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CELINA REBECA VALENÇA CARNEIRO

INFLUENCE OF SEAWATER CARBONATE CHEMISTRY ON THE COCCOLITH GEOMETRY OF THREE COCCOLITHOPHORE ECOTYPES

Recife
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Dissertação apresentada ao Programa de Pós-Graduação em Oceanografia da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de mestre em Oceanografia. Área de concentração: Oceanografia Biológica.

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Resumo

A concentração de dióxido de carbono (CO_2) nos oceanos aumentou desde a Revolução Industrial, provocando alterações no sistema de carbonato que resultam na acidificação oceânica. Uma das regiões mais afetadas é o Oceano Antártico, devido à sua maior solubilidade do CO_2, onde o cocolitóforo *Emiliania huxleyi* é amplamente distribuído. Os cocolitóforos participam da bomba biológica de carbono do oceano pelágico, sendo importantes para nosso entendimento sobre a capacidade da superfície oceânica em sequestrar CO_2 atmosférico. Portanto, foi investigada a influência do sistema de carbonato na geometria e estado de calcificação dos cocólitos de três ecótipos de *E. huxleyi* do Oceano Antártico. Adicionalmente, foi realizada uma análise comparativa entre três diferentes métodos de mensuração de massa dos cocólitos: medidas geométricas, Coulter Multisizer e SYRACO. Foram utilizadas imagens de microscopia eletrônica dos seguintes ecótipos de *E. huxleyi,* cada um com morfologia diferente: A (oceânico), A o/c (costeiro) e B/C (oceânico). Em seguida, cada ecótipo foi analisado utilizando o software “Image J”. Assim sendo, os parâmetros geométricos medidos foram: comprimento do escudo distal (DSL), largura do escudo distal (DSW), comprimento da área central (CAL), largura da área central (CAW), área do escudo distal (DAS) e área da área central (CAA). Os três ecótipos foram sensíveis às mudanças na química do sistema de carbonato. O ecótipo B/C foi aquele mais sensível, curiosamente este ecótipo possui a estrutura de seus cocólitos mais delicada, com quantidade relativamente baixa de carbonato de cálcio. Além disso, o ecótipo A, também de origem oceânica, foi bastante sensível. No entanto, o ecótipo A o/c, foi o menos sensível às mudanças no sistema de carbonato, o que pode indicar que cepas provenientes de diferentes regiões exibem diferenças em sua capacidade de se aclimatar às mudanças no sistema de carbonato. Os três métodos de obtenção da massa dos cocólitos tiveram valores absolutos diferentes, no entanto, apenas o SYRACO parece superestimar os valores de massa reais. Assim sendo, as alterações na geometria dos cocólitos indicam que *E. huxleyi* pode ter seu processo de calcificação afetado em um cenário de acidificação oceânica. Além disso, a escolha do método de estimação da massa dos cocólitos deve ser feita de forma cuidadosa.

Palavras-chave: *Emiliania huxleyi*; calcificação; Oceano Antártico; sistema de carbonato; massa dos cocólitos.

Abstract

The concentration of carbon dioxide (CO_2) in the oceans has increased since the Industrial Revolution, causing changes in the carbonate system that result in ocean acidification. One of the most affected regions is the Southern Ocean, due to its higher CO_2 solubility, where the coccolithophore *Emiliania huxleyi* is widely distributed. Coccolithophores participate in the biological carbon pump of the pelagic ocean and are important for our understanding of the ocean surface's ability to sequester atmospheric CO_2. Therefore, the influence of the carbonate system on the geometry and calcification status of coccoliths of three ecotypes of *E. huxleyi* from the Southern Ocean was investigated. Additionally, a comparative analysis was performed between three different methods of measuring coccolith mass: geometric measurements, Coulter Multisizer™ 4 and SYRACO. In all, 556 scanning electron microscopy of the following *E. huxleyi* ecotypes were used, each with different morphology: A (oceanic), A o/c (coastal) and B/C (oceanic). Then, each ecotype was analyzed using the “Image J” software. Therefore, the geometric parameters measured were: distal shield length (DSL), distal shield width (DSW), central area length (CAL), central area width (CAW), distal shield area (DSA) and central area area (CAA). The three ecotypes were sensitive to changes in the carbonate chemistry. The B/C ecotype was the most sensitive, curiously this ecotype has the most delicate structure of its coccoliths, with a relatively low amount of calcium carbonate. In addition, ecotype A, also of oceanic origin, was quite sensitive. However, the A o/c ecotype (coastal origin) was the least sensitive to changes in the carbonate system, which may indicate that strains from different regions exhibit differences in their ability to acclimate to changes in the carbonate system. The three methods of obtaining the mass of coccoliths had different absolute values, however, only SYRACO seems to overestimate the actual mass values. Therefore, changes in coccolith geometry indicate that *E. huxleyi* may have its calcification process affected in a scenario of oceanic acidification. In addition, the choice of method for estimating the mass of coccoliths must be done carefully.

Keywords: *Emiliania huxleyi*; calcification; Southern Ocean; carbonate system; coccolith mass.

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CAA	Central Area Area
CAL	Central Area Length
CAW	Central Area Width
$CaCO_3$	Calcium Carbonate
CM	Coulter Multisizer
CO_2	Carbon Dioxide
CO_3^2	Carbonate Ion
DIC	Dissolved Inorganic Carbon
DSA	Distal Shield Area
DSL	Distal Shield Length
DSW	Distal Shield Width
g/cm^3	Gram per Cubic Centimetre
GEO	Geometric Measures
HCl	Hydrochloric Acid
HCO^-_3	Bicarbonate
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
o/c	Overcalcified
*p*CO2	Partial Pressure of Carbon Dioxide
pg	Picogram
SEM	Scanning Electron Microscopy
SY	SYRACO
TA	Total Alkalinity
µatm	Microatmosphere
µm	Micrometre
Ω	Calcite Saturation

Sumário

1 INTRODUCTION

Since the beginning of the industrial revolution, the anthropogenic emission of atmospheric carbon dioxide has increased and, consequently, more carbon dioxide (CO_2) has been absorbed by the ocean (approximately 30%). Since then, the oceanic pH has already decreased by 0.1 units, corresponding to an increase of approximately 30% in the concentration of hydrogen ions (Guinotte & Fabry, 2008). This entry of CO_2 into the ocean is a threat to marine biodiversity because it quickly reacts with water and modifies the chemistry of the seawater carbonate system, for example by increasing dissolved inorganic carbon (DIC), $[CO_2]$ and $[HCO^-_3]$, lowering the pH and CO_3 and while keeping the total alkalinity constant (Zeebe; Wolf-Gladrow, 2001; Hönisch et al., 2012; Müller et al., 2015).

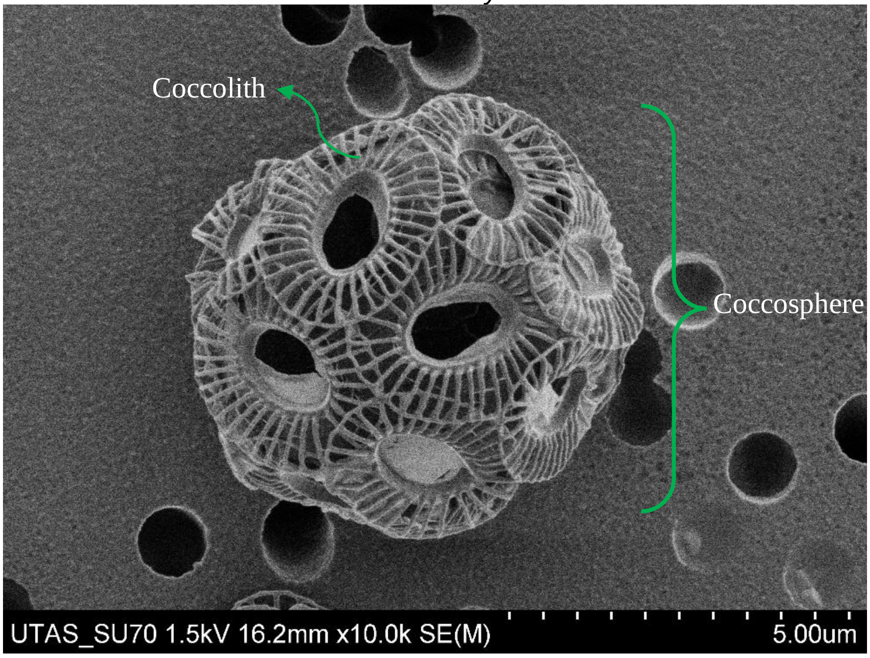
This reduction in pH in the oceans resulting from increased absorption of atmospheric CO_2 is termed ocean acidification and is predicted to occur first in the Southern Ocean, mainly due to its greater solubility of carbon dioxide (Sabine et al., 2004; Orr et al., 2005; Roden et al., 2013). In general, seawater pH ranges between 7.8 - 8.2 and research indicates that oceanic pH will decrease between 0.3 and 0.4 by 2100 (Caldeira and Wickett, 2003).

The ocean acidification, caused by the increase in the concentration of CO_2, can be detrimental for planktonic species that have calcified structures, as it is the case of coccolithophores. This calcification process and species distribution can change in response to an increase in CO_2 atmospheric levels and, consequently, to a decrease in pH and less availability of carbonate ions (CO_3) for the production of their coccoliths (McNeil & Matear, 2008; Hutchins, 2011).

Coccolithophores are unicellular marine microalgae characterized as a functional group capable of producing coccoliths (small structures of biogenic calcium carbonate), covering their cell and forming a coccosphere (Figure 1). Besides, they are important in carbon cycling dynamics, contributing to both the organic carbon pump and the carbonate counter pump (through the processes of photosynthesis and calcification, respectively), where the reduction of surface CO_2 due to the production of organic carbon is partially compensated by pelagic calcification (Rost & Riebesell, 2004).

The carbon chemistry occurs through reactions of carbon in seawater when atmospheric carbon dioxide (CO_2) enters the sea surface. Then, in the sunlight zone, coccolithophores assimilate CO_2 to produce organic carbon through photosynthesis.

Figure 1: Scanning electron microscopy image of the coccoliths and coccosphere of the ecotype B/C of *E. huxleyi*.



Fonte: A autora (2022).

However, the atmospheric CO_2 reacts with water (H_2O), resulting in the formation of bicarbonate ions (HCO^-_3) and the release of protons (H^+) — lowering the pH of surface waters.

Thereon, the coccolithophores also perform calcification reactions converting HCO^-_3 into calcium carbonate ($CaCO_3$) and CO_2 according to the following reaction (Figure 2):

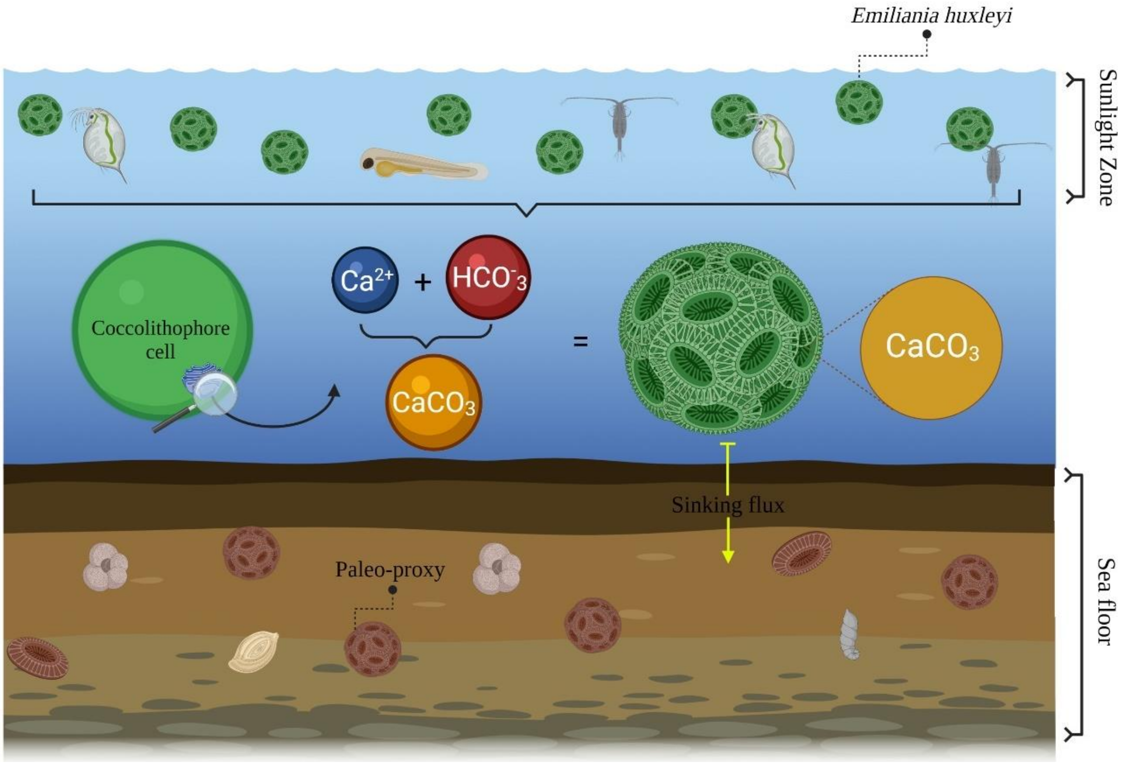
$$Ca^{2+} + 2HCO^-_3 \longrightarrow CaCO_3 + CO_2 + H_2O$$

Subsequently, $CaCO_3$ is used as raw material for coccoliths that can be classified into holococcoliths and heterococcoliths. This classification is based on criteria such as the form of biomineralization, the amount and shape of the crystals and the phase of the life cycle they are associated (Rowson et al., 1986; Young & Henriksen, 2003).

The coccoliths have a still controversial function, with hypothesis ranging from virus protection to metabolic calcium regulation (Winter & Siesser, 2006; Müller, 2019). For example, the function of a defense mechanism against predation (mainly of microzooplankton) has been tested and obtained controversial results, as is the case of copepods preying on the calcified coccolithophore *Emiliania huxleyi* both in the laboratory and in mesocosms (Sikes & Wilbur 1982; Harris 1994; Nejstgaard et al. al., 1997).

Furthermore, Hansen et al. (1996) investigated the preference of grazing *Oxyrrhis marina* (heterotrophic dinoflagellate) for calcified *E. huxleyi* cells and, on the other hand, the decrease in the grazing pressure of *E. huxleyi* through the decrease in the growth rate of *Oxyrrhis marina* has been observed in laboratory experiment (Harvey; Bidle; Johnson, 2015).

Figure 2: Graphic representation of the calcification of *E. huxleyi* and deposition of inorganic carbon on the sea floor.



Fonte: A autora (2022).

The species *Emiliania huxleyi* (Lohmann) W.W.Hay & H.P.Mohler, 1967 is the most studied and among the numerically most abundant and geographically distributed representatives of this functional group, being an important model species in context of the global carbon cycle (Paasche, 2019). *Emiliania huxleyi* has only heterococcoliths which are precipitated intracellularly, formed in coccolith vesicles derived from the Golgi apparatus and finally are exported out of the coccolith cell by exocytosis (Balch; Holligan; Kilpatrick, 1992; Paasche, 2001; Hutchins, 2011).

Emiliania huxleyi is a cosmopolitan species, found in coastal and ocean waters, where it is able to form blooms that exceed 100,000 km^2 (Brown & Yoder, 1994). In the Southern Ocean, the most dominant ecotypes are: A, A o/c (overcalcified) and B/C. These different ecotypes are distributed from north to south on a gradient that starts further north with A o/c (48° S), passes through A (43 – 53° S) and ends further south with B/C (43 – 65° S) (Cubillos et al. 2007). The distribution of these ecotypes is determined by temperature, influence of the Antarctic Polar Front (which is capable of reducing the possibility of phytoplankton genetic recombination) and possibly also influenced by seawater carbonate chemistry (Mohan et al., 2008; Winter et al., 2014; Cubillos et al., 2007; Müller et al., 2015).

To determine the differences between the ecotypes, the criteria of morphological characterization (structure and geometry of the coccoliths), genetic, light harvesting pigments, their ecological niche and geographic variability are used. The ecotype A is commonly found in blooms and globally the most widespread forming; ecotype A o/c is similar to the ecotype A, but with heavily calcified shields without openings; and the B/C ecotype is the one with the lowest amount of calcium carbonate in its structures, in addition to being commonly found in the Southern Ocean (Paasche, 2001; Young et al., 2003; Cubillos et al., 2007; Cook, 2010; Cook et al, 2011 and 2013; Poulton et al., 2013; Krueger-Hadfield et al., 2014).

In addition, the flux of particle rain to the seafloor results in the sinking of coccoliths and coccospheres into the sediment. The existence of coccolithophore sediment records, which accumulate in the oceans over the time, makes them useful in reconstructing past climate and oceanographic conditions. Therefore, coccolithophores are also used as a paleo-proxy — mainly for the Jurassic and Cretaceous (Tyrrell & Young, 2009).

Paleo-proxy functions as a "natural archive", providing evidence that can be used to reconstruct past physical, chemical and climate conditions (Sorooshian & Martinson, 1995). These records occur both organically (e.g. molecular fossils or biomarkers) and inorganically (e.g. calcium carbonate). The formation of coccoliths is affected by several environmental factors (e.g. changes in its geometric parameters), for example: temperature, carbonate chemistry, salinity, light and nutrients (Paasche, 1998; Riebesell et al., 2000; Zondervan et al., 2007; Müller et al., 2011, 2012, 2014).

Therefore, coccoliths can be used as geochemical paleo-proxies (analyzing the chemical signature of $CaCO_3$). In addition, there is a growing number of studies that aim to use the geometric parameters of coccoliths as indicators of past and current oceanic conditions. For example, Henderiks & Pagani (2008) investigated the relationship between the size of coccolithophore cells and the Paleogene decline in atmospheric CO_2; Henderiks (2008) discusses calcite quota (per calcite per cell) and morphometric data from different coccolithophore genera in order to facilitate the estimation of calcite quota, cell and coccosphere diameters from individual coccoliths; and Müller et al. (2015) investigated the sensitivity of three *E. huxleyi* ecotypes to changing seawater carbonate chemistry. However, there is a detriment of information on strains from the Southern Hemisphere when compared to studies from the Northern Hemisphere.

Therefore, this study compared the different geometric parameters of Southern Ocean ecotypes of *E. huxleyi* with changes in seawater carbonate chemistry. Additionally, different methods used to estimate coccolith mass were also compared.

2 GOALS

2.1 Main goal

- To investigate the influence of seawater carbonate chemistry on the geometry of coccoliths from three different ecotypes of *Emiliania huxleyi*.

2.2 Specific goals

- To analyze scanning electron microscopy images of the coccoliths geometry obtained in an experiment with three ecotypes of *E. huxleyi* (A, Ao/c and B/C);
- To compare different methods that assess the mass of coccoliths;
- To relate the geometry of coccoliths to enviromental conditions and the physiology of *E. huxleyi*;
- To elaborate a bibliographic survey of the geometry of the Southern Ocean coccolithophore *E. huxleyi*.

3 MATERIALS AND METHODS

The scanning electron microscopy (SEM) images analyzed in the study are related to a laboratory experiment published by Müller et al. (2015), where future scenarios of ocean acidification were simulated. In this experiment, two strains of each ecotype (A, A o/c and B/C) were exposed to 6 different $p\mathrm{CO_2}$/pH treatments.

Experimental incubations were carried out in 500 mL autoclaved borosilicate flasks containing growing cultures of *Emiliania huxleyi* and already acclimated to experimental conditions. The carbonate chemistry speciation was adjusted by additions of HCl, NaOH and $NaHCO_3$, resulting in a partial pressure of carbon dioxide ($p\mathrm{CO_2}$) that ranged from 240 to 1750 µatm. The temperature of the experiments was 14ºC, which is the average annual temperature in the regions where the 3 ecotypes co-occur (Cubillos et al., 2007).

At the beginning and at the end of the experiments, the inorganic dissolved carbon, total alkalinity and coccolith volume were measured. Furthermore, scanning electron microscopy (SEM) was performed by filtering samples onto polycarbonate filters (0.8 µm pore size) that were subsequently dried and stored 60ºC, pending analyses on a Hitachi SU-70 field emission at CSL-UTAS (Müller et al., 2015).

3.1 Scanning Electron Microscopy

A total of 556 SEM images distributed among the three ecotypes were analyzed (Table 1). The images of scanning electron microscopy (SEM) were taken at 1500x magnification and were analyzed using the 'Image J' software (Ferreira & Rasband, 2012) to measure the dimensions of the each individual coccolith plates.

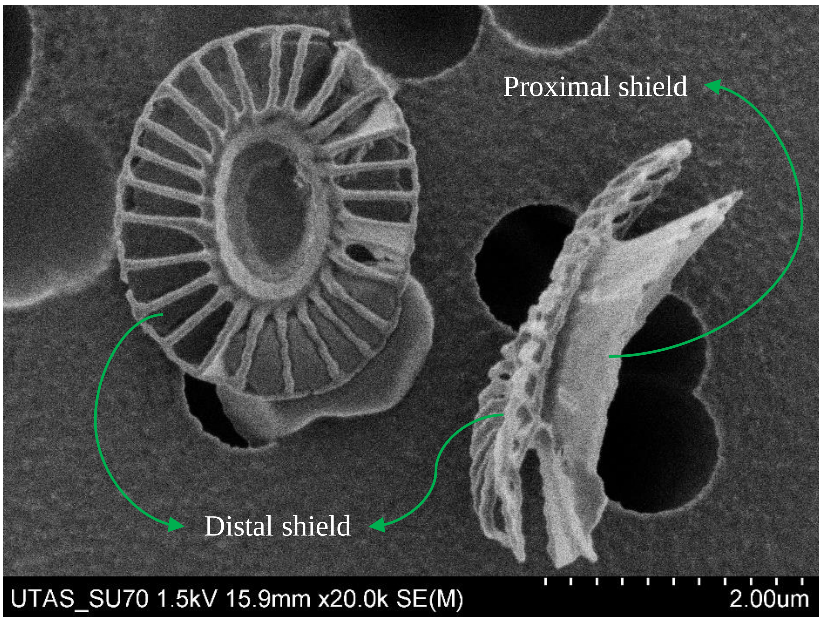
Table 1. Description of the number of SEM images and coccoliths analyzed for each ecotype.

	Ecotype A	**Ecotype A o/c**	**Ecotype B/C**
Nº Treatment	12	12	10
Nº SEM Images	151	158	247
Nº Coccolith	1.199	1.067	896

Fonte: A autora (2022).

The Image J has been calibrated using the size bars given on the images. Besides, were measured only the coccoliths found lying face up (corresponding to measuring only the distal shield) and those upside down were ignored for all measurements.

Figure 3: SEM of *E. huxleyi* coccoliths built up by distal and proximal shield.



Fonte: A autora (2022).

The scanning electron microscopy (SEM) is capable of producing high-resolution three-dimensional images of a sample's surface. Besides to adding the topographical aspects of the surface of the sample (Vernon-Parry, 2000).

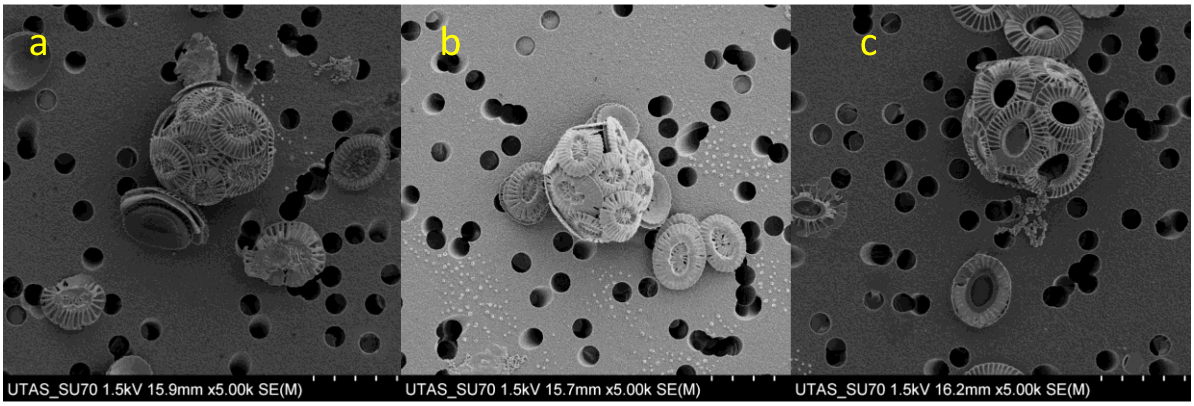
Therefore, some of its advantages over optical microscopy are:

- Much higher magnifications can be achieved with a final resolution of 1 nm (up to 1.000.000x);
- Greater depth of field, being possible that a larger part of the sample surface is in focus;
- Allows a non-destructive evaluation of the sample.

3.2 Ecotypes

The differences in response to changes in the carbonate system were verified using two strains of three different ecotypes of *Emiliania huxleyi* (A, A o/c and B/C) that were collected from Southern Ocean sites and the Tasmanian Coast (Figure 1 and Table 2).

Figure 4: Scanning electron microscopy images of the three ecotypes of *E. huxleyi*, A (a), A o/c (b) and B/C (c).



Fonte: A autora (2022).

Table 2. Ecotypes used and their respective strains and origin.

Strain code	Ecotype	Origin
EHSO 5.14	A	Southern Ocean (50° S, 149°E)
EHSO 5.30	A	Southern Ocean (50° S, 149°E)
EHTB 11.15	A o/c	Tasmanian Coast (43° S, 147°E)
EHBH 13.28	A o/c	Tasmanian Coast (42º S, 148°E)
EHSO 5.11	B/C	Southern Ocean (50° S, 149°E)
EHSO 8.15	B/C	Southern Ocean (54° S, 146°E)

Fonte: Müller et al. (2015).

3.3 Coccolith Geometry of *Emiliania huxleyi*

The geometric parameters measured are illustrated in Figure 5 and include the Distal Shield Length (DSL), Distal Shield Width (DSW), Central Area Length (CAL), Central Area Width (CAW), Distal Shield Area (DSA) and the Central Area Area (CAA).

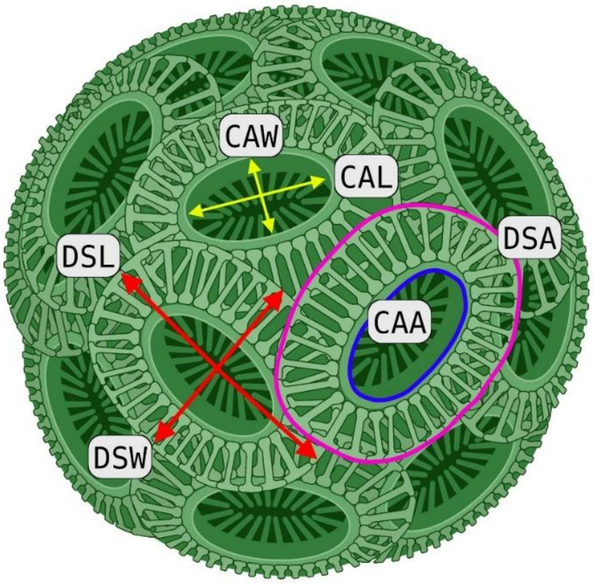
From these measurements, both the DSA and the CAA were calculated assuming that the shields are resembled in an elliptical shape (Bach et al., 2012):

$$DSA = \pi * \frac{DSL*DSW}{4} \qquad CAA = \pi * \frac{CAL*CAW}{4}$$

The values of geometric measures are represented by their absolute values and their normalized values.

Finally, geometric measurements were correlated with carbonate chemical parameters (e.g. pH, total alkalinity, Ω (calcite), dissolved inorganic carbon (DIC) and concentrations of pCO_2, CO_2, CO_3^2 and HCO_3^-), data were obtained from Müller et al. (2015).

Figure 5. Representation of the coccolithophore *Emiliania huxleyi* demonstrating the measurements performed on the coccoliths (DSL = Distal Shield Length, DSW = Distal Shield Width, DSA = Distal Shield Area, CAL = Central Area Length, CAW = Central Area Width, CAA = Central Area Area).



Fonte: A autora (2022).

3.4 Methods of Estimating Coccolith's Mass

Three methods were used to estimate the coccolith mass:

- <u>Geometrical measurements</u>

  The scales defined in the SEM images were used and the images were enlarged by 1500x to measure the dimensions of each coccolith's distal shield, using the Image J software (Ferreira & Rasband, 2012). The geometrical measurements were transformed into the mass of coccolith according to the equation $Mass = 2.7 * ks * l^3$, where 2.7 is the density of calcite in g/cm^3, ks is a constant dependent on the shape and l is the length of the coccolith's distal shield (Young and Ziveri, 2000).

- Coulter Multisizer ™ 4

  The Coulter method is a system for counting and characterizing particles through electrical zone detection, regardless of the nature or optical properties of the particles. In this method, particles are suspended in a weak electrolyte and pass through a small diameter opening defined between two electrodes with an electrical current. When a particle passes through the opening, it displaces an amount of electrolyte that is capable of changing the voltage and causing a pulse in the electrical current. Finally, the pulse height is then proportional to particle volume. The data used were extracted from the publication by Müller et al., 2015. For this, the volume of coccoliths obtained with a Coulter Multisizer ™ 4 were transformed into the mass of coccolith according to the equation $Mass = 2.7 * volume$ (Young and Ziveri, 2000), where 2.7 corresponds to the density of calcite in g/cm^3.

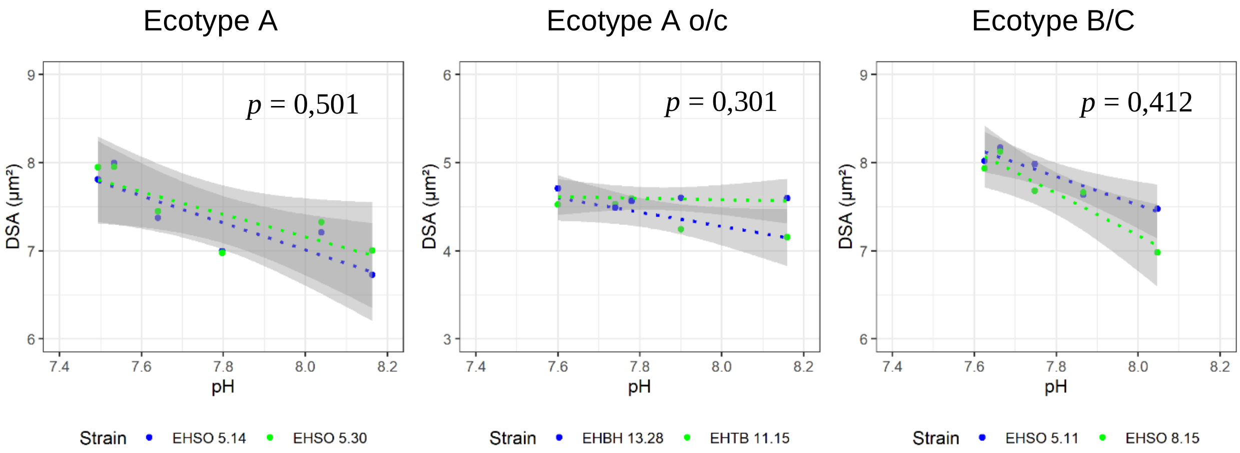
- Système de Reconnaissance Automatique de Coccolithes (SYRACO)

  SYRACO is an automatic coccolith recognition system based on a database of images of structures containing different amounts of calcite. This method uses pattern recognition capable of separating and recognizing coccoliths from particles that are not coccoliths (Beaufort and Dollfus, 2004). The SYRACO method avoids counting problems (*e. g.* insufficient sample counts to obtain statistically sound results and divergences between counts by different researchers) in addition to automating the identification and counting of coccoliths. The SYRACO analyzes used were performed by Luc Beaufort at Aix Marseille Université, Marseille, France.

3.5 Statistical analysis

For statistical analysis between strains of each ecotype, an ANCOVA was performed to test the statistical significance of the slopes for the various DSA values of the strains of each ecotype at the 90% confidence level or higher (Figure 6). For this, the Comparison of Regression Lines function of STATGRAPHICS Centurion 19 was used. The strains are not significantly different, so strains from each ecotype were analyzed together. For this, the data of each ecotype were combined and had their values normalized.

Figure 6. Correlation between pH and DSA of *E. huxleyi* strains of each ecotype. The gray areas correspond to the 95% confidence interval. The p-value corresponds to the significance of the ANCOVA performed for the slope variable, indicating no significant difference between the two strains of each ecotype.



Fonte: A autora (2022).

For all the others statistical analysis the R program was used. To normalize the values, the rescale function of the scales package was used, which resulted in a normalization that varied between 0 and 1. Besides, linear regressions were used to verify the correlations between the geometric measurements of *Emiliania huxleyi* and the carbonate system parameters for each ecotype, between the geometric measurements for each ecotype, and between the different methods of obtaining the mass of coccolith. In all analyses, the r^2 correlation coefficients and associated *p*-values (the level of significance was set to $p < 0.05$) were used to determine any significant relationships. In addition, for a better visualization of the figures, in the results section only the significant regression lines were plotted.

4 RESULTS

4.1 Ecotypes Responses to Seawater Carbonate Chemistry

The changes in coccolith geometry in relation to a pH gradient (from 7.48 to 8.17) and a pCO_2 gradient (from 296 to 1683 µatm) were greater in the ecotype B/C compared to the ecotypes A and A o/c from the Southern Ocean, with the ecotype B/C being is the most sensitive to changes in the carbonate system of seawater and the ecotype A o/c is the least sensitive, indicated by the total number of sensitive geometric parameters (Tables 3 and 4).

Table 3. Number of geometric measurements of *E.huxleyi* significantly correlated with their respective carbonate system parameter.

	Ecotype A		**Ecotype A o/c**		**Ecotype B/C**	
Carbonate System Parameter	**Geometric Parameters**	Nº Total	**Geometric Parameters**	Nº Total	**Geometric Parameters**	Nº Total
pCO_2	DSL, DSW, CAL, CAW, DSA, CAA	**6**	CAL, CAA, CAA:DSA	**3**	DSL, DSA, CAL, CAW, CAA, CAA:DSA	**6**
pH	DSL, DSW, CAW, DSA, CAA	**5**	CAA:DSA	**1**	DSL, DSW, DSA, CAL, CAW, DSA, CAA, CAA:DSA	**8**
CO_2	DSL, DSW, CAL, CAW, DSA, CAA	**6**	CAL, CAA, CAA:DSA	**3**	DSL, DSA, CAL, CAW, CAA, CAA:DSA	**6**
CO_3	DSL, DSW, CAW, DSA, CAA	**5**	CAA:DAS	**1**	DSL, DSW, DSA, CAL, CAW, CAA, CAA:DSA	**7**
DIC	DSL, DSW, CAW, DSA	**4**	CAA:DSA	**1**	DSL, DSW, CAL, CAW, DSA, CAA, CAA:DSA	**7**
HCO_3	DSL, DSW, CAW, DSA	**4**	CAA:DSA	**1**	DSL, DSW, DSA, CAL, CAW, CAA, CAA:DSA	**7**
TA	-	-	-	-	-	-
Ω	DSL, DSW, CAW, DSA	**4**	CAA:DAS	**1**	DSL, DSW, CAL, CAW, DSA, CAA, CAA:DSA	**7**

Fonte: A autora (2022).

The absolute mean values of the geometric parameters of *E. huxleyi* coccoliths varied among the different ecotypes. The DSL ranged between 2.73 and 3.44 µm, the DSW between 2.10 and 2.87 µm and the DSA between 4.57 and 7.85 µm², with the highest values attributed to the B/C ecotype. The CAL varied between 1.34 and 1.73 µm, the CAW between 0.78 and 1.17 µm, the CAA between 0.84 and 1.80 µm² and the CAA:DAS ratio between 0.17 and 0.22, with the highest values attributed to the ecotype A (Figures 7 and 9).

The ecotype B/C had all its geometric parameters significantly correlated with pH (Figures 7 and 9).

The ecotype A had DSL, DSW, DSA, CAW and CAA significantly correlated with pH and in the ecotype A o/c only the CAA:DSA of was significantly correlated with pH (Figures 7 and 9).

The ecotypes A and B/C had their overall coccolith size (DSA) increased at lower pH and at higher pCO_2 values. The overall coccolith size (DSA) of ecotype A o/c increased at higher pH and at lower pCO_2 values (Figures 7c and 9c).

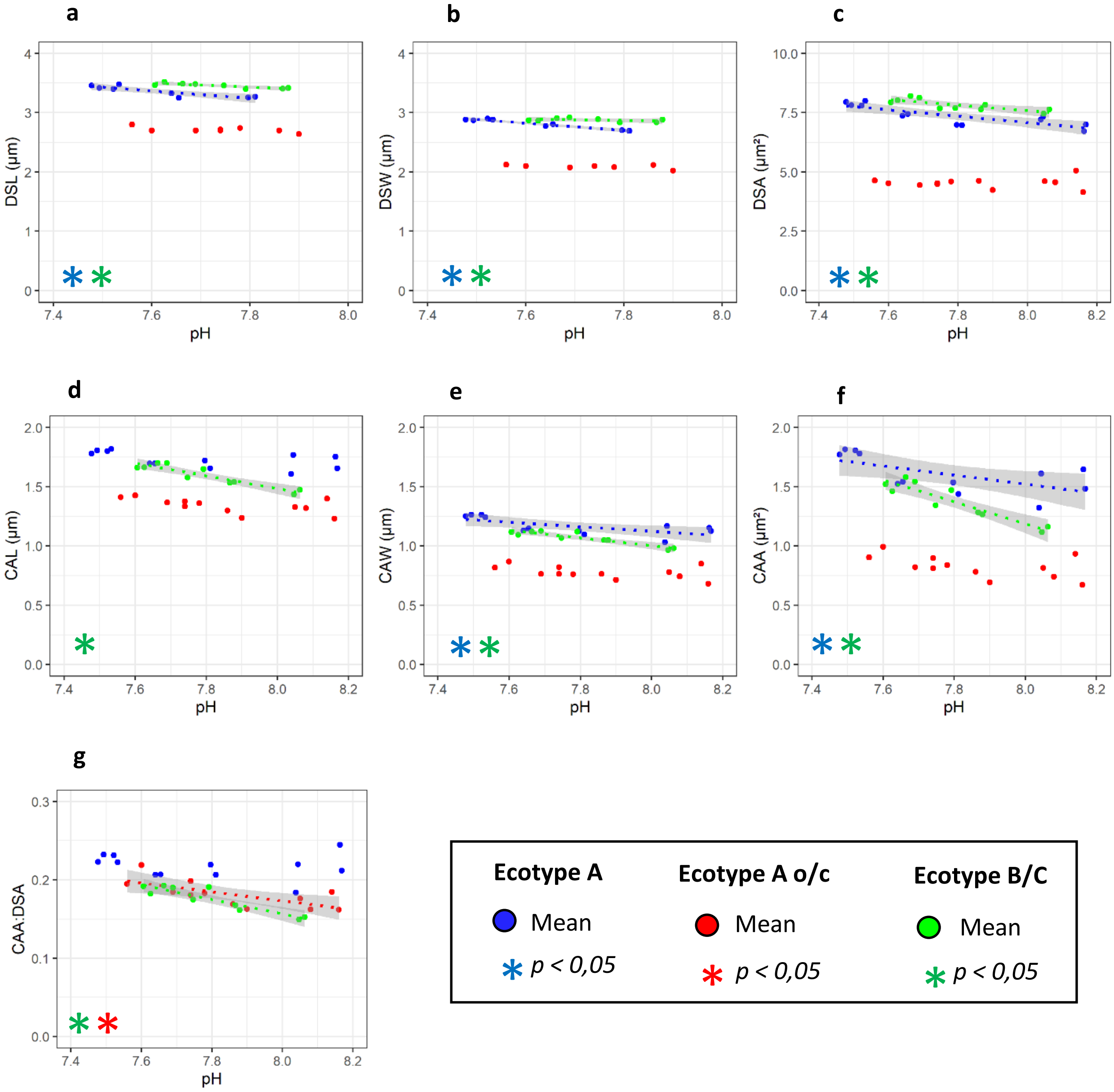
Distal Shield Area and Central Area of all ecotypes were significantly correlated with each other (Figures 8 and 10).

Table 4. Linear regression to verify correlations between pH and geometric measures of *E. huxleyi*.

	Geometric Parameter	**r^2**	***p*-value**
Ecotype A	DSL	0.587	0.003
	DSW	0.679	0.001
	DSA	0.675	0.001
	CAL	0.252	0.055
	CAW	0.416	0.014
	CAA	0.341	0.027
	CAA:DSA	0.066	0.583
Ecotype A o/c	DSL	0.095	0.838
	DSW	0.078	0.663
	DSA	0.087	0.737
	CAL	0.236	0.062
	CAW	0.076	0.198
	CAA	0.155	0.113
	CAA:DSA	0.448	0.010
Ecotype B/C	DSL	0.803	0.0002
	DSW	0.357	0.040
	DSA	0.583	0.006
	CAL	0.843	0.0001
	CAW	0.813	0.0002
	CAA	0.835	0.0001
	CAA:DSA	0.808	0.0002

Fonte: A autora (2022).

Figure 7. Linear regressions between pH and geometric measurements of *E. huxleyi* coccoliths. Gray areas correspond to 95% confidence interval.



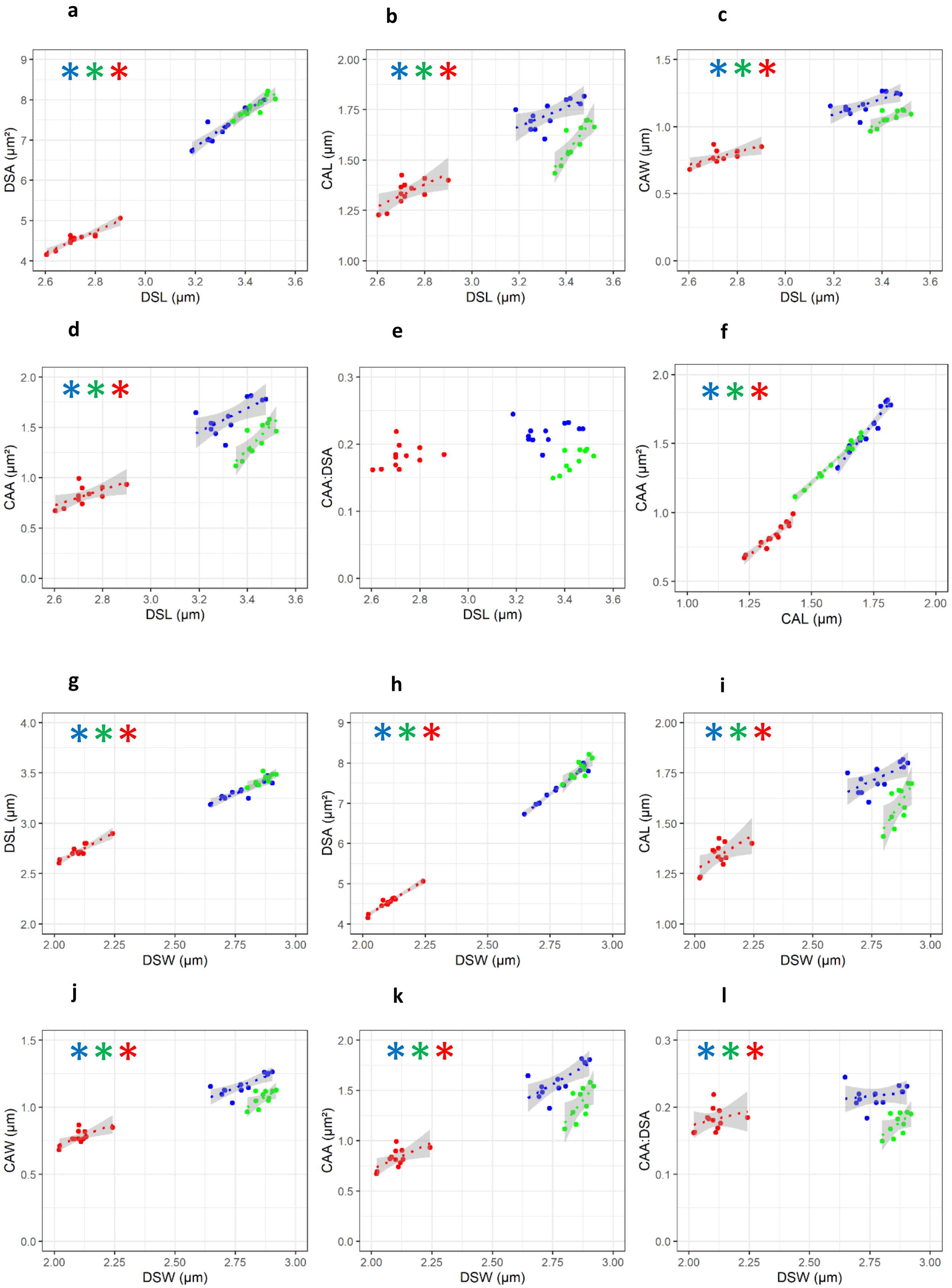
Fonte: A autora (2022).

Table 5. Linear regression to verify correlations between geometric measures of *E. huxleyi*.

Correlations	**Ecotype A**		**Ecotype A o/c**		**Ecotype B/C**	
	r²	***p*-value**	**r²**	***p*-value**	**r²**	***p*-value**
DSW x **DSL**	0.801	5.166e-05	0.838	1.835e-05	0.539	0.009
DSW x **DSA**	0.971	3.101e-09	0.947	6.866e-08	0.635	0.004
DSW x **CAW**	0.603	0.002	0.465	0.009	0.353	0.041
DSW x **CAL**	0.394	0.017	0.343	0.027	0.413	0.026
DSW x **CAA**	0.498	0.006	0.342	0.027	0.376	0.035
DSW x **CAA:DSA**	0.039	0.538	0.089	0.346	0.247	0.082
DSL x **DSA**	0.877	4.433e-06	0.866	6.837e-06	0.727	0.001
DSL x **CAW**	0.470	0.008	0.409	0.015	0.528	0.010
DSL x **CAL**	0.341	0.027	0.388	0.018	0.681	0.002
DSL x **CAA**	0.399	0.016	0.339	0.028	0.618	0.004
DSL x **CAA:DSA**	0.021	0.656	0.008	0.320	0.496	0.013
DSA x **CAW**	0.557	0.003	0.474	0.008	0.559	0.008
DSA x **CAL**	0.375	0.020	0.405	0.015	0.724	0.001
DSA x **CAA**	0.4625	0.009	0.380	0.019	0.698	0.002
DSA x **CAA:DSA**	0.02469	0.626	0.014	0.307	0.506	0.013
CAW x **CAL**	0.8586	9.13e-06	0.830	2.301e-05	0.939	2.347e-06
CAW x **CAA**	0.9703	3.581e-09	0.952	3.807e-08	0.936	2.892e-06
CAW x **CAA:DSA**	0.4881	0.007	0.722	0.0002	0.949	1.138e-06
CAL x **CAA**	0.9285	2.929e-07	0.902	1.405e-06	0.983	1.53e-08
CAL x **CAA:DSA**	0.6159	0.616	0.715	0.0003	0.936	2.989e-06
CAA x **CAA:DSA**	0.608	0.002	0.837	1.86e-05	0.964	3.017e-07

Fonte: A autora (2022).

Figure 8. Linear regressions between geometric measurements of *E. huxleyi* coccoliths. Gray areas correspond to 95% confidence interval.



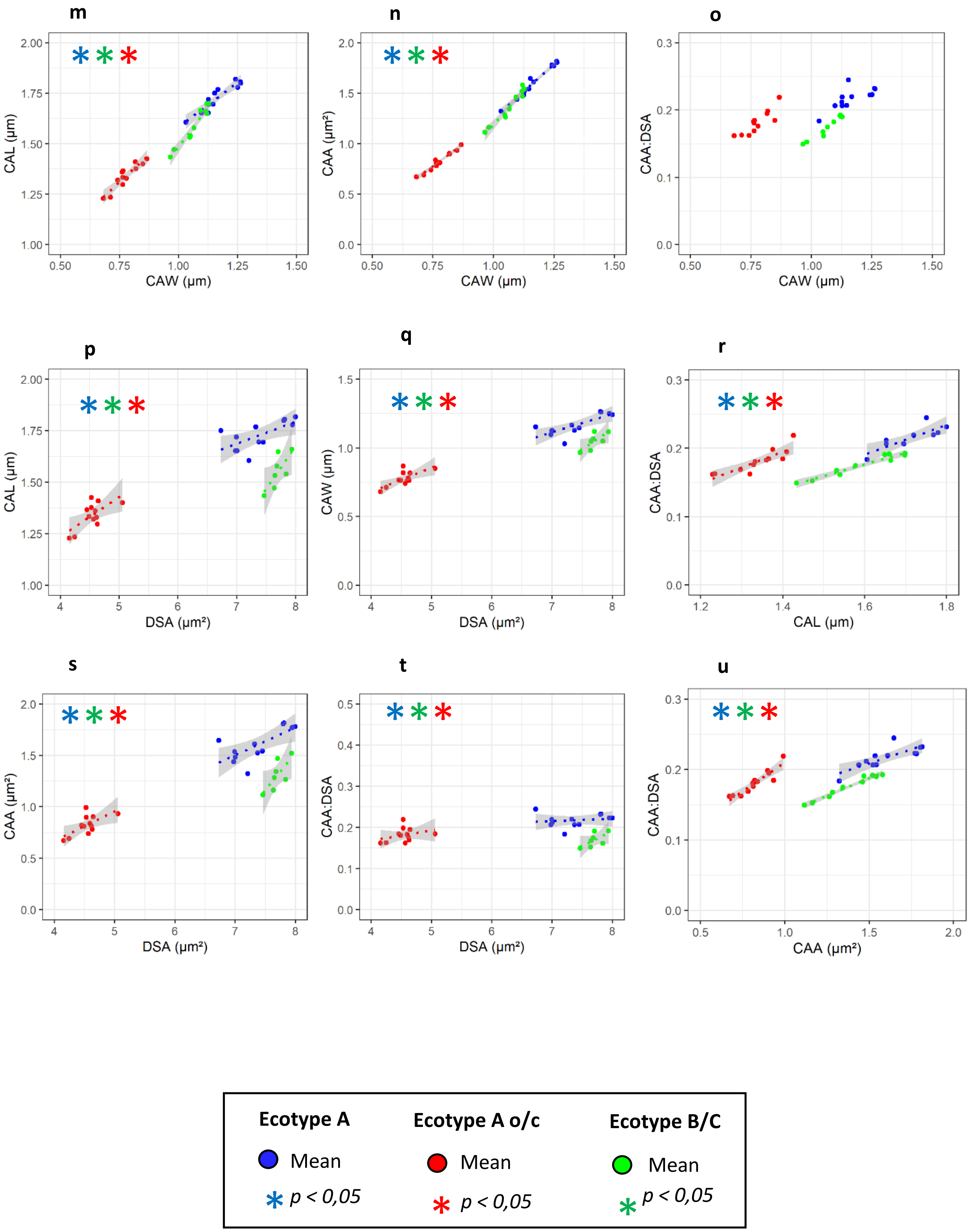
Fonte: A autora (2022).

Ecotype A
● Mean
* *p < 0,05*

Ecotype A o/c
● Mean
* *p < 0,05*

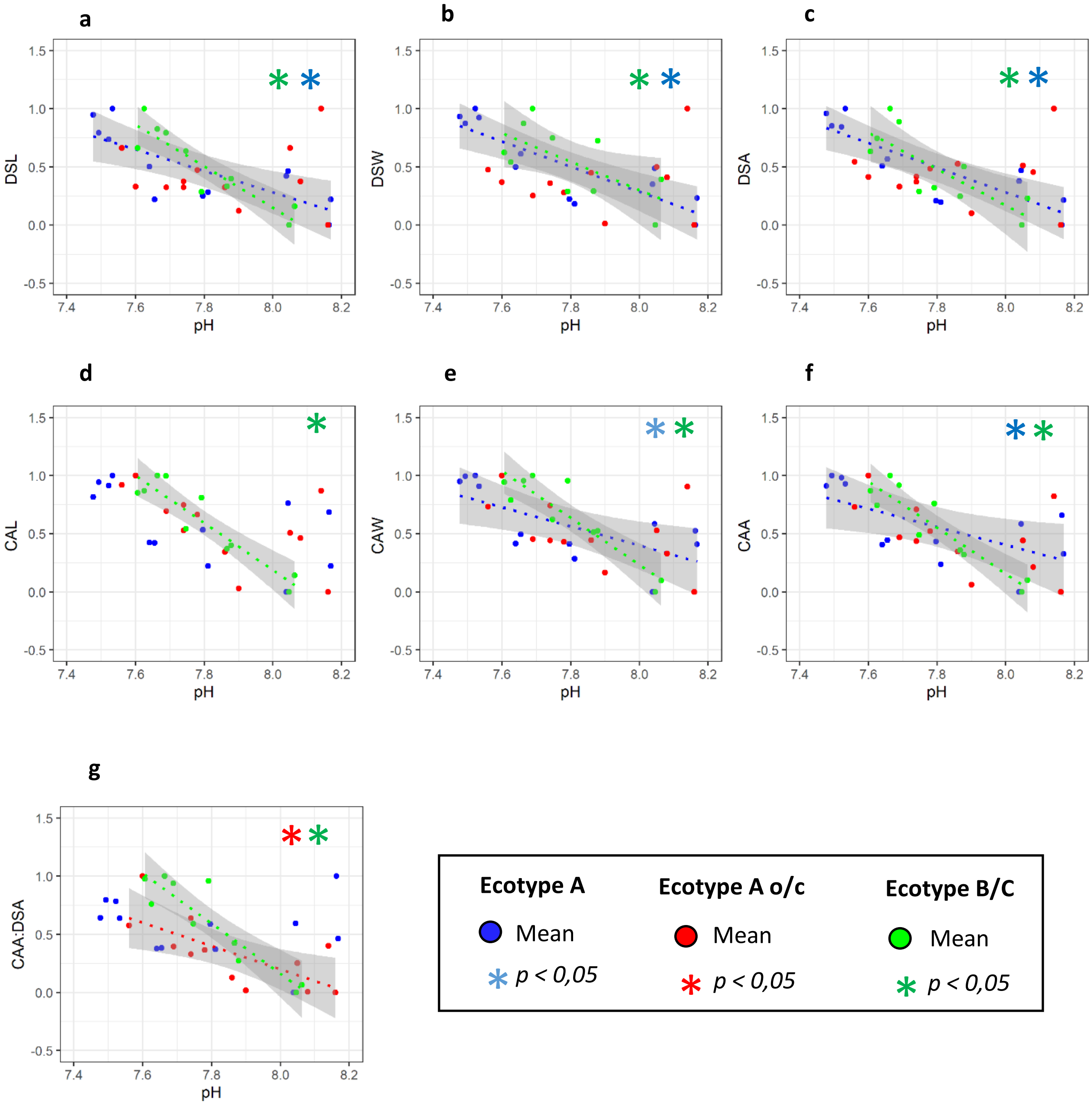
Ecotype B/C
● Mean
* *p < 0,05*

Figure 8. Linear regressions between geometric measurements of *E. huxleyi* coccoliths. Gray areas correspond to 95% confidence interval.



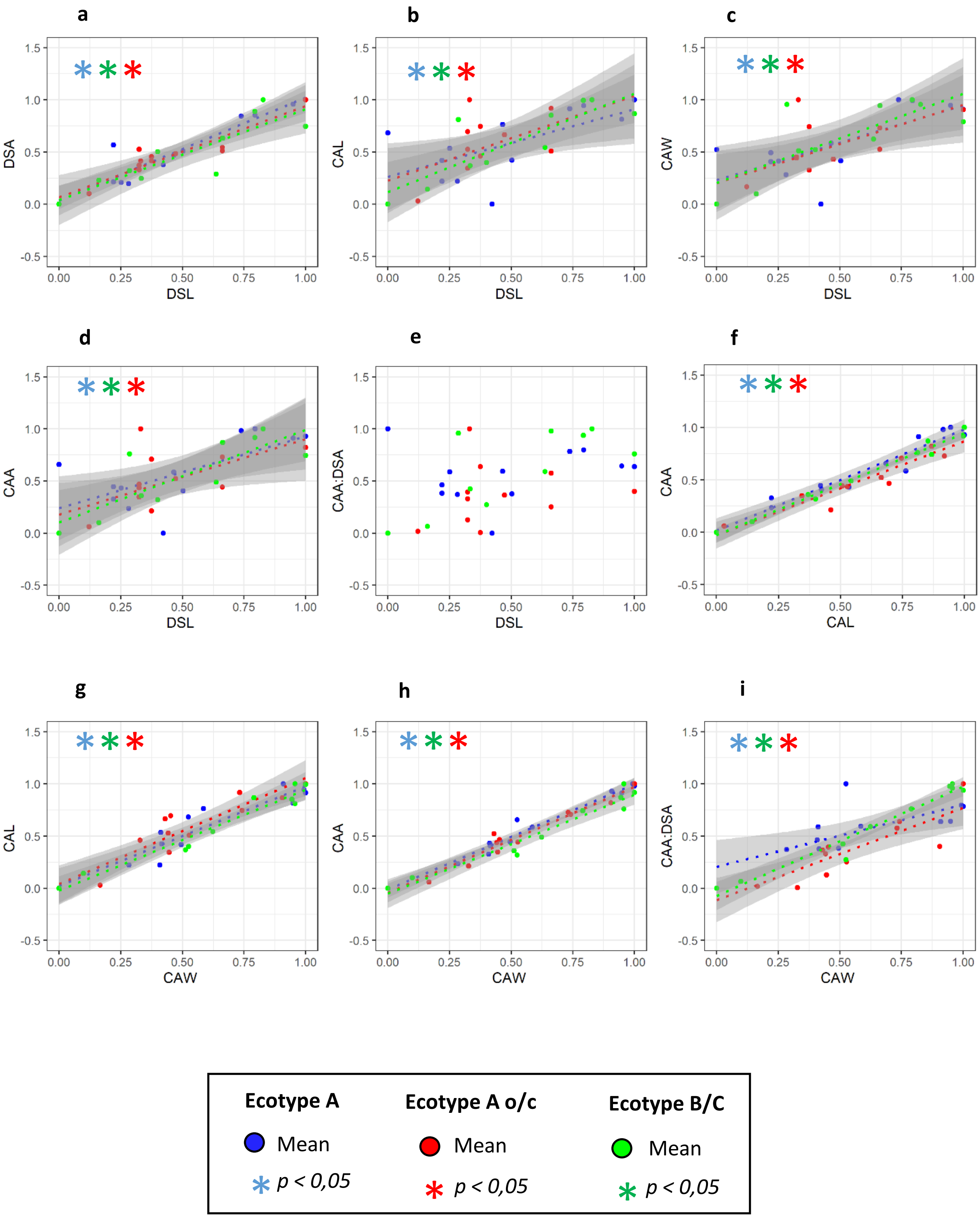
Fonte: A autora

Figure 9. Linear regressions between pH and geometric measurements of *E. huxleyi* coccoliths with normalized values. Gray areas correspond to 95% confidence interval.



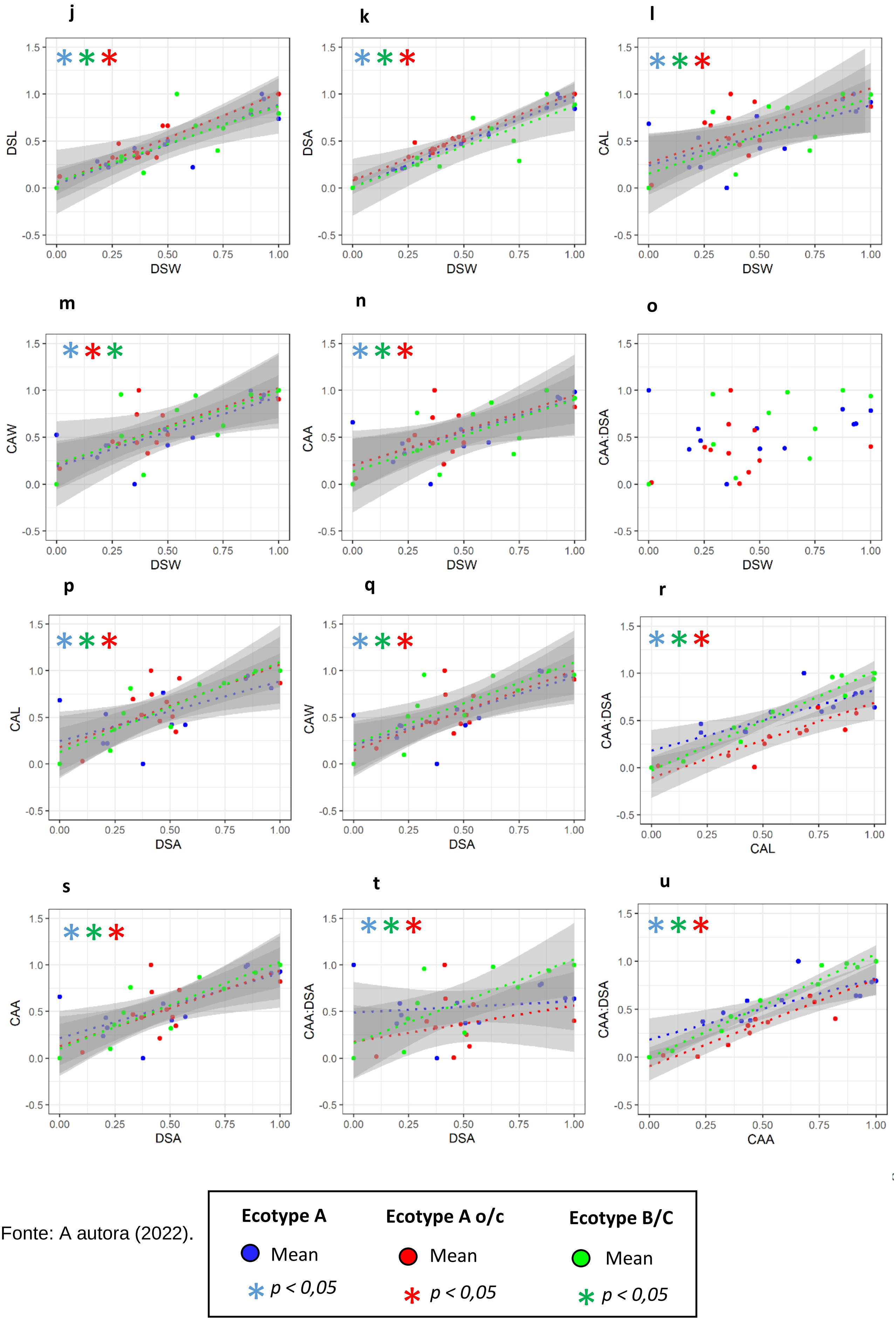
Fonte: A autora (2022).

Figure 10. Linear regressions between geometric measurements of *E. huxleyi* coccoliths with normalized values. Gray areas correspond to 95% confidence interval.



Fonte: A autora (2022).

Figure 10. Linear regressions between geometric measurements of *E. huxleyi* coccoliths with normalized values. Gray areas correspond to 95% confidence interval.



Fonte: A autora (2022).

4.2 Mass of Coccoliths

The different methods to estimate coccolith's mass resulted in divergent values. The coccolith masses (absolute values) that were calculated using the data obtained from geometric measurements and the Coulter Multisizer were closer when compared to the coccolith masses obtained using the SYRACO and the masses obtained by SYRACO are greater than those obtained by geometric measurements and Coulter Multisizer (Table 6).

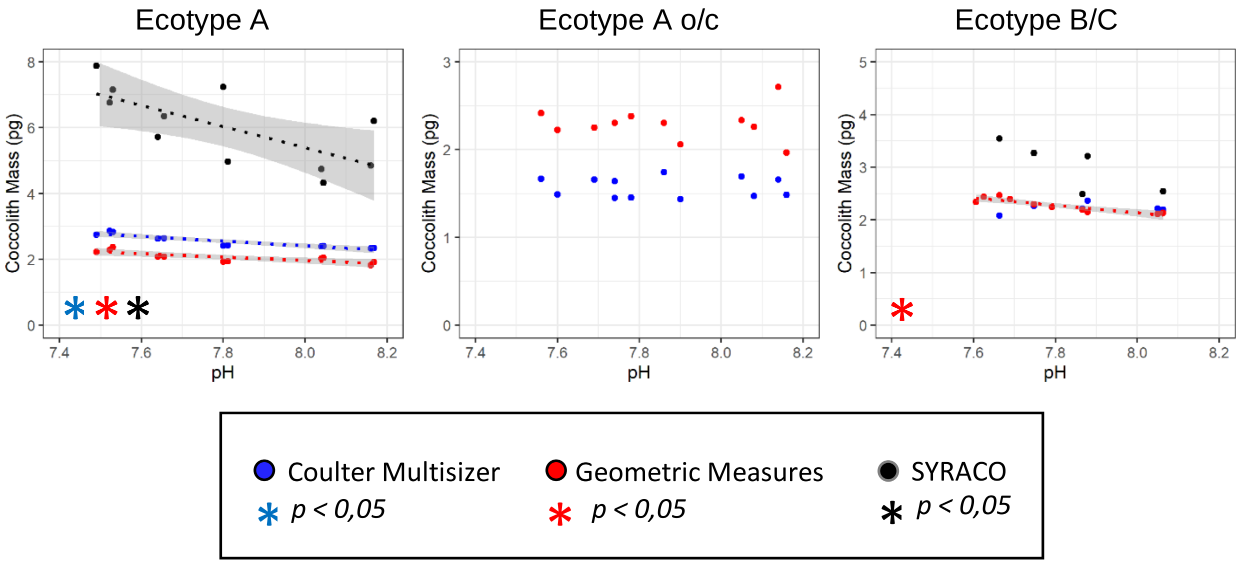
Table 6. Absolute coccolith mass values (pg) of the different ecotypes of *E. huxleyi* (CM = Coulter Multisizer, GEO = Geometric Measures, SY = SYRACO).

Ecotype A				**Ecotype A o/c**			**Ecotype B/C**			
pH	CM	GEO	SY	**pH**	CM	GEO	**pH**	CM	GEO	SY
8.16	2.32	1.82	4.86	**8.16**	1.48	1.96	**7.87**	2.21	2.19	2.49
8.04	2.39	2.02	4.75	**8.08**	1.47	2.25	**7.75**	2.28	2.29	3.27
7.80	2.41	1.92	7.23	**7.90**	1.43	2.06	**7.66**	2.08	2.46	3.54
7.64	2.63	2.07	5.72	**7.78**	1.45	2.37	**8.06**	2.19	2.13	2.54
7.53	2.83	2.36	7.16	**7.74**	1.44	2.30	**7.88**	2.36	2.15	3.21
7.49	2.75	2.22	7.88	**7.60**	1.49	2.22	**8.05**	2.21	2.11	-
8.17	2.34	1.91	6.21	**8.14**	1.65	2.71	**7.63**	-	2.43	-
8.04	2.40	2.04	4.33	**8.05**	1.69	2.33	**7.79**	-	2.24	-
7.81	2.42	1.93	4.98	**7.86**	1.74	2.30	**7.69**	-	2.39	-
7.66	2.64	2.08	6.35	**7.74**	1.64	2.30	**7.61**	-	2.33	-
7.52	2.87	2.28	6.77	**7.69**	1.65	2.24	**-**	-	-	-
-	-	-	-	**7.56**	1.66	2.41	**-**	-	-	-

Fonte: A autora (2022).

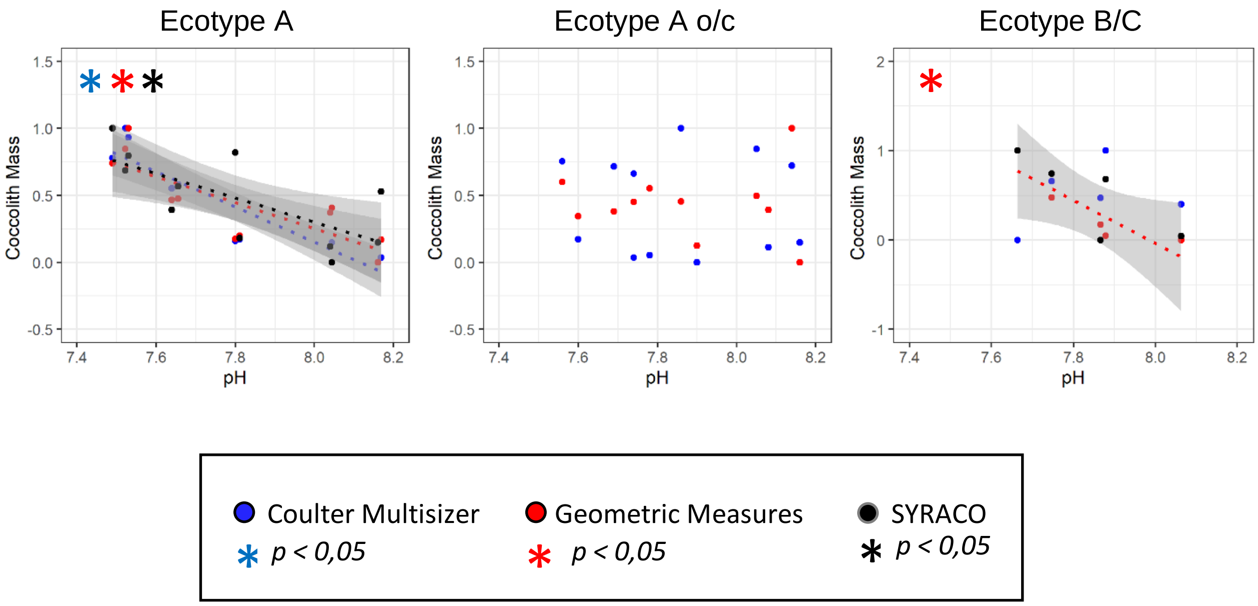
The relative changes in the masses of ecotype A coccoliths in relation to the pH gradient are significant when calculated from data obtained using the Coulter Multisizer, SYRACO, and geometric measurements. On the other hand, the relative changes in the masses of the ecotype B/C coccoliths in relation to the pH gradient are significant when calculated specifically from the data obtained through geometric measurements ($p < 0.05$). However, the ecotype A o/c does not have SYRACO data and does not have significant changes in its mass (calculated only from Coulter Multisizer and geometric measurements) in relation to the pH gradient (Figures 11 and 12).

Figure 11. Linear regressions between pH and the different methods analyzed to calculate the coccolith mass. Gray areas correspond to 95% confidence interval.



Fonte: A autora (2022).

Figure 12. Linear regressions between pH and the different methods analyzed to calculate the coccolith mass with normalized values. Gray areas correspond to 95% confidence interval.



Fonte: A autora (2022).

Table 7. Linear regression to verify correlations between pH and methods to calculate coccolith mass (CM = Coulter Multisizer, GEO = Geometric Measures, SY = SYRACO).

Ecotype	Method	r²	*p*-value
A	CM	0.826	6.642e-05
	GEO	0.609	0.003
	SY	0.446	0.015
A o/c	CM	0.002	0.885
	GEO	0.001	0.943
B/C	CM	0.099	0.605
	GEO	0.707	0.047
	SY	0.535	0.099

Fonte: A autora (2022).

5 DISCUSSION

The results indicate that the three analyzed ecotypes have different sensitivities to changes in the seawater carbonate chemistry, for example, by the experimental decrease in pH, as would happen in a future scenario of oceanic acidification.

The sensitivities of each ecotype to changes in seawater carbonate chemistry suggest different physiological responses of coccolithophores to ocean acidification, such as the significant changes in the geometric parameters of *E. huxleyi* coccoliths ($p < 0.05$). Therefore, the ecotype B/C (oceanic origin) was the most sensitive (highest slope values of linear regressions). This greater sensitivity of ecotype B/C can be explained by the structure of its coccoliths, which are more delicate, with a low amount of calcium carbonate, as it contains about 50% less calcite in its coccolith (Poulton et al., 2013).

The ecotype A had most of its geometric parameters significantly correlated with changes in seawater carbonate chemical parameters, this is an ecotype of oceanic origin, which may indicate that oceanic strains are more sensitive to changes caused in the laboratory experiment.

Interestingly, ecotype A o/c (coastal origin) did not have significant correlations between DSA and the parameters $p\mathrm{CO}_2$ and pH. This may indicate that strains from different regions exhibit differences in their ability to cope with changes in the carbonate system. For example, strains from regions with greater variation in the carbonate system (such as coastal regions) may exhibit adaptations, being more resistant to laboratory experiments where CO_2 and pH are manipulated (Beaufort et al., 2011).

The increase in atmospheric CO_2 levels causes significant changes in ocean surface pH and, consequently, in carbonate chemistry. This increase in CO_2, associated with the decrease in seawater pH, can cause significant effects on the calcification of coccolithophores, having implications for global biogeochemical processes. For example, the increase in CO_2 associated with a decrease in pH causes carbonate ions (CO_3^{2-}) to no longer combine with calcium ions (Ca2+) to form calcium carbonate ($CaCO_3$) — the raw material of coccoliths. Instead, carbonate ions (CO_3^{2-}) combine with hydrogen ions (H^+) to form bicarbonate (HCO_3) and the raw material of coccoliths is not formed.

Consequently, the increase in the overall size of coccoliths (DSA) under high $p\mathrm{CO}_2$ concentrations and associated with a decrease in pH seems counterintuitive, but it can be explained by the rate of calcification of coccolithophores which has already been shown to decrease with ocean acidification – becoming slower and with less production of coccoliths per cell, however with a greater coccolith size –, which may indicate that the calcification process can be slowed down during adverse conditions and severe physiological stress (Beaufort et al., 2011; Müller et al., 2015).

Furthermore, the growth rate is the parameter that tells us whether the organism is growing, stable or decreasing and how quickly these changes occur. In this sense, the growth rate plays a central role both in correctly predicting future trends and in our understanding of environmental stress — such as changes in the seawater carbonate system (Sibly and Hone, 2002). Müller et al. (2015) observed that the growth rate of ecotypes A and B/C decreases with decreasing pH. On the other hand, the growth rate of ecotype A remains constant along the simulated pH gradient in a laboratory experiment. When comparing the geometry data of *E. huxleyi* from the present study with the growth rates obtained by Müller et al. (2015), it is observed that all geometric parameters of ecotypes A and B/C have their largest sizes when exposed to the lowest pH values (except CAL, which also obtained p values close to 0.05). This indicates that the size of the geometric parameters is inversely proportional to the growth rates in these ecotypes.

This decrease in the calcification rate of *E. huxleyi* is interesting, as the coccosphere is responsible for providing the ecological role of protection against one of the main causes of phytoplankton death in the ocean: predation by zooplankton. The B/C ecotype of *E. huxleyi* has already been shown to decrease the production of its coccoliths in regions with low pH (Müller et al., 2015). Furthermore, in the present study, ecotype A was also shown to decrease its calcification with deacreasing pH. Therefore, in a future scenario where the oceanic pH continues to decrease at a similar rate to the current one, the B/C and A ecotypes may experience a higher herbivory pressure.

There is a large number of studies carried out with strains from the north to the detriment of those studies that used strains from the Southern Ocean. For example, Bach et al. (2012) investigated the influence of changes in carbonate chemistry on the morphology of coccoliths produced by an ecotype A strain of *E. huxleyi*. Among the results is the high sensitivity of their coccoliths, which had their DSL, DSW, CAW, DSA and CAA parameters significantly correlated with changes in pH — corroborating with the ecotype A of the present study. However, the same ecotype A used by Bach et al. (2012) had no significant correlation between the overall growth of the coccolith and the central area of the coccolith. On the other hand, all 3 ecotypes used in the present study had a positive correlation between the growth of the total distal part and the central part ($p < 0.05$), which indicates that the growth of the central area normally increases proportionally with its total size (For example: Figure 8b and c).

In addition, *E. huxleyi* coccoliths are found in sedimentary records dating back approximately 270.000 years (Müller et al., 2020). Therefore, the geometry of coccoliths can be used as a paleo-proxy, since the size of these organisms is an important characteristic when correlated with several ecophysiological processes, energy transfer and biogeochemical cycling (Henderiks & Pagani, 2008; Müller et al., 2020).

However, natural populations of *E. huxleyi* are composed of multiple genetic lineages (Iglesias-Rodríguez et al., 2006), which makes it difficult to directly apply geometric measurements of their coccoliths as paleo-proxies. In this sense, by using different ecotypes of *E. huxleyi* that co-occur in the Southern Ocean — that is, using individuals of the same species, but with different ecological niches, the present study provides an interesting tool for interpreting the change in coccolith geometry in relationship with the change in seawater carbonate chemistry, which indicates that the combination of laboratory experiments and the analysis of coccoliths present in the fossil record is a tool that should be used to identify which factors act on changes in coccolith geometry (Gibbs et al., 2013; Müller et al., 2013; Müller et al. al., 2020).

The coccolith mass was measured in different ways: through the volume obtained by the Coulter Multisizer™ 4 multiplied by the calcite density (using the Coulter principle, in which an electric field is used to count and size particles that are suspended in a liquid conductor); through SYRACO (a tool for automatic recognition of coccoliths by neural networks) and through the geometric measurements themselves (Young and Ziveri, 2000; Beaufort & Dollfus, 2004).

Interestingly, the coccolith mass values obtained by the different methods were divergent. For all ecotypes, the masses calculated using Coulter Multisizer™ 4 and geometric measurements had similar values. On the other hand, in the A and B/C ecotypes, the mass values obtained by SYRACO were overestimated when compared to the masses obtained by the other tools — which suggests that greater attention should be paid to the choice of the method that will be used. Furthermore, only the A o/c ecotype had no significant correlation between the coccoliths mass and the pH, which corroborates its lower sensitivity to the parameters of the carbonate system (demonstrated in this study).

6 CONCLUSION

The three ecotypes of *E. huxleyi* were sensitive to changes in the chemistry of the carbonate system. In general, changes in coccolith geometry correlated with pH values, indicating that *E. huxleyi* may have its calcification process affected in an oceanic acidification scenario. Furthermore, ecotypes from different regions showed different sensitivities to changes in chemical parameter gradients. In addition, the different results obtained by the methods for estimating the mass of coccoliths suggest that, only for the use of absolute values, the choice of method must be done with care, because the values obtained differ from each other and may not represent actual values.

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