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Short Communication

Green tides select for fast expanding *Ulva* strains

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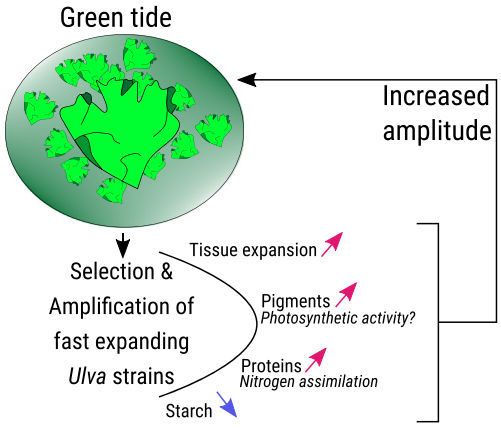
HIGHLIGHTS

- Green tide *Ulva* strains display different growth and metabolic characteristics.
- Differences indicate a selective pressure in green tide areas.
- Green tides events might worsen in future even if eutrophication stabilises.
- Those elite green tide strains could be used in aquaculture.

GRAPHICAL ABSTRACT



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ABSTRACT

Green tides, the phenomenon whereby large volume of marine environment is taken over by the sea lettuce *Ulva* spp, are a seasonal occurrence thought to be caused mainly by anthropogenic eutrophication. The aggravation of green tide occurrence since the 1970s could however be due to the amplification of fast-growing strains within these areas. In this study, we compared the growth and metabolite content of 28 green tide *Ulva* strains against 100 non-green tide strains, under conditions close to those encountered in green tides areas. The aim was to determine whether the presence of specific characteristics intrinsic to green tide strains could in itself be a major factor for their reoccurrence. We confirmed that green tide strains have specific characteristics, with faster tissue expansion, higher protein and pigments, and lower starch content compared to non-green tide ones, thus highlighting a genetic component specific to green tide strains. Dry biomass accumulation, however, was not different between the two types of *Ulva* strains. Hence, we hypothesise that the selective pressure in green tide areas leads to the amplification of *Ulva* genotypes best adapted for this environment. Such selection of fast-growing strains would indicate that green tides are likely to become more prevalent and of higher magnitude over the coming years.

1. Introduction

Coastal eutrophication is an increasingly important problem worldwide, where rising nutrients levels in seawater (mainly nitrogen and phosphorus) threaten natural ecosystems (Smith et al., 1999; Diaz and

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Rosenberg, 2008). Eutrophication is often directly linked to human activities, largely as a result of fertilisers, municipal pollution and slurry leakage into freshwater streams eventually reaching coastal areas (Kiedrzyńska et al., 2014; Nixon, 1995). Eutrophication, on its own or in combination with other anthropogenic stressors, can have devastating consequences on the ecosystem (Duarte and Krause-Jensen, 2018; Heiskanen et al., 2019; Ceccherelli et al., 2018). For example, algal blooms and their subsequent collapses lead to the development of hypoxia, causing direct effects on the trophic chain (Smith et al., 1999), leading to community structure changes (Lyons et al., 2014), biodiversity and habitat loss (Cook et al., 2018; Deegan et al., 2012) and overall impoverished ecosystems (Mineur et al., 2014). The economic implications of algal blooms are also serious, with negative effects on tourism and fisheries (Le Luherne et al., 2017) and cleaning operations of the affected areas are costly (Charlier et al., 2008).

The sea lettuce *Ulva* spp. is one of the macroalgal species most commonly linked to the formation of "green tides", where large swaths of the coastal environment are taken over by rapidly proliferating *Ulva* biomass (Smetacek and Zingone, 2013). Due to this opportunistic behaviour and tolerance to a variety of environmental parameters, 'green tide' algae such as *Ulva* spp. are usually regarded as pollution indicators (Largo et al., 2004; Taylor et al., 2001). Of all the environmental factors that can lead to the development of green tides, eutrophication caused by excessive loadings of anthropogenic nitrogen is regarded as the most important (Zhang et al., 2013b; Perrot et al., 2014), and massive green tide events are becoming an annual occurrence in numerous areas with an aggravation year-on-year (Xu et al., 2014; Perrot et al., 2014). Nonetheless, it is possible that factors other than eutrophication are responsible for this aggravation and that a decrease in nutrient loads in coastal and transitional waters might not be sufficient to reduce the worldwide occurrence of green tides (Yabe et al., 2009). Other environmental envelope conditions such as irradiance, salinity and temperature could also play a role in the development and severity of green tides, as demonstrated by laboratory experiments (Kim et al., 2011; Kang and Kim, 2016; Xiao et al., 2016).

Growth and other phenotypic traits are controlled by two major factors: the genotype (G), and the environment (E). For an individual grown under a given set of environmental parameters, the expressed phenotype is the result of those two factors (G and E), and their interaction (G X E). G X E is also known as phenotypic plasticity, where the phenotypic response of a set of genotypes varies depending of the environment (reviewed in (Marais et al., 2013). In order to unravel whether green tides strains have specific genotypes (G) over their non-green tide counterparts, it is then necessary to remove the plasticity conferred by the environment (E and G X E), by the use of common garden experiments (reviewed in (de Villemereuil et al., 2016)). Common garden experiments allow researchers to specifically detect whether genetic variations among individuals lead to phenotypic variation, and whether populations can be discriminated phenotypically due to their genetic makeup. The genetic component in phenotypes (heritability) between populations can be significant, with for instance up to 63% of phenotypic variation among *Arabidopsis thaliana* accessions being explained by genotype diversity alone, as inferred by a common garden experiment (Brachi et al., 2013). While studies have shown that green tides could be comprised of several species of *Ulva* (Le Luherne et al., 2017; Largo et al., 2004; Bermejo et al., 2019; Taylor et al., 2001), to our knowledge, no study has compared the performance of green tide strains over non green tide strains with enough individual strains to infer whether genotype (G) factors could influence the phenotype of green tide strains, affecting the occurrence and/or the magnitude of green tides.

Here, we investigated the growth and metabolite content of 28 green tides strains versus 100 non-green tide strains, grown under a single growth condition close to those encountered in green tide areas. The aim of the study was to infer whether green tide strains display specific phenotypic characteristics compared to non-green tide strains when grown under the same conditions, thus unravelling the presence, or absence, of a genetic component specific to green tide strains. Such study is important because the presence of genetic factors specific to green tide strains would suggest that green tides act as a selective mechanism, which could partly account for the aggravation and increase in frequency of green-tide events over the last five decades.

2. Materials and methods

2.1. Algae material

Intertidal and subtidal laminar *Ulva* samples from green tide and non-green tide areas were collected at various sampling sites in Ireland, the Netherlands, Brittany (France), Portugal and Spain (for the list of samples and their geographical locations, see Supplementary Dataset 1). Immediately after collection, samples were placed in bags filled with seawater and brought back to the lab in a coolbox. Samples collected outside Ireland were shipped in individual falcon tubes filled with seawater inside coolboxes. Upon arrival to the lab at NUI Galway, all *Ulva* samples were maintained in vegetative growth as per Fort et al. (2019) for at least three weeks prior to phenotyping, to ensure adequate acclimation of all strains to the growth conditions used in this study. Indeed, with a protein turnover in photosynthetic organisms between 6 and 42%.day^{-1} (Huffaker and Peterson, 1974), a growth rate of ~20%.day^{-1}, and an almost complete depletion of internal nitrate reserves at the end of each day (Fort et al., 2019), most if not all proteins, biomass and nitrogen reserves in the acclimated *Ulva* samples will have been generated under the culture conditions used in this study.

2.2. Genetic barcoding analysis

A 2 × 2 cm portion of each *Ulva* sample was placed artificial seawater and kept in the dark for three days to reduce polysaccharide content and generate higher quality DNA. The samples were then flash frozen in liquid nitrogen and freeze dried. Freeze dried samples were ground into fine power using a ball mill (QIAGEN TissueLyser II), and DNA extractions were carried out using ~7 mg of biomass powder and the adapted NucleoMag Plant kit (Macherey-Nagel) protocol described in Fort et al. (2018). For barcoding, a PCR amplifying the *RbcL* gene using the primers SHF1 and SHR4 (Heesch et al., 2009) was performed using 1 μL of undiluted DNA in 25 μL final reaction volume. Amplicons were sequenced via Sanger sequencing by LGC Genomics GmbH (Germany). Sequencing traces (as well as *RbcL* sequences from the most likely species from the NCBI database) were aligned using the MAFFT algorithm (Katoh et al., 2002). Alignments were manually corrected using JalView (Waterhouse et al., 2009), and species delimitation was performed using the BEAST software (Bouckaert et al., 2014), under a Generalized Mixed Yule Coalescent (GMYC) model, and analysed in R using the *splits* package (Fujisawa and Barraclough, 2013). The resulting phylogenetic tree was visualised using the FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. Growth monitoring and metabolic analysis

Ulva strain growth was monitored using a custom made phenotyping platform (Fort et al., 2019) whereby discs from *Ulva* thalli were used as proxy for thallus growth. Discs (18 to 24 per *Ulva* strain, their position randomised across eight aquarium tanks) were grown under a photoperiod of 12 h of light and 12 h of darkness for six to seven days. The light intensity was set at 200 μmol.m^{-2} s^{-1} Photosynthetically Active Radiation (PAR), obtained using near-sunlight spectrum LED lights (Spectron 20W T8 GB white, Hydrogarden, UK). The temperature was set at 15 ± 1 °C, to best reflect the average temperature of the water during spring to autumn in the sampled areas. The growth media consisted of artificial seawater (35 ppt salinity) and 1× Cell-HI F2P vitamins and nutrients (Varicon aqua, UK), in order to obtain a concentration of nutrients consistent with values recorded in polluted waters in

the NE Atlantic, e.g. above 8 mg l^{-1} (European Environment Agency, 2019). Tissue expansion expressed as Area Specific Growth Rate (Area SGR) in %. $period^{-1}$ was monitored using raspberry pi cameras for each individual disc. Biomass accumulation expressed as Relative Growth Rate (RGR, in $mg.mg^{-1}.day^{-1}$) was calculated by comparing the fresh or dry weight of discs before (3 discs) and after the growth period with 6 to 8 pools of three discs per strain, half being harvested at the end of day and the other half at the end of night, using a randomised design. Each pool of three discs was rinsed in distilled water, blotted dry with tissue paper, placed in pre-weighted tubes and flash frozen in liquid nitrogen. The fresh weight of the pools of discs was then measured before freeze drying. The weight of lyophilised pools was determined, and the water content of each pool was estimated by comparing the fresh weight of the pools upon harvest and the dry weight of the pools after freeze-drying. Net Assimilation Rate (NAR) and Specific Leaf Area (SLA) were calculated according to Fort et al. (2019).

Freeze-dried disc pools were ground into powder using a ball mill (Qiagen TissueLyser II), and metabolic analysis was performed on ~5 mg aliquots, with three to four pools of three discs per timepoint per strain. Soluble metabolites were extracted from the 5 mg aliquots by sequential incubation for 30 min at 90 °C in 100% Ethanol, 80% Ethanol and 50% Ethanol (Esteves-Ferreira et al., 2017). The supernatants of the three extractions were pooled and used to determine pigments (Porra et al., 1989), nitrite and nitrate (Tsikas, 2007), and amino acids (Yemm and Cocking, 1955). The ethanol-insoluble pellet was incubated in 0.1 M NaOH at 90 °C for 1 h, and the supernatant used to determine proteins (Lowry et al., 1951), and starch content (Smith and Zeeman, 2006). In order to ensure equivalent extraction and determination efficiencies between all samples, for each batch of samples analysed for metabolite contents, two samples containing ~5 mg of an *Ulva* biomass master mix were added to separate tubes, and their metabolic content determined.

2.4. Data analysis

All data was analysed using R (R Development Core Team, 2011). Statistical differences in the clustering of green tide versus non-green tide strains was determined using a Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) (Anderson, 2017) using the *adonis* function in the *vegan* package (Dixon, 2003). To identify statistical differences between green tide and non-green tide strains for each trait, we used a permutation test of independence using the *Coin* package in R (Hothorn et al., 2008), taking the mean for each strain's trait as dependent variable, and the type of strain as independent variable. Both PERMANOVA and permutation test of independence allow for comparisons of groups regardless of normality and unequal sample sizes between two groups (Anderson, 2017; Ernst, 2004).

3. Results

3.1. Green tide strains belong to several laminar Ulva species

We sampled laminar *Ulva* individuals in green tide and non-green tide locations in Ireland, France, Portugal, Spain and the Netherlands (Supplementary Dataset 1, Fig. 1A). A total of 28 green tide and 100 non-green tide individuals were sampled during spring to summer 2018. First, we determined which species of *Ulva* were present in our dataset of 128 *Ulva* strains by comparing the sequence of each strains' *RbcL* gene under a GMYC model (Fig. 1B). Six distinct *Ulva* species, as well as strains belonging to *Umbraulva* spp. were identified. The *Ulva*

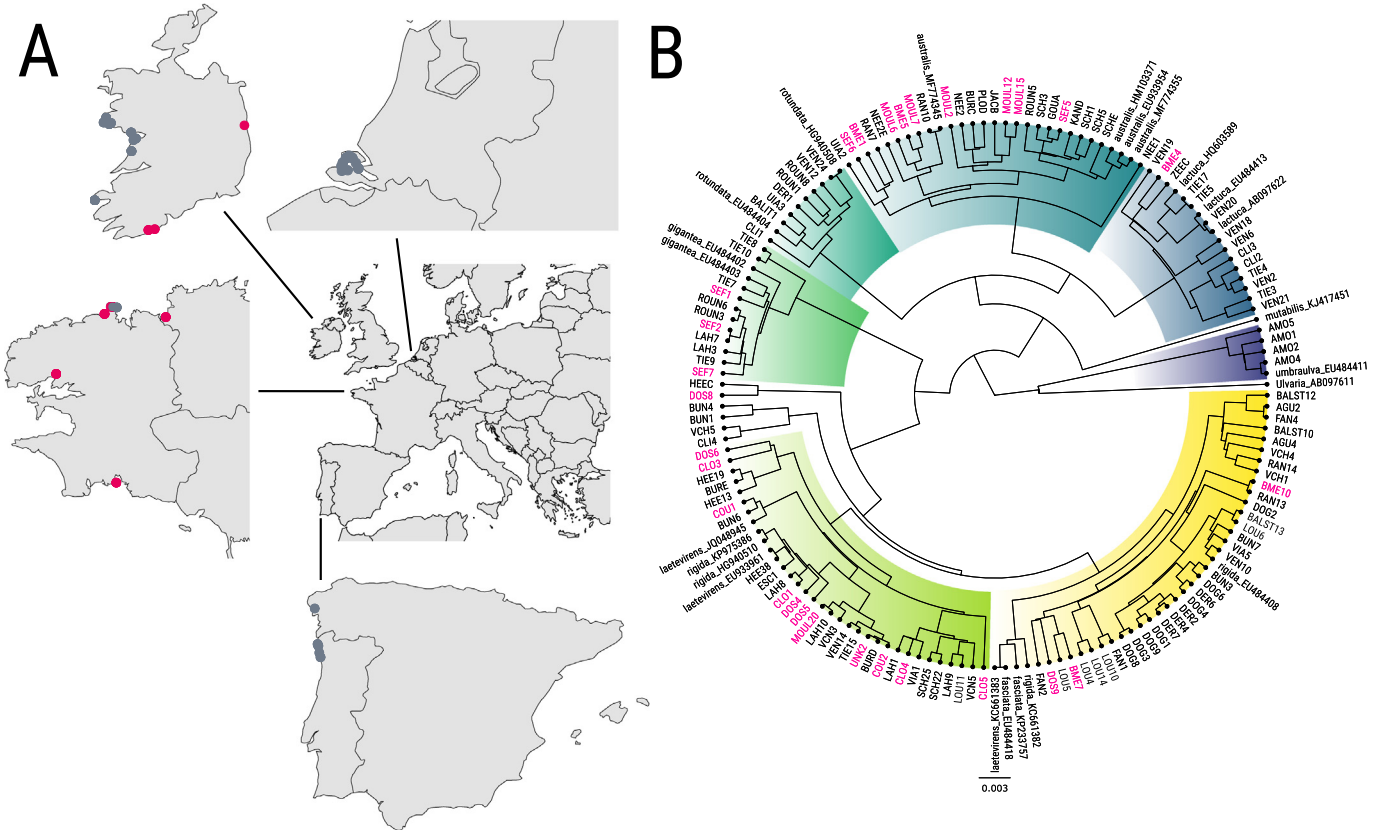

Fig. 1. Sampling sites and species identification of the *Ulva* strains used in this study. A) Location of the sampling sites in Ireland, Brittany (France), the Netherlands, Portugal and Spain. Dots in blue represent "normal" sampling sites, dots in pink represent green tide sampling sites. B) Phylogeny and species identification of the *Ulva* strains used in this study, using *RbcL* barcode and a GMYC model for species delimitation. Strains names in pink represent green tide strains, colour represents species clusters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species present within our dataset were *Ulva lactuca*, *Ulva australis*, *Ulva rotundata*, and *Ulva gigantea*. Two more species clusters were present but could not be attributed to an *Ulva* species based on their closest matches in the NCBI database. Indeed, while the GMYC model predicted two distinct species, both species clusters were attributed to *Ulva laetevirens* and *Ulva rigida* sequences. Such discrepancy is likely due to the mis-classification of *Ulva* species in the public databases and/or the difficulty to discriminate some *Ulva* species based on a single barcode. Six strains could not be attributed to a specific cluster, but were closest to the *laetevirens*/*ridiga* clusters. Green tide-originating strains fell within five of the six *Ulva* species in the dataset (Fig. 1B), indicating that green tide areas contained several laminar *Ulva* species and that most have similar potential for the formation of green tides. These results further confirm previous reports showing coexistence of several *Ulva* species within the same green tide areas (Yabe et al., 2009; Bermejo et al., 2019; Kang et al., 2014).

3.2. Ulva strains from green tide areas have distinct growth and metabolic features

Each strain of *Ulva* was grown on the phenotyping platform, and their growth, physiological and metabolic characteristics are available in Supplementary Dataset 1. To determine whether strains originating from green tide areas have specific growth or metabolic characteristics, we performed a Principal Component Analysis (PCA) on the mean of each strain's traits (Fig. 2). We observed a significant clustering of strains based on their type (green tide or non-green tide, PERMANOVA, $p < 0.001$). Protein content, nitrite content, pigments, starch, water content, tissue expansion (Area SGRs) and Fresh Weight accumulation (Fresh Weight RGR) were the main contributing factors to the clustering. On the other hand, amino acids, Net Assimilation Rate (NAR), Specific Leaf Area (SLA), dry biomass accumulation (Dry Weight RGR) and nitrate delta (difference between end of day and end of night in *Ulva* tissue) did not contribute to the separation between green tide and non-green tide strains. To confirm those results and precisely quantify differences between green tide and non-green tide *Ulva* strains, we performed a permutation test of independence between each investigated trait and the strains's origin (Table 1). We found significant differences for 12 out of the 20 traits measured. Notably, green tide strains discs expanded (Area SGRs) on average ~28% faster than non-green tide ones, with 3% higher water content, ~25% faster fresh weight RGR, 55% higher protein content, 30% more nitrite and ~75% higher pigment content. In contrast, starch content at the end of day and end of night were ~25% lower in green tide strains, while dry biomass accumulation (Dry Weight RGR), amino acids and nitrate content were not significantly different ($p > 0.05$). Our data demonstrate that regardless of their species, *Ulva* strains from green tide areas possess specific growth and metabolic characteristics compared with strains from non-green tide areas when grown under the same conditions.

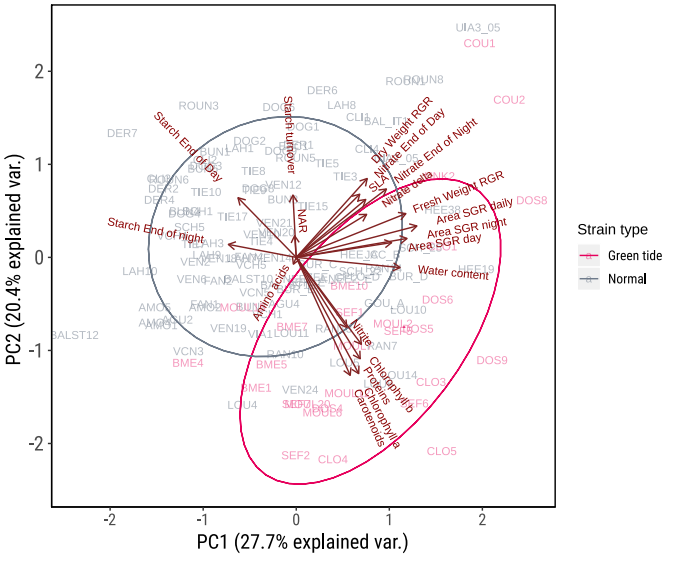

Fig. 2. Clustering of strains based on their green tide or non-green tide origin. Principal Component Analysis of *Ulva* traits based on their sampling location.

Table 1
Growth and metabolite differences between green tides and normal *Ulva* strains.

Data represent the mean trait data ± s.d between strains type. p-Values are inferred by permutation test of independence. All mean data per strain is available in Supplementary Dataset 1.

Trait	Strain origin		Fold change	p-Value
	Normal, *n* = 100	Green tide, *n* = 28		
Starch turnover	205.41 ± 154.11	144.49 ± 111.84	0.7	0.0537
Starch end of day	**504.8 ± 189.91**	**374.28 ± 141.55**	**0.74**	**0.0012**
Starch end of night	**299.39 ± 160.38**	**229.79 ± 115.76**	**0.77**	**0.0346**
Nitrate end of night	3.37 ± 1.62	3.03 ± 1.32	0.9	0.301
Nitrate delta	2.63 ± 1.3	2.36 ± 0.92	0.9	0.2987
Amino acids	67.61 ± 22.7	61.62 ± 26.4	0.91	0.2348
Nitrate end of day	0.74 ± 0.88	0.67 ± 0.72	0.91	0.6958
NAR	0.01 ± 0	0.01 ± 0	1	0.9804
Water content	**79.17 ± 3.12**	**81.43 ± 2.8**	**1.03**	**< 0.001**
SLA	31.03 ± 8.71	32.56 ± 9.2	1.05	0.4155
Dry weight RGR	0.18 ± 0.06	0.2 ± 0.07	1.11	0.2254
Fresh weight RGR	**0.2 ± 0.05**	**0.25 ± 0.07**	**1.25**	**< 0.001**
Day area SGR	**5.5 ± 2.15**	**6.96 ± 3.09**	**1.27**	**0.006**
Daily area SGR	**13.18 ± 4.98**	**16.73 ± 6.31**	**1.27**	**0.002**
Night area SGR	**8.32 ± 3.14**	**10.84 ± 3.43**	**1.3**	**< 0.001**
Nitrite	**0.13 ± 0.05**	**0.18 ± 0.06**	**1.38**	**< 0.001**
Proteins	**89.38 ± 29.83**	**138.9 ± 34.49**	**1.55**	**< 0.001**
Carotenoids	**0.21 ± 0.12**	**0.36 ± 0.11**	**1.71**	**< 0.001**
Chlorophyll a	**0.18 ± 0.09**	**0.31 ± 0.1**	**1.72**	**< 0.001**
Chlorophyll b	**0.37 ± 0.17**	**0.66 ± 0.21**	**1.78**	**< 0.001**

4. Discussion

The occurrence of macroalgae blooms is generally linked to anthropogenic eutrophication of the coastal environment (Valiela et al., 1997), with green tide occurrences steadily on the rise since the 1970s (Charlier et al., 2008). Indeed, with higher nitrogen supply, *Ulva* growth rate increases (Luo et al., 2012). Mitigating the occurrence of *Ulva* blooms is a critical challenge worldwide (Smetacek and Zingone, 2013), the reduction of the nutrient load from agriculture to the environment being the most evident target to achieve it (Diaz et al., 2013; Wang et al., 2018). However, reducing the nutrient loads on the coastal environment may not be sufficient to limit green-tide occurrence and promote ecological recovery (Deegan et al., 2012). Indeed, it has been demonstrated that, even after eutrophication events, pristine conditions are not recovered, e.g. in the Black Sea, (Oguz and Velikova, 2010). Indeed, *Ulva* biomass decaying after the bloom release nutrients back to the environment which could be opportunistically reused by the algae during the next bloom (Charlier et al., 2008). Furthermore, *Ulva* rafts are less affected by their natural grazers e.g. amphipods that normally live on the seafloor (Geertz-Hansen et al., 1993). Hence, without careful removal of the excess biomass produced during the blooms, coupled with further restorative approaches that would increase flushing of the excess nutrient loads while reducing agricultural and urban nutrient inputs, ecological recovery could be difficult, especially when cumulative effects from climate change are considered (Duarte and Krause-Jensen, 2018).

While environmental factors and their possible mitigation has been studied extensively, genetic factor(s) intrinsic to green tides strains could also impact green tides occurrence and magnitude. Indeed,

despite green tide forming *Ulva* species having been studied under various environmental conditions, both in situ and in laboratories (Gao et al., 2017; Li et al., 2016; Fan et al., 2014; Merceron et al., 2007; Zhang et al., 2013a), the comparison of *Ulva* strains originating from green tide versus non-green tide areas had not been performed to date. Such comparison is of importance to infer a possible genetic basis to green tide occurrence/magnitude. The clustering found in Fig. 2 and the identification of numerous traits with significant differences between green tide and non-green tide strains (Table 1) when grown under the same environmental conditions demonstrate the presence of genotype effects separating green tide strains from the rest of the *Ulva* population. Deciphering those genetic effects, i.e. which alleles / genes / genetic interactions are explaining the phenotypic differences, represent exciting avenues for algae physiology, ecophysiology and more generally for the understanding of the evolution of this financially and environmentally important macroalgae. Future studies should also focus on the interactions between green tide genotypes and their environment. For instance, whether green tides strains would display the same phenotypic differences over non-green tides ones under limiting conditions of light and nutrients remains to be investigated and will provide invaluable information for the prediction of the occurrence of green tides in the future.

In terms of which traits are selected for in a green tide environment, our study demonstrates that laminar *Ulva* strains originating from green tide areas tend to expand faster than their non-green tide counterparts when grown under the same conditions. Fast thallus expansion is likely an adaptation to the highly competitive green tide environment, were each *Ulva* thallus competes for light and nutrient capture. Notably, we found that green tide *Ulva* strains display ~75% higher pigment content over their non-green tide counterparts, further corroborating our previous finding of pigment levels significantly correlating with tissue expansion (Fort et al., 2019). These results are probably indicative of higher photosynthetic capability per thallus area and thus higher efficiency for light use in *Ulva* spp. green tide strains. Increased photosynthetic capability per thallus area can be advantageous in a crowded environment, rendering the individual able to overtake its competitors. Higher dry biomass accumulation (Dry Weight RGR), on the other hand, is unlikely an advantageous adaptation in a competitive environment for light and nutrients unless associated with fast expansion. Concomitantly, the higher water content in green tide strains indicates a possible beneficial adaptation whereby these strains increase thallus volume without producing dry biomass. In good agreement, water content correlates positively with tissue expansion in this study and in Fort et al. (2019). Such hypotheses would explain the over-representation of fast expanding strains within green tide sampling sites, and the absence of a significant difference when dry biomass accumulation alone is considered (Table 1).

In addition, while we found that differences in nitrate content between end of day and end of night in *Ulva* correlate with growth (i.e. biomass and tissue expansion; (Fort et al., 2019), this study), the differences for this trait between green tide or non-green tide strains were not statistically significant (Table 1). It is possible that large increases in protein amount in green tide strains (55% more than non-green tide ones) indicate a more efficient nitrogen flux towards protein synthesis, and a higher amount of RubisCo, which is the most abundant protein in autotrophic organisms (Raven, 2013). Indeed, capturing and processing nitrogen faster could lead to a similar steady-state internal nitrate level, but a higher protein content. A larger amount of RubisCo in green tide strains could also provide higher photosynthetic capacity. Thus, alongside faster thallus expansion and possibly higher photosynthesis per unit area, nitrogen uptake and utilisation by green tide *Ulva* strains likely represent an advantage in a green tide environment. Worryingly, the selective pressure from green tide environments may lead to the amplification of "elite" fast expanding strains, and could cause year-on-year increase in the magnitude of *Ulva* blooms, regardless of de-eutrophication efforts (Yabe et al., 2009).

Finally, these findings could also prove of commercial interest for the growing seaweed aquaculture industry, considering that green tide sea lettuce strains possess on average 55% more proteins and 25% less starch than their non-green tide counterparts. The use of green tide *Ulva* spp. strains for aquaculture would likely to lead to a more valuable crop with higher protein yield and lower starch content.

5. Conclusions

Overall our study demonstrates that *Ulva* spp. strains are genetically different from non-green tide strains, as demonstrated by the significant clustering of green tides and non-green tides strains based on their phenotypes, when grown under the same growth conditions. Such genetic effect indicates that the selective pressure within green tides favours the apparition and/or amplification of *Ulva* strains with specific genetic makeup, rendering them capable of fast tissue expansion, high content in pigments and proteins, and low starch. We hypothesise that those traits represent a fitness advantage within the competitive green tide environment. Finally, the yearly selection and amplification of fast-expanding strains could lead to more intense green tide events in the future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134337.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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