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Physiological response of evolutionary divergent *Symbiodinium* clades (A1-D1) to alterations in seawater physical-chemical parameters

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Monografia apresentada ao Curso de Bacharelado em Oceanografia do Instituto Oceanográfico da Universidade de São Paulo, como parte dos requisitos para a conclusão da disciplina Trabalho de Graduação II.

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ABSTRACT

S*ymbiodinium* are photosynthetic dinoflagellates that can be found in two different life modes. Most commonly, they are associated with a host in a mutualistic relationship. Alternatively, they can also be found in the water column as free-living cells. Only recently, the *Symbiodinium* genus was divided into 9 different clades which, as endosymbionts, present distinct tolerance levels to fluctuations in abiotic parameters (*i.e*., light, temperature and pH). However, there is still a lack of information about the free-living mode for each clade. Here we studied the population growth rate responses of free-living *Symbiodinium* clades (A1-D1) to individual changes in seawater pH, calcium and distinct light conditions. Although Ca^{2+} experiments were only performed for clades A1 and B1, we noticed that all clades presented distinct growth rate responses and boundaries to pH and light availability. Results provided a mechanistic understanding of *Symbiodinium* clades growth and they are complementary for host-symbiont research. Understanding *Symbiodinium* responses facilitates projections of possible distributional and population structure changes in face of current climate change scenarios (*i.e*., ocean acidification, stratification of the mixing layer, higher light availability).

Key words: Carbonate chemistry, *Symbiodinium,* coral-reefs, physiological response, mechanistic understanding.

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LIST OF SYMBOLS

T Temperature

S Salinity

t Time

IMAS Institute for marine and Antarctic sciences

IOUSP Instituto Oceanográfico da USP

DIC Dissolved inorganic carbon

TA Total alkalinity

D/L Photoperiod (Dark/Light cycle)

Chl-*a* Chlorophyll-*a*

μ Growth rate

c Number of cells

F_b Fluorescence read before addition of hydrochloric acid

F_a Fluorescence read after addition of hydrochloric acid

F_0 Relative fluorescence

K_x Linear correlation factor

V_E Volume of extract

V_s Volume of filtered sample

1. INTRODUCTION :

Symbiodinium, also known as *zooxanthellae*, are photosynthetic dinoflagellates found as yellow-brownish vegetative cysts in a mutualistic endosymbiosis with many marine invertebrates and protists, including, flatworms, molluscs, sponges, ciliates and foraminiferans and most importantly, corals (Pawlowski *et al*., 2001; Stat *et al.,* 2006). When associated with scleractinian corals, their photosynthetic products enable coral growth, reproduction and maintenance (Muscatine & Porter, 1977), underpinning reef productivity and skeleton calcification. It is also known that 80% of scleractinian corals depend on horizontal transmission of *Symbiodinium* (Baird *et al*.,2009), which means that they acquire the dinoflagellate from the water column and into their tissue. It has been widely reported that this order of corals can shift their endosymbiont population to distinct clades (Baker, 2001; Berkelmanns & Van Oppen, 2006; Jones *et al*., 2008), which means that *Symbiodinium* are acquired from the free-living mode into the host.

Not surprisingly, recent studies have also shown large *Symbiodinium* populations as free-living cells in the plankton (Manning & Gates, 2008; Pochon & Gates, 2010; Takabayashi *et al.*, 2012). These organisms have recently been classified into nine clades (A-I) (Pochon & Gates, 2010), and each shows some degree of host-specificity and different tolerances to environmental conditions (Toller *et al.*, 2001; Baker, 2003; Chen *et al*., 2003; Coffroth & Santos, 2005; LaJeunesse, 2005; Berkelmans & van Oppen, 2006; Goulet, 2006).

Symbiodinium's unique role in coral reef systems has led to a recent intense research on the strength of this mutualistic relationship in response to predicted changes in seawater chemistry (Davy & Cook, 2001; Cervino *et al*., 2004; Barneah *et al*.,2007; Hoegh-Guldberg *et al.,*2007), but little still known about what may happen when they are found in the free-living mode.

Seawater geochemical parameters have been changing throughout geological time (Hönisch *et al.* 2012) and are predicted to change in the future. In fact, in such a short time great alterations are expected regarding the carbonate chemistry system due to

anthropogenic CO_2 emissions (Gattuso *et a*l., 2015). In addition, continuous changes in the marine calcium concentration, associated with changes in temperature, light, total dissolved inorganic carbon, pH and alkalinity, are believed to be a major driving force for biological evolution throughout the history of Earth (Kazmierczak & Denges, 1986; Kempe & Kazmierczak, 1994; Kazmierczak & Kempe, 2004). Besides calcium, magnesium and the whole carbonate chemistry have also substantially changed overtime (Horita *et al*., 2002). This has had a major effect on the physiology of several phytoplankton species, such as coccolithophores (Müller *et al*., 2011; Müller *et a*l., 2015).

The present study targets to understand the growth rate response of *Symbiodinum* to changing environmental conditions providing a mechanistic understanding of the organism's physiology. For this, we performed experiments manipulating individual gradients that consider alterations in seawater parameters since the Cretaceous – Tertiary boundary (K-T boundary, 65MY) based on the model compilation of Calcium and seawater carbonate chemistry speciation of Hönisch *et al.* (2012). The K-T boundary is of paramount importance since it was the period when *Symbiodinium* clades most likely started to diverge from its ancestor clade A (Tchernov *et al*., 2004). For future climate change scenarios, we also included elevated pCO_2 levels together with lower pH and increased light intensity that are expected due to anthropogenic carbon dioxide emissions (Gattuso *et a*l., 2015). The results help to comprehend the processes that allowed *Symbiodinium* clades to withstand changes in seawater chemistry in the past and how it may behave in the future. Nevertheless, the findings are also important to understand past and present ocean physical-chemical conditions related to coral reef ecology.

2. HYPOTHESES:

Symbiodinium clades A1, B1, C1 and D1 are the most abundant and important clades for coral reef biology and very little is known about their physiological response to abiotic variables during the free-living stage. Therefore, our research is focused on answering the question below:

"How the individual variation in concentrations of pH, light and calcium influence population growth rates of Symbiodinium clades (A1-D1)?

Different clades may present distinct responses to exposure to seawater containing different gradients of abiotic variables (pH, light and calcium). because they were suggested to exhibit different ranges of tolerance and adaptation to environmental changes, which could also explain why they associate with hosts that occupy a wide range of niches (Iglesias-Prietto et al., 2004).

3. METHODS:

Six experiments were performed. The measured parameters are shown on Table 1, together with a summary of the clades selected and in which facility they took place. Light source was based on cool white fluorescent tubing. The growth rates were used as a proxy for the physiological response of *zooxanthellae*. In some experiments, chlorophyll-*a* production was also measured and used as an additional proxy.

Table 1 – Summary of performed experiments

Experiment	Clade(s)	Sampled parameters	Facility
Control - Natural & Artificial SW	A1,B1,C1,D1	Cell density [1]	IOUSP
Ca gradient (Mg ~ 20mM/kgSW)	A1 & B1	TA & DIC[4]	IOUSP
pH gradient	A1	Cell density [2]; TA; DIC[5] and Chlorophyll	IMAS
Ca gradient (Mg ~ 40mM/kgSW) - Lower pH	A1	Cell density [2]; TA; DIC[5]; Chlorophyll; Ca & Mg concentrations	IMAS
pH gradient	A1,B1,C1,D1	Cell density[2,3]; TA; DIC[4] and Chlorophyll	IOUSP
Light gradient	A1,B1,C1,D1	Cell density[2,3]; TA; DIC[4] and Chlorophyll	IOUSP

Different methods :1- Conting chamber ; 2 – Coulter Counter ; 3 – Flow cytometer ; 4- LICOR-AIRICA Marinda DIC analyzer ; 5- Apollo SciTech DIC analyzer; IOUSP ~ Instituto Oceanográfico da USP and IMAS ~ Institute for Marine and Antarctic Sciences.

3.1 Culture Conditions:

Symbiodinium monoclade cultures (A1, B1, C1 and D1) were obtained from two collections:

Cultures used in the experiments performed at IOUSP came from University at Buffalo (NY-USA) and kept at the Aidar & Kutner Microorganism Collection Bank (BMA&K, IO-USP). They were maintained in natural seawater in f/2 nutrient conditions (Guillard & Ryther, 1962) at 22ºC. Light intensity varied from 80 to 90 µmol photons/m^2s with a photoperiod of 12L:12D.

Cultures used in the experiments performed at IMAS (Institute for Marine and Antarctic Science – University of Tasmania) came from their algae collection facility. Organisms are maintained in natural sea water in f/2 nutrient conditions (Guillard & Ryther, 1962) at 25ºC. Light intensity varies from 250 to 3000 µmol photons/m^2 s with a photoperiod of 12 Light : 12 Dark.

3.2 Seawater preparation:

Natural seawater was obtained from Saco do Ribeira (Ubatuba - SP) and Bruny Island (Tasmania) for the experiments performed at IOUSP and IMAS, respectively. For the control and the first calcium gradient experiment artificial seawater was used. It was prepared according to Kester et al. (1967) in order to guarantee constant conditions in seawater ionic strength.

In both cases, macro- and micronutrients concentration followed conditions present in f/20 medium (Guillard, 1975). Nitrate concentration was about 88µmol/kgSW and phosphate around 36µmol/kgSW. After preparation, the water was filtered with a 0.22 µm filter to avoid contamination and guarantee clean experiment monocultures.

3.3 Experimental design:

3.3.1 Control Experiment: The normal growth rates of the four clades (A1-D1) were estimated in both natural and artificial seawater by cell density determination. Photoperiod was 12:12 D/L; Light 70-80 µmol photons/m^2s and 22ºC.

3.3.2 pH Experiment

3.3.2.1 Part I – IMAS: pH treatments went from 7.5 to 8.3 (7.5 ; 7.7; 7.9; 8.1 and 8.3). Seawater carbonate chemistry changes were simulated through bicarbonate and chloridric acid (HCl) addition according to Fabry *et. al*. (2010). Carbonate chemistry was also aimed to be stable throughout the experiments to measure its effects on the growth rates and chlorophyll-*a* production. Only clade A1 was used. Photoperiod was12:12 D/L; light 280-333µmol photons/m^2s; 25ºC. Cell density, chlorophyll-*a* and Total Alkalinity (TA) and Dissolved Inorganic Carbon (DIC) were measured.

3.3.2.2 Part II – IOUSP: Clades A1, B1, C1 and D1 experiments followed the same set up mentioned above. However, under different photoperiod, light and temperature (12:12 D/L;

Light 70-80 µmol photons/m^2s and 22ºC, respectively). Cell density, chlorophyll-*a* and TA and DIC were measured.

3.3.3 Light Experiment

Clades A1, B1, C1 and D1 were used. Photoperiod was 12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. The light intensities were chosen according to the space availability inside BMA&K (IO-USP). We build up the experiment placing the bottles at distinct distances from the light source. Light treatments went from 70 to 270µmol photons/m^2s (70; 90; 135; 155 and 270). Cell density, chlorophyll-*a* and TA and DIC were measured.

3.3.4 Calcium Experiment

3.3.4.1 Part I – IO USP: Clades A1 and B1 were invetsigated. Artificial seawater was used. Calcium concentrations ranged from 1 to 50 mM/kgSW (1; 10; 20; 30; 40; 50). Abiotic conditions were 12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC.Only TA and DIC were measured.

3.3.4.2 Part II – IMAS: Only clade A1 was investigated. Natural seawater was used and calcium gradient ranged from 10 to 40 (10; 20; 30; 40). Photoperiod was 12:12 D/L; Light 70-80 µmol photons/m^2/s and 22ºC. Cell density, chlorophyll-*a*, calcium concentrations, plus TA and DIC were measured.

3.4 Population growth rates:

Due to logistic limitations, 3 methods were used to cell number density acquisition to estimate population growth rates.

At IOUSP the cell numbers were determined with a Neubauer counting chamber under the microscope. A flow cytometer Attune Nxt was also utilized when it was available.

At IMAS, a Multisizer- 4 Coulter counter was utilized. Organisms from cultures were counted 3 times and the mean was used as de density number.

For all 3 cases, the population growth rates (***µ***) was calculated with the equation below:

$$\mu = \frac{(\ln c_1 - \ln c_0)}{t_1 - t_0}$$

where c_0 and c_1 denote the number of cells at the start (t_0) and end (t_1) of the incubation period (expressed in days).

More information about limitations and differences between the three methods can be found in the discussion.

3.4.1 Counting chamber, Coulter counter and cytometer:

The Neubauer counting chamber is based on manual count of cells inside a known volume of sample (Willén, 1976). Unfortunately, it requires higher number of cells counting to be accurate and precise, which requires large amounts of time by the user. In this case the frequency of human errors is, therefore, inevitably higher.

The coulter counter measures the impedance pulses produced as particles suspending an electrolyte solution pass through a small orifice in a glass tube through which current is flowing through internal and external electrodes (Graham, 2003). As the impedance pulses are proportional to the particle volume, the instrument provides information about particle volumes as well as numbers of cells with high precision. It has been used for counting of various types of algal cells (Marie *et. al*., 2005; Hennige *et. al*., 2009 and Córdoba-Matson, 2010).

The cytometer is based on the principle of individual particles passing through the point of laser illumination allowing measurement of each particle size (via front scattering and side scattering) and fluorescence. The endogenous chlorophyll fluorescence of cells can be used to discriminate the from non-algal cells and debris in the suspension fluid analyzed. When large numbers of cells can be counted, it provides results with high precision. Several types of cytometer have been used to quantify algae cells (Johnson & Zinser, 2006; Littman *et. al*., 2008 and Lee *at. al*., 2012).

3.5 Carbonate Chemistry:

Dissolved inorganic carbon (DIC) was analyzed in triplicate by infrared detection with an Apollo SciTech DIC analyzer and a LICOR-AIRICA MARIANDA at IMAS and at IOUSP, respectively. Total Alkalinity (TA) was also analyzed in triplicates by potentiometric titration (Metrohm Titrino) (Dickson et al., 2003). Data was corrected using certified reference materials (Scripps Institution of Oceanography, USA). The different carbonate system species were calculated using equations from Zeebe and WolfGladrow (2001) with dissociation constants for carbonic acid after Roy et al. (1993), modified with sensitivity parameters for [Na^{+}], [Mg^{2+}] and [Ca^{2+}] (Ben-Yaakov and Goldhaber, 1973). TA and DIC samples were taken in the beginning and at the end of each experiment. "CO_2 Calc Software" was used to perform the calculations of the additional parameters of the carbonate system under known values of salinity (S), temperature(T), nutrients and atmospheric pressure.

3.6 Chlorophyll-*a* analysis:

Determination of chlorophyll-*a* was conducted on a Turner 10AU Fluorimeter using Holm-Hansen and Riemann (1978) and Wasmund et al (2006) protocols at IMAS and IOUSP, respectively.

In Holm-Hansen and Riemann (1978) protocol, the bottles of samples were filtered under slight negative pressure (-0.2milibar) onto a 47mm Whatman GF/F filter; wrapped in aluminum foil and frozen at -20^{o}C for 3 weeks before extraction. Chlorophyll-*a* was extracted in a centrifuge tube by submerging filters in 10 ml of 100% methanol for 12hours at 4^{o}C. Tubes were then spun at 2500 x g for 5min to pellet any particulate matter. Supernatant was removed and placed into a glass tube and fluorescence read (F_b), before the addition of 100 μL 1M hydrochloric acid to remove phaeophytin. Following acid addition, a second fluorescence (F_a) measurement was taken and chlorophyll concentration calculated (Equation 1).

$$chla = \frac{F_b - F_a}{Sample\ Volume\ (ml)/Extract\ Volume\ (ml)}$$

In Wasmund et al (2006) protocol, chlorophyll-*a* was extracted with 90% acetone after being filtered under slight negative pressure onto a 47mm Whatman GF/F filter and also wrapped in aluminum foil and frozen at -20ºC. The extracts were measured in the fluorometer, relative fluorescence is calculated (F_0) discarding blank values. Chlorophyll concentration is estimated also utilizing a linear correlation factor (Kx) obtained from fluorimeter calibration (Equation 2).

$$\text{chl}\,a \text{ total } [\text{mg m}^{-3}] = F_0\ K_x\ V_E\ V_S^{-1},$$

Where: V_E = Volume of the extract;

Vs = Volume of the filtered sample.

3.7 Calcium ion concentration analysis:

Sea water calcium concentrations were determined at the start of the experiments via chelation ion chromatography (Meléndez et al., 2013) with adjustments to match the different calcium concentrations (precision of ±1.4 %). Analysis were done at ACRSS (UTAS) by professor Pavel Nesterenko.

3.8 Redfield ratio and nutrient availability estimations:

Phytoplankton Redfield ratio (Redfield, 1958) was used to estimate nitrogen and phosphorus consumption based on DIC consumption/ biomass built-up in each experiment bottles.

3.9 Statistics:

All the experiments were performed in duplicates and analyzed with a non-linear regression curve. Our question was based on finding the differences between the growth rate (in some cases also chlorophyll-*a*) curves, so a higher number of treatments was preferred, rather than a higher number of replicates, such as previously done in other phytoplankton studies (*i.e*., Bach *et. al*.,2015). In the calcium experiments, however, four replicates were available and ANOVA-ONE WAY statistics was applied.

4. RESULTS:

Although 6 experiments were performed, not all of them succeeded. A summary is shown on Table 2, where the ones in red had unexpected performance problems.

Table 2 – Summary of performed experiments. The ones in red had unexpected performance problems.

Experiment	Clade(s)	Sampled parameters	Facility
Control - Natural & Artificial SW	A1,B1,C1,D1	Cell density [1]	IOUSP
Ca gradient (Mg ~ 20mM/KGSW)	A1 & B1	TA & DIC[4]	IOUSP
pH gradient	A1	Cell density [2]; TA; DIC[5] and Chlorophyll	IMAS
Ca gradient (Mg ~ 60mM/KGSW) - Lower pH	A1	Cell density [2]; TA; DIC[5]; Chlorophyll[6]; Ca & Mg concentrations	IMAS
pH gradient	A1,B1,C1,D1	Cell density[2,3]; TA; DIC[4] and Chlorophyll[7]	IOUSP
Light gradient	A1,B1,C1,D1	Cell density[2,3]; TA; DIC[4] and Chlorophyll[7]	IOUSP

4.1 Control Experiment:

Table 3 – Growth rates responses of clades A1-D1 in artificial and natural sea water. Photoperiod of 12:12 D/L; Light 70-80µmol photons/m^2/s and 22ºC.

	Artificial SW	Natural SW
Clade A1	0.40	0.41
Clade B1	0.30	0.39
Clade C1	0.12	0.13
Clade D1	0.15	0.16

4.2 Calcium Gradient Experiment:

4.2.1 PART I – IOUSP:

4.2.1.1 Carbonate Chemistry:

Carbonate chemistry of clade B1 changed substantially in the experiment (DIC consumption around 45% and TA changes below 4%) as shown in Table 4. However, clade A1 showed abrupt changes in the system (Table 5). The ending values of CO_2 show that the experiment was completely carbon depleted. Additionally, according to Table 21, it was also nitrogen limited.

In this experiment, it was also first noticed the presence of small green cells inside the experiment bottles, which apparently bloomed, mainly in clade A1 when exposed to higher calcium concentrations (Figure 1).

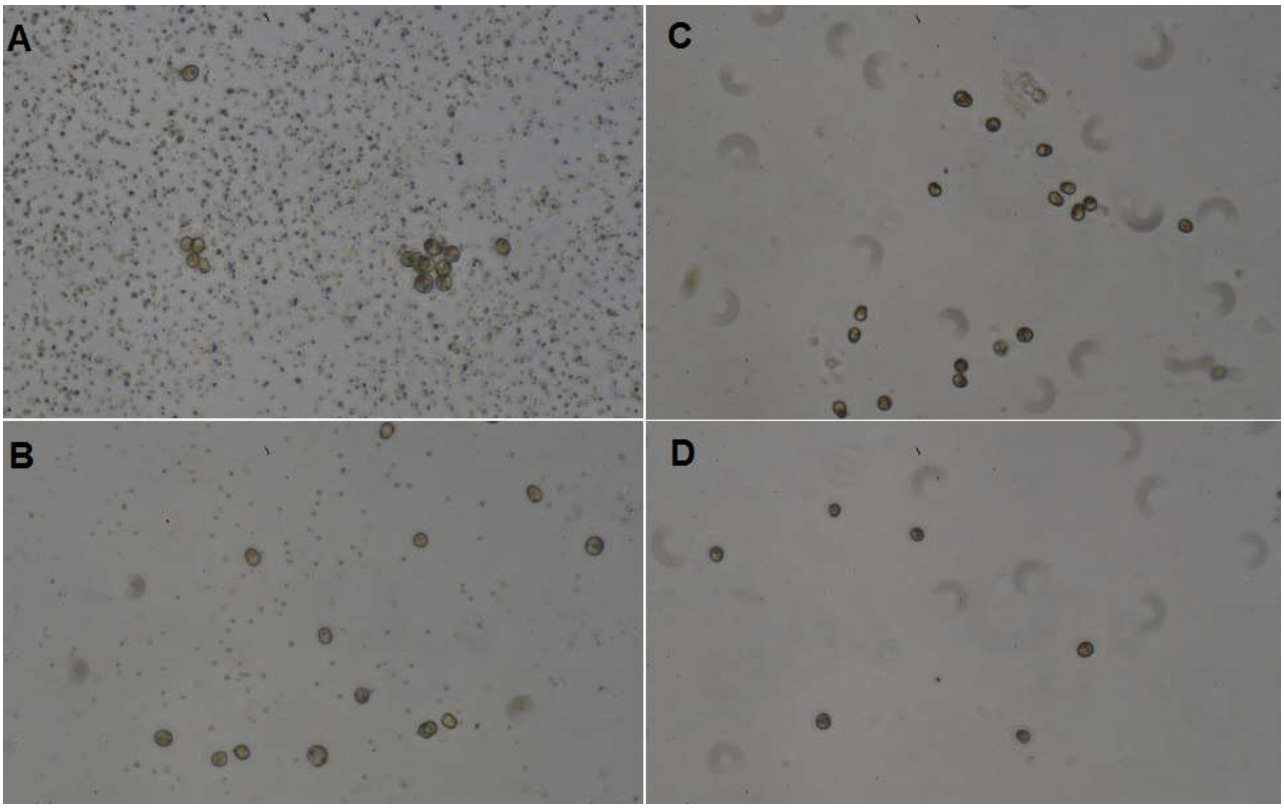

Figure 1: Ca gradient experiment with clades A1 and B1 pictured inside the bottles (200x magnification). **A** – Clade A1 [Ca^{2+}]= 30mM/kgSW ; **B**- Clade A1 [Ca^{2+}]=10mM/kgSW; **C** – Clade B1 [Ca^{2+}]=30mM/kgSW and **D**- Clade B1 [Ca^{2+}]=10mM/kgSW.

Table 4: Carbonate Chemistry variations of Clade A1 calcium gradient experiment performed in artificial sea water under conditions of 12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
Ca 1	2178	2360	2047	1046	1047	1047	937.31	2.53	7.70	9.41	1915.15	270.09	105.33	776.84	26.53	0.07
Ca 10	2286	2410	2134	1106	1059	1083	885.20	2.76	7.74	9.4	1988.69	285.75	120.26	797.17	25.05	0.08
Ca 20	2126	2469	2078	233	188	211	1654.37	0.01	7.46	10.42	1968.15	6.96	63.02	204.04	46.82	0
Ca 30	2175	2248	2078	678	358	518	1181.72	0.06	7.60	10.13	1957.29	32.7	87.26	485.29	33.44	0
Ca 50	2020	2051	1865	236	228	232	708.28	0.01	7.77	10.28	1731.07	10.49	113.88	221.51	20.05	0

Table 5: Carbonate Chemistry variations of Clade B1 calcium gradient experiment performed in artificial sea water under conditions of 12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
Ca 1	2178	2249	2047	2014	2047	2031	937.31	586.34	7.70	7.89	1915.15	1856.23	105.33	158.17	26.53	16.59
Ca 10	2286	2290	2134	2025	2018	2022	885.20	465.68	7.74	7.98	1988.69	1818.29	120.26	190.49	25.05	13.22
Ca 20	2126	2302	2078	1911	1924	1918	1654.37	285.53	7.46	8.15	1968.15	1655.04	63.02	260.96	46.82	8
Ca 30	2175	2399	2078	1915	2002	1959	1181.72	238.67	7.60	8.22	1957.29	1646.5	87.26	305.75	33.44	6.75
Ca 50	2020	2036	1865	1916	1923	1920	708.28	936.97	7.77	7.67	1731.07	1800.37	113.88	93.12	20.05	26.52

Table 6: Carbonate Chemistry variations of Clade A1 performed in natural sea water under conditions of 12:12 D/L; light 280-333µmol photons/m^2s; 25ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pH st	pH end	pCO_2 st	pCO_2 end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
Ca 10	2223	2281	2027	1924	1925	1924	7.83	8.11	683.45	314.52	1865.11	1671.15	143.23	244.51	19.25	8.86
Ca20	2204	2315	2020	1899	1887	1893	7.81	8.20	707.27	243.91	1864.58	1598.81	135.51	287.82	19.92	6.87
Ca 30	2191	2301	2021	1936	1928	1932	7.77	8.13	790.45	305.13	1870.66	1670.58	128.04	253.33	22.30	8.59
Ca 40	2242	2297	2016	1912	1920	1916	7.89	8.15	584.54	287.40	1838.27	1647.15	161.64	260.76	16.46	8.10

4.2.2 PART II – IMAS:

4.2.2.1 Carbonate Chemistry:

The experiment was only performed with clade A1. DIC changes in the carbonate system were around 5% and TA below 4% (Table 6). The real calcium values referred to the treatments are shown further on item 4.2.2.1.

4.2.2.2 Growth Rate Response:

Growth rate response showed a linear tendency (Figure 2). ANOVA ONE-WAY test was used to analyze differences between the treatments showing p>0.005 (Table 7). Therefore, the treatments did not show any statistically relevant distinction on clade A1 growth rate.

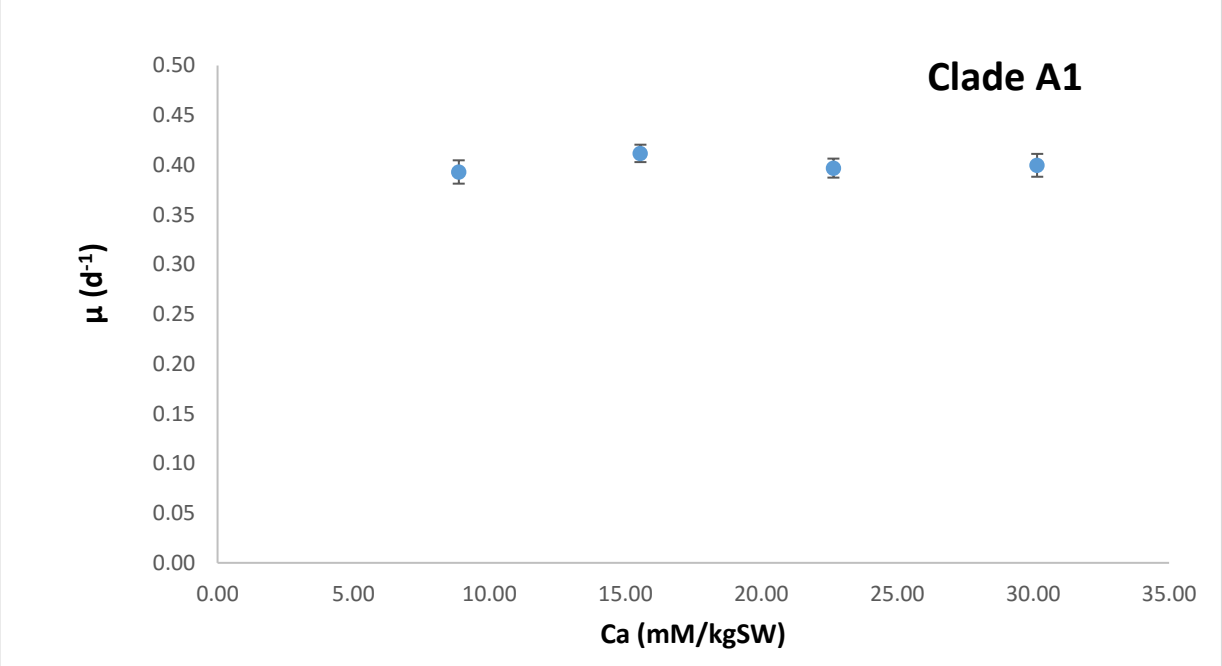

Figure 2: Clade A1 growth rate response under distinct calcium concentrations (item 4.2.2.4). The experiment was performed with natural SW in quadruplicates under conditions of S=35.5;12:12 D/L; light 280-333μmol photons/m^2/s; 25ºC.Graph shows average points with its respective standard deviations (n=4).

Table 7: ANOVA ONE WAY test summary for clade A1 calcium gradient experiment.

ANOVA Summary		4			
Source	SS	df	MS	F	P
Treatment [between groups]	0.00065	3	0.000217	1.49	0.267080
Error	0.00175	12	0.000146		
Ss/Bl					Graph Maker
Total	0.0024	15			

4.2.2.3 Chlorophyll-*a* Production:

Clade A1 chlorophyll-*a* production per day was not much variable among the calcium treatments (Figure 3), showing a similar tendency of increasing growth rates in which, also, no significant difference was noticed.

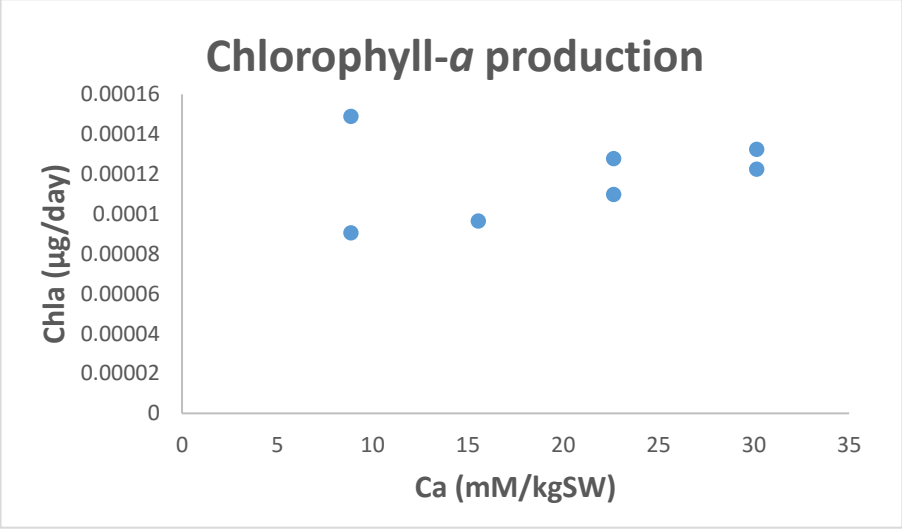

Figure 3: Chlorophyll-*a* production of clade A1 under the calcium gradient (n=2).The experiment was performed with natural SW under conditions of S=35.5;12:12 D/L; light 280-333µmol photons/m^2s; 25ºC. Graph scale had to be distinct from the other chlorophyll graphs in order to create visible data points.

4.2.2.4 Real Calcium and Magnesium concentrations:

Real calcium concentrations were a bit lower than expected (Table 8).

Table 8: Real calcium and magnesium concentrations in each desired treatment.

Desired Treatment [mM/kgSW]	Ca (mM/kgSW)
Ca10	8.87
Ca20	15.55
Ca30	22.65
Ca40	30.15

4.3 pH Gradient Experiment:

4.3.1 PART I – IMAS

4.3.1.1 Carbonate Chemistry

The experiment was only performed with clade A1. Carbonate chemistry presented variations around 5% and below 3% of DIC and TA, respectively (Table 9).

4.3.1.2 Growth Rate Response

Growth rate response of clade A1 showed slight distinctions between treatments and a tendency of decreasing growth rates with lower pH values. Duplicates resulted in a curve with R=0.8556.

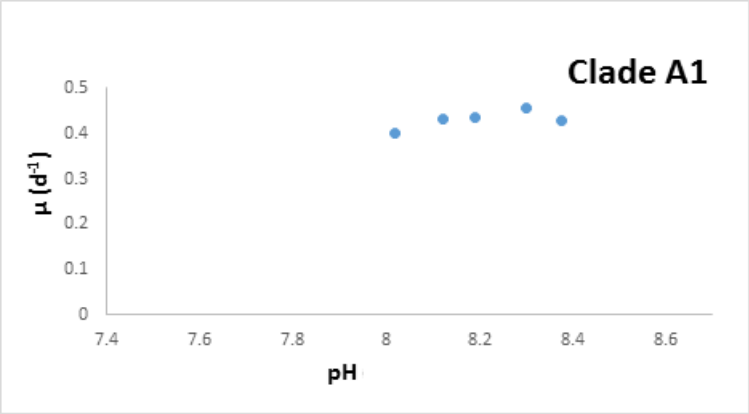

Figure 4: Growth rate response curve of clade A1 under five different pH treatments (referred values on Table 9). Regression curve was obtained with n=2.

4.3.1.2 Chlorophyll-*a* Production:

Clade A1 chlorophyll-*a* production per day was not much variable along the pH gradient (Graph 3) and the amounts produced were all very low.

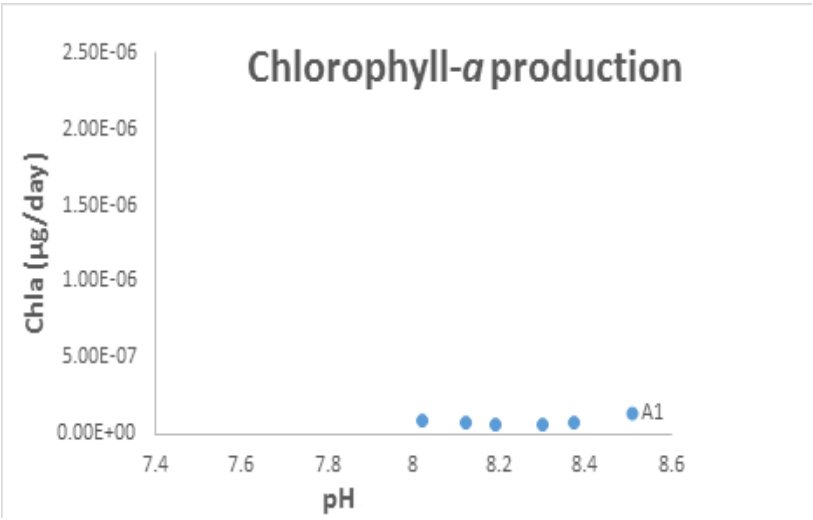

Figure 5: Chlorophyll-*a* production of clade A1 under the pH gradient (n=1). The experiment was performed with natural SW under conditions of 12:12 D/L; light 280-333μmol photons/m^2s; 25ºC.

4.3.2 PART II – IOUSP

4.3.2.1 Carbonate Chemistry:

Carbonate chemistry variations for all the clades were higher than in the experiment performed at IMAS. Average DIC consumption in the four clades was around 10% and TA changed about (Tables 10-13). The experiment had variable quantifiable numbers of the small green cells (Figure 8) which have contributed, together with *Symbiodinium* for carbonate chemistry alterations. Table also shows that clade C treatments were under nitrogen depleted conditions due to high biomass build-up.

Table 9: Carbonate Chemistry variations of Clade A1 performed in natural sea water under conditions of 12:12 D/L; light 280-333µmol photons/m^2s; 25ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
pH1	2575	2688	2135	1922	1861	1891	8.18	8.57	289.67	87.25	1814.65	1330.96	312.2	557.59	8.16	2.46
pH2	2593	2688	2253	1843	1952	1898	8.04	8.56	442.39	89.52	1993.78	1342.54	246.77	552.94	12.46	2.52
pH3	2669	2690	2386	2032	1997	2014	7.94	8.44	602.08	135.45	2156.86	1533.44	212.19	476.75	16.95	3.81
pH4	2675	2728	2466	2059	2041	2050	7.81	8.43	860.67	140.08	2276.42	1565.56	165.35	480.5	24.23	3.94
pH5	2674	2688	2511	2157	2067	2112	7.71	8.33	1095.82	193.42	2342.65	1697.45	137.53	409.1	30.85	5.45

Table 10: Carbonate Chemistry variations of Clade A1 performed in natural sea water under conditions of 12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.2.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
pH1	2584	2703	2602	2282	2288	2285	3196.40	345.09	7.27	8.14	2460.45	1971.01	51.18	304.24	90.37	9.76
pH2	2569	2793	2523	2311	2344	2328	2085.30	310.33	7.44	8.19	2390.02	1979.17	74.03	340.03	58.96	8.8
pH3	2528	2685	2272	2164	2160	2162	603.10	227.94	7.92	8.28	2064.06	1780.76	190.89	374.78	17.05	6.46
pH4	2485	2722	2114	2058	2059	2059	346.70	143.36	8.11	8.43	1840.22	1582.72	263.98	472.23	9.8	4.05
pH5	2471	2802	2024	1947	1929	1938	247.90	76.83	8.22	8.62	1703.48	1321.51	313.45	614.32	7.07	2.17

Table 11: Carbonate Chemistry variations of Clade B1 performed in natural sea water under conditions of 12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.2

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
pH1	2584	2654	2602	2245	2249	2247	3196.40	345.85	7.27	8.13	2460.45	1942.4	51.18	294.82	90.37	9.78
pH2	2569	2680	2523	2269	2583	2426	2085.30	680.09	7.44	7.9	2390.02	2212.29	74.03	194.48	58.96	19.23
pH3	2528	2771	2272	2122	2118	2120	603.10	157/32	7.92	8.41	2064.06	1648.63	190.89	466.92	17.05	4.45
pH4	2485	2707	2114	1955	1981	1968	346.70	107.5	8.11	8.51	1840.22	1442.11	263.98	522.85	9.8	3.04
pH5	2471	2640	2024	1747	1785	1766	247.90	59.37	8.22	8.67	1703.48	1156.97	313.45	607.34	7.07	1.68

Table 12: Carbonate Chemistry variations of Clade C1 performed in natural sea water under conditions of12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.2.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
pH1	2584	2699	2602	2059	1998	2028.5	3196.40	136.2	7.27	8.44	2460.45	1548.73	51.18	453.74	90.37	3.85
pH2	2569	2683	2523	1817	1893	1855	2085.30	75.35	7.44	8.61	2390.02	1272.15	74.03	580.72	58.96	2.13
pH3	2528	2645	2272	1794	1689	1741.5	603.10	53.29	7.92	8.7	2064.06	1113.02	190.89	626.97	17.05	1.51
pH4	2485	2669	2114	1676	1672	1674	346.70	38.11	8.11	8.8	1840.22	984.94	263.98	687.98	9.8	1.08
pH5	2471	2685	2024	1600	1602	1601	247.90	26.53	8.22	8.89	1703.48	855.18	313.45	745.07	7.07	0.75

Table 13: Carbonate Chemistry variations of Clade D1 performed in natural sea water under conditions of 12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.2.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
pH1	2584	2720	2602	2544	2515	2530	3196.40	958.48	7.27	7.77	2460.45	2347.52	51.18	155.38	90.37	27.1
pH2	2569	2665	2523	2268	2308	2288	2085.30	392.39	7.44	8.09	2390.02	2001.77	74.03	275.1	58.96	11.13
pH3	2528	2625	2272	2264	2245	2255	603.10	390.53	7.92	8.09	2064.06	1975.04	190.89	268.88	17.05	11.08
pH4	2485	2684	2114	2034	2030	2032	346.70	143.23	8.11	8.42	1840.22	1565.51	263.98	462.44	9.8	4.05
pH5	2471	2675	2024	1930	1931	1931	247.90	101.7	8.22	8.53	1703.48	1404.15	313.45	523.98	7.07	2.88

4.3.2.2 Growth Rate Response:

Figure 6 shows the growth rates of the four clades with its respective "R²" values of the regression analysis. Response curves were different between clades and they showed distinct cell numbers of green cells (Figure 11). Clade A1 had the lowest growth rate variations along the pH gradient and it showed an optimum response very similar to clade B1 (7.8 and 7.78, respectively). Clade C1 had its optimum on pH 8.3 and D1 on pH 8.01

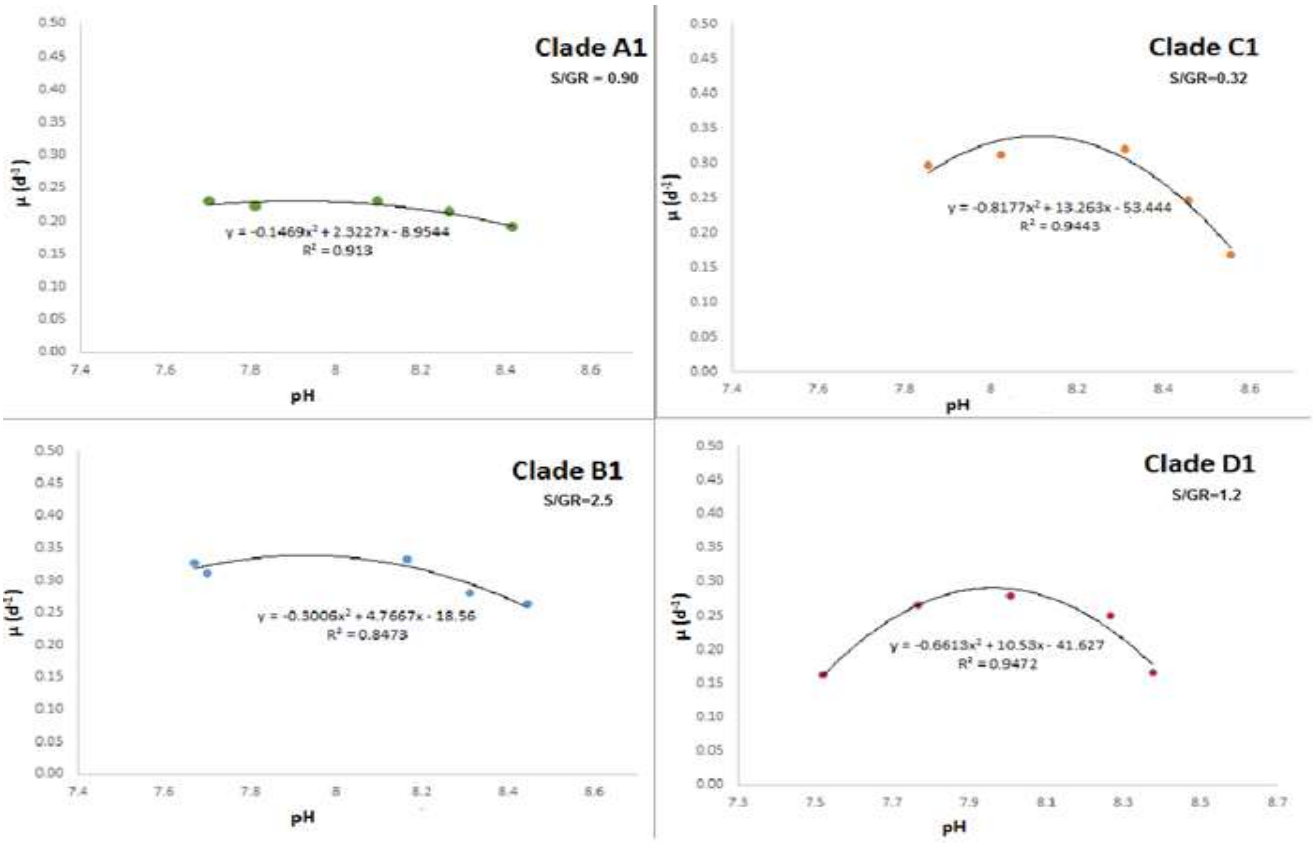

Figure 6: Growth rate response curves of A1, B1, C1 and D1 clades. Regression curve was calculated with n=2. S/GC numbers represent the averages of the ratios between *Symbiodinium* and green cell that each clade has presented in the experiment. pH is shown in seawater scale.

4.3.2.3 Chlorophyll-*a* Production:

Clades A1- D1 chlorophyll-*a* production per day was different (Figure 3). Clades A1 and B1 showed similar response but low production. Clade C1 had the highest production and showed a tendency of increasing along with pH increase. Clade D1 had the lowest production rate.

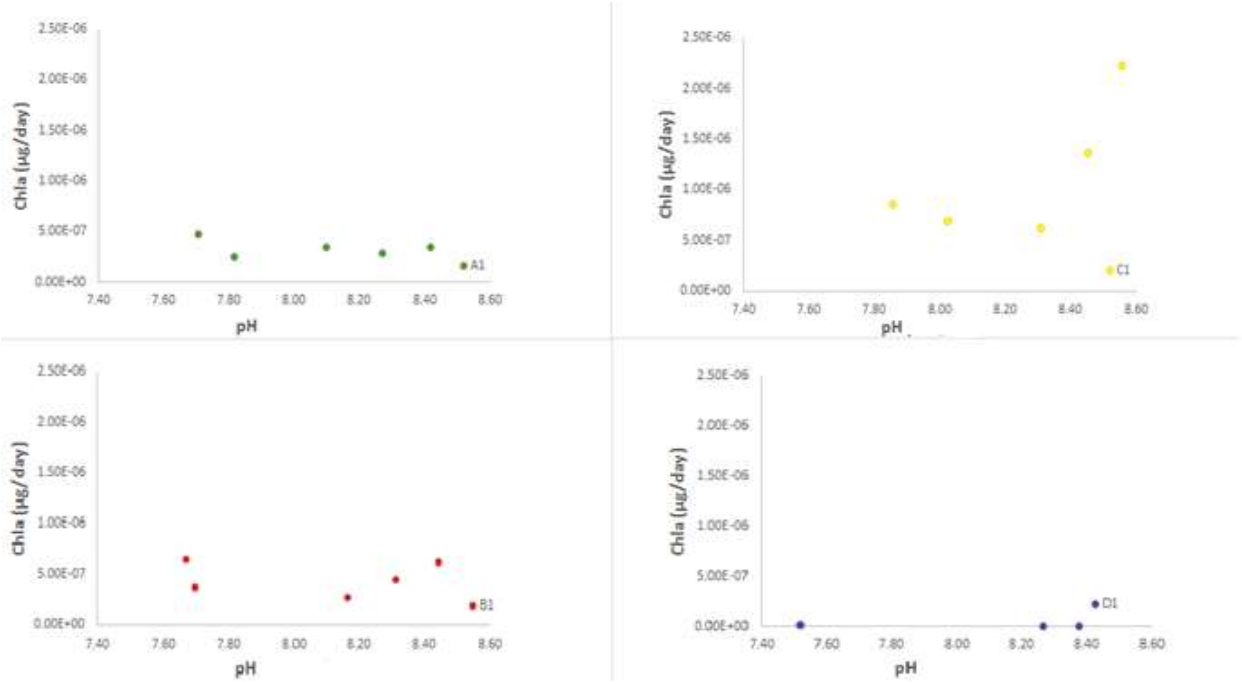

Figure 7: Chlorophyll-*a* production of clades A1-D1 under the pH gradients (n=1). The experiment was performed in natural sea water under conditions of conditions12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. pH is shown in seawater scale.

4.4 Light Gradient Experiment:

4.4.1 Carbonate Chemistry:

Average DIC variation was the highest in all the experiments (around17%) and TA around 3% (Tables 15 to 18). Clade C1 was the one with highest DIC consumption (981μmol) and according to Table 21 it was also under nitrogen depleted conditions. The experiment had variable quantifiable numbers of the small green cells (Figure 8) which have contributed, together with *Symbiodinium* for carbonate chemistry alterations. Again, clade C1 was the one with higher absolute number of green cells. Table 14 shows the light intensities conditions.

Table 14: Light Intensities of the five treatments.

Light treatment	μmol photons/m²s
1	70
2	90
3	135
4	155
5	270

Table 15: Carbonate Chemistry variations of Clade A1 performed in natural sea water under 12:12 D/L and 22ºC. The respective light intensities referred to the treatments are shown above on Table 14. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.2.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
1	2950	2998	2500	2450	2453	2452	372.97	277.24	8.15	8.25	2153.4	2038.92	336.01	405.24	10.54	7.84
2	2950	3031	2500	2337	2336	2337	372.97	175.16	8.15	8.4	2153.4	1820.64	336.01	511.41	10.54	4.95
3	2950	3034	2500	2284	2285	2285	372.97	147	8.15	8.46	2153.4	1730.39	336.01	550.46	10.54	4.16
4	2950	3042	2500	2203	2203	2203	372.97	110.23	8.15	8.54	2153.4	1585.52	336.01	614.36	10.54	3.13
5	2950	3038	2500	2044	2049	2047	372.97	65.94	8.15	8.69	2153.4	1325.29	336.01	719.85	10.54	1.86

Table 16: Carbonate Chemistry variations of Clade B1 performed in natural sea water under 12:12 D/L and 22ºC. The respective light intensities referred to the treatments are shown above on Table 14. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at T°C = 25 ; S=35.2.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
1	2950	3012	2500	2361	2340	2351	372.97	191.64	8.15	8.37	2153.4	1858.5	336.01	487.08	10.54	5.42
2	2950	3038	2500	2240	2172	2206	372.97	112.67	8.15	8.54	2153.4	1593.64	336.01	609.18	10.54	3.19
3	2950	3059	2500		2066	2066	372.97	67.24	8.15	8.68	2153.4	1341.17	336.01	722.93	10.54	1.9
4	2950	3064	2500	2046	2001	2024	372.97	57.39	8.15	8.73	2153.4	1266.73	336.01	755.65	10.54	1.62
5	2950	3099	2500		1982	1982	372.97	45.57	8.15	8.79	2153.4	1169.53	336.01	811.19	10.54	1.29

Table 17: Carbonate Chemistry variations of Clade C1 performed in natural sea water under 12:12 D/L and 22ºC. The respective light intensities referred to the treatments are shown above on Table 14. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at TºC = 25 ; S=35.2.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
1	2950	3067	2500	1704	1751	1728	372.97	17.2	8.15	9.04	2153.4	777.63	336.01	948.88	10.54	0.49
2	2950	3080	2500	1613	1552	1583	372.97	7.76	8.15	9.23	2153.4	545.79	336.01	1036.99	10.54	0.22
3	2950	3086	2500	1405	1470	1438	372.97	2.98	8.15	9.45	2153.4	347.01	336.01	1090.91	10.54	0.08
4	2950	3069	2500	1440	1457	1449	372.97	3.43	8.15	9.42	2153.4	369.87	336.01	1079.03	10.54	0.1
5	2950	3080	2500	1394	1407	1401	372.97	2.34	8.15	9.5	2153.4	307.41	336.01	1093.52	10.54	0.007

Table 18: Carbonate Chemistry variations of Clade D1 performed in natural sea water under 12:12 D/L and 22ºC. The respective light intensities referred to the treatments are shown above on Table 14. Starting(st) and ending(end) conditions were calculated from TA and DIC measurements. Data compilation at TºC = 25 ; S=35.2.

Treatment	TA st	TA end	DIC st	DIC A end	DIC B end	Avg DIC end	pCO_2 st	pCO_2 end	pH st	pH end	HCO_3^- st	HCO_3^- end	CO_3^{2-} st	CO_3^{2-} end	CO_2 st	CO_2 end
1	2950	2985	2500	2486	2482	2484	372.97	320.05	8.15	8.2	2153.4	2101.9	336.01	373.05	10.54	9.05
2	2950	2961	2500	2488	2471	2480	372.97	336.79	8.15	8.18	2153.4	2112.41	336.01	358.07	10.54	9.52
3	2950	2990	2500	2454	2363	2409	372.97	244.8	8.15	8.29	2153.4	1272.54	336.01	429.54	10.54	6.92
4	2950	2967	2500	2340	2342	2341	372.97	206.51	8.15	8.34	2153.4	1875.05	336.01	460.11	10.54	5.84
5	2950	2969	2500	2268	2270	2269	372.97	161.98	8.15	8.42	2153.4	1752.19	336.01	512.23	10.54	4.58

4.4.2 Growth Rate Response:

Figure 4 shows that growth rate responses were also different between the four clades. For clades A1 and B1 it was possible to estimate Michaelis-Menten optimum kinetics curve. However, only clade A1 data points fit well into its curve. Clade B1 presented a non-expected growth rate of 0.41 at intensity of 270 µmol photons/m^2s, but it was also nearly a nitrogen depleted condition (Table 21).

According to its curve, clade C1 presented the lowest growth rates in all light intensities. However, all experiment bottles were under nitrogen limited conditions (Table 21). Clade D1 showed no specific behavior and we believe the data points are not consistent due to its low counting on estimation of cell densities.

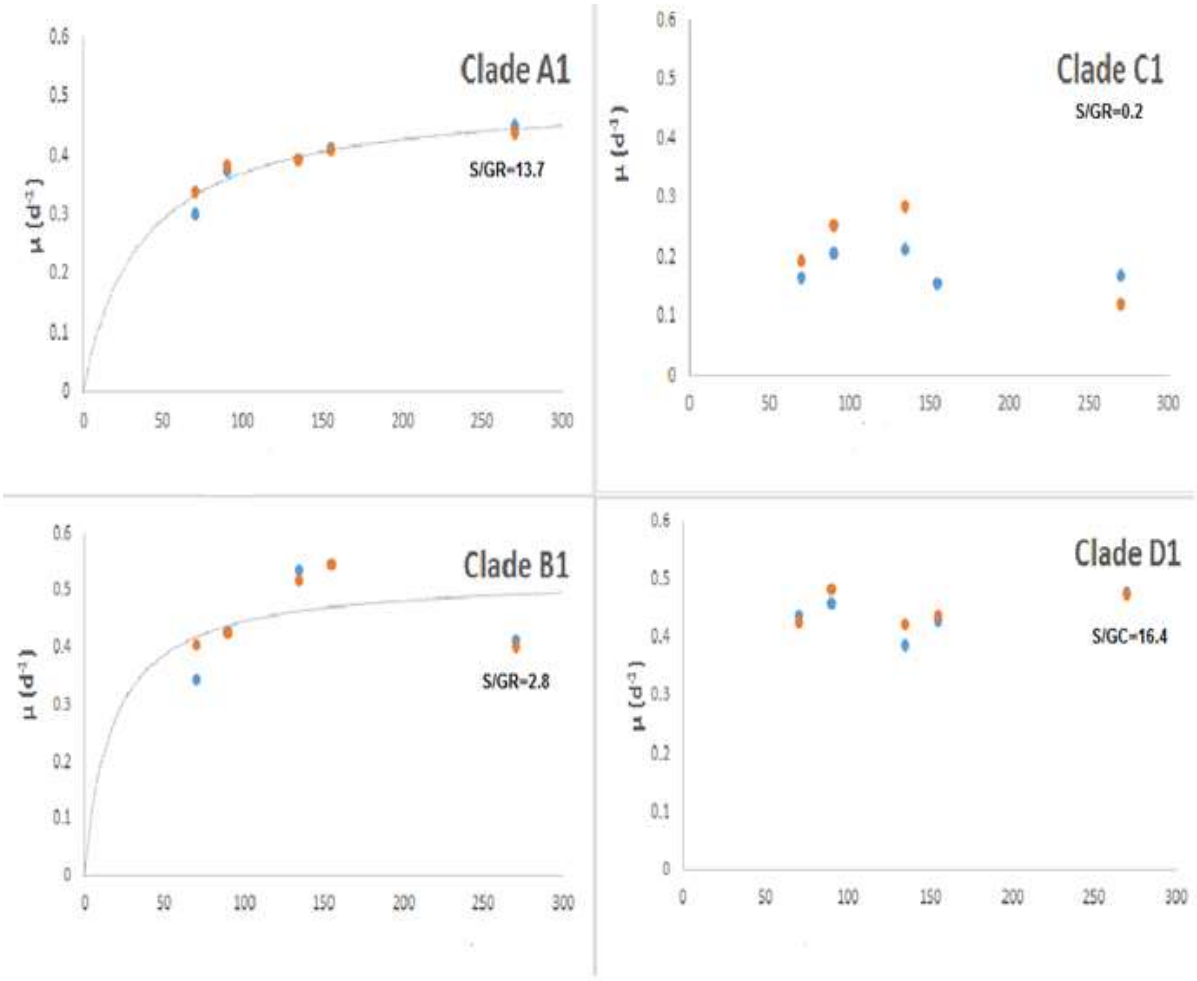

Figure 8: Growth rate response curves of A1, B1, C1 and D1 clades. Michaelis Menten Kinetics curve was estimated for clades A1 and B1 and its respective Km and Vm values are presented on table 19. X axis represents light intensity μmol photons/m^2s. S/GC numbers represent the averages of the ratios between *Symbiodinium* and green cells that each clade has presented in the experiment.

Table 19: Km and Vm values for Michaelis-Menten Kinetics curves estimated on Figure 3.

Kinetics Curve	Km	Vmax
Clade A1	36.40	0.51
Clade B1	17.91	0.52

4.4.3 Chlorophyll-*a* Production:

Clades A1- D1 chlorophyll-*a* production per day was different (Figure 5). Although it is only noticeable in clade C1 due to the graphs scales, there was a tendency of lower production at higher light intensities also for clades A1 and B1. Clade D1 was the one with the lowest production rates.

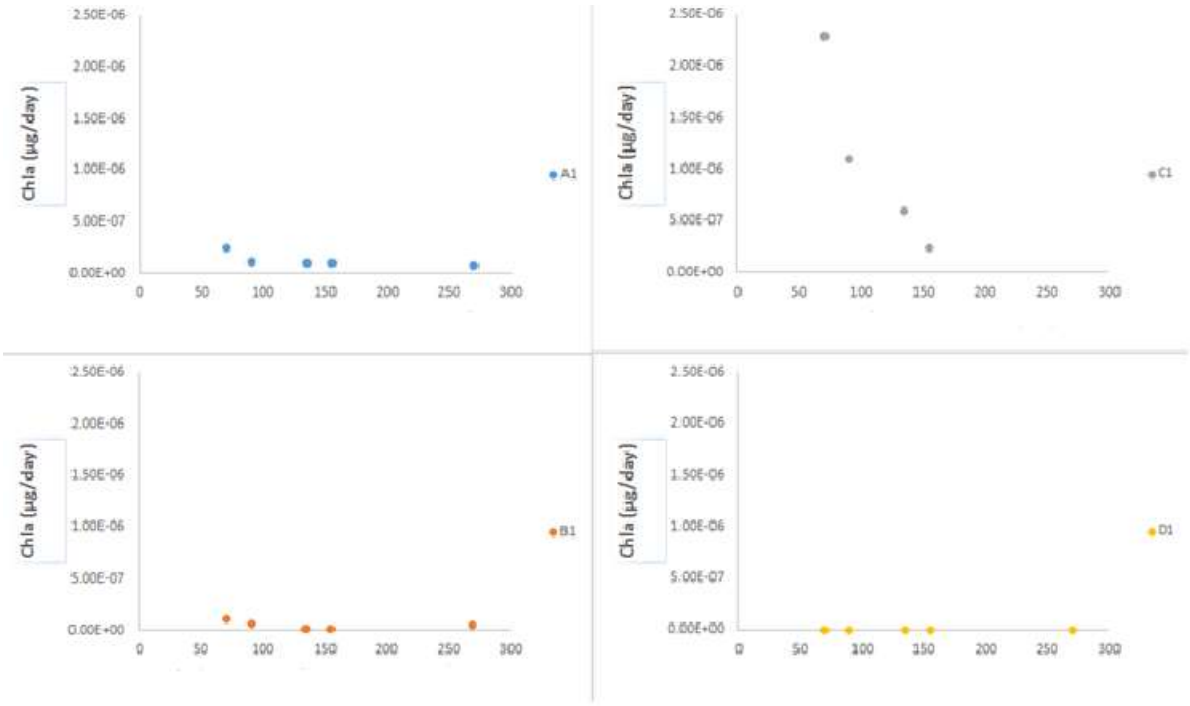

Figure 9: Chlorophyll-*a* production of clades A1-D1 under different light intensities (n=1).The experiment was performed in natural sea water under conditions of conditions12:12 D/L; Light 70-80µmol photons/m^2s and 22ºC. X axis represents light intensity µmol photons/m^2s.

4.5 Green Cells Observations:

All experiments presented unexpected small green cells (Figure 6) growing together with *Symbiodonium*. There were different numbers of cells in the experiments but clade C1 always showed the highest densities and the lowest *Symbiodinium*/Green Cells ratio (Figure 7). Initial cell numbers of green cells could not be estimated because, at IOUSP, all initial densities of the experiments were done with a counting chamber where the presence of the smaller organism is barely detectable.

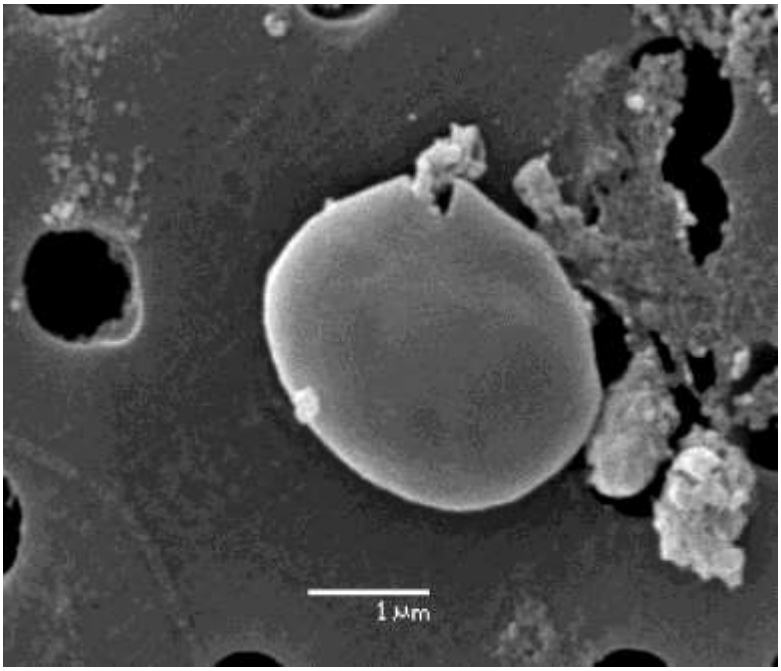

Figure 10: Electronic microscope capture of green cells.

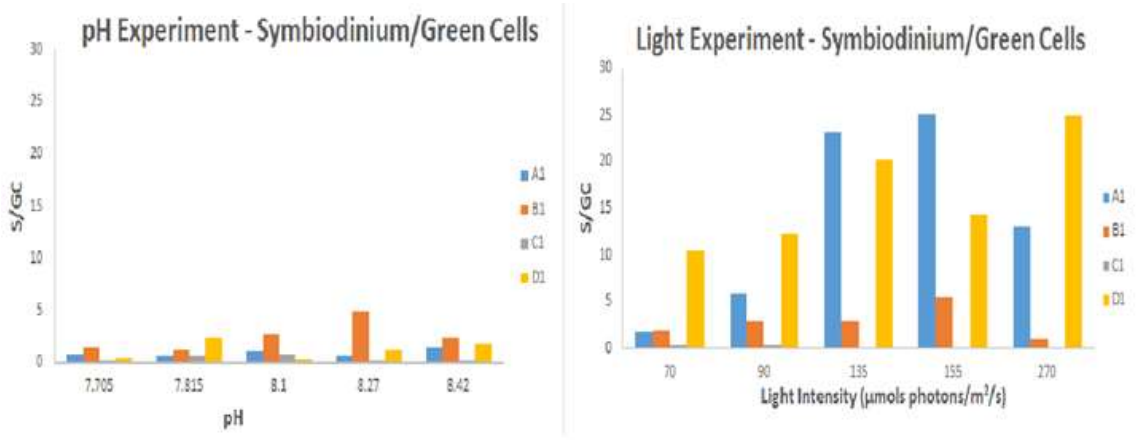

Figure 11: *Symbiodinium*/Green Cells ratio graphs from pH and light experiments.

4.6 Redfield Ratio and Nutrients Availability Estimations:

We estimated the total nitrogen and phosphorus consumption in each experiment bottle (Table 21) based on DIC consumption (biomass build-up) data from carbonate chemistry (Tables 4-6, 9, 10-13 and 15-18) and the Redfield ratio considered for phytoplankton. Initial nutrient f/20 media showed on Table 20 was utilized for the calculations.

Experiments performed with clade C1 for both light and pH gradients showed the highest nitrogen depletion conditions. In addition, in the calcium gradient experiment (pH~8.3 & Mg~20mM/kgSW), clade A1 also exhibited nitrogen depletion.

Table 20: f/20 media nitrate and phosphate concentrations.

f/20 media	µmol/kgSW
NO_3^-	88
PO_4^{3-}	36

Table 21: Estimations of total consumed nitrogen and phosphorus in the experiments. Estimations were done based on the DIC variations from carbonate chemistry data and the Redfield ratio estimated for phytoplankton. The Treatments* refers to data presented in the carbonate chemistry data tables from each experiment. Conditions marked in red denote nitrogen limited scenarios, while blue marks denote near nitrogen limitation scenarios. In both scenarios the Symbiodinium/ Green Cells ratios were lower than one.

			REDFIELD RATIO			
			C	N	P	
			106	16	1	
Experiment	**Clade**	**Treatment***	**Δ DIC**	**Estimated NO_3^- availability**	**Estimated PO_4^{3-} availability**	***Symbiodinium*/Green Cells**
Light	A1	1	-48.5	80.7	35.5	1.73
	A1	2	-163.5	63.3	34.5	5.77
	A1	3	-215.5	55.5	34.0	23.17
	A1	4	-297.0	43.2	33.2	25.19
	A1	5	-453.5	19.5	31.7	12.95
	B1	1	-149.5	65.4	34.6	2.02
	B1	2	-294.0	43.6	33.2	2.91
	B1	3	-434.0	22.5	31.9	2.93
	B1	4	-476.5	16.1	31.5	5.50
	B1	5	-518.0	9.8	31.1	0.91

	C1	1	-772.5	-28.6	28.7	0.37
	C1	2	-917.5	-50.5	27.3	0.37
	C1	3	-1062.5	-72.4	26.0	0.16
	C1	4	-1051.5	-70.7	26.1	0.13
	C1	5	-1099.5	-78.0	25.6	0.06
	D1	1	-16.0	85.6	35.8	10.50
	D1	2	-20.5	84.9	35.8	12.25
	D1	3	-91.5	74.2	35.1	20.18
	D1	4	-159.0	64.0	34.5	14.30
	D1	5	-231.0	53.1	33.8	25.00
pH	A1	pH 1	-317	40.2	33.0	0.74
	A1	pH 2	-195.5	58.5	34.2	0.56
	A1	pH 3	-110	71.4	35.0	1.07
	A1	pH 4	-55.5	79.6	35.5	0.67
	A1	pH 5	-86	75.0	35.2	1.47
	B1	pH 1	-355	34.4	32.7	1.46
	B1	pH 2	-97	73.4	35.1	1.23
	B1	pH 3	-152	65.1	34.6	2.60
	B1	pH 4	-146	66.0	34.6	4.90
	B1	pH 5	-258	49.1	33.6	2.42
	C1	pH 1	-573.5	1.4	30.6	0.13
	C1	pH 2	-668	-12.8	29.7	0.59
	C1	pH 3	-530.5	7.9	31.0	0.71
	C1	pH 4	-440	21.6	31.8	0.11
	C1	pH 5	-423	24.2	32.0	0.10
	D1	pH 1	-72.5	77.1	35.3	0.49
	D1	pH 2	-235	52.5	33.8	2.38
	D1	pH 3	-17.5	85.4	35.8	0.39
	D1	pH 4	-82	75.6	35.2	1.16
	D1	pH 5	-93.5	73.9	35.1	1.80
Ca (pH~7.9 & Mg~60mM/kgSW)	A1	Ca 10	-102.82	72.5	35.0	N/A
	A1	Ca 20	-126.875	68.8	34.8	N/A
	A1	Ca 30	-88.495	74.6	35.2	N/A
	A1	Ca 40	-100.1	72.9	35.1	N/A
pH	A1	pH 1	-244.16	51.1	33.7	N/A
	A1	pH 2	-355.33	34.4	32.6	N/A
	A1	pH 3	-371.61	31.9	32.5	N/A
	A1	pH 4	-415.875	25.2	32.1	N/A
	A1	pH 5	-399.055	27.8	32.2	N/A
Ca (pH~8.3 & Mg~20mM/kgSW)	A1	Ca 1	-1000.5	-63.0	26.6	N/A
	A1	Ca 10	-1051.5	-70.7	26.1	N/A
	A1	Ca 20	-1867.5	-193.9	18.4	N/A
	A1	Ca 30	-1560	-147.5	21.3	N/A

	A1	Ca 50	-1633	-158.5	20.6	N/A
	B1	Ca 1	-16.5	85.5	35.8	N/A
	B1	Ca 10	-112.5	71.0	34.9	N/A
	B1	Ca 20	-160.5	63.8	34.5	N/A
	B1	Ca 30	-119.5	70.0	34.9	N/A
	B1	Ca 50	54.5	96.2	36.5	N/A

5. DISCUSSION:

In this research, we aimed to discover how do individual varying concentrations of pH, light and calcium influence population growth rates of free-living *Symbiodinium* clades (A1-D1). The results showed that they present distinct optimum curves and specificities to pH and light (Figures 6 and 8, respectively). However, calcium was only studied for clades A1 and B1 producing insufficient data, so the objective failed for calcium influence.

pH and light experiments also presented challenging issues regarding its different methods utilization due to logistics and financial challenges. It would be ideal if all the experiments in this research have used the same cell density estimation method, so that the errors would be the same in every counting, therefore, the desired growth rates (based on cell density variations) would be likely to be more accurate. We discuss below, how do each method error could have influenced in our cell densities estimations and its implications accomplishing the research objectives. Furthermore, we add about each experiment results.

5.1 Methods considerations:

5.1.2 Cell density estimations:

Krediet *et. al*., (2015) showed a comparison of *Symbiodinium* counts utilizing different instruments, where a comparison between flow cytometer (Guava) and Coulter Counter was reported. Both seemed to be very precise and accurate methods. However, higher counts can be obtained with the Coulter Counter reflecting some counting of non-*Symbiodinium* debris, whereas a failure to count some *Symbiodinium* cells of low fluorescence is plausible utilizing the flow cytometer. As a consequence, by utilizing the Coulter Counter, the real cell density numbers tend to be super estimated, contrasting with the flow cytometer counts that tend to under estimate it.

In our case, it is most likely that the data of *Symbiodinium* cell densities from the flow cytometer estimations presented the lowest precision and accuracy. Only 1.5mL was sampled from each experiment bottle, consequently the number of counts was mostly low. Low cell densities were aimed to avoid great biomass build-up, which would cause variations in the carbonate chemistry. Besides, inside the experiment bottles, not only *Symbiodinium* was present, but there were also variable quantities of small green organisms (Figure 12). Fortunately, they presented completely different sizes (*Symbiodinium* between 10-12µm and green unicellular organisms smaller than 4µm) so that they could be well separated in different gates utilizing front scatter and side scatter graphs (Figure 14), due to its indication of particle volume and size by the flow cytometer. As exemplified in Figure 14, when the estimated densities were higher (>4.000cells/mL), the data plots fit better into the gates. Nonetheless, in many cases, the densities were too low and/or aggregations of cells were present, resulting in less satisfactory counts where populations were overlapping each other (Figure 15). When Coulter Counter was utilized, (experiments performed at IMAS ~ Figures 2&4; Tables 6&9) the green unicellular organisms were also detected by a peak (*Symbiodinium*) and a great aggregation of particles smaller than 4µm. Additionally, *Symbiodinium* cells densities were more consistent in the triplicate measurements, but the densities of the smaller organisms could not be estimated due to limitations of volume range in the equipment (Figure 13).

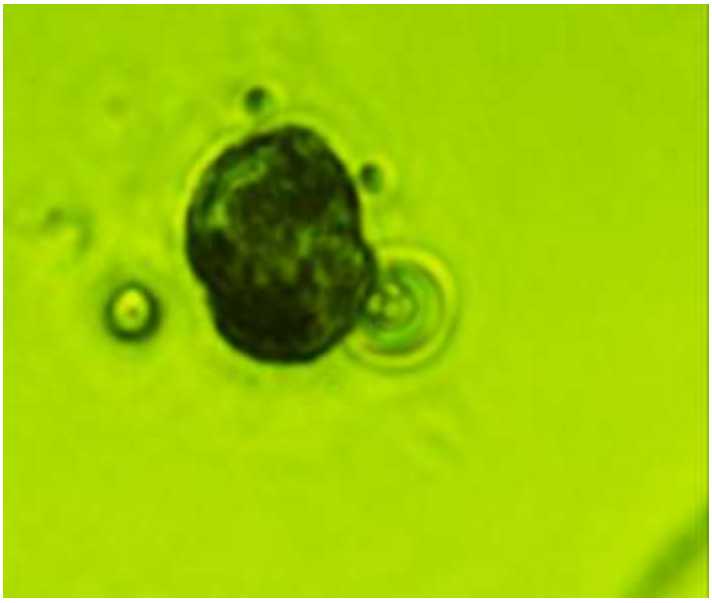

Figure 12: Clade A1 Symbiodinium growing together with little green unicellular organisms (1000x magnification in the microscope).

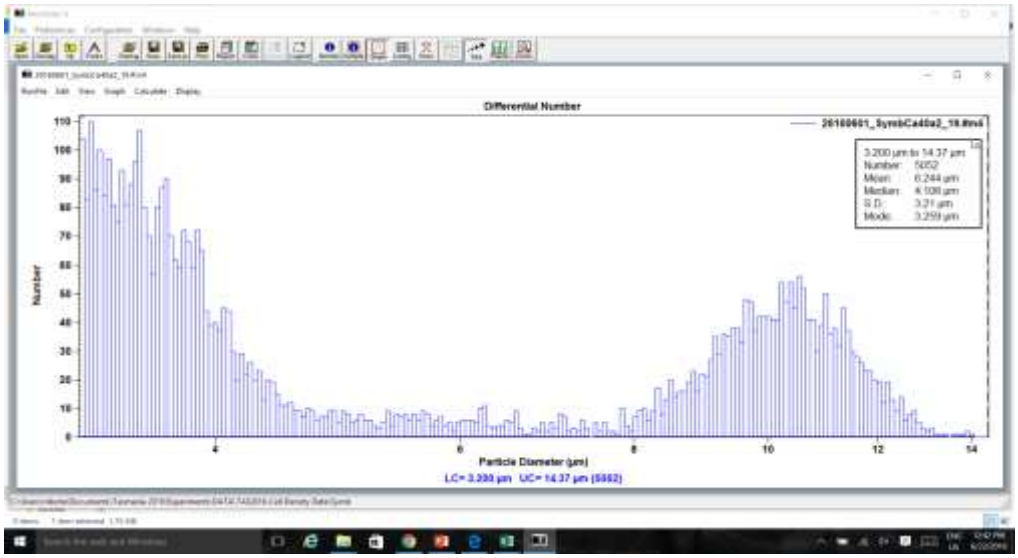

Figure 13: Clade A1 (under [Ca^{2+}]= 40Mm/kgSW and pH=7.94 cell density analysis showing one peak and one great aggregation (<4µm).The aggregation refers to the green unicellular organisms and total amount could not be precisely stablished because size range does not go below 0µm. The peak (8-14µm) refers to *Symbiodinium* cells.

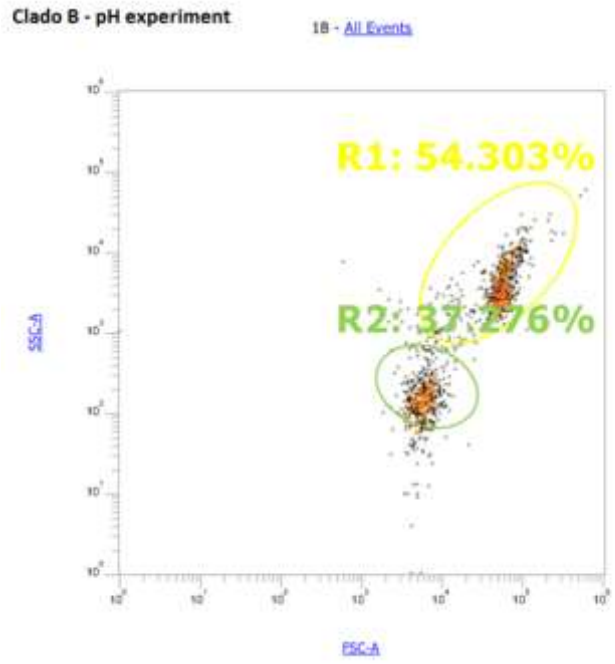

Figure 14: Clade B cell density data points from pH experiment (pH 3 = 7.92) utilizing front scatter and side scatter view. Estimated densities of *Symbiodinium* cells were 10140 cells/mL (R1) and 4000 cells/mL (R2), respectively. Data plots were well stablished inside the gates.

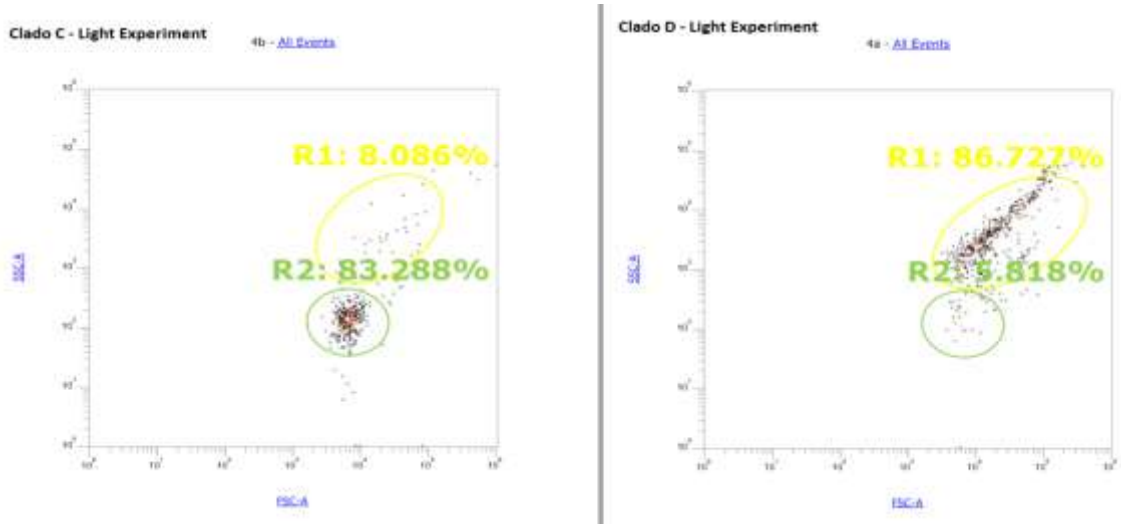

Figure 15: Clades C and D density data plots from light experiment (Treatment 3 - 135 µmols photon/m^2s) utilizing front scatter and side scatter view. Estimated densities of *Symbiodinium* cells were 680 cells/mL (R1) and 3230 cells/mL (R2), respectively. In both cases the data plots were not well stablished inside the gates. In case of the fist image, due to low cell density; also, due to aggregations in the second image.

5.2 Calcium Gradient Experiment:

Great biomass build-up caused variations in the carbonate chemistry in this experiments. In addition to this fact, absence of either growth rate and chlorophyll-*a* production for clades A1 and B1 made the experiment not conclusive. However, the experiment performed at IMAS with clade A1 showed no significant variations in either chlorophyll-*a* production and growth rate in different calcium treatments, confronting Müller *et. al.* (2015). Therefore, differently from coccolithophores, *Symbiodinium* may present no “calcium poisoning” indications.

5.3 pH Gradient Experiment:

Clades showed distinct curves (Figure 6) and optimum growth rate responses in the pH gradient. However, clade A1 curves from IMAS and IOUSP experiments showed opposite behaviors. The first one (Figure 4) showed a tendency of growth rate reduction with lower pH, however the second showed a linear tendency with no great variation in the treatments. The second experiment data goes along with Brading *et. al*. (2011). They also found that the growth rates of clade A1 were not affected by high pCO_2 in cultures growing at 26 ºC; also, its photosynthetic capacity was increased with higher pCO_2. Our chlorophyll-*a* data did not show any evidence of that. Chlorophyll-*a* production barely varied between the treatments (Figure 7), as well as the growth rates, therefore, they all showed evidence of using chlorophyll in a similar way. The same study had the same observations for both clade A1 and B1, but in our case, we could see a slight tendency towards growth rate reduction with higher pCO_2.

For both clades C1 and D1, nothing was found in the literature regarding these *Symbiodinium* clades while in the free-living mode. We also must say that our data for both is not conclusive because in clade C1, carbonate chemistry variations were extremely high and a great number of green cells was noticed (Table 21 and Figure 11, respectively). Therefore, we cannot attribute neither the chlorophyll-*a* production or the growth rate data, exclusively to the pH/ pCO_2 gradient. In the case of clade D, cell number estimations were less accurate because the densities were very low, thus, data from this experiment might be biased due to its methods limitations mentioned on the item above.

5.4 Light Gradient Experiment:

Light saturation curve and its boundaries for *Symbiodinium* growth rates while free-living are not known in the literature. In our study, only clades A1 and B1 followed the general phytoplankton kinetics model, such as observed in previous studies (*i.e*., Leonardos *et. al*.,2006). For both clades, parameters Vm and Km were estimated. Vm refers to the maximum estimated growth rate and Km refers to the light intensity at which the growth is half of Vm. Vm values were the same considering the standard deviations: 0.51 for clade

A1 and 0.52 for clade B1, indicating the same growth maximums. Nevertheless, the Km values were distinct, 36.40 and 17.91 for A1 and B1, respectively. It is an indication that clade B1 growth is more efficient to light than clade A1. It also may be an indication that clade B1 is more light sensitive, because it becomes light saturated at lower light intensities. A number of coral species have been shown to associate with different symbionts depending on the depth of the coral colony (Baker and Rowan, 1997; Rowan, 1998; LaJeunesse et al., 2003, 2004b; LaJeunesse, 2005). Combining our results with the previous studies, we suggest that clade B1 might be mostly found in deeper water corals, so as it has been reported in Stat *et. al*.(2006).

Although our growth rate data for clade C was not conclusive due to great biomass built-up the chlorophyll-*a* production data showed the general behavior of decrease in chlorophyll-*a* production with light increase, also shown in Leonardos *et. al.* (2006). The higher the irradiance, less pigment is required to result in the same photosynthetic efficiency.

5.5 Green Cells Presence:

The presence of green non-identified cells was noticed in different amounts in our experiments as shown in Figure 11. They have influenced the DIC consumptions and they surely influenced all data produced. Personal contact with T. LaJaunesse, C. Langdon and A. Baker have said that they might be a common contaminant in *Symbiodinium* cultures, but they are not that representative in cultures growing under no nutrient limitations and “no-stress” conditions (such as higher pCO_2 and higher calcium concentrations). The only real conclusion is that they were abundant under nitrogen depleted scenarios, as shown in Table 21, where the *Symbiodinium*/Green Cells ratios are lower than one.

Genetic investigation was done (with 10 clones, only) and Planctomyces sp; Marinobacter; Phaeomarinobacter; Roseovarius; Limnobacter and Filo Spirochaetes were found using prokaryote primers. We have not come yet to a conclusion regarding what this organism is and further investigation is utterly necessary.

Table 22: Meta-analysis aggregation of literature about free-living *Symbiodinium* and their diversity of abiotic conditions.

Clade(s)	Nutrient	Light conditions	Photoperiod	Acclimation	Temperature	pCO_2	Observations	Reference
A1 & B1	f/2	350µmol photons/m^2s	14:10(L/D)	Yes - 2 weeks	26 °C	390/ 800µatm	High CO_2 induced no alteration in their µ	Brading *et. al*., 2011
A1	f/2	75-80µmol photons/m^2s	12:12 (L/D)	Yes - 1 week	26/28/31°C	N/A	µ ↓ with both ↑ T°C and Sediment presence	Nitschke *et. al*., 2015
A	f/2	100µmol photons/m^2s	12:12 (L/D)	N/A	24/27/30°C	N/A	↓O_2 production with ↑ T°C	Sorek&Levy, 2012
A1&B1	f/2	100-120µmol photons/m^2s	12:12 (L/D)	None	25/32/34°C	N/A	A1Thermal tolerant and B1 thermal sensitive	Hawkings& Davy, 2012

6. FINAL CONSIDERATIONS:

Growth rate responses of clades (A1-D1) showed distinct optimum curves for pH and light. The experiments did not go through an acclimation period, therefore, no speculations about adaptation and evolution can be done. However, we could notice how they might respond to abrupt changes in sea water carbonate chemistry and light increment. Results, therefore, implicate on present ocean and coral reef environments vulnerable to ocean acidification and possible light availability alterations due to anthropogenic carbon dioxide increment in the atmosphere.

Clade A1 might exhibit a broader ecological niche, since its growth rates did not change significantly with variations in pH. In addition, its Km values indicates that it is less sensitive to light increment. Also, Table 22 show studies where this clade was reported to be thermal tolerant, thus even in the future, when higher temperatures are predicted, clade A1 will probably still succeed.

The other clades might show narrower ecological niches. Clade D is probably the narrowest because its growth rate greatly decrease beyond the boundaries of pH=8.01±0.1. It might be the most sensitive to ocean acidification, but it had been reported to be the most “thermal

tolerant" (LaJaunesse *et. al*, 2014). Therefore, in the future when warm scenarios are expected together with lower pH, it is hard to speculate what will happen. For that, the effects of both parameters in the organism's physiology must be better understood.

Finally, regarding experimental designs, it is important to mention that when organisms are exposed to gradients of abiotic variables, mechanistic understanding of their physiological response outcomes from research, therefore, population structures speculations can be done. Rather than studies with "scenarios approach", in which comparisons between them tend to be imprecise and general assumptions implausible. Table 22 shows a meta-analysis aggregation of the previous studies about free-living *Symbiodinium* and how their abiotic conditions varied from one to another. When differences are found for the same clade, regarding the same stressor parameter, it might be just because they were performed under different light and temperature conditions.

We aim to keep with the mechanistic understanding approach and repeat the experiments, all with the same cell density estimation method and better controlling of the carbonate system. For further research, temperature will be also included as another factor, considering its unique importance in organism's physiology and its increasing predictions for the future.

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