Nuno Tavares Martins

Physiological responses of *Ulva fasciata* Delile (Ulvales, Chlorophyta): comparison of two populations from thermally distinct sites from Brazilian coast.

Respostas fisiológicas de *Ulva fasciata* Delile (Ulvales, Chlorophyta): comparação de duas populações de locais termicamente distintos do litoral brasileiro.

São Paulo
2016

Nuno Tavares Martins

Physiological responses of *Ulva fasciata* Delile (Ulvales, Chlorophyta): comparison of two populations from thermally distinct sites from Brazilian coast.

Respostas fisiológicas de *Ulva fasciata* Delile (Ulvales, Chlorophyta): comparação de duas populações de locais termicamente distintos do litoral brasileiro.

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo para obtenção do Título de Mestrado em Botânica

Dissertation presented to the Institute of Biosciences of the University of São Paulo to obtain MSc degree in Botany

Supervisor: Prof. Dr. Estela M. Plastino
Co-supervisor: Prof. Dr. Carlos Frederico D. Gurgel

São Paulo
2016

Catalogação na publicação
Serviço de Biblioteca e Documentação
Instituto de Biociências da Universidade de São Paulo

Martins, Nuno Tavares
Respostas fisiológicas de *Ulva fasciata* Delile (Ulvales, Chlorophyta): comparação de duas populações de locais termicamente distintos do litoral brasileiro / Nuno Tavares Martins – São Paulo: N.T.M., 2016, 73 pp.

Dissertação (Mestrado) – Universidade de São Paulo, Instituto de Biociências, Departamento de Botânica, 2016.

1. Aquecimento global, 2. Clorofila, 3. Eventos extremos, 4. Fluorescência, 5. Fv/Fm, 6. Onda de calor, 7. PAM, 8. Pigmentos, 9. Ressurgência, 10. Temperatura

I. Universidade de São Paulo. Instituto de Biociências, Departamento de Botânica

Comissão Julgadora:

_____ _____

Prof. (a) Dr. (a) Prof. (a) Dr. (a)

_____

Profa. Dra. Estela M. Plastino
Orientadora

O lar
do passarinho
é
o ar
não
é
o ninho.
Braulio Tavares

Choose a job you love, and you will never have to work a day in your life.
Confucius

Acknowledgements

Agradecimentos

Eu gostaria de expressar minha gratidão à minha orientadora Prof. Dra. Estela Maria Plastino, quem me ensinou a andar, quando eu pensava que podia voar.

Ao Prof. Dr. Carlos Frederico Gurgel, pela hospitalidade, por todos os comentários, observações e, além da coorientação, pela amizade que nasceu lá em "the land down under".

Ao apoio financeiro fornecido pelo CNPq (130061/2015-8) e FAPESP (2014/22349-8).

Ao Prof. Dr. Paulo Polito, pela ajuda com os dados de satélite de temperatura dos locais de coleta.

Aos técnicos do laboratório: Rosário Petti e Willian Oliveira, por toda ajuda fornecida durante os experimentos.

Aos colegas: André Faria, pela amigável companhia e ajuda durante a coleta; Caroline Ximenes e Fábio Nauer, por toda paciência e dedicação em me ensinar biologia molecular e; Samuel Galvão, pela ajuda com o R e estatísticas.

A todos os professores do "Laboratório de Algas Marinhas – Édison José de Paula" pela ótima convivência.

A todos os amigos do LAM pela recepção, companheirismo diário e pelos longos e divertidos momentos de café.

A todos os funcionários do Instituto de Biociências, mas em particular à Erika Takamoto, pela paciência em sanar minhas dúvidas.

Também gostaria de agradecer ao Prof. Dr. Vinícius Peruzzi de Oliveira por me introduzir à ciência, despertar em mim o amor pela pesquisa e por sua amizade.

À minha família: meus pais, Sandra Tavares e Paulo Martins, meu irmão Daniel e minha irmã Paula, por todo apoio ao longo da vida.

Por ultimo, mas não menos importante, à minha amada esposa Thayssa Campista Martins, por seu apoio e por estar ao meu lado em todos os momentos. Serei sempre grato por seu amor.

Summary

Sumário

ABSTRACT

In a global warming scenario, an increase temperature is expected in addition to the occurrence and intensity of extreme climate events. One example of extreme events is the marine heat waves, which are a major threat to marine macroalgae. *Ulva fasciata* is a cosmopolitan species that occur in the whole Brazilian coast. This study was performed in two regions of Rio de Janeiro State (RJ) coast. Both regions are tropical, however, Arraial do Cabo/RJ is naturally colder than Niterói/RJ due upwelling phenomenon. This study aimed to: (i) confirm that *U. fasciata* individuals from these two Brazilian coast regions are of the same species; and (ii), physiologically analyze individuals of *U. fasciata* in the field and under in-laboratory controlled temperature experiment. We hypothesized that *U. fasciata* populations grown at thermally different locations would present distinct ecophysiological responses. In the field, it was accessed maximum quantum yield (Fv/Fm) and pigment content, and in laboratory, it was also evaluated growth rate. The in-laboratory controlled experiment comprised three phases: (i) a temperature gradient; (ii) a 5-day heat wave (+ 5 °C); and (iii) a 5-day recovery (- 5 °C). The molecular data allow us to state that the two populations belong to the same species. No differences of the fluorescence-derived factors were observed between individuals from both populations in the field, suggesting acclimation. However, differences were detected along all three experimental phases. The analysis of pigment content field data evidenced that individuals from the population of Niterói (warmer site) had higher concentrations of chlorophyll *a* than individuals from Arraial do Cabo (colder site). However, individuals of population from Niterói when cultured at 21 °C showed the lowest values of pigment. The differences observed suggest ecotypes. In conclusion, as the planet becomes warmer and extreme weather events become more frequent, the likelihood that heat wave to occur is higher. Therefore, *U. fasciata* from Arraial do Cabo showed better physiological responses to the effects of heat wave, what could confer them higher competitiveness ability to overcome thermal stress.

KEY WORDS: Chlorophyll, extreme events, fluorescence, Fv/Fm, global warming, heat wave, PAM, pigments, temperature, upwelling.

RESUMO

Num cenário de aquecimento global, um aumento da temperatura é esperado, assim como a ocorrência e intensidade de eventos climáticos extremos. Um exemplo de evento extremo são as ondas de calor marinhas, que são a principal ameaça a macroalgas marinhas. *Ulva fasciata* é uma espécie cosmopolita que ocorre em toda costa brasileira. Esse estudo foi realizado em duas regiões da costa do Estado do Rio de Janeiro (RJ). Ambas regiões são tropicais, mas Arraial do Cabo/RJ é naturalmente mais fria que Niterói/RJ devido ao fenômeno de ressurgência. Esse estudo objetivou: (i), confirmar que os indivíduos de *U. fasciata* dessas duas localidades da costa brasileira são da mesma espécie; e (ii) analisar fisiologicamente indivíduos de *U. fasciata* em campo e em experimentos de temperatura em condições controladas de laboratório. Nossa hipótese era de que populações de *U. fasciata* procedentes de localidades termicamente diferentes iriam apresentar respostas ecofisiológicas distintas . Em campo, foi acessado o rendimento quântico máximo (Fv/Fm) e o conteúdo pigmentar, e em laboratório, foi também avaliada a taxa de crescimento. O experimento em condições controladas de laboratório consistiu de três fases: (i) gradiente de temperatura; (ii) onda de calor (+5 °C) de 5 dias; e (iii) recuperação (- 4 °C) de 5 dias. Os dados moleculares permitiram afirmar que as duas populações pertencem à mesma espécie. Não foram detectadas diferenças nos fatores derivados da fluorescência entre os indivíduos das duas populações avaliadas em campo, sugerindo aclimatação. Contudo, foram detectadas diferenças ao longo das três fases experimentais. A análise do conteúdo pigmentar em campo evidenciou que os indivíduos da população de Niterói (região mais quente) tinham mais clorofila *a* do que os indivíduos de Arraial do Cabo (região mais fria). No entanto, indivíduos da população de Niterói, quando cultivados em 21 °C, mostraram valores menores de pigmentos. As diferenças observadas sugerem ecótipos. Em conclusão, conforme o planeta se torna mais quente e eventos extremos climáticos se tornam mais frequentes, a probabilidade de ocorrência de ondas de calor é maior. Dessa forma, *U. fasciata* de Arraial do Cabo mostro melhor resposta fisiológica aos efeitos da onda de calor, o que lhe pode conferir maior capacidade de competição para superar estresses térmicos.

PALAVRAS-CHAVE: Aquecimento global, clorofila, eventos extremos, fluorescência, Fv/Fm, onda de calor, PAM, pigmentos, ressurgência, temperatura.

Chapter 1

General introduction

Introdução geral

BACKGROUND

Temperature

Anthropogenic climate change is any change in climate caused by the effect of human activity, such as the increase of greenhouse gases in the atmosphere. The magnitude of anthropogenic global climate change is currently considered irreversible at human time scales (Turra et al. 2013). Global warming is one of the major processes resulting from climate change in the marine environment. The warming has been confirmed by ocean temperature data recorded in recent years (Field et al. 2014, Vergés et al. 2014). A rise of 2-4 °C from the average temperature of the planet, including the oceans is speculated to 2100 (Field et al. 2014, Vergés et al. 2014).

Temperature dramatically influences biological processes, acting from molecules to the whole biota (Turra et al. 2013, Ferreira et al. 2014). Thus, global warming is expected to produce major changes in the marine environment, such as changes in the distribution and abundance of species and also changes in the structure of communities, including local extinctions (Harley et al. 2012, Turra et al. 2013, Ferreira et al. 2014). Hereof, recent studies show that climate change is a major threat to marine macroalgae (Wernberg et al. 2011, Harley et al. 2012, Ferreira et al. 2014).

Marine macroalgae are key components of benthic marine ecosystems and their abundance and diversity have fundamental implications for ecosystem services and life in the coastal zone (Dayton and Tegner 1984). Although some macroalgae species have shown high tolerance, or even have benefited from, the global warming increase in temperature tends to bring drastic changes to benthic communities (Mayer-Pinto et al. 2012). Abiotic ecological processes influence marine macroalgae, however, we still lack knowledge on how distinct temperatures promote population structure, geographic differentiation and acclimation/adaptation (Poloczanska et al. 2013).

The effects of temperature on chemical reactions, molecular structures, and physiology of algae are well documented (see Raven and Geider 1988, Davison et al. 1996), although not so well elucidated. The gaps in knowledge are attributed to the difficulty in isolating the factor temperature from other environmental factors (Oliveira et al. 2013). In most cases, due to effects on chemical and molecular levels, macroalgae are physiologically benefited by the increase in temperature (Davison 1991, Wang et al. 2012). The increase in temperature may show no differences on physiology of macroalgae that have their maximum yield close to their physiological limit, which can wrongly suggest toleration of such a situation (Davison 1991, Pearson and Davison 1996, Necchi 2004, Chaloub et al. 2010).

Species naturally exposed to a wider temperature range between summer and winter (temperate species) generally have a higher thermal tolerance when compared to individuals from environments with lower annual thermal amplitude (tropical species) (Padilla-Gamiño and Carpenter 2007). At a smaller scale, marine species that occur in habitats characterized by large temperature variations (*e.g.*, supra and mesolittoral) tend to live closer to their physiological temperature limits, so they may be more vulnerable to global warming than species less tolerant to temperature rising, such as those present on the infra-littoral (Stillman 2003, Ferreira et al. 2014). Under a background of global warming, organisms living close to their physiological limits are likely to be the first to be affected (*i.e.*, tropical species habiting mesolittoral, *e.g.*, species of the genus *Ulva*).

Ulva spp.

Among marine macroalgae, *Ulva* spp. is probably the most studied genus, due to its cosmopolitan distribution and easy collection (inhabiting the upper mesolittoral) (Joly 1965, Villaça et al. 2010). *Ulva* species have prominent ecological and economic importance such as applications in bioremediation (Neori et al. 1991, Vijayaraghavan and Joshi 2014, Oliveira et al. 2016), production of noxious blooms (Kong et al. 2011, Wang et al. 2011, Guidone and Thornber 2013), study of the bacterial-algae interaction (Provasoli and Pinter 1980), as bioindicators of eutrophication conditions (Kozhenkova et al. 2006), potential source of biofuels (Li et al. 2013), and as a source of food (Mabeau and Fleurence 1993). *Ulva* species also produce bioactive molecules with biomedical applications on cancer and other therapies (Ryu et al. 2013, Wang et al. 2013). Moreover, *Ulva* species are also often

used as a model organism in studies of photosystem II fluorescence, photochemistry, algal productivity (Beer et al. 2000, Longstaff et al. 2002, Liu et al. 2012), and temperature (Rautenberger and Bischof 2006, Chaloub et al. 2010, Teichberg et al. 2010).

Temperature is one of the most important factors in the metabolism of *Ulva* spp. (Steffensen 1976), where an increase of 5 °C showed to be harmful and an increase of 10 °C lethal (Steffensen 1976, Fortes and Lüning 1980). Thus, the optimal temperature for *Ulva* growth often coincides with the average temperature of the environment (Steffensen 1976, Fortes and Lüning 1980, Han and Choi 2005). The temperature decrease of 5 °C, albeit harmful, has been shown to stimulate reproduction in *Ulva fasciata* (Mohsen et al. 1972) and *U. pertusa* Kjellman (Han and Choi 2005). No spore release was observed when temperatures dropped by 10 °C (Han and Choi 2005). Mohsen et al. (1972) cultivated individuals of *U. fasciata* from the Mediterranean Sea, in laboratory under a temperature gradient of 15 - 35 °C and observed that: i) maximum growth occurred at 25 °C (same as local average); ii) a temperature drop of 10 °C promoted reduction of mass and total nitrogen; iii) the temperature of 35 °C was harmful; and iv) gamete formation occurred at 15 °C. Another work in which *U. fasciata* from India was cultured on a temperature gradient (15-35 °C), the maximum growth was observed between 25 °C and 30 °C (the local average was 25 °C), and the treatment at 35 °C was harmful (Mantri et al. 2011).

Although widely studied, the genus *Ulva* currently forms a large species complex. Two of the first molecular studies pointed out that Linneaus was right: the genus *Ulva* grouped with the genus *Enteromorpha* (until then distinct), and the two genera were merged (Hayden et al. 2003, Shimada et al. 2003). Shimada et al. (2003) when analyzing specimens from Japan, separated *U. fasciata* and *U. lactuca* Linneaus based on molecular data. Nevertheless, the existence of inconsistencies in the taxonomy of *U. lactuca* around the world was warned: many specimens that were referred to *U. lactuca* were receiving the wrong epithet while specimens belonging to the true *U. lactuca* would be erroneously receiving other epithets (Butler 2007). After this alert, O'Kelly et al. (2010) stated, based on molecular data, that *U. fasciata* from Hawaii (USA) should be referred to as *U. lactuca*. After Butler (2007) and O'Kelly et al. (2010), several articles considered both species (*U. fasciata* and *U. lactuca*) as a single entity (*U. lactuca*). Concomitantly, two papers using on molecular data indicated probable taxonomic errors regarding *U. lactuca* in Australia, (Kraft et al.

2010, Kirkendale et al. 2013). However, in a later review, Comarci et al. (2014) based on Butler (2007) and O'Kelly et al. (2010) suggested that only *U. fasciata* from Hawaii should be referred to *U. lactuca*, and therefore, while new studies are not published, *U. fasciata* and *U. lactuca* should be considered as two distinct and valid species. It is noteworthy that *U. fasciata* and *U. lactuca* can co-occur in the same site in Brazil (Yoneshigue 1985).

Ulva fasciata Delile has isomorphic diplobiont life history (Figure 1.1) and the phases show similar physiological performance (Beach et al. 1995, Wichard 2015). Diploid sporophytes produce haploid zoospores by meiosis. These zoospores, when under favorable conditions, migrate towards the substrate, where will settle and give rise to gametophytes that produce gametes by mitosis. After the fertilization of gametes and formation of the zygote, the sporophyte will be generated, restarting the historic (Beach et al. 1995, Wichard 2015). *U. fasciata* has simple morphology (two thin layers of cells), abundance and global distribution, including the whole Brazilian coast (Joly 1965, Kraft et al. 2010, Villaça et al. 2010). Because of these characteristics, the species can be used as a model in physiological studies that take into account factors such as temperature.

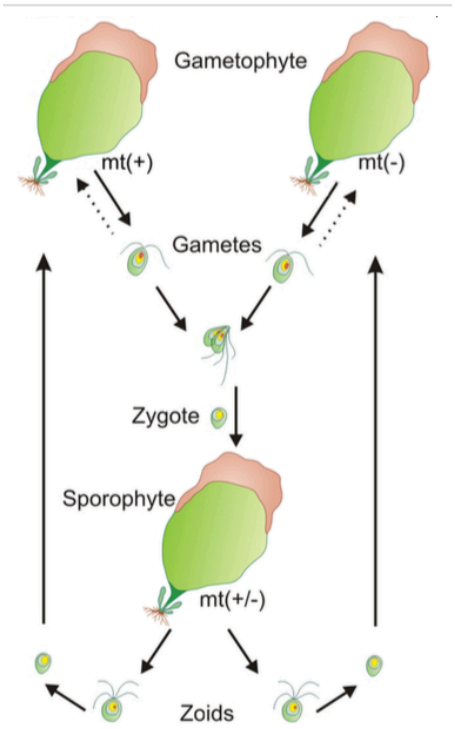

Figure 1.1: The isomorphic diplobiont life history of *Ulva* spp.. The green color of the thalli represents vegetative region; the brownish color represents a fertile region. Dashed arrows indicate rare parthenogenic events. (+) and (−) indicate mating types (mt) (Wichard 2015).

Differences in physiological responses of a species and its populations may result from processes of acclimation or adaptation. However, physiological studies in natural populations alone, do not allow the distinction between these processes, since several environmental variables could mask possible conclusions about the effects of certain abiotic factors (Plastino and Guimarães 2001, Ferreira et al. 2014). Therefore, to study populations in nature together to laboratory-controlled temperature variation is crucial to determine patterns of physiological response by individuals from different populations in response to increase temperature of 4 °C. The data obtained in this study, using *Ulva fasciata* as a model organism, should help better predict the effects of rising temperatures on the future of marine communities under a global warming scenarios.

OBJECTIVES

This study aimed to analyze the effect of temperature on individuals from two tropical populations of *Ulva fasciata* occurring in thermally distinct environments.

Although both regions are located in a tropical environment, one is naturally colder than the other due to a coastal deep-water upwelling phenomenon. By analyzing populations occurring under different thermal conditions, we intend to investigate their local acclimation and adaptation to distinct climate scenarios.

SPECIFIC OBJECTIVES

- Confirm that *Ulva fasciata* individuals from two Brazilian coast regions are the same species;
- Identify *in situ* physiological differences between two populations with different thermal characteristics, based on photosynthetic performance and pigment content;
- Evaluate growth rates, chlorophyll *a* fluorescence and pigment content of specimens of two populations when exposed to a temperature gradient (16 - 31 °C) under laboratorial controlled conditions; and
- Evaluate the effects caused by a sudden temperature increase (simulated heat wave) and the recoverability of specimens from distinct populations, considering growth rates and maximum quantum yield as dependent variables.

HYPOTHESES

- Individuals of *Ulva fasciata* from two populations with distinct thermal characteristics have different photosynthetic performances and pigment content when evaluated in the field;
- Individuals of *Ulva fasciata* from two distinct populations with distinct thermal characteristics respond differently when subjected to the same controlled conditions in the laboratory, characterizing themselves as ecotypes.

GENERAL APPROACHES

Specimens

Ulva fasciata was collected on the upper mesolittoral zone, at two thermally distinct sites on the Rio de Janeiro State (RJ) coast, Brazil (Niterói and Arraial do Cabo). Five transects, three meter long each, were placed perpendicularly to the coastline, three meters away from each other and over the target population. On each transect one healthy individual was collected from four randomly selected points

totaling 20 specimens per population. After macroscopic epibionts were removed, thalli were transported to the laboratory in seawater soaked paper inside a thermal box. Voucher specimens from Niterói and Arraial do Cabo populations were deposited in the herbarium of the Institute of Bioscience, University of São Paulo (SPF-57878 and SPF-57877, respectively).

Niterói site (NI)

The NI population refers to the Itacoatiara beach (22°58' S and 43°02' W) and was sampled on 5th February 2015 and then on 8th February 2016. Samples from 2015 were used in laboratory experiments while samples collected in 2016 were sued to obtain field data. Itacoatiara beach has very low anthropogenic impact including the absence of any nearby sewage influence (Carneiro et al. 1987, Teixeira et al. 1987, Catanzaro et al. 2004). However, small levels of localized impact by sunbathers are occasionally observed, mainly during the summer. The sea surface temperature ranges from 21 to 28 °C along the year, averaging 24 °C (Marazzo and Nogueira 1996, Catanzaro et al. 2004). This site is characterized as a non-upwelling region.

Arraial do Cabo site (AC)

The AC population refers to Prainha beach (22°58' S and 42°02' W) and was sampled on 4th February 2015 and on 7th February 2016. Samples collected in 2015 were used in laboratory experiments while collections from 2016 were used to obtain field data. The region is characterized by the occurrence of the southeastern Brazil coastal upwelling phenomenon (Valentin et al. 1987). This upwelling is a result of the combination of northeast winds, the proximity of the continental shelf break, an abrupt change in coastline, and also the Earth rotation itself (Valentin et al. 1987). Low temperatures and high amounts of nutrients characterize the upwelled waters. When the upwelling phenomenon is on its maximum (January-March) sea surface temperatures reach values as low as 15 °C. The maximum sea surface temperature in the AC site is 28 °C and the annual average is 20 °C (Guimaraens and Coutinho 1996, 2000). The algal collection in this site occurred during the austral summer when upwelling is the strongest (Valentin et al. 1987).

General culture conditions

Unialgal non-axenic cultures of *U. fasciata* were obtained for five haphazardly selected individual from the 20 individuals sampled per site following Plastino and Oliveira (1990) protocol. They were cultivated in 500 ml Erlenmeyer flasks filled with 500 ml of a modified Von Stosch medium (Ursi and Plastino 2001) concentrated to 200 % in sterile seawater (32 ppt salinity) for all algal cultures. The medium was renewed weekly and the cultures were not aerated. Specimens were kept in a temperature-controlled room at 24 ± 1 °C with a photoperiod of 14 hours. Photosynthetically active radiation (PAR) was kept at 70 µmol photons $m^{-2}s^{-1}$ provided by Osram 40 W daylight fluorescent tubes and measured with an spherical Li-COR sensor (model L1-193) connected to a quantameter (Li-COR, model L1-185). The 5-individual macroalgal cultures were acclimated for 6 months in laboratory under the general culture conditions before the beginning of the experiment.

Physiological indexes

Growth rates (GR)

Thalli were blotted with paper towel prior to weighting to remove excess seawater. Growth rates were calculated according to Mtolera et al. (1995)'s equation recognized to contain the smallest intrinsic error for macroalgae (Yong et al. 2013):

$$GR\ (\%\ day^{-1}) = \left[\left(\frac{M_f}{M_i}\right)^{\frac{1}{t}} - 1\right] * 100$$

where *GR* is growth rate, *Mf* is the final mass, *Mi* is the initial mass, and *t* is the time. Macroalga fresh mass was calculated in grams in a digital scale with four decimals.

***In vivo* Chlorophyll *a* fluorescence**

In vivo chlorophyll *a* fluorescence measurements were performed using an underwater Walz Diving-PAM fluorometer. Dark-acclimated measurements (*i.e.*, maximum quantum yield; Fv/Fm) were performed in thalli dark-acclimated for 15 minutes inside falcon tubes wrapped in aluminum foil (Schreiber et al. 1995).

Pigment content

In the laboratory, 10 mg (fresh weight) samples were washed in distilled water; paper blotted to remove water excess, and stored in Eppendorffs tubes at -80

°C for later use. Pigment characterization was performed by means of extraction, absorbance curve analysis, and quantification of the pigments chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and total carotenoids.

For pigment extraction, 2 mL of N, N-dimethylformamide (DMF 99.8%) were added to each Eppendorf's tubes containing the samples, followed by incubation at 4 °C in the dark for 24 hours. Subsequently, the tubes were centrifuged at 19,000 g for 20 minutes at 4 °C. The supernatant containing chl *a*, *b* and total carotenoids were transferred to cuvettes for spectrophotometer reading (Wellburn 1994). Absorption spectra were obtained on a Hewlett Packard 8452A UV - Visible Spectrophotometer or Spectrophotometer UV-VIS EPOCH (Biotek Instruments, EUA) using 10 mm optical path quartz cuvettes. The calibration of the apparatus and sample blanks were performed with pure DMF (99.8%). The absorption spectrum was recorded between 400 to 700 nm. chl *a* and *b* concentrations were calculated from the equation described by Inskeep and Bloom (1985) and the total carotenoids concentration was obtained using Wellburn (1994) equations:

Chl *a* ($\mu g/g^{-1}$ FM) = [(12.70 * A_{664}) - (3.11 * A_{647})] * [V(ml) / FM(g)] (Inskeep and Bloom 1985)

Chl *b* ($\mu g/g^{-1}$ FM) = [(20.78 * A_{647}) - (4.88 * A_{664})] * [V(ml) / FM(g)] (Inskeep and Bloom 1985)

Total carotenoids ($\mu g/g^{-1}$ FM) = [(A_{480} * 1000) - (1.12 * chl *a*) - (34.07 * chl *b*)] * [V(ml) / FM(g)] (Wellburn 1994)

where, FM = fresh mass and A = absorbance.

Statistics

Normality and homogeneity assumptions of variances were tested using Shapiro-Wilk and modified robust Brown-Forsythe Levene-type test, respectively (Zar 1999). Analysis of variance (ANOVA) with type-3 sum of squares was used to test the factors effects. ANOVA was used even if the data failed the Shapiro-Wilk normality test, considering that parametric tests usually have more statistical power

than nonparametric tests, and ANOVA is robust against some deviations from normality (Schmider et al. 2010). During laboratory experimental tests, some replicates evidenced fertile thalli, thus, logistic regression (function *glm* in R) was performed, on the presence/absence of fertility activity data, to test the effect of the factors temperature and population on reproductive activity. In all cases the Student Newman–Keuls (SNK) test was used as a *post hoc* test whenever a significant difference between means has been detected in the ANOVA. The SNK test was also used even on non-parametric data because the test is also quite robust to violations of normality, although the assumptions are essentially the same as for an independent groups t-tests (normality, homogeneity of variance, and independent observations; Muth 2014). Statistical analyses were done in R (RCoreTeam 2014) adopting an $p < 0.05$.

Chapter 2

In situ chlorophyll *a* fluorescence and pigment content of *Ulva fasciata* Delile (Ulvales, Chlorophyta) from Brazilian thermally distinct sites.

Fluorescência da clorofila *a in situ* e conteúdo pigmentar de *Ulva fasciata* Delile (Ulvales, Chlorophyta) de dois locais termicamente distintos da costa brasileira.

Abstract: In macroalgae, abiotic factors such as solar radiation, temperature, and nutrient availability have been referred to as the main factors responsible for physiological process shifts such as photosynthetic rates and pigment levels. Analyses of physiological characteristics performed among species or within the same species, as in distinct populations, allow the knowledge of the functional diversity of macroalgae. *Ulva fasciata* is a cosmopolitan species that occur in the whole Brazilian coast. This study was performed in two regions of Rio de Janeiro State (RJ) coast. Although both sites are located in a tropical region, Arraial do Cabo/RJ is naturally colder than Niterói/RJ due to the deep-water upwelling effect. We hypothesized that *U. fasciata* populations grown at thermally different locations would present distinct *in situ* ecophysiological responses. Therefore, this work aimed to (i) confirm that *U. fasciata* individuals belongs the same species; and (ii) evaluate photosynthesis chlorophyll *a* fluorescence-derived parameters and pigment contents (chlorophyll *a* and *b* and total carotenoids) of these individuals in nature. All ten Brazilian samples (five from each population) formed a single well-supported clade (bootstrap = 86 %) together with public sequences previously identified as *U. fasciata* from Australia and Italy. The molecular data obtained in this study allow us stating that the two populations belong to the same species. No differences of the fluorescence-derived factors were observed between individuals from the two populations, however, the analysis of pigment contents evidenced that individuals from the population of Niterói (warmer site) had higher concentrations of chlorophyll *a* than individuals from Arraial do Cabo (colder site). Our hypothesis that populations grown at locations with different thermal regimes would present distinct ecophysiological responses was not

rejected, due to differences in chlorophyll *a* content; however, no differences could be detected for fluorescence-derived parameters. We did the experiments when the abiotic differences between collecting sites were more accentuated (*i.e.*, when the upwelling phenomenon was at its peak, in January). Therefore we expected to detect more abrupt differences between populations. Thus, further studies considering controlled laboratory conditions should be done to better interpret the role of temperature on these populations.

Resumo: Em macroalgas, fatores abióticos como irradiação solar, temperatura e disponibilidade de nutrientes, têm sido referidos como os principais fatores responsáveis nos processos de mudanças fisiológicas, como as taxas fotossintetizantes e a quantidade de pigmentos. Análises de características fisiológicas realizadas entre espécies ou dentro de uma mesma espécie, mas em populações distintas, permitem o entendimento da diversidade funcional das macroalgas. *Ulva fasciata* é uma espécie cosmopolita que ocorre em toda a costa brasileira. Esse estudo foi realizado em duas regiões da costa do Estado do Rio de Janeiro (RJ). Apesar de ambas regiões estarem localizadas nos trópicos, Arraial do Cabo/RJ é naturalmente mais fria do que Niterói/RJ devido ao efeito de ressurgência de águas profundas. Nossa hipótese era de que as populações de *U. fasciata* que crescem em locais termicamente diferentes iriam apresentar respostas ecofisiológicas distintas *in situ.* Dessa forma, esse trabalho objetivou: (i) confirmar que indivíduos de *U. fasciata* das duas populações selecionadas pertencem à mesma espécie; e (ii) avaliar parâmetros fotossintetizantes derivados de fluorescência da clorofila *a* e conteúdo pigmentar (clorofila *a,* clorofila *b* e carotenoides totais) desses indivíduos, na natureza. Todas as dez sequências da costa brasileira (cinco de cada população) formaram um único clado (bootstrap = 86 %), juntamente com sequências previamente identificadas como *U. fasciata* da Itália de Austrália. Os dados moleculares nos permite afirmar que as duas populações pertencem à mesma espécie. Não foram identificadas diferenças nos parâmetros derivados de fluorescência, contudo, as análises do conteúdo pigmentar evidenciaram que indivíduos de Niterói (região mais quente) possuíam maiores concentrações de clorofila *a* quando comparados aos de Arraial do Cabo (região mais fria). Nossa hipótese de que populações que ocorrem em localidades com regimes térmicos diferentes iriam apresentar respostas fisiológicas distintas não foi rejeitada, devido à diferença no conteúdo de clorofila *a;* contudo, não foram identificadas diferenças em

parâmetros derivados da fluorescência. Nós realizamos os experimentos quando as diferenças abióticas entre os dois locais de coleta eram mais acentuadas (*i.e.*, quando o fenômeno da ressurgência está no máximo – Janeiro). Desse modo, nós esperávamos encontrar diferenças mais drásticas entre as populações. Sendo assim, estudos futuros, considerando condições controladas de laboratório, devem ser realizados para melhor interpretar o papel da temperatura nessas populações.

Key words: carotenoids, chlorophyll *a,* chlorophyll *b,* effective quantum yield, fluorescence, PAM, temperature, upwelling.

Palavras-chave: carotenoides, clorofila *a*, clorofila *b*, fluorescência, PAM, rendimento quântico efetivo, ressurgência, temperatura.

INTRODUCTION

Photosynthesis is the biological reaction that converts light into chemical energy, and the main way in which the carbon fixation and storage is guided into organic compounds (Ballottari et al. 2012, Falkowski and Raven 2013). Photosynthesis is also regarded the most important indicator of physiological adaptation, stress, and performance in plants - for both terrestrial and marine taxa (Maxwell and Johnson 2000, Carr and Bjork 2003). Consequently, measurements of photosynthetic efficiency and photosynthetic rates are two long-standing endeavors in plant ecological and physiological research (Longstaff et al. 2002).

Measurements of macroalgal photosynthetic rates in the laboratory have been usually determined by assessing oxygen (O_2) or carbon dioxide (CO_2) flux using metabolic chambers (Maxwell and Johnson 2000). However, the development of a wide variety of fluorescence-based techniques, allowed the precisely and easily measuring photosynthetic assessments in the both laboratory and field (Baker 2008, Klughammer and Schreiber 2008). The development of methods to measure chlorophyll *a* fluorescence via pulse amplitude modulation (PAM) techniques provide non-destructive, fast, and real time results (Baker 2008, Chaloub et al. 2010).

In macroalgae, seasonal changes in abiotic factors such as solar radiation, temperature, and nutrient availability have been recognized as the main factors responsible driving biochemical or physiological processes such as photosynthetic rates and pigment content. Physiological changes occur as a mechanism to protect the organism's vital machinery and reestablish homeostasis (Kain 1989, Franklin and Forster 1997, Cruces et al. 2012). Pigment content can give fundamental information on the light harvesting capacity of the algae, however, pigment accumulation and light harvesting capacity are usually uncoupled (Cordi et al. 1997). For example, irradiance-dependent changes in pigmentation are known to be acclimation process: more pigments are allocated to antenna complexes at low growth irradiance to compensate the reduced light (Duke et al. 1986, Demmig-Adams and Adams 1992, Häder and Figueroa 1997).

Physiological variations may occur in the same population or between different populations (Innes 1984) due to differences in either distinct physiological acclimation or evolutionary adaptation (Plastino and Guimarães 2001). Temperature ecotypes have been described for *Urospora penicilliformis* (Roth) Areschoug from artic populations (Bischoff and Wiencke 1995), where cold-temperate strains had

relatively higher optimal growth rates than polar strains. In *Valonia utricularis* (Roth) C.Agardh, temperature-related ecotypes were identified after comparing individuals from Mediterranean Sea (low temperatures) with individuals from tropical India (higher temperatures) (Eggert et al. 2003). Irradiance ecotypes have been identified along the Brazilian coast for *Gracilaria* spp. (Ursi et al. 2003, 2013, Araújo et al. 2014, Ayres-Ostrock and Plastino 2014a, 2014b). Photosynthesis can be used to detect ecotypes (*e.g.*, Eggert et al. 2003) that is, isolated populations on the path to speciation (Lowry 2012). This work firstly aimed to confirm that *Ulva fasciata* individuals from two populations of the Brazilian coast regions are of the same species. Then, aimed at characterizing these individuals of *U. fasciata* from both populations with different thermal characteristics, evaluating *in situ* photosynthetic performance and pigment content (chlorophyll *a* and *b* and total carotenoids). Although both regions are located in the tropics, one is naturally colder than the other due to a deep-water upwelling phenomenon. Our expectation was that *U. fasciata* populations grown at thermally different locations would present distinct *in situ* ecophysiological responses, concerning photosynthetic performances and pigment content.

MATERIAL AND METHODS

Specimens

Ulva fasciata specimens were collected from two different populations situated on the Rio de Janeiro State, Brazil: Niterói, and Arraial do Cabo. Sampling occurred at the mesolittoral. Five transects set perpendicular to the coastline were assigned to each target population. Three meter long transects were spaced three meters apart from each other. On each transect four specimens were collected from four randomized points, thus, 20 individuals were randomly collected from each population. Collections occurred in 8th February 2016 in Niterói and 7th February 2016 in Arraial do Cabo. The monthly average sea surface temperature before sampling (*i.e.*, January 2016) was 27.03 ± 0.35 °C and 24.02 ± 0.35 °C for Niterói and Arraial do Cabo, respectively. Satellite data used to obtain sea surface temperature was the Visible Infrared Imaging Radiometer Suite (VIIRS) operated by NASA.

Molecular taxonomy

Ten (five from each population) haphazardly selected specimens from the original pool of 20 were used for molecular analysis. DNA was extracted from 50 mg of fresh thallus pulverized with liquid nitrogen inside an Eppendorf's microtubes using a plastic pestle. DNA extraction protocol followed a modified Chelex resin extraction of Goff and Moon (1993) where 350 ml of Chelex resin was added and mixed to the pulverized algal material, heated to 90 °C for 10 minutes, then transferred at room temperature for 10 minutes, followed by an incubation in ice for 15 minutes. After that, the material was centrifuged at 13,000 g at 4 °C for five minutes. The supernatant containing the isolated DNA was collected and stored at -80 °C for further manipulations.

The plastid *tuf*A gene was amplified with the primer pairs *tuf*AF and *tuf*AR from (Famà et al. 2002). PCR was executed in 50 μL reactions using 37.25 μL of milli-Q autoclaved water, 5 μL of 10x PCR buffer, 1.5 μL 50mM $MgCl_2$, 1μL of dNTP, 1 μl of 0.2 μM each primer, 3 μL of total DNA, and 1.25 μL of *Taq* DNA polymerase (1.25 U; Promega Corp., Madison, WI, USA). Amplification reactions were performed in a Techne TC-4000 termocycler (Bibby Scientific Ltd., Staffordshire, UK). PCR products were purified using the Illustra GFX^{TM} PCR DNA and Gel Band Purification (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Purified amplicons were sequenced in both directions using the same primers mentioned above and the BigDye^{TM} Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, USA) on ABI PRISM^{TM} 3100 or ABI PRISM^{TM} 3730 Genetic Analyzer (Applied Biosystems) available in the Botany Department, USP.

We performed maximum likelihood to estimate the phylogenetic relationship of *U. fasciata* tuf*A* sequences. Levels of intra-population genetic diversity were assessed by calculating total number of segregating sites, number of haplotypes, haplotype diversity, and nucleotide diversity. Tajima's D (Tajima 1989) neutrality tests was implemented to detect the presence of selective sweeps or population expansion. These analyses were performed using DnaSP v5.10.01 (Librado and Rozas 2009).

Chlorophyll *a in vivo* fluorescence

From the original pool of 20 selected individuals, another randomization event was performed to pick 10 specimens for fluorescence measurements. Fluorescence was measured using the Walz Diving-PAM underwater fluorometer with an optical fiber model Diving-F, and a magnetic sample holder. Photosynthetic performance was estimated by relative electron transport rate (rETR) and derived fluorescence-based parameters (Genty et al. 1989, Runcie and Durako 2004). The relative electron transport rate (rETR) was calculated following the formula (Genty et al. 1989):

$$rETR = Y(II) * PAR * 0.5$$

where Y(II) is the photosystem II effective quantum yield; PAR is the photosynthetically active radiation to increasing light pulses and; 0.5 considered an equal share of energy between PSII and PSI (Gilbert et al. 2000). Relative ETR (rETR) values, *i.e.*, ETR calculations without the tissue light absorption at PAR range ($A_L(PAR)$) components of equation, cannot be compared with data outside the respective study (Martins et al. 2014).

Rapid light curve (RLC) protocol was implemented to obtain the rETR, which comprises the calculation of ETR values along a pre-programmed set of increasing short-time irradiance (PAR) values, obtaining photosynthesizing quantum yield measurements at the end of each step (White and Critchley 1999). Twelve irradiance steps were implemented: 0, 6, 21, 44, 81, 123, 172, 258, 357, 547, 815, 1,219, and 2,048 μmol photons.$m^{-2}s^{-1}$. The light exposure time at each irradiance step was 20 seconds. The Photosynthesis vs. Irradiance (PI) curves were generated based on the values of rETR. The photosynthesizing efficiency (alpha; α), maximum photosynthesis (ETRmax), light saturation (Ik), effective quantum yield (Y(II)), and the photoinhibition (betta; β) parameters were calculated following Jassby and Platt (1976) and Platt et al. (1980)'s equations:

$$P = ETRmax * \tanh (\alpha * I / Pmax)$$ (Jassby and Platt 1976)

$$P = PS * [1 - \exp (-\alpha * I / PS)] * [\exp (-\beta * I / PS)]$$ (Platt et al. 1980)

ETRmax = PS * [α / (α + β)] * [β / (α + β)] ^ β/α (Platt et al. 1980)

where, P = photosynthesis; ETRmax = maximum ETR; tanh= hyperbolic tangent; PS= photosynthesis saturation; α = photosynthetic efficiency; β = photoinhibition, and I = irradiance. Each sample was adjusted by Jassby and Platt (1976)'s equation (without photoinhibition) and the Platt et al. (1980)'s equation (with photoinhibition). The parameters were then, calculated, with the curve with highest R-square value.

Pigments content

For pigment quantification, from the original pool of 20 specimens 8 clean, epibiont-free, and healthy individuals were selected, and transported to the laboratory. Pigment characterization was performed by means of extraction, absorbance curve analysis, and quantification of chlorophyll *a* and *b*, and total carotenoids.

Statistics

Pigment content and PAM fluorescence parameters were first tested for parametricity using the Shapiro-Wilk test and for homocedasticity (Levene Test). Pigment content and each of the PAM fluorescence parameters was tested by SNK, adopting the pigment content and fluorescence-derived parameters as dependent variable and populations as independent variable. All analyses were performed using R (RCoreTeam 2014) and adopting $p < 0.05$ as significance level.

RESULTS

Molecular taxonomy

Levels of pairwise genetic diversity varied between 0.00 to 1.4 % and 0.00 to 2.7 % in NI and AC populations, respectively. All ten Brazilian samples (five from each population) form a single well-supported clade (bootstrap = 86 %) together with public sequences previously identified as *Ulva fasciata* from Australia and Italy (Figure 1.2). Number of haplotypes found within each population varied from 2 in NI to five in AC. Overall pairwise genetic diversity among all 10 samples varied between 0.3 - 2.7 % with a total of 6 haplotypes. Tajima's D produced a non-significant negative values (*i.e.,* effectively D is not different from zero) for both populations confirming that the populations are in equilibrium.

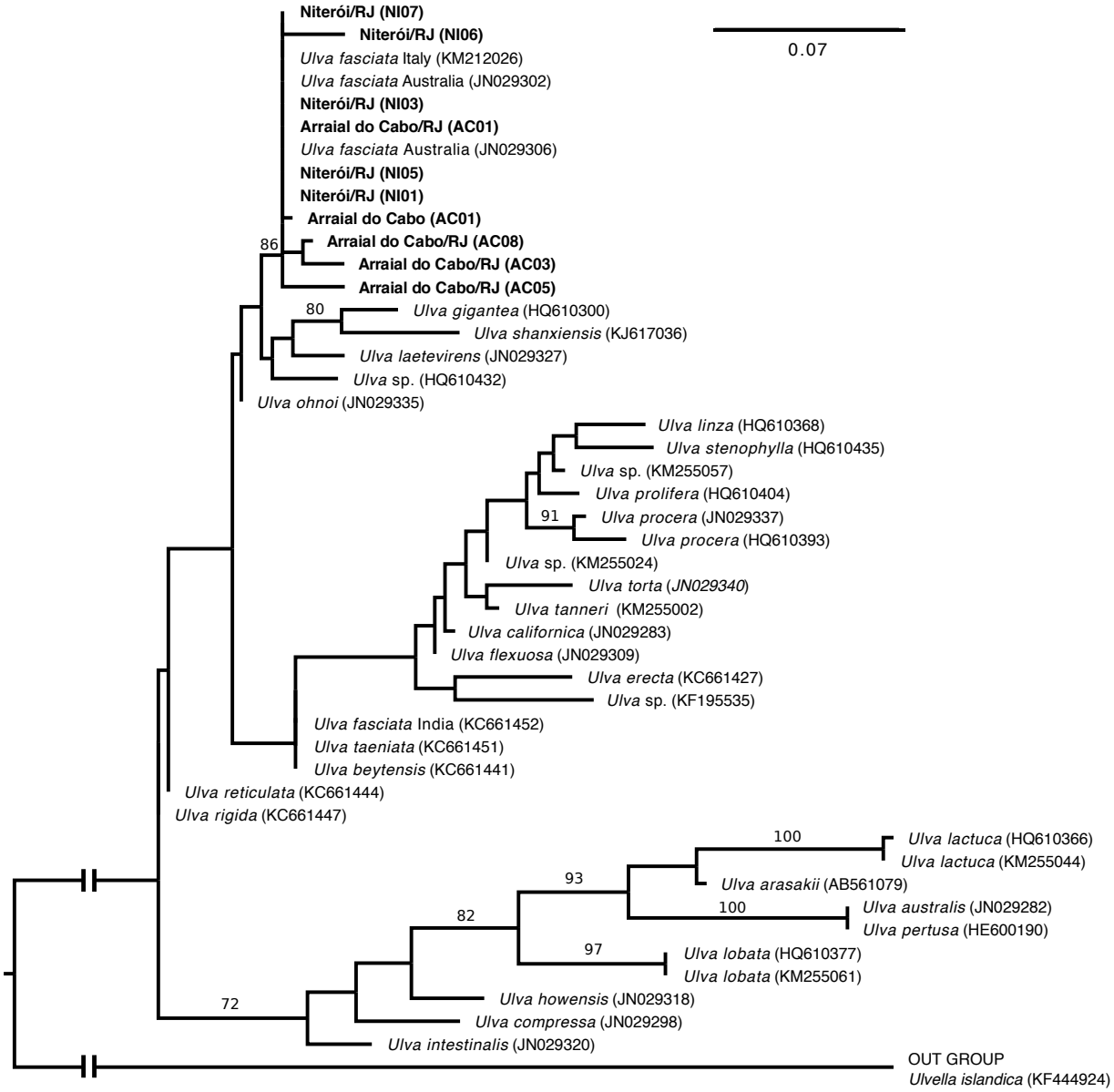

Figure 1.2: Maximum likelihood phylogenetic tree based on tuf*A* DNA sequences of *Ulva* spp. (Chlorophyta). For the construction of this tree, a sequence of each species of *Ulva* deposited in GenBank and all sequences generated in this study were used. Out group = *Ulvella islandica*. The bootstrap values (> 70) are shown over the branches. GenBank codes cited between brackets. Bold indicates newly generated sequences.

Fluorescence data

Data did not pass the normality test ($p > 0.05$) but passed the homogeneity test ($p > 0.05$). No statistical differences in fluorescence-derived parameters were

observed between the two populations of *U. fasciata* (SNK tests, $p > 0.05$), *i.e.*, rETRmax, alpha, Ik, Y(II) and betta (Table 2.1). For the individuals from NI, 70% of the samples obtained higher R-square for Platt et al. (1981) equation, *i.e.*, with photoinhibition. Similar pattern was observed for individuals from AC, where 22% evidenced photoinhibition. Note that one sample from AC was not considered due to technical problem. Betta average did not take into account those without photoinhibition (Table 2.1).

Pigments content

Pigment content (Table 2.2) showed differences on chl *a* content between Niterói and Arraial do Cabo populations (SNK test, $p < 0.01$), although no differences could be detected between chl *b* and total carotenoid content (SNK tests, $p > 0.05$).

Table 2.1: Mean and standard deviations of fluorescence-derived parameters for individuals of *Ulva fasciata* from two populations, Niterói/RJ (n = 10) and Arraial do Cabo/RJ (n = 9), Brazil. Collection occurred in 8th February 2016 in Niterói and 7th February 2016 in Arraial do Cabo. Different letters indicate statistically significant difference ($p < 0.05$).

PAM fluorescence parameters	**Niterói**	**Arraial do Cabo**
rETRmax	330 ± 126^{a}	244 ± 119^{a}
Alpha (α)	$0.29 \pm 0.06^{\mathbf{a}}$	$0.28 \pm 0.10^{\mathbf{a}}$
Ik	1194 ± 575^{a}	859 ± 200^{a}
Y(II)	$0.744 \pm 0.040^{\underline{a}}$	$0.618 \pm 0.230^{\underline{a}}$
Betta (β)	$0.123 \pm 0.120^{\boldsymbol{a}}$	$0.083 \pm 0.040^{\boldsymbol{a}}$

Table 2.2: Mean and standard deviations of pigments for individuals of *Ulva fasciata* (n = 10) from two populations, Niterói/RJ and Arraial do Cabo/RJ, Brazil. Collection occurred in 8th February 2016 in Niterói and 7th February 2016 in Arraial do Cabo. Different letters indicate statistically significant difference ($p < 0.05$).

Pigments ($\mu g.g^{-1}$ FW)	**Niterói**	**Arraial do Cabo**
Chlorophyll *a*	668 ± 251^{a}	464 ± 193^{b}
Chlorophyll *b*	$422 \pm 242^{\mathbf{a}}$	$463 \pm 122^{\mathbf{a}}$
Total carotenoids	120 ± 70^{a}	97 ± 30^{a}

DISCUSSION

To our knowledge, there are no public available *U. fasciata* DNA sequences from the type locality (Egypt, Mediterranean Sea) (Guiry and Guiry 2016). The lack of DNA sequences from the type locality casts doubts about the species' true identity. Consequently, more studies are needed to confirm if the Brazilian populations that have been morphologically treated as *U. fasciata* (*e.g.*, Joly 1965, Yoneshigue 1985) match the true *U. fasciata*. However, the data obtained in this study allow us stating that the two populations belong to the same species. *Tuf*A marker has been used as barcode for Chlorophyta (Famà et al. 2002). We chose this marked because our main aim was to state if the studied populations belong to the same species; and this was not rejected. Our intra- and inter-population genetic analyses revealed that the number of haplotypes and mutations found in the Arraial do Cabo populations was twice larger than those found for Niterói. However, the differences between populations are not great enough to propose population differentiation, suggesting panmixia (Tajima's D is not different from zero).

Individuals of *U. fasciata* from NI (warmer site) had 30.5% more chl *a* than individuals from AC (colder site). However, both populations output the same photosynthetic efficiencies. Our results are not in agreement to what was observed for two *Ulva* species (*U. prolifera* O.F.Müller and *U. intestinalis* Linnaeus), in which species with more pigment content (*U. prolifera*) showed higher values of maximum quantum yield (Wang et al. 2012). Moreover, the highest chl *a* content in *U. fasciata* from warmer site is opposite to what was described for three brown alga canopy-forming species (*Ecklonia radiata* (C.Agardh) J.Agardh, *Scytothalia dorycarpa* (Turner) Greville, and *Sargassum fallax* Sonder) collected at warmer and colder locations in western Australia (Wernberg et al. 2016). The authors observed inter-population pigment content differences for the same species showing lower chl *a* content in warmer regions, and these differences resulted in differences in fluorescence parameters.

A possible explanation to the differences in chl *a* might be due to the characteristic of upwelled waters. We have detected higher chl *a* concentrations in individuals of *U. fasciata* from NI (non-upwelling site) than in individuals from AC (upwelling site). Upwelled waters are also characterized by low turbidity in addition to temperature and nutrient aspect (Valentin et al. 1987), resulting in higher solar irradiance on benthic organisms while submerged, when comparing to non-upwelling

sites. Our results corroborate previous irradiance experiments in the field (Ramus et al. 1976a, 1976b, 1977) and in the laboratory, which have also demonstrated an inverse correlation between thallus pigment content and incident irradiance (Lapointe 1981, Lapointe and Duke 1984, Lapointe et al. 1984, Duke et al. 1986).

In this study, the differences in pigment content of *U. fasciata* did not result in differences in fluorescence-derived parameters (rETRmax, alpha, Ik, Y(II), and betta) suggesting that the two populations of *U. fasciata* were photosynthetically acclimated to local field conditions, at the moment of collection. Similar results were observed for *U. rotundata* Bliding - *armoricana* P.Dion, B.deReviers & G.Coat species complex in which was found similar values of fluorescence-derived parameters in three distinct sites along the Atlantic coast of France (Merceron et al. 2007). In addition, several studies have observed similar values of maximum photosynthesis (obtained from oxygen evolution) for a range of *Ulva* species exposed to several distinct abiotic conditions, such as wave protected/exposed, shallow (< 3 meters deep) and deep (> 3 – 10 meters deep) waters and latitudinal/longitudinal distant populations (*e.g.*, Menéndez et al. 2001, Brush and Nixon 2003, Han et al. 2003, Plus et al. 2005, Merceron et al. 2007). Our findings together with data observed in the literature, suggest that maximum photosynthesis in *Ulva* spp. seems to be closely related and widespread similar throughout the genus for a wide variety of abiotic conditions.

Similarities of fluorescence parameters between the two populations of *U. fasciata* could be also explained by their close geographic location, such as similar latitude, *i.e.*, similar solar irradiance when exposed to air. In macroalgae exposed to extreme abiotic fluctuations and conditions, especially those occurring in mesolittoral in tropical areas, the photoperiod is a determinant of photosynthesis performance (Henley and Ramus 1989, Plastino and Oliveira 2002). *Ulva* spp. are able to adjust their chlorophyll content to irradiance variations linked to the location and the season of year (Merceron et al. 2007). Hence, at the moment of our fluorescence measurements, solar irradiance or microhabitat parameters might have had more influence on photosynthesis performance than other biotic or abiotic factors such as temperature. The capability of acclimation to abiotic condition can mask possible physiological differences.

Another abiotic factor that could be acting in *U. fasciata* is the nutrient concentration, since individuals from Arraial do Cabo (upwelling site) are exposed to

larger amount of nutrients along the year when comparing to Niterói (non-upwelling site) (Valentin et al. 1987). The differences in chl *a* then, could be due to nutrient variation between sites. At the moment of collection, however, the individuals from Arraial do Cabo were visually bleacher than individuals from Niterói, suggesting that there should have occurred a local event in Arraial do Cabo that resulted in such observation. An explanation could be temperature (upwelling). The decrease in temperature is a strong abiotic signal to fertility, and then vegetative cells are transformed in reproductive cells (Mohsen et al. 1972, Steffensen 1976, Beach et al. 1995, Mantri et al. 2011, Wichard 2015). Fertility often results in bleached cells (Wichard 2015). However, further studies are needed to better understand the effects of abiotic factors on both populations of *U. fasciata* including details of *in situ* microhabitat variation along all seasons of the year and, perhaps in consecutive years. In example, Khairy and El-Shafay (2013) observed seasonal variability in the biochemical composition of three macroalgal species from Alexandria coast, where they detected more protein and lipid during spring and more carbohydrate during the summer, for *U. lactuca* Linnaeus. Although having information on how pigment content varies along the year would have been beneficial to our study, here we focused on detecting differences during a specific time of the year, when temperatures differences between sites were at their largest, considering the upwelling phenomenon.

In the present study, we hypothesized that populations grown at locations with different thermal climate regimes would present distinct ecophysiological responses. Our hypothesis was not rejected, although no differences could be detected for fluorescence-derived parameters. The existence of baseline data on how the dependent variable herein measured varies across the year should be next phase in research. We have examined only once, however, our collections occurred when the upwelling phenomenon was at its peak (*i.e.*, January). Therefore if there were physiological differences between these two populations, those differences were supposed to be more evident during our collecting period. Thus, further studies considering controlled laboratory conditions should be done to better interpret the role of temperature on these populations.

Chapter 3

Effects of temperature and simulated heat wave on physiology of *Ulva fasciata* Delile (Ulvales, Chlorophyta) from Brazilian thermally distinct sites.

Efeitos da temperatura e simulação de onda de calor na fisiologia de *Ulva fasciata* Delile (Ulvales, Chlorophyta) de dois locais termicamente distintos da costa brasileira.

Abstract: One example of extreme events is the marine heat waves, which have been observed around the world. As consequence, climate change is a major threat to several species of macroalgae, many of which play crucial roles in several marine habitats. This study aimed to analyze the effects of temperature on growth rates and maximum quantum yield (Fv/Fm) of individuals from two populations of *Ulva fasciata* from thermally distinct environments of Brazilian coast: Niterói (NI) and Arraial do Cabo (AC). Both population sites are located in the tropics, but one is naturally colder (AC) than the other (NI) due to the upwelling phenomenon of cold and nutrient-rich deep water. The in-laboratory controlled experiment comprised three phases: (i) a temperature gradient; (ii) a 5-days heat wave simulation; and (iii), a 5-days heat wave recovery period. At the first 24 hours of heat wave and recovery phase were made 8 measurements (each phase) of Fv/Fm to better understand both shorter and longer-term effects of temperature stress on individuals of both populations. Differences in individuals between populations of *U. fasciata* were detected for pigment content during the temperature gradient and for growth rates and Fv/Fm in all three experimental conditions, suggesting that the populations are ecotypes. Physiological stress caused by a five day of exposure to heat wave, did not recover to previous physiological status when plants were returned to the lower temperatures. Individuals from AC presented better physiological responses during heat wave, . This characteristic could confer to AC individual higher competitiveness ability to overcome thermal stress (warming). Negative physiological effects on *U. fasciata* during thermal stress seemed to be more pronounced during the night than during the

day. Our results also showed that the decrease in temperature induced *U. fasciata* gamete production in both NI and AC populations under low temperature. In conclusion, the observed differences in thermal responses between the two populations could likely contribute to gradual changes in the spatial distribution along the two collecting sites.

Resumo: Um exemplo de evento extremo são as ondas de calor, que têm sido observadas ao redor do globo. Como consequência, o aquecimento global é a maior ameaça às diversas espécies de macroalgas, muitas das quais possuem papel crucial em diversos habitats marinhos. O presente estudo objetivou analisar os efeitos da temperatura no crescimento e no rendimento quântico máximo (Fv/Fm) de indivíduos de duas populações de *Ulva fasciata* de locais termicamente distintos da costa brasileira: Niterói (NI) e Arraial do Cabo (AC). Ambas regiões estão localizadas nos trópicos, mas uma (AC) é naturalmente mais fria que a outra (NI) devido ao fenômeno de ressurgência de águas profundas. O experimento em condições controladas de laboratório consistiu de três fases: (i) gradiente de temperaturas; (ii) simulação de onda de calor por 5 dias; e (iii) período de recuperação à onda de calor por 5 dias. Nas primeiras 24 horas das fases de onda de calor e recuperação foram realizadas 8 medidas (cada fase) de Fv/Fm para melhor compreender os efeitos do estresse de temperatura em curto e longo prazos nos indivíduos de ambas populações. Diferenças nos indivíduos de *U. fasciata* das duas localidades foram detectadas para conteúdo pigmentar na fase 1 e para taxa de crescimento e Fv/Fm em todas as três fases experimentais, sugerindo que as populações sejam ecótipos. O estresse fisiológico, causado por cinco dias de exposição à onda de calor, não mostrou recuperação das plantas ao estado anterior quando foram retornadas às temperaturas mais baixas. Indivíduos de AC apresentaram melhores respostas fisiológicas durante a onda de calor. Essa característica pode conferir aos indivíduos de AC maior capacidade de competição para superar estresse térmico (aquecimento). Efeitos fisiológicos negativos em *U. fasciata* durante o estresse térmico se mostrou mais pronunciado a noite do que de dia. Nossos resultados mostraram também que o decréscimo de temperatura induziu à formação de gametas em indivíduos de ambas populações, sob baixas temperaturas. Em conclusão, as diferenças observadas nas respostas térmicas entre as duas populações provavelmente contribuirão para graduais alterações na distribuição das duas populações.

Key words: extreme events, fluorescence, Fv/Fm, global warming, heat wave, PAM, quantum yield, recovery, temperature.

Palavras-chave: aquecimento global, eventos extremos, fluorescência, Fv/Fm, onda de calor, PAM, recuperação, rendimento quântico, temperatura.

INTRODUCTION

Temperature can drastically influence biological processes, acting on multiple scales from molecules to whole biotas (Turra et al. 2013, Ferreira et al. 2014). Global warming is expected to produce an increase of 1°C to 4 °C in the world's average temperature by the end of this century (Field et al. 2014), causing large changes in the marine environment such as shifts in species distribution, community structure, and the rate of local extinctions (Harley et al. 2012, Turra et al. 2013, Ferreira et al. 2014). Recent studies have shown that climate change is a major threat to several species of marine macroalgae, many of which play crucial roles in several marine habitats (*e.g.*, Wernberg et al. 2011, Harley et al. 2012, Ferreira et al. 2014).

Seaweeds are the ecological basis of coastal marine ecosystems. Their diversity has fundamental implications for life and the maintenance of ecosystem services along coastal zones (Dayton and Tegner 1984). Although some species of macroalgae have shown high tolerance, or even to benefit from processes associated to climate change, the increase in temperature tends to bring a pronounced changes to benthic communities (Mayer-Pinto et al. 2012). Several aspects of marine macroalgal ecology have been well characterized, however, very few studies have focused on species acclimation/adaptation processes, particularly in relation to spatial distribution (geography) and global warming (Poloczanska et al. 2013).

Most physiological responses to changes in abiotic conditions such as temperature, light, salinity, and pH, involve acclimation or adaptation processes (Hellberg et al. 2002). Among marine macroalgae, the green algal genus *Ulva* (Ulvales, Chlorophyta) is probably the most studied taxon due to a cosmopolitan distribution, high abundance in the upper intertidal zone, effortless handling, easy taxonomic identification in the field, and for being recognized as a bioindicator of eutrophic habitats (Joly 1965, Kozhenkova et al. 2006, Villaça et al. 2010). Furthermore, *Ulva* has been widely used as a model-organism in several temperature related studies such as Rautenberger and Bischof (2006), Chaloub et al. (2010) and Teichberg et al. (2010). *Ulva* species are known for presenting the capacity to form blooms (*e.g.*, Kong et al. 2011, Wang et al. 2011, Guidone and Thornber 2013) or to act as scrubbers in bioremediation projects (Oliveira et al. 2016). *Ulva* is also recognized as source of human food (Mabeau and Fleurence 1993), biofuels (Li et al. 2013), and biomedical molecules (Ryu et al. 2013, Wang et al. 2013).

An increase in the occurrence and intensity of extreme climate events is expected as a result of climate change (Field et al. 2014). Extreme events represent discrete pulse disturbances that cause abrupt changes to the abiotic environment relative to the life history of most organisms or ecological processes (Vergés et al. 2014). Hence, such short-term extreme variation in climatic conditions can be biologically more significant than longer-term trends of change to which organisms have greater probability of acclimation, adaptation and, ultimately, evolution (Wernberg et al. 2011, Vergés et al. 2014). Marine heat waves represent one kind of extreme climate events that have been observed around the world (Hobday et al. 2016a, 2016b). Recently marine heat waves were defined as an increase in temperature of at least 4 °C lasting at least 5 days over the maximum observed for the local temperature (Hobday et al. 2016a).

To test how populations adapted to distinct environmental conditions will respond to processes associated to global warming is important to better understand the physiology, ecology and, ultimately, predict the performance of the populations (Plastino and Guimarães 2001, Ferreira et al. 2014). To study the effects of temperature variation under controlled conditions is essential to determine the physiological variation expressed by different populations, considering the difficulties in evaluating temperature effects in the field (Plastino and Guimarães 2001, Ferreira et al. 2014).

This study aimed to analyze *in vitro* the effects of distinct temperature parameters on the physiology of individuals from two populations of *Ulva fasciata* originated from two thermally distinct environments of the Brazilian coast. Although both population sites are located in the tropics, one is naturally colder than the other due to the upwelling phenomenon of cold and nutrient-rich deep water. We hypothesized that specimens of the warmer site would show better physiological responses at higher temperatures while the individuals from the colder site would be healthier at lower temperatures. To test this hypothesis, individuals from both populations were grown at a range of different temperatures (16-31 °C), followed by a heat wave simulation and a recovery period. Growth rates, chlorophyll *a* fluorescence maximum quantum yield, and pigment content were measured to assess population adaptation, ecophysiological performance and stress.

MATERIAL AND METHODS

Experimental design

After a 6-monsths *in vitro* acclimation period in the laboratory, unialgal non-axenic cultures of five individuals (replicates) were haphazardly obtained from each population (Niterói and Arraial do Cabo). Four fragments (clones) were obtained for each individual of *U. fasciata*, therefore totaling 20 fragments. The fragments were then cultivated in 500 ml Erlenmeyer flasks containing 500 ml of enriched seawater (one 50 ± 1 mg macroalgal fragment per flask) under general culture conditions (n = 5 for each population). According to our experimental design, plants received four treatments: 16 °C, 21 °C, 26 °C, and 31 °C (temperature gradient). Otherwise, general culture conditions remained the same as those during acclimation period. Each distinct individual represented a different replicate in each treatment and each population; hence, each specimen was submitted to all four-temperature level. Four biochemical oxygen demand chambers (BOD) were used to create the temperature gradient. During the experiment, the culture medium was renewed every five days.

The experiment lasted 25 days and comprised three phases (Figure 3.1): (i) a temperature gradient; (ii) a 5-day heat wave simulation; and (iii) a 5-day heat wave recovery period. Phases 2 and 3 started immediately after the end of the previous phase with the very same individuals used in the previous phases. Only specimens that presented ≥ 50 ±1 mg fresh mass at the end of one phase were used in the subsequent phase. Phase 1 was designed to identify whether individuals from NI and AC presented different growth rate temperature optima. Phase 2 was designed to test if individuals from NI and AC populations would respond differently to a five-day thermal stress as observed during putative marine heat waves. Phase 3 was designed to test whether there were differences in the way individuals from NI and AC populations would recover from thermal stress. Macroalga fresh mass and chlorophyll *a* fluorescence maximum quantum yield (Fv/Fm) were used as response variables and as proxy for physiological stress. Additionally, pigment content was available at the end of phase 1.

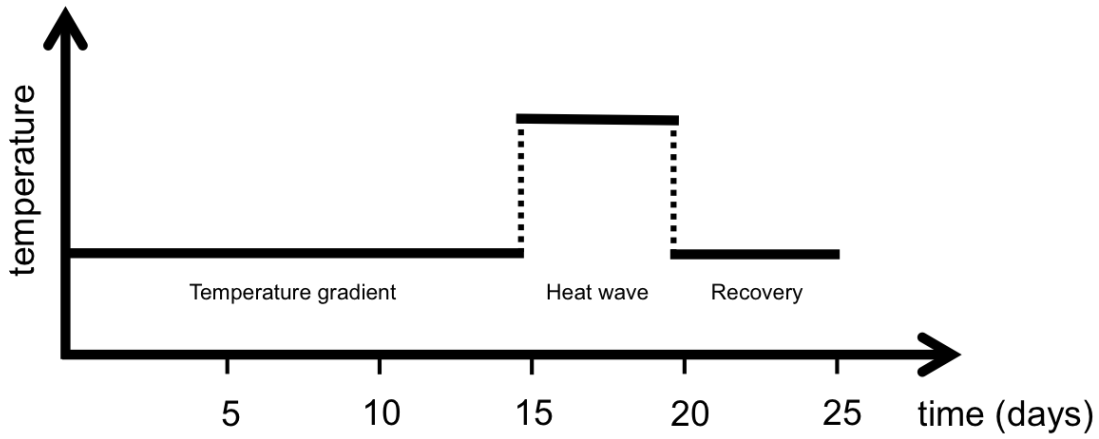

Figure 3.1: Experimental design showing the order, duration and different phases of the experiment of individuals from two populations of *Ulva fasciata* submitted to: temperature gradient (phase 1), heat wave (phase 2), and recovery (phase 3).

Phase 1: temperature gradient. The temperature gradient was composed of four temperature levels: 16 ± 1 °C, 21 ± 1 °C, 26 ±1 °C, and 31 ± 1 °C. Temperature values were chosen based on maximum and mean sea surface temperature from both collecting sites (Marazzo and Nogueira 1996, Guimaraens and Coutinho 2000, Franchito et al. 2008). Fresh mass and maximum quantum yield (Fv/Fm) were measured at the beginning of the phase 1 and every five days. This phase lasted 15 days.

Phase 2: heat wave. At the end of phase 1, each temperature level was increased by 4 °C. Thalli that became fertile with the concomitant reduction in mass below 50 mg were unable to be used in the subsequent experimental phases. Consequently, only specimens cultivated at 26 °C and 31 °C during phase 1 (*i.e.*, the temperature gradient) were used during phase 2. Heat wave temperature levels were 30 °C and 35 °C, related to 26 °C and 31 °C from the previous phase, respectively, and lasted 5 days. The 4 °C increase and the five days length for phase 2 were chosen based on the current definition of what a marine heat wave entails as described in (Hobday et al. 2016a).

Fresh mass was measured at the beginning and at the end of the phase 2. Fv/Fm was measured at the first two and last two days (totaling fours days). In addition, during the first experimental day of heat wave, Fv/Fm was measured four times during the day and four times during the night, as illustrated in Figure 3.2,

(totaling eight Fv/Fm measurements). This larger number of fluorescence measurements was implemented to better understand both shorter (< 24 hours) and longer-term (5 days) effects of temperature stress on both populations.

Phase 3: recovery. Specimens that survived phase 2 with a final fresh mass ≥ 50 mg had their temperatures returned to phase 1 conditions (*i.e.*, a reduction in 4 °C, respectively). This final recovery gradient also lasted 5 days. Fresh mass and Fv/Fm were measured at the beginning and at the end of the phase 3. Eight Fv/Fm measurements were made at first day of phase 3. During the first experimental day of recovery, Fv/Fm measurements were performed as described for heat wave (Figure 3.2), four times during the day and four times during the night.

Physiological analysis

Growth rates (GR)

Growth rates were calculated using Mtolera et al. (1995)'s equation as described in Chapter 1 (see General Approaches).

In vivo Chlorophyll *a* fluorescence

In vivo chlorophyll *a* fluorescence measurements were performed using an underwater Walz Diving-PAM fluorometer, as described in Chapter 1 (see General Approaches). Maximum quantum yield (Fv/Fm) measurements taken during the day were performed in thalli acclimated for 15 minutes in the dark inside falcon tubes wrapped in aluminum foil prior to (Schreiber et al. 1995). Night measurements did not require dark acclimation.

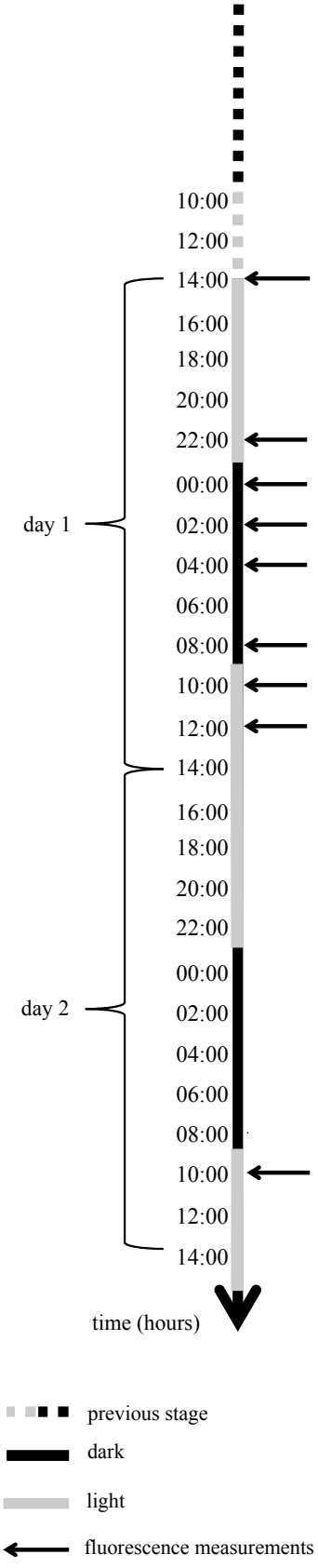

Figure 3.2: Representative scheme of phases 2 (heat wave) and 3 (recovery) of the temperature experiment of individuals from two populations of *Ulva fasciata,* detailing the first two experimental days. The photoperiod was 14 hours.

Observation of fertile thalli

Whenever the culture medium was renewed, presence/absence of reproductive structure (fertile thalli) was recorded to access reproductive status. The observation of thallus fertility can be easily recognized by the presence of brownish region or the presence of green clouds of swimming propagules (Wichard 2015). The data were used to test if temperature, population or time affected reproductive activity.

Pigment content analysis

Pigment characterization was performed by means of extraction, absorbance curve analysis, and quantification of the pigments: chlorophyll *a* and *b* (chl *a* and chl *b*) and total carotenoids (see Chapter 1, General Approaches). Samples of 10 mg of fresh mass were selected and washed in distilled water. Thalli were blotted with paper towel prior to weighting to remove excess of water. The samples were stored in Eppendorf's tubes; frozen in liquid nitrogen then stored at -80 °C until extraction.

At the end of each phase of the experiment, there was not enough fresh mass to pigment extraction. Therefore, another experiment was performed. This second experiment occurred 18 months after collection (*i.e.,* 18 months acclimated in laboratory conditions), constituted of four replicates (instead of five), the initial fresh mass was 200 ± 5 mg (instead of 50 ± 1 mg) and comprised only phase 1 (temperature gradient). This second experiment was conducted only by means of pigment extraction.

Statistics

Statistical analysis was performed as described in Chapter 1 (see General Approaches). The data were firstly tested to normality and homogeneity deviations, then, for each experimental phase, three-way Analysis of Variance (ANOVA) was used to test the effects of temperature, time, and population factors. Dependent variables were growth rates, fluorescence data and pigments content. During the first (temperature gradient) and third (recovery) phases, some replicates evidenced fertile thalli, thus, logistic regression (function *glm* in R) was performed, on the presence/absence data, to test the effect of temperature, population, and time on reproductive activity. In all cases the Student Newman–Keuls (SNK) test was used as a *post hoc* test whenever a significant difference between means has been revealed by

the analysis of variance (ANOVA). Statistical analyses were done in R (RCoreTeam 2014) adopting an $p < 0.05$.

RESULTS

Temperature gradient (phase 1)

Growth rates and fluorescence data did not pass the normality test ($p > 0.05$) but passed the homogeneity test ($p > 0.05$). ANOVA analysis of growth rates data detected a two-way interaction between temperature and population ($F_{3, 16} = 490.93$, $p < 0.01$). Within the NI population, specimens grown at 26 °C (19.02 ± 1.74 %.day^{-1}) showed the highest growth rates while in the AC population, highest growth rates were observed at both 26°C (9.33 ± 3.84 %.day^{-1}) and 31 °C (9.86, ± 3.22 %.day^{-1}). Growth rates of individuals from AC at 26 and 31 °C were not different from NI at 21 °C (9.85 ± 3.00%.day^{-1}) and NI at 31 °C (11.75 ± 1.36 %.day^{-1}). The majority of the individuals from both NI and AC at 16 °C and 21 °C became fertile during phase 1. Despite presenting signs of loss in fresh mass due to reproductive activity, individuals from NI at 21 °C expressed growth rates as high as some specimens that did not became fertile (*e.g.*, NI at 31 °C, AC at 26 °C and 31 °C). The growth rates from NI at 21 °C were higher than NI at 16 °C (1.67 ± 3.84 %.day^{-1}), AC at 16 °C (-3.17 ± 2.61 %.day^{-1}), and AC at 21 °C. Thalli of individuals from AC at 21 °C became completely fertile after 15 days of experiment, becoming unable to measure fresh mass (Figure 3.3).

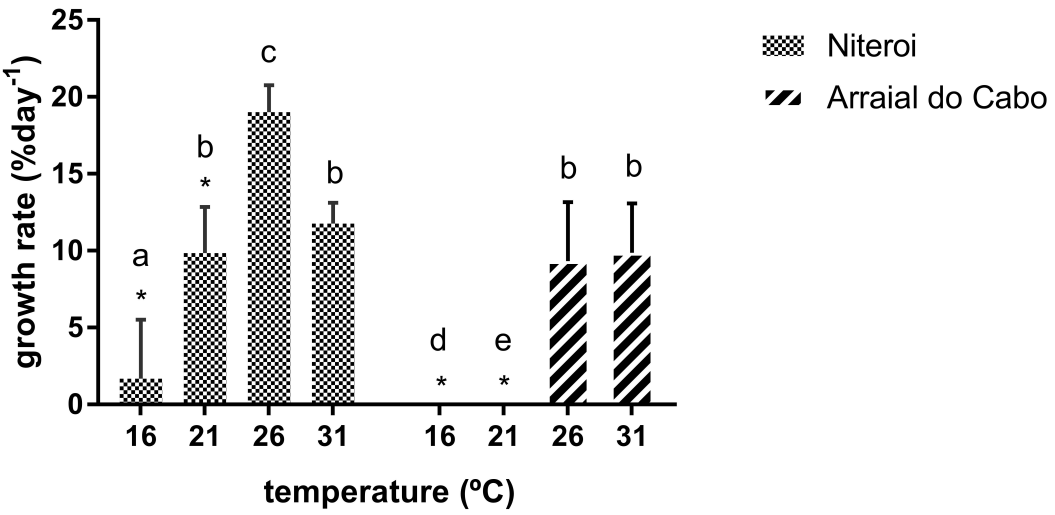

Figure 3.3: Growth rates of *Ulva fasciata* individuals from Niterói/RJ and Arraial do Cabo/RJ, Brazil, obtained from fresh mass (mg). Plants (n = 5) cultivated during 15 days (phase 1) at temperature gradient (16 °C, 21 °C, 26 °C and 31 °C). No difference could be detected among time ($p > 0.05$) hence it was merged. Different letters indicate significant differences according to SNK test ($p < 0.05$). * indicates observation of fertile.

There was an interaction of Fv/Fm between temperature and population ($F_{3, 83} = 7.65$, $p < 0.001$). Fv/Fm average values of AC at 21 °C (Fv/Fm = 0.727 ± 0.064) and NI at 16 °C (Fv/Fm = 0.735 ± 0.064) were the lowest two and not different from each other, but differed from all the other treatment combinations, which in turn were similar among them (Figure 3.4).

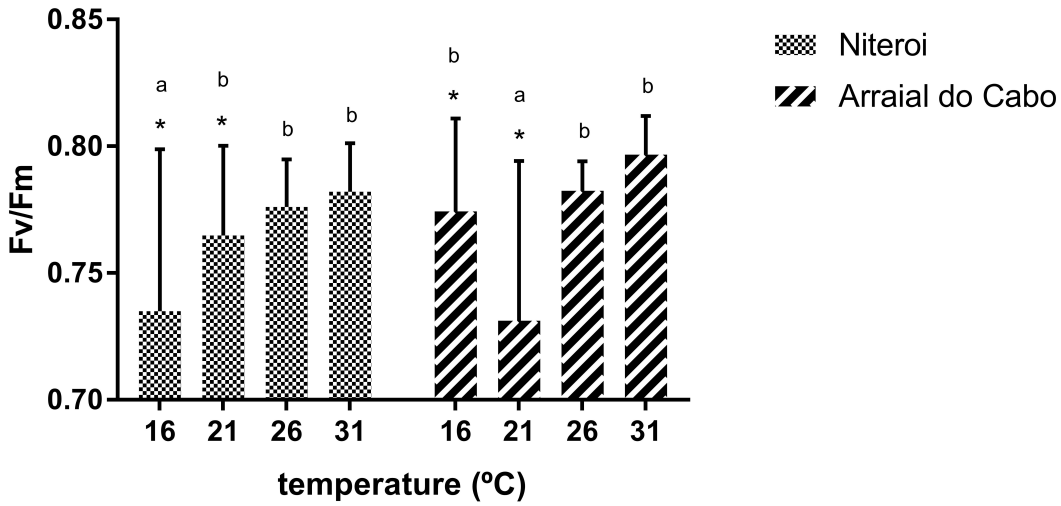

Figure 3.4: Maximum quantum yield (Fv/Fm) of *Ulva fasciata* individuals from Niterói/RJ and Arraial do Cabo/RJ, Brazil. Plants (n = 5) cultivated during 15 days (phase 1) at temperature gradient (16 °C, 21 °C, 26 °C and 31 °C). No difference could be detected between times ($p > 0.05$) hence it was merged. Different letters indicate significant differences according to SNK test ($p < 0.05$). Samples acclimated in dark for 15 minutes in Falcon tubes wrapped in aluminum foil prior to measurements. * indicates observation of fertile thalli.

During phase 1, however, the majority of the specimens submitted to 16 °C and 21 °C became fertile resulting in the complete dilapidation of the vegetative thallus. Across the entire experiment, whenever plants became fertile and released propagules in the medium, propagules swam upwards towards the light and no plantlets were found growing on the vials' walls during the experiment. The logistic regression evidenced different responses of reproductive activities among temperatures ($p < 0.001$) but not between populations or time ($p > 0.05$). Therefore, these both factors were not take into account in the analysis. Total number of observations was 30 for each temperature. *Post hoc* comparisons revealed difference between 16 °C (30% of fertility observations) and all the other temperature treatments, regardless of collecting site and time. The highest number of fertility observations was obtained at 21 °C (58.3% of fertility observations) and was different from all the other temperature treatments. No fertile individuals could be observed on 26 °C and only one fertility observation at 31 °C (0.03% of fertility observation)

throughout the whole 15 days of phase 1. The occurrence of fertility observed at 26 °C and 31 °C was not different from each other (Figure 3.5).

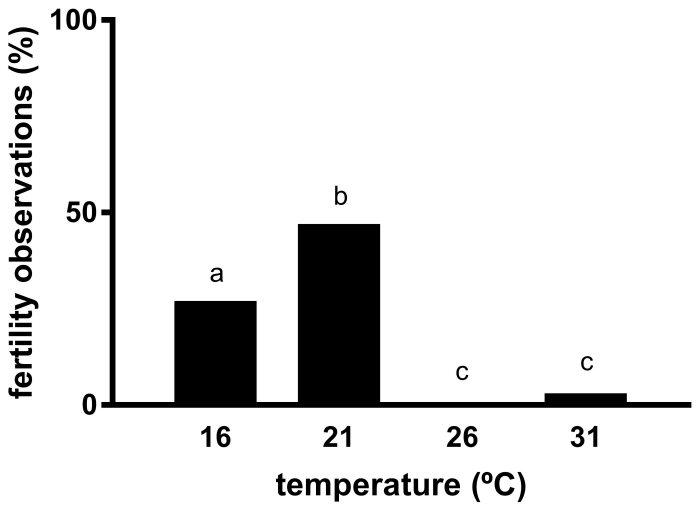

Figure 3.5: Percentage of fertility observations on individuals from Niterói/RJ and Arraial do Cabo/RJ (Brazil) of *Ulva fasciata.* Plants cultivated during 15 days (phase 1) at temperature gradient (16 °C, 21 °C, 26 °C and 31 °C). No difference could be detected between populations or time ($p > 0.05$) hence they were merged. Total number of observations was 30 for each temperature. Different letters indicate significant differences according to SNK test ($p < 0.05$).

The ANOVA analysis of pigments content data detected interaction between population and temperature for chl *a* ($F_{2, 16} = 9.07$, $p = 0.02$), chl *b* ($F_{2, 16} = 7.09$, $p = 0.04$) and carotenoids ($F_{2, 16} = 7.96$, $p = 0.04$). For all three pigments, *post hoc* tests identified that; individuals from NI at 21 °C were lower from all the other treatments (Table 3.1). The tissue of most individuals cultivated at 16 °C was bleached due to fertility and there was not enough mass to estimate their pigments.

Table 3.1: Mean and standard deviations of pigments for individuals of *Ulva fasciata* from two populations, Niterói/RJ and Arraial do Cabo/RJ, Brazil. Plants (n = 3 - 4) cultivated for 15 days at temperature gradient (phase 1) at 21 °C, 26 °C, and 31 °C. Individuals cultivated at 16 °C did not obtained enough mass to estimate pigments. Different letters indicate statistically significant difference ($p < 0.05$).

Populations	**Niterói**			**Arraial do Cabo**		
Temperature (°C)	21	26	31	21	26	31
Chlorophyll a (μg.g^{-1} FW)	566 ± 427^{a}	1384 ± 400^{b}	1695 ± 327^{b}	1126 ± 116^{b}	1184 ± 233^{b}	1011 ± 9^{b}
Chlorophyll b (μg.g^{-1} FW)	$450 \pm 325^{\mathbf{a}}$	$1071 \pm 304^{\mathbf{b}}$	$1166 \pm 220^{\mathbf{b}}$	$901 \pm 121^{\mathbf{b}}$	$920 \pm 242^{\mathbf{b}}$	$715 \pm 68^{\mathbf{b}}$
Carotenoids (μg.g^{-1} FW)	72 ± 55^{a}	181 ± 46^{b}	219 ± 51^{b}	143 ± 26^{b}	163 ± 35^{b}	129 ± 7^{b}

Heat wave (phase 2)

During phase 2, there was no observation of fertility. Growth rates and fluorescence data did not pass the normality test ($p > 0.05$) but passed the homogeneity test ($p > 0.05$). Only specimens submitted to 26 and 31 °C during experimental phase 1 were used in the heat wave phase because most specimens at 16 °C and 21 °C became fertile during phase 1. During heat wave phase, there was an interaction between population and temperature considering growth rates data (ANOVA, $F_{1, 8} = 7.26$, $p = 0.03$). The highest growth rates were observed for individuals from AC at 30 °C (15.43 ± 2.14 %.day^{-1}, Figure 3.6). *Post hoc* test detected differences between AC at 30 °C and at 35 °C (1.93 ± 1.27 %.day^{-1}), and also different when comparing to individuals from NI at 30 °C (7.30 ± 7.70 %.day^{-1}) and 35 °C (0.20 ± 0.96 %.day^{-1}).

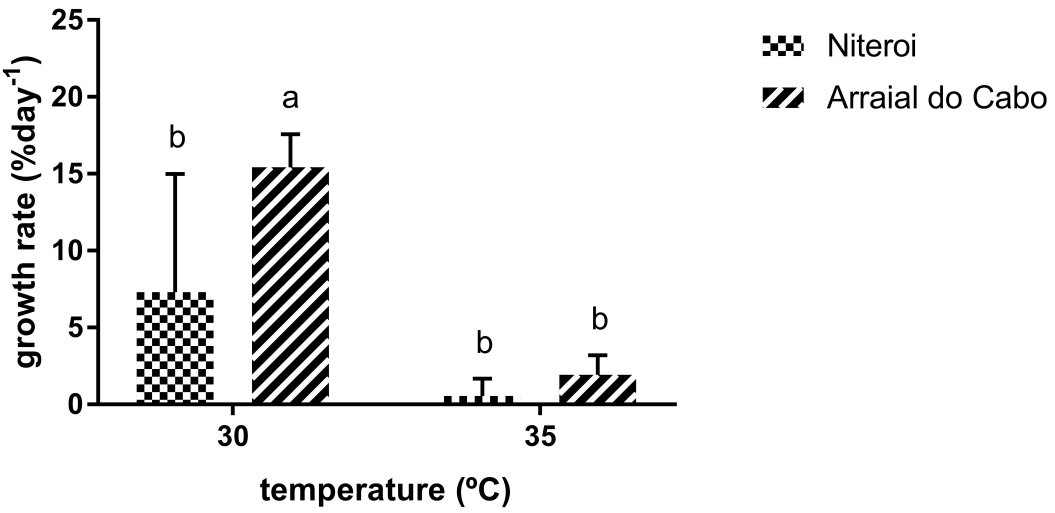

Figure 3.6: Growth rates of *Ulva fasciata* individuals from Niterói/RJ and Arraial do Cabo/RJ (Brazil) obtained from fresh mass (mg). Plants (n = 3 - 5) cultivated during 5 days at 30 °C and 35 °C after a sudden increase in temperature by 4 °C. Different letters indicate significant differences according to SNK test ($p < 0.05$).

Interaction was observed between temperature and time for the Fv/Fm data (ANOVA, $F_{3, 33} = 114.26$, $p < 0.001$) but no differences were detected between populations. At 30 °C, the Fv/Fm remained the same throughout the whole heat wave phase, however, there was detected a decrease of Fv/Fm values at fourth (Fv/Fm = 0.792 ± 0.010) and fifth (Fv/Fm = 0.792 ± 0.007) days, evidencing a decrease in photosynthesis performance as phase 2 progressed at 35 °C (Figure 3.7).

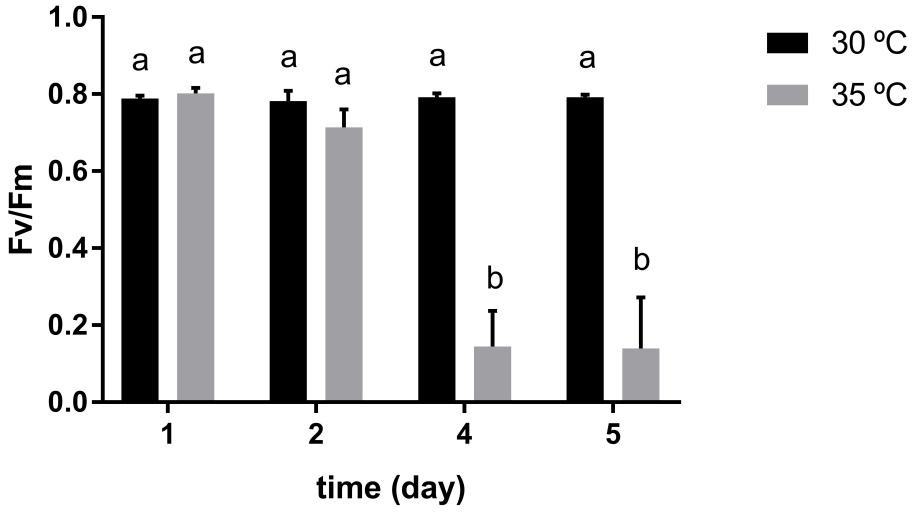

Figure 3.7: Maximum quantum yield (Fv/Fm) of *Ulva fasciata* individuals from Niterói/RJ and Arraial do Cabo/RJ, Brazil. Plants (n = 3 -5) cultivated during 5 days at 30 °C and 35 °C after a 4 °C sudden increase in temperature. No difference could be detected between populations ($p > 0.05$) hence population origin was merged. Different letters indicate significant differences according to SNK test ($p < 0.05$). Samples acclimated in dark for 15 minutes in Falcon tubes wrapped in aluminum foil prior to measurements. X axis is not in linear scale.

Results from Fv/Fm data measured during the first 24 hours into phase 2 (Figure 3.8) showed interactions between temperature and time (ANOVA, $F_{7, 96} = 3.22$, $p = 0.004$), and between temperature and population (ANOVA, $F_{1, 96} = 6.31$, $p = 0.01$), however, there was not a three-way interaction among temperature, time and population. The AC population at 35 °C displayed the highest values of Fv/Fm during the first 24 hours of thermal stress (Fv/Fm = 0.784 ± 0.021). Considering the temperature and population interaction, individuals from AC at 35 °C (Fv/Fm = 0.784 ± 0.021) obtained the highest value of Fv/Fm. Individuals from AC at 30 °C showed intermediary values of Fv/Fm (Fv/Fm = 0.775 ± 0.018) being not different to any other treatment. The individual from NI at 30 °C (Fv/Fm = 0.767 ± 0.018) and 35 °C (Fv/Fm = 0.768 ± 0.022), had lowest values of Fv/Fm and not different from each other (Figure 3.8).

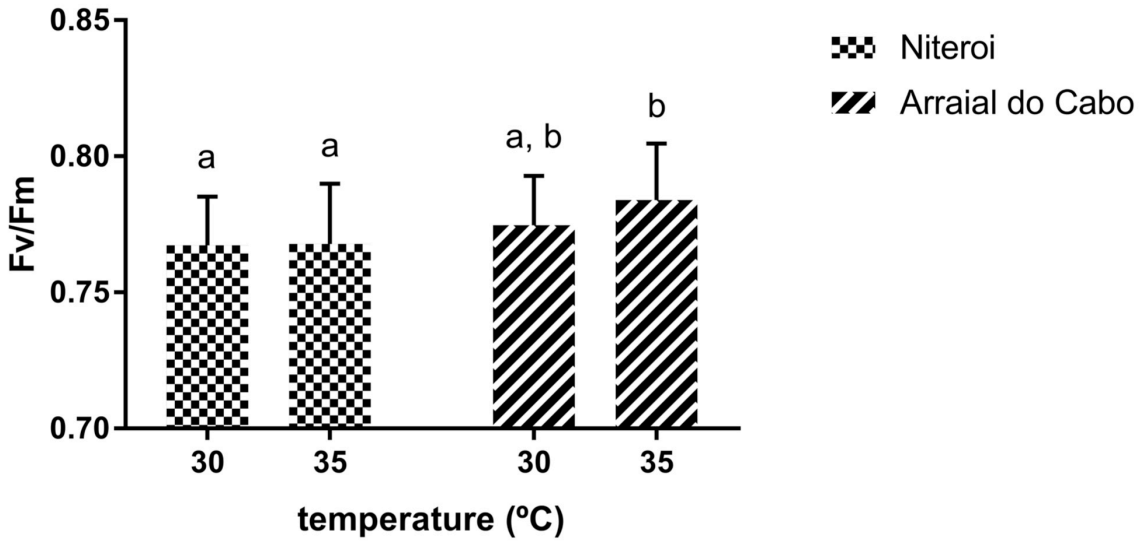

Figure 3.8: The maximum quantum yield (Fv/Fm) of *Ulva fasciata* individuals (n = 3-5) from Niterói/RJ and Arraial do Cabo/RJ, Brazil. Data obtained from the eight Fv/Fm measurements at the first 24 hours exposure to 30 °C and 35 °C, corresponding to a sudden temperature increased of 4 °C. No difference could be detected between times ($p > 0.05$) hence it was merged. Different letters indicate significant differences according to SNK test ($p < 0.05$). Samples acclimated in dark for 15 minutes in Falcon tubes wrapped in aluminum foil prior to measurements.

The SNK test for the interaction between temperature and time for Fv/Fm data, during the first 24 hours, evidenced a drop in Fv/Fm during the night period (Figure 3.9). To better understand if this drop indeed occurred during the night (dark period) another test was performed. We performed a three-way ANOVA considering population, time and the period of the day (a three level factor: before the night, during the night, and after night) as independent factors. It was detected differences among period of the day ($F_{2, 116} = 9.681$, $p < 0.001$). *Post hoc* tests evidenced differences among all three periods. The values during the night (Fv/Fm = 0.758 ± 0.016) were 4.47% lower than before the night period (Fv/Fm = 0.794 ± 0.011), evidencing a clear and sharp drop. After the period of the night, the values increased 3.42% (Fv/Fm = 0.784 ± 0.012), however 1.20% lower than before the period of the night (Figure 3.9).

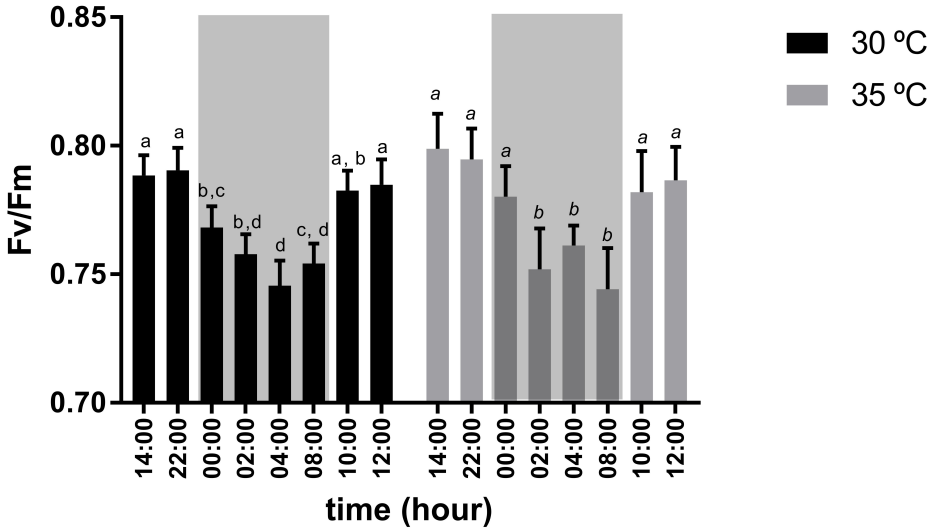

Figure 3.9: The maximum quantum yield (Fv/Fm) of *Ulva fasciata* individuals (n = 3-5) from Niterói/RJ and Arraial do Cabo/RJ, Brazil. Data obtained during the first 24 hours of plants (n = 3 – 5) cultivated at 30 °C and 35 °C after a 4 °C sudden increase in temperature. No difference could be detected between populations ($p > 0.05$) hence data from both populations were merged. Different letters indicate significant differences according to SNK test ($p < 0.05$). Measurements during the day were acclimated in dark for 15 minutes. Night measurements did not require dark acclimation. Grey zone represents the period of the night. X axis is not in linear scale.

Recovery (phase 3)

Growth rates and fluorescence data did not pass the normality test ($p > 0.05$) but passed the homogeneity test ($p > 0.05$). During the recovery period, 81% of the all plants (regardless of temperature and population; n = 13) became fertile after 5 days of recovery phase. However, the logistic regression did not detect differences on fertility between treatments ($p > 0.05$). For growth rates data, no differences could be detected among any treatments. But, for the 5-day Fv/Fm data, a three-way interaction among temperature, population, and time was detected (ANOVA, $F_{3, 40} = 2.99$, $p = 0.04$). Fv/Fm data for the first 24 recovery hours (Figures 3.10 and 3.11) showed differences between 26 °C (Fv/Fm = 0.784 ± 0.019) and 31 °C (Fv/Fm = 0.187 ± 0.134) temperatures (ANOVA, $F_{7, 88} = 1009.51$, $p < 0.001$) and between individuals from NI (Fv/Fm = 0.392 ± 0.313) and AC (Fv/Fm = 0.550 ± 0.297) populations (ANOVA, $F_{1, 88} = 4.88$, $p = 0.03$), however, no interaction could be detected between

temperature and population. The values of Fv/Fm for the first 24 hours during recovery evidenced that at 26 °C were higher (Fv/Fm = 0.784 ± 0.019) than at 31 °C (Fv/Fm = 0.187 ± 0.134).

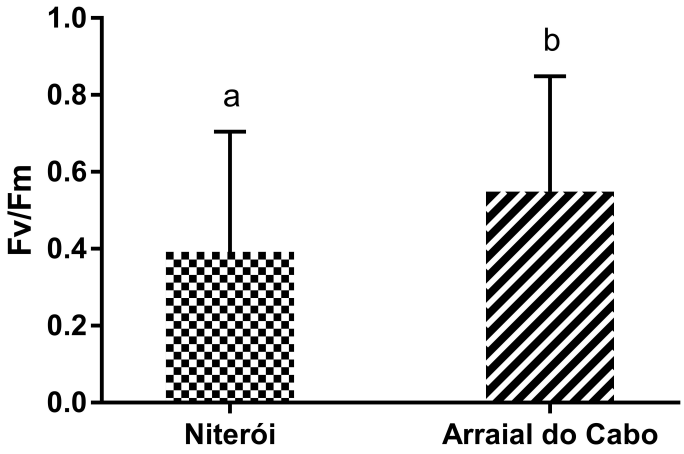

Figure 3.10: Maximum quantum yield (Fv/Fm) of *Ulva fasciata* individuals (n = 3-5) from Niterói/RJ and Arraial do Cabo/RJ populations, Brazil. Data obtained after a 24 hours recovery period (herein at 26 °C and 31 °C) after a 5-day at 4 °C increased temperature. No difference could be detected among temperatures ($p > 0.05$) hence population origin was merged. Different letters indicate significant differences according to SNK test ($p < 0.05$). Samples acclimated in dark for 15 minutes in Falcon tubes wrapped in aluminum foil prior to measurements.

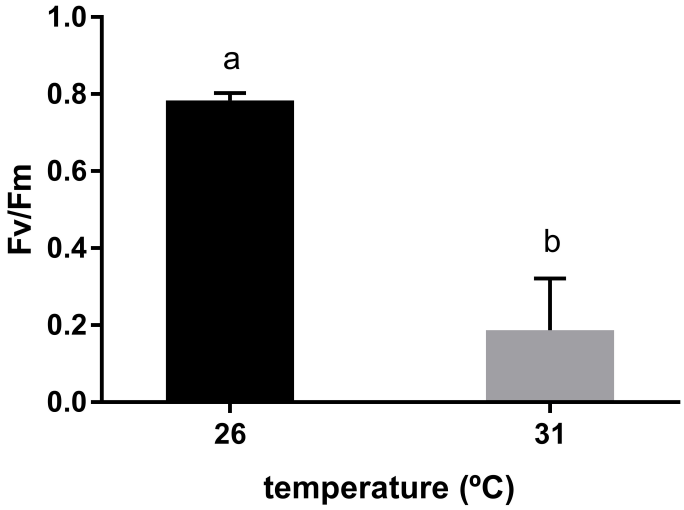

Figure 3.11: Maximum quantum yield (Fv/Fm) of *Ulva fasciata* individuals (n = 3-5) from Niterói/RJ and Arraial do Cabo/RJ populations, Brazil. Data obtained after a 24 hours recovery period (herein at 26 °C and 31 °C) after a 5-day at 4 °C increased temperature. No difference could be detected between populations ($p > 0.05$) hence population origin was merged. Different letters indicate significant differences according to SNK test ($p < 0.05$). Samples acclimated in dark for 15 minutes in Falcon tubes wrapped in aluminum foil prior to measurements.

DISCUSSION

Growth rates and maximum quantum yield differences between NI and AC populations were detected for *Ulva fasciata* specimens for all three experimental phases: the temperature gradient, the heat wave simulation, and the recovery period. Thus, our results suggest the presence of not only spatial ecological acclimation but also possible local genetic adaptation. Specimens from NI displayed the highest growth rates at 26 °C during the temperature gradient experiments, whilst individuals from AC obtained highest growth rates at 26 °C and 31 °C, 46% lower when compared to NI at 26 °C. Therefore, the maximum growth rates at NI occurred close to nature's annual average (24 °C) while in AC the maximum growth rates were observed for 26 °C and 31 °C, being 6 °C and 11 °C higher than the nature's annual average (20 °C) respectively. In laboratory, maximum growth rates at similar temperature to nature's annual average were previously observed for *U. fasciata* from Mediterranean Sea (Mohsen et al. 1972) and India (Mantri et al. 2011). However, in

our study, different results were observed for individuals from AC - the population from the upwelling site. This observation is in agreement to what was discussed by Guimaraens et al. (2005): they argue that the decrease in temperature is a stress to *Ulva* spp. and the high coverage observed in field is due to upwelled nutrients.

During the heat wave, individuals of *U. fasciata* from AC at 30 °C obtained the highest growth rates, 52% higher than NI at 30 °C. On the other hand, the treatment at 35 °C for individuals from both populations did not evidence growth. This is in agreement to what was described in literature. Mohsen et al. (1972) cultivated individuals of *U. fasciata* from the Mediterranean Sea (nature's annual average is 25 °C), under a temperature gradient of 15 - 35 °C and observed that the temperature of 35 °C was harmful. In another work, *U. fasciata* organisms, from India (nature's annual average is 25 °C), were also cultured on a temperature gradient (15 - 35 ° C), and it was also observed that the 35 °C treatment was harmful (Mantri et al. 2011). These two studies, together to our findings, suggest that *U. fasciata* does not tolerate an increase of 10 °C above nature's annual average.

Heat wave and recovery results were opposite to what we expected. We hypothesized that individuals from NI would obtain higher photosynthetic performance at higher temperatures, because the NI population experiences higher temperatures year-round (averaging 24 °C in comparison to 20 °C in Arraial do Cabo) along the year. Moreover previous studies showed that the optimal temperature of *Ulva* spp. often coincides with the average of the environment (Steffensen 1976, Fortes and Lüning 1980, Han and Choi 2005). Instead, *U. fasciata* from AC had higher competitive ability to overcome thermal stress (warming) than individuals from NI, which can have implications for the distribution and conservation of different genetic stocks in a warmer future. Our results corroborate studies on three marine macroalgal species (*Ecklonia radiata* (C.Agardh) J.Agardh*, Scytothalia dorycarpa* (Turner) Greville*,* and *Sargassum fallax* Sonder) collected from colder locations (Wernberg et al. 2016), which had higher photosynthetic performance compared to specimens collected at warmer locations when cultivated under a temperature gradient (*i.e.*, from 10°C to 30 °C).

Our results showed that the physiological stress of *U. fasciata* caused by the heat wave does not recover to previous physiological status when plants are returned to lower temperatures, regardless of population. Yet, during the recovery, there were

further negative growth rates in all treatments and a decrease in Fv/Fm values, suggesting that a consecutive temperature shift acted as another source of stress. Still, individuals from AC had better photosynthetic performance during recovery (8% higher than individuals from NI).

Negative physiological effects on *U. fasciata* during thermal stress seemed to be more pronounced during the night than during the day. Although there was detected a recovery of 1.20% after three hours return to light, this was not similar the light period before night. Similar results have been observed for terrestrial plants (Buchner et al. 2013) suggesting the existence of an ameliorating effect on light acclimated tissue during heat stress. (Buchner et al. 2013) results for vascular plants and our results for *U. fasciata* suggest that light-ameliorating effects on short-term stress could be phylogenetically widespread within the Chloroplastida or even across all autotrophs. To date and to our knowledge, no other study has tested the effect of temperature during the night period on any other autotrophic taxa. Still, we only examined two populations of a single species from southeastern Brazil. Generalizations should therefore be considered with care until further evidences, including temperate, deep-water taxa, and other taxa experiments are presented.

Decrease in temperature induced *U. fasciata* propagule production in both NI and AC populations (16 °C had 30% and 21 °C showed 58.33% of fertility, regardless of population). Processes involved in algal reproduction resulting in the formation of spores and gametes are highly affected by temperature (Lünning 1990). Our results evidenced that the temperature decreases of stimulate the fertility in individuals, and the same was previously observed in a temperature drop of 5 °C for *U. fasciata* (Mohsen et al. 1972) and *U. pertusa* Kjellman (Han and Choi 2005), although no spore release was observed when the temperature dropped by 10 °C in *U. pertusa* (Han and Choi 2005). The swimming orientation of the propagules of *U. fasciata* and the lack of plantlets suggest that all propagules were gametes, and hence all the individuals used in experiments were gametophytes. The patterns of swimming orientation and lack of plantlets were previously described for gametophytes in *Ulva* spp. (Beach et al. 1995, Wichard 2015).

Specimens of *U. fasciata* cultivated at 16 °C led to overall lower growth rates (NI: 1.67 ± 3.84 ± 1.36 %.day^{-1}; AC: -3.17 ± 2.61 %.day^{-1}) and lower gamete production when compared to treatments at 21 °C (*e.g.*, 30% of thallus turned into

gametes at 16°C compared to 58.33% at 21 °C). Incubation at 21 °C also stimulated gamete production together with positive thallus' growth. In macroalgae, lower temperature values have been shown to reduce enzyme activities and growth rates for a range of distinct species (Lobban and Harrison 1994). For example, no signs of growth were observed on *U. fasciata* from Mediterranean Sea incubated below optimum temperatures (Mohsen et al. 1972). Our results corroborate observations that temperature seems to be the strongest environmental process driving reproduction in tropical marine macroalgae growing in upwelled waters (Guimaraens et al. 2005), and showed that the same pattern occurred for the population from the non-upwelling site (Niterói).

We detected small yet significant lower concentrations of all the three pigments analyzed (chl *a*, chl *b,* and total carotenoid) of individuals from Niterói when cultured at 21 °C and all the other treatment for individuals. Temperature damage could lead to pigment degradation. The pigment degradation provides organic components (carbon sources) and inorganic (nitrogen sources) components that can be translocate to form new compounds that will allow the maintenance and defense of the cells (Foyer and Shigeoka 2011). Thus, suggesting that the temperature of 21 °C is a stronger stress on pigments for individuals from NI than for individuals from AC, as expected because NI experiences higher local temperature average than AC (24 °C and 21 °C, respectively).

Novel data on how different organisms respond to distinct thermal conditions, such as this experiment, have received renewed interested due to our need to better understand the relationship between population dynamics, the effect of local (*e.g.*, the upwelling phenomenon) and global processes (*e.g.,* global warming), and also to calibrate ecological models (*e.g.*, Guimaraens et al. 2005, Cheung et al. 2009, Harley et al. 2012). We observed for both populations of *U. fasciata* a clear increase in thermal stress during the night, and this pattern was even more pronounced in individuals previously acclimated to higher temperatures (*i.e.*, 31 °C during phase 1 elevated to 35 °C during phase 2). As the planet becomes warmer and extreme weather events become more frequent (Field et al. 2014) the likelihood that 1-4 °C warmer sites will experience 4-5 °C heat waves are more likely (Smale and Wernberg 2013, Field et al. 2014, Hobday et al. 2016b). *U. fasciata* from NI showed better physiological responses during 15 days cultured in a temperature gradient, however,

individuals from AC showed better physiological responses to thermal stress (heat wave and recovery). The observed differences in thermal physiology between the two populations could likely contribute to the gradual change in the spatial distribution along the two collecting sites. Further studies will help separate pheno- and genotypic components of these responses and further improve our understanding of the physiological ecology of *U. fasciata*.

Chapter 4

Final considerations

Considerações finais

The main aim of this study was to analyze the effect of temperature on physiological processes of individuals from two tropical populations of *Ulva fasciata* occurring in thermally distinct environments using both field and laboratory controlled experiments. Molecular data confirmed that the two populations belong to the same species, with low genetic differentiation between them. In the laboratory, specimens were cultured under a temperature gradient (16 °C, 21 °C, 26 °C and 31 °C) followed by a heat wave (+ 4 °C) and recovery period (- 4 °C). We analyzed growth rates, fluorescence and pigment content. In the field, we analyzed fluorescence and pigment content from both populations within a 24-hour time difference between assessments. By comparing populations occurring under different thermal conditions, we intended to discuss and generate knowledge about thermal acclimation or adaptation of this species under warming scenarios. Novel data on how different organisms respond to distinct thermal conditions, such as those presented in this study, have received renewed interested due to our need to better understand the relationship among population dynamics, the effect of local (e.g., the upwelling phenomenon) and global processes (*e.g.*, global warming) and also to calibrate ecological models (Guimaraens et al. 2005, Cheung et al. 2009, Harley et al. 2012).

The analysis of field data evidenced more chl *a* in individuals of *U. fasciata* from Niterói (warmer site) than individuals from Arraial do Cabo (colder site), although no differences in fluorescence parameters could be detected between both populations. The differences in chl *a* concentration might be due to differences in turbidity between sites. The upwelled waters have less turbidity (Valentin et al. 1987), and then, there is more solar irradiance available for benthic organisms, when they are submerged, and an inverse correlation between pigment content and irradiance has been observed (Ramus et al. 1976a, 1976b, 1977). However, the water temperature could also be affecting the chl *a* concentration, since low temperature can be damage

to *Ulva* species, promoting a reduction on pigment content (Mohsen et al. 1972, Mantri et al. 2011).

The similar values of fluorescence-derived parameters contents suggest that these two populations were well acclimated to field conditions at the moment of measurements. We speculate that both populations being located at similar latitude, thus, under similar solar irradiance, could explain this pattern. In macroalgae exposed to extreme abiotic fluctuations and conditions, especially those occurring in mesolittoral in tropical areas, the photoperiod is determinant to photosynthesis performance (Henley and Ramus 1989, Plastino and Oliveira 2002). The comparison between our data to the literature suggests that the putative differences between both collecting sites are not enough to create detect disparity in photosynthetic performances. *Ulva* spp. was described to adjust its chlorophyll content to irradiance variations linked to location and time of the year (Merceron et al. 2007). Although we have examined only once, our collection occurred when the upwelling phenomenon is described to be on its maximum (January – March) (Valentin et al. 1987). Because of that, we expected physiological differences to be more pronounced between populations.

Although no differences could be detected for fluorescence field data, there were differences on maximum quantum yield between individuals from NI and AC populations of *U. fasciata* considering specimens in all three laboratorial experimental phases: the temperature gradient, the heat wave simulation, and the recovery period. The fluorescence data for laboratory experiments were opposite to what we expected for heat wave and recovery results. Results strongly suggested that individuals from AC have higher competitive ability to overcome thermal stress (warming) than individuals from NI, which can have implications for the distribution and conservation of different genetic stocks in a warmer future. Furthermore, we expected that individuals from NI would obtain higher photosynthetic performance at higher temperatures, considering that the NI population experiences higher temperatures along the year due to the absence of the upwelling phenomenon and, also because the optimal temperature of *Ulva* often coincides with the environment average (Steffensen 1976, Fortes and Lüning 1980, Han and Choi 2005). Indeed, maximum growth rates for individuals from Niterói were observed at 26 °C (2 °C higher than the 24 °C annual average), however, for individuals from AC, the maximum growth rates

occurred at 26 °C and 31 °C (6 °C and 11 °C higher than the 20°C annual average, respectively).

During heat wave, however, maximum growth rates of *U. fasciata* were observed for individuals from AC (at 30 °C), while the temperature of 35 °C did not promote growth in both populations. In addition, the maximum quantum yield data revealed negative physiological effects on *U. fasciata* during the heat wave simulation being more pronounced during the night than during the day. The period of the night promoted a sharp drop in Fv/Fm values, which was not recovered when returned to light. This pattern was even more pronounced in individuals previously acclimated to higher temperatures. Similar results have been previously observed for terrestrial alpine shrubs (Buchner et al. 2013), therefore, the ameliorating effects to heat stress could be phylogenetically widespread within the autotrophs. However, due to the lack of studies supporting this affirmation, generalizations should be considered with care.

Decrease in temperature induced *U. fasciata* gametes production in both NI and AC populations in laboratory experiments. We suppose all the individuals used in experiments were gametophytes due to the swimming orientation of the propagules and the lack of plantlets growing in vials' walls. Lower temperature is considered an environmental stress for tropical species, thus formation of spores and gamete release can be considered an adaptation to avoid unfavorable environmental conditions such as the decrease in temperature for tropical species, in other words, an escape strategy.

In conclusion, we could not detect drastic physiological differences when characterizing individuals of *U. fasciata* from two thermally distinct populations of Rio de Janeiro State coast. However, when individuals from these two populations were brought to laboratory and cultivated under similar conditions, physiological differences could be detected. As the planet becomes warmer and extreme weather events become more frequent (Field et al. 2014) the likelihood that 1 - 4 °C warmer sites will experience 4 - 5 °C heat waves are more likely (Smale and Wernberg 2013, Field et al. 2014, Hobday et al. 2016a). *U. fasciata* from AC showed better physiological responses to the effects of heat wave. In a global warming scenario, gradual changes in the spatial distribution of ecotypes of *U. fasciata* along the two collecting sites is likely to occur due to the observed differences in thermal responses between the two populations. Further studies should help better understand phenotypic and genotypic components of these responses of *U. fasciata* and also

improve our knowledge on physiological ecology of organisms inhabiting regions impacted by upwelling waters.

References

Araújo, F.O., Ursi, S. & Plastino, E.M. 2014. Intraspecific variation in *Gracilaria caudata* (Gracilariales, Rhodophyta): growth, pigment content, and photosynthesis. *Journal of Applied Phycology*. 26:849–58.

Ayres-Ostrock, L.M. & Plastino, E.M. 2014a. Effects of UV-B radiation on growth rates, pigment content and ultrastructure of red (wild type), greenish-brown and green strains of *Gracilaria birdiae* (Gracilariales, Rhodophyta). *European Journal of Phycology*. 49:197–212.

Ayres-Ostrock, L.M. & Plastino, E.M. 2014b. Effects of short-term exposure to ultraviolet-B radiation on photosynthesis and pigment content of red (wild types), greenish-brown, and green strains of *Gracilaria birdiae* (Gracilariales, Rhodophyta). *Journal of Applied Phycology*. 26:867–79.

Baker, N.R. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annual Peview of Plant Biology*. 59:89–113.

Ballottari, M., Girardon, J., Dall'osto, L. & Bassi, R. 2012. Evolution and functional properties of photosystem II light harvesting complexes in eukaryotes. *Biochimica et Biophysica Acta*. 1817:143–57.

Beach, K., Smith, C., Michael, T. & Shin, H. 1995. Photosynthesis in reproductive unicells of *Ulva fasciata* and *Enteromorpha flexuosa*: implications for ecological success. *Marine Ecology and Progress Series*. 125:229–37.

Beer, S., Larsson, C., Poryan, O. & Axelsson, L. 2000. Photosynthesis rates of *Ulva* (Chlorophyta) measured by pulse amplitude modulated (PAM) fluorometry. *European Journal of Phycology*. 35:69–74.

Bischoff, B. & Wiencke, C. 1995. Temperature ecotypes and biogeography of Acrosiphoniales (Chlorophyta) with Arctic-Antarctic disjunct and Arctic/cold-temperature distributions. *European Journal of Phycology*. 30:19–27.

Brush, M.J. & Nixon, S.W. 2003. Biomass layering and metabolism in mats of the macroalga *Ulva lactuca* L. *Estuaries*. 26:916–26.

Buchner, O., Karadar, M., Bauer, I. & Neuner, G. 2013. A novel system for in situ determination of heat tolerance of plants: first results on alpine dwarf shrubs. *Plant Methods*. 9:7.

Butler, D. 2007. Ancient algal mixup sorted. *Nature*. 10–2.

Carneiro, M.E.R., Marques, A.N., Pereira, R.C., Cabral, M.M.O. & Teixeira, V.L. 1987. Estudos populacionais de *Ulva fasciata* Delile, indicadora de poluição na Baia de Guanabara. *Nerítica, Pontal do Sul, PR*. 2:201–11.

Carr, H. & Bjork, M. 2003. A methodological comparison of photosynthetic oxygen evolution and estimated electron transport rate in tropical *Ulva* (Chlorophyceae) species under different light and inorganic carbon conditions. *Journal of Phycology*. 39:1125–31.

Catanzaro, L.F., Baptista Neto, J.A., Guimarães, M.S.D. & Silva, C.G. 2004. Distinctive sedimentary processes in Guanabara Bay – SE/Brazil, based on the analysis of echo-character (7.0 kHz). *Revista Brasileira de Geofísica*. 22:69–83.

Chaloub, R., Reinert, F. & Nassar, C. 2010. Photosynthetic properties of three Brazilian seaweeds. *Revista Brasileira de Botânica*. 33:371–4.

Cheung, W.W.L., Lam, V.W.Y., Sarmiento, J.L., Kearney, K., Watson, R. & Pauly, D. 2009. Projecting global marine biodiversity impacts under climate change scenarios. *Fish and Fisheries*. 10:235–51.

Comarci, M., Furnari, G. & Alongi, G. 2014. Flora marina bentonica del Mediterraneo: Chlorophyta. *Bolletino Academia Gioenia Sciencia Naturale*. 47:11–426.

Cordi, B., Depledge, M.H., Price, D.N., Salter, L.F. & Donkin, M.E. 1997. Evaluation of chlorophyll fluorescence, in vivo spectrophotometric pigment absorption and ion leakage as biomarkers of UV-B exposure in marine macroalgae. *Marine Biology*. 130:41–9.

Cruces, E., Huovinen, P. & Gómez, I. 2012. Stress proteins and auxiliary anti-stress compounds in intertidal macroalgae. *Latin American Journal of Aquatic Research*. 40:822–34.

Davison, I.R. 1991. Environmental effects on algal photosynthesis: temperature. *Journal of Phycology*. 27:2–8.

Dayton, P.P.K. & Tegner, M.M.J. 1984. The importance of scale in community ecology: a kelp forest example with terrestrial analogs. *In* Price, P., Slobodchikoff, C. & Gaud, W. [Eds.] *A New Ecology: Novel Approacher to Interactive Systems*. Wiley, New York, pp. 457–81.

Demmig-Adams, B. & Adams, W.W. 1992. Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology*. 43:599–626.

Duke, C.S., Litaker, W. & Ramus, J. 1986. Effect of temperature on nitrogen-limited growth rate and chemical composition of *Ulva curvata* (Ulvales: Chlorophyta). *Marine Biology*. 162:157–62.

Eggert, A., van Hasselt, P.R. & Breeman, A.M. 2003. Differences in thermal acclimation of chloroplast functioning in two ecotypes of *Valonia utricularis* (Chlorophyta). *European Journal of Phycology*. 38:123–31.

Falkowski, P.G. & Raven, J.A. 2013. Aquatic photosynthesis. Princeton University Press.

Famà, P., Wysor, B., Kooistra, W.H.C.F. & Zuccarello, G.C. 2002. Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloropplast *tuf*A gene. *Journal of Phycology*. 38:1040–50.

Ferreira, J.G., Arenas, F., Martínez, B., Hawkins, S.J. & Jenkins, S.R. 2014. Physiological response of fucoid algae to environmental stress: Comparing range centre and southern populations. *New Phytologist*. 202:1157–72.

Field, C.B., Barros, V.R., Dokken, D.J., Mach, K.J., Mastrandrea, M.D., Bilir, T.E., Chatterjee, M. et al. 2014. IPCC, 2014: Climate Change 2014: Impacts, adaptation, and vulnerability. Part A: Global and sectoral aspects. Contribution of working group II to the fifth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Fortes, M.D. & Lüning, K. 1980. Growth rates of North Sea macroalgae in relation to temperature, irradiance and photoperiod. *Helgoländer Meeresuntersuchungen*. 34:15–29.

Foyer, C.H. & Shigeoka, S. 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology*. 155:93–100.

Franchito, S.H., Oda, T.O., Rao, V.B. & Kayano, M.T. 2008. Interaction between coastal upwelling and local winds at Cabo Frio, Brazil: an observational study. *Journal of Applied Meterology and Climatology*. 47:1590–8.

Franklin, L. & Forster, R. 1997. The changing irradiance environment: consequences for marine macrophyte physiology, productivity and ecology. *European Journal of Phycology*. 32:207–32.

Genty, B., Briantais, J.-M. & Baker, N.R. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta*. 990:87–92.

Gilbert, M., Wilhelm, C. & Richter, M. 2000. Bio-optical modelling of oxygen evolution using in vivo fluorescence: Comparison of measured and calculated photosynthesis/irradiance (P-I) curves in four representative phytoplankton species. *Journal of Plant Physiology*. 157:307–14.

Goff, L. & Moon, D. 1993. Pcr amplification of nuclear and plastid genes from algal herbarium specimens and algal spores. *Journal of Phycology*. 29:381–4.

Guidone, M. & Thornber, C.S. 2013. Examination of *Ulva* bloom species richness and relative abundance reveals two cryptically co-occurring bloom species in Narragansett Bay, Rhode Island. *Harmful Algae*. 24:1–9.

Guimaraens, M.A. & Coutinho, R. 1996. Spatial and temporal variation of benthic marine algae at the Cabo Frio upwelling region, Rio de Janeiro, Brazil. *Aquatic Botany*. 52:283–99.

Guimaraens, M.A., Paiva, A.D.M. & Coutinho, R. 2005. Modeling *Ulva* spp. dynamics in a tropical upwelling region. *Ecological Modelling*. 188:448–60.

Guimaraens, M. & Coutinho, R. 2000. Temporal and spatial variation of *Ulva* spp. and water properties in the Cabo Frio upwelling region of Brazil. *Aquatic Botany*. 66:101–14.

Guiry, M.D. & Guiry, G.M. 2016. World-wide eletronic publication. Available at: www.algaebase.org (last accessed May 2, 2016).

Häder, D.-P. & Figueroa, F.L. 1997. Photoecophysiology of marine macroalgae. *Photochemistry and Photobiology*. 66:1–14.

Han, T. & Choi, G.-W. 2005. A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga *Ulva pertusa* (Chlorophyta). *Aquatic Toxicology*. 75:202–12.

Han, T., Han, Y.S., Kim, K.Y., Kim, J.H., Shin, H.W., Kain, J.M., Callow, J. a. et al. 2003. Influences of light and UV-B on growth and sporulation of the green alga *Ulva pertusa* Kjellman. *Journal of Experimental Marine Biology and Ecology*. 290:115–31.

Harley, C.D.G., Anderson, K.M., Demes, K.W., Jorve, J.P., Kordas, R.L., Coyle, T.A. & Graham, M.H. 2012. Effects of climate change on global seaweed communities. *Journal of Phycology*. 48:1064–78.

Hayden, H.S., Blomster, J., Maggs, C.A., Silva, P.C., Stanhope, M.J., Waaland, R. & Waaland, J.R. 2003. Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European Journal of Phycology*. 38:277–94.

Hellberg, M.E., Burton, R.S., Neigel, J.E. & Palumbi, S.R. 2002. Genetic assessment of connectivity among marine populations. *Bulletin of Marine Science*. 70:273–90.

Henley, W.J. & Ramus, J. 1989. Optimization of pigment content and the limits of photoacclimation for *Ulva rotundata* (Chlorophyta). *Marine Biology*. 103:267–74.

Hobday, A.J., Alexander, L. V., Perkins, S.E., Smale, D.A., Straub, S.C., Oliver, E.C.J., Benthuysen, J.A. et al. 2016a. A hierarchical approach to defining marine heatwaves. *Progress in Oceanography*. 141:227–38.

Hobday, A.J., Cochrane, K., Downey-Breedt, N., Howard, J., Aswani, S., Byfield, V., Duggan, G. et al. 2016b. Planning adaptation to climate change in fast-warming marine regions with seafood-dependent coastal communities. *Reviews in Fish Biology and Fisheries*. 26:249–64.

Innes, D.J. 1984. Genetic differentiation among populations of marine algae. *Helgoländer Meeresuntersuchungen*. 38:401–17.

Inskeep, W.P. & Bloom, P.R. 1985. Extinction coefficients of chlorophyll a and b in N,N-Dimethylformamide and 80% acetone. *Plant Physiology*. 77:483–5.

Jassby, A.D. & Platt, T. 1976. Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. *Limnology and Oceanography*. 21:540–7.

Joly, A.B. 1965. Flora marinha do litoral norte do Estado de São Paulo e regiões circunvizinhas. *Boletim da Faculdade de Filosofia, Ciências e Letreas da Universidade de São Paulo, Botânica*. 21:393–5.

Kain, J.M. 1989. The seasons in the subtidal. *British Phycological Journal*. 24:203–15.

Khairy, H.M. & El-Shafay, S.M. 2013. Seasonal variations in the biochemical composition of some common seaweed species from the coast of Abu Qir Bay, Alexandria, Egypt. *Oceanologia*. 55:435–52.

Kirkendale, L., Saunders, G.W. & Winberg, P. 2013. A Molecular survey of *Ulva* (Chlorophyta) in temperate Australia reveals enhanced levels of cosmopolitanism. *Journal of Phycology*. 49:69–81.

Klughammer, C. & Schreiber, U. 2008. Complementary PS II quantum yields calculated from simple fluorescence parameters measured by PAM fluorometry and the Saturation Pulse method. *PAM Application Notes*. 1:27–35.

Kong, F., Mao, Y., Cui, F., Zhang, X. & Gao, Z. 2011. Morphology and molecular identification of *Ulva* forming green tides in Qingdao, China. *Journal of Ocean University of China*. 10:73–9.

Kozhenkova, S.I., Chernova, E.N. & Shulkin, V.M. 2006. Microelement composition of the green alga *Ulva fenestrata* from Peter the Great Bay, Sea of Japan. *Russian Journal of Marine Biology*. 32:289–96.

Kraft, L.G.K., Kraft, G.T. & Waller, R.F. 2010. Investigations into southern Australian *Ulva* (Ulvophyceae, Chlorophyta) taxonomy and molecular phylogeny indicate both cosmopolitanism and endemic Cryptic Species. *Journal of Phycology*. 46:1257–77.

Lapointe, B.E. 1981. The effects of light and nitrogen on growth, pigment content, and biochemical composition of *Gracilaria foliifera* v. *angustissima* (Gigartinales, Rhodophyta). *Journal of Phycology*. 17:90–5.

Lapointe, B.E. & Duke, C.S. 1984. Biochemical strategies for growth of *Gracilaria tikvahiae* (Rhodophyta) in relation to light intensity and nitrogen availability. *Journal of Phycology*. 20:488–95.

Lapointe, B.E., Tenore, K.R. & Dawes, C.J. 1984. Interactions between light and temperature on the physiological ecology of *Gracilaria tikvahiae* (Gigartinales: Rhodophyta) - I. Growth, photosynthesis and respiration. *Marine Biology*. 80:161–70.

Li, X.C., Gu, X.H., Zhang, G.C., Mu, C.X. & Zhang, Q. 2013. Effects of fermentation temperature and waste paper (as auxiliary feedstock) on biogas yield of *Ulva pertusa*. *Advanced Materials Research*. 666:43–9.

Librado, P. & Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*. 25:1451–2.

Liu, Y., Xu, J. & Gao, K. 2012. CO2-driven seawater acidification increases photochemical stress in a green alga. *Phycologia*. 51:562–6.

Lobban, C.S. & Harrison, P.J. 1994. Seaweed ecology and physiology. Cambridge University Press.

Longstaff, B.J., Kildea, T., Runcie, J.W., Cheshire, A., Dennison, W.C., Hurd, C., Kana, T. et al. 2002. An in situ study of photosynthetic oxygen exchange and electron transport rate in the marine macroalga *Ulva lactuca* (Chlorophyta). *Photosynthesis Research*. 74:281–93.

Lowry, D.B. 2012. Ecotypes and the controversy over stages in the formation of new

species. *Biological Journal of the Linnean Society*. 106:241–57.

Lünning, K. 1990. Seaweeds: Their environment, biogeography, and ecophysiology. *Wiley-Interscience, New York, USA*. 527 pp.

Mabeau, S. & Fleurence, J. 1993. Seaweed in food products: biochemical and nutritional aspects. *Trends in Food Science & Technology*. 4:927–9.

Mantri, V.A., Singh, R.P., Bijo, A.J., Kumari, P., Reddy, C.R.K. & Jha, B. 2011. Differential response of varying salinity and temperature on zoospore induction, regeneration and daily growth rate in *Ulva fasciata* (Chlorophyta, Ulvales). *Journal of Applied Phycology*. 23:243–50.

Marazzo, A. & Nogueira, C.S.R. 1996. Composition, spatial and temporal variations of Chaetognatha in Guanabara Bay, Brazil. *Journal of Plankton Research*. 18:2367–76.

Martins, N.T., Runcie, J.W. & Gurgel, C.F.D. 2014. light absorptance values: A case study with *Ulva australis*. *Aquatic Botany*. 120:8–11.

Maxwell, K. & Johnson, G.N. 2000. Chlorophyll fluorescence - a practical guide. *Journal of Experimental Botany*. 51:659–68.

Mayer-Pinto, M., Ignacio, B.L., Széchy, M.T.M., Viana, M.S., Curbelo-Fernandez, M.P., Lavrado, H.P., Junqueira, A.O.R. et al. 2012. How much is too little to detect impacts? A case study of a nuclear power plant. *PLoS ONE*. 7:1–9.

Menéndez, M., Martínez, M. & Comín, F.A. 2001. A comparative study of the effect of pH and inorganic carbon resources on the photosynthesis of three floating macroalgae species of a Mediterranean coastal lagoon. *Journal of Experimental Marine Biology and Ecology*. 256:123–36.

Merceron, M., Antoine, V., Auby, I. & Morand, P. 2007. In situ growth potential of the subtidal part of green tide forming *Ulva* spp. stocks. *Science of the Total Environment*. 384:293–305.

Mohsen, A.F., Nasr, A.H. & Metwalli, A.M. 1972. Effect of different salinities on growth, reproduction, amino acid synthesis, fat and sugar content in *Ulva fasciata* Delile. *Botanica Marina*. 15:177–81.

Mtolera, M.S.P., Collén, J., Pedersén, M. & Semesi, A.K. 1995. (Rhodophyta) during stress caused by elevated pH, high light intensities and competition with other species. *European Journal of Phycology*. 30:289–97.

Muth, J.E. 2014. Basic statistics and pharmaceutical statistical applications. 3rd ed. CRC Press, Florida (USA).

Necchi, O. 2004. Photosynthetic responses to temperature in tropical lotic macroalgae. *Phycological Research*. 52:140–8.

Neori, A., Cohen, I. & Gordin, H. 1991. *Ulva lactuca* biofilters for marine fishpond effluents. II. Growth rate, yield and C:N ratio. *Botanica Marina*. 34:483–9.

O'Kelly, C.J., Kurihara, A., Shipley, T.C. & Sherwood, A.R. 2010. Molecular assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the hawaiian islands. *Journal of Phycology*. 46:728–35.

Oliveira, V.P., Martins, N.T., Guedes, P. de S., Pollery, R.C.G. & Enrich-Prast, A. 2016. Bioremediation of nitrogenous compounds from oilfield wastewater by *Ulva lactuca* (Chlorophyta). *Bioremediation Journal*. 20:1–9.

Oliveira, V.P., Martins, N.T., Yoneshigue-Valentin, Y. & Enrich-Prast, A. 2013. Chlorophyll-*a* fluorescence in ecology: Theoretical considerations and examples around marine macroalgae. *Journal of Environmental Science and Engineering*. 2:606–16.

Padilla-Gamiño, J.L. & Carpenter, R.C. 2007. Seasonal acclimatization of *Asparagopsis taxiformis* (Rhodophyta) from different biogeographic regions. *Limnology and Oceanography*. 52:833–42.

Pearson, G.A. & Davison, I.R. 1996. Review: Stress tolerance in intertidal seaweeds. *Journal of Phycology*. 32:197–211.

Plastino, E.M. & Guimarães, M. 2001. Diversidad intraespecifica. *Sustentabilidad de la biodiversidad, un problema actual. Bases cientifico-tecnicas, teorización y proyecciones. Universidad de Concepción, Concepción-Chile*. 19–27.

Plastino, E.M. & Oliveira, E.C. 1990. Crossing experiments as an aid to the taxonomic recognition of the agarophytes *Gracilaria*. *In Cultivation of Seaweeds in Latin America*. Universidade de São Paulo, São Paulo, pp. 127–33.

Plastino, E.M. & Oliveira, E.C. 2002. *Gracilaria birdiae* (Gracilariales, Rhodophyta), a new species from the tropical South American Atlantic with a terete frond and deep spermatangial conceptacles. *Phycologia*. 41:389–96.

Platt, T., Gallegos, C.L. & Harrison, W.G. 1980. Photoinibition of photosynthesis in natural assemblages of marine phytoplankton. *Journal of Marine Research*. 38:687–701.

Plus, M., Auby, I., Verlaque, M. & Levavasseur, G. 2005. Seasonal variations in photosynthetic irradiance response curves of macrophytes from a Mediterranean coastal lagoon. *Aquatic Botany*. 81:157–73.

Poloczanska, E.S., Brown, C.J., Sydeman, W.J., Kiessling, W., Schoeman, D.S., Moore, P.J., Brander, K. et al. 2013. Global imprint of climate change on marine life. *Nature Climate Change*. 3:919–25.

Provasoli, L. & Pinter, I.J. 1980. Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). *Journal of Phycology*. 16:196–201.

Ramus, J., Beale, S.I. & Mauzerall, D. 1976a. Correlation of changes in pigment content with photosynthetic capacity of seaweeds as a function of water depth. *Marine Biology*. 37:231–8.

Ramus, J., Beale, S.I., Mauzerall, D. & Howard, K.L. 1976b. Changes in photosynthetic pigment concentration in seaweeds as a function of water depth. *Marine Biology*. 37:223–9.

Ramus, J., Lemons, F. & Zimmerman, C. 1977. Adaptation of light-harvesting pigments to downwelling light and the consequent photosynthetic performance of the eulittoral rockweeds *Ascophyllum nodosum* and *Fucus vesiculosus*. *Marine Biology*. 42:293–303.

Rautenberger, R. & Bischof, K. 2006. Impact of temperature on UV-susceptibility of two *Ulva* (Chlorophyta) species from Antarctic and Subantarctic regions. *Polar Biology*. 29:988–96.

Raven, J. a & Geider, R.J. 1988. Temperature and algal growth. *New Phytologist*. 110:441–61.

RCoreTeam 2014. R: A Language and Environment for Statistical Computing. Available at: http://www.r-project.org/.

Runcie, J.W. & Durako, M.J. 2004. Among-shoot variability and leaf-specific absorptance characteristics affect diel estimates of in situ electron transport of *Posidonia australis*. *Aquatic Botany*. 80:209–20.

Ryu, M.J., Kim, A.D., Kang, K.A., Chung, H.S., Kim, H.S., Suh, I.S., Chang, W.Y. et al. 2013. The green algae *Ulva fasciata* Delile extract induces apoptotic cell death in human colon cancer cells. *In vitro Cellular & Developmental Biology. Animal*. 49:74–81.

Schmider, E., Ziegler, M., Danay, E., Beyer, L. & Bühner, M. 2010. Is It Really Robust?: Reinvestigating the robustness of ANOVA against violations of the normal distribution assumption. *Methodology*. 6:147–51.

Schreiber, U., Endo, T., Mi, H. & Asada, K. 1995. Quenching analysis of chlorophyll

fluorescence by the saturation pulse method: particular aspects relating to the study of eukaryotic algae and cyanobacteria. *Plant and cell physiology*. 36:873.

Shimada, S., Hiraoka, M., Nabata, S., Iima, M. & Masuda, M. 2003. Molecular phylogenetic analyses of the Japanese *Ulva* and *Enteromorpha* (Ulvales, Ulvophyceae), with special reference to the free-floating *Ulva*. *Phycological Research*. 51:99–108.

Smale, D.A. & Wernberg, T. 2013. Extreme climatic event drives range contraction of a habitat-forming species. *Proceedings. Biological sciences / The Royal Society*. 280:20122829.

Steffensen, D.A. 1976. The effect of nutrient enrichment and temperature on the growth in culture of *Ulva lactuca* L. *Aquatic Botany*. 2:337–51.

Stillman, J.H. 2003. Acclimation capacity underlies susceptibility to climate change. *Science*. 301:65.

Tajima, F. 1989. Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. *Genetics*. 123:585–95.

Teichberg, M., Fox, S.E., Olsen, Y.S., Valiela, I., Martinetto, P., Iribarne, O., Muto, E.Y. et al. 2010. Eutrophication and macroalgal blooms in temperate and tropical coastal waters: Nutrient enrichment experiments with *Ulva* spp. *Global Change Biology*. 16:2624–37.

Teixeira, V.L., Pereira, R.C., Marques, A.N., Leitao, C.M. & Silva, C.A.R. 1987. Seasonal variations in infralitoral seaweed communities under a pollution gradient in Baía de Guanabara, Rio de Janeiro (Brazil). *Ciência e Cultura*. 39:423–8.

Turra, A., Cróquer, A., Carranza, A., Mansilla, A., Areces, A.J., Werlinger, C., Martínez-Bayón, C. et al. 2013. Global environmental changes: Setting priorities for Latin American coastal habitats. *Global Change Biology*. 19:1965–9.

Ursi, S., Costa, V.L., Hayashi, L., Pereira, R.T.L., Paula, E.J. & Plastino, E.M. 2013. Intraspecific variation in *Gracilaria birdiae* (Gracilariales, Rhodophyta): Growth, and agar yield and quality of color strains under aquaculture. *Botanica Marina*. 56:241–8.

Ursi, S., Pedersén, M., Plastino, E. & Snoeijs, P. 2003. Intraspecific variation of photosynthesis, respiration and photoprotective carotenoids in *Gracilaria birdiae* (Gracilariales: Rhodophyta). *Marine Biology*. 142:997–1007.

Ursi, S. & Plastino, E.M. 2001. Crescimento in vitro de linhagens de coloração

vermelha e verde clara de *Gracilaria birdiae* (Gracilariales, Rhodophyta) em dois meios de cultura: análise de diferentes estádios reprodutivos. *Revista Brasileira de Botânica*. 24:587–94.

Valentin, J.L., Andre, D.L. & Jacob, S.A. 1987. Hydrobiology in the Cabo Frio (Brazil) upwelling: two-dimensional structure and variability during a wind cycle. *Continental Shelf Research*. 7:77–88.

Vergés, A., Steinberg, P.D., Hay, M.E., Poore, A.G.B., Campbell, A.H., Ballesteros, E., Heck, K.L. et al. 2014. The tropicalization of temperate marine ecosystems: climate-mediated changes in herbivory and community phase shifts. *Proceedings. Biological sciences / The Royal Society*. 281:20140846.

Vijayaraghavan, K. & Joshi, U.M. 2014. Application of Ulva sp. biomass for single and binary biosorption of chromium(III) and manganese(II) ions: Equilibrium modeling. *Environmental Progress and Sustainable Energy*. 33:147–53.

Villaça, R., Fonseca, A.C., Jensen, V.K. & Knoppers, B. 2010. Species composition and distribution of macroalgae on Atol das Rocas, Brazil, SW Atlantic. *Botanica Marina*. 53:113–22.

Wang, C., Yu, R. & Zhou, M. 2011. Acute toxicity of live and decomposing green alga *Ulva* (Enteromorpha) *prolifera* to abalone *Haliotis discus hannai*. *Chinese Journal of Oceanology and Limnology*. 29:541–6.

Wang, R., Paul, V.J. & Luesch, H. 2013. Seaweed extracts and unsaturated fatty acid constituents from the green alga *Ulva lactuca* as activators of the cytoprotective Nrf2-ARE pathway. *Free Radical Biology & Medicine*. 57:141–53.

Wang, Y., Wang, Y., Zhu, L., Zhou, B. & Tang, X. 2012. Comparative studies on the ecophysiological differences of two green tide macroalgae under controlled laboratory conditions. *PloS one*. 7:e38245.

Wellburn, A.R. 1994. The spectral determination of Chlorophylls *a* and *b*, as well as Total Carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology*. 144:307–13.

Wernberg, T., Bettignies, T., Joy, B.A. & Finnegan, P.M. 2016. Physiological responses of habitat-forming seaweeds to increasing temperatures. *Limnology and Oceanography*. 1–11.

Wernberg, T., Russell, B. & Moore, P. 2011. Impacts of climate change in a global hotspot for temperate marine biodiversity and ocean warming. *Journal of Experimental Marine Biology and Ecology*. 400:7–16.

White, A.J. & Critchley, C. 1999. Rapid light curves: A new fluorescence method to assess the state of the photosynthetic apparatus. *Photosynthesis Research*. 59:63–72.

Wichard, T. 2015. Exploring bacteria-induced growth and morphogenesis in the green macroalga order Ulvales (Chlorophyta). *Frontiers in Plant Science*. 6:86.

Yoneshigue, Y. 1985. Taxonomie et ecologie des algues marines dans la region de Cabo Frio (Rio de Janeiro, Bresil). PhD Thesis, Universite d'Aix-Marseille, Marseille (FR).

Yong, Y.S., Yong, W.T.L. & Anton, A. 2013. Analysis of formulae for determination of seaweed growth rate. *Journal of Applied Phycology*. 25:1831–4.

Zar, J.H. 1999. Biostatistical analysis. Pearson Education India, India.