and biochemical content of green gram (Vigna radiata (L.) R. Wilczek). *Recent Research in Science and Technology,* 4.

KUMAR, V., ZOZAYA -VALLEJOS, M., KJELLEBERG, S. 2016. Multiple opportunistic pathogens can cause a bleaching disease in the red seaweed Delisea pulchra. *Environmental microbiology,* 18**,** 3962-3975.

KUMARI, R., KAUR, I. & BHATNAGAR, A. 2011. Effect of aqueous extract of Sargassum johnstonii Setchell & Gardner on growth, yield and quality of Lycopersicon esculentum Mill. *Journal of Applied Phycology,* 23**,** 623-633.

KWON, H. K., KANG, H., OH, Y. H., PARK, S. R. & KIM, G. 2017. Green tide development associated with submarine groundwater discharge in a coastal harbor, Jeju, Korea. *Scientific reports,* 7**,** 6325.

LACHNIT, T., BLÜMEL, M., IMHOFF, J. F. & WAHL, M. 2009. Specific epibacterial communities on macroalgae: phylogeny matters more than habitat. *Aquatic Biology,* 5**,** 181-186.

LACHNIT, T., MESKE, D., WAHL, M., HARDER, T. & SCHMITZ, R. 2011. Epibacterial community patterns on marine macroalgae are host -specific but t *Environmental microbiology,* 13**,** 655-665.

LAFFOLEY, D. D. A., ROE, H., ANGEL, M., ARDRON, J., BATES, N., BOYD, I., BROOKE, S., BUCK, K., CARLSON, C. & CAUSEY, B. 2011. The Protection and Management of the Sargasso Sea. Sargasso Sea Alliance.

LAHAYE, M. & RAY, B. 1996. Cell-wall polysaccharides from the marine green alga Ulva "rigida"(Ulvales, Chlorophyta)—NMR analysis of ulvan oligosaccharides. *Carbohydrate research,* 283**,** 161-173.

LAHAYE, M. & ROBIC, A. 2007. Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules,* 8**,** 1765-1774.

LARGO, D. B., SEMBRANO, J., HIRAOKA, M. & OHNO, M. 2004. Taxonomic and ecological profile ofgreen tide'species of Ulva (Ulvales, Chlorophyta) in central Philippines. *Hydrobiologia,* 512**,** 247-253.

LATEF, A. A. H. A., SRIVASTAVA, A. K., SABER, H., ALWALEED, E. A. & TRAN, L.-S. P. 2017. Sargassum muticum and Jania rubens regulate amino acid metabolism to improve growth and alleviate salinity in chickpea. *Scientific Reports,* 7**,** 10537.

LAWTON, R. J., MATA, L., DE NYS, R. & PAUL, N. A. 2013. Algal bioremediation of waste waters from land-based aquaculture using Ulva: selecting target species and strains. *PLoS One,* 8**,** e77344.

LAZZARI, R. & BALDISSEROTTO, B. 2008. Nitrogen and phosphorus waste in fish farming. *Boletim do Instituto de Pesca,* 34**,** 591-600.

LEE, W.-Y. & WANG, W.-X. 2001. Metal accumulation in the green macroalga Ulva fasciata: effects of nitrate, ammonium and phosphate. *Science of the total environment,* 278**,** 11-22.

LELIAERT, F., SMITH, D. R., MOREAU, H., HERRON, M. D., VERBRUGGEN, H., DELWICHE, C. F. & DE CLERCK, O. 2012. Phylogeny and molecular evolution of the green algae. *Critical Reviews in Plant Sciences,* 31**,** 1-46.

LEUNG, J., BOUVIER-DURAND, M., MORRIS, P.-C., GUERRIER, D., CHEFDOR, F. & GIRAUDAT, J. 1994. Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. *Science-AAAS-Weekly Paper Edition-including Guide to Scientific Information,* 264**,** 1448-1451.

LEUNG, J., MERLOT, S., & GIRAUDAT, J. (1997). The *Arabidopsis* ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *The Plant Cell*, 9(5), 759-771.LYNCH, J. 1995. Root architecture and plant productivity. *Plant physiology,* 109**,** 7-13.

LEYSER, H. O., LINCOLN, C. A., TIMPTE, C., LAMMER, D., TURNER, J. & ESTELLE, M. 1993. Arabidopsis auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature,* 364**,** 161.

LI, H.-T., LIU, H., GAO, X.-S. & ZHANG, H. 2009. Knock-out of Arabidopsis AtNHX4 gene enhances tolerance to salt stress. *Biochemical and biophysical research communications,* 382**,** 637-641.

LI, Q., LI, B. H., KRONZUCKER, H. J. & SHI, W. M. 2010a. Root growth inhibition by NH4+ in Arabidopsis is mediated by the root tip and is linked to NH4+ efflux and GMPase activity. *Plant, Cell & Environment,* 33**,** 1529-1542.

LI, X.-J., YANG, M.-F., CHEN, H., QU, L.-Q., CHEN, F. & SHEN, S.-H. 2010b. Abscisic acid pretreatment enhances salt tolerance of rice seedlings: proteomic evidence. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics,* 1804**,** 929-940.

LI, X., CHEN, L., FORDE, B. G. & DAVIES, W. J. 2017. The Biphasic Root Growth Response to Abscisic Acid in Arabidopsis Involves Interaction with Ethylene and Auxin Signalling Pathways. *Frontiers in Plant Science,* 8**,** 1493.

LILLEY, S. A. & SCHIEL, D. R. 2006. Community effects following the deletion of a habitat-forming alga from rocky marine shores. *Oecologia,* 148**,** 672-681.

LINCOLN, C., BRITTON, J. H. & ESTELLE, M. 1990. Growth and development of the axr1 mutants of Arabidopsis. *The Plant Cell Online,* 2**,** 1071-1080.

LINK, H. 1820. Epistola de algis aquaticis in genera disponendis. *Horae physicae berolinenses,* 1**,** pl. 1.

LINKOHR, B. I., WILLIAMSON, L. C., FITTER, A. H. & LEYSER, H. 2002. Nitrate and phosphate availability and distribution have different effects on root system architecture of Arabidopsis. *The Plant Journal,* 29**,** 751-760.

LIU, D., KEESING, J. K., HE, P., WANG, Z., SHI, Y. & WANG, Y. 2013. The world's largest macroalgal bloom in the Yellow Sea, China: formation and implications. *Estuarine, Coastal and Shelf Science,* 129**,** 2-10.

LOBBAN, C. & HARRISON, P. 1995. Seaweed ecology and physiology. *Oceanographic Literature Review,* 9**,** 774.

LOLA-LUZ, T., HENNEQUART, F. & GAFFNEY, M. 2013. Enhancement of phenolic and flavonoid compounds in cabbage (Brassica oleraceae) following application of commercial seaweed extracts of the brown seaweed,(Ascophyllum nodosum). *Agricultural and Food Science,* 22**,** 288-295.

LOLA-LUZ, T., HENNEQUART, F. & GAFFNEY, M. 2014. Effect on health promoting phytochemicals following seaweed application, in potato and onion crops grown under a low input agricultural system. *Scientia Horticulturae,* 170**,** 224-227.

LONGFORD, S. R., TUJULA, N. A., CROCETTI, G. R., HOLMES, A. J., HOLMSTRÖM, C., KJELLEBERG, S., STEINBERG, P. D. & TAYLOR, M. W. 2007. Comparisons of diversity of bacterial communities associated with three sessile marine eukaryotes. *Aquatic Microbial Ecology,* 48**,** 217-229.

LOQUÉ, D., YUAN, L., KOJIMA, S., GOJON, A., WIRTH, J., GAZZARRINI, S., ISHIYAMA, K., TAKAHASHI, H. & VON WIRÉN, N. 2006. Additive contribution of AMT1; 1 and AMT1; 3 to high -affinity ammon
deficient Arabidopsis roots. *The Plant Journal,* 48**,** 522-534.

LÖVLIE, A. 1963. Genetic control of division rate and morphogenesis in Ulva mutabilis Føyn. *Comptes-rendus des travaux du Laboratoire Carlsberg,* 34**,** 77-168.

LØVLIE, A. 1964. Genetic control of division rate and morphogenesis in *Ulva mutabilis* Føyn. *C. R. Trav. Lab. Carlsberg* 34**,** 77-168.

LØVLIE, A. & BRYHNI, E. 1978. On the relation between sexual and parthenogenetic reproduction in haplo-diplontic algae. *Botanica Marina,* 21**,** 155-164.

MACDONALD, H. 1997. Auxin perception and signal transduction. *Physiologia Plantarum,* 100**,** 423-430.

MADHUSUDAN, C., MANOJ, S., RAHUL, K. & RISHI, C. M. 2011. Seaweeds: A diet with nutritional, medicinal and industrial value. *Research Journal of Medicinal Plant,* 5**,** 153-157.

MAGGS, C. A. & CALLOW, M. E. 2003. Algal spores. *eLS*.

MAIRHOFER, S., PRIDMORE, T., JOHNSON, J., WELLS, D. M., BENNETT, M. J., MOONEY, S. J. & STURROCK, C. J. 2017. X -Ray Compute
Systems Grown in Soil. *Current Protocols in Plant Biology,* 2**,** 270-286.

MANCUSO, S., AZZARELLO, E., MUGNAI, S. & BRIAND, X. 2006. Marine bioactive substances (IPA extract) improve foliar ion uptake and water stress tolerance in potted Vitis vinifera plants. *Advances in Horticultural Science,* 156-161.

MARIYA, V. & RAVINDRAN, V. S. 2013. Biomedical and Pharmacological significance of marine macro algae-review.

MARSHALL, K., JOINT, I., CALLOW, M. E. & CALLOW, J. A. 2006. Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. *Microbial ecology,* 52**,** 302-310.

MARSHALL, K. A. 2004. *The influence of periphytic bacteria on growth and morphology of Ulva linza (syn. Enteromorpha linza).* University of Birmingham.

MARTÍNEZ-ARAGÓN, J., HERNÁNDEZ, I., PÉREZ-LLORÉNS, J., VÁZQUEZ, R. & VERGARA, J. 2002. Biofiltering efficiency in removal of dissolved nutrients by three species of estuarine macroalgae cultivated with sea bass (Dicentrarchus labrax) waste waters 1. Phosphate. *Journal of Applied Phycology,* 14**,** 365-374.

MATSUO, Y., IMAGAWA, H., NISHIZAWA, M. & SHIZURI, Y. 2005. Isolation of an algal morphogenesis inducer from a marine bacterium. *Science,* 307**,** 1598-1598.

MAYDEN, R. L. 1997. A hierarchy of species concepts: the denouement in the saga of the species problem.

MAYR, E. 1999. *Systematics and the origin of species, from the viewpoint of a zoologist*, Harvard University Press.

MCGUIRE, S. 2015. FAO, IFAD, and WFP. The state of food insecurity in the World 2015: Meeting the 2015 international hunger targets: taking stock of uneven progress. Rome: FAO, 2015. Oxford University Press.

MEDRANO, H., FLEXAS, J. & GALMÉS, J. 2009. Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. *Plant and Soil,* 317**,** 17-29.

MÉNESGUEN, A. & PIRIOU, J.-Y. 1995. Nitrogen loadings and macroalgal (Ulva sp.) mass accumulation in Brittany (France). *Ophelia,* 42**,** 227-237.

MEYER, K., LEUBE, M. P. & GRILL, E. 1994. A protein phosphatase 2C involved in ABA signal transduction in Arabidopsis thaliana. *Science-AAAS-Weekly Paper Edition-including Guide to Scientific Information,* 264**,** 1452-1454.

MEYEROWITZ, E. M. & PRUITT, R. E. 1985. Arabidopsis thaliana and plant molecular genetics. *Science,* 229**,** 1214-1218.

MHLW 2014. The National Health and Nutrition Survey in Japan, 2004–2014. .

MIYAZONO, K. I., MIYAKAWA, T., SAWANO, Y., KUBOTA, K., KANG, H. J., ASANO, A., ... & YOSHIDA, T. (2009). Structural basis of abscisic acid signalling. *Nature*, 462(7273), 609.

MILLEDGE, J. & HARVEY, P. 2016. Golden Tides: Problem or golden opportunity? The valorisation of Sargassum from beach inundations. *Journal of Marine Science and Engineering,* 4**,** 60.

MILTON, R. 1952. Improvements in or relating to horticultural and agricultural fertilizers. *British Patent,* 664989.

MITCHELL, R. 1963. Addition of fungal cell-wall components to soil for biological disease control. *Phytopathology,* 53**,** 1068-1071.

MOODY, L. A., SAIDI, Y., GIBBS, D. J., CHOUDHARY, A., HOLLOWAY, D., VESTY, E. F., BANSAL, K. K., BRADSHAW, S. J. & COATES, J. C. 2016. An ancient and conserved function for Armadillo -related protein abscisic acid. *New Phytologist,* 211**,** 940-951.

MOORE, K. K. 2004. Using Seaweed Compost To Grow Bedding Plants. *BioCycle,* 45.

MORAND, P., CARPENTIER, B., CHARLIER, R. H., MAZÉ, J., ORLANDINI, M., PLUNKETT, B. A. & DE WAART, J. 1991. Bioconversion of seaweeds. *Seaweed Resources in Europe: Uses and Potential. John Wiley & Sons, Chichester***,** 95-148.

MORRIS, R. M., RAPPÉ, M. S., CONNON, S. A., VERGIN, K. L., SIEBOLD, W. A., CARLSON, C. A. & GIOVANNONI, S. J. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. *Nature,* 420**,** 806.

MSUYA, F. E. & NEORI, A. 2008. Effect of water aeration and nutrient load level on biomass yield, N uptake and protein content of the seaweed Ulva lactuca cultured in seawater tanks. *Journal of Applied Phycology,* 20**,** 1021-1031.

MURPHY, J. & RILEY, J. P. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica chimica acta,* 27**,** 31-36.

MÜSSIG, K. 2009. Iodine-induced toxic effects due to seaweed consumption. *Comprehensive handbook of iodine***,** 897-908.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and environmental microbiology,* 59**,** 695-700.

NABATI, D., SCHMIDT, R. & PARRISH, D. 1994. Alleviation of salinity stress in Kentucky bluegrass by plant growth regulators and iron. *Crop science,* 34**,** 198-202.

NAKAMURA, Y., SASAKI, N., KOBAYASHI, M., OJIMA, N., YASUIKE, M., SHIGENOBU, Y., SATOMI, M., FUKUMA, Y., SHIWAKU, K. & TSUJIMOTO, A. 2013. The first symbiont-free genome sequence of marine red alga, Susabi-nori (Pyropia yezoensis). PloS one, 8, e57122.

NAKANISHI, K., NISHIJIMA, M., NISHIMURA, M., KUWANO, K. & SAGA, N. 1996. Bacteria that induce morphogenesis in Ulva pertusa (Chlorophyta) grown under axenic conditions. *Journal of Phycology,* 32**,** 479-482.

NAYLOR, R. L., GOLDBURG, R. J., PRIMAVERA, J. H., KAUTSKY, N., BEVERIDGE, M. C., CLAY, J., FOLKE, C., LUBCHENCO, J., MOONEY, H. & TROELL, M. 2000. Effect of aquaculture on world fish supplies. *Nature,* 405**,** 1017.

NEORI, A., CHOPIN, T., TROELL, M., BUSCHMANN, A. H., KRAEMER, G. P., HALLING, C., SHPIGEL, M. & YARISH, C. 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. *Aquaculture,* 231**,** 361-391.

NEORI, A., KROM, M. D., ELLNER, S. P., BOYD, C. E., POPPER, D., RABINOVITCH, R., DAVISON, P. J., DVIR, O., ZUBER, D. & UCKO, M. 1996. Seaweed biofilters as regulators of water quality in integrated fish-seaweed culture units. *Aquaculture,* 141**,** 183-199.

NEORI, A., MSUYA, F. E., SHAULI, L., SCHUENHOFF, A., KOPEL, F. & SHPIGEL, M. 2003. A novel three-stage seaweed (Ulva lactuca) biofilter design for integrated mariculture. *Journal of Applied Phycology,* 15**,** 543-553.

NIKOLAISEN, L., DAUGBJERG JENSEN, P., SVANE BECH, K., DAHL, J., BUSK, J., BRØDSGAARD, T., RASMUSSEN, M. B., BRUHN, A., BJERRE, A.-B. & BANGSØ NIELSEN, H. 2011. Energy production from marine biomass (Ulva lactuca). Danish Technological Institute.

NIXON, S. W. 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. *Ophelia,* 41**,** 199-219.

NOBRE, A., ROBERTSON-ANDERSSON, D., NEORI, A. & SANKAR, K. 2010. Ecological–economic assessment of aquaculture options: comparison between abalone monoculture and integrated multi-trophic aquaculture of abalone and seaweeds. *Aquaculture,* 306**,** 116-126.

NOTOYA, M. 2010. Production of biofuel by macroalgae with preservation of marine resources and environment. *Seaweeds and their role in globally changing environments.* Springer.

OERTEL, W., WICHARD, T. & WEISSGERBER, A. 2015. Transformation of Ulva mutabilis (Chlorophyta) by vector plasmids integrating into the genome. *Journal of phycology,* 51**,** 963-979.

OLSEN, L. M., HOLMER, M. & OLSEN, Y. 2008. Perspectives of nutrient emission from fish aquaculture in coastal waters. *Literature review with evaluated state of knowledge. FHF project,* 542014**,** 87.

PAINTER, T. J. 1983. Algal polysaccharides. *The polysaccharides.* Elsevier.

PANDA, S. K., BALUŠKA, F. & MATSUMOTO, H. 2009. Aluminum stress signaling in plants. *Plant signaling & behavior,* 4**,** 592-597.

PARINOV, S. & SUNDARESAN, V. 2000. Functional genomics in Arabidopsis: large-scale insertional mutagenesis complements the genome sequencing project. *Current Opinion in Biotechnology,* 11**,** 157-161.

PATEY, M. D., RIJKENBERG, M. J., STATHAM, P. J., STINCHCOMBE, M. C., ACHTERBERG, E. P. & MOWLEM, M. 2008. Determination of nitrate and phosphate in seawater at nanomolar concentrations. *TrAC Trends in Analytical Chemistry,* 27**,** 169-182.

PATIER, P., YVIN, J.-C., KLOAREG, B., LIÉNART, Y. & ROCHAS, C. 1993. Seaweed liquid fertilizer fromAscophyllum nodosum contains elicitors of plant d-glycanases. *Journal of applied phycology,* 5**,** 343-349.

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

PETERS, A. F., MARIE, D., SCORNET, D., KLOAREG, B. & MARK COCK, J. 2004. PROPOSAL OF ECTOCARPUS SILICULOSUS (ECTOCARPALES,

PHAEOPHYCEAE) AS A MODEL ORGANISM FOR BROWN ALGAL GENETICS AND GENOMICS 1, 2. *Journal of Phycology,* 40**,** 1079-1088.

PHILLIPS, J. 1990. Life history studies of Ulva rigida C. Ag. and Ulva stenophylla S. et G.(Ulvaceae, Chlorophyta) in Southern Australia. *Botanica marina,* 33**,** 79-84.

POSTGATE, J. R. 1982. *The fundamentals of nitrogen fixation*, CUP Archive.

PRETTY, J. 2008. Agricultural sustainability: concepts, principles and evidence. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 363**,** 447-465.

PROVASOLI, L. & PINTNER, I. J. 1980. Bacteria induced polymorphism in an axenic laboratory strain of Ulva lactuca (Chlorophyceae). *Journal of Phycology,* 16**,** 196-201.

QIN, F., SHINOZAKI, K. & YAMAGUCHI-SHINOZAKI, K. 2011. Achievements and challenges in understanding plant abiotic stress responses and tolerance. *Plant and Cell Physiology,* 52**,** 1569-1582.

RABALAIS, N. N., TURNER, R. E., DIAZ, R. J. & JUSTIĆ, D. 2009. Global change and eutrophication of coastal waters. *ICES Journal of Marine Science,* 66**,** 1528-1537.

RAMANAN, R., KIM, B.-H., CHO, D.-H., OH, H.-M. & KIM, H.-S. 2016. Algae–bacteria interactions: evolution, ecology and emerging applications. *Biotechnology advances,* 34**,** 14-29.

RAO, D., WEBB, J. S., HOLMSTRÖM, C., CASE, R., LOW, A., STEINBERG, P. & KJELLEBERG, S. 2007. Low densities of epiphytic bacteria from the marine alga Ulva australis inhibit settlement of fouling organisms. *Applied and environmental microbiology,* 73**,** 7844-7852.

RAO, G. N. & CHATTERJEE, R. 2014. Effect of Seaweed Liquid Fertilizer from Gracilaria textorii and Hypnea musciformis on Seed germination and productivity of some vegetable crops. *Universal Journal of Plant Science,* 2**,** 115-120.

RAYIRATH, P., BENKEL, B., HODGES, D. M., ALLAN-WOJTAS, P., MACKINNON, S., CRITCHLEY, A. T. & PRITHIVIRAJ, B. 2009. Lipophilic components of the brown seaweed, Ascophyllum nodosum, enhance freezing tolerance in Arabidopsis thaliana. *Planta,* 230**,** 135-147.

RAYORATH, P., JITHESH, M. N., FARID, A., KHAN, W., PALANISAMY, R., HANKINS, S. D., CRITCHLEY, A. T. & PRITHIVIRAJ, B. 2008. Rapid bioassays to evaluate the plant growth promoting activity of Ascophyllum nodosum (L.) Le Jol. using a model plant, Arabidopsis thaliana (L.) Heynh. *Journal of Applied Phycology,* 20**,** 423-429.

RIDLER, N., WOWCHUK, M., ROBINSON, B., BARRINGTON, K., CHOPIN, T., ROBINSON, S., PAGE, F., REID, G., SZEMERDA, M. & SEWUSTER, J. 2007. Integrated multi‐trophic aquaculture (IMTA): A potential strategic choice for farmers. *Aquaculture Economics & Management,* 11**,** 99-110.

ROBERTSON-ANDERSSON, D. V., POTGIETER, M., HANSEN, J., BOLTON, J. J., TROELL, M., ANDERSON, R. J., HALLING, C. & PROBYN, T. 2008. Integrated seaweed cultivation on an abalone farm in South Africa. *Journal of Applied Phycology,* 20**,** 579-595.

ROMANENKO, L. A., UCHINO, M., KALINOVSKAYA, N. I. & MIKHAILOV, V. V. 2008. Isolation, phylogenetic analysis and screening of marine mollusc-associated bacteria for antimicrobial, hemolytic and surface activities. *Microbiological research,* 163**,** 633-644.

RUEGGER, M., DEWEY, E., GRAY, W. M., HOBBIE, L., TURNER, J. & ESTELLE, M. 1998. The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes & development,* 12**,** 198-207.

RUEGGER, M., DEWEY, E., HOBBIE, L., BROWN, D., BERNASCONI, P., TURNER, J., MUDAY, G. & ESTELLE, M. 1997. Reduced naphthylphthalamic acid binding in the tir3 mutant of Arabidopsis is associated with a reduction in polar auxin transport and diverse morphological defects. *The Plant Cell,* 9**,** 745-757.

SAH, S. K., REDDY, K. R. & LI, J. 2016. Abscisic acid and abiotic stress tolerance in crop plants. *Frontiers in plant science,* 7.

SAKAI, H., HUA, J., CHEN, Q. G., CHANG, C., MEDRANO, L. J., BLEECKER, A. B., & MEYEROWITZ, E. M. 1998. ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. *Proceedings of the National Academy of Sciences*, 95(10), 5812-5817.

SALAÜN, S., KERVAREC, N., POTIN, P., HARAS, D., PIOTTO, M. & LA BARRE, S. 2010. Whole-cell spectroscopy is a convenient tool to assist molecular identification of cultivatable marine bacteria and to investigate their adaptive metabolism. *Talanta,* 80**,** 1758-1770.

SALAÜN, S., LA BARRE, S., DOS SANTOS-GONCALVEZ, M., POTIN, P., HARAS, D. & BAZIRE, A. 2012. Influence of exudates of the kelp Laminaria digitata on biofilm formation of associated and exogenous bacterial epiphytes. *Microbial ecology,* 64**,** 359-369.

SALE, P. 1976. Reef fish lottery. *Nat History* 85**,** 60-65.4.

SALTA, M., WHARTON, J. A., BLACHE, Y., STOKES, K. R. & BRIAND, J. F. 2013. Marine biofilms on artificial surfaces: structure and dynamics. *Environmental microbiology,* 15**,** 2879-2893.

SANTANIELLO, A., SCARTAZZA, A., GRESTA, F., LORETI, E., BIASONE, A., DI TOMMASO, D., PIAGGESI, A. & PERATA, P. 2017. Ascophyllum nodosum seaweed extract alleviates drought stress in Arabidopsis by affecting photosynthetic performance and related gene expression. *Frontiers in plant science,* 8**,** 1362.

SAUNDERS, G. W. 2010. An evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. *Crypt. Algol.,* 31**,** 487-528.

SCHALLER, G. E., BISHOPP, A. & KIEBER, J. J. 2015. The yin-yang of hormones: cytokinin and auxin interactions in plant development. *The Plant Cell,* 27**,** 44-63.

SCHELLNHUBER, H. J., HARE, W., SERDECZNY, O., ADAMS, S., COUMOU, D., FRIELER, K., MARTIN, M., OTTO, I. M., PERRETTE, M. & ROBINSON, A. 2012. Turn down the heat: why a 4 C warmer world must be avoided. World Bank.

SCHIEL, D. R. & LILLEY, S. A. 2007. Gradients of disturbance to an algal canopy and the modification of an intertidal community. *Marine Ecology Progress Series,* 339**,** 1-11.

SCHRAMM, W. 1996. *Marine benthic vegetation: recent changes and the effects of eutrophication,* Springer Science & Business Media.

SELVAM, G. G. & SIVAKUMAR, K. 2013. Effect of foliar spray from seaweed liquid fertilizer of Ulva reticulata (Forsk.) on Vigna mungo L. and their elemental composition using SEM–energy dispersive spectroscopic analysis. *Asian Pacific Journal of Reproduction,* 2**,** 119-125.

SHENG, X.-F. & XIA, J.-J. 2006. Improvement of rape (Brassica napus) plant growth and cadmium uptake by cadmium-resistant bacteria. *Chemosphere,* 64**,** 1036-1042.

SHEPHERD, A., MITCHELL, T., LEWIS, K., LENHARDT, A. J., LINDSEY, S. L. & MUIR-WOOD, R. 2013. The geography of poverty, disaster and climate extremes in 2030.

SHI, H., LEE, B.-H., WU, S.-J. & ZHU, J.-K. 2003. Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. *Nature biotechnology,* 21**,** 81-85.

SHPIGEL, M., GUTTMAN, L., SHAULI, L., ODINTSOV, V., BEN-EZRA, D. & HARPAZ, S. 2017. Ulva lactuca from an integrated multi-trophic aquaculture (IMTA) biofilter system as a protein supplement in gilthead seabream (Sparus aurata) diet. *Aquaculture,* 481**,** 112-118.

SHPIGEL, M., MCBRIDE, S. C., MARCIANO, S., RON, S. & BEN-AMOTZ, A. 2005. Improving gonad colour and somatic index in the European sea urchin Paracentrotus lividus. *Aquaculture,* 245**,** 101-109.

SIEGEL, B. & SIEGEL, S. 1973. The chemical composition of algal cell walls. *CRC Critical Reviews in Microbiology,* 3**,** 1-26.

SILVA, P. C., BASSON, P. W. & MOE, R. L. 1996. *Catalogue of the benthic marine algae of the Indian Ocean*, Univ of California Press.

SINGH, R. P., MANTRI, V. A., REDDY, C. & JHA, B. 2011. Isolation of seaweed-associated bacteria and their morphogenesis-inducing capability in axenic cultures of the green alga Ulva fasciata. *Aquatic Biology,* 12**,** 13-21.

SINGH, R. P. & REDDY, C. 2014. Seaweed–microbial interactions: key functions of seaweed-associated bacteria. *FEMS microbiology ecology,* 88**,** 213-230.

SIVRITEPE, N. 2008. Organic priming with seaweed extract (Ascophyllum nodosum) affects viability of pepper seeds. *Asian Journal of Chemistry,* 20**,** 5689.

SKLADANY, M., CLAUSEN, R. & BELTON, B. 2007. Offshore aquaculture: the frontier of redefining oceanic property. *Society & natural resources,* 20**,** 169-176.

SLAMA, I., ABDELLY, C., BOUCHEREAU, A., FLOWERS, T. & SAVOURÉ, A. 2015. Diversity, distribution and roles of osmoprotective compounds accumulated in halophytes under abiotic stress. *Annals of Botany,* 115**,** 433-447.

SMEEKENS, S., MA, J., HANSON, J. & ROLLAND, F. 2010. Sugar signals and molecular networks controlling plant growth. *Current opinion in plant biology,* 13**,** 273-278.

SMETACEK, V. & ZINGONE, A. 2013. Green and golden seaweed tides on the rise. *Nature,* 504**,** 84.

SMIL, V. 2000. Phosphorus in the environment: natural flows and human interferences. *Annual review of energy and the environment,* 25**,** 53-88.

SMITH, B. E. 2002. Nitrogenase reveals its inner secrets. *Science,* 297**,** 1654-1655.

SMYTH, P. P. 2003. The thyroid, iodine and breast cancer. *Breast Cancer Research,* 5**,** 235.

SODE, S., BRUHN, A., BALSBY, T. J., LARSEN, M. M., GOTFREDSEN, A. & RASMUSSEN, M. B. 2013. Bioremediation of reject water from anaerobically digested waste water sludge with macroalgae (*Ulva lactuca*, Chlorophyta). *Bioresource technology,* 146**,** 426-435.

SOHLENKAMP, C., WOOD, C. C., ROEB, G. W. & UDVARDI, M. K. 2002. Characterization of Arabidopsis AtAMT2, a high-affinity ammonium transporter of the plasma membrane. *Plant physiology,* 130**,** 1788-1796.

SOTO, D. 2009. *Integrated mariculture: a global review*, Food and Agriculture Organization of the United Nations (FAO).

SPOERNER, M., WICHARD, T., BACHHUBER, T., STRATMANN, J. & OERTEL, W. 2012. Growth and thallus morphogenesis of *Ulva mutabilis* (Chlorophyta) depends on a combination of two bacterial species excreting regulatory factors. *Journal of phycology,* 48**,** 1433-1447.

SREENIVASULU, N., RADCHUK, V., ALAWADY, A., BORISJUK, L., WEIER, D., STAROSKE, N., FUCHS, J., MIERSCH, O., STRICKERT, M. & USADEL, B. 2010. De -regulation of a barley mutant seg8. *The Plant Journal,* 64**,** 589-603.

SRIDHAR, S. & RENGASAMY, R. 2010. Significance of seaweed liquid fertilizers for minimizing chemical fertilizers and improving yield of Arachis hypogaea under field trial. *Recent Research in Science and Technology,* 2.

STEINHAGEN, S., BARCO, A., WICHARD, T. & WEINBERGER, F. 2019a. Conspecificity of the model organism Ulva mutabilis and Ulva compressa (Ulvophyceae, Chlorophyta). *Journal of phycology,* 55**,** 25-36.

STEINHAGEN, S., WEINBERGER, F. & KAREZ, R. 2019b. Molecular analysis of Ulva compressa (Chlorophyta, Ulvales) reveals its morphological plasticity, distribution and potential invasiveness on German North Sea and Baltic Sea coasts. *European Journal of Phycology,* 54**,** 102-114.

STIRK, W., ARTHUR, G., LOURENS, A., NOVAK, O., STRNAD, M. & VAN STADEN, J. 2004. Changes in cytokinin and auxin concentrations in seaweed concentrates when stored at an elevated temperature. *Journal of Applied Phycology,* 16**,** 31-39.

STIRK, W., NOVÁK, O., STRNAD, M. & VAN STADEN, J. 2003. Cytokinins in macroalgae. *Plant Growth Regulation,* 41**,** 13-24.

STIRK, W. & VAN STADEN, J. 1997. Isolation and identification of cytokinins in a new commercial seaweed product made from Fucus serratus L. *Journal of applied phycology,* 9**,** 327-330.

STRATMANN, J., PAPUTSOGLU, G. & OERTEL, W. 1996. Differentiation of *Ulva mutabilis* (chlorophyta) gametangia and gamete release are controlled by extracellular inhibitors. *Journal of Phycology,* 32**,** 1009-1021.

SUN, P., TIAN, Q.-Y., CHEN, J. & ZHANG, W.-H. 2009. Aluminium-induced inhibition of root elongation in Arabidopsis is mediated by ethylene and auxin. *Journal of Experimental Botany,* 61**,** 347-356.

SUSSMAN, M. R., AMASINO, R. M., YOUNG, J. C., KRYSAN, P. J. & AUSTIN-PHILLIPS, S. 2000. The Arabidopsis knockout facility at the University of Wisconsin–Madison. *Plant Physiology,* 124**,** 1465-1467.

SUZUKI, N., RIVERO, R. M., SHULAEV, V., BLUMWALD, E. & MITTLER, R. 2014. Abiotic and biotic stress combinations. *New Phytologist,* 203**,** 32-43.

SUZUKI, Y., KAMETANI, T. & MARUYAMA, T. 2005. Removal of heavy metals from aqueous solution by nonliving Ulva seaweed as biosorbent. *Water Research,* 39**,** 1803-1808.

TAIT, K., WILLIAMSON, H., ATKINSON, S., WILLIAMS, P., CÁMARA, M. & JOINT, I. 2009. Turnover of quorum sensing signal molecules modulates cross -kingdom signa *Environmental microbiology,* 11**,** 1792-1802.

TAN, I. H., BLOMSTER, J., HANSEN, G., LESKINEN, E., MAGGS, C. A., MANN, D. G., SLUIMAN, H. J. & STANHOPE, M. J. 1999. Molecular phylogenetic evidence for a reversible morphogenetic switch controlling the gross morphology of two common genera of green seaweeds, Ulva and Enteromorpha. *Molecular biology and evolution,* 16**,** 1011-1018.

TANG, H.-F., YI, Y.-H., YAO, X.-S., XU, Q.-Z., ZHANG, S.-Y. & LIN, H.-W. 2002. Bioactive steroids from the brown alga Sargassum carpophyllum. *Journal of Asian natural products research,* 4**,** 95-101.

TAY, S., PALNI, L. & MACLEOD, J. 1987. Identification of cytokinin glucosides in a seaweed extract. *Journal of Plant Growth Regulation,* 5**,** 133-138.

TAY, S. A., MACLEOD, J. K., PALNI, L. M. S. & LETHAM, D. S. 1985. Detection of cytokinins in a seaweed extract. *Phytochemistry,* 24**,** 2611-2614.

TCHOUNWOU, P. B., YEDJOU, C. G., PATLOLLA, A. K. & SUTTON, D. J. 2012. Heavy metal toxicity and the environment. *Molecular, clinical and environmental toxicology.* Springer.

THAKUR, M. & SOHAL, B. S. 2013. Role of elicitors in inducing resistance in plants against pathogen infection: a review. *ISRN biochemistry,* 2013.

THOMAS, C., ALCOCK, T., GRAHAM, N., HAYDEN, R., MATTERSON, S., WILSON, L., YOUNG, S., DUPUY, L., WHITE, P. J. & HAMMOND, J. 2016. Root morphology and seed and leaf ionomic traits in a Brassica napus L. diversity panel show wide phenotypic variation and are characteristic of crop habit. *BMC plant biology,* 16**,** 214.

THOMAS, R. & ALLSOPP, D. effects of certain periphytic marine bacteria upon the settlement and growth of Enteromorpha, a fouling alga. Biodeterioration 5: papers presented at the 5th International Biodeterioration Symposium, Aberdeen, September, 1981/edited by TA Oxley and S. Barry, 1983. Chichester [Sussex]: Wiley, c1983.

THOMAS, T., EVANS, F. F., SCHLEHECK, D., MAI-PROCHNOW, A., BURKE, C., PENESYAN, A., DALISAY, D. S., STELZER-BRAID, S., SAUNDERS, N. & JOHNSON, J. 2008. Analysis of the Pseudoalteromonas tunicata genome reveals properties of a surface-associated life style in the marine environment. *PLoS One,* 3**,** e3252.

TIETZ, A., RUTTKOWSKI, U., KOHLER, R. & KASPRIK, W. 1989. Further investigations on the occurrence and the effects of abscisic acid in algae. *Biochemie und Physiologie der Pflanzen,* 184**,** 259-266.

TILMAN, D., BALZER, C., HILL, J. & BEFORT, B. L. 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences,* 108**,** 20260-20264.

TISSIER, A. F., MARILLONNET, S., KLIMYUK, V., PATEL, K., TORRES, M. A., MURPHY, G. & JONES, J. D. 1999. Multiple independent defective suppressor-mutator transposon insertions in Arabidopsis: a tool for functional genomics. *The Plant Cell Online,* 11**,** 1841-1852.

TROELL, M. & BERG, H. 1997. Cage fish farming in the tropical Lake Kariba, Zimbabwe: impact and biogeochemical changes in sediment. *Aquaculture research,* 28**,** 527-544.

TSAGKAMILIS, P., DANIELIDIS, D., DRING, M. J. & KATSAROS, C. 2010. Removal of phosphate by the green seaweed *Ulva lactuca* in a small-scale sewage treatment plant (Ios Island, Aegean Sea, Greece). *Journal of Applied Phycology,* 22**,** 331-339.

TSAY, Y.-F., SCHROEDER, J. I., FELDMANN, K. A. & CRAWFORD, N. M. 1993. The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. *Cell,* 72**,** 705-713.

TUJULA, N. A., CROCETTI, G. R., BURKE, C., THOMAS, T., HOLMSTRÖM, C. & KJELLEBERG, S. 2010. Variability and abundance of the epiphytic bacterial community associated with a green marine Ulvacean alga. *The ISME journal,* 4**,** 301.

TURCIOS, A. E. & PAPENBROCK, J. 2014. Sustainable treatment of aquaculture effluents—what can we learn from the past for the future? *Sustainability,* 6**,** 836-856.

VALENTE, L., GOUVEIA, A., REMA, P., MATOS, J., GOMES, E. & PINTO, I. 2006. Evaluation of three seaweeds Gracilaria bursa-pastoris, Ulva rigida and Gracilaria cornea as dietary ingredients in European sea bass (Dicentrarchus labrax) juveniles. *Aquaculture,* 252**,** 85-91.

VALIELA, I., MCCLELLAND, J., HAUXWELL, J., BEHR, P. J., HERSH, D. & FOREMAN, K. 1997. Macroalgal blooms in shallow estuaries: controls and ecophysiological and ecosystem consequences. *Limnology and oceanography,* 42**,** 1105-1118.

VAN DER WAL, H., SPERBER, B. L., HOUWELING-TAN, B., BAKKER, R. R., BRANDENBURG, W. & LÓPEZ-CONTRERAS, A. M. 2013. Production of acetone, butanol, and ethanol from biomass of the green seaweed Ulva lactuca. *Bioresource technology,* 128**,** 431-437.

VANNESTE, S. & FRIML, J. 2009. Auxin: a trigger for change in plant development. *Cell,* 136**,** 1005-1016.

VERNIERI, P., BORGHESI, E., FERRANTE, A. & MAGNANI, G. 2005. Application of biostimulants in floating system for improving rocket quality. *Journal of Food Agriculture and Environment,* 3**,** 86.

VESTY, E. F., KESSLER, R. W., WICHARD, T. & COATES, J. C. 2015. Regulation of gametogenesis and zoosporogenesis in Ulva linza (Chlorophyta): comparison with Ulva mutabilis and potential for laboratory culture. *Frontiers in plant science,* 6**,** 15.

VON HORN, J. & SARTORIUS, C. 2009. *International Conference on Nutrient Recovery From Wastewater Streams. In: Ashley K, Mavinic D, Koch F. IWA, London, 2009,* IWA Publishing.

WALLY, O. S., CRITCHLEY, A. T., HILTZ, D., CRAIGIE, J. S., HAN, X., ZAHARIA, L. I., ABRAMS, S. R. & PRITHIVIRAJ, B. 2013. Regulation of phytohormone biosynthesis and accumulation in Arabidopsis following treatment with commercial extract from the marine macroalga Ascophyllum nodosum. *Journal of plant growth regulation,* 32**,** 324-339.

WALZ, E. 1999. Final results of the third biennial national organic farmers' survey.

WANG, R. & CRAWFORD, N. M. 1996. Genetic identification of a gene involved in constitutive, high-affinity nitrate transport in higher plants. *Proceedings of the National Academy of Sciences,* 93**,** 9297-9301.

WANG, W., VINOCUR, B. & ALTMAN, A. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta,* 218**,** 1-14.

Wang, M., Zheng, Q., Shen, Q., & Guo, S. 2013. The critical role of potassium in plant stress response. *International journal of molecular sciences,* 14, 7370-7390.

WANI, S. H., KUMAR, V., SHRIRAM, V. & SAH, S. K. 2016. Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *The Crop Journal,* 4**,** 162-176.

WASILEWSKA, A., VLAD, F., SIRICHANDRA, C., REDKO, Y., JAMMES, F., VALON, C., DIT FREY, N. F. & LEUNG, J. 2008. An update on abscisic acid signaling in plants and more…. *Molecular plant,* 1**,** 198-217.

WATTS, S., RODRIGUEZ, J., EVANS, S. E. & DAVIES, W. 1981. Root and shoot growth of plants treated with abscisic acid. *Annals of Botany,* 47**,** 595-602.

WEIGEL, D., & GLAZEBROOK, J. (2002). *Arabidopsis*: a laboratory manual. CSHL Press.

WEISS, A., COSTA, R. & WICHARD, T. 2017. Morphogenesis of Ulva mutabilis (Chlorophyta) induced by Maribacter species (Bacteroidetes, Flavobacteriaceae). *Botanica Marina,* 60**,** 197-206.

WELLS, M. L., POTIN, P., CRAIGIE, J. S., RAVEN, J. A., MERCHANT, S. S., HELLIWELL, K. E., SMITH, A. G., CAMIRE, M. E. & BRAWLEY, S. H. 2017. Algae as nutritional and functional food sources: revisiting our understanding. *Journal of applied phycology,* 29**,** 949-982.

WENDT, I., ARRHENIUS, Å., BACKHAUS, T., HILVARSSON, A., HOLM, K., LANGFORD, K., TUNOVIC, T. & BLANCK, H. 2013. Effects of five antifouling biocides on settlement and growth of zoospores from the marine macroalga Ulva lactuca L. *Bulletin of environmental contamination and toxicology,* 91**,** 426-432.

WEST, G., INZÉ, D. & BEEMSTER, G. T. 2004. Cell cycle modulation in the response of the primary root of Arabidopsis to salt stress. *Plant physiology,* 135**,** 1050-1058.

WHEELER, G. L., TAIT, K., TAYLOR, A., BROWNLEE, C., & JOINT, I. A. N. 2006. Acyl - homoserine lactones modulate the settlement rate of zoospores of the marine alga Ulva intestinalis via a novel chemokinetic mechanism. *Plant, cell & environment,* 29, 608-618.

WICHARD, T. 2015. Exploring bacteria-induced growth and morphogenesis in the green macroalga order Ulvales (Chlorophyta). *Frontiers in plant science,* 6**,** 86.

WICHARD, T. & BEEMELMANNS, C. 2018. Role of chemical mediators in aquatic interactions across the prokaryote–eukaryote boundary. *Journal of chemical ecology***,** in press.

WICHARD, T., CHARRIER, B., MINEUR, F., BOTHWELL, J. H., CLERCK, O. D. & COATES, J. C. 2015. The green seaweed Ulva: a model system to study morphogenesis. *Frontiers in plant science,* 6**,** 72.

WICHARD, T. & OERTEL, W. 2010. Gametogenesis and gamete release of *Ulva mutabilis* and *Ulva lactuca* (Chlorophyta): regulatory effects and chemical characterization of the "swarming inhibitor". *Journal of Phycology,* 46**,** 248-259.

WIESE, J., THIEL, V., NAGEL, K., STAUFENBERGER, T. & IMHOFF, J. F. 2009. Diversity of antibiotic-active bacteria associated with the brown alga Laminaria saccharina from the Baltic Sea. *Marine Biotechnology,* 11**,** 287-300.

WILSON, K. A., ABLE, K. W. & HECK JR, K. L. 1990. Predation rates on juvenile blue crabs in estuarine nursery habitats: evidence for the importance of macroalgae (Ulva lactuca). *Marine Ecology Progress Series***,** 243-251.

WOESTE, K. E., YE, C. & KIEBER, J. J. 1999. Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. *Plant Physiology,* 119**,** 521-530.

WOLF, M. A., SCIUTO, K., ANDREOLI, C. & MORO, I. 2012. Ulva (Chlorophyta, Ulvales) biodiversity in the North Adriatic Sea (Mediterranean, Italy): cryptic species and new introductions. *Journal of Phycology,* 48**,** 1510-1521.

WOOD, E. D., ARMSTRONG, F. & RICHARDS, F. A. 1967. Determination of nitrate in sea water by cadmium-copper reduction to nitrite. *Journal of the Marine Biological Association of the United Kingdom,* 47**,** 23-31.

WOODWARD, A. W. & BARTEL, B. 2005. Auxin: regulation, action, and interaction. *Annals of botany,* 95**,** 707-735.

WRIGHT, S. 1940. The statistical consequences of Mendelian heredity in relation to speciation. *The new systematics***,** 161-183.

XIONG, L. & ZHU, J.-K. 2003. Regulation of abscisic acid biosynthesis. *Plant physiology,* 133**,** 29-36.

XU, W., JIA, L., SHI, W., LIANG, J., ZHOU, F., LI, Q. & ZHANG, J. 2013. Abscisic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress. *New Phytologist,* 197**,** 139-150.

YABE, T., ISHII, Y., AMANO, Y., KOGA, T., HAYASHI, S., NOHARA, S. & TATSUMOTO, H. 2009. Green tide formed by free-floating Ulva spp. at Yatsu tidal flat, Japan. *Limnology,* 10**,** 239-245.

YAMAMOTO, H. 2000. Viable but nonculturable state as a general phenomenon of non-spore-forming bacteria, and its modeling. *Journal of Infection and Chemotherapy,* 6**,** 112-114.

YAN, J. 1993. Influence of plant growth regulators on turfgrass polar lipid composition, tolerance to drought and salinity stresses, and nutrient efficiency. *Virginia Tech*.

YANG, Z. B., LIU, G., LIU, J., ZHANG, B., MENG, W., MÜLLER, B., HAYASHI, K. I., ZHANG, X., ZHAO, Z. & DE SMET, I. 2017. Synergistic action of auxin and cytokinin mediates aluminum *EMBO reports,* growth inhibitio e201643806.

YEH, T. S., HUNG, N. H. & LIN, T. C. 2014. Analysis of iodine content in seaweed by GC-ECD and estimation of iodine intake. *journal of food and drug analysis,* 22**,** 189-196.

YOKOI, S., QUINTERO, F. J., CUBERO, B., RUIZ, M. T., BRESSAN, R. A., HASEGAWA, P. M. & PARDO, J. M. 2002. Differential expression and function of Arabidopsis thaliana NHX Na+/H+ antiporters in the salt stress response. *The Plant Journal,* 30**,** 529-539.

YOSHIDA, G., UCHIMURA, M. & HIRAOKA, M. 2015. Persistent occurrence of floating Ulva green tide in Hiroshima Bay, Japan: seasonal succession and growth patterns of Ulva pertusa and Ulva spp.(Chlorophyta, Ulvales). *Hydrobiologia,* 758**,** 223-233.

YOUNUS, H. 2018. Therapeutic potentials of superoxide dismutase. *International journal of health sciences,* 12**,** 88.

YUAN, H.-M., XU, H.-H., LIU, W.-C. & LU, Y.-T. 2013. Copper regulates primary root elongation through PIN1-mediated auxin redistribution. *Plant and Cell Physiology,* 54**,** 766-778.

ZHAO, C., LIU, B., PIAO, S., WANG, X., LOBELL, D. B., HUANG, Y., HUANG, M., YAO, Y., BASSU, S. & CIAIS, P. 2017. Temperature increase reduces global yields of major crops in four independent estimates. *Proceedings of the National Academy of Sciences,* 114**,** 9326-9331.

ZHANG, H. & FORDE, B. G. 2000. Regulation of Arabidopsis root development by nitrate availability. *Journal of experimental botany***,** 51-59.

ZHANG, X. & SCHMIDT, R. 2000. Hormone-containing products' impact on antioxidant status of tall fescue and creeping bentgrass subjected to drought. *Crop Science,* 40**,** 1344-1349.

ZHANG, H., JOHNSON, G. V. & FRAM, M. 2002. *Managing phosphorus from animal manure,* Division of Agricultural Sciences and Natural Resources, Oklahoma State University Stillwater.

ZHANG, X., ERVIN, E. & SCHMIDT, R. 2003. Physiological effects of liquid applications of a seaweed extract and a humic acid on creeping bentgrass. *Journal of the American Society for Horticultural Science,* 128**,** 492-496.

ZHANG, W., LI, Z., MIAO, X. & ZHANG, F. 2009. The screening of antimicrobial bacteria with diverse novel nonribosomal peptide synthetase (NRPS) genes from South China Sea sponges. *Marine biotechnology,* 11**,** 346.

ZHANG, D.-P. 2014. *Abscisic acid: metabolism, transport and signaling,* Springer.

ZHANG, J., KIM, J. K., YARISH, C. & HE, P. 2016. The expansion of Ulva prolifera OF Müller macroalgal blooms in the Yellow Sea, PR China, through asexual reproduction. *Marine pollution bulletin,* 104**,** 101-106.

ZHANG, J., WEI, J., LI, D., KONG, X., RENGEL, Z., CHEN, L., YANG, Y., CUI, X. & CHEN, Q. 2017. The Role of the Plasma Membrane H+-ATPase in Plant Responses to Aluminum Toxicity. *Frontiers in Plant Science,* 8**,** 1757.

ZHANG, Y., ZHANG, J., GUO, J., ZHOU, F., SINGH, S., XU, X., ... & HUANG, C. F. 2019. F-box protein RAE1 regulates the stability of the aluminum-resistance transcription factor STOP1 in Arabidopsis. *Proceedings of the National Academy of Sciences*, 116, 319-327.

ZHENG, S., JIANG, J., HE, M., ZOU, S. & WANG, C. 2016. Effect of Kelp Waste Extracts on the Growth and Development of Pakchoi (Brassica chinensis L.). *Scientific reports,* 6.

ZHU, J.-K. 2016. Abiotic stress signaling and responses in plants. *Cell,* 167**,** 313-324.

ZHU, Z. & GUO, H. 2008. Genetic basis of ethylene perception and signal transduction in Arabidopsis. *Journal of integrative plant biology,* 50**,** 808-815.