MR. PABLO LEAL (Orcid ID : 0000-0002-7616-1850)

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Ocean acidification and kelp development: Reduced pH has no negative effects on meiospore germination and gametophyte development of *Macrocystis pyrifera* and *Undaria pinnatifida*[1]

Pablo P. Leal [2]

Department of Botany, University of Otago, 479 Great King Street, Dunedin 9016, New Zealand and Institute for Marine and Antarctic Studies, University of Tasmania, 20 Castray Esplanade, Battery Point, Hobart, Tasmania 7004, Australia

Catriona L. Hurd

Department of Botany, University of Otago, 479 Great King Street, Dunedin 9016, New Zealand and Institute for Marine and Antarctic Studies, University of Tasmania, 20 Castray Esplanade, Battery Point, Hobart, Tasmania 7004, Australia

Pamela A. Fernández

Department of Botany, University of Otago, 479 Great King Street, Dunedin 9016, New Zealand and Institute for Marine and Antarctic Studies, University of Tasmania, 20 Castray Esplanade, Battery Point, Hobart, Tasmania 7004, Australia

Michael Y. Roleda

Department of Botany, University of Otago, 479 Great King Street, Dunedin 9016, New Zealand and Norwegian Institute of Bioeconomy Research, Kudalsveien 6, 8049 Bodø, Norway

[2]Corresponding author email: pablo.sandoval@otago.ac.nz

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ABSTRACT

The absorption of anthropogenic CO_2 by the oceans is causing a reduction in the pH of the surface waters termed ocean acidification (OA). This could have substantial effects on marine coastal environments where fleshy (non-calcareous) macroalgae are dominant primary producers and ecosystem engineers. Few OA studies have focused on the early life stages of large macroalgae such as kelps. This study evaluated the effects of seawater pH on the ontogenic development of meiospores of the native kelp *Macrocystis pyrifera* and the invasive kelp *Undaria pinnatifida*, in south-eastern New Zealand. Meiospores of both kelps were released into four seawater pH treatments (pH_T 7.20, extreme OA predicted for 2300; pH_T 7.65, OA predicted for 2100; pH_T 8.01, ambient pH; and pH_T 8.40, pre-industrial pH) and cultured for 15 d. Meiospore germination, germling growth rate, and gametophyte size and sex ratio were monitored and measured. Exposure to reduced pH_T (7.20 and 7.65) had

positive effects on germling growth rate and gametophyte size in both *M. pyrifera* and *U. pinnatifida*, whereas, higher pH_T (8.01 and 8.40) reduced the gametophyte size in both kelps. Sex ratio of gametophytes of both kelps was biased towards females under all pH_T treatments, except for *U. pinnatifida* at pH_T 7.65. Germling growth rate under OA was significantly higher in *M. pyrifera* compared to *U. pinnatifida* but gametophyte development was equal for both kelps under all seawater pH_T treatments, indicating that the microscopic stages of the native *M. pyrifera* and the invasive *U. pinnatifida* will respond similarly to OA.

Keywords: gametophyte, germination, hydrogen ion concentration, Laminariales, microscopic stages, *Macrocystis*, *Undaria*

Abbreviations: ANOVA, analyses of variances; CA, carbonic anhydrase; CCMs, carbon concentrating mechanisms; CO_2, carbon dioxide; H^+, hydrogen proton; HCl, hydrochloric acid; HCO_3^-, bicarbonate; $NaHCO_3$, sodium bicarbonate; NaOH, sodium hydroxide; OA, ocean acidification; pH_T, pH measured on the total scale; AT, total alkalinity; DIC, dissolved inorganic carbon

INTRODUCTION

Marine fleshy (non-calcifying) macroalgae are important components of coastal environments due to their high productivity and ecological function as ecosystem engineers, a source of food to higher trophic levels and refuge providers (Dayton 1985, Graham et al. 2007, Hurd et al. 2014). Macroalgal cells fix CO_2 using RuBisCO but due to its low affinity for CO_2 (Raven 1997, Giordano et al. 2005), most macroalgae have developed carbon

concentrating mechanisms (CCMs). In macroalgae with a CCM, HCO_3^- can be actively taken across the cell membrane, through a plasmalemma-located anion exchange protein, and subsequently converted to CO_2 by the enzyme carbonic anhydrase (CA) within the cell and/or HCO_3^- can be dehydrated to CO_2 by an external CA and the CO_2 can be actively or passively transported into the cell (Giordano et al. 2005, Raven et al. 2008, Fernández et al. 2014). A number of macroalgae lack a CCM and depend on passive diffusion of CO_2 which does not involve energy costs (Raven et al. 2005, Cornwall et al. 2015). Accordingly, the 200% increase in $CO_{2(aq)}$ associated with the predicted OA (Koch et al. 2013) may have positive effects on fleshy macroalgae because the energy cost for photosynthesis might be reduced (Raven 1997, Kroeker et al. 2013). However, the decrease in seawater pH that is associated with an increase of 151% in $[H^+]$ by 2100 (Guinotte and Fabry 2008) may have negative impacts on cellular metabolism (Roleda et al. 2012a, Taylor et al. 2012), which may affect the physiology, growth and development of macroalgae (Gail 1919, Sorokin 1962a, 1962b, Roleda et al. 2012a).

Aside from OA-mediated changes in seawater carbonate chemistry, macroalgal populations are themselves able to modify the pH and carbonate chemistry of the surrounding seawater due to photosynthesis and respiration which increases and decreases the seawater pH, respectively (Delille et al. 2000, Hofmann et al. 2011, Cornwall et al. 2013, Krause-Jensen et al. 2015, Roleda et al. 2015). For instance, in situ pH measurements inside forests of the kelp *Macrocystis pyrifera* (Linnaeus) C.Agardh indicate that seawater pH can fluctuate between ~7.7 at night-time and ~9.1 during the day-time (Delille et al. 2000, Hofmann et al. 2011, Cornwall et al. 2013). The lowest proximate seawater pH associated with the natural pH fluctuation is comparable to that predicted due to OA. However, exposure to persistent reduced pH under future OA may compromise the physiological tolerance of macroalgal

communities (Wernberg et al. 2011, Kroeker et al. 2013, Roleda et al. 2015) by affecting the development of their early life history stages (Roleda et al. 2012a, 2015, Gaitán-Espitia et al. 2014).

Climate change-related environmental factors such as temperature, light/UVR and oceanic pH have been proposed to facilitate the establishment of invasive species in marine coastal environments (Wernberg et al. 2011). For example, the invasive red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss has successfully colonized most of the coastal areas of Europe as far north as Sweden and Norway since its introduction from Japan due to its wide tolerance to changes in salinity, temperature and light (Nejrup and Pedersen 2010, Roleda et al. 2012b). In addition, the early life history stages of *Mastocarpus stellatus* Stackhouse (Guiry), a species accidentally introduced from Iceland to Helgoland, Germany, were more tolerant to an environmental stress factor (i.e., UVR) compared to those of the native *Chondrus crispus* Stackhouse (Roleda et al. 2004). The successful colonization of early life history stages of invasive macroalgae on natural and artificial substrata might lead to a change in the abundance, biomass and productivity of native species through competition (e.g., space monopolization), thereby affecting trophic dynamics and ecosystem biodiversity (Bax et al. 2003, Casas et al. 2004, Schaffelke and Hewitt 2007, Jiménez et al. 2015).

There are fewer studies addressing the response of macroalgae to climate change (22%) compared to other organisms such as benthic invertebrates (75%; Wernberg et al. 2012). Moreover, these macroalgal studies focused on the macroscopic adult stages which have been reported to be less sensitive to environmental stress compared to their respective microscopic early life history stages. For example, comparative responses between different macroalgal life history stages showed that early stages are more sensitive to high PAR and UVR (Roleda et al. 2004, 2009, 2012b, Fredersdorf et al. 2009), and low temperature and high trace metal concentration (e.g., copper; Nielsen et al. 2014) than the later developmental

stages. In this regard, the survival of early life stages is important because non-completion of the life cycle will have a flow on effects on the adult population and ecosystem dynamics and function (Reed and Foster 1984, Dayton 1985, Dean et al. 1989, Buschmann et al. 2006).

This study focuses on two ecologically important kelp species belonging to the order Laminariales, the giant *M. pyrifera* and the invasive kelp *Undaria pinnatifida*. *Macrocystis pyrifera* is a dominant species in the Pacific Ocean coasts of northern and southern America and sub-Antarctic islands (Hay 1990a, Graham et al. 2007). *Undaria pinnatifida* (Harvey) Suringar is native to northeast Asia but has spread to coastal areas of Europe, Australia, New Zealand and America, where its populations are now well established (Pérez et al. 1981, Hay 1990b, Sanderson 1990, Minchin and Nunn 2014). In Europe, *U. pinnatifida* is listed as one of the top 10 invasive species (Gallardo 2014), colonizing artificial substrates in harbours along southern coast of England (Arenas et al. 2006). In New Zealand, *M. pyrifera* is a native species and co-habits with *U. pinnatifida* in coastal areas in the South and North Islands (Pérez et al. 1981, Hay et al. 1985, Hay 1990a, 1990b, Sanderson 1990, Minchin and Nunn 2014). Invasive macroalgal species have been thought to have negative effects on local communities by displacing native macroalgal species (Bax et al. 2003, Schaffelke and Hewitt 2007, Williams and Smith 2007). For example, *U. pinnatifida* has been reported to decrease macroalgal richness by displacing native species such as *M. pyrifera* populations in Argentina (Raffo et al. 2009). However, in New Zealand, *U. pinnatifida* appears to have only a slightly negative effect on native intertidal macroalgal communities, and positively affected community primary productivity (South et al. 2016).

The life cycle of both *M. pyrifera* and *U. pinnatifida* consist of an alternation of haploid microscopic and diploid macroscopic stages. The macroscopic sporophytes bear specialized laminae (i.e., sporophylls) that contain a dense aggregation of binocular sporangia (i.e., sori) where haploid meiospores are produced. After release, meiospores settle,

germinate and develop into haploid male or female gametophytes which produce sperm and egg, respectively. Fertilization produces the zygote that develops into the diploid macroscopic sporophyte (Bartsch et al. 2008, Leal et al. 2014). In terms of carbon physiology, both kelp species have a CCM and are able to use CO_2 and HCO_3^- (Zhang et al. 2006, Fernández et al. 2014) which suggests that their physiology will not be substantially affected by future elevated $CO_{2(aq)}$ concentrations (Koch et al. 2013, Fernández et al. 2015).

The effects of a range of seawater pH from extremely reduced pH 7.20 to pre-industrial values of 8.40 on the ontogenic development of meiospores of the native *M. pyrifera* and the invasive *U. pinnatifida*, from south-eastern New Zealand, were studied. We hypothesized that: 1) meiospore development (i.e., germination, germling growth, gametophyte development and sexual differentiation) of both kelps will not be negatively affected by reduced seawater pH/high $[H^+]$; and 2) meiospore development under reduced pH/high $[H^+]$ will not be different between the native *M. pyrifera* and the invasive *U. pinnatifida*.

MATERIAL AND METHODS

Sporophyll collection. Ten reproductive sporophytes of *M. pyrifera* and *U. pinnatifida* were collected during low tide from the upper sub-tidal of Hamilton Bay (45°47′ 51″ S; 170°38′ 39″ E), Otago Harbour, New Zealand, in April 2014. From each *M. pyrifera* sporophyte, five to twelve sporophylls with mature sori were selected and from each *U. pinnatifida* sporophyte, the entire wing sporophyll with mature sori was selected. Sporophylls from each individual of each and species were separately packed and transported in a cool box to the laboratory within 1 h of collection. Sporophylls were lightly brushed and cleaned of visible epibionts under filtered seawater (0.2 µm, Whatman™ Polycap™ TC filter capsule,

GE Healthcare Life Sciences, UK), blotted dry, wrapped in tissue paper and kept overnight at 4°C. The next day, partial meiospore release, as staining the moist tissue paper, was observed in all individuals. Thereafter, meiospore release was induced by a thermal (12°C) rehydration shock described below.

Seawater pH treatments. The seawater used in the experiment was collected at the same time as the sporophylls. The seawater was filtered (0.2 µm, Whatman™ Polycap™ TC filter capsule, GE Healthcare Life Sciences, UK) and kept in previously sterilized 2 L-bottles at 12°C (experimental temperature) overnight before use. The ambient seawater pH_T after filtration and nutrient addition (10 µM $NaNO_3$ and 1 µM NaH_2PO_4) was 8.01 and the salinity was 35‰. To adjust the seawater pH, equal volumes of 0.5 M HCl and 0.5 M $NaHCO_3$ were used to reduce the ambient seawater pH_T to extreme (pH_T 7.20) and moderate (pH_T 7.65) OA, and equal volumes of 0.5 M NaOH and 0.5 M $NaHCO_3$ were added to obtain the pre-industrial treatment (pH_T 8.40; Riebesell et al. 2010, Roleda et al. 2012a). Acid/base modified seawater corresponding to the below four pH_T treatments were prepared fresh every d to renew the culture medium.

Seawater pH measurements. The seawater pH during the experiment was measured as pH_T at 12°C using a pH electrode (Orion ROSS Sure-Flow semi-micro, ORI8175BNWPW) connected to a pH meter (Thermo Scientific Orion 720A pH/ION Meter). The electrode slope was determined using temperature equilibrated pH 7 and pH 9 buffers (colour coded, NIST traceable). A TRIS buffer, which was standardized against a seawater buffer was then used to measure pH on the total scale. Seawater samples representing the four pH_T treatments were collected and fixed with mercuric chloride for determining carbonate chemistry. AT was measured using the closed-cell titration method and DIC was measured directly by acidifying

the sample (Dickson et al. 2007). AT, DIC, pH, salinity and temperature were used to calculate seawater carbonate chemistry parameters (Table 1) using the programme SWCO2 (Hunter 2007).

Effect of seawater pH on meiospore germination and development. Meiospore release and cultivation was performed according to Leal et al. (2014) with modifications. At least one-2 cm^2 disc of mature sorus per sporophyll was excised from the *M. pyrifera* (10 individuals × 5-12 sporophylls) using a cork borer while the whole wing sporophylls of *U. pinnatifida* (10 individuals, 21 ± 6.0 cm long × 6 ± 1.5 cm width sporophyll) were dissected using a scalpel. Due to the extent of experimental work, for each species, a subsample of excised sorus from each sporophyte was pooled (total of 50 g of sori) to perform meiospore release under each of the four pH_T treatments (7.20, 7.65, 8.01 and 8.40). Sori were immersed for 15 min and removed to obtain stock meiospore suspensions representing 10 sporophytes per seawater pH_T level. Initial meiospore density released per seawater pH_T level was calculated using a 0.1 mm depth-haemocytometer (Neubauer improved bright-line, Marienfeld, Germany), then equally adjusted to 25,000 cell · mL^{-1} and separately dispensed onto each compartment (final volume = 12 mL) of six-well polystyrene tissue culture vessels (Costar 3516; Corning Inc., New York, USA) containing seawater with the corresponding pH_T treatment (n = 6 independent replicates per pH_T treatment). Meiospores were allowed to settle for 3 h. Then, the culture medium was renewed to eliminate unsettled meiospores and detritus. Throughout the experiment, culture vessels were sealed using a plastic film (Parafilm™ M, Pechiney Plastic Packaging, Chicago, IL, USA) after every renewal of the culture medium. Meiospores were cultivated for 15 d in a temperature-controlled room at 12°C, with a photoperiod of 12 h light:12 h dark and 52 ± 3 µmol photons · m^{-2} · s^{-1} of PAR which is within the range (40 to 70 µmol photons · m^{-2} · s^{-1}) of saturating irradiances for kelp gametophytes and embryonic sporophytes (Fain and Murray 1982). Light was provided by

metal halide lamps (Philips HPI- T 400 W quartz) and measured with a spherical quantum sensor (LI-193, LI-COR, Lincoln Nebraska) connected to a light meter (LI-250A, LI-COR, Lincoln Nebraska). Control cultures (without kelp meiospores) corresponding to each pH_T treatment were also prepared and their pH_T monitored. The culture media of the treatment (four pH_T levels) and control experimental units were renewed every 12 h to avoid nutrient depletion and monitor metabolism-induced changes in the seawater carbonate chemistry. pH_T of the culture medium was measured every 12 h, within the last 2 h of light and dark conditions, before each medium renewal.

Meiospore germination and development. Photographs (5.1M CMOS camera, UCMOS0510KPA) from at least five randomly chosen visual fields were taken every two d, using an inverted microscope (20×, Olympus CK2; Olympus Optical Co. Ltd., Tokyo, Japan). Photographs were analysed using the digital camera software ToupView 3.5. Meiospores with visible germ tubes were considered germinated and the germination (%) was calculated from 350 individuals per replicate after 5 d of culture. The size of sexually ambiguous growing meiospores (germlings) and sexually-differentiated male and female gametophytes was obtained from an average of 30 individuals per replicate after 13 and 15 d of culture, respectively. Germling growth rate was calculated as (Yong et al. 2013): Growth rate ($\% \cdot d^{-1}$) = $[(W_t/W_0)^{1/t}-1]\times100$, where W_0 is the initial size, W_t is the final size, and t is d of culture. At day 15, when sexual ambiguity was resolved, male and female gametophytes were counted and the sex ratio, expressed as frequency of males per progeny, was calculated as (Roleda et al. 2012a): sex ratio = number of males/(number of males + number of females).

Statistical analyses. Percentage germination and germling growth rate ($\% \cdot d^{-1}$) were logit transformed (Warton and Hui 2011), and the gametophyte sex ratio was rank transformed (Potvin and Roff 1993) to satisfy ANOVA assumptions of normality (Kolgomorov-Smirnow test) and homogeneity of variances (Levene's test). The statistical significance of differences in meiospore germination, germling growth rate and gametophyte size and gametophyte sex ratio between species, seawater pH_T treatments and their interaction were tested using the two-way ANOVA ($P < 0.05$). A post hoc Tukey test ($P < 0.05$) was applied when significant differences were obtained. The relation between gametophyte size of both kelp species and seawater pH_T treatments was fitted using a non-linear regression model ($y = ae^{-bx}$). The software SigmaPlot version 12.0 (Systat Software, Inc., San Jose, CA) was used to run all the statistical analyses.

RESULTS

Meiospore germination. After 5 d, the germination of meiospores in the different seawater pH_T treatments ranged from 89 to 93 % and 85 to 93 % for *M. pyrifera* and *U. pinnatifida*, respectively (Fig. 1). Meiospore germination was not significantly different between kelp species but was significantly higher at reduced pH (Table 2). The effect of seawater pH on meiospore germination did not differ significantly between the two kelp species (Table 2).

Germling growth rate. After 13 d, the growth rate of germlings in the different seawater pH_T treatments ranged from 17 to 22 $\% \cdot d^{-1}$ and 15 to 20 $\% \cdot d^{-1}$ for *M. pyrifera* and *U. pinnatifida*, respectively (Fig. 2). Germling growth rate was significantly faster for *M. pyrifera* than to *U. pinnatifida* (Table 2). Germling growth rates under reduced seawater pH

were significantly faster for *M. pyrifera* compared to *U. pinnatifida* (Table 2) and the significantly different subgroups are denoted by different letters in Fig. 2 (Tukey, $P < 0.05$).

Gametophyte size. Gametophyte sexual differentiation in both kelp species occurred on the 15th d of culture. For *M. pyrifera*, male gametophyte size ranged from 315 to 690 μm^2 and for *U. pinnatifida* ranged from 361 to 651 μm^2 (Fig. 3a). Male gametophyte size did not differ significantly between kelp species but was significantly higher at reduced seawater pH_T treatments (Table 2). The effects of seawater pH_T on male gametophyte size did not differ significantly between the two kelp species (Table 2).

Female gametophyte size ranged from 279 to 449 μm^2 and from 253 to 437 μm^2 for *M. pyrifera* and *U. pinnatifida*, respectively (Fig. 3b). Female gametophytes were significantly larger for *M. pyrifera* compared to *U. pinnatifida*, and were also larger at reduced seawater pH_T (Table 2). Female gametophyte of *M. pyrifera* were significantly larger under reduced seawater pH_T compared to *U. pinnatifida* (Table 2). Significantly different subgroups are denoted by different letters in Fig. 3b (Tukey, $P < 0.05$).

Non-linear regression analysis showed that male and female gametophyte size of both kelp species significantly and exponentially decreased with increasing seawater pH_T (Fig. S1 in the Supporting Information). Male gametophyte exponential curve fitness ($R^2 = 0.89$, $P<0.01$, df = 5, Fig. S1a; $R^2 = 0.81$, $P<0.01$, df = 5, Fig. S1b) were substantially higher than those of female gametophyte ($R^2 = 0.64$, $P<0.05$, df = 5, Fig. S1a; $R^2 = 0.79$, $P<0.01$, df = 5, Fig. S1b) of both kelp species.

Gametophyte sex ratio. After 15 d, gametophyte sex ratios in the different seawater pH_T treatments ranged from 0.44 to 0.49 and from 0.43 to 0.50 in *M. pyrifera* and *U. pinnatifida*, respectively (Fig. 4). Gametophyte sex ratio did not differ significantly between kelp species but increased with reducing seawater pH_T treatments (Table 2). The effects of seawater pH_T on gametophyte sex ratio did not differ significantly between the two kelp species (Table 2).

Seawater pH_T in meiospore cultures. During the 15 d of cultivation in the different seawater pH_T treatments of 7.20, 7.65, 8.01 and 8.40 the pH_T of the culture medium for both *M. pyrifera* and *U. pinnatifida* meiospores increased after each daily 12 h light period (Fig. S2 in the Supporting Information). In the pH_T 7.20 treatment, the pH_T of the culture medium increased from 7.20 to 7.70 – 7.73; in the pH_T 7.65 treatment, the pH_T increased from 7.65 to 7.74 – 8.02; in the pH_T 8.01 treatment, from 8.01 to 8.36; and in the pH_T 8.40 treatment, from 8.40 to 8.56 – 8.58. At the end of each daily 12 h dark period, the pH_T of the culture media was more stable over the 15 d experiment: in the pH_T 7.20 treatment, the pH_T of the culture medium decreased from 7.20 to 7.14 – 7.19; in the pH_T 7.65 treatment, the pH_T decreased from 7.65 to 7.62 – 7.64; in the pH_T 8.01 treatment, from 8.01 to 7.95 – 8.00; and in the pH_T 8.40 treatment, from 8.40 to 8.25 – 8.39 (Fig. S2).

DISCUSSION

The first hypothesis that reduced seawater pH/high $[H^+]$ will not be detrimental for the development of early life history stages of *M. pyrifera* and *U. pinnatifida* was supported by our results. For both kelp species, meiospore germination was not affected by seawater pH, whereas reduced seawater pH_T (7.20 and 7.65) enhanced germling growth rate and

gametophyte size compared to the ambient treatment (pH_T 8.01). Gametophyte size was significantly reduced in the pre-industrial seawater treatment of pH_T 8.40.

Germination of both *M. pyrifera* and *U. pinnatifida* meiospores was insensitive to the seawater pH_T treatments. Successful meiospore germination across the range of seawater pH investigated may be related to the low photosynthetic capacity of kelp meiospores. Upon release, kelp meiospores contain only one or two chloroplasts (Henry and Cole 1982, Steinhoff et al. 2011) which enables them to photosynthesize but with a very low net photosynthetic rate (e.g., 161 nL $O_2 \cdot mL^{-1} \cdot h^{-1} \cdot mg\ C^{-1}$ for *M. pyrifera* meiospores; Amsler and Neushul 1991). Therefore, it is most likely that the metabolic activity during swimming, settlement and subsequent germination are mainly supported by internal carbon reserves (i.e., neutral lipid triacylglycerol) that are stored inside vesicles (Brzezinski et al. 1993, Reed et al. 1999, Bartsch et al. 2008). In another kelp species *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders, meiospore development was sustained by lipid reserves during the first ten d before becoming photosynthetically autonomous (Steinhoff et al. 2011). The same dependence on internal energy reserves may be operating in *Fucus* sp. Linnaeus, where successful embryogenesis and subsequent germination (40 – 95%) was observed within a range of seawater pH from 7.40 to 8.60 but maximum germination occurred at pH 8.00 – 8.20; negative effects were only observed at seawater pH < 7.40 (Gail 1919). With low DIC uptake associated with low photosynthetic rate, the seawater carbonate chemistry is likely to have a limited impact on the carbon physiology of germinating meiospores and embryos. However, the effects of proximate seawater $[H^+]$, that is higher under OA on cellular membrane integrity and cellular processes remains to be investigated. In microalgae, low pH was reported to inhibit cell division (Sorokin 1962b, 1964), but this was not the case in the early life history stages of kelps where meiospores successfully undergo cell division and developed into gametophytes (Roleda et al. 2012a, this study).

When internal energy reserves are exhausted and germinating spores are completely photosynthetically autonomous, they are directly dependent on exogenous carbon source for photosynthesis, growth and development (Steinhoff et al. 2011). The higher availability of $CO_{2(aq)}$ under lower seawater pH_T (7.20 and 7.65) appeared to increase rates of growth of germlings and gametophytes of both species *M. pyrifera* and *U. pinnatifida* compared to the high pH_T treatments (8.01 and 8.40). Members of the order Laminariales are known to be mixed CO_2/HCO_3^- users. Under OA, CO_2 uptake by *M. pyrifera* sporophytes significantly increased while HCO_3^- uptake remained the same (Fernández et al. 2015). On the other hand, gametophytes of *U. pinnatifida* have been reported to have a higher affinity for $CO_{2(aq)}$ than HCO_3^- to support photosynthesis (Zhang et al. 2006). Therefore, as passive diffusion of CO_2 is energetically less expensive compared to HCO_3^- uptake by a CCM (Raven et al. 2008), the greater higher availability of $CO_{2(aq)}$ under the OA treatments might have favoured the growth of germlings and gametophytes of both *M. pyrifera* and *U. pinnatifida*. Under pre-industrial seawater pH, extracellular enzymatic conversion of HCO_3^- to CO_2 by the enzyme CA (Zhang et al. 2006, Fernández et al. 2014), may be activated to compensate for the lower available $CO_{2(aq)}$ concentration. The energy cost of this enzymatic reaction may have compromised germling and gametophyte growth rates at seawater pH_T 8.40.

Changes in pH of the proximate seawater can also indicate the DIC source that is being taken up by the algal cell (Gail 1919, Roleda et al. 2012a, Fernández et al. 2015). In the closed culture system used in this experiment, DIC uptake (most likely CO_2) and photosynthesis by the microscopic stages of *M. pyrifera* and *U. pinnatifida* following 12 h of light induced a pH_T increase in the culture medium. The magnitude of the increase of seawater pH_T was greatest (by 0.5 pH units) in the pH_T 7.20 treatment after day 7 compared to the first 5 d, for both kelp species. Likewise, highest germling growth rate and gametophyte size of both kelp species were observed under this that pH_T treatment. This

strongly suggests that the higher photosynthetic rates under reduced seawater pH_T produce bigger germlings and gametophytes in a high $CO_{2(aq)}$ culture medium

The earliest oxygenic eukaryotic algae may have evolved 2 billion years ago (i.e., early Proterozoic), when atmospheric CO_2 concentrations were around 100-fold higher than in the present-day and O_2 concentrations were close to zero (Falkowski and Raven 1997, Beardall and Raven 2004). It has been proposed that the evolution of RuBisCO was favored (i.e., positive selection) by equilibration of the atmospheric CO_2 concentrations with the surface oceanic waters (Young et al. 2012). The poor affinity of RuBisCO for CO_2 in that ancient environment may have been alleviated by the saturating concentrations of CO_2 and low O_2 concentrations (Falkowski and Raven 1997, Beardall and Raven 2004). Thus, in terms of evolution, the enhanced performance of RuBisCO under high CO_2 conditions led to a decrease in atmospheric CO_2 and a rise of O_2, which in turn limited the fixation of CO_2 by RuBisCO (Falkowski and Raven 1997, Beardall and Raven 2004, Young et al. 2012). This photosynthetically-induced change in atmospheric composition may have constituted a selective factor for the evolution of CCM in algal cells (Beardall and Raven 2004, Giordano et al. 2005). Therefore, it is possible that macroalgae (including their early life stages) have genetically imprinted tolerance to high CO_2 oceans such that expected for the end of the century, and this may explain the positive response of *M. pyrifera* and *U. pinnatifida* microscopic stages to OA.

In natural populations, under favourable environmental conditions, sex ratios are often present in equal proportions (i.e., 0.5 = 1 male: 1 female; Uller et al. 2007). In the present study, however, the formation of more female than male gametophytes (sex ratio < 0.5) was observed in both *M. pyrifera* and *U. pinnatifida* under all pH_T treatments (except for *U. pinnatifida* gametophytes at pH_T 7.65 where sex ratio was 0.5). These findings are opposite to those of Roleda et al. (2012a) who found for a nearby population of *M. pyrifera* a sex ratio

slightly biased towards males (> 0.5) under OA (pH 7.61 and 7.86) and ambient (pH 8.19) conditions. These differences may be related to the season when sampling was performed: autumn (present study) and summer (Roleda et al. 2012a) because, although sex ratio in kelp species is mainly controlled by chromosomes, the interaction between genetic and temperature can modulate the expression of sex (Bartsch et al. 2008, Oppliger et al. 2011). For instance, the number of male gametophytes increased with low temperature and decreased with high temperature in populations of *Lessonia nigrescens* Bory de Saint-Vincent adapted to warm seawaters (northern Chile) and cold waters (southern Chile), respectively (Oppliger et al. 2011). Temperature-dependence has also been reported in the sex ratio in *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders [= *Laminaria saccharina* (Linnaeus) J.V.Lamouroux] and (Lee and Brinkhuis 1988) and *Lessonia variegata* J.Agardh (Nelson 2005) where the number of male gametophytes was greater with increasing temperature, and in *Saccharina religiosa* (Miyabe) C.E.Lane, C.Mayes, Druehl & G.W.Saunders [= *L. religiosa* Miyabe] with a lower proportion of males at high and low temperatures (Funano 1983). Conversely, no significant effect of temperature was observed on gametophyte sex ratio of the perennial *Laminaria ochroleuca* Bachelot de la Pylaie and the annual *Saccorhiza polyschides* (Lightfoot) Batters (Pereira et al. 2011). Other stress factors (e.g., salinity) may alter the mechanisms of sex determination and/or favour the survival of more male gametophytes in kelp species (cf. Bartsch et al. 2008 and references therein) altering the sex ratio biased towards males. This information indicates that the interaction of local environmental factors could have indirect species-specific effects on sex ratio of kelp species.

The interaction of reduced pH with other environmental factors can change the responses of macroalgae to OA. For example, experimental exposure to OA had no effect on growth and photosynthesis of *M. pyrifera* sporophytes (Brown et al. 2014, Fernández et al.

2015) but when the OA interacted with temperature (3°C higher) a significant increase in both response variables were observed (Brown et al. 2014). In contrast, germination of *M. pyrifera* meiospores decreased by 10% under OA conditions (pH 7.46, 13°C) but by 40% under OA and higher temperature (15°C; Gaitán-Espitia et al. 2014). These different responses observed for early and later life history stages of *M. pyrifera* reflect the complexity of the physiological response of kelps to climate change-related factors such as OA and temperature and the importance of studying the response of different life stages of macroalgae to different abiotic factors in isolation and interaction.

Our results supported the hypothesis that meiospore development of both *M. pyrifera* and *U. pinnatifida* under reduced pH/high H^+ will not differ. *Macrocystis pyrifera* had higher germling growth rates compared to *U. pinnatifida* under the pH_T 7.20, 7.65 and 8.40 treatments but gametophyte development and sexual differentiation were the same for both kelps under all pH_T treatments. This suggests that both kelp species will be positively/neutrally affected by future OA, at least during early life history stages. However, the reproduction and early developmental stages of kelps may be negatively affected by other climate change-related factors such as temperature, among others. For example, the abundance of brown macroalgal forests, including *M. pyrifera*, has been projected to decline as a consequence of ocean warming (1 to 3°C), shifting to ecosystems dominated by opportunistic seaweeds (Johnson et al. 2011, Wernberg et al. 2016). However, sexual and asexual reproduction, i.e., gametogenesis and sporogenesis, respectively, may be more susceptible to climate change drivers compared to the physiological responses of the early life history stages such as meiospores and gametophytes (Roleda 2015). *Undaria pinnatifida* is an opportunistic invader, with early life stages colonizing free space after natural or experimental native canopy removal but the density of this invasive kelp declines with increasing dominance of native algal species (Reed and Foster 1984, Valentine and Johnson

2003, 2004, Schiel and Thompson 2012). In addition, *U. pinnatifida* has a broad range of thermal tolerance (0.1 – 15°C) and, therefore, its global distribution is projected to increase in a future 4°C-warmer ocean (James et al. 2015). Although no differences in microscopic stage development between *M. pyrifera* and *U. pinnatifida* were detected in response to OA in this study, there is a clear need for future competition experiments, in which the meiospores of both species are grown together under climate change drivers (independent and multiple-driver experiments) where their development (germination, growth and gametogenesis) is followed.

For both the native *M. pyrifera* and the invasive *U. pinnatifida*, germination was insensitive to the seawater pH_T treatments, suggesting that meiospores do not depend on external DIC concentrations but mainly on internal carbon reserves to support metabolism during germination. The positive effect of OA on germling growth rate and gametophyte (male and female) size for both species suggest that the greater availability of CO2(aq) supported higher (albeit non-significant) rates of photosynthesis and growth in these developmental stages as observed in adult sporophyte blades (Fernández et al. 2015). In contrast, the negative effect of pre-industrial pH on gametophyte size of both kelp species indicate a higher energetic cost when using HCO_3^- as DIC source which requires enzymatic reactions to dehydrate HCO_3^- to CO_2 to compensate for the lower $CO_{2(aq)}$ availability at seawater pH_T 8.40. Finally, we demonstrate that the native *M. pyrifera* and the invasive *U. pinnatifida* have similar response to seawater pH, indicating that the invasive kelp will not have a competitive advantage in a projected acidified ocean. However, further studies on carbon physiology, specifically on the presence and role of different isozymes of CA (e.g., Rautenberger et al. 2015) on internal pH homeostasis, photosynthesis and growth of early life

history stages of both *M. pyrifera* and *U. pinnatifida* will help to understand the relative tolerance of kelps to OA in isolation. Also, the interaction of OA with other climate change factors such as temperature and eutrophication may have different effects on the physiology of macroalgal microscopic stages.

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FIGURE CAPTIONS

Figure 1. Percentage meiospore germination for *M. pyrifera* and *U. pinnatifida* after 5 d of culture in four pH_T seawater treatments (pH_T 7.20, 7.65, 8.01 and 8.40). Bars represent mean ± SD (n = 6). Different letters indicate a significant difference between subgroups (Tukey, $P < 0.05$) according to the independent factor pH_T treatment (Table 2). Note that y-axis start at 50%.

Figure 2. Growth rate of germlings of *M. pyrifera* and *U. pinnatifida* after 13 d of culture in four pH_T seawater treatments (pH_T 7.20, 7.65, 8.01 and 8.40). Bars represent mean ± SD (n = 6). Different letters indicate a significant difference between subgroups (Tukey, $P < 0.05$) according to the two-way interaction species × pH_T treatment (Table 2).

Figure 3. Size of (a) male and (b) female gametophytes of *M. pyrifera* and *U. pinnatifida* at the 15th d of culture in four pH_T seawater treatments (pH_T 7.20, 7.65, 8.01 and 8.40). Bars represent mean ± SD (n = 6). Different letters indicate a significant difference between subgroups (Tukey, $P < 0.05$) according to the independent factor species and to the two-way interaction species × pH_T treatment, for male and female gametophyte size, respectively (Table 2).

Figure 4. Sex ratio of male and female gametophytes of *M. pyrifera* and *U. pinnatifida* at the 15th d of culture in four pH_T seawater treatments (pH_T 7.20, 7.65, 8.01 and 8.40). Circles and squares represent mean ± SD (n = 6). Different letters indicate a significant difference between subgroups (Tukey, $P < 0.05$) according to the independent factor pH_T treatment.

Figure S1. Non-linear regression ($y = ae^{-bx}$) between seawater pH_T treatment (pH_T 7.20, 7.65, 8.01 and 8.40) and size of male and female gametophyte of (a) *M. pyrifera* and (b) *U. pinnatifida*. The corresponding non-linear regression equations and coefficient of determination (R^2) are shown. Black and white circles are individual replicates (n = 6) within a pH_T treatment.

Figure S2. Seawater pH_T in the culture medium of *M. pyrifera* and *U. pinnatifida* during 15 d of culture under pH_T seawater treatments (pH_T 7.20, 7.65, 8.01 and 8.40). Measurements were performed every 12 h in dark (D) and light (L) conditions before culture medium renewal. The meiospores germinated into 'germlings' (i.e., sexually un-differentiated gametophytes) on day 5, and sexual differentiation of gametophytes was apparent on day 15. Symbols represent mean ± SD (n = 6). Note that the y-axis scales are different between pH_T treatments.

Table 1. Carbonate chemistry parameters were calculated form total alkalinity (AT) and dissolved inorganic carbon (DIC) measurements of seawater corresponding to each pH_T treatment: extreme OA predicted for 2300, pH_T 7.20; OA predicted for 2100, pH_T 7.65; ambient pH_T 8.01 and pre-industrial pH_T 8.40. Temperature (12°C) and salinity (35‰) were stable during the experiment period. Mean ± SD (n = 3) are reported for each seawater pH_T treatment.

	Seawater pH_T treatments			
Seawater parameter	7.20	7.65	8.01	8.40
pH_T	7.20 ± 0.007	7.65 ± 0.006	8.01 ± 0.021	8.39 ± 0.004
DIC ($\mu mol \cdot Kg^{-1}$)	2385.22 ± 66.6	2166.94 ± 44.50	2102.42 ± 10.87	2064.26 ± 34.25
AT ($\mu mol \cdot Kg^{-1}$)	2288.86 ± 63.58	2216.23 ± 44.88	2278.25 ± 11.44	2484.37 ± 39.03
HCO_3^- ($\mu mol \cdot Kg^{-1}$)	2230.31 ± 62.27	2062.53 ± 42.36	1954.17 ± 10.10	1763.08 ± 29.25
$H_2CO_3^-$ ($\mu mol \cdot Kg^{-1}$)	131.39 ± 3.67	43.11 ± 0.89	18.25 ± 0.09	6.55 ± 0.11
CO_3^{2-} ($\mu mol \cdot Kg^{-1}$)	23.52 ± 0.66	61.29 ± 1.26	130.01 ± 0.67	294.63 ± 4.89
pCO_2 (µatm)	3207.31 ± 89.50	1052.36 ± 21.61	445.37 ± 2.31	159.97 ± 2.65

Table 2. Two-way ANOVA and significance values for effects of seawater pH_T treatments on meiospore germination, germling growth rate, gametophyte size and gametophyte sex ratio of *Macrocystis pyrifera* and *Undaria pinnatifida*.

Variable	Source of variation	Degree of freedom	Sum of squares	Mean square	*F*	*P*
Meiospore germination	Species	1	0.0688	0.0688	0.856	0.360
	pH_T treatment	3	0.6870	0.2290	2.850	0.049
	Species × pH_T treatment	3	0.3940	0.1310	1.635	0.196
	Residual	40	3.2150	0.0804		
	Total	47	4.3660	0.0929		
Germlings growth rate	Species	1	0.0760	0.07600	60.151	< 0.001
	pH_T treatment	3	0.1210	0.04040	32.019	< 0.001
	Species × pH_T treatment	3	0.0227	0.00757	5.998	0.002
	Residual	40	0.0505	0.00126		
	Total	47	0.2710	0.00576		
Male gametophyte size	Species	1	4511.933	4511.933	1.214	0.277

	pH_T treatment	3	764336.852	254778.951	68.564	< 0.001
	Species × pH_T treatment	3	22125.959	7375.320	1.985	0.132
	Residual	40	148637.640	3715.941		
	Total	47	939612.384	19991.753		
Female gametophyte size	Species	1	8910.597	8910.597	4.192	0.047
	pH_T treatment	3	211719.184	70573.061	33.205	< 0.001
	Species × pH_T treatment	3	2397.721	799.240	0.376	0.771
	Residual	40	85014.696	2125.367		
	Total	47	308042.199	6554.089		
Gametophyte sex ratio	Species	1	204.188	204.188	1.341	0.254
	pH_T treatment	3	2670.375	890.125	5.847	0.002
	Species × pH_T treatment	3	247.688	82.563	0.542	0.656
	Residual	40	6089.250	152.231		
	Total	47	9211.500	195.989