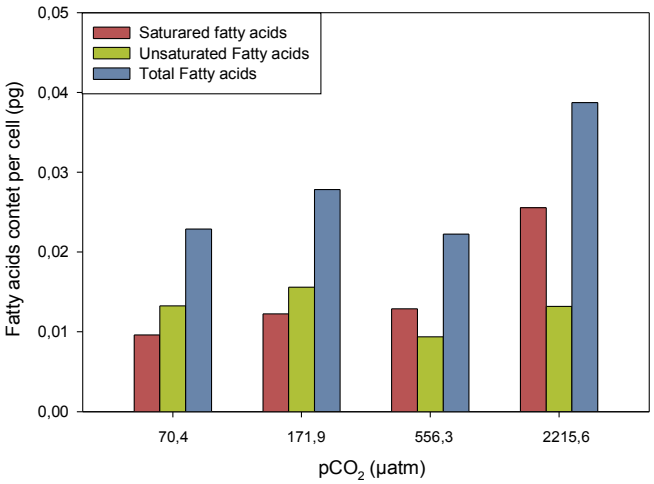
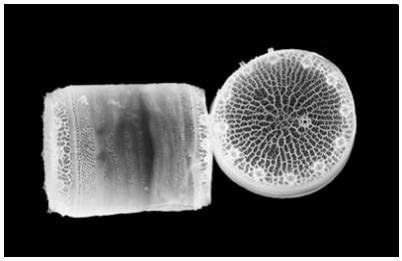
IFM-GEOMAR
Leibniz-Institut für Meereswissenschaften
an der Universität Kiel

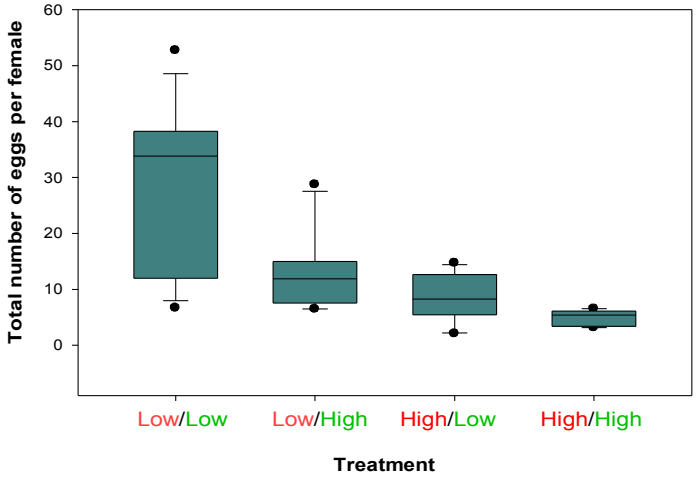
Effect of CO_2 on elemental composition and fatty acids of diatoms and concomitant effects on copepods.

Jorge Rafael Bermúdez Monsalve

Master Thesis







IFM-GEOMAR
Leibniz-Institut für Meereswissenschaften
an der Universität Kiel

Effect of CO_2 on elemental composition and fatty acids of diatoms and concomitant effects on copepods.

Master Thesis

Submitted by

J. Rafael Bermúdez M.

Supervised by

Prof. Dr. rer. nat. Ulf Riebesell

and

Dr. rer. nat. Kai Schulz

Faculty of Mathematics and Natural Sciences

Kiel 2010

A mi familia

Table of Contents

Figures

fatty acids in the diatom *T. pseudonana* culture under two different pCO_2 (µatm) levels. The fatty acids that show an significant difference are mark with an ***** (see text for details).

Figure 11.- Relative copepodite stage development analysed at three different days.

Figure 12.- Number of eggs produced per female of *A. tonsa* in a period of 24 hours, grown under two different pCO_2 levels (~350 and ~740 µatm) and feed with the diatom *T. pseudonana* growth under similar pCO_2 conditions as the copepods.

Figure 13.- Left: content of total fatty acids per female of *A. tonsa*. Right: Average content and standard deviation of saturated and unsaturated fatty acids content per female copepod.

Figure 14.- Correlation between total fatty acid per female and eggs produced in 24 hours in the for different treatments.

Figure 15.- Genera scheme of fatty acid biosynthesis (from Harwood 2010).

Figure 16.- A: Current model of Carbon Concentration Mechanism (CCM) in eukaryotic phytoplankton where is show the release of H^+ that could modify the cytoplasm pH (modified from the original of McGinn et al. 2008). B: The increase in the pH of the media could break the double bounds of the carbon chains in membrane fatty acids.

Figure 17.- Relative content of saturated and unsaturated fatty acids in different species of phytoplankton expressed as percentage of the total in panels A (this study), B (Riebesell et al. 2000) and C (Yongmanitchai et al. 1991), and as mol percentage in panels D, E, F and G (Tsuzuki et al. 1990). Note the reduction in the relative amount of unsaturated fatty acids under higher CO_2 concentrations.

Figure 18.- Relative content of saturated and unsaturated fatty acids in different species of phytoplankton expressed as percentage of the total measured. Left panel (Chu et al. 1996) the diatom *Nitzchia inconspicua*, and in the right panel (Tang et al. 2010) the green algae *Scenedeismus obliquus*. Note the increase of the relative amount of unsaturated fatty acids under higher CO_2 concentrations.

Figure 19.- Changes in fatty acid composition of the green algae *Chlorella kessleri* after shift of CO_2 concentration from 2 to 0.04% in panels A and B, and from 0.04 to 2% in panels C and D (Tzusuki et al. 1990).

Tables

Table 1.- Measured alkalinity, DIC, calculated pCO2 and pH of each phytoplankton specie used in the study. The DIC of the copepods feeding experiment was calculated from the DIC measurements using the CO2sys software. The K1 and K2 for the calculations are from Roy et al. (1993).

Table 2.- Nitrogen phosphate and silicate measured in each experiment in this study

Table 3.- Correlation between nutrient content per cell (picomol) and final cell number. At the top are the r values and under them the confidence p values (n= 11).

Table 4.- Correlation between nutrient content per cell (picomol) and the gradient of calculated pCO_2 (µatm) in each culture bottle (n= 11)

Table 5.- Saturated and unsaturated fatty acids detected and measured in the diatom *T. pseudonana* cultured under a gradient of pCO_2 (µatm) and constant alkalinity. The concentration is expressed as femtograms of fatty acid per cell (fg $cell^{-1}$) . Note that some saturated fatty acids are more abundant at higher pCO_2 concentrations.

Table 6.- Specific saturated and unsaturated fatty acids per cell (fg $cell^{-1}$) in *T. pseudonana* used to feed the copepod *A. tonsa* cultured at two different pCO_2 (µatm) levels. The values are averages of five different measurements at low pCO_2 and three measurements at high pCO_2.

Table 7.- Average concentration (ng $ind.^{-1}$) and standard deviation of saturated and unsaturated fatty acids per copepod female measured in each treatment. Note that treatment High/High show more saturated fatty acids and treatment Low/Low show more unsaturated fatty acids.

Table 8.- Correlation analysis and its respective significance between the content of specific saturated and unsaturated fatty acids per female (ng $ind.^{-1}$) and the average number of eggs produced per female. The significant values are highlighted in red.

Summary

Since the beginning of the industrial revolution the atmospheric partial pressure of CO_2 (pCO_2) has increased exponentially, reaching 380 μatm nowadays, and is expected to rise to values up to 700 μatm by the end of this century. These changes affect marine plankton in various ways, positively as for cyanobacteria, or in most cases, negatively as for coccolithophores. However there is a lack in the understanding of the effect of this increase in carbon for some important organisms as diatoms, an important primary producer in the ocean. Diatoms have not been reported as affected by ocean acidification, although several studies have reported a change of the total lipid content in some diatoms when cultured at high CO_2 conditions. With this perspective, a set of two experiments were designed; the first was intended to determine if the amount of different fatty acids (the building blocks of lipids) of the diatom *Thalassiosira pseudonana* is altered when cultures under diverse CO_2 conditions; while the second experiment was intended to determine the possible effects of the change in the fatty acids of *T. pseudonana* on the life cycle of the copepod *Acartia tonsa* when feed with this diatom. The first experiment showed that the fatty acid content of *T. pseudonana* change toward high CO_2 levels, with an increase in the amount of saturated fatty acids and a decrease of unsaturated fatty acids content. The second experiment showed that the growth rate, amount of egg produced per female, and fatty acid content per female are reduced when feed with *T. pseudonana* cultured at high CO_2 conditions. Our results show that CO_2 actually affects the fatty acid composition of *T. pseudonana* and that this fatty acid alteration in the diatom have a significant influence on the life cycle of *A. tonsa*. However, further studies are required to determine if the effects observed in this study also take place in the environment.

Effect of CO_2 on elemental composition and fatty acids of diatoms and concomitant effects on copepods

1. Introduction

1.1 Carbonate system

1.1.1 The Carbonate System of the Ocean

The ocean represents the largest carbon reservoir (~38,000 Pg C) of Earth's carbon cycle. The mean concentration of inorganic carbon in the ocean is about 2.3 mmol kg^{-1}; its residence time is ~200 ka. Ocean and atmosphere exchange carbon in the form of carbon dioxide (CO_2). Atmospheric CO_2 is therefore strongly coupled to the oceanic reservoir. The total amount of dissolved inorganic carbon in the modern ocean is about sixty times larger than of the pre-anthropogenic atmosphere (Zeebe and Wolf-Gladrow, 2001).

Dissolved carbon dioxide in seawater occurs mainly in three inorganic forms (Figure i, left panel): free aqueous carbon dioxide ($CO_{2,aq}$), bicarbonate (HCO_3^-), and carbonate ions (CO_3^{2-}). Typical concentrations of dissolved carbonate species in seawater as a function of pH. The majority of dissolved inorganic carbon in the ocean is in the form of HCO_3^- (>85%) (Zeebe and Wolf-Gladrow, 2001). Gaseous carbon dioxide ($CO_{2,g}$), and [CO_2] are related by Henry's law in thermodynamic equilibrium:

$$CO_{2,g} = CO_2 \; ; \quad K_0$$

where K_0 is the solubility coefficient of CO_2 in seawater. The concentration of dissolved CO_2 and the fugacity of gaseous CO_2, fCO_2, then obey the equation $[CO_2] = K_0 \times fCO_2$. The fugacity is practically equal to the partial pressure, pCO_2 (within ~1%) (Zeebe and Wolf-Gladrow, 2001). The dissolved carbonate species are related by:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \; ; \quad K^*_1$$
$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \; ; \quad K^*_2$$

The pK*'s (= −log(K*)) of the stoichiometric dissociation constants of carbonic acid in seawater are pK*1 = 5.94 and pK*2 = 9.13 at temperature T=15°C, salinity S=35, and surface pressure P=1 atm on the total pH scale. At typical surface seawater pH of 8.2, the speciation between $[CO_2]$, $[HCO_3^-]$, and $[CO_3^{2-}]$ hence is 0.5%, 89%, and 10.5% (Zeebe and Wolf-Gladrow, 2001). The sum of the dissolved carbonate species is denoted as total dissolved inorganic carbon (DIC ≡ ΣCO2 ≡ TCO2 ≡ CT):

$$TCO_2 = [CO_2] + [HCO_3^-] + [CO_3^{2-}]$$

Another critical parameter to describe the marine carbonate system is the total alkalinity (TA). Total alkalinity is is related to the charge balance in seawater and can be defined as the excess of proton (H^+ ion) acceptors over proton donators (with respect to a zero level of protons) (Zeebe and Wolf-Gladrow, 2001):

$$TA = [HCO_3^-] + 2\,[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] - [H^+] + \text{minor compounds}$$

Of the carbonate system parameters, pCO_2, pH, TCO_2, and TA can be determined analytically. However, if any two parameters and total dissolved boron are known, all parameters (pCO_2, $[CO_2]$, $[HCO_3^-]$, $[CO_3^{2-}]$, pH, TCO_2, and TA) can be calculated at given temperature, salinity and pressure (Zeebe and Wolf-Gladrow, 2001).

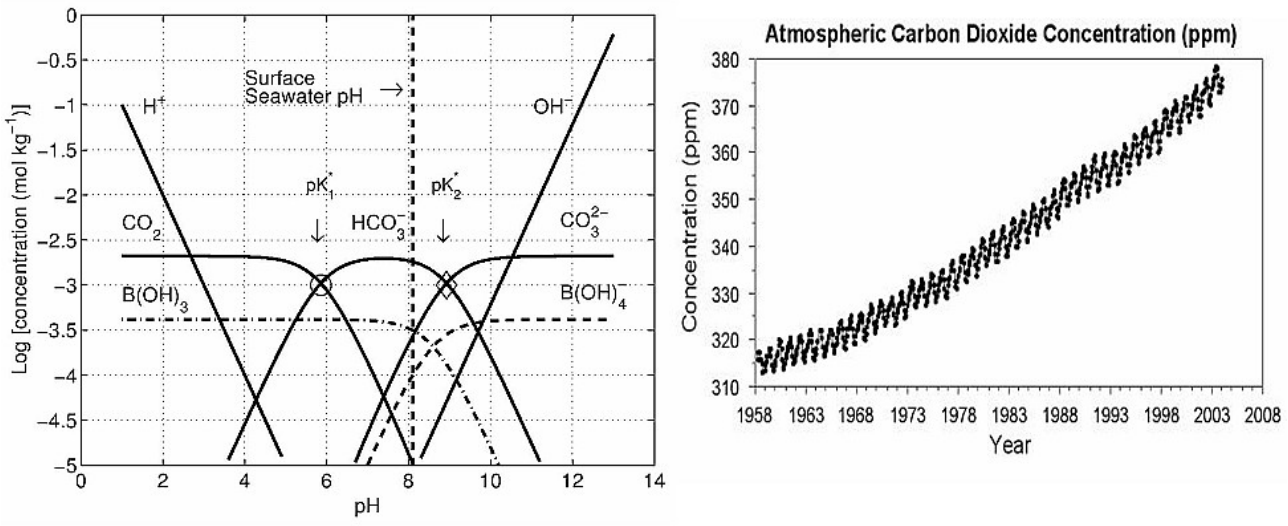


Figure i.- Left: Typical concentrations of dissolved carbonate species in seawater as a function of pH. (Zeebe and Wolf-Gladrow, 2001). Right: increase in the CO_2 concentration in the atmosphere in the last fifty years (Keeling et al. 2004).

1.1.2 Anthropogenic CO2

Since the beginning of the industrial revolution the atmospheric partial pressure of CO_2 (pCO_2) has increased exponentially (Figure i, right panel) reaching 380 μatm nowadays, and is expected to rise to values up to 700 μatm by the end of this century if the rate of CO_2 emissions continues according to a "business as usual" scenario (IPCC Scenario IS92a. 2007; Houghton et al. 2001; Raven et al. 2005; Raupach et al. 2007).

A pH value of 8.1 is nowadays typical for the surface ocean. At this value, less than 1% of the CO_2 coming from the atmosphere remains as dissolved CO_2, while the rest is converted into HCO_3^- (~90%) and CO_3^{2-} (~9%). The reaction of CO_2 with water generates one proton (H^+) for each HCO_3^- and two protons for each CO_3^{2-} formed. This acidification causes a shift of the pH-dependent equilibrium between CO_2, HCO_3^- and CO_3^{2-} causing a higher CO_2 concentration, a slight increase in the concentrations of HCO_3^- and a lower carbonate ion concentrations [CO_3^{2-}]. (Murray, 2002; Riebesell, 2004). It is expected that the projected CO_2 emissions will lead to a decrease of ~0.3 units of surface ocean pH by the end of this century, along with a threefold increase in the concentration of CO_2, and a decrease of the CO_3^{2-} ion concentration by nearly 50% (Riebesell, 2004).

These changes affect marine plankton in various ways. For example at cellular level, a moderate increase of CO_2 might facilitate photosynthetic carbon fixation of some phytoplankton groups like nitrogen fixing cyanobacteria (Kranz et al, 2009; Barcelos e Ramos 2007). However there is a lack in the understanding of the effect of this increase in carbon for some important organisms as diatoms, fast growing algae and important primary producer in the ocean.

1.2 Diatoms

The most diverse group of phytoplankton is the microscopic, eukaryotic microorganisms known as diatoms; with an estimated 200.000 different species, ranging in size from a few micrometers to a few millimeters and existing either as single cells or as chains of connected cells (Kooistra et al, 2007).

Diatoms are quickly bloomers, increasing in cell number by many orders of magnitude in just a few days (Armbrust , 2009), primarily by mitotic divisions, interrupted infrequently by sexual events (Jewson, 1992).

Diatoms tend to dominate phytoplankton communities in well-mixed coastal and upwelling regions, as well as along the sea-ice edge, where sufficient light, inorganic nitrogen, phosphorus, silicon and trace elements are available to sustain their growth (Morel and Price, 2003). About one-fifth of the photosynthesis on Earth is carried out by this microorganisms, producing about as much organic carbon as all the terrestrial rain-forests combined (Nelson et al, 1995). This organic carbon produced by diatoms is consumed rapidly and serves as a base for marine food webs. In coastal waters, diatoms support the most productive fisheries. In the open ocean, a relatively large proportion of diatom organic matter sinks rapidly from the surface, becoming food for deep-water organisms (Sarthou et al, 2005). In polar environments, where glaciers and permafrost limit photosynthesis on land, diatoms are critical components of the food webs that sustain both marine and terrestrial ecosystems (Armbrust , 2009).

1.3 Copepods

The copepods, small aquatic crustaceans, classifiedd in the subclass Copepoda, comprise over 14.000 species (both valid and invalid, including senior and junior synonyms), being the most numerous metazoans in the water community. Their habitat range from the shallow coastal line to the deepest ocean trenches and from the cold polar ice-water interface to the hot active hydrothermal vents (Mauchline, 1998).

Copepods may be free-living, symbiotic, or internal or external parasites on almost every phylum of animals in water. The usual length of adults is 1-2 mm, but some species may be as short as 0.2mm and others may be as long as 10mm or even longer in the case of parasites (Mauchline, 1998).

Ecologically planktonic copepods are important links in the aquatic food chain, connecting microscopic algal cells, to juvenile fish, to whales (Mauchline, 1998).

1.4 Fatty acids

NOTE: In this manuscript, for purposes of simplicity, the fatty acids were classified in saturated and unsaturated (regardless of the number of double bonds).

Fatty acids are long hydrocarbon chains of diverse length (number of carbon atoms) that can contain singles or double bonds between the carbon atoms, with a carboxyl ($-COOH$) group attach in one extreme and a methyl group (CH_3) in the other; being the first the hydrophilic part second the hydrophobic (Mouritsen, 2005).

The plainest way to classify fatty acids is to write the number of carbon atoms, followed by the number of double bonds, follow with an *n* (or ω, omega) that refers to the position of the double bond nearest to the methyl end of the molecule. eg: linoleic acid is notated as 18:2*n*-6. According to the number of double bonds the fatty acids are called: Saturated (no double bonds), Mono-unsaturated (one double bond), Poly-unsaturated (one to four) and Super-unsaturated (five or more) (Mouritsen, 2005).

Fatty acids are the fundamental building blocks of cell membranes and, naturally, of all lipids in living matter. Plants and animals use a variety of fatty acids with chain lengths ranging from two to thirty-six. The most common chain lengths fall between fourteen and twenty-two; the length of the chain is likely to be controlled by the need of cells to have membranes with a certain thickness in order to function properly. In animals and plants most of the fatty acid chains are unsaturated, most frequently with a single double bond and in some cases with as many as six double bonds (Mouritsen, 2005).

Short chain fatty acids can be produced by electrical discharges out of inorganic compounds, eg: CO_2 and methane. Intermediate- and long-chain fatty acids are believe to be produced only by biochemical synthesis in living organisms. Animals acquire fatty acids through the diet, and are able to transform saturated fatty acids in mono-unsaturated fatty acids with a double bond in position 9 along the chain, but are unable to make unsaturated bonds in the positions 12 and 15, only plants have the capacity to do so. Since animals need to get them from their diet, they are call *essential fatty acids*. This essential fatty acids are the linoleic acid 18:2*n*-6 and the α-linoleic acid 18:3*n*-3,

with two and three double bonds respectively. From these fatty acids, two families of polyunsaturated and super-unsaturated fatty acids can be formed by elongation and desaturation: the *n*-6 (or ω-6) and *n*-3 (also ω-3) (Appendix, Figure 1). Linoleic acid is found in oils from various seeds (as sunflower) and the α-linoleic acid is synthesized only in higher plants, algae and phytoplankton. From the the fatty acids formed by elongation and desaturation the two most important are the docosahexaenoic or DHA (22:6*n*-3), arachidonic or AA (20:4*n*-6) both important constituents of the neural tissue, and the eicosapentaenoic acid (EPA; 20:5*n*-3), precursor of DHA (Mouritsen, 2005).

1.5 Variation of the C:N:P stoichiometry and macromolecular partitioning in diatoms

The availability of nutrients have an important influence on the elements stoichiometry of phytoplankton (Hecky et al, 1993); in response to different nutrient concentrations, diatoms, like other phytoplankton cells, could show a different C:N:P ratio (Burkhardt et al. 1999). Alterations in the nutrient ratio produce a metabolic acclimatisation, that is changes in the cellular composition of macromolecules in the cells (Wilhelm et al, 2006). For example nitrogen limitation reduces the ability to use photosynthetically fixed carbon for protein synthesis, but does not prevent the formation of photosynthetic storage products (Berges et al. 1996; Granum et al. 2002). Consequently, a gradual increase in cellular carbon reserves without a concomitant increase in organic nitrogen (expressed as increased C:N ratio) as well as a decline of Chl-*a* and of photochemical efficiency are observable (Berges and Falkowski 1998; Lippemeier et al. 2001).

Recently, Hein & Sand-Jensen (1997) Engel et al. (2002) and Riebesell et al. (2007) reported an increase in photosynthetic carbon uptake under elevated CO_2 concentrations in sea water; particularly Riebesell et al. (2007), in a natural assemblage dominated by diatoms, report a shift in the exported organic matter C:N ratio from 6,0 at low CO_2 to 8,0 at high CO_2 concentrations, thus exceeding the today's ocean ratio of 6,6. Also Burkhardt et al. (1999) during a study on the effect of variable concentrations of dissolved carbon dioxide (CO_2), on C:N:P ratios in marine phytoplankton in batch cultures, reported that the elemental composition in six out of seven species tested was affected by variations in CO_2. Either an increase or a decrease in the C:N and C:P ratios with increasing CO_2 concentrations was observed, depending on the species tested.

Actually, some authors working on phytoplankton species used in aquaculture found a change in the macromolecular composition under elevated CO_2 concentrations. Castro Araújo et al. (2005) showed that in the diatom *Chaetoceros wighamii* an increase in protein and a reduction of carbohydrates content cultured under high CO_2 conditions, but no effect on lipids. Brown et al. (1997), in an study with over 40 species, grown in media enriched with 1% carbon dioxide, noticed increases in the average protein concentration of about 10%. Chrismadha & Borowitzka (1994), reported that the protein content was increased with carbon dioxide additions in the diatom *Phaeodactylum tricornutum.* Finally, Chu et al. (1996) observed an opposite effect in the diatom *Nitzschia inconspicua*, with increases in lipids and carbohydrates at protein expenses when the culture was enriched with 5% (v/v) of carbon dioxide.

1.6 Copepods dietary requirements and the influence of prey as food source

Phytoplankton is mainly consumed by zooplankton species such as calanoid copepods, which themselves are the food source for higher trophic levels, such as fish. As a consequence, interactions between phytoplankton and zooplankton determine the trophic transfer and the energy flow in aquatic environments (Wichard et al. 2007).

It is often argued that the macronutrient stoichiometry of the food (C:N:P ratio) is an important factor in the trophic transfer in marine food webs (Jones and Flynn, 2005; Mitra and Flynn, 2005). Thus, minor changes in the prey's stoichiometry is traduced in changes in its macromolecular partitioning, that have been associated with more significant changes in prey quality (Mitra and Flynn, 2005). These changes may have a disproportionate effect on the predator's growth efficiency (Mitra and Flynn, 2005), egg production and hatching success (Jónasdóttir, 1994; Jónasdóttir and Kiørboe 1996). In this respect, one of the main aspects of food quality under study in marine systems is the biochemical composition of the food, for instance the Carbon:Nitrogen ratio (C:N) that has a crucial influence on the fatty acid spectrum of different algal groups (Klein Breteler et al, 2005).

Studies on the nutritional requirements of crustaceans indicate that fertility and development may require specific fatty acids (Jónasdóttir, 1994; Jónasdóttir and Kiørboe 1996) because they do

not or cannot easily biosynthesize the polyunsaturated fatty acids (PUFAs) *n*3 and *n*6, being found in the crustaceans in proportion to their availability in its diet (Fraser et al. 1989). Between the different PUFAs, the long chain *n*3 fatty acids are recognized to have a high nutritional value for copepods, especially the eicosapentaenoic acid (EPA; 20:5*n*-3) and the docosahexaenoic acid (DHA; 22:6*n*-3) (Moreno et al., 1979). Particularly PUFAs and copepod reproduction have been reported to be correlated; Arent et al. (2005) report a positive relationship between the amount of EPA in the diatom *Thalassiosira weissflogii* used to feed the copepod *Temora longicornis* and its egg production and hatching success. Jónasdóttir (1994) reported that the egg production of *Acartia tonsa* and *Acartia hudsonica* was correlated with specific fatty acids [16:1*n*-7 (negative), 20:5*n*-3, 22:6*n*-3, and 18:0 (positive)] present in young cultures of *T. weissflogii*.

Nutrient variations alter the stoichiometry of diatoms and affect strongly its lipid composition; Klein Breteler et al. (2005) reported that young copepodite stages of *Temora longicornis* and *Pseudocalanus elongatus* developed at significantly reduced rates (1,5 to 4 days delay in relation to the control culture) when fed with *T. weissflogii* grown under different levels of nitrogen and phosphorus limitation and that the proportion and the content of PUFAs were reduced, particularly under phosphorus limitation.

As is show above, CO_2 can change the C:N:P ratio and macromolecular partitioning in phytoplankton, affecting the development of other organisms as copepods. Under this perspective, become of great importance the study of the biochemical composition of phytoplankton species under the predicted future CO_2 scenario, and the consequences this could have on other organisms of higher trophic levels.

To assess these consequences, two sets of experiments were performed, one with focus on the variation in the C:N:P ratio and lipid composition of diatoms under a range of different CO_2 levels (seven), and a second focus on the lipid content, egg production and growth rate of copepods cultured under two different pCO_2 (380 and 750 pCO_2) and feed with diatoms growth under the same two CO_2 levels.

2. Hypothesis

H_1 Diatoms experiment: The C:N:P ratio and lipid composition in diatoms change under different CO_2 levels.

H_1 Copepods experiment: The growth, egg production, and lipid content of copepods is affected as consequence of the change in the lipid composition of its food source.

The objectives of this study are:

- Determine the change in the C:N:P ratio and macromolecular composition of diatoms in terms of fatty acids variations −with emphasis on unsaturated fatty acids− under a gradient of pCO_2 and nutrient repleted conditions.

- Determine if the variations in the diatoms macromolecular composition affect copepods development in terms of growth rate, egg production and fatty acids composition, as consequence of its use as food source.

3. Materials and methods

3.1 Diatoms cultured in a pCO_2 gradient

The centric diatom *Thalassiosira pseudonana,* a well studied species was used in the experimental cultures.

A set of one laboratory batch culture with a constant alkalinity of 2350 μmol kg^{-1}, typical for open ocean surface conditions (Sarmiento & Gruber, 2006) was carried under nutrient replete conditions and seven different dissolved inorganic carbon (DIC) concentrations, (Appendix, Figure 2).

The range of DIC set for the nutrient-replete cultures under the above mentioned alkalinity was: 1550, 1702, 1854, 2007, 2159, 2311 and 2463 μmol kg^{-1}.

3.1.1 Culture conditions

The Pre-cultures and experimental cultures were kept in an incubation chamber with an incident photon flux density of 150 μmol photons $m^{-2}s^{-1}$, on a 16:8 light:dark cycle, and a temperature of ~15 ^{o}C.

Artificial seawater (ASW) was the culture media, prepared following the protocol by Kester et al. (1967) with a salinity of 35 ‰. The advantage of using ASW is the possibility of a more easy carbonate system manipulation and the exclusion of possible contaminants presented in seawater that could alter the physiological condition of the cultures. Nitrate, silicate, and phosphate were be added to concentrations of ca. 64, 64, and 4 μmol kg^{-1} respectively, to avoid nutrient limitation. Trace metals and vitamins were added at concentrations of f/2 medium, following the protocol of Guillard & Ryther (1962). Also 10 ml per liter of natural seawater was added to avoid micro-nutrient limitation.

3.1.2 DIC manipulation

To adjust the above mentioned CO_2 range in the culture media, varying amounts of DIC were added in form of sodium carbonate (Na_2CO_3) solution. After the addition of sodium carbonate, a precise amount of 3,571 molar HCl was added to regulate the alkalinity and keep it constant (2350 µmol kg^{-1}). This is simulating the ongoing accumulation of DIC at constant alkalinity.

3.1.3 Cultures

An aliquot of the culture was inoculated in a 600 ml polycarbonate flask with artificial seawater to acclimate the cells to this media for ~10 generations. Here, the DIC and alkalinity was set at ~2100 and ~2350 µmol kg^{-1} respectively, both representative values of today's ocean. Afterward, an aliquot of the culture was inoculated in 7 different bottles with the above mentioned CO_2 gradient, to acclimate the cells to different carbonate chemistry for about ~10 generations. Finally the experiment began with the transfer of culture aliquots to 2,4 liter polycarbonate bottles filled with culture media and the respective carbonate chemistry (Appendix, Fig. 1).

The incubations were performed in dilute batch cultures, with a low initial cell inoculation. The cultures were harvested after 7 or 8 generations, to not exceed a cell concentration of 50.000 cells ml^{-1} to avoid an excessive perturbation of the carbonate system, also the exponential growth phase has been reported as the period with the highest nutritional value in terms of fatty acids (Jónasdóttir, 1994).

The growth rate was determined by cell counts using a Coulter Counter (Z2 Coulter® Particle Count and Size Analyzer, Beckman Coulter™). The specific growth rate (µ) was calculated as $\mu=(\ln C_1 - \ln C_0)/(t_1 - t_0)$, where C_0 and C_1 represent the cell concentrations at t_0 (initial time) and t_1(final time, during sampling) respectively. (The objective is to avoid a significant alteration of the carbonate chemistry and growth conditions by the growing cells.

3.1.4 DIC and Alkalinity measurements

To minimize the exchange of CO_2 in the samples with the atmosphere, DIC samples were take first, filtering 10 ml of water through a disposable sterile filter (Whatman® 0,2 μm Puradisc™ 25 AS filter) with a syringe, into 4ml vials sealed air-tight with teflon coated septa, and finally sealed with parafilm® and kept at 4°C until analysis. The DIC concentration was measured according to the photometric method of Stoll et al. (2001).

500 ml of water were filtered through precombusted (450 °C, 6 h) glass-fiber filters (GF/F, nominal pore size of 0,7 μm) and kept at 4 °C until analysis. The samples were not poisoned with mercury-chloride ($HgCl_2$) as recommended because they were measured shortly after recollection. A potentiometric titration was performed following the method of Dickson et al. (2003).

3.1.5 Dissolved inorganic nutrient determination

At the end of the experiments, 100 ml of water was taken in plastic bottles and kept at -20°C until analysis. A photometrical determination of NO_3^- plus NO_2^-, PO_4^{3-} and SiO_4^{4-} was achieved by the method of Hansen & Korolef (1999) to ensure that the cultures did not reach nutrient limited condition.

3.1.6 Particulate matter

A certain volume of of culture was filtered through precombusted (6 hours at 450°) glass-fiber filters (GF/F, nominal pore size of 0,7 μm) to determine particulate carbon, nitrogen (400 ml), silicate and phosphate (100 ml each), and kept at -20°C. The analysis of POC, PON was performed by the Ehrhardt & Koeve (1999) method, POP by Hansen & Korolef (1999), and BSi by Strickland & Parsons (1972).

3.1.7 Lipid analysis

The fatty acids were measured as fatty acid methyl esters (FAMEs). 400 or 200 ml of water, depending of diatom cell abundance, were filtered on precombusted GF/F filters 0.7 µm nominal pore size and stored at -80°C until analysis. Lipids were extracted over night from the filters using 3ml of a solvent mixture (dichloromethane:methanol:chloroform in 1:1:1 volume ratios). As internal standard, a five component FAME Mix (company Restek, Bad Homburg, Germany; c= 18.09 ng component^{-1}µl^{-1}) was added, and a C23 FA standard (c= 25.1 ng µl-1) was used as an esterification efficiency control (usually 80-85%). Water-soluble fractions were removed by washing with 2.25 ml of KCl solution (c= 1 mol L^{-1}), and the remainder dried by addition of $NaSO_4$. The solvent was evaporated to dryness in a rotary film evaporator (100-150mbar), redissolved in Chloroform and transferred into a glass cocoon. Again, the solvent was evaporated (10-30mbar), and esterification was performed over night using 200µl 1% H_2SO_4 (in CH3OH) and 100µl toluene at 50°C. Phases were split using 300µl 5% sodium chloride solution, and FAMEs were separated using n-Hexane, transferred into a new cocoon, evaporated, and 100µl (final volume) added. All solvents used were gas chromatography (GC) grade. FAMEs were analyzed by a Thermo GC Ultra gas chromatograph equipped with a nonpolar column (RXI1-SIL-MS 0.32µm, 30m, company Restek) using a FID. The column oven was initially set to 100°C, and heated to 220°C at 2 °C min^{-1}. The carrier gas was helium at a constant flow of 2ml min^{-1}. The flame ionization detector was set to 280°C, with a gas flow of 350, 35 and 30 ml min^{-1} of synthetic air, hydrogen and helium, respectively. Injected were 1-µl aliquots of the samples. The system was calibrated with a 37-component FAME-mix (company Supelco, Germany) and chromatograms were analyzed using Chrom-Card Trace-Focus GC software (Klein Breteler et al. 1999).

3.2 Diatom feeding of copepods at two pCO2 levels

3.2.1 Diatoms for copepod feeding

Once again the diatom used for this experiment was *Thalassiosira pseudonana*, because it is a well studied specie, its size (~5 µm) is suitable to be consumed by the copepods, and have been used as prey in previous copepod feeding experiments (Jones and Flynn, 2005).

3.2.1.1 Cultures

A set of one laboratory batch cultures with a constant alkalinity of 2014 µmol kg^{-1}, to

reproduce the same in the copepods growth media, was carried in nutrient replete conditions and two different Dissolved Inorganic Carbon (DIC) concentrations (Appendix, Figure3).

The pre-cultures and cultures were kept in a culture room with an incident photon flux of 150 µmol photons $m^{-2}s^{-1}$, on a 18:6 light:dark cycle, and a temperature of 18 ^{o}C.

Artificial seawater (ASW) was the culture media, prepared following the protocol by Kester et al. (1967) with a salinity of 18 ‰ to simulate the same of the copepod´s growth media. Nutrients, vitamins and micro-nutrients were added as mentioned in section 3.1.1.

A portion of the culture was inoculated in a 600 ml polycarbonate flask with artificial seawater to acclimate the cells to this media and salinity for ~20 generations. Here, the DIC and alkalinity was set at ~1950 and ~2014 µmol kg^{-1} respectively, as was in the copepods culture media. Afterward, a portion of the culture was inoculated in 2 different bottles with the above two mentioned DIC values to acclimate the cells to different carbonate chemistry for about ~10 generations. Finally the experiment began with the transference of the culture to 25 liter polycarbonate bottles filled with culture media and the respective carbonate chemistry (Appendix, Figure 3). The incubation and cell number estimation was made as mention in section 3.1.3.

Finally the cells were concentrated via gravity filtration (to avoid cell damage) through a 2,5 cm-diameter, 3 µm pore size Policarbonate filters, until reaching the wanted amount of cells per milliliter in terms of carbon wanted for copepods feeding (~600 µg per liter of copepod media).

The nutrients, particulate matter and lipid analysis were perform in the same way as in mention in section 3.1.5 and 3.1.6.

3.2.1.2 DIC manipulation

The range of DIC set for the cultures was: 1908.7±20 in the low DIC bottles and 1988.4±40 µmol kg^{-1} in the high DIC bottles.

To adjust the above mentioned DIC range in the culture media, varying amounts of DIC were add in form of sodium carbonate (Na_2CO_3) solution. After the addition of sodium carbonate, a precise amount of 3,571 molar HCl is added to regulate the alkalinity and keep it constant (2014 µmol kg^{-1}).

3.2.2 *Acartia tonsa* culture

The species selected to perform the experiment was *Acartia tonsa*, a fast growing (~2 weeks at 18 °C) and easy maintenance calanoid copepod, that has been used in several experiments (Jones and Flynn, 2005; Jónasdóttir, 1994; Jónasdóttir and Kiørboe 1996, Kleepel et al. 1998); and most important, the females, unlike other species, release its eggs in the media, allowing an easier concentration and counting.

To determine if only the pCO_2, only the food or a combination of both could have influence on copepod development, a set of four treatments, each in triplicates, was implemented (Appendix, figure 2):

Low/Low: Copepods grown at low pCO_2 feed with diatoms cultured at low pCO_2 (380/380 pCO_2).
Low/High: Copepods grown at low pCO_2 feed with diatoms cultured at high pCO_2 (380/740 pCO_2).
High/Low: Copepods grown at high pCO_2 feed with diatoms cultured at low pCO_2 (740/380 pCO_2).
High/High: Copepods grown at high pCO_2 feed with diatoms cultured at high pCO_2 (740/740 pCO_2).

(Color code: Red is for the pCO_2 in the copepods media and Green for the diatoms pCO_2 media).

3.2.2.1 Cultures conditions and DIC manipulation

The cultures were kept in a culture room with an incident photon flux of 150 µmol photons $m^{-2}s^{-1}$, on a 14:10 light:dark cycle, and a temperature of 18 °C.

Natural seawater (NSW) collected in the Kiel fjord, with a salinity of 18 ‰ and filtered by a 0,2 μm pore size filter was the culture media.

The required pCO_2 was achieved by bubbling of the water during 5 days prior the beginning of the experiment with normal air to reach the actual environmental pCO_2 in the case of the 380 pCO_2, and air enriched with CO_2 in the case of the 740 pCO_2.

3.2.2.2 *Rhodomonas* culture

Right after hatching, the first copepodite stage was feed with *Rhodomonas sp*. The feeding with *Rhodomonas* is intended to avoid a lack of essential nutrients that have been attributed to diatoms and affect the first development stages and produce early mortality (Jones and Flynn, 2005).

Two high cell concentration *Rhodomonas* cultures were grown using f/2 medium, following the protocol of Guillard & Ryther (1962), these cultures were in continuous bubbling with the same air and pCO_2 used for the copepods media.

3.2.2.3 Cultures

A set of *A. tonsa* eggs from the island of Helgoland was kept in a 200 liter NSW tank to hatch and until the individuals reached copepodite stage I or II. After this point the copepodites were transfer to 2 liters polycarbonate bottles in an initial concentration of 1000 individuals per liter according to the recommendations of Medina and Barata (2004), expecting that only ~1/4 of the total initial amount will reach adult stage.

The copepodites were feed with *Rhodomonas sp.* every second day until reaching stage 6 (2 times, 6 days after hatching). After this point the copepods were feed with *T. pseudonana* until reaching sexual mature stage and the beginning of egg production. In every feeding a calculated minimum of 600 μg of carbon per liter of copepod media, calculated from the carbon cell content in the diatoms was given to the copepods.

3.2.2.4 Copepods growth, egg production and fatty acid content

Along with the replacement of the media, an small sample of copepods was taken for the determination of its stage of development by microscopy counts.

Two days after the copepods had reached the adult stage, the egg production experiment was prepared (Appendix, Figure 3). From each experimental unit (2 l bottles) a set of four 500 ml bottles containing 5 female copepods and a small amount of algae (to avoid starvation) was incubated during 24 hours at the same conditions as the culture. Each 500 ml bottle was equipped with a 100 μm net to separate eggs and females to avoid predation on eggs. After this period the females were removed and the eggs concentrated and counted.

As the female copepods are the egg spawner and therefore its lipid content and composition have a direct influence on the amount of the spawned eggs, a total of 45 individuals from each experimental unit, including the females used for the egg production experiment, were collected and analyzed to determine its lipid content and composition. The method was the same used for the lipid measurement in diatoms, described in section 3.1.7.

4. Results

4.1 Alkalinity, DIC and pH

The measured alkalinity in the *T. pseudonana* batch culture under a pCO_2 gradient at the end of the experiments was 2386,8 ± 60,5 μmol kg^{-1}. In the *T. pseudonana* for copepod feeding experiment the alkalinity was 2041,6 ± 48,99 μmol kg^{-1}. The measured DIC, calculated pCO_2 and pH is summarized in the Appendix, Table 1.

The DIC and pCO_2 in the copepod feeding experiment was not directly measured in the samples but calculated from alkalinity measurements of each sample using the software CO_2sys (Appendix, Table 1). The average calculated DIC show significant differences (t-test, t= -3,42; $p < 0,05$) between the two treatment levels.

4.2 Nutrients

The nutrients measured after the experiments are summarized in the Appendix, Table 2. Any of the samples was below the detection level of the methods used for its determination.

4.3 Growth rate of *T. pseudonana*

The analysis of the calculated specific growth rate (μ) in *T. pseudonana* culture under a pCO_2 gradient show a statistically significant Log Normal distribution (r = 0,909; Chi square= 16,69; $p<0,05$; Figure 4, left panel). When analyzed the triplicate cultures inside the gradient at 422 ± 36 and 536 ± 17 μatm pCO_2, the second group show a minor growth rate than the firsts, however this difference is not significant (t test, t= 2,75; p= 0,0509; Figure 4, right panel).

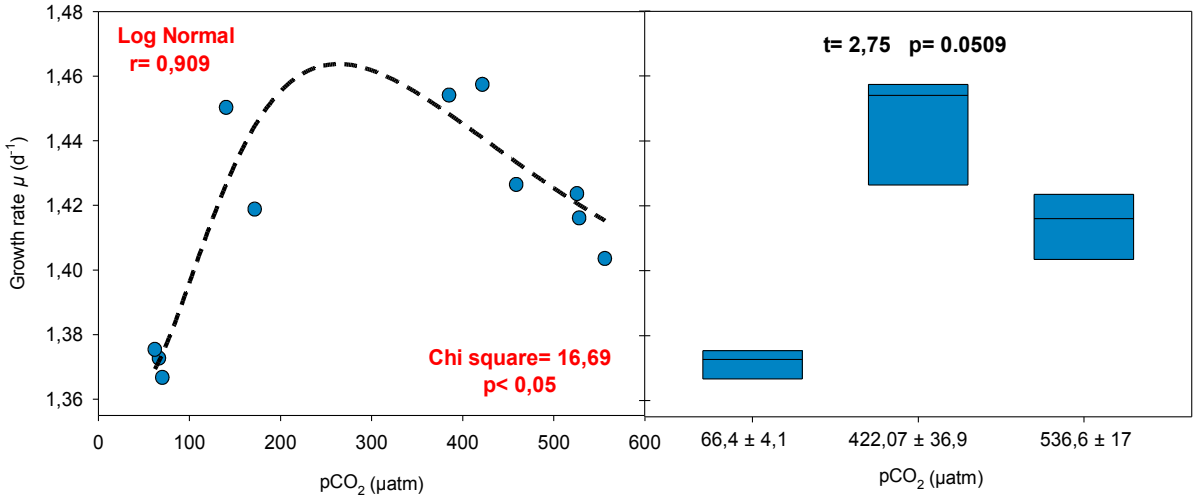


Figure 4.- Growth rate vs. pCO_2 of *T. pseudonana*. Left panel: growth rate in the gradient; right panel: growth rate of the triplicates inside the gradient.

However the analysis of the growth rate in the replicated cultures of *T. pseudonana* used for copepod feeding show show the same trend, altough also not significant (t-test, t= 1,70; p > 0,05; Figure 5) relation between the growth rate and the DIC concentration in the media.

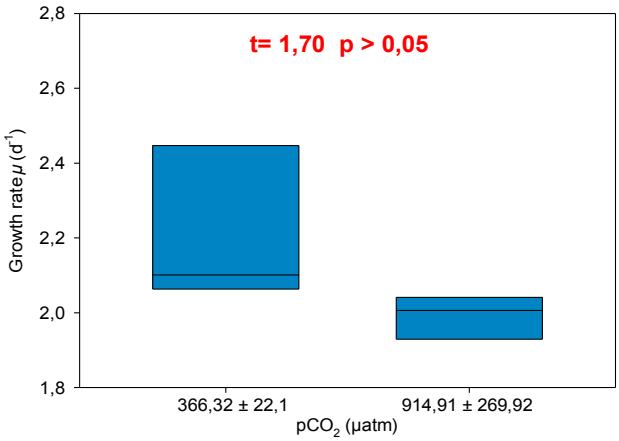


Figure 5.- Growth rates of *T. pseudonana* under two different DIC concentrations.

4.4 Nutrients ratio

4.4.1 Cellular quota

The nutrient cellular quota and its relation with the final cell number was revised in order to determine the possible formation of precipitates that could have been measured as particulate matter. In Table 3 the correlation analysis for *T. pseudonana* is show, the phosphate have formed precipitates in the culture and appears with a significant correlation with the cell number. Therefore was not consider in the analysis of nutrient ratios.

Table 3.- Correlation between nutrient content per cell (picomol) and final cell number. At the top are the r values and under them the confidence p values (n= 11).

Final Cell N°	Si	N	C	P
T. pseudonana	-0,2300 p= 0,472	-0,2118 p= 0,509	-0,0238 p= 0,942	-0,9165 p= 0,000

The correlation between the cellular quota and pCO_2 show that the nitrogen content per cell increase with higher pCO_2 levels. The results for each nutrient are summarized in Table 4.

Table 4.- Correlation between nutrient content per cell (picomol) and the gradient of calculated pCO_2 (µatm) in each culture bottle (n= 11)

DIC	Si	N	C
T. pseudonana	-0,1165 p=0,733	0,7026 P= 0,016	0,1350 p= 0,692

4.4.2 C:N:Si Ratios

The carbon-to-nitrogen ratio vs. in response to pCO_2 showed a negative and significant trend in *T. pseudonana* (Pearson r = -0,73; p< 0,05; Figure 6, top panel). The carbon-to-silicate ratio is positive but not significant(Pearson r = 0,33; p> 0,05; Figure 6, middle panel). Finally, the silicate-

to-nitrogen is negative and significant (Pearson r = -0,70; p< 0,05; Figure 6, bottom panel).

The carbon-to-nitrogen ratio in *T. pseudonana* used for copepod feeding show no significant difference between the two pCO_2 treatments (t-test, t= 0,87, p > 0,05), however the average values are lower than the lowest C:N ratio of the samples from the DIC gradient, being 5,45±0,31 for the low DIC and 5,690±0,36 for the high DIC treatment.

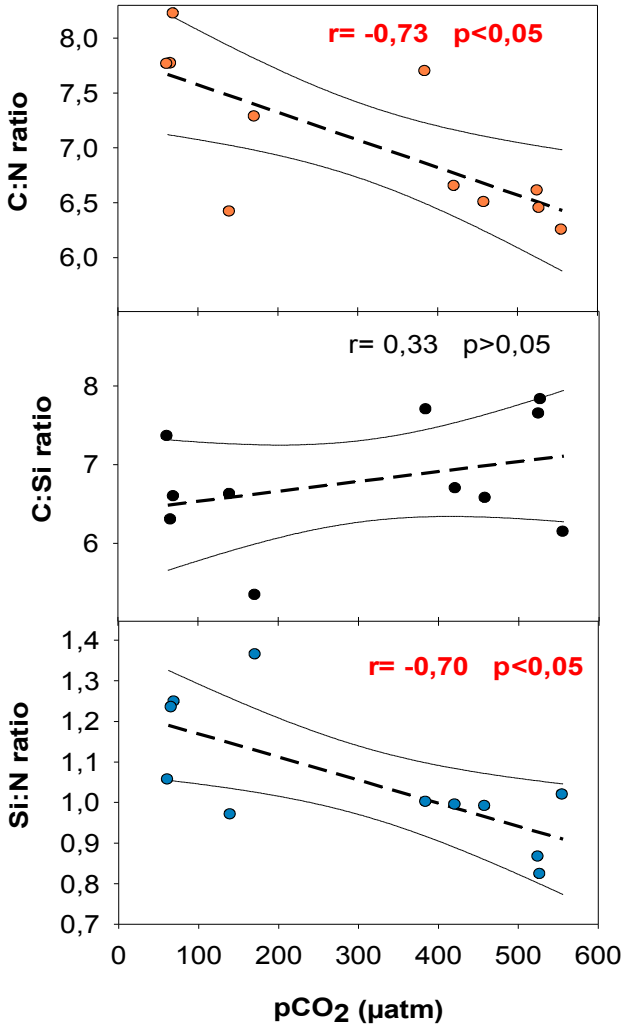


Figure 6.- The carbon-to-nitrogen (top), carbon-to-silicate (middle) and silicate-to-nitrogen (bottom) ratios in response to pCO_2 of *Thalassiosira pseudonana*. All the correlations (Pearson) and its significance are show in each plot, being significant the carbon-to-nitrogen ratio (top) and silicate-to-nitrogen (bottom) ratios.

4.5 Fatty acid composition of T. pseudonana in response to pCO_2

The total ammount of saturated and unsaturated fatty acids per cell of *T. pseudonana* is presented in Figure 7. The data show a positive but no significant correlation between the total fatty acid content and pCO_2 (Pearson, r= 0,894; p > 0,05). The totals unsaturated fatty acids show no relation with the pCO_2 content (Pearson, r= -0,093; p > 0,05), however the total saturated fatty acid content show a high positive and significant correlation (Pearson, r= 0,991; p< 0,05).

Is also interesting that the amount of saturated and unsaturated fatty shift with the pCO_2, whit the first becoming more abundant at increasing pCO_2 concentrations (Figure7).

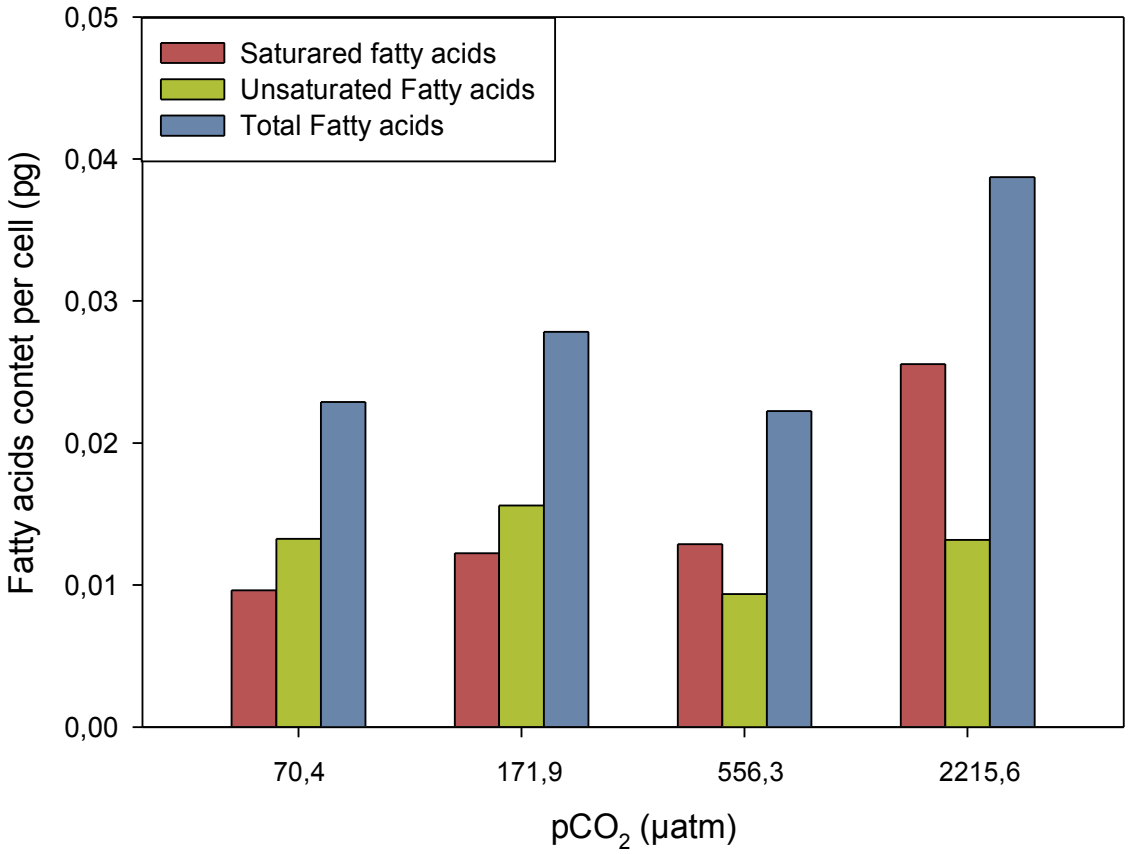


Figure 7.- The saturated, unsaturated and total fatty acid content of *T. pseudonana* growth under different pCO_2 concentrations.

4.5.1 Specific saturated and unsaturated fatty acids.

A total of twelve fatty acids where detected and quantified; four saturated and eight unsaturated fatty acids.

The myristic (14:0), palmitic (16:0) and stearic acid (18:0) were present in all the samples, being more abundant at high DIC concentrations (Table 5).

From the total unsaturated fatty acids, four were present in all the samples: palmitoleic (16:1), g-linolenic (18:3*n*-6), the tandem (measured together due to co-elution) arachidonic (20:4*n*-6) - eicosapentaenoic or EPA (20:5*n*-3) and docosahexaenoic or DHA (22:6*n*-3) (Table 5).

Table 5.- Saturated and unsaturated fatty acids detected and measured in the diatom *T. pseudonana* cultured under a gradient of pCO_2 (µatm) and constant alkalinity. The concentration is expressed as femtograms of fatty acid per cell (fg $cell^{-1}$) . Note that some saturated fatty acids are more abundant at higher pCO_2 concentrations.

	pCO_2			
	70,40	171,90	556,30	2215,60
Saturated fatty acids				
14:0	3,56	3,51	4,76	8,07
16:0	4,97	6,48	6,67	13,23
18:0	1,09	2,23	0,93	3,11
22:0	--	--	0,53	1,15
Unsaturated fatty acids				
16:1	5,19	5,65	4,27	5,92
18:3*n*-6	2,48	2,84	1,62	2,45
18:1*n*-9t	0,47	--	0,27	1,11
18:3*n*-3, 18:2*n*-6	0,28	0,46	--	--
18:1*n*-9c, 18:2*n*	0,09	1,24	--	--
20:4*n*-6, 20:5*n*-3	3,96	4,57	2,78	3,69
20:3*n*-6	0,05	--	--	--
22:6*n*-3	0,74	0,84	0,42	--
TOTAL	22,878	27,836	22,238	38,734

4.5.2 pCO_2 and relative content of saturated and unsaturated fatty acids

Is important to note that the relative amount of fatty acid in relation to the others in each sample present a decreasing content towards higher pCO_2 in the case of unsaturated fatty acids (Figure 8, left panel); while the saturated fatty acids show the opposite trend, with a higher proportion toward higher pCO_2 (Figure 8, right panel).

A significant positive correlation between pCO_2 and the saturated myristic (14:0) (Pearson r= 0,996; p< 0,05) and palmitic (16:0) (Pearson r= 0,988; p< 0,05) fatty acids was observed.

From the unsaturated fatty acids only the DHA (22:6*n*-3) show a significant negative correlation with the pCO_2 (Pearson r= -0,952; p< 0,05).

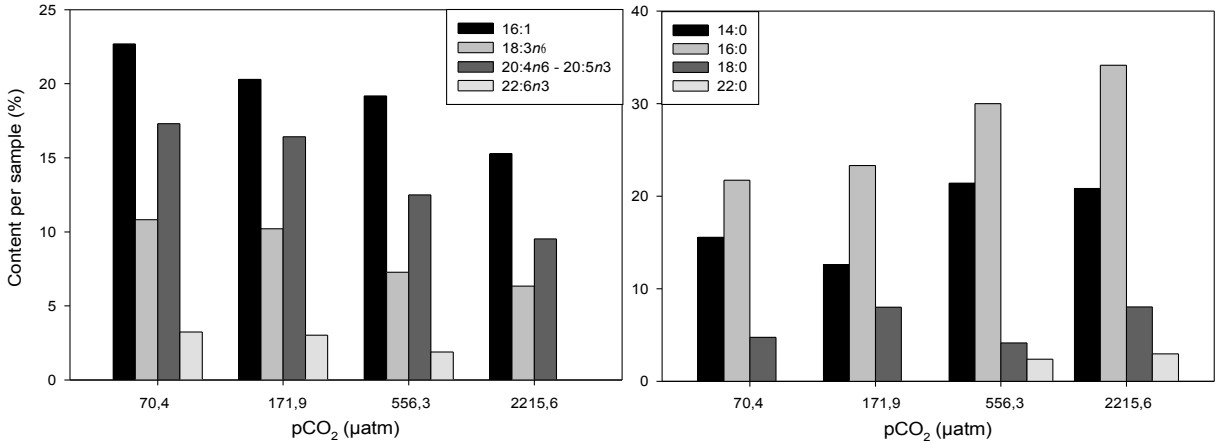


Figure 8.- Relative content of unsaturated (left) and saturated (right) fatty acids in each sample. Note that Y axis are in a different scale.

4.6 Fatty acids composition in *T. pseudonana* used for *A. tonsa* feeding

One of the cultures intended to be growth at a high pCO_2 concentration, after analysis, appeared with a lower pCO_2 than the one expected (740 Day 2, Table 1); this may be a consequence of an error in the set up of the carbonate system. After a very, very careful analysis of the pCO_2

calculations and fatty acid data, was conclude that the sample presented all the characteristics of the low pCO_2 samples, thus, it was analyzed as a part of this group, since one of the objetives of this study was to determine if with a given pCO_2 the fatty acids in T. Pseudonana change, and as is show in the following lines that efectively hapen. However the implications of feeding the high pCO_2 copepods (Treatments High/Low and High/High) with this diatoms, as can be consider a unexpected factor affecting the results of the experiment, is discussed in section 5.4.

The total concentration of fatty acids in the diatoms cultured at lower pCO_2 concentrations is higher that the one observed for the diatoms under high pCO_2 (Figure 9, left panel) , but not significant (t-test, t= 1,84; p > 0,05). However the total content of unsaturated fatty acids in the low pCO_2 diatoms is significantly higher that the total saturated fatty acids in the same sample (log data, t-test, t= -3,62; p< 0,05; Figure 9, right panel), and than the total unsaturated fatty acids in the diatoms under high DIC (t-test, t= 2,69; p< 0,05; Figure 9, right panel).

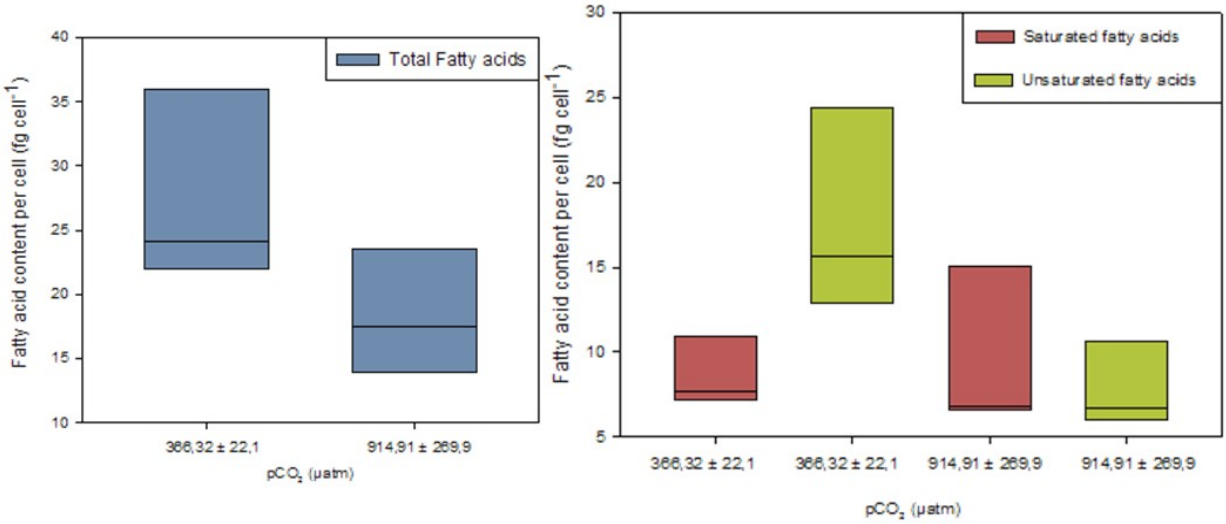


Figure 9.- Total (left panel), saturated and unsaturated (right panel) fatty acids in *T. pseudonana* growth at two different pCO_2.

4.6.1 Specific saturated and unsaturated fatty acids

A total of seventeen fatty acids were detected and measured, five saturated and twelve unsaturated (Table 6).

The lauric (12:0), myristic (14:0), palmitic (16:0) and stearic (18:0) acids were present in all the samples at both pCO_2 levels. In reference to the unsaturated fatty acids, the palmitoleic (16:1), g-linolenic (18:3*n*-6), linolenic, α-linolenic (18:2n-6 and 18:3n-3 respectively, measured in tandem due to co-elution), oleic (18:1*n*-9c), eladic (18:1*n*-9t), arachidonic-EPA (20:4*n*-6, 20:5*n*-3 respectively, measured in tandem due to co-elution), dihomo-g-linolenic (20:3*n*-6), DHA (22:6*n*-3), and nervonic acid (24:1*n*-9) were present in all the samples, being more abundant at low pCO_2.

The most representative of the unsaturated fatty acids, DHA (22:6*n*-3), show a significant difference in its concentration between the low and high pCO_2 cultures (t-test, t= 2,5; p< 0,05; Figure 10, panel F) as well as the linolenic acid (18:3*n*-6) (t-test, t= 2,60; p<0,05; Figure 10, panel E), the group arachidonic-EPA (20:4*n*-6, 20:5*n*-3) although showing a big diference, this is not significant (t-test, t= 2,01; p > 0,05) (Figure 10, panel D).

The most representative saturated fatty acids did not show any significant diference between low and high pCO_2 treatments, those are: palmitic (16:0) (t= -0,42; p> 0,05;), myristic (14:0) (t= -0,36; p> 0,05;) and stearic (18:0) (t= -0,68; p> 0,05) (Figure 10, panel A, B and C respectively).

Table 6.- Specific saturated and unsaturated fatty acids per cell (fg cell^{-1}) in *T. pseudonana* used to feed the copepod *A. tonsa* cultured at two different pCO_2 (µatm) levels. The values are averages of five different measurements at low pCO_2 and three measurements at high pCO_2.

	pCO_2	
	366,3 ± 22	**914,9 ± 269**
Saturated fatty acids		
12:0	0,015 ± 0,030	0,015 ± 0,018
14:0	2,976 ± 0,928	3,217 ± 0,314
16:0	4,878 ± 2,043	4,987 ± 3,778
18:0	0,897 ± 0,362	1,235 ± 1,053
24:0	--	0,046 ± 0,079
Unsaturated fatty acids		
14:1	0,003 ± 0,01	0,007 ± 0,012
15:1	0,002 ± 0,01	--
16:1	5,534 ± 1,49	3,261 ± 1,273
18:3*n*-6	3,053 ± 0,97	1,337 ± 0,729
18:3*n*-3, 18:2*n*-6	1,856 ± 0,56	1,001 ± 0,646
18:1*n*-9c, 18:2*n*	0,415 ± 0,41	0,105 ± 0,181
18:1*n*-9t	0,095 ± 0,14	0,074 ± 0,129
20:4*n*-6, 20:5*n*-3	5,806 ± 3,49	1,564 ± 0,677
20:3*n*-6	0,054 ± 0,08	0,029 ± 0,050
22:6*n*-3	0,956 ± 0,33	0,328 ± 0,362
22:1*n*-9	0,196 ± 0,22	--
24:1*n*-9	0,057 ± 0,07	0,121 ± 0,122

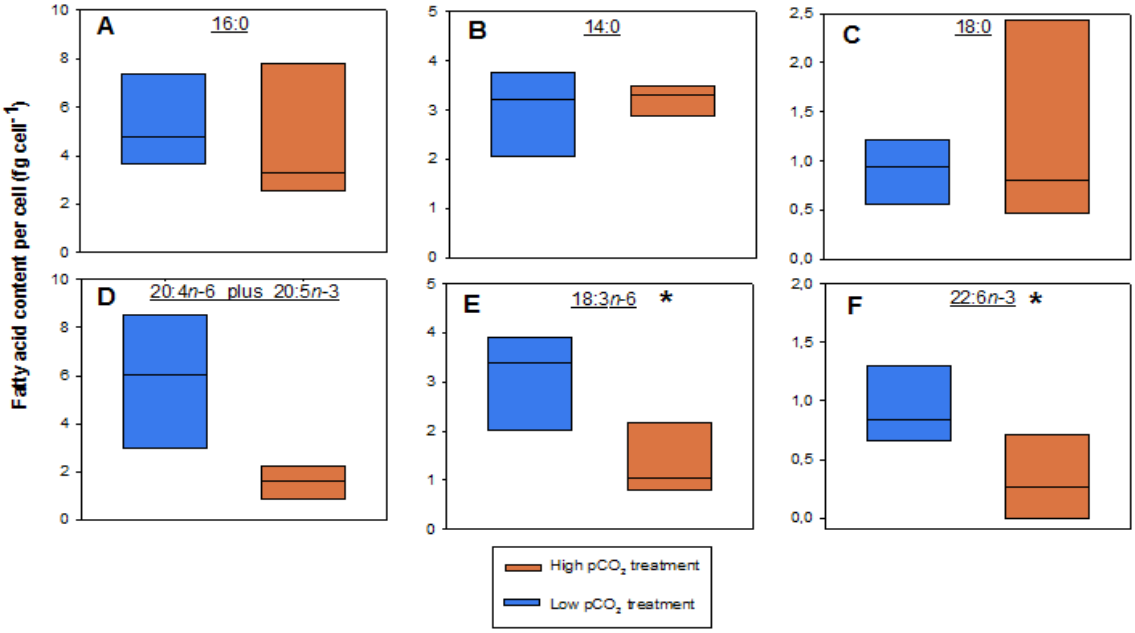


Figure 10.- Average and standard deviation of some saturated (A, B, C) and unsaturated (D, E, F) fatty acids in the diatom *T. pseudonana* culture under two different pCO_2 (µatm) levels. The fatty acids that show an significant difference are mark with an * (see text for details).

4.7 *A. tonsa* growth (data obtained by Dennis Rossoll)

The relative copepod stage development showed an apparent difference between the treatments (Figure 10), with the treatments Low/Low (Low pCO_2 copepods - Low pCO_2 diatoms) and Low/High B (Low pCO_2 copepods - High pCO_2 diatoms) showing advanced copepods development stages than treatments High/Low (High pCO_2 copepods - Low pCO_2 diatoms) and High/High (Low pCO_2 copepods - Low pCO_2 diatoms) in all the analyzed days during the culture period.

On Day 5 about 80% of the individuals had reached copepodite 3 stage in treatment Low/Low and Low/High, while in High/Low and High/High only a 50% had reached this stage (Figure 11, top panel). On Day 9, 80% of the individuals in treatment Low/Low and Low/High had reached the copepodite 6 stage, and in treatments High/Low and High/High only about a 25% (Figure 10, middle panel). However on Day 12 the amount of individuals in the copepodite stage 6 is similar in all treatments (Figure 10, bottom panel). (This change in the copepodites development is analyzed in section 5.4).

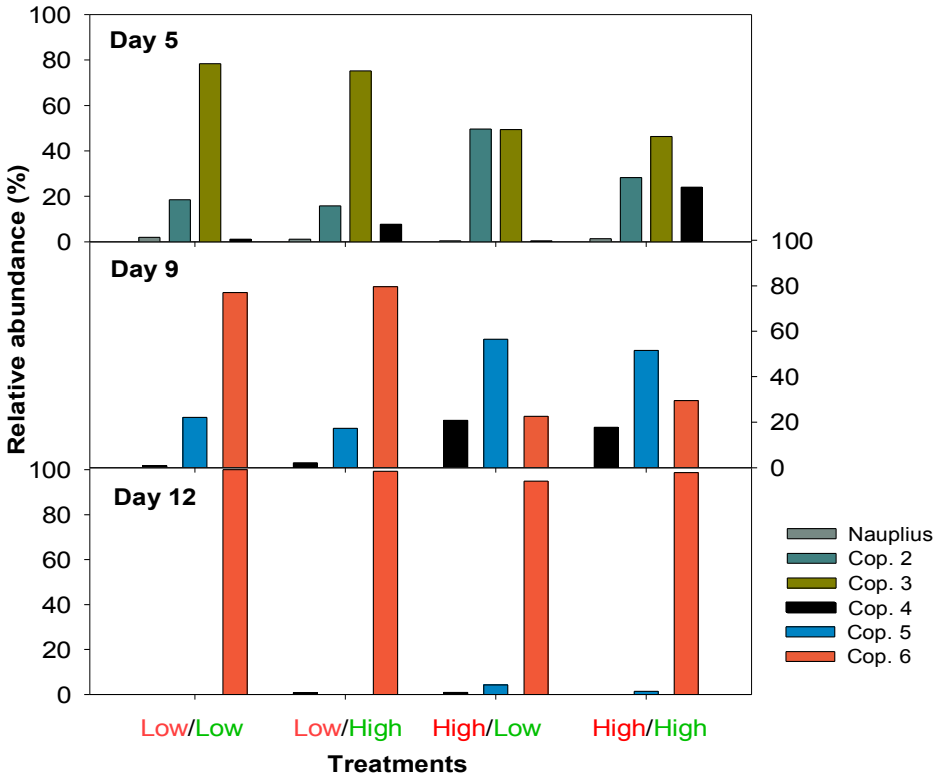


Figure 11.- Relative copepodite stage development analysed at three different days.

4.8 *A. tonsa* egg production (data obtained by Dennis Rossoll)

The *A. tonsa* egg production experiment, realized with 12 replicates from each treatment level, four per experimental bottle (Appendix, Figure 3), was intended to determine the reproductive success of the copepods, measured as the number of eggs produced per female in a period of 24 hours (eggs female^{-1} day^{-1}).

The result showed a decreasing trend in the production of eggs (Figure 12), with a significant (Mann Whitney, U= 0,00; Z= 4,156; $p < 0,001$) difference in the amount of eggs produced between the two extreme treatments Low/Low (28,8±14,6 eggs/female) and High/High (4,9±1,3 eggs/female). In treatment Low/High (13,3±6,9 eggs/female) the amount of eggs produced was slightly superior than in treatment High/Low (8,4±4,16 egg/female); the possible causes of this apparently counter-intuitive result as a mayor egg production in treatment Low/High is analyzed in section 5.4.

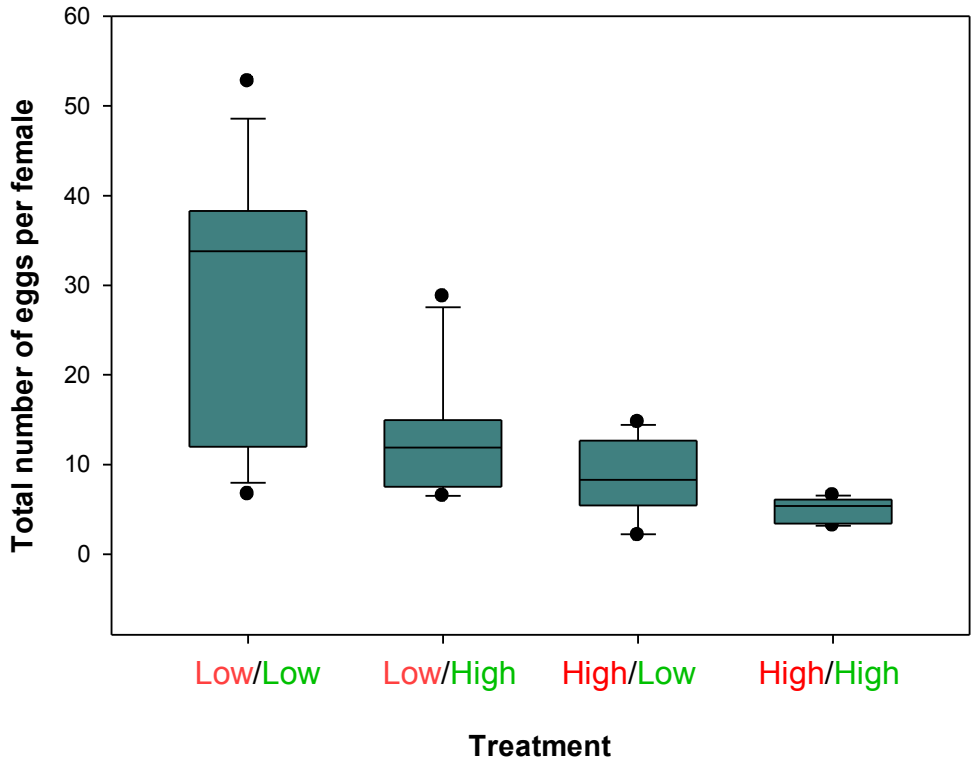


Figure 12.- Number of eggs produced per female of *A. tonsa* in a period of 24 hours, grown under two different pCO_2 levels (~350 and ~740 µatm) and feed with the diatom *T. pseudonana* growth under similar pCO_2 conditions as the copepods.

4.9 *A. tonsa* fatty acid composition

The analysis of the fatty acids composition of female *A. tonsa* copepods realized after the beginning of the egg production show a marked decrease in the lipid content per female (Figure 13, left panel) when cultured at high pCO_2. The difference is significant (t-test with Log data, t= 5,37; p < 0,01) between the extreme treatments: Low/Low (8,9±5,7 ngFA/female) and High/High (0,85±0,24 ngFA/female).

Between the treatments Low/High (3,9±0,95 ngFA/female) and High/Low (0,79±0,17 ngFA/female) there is a significant difference (t-test with Log data, t= 7,85; p < 0,01). Finally there is no significant difference between treatment Low/Low and Low/High (t= 1,76; p > 0,05) and also between High/Low and High/High (t= 0,27; p > 0,05).

The concentrations of saturated and unsaturated fatty acids per female copepod also show variation between treatments (Figure 13, right panel). The unsaturated fatty acids were more abundant in the treatments Low/Low and Low/High, however the difference is not significant in Low/Low (t= -0,72; p > 0,05) and Low/High (t= -0,2; p > 0,05). On the other hand treatments High/Low and High/High showed the opposite, with more saturated than unsaturated fatty acids, being this difference in content significant in both, High/Low(t= 6,99; p < 0,05) and High/High (t= 4,11; p < 0,05).

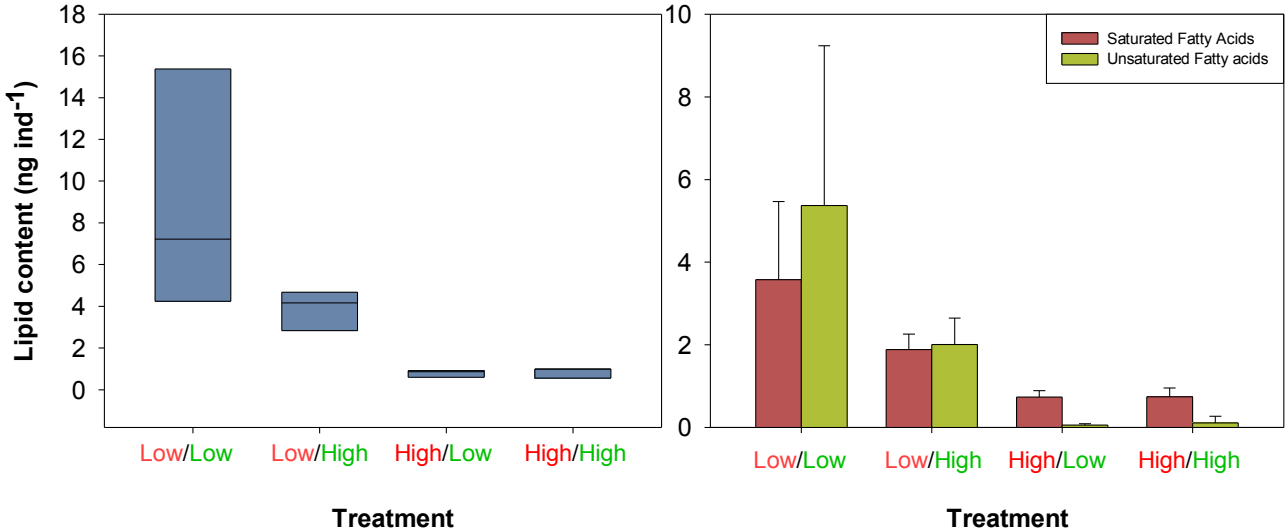


Figure 13.- Left: content of total fatty acids per female of *A. tonsa*. Right: Average content and standard deviation of saturated and unsaturated fatty acids content per female copepod.

4.9.1 Specific saturated and unsaturated fatty acids.

A total of 18 fatty acids where detected in the female copepods, from those, seven where saturated and eleven unsaturated fatty acids (Table 7). However, not all the fatty acids where present in all samples. All the seven measured saturated fatty acids where present in treatment High/High, while in treatment Low/Low only five were detected, finally in Low/High and High/High treatments only four where detected. The myristic (14:0), palmitic (16:0), stearic (18:0) and lignoceric acids (24:0), were present in all samples, being far more abundant in treatments Low/Low and Low/High. On the other hand, from the unsaturated fatty acids, ten out of eleven where present in treatment Low/Low, eight in treatment Low/High, five in treatment High/Low, and seven in treatment High/High; here the linolenic, α-linolenic (18:2*n*-6 and 18:3*n*-3 respectively) gondoic (22:1*n*-9) and nervonic acid (24:1*n*-9) are present in all the samples, being more abundant in treatment Low/Low and Low/High. (Table 7).

Table 7.- Average concentration (ng ind.$^{-1}$) and standard deviation of saturated and unsaturated fatty acids per copepod female measured in each treatment. Note that treatment High/High show more saturated fatty acids and treatment Low/Low show more unsaturated fatty acids.

	Low/Low	Low/High	High/Low	High/High
Saturated fatty acids				
12:0	--	--	--	0,003 ± 0,005
14:0	1,122 ± 0,819	0,400 ± 0,077	0,090 ± 0,024	0,072 ± 0,009
16:0	1,910 ± 0,983	1,028 ± 0,211	0,440 ± 0,100	0,415 ± 0,119
18:0	0,376 ± 0,089	0,282 ± 0,063	0,174 ± 0,028	0,208 ± 0,089
20:0	0,014 ± 0,024	--	--	0,003 ± 0,005
22:0	--	--	--	0,003 ± 0,006
24:0	0,150 ± 0,035	0,175 ± 0,029	0,025 ± 0,033	0,037 ± 0,045
Unsaturated fatty acids				
16:1	1,847 ± 1,667	0,407 ± 0,153	--	0,005 ± 0,008
17:1	0,005 ± 0,008	--	--	0,001 ± 0,003
18:3*n*-6	0,433 ± 0,469	0,074 ± 0,033	--	--
18:3*n*-3, 18:2*n*-6	0,052 ± 0,035	0,031 ± 0,012	0,008 ± 0,004	0,010 ± 0,001
18:1*n*-9c, 18:2*n*	0,204 ± 0,128	0,088 ± 0,028	0,003 ± 0,005	--
18:1*n*-9t	--	--	0,010 ± 0,003	0,009 ± 0,007
20:4*n*-6, 20:5*n*-3	1,529 ± 1,105	0,580 ± 0,238	--	--
20:3*n*-6	0,004 ± 0,021	--	--	--
22:6*n*-3	1,019 ± 0,438	0,561 ± 0,166	--	0,035 ± 0,061
22:1*n*-9	0,031 ± 0,032	0,029 ± 0,005	0,017 ± 0,015	0,008 ± 0,013
24:1*n*-9	0,234 ± 0,049	0,236 ± 0,044	0,020 ± 0,034	0,041 ± 0,071
TOTAL	8,942 ± 5,764	3,892 ± 0,950	0,786 ± 0,172	0,850 ± 0,245

4.10 *A. tonsa* fatty acid and egg production correlation

The correlation between the total fatty acids content per female and the egg produced show a significant positive correlation (Pearson r=0,871; $p < 0,01$; Figure 14) A correlation between the average egg number produced per female and the specific saturated and unsaturated fatty acids per female was positively and significantly correlated with three saturated and five unsaturated fatty acids (Table 8). After performing an univariate test of significance for the above mentioned significant correlations all the results where highly significant (Table 8). However, the most significant between them were the palmitic (16:0) and the tandem oleic (18:1*n*-9c) linoleic acids (18:2*n*) (Table 8).

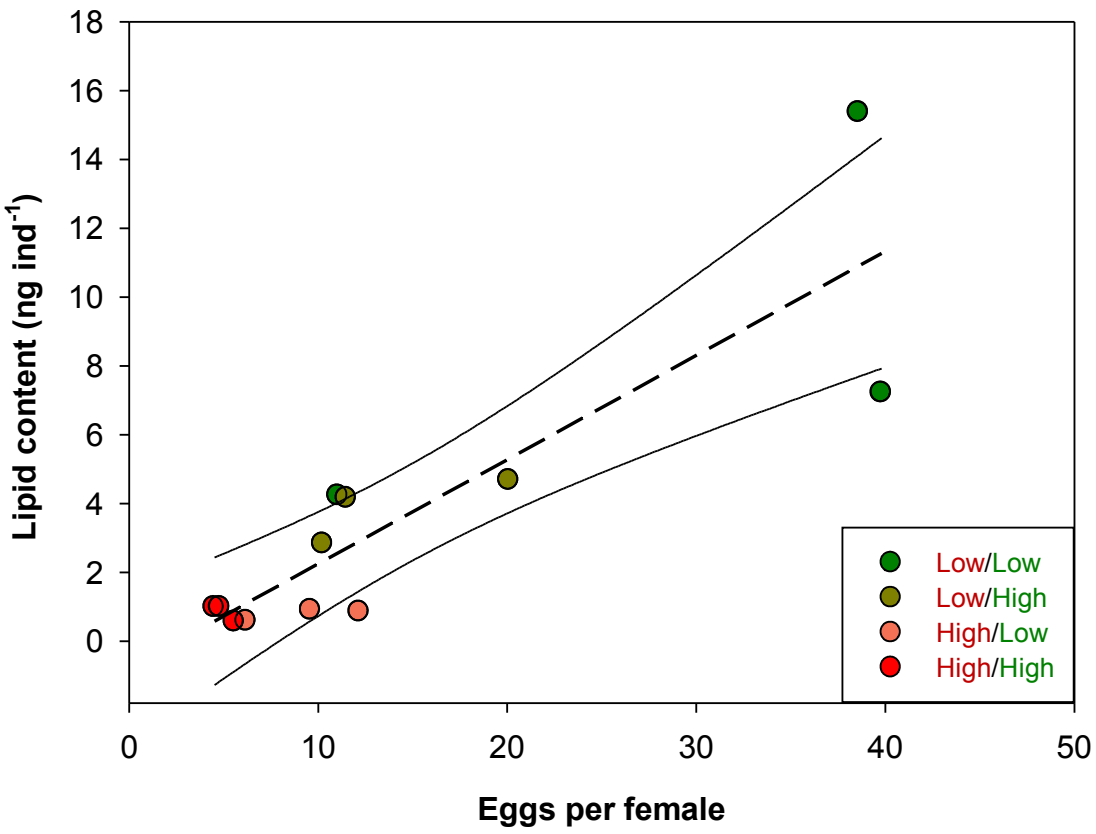


Figure 14.- Correlation between total fatty acid per female and eggs produced in 24 hours in the for different treatments.

Table 8.- Correlation analysis and its respective significance between the content of specific saturated and unsaturated fatty acids per female (ng ind.$^{-1}$) and the average number of eggs produced per female. The significant values are highlighted in red.

	Correlation		Significance	
	r	p	F	p
Saturated fatty acids				
12:0	-0,25	P=0,436	--	--
14:0	0,85	P=0,000	26,67	4,22E-004
16:0	0,90	P=0,000	40,61	8,12E-005
18:0	0,81	P=0,001	19,47	1,31E-003
20:0	0,57	P=0,055	--	--
22:0	-0,23	P=0,474	--	--
24:0	0,59	P=0,046	--	--
Unsaturated fatty acids				
16:1	0,83	P=0,001	22,24	8,21E-004
17:1	0,56	P=0,060	--	--
18:3*n*-6	0,75	P=0,005	--	--
18:3*n*-3, 18:2*n*-6	0,86	P=0,000	29,67	2,82E-004
18:1*n*-9c, 18:2*n*	0,91	P=0,000	47,40	4,27E-005
18:1*n*-9t	-0,50	P=0,099	--	--
20:4*n*-6, 20:5*n*-3	0,86	P=0,000	28,18	3,43E-004
20:3*n*-6	0,59	P=0,043	--	--
22:6*n*-3	0,88	P=0,000	28,18	3,43E-004
22:1*n*-9	0,32	P=0,305	--	--
24:1*n*-9	0,69	P=0,013	--	--

5. Discussion

5.1 Growth rate of *T. pseudonana*

CO_2 is an important factor in autotrophic plankton physiology, because it is the source of inorganic carbon that is fixed by photosynthesis in a molecule of glucose (Falkowski and Raven 2007).

The typical seawater CO_2 concentrations range between 10 and 25 μmol kg^{-1} is not enough to satisfy the phytoplankton's carbon requirements (Riebesell, 2004). Most of the inorganic carbon is found as HCO_3^- in the actual ocean, but this form of carbon does not diffuse through the cell walls and has to be taken up actively. For that purpose marine phytoplankton have developed so-called Carbon Concentration Mechanisms (CCMs) (Figure 16, A), which vary between taxonomical groups (Giordano et al, 2005). Its function is to increase the carboxylation reaction of the enzyme ribulose-1,5-*bis*phosphate carboxylase/ oxygenase (RubisCO), that fixes CO_2 in to organic matter. RubisCO uses CO_2 as the only carbon substrate and has a high half-saturation constant (20 to 70 μmol kg^{-1} of CO_2), that vary among phytoplankton species (Badger et al, 1998).

CCM activity is driven by adenosine triphosphate (ATP) and is regulated by a number of environmental factors, including light intensity and spectral quality, nutrient status and, importantly, by the availability of CO_2 (Giordano et al, 2005).

A central component of a CCM are Carbonic Anhydrases (CA), a group of zinc-containing enzymes that catalyze the slow and energy consuming reaction (uncatalyzed half-life ~30 s) between CO_2 and HCO_3^- (Sültemeyer et al, 1998). Several authors have reported an up and down regulation of CA in terms of its activity or gene expression, under diverse CO_2 conditions. For example Satoh et al. (2001) determined a reduction in the intracellular carbonic anhydrase (iCA) concentration in high CO_2-grown cells of the marine diatom *Phaeodactylum tricornutum*. Rost et al (2003) reported in the diatom *Skeletonema costatum* an increase of extracellular carbonic anhydrase (eCA) activity with decreasing CO_2 concentrations, while iCA did not show a clear trend. Trimborn et al (2008, 2009) also observed that eCA activity is strongly increased with decreasing CO_2 supply in the diatoms *Eucampia zodiacus, Skeletonema costatum*, *Thalassionema nitzchioides, Pseudo-nitzschia multiseries* and *Stellarima stellaris*. Finally Burkhardt et al. (2001) reported that

Thalassiosira weissflogii and *Phaeodactylum tricornutum* respond to diminishing CO_2 supply with an increase in eCA and iCA activity.

Another factor still controversial in the CCM of diatoms is its cataloging as owners of a biochemical C_3 pathway. Recently another biochemical pathway has been proposed, the so-called C_4 pathway (Reinfelder et al., 2000; Granum et al, 2005; Roberts et al, 2007; Kroth et al, 2008) adding another enzyme that is regulated by the concentration of CO_2. Have been shown that the enzyme phosphoenolpyruvate carboxylase (PEPC), belonging to a putative C_4 mechanism in diatoms, is up-regulated under low CO_2 levels in *Thalassiosira weissflogii, Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Roberts et al., 2007; McGinn and Morel, 2008). As part of a putative CCM, the PEPC gene expression can be used to interpret the CCM activity in these diatoms, since its up regulation imply a mayor activity of this mechanism.

From those reports is possible to suggest that an increase in the CO_2 concentration increases its diffusivity through the cell and reduces its leakage from the cell's interior, therefore the activity of the CA and PEPC enzymes are reduced and its energy consumption is diminished. This has already been proposed by Burkhardt et al. (2001) who speculated that CO_2 transport may be less energetically demanding than that of $HC0_3^-$, so the enhanced operation of a CO_2 transport system rather than a $HC0_3^-$ transport system may provide energetic savings to cells as external CO_2 levels rise (Beardall & Raven, 2004).

However, it is still not clear what the consequences of this down-regulation are in terms of energy efficiency and resource utilization. One of the suggested possible outcomes of it is an increase in the growth rate of diatoms in response of energy saving (Beardall & Raven, 2004). This have been reported by Tortell et al. (2008) who showed that elevated CO_2 concentrations led to an increase in phytoplankton productivity and promoted the growth of larger chain forming diatoms in natural phytoplankton assemblages in the Southern Ocean. An increase in the growth rate can be considered as a signal of additional energy utilization by this process in diatoms. Actually Wu et al. (2010) observed that the diatom *Phaeodactylum tricornutum*, when cultured at elevated CO_2 conditions, increased his growth rate by 5%. They observed that in the high CO_2 grown cells, the electron transport rate from photosystem II (PSII) was photoinhibited to a greater extent at high levels of photosynthetically active radiation, while non-photochemical quenching was reduced

compared to low CO_2 grown cells, suggesting that this was probably due to the down-regulation of CCMs activity, which could serves as a sink for excessive energy.

Nevertheless this seems to be an species specific effect and not all diatoms react in the same way to a high CO_2 concentrations (Beardall & Raven, 2004) and seems that each species have a preference for an specific form of inorganic carbon source (Trimborn et al. 2009). Elzenga et al. (2000) characterized *Thalassiosira pseudonana* as relying solely in HCO_3^- as source of carbon. This may imply that at higher pCO2 levels, and as HCO_3^- become more scarce, this species up-regulate the CCM to supply its carbon requirements, therefore consuming more energy in the process and reducing its growth rate. This could explain the results observed in our experiment where the diatom show the highest growth rates at current CO_2 concentrations and its decrease toward higher levels. However, this results are contradictory with the observations of Trimborn et al. (2009) that show an independence of the carbon source in this diatom, using any of the available sources. A more detailed research is needed to determine the effect of the future CO_2 scenarios over this species and others.

5.2 Nutrients ratio

The most interesting result is the increase in the nitrogen cell quota under higher pCO_2 concentrations of *T. pseudonana*. This increase result in the negative C:N and Si:N correlations with pCO_2, that can be interpreted as the cell having more nitrogen at high pCO_2 rather than less carbon or silicate. The reason under the nitrogen accumulation could be a physiological adaptation to the high dissolved CO2 or lower pH, however to elucidate the reason under this process further investigation is needed.

The present study is, as far as we know, the first in report an effect of CO_2 on the C:N ratio of *T. pseudonana*. Burkhardt, et al. (1999), reported that the C:N in two diatoms of the gender *Thalassiosira, weissflogii* and *puctigera,* however only the first show a similar trend that the observed in *T. pseudonana*, although less pronounced, while the second show the opposite, whit the lowest C:N at highest $CO_{2,aq}$ concentrations. Therefore it is not possible to generalize a trend observed in one species to all the other species of the same gender.

Burkhardt & Riebesell, (1997) and Burkhardt, et al. (1999) were not able to identify the physiological reason for the variations in the C:N, but Burkhardt, et al. (1999) proposed that the variation could be a consequence of a change in the cellular content of pigment-associated proteins or RubisCO, in the ability to synthesize storage carbohydrates or polyphosphates, or in the loss rates of dissolved organic carbon compounds.

The amount of carbon contained in the fatty acids increased towards higher DIC concentrations, however the increase is not enough to compensate the marked nitrogen accumulation in the cell at higher DIC concentrations. Also the amount of carbon contained in the fatty acids is too small in comparison to the measured POC per cell, representing only about 0,01% of the total. This also implies that its increase at high DIC have a influence in the C:N ratio, although can be neglected, and therefore other molecules, as the mentions by Burkhardt, et al. (1999), could be the main drivers of the C:N ratio.

The above observations also imply that the saturated fatty acid content appears inversely correlated to the C:N ration in *T. pseudonana*, with highest amounts of saturated fatty acids when lowest C:N ratios are observed; the opposite situation applies for the unsaturated fatty acids. This however may apply only for nutrient repleted conditions, as Klein Breteler et al. (2005) report that a high C:N ration caused for a N limitation in the diatom *Thalassiosira weissflogii*, produce an increase in the content of saturated fatty acids in this species; this may also apply for *T. pseudonana* that show a similar trend in the C:N ratio under nutrient repleted conditions.

Another interesting finding was the significant negative correlation between the silicate-to-nitrogen vs. pCO_2 in *T. pseudonana*. Nitrogen has a influence on the silicate content when N stress results in an increase in the duration of G1 and G2 interphases of the cell division, prolonging the time of silicification, resulting in high Si cell quotas (Flynn & Martin-Jézéquel, 2000). Our cultures apparently were not under nitrogen stress (Appendix, table 2), as the negative correlation clearly show, its significance is consequence of the increase nitrogen cell content under higher DIC.

5.3 Fatty acid composition of *T. pseudonana* and other species in response to pCO2

To understand the mechanisms regulating the relative content of saturated and unsaturated

fatty acids in a cell it is important to understand how fatty acids are formed.

Long chain fatty acids are synthesized from small precursors, in general photosynthate derivates, for example stored carbohydrates. Two enzyme systems are utilized, acetyl-CoA carboxylase and Fatty Acid Synthases (FAS). The end products of this synthesis are usually the saturated fatty acids palmitic (16:0) and stearic acid (18:0). Once the long chain acids have been produced they can be subject to elongation done by elongases and desaturation by desaturases (Appendix, Figure 15) (Harwood, 2010). After its formation the fatty acids are incorporated in different kinds of lipids that are constituent parts of the cell membranes (Harwood, 2010).

A similar effect than the one observed in this study over the fatty acids of *T. pseudonana* cultured at high CO_2 was observed by Sato et al. (2003) in the green algae *Chlorella kessleri*, detecting higher saturation levels of the fatty acids in lipids of chloroplast and extrachloroplast membranes when cultured under high-CO_2 conditions (2% enrichment).

Our study and the observations by Sato et al. (2003) show that CO_2 may influence the saturation state of the fatty acids in this two species. We propose that CO_2 influence the mechanism involve in the fatty acid metabolism by the alteration of the cell's pH and fatty acid structure.

A.- Reduction of the cell pH

An increase in the CO_2 concentration of the surrounding media augments CO_2 diffusivity through the cell and reduces its leakage from the cell's interior. But the CO_2 that enters the cell reacts with the water in the cytoplasm (depending of the pH), forming HCO_3^- and releasing a proton H^+, therefore reducing the pH of the cytoplasm, that in general is keep in tight values by most organisms. Indeed this reaction is the reason for the presence of internal Carbonic Anhydrase proteins (iCA), to convert this HCO_3^- in CO_2 to be transported in to the chloroplast where RubisCO is located (Figure 16, A).

B.- Membrane damage

As was mention before, fatty acids are the building blocks of lipids, the constituents of cell walls. Unsaturated fatty acids have a mayor number of double bound in its carbon chains that could be easily broken at lower pH values (Figure 16, B), producing damages in the cell wall and the lost

of its properties.

The cell, to cope with this two effects that may cause CO_2 would start to produce cell membranes with a major proportion of saturated fatty acids. Shorter-chain fatty acids are more stiff, less viscous and have higher melting points; a membrane built with short chain fatty acids would be less fluid and permeable, reducing the influx of CO_2 in the cell; also would be less susceptible to damage by the breaking of the double bonds commonly present in unsaturated fatty acids as consequence of the low pH in the media.

A mechanism explaining how pH can affect the fatty acid production have been proposed very recently by Young et al. (2010) who determined that the the pH in the cytoplasm of baker's yeast cells, *Saccharomyces cerevisiae*, act as a signal that regulate the synthesis of cell membranes by controlling the production of enzymes that synthesize them. In general terms the authors propose that the acidity in the cell can play an important role in regulating Opi1, a central regulatory protein that can inhibit the production of a number of membrane synthesis proteins, the authors also propose that the elucidated mechanism is so simple and universal that it is highly likely to be widely distributed in the nature.

We hypothesize that a pH-dependent mechanism regulates the saturation state of the fatty acids in lipid membranes of phytoplanktonic organisms by the down regulation in the activity of elongases and desaturases enzymes, producing a cell membrane with a higher amount of saturated fatty acids.

In *T. pseudonana* a set of putative desaturases have been described by Tonon et al. (2005) but its regulation mechanism is unknown. We suggest that the reduction in the unsaturation of the fatty acids in this specie could be produce by the down regulation in the activity of the elongases and desaturases enzymes, diminishing the desaturation process of the newly formed saturated fatty acids and favoring its accumulation in the cell, this could also explain the reason for the increase of totals saturated fatty acids content towards higher CO_2 concentrations (Figure 7).

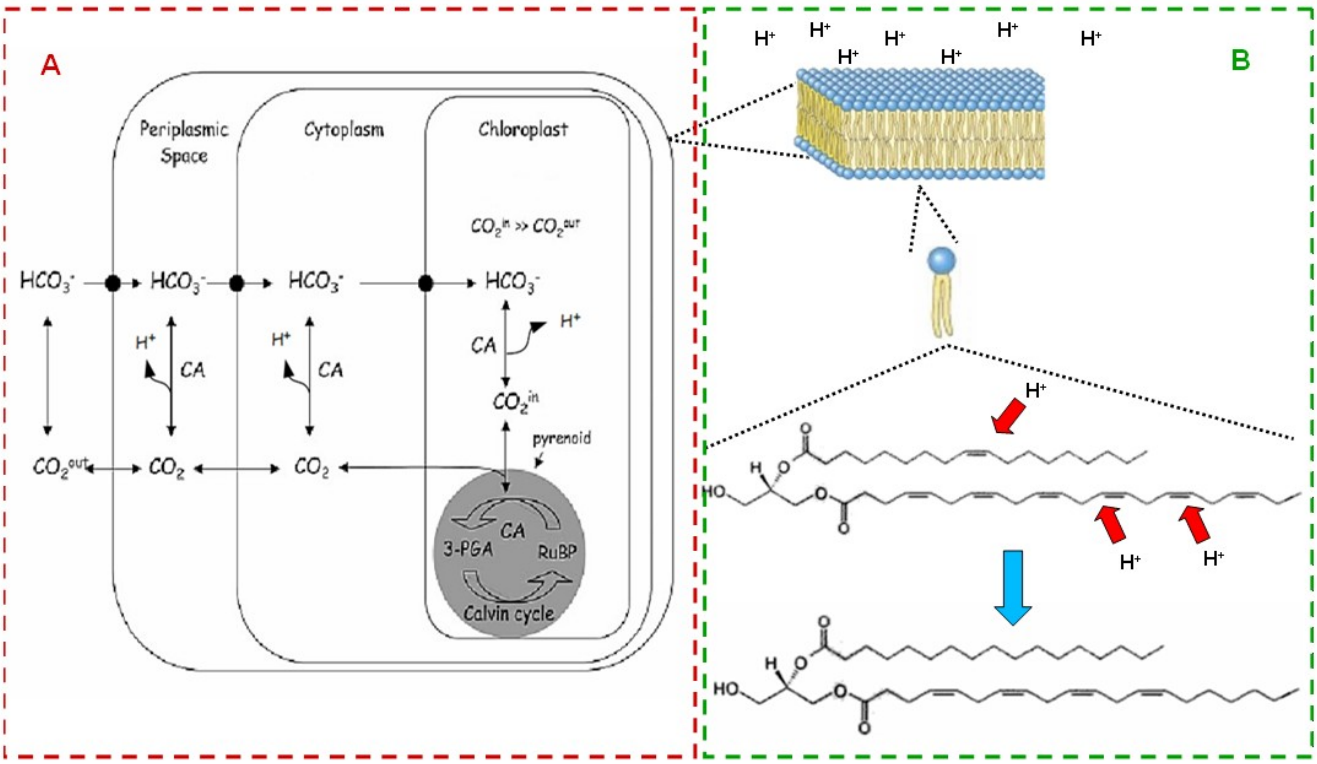


Figure 16.- A: Current model of Carbon Concentration Mechanism (CCM) in eukaryotic phytoplankton where is show the release of H^+ that could modify the cytoplasm pH (modified from the original of McGinn et al. 2008). B: The increase in the pH of the media could break the double bounds of the carbon chains in membrane fatty acids.

The shift in the relative content of saturated and unsaturated fatty acids in *T. pseudonana* as consequence of CO_2 (Figure 16, panel A) can be also observe in other phytoplankton species cultured under different carbon concentrations. After analyzing the data presented by Yongmanitchai and Ward (1991), the diatom *Phaeodactylus tricornutum* show a slight decrease in the relative concentration of the polyunsaturated eicosapentaenoic acid (EPA; 20:5*n*-3) at increasing amounts of dissolved CO_2 (Figure 17, panel C). This trend can be also observed in the coccolithophores *Emiliania huxleyi* (Riebesell et al., 2000) that, when cultured under a broad dissolved CO_2 gradient, show a decrease in the relative amount of unsaturated fatty acids toward higher CO_2 concentrations (Figure 17, panel B). The green algae *Chlamydomonas reinhardtii*, *Dunalliela tertiolecta Euglena gracilis* and *Chlorella vulgaris*, (Tsuzuki et al. 1990) cultured in a media bubbled with air enriched at 2, 4 or 5% CO_2 also show the above mentioned trend (Figure 17, panels C, D, E, F respectively).

However, in all the above mentioned studies the shift in the fatty acid composition is not as

pronounced as in *T. pseudonana*; and although being perceptible in different taxons as Bacillariophytes, Haptophytes and Chlorophytes, this process seems to be species specific, and even the opposite trend can be observed in other species. As show the analysis of the data of Chu et al. (1996) for the diatom *Nitzchia inconspicua*, an increase in the relative content of unsaturated fatty acids and a decrease in the saturate fatty acids can be observed when culture in a 5% air bubbled CO_2 enriched media (Figure 18, left panel). This is also detected in the green algae *Scenedesmus obliquus* when cultured in a CO_2 enriched media (Tang et al. 2010) (Figure 18, right panel).

Must be consider that most of the above mentioned publications were intend for aquaculture studies and not for the understanding of the future CO_2 scenarios, therefore the ranges of CO_2 utilized in those experiments is far over any of the predicted ocean acidification scenarios (as Tang et al. 2010). They are show here with the intention of demonstrate that CO_2 actually have influence in the fatty acid composition of phytoplankton.

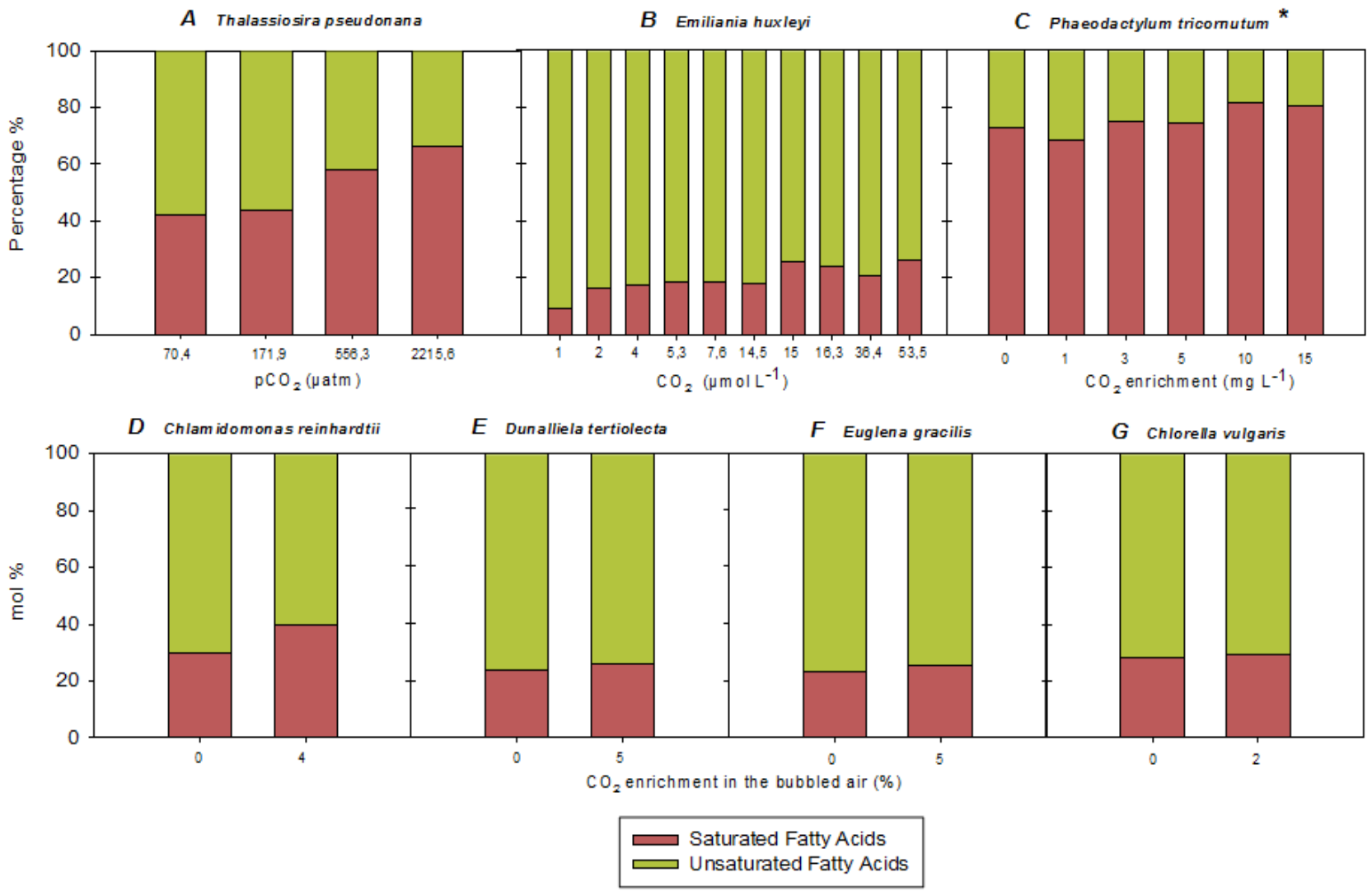


Figure 17.- Relative content of saturated and unsaturated fatty acids in different species of phytoplankton expressed as percentage of the total in panels A (this study), B (Riebesell et al. 2000) and C (Yongmanitchai et al. 1991), and as mol percentage in panels D, E, F and G (Tsuzuki et al. 1990). Note the reduction in the relative amount of unsaturated fatty acids under higher CO_2 concentrations.

Apparently diatoms have a higher amount of saturated fatty acids at current CO_2 conditions, for example in *Nitzchia inconspicua* around of 50% of the fatty acids are saturated under normal CO_2 conditions (Chu et al. 1996) (Figure 18, left panel), in *Chaetoceros sp*. around a 50%; between 56-37% in *Phaeodactylus tricornutum* (Esquivel et al. 1993), a 65-70% in *Thalassiosira weissflogii* (Jónasdóttir, 1994) and finally *T. pseudonana* with a 40% (this study). Under this perspective could be possible that the effect of high CO_2 would modify more drastically the proportion of saturated and unsaturated fatty acids in diatoms when compared with other taxa, that at atmospheric CO_2 conditions have around a 20% of saturated fatty acids (Figure 17, panels B, C,D,E,F),

Since the observed influence in the fatty acids is broad distributed and apparently affects each species in a particular form, a community analysis become necessary to estimate the possible consequences of this effect on the fatty acids under the predicted possible future CO_2 scenarios.

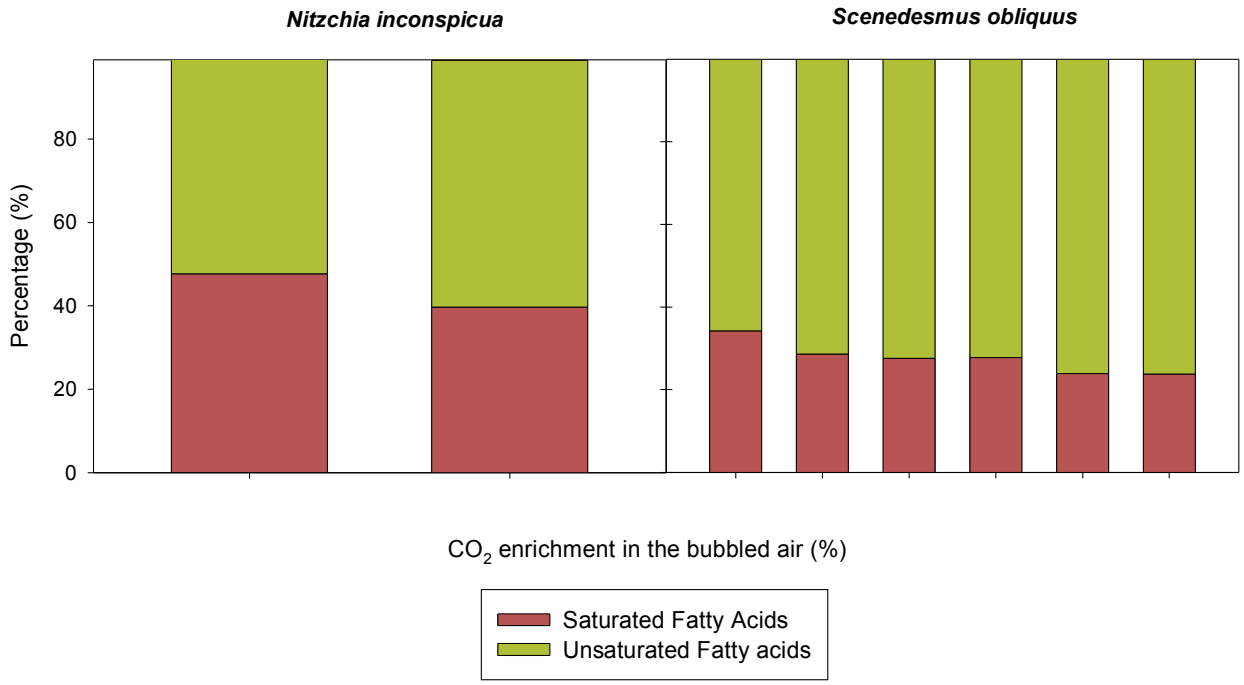


Figure 18.- Relative content of saturated and unsaturated fatty acids in different species of phytoplankton expressed as percentage of the total measured. Left panel (Chu et al. 1996) the diatom *Nitzchia inconspicua*, and in the right panel (Tang et al. 2010) the green algae *Scenedeismus obliquus*. Note the increase of the relative amount of unsaturated fatty acids under higher CO_2 concentrations.

5.3.1 pCO_2 and shift in the fatty acid content

The diatoms cultured to feed *A. tonsa* show the same trend in the fatty acids composition than the ones cultured in a CO_2 gradient, with less unsaturated fatty acids at higher CO_2 concentrations (Figure 9), particularly some of the fatty acids consider essentials for heterotrophic organisms, as linolenic acid (18:3*n*-6) and DHA (22:6*n*-3), with a significant difference between both treatments, showing that the content of them actually decline at higher CO_2 levels, and being practically absent in the copepods maintained at high CO_2 in treatments High/Low and High/High (Table 3).

However this make hard to explain the observed fatty acids content of the copepods in treatment Low/High (Low pCO_2 copepods - High pCO_2 diatoms) (Figure 13, right panel) which are higher than the ones observed in the diatoms used to feed them considering that copepods reflect the fatty acids of their preys (Fraser et al. 1989).

This high content of fatty acids in the copepods of treatment Low/High could be explained by the fast adaptation of phytoplankton to different CO_2 concentrations.

The increase in the saturated fatty acid content is a transitory adaptation of the cell to high CO_2 conditions. Tsuzuki et al. (1990) and Sato et al. (2003) have show that the green algae *Chlorella kessleri* shift its relative content of saturated and unsaturated fatty acids within hours of its transference to a different dissolved CO_2 concentration (Figure 19). This apparently happen because, as the cells divided in the new CO_2 media and therefore different pH environment, they modify its fatty acid content to deal with the new conditions.

We hypothesize that the observed increase in the fatty acid content of the copepods of treatment Low/High could be a consequence of the change in the fatty acid condition of its prey, *T. pseudonana*, adapted to deal with the new CO_2 and pH. This also apply for copepods in treatment High/Low.

Summarizing, *T. pseudonana* culture with a low pCO_2 that is shift to high pCO_2 may increase its content of saturated fatty acids in relation to the unsaturated ones. And the cells from high pCO_2 transferred to low pCO_2 would decrease its content of saturated fatty acids.

However this shift is seems to be dependent of the division rate of the phytoplankton, taking several generations and the disappearance of the oldest cells to appreciate the change in the fatty acids of the culture.

An experimental test is required to determine the speed and magnitude of the change in the diatom.

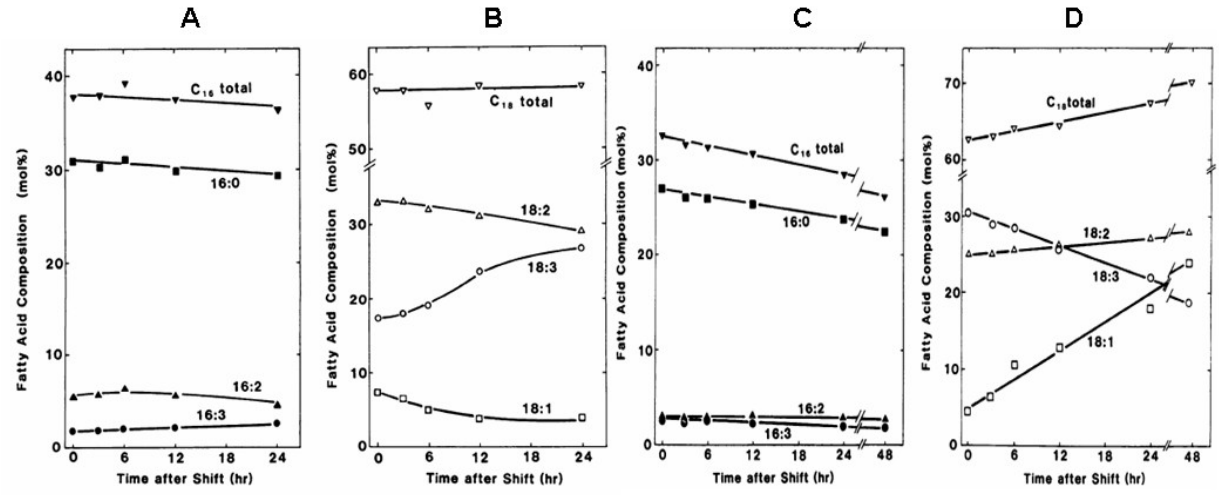


Figure 19.- Changes in fatty acid composition of the green algae *Chlorella kessleri* after shift of CO_2 concentration from 2 to 0.04% in panels A and B, and from 0.04 to 2% in panels C and D (Tzusuki et al. 1990).

5.3.2 Denaturation of unsaturated fatty acids in to aldehydes.

The transfer of lipids and fatty acids from algal cells to higher trophic levels has been typically studied by using simple extraction and quantification approaches (Jónasdóttir, 1994), that assume a transfer of the total nutrient content of the cell to the grazer, but predators as copepods do not always "swallow" the entire cell, many species disrupt the prey for its consumption, breaking and releasing its content. Several authors have shown, in diatoms, that the cell disruption induces the transformation of the contained unsaturated fatty acids in to aldehydes (Pohert et al. 2002a;

Pohert et al. 2004; Wichard et al. 2007) that have adverse effects on the copepods hatch success and develop (Pohert et al. 2002b).

Moreover, Wichard et al. (2007) have determined that the cell disruption of different diatom species change drastically its lipid and fatty acid content, reducing its quality as food source. For example in the diatom *Thalassiosira rotula* a depletion of 70% in its EPA (20:5*n*-3) content just 3 minutes after its breakage. This fatty acid is transformed to (2E,4E/Z)- hepta-2,4-dienal, (2E,4E/Z,7Z)-deca-2,4,7-trienal and (5Z,8E/ Z,10E)-12-oxododeca-5,8,10-trienoic acids. The transformation is mediated by hydroperoxide lyases, halolyases and mainly lipoxygenases, these apparently being pH sensitive, with maximal activity at certain specific pH levels (Chedea et al. 2008) depending of the enzyme.

Although could be consider a lost of unsaturated fatty acids due to its denaturation in aldehydes after cell rupture by the copepod for its ingestion, *T. pseudonana* have been reported as unable of this response (i.e. after its rupture not produce alhehydes) (Wichard et al. 2007), with a lost of only around of 15% of the unsaturated fatty acids content after 30 minutes of the cell rupture. Then, would not be possible to attribute to this mechanism the observed results in development and egg production of the copepods.

Anyway, considering that the enzymatic degradation occurs in the seawater (Wichard et al. 2007), the future expected ocean pH could affect this process, increasing or reducing the denaturation of fatty acids, affecting different trophic levels. This difference in activity caused by specific pH requirements could be also the reason of the discrepancy observed between some publications that report a neutral (Dutz et al. 2008) or negative (Turner et al. 2001) effect of the same diatom, *Thalassiosira rotula,* on the hatching success and larvae survival of copepods.

5.4 Copepods, fatty acids and its life cycle

Between the objectives of this study was to determine if the variations in the diatoms macromolecular composition affect copepods development in terms of growth rate, egg production and fatty acids composition as consequence of its use as food source.

The detected change in the macromolecular fatty acid composition of *T. pseudonana* used to feed *A. tonsa*, as consequence of CO_2; particularly the significant differences in content of unsaturated fatty acids as linolenic acid (18:3*n*-6), precursor of highly unsaturated fatty acids, eicosapentaenoic acid (20:5*n*3, EPA) and docosahexaenoic acid (22:6ω3, DHA) (Figure 10) important component of the neural system, apparently have affected the growth rate, egg production and fatty acids composition of the copepod.

Klein Breteler et al. (2005) report that the copepods *Temora longicornis* and *Pseudocalanus elongatus* show an increase of the average duration of the copepodite stages in around 1 or two days. when feed with the diatom *Thalassiosira weissflogii* that show a low content of unsaturated fatty acids as consequence of nutrient limitation. This trend was also observed in our study, where the copepodites feed with low unsaturated fatty acid diatoms (treatment High/High) show an smaller number of individuals in advanced copepodite stages in relation with the copepodites feed with high unsaturated fatty acid diatoms (treatment Low/Low) (Figure 11). This delay in the copepodite development could be a consequence of the lack of enough unsaturated fatty acids that are used for example in the formation of neural tissue (Mouritsen, 2005).

In treatments Low/High (Low pCO_2 copepods feed with High pCO_2 diatoms) and High/Low (High pCO_2 copepods feed with Low pCO_2 diatoms) a different trend in the relative development was expected, with a lower development in treatment Low/High as this was feed with diatoms cultured at high pCO_2, and a higher development in treatment High/Low that was feed with diatoms cultured at low pCO_2. But as was already analyzed in section 5.3.1 this contradictory effect could be caused by the diatoms that may undergo a shift in its fatty acid composition after being transfer to a different pCO_2, increasing (low pCO_2) or diminishing (High pCO_2) its unsaturated fatty acid content.

Probably the most interesting result in this study is the high and significant difference in the egg production rate between the treatments Low/Low and High/High, that apparently was produce for the difference in the fatty acids composition of the diatoms used as food source, with a higher egg production rate when the content of unsaturated fatty acids was high (treatment Low/Low). The correlation in the fatty acid content per female and egg production rate show that the most important fatty acids are the 18:1*n*-9c and 16:0 (Table 4).

This correlation between egg production rate and fatty acids in *A. tonsa* have been already described by Jónasdóttir (1994) who showed that the egg production rate was correlated with the the fatty acids 16:1*n*-7 (negative), 20:5*n*-3, 22:6*n*-3, and 18:0 (positive). Also Jónasdóttir & Kiorboe (1996) reported a similar trend in *A. tonsa*, as well, where the egg production rate was high when both *n*3:*n*6 and *22:6n-3* versus 20:5*n-3* fatty acid in the diet were high.

In treatments Low/High and High/Low an opposite trend was expected in terms of the egg production rate. A lower egg production rate in treatment Low/High as this was feed with diatoms cultured at high pCO_2, and a higher egg production rate in treatment High/Low that was feed with diatoms cultured at low pCO_2. But as was already analyzed in section 5.3.1 and explained for the copepodited development, this contradictory effect could be caused by the diatoms that may undergo a relative fast shift in its fatty acid composition after being transfer to a different pCO_2.

Finally the fatty acid content in the female copepods closely resembles the profile of the fatty acids in the diatoms. This resemblance have been already observed in *A. tonsa* by Hazzard & Kleppel (2003), who reported that for example 40 to 60% of the ingested omega-3 fatty acids in adult copepods were originally from its prey.

This resemblance in the fatty acid profile of the predator to the one in its prey can go high in the food web as reported by Fraser et al. (1989) who determined that fatty acids originated in phytoplankton were shown to be sequentially incorporated into total lipid of zooplankton and triacylglycerol of herring (*Clupea harengus*) larvae.

The feeding of the high pCO_2 copepods in treatment High/High once with low pCO_2 diatoms by an error in the initial manipulation of the carbonate system in the diatom culture seems that did not have affected the overall outcome of the experiment as can be inferred from the observed data. An effect would imply an increase in the egg production or fatty acids, that can not be observed in the data. The fact that the culture media was replaced every other day and then replenishment with fresh diatoms, apparently avoided an impact of this error in the overall experiment.

6. Conclusions

- CO_2 can modify the C:N:Si ratio in nutrient repleted conditions.
- CO_2 can influence the growth rate of diatoms.
- CO_2 affect the fatty acid composition of Thalassiosira pseudonana.
- The change in the fatty acid composition caused by CO_2 seems to take place in other species and is specie specific. The quality of phytoplankton as food at high CO_2 require further investigation.
- The fatty acids alteration in diatoms have a significant influence in the development cycle of copepods, however further study is required to assert the possible impacts through the trophic webs.

Finished the 19/09/2010, 05:32 am.

7. Appendix

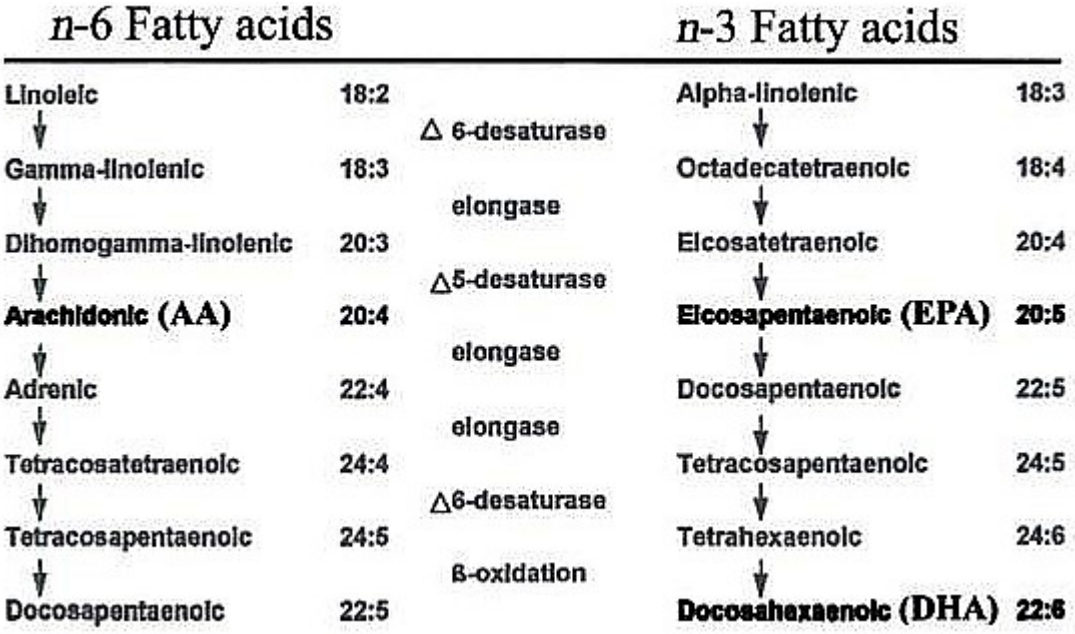


Figure 1.- Elongation and desaturation pathway for the essential fatty acids, linoleic acid 18:2*n*-6 and the α-linoleic acid 18:3*n*-3. The three highlighted fatty acids, arachidonic acid (20:4*n*-6), eicosapentaenoic acid (EPA; 20:5*n*-3), and docosahexaenoic or DHA (22:6*n*-3), are important for neural membranes and the brain (figure from Mouritsen O. G. 2005)

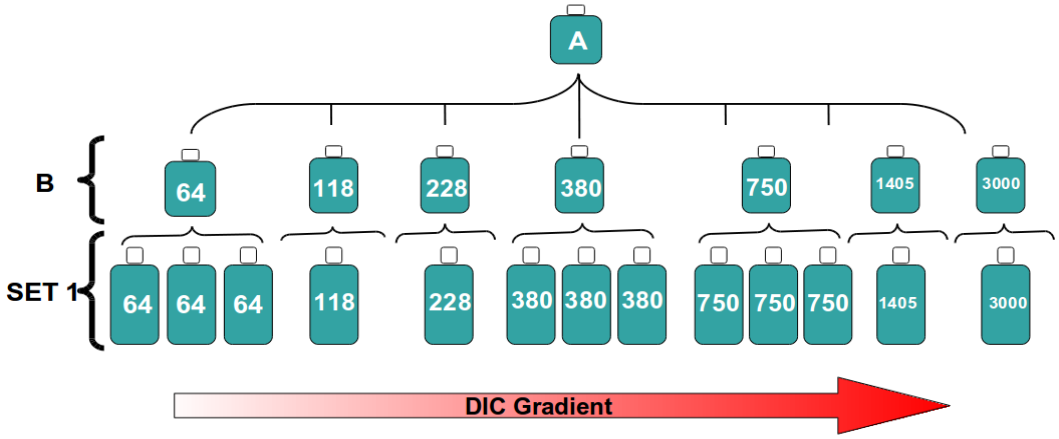


Figure 2.- Experimental setup of the diatom batch cultures. **A bottle**, 600 ml polycarbonate flask with artificial seawater; diatoms were cultured during ~10 generations with a pCO_2 and alkalinity set to ~380 (2100 µmol kg^{-1} DIC) and ~2350 µmol kg^{-1} respectively. **B bottles**, set with a pCO_2 gradient between 64 to 3000 (1550 to 2463 µmol kg^{-1} DIC) to acclimate the cells to different carbonate chemistry, cultured for about ~10 generations. **Set 1** culture in 2,4 liter polycarbonate bottles filled with culture media and the respective DIC concentration.

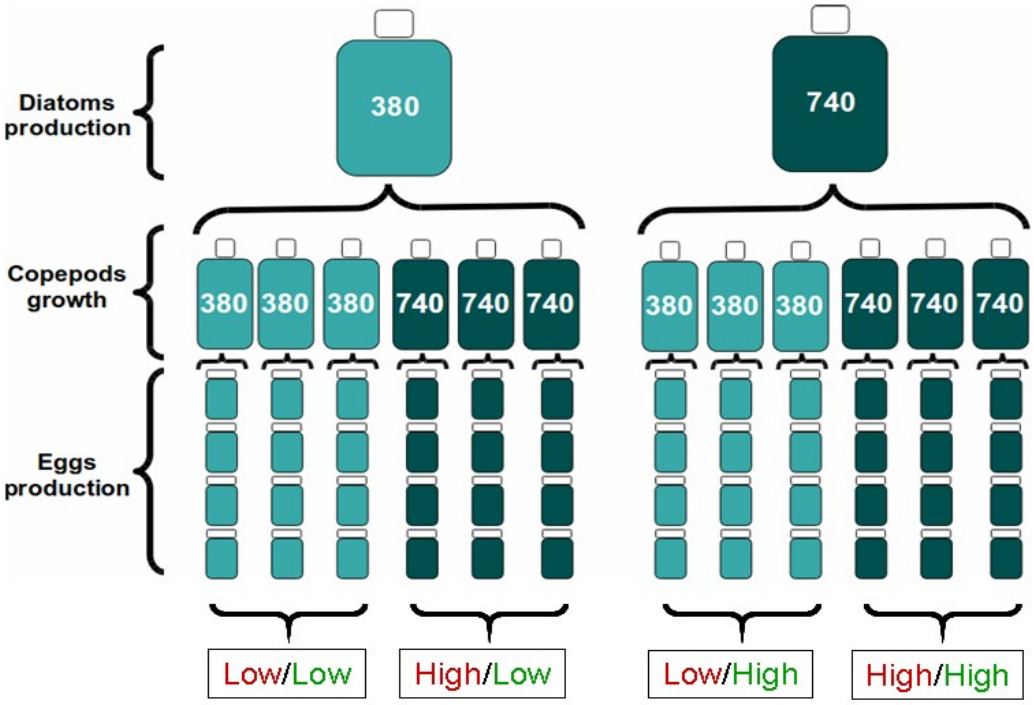


Figure 3.- Experimental setup of the copepods cultures. **Diatoms production**, 25 liters polycarbonate flask with artificial seawater; diatoms were cultured with a pCO_2 set at ~380 and ~740 (2100 µmol kg^{-1} DIC) and alkalinity at ~2014 µmol kg^{-1} respectively. **Copepods growth**, culture in 2 liter polycarbonate bottles filled with culture media and the respective pCO_2. **Egg production,** 500 ml bottles with 5 copepod females and an small amount of diatoms, each in quadruplicate.

The treatments are: Low/Low**,** Low pCO_2 copepods - Low pCO_2 diatoms; Low/High, Low pCO_2 copepods - High pCO_2 diatoms; High/Low, High pCO_2 copepods - Low pCO_2 diatoms; and High/High, High pCO_2 copepods - High pCO_2 diatoms.

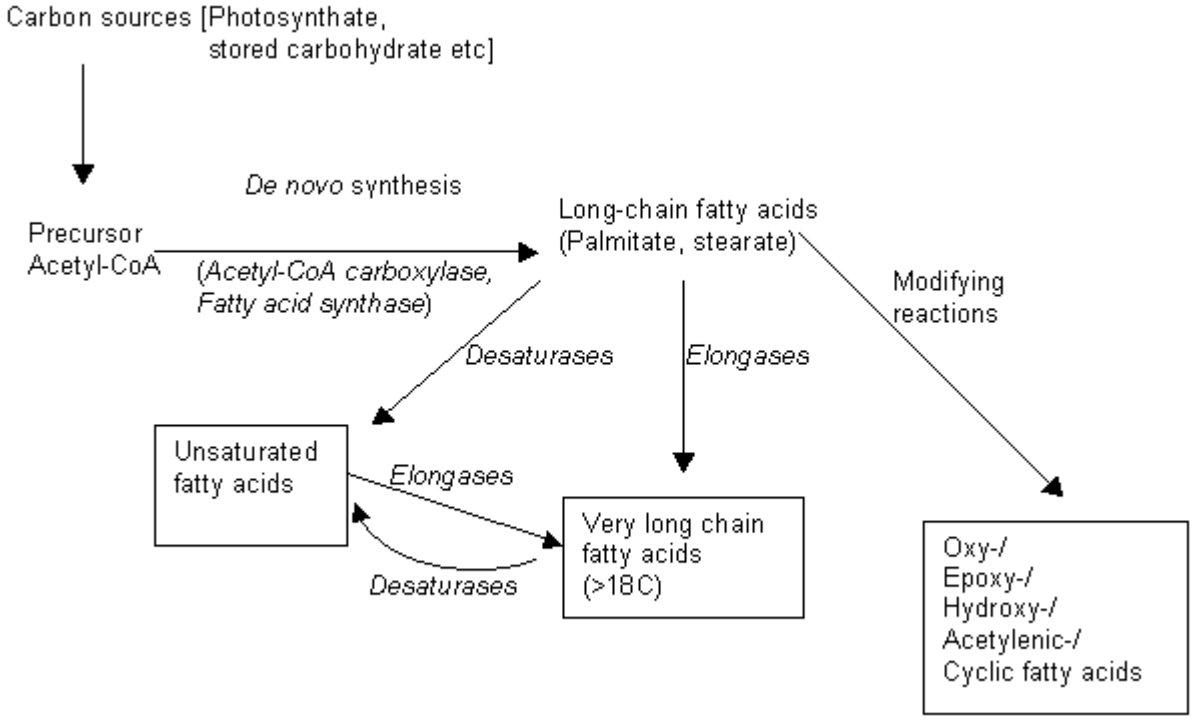
Table 1.- Measured alkalinity, DIC, calculated pCO2 and pH of each phytoplankton specie used in the study. The DIC of the copepods feeding experiment was calculated from the DIC measurements using the CO2sys software. The K1 and K2 for the calculations are from Roy et al. (1993).

	Bottle °N (2000ml)	DIC (µmol/kg)	Alkalinity (µmol/kg)	pCO2	pH free scale (mol/kg)
T. pseudonana	1a	1750,3	2410,1	70,4	8,720
	1b	1712,8	2376,2	66,8	8,731
	1c	1721,3	2414,2	62,2	8,757
	2,0	1966,9	2404,4	171,9	8,439
	3,0	1880,0	2352,7	140,5	8,498
	4a	2081,7	2307,5	421,9	8,110
	4b	2078,0	2323,3	385,3	8,146
	4c	2115,6	2329,3	459,1	8,082
	5a	2199,1	2386,2	556,3	8,019
	5b	2201,3	2398,7	528,1	8,041
	5c	2205,7	2406,1	525,7	8,044
	7,0	2558,4	2550,1	2215,6	7,495
T. pseudonana for *A. tonsa* feeding experiment	380 Day 1	1895,1	2043,4	379,2	8,159
	380 Day 2	1921,1	2073,5	350,2	8,165
	380 Day 3	1885,5	2032,6	390,8	8,157
	380 Day 4	1926,7	2079,1	345,2	8,166
	740 Day 1	1929,4	1993,2	1003,5	7,888
	740 Day 2	1884,4	1943,8	1255,9	7,878
	740 Day 3	1999,5	2069,2	733,1	7,903
	740 Day 4	2023,3	2094,6	667,1	7,908

Table 2.- Nitrogen phosphate and silicate measured in each experiment in this study

Phytoplankton specie	Bottle °N (2000ml)	Nitrogen (µmol/l)	Phosphate (µmol/l)	Silicate (µmol/l)
T. pseudonana	1a	8,161	0,942	29,192
	1b	8,186	0,737	13,125
	1c	11,903	0,853	16,988
	2	11,325	0,867	21,184
	3	11,162	1,291	25,150
	4a	10,371	1,571	25,585
	4b	9,429	1,421	25,073
	4c	11,802	1,345	21,415
	5a	9,492	1,455	27,427
	5b	7,759	1,985	27,529
	5c	6,504	0,963	16,323
	6	12,066	0,799	16,630
	7	11,664	1,127	17,142
T. pseudonana for *A. Tonsa* feeding experiment	740 Day 1	6,931	0,658	22,489
	740 Day 2	1,959	0,084	16,246
	740 Day 3	13,133	1,031	21,466
	740 Day 4	8,965	0,658	16,886
	380 Day 1	4,470	1,629	18,089
	380 Day 2	5,512	1,205	18,165
	380 Day 3	11,915	1,233	18,421
	380 Day 4	14,477	0,867	17,423

Figure 15.- Genera scheme of fatty acid biosynthesis (from Harwood 2010).



8. Bibliography

Ambler, J. 1986. Effect of food quantity and quality on egg production of *Acartia tonsa* Dana from East Lagoon, Galveston, Texas. Est. Coast. Shelf Sci. 23: 183–196.

Anderson, I. Backlund, A. 2008. Structure and function of RubisCO. Plant Physiology and Biochemistry 46: 275-291.

Armburst E.V. 2009. The life of diatoms in the world's oceans . Nature. 459: 185-192.

Augustin, C., Boersma, M. 2006. Effects of nitrogen stressed algae on different Acartia species. Journal of Plankton Research 28: 429-436.

Badger, M. Andrews, T. Whitney, S. Ludwig, M. Yellowlees, D. Leggat, W. Price, G. 1998. The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO_2-concentrating mechanisms in algae. Canadian Journal of Botany. 76: 1052–1071.

Barcelos e Ramos, J. Biswas, H. Schulz, K. La Roche, J. Riebesell, U. 2007. Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*. Global Biogeochemical Cycles 21, GB2028.

Beardall, J., Raven, J. 2004. The potential effects of global climate change on microalgal photosynthesis, growth and ecology. Phycologia. 43, 1: 26-40.

Berges, J. Falkowski, P. 1998. Physiological stress and cell death in marine phytoplankton: Induction of protease in response to nitrogen or light limitation. Limnology and Oceanography. 43: 129-135.

Berges, J. Charlebois, D. Mauzerall, D. Falkowski, P. 1996. Differential effects of nitrogen limitation on photosynthetic efficiency of photosystem I and II in microalgae. Plant Physiologhy 110: 689-696.

Biddanda, B. Benner, R. 1997. Carbon, nitrogen and carbohydrate fluxes during the production of particulate dissolved organic matter by marine phztoplankton. Limnology and Oceanography . 42: 506-518.

Breteler, W., Schogt, N., Rampen, S., 2005. Effect of diatom nutrient limitation on copepod development: role of essential lipids. Marine Ecology Progress Series 291, 125–133.

Burkhardt, S. Riebesell, U. 1997. CO_2 availability affects elemental composition of the diatom *Skeletonema costatum*. Marine Ecology Progress Series 155: 67-76.

Burkhardt, S. Zondervan, I. Riebesell, U. 1999. Effect of CO_2 concentration on C:N:P ratio in marine phytoplankton: A species comparison. Limnology and Oceanography. 44: 683–690.

Burkhardt, S. Amoroso, G. Riebesell, U. Soltemeyer, D. 2001. CO_2 and $HC0_3^-$ uptake in marine diatoms acclimated to different CO_2 concentrations. Limnology and Oceanography 46: 1378- 1391.

Brown, M. Jeffrey, S. Volkman, J. Dunstan, G. 1997. Nutritional properties of microalgae for mariculture. Aquaculture 151: 315-331.

Castro Araújo. Tavano Garcia. 2005. Growth and biochemical composition of the diatom *Chaetoceros cf. wighamii* brightwell under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids Aquaculture 246: 405-412.

Chedea, V. Vicas, S. Socaciu, C. 2007. Kinetics of soybean lipoxygenases are related to pH, substrate availability and

extraction procedures. Journal of Food Biochemistry. 32: 153-172.

Chen, X. Gao, K. 2003. Effect of CO_2 concentrations on the activity of photosynthetic CO2 fixation and extracelluar carbonic anhydrase in the marine diatom *Skeletonema costatum.* Chinese Science Bulletin. 48, 23: 2616-2620.

Chrismadha, T. Borowitzka, M. 1994. Effect of cell-density and irradiance on growth, proximate composition and eicosapentaenoic acid production of *Phaeodactylum tricornutum* grown in a tubular photobioreactor. Journal of Applied Phycology. 6: 67-74.

Chu, W. Phang, S. Goh, S. 1996. Environmental effects on growth and biochemical composition of *Nitzschia inconspicua* Grunow. Journal of Applied Phycology. 8: 389-396.

Dickson, A. Afghan, J. Anderson, C. 2003. Reference materials for oceanic CO_2 analysis: a method for the certification of total alkalinity. Marine Chemistry. 80:185-197.

Dutz, J. Koski, M. Jónasdóttir, S. 2008. Copepod reproduction is unaffected by diatom aldehydes or lipid composition. Limnology and Oceanography. 53: 225-235.

Ehrhardt, M. Koeve, J. 1999. Determination of particulate organic carbon and nitrogen, p. 437–444. In K. Grasshoff, K. Kremling and M. Erhardt [eds.], Methods of seawater analysis, 3rd ed. Wiley-VCH.

Elzenga, J. Prins, H. Stefels, J. 2000. The role of extracellular carbonic anhydrase activity in inorganic carbon utilization of *Phaeocystis globosa* (Prymnesiophyceae): a comparison with other marine algae using the isotope disequili- brium technique. Limnology and Oceanography. 45: 372-380.

Engel, A. 2002. Direct relationship between CO_2 uptake and transparent exopolymer particles production in natural phytoplankton. Journal of Plankton Research, 24: 49-54.

Esquivel B., Lobina, D., Correa, F. 1993. The biochemical composition of two diatoms after different preservation techniques. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. 105, 2: 369-373.

Falkowski, P. Raven. J. 2007. Aquatic photosynthesis. Blackwell publishing group.

Flynn, K., Martin-Jézéquel, V. 2000. Modelling Si–N-limited growth of diatoms. Journal of Plankton Research. 22; 3:447-472.

Fraser, A. Sargent, J. Gamble, J. Seaton, D. 1989. Formation and transfer of fatty acids in an enclosed marine food chain compris- ing phytoplankton, zooplankton and herring (*Clupea harengus* L.) larvae. Marine Chemistry 27:1-18.

Giordano, M. Beardall, J. Raven, J. 2005. CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Annual Review of Plant Biology 56: 99-131.

Gomez-Gutierrez, J. Peterson, W. 1999. Egg production rates of eight calanoid copepod species during summer 1997 off Newport, Oregon, USA. Journal of Plankton Research. 21: 637–657.

Granum, E. Kirkvold, S. Myklestad, S. 2002, Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion. Marine Ecology Progress Series. 242: 82-93.

Granum, E. Raven, J. Leegood, R. 2005. How do marine diatoms fix 10 billion tonnes of anorganic carbon per year. Canadian Journal of Botany. 83: 898–908.

Guillard, R. Ryther, J. 1962. Studies of marine planktonic diatoms. Canadian Journal of Microbiology. 8: 229–239.

Hansen, H. Koroleff, F. 1999. Determination of nutrients, in: Methods of seawater analysis, 3. Ed., edited by: Grasshoff, K., Kremling, K., and Ehrhardt, M., Wiley, V., Weinheim, 159-228.

Harwood, J. 2010. Plant fatty acid synthesis. In "The AOCS Lipid Library" Electronic resource http://lipidlibrary.aocs.org/.

Hecky, R. Campbell, P. Hendzel L. 1993. The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. Limnology and. Oceanography. 38: 709-724.

Hein, M. Sand-Jensen, K. 1997: CO_2 increases oceanic primary production. Nature 388: 526–527.

Holmes, R. M., Aminot, A., Kérouel, R., Hooker, B. A., Peterson, B. J. 1999. A simple and precise method for measuring ammonium in marine and freshwater ecosystems, Can. J. Fish. Aquat. Sci., 56, 1801–1808.

Houghton, J. Ding, Y. Griggs, D. Nouger, M. and others. 2001. Climate change 2001: the scientific basis. Contribution of Working Group I to the 3rd assessment report of the Intergovernmental Panel of Climate Change. Cambridge University Press, Cambridge

Intergovernmental Panel on Climate Change, (IPCC). 2007. Working Group I report 'The physical science basis'

Jewson, D. 1992. Size Reduction, Reproductive Strategy and the Life Cycle of a Centric Diatom. Philosophical Transactions: Biological Sciences. 336: 1277.

John, D. Patterson, S. Paul, J. 2007. Phytoplankton-Group Specific Quantitative Polymerase Chain Reaction Assays for RuBisCO mRNA Transcripts in Seawater. Marine Biotechnology. 9: 747-759.

John, D. Wang, Z. Liu, X. Byrne, R. Corredor, J. López, J. Cabrera, A. Bronk, D. Tabita F. Paul, J. 2007. Phytoplankton carbon fixation gene (RuBisCO) transcripts and air-sea CO_2 flux in the Mississippi River plume. ISME Journal. 1: 517-531.

Jónasdóttir, S. 1994. Effects of food quality on the reproductive success of *Acartia tonsa* and *Acartia hudsonica* : laboratory observations. Marine Biology. 121: 67- 81.

Jónasdóttir, S. Kiørboe, T. 1996. Copepod recruitment and food composition : do diatoms affect hatching success? Marine Biology. 125: 743-750.

Jones, R. Flynn, K. 2005. Nutritional status and diet composition affect the value of diatoms as copepod prey. Science 307: 1457-1459.

Kester, D. Duedall, I. Connors, I. Pytkowicz M. 1967. Preparation of artificial seawater. Limnology and. Oceanography. 12: 176-178.

Klein Breteler, W, Schogt, N. Baas, M. Schouten, S. Kraay, G. 1999. Trophic upgrading of food quality by protozoans enhancing copepod growth: role of essential lipids. Marine Biology. 135:191–198.

Klein Breteler, W. Koski, M. Rampen, S. 2004. Role of essential lipids in copepod cultivation: no evidence for trophic upgrading of food quality by a marine ciliate. Marine Ecology Progress Series. 274:199–208.

Klein Breteler, W. Schogt, N. Rampe, S. 2005. Effect of diatom nutrient limitation on copepod development: role of

essential lipids. Marine Ecology Progress Series. 291: 125–133.

Kleepel, G. Burkhart, C. Houchin, L. 1998. Nutrition and the regulation of egg production in the calanoid copepod Acartia tonsa. Limnology and. Oceanography. 43: 1000–1007.

Kooistra, W. Gersonde, C. Medlin, R. Mann, L. 2007. Evolution of Primary Producers in the Sea. Elsevier . Falkowski, P. G. & Knoll, A. H. eds. 207–249.

Kranz S A , Sültemeyer D, Richter KU, Rost B. 2009. Carbon acquisition by *Trichodesmium*: The effect of pCO_2 and diurnal changes. Limnology and. Oceanography. 54: 548–559.

Kroth, P. Chiovitti, A., Gruber, A., Martin-Jezequel, V., Mock, T., Parker, M. Schnitzler, Stanley, M.S., Kaplan, A., Caron, L.,Weber, T., Maheswari, U., Armbrust, E., Bowler, C. 2008. A model for carbohydrate metabolism in the diatom *Phaeodactylum tricornutum* deduced from comparative whole genome analysis. PLoS ONE 3: 1426.

Lippemeier, S. Hitze, R. Vaselow, K. Hartig, P. Colijn, F. 2001. In-line recording of PAM fluorescence of phytoplankton cultures as a new tool for studying effects of fluctuating nutrient supply on photosynthesis. European Journal of Phycology. 36: 89-100.

Mauchline, J. 1998. The Biology of Calanoid Copepods, Volume 33 (Advances in Marine Biology). Academic Press; first edition; 710 pp.

McGinn, J. Morel, F. 2008. Expression and Inhibition of the Carboxylating and Decarboxylating Enzymes in the Photosynthetic C4 Pathway of Marine Diatoms. Plant Physiology 146: 300-309.

Medina, M. Barata, C. 2004. Static-renewal culture of *Acartia tonsa* (Copepoda: Calanoida) for ecotoxicological testing . Aquaculture 229: 203-213.

Mitra, A. Flynn, K. 2005. Predator–prey interactions: is 'ecological stoichiometry' sufficient when good food goes bad? Journal of Plankton Research. 27: 393-399.

Moreno, V. De Moreno, J. Brenner, R. 1979. Biosynthesis of unsaturated fatty acids in the diatom *Phaeodactylum tricornutum*. Lipids 14: 15-19.

Morel, F. Price, N. 2003. The biogeochemical cycles of trace elements in the oceans. Science 300: 944–947.

Mouritsen, O. 2005. Life - As a Matter of Fat. Springer Berlin. 276 p. ISBN: 978-3-540-23248-3.

Murray, J. 2002. on: The ocean carbon cycle and climate. Mick Follows, Temel Oguz Eds. Springer Verlag ISBN: 1402020856, 395 pp.

Nelson, D. M., Treguer, P., Brzezinski, M. A., Leynaert, A. & Queguiner, B. 1995. Production and dissolution of biogenic silica in the ocean: revised global estimates, comparison with regional data and relationship to biogenic sedimentation. Global Biogeochemical Cycles. 9: 359-372.

Pohnert, G. 2002a. Phospholipase A 2 Activity Triggers the Wound-Activated Chemical Defense in the Diatom *Thalassiosira rotula*. Plant Physiology 129: 103-111.

Pohnert, G., Lumineau, O., Cueff, A., Adolph, A., Cordevant, C., Lange, M., Poulet, S. 2002b. Are volatile unsaturated aldehydes from diatoms the main line of chemical defence against copepods?. Marine Ecology Progress Series 245: 33-45.

Pohnert, G., Adolph, S., Wichard, T. 2004. Short synthesis of labeled and unlabeled 6Z,9Z,12Z,15-hexadecatetraenoic acid as metabolic probes for biosynthetic studies on diatoms. Chemistry and physics of lipids. 131: 159-66.

Raupach, M. Marland, G. Ciais, P. Le Quéré,.C. Canadell, J. Klepper, G. Field, C. 2007. Global and regional drivers of accelerating CO_2 emissions. PNAS. 104:10288–10293.

Raven, J. Caldeira, K. Elderfield, H. Hoeg-Guldberg, O. and others. 2005. Ocean acidification due to increasing atmospheric carbon dioxide. Policy Document 12/05, The Royal Society, London . Available at: www.royalsoc.ac.uk Intergovernmental Panel on Climate Change (2007) Working Group I report 'The physical science basis'.

Reinfelder, J. Kraepiel, A. Morel, F. 2000. Unicellular C_4 photosynthesis in a marine diatom. Nature 407: 996–999.

Riebesell, U., Revill, A., Holdsworth, D., Volkman, J. 2000. The effects of varying CO_2 concentration on lipid composition and carbon isotope fractionation in *Emiliania huxleyi*. Geochimica et Cosmochimica Acta 64, 24: 4179-4192.

Riebesell, U. 2004. Effects of CO_2 Enrichment on Marine Phytoplankton. Journal of Oceanography, 60: 719-729.

Riebesell, U., Schulz, K. Bellerby, R. Botros, M. Fritsche, P. Meyerhöfer, M. Neill, C. Nondal, G. Oschlies, A. Wohlers J. Zöllner, E. 2007. Enhanced biological carbon consumption in a high CO_2 ocean. Nature. 450: 545-548.

Roberts, K. Granum, E. Leegood, R. Raven, J. 2007. C_3 and C_4 Pathways of Photosynthetic Carbon Assimilation in Marine Diatoms Are under Genetic, Not Environmental, Control. Plant Physiology 145: 230-235.

Rost, B., Riebesell, U. Burkhardt, S. Sültemeyer, D. 2003. Carbon acquisition of bloom-forming marine phytoplankton. Limnology and Oceanography. 48: 55-67.

Sarmiento, J. Gruber, N. 2006. Ocean Biogeochemical Dynamics. Princeton, Woodstock: Princeton University Press.

Sarthou, G., Timmermans, K. Blain, S. Treguer, P. 2005. Growth physiology and fate of diatoms in the ocean: a review. Journal of Sea Research. 53: 25–42.

Sato, N., Tsuzuki M., Kawaguchi A. 2003. Glycerolipid synthesis in *Chlorella kessleri* 11 h II . Effect of the CO_2 concentration during growth. Biochimica et Biophysica Acta. 1633: 35-42.

Satoh, D., Hiraoka, Y. Colman, B., Matsuda, Y., 2001. Physiological and molecular biological characterization of intracellular carbonic anhydrase from the marine diatom *Phaeodactylum tricornutum*. Plant Physiol. 126:1459-70.

Stoll, M., Bakker, K., Nobbe, G., Haese, R. 2001. Continuous-Flow analysis of dissolved inorganic carbon content in seawater. Analytical chemistry 73: 4111-4116.

Strickland, J., Parsons, T. 1972. A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada, 310p.

Sültemeyer, D. 1998. Carbonic anhydrase in eukaryotic algae: characterization, regulation, and possible function during photosynthesis. Canadian Jmurnal of Botany 76: 962–972.

Tang, D., Han, W., Li, P., Miao, X., Zhong, J. 2010. CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. Bioresource technology (October). doi:10.1016/j.biortech.2010.10.047. In press.

Tonon, T. Sayanova, O. Michaelson, L. et al. 2005. Fatty acid desaturases from the microalga *Thalassiosira*

pseudonana. FEBS Journal. 272: 3401-3412.

Tortell, P., Payne, C., Li, Y., Trimborn, S. and others. 2008. The CO_2 response of Southern Ocean phytoplankton. Geophysical Research Letters 35, L04605, doi: 10.1029/2007GL032583

Trimborn, S., Lundholm, N., Thoms, S., Richter, K., Krock, B., Hansen, P., Rost, B., 2008. Inorganic carbon acquisition in potentially toxic and non-toxic diatoms: the effect of pH-induced changes in the carbonate chemistry. Plant Physiology. 133: 92-105.

Trimborn, S., Wolf-Gladrow, D., Richter, K., Rost, B. 2009. The effect of pCO_2 on carbon acquisition and intracellular assimilation in four marine diatoms. Journal of Experimental Marine Biology and Ecology. 376: 26-36.

Tsuzuki, M., Ohnuma E., Sato, N., Takaku, T., Kawaguchi, A. 1990. Effects of CO_2 Concentration during Growth on Fatty Acid Composition in Microalgae. Plant physiology 93: 851-856.

Turner, J., Ianora, A., Miralto, A., Laabir, M., Esposito, F. 2001. Decoupling of copepod grazing rates , fecundity and egg-hatching success on mixed and alternating diatom and dinoflagellate diets. Marine Ecology Progress Series 220: 187-199.

Waller RF, et al. 1998. Nuclear-encoded proteins target to the plastid in Toxoplasma gondii and Plasmodium falciparum. Proc. Natl. Acad. Sci. USA 95: 12352—12357.

Wichard, T., Gerecht, A., Boersma, M., Poulet, S., Wiltshire, K., Pohnert, G. 2007. Lipid and fatty acid composition of diatoms revisited: rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success. Chembiochem: a European journal of chemical biology. 8: 1146-53.

Wilhelm, C., Büchel, C., Fisahn, J., Goss, R., Jakob, T., LaRoche, J., Lavaud, J., Lohr, M., Riebesell, U., Stehfest, K. 2006. The regulation of carbon and nutrient assimilation in diatoms is significantly different from green algae. Protist. 157: 91–124.

Wu, Y., Gao, K., Riebesell U. 2010. CO_2-induced seawater acidification affects physiological performance of the marine diatom *Phaeodactylum tricornutum.* Biogeosciences, 7: 2915–2923.

Yongmanitchai, W., Ward, P. 1991. Growth of and Omega-3 Fatty Acid Production by *Phaeodactylum tricornutum* under Different Culture Conditions. Microbiology. 57: 419-425.

Young, B., Shin, J., Orij, H., Chao, R., Li, J., Guan, S., Khong, X., Jan, A., Wenk, E., Prinz, M., Smits, W., Loewen C. 2010. Phosphatidic Acid Is a pH Biosensor That Links Membrane Biogenesis to Metabolism. Science. 329: 1085-1088.

Zeebe, R., Wolf-Gladrow, D. 2001. CO_2 in Seawater: Equilibrium, Kinetics, Isotopes, vol. 65 of Elsevier Oceanography Book Series, Elsevier Science Publishing.

Thanks

To Dr. Prof. Ulf Riebesell and Dr. Kai Schulz for the oportunity of developin this thesis.

To Dennis Rossoll and Helena Hauss without whose help this study would have been impossible.

To all the people in the Forschungsbereich 2 for their help in the dificult moments.

To my study colleagues Franziska Werner, Helen Moor, Isabel Keller, Luisa Federwish and Nikos Alexandridis, this two years have been great.

Rafael.

Statement

Herewith I certify that the present thesis, apart from the consultation of my supervisors, was independently prepared by me. N another than the indicated resources and references were used. This thesis was presented to no other place within the scope of an examination procedure. The written thesis is identical to the electronic one.

I agree on including this thesis in the library of the Leibniz Institute of Marine Sciences as well in the library of the Christian-Albrechts- Univesität zu Kiel.

Kiel, at

J. Rafael Bermúdez M.