Primary producers and future ocean scenarios: Effect of environmental change on the biomolecular composition of phytoplankton and transference to higher trophic levels

Dissertation

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1. Summary

An increasing body of research emphasizes that various biological processes in marine organisms are affected due to the uptake of anthropogenic atmospheric CO_2 by the ocean in a process termed as ocean acidification (OA). The magnitude and direction of OA effects varies greatly among species and genotypes, highlighting different capabilities to adapt to increasing CO_2. Direct OA impacts can be expected in the biochemical and elemental composition of primary producers (PP), which may be transferred to higher trophic levels, while indirect impacts can derive from altered trophic interactions as OA can modify plankton community composition.

Fatty acids (FA) are the main component of lipids and cell membranes, with polyunsaturated fatty acids (PUFA) having additional important physiological and metabolic roles. Phytoplankton is the main source of essential biomolecules for heterotrophs as they cannot synthesize them *de novo*. Transference of organic essential macromolecules, in particular PUFA from phytoplankton-to-zooplankton-to-fish is a key factor influencing the life cycle of many organisms including humans.

In the present work was investigated how OA influences the food quality of primary producers in terms of their fatty acid makeup at specie and community level, and how these OA-driven changes in the algae affect the fatty acid profile and life cycle of consumers. A combination of short- and long-term experiments on individual algal species, interaction between a single primary producer and one consumer, and natural plankton communities encompassing several producers and consumers were conducted in laboratory and natural conditions.

In the short-term experiments at species level, the first and second laboratory study showed that CO_2 can affect the biochemical composition of the diatoms *Thalassiosira pseudonana* and *Cylindrotheca fusiformis*, reducing their PUFA content; additionally the second diatom showed a reduced amount of amino acids. The interaction between a single primary producer and one consumer showed that when *T. pseudonana* cultured under high CO_2 was used to feed the copepod *Acartia tonsa*, it affected their FA composition, severely impaired development and egg production rates. This demonstrated that a direct OA-driven shift in algal food quality can influence the reproduction success of upper trophic levels.

At the community level, the third study conducted in a North Sea natural plankton assemblage subjected to a CO_2 gradient showed that OA can modify phytoplankton community structures by favoring small phytoplankton cells with a comparatively low

PUFA content. This community shift reduced PUFA content in primary producers was linked to a gradual PUFA decline in the dominant copepod species *Calanus finmarchicus*. In contrary, the fourth study revealed that the natural plankton community of the Baltic Sea experienced small differences in the algal community composition between CO_2 treatments. The PUFA profile of the PP was influenced by phosphorus availability in the mesocosms, which was reflected by the PUFA composition of the copepod *Acartia tonsa* and *Eurytemora affinis*, but showed no significant CO_2-related changes. This indicates that OA can affect the plankton community composition and its associated PUFA content, however this effect is lower in environments where communities are exposed to natural occurring high CO_2 fluctuations like in the Baltic Sea, and that other essential nutrients have a stronger influence in the algal FA profile when present in limited amounts.

In the long term experiments at species level, the fourth study determined that the coccolithophore *Emiliania huxleyi* and the diazotrophic cyanobacterium *Trichodesmium* sp. cultured over a thousand generations at high CO_2 conditions showed a change in their FA content and composition. The FA profile of both algae presented a differentiate adaptation to high CO_2 and particularly PUFA, which have metabolic functions in the cells, displayed evidence of adaptive evolution in both algae.

These results highlight the diversity of OA responses among single plankton species and communities and that changes in biomolecular composition at the base of the marine food web are transferred to primary consumers. The thesis also highlights that the magnitude and direction of CO_2-effects likely depends on the CO_2 conditions and fluctuations the organisms are adapted to.

2. Zusammenfassung

Immer mehr wissenschaftliche Studien zeigen, dass Ozeanversauerung, das heißt die Aufnahme des anthropogenen CO_2 aus der Atmosphäre in den Ozean, viele biologische Prozesse in marinen Organismen beeinflusst. Sowohl Stärke als auch Richtung dieser Ozeanversauerungseffekte können sehr variieren, je nach Arten und Genotypen, und zeigen damit unterschiedliche Anpassungsfähigkeiten an steigende CO_2-Konzentrationen. Es wird erwartet, dass Ozeanversauerung sich direkt auf die Biochemie und Elementarzusammensetzung von Primärproduzenten auswirkt und diese Effekte auf höhere trophische Ebenen übertragen werden. Weiterhin könnte Ozeanversauerung indirekt trophische Interaktionen beeinflussen, indem sie die Zusammensetzung der Planktongemeinschaft verändert.

Fettsäuren sind die Hauptbestandteile von Lipiden und Zellmembranen. Mehrfach ungesättigte Fettsäuren (*engl. Polyunsaturated fatty acids*, PUFA) spielen außerdem eine zentrale physiologische und metabolische Rolle. Phytoplankton ist die wichtigste Quelle essentieller Biomoleküle für heterotrophe Organismen, die diese selbst nicht *de novo* synthetisieren können. Der Transfer dieser essentiellen, organischen Makromoleküle, speziell PUFA, von Phytoplankton auf Zooplankton und Fische ist ein Schlüsselfaktor für den Lebenszyklus vieler Organismen, auch für den des Menschen.

Diese Arbeit untersucht den Einfluss der Ozeanversauerung auf die Zusammensetzung und Nahrungsqualität der Primärproduzenten bezogen auf den Aufbau und Transfer von Fettsäuren in höhere trophische Ebenen. Hierfür wurden Kurz- und Langzeitexperimente durchgeführt mit einzelnen Algenarten (Studien I, II und V), zu Interaktion zwischen einem Primärproduzenten und einem Konsumenten (Studie II) sowie mit natürlichen Planktongemeinschaften bestehend aus zahlreichen Produzenten und Konsumenten (Studien II und IV).

Die ersten beiden Kurzzeitexperimente im Labor zeigen, dass eine Erhöhung des CO_2 die biochemische Zusammensetzung der beiden Diatomeen *Thalassiosira pseudonana* und *Cylindrotheca fusiformis* verändert und ihren PUFA-Gehalt reduziert. Außerdem verringer sich der Anteil an Aminosäuren in *C. fusiformis*. *Thalassiosira pseudonana*, die unter erhöhten CO_2-Konzentrationen kultiviert wurden, wurden anschließend an den Ruderfußkrebs *Arcatia tonsa* verfüttert. Infolgedessen veränderte sich die Fettsäurezusammensetzung der Konsumenten, was Entwicklung und Eiproduktionsraten erheblich beeinträchtigte. Diese Ergebnisse zeigen, dass eine direkte Veränderung der Nahrungsqualität von Algen aufgrund von Ozeanversauerung den Fortpflanzungserfolg höherer trophischer Ebenen beeinflussen kann.

In der dritten Studie wurde eine natürliche Planktongemeinschaft der Nordsee einem CO_2-Gradienten ausgesetzt. Aufgrund der Versauerung setzten sich vor allem Phytoplankton-Arten mit kleinen Zellgrößen und vergleichsweise geringem PUFA-Gehalt durch. Gleichzeitig mit der PUFA-Abnahme in den Primärproduzenten verringerte sich auch sukzessive der PUFA-Gehalt der dominanten Ruderfußkrebsart *Calanus finmarchicus*. Im Gegensatz dazu wies die Ostsee-Planktongemeinschaft der vierten Studie nur kleine CO_2-bedingte Änderungen in ihrer Zusammensetzung auf. Das PUFA-Profil der Primärproduzenten wurde durch die Phosphorverfügbarkeit in den Mesokosmen, aber nicht signifikant durch die CO_2-Konzentration beeinflusst. Dies zeigte sich auch in der PUFA-Zusammensetzung der Ruderfußkrebse *Acartia tonsa* und *Eurytemora affinis.* Diese Ergebnisse deuten darauf hin, dass Ozeanversauerung die Zusammensetzung der Planktongemeinschaft und deren PUFA-Gehalt verändern kann. Gleichzeitig wird aber auch deutlich, dass dieser Effekt schwächer ist in Regionen wie der Ostsee, die natürlichen hohen CO_2-Schwankungen unterliegen. Essentielle Nährstoffe scheinen weitaus bedeutender zu sein für die Fettsäurezusammensetzung von Algen, sobald sie limitierend sind.

Während der fünften Studie, einem Langzeitexperiment, wurden die Kalkalge *Emiliania huxleyi* und die stickstofffixierende Blaualge *Trichodesmium* sp. über mehr als 1000 Generationen unter erhöhten CO_2-Konzentrationen kultiviert. Beide reagierten mit einer Veränderung von Fettsäuregehalt und -zusammensetzung und zeigten eine klare Adaptation an die hohen CO_2-Bedingungen. Besonders die Veränderungen der PUFA, die eine wichtige Rolle im Zellmetabolismus spielen, zeigten deutlich erkennbar die adaptive Evolution beider Algen.

Die Ergebnisse dieser Arbeit belegen die Vielfältigkeit der Reaktionen von einzelnen Planktonarten und -gemeinschaften auf Ozeanversauerung. Jede Änderung der biomolekular Zusammensetzung an der Basis des marinen Nahrungsnetzes überträgt sich auf Primärkonsumenten. Weiterhin zeigt diese Dissertation auf, dass Stärke und Richtung der CO_2-Effekte je nach Art und Gemeinschaft sehr unterschiedlich sind und wahrscheinlich davon abhängen, an welche CO_2-Bedingungen und -schwankungen die Organismen angepasst sind.

3. Introduction

3.1 Anthropogenic CO_2 and the ocean

Since the industrial era, anthropogenic carbon dioxide (CO_2) emissions from fossil fuel burning, cement manufacturing and land-use change have increased the CO_2 concentration in the Earth's atmosphere by ~40% (IPCC, 2013). The combined fossil fuel and cement emissions reached a record high in 2010 of 9.1PgCyr^{-1} (1Pg=1015 g) (Peters et al., 2012; Zeebe & Zachos, 2013), this value is higher than what was predicted 20 years ago under business-as-usual scenarios for the year 2010 (8.7PgCyr−1, IS92a scenario) (Pepper et al., 1992; Zeebe & Zachos, 2013). A direct impact of anthropogenic CO_2 emissions is ocean acidification (OA), which refers to the uptake of atmospheric CO_2 by the ocean causing a decline in pH and reduction in the ocean's carbonate saturation state, with negative consequences for marine life (Zeebe & Zachos, 2013; Kroeker et al., 2010). The magnitude and direction of OA effects on marine organisms however varies greatly among species and genotypes, highlighting a differential sensitivity and capacity for adaptation. Calcifying organisms respond particular sensitively to elevated CO_2 levels, whereas biological effects of OA on non-calcifying organisms are mixed and often highly species specific (Doney et al., 2009; Kroeker et al., 2012).

3.2 Marine carbonate system

The largest carbon reservoir of Earth's carbon cycle is the ocean (~38,000 Pg C) (Zeebe & Wolf-Gladrow, 2001). The mean concentration of inorganic carbon in the ocean is about 2.3 mmol kg^{-1} and its residence time is ~200 ky. The total amount of dissolved inorganic carbon (DIC) in the modern ocean is about sixty times larger than of the pre-anthropogenic atmosphere (Zeebe & Wolf-Gladrow, 2001).

When atmospheric CO_2 dissolves in seawater it reacts with water (H_2O) and forms carbonic acid (H_2CO_3), which immediately dissociates into bicarbonate (HCO_3^-) and

further into carbonate (CO_3^{2-}) by releasing two H^+ (equation 1). Carbonic acid represents a negligible proportion that is chemically indistinguishable from CO_2 and therefore the concentrations of CO_2 and H_2CO_3 are combined and expressed as the hypothetical carbon species CO_2* (Zeebe & Wolf-Gladrow, 2001; Dickson, 2007). Therefore the chemical equilibrium of the Ocean carbonate system is as follow:

$$CO_2^* + H_2O \overset{K_1}{\rightleftharpoons} HCO_3^- + H^+ \overset{K_2}{\rightleftharpoons} CO_3^{2-} + 2H^+ \qquad (1)$$

The equilibrium concentrations of CO_2, HCO_3^- and CO_3^{2-} can be calculated using the temperature, salinity and pressure dependent stoichiometric equilibrium constants K1 and K2 (equation 2 and 3) (Roy et al. 1993; Zeebe & Wolf-Gladrow, 2001):

$$K_1 = \frac{[HCO_3^-][H^+]}{[CO_2]} \qquad (2)$$

$$K_2 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]} \qquad (3)$$

The carbonate system of seawater is by definition extended with the conceptual parameters dissolved inorganic carbon (DIC) (equation 4) and total alkalinity (TA) (equation 5) (Zeebe & Wolf-Gladrow, 2001). DIC represents the sum of all dissolved inorganic carbon species while TA is defined as the amount of excess hydrogen ion equivalents to excess proton acceptors (Dickson, 2007).

$$DIC = [CO_2^*] + [HCO_3^-] + [CO_3^{2-}] \qquad (4)$$

$$TA = [HCO_3^-] + 2\,[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] - [H] + [HPO_4^{2-}] + 2[PO_3^{4-}] + [SiO(OH)_3^-] + [NH_3] + [HS^-] - [H^+] - [HSO_4^-] - [HF] - [H_3PO_4] \qquad (5)$$

Of the carbonate system parameters, pCO_2, pH, TCO_2, and TA can be determined analytically. However, if any other two parameters and total dissolved boron are known,

all parameters (pCO2, $[CO_2]$, $[HCO_3^-]$, $[CO_3^{2-}]$, pH, TCO_2, and TA) can be calculated at given temperature, salinity and pressure (Zeebe & Wolf-Gladrow, 2001).

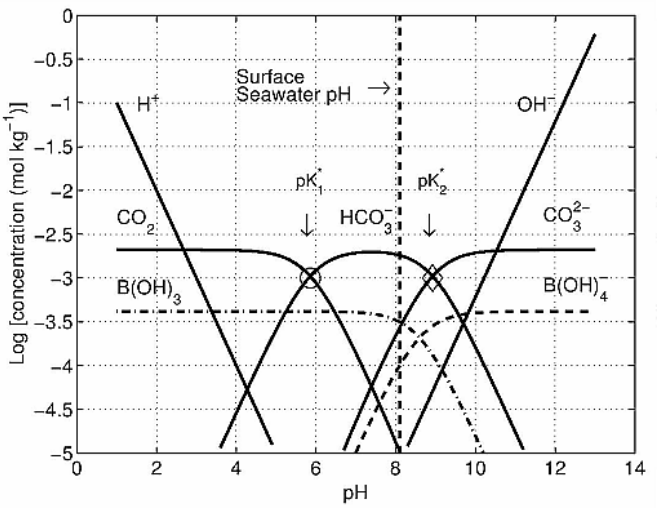

Figure 1. Typical concentrations (log scale) of dissolved carbonate species in seawater as a function of pH. Graph is taken from Zeebe and Wolf-Gladrow (2001).

The current concentrations of dissolved carbonate species in seawater are a function of pH. Under the current pH of 8.1 the majority of DIC in the ocean is in the form of HCO_3^- (>85%) followed by CO_3^{2-} (~9%) and a very small part is in the form of CO_2 (<1%) (Zeebe & Wolf-Gladrow, 2001). It is expected that the projected increased of atmospheric CO_2 of 700-1000 ppm will reduce the mean ocean surface pH by 0.14-0.35 units by the end of this century (Orr et al. 2005; IPCC 2013), 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).

3.3 Biological impacts of ocean acidification

Ocean acidification is a ubiquitous stressor in the marine environment that can affect many organisms with profound ecological consequences (Doney et al., 2009; Kroeker et al., 2010). A myriad of biological responses to OA have been measured across a range of taxa, which have shown negative effects on survival, calcification, growth and reproduction (Kroeker et al., 2010). At specie level there is significant variation in the sensitivity of marine organisms to OA. Calcifying organisms generally exhibit larger

negative responses than non-calcifying organisms across numerous variables (Riebesell el al., 2000; Doney et al., 2009; Kroeker et al., 2010). However, the sensitivity of non-calcifying organisms, although showing variation, is dependent on the taxonomic group (Doney et al., 2009; Kroeker et al., 2010). This suggests that the variability on sensitivity among organisms and taxa have important implications for ecosystem responses to OA (Kroeker et al., 2010).

3.4 Essential biomolecules in the marine environment

Lipids and proteins have important physiological and structural functions in all living organisms. They are composed of molecular subunits known as fatty (FA) and amino acids (AA), respectively. The FA consist of hydrocarbon chains with a carboxyl group (-COOH) at one end, diverse length and saturation degree defined as the number of double bonds between carbon atoms in the chain; they are classified according to their saturation degree in saturated (SFA) with no double bonds, monounsaturated (MUFA) with one double bond, and polyunsaturated (PUFA) with two or more double bonds (Mouritsen, 2005). The AA are composed of a carbon chain with an amine ($-NH_2$) and carboxyl (-COOH) functional groups with a side-chain specific to each AA (Wagner, 1983). There are several forms of AA classification, however from an ecological/nutritional perspective they can be divided in essential (EA) and non essential (NEA) (Belitz et al., 2009).

PUFA and EA have a particular ecological relevance as phytoplankton produce them in high amounts and are fundamental components in the life cycle of heterotrophs (Helland et al., 2003; Arts et al., 2009). Transference of essential FA and AA, from phytoplankton-to-zooplankton-to-fish is a key factor influencing consumer growth and reporduction (Jónasdóttir et al., 2009; Izquierdo et al., 2001). However, there are several factors that determine the abundance of these essential compounds in algae and their transference to higher trophic levels, all of which are affected by OA, and are discussed below.

3.4.1 Algal metabolism of essential biomolecules and CO_2

In the ocean there is a continuous change in light, nutrients and temperature conditions that force a constant modulation of the metabolism in the cells. Marine phytoplankton depends on inorganic carbon to form biomass, growth and ultimately reproduce (divide). In order to successfully fulfill these biological processes, the algal cells conduct a set of metabolic steps to accomplish an effective uptake and fixation into biomolecules of environmental inorganic carbon, which are grouped in the so called Carbon Metabolic Mechanisms (Smith et al., 2012).

Based in a model of carbon metabolism for diatoms proposed by Smith et al. (2012) a more general model that fit most of the algal groups can be designed. The model consists of 3 basic parts common to most photoautotrophs: a) inorganic carbon uptake, b) carbon fixation (photosynthesis), and c) synthesis of biomolecules, which include FA, AA and carbohydrates (Fig. 2). In general terms, carbon metabolism in the majority of algae species starts with DIC uptake from the environment, known as carbon-concentrating-mechanisms (CCM) (Giordano et al., 2005) (Fig. 2a). The CCM is an energy consuming process whose function is to actively transport and accumulate DIC (HCO_3^- and CO_2) within the cell where the C pool is utilized to provide elevated CO_2 concentrations around the biphosphate carboxylase oxygenase enzyme (Rubisco). The Rubisco enzyme, which is located inside micro-compartments known as carboxysomes in cyanobacteria and as pyrenoids in eukaryotic algae, fixes inorganic carbon to organic compounds. There are several classes of Rubisco enzyme in each algae taxa, and in general these enzymes are notoriously inefficient due to their very slow catalytic rate and low affinity for CO_2 (Spreitzer & Salvucci, 2002) (Fig. 2b). The immediate product of CO_2 fixation by Rubisco is 3-Phosphoglycerate (3PG), which can be used for carbohydrates and secondary metabolites in the Gluconeogenesis metabolic pathway, or for acetyl-CoA synthesis through the Glycolysis mechanism. Acetyl-CoA then can be used in the plastids for the production of FA (Ohlrogge & Jaworski, 1997) and/or in the ribosomes for AA anabolism (Fig. 2c).

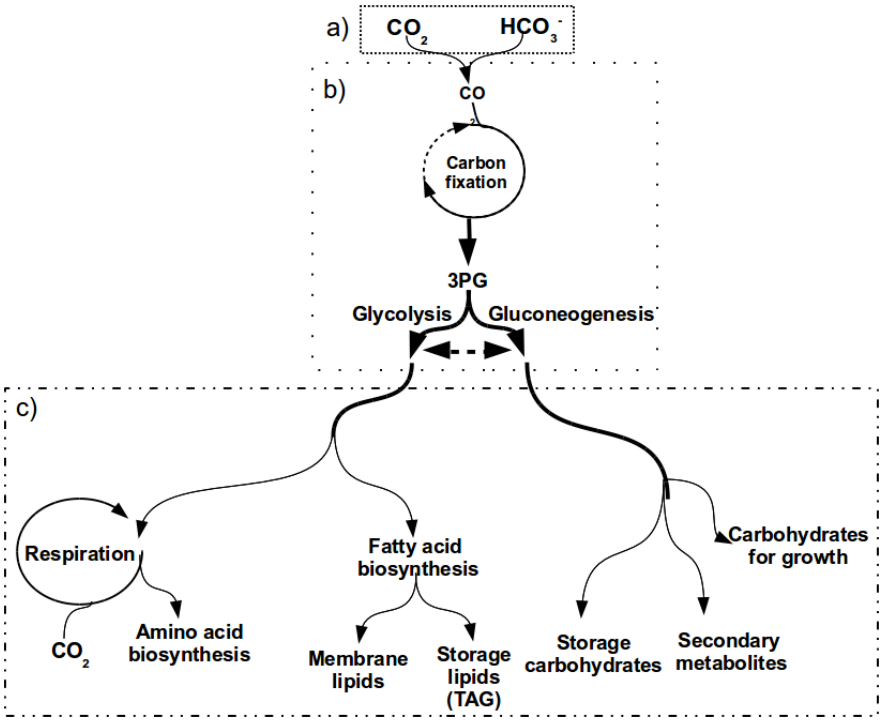

Figure 2. General carbon metabolism model in algae. The model consist of: a) Dissolved inorganic carbon (DIC) uptake, b) DIC fixation into particulate organic carbon, c) Synthesis of biomolecules which include fatty acids, amino acids and carbohydrates. Modified after Smith et al. (2012)

The biomolecular profile of individual algae species can be altered by CO_2 (Sato, 1989; Tsuzuki et al., 1990; Sato et al., 2003; Torstensson et al., 2013). Particularly, high CO_2 has been show to affect algal FA content, especially PUFA (Tsuzuki et al., 1990; Riebesell et al., 2000; Torstensson et al., 2013). The mechanisms through which CO_2 affects algal FA are unclear, however Beardall & Raven (2004) have suggested that elevated CO_2 concentration will suppress the activity of the CCM due to a higher diffusive CO_2 entry in the cell, which would saturate the enzyme Rubisco; the CO_2 fixation rate per Rubisco unit would go up, producing more 3PG, which would translate in the accumulation of biomolecules in the cell (Table 1).

Table 1. Expected effect of CO_2 in the activity of the different processes in a general carbon metabolism model based on Smith et al. (2012). ↑ Up-regulation, ↓ Down-regulation – No change.

	a) Active Carbon uptake	b) Carbon fixation rate	c) Biomolecules synthesis
Low CO_2	↑	--	↓
High CO_2	↓	↑	↑

However, the algal PUFA response to high CO_2 in terms of abundance seems to be diverse and highly species specific, going from positive to deleterious effects (Tsuzuki et al., 1990; Riebesell et al., 2000; Fiorini et al., 2010; Torstensson et al., 2013). In terms of EA synthesis and CO_2 there is very little information available, although EA seem to be more abundant at low CO_2 conditions (Renberg et al., 2010). The diversity of results in FA and the lack of information in AA synthesis highlight the necessity of a better understanding of the consequences of the OA effects in the biomolecular profile of primary producer due to their effects on higher trophic levels as is discussed in sections 3.4.2 and 3.4.3.

3.4.2 Trophic transfer of essential biomolecules

Transference of essential biomolecules from phytoplankton to zooplankton makes available highly nutritious food for upper trophic levels and is a key factor influencing the zooplankton life cycle. Aside from having fundamental metabolic and structural roles in primary producers (Fig. 3), essential biomolecules are physiologically required by heterotrophs who cannot synthesize them *de novo* or cannot produce them in sufficient amounts to meet their requirements for somatic growth, reproduction, and survival (Goulden & Place, 1990; Tocher, 2003; Kainz & Fisk, 2009). Essential FA and AA are recognized to be among the most important nutritional factors that affect the fitness of aquatic organisms (Müller-Navarra et al., 2000; Kainz & Fisk, 2009) and are retained and accumulated within the consumer's organism (Jónasdóttir, 1994; Kainz & Fisk, 2009) (Fig. 3). Furthermore it has been shown that the FA composition of consumers often closely reflects the algae FA profile they graze on (Ishida et al., 1998; Caramujo et al., 2007). As mentioned above, CO_2 can modify the FA profile of algae, and therefore any CO_2 related effect in the biomolecular profile of primary producers will affect the grazer feeding on

them (Fig. 3). Thus, a better knowledge of the CO_2 effects in primary producers and trophic transfer of essential biomolecules from algae to upper trophic levels is critical for the understanding of food web interactions in future OA scenarios.

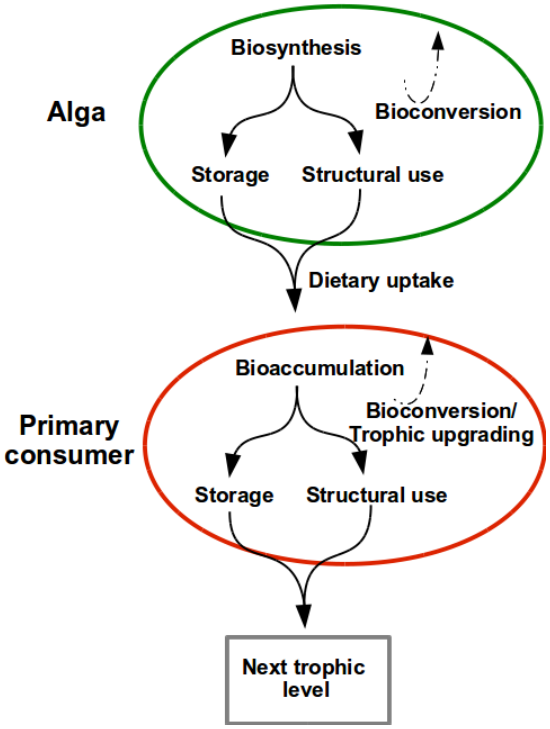

Figure 3. Schematic of the production and transference of essential biomolecules through algae producers and primary consumers. The relative importance of each process can vary widely with the biomolecules and organisms in question. Processes and pathways in the primary consumer are similar in secondary and higher level consumers. Modified after Kainz & Fisk (2009).

3.4.3 Biomolecular composition of primary producers and transference in food webs

Phytoplankton are at the base of aquatic food webs and of global importance for ecosystem functioning and services (Winder & Sommer, 2012). Sommer & Stibor (2002) have summarized three typical plankton compositions in the current oceans that have a bottom-up influence in higher trophic levels: In environments with a high abundance of by picoplankton, herbivores are dominated by protozoa and by tunicates, with copepods grazing on them being located in a higher tropic position because they cannot feed on the picoplankton (Fig. 4). In environments composed by diatoms at the base, herbivory is predominantly by copepods, cladocerans and krill, with tunicates in a secondary role (Fig. 4). Finally, an environment controlled by nuisance algae (harmful algae, eg.

Alexandrium sp.) remains ungrazed to a great extent and produces dissolved organic carbon (DOC) upon decay, which benefits bacteria feeders, such as heterotrophic nanoflagellates (HNF) and tunicates while copepods will feed on them (Fig. 4).

At community level OA can affect the species composition and size structure of natural phytoplankton assemblages (Hare et al., 2007; Biswas et al., 2011; Brussaard et al., 2013). This can be due to species with traits best adapted to the changing conditions being favored in the new altered environment (Winder & Sommer, 2012). Different algae groups at the base of the food web can affect the food quality of the entire community itself due to diverse plankton taxa having different FA profiles (Dunstan et al., 1992; Viso & Marty, 1993; Zhukova & Aizdaicher, 1995). Therefore the quantity and quality of the essential biomolecules, like FA, transferred to higher trophic levels will depend on the dominant algae in the community and its effect on subsequent trophic links. Hence, OA-driven shifts in phytoplankton may have far-reaching consequences for ecosystem structure and functioning (Winder & Sommer, 2012) as higher trophic levels like fish, heavily rely on their prey as a source of food and essential macromolecules (St. John et al., 2001; Izquierdo et al., 2001). Under this perspective it is crucial to extend the current research of OA effects on food web structure and essential macromolecule origin and transference to several trophic levels and species.

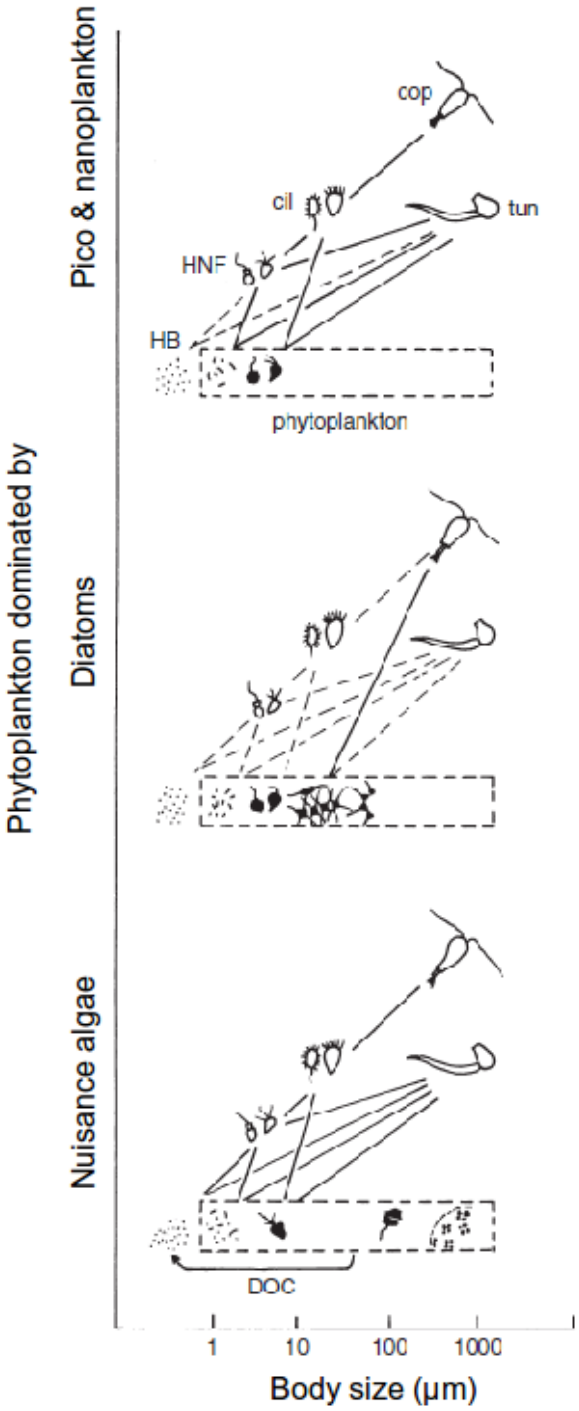

Figure 4. Bottom-up effect of the primary producer community composition over the structure of the lower marine pelagic food web: Main (—) and secondary (--) pathways of carbon and energy flow to the calanoid copepods (cop) in marine pelagic systems with different phytoplankton dominance patterns. Tunicates (tun) are represented by appendicularians, salps and doliolids. cil, ciliates; DOC, dissolved organic carbon; HB, heterotrophic bacteria; HNF, heterotrophic nanoflagellate. Graph is taken from Sommer & Stibor (2002)

3.4.4 Evolution of primary producers under high CO_2 and essential biomolecules

Evolution can be defined as the change in the inherited characteristics of biological populations over successive generations. Evolutionary processes give rise to diversity at every level of biological organization, including species, individual organisms and molecules such as DNA, lipids and proteins (Hall & Hallgrímsson, 2008).

Marine species might have the capacity to adapt to OA by evolutionary change through the standing genetic variation within populations (Sunday et al., 2014). However, the current OA research mostly addresses coastal species that are already exposed to fluctuations in temperature, pH and oxygen levels (Reusch, 2013). These kind of studies focus on environmental tolerances, which correspond to phenotypic buffering, a plasticity type that maintains a functional phenotype despite external disturbance (Reusch, 2013).

In general there is little research about long-term adaptation in marine algae and no available studies about essential biomolecules and evolution in primary producers. Nevertheless, given that marine microalgae live in a relatively stable environment and reproduce quickly, they should be particularly prone to respond to ocean changes by adaptive evolution (Lohbeck et al., 2012). It has been shown recently that a fast growing coccolithophore is able to partly restore its calcification and growth rate through adaptive evolution when kept for several hundreds of generations under high CO_2 levels (Lohbeck et al., 2012).

As mentioned in section 3.4.1, algae show a strong variation in their FA profile in response to high CO_2 levels (Tsuzuki et al., 1990; Riebesell et al., 2000; Fiorini et al., 2010; Torstensson et al., 2013). Therefore, considering that algal FA profiles have a strong influence on consumers who rely on them as source of essential biomolecules, results ecologically relevant to determine if any CO_2 effect in the algal FA, either positive or deleterious, are a form of phenotypic buffering or a constant trait in the algae on future OA scenarios.

3.5 Thesis objectives and scopes

The goal of the present thesis was to investigate how OA influences the food quality of primary producers in terms of their fatty acid makeup at species and community level, and how these OA-driven changes in the algae affect the fatty acid profile and life cycle of consumers. A combination of short- and long-term experiments on individual algal species, interaction between a single primary producer and one consumer, and natural plankton communities encompassing several producers and consumers were conducted in laboratory and natural conditions.

- Article I: At single species level, the interactive effect of CO_2 and temperature on the relative FA and AA composition were investigated in a marine algae, with emphasis on PUFA and EA.

- Article II: At predator-prey interactions, a single algae was subjected to elevated CO_2 to test potential OA effects on its FA composition and its trophic transference to a copepod grazer in order to determine indirect CO_2 effects in its reproduction and growth.

- Articles III and IV: At community level, the FA response was determined in two natural plankton assemblages subjected to elevated CO_2 conditions and its transference to primary consumers.

- Article V: At single species level, the FA composition of two algae subjected to long-term elevated CO_2 conditions was analyzed to elucidate any adaptive potential to OA in terms of biochemical composition.

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4. Articles

4.1 Author contributions

Article I: **Bermúdez JR**, Feng Y, Roleda M, Larsen T, Riebesell U, Winder M. Effect of CO_2 and temperature on the amino and fatty acid makeup of the diatom *Cylindrotheca fusiformis*. Contribution: Sample analysis, data analysis, manuscript writing.

Article II: Rossoll D, **Bermúdez JR**, Hauss H, Schulz KG, Riebesell U, Sommer U, Winder M. (2012). Ocean Acidification-Induced Food Quality Deterioration Constrains Trophic Transfer. PLoS ONE 7(4): e34737doi: 10.1371/journal.pone.0034737. Contribution: Original idea, experimental design, experimental work, data analysis, manuscript writing.

Article III: **Bermúdez JR**, Winder M, Larsen A, Riebesell U. Ocean acidification adversely affects trophic interactions and transfer of essential biochemical compounds in a natural plankton community. Contribution: Sampling experimental design, experimental work, data analysis, manuscript writing.

Article IV: **Bermúdez JR**, Winder M, Riebesell U. Effect of ocean acidification on the structure and fatty acid composition of a natural plankton community in the Baltic Sea. Contribution: Sampling experimental design, experimental work, data analysis, manuscript writing.

Article V: **Bermúdez JR**, Lohbeck K, Fu F, Hutchins D, Reusch T, Riebesell U, Winder M. Effect of long-term high CO_2 exposure on the fatty acid composition of two key marine phytoplankton species. Contribution: Sample analysis, data analysis, manuscript writing.

4.2 Effect of CO_2 and temperature on the amino and fatty acid makeup of the diatom C*ylindrotheca fusiformis*

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Abstract

It is predicted that anthropogenic CO_2 emissions will have a strong influence in the oceans by increasing the mean sea-surface temperature by 4°C and causing a pH decline of 0.3 units by the year 2100. Temperature and CO_2/pH can influence the amino (AA) and fatty (FA) acid composition of marine primary producers (PP), specially their essential amino acid (EA) and polyunsaturated fatty acid (PUFA) content. EA and PUFA are of particular importance due to their nutritious value to higher trophic levels. In order to determine the effect of CO_2-temperature interactions on PP we analyze the relative PUFA and EA composition of the diatom C*ylindrotheca fusiformis* cultured under diverse temperature and CO_2 conditions. Our results showed a decline of ~3% and ~6% in PUFA and EA content in algae kept at 750 µatm pCO_2 (high) in relation to 380 µatm pCO_2 (intermediate) treatments under a 14°C temperature regime. Cultures kept at 19°C

displayed a ~3% lower PUFA content under high CO_2 in relation with the intermediate, while EA did not show differences. Algae at 180 µatm pCO_2 (low) had a lower PUFA and EA in relation to the intermediate and high CO_2 treatments at 14°C, but no differences in EA at 19°C. The present study is the first to report negative effects of temperature and CO_2 in the EA content of a primary producer and corroborates previous observations on PUFA. Considering that only ~20% of essential biomolecules like PUFA (and possibly EA) are incorporated into new biomass of organisms of the next trophic level (Gladyshev et al., 2011), the consequences of deleterious temperature and CO_2 effects in primary producers can be far reaching in marine food webs as higher trophic organisms rely on PP as source of essential biomolecules.

Introduction

The steady rises of carbon dioxide (CO_2) since the beginning of the industrial era due to anthropogenic emission have caused a double in its atmospheric concentration (IPCC, 2007). This CO_2 emissions are predicted to have a strong influence in the ocean physical environment by increasing the mean sea surface temperature by 1–4°C and, when absorbed by the sea water, alter its carbon chemistry causing a pH decline of 0.3 units by the year 2100 (IPCC, 2007). The rising temperatures and lowered pH/high dissolve CO_2 processes, termed as global warming (GW) and ocean acidification (OA) respectively, can alter the biochemistry of a wide range of marine organisms and environments (Doney et al., 2009; Kroeker et al., 2010). GW and OA can as well affect the macromolecular composition of primary producers (Rousch et al., 2003; Torstensson et al., 2013) and its tropic transfer to higher tropic levels that depend upon them as a source of essential biomolecules (Winder & Schindler, 2004; Rossoll et al., 2012; Winder & Sommer, 2012).

Lipids and proteins have crucial structural and physiological roles in all living organisms and both are composed of subunits known as fatty (FA) and amino acids (AA). FA consists of hydrocarbon chains of different length and saturation (number of double bonds); they are generally classified in saturated (SFA, no double bonds), monounsaturated (MUFA, one double bond) and polyunsaturated (PUFA, with two or

more double bonds) (Mouritsen, 2005). AA are composed of a carbon chain with a side-chain specific to each AA (Wagner, 1983). Although AA can be classified in several ways, they can be divided from an ecological, nutritional, and physiological perspective in essential (EA) and non-essential (NEA) components (Belitz et al., 2009). The PUFA and EA have a particular ecological relevance as they cannot be synthesized *de novo* by heterotrophs and have to be acquired from dietary sources (Helland et al., 2003; Arts et al., 2009).

The macromolecular profile of individual algae species can be altered by environmental variables such as nutrients (Harrison et al., 1990; Reitan et al., 1994), temperature (Rousch et al., 2003; Torstensson et al., 2013), and CO_2 (Tsuzuki et al., 1990; Torstensson et al., 2013). The FA profile of marine algae, especially PUFA, can be affected by temperature and CO_2. Under temperature-related stress, marine algae regulate their FA composition and desaturation degree in order to keep a steady membrane fluidity at different temperatures (Mouritsen, 2005), with the amount of PUFA being generally inversely proportional to temperature (Van Wagener et al., 2012). Whereas the high CO_2 effect on algal FA content, particularly PUFA, seems to be more diverse and specie related, ranging from declining to increasing PUFA concentration (Tsuzuki et al., 1990; Riebesell et al., 2000; Fiorini et al., 2010; Torstensson et al., 2013). The mechanisms through which CO_2 affects algal FA are unclear, however it has been suggested that high-CO_2 levels enhance SFA synthesis and accumulation (Sato et al., 2003).

There is little information regarding the effects of temperature and CO_2 in algal AA composition. Higher temperatures can increase the protein content in algae (Rousch et al., 2003; Castro Araujo et al., 2005), while EA show an optimum curve, increasing up to a point at which they begin to decline with greater increases (James et al., 1989). Some studies have shown that CO_2 can affect the protein content of marine algae (Castro Araujo et al., 2005) and that EA seems to be more abundant at low CO_2 conditions (Renberg et al., 2010). This CO_2-induced change in AA have been attributed to reduced amounts of protein content, particularly of the ones related with active CO_2 uptake (Renberg et al., 2010). However the above mentioned experiments were conducted using extremely high CO_2 values that are unlikely in natural conditions and above

forecasted CO_2 levels by the end of this century (IPCC, 2007). Therefore their results cannot be easily extrapolated to understand AA metabolism in future OA scenarios.

Temperature and CO_2-driven changes on the algal FA and AA composition are ecologically relevant as qualitative traits in resource biomass are often more important than resource quantity in trophic transfer efficiency and production at higher trophic levels (Müller-Navarra et al. 2000; 2004). Several laboratory and field studies have shown that a decline of PUFA and/or AA in marine primary producers can directly affect intermediate level grazer in food webs as for instance copepods, and have serious deleterious consequences on their development and egg production rates (Helland et al., 2003; Jónasdóttir et al., 2009; Fink et al., 2012; Rossoll et al., 2012). Furthermore, it has been show that the FA and AA composition of primary producers and secondary consumers escalate to upper trophic levels in the food web and can affect the physiology of larval fish, which heavily rely on their prey as a source of essential macromolecules (St. John et al., 2001; Izquierdo et al., 2001; Moksness et al., 2008).

In spite of the importance of marine food webs as a source of essential nutrients for humans (FAO, 2010), the understanding of GW and OA influence over primary producers in terms of their quality as a source of essential macromolecules is very limited. To fill this knowledge gap we investigated the effect of CO_2 and temperature interactions on the relative FA and AA composition in marine algae, with emphasis on PUFA and EA. A freshly isolated strain of the diatom C*ylindrotheca fusiformis* was kept in a factorial design at three CO_2 levels and two temperature regimes for >250 generations and their FA and AA profiles compared. A diatom was selected as they are arguably one of the most abundant marine taxa and is the algal group at the base of some of the most productive ecosystems in the world.

Materials and Methods

Experimental set up

We analyzed fatty and amino acid profiles of the diatom C*ylindrotheca fusiformis* Reimann and Lewin 1964 collected off the coast of the city of Dunedin on the South Island of New Zealand during January of 2011. A factorial design with triplicates for each treatment level, consisting of three CO_2 concentrations: 180, 380 and 750 µatm pCO_2, and two temperatures: 14 and 19 °C were set. The CO_2 levels correspond to pre-industrial, current and future (year 2050) projected scenarios (IPCC, 2007). A temperature of 14°C is typical for the ocean during summer at the collection site of *C. fusiformis*, the +5°C manipulation was selected based on predicted sea surface warming from the IPCC (2007). The algae were kept in active growth using semi-continuous culture methods for over 250 generation before sampling. A 100 ml sample for FA and AA from each replicate was taken in a polycarbonate filter and stored at -80°C until analysis at the end of the culture period.

Culture conditions

The alga were keep in natural sea water collected approximately 3 km offshore from Tairoa Head at the mouth of Otago Harbour halfway to Munida (45, 45.09° S 170, 48.6° E). The ambient sea surface temperature was 14.8°C. All water was combined into an approximately 500 l container and sub-sampled after filtering through 80 µm mesh to remove large zooplankton. A volume of 800 ml were added to triplicate polycarbonate bottles and spiked with an f/50 nutrient derivative (10 µM $NaNO_3^-$, 0.8 µM $NaH_2PO_4^{3-}$, 10 µM Na_2SiO_3 and f/50 vitamin and trace metal concentrations [Guillard, 1962; Guillard, 1975]) to promote diatom growth. Triplicate sterilized 1 l polycarbonate bottles were gently bubbled at each temperature using commercially prepared air/ CO_2 mixtures (Alphagaz, Air Liquide) following the protocols of Collins & Bell (2006), Hare et al. (2007) and Sun et al. (2011). The bottles were incubated on a 12 L : 12 D cycle under 140 µE of cool white fluorescent illumination in free-standing laboratory incubators. The six pCO_2/temperature treatments were maintained in active growth using semi-continuous culture methods (Tatters et al., 2013). Each bottle was diluted to the original time-zero

in vivo chlorophyll-a fluorescence value every second day with nutrient-amended 0.2 µm- filtered seawater. Aliquots were removed initially and after one and two weeks for examination of carbonate system parameters and community structure using microscopic cell counts. Cellular abundances in an un-bubbled control treatment did not significantly deviate from results of the current pCO_2-bubbled treatment (data not shown).

Cell abundance

Algal samples for cell counts were collected in 30 ml borosilicate glass scintillation vials, preserved with acidified Lugol's solution and enumerated using an Accu-Scope v. 3032 inverted microscope using the Utermöhl method (1931). Samples were obtained before and after dilution and upon termination of the incubations to determine abundances. Cell-specific growth rates for each treatment level were calculated as $\mu=\ln(N_t/N_o)/(t_1-t_0)$, where N is the number of cells at time t_1 and t_0 (in days).

Carbonate system

Samples for carbonate system parameter analysis were taken at the time of the natural sample collection and at the termination of the experiments. Spectrophotometric pH for the initial community incubations was measured after Zhang & Byrne (1996) as described in McGraw et al. (2010) using a UV–vis spectrophotometer (Ocean Optics USB4000). Temperature was monitored using standard laboratory incubator thermometers and salinity by conductivity with an interchangeable probe using an Orion 5-star plus pH meter. For pH measurements, temperature and salinity values for the initial experiment were 23.6°C and 35 PSU, respectively. Experimental pCO_2 was calculated using CO2SYS software (Müller et al., 2010) with dissociation constants from Dickson & Millero (1987) using the combined data of Mehrbach et al. (1973) and Hansson (1973) and KSO_4 from Dickson (1990).

Fatty acid quantification

The FA were measured by gas chromatography as fatty acid methyl esters (FAME) following Klein-Breteler et al. (1999). 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, FAME C19:0 (Restek, Bad Homburg, Germany; c= 20.0 ng component^{-1}µl^{-1}) was added, and a C23:0 FA standard (c= 25.1 ng µl^{-1}) 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 (100-150 mbar), re-dissolved in Chloroform and transferred into a glass cocoon. The solvent was evaporated again (10-30 mbar), and esterification was performed overnight using 200µl 1% H_2SO_4 (in CH_3OH) 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 non-polar column (RXI1-SIL-MS 0.32 µm, 30 m, company Restek) using a Flame ionization detector. 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. A 1 µl aliquot of the sample was injected. The system was calibrated with a 37-component FAME-mix (Supelco, Germany) and chromatograms were analyzed using Chrom-Card Trace-Focus GC software (Klein-Breteler et al., 1999) and the fatty acids were clustered according to their degree of saturation: Saturated (SFA), Monounsaturated (MUFA) and Polyunsaturated (PUFA) and calculated in terms of their relative abundance (%).

Amino acid quantification

Amino acids were quantified with the PhenomenexEZ:faast^{TM} kit for a GC flame ionization detector (Torrance, CA, USA) following the protocol by Badawi et al. (2012). The samples were thoroughly washed off the polycarbonate filters in to Pyrex culture tubes (13 × 100 mm), hydrolyzed with 1 ml 6 N HCl (37% HCl diluted with purified water) flushed with N_2, sealed with PTFE lined heat resistant caps, and placed in a heating block at 150 ºC for 70 minutes. After hydrolysis, the samples were dried in a heating block at 110 ºC for 30 minutes under a gentle stream of N_2. Before derivatization, 200 µM norvaline in an acidified solution were added as an internal standard, then the samples

were cleaned with a proprietary cation-exchange mechanism (solid-phase extraction) from Phenomenex. The samples were then washed with 1-propanol and H_2O, eluted with a solution of aqueous NaOH, 1-propanol and 3-picoline, derivatized with a solution of $CHCl_3$, 2,2,4-trimethylpentane and propylchloroformate. In the final step, the derivatized amino acids were diluted with a HCl solution (Badawy, 2012). The final solution was injected into an Agilent 6890N GC with a 10 m x 0.25mm Zebron EZ-AAA column and a flame ionization detector. The GC settings were as follows: 2 µl of each sample was injected with a 1:15 ratio into a 250°C liner, the carrier gas was set to constant flow at 1.5 ml H_2 min-1 and the oven program was 32°C min^{-1} from 110°C to 320°C. A standard solution with 23 amino acids (Phenomenex) was used as an internal reference. Of the amino acids we were able to analyze the following were defined as non-essential: alanine (Ala), asparagine/aspartic acid (Asx), glutamine/glutamic acid (Glx), glycine (Gly), and tyrosine (Tyr). The following were defined as essential: histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), and valine (Val).

Statistical analysis

Statistical analyses consisted of multi-factorial ANOVA (MANOVA). Homogeneity of variance was check with a Bartlett or Fligner test, and normality of distribution with a Shapiro test. A principal component analysis (PCA) was used to determine the difference in individual FA and AA composition of algae across the treatment combinations. To reduce unexplained variance in the PCA, a threshold of >1% in relative concentration of the individual FA and AA was set for its inclusion it the analysis. All statistical analyses were done using the R software environment 3.0.1 (R Development Core Team 2005).

Results

pH and growth rate

The measured pH in the cultures of the diatom *Cylindrotheca fusiformis* did show a significant difference between the CO_2 treatments (MANOVA, F= 69.5, p<0.0001), while there were no temperature-related effects (p>0.05) (Fig. 1a). The growth rate showed a rather large variability, which translates in the absence of significant differences between the CO_2 treatments (p>0.05). Although the diatom showed a higher growth rate at 19°C, this difference was not significant in relation to the 14°C cultures (p>0.05) (Fig. 1b).

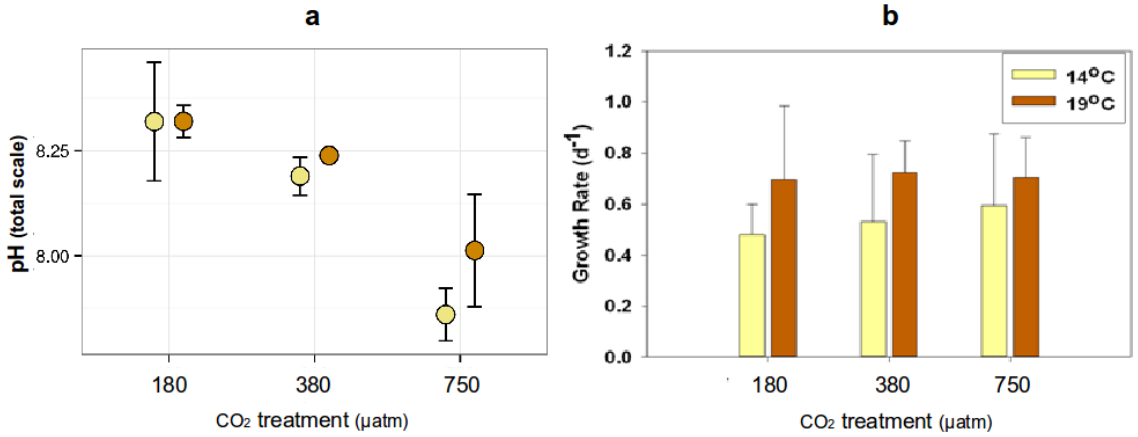

Figure 1. a) Measured pH in the different treatments of the diatom *Cylindrotheca fusiformis* cultured under different CO_2 conditions and temperatures for >250 generations (n=3). There is a significant difference between pH in the CO_2 treatments (MANOVA, F=69.5, p<0.0001), while there was no significant difference with the temperature treatments. b) Growth rate of the diatom *Cylindrotheca fusiformis* (n=3). There is no significant difference between the CO_2 treatments (Two way ANOVA, p>0.05), and although the diatom showed a higher growth rate at 19°C, there was also not significant difference with the 14°C cultures (t-test, p>0.05). Error bars show standard deviation.

Fatty acids

A total of 24 individual FA were identified and measured in the diatom *C. fusiformis*. The FA profile consisted of ~27% PUFA, ~ 23% MUFA and ~50% SFA. The analysis of the FA groups showed that the relative PUFA content was significantly affected by CO_2 (MANOVA, F= 16.32, p<0.001, df=2) with a maximum concentration at 380 µatm CO_2. Although temperature showed a significant difference (MANOVA, F=8.3, p=0.013, df=1),

this is only true in the 180 µatm CO_2 treatment with a higher PUFA composition at 19°C compared to 14°C (Fig. 2a). MUFA did not show significant differences between the treatments (p> 0.05) (Fig. 2b). The SFA relative content differed between CO_2 treatments (MANOVA, F=13.24, p<0.001, df=2) with the lowest concentration at 380 µatm CO_2, although did not show significant temperature related effects (p>0.05) (Fig. 2c). The most abundant PUFA was Eicosapentaenoic acid (EPA, 20:5n3c), which amounted almost to half of the total PUFA in the samples and was strongly influenced by CO_2 (Fig. S1a).

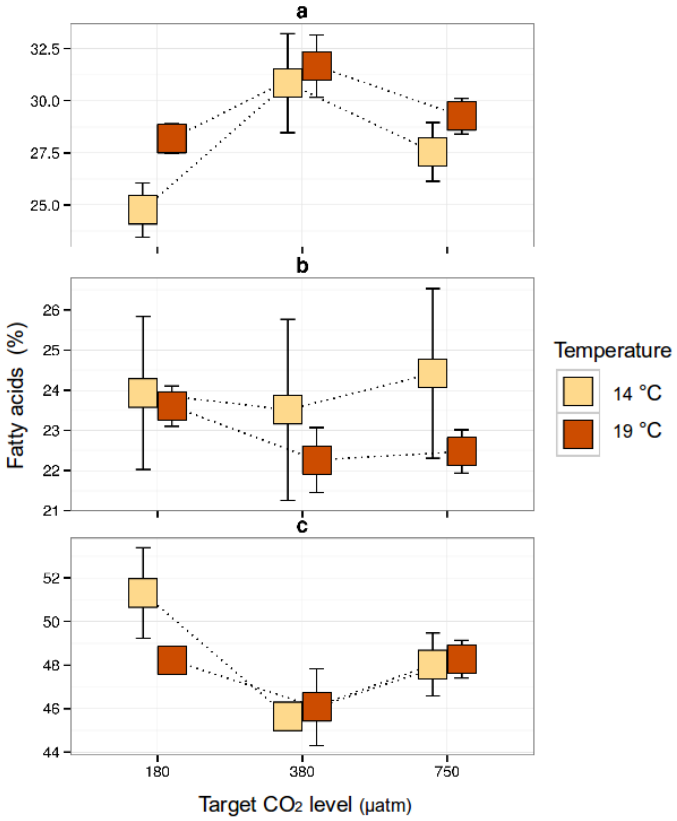

Figure 2. Relative content of (a) Polyunsaturated [PUFA], (b) Monounsaturated [MUFA] and (c) Saturated [SFA] in the diatom Cylindrotheca fusiformis cultured under three CO_2 levels and two temperatures conditions for >250 generations. a) PUFA showed a significant CO_2 (MANOVA, F= 16.32, p=0.0003, df=2) and temperature (MANOVA, F=8.3, p=0.013, df=1) effect with a maximum at the intermediate CO_2 treatment and an overall higher concentrations at 19°C. b) MUFA showed no differences between the treatments (p> 0.05). c) SFA showed a significant difference related to CO_2 (MANOVA, F=13.24, p<0.001, df=2) but no temperature effects (p>0.05). Error bars show standard deviation (n=3).

The PCA of the individual algal FA compounds showed that PUFA were more similar at high temperature (PC1, 44.4%) and intermediate CO_2 level (PC2, 24.7%), however, the influence of the former was lower (Fig. 3a). The axis loads of the PCA analysis showed that the PUFA had a strong influence in the explained variance of both axis (Fig. 3b).

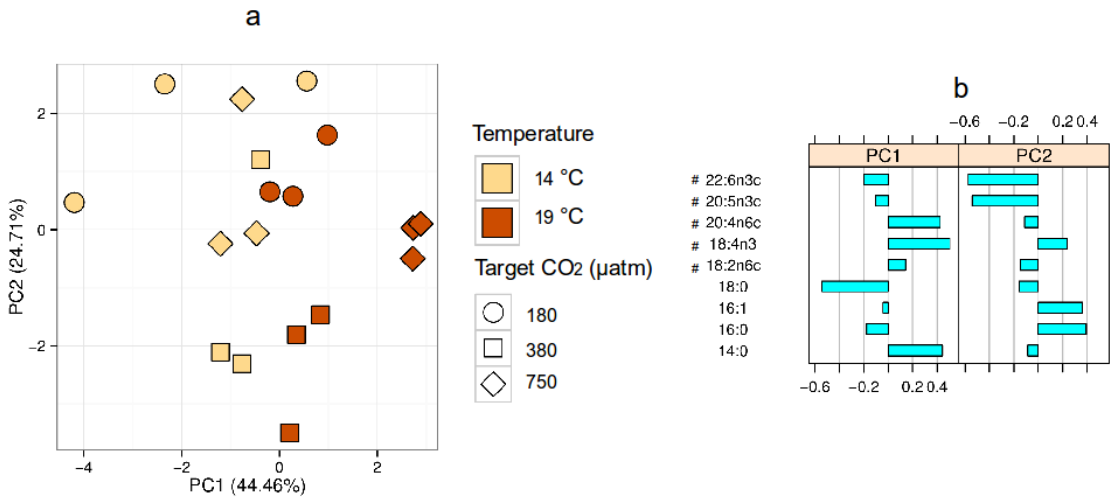

Figure 3. a) Principal component analysis [PCA] of the different fatty acids [FA] above 1% in abundance of the diatom *Cylindrotheca fusiformis* cultured under different CO_2 conditions and temperatures for >250 generations. The FA profile showed that the similarity of essential polyunsaturated fatty acids [PUFA] was higher at 19°C (PC1) and intermediate CO_2 level (PC2), however the influence of the former was lower. b) Axis loads of the PCA analysis. The PUFA had a strong influence in the variance of both axis. # indicate PUFA.

Amino acids

In the diatom samples a total of 12 AA were identified and quantified. The analysis of the specific AA groups in the diatom showed that EA represented ~40% and NEA the remaining ~60%. The relative content of both of the EA and NEA groups was significantly affected by temperature (MANOVA, F=8.3, p=0.015, df=1, for both groups), and although CO_2 affected their concentration this was not statistically significant (MANOVA, F=3.2, p= 0.07, df=2, for both). Furthermore the interaction of CO_2 and temperature in the EA and NEA relative abundances was significant (MANOVA, F=6.1, p= 0.016, df=2, in both) (Fig. 4a,b). At 380 µatm CO_2, EA showed their maximum relative concentration at 14°C compared to the other CO_2 treatments (Fig. 2a), however at 19°C their concentration was higher at 180 and 750 µatm CO_2, while the NEA followed the opposite trend (Fig. 4b). Leucine (Leu) was the most abundant EA and represented ~10% of the total AA and ~¼ of the EA (Fig. S2).

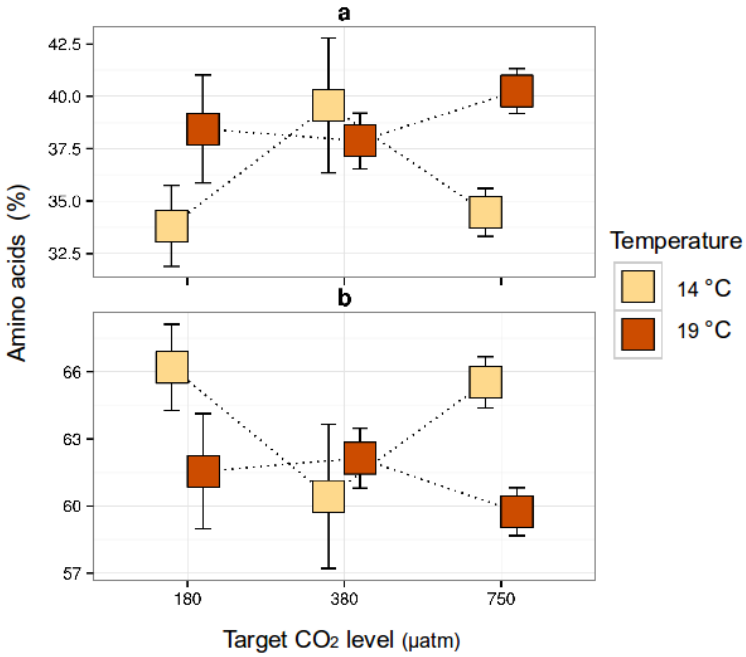

Figure 4. Relative content of (a) Essential [EA] and (b) Non essential [NEA] amino acids in the diatom *Cylindrotheca fusiformis* cultured under different CO_2 conditions and temperatures for >250 generations. a) EA showed a significant difference with temperature (MANOVA, F=8.3, p=0.015, df=1), and although CO_2 affected its concentration, it was no significant (MANOVA, F=3.2, p= 0.07, df=2); furthermore the CO_2-temperature interaction on EA was significant (MANOVA, F=6.1, p= 0.016, df=2). Bars show standard deviation (n=3, with exception of the 180 CO_2-19°C treatment where n=2).

The PCA of the AA composition of the diatom showed a higher similarity EA composition at high temperature (PC1, 39%) and intermediate CO_2 level, although its influence was comparatively smaller (PC2, 27%) (Fig. 5a). The axis loads showed that EA abundance strongly drove the differences in the AA profile composition of the treatments on both axis (Fig. 5b).

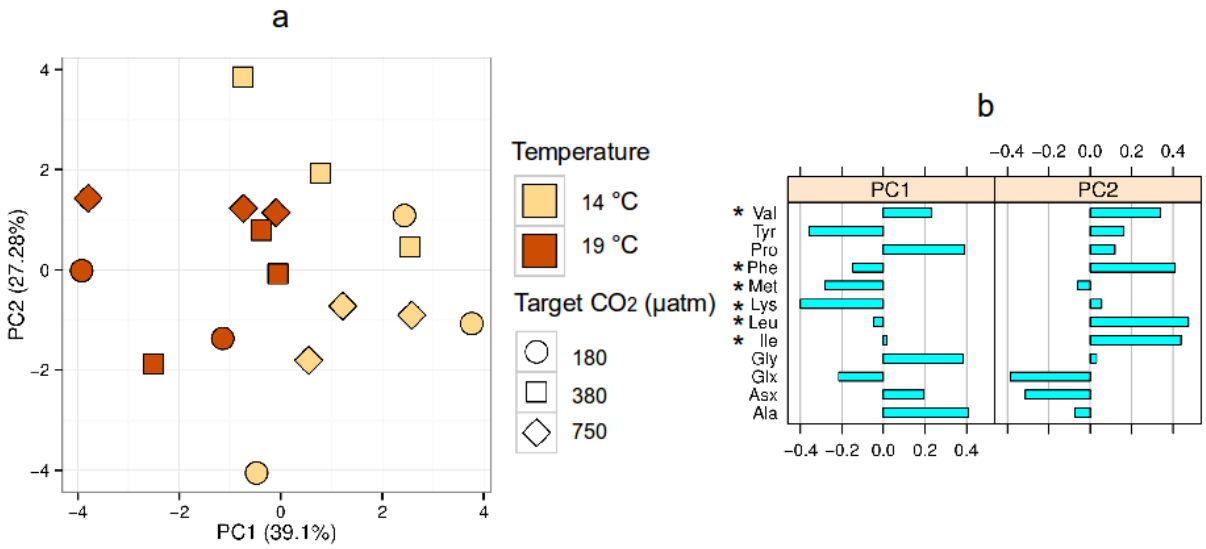

Figure 5. a) Principal component analysis [PCA] of the amino acids [AA] above 1% in abundance of the diatom *Cylindrotheca fusiformis* cultured under different CO_2 conditions and temperatures for >250 generations. The AA profile showed that a higher simmilarity of essential amino acids [EA] composition at high temperature (PC1) and intermediate CO_2 level (PC2), although the CO_2 influence was comparatively smaller. b) Axis loads of the PCA analysis. The EA had an strong influence in the variance of both axis. *indicate EA.

Discussion

Our results showed that the relative FA and AA content in the diatom *C. fusiformis* are affected by CO_2 and temperature, producing contrasting biochemical profiles between the different treatments.

Growth rates

The growth rate of *C. fusiformis* was constant between the different treatments, indicating that the cells were able to compensate for the environmental stress that caused low CO_2 levels. This is consistent with observations of the marine diatoms *Stephanopyxis palmeriana*, *Ditylum brightwellij* and *Coscinodiscus sp*., which were kept a constant growth rate even at very low CO_2 concentrations (Goldman, 1999). This resilience in growth rate under low CO_2 conditions can be attributed to the presence of a highly efficient carbon concentration mechanism (CCM) in the diatoms (Burkhardt et al., 2001). The CCM is an energy consuming process whose function is to transport and accumulate dissolved organic carbon (DIC) (CO_2 and HCO_3^-) within the cell, where DIC is

fixed into organic compounds by the Rubisco enzyme (Giordano et al., 2005). We would expect that an up-regulated CCM in *C. fusiformis* due to low CO_2 levels have help in keeping up constant growth rates between treatments (Burkhardt et al., 2001). Although temperature slightly increased the growth rates of the algae this difference was not significant.

Fatty acids

The PUFA group showed a maximum in abundance at current environmental CO_2 and temperature conditions, decreasing at values above or under them. This indicates that CO_2 had a stronger influence than temperature on the relative algal PUFA content. Our observations of the *C. fusiformis* FA profile are somewhat consistent with results reported in other diatoms. A recent study showed that elevated CO_2 significantly changed the FA concentration and composition of the diatom *Thalassiosira pseudonana*, causing a significant PUFA decline of ~20% in the algae cultured under elevated (750 µatm) compared to present day (380 µatm) CO_2 levels (Rossoll et al., 2012). Along the same lines, a recent report on Antarctic sea ice diatom *Nitzschia lecointei* on the synergism between temperature and CO_2 in the algae showed that cellular PUFA content was reduced by a 40% at 960 µatm CO_2 relative to 390 µatm pCO_2 when cultured at −1.8 °C, and the PUFA content was reduced by a 50% at 2.5°C cultures in relation to the −1.8 °C treatment, although there were no differences between both CO_2 levels at 2.5◦C (Torstensson et al., 2013). Our results show a decay of ~3% in PUFA content in the 750 µatm manipulation in relation to the 380 µatm CO_2 treatment on both temperature levels which is much smaller in relation to the above mentioned studies. The reason for such a difference can be due to species-specific responses to CO_2 within the same taxonomic group, which would help to compensate the deleterious CO_2 effect in the algal PUFA content (Fabry, 2008). A similar situation was observed in several green algae on a study by Tsuzuki et al. (1990) who reported a strong influence of high CO_2 in the FA of *ChIorella vulgaris* 11 h cells and similar but smaller changes in *Chiamydomonas reinhardtii* and *Dunaliella tertiolecta*. The low amount of PUFA observed in the 180 µatm CO_2 treatment in relation to the 380 µatm CO_2 cultures (difference of ~6% at 14°C and ~4% at 19°C) can be attributed to carbon limitation at low CO_2 levels, which may force a relocation of the limited carbon available towards other

essential processes like cell division, which was kept constant by *C. fusiformis* among the different treatments. This relocation might be done even if this is in expense of physiologically important biomolecules as PUFA and EA (see Fig. 2b). Although the mechanisms through which CO_2 affects algal FA are unclear, it has been proposed that an elevate SFA level in cells grown at high-CO_2 conditions is due to enhanced FA synthesis and accumulation (Sato et al., 2003). It has been observe that environmentally high CO_2 levels and related low pH can severely alter the cellular internal pH and therefore the tightly regulated homeostasis (Lane et al., 1981; Suffrian et al., 2011). Therefore an elevate SFA synthesis under high CO_2 conditions can be a mechanism to produce more rigid cell wall, with membranes built of short-chain FA, making them less fluid and permeable, helping in the regulation of the cell internal homeostasis (Rossoll et al., 2012).

Our results in general did not show a significant temperature-related effect in the FA composition of *C. fusiformis*, at 380 and 750 µatm CO_2. However, the 180 µatm CO_2 treatment showed a higher amount of PUFA (and lower SFA) at 19°C in relation to the 14°C treatment; this observation agrees with results in the diatom *Chaetoceros muelleri* that exhibited a decrease in relative PUFA content when cultured at 35°C in contrast to 40°C treatments (Rousch et al., 2003). These results are contradictory with observations of PUFA concentrations being in general inversely proportional to temperature (Sukenik et al., 1993; Torstensson et al., 2013, Van Wagener et al., 2013). This discrepancy may be due to the stress in the algae due to the low DIC in the culture media. Further research is required to clarify the underlying mechanisms of FA composition response to temperature effects.

Amino acids

Overall there is scarce data regarding the effects of CO_2 in algal AA composition, and none within the range of future CO_2 projections. The analysis of *C. fusiformis* showed a higher EA content at 380 µatm CO_2 levels in relation to the 750 µatm CO_2 treatment (~6%) at 14°C. This somewhat agree with observations of the green algae *Chlamydomonas reinhardtii* when comparing low (0.04%) to high (5%) CO_2 enriched cultures, which showed that five out of six EA were significantly lower at the high CO_2

treatments (Renberg et al., 2010). This EA change in *C. reinhardtii* was attributed to a down-regulation of the proteins related to the CCM mechanisms at high CO_2 levels (Renberg et al., 2010). We would expect a similar mechanism behind the EA shift observed in *C. fusiformis* as diatoms have been described as possessing highly efficient CCM, which are effectively down-regulated by elevated CO_2 concentrations (Burkhardt et al., 2001). It is noteworthy that there were lower EA amounts at the 180 µatm CO_2 level than in the other CO_2 treatments; this result, like in the case of PUFA, can be due to carbon limitation and relocation into other essential biological processes. Although in this case it is more likely that the down-regulated proteins did not belong to the algal CCM complex in order to keep up a high DIC uptake and constant growth. A more detailed study would be required to clarify the AA metabolic state of the algae under low CO_2 conditions.

Our observations regarding the temperature effects in the relative *C. fusiformis* AA composition showed that EA were more abundant at 19°C in relation to the 14°C. These results agree with findings in the green algae *Chlorella* strain MFD-1 and *Nannochloropsis* strain MFD-2, both of which showed a continuous increase in relative EA concentration in a temperature gradient ranging from a ~31% and a ~33% at 15°C respectively, up to ~36% at 25°C in the first algae, and to ~38% at 35°C in the second (James et al., 1989). The results of both studies indicate that higher temperatures increase the EA content in algae.

The lack of CO_2-related differences in relative EA content between the treatments at 19°C indicates that temperature have a greater influence in the algal EA profile than CO_2 and can overrun any CO_2-related effect in the relative algal EA content below and above the current 380 µatm CO_2 levels. This could be due to temperature regulating a higher number of physiological processes in the cell than CO_2. Consequently the amount of synthesized protein and AA by the temperature-dependent pathways might be higher than the produce by CO_2-dependent mechanisms. For instance, three putative sequences of small heat shock proteins (sHSPs) were identified within the genome of the diatom *Thalassiosira pseudonana* (Waters & Rioflorido, 2007). These proteins are synthesized when the cells are subjected to temperature stress and seem to act as

chaperones that can protect other proteins against heat-induced denaturation and aggregation, and also have important non-stress roles within the cell (Waters & Rioflorido, 2007). Nevertheless all this does not explain why proportionally more EA are produced at higher temperatures, as for instance the identified sHSPs in *T. pseudonana* have roughly a 50% EA content (Waters & Rioflorido, 2007, data not shown). A detailed study of the AA metabolism in the algae will be required to clarify this effect.

In general the observed CO_2 influence on the FA profile of *C. fusiformis* is comparatively small in relation with previous studies. A possible reason for such a result is that the diatom has undergone adaptive evolution. Recently Lohbeck et al. (2012) showed that the CO_2 sensitive coccolithophore *Emiliania huxleyi* was able to partly restore its calcification and growth rates through adaptive evolution when kept over 500 generations under high CO_2 levels. Our cultures were kept for >250 generations at high CO_2, therefore there is the possibility that any deleterious high CO_2-related effect in the algal FA and EA profiles might have been compensate through adaptive evolution (Lohbeck et al., 2012) and the algae have been able to eventually restore its original FA profile. However, if that is the case, the restoration was not total as there were significant differences between the different CO_2 and temperature treatments in both FA and EA.

It has been shown that a PUFA decrease of 19% in the diatom *Thalassiosira pseudonana* kept at 750 µatm CO_2, constrained growth and reproduction of the copepod *Acartia tonsa* when feed with them. The PUFA decay by a 29% in the copepod and caused a decrease in both somatic growth and egg production of ~85% (Rossoll et al., 2012). In the same line, EA have an strong influence on aquatic grazers as observed in the copepods *Calanus finmarchicus* subjected to different diets. It has been shown that the highest cumulative egg production typically correlates with the pool of EA in the food source, and that a high abundance of EA in the copepod diet promoted high fecundity and egg hatching success (Helland et al., 2003). The present manuscript is the first to report deleterious effects of temperature and CO_2 in the EA of a primary producer, and corroborate previous finding of their effects on essential PUFA. Although the magnitude of these effects in the *C. fusiformis* FA are comparatively modest in relation to previous

reports, the consequences of even smaller changes in primary producers can be amplified in higher trophic levels. This can be observed in the results from Rossoll et al. (2012) where the CO_2 impact in the prey diatom PUFA was amplified 1.5 times in the copepod PUFA. Furthermore only about 20% of essential macromolecules like PUFA (and possibly EA) are incorporated into new biomass of organisms of the next trophic level (Gladyshev et al., 2011). Thus, the consequences of deleterious temperature and CO_2 effects in primary producers can be far reaching in marine food webs since essential macromolecules are sequentially incorporated into larval fish (Fraser et al., 1989; Izquierdo et al., 2001).

Nonetheless, the effect of temperature and CO_2 on the algal food quality for higher trophic levels will depend on the sensitivity of the different primary producers and on how OA affects the specie composition of plankton assemblages because the different algae taxa posses contrasting biomolecular compositions (Dunstan et al., 1992; Viso & Marty, 1993; Zhukova & Aizdaicher, 1995) and sensitivities to CO_2 (Sato, 1989; Tsuzuki et al., 1990; Sato et al., 2003).

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Supplementary information

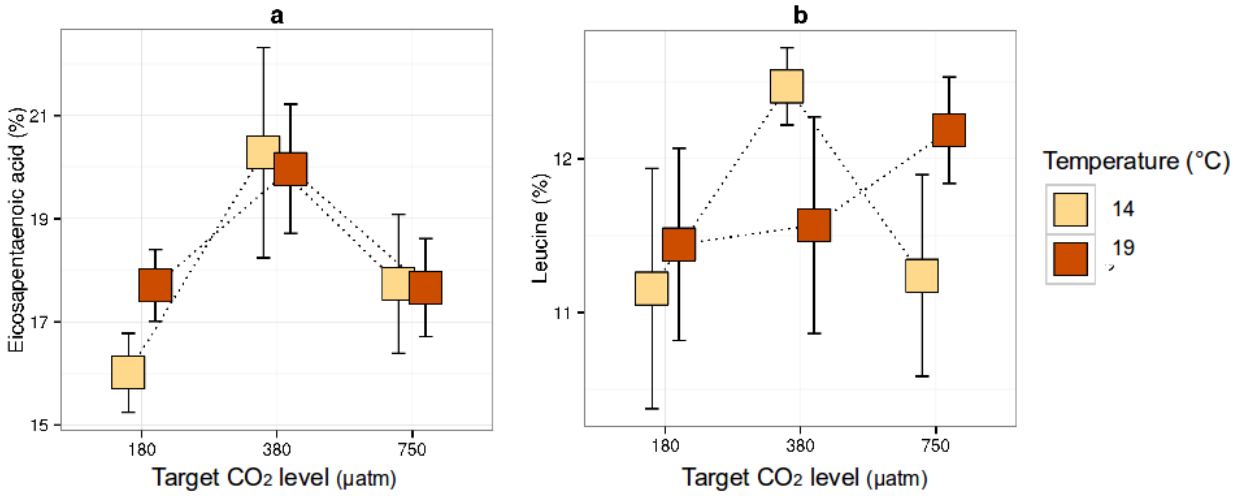

Figure S1. Relative content of (a) Eicosapentaenoic acid [EPA] and (b) Leucine [Leu], the most abundant PUFA and EA, respectively, in the diatom *Cylindrotheca fusiformis* cultured under different CO_2 conditions and temperatures for >250 generations. The EPA (a) showed significant differences in relation with CO_2 (MANOVA, F=11.02, p= 0.001922, df=2), while temperature and its interaction with CO_2 were not significant (p>0.05). The Leucine (b) showed no significant differences between temperature or CO_2 (p>0.05), however the interaction of temperature and CO_2 was significant (MANOVA, F=3.7, p= 0.057, df=2). Bars show standard deviation (n=3, with exception of the 180 CO_2-19°C treatment in (b) where n=2).

4.3 Ocean acidification-induced food quality deterioration constrains trophic transfer

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Ocean Acidification-Induced Food Quality Deterioration Constrains Trophic Transfer

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Abstract

Our present understanding of ocean acidification (OA) impacts on marine organisms caused by rapidly rising atmospheric carbon dioxide (CO_2) concentration is almost entirely limited to single species responses. OA consequences for food web interactions are, however, still unknown. Indirect OA effects can be expected for consumers by changing the nutritional quality of their prey. We used a laboratory experiment to test potential OA effects on algal fatty acid (FA) composition and resulting copepod growth. We show that elevated CO_2 significantly changed the FA concentration and composition of the diatom *Thalassiosira pseudonana*, which constrained growth and reproduction of the copepod *Acartia tonsa*. A significant decline in both total FAs (28.1 to 17.4 fg cell^{-1}) and the ratio of long-chain polyunsaturated to saturated fatty acids (PUFA:SFA) of food algae cultured under elevated (750 µatm) compared to present day (380 µatm) pCO_2 was directly translated to copepods. The proportion of total essential FAs declined almost tenfold in copepods and the contribution of saturated fatty acids (SFAs) tripled at high CO_2. This rapid and reversible CO_2-dependent shift in FA concentration and composition caused a decrease in both copepod somatic growth and egg production from 34 to 5 eggs female^{-1} day^{-1}. Because the diatom-copepod link supports some of the most productive ecosystems in the world, our study demonstrates that OA can have far-reaching consequences for ocean food webs by changing the nutritional quality of essential macromolecules in primary producers that cascade up the food web.

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Introduction

Anthropogenic emissions of carbon dioxide (CO_2) and its uptake by the surface ocean cause profound changes in marine carbonate chemistry, including seawater acidification and lowering of the calcium carbonate saturation state [1], [2]. Contemporary surface ocean pH has decreased on average by 0.1 units due to CO_2 invasion since preindustrial times. According to IPCC projections atmospheric partial pressure of CO_2 (pCO_2) is expected to further increase from current ~390 µatm to ~760 µatm, corresponding to a drop in mean oceanic surface pH by 0.3 to 0.4 units until the end of the 21st century ('business-as-usual scenario' [3], [4]). This change in carbonate chemistry, termed ocean acidification (OA), is thought to primarily affect calcifying organisms building their shells and skeletons of calcium carbonate [5], [6], [7]. Biological effects of OA on non-calcifying organisms are diverse and often highly species-specific [8].

Our present understanding of potential OA impacts is almost entirely limited to single species responses, while OA consequences for food web interactions remain poorly understood. Indirect impacts through trophic interactions are expected because OA may change the biochemical composition of primary producers that affects nutritional food quality for consumers. Increased CO_2 can stimulate carbon fixation by photosynthetic organisms and thereby reduce the nutrient content relative to carbon [9], [10], [11], which determines the food quality for herbivores [12]. Enhanced carbon consumption relative to nutrients under elevated CO_2 conditions [13], [14] can cause an imbalance between phytoplankton stoichiometric composition and consumer nutrient demand for somatic growth [11]. Besides elemental stoichiometry, fatty acid (FA) associated food quality is a critical factor that regulates the energy transfer between primary producers and consumers [15], [16], because essential FAs cannot be synthesized *de novo* by heterotrophic organisms and have to be acquired through the diet. In particular long-chain polyunsaturated FAs (PUFAs) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) play an important role in growth, development and reproduction success in heterotrophs [17], [15]. OA may impact phytoplankton FA synthesis because extracellular pH is known to affect various intracellular physiological parameters [18] that influences enzyme activity.

The classic diatom-copepod-fish link in the ocean supports some of the most productive ecosystems in the world and is an important source of highly nutritious food for upper trophic levels.

Experimental studies indicate a weak sensitivity of primary production to CO_2 [14] and no direct effects on copepod growth and hatching success at CO_2 levels within the range expected by the end of this century [19], [20]. However, CO_2 may indirectly affect zooplankton growth through its potential impact on the nutritional quality of phytoplankton, their major food source. To test this hypothesis, we independently manipulated CO_2 concentration in both diatoms used as food algae and copepod cultures, and investigated dietary OA effects on copepod growth and reproduction. The experiment consisted of a two-by-two factorial design crossing two CO_2 levels in food algae media and seawater used for copepod growth. The cryptophyte *Rhodomonas* sp. and diatom *Thalassiosira pseudonana* were used as food source and the copepod *Acartia tonsa* as consumer. We determined resulting FA composition of both alga and copepod as well as copepod development and reproduction. Our experiment showed that elevated CO_2 affected biochemical composition of the diatom that constrained copepod growth performance.

Methods

CO_2 manipulation and experimental design

The target values for experimental CO_2 manipulation were 380 µatm for the low (L) and 740 µatm for the high (H) CO_2 treatment. Phytoplankton (P) was grown at both L and H pCO_2 concentrations and fed to copepod zooplankton (Z) grown in seawater at the same L and H target levels in a crossed design. It is important to note that biological activity, such as photosynthesis and respiration, alter the carbonate system. In addition, water exchange and combining treatments with low and high CO_2, as was done in the copepod growth experiment, can result in deviations from the target CO_2 levels. Nevertheless, pCO_2 levels of L and H treatments were maintained close to target values and differences among treatments persisted throughout the experiment (Figure S1).

Rhodomonas sp. and *T. pseudonana* were cultured as food sources in artificial seawater at pCO_2 of ~495±100 SD (L) and ~760±110 (H) for *Rhodomonas* sp. and ~365±120 (L) and ~915±270 (H) µatm for *T. pseudonana*, respectively. Juvenile copepods were fed with *Rhodomonas* to ensure optimal growth of the first developmental stages and *T. pseudonana* was used as food source after copepodite stage 1. The carbonate system of *T. pseudonana* cultures was manipulated by combined additions of sodium carbonate (Na_2CO_3) and hydrogen chloride (HCl) at constant alkalinity; the two CO_2 treatments for *Rhodomonas* cultures were continuously aerated with CO_2-enriched air. Algae were grown in laboratory batch cultures on a 18:6 light:dark cycle with replete nutrients. To investigate the response time of algae fatty acid composition alterations to changing pCO_2, *T. pseudonana* was grown at high (~1120 µatm) pCO_2 for five days and then transferred to a low (~380 µatm) pCO_2 media. FA concentration was measured every five hours over a 30 h time period.

Acartia tonsa eggs were hatched in seawater under pCO_2 conditions of ~380 µatm. After the nauplii reached developmental stage 2, they were transferred into 2-L NALGENE bottles (1000 individuals L^{-1}) filled with seawater (salinity 18.2) from a tank that was aerated continuously with appropriately CO_2-enriched air of ~495±100 (L) and ~760±110 (H) µatm pCO_2, respectively. Copepod zooplankton (Z) were fed with CO_2 preconditioned phytoplankton (P) at about 1000 µg C L^{-1} in a factorial design with four treatment combinations: P_L/Z_L, P_L/Z_H, P_H/Z_L and P_H/Z_H, each with three replicates. Water and food during the copepod growth experiment were replaced every other day. All replicates were randomly placed in a temperature-controlled culture room at 18°C and 14:10h light:dark cycle until the copepods reached adult stage. Over the course of the experiment, developmental stages were identified and at the end of the growth experiment egg production of females measured over 24 h and hatching success of eggs and nauplii morphological formation observed for two days. Species involved for this experiment were lab cultures and thus no specific permits were required for the sample collection.

Dissolved inorganic carbon (DIC) was measured after every water exchange and pH was recorded daily during the copepod growth experiment. For DIC the water was smoothly filtered via syringe and a 0.2 µm pre-filter and stored in 4 ml borosilicate flasks at 4°C. The sample flasks were closed with a plastic screw cap and a Teflon septum. DIC was determined photometrically with an auto-analyzer (QUAATRO, Bran & Lübbe) at a precision of ±20 µmol kg^{-1} [21], [22]. DIC and pH were used for seawater carbonate system calculations (Text S1). During the copepod growth experiment measured mean (±SD) pH values were 8.14±0.12 and 7.94±0.08, and for DIC 480±110 and 725±140 µatm CO_2 in the L and H treatment, respectively (Figure S1). DIC values in the crossed treatments were 485±80 (P_H/Z_L) and 745±80 (P_L/Z_H) µatm CO_2. Due to the fact that NBS based pH measurements are rather weak for reliable carbonate chemistry calculations, total alkalinity (TA) measurements (Text S1) were taken two times per week for crosscheck calculations. Values for pCO_2 calculated from pH and DIC differed from pCO_2 calculations using DIC and TA on average ~110 and ~210 µatm at the low and high CO_2 treatment level, respectively, over the duration of the experiment. These uncertainties are probably higher than the real error since they were caused by outliers in TA and DIC measurements to which the carbonate system is relative insensitive when pH is involved in the calculations.

FA composition of *T. pseudonana* was analyzed from the stock culture during exponential growth phase and of copepod females at the end of the experiment. FAs were measured as fatty acid methyl esters (FAMEs) with a Thermo GC Ultra gas chromatograph equipped with a nonpolar column (RXI1-SIL-MS 0.32 µm, 30 m) using a flame ionization detector (FID). A complete method description is provided in Text S1.

Statistical analysis

Algal responses to experimental conditions were assessed using two-tailed *t*-tests. Differences in copepod FA classes and egg production between treatments were tested using analysis of variance (ANOVA). A Tukey HSD post hoc test was used to assess differences among treatments in egg production. Generalized linear models (GLM) were used to examine the effect of the seawater pCO_2 used for algal and copepod cultures on the relative proportion of FA classes in copepods. Principal component analysis (PCA) was used to assess the difference in individual FA composition of the diet algae and copepods across the treatment combinations. For algal food, log-transformed FA concentration per cell and for copepods arcsine-square root transformed percentage of total FA was used since the proportion of FA classes varies between *T. pseudonana* and *A. tonsa* (see Figure 1). Each FA was standardized by subtracting its mean and dividing by its standard deviation, assembling the resulting standardized series into a 15-FA by 20-treatment combination data matrix. The PCA used a covariance matrix and Varimax rotation. This analysis identified FA that explained most to the observed variance. Statistical analyses were performed using Statistica and the R software environment 2.14.1 [23].

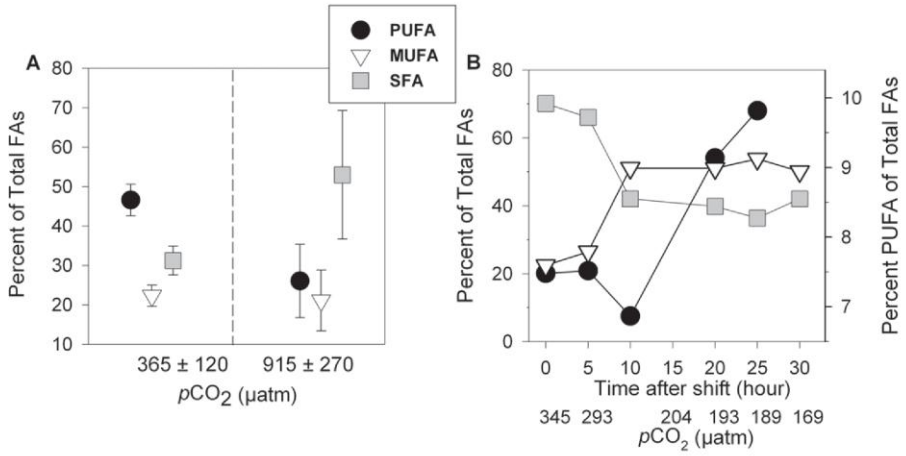

Figure 1. Fatty acid composition and concentration of *Thalassiorira pseudonana* cultured at different CO_2 treatments. A) Percentage of polyunsaturated (PUFA), monounsaturated (MUFA), and saturated (SFA) fatty acids relative to total fatty acids during the exponential growth phase cultured at low (realized value of 365 µatm pCO_2, n = 5) and high (realized value of 915 µatm pCO_2, n = 3) CO_2 treatments used as copepod food source. **B**) Change in the fatty acid composition in *T. pseudonana* after a shift from high to low pCO_2 conditions (n = 1 per treatment level). Time 0 are measured values before the culture media shift. Error bars indicate standard errors.
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Results and Discussion

Our experiment showed that CO_2 concentration significantly changed FA concentration and composition in the diatom *T. pseudonana* used for copepod diet. The relative amount of PUFAs was significantly lower ($t = 4.48$, $p = 0.004$) and the amount of SFAs higher ($t = -3.37$, $p = 0.015$) at high pCO_2 compared to the low pCO_2 treatment (Figure 1A). Essential PUFA concentrations were significantly reduced at high pCO_2 (Table S1), specifically DHA (22:6*n*-3; $t = 2.81$, $p = 0.03$) and the group ARA-EPA (20:4*n*-6, 20:5*n*-3; $t = 6.63$, $p < 0.001$). A shift in FA composition at projected future CO_2 levels is consistent with observations in the coccolithophorid *Emiliania huxleyi* [24] and with green algae and prymnesiophyte experiments conducted at extreme CO_2 changes [25], [26], [27].

A separate experiment confirmed that the shift in FA occurred rapidly in response to changing pCO_2 in the diatom *T. pseudonana*. When transferred from high to low CO_2, FA composition was already significantly different from its initial composition after 15 h (Figure 1B) and FA components changed in the same direction as observed at constant high and low pCO_2 treatments. Similarly, a rapid transition in FA composition can be expected when algae are transferred from low to high pCO_2, which was, however, not tested in our experiment. Though, a rapid reversible FA response to changing pCO_2 concentration has been reported in green algae [26]. The higher unsaturation levels of FAs in algae cells cultured at low pCO_2 compared to cells at high pCO_2 has been suggested to be partially a consequence of repressed FA synthesis, which promotes the desaturation of pre-existing SFAs [26]. Recently it has been proposed that pH might act as a regulation signal for the formation of cell membranes, which are mainly composed of fatty acids, by controlling the production of its synthesizing enzymes [28]. A high environmental pCO_2 (low pH) can decrease the internal cell-pH [29]. Therefore the increased amount of SFAs could be a mechanism to control the internal cell-pH, as a membrane built of short-chain FAs is less fluid and permeable to CO_2. However, the cellular processes involved in FA synthesis under changing pH or pCO_2 levels are not fully understood.

Similar to FA modification in algal food, FA concentration and composition of adult copepods varied significantly between CO_2 treatments. The mean ±SD total amount of FAs in *A. tonsa* was significantly different across treatments ($F_{(3,\ 8)} = 5.15$, $p = 0.028$) and higher when raised and fed with algae cultured at low pCO_2, with 8.9±5.6 ng ind.$^{-1}$ compared to 0.8±0.2 ng ind.$^{-1}$ when both copepods and algal diet were cultured at high pCO_2 and to 2.3±0.5 ng ind.$^{-1}$ in the crossed treatment combinations (Table S1). Copepods raised and fed with algae at low pCO_2 contained high proportions of PUFAs relative to total FAs that are in the same range with reports in marine calanoids [30]. The PUFA fraction in copepods decreased from more than 30% at low pCO_2 to less than 5% at high pCO_2 ($F_{(3,\ 8)} = 54.51$, $p < 0.001$) (Figure 2A). The long-chain highly unsaturated FAs DHA and ARA-EPA, which are important components for growth and reproduction of consumers [31], decreased from 15% in copepods raised at low pCO_2 below detection limit in those at high pCO_2 (Table S1). Similarly, the proportion of MUFAs (monounsaturated fatty acids) varied significantly across treatments ($F_{(3,\ 8)} = 8.2$, $p = 0.008$) and decreased from around 20% at low pCO_2 to less than 10% at high pCO_2. On the other hand, the relative amount of SFAs tripled in copepods at high pCO_2 (Figure 2A) and FA compositions were different between treatments ($F_{(3,\ 8)} = 26.22$, $p = <0.001$).

Contrary to our expectation, FA composition in copepods differed between individuals raised at low and high seawater pCO_2, irrespective of the CO_2 level of their algal diet (Figure 2A). Because consumers are unable to synthesize PUFAs we expected that copepod FA composition in the crossed pCO_2 treatments of copepod culture and food algae (P_L/Z_H, P_H/Z_L) would reflect changes in FA of their diet. Principal component analysis (PCA) of individual FAs in diet algae and copepods also showed distinctive clustering of the copepod groups raised at low and high pCO_2 treatments, irrespective of the CO_2 conditions of their diet algal culture (Figure 2B), which was mainly explained by PUFAs and SFAs (Figure S2). A GLM model supported that CO_2 concentration of the seawater used to raise copepods significantly negatively affected the relative proportion of PUFAs ($p < 0.001$) and positively affected the proportion of SFAs in copepods ($p < 0.001$), which was

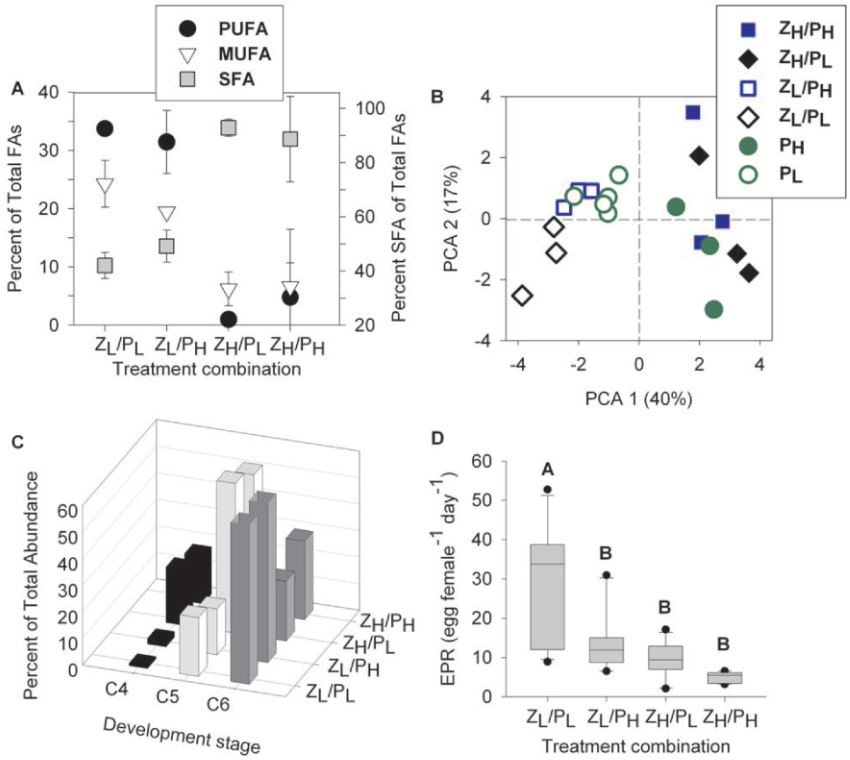

Figure 2. Fatty acid composition, somatic growth and reproduction of *Acartia tonsa* across CO_2 treatment combinations. A) Percentage of polyunsaturated (PUFA), monounsaturated (MUFA), and saturated (SFA) fatty acids relative to total fatty acids in female copepods. **B**) Principal component analysis (PCA) of fatty acid composition for the dietary algae *Thalassiorira pseudonana* and *A. tonsa* of the different treatment combinations. PCA scores 1 explained 40% of the variability (see *x*-axis of c) and was highly negatively correlated with 22:6*n*-3 ($r^2 = 0.73$), 20:4*n*-6+20:5*n*-3 ($r^2 = 0.85$), 18:3*n*-6 ($r^2 = 0.73$) and 16:1 ($r^2 = 0.79$), and positively with 22:1*n*-9 ($r^2 = 0.25$) and 18:1*n*-9*t* ($r^2 = 0.57$). PCA score 2 explained 17% of the overall variability (see *y*-axis of c) and was strongest positively correlated with 24:0 ($r^2 = 0.84$). Loadings of the PC scores are shown in Figure S2). **C**) Stage distribution of *A. tonsa* individuals at day 10. C4, C5, C6 = copepodite stage 4, 5, and adult, respectively. **D**) Egg production rate (EPR) of incubated females (n = 12 per treatment level). EPR was significantly different between treatments ($F_{(3, 44)} = 18.02$, $p<0.001$). Different letters above bars represent significant differences from a Tukey HSD test. The bars represent the 25th, 50th and 75th percentiles, whiskers stand for the 10th and the 90th percentiles and black points show outliers. Legend refers to treatment combinations of copepod zooplankton (Z) and phytoplankton food source (P) at low (L) and high (H) pCO_2.
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consistent across combinations and not dependent on the pCO_2 level of the algal culture.

These findings suggests that the FA composition of algae changed rapidly when transferred from low pCO_2 culture media to high pCO_2 seawater used to raise copepods and *vice versa*. Since consumers are unable to synthesize PUFAs [30] and previous experiments showed that copepod growth is rather insensitive to CO_2 levels within OA predictions [19], [20], direct CO_2 effects on copepod FA synthesis seem unlikely. In our experiment, water and food was exchanged every second day and algae were in their exponential growth. Thus, we rather expect that high turnover rates and the ability of *T. pseudonana* to rapidly change the FA composition in a variable pCO_2 environment (Figure 1B) are responsible for an adjustment in FA composition in the crossed treatments within the first day. Rapid modification in algae FA and the fact that *A. tonsa* has no lipid reserves [32] likely explains the absence of the influence from the algae culture media pCO_2 on copepod FA composition within both crossed treatment combinations.

The CO_2-dependent dietary shift in FAs had a significant effect on *A. tonsa* growth and development. Copepods of the same age (10 d) showed a delay in stage development of 1 to 2 days at high pCO_2 (Figure 2C). Egg production decreased from a median of 34 eggs female^{-1} d^{-1} at low water and food pCO_2 to less than 12 eggs female^{-1} d^{-1} in all other treatments, with the lowest production (5 eggs female^{-1} d^{-1}) at high water and food pCO_2 (Figure 2D). The egg production rate was significantly related to the ratio of PUFA:SFA and the content of DHA and ARA-EPA within the female copepods (Table 1), consistent with other observations in zooplankton [33]. Copepod egg production raised at low pCO_2 and fed with algae grown at high pCO_2 produced significantly less eggs compared to copepods in the low pCO_2 treatment combination (Figure 2D). This significant decline is most likely a result of the overall lower copepod FA quantity when fed with algae cultured at high CO_2 compared to food at low CO_2 (Table S1). Given that adult *A. tonsa* females invest the majority of their lipids into reproduction [34], the significant decrease of essential PUFAs due to low quality food algae is most likely the reason for

Table 1. Regression statistics of *Acartia tonsa* egg production as a linear function of fatty acid composition.

Fatty acid	Slope	*Y*-intercept	r^2	*p-value*
PUFA (%)	0.03	1.9	0.52	0.013
MUFA (%)	0.07	1.6	0.73	**<0.001**
SFA (%)	−0.02	3.8	0.60	**0.005**
PUFA:SFA	1.3	1.9	0.59	**0.006**
ARA-EPA (ng cop^{-1})	0.68	2.06	0.67	**0.002**
DHA (ng cop^{-1})	1.23	1.92	0.77	**<0.001**

Bonferroni-corrected significance levels for multiple fatty acid comparisons were $\alpha = 0.008$ (0.05/6). Significant correlations are highlighted in bold; n = 11. PUFA = polyunsaturated fatty acid; MUFA = monounsaturated fatty acid; SFA = saturated fatty acid; ARA-EPA = 20:5*n*3; DHA = docosahexaenoic acid (22:6*n*3).
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the considerable decline in egg production observed in the high pCO_2 treatment combinations (Figure 2).

Some studies showed that the disruption of diatom cells induced by feeding triggered the transformation of unsaturated FAs into aldehydes causing adverse effects on copepod development, egg production and hatching success [35]. Even though several diatom species are known to possess these deleterious effects, *T. pseudonana* has recently been reported not to produce aldehydes [36]. We also could not find any malformations of the hatched nauplii by microscopic observations (data not shown), suggesting that negative aldehyde effects were not present.

Our study suggests that OA can have important consequences for consumer growth and production by affecting the nutritional quality of primary producers that translates to higher trophic levels. These results are consistent with experiments on freshwater cladocerans, fed with algae from an acidic lake [37], suggesting that our results are not restricted to monospecific laboratory cultures and may be expected at community level. However, future experimental manipulations are required to clarify the widespread response of phytoplankton biochemical composition to ocean acidification at relevant pCO_2 levels in other taxonomic groups and natural communities. It can be expected that trophic upgrading and differential algae sensitivity to pCO_2 at the community and ecosystem level may compensate for low food quality observed at the single species level. Moreover, the tolerance to pCO_2 and pH might be lower for monocultures compared to natural populations, which have high ecophysiological variability [38] and genetic diversity, important for adaption to various environmental factors [39]. Nonetheless, shifts in FA composition as a response to changing CO_2 have been documented in other phytoplankton species [26], [40], and FA-responses in phytoplankton as observed here might be important during bloom periods if CO_2 sensitive organisms dominate.

The effect of OA on nutritional quality in the diatom-copepod food chain relationship observed in our study may have far reaching consequences for food webs since FAs originating in phytoplankton are sequentially incorporated into the total lipid fraction of zooplankton and triacylglycerol of larval fish [41]. Given that fish is a critical natural resource [42], acidification-driven food quality deterioration may impair fish production by changing the biochemical composition of food algae and its transfer to higher trophic levels [43], [44]. While it is difficult to extrapolate from monocultures to community level, these results point to the likelihood that OA consequences go beyond direct physiological impacts and that indirect effects through trophic interactions need to be considered.

Supporting Information

Figure S1 Carbonate system over the course of the copepod growth experiment.
(DOCX)

Figure S2 Loadings for Principal Component Analysis (PCA) of fatty acids for *Thalassiosira pseudonana* and *Acartia tonsa*.
(DOCX)

Text S1 Full material and methods description.
(DOCX)

Table S1 Amount of fatty acids of the food algae *Thalassiosira pseudonana* and the copepod consumer *Acartia tonsa* at different CO_2 treatment combinations.
(DOCX)

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Author Contributions

Conceived and designed the experiments: DR RB KS UR US MW. Performed the experiments: DR RB. Analyzed the data: DR RB HH MW. Contributed reagents/materials/analysis tools: DR RB HH MW. Wrote the paper: DR RB MW. Helped with writing the manuscript: HH KS UR US..

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Suplemmentary information

Text S1. Full material and methods description

Manipulation of the carbonate chemistry

The target values for experimental CO_2 manipulation were 380 µatm in the low (L) and 740 µatm pCO_2 in the high (H) CO_2 treatment. To adjust seawater and the culture media to the different pCO_2 levels, the carbonate system was manipulated using two methods, depending on the sensitivity of the organisms to pCO_2 manipulation [1]: 1) For *Thalassiosira pseudonana* cultures, carbonate chemistry was adjusted by combined additions of sodium carbonate (Na_2CO_3) and hydrogen chloride (HCl), manipulating dissolved inorganic carbon (DIC) while keeping total alkalinity (TA) constant because diatoms can be negatively affected by aerating. Artificial seawater was used as culture media (recipe after [2]). 2) For copepods and *Rhodomonas salina* used as nauplii diet, pCO_2 was manipulated by aeration of seawater and culture media at a target pCO_2 concentration. This was achieved by bubbling culture vessels with a mixture of CO_2 and air with the respective target values. The difference between acid addition and bubbling is a small difference in HCO_3 concentration, while pH and concentrations of CO_2 and CO_3 are the same [1].

<u>CO_2 enrichment of seawater stock solution for the copepod growth experiment</u>

Baltic seawater from Kiel Fjord was filtered with a 0.2 µm SartobranP sterile MidiCap membrane filter and stored in two 300 L tanks, bubbled continuously with aeration stones using ambient air (pCO_2 = 380 ppm) and CO_2-enriched air (pCO_2 = 740 ppm). All CO_2 enrichments were undertaken with a CO_2-gas mixing system (Linde GMZ 750 IRA infrarot absorption sensor). The seawater tanks (salinity 18.2) were closed with a rounded plexiglass lid (Adolf Richter GmbH) and stored in darkness in a temperature-controlled culture room at 18°C. Water pH was monitored daily using a WTW 340i pH-analyzer connected to a SENTIX-81 electrode. The electrode was calibrated with standard WTW buffer 4 and 7 before each measurement set.

Copepod growth experimental set-up

The copepod *Acartia tonsa* was used as consumer. *A. tonsa* eggs obtained from the Alfred Wegener Institute for Polar and Marine Research (Helgoland) were initially incubated for 3 days within a 200 L tank filled with 0.2 µm filtered seawater from Kiel Fjord (salinity 18.2, temperature 18 °C). After the hatched nauplii reached the second developmental stage, individuals were transferred from the stock solution to 2-L NALGENE bottles filled with CO_2 pre-treated water to reach an approximate density of 1000 individuals L^{-1} in each treatment and replicate (12 bottles total). In a full factorial design, nauplii were grown under two different levels of CO_2 aerated seawater (low and high; see above) and fed with algae at a concentration of 1000 µg C L^{-1} cultured under low and high CO_2 levels (see below). Nauplii were fed with CO_2 aerated stock *R. salina* to ensure optimal growth and development of the first developmental stages. After the copepods reached copepodide stage 1, *T. pseudonana* was used as food source. The CO_2 manipulation resulted in a mean (±SD) pCO_2 concentration in the L and H treatment of 533 (±28) and 674 (±57) µatm during the *R. salina* food addition and 388 (±233) (L) and 838 (±70) (H) µatm during the *T. pseudonana* addition. Four treatment combinations for copepod zooplankton (Z) and phytoplankton diet (P) were used: Z_L/P_L, Z_L/P_H, Z_H/P_L and Z_H/P_H (each in triplicate). All replicates were randomly placed in a temperature-controlled culture room for 16 days at 18 °C and 14 h:10 h light:dark cycle until the copepods reached the adult stage.

After the copepods reached copepodite stage 1 the total water of all incubation bottles was exchanged. Therefore the content of each flask was slowly run through a 41 µm mesh to separate the copepods from *R. salina*. The copepods were then smoothly rinsed and transferred into clean incubation bottles, refilled with CO_2 aerated seawater. For the following growth phase (copepodite stage 2 to adult), *T. pseudonana* stock culture was added as diet following the CO_2 treatment combinations. Microscopic inspection of water samples taken directly after the refilling process from each incubation bottle ensured no further contamination with *R. salina*.

During the course of the copepod growth experiment, 80–90 % incubation water was exchanged with CO_2-enriched seawater from the stock solution tanks every second day to ensure a constant CO_2 environment. Water was removed by reverse filtration using an 80 cm silicone tube with a net ending of 41 µm mesh.

Carbonate chemistry sampling and analysis

Water pH was monitored daily over the course of the copepod growth experiment. Water samples for carbonate chemistry analysis were taken every second day for DIC and two times per week for TA, respectively directly after water exchange. For DIC, the water was smoothly filtered via syringe and a 0.2 µm pre-filter and stored in 2 ml brown flasks at 4°C. The sample flasks were closed with a plastic screw cap and a Teflon septum in between to minimize outgassing. DIC was determined photometrically with an auto-analyzer (QUAATRO, Bran & Lübbe) at a precision of ±20 µmol kg^{-1} [3], [4]. Samples for TA measurements were filtered, poisioned with 1 ml of a $HgCl_2$ solution (35 g L^{-1}) and stored in 300 ml borosilicate flasks. TA was calculated from linear Gran plots [5] after duplicate potentiometric titration [6].

The values of DIC and pH (NBS scale), combined with temperature and salinity, were used to calculate carbonate system parameters in the seawater. CO_2 calculations were conducted with an excel macro of the CO2SYS.EXE program [7] using the constants after [8] as refitted after [9] and [10]. pH measurements were checked with alkalinity measurements two times per week by calculating pH from measurements of TA and DIC using the software CO2sys (see above). Calculated pH values agreed with pH measurements with a maximum deviation of ±110 and ±210 µatm for the low and high CO_2 treatment level, respectively.

Egg production and hatching experiments

After copepods reached adult stage, *A. tonsa* females were sorted from each incubation bottle and transferred into 500 ml chambers filled with CO_2-treated seawater and *T. pseudonana* (1000 µg C L^{-1}) and closed leaving no headspace. To separate the copepods

from produced eggs, a mesh of 250 µm separated the chambers. For each experimental replicate, 4 egg chambers each with 5 females were set up (total 48 egg chambers).

After 24h incubation time all living female copepods were separated from the egg chambers and stored under -80°C for fatty acid analysis. The eggs and hatched nauplii inside the egg chambers were concentrated and transferred into 20 ml airtight hatching chambers for another incubation of 48h, followed by formalin preservation. The content of the hatching chambers was analyzed and categorized into nauplii, hatched and empty eggs; nauplii were further examined for visible mutation or developmental malfunctions. All procedures of the egg production and hatching experiment took place within a temperature-controlled culture room at constant temperature conditions (18°C). Copepod sorting and analysis took place with a Leica MS 5 microscope (10–40x).

Preparation of algae stock cultures

The cryptophyte *R. salina* and the diatom *T. pseudonana* were used as food sources for *A. tonsa*. *R. salina* was inoculated and grown in two flasks with a modified f/2 media (salinity 18.2) and bubbled continuously with CO_2 air of 380 ppm (low) and 740 ppm (high), respectively under constant light. *T. pseudonana* was grown in a set of laboratory batch cultures with artificial seawater (ASW) as culture media [2] at a salinity of 18 and constant alkalinity of 2041 µmol kg^{-1} to simulate the parameters measured in the copepod media. TA and DIC were measured in the *T. pseudonana* cultures before its use for copepod feeding. The carbonate system was calculated in the same way as was done in the copepods media, resulting in a mean (±SD) pCO_2 of 366±120 and 915±270 µatm in the L and H treatment, respectively. The relatively high pCO_2 variability within each treatment is expected to be a consequence of DIC losses and slight changes in alkalinity in the culture media due to cell growth. Incubations were conducted at a photon flux density of 150 µmol photons m^{-2} s^{-1} induced by six fluorescent tubes [T5, types 5 JBL Solar Tropic (4,000 K), 1 JBL Solar Natur (9000 K)] on a 18:6h light:dark cycle, and a temperature of 18°C. Nitrate, silicate, and phosphate were added at final concentrations of approximately 64, 64, and 4 µmol kg^{-1}, respectively; trace metals and vitamins were added at f/2 medium concentrations [11].

Algae CO_2 shift experiment

To investigate the response through time of the algae fatty acid composition to changing pCO_2, *T. pseudonana* was grown in a batch culture at 1120 µatm pCO_2 under the same light and temperature conditions as the copepod experiment. Nutrients were set at the same concentrations used in the algae cultures for copepod feeding. After 5 days the culture was transferred to a medium with 380 µatm pCO_2. The further decline in pCO_2 after the transfer to low pCO_2 was a consequence of the carbon dioxide uptake by the algae and was accentuated by sporadic vacuum pumping of the culture bottle overhead space; the pCO_2 varied between 345 µatm at the beginning to 169 µatm at the end of the experiment. TA and DIC were measured and the carbonate system calculated as was done in the copepods media. Samples for fatty acids were taken every 5 hours over a 30h period, with a pause at 15 hour during the dark phase of the culture. Cells were harvested during the exponential growth phase of the culture. Algal density was determined using a Coulter Counter (Z2 Coulter® Particle Count and Size Analyzer, Beckman Coulter™).

Fatty acid analysis

Fatty acids of the copepods and algae food culture were measured as fatty acid methyl esters (FAMEs) at the end of the egg production experiment. 250 or 500ml of water, depending on the concentration of phytoplankton, were filtered on precombusted 0.2 µm GF/F filters and stored at -80°C until analysis. 45-50 adult copepods from each incubation bottle were sampled into 2ml scintivials 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 2.25ml 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 rotaryfilm evaporator (100-150mbar), redissolved in Chloroform and transferred into a glass cocoon. Again,

the solvent was evaporated to dryness (10-30mbar), and esterification was performed overnight 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°Cmin^{-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 30ml 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.

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Table S1. Amount of fatty acids in *Thalassiosira pseudonana* (Phytoplankton, P) and in copepod zooplankton (*Acartia tonsa*, Z) cultured in seawater at low (L) and high (H) CO_2 concentration in a full factorial design.

		P_L		Acartia (Z_L/P_L)		Acartia (Z_H/P_L)		P_H		Acartia (Z_L/P_H)		Acartia (Z_H/P_H)	
Fatty acid	Common name	%	fg cell^{-1}	%	ng cop^{-1}	%	ng cop^{-1}	%	fg cell^{-1}	%	ng cop^{-1}	%	ng cop^{-1}
14:00	Tetradecanoic acid	11.1	2.98	12	1.12	11.4	0.09	19	3.22	10.4	0.4	8.9	0.07
16:00	Hexadecanoic acid	18.2	4.88	23	1.91	56	0.44	29	4.99	26.7	1.03	50	0.41
16:01	Hexadecenoic acid	20.7	5.53	18	1.85	--	--	19	3.26	10.2	0.41	0.5	0
17:01	Heptadecenoic acid	--	--	0.1	0	--	--	--	--	--	--	0.1	0
18:00	Octadecanoic acid	3.32	0.89	5	0.38	22.5	0.17	7.2	1.24	7.4	0.28	24.8	0.21
18:1n9t	Oleic acid	0.37	0.1	--	--	1.2	0.01	0.4	0.07	--	--	1	0.01
18:1n9c, 18:2n	Oleic acid	1.57	0.42	2.3	0.2	0.3		0.6	0.11	2.2	0.09	--	--
18:3n6	Gamma-linolenic acid	11.4	3.05	4	0.43	--	--	7.7	1.34	1.9	0.07	--	--
18:3n3, 18:2n6	Linolenic acid	6.95	1.86	0.6	0.05	1	0.01	5.8	1	0.8	0.03	1.2	0.01
20:00	Eicosanoic acid	--	--	0.1	0.01	--	--	--	--	--	--	0.3	0
20:3n6	Dihomo-gamma-linolenic acid	0.19	0.05	0	0.02	--	--	0.2	0.03	--	--	--	--
20:4n6, 20:5n3	ARA-EPA	21.7	5.81	17	1.53	--	--	9	1.56	14.5	0.58	--	--
22:00	Docosanoic acid	--	--	--	--	--	--	--	--	--	--	0.6	0
22:1n9	Erucic acid	0.71	0.19	0.4	0.03	2.4	0.02	--	--	0.8	0.03	0.8	0.01
22:6n3	Docosahexaenoic acid	3.58	0.96	13	1.02	--	--	1.9	0.33	14.3	0.56	3.5	0.04
24:00:00	lignoceric acid	--	--	2.2	0.15	2.9	0.02	0.3	0.05	4.6	0.18	3.9	0.04
24:1n9	Nervonic acid	0.22	0.06	3.3	0.23	2.3	0.02	0.7	0.12	6.2	0.24	4.2	0.04
SFAs		32.7	8.75	42	3.57	92.8	0.72	55	9.5	49.1	1.89	88.5	0.73
MUFAs		23.5	6.3	24	2.31	6.2	0.05	21	3.56	19.4	0.77	6.6	0.06
PUFAs		43.8	11.73	34	3.05	1	0.01	25	4.26	31.5	1.24	4.7	0.05
TOTAL			26.78		8.93		0.78		17.32		3.9		0.84

Realized pCO_2 values were 497 µatm ± 98 sd for the low (Z_L/P_L) and 761 µatm ± 113 for the high (Z_H/P_H) treatment. Shown are percentage of total fatty acids and concentration per cell (fg) and per copepod (ng), respectively

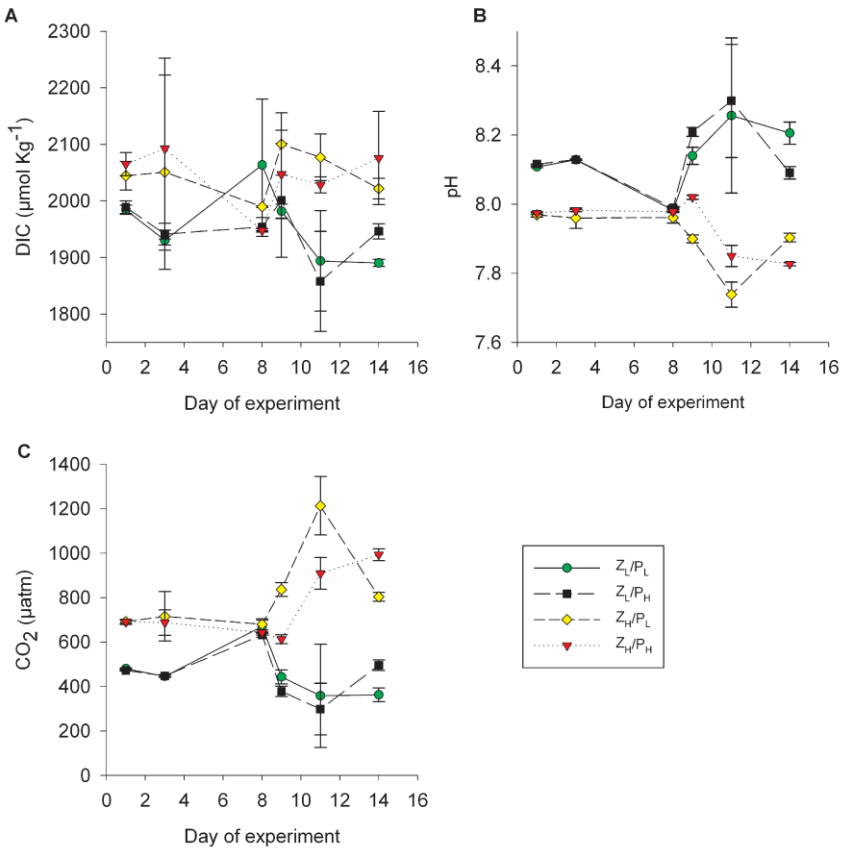

Figure S1. Carbonate system over the course of the copepod growth experiment.

A) Dissolved inorganic carbon (DIC) within CO_2 treatments during experimental growth phase of the copepods with a standard deviation of 47-91 µmol kg^{-1} within replicates of each treatment combination. On the first and the last day, all treatments were well separated with some overlap on days 8 and 9. In the high CO_2 treatment (Z_H/P_H), incubation bottle 740-4 displayed maximum deviation of DIC with 130-210 µmol kg^{-1} relative to the other treatment replicates. **B)** Mean pH values during experimental growth phase of the copepods were well separated between the two main CO_2 seawater

treatments 8.14 and 7.94 in the Z_H and Z_L treatment, respectively, with standard deviations ranging from 0.08 to 0.12. **C)** Calculated mean CO_2 values in Z_L and Z_H were 460 and 755 µatm, respectively, with a standard deviation of 3-57 µatm between replicates during the first feeding period with *Rhodomona salinas*. From day 8 on, after the food algae was changed from *R. salinas* to *Thalassiosira pseudonana*, the standard deviations of the mean CO_2 values in all treatment combinations were higher, ranging from 20 to 232 µatm. The CO_2 concentrations of the mixed treatment combinations Z_L/P_H and Z_H/P_L were closer to the CO_2 target levels with 454 and 823 µatm, respectively. Error bars indicate standard deviations.

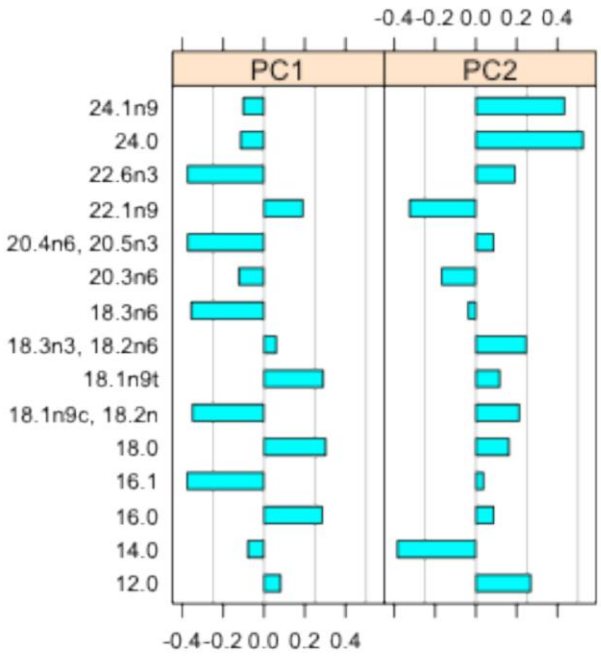

Figure S2. Loadings for Principal Component Analysis (PCA) of fatty acids for *Thalassiosira pseudonana* and *Acartia tonsa*.

FAs 22.6n3, 20.4n6.20.5n3, 18.3n6, 18.3n3-18.2n6, and 16.1 contributed most to Axis 1, and 24.0 and 14.0 to Axis 2. PCA is shown in Figure 2b.

4.4 Ocean acidification adversely affects trophic interactions and transfer of essential biochemical compounds in a natural plankton community

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Abstract

The change of seawater chemistry towards reduced pH as a result of increasing atmospheric carbon dioxide (CO_2) uptake in a process termed Ocean acidification (OA) is affecting a wide range of marine organisms. In order to determine if an OA-induced community shift at the base of marine food webs can affect the biochemical composition of primary producers and subsequently consumers, a set of mesocosm containing a North Sea natural plankton assemblage was subjected to different pCO_2 levels. The community showed a change in species composition of primary producers of towards an increase of small-sized cells, particularly picoeukaryotes at high CO_2 values. This change in plankton community composition and size was related to a decline in the relative polyunsaturated fatty acids (PUFA) content of the nano- and picophytoplankton size fraction at high CO_2 conditions. The PUFA decrease in the nanoplankton was linked to a gradual reduction in the relative PUFA content of the copepod *Calanus finmarchicus*, which primarily fed on this size class. This study shows that a shift in community composition of primary producers as a result of increasing CO_2 and an associated change in biochemical composition can affect the transfer of essential compounds to upper trophic levels which heavily rely on their prey as a source of essential macromolecules, ranging from fish and up to human populations.

Introduction

An increasing body of research emphasizes that various biological processes of marine organisms are affected by ocean acidification (OA), caused by the uptake of anthropogenic CO_2 by the ocean (IPCC, 2013). OA has been shown to affect various biological processes of marine organisms with a magnitude and direction that varies greatly among species (Doney et al., 2009; Kroeker et al., 2010). Currently there a is better understanding of direct biological OA effects in calcifying organisms, however its impact on non-calcifying species are diverse and not well understood. Direct OA impacts can be expected in the biochemical and elemental composition of primary producers (PP) which are later transferred to higher trophic levels (Rossoll et al., 2012), while indirect impacts can derive from altered trophic interactions as OA can modify plankton community composition (Hare et al., 2007; Biswas et al., 2011; Brussaard et al., 2013). This is important as qualitative traits in resource biomass are often more important than resource quantity and can limit trophic transfer efficiency and the production at higher trophic levels (Müller-Navarra et al., 2004). However, our understanding of OA consequences for food web interactions and transfer of essential compounds from primary producers to upper trophic levels is mainly limited to single species responses or one producer-one consumer interactions (Kroeker et al., 2010; Rossoll et al., 2012).

Phytoplankton are a key source of essential biomolecules that fuel secondary production as consumers cannot easily synthesize these compounds *de novo* and have to acquire them through their diet (Arts et al., 2009). Essential organic macromolecules, such as fatty acids (FA), and its transfer from phytoplankton to zooplankton and further up to fish is the source of highly nutritious food for upper trophic levels. FA are the main component of lipids and cell membranes, and therefore have an important physiological role (Mouritsen, 2005). Fatty acids consist of hydrocarbon chains of different length and saturation (number of double bonds) and are generally classified according to their saturation degree. In particular, polyunsaturated fatty acids (PUFA, with two or more double bonds), produced in high amounts by algae, are considered essential metabolites for zooplanktonic grazers, like copepods (Breteler et al., 2005; Jónasdóttir et al., 2009; Vargas et al., 2009).

At community level, the different species present in a natural plankton assemblage determines its food quality properties as distinct algal taxonomic groups have contrasting FA compositions (Dunstan et al., 1992; Viso and Marty, 1993; Zhukova and Aizdaicher, 1995). Increasing seawater CO_2 can modify phytoplankton species composition by favoring certain taxa (Graeme et al., 2005; Brussaard et al., 2013, Schulz et al., 2013). In particular, small-sized cells like picoeukaryotes and cyanobacteria benefit from high CO_2 conditions (Hare et al., 2007; Biswas et al., 2011; Brussaard et al., 2013). Cyanobacteria are a poor source of essential FA (Demott & Müller-Navarra, 1997) and a reduction of mean prey-size towards small-sized cells reduces feeding efficiency of large copepods irrespective of their feeding mechanism (Kiørboe, 2011). This suggests that a shift in phytoplankton species composition as a result of changing CO_2 can affect the quality and quantity of food available for upper trophic levels.

In addition to taxonomic composition, environmental growth conditions affect elemental and biochemical status of primary producers. Several studies showed that temperature, inorganic nutrient concentrations and CO_2 (Reitan et al., 1994; Rousch et al., 2003; Torstensson et al., 2013) can alter the FA profile of individual algal species. Laboratory studies with monospecific cultures showed that the effects of high CO_2 on algal FA, particularly their PUFA content, is highly species-specific, varying from increasing (Hoshida et al., 2005; Fiorini et al., 2010) to no effects (Tsuzuki et al., 1990), to a reduction in PUFA content at high CO_2 (Tsuzuki et al., 1990; Riebesell et al., 2000a; Carvalho & Malcata, 2005; Rossoll et al., 2012; Torstensson et al., 2013). These observations are relevant as changes in algal food quality can be detrimental for grazers as has been shown in laboratory and field studies (Locke & Sprules, 2000; Rossoll et al., 2012). Nevertheless, our current knowledge on the direct and indirect effects of OA in the physiology and food quality of planktonic communities is limited to a few species and the question remains whether plankton communities consisting of diverse taxonomic groups compensate for biochemical deficiencies of individual taxa and therefore dampen CO_2 effects on food quality at the community level (Rossoll et al., 2013).

The goal of the present study was to determine if an increase in CO_2 affects the FA makeup of primary producers and if so, whether this is transferred up the food web in a natural plankton community. An off-shore mesocosm CO_2 perturbation experiment enclosing a natural plankton assemblage in the North Sea were used as study units. The CO_2 levels ranged from current to projected end of this century values (IPCC, 2013) plus two extreme treatments as test cases. Algal FA was measured in three size fractions and zooplankton FA was measured in the copepod *Calanus finmarchicus*, an important prey item for fish and the most abundant grazer in the mesocosms during our experiment.

Material and Methods

Experimental set-up and CO_2 manipulation

Our study was conducted during an off-shore mesocosm CO_2 perturbation experiment in Raunefjord, southern Norway during late spring 2011 using nine enclosures with a length of 25m containing ~75m^3 of natural seawater. The mesocosms were set up and manipulated as described in detail by Riebesell et al. (2013). Carbon dioxide enrichment was achieved through the addition of CO_2-saturated seawater to seven out of nine mesocosms in five steps between day 1 and day 5 to achieve target values of 380, 560, 840, 1120, 1400, 2000 and 3000 µatm plus one control unit with a natural concentration of 280 µatm. The initial and final measurements of CO_2, pH and inorganic nutrients for all nine mesocosms are provided in Table S1. On day 14, nitrate and phosphate were added to all mesocosms to a final concentration of 5 and 0.31 µmol l^{-1}, respectively. Samples for phytoplankton counts and flow cytometry were taken every second day and for fatty acids every fourth day using a depth-integrated water sampler (Hydrobios, Kiel, Germany) covering the upper 20m of the water column; integrated zooplankton net tows were taken every 7th day starting the day before the CO_2 manipulation (day -1).

Phytoplankton abundance and biomass calculation

Phytoplankton cell counts were carried out from 50ml sample water, fixed with alkaline Lugol's iodine (1% final concentration) using the Utermöhl's (1958) method with an inverted microscope (ZEISS Axiovert 100). At 200 times magnification, cells larger than 12µm were counted on half of the chamber area, while smaller cells were counted at

400 times magnification on two radial strips; ciliates and large (>50µm) less abundant cells were counted in the entire chamber with a magnification of 50. The plankton was identified to the genus or species level according to Tomas (1997), Hoppenrath et al. (2009), Kraberg et al. (2010) and von Quillfeldt (1996). Picoplankton cells were enumerated using a bench-top Becton Dickinson FACSCalibur flow cytometer (FCM) equipped with a 488nm argon laser. Eukaryotic photosynthetic organisms and cyanobacteria cells were counted fresh and discriminated by their pigment autofluorescence and side scatter signal (Marie et al., 1999). Biovolume was calculated according to geometric shapes and converted to cellular organic carbon using taxon-specific conversion equations [for phytoplankton Menden-Deuer (2000), for picophytoplankton Worden et al. (2004) and for ciliates Putt & Stoecker (1989)].

Fatty acid composition

For algal FA, 1L of seawater was filtered in three size fractions: 100 to 10µm (micro), 10 to 2.7µm (nano) and 2.7 to 0.3µm (pico) by using pre-combusted (450 °C, 6 h) 25 mm Advantec Grade GF 75 (0.3 µm ø pore), Whatman GF/D (2.7 µm ø pore) and non-combusted Millipore NY1002500 (10 µm ø pore) filters, respectively. Individuals of the copepod *Calanus finmarchicus* (copepodite stage V) were sorted for FA measurement. All samples were immediately stored at -80°C until analysis.

The FA were measured by gas chromatography as fatty acid methyl esters (FAME) following Klein Breteler et al. (1999). 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, FAME C19:0 (Restek, Bad Homburg, Germany; c= 20ng component^{-1}µl^{-1}) was added, and a C23:0 FA standard (c= 25.1 ng µl^{-1}) 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 (100-150 mbar), re-dissolved in chloroform and transferred into a glass cocoon. The solvent was evaporated again (10-30 mbar), and esterification was performed over night using 200µl 1% H_2SO_4 (in CH_3OH) 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 non-polar column (RXI1-SIL-MS 0.32µm, 30m, company Restek) and Flame ionization detector. 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. A 1µl aliquot of the sample was injected. The system was calibrated with a 37-component FAME-mix (Supelco, Germany) and chromatograms were analyzed using Chrom-Card Trace-Focus GC software (Breteler & Schouten, 1999) and the fatty acids were clustered according to their degree of saturation: saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA).

Statistical analyses

To identify differences in the relative content of FAs between the pCO_2 treatments, absolute deviations from the overall mean were calculated in each mesocosm for the different FA groups (SFA, MUFA, PUFA). Therefore, the residuals (RE) were calculated by subtracting the arithmetic mean of all mesocosm observations per time-point from each mesocosm observation at that time-point, according to Engel et al. (2013). The RE were analyzed by a nested mixed effects model (MEM) to determine the differences in relative fatty acid content (%) between the CO_2 treatments (µatm) with the treatment level as nested random variable (random distribution of CO_2 treatments among the mesocosm). Average mesocosm CO_2 was calculated for the total duration of the sampling period (day 1 to 25 for seston and day -1 to 33 for zooplankton). Mann-Kendall trend test was applied in monotonic distributed date to determine the temporal change for plankton taxonomic groups in terms of calculated biomass throughout the experiment. Slopes were expressed as % day^{-1} by dividing by the experimental mean of the variable; when the biomass distribution was non monotonic and MEM analysis as described above was performed. The similarity in the structure of the plankton community between the treatments in terms of calculated specie biomass in the mesocosms was analyzed by a Non Metrical Multidimensional Scaling (NMDS) test with Bray distance, auto-transformation and 4 dimensions (k=4). An analysis of similarity

(ANOSIM) using a Bray-Curtis distance matrix and 5000 permutations was used to corroborate the NMDS results. All statistical analyses were done using the R software environment 3.0.1 (R Development Core Team 2005).

Results

Plankton community composition

The initial algal community consisted of typical post-bloom species dominated by small-sized flagellates. Phytoplankton biomass showed an small bloom at the start of the experiment (Fig. 1). Nutrient addition at day 14 triggered a second short-lived bloom, which reached a peak around day 20. Chlorophyta were the most abundant phytoplankton group in all mesocosms throughout the experiment, particularly at low pCO_2 levels, while picoeukaryotes made up a large fraction of total biomass at high CO_2 levels (Fig. 1). Microzooplankton, dominated by the ciliate *Strombidium* sp., followed the phytoplankton bloom and reached the highest abundances at intermediate CO_2 conditions (Fig. 1).

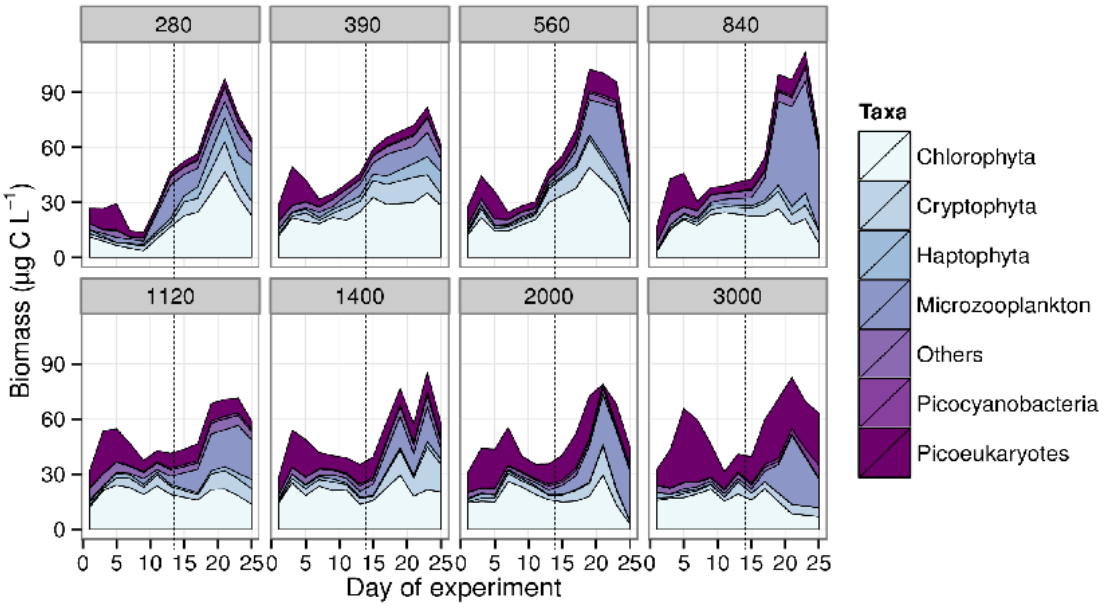

Figure 1. Calculated biomass after cell counts of the main plankton taxonomic groups in the different CO_2 treatment. Each treatment is label with its target CO_2 level (top). The dotted line shows the point of nutrient addition at day 14.

Trends over the duration of the experiment revealed differential responses of major taxonomic groups to CO_2 manipulation (Fig. 2a). Chlorophyta increased significantly (p<0.05) at low CO_2 treatments over the duration of the experiment, but did not change at intermediate and high CO_2 conditions even after nutrient addition. Picocyanobacteria and Microzooplankton increased significantly in all mesocosms along with rising CO_2 levels, however the second showed a maximum at intermediated CO_2 conditions. Cryptophyta increased significantly at all CO_2 although did not show a consistent pattern between treatments. The Haptophyta, composed mostly by the coccolithophore *Emiliania huxleyi*, increased significantly at low CO_2 levels, were unaffected at intermediate values and almost disappeared at high CO_2, with a significant negative trend (Fig. 2a). The same pattern was observed for the sum of miscellaneous phytoplankton taxa (Euglenophyta, Heterokontophyta and Dinophyta), which comprised a small fraction of algal biomass in all mesocosms (Fig. 2a). The Picoeukaryotes biomass followed the treatment gradient and were the most dominant taxa at elevated CO_2; due to the non-monotonic distribution of the biomass data, it was analyzed with a mixed model effect (MEM) which showed a significantly higher biomass at elevated CO_2 conditions (MEM, F=86.21 , p<0.0001, df=94) (Fig. 2b). A MEM analysis performed by size fraction revealed that plankton biomass in the micro- and nano-size fractions was significantly higher at low CO_2 treatments (Fig. S1) (MEM, micro: F=9.36, p=0.003, df=95; nano: F=6.16 , p=0.014, df=95), while the pico-size fraction biomass was significantly higher at elevated CO_2 levels (MEM, pico F=67.23, p <0.0001, df=95) (Fig. S1).

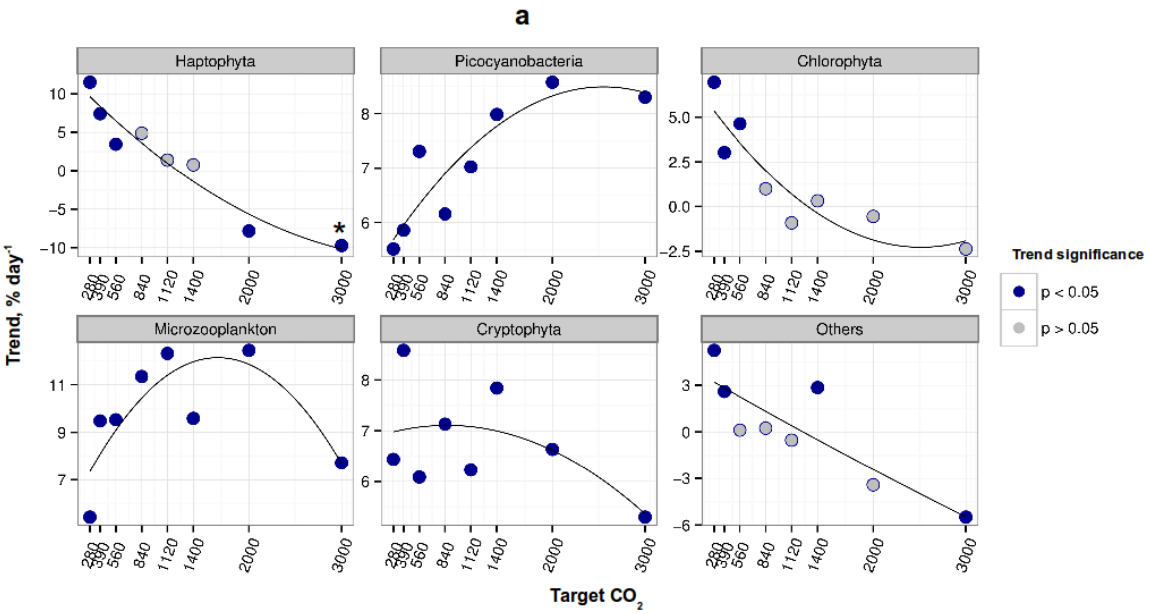

Figure 2. a) Mann Kendall trend analysis of the percentage of change per day of the calculated phytoplankton biomass of major plankton taxonomic groups over the duration of the experiment across mesocosms. Trends are shown as percent change per day (Trend $\%day^{-1}$); blue dots indicate significant increase or decrease trends ($p< 0.05$), grey dots no significant change. (*p-value= 0.059). b) Mean of the picoekaryotes calculated biomass during the experiment. Due to the non monotonic distribution of the picoeukaryotes biomass a mixed effect model analysis was performed in the CO_2 gradient treatments and showed a significant difference in the biomass between the different treatments ($F=86.21$, $p<0.0001$, $df=94$, $n=13$), bars shows standard error. Lines display a Loess smooth curve across CO_2 level.
The x-axis displays the target CO_2 level in each mesocosm.

The NMDS analysis of the entire algal plankton community in terms of biomass showed a strong gradual change in community composition over time, while the change associated with CO_2 was comparatively smaller (Fig. S2). The temporal variation in community composition was confirmed by ANOSIM ($R= 0.41$; $p<0.001$) whereas the effect of CO_2 manipulation was small, although significant ($R= 0.17$; $p< 0.001$), and strongly driven by deviation of the highest CO_2 treatments after nutrient addition.

Seston fatty acid composition

Fatty acid composition of SFA and MUFA showed small variation with no consistent patterns in all size fractions across CO_2 treatments (Fig. S3). The relative PUFA content revealed marked differences between size fractions, representing ~7% (3 ± 2ng L^{-1}) of the total lipids in the micro-, ~34% (39 ± 14ng L^{-1}) in the nano- and ~20% (21 ± 15ng L^{-1}) in the pico-size fraction.

PUFA content of the micro-size fraction did not change with CO_2, while there was a significant negative CO_2 effect at smaller size fractions (MEM, nano: F=14.70, p<0.001, df=44; pico: F= 6.58, p= 0.013, df= 42) (Fig. 3). At low CO_2 treatments, the nano- and pico-size fractions had particularly higher relative amounts of Eicosapentaenoic (20:5n3, EPA), Docosahexaenoic (22:6n3, DHA), Linoleic (18:2n6) and Arachidonic (20:4n6) acids. The CO_2-related PUFA decrease in the nano- and pico-size fraction was associated with an increase of both SFA and mostly MUFA.

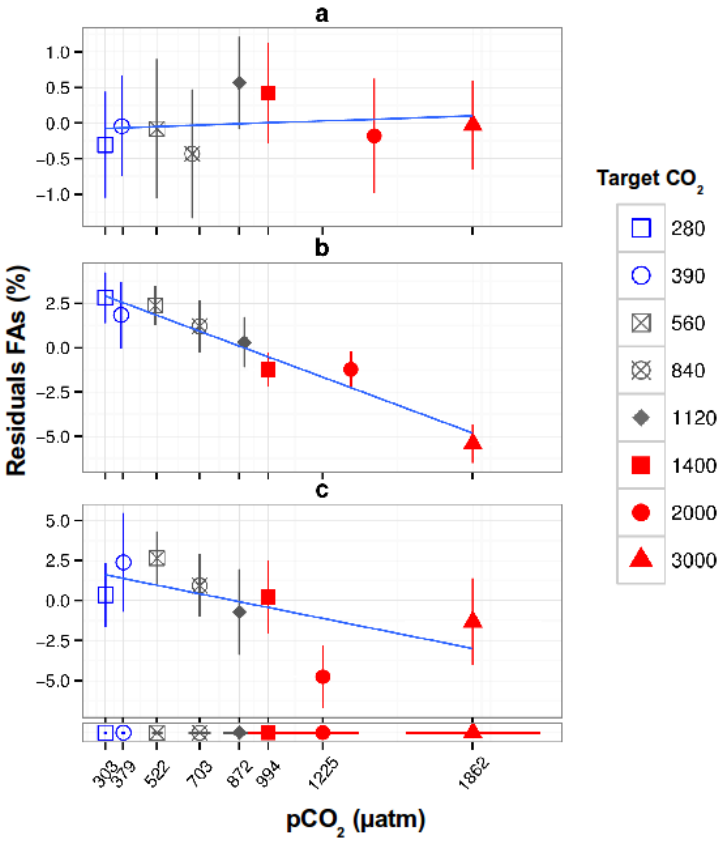

Figure 3. Residuals of the relative PUFAs content in the (a) Micro- (no significant difference), (b) Nano- (MEM, F=14.70, p<0.001, df=44) and (c) Pico- (MEM, F=6.58, p=0.013, df=42) size seston fraction in the CO_2 gradient treatments. The x-axis show the mean pCO_2 measured during the sampling period from day 1-25, bars shows standard error.

The seston PUFA dynamic showed two distinguishable phases, corresponding to CO_2 manipulation and nutrient addition (Fig. S4). During the first phase, when CO_2 was added, PUFA content showed a steep decline in the nano-size fraction and increased in the micro- and especially in the pico-size fraction. These trends were reversed after nutrient addition and PUFA content increased slightly in the nano-size fraction coincident with the biomass bloom, while kept a constant level in the pico-size fraction.

Copepod fatty acids

The overall PUFA content of the copepod *C. finmarchicus* represented ~32% (120 ± 40ng ind^{-1}) of total FAs, and the relative PUFA content decreased significantly with higher CO_2 levels (MEM, F=80.74, p=0.001, df=31) (Fig. 4a). The PUFA dynamics through time showed a gradual and significant decrease at CO_2 treatments of 1120 µatm and higher (Fig. 4b), with an average loss of ~4% and a strong decline (~10%) at the highest CO_2 treatment. In contrast, the relative PUFA content of *C. finmarchicus* was constant at low CO_2 levels throughout the experiment (Fig. 4b). The relative PUFA decline was associated with an increase of content of both MUFA and SFA (Fig. S5).

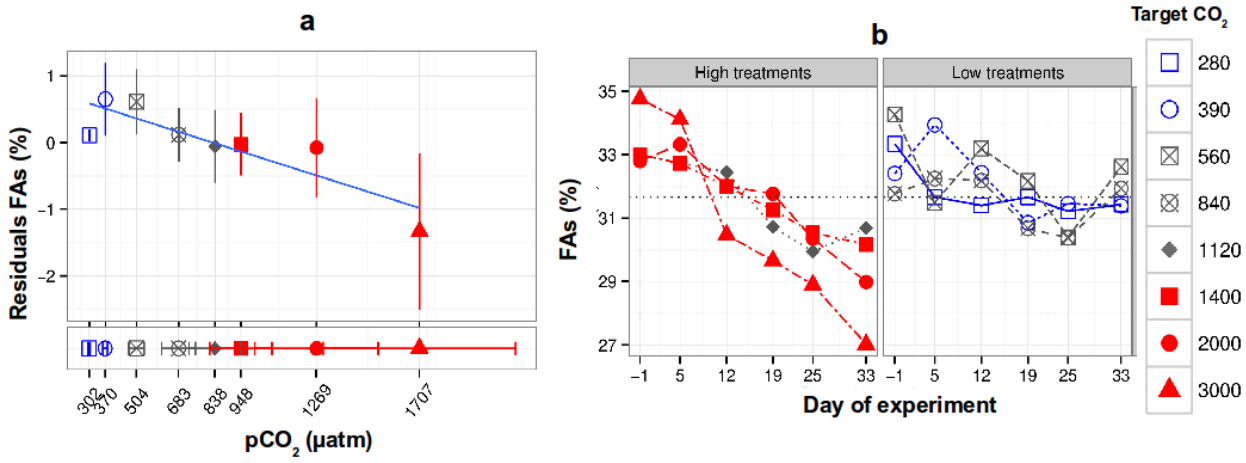

Figure 4. a) Residual of the relative PUFA content in the copepod *C. finmarchicus* for the different CO_2 treatments (MEM, F= 80.74, p=0.001,df=31). The x-axis legend show the mean pCO_2 measure during the sampling period between days 1-33, bars shows standard error. b) Relative PUFAs content of *C. finmarchicus* over experimental duration across CO_2 treatments. High CO_2 treatments (left) showed a significant decrease through time (Linear regression, 1120: R^2=0.73, p=0.017; 1400: R^2=0.97, p=<0.001; 2000: R^2=0.86, p<0.01; 3000: R^2=0.93, p=0.001). Horizontal dashed line indicates the position of the overall mean PUFA value.

The overall ratio of the different FA classes (PUFA, MUFA, and SFA) in copepods resembled closely the proportions of the nano-size phytoplankton fraction (Table 1), and this pattern was consistent for the duration of the experiment (Table S1). Also, the relative copepod PUFA content was related to the nano-size fraction at the two highest CO_2 treatments (Fig. S6), indicating that *C. finmarchicus* strongly relied on this size fraction as a PUFA resource.

Table 1. Ratios between different Fatty Acid (FA) classes: Saturated (SFA), Monounsaturated (MUFA) and Polyunsaturated (PUFA) in the copepod *Calanus finmarchicus* and different phytoplankton size fractions (Micro: 10-100 µm, Nano: 2.7-10 µm, Pico: 0.3-2.7 µm). The nano-size fraction is highlighted to show the similarity between this group and *C. finmarchicus*.

	SFA:PUFA	sd	SFA:MUFA	sd	MUFA:PUFA	sd
Calanus	**1.67**	**0.14**	**3.48**	**1.03**	**0.48**	**0.1**
Micro-fraction	12.13	4.32	5.74	2.1	2.3	0.96
Nano-fraction	**1.41**	**0.33**	**3.19**	**1.37**	**0.52**	**0.22**
Pico-fraction	3.2	1.24	3.1	1.36	1.12	0.54

Discussion

Community composition

The phytoplankton community composition showed a CO_2 effect in the biomass of different plankton groups. Large and intermediate cell-sized species decreased while small-sized cells, especially picoeukaryotes increased at high CO_2 levels. These observations are consistent with previous studies showing small-sized phytoplankton to benefit from high CO_2 levels in terms of growth rate and biomass accumulation (Hare et al., 2007; Biswas et al., 2011; Schulz et al., 2013; Brussaard et al., 2013). In general CO_2 is not considered a limiting nutrient in the ocean. However, under sufficient nutrient availability, high CO_2 conditions are expected to give a competitive advantage to certain algal taxa due to their inherent physiological characteristics for carbon uptake and fixation (Tortell et al., 2008; Low-Décarie et al., 2011; Brussaard, et al. 2013; Schulz et al., 2013). Rubisco, the enzyme that fixes inorganic carbon to organic compounds in photosynthetic organisms, is notoriously inefficient due to its very slow catalytic rate and low affinity for CO_2 (Spreitzer & Salvucci, 2002). To compensate for the low

performance of Rubisco most algae species keep active, energy consuming carbon concentration mechanisms (CCM) under low CO_2 conditions (Giordano et al., 2005; Price et al., 2007). High CO_2 can give a competitive advantage to picoplankton cells over larger algae due to their size (Reinfelder, 2011). For cells with an small radii, CO_2 diffusion can support high specific carbon fixation rates at low seawater CO_2 concentrations, while diffusion-supported specific carbon fixation rates drop sharply as cell radius increases (Reinfelder, 2011). An elevated CO_2 concentration would allow a down-regulation of the CCM, which may translate into a metabolic energy saving that can be allocated to other biological processes such as growth (Raven & Beardall, 2003; Rost et al., 2008).

The negative CO_2 effect on coccolithophores (Haptophyta), especially *Emiliania huxleyi*, observed in the present experiment is consistent with other mesocosm studies that showed a diminished abundance of calcifying algae at high CO_2 levels (Engel et al., 2005). Coccolithophores have typically reduced growth rates at increased CO_2 conditions (Riebesell et al., 2000 b) due to adverse effects of low pH on their physiology (Bach et al., 2011).

Seston FAs

The negative effect of CO_2 on the PUFA content in the nano-size fraction is a combination of a change in plankton community composition and a direct CO_2 effect on cell physiology. This size fraction reached the highest amount of PUFA of all fractions, and was mainly composed of Haptophyta and Chlorophyta. Green algae (Chlorophyta) have high concentrations of C_{16} and C_{18} PUFAs (Dunstan et al., 1992; Zhukova and Aizdaicher, 1995) and coccolithophores are a good sources of PUFA (Pond & Harris, 1996; Volkman et al., 1981). The abundance of these two taxa was strongly influenced by CO_2 and both of them decreased at high levels, which potentially contributed to the lower PUFA content of this size fraction at high CO_2 compared to low CO_2 treatments.

Although our experiment did not allow distinguishing between an indirect effect on PUFA concentration through shift in species composition and a direct CO_2 response, an effect on cell physiology cannot be excluded. It has been shown that some green algae have reduced amounts of PUFA when cultured at high CO_2 (Tsuzuki et al., 1990; Sato et

al., 2003), and the same effect was observed in *Emiliania huxleyi* (Riebesell et al., 2000a), the most abundant Haptophyta during our experiment. Direct CO_2-driven changes in PUFA concentration can occur at very short time scales (Tsuzuki et al., 1990; Rossoll et al., 2012), and likely explain the steep decrease in the nano-size PUFA content immediately after CO_2 addition with a subtle biomass change. Although the mechanisms through which CO_2 affects algal FA are unclear, Sato et al. (2003) suggested that an elevate SFA level in cells grown at high-CO_2 conditions is due to enhanced FA synthesis and accumulation. Also have been suggested that an elevated SFA content under high CO_2/low pH is a mechanism to help in the maintenance of the cellular internal homeostasis (Lane et al., 1981; Burkhardt et al., 2001; Rossoll et al., 2012), as a more rigid cell wall with membranes built of short-chain FA makes them less fluid and permeable (Rossoll et al., 2012).

The reduced PUFA content in the pico-size fraction at elevated CO_2 is more difficult to interpret due to the fraction biomass increases along with CO_2; the high abundance of picoeukaryotes at elevated CO_2 allows the assumption that more of the relative PUFA loss occurs in this group. Significant changes in picoeukaryotic community composition have been observed in plankton assemblages in relation to CO_2, with the Chlorophyta *Micromonas* sp. thriving under elevated CO_2 conditions (Newbold et al., 2012). This suggest that, like in the nano-size fraction, the relative PUFA decrease is most likely a combination of a change community composition and a direct CO_2 effect on cell physiology, however further research is needed in the effects of CO_2 on picoplankton FA and community composition.

A recent laboratory study reported a significant decline of ~20% in relative PUFA content of a diatom cultured under elevated CO_2 (750µatm) compared to present day levels (380µatm) (Rossoll et al., 2012). Our results are concordant on the direction of the CO_2 effect in the nano- and pico-size fractions, however the observed magnitude is smaller with a decrease of ~10% in both. Diatoms were a small part of the plankton biomass in our study and the lower magnitude of the CO_2 effect can be due to differential algal sensitivity to CO_2 and a higher genetic diversity of the natural plankton community,

which may counteract extreme negative CO_2 effects compared to monocultures (Nielsen et al., 2010; Rossoll et al., 2013).

Our results of a declining seston PUFA at high CO_2 are contrasting with the response of an Arctic plankton community, which showed a relative PUFA increase at elevated CO_2 levels that was attributed to a rise in dinoflagellate abundance (Leu et al., 2013). The divergent results between these experiments maybe can attributed to the different plankton community compositions and related FA profiles (Dunstan et al., 1992; Viso & Marty, 1993; Zhukova & Aizdaicher, 1995). However, FA analysis of the entire phytoplankton community, as performed by Leu et al. (2013), might mask changes in certain size or taxonomic groups. Our results showed that the FA response to CO_2 differ among size fractions, with no change in the largest and decreasing PUFA in the smaller size fractions at higher CO_2. The PUFA increase of the Arctic community could be a consequence of the increasing contribution of large-sized dinoflagellates (Leu et al., 2013), rather than a net positive CO_2 effect on the entire community. Therefore assessing a natural plankton community as a whole may overlook relevant biochemical changes of primary producers that can affect higher trophic levels. Considering that size-dependent patterns in FA concentration are important for ecosystem function because most aquatic predators are size-selective (Kainz et al., 2004), the presence of PUFA in a specific, possibly narrow size range can be essential for grazers.

Copepod fatty acids

PUFA concentration of the dominating copepod species *C. finmarchicus* markedly declined at CO_2 levels of 1120 µatm and above, with the strongest decrease at the highest CO_2 conditions. The FA change of the copepod mimics the decline in the nano-size phytoplankton fraction, suggesting that *C. finmarchicus* primarily feeds on this size fraction. This is consistent with reports by Nejstgaard et al. (1997) and Harris et al. (2000) of a high grazing efficiency of *C. finmarchicus* during nano-phytoplankton blooms, with small algae being a significant part of their diet.

The copepod's prey-dependent FA profile is consistent with results of other studies showing that copepods strongly rely on their diet as source of essential PUFAs and that

their composition mirrors the algae they graze on (Ishida et al., 1998; Caramujo et al., 2007; Rossoll et al., 2012). Although *sensu stricto* a direct CO_2 effects on copepod FA synthesis cannot be excluded, it seems quite unlikely. Previous experiments showed that *C. finmarchicus* is rather insensitive to projected high CO_2 scenarios (Mayor et al., 2007). Other factors that may have influenced FA transfer and possibly the copepod abundance in the present study is the shift in size of the plankton community, with the pico-size fraction becoming more dominant at high CO_2. A reduction of mean prey size towards small cells can cause a decline in feeding efficiency of large copepods, such as *C. finmarchicus,* irrespective of their feeding mechanism (Kiørboe, 2011). Therefore the observed decrease in the *C. finmarchicus* PUFA content at high CO_2 conditions may be a combined consequence of a CO_2-driven deterioration of the food quality and prey availability within each treatment level.

Although the present study provides the first evidence of a negative effect of OA on the transfer of essential organic macromolecules between trophic links in a natural marine assemblage, similar results have been reported in experiments on freshwater cladocerans fed with algae from an acidic lake (Locke & Sprules, 2000). This may demonstrate that this deleterious effect could be more widespread than previously thought. Nonetheless, the plankton community response to OA will strongly depend on how CO_2 affects species composition of primary producers as some taxa seem to be unaffected by CO_2 (Tsuzuki et al., 1990; Fiorini et al., 2010), which along with high genetic and physiological diversity in natural assemblages and may buffer negative CO_2 effects (Leu et al., 2013; Rossoll et al., 2013).

The diminished food quality of the copepod in terms of PUFA content in response to elevated pCO_2 may have implications higher up in the food web. Since PUFA are essential dietary compounds for fish, which heavily rely on their prey as a source of essential macromolecules (St. John et al., 2001). Given that fish is a critical natural resource (FAO, 2010), acidification-driven food quality deterioration can be a threat for human populations who rely on fisheries as an important food source (Arts et al., 2001; Kang, 2011)

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Supplementary information

Table S1. Ratios between the different fatty acids classes: Saturated (SFA), Monounsaturated (MUFA) and Polyunsaturated (PUFA) in each analyzed group: the copepod *Calanus finmarchicus*, Microplankton (10-100µm), Nanoplankton (2.7-10µm) and Picoplankton (0.3-2.7µm) in Day 1 and Day 25. The nano-size fraction is highlighted to show the similarity between this group and *C. finmarchicus*.

Day 1	SFA:PUFA	sd	SFA:MUFA	sd	MUFA:PUFA	sd
Calanus	**1.56**	**0.12**	**3.58**	**0.28**	**0.44**	**0.04**
Micro-fraction	14.69	3.7	8.05	1.39	1.75	0.45
Nano-fraction	**1.38**	**0.21**	**4.67**	**0.54**	**0.29**	**0.02**
Pico-fraction	5.06	1.37	4.35	1.06	1.25	0.59
Day 25						
Calanus	**1.81**	**0.04**	**3.82**	**0.33**	**0.48**	0.05
Micro-fraction	13.28	3.01	6.79	1.25	1.95	0.16
Nano-fraction	**1.74**	**0.26**	**4.45**	**1.09**	**0.41**	**0.12**
Pico-fraction	3.44	0.78	3.43	1.58	1.19	0.62

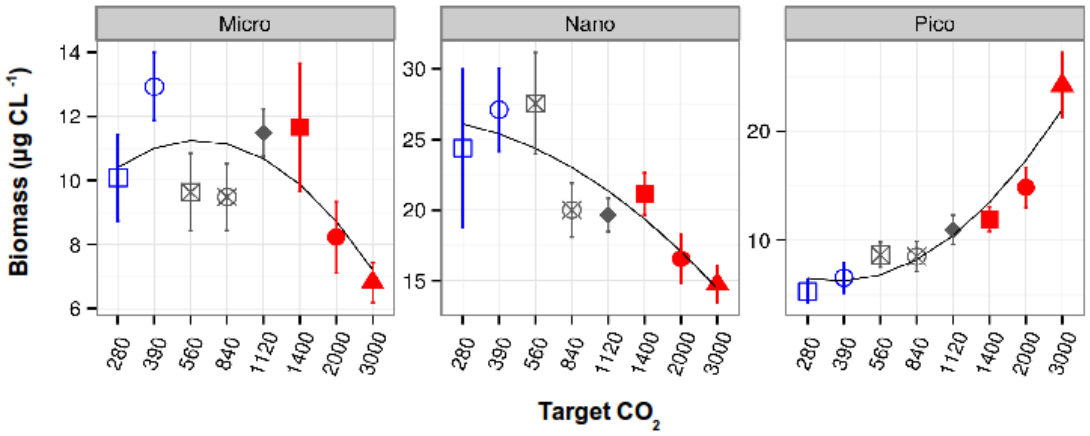

Figure S1. Mixed effect model analysis of the calculated phytoplankton biomass by size fraction: Microplankton (Micro, >10 µm) Nanoplankton (Nano, 10-2.7 µm) and Picoplankton (Pico, >2.7 µm) in the CO_2 gradient treatments during the experiment. All size fractions showed a significant difference in biomass between the treatments (Micro: F=9.36, p=0.003, df=95; Nano: F=6.16 , p<0.014, df=95; Pico F=67.23, p <.0001, df=95) (n=13) Bars show standard error.

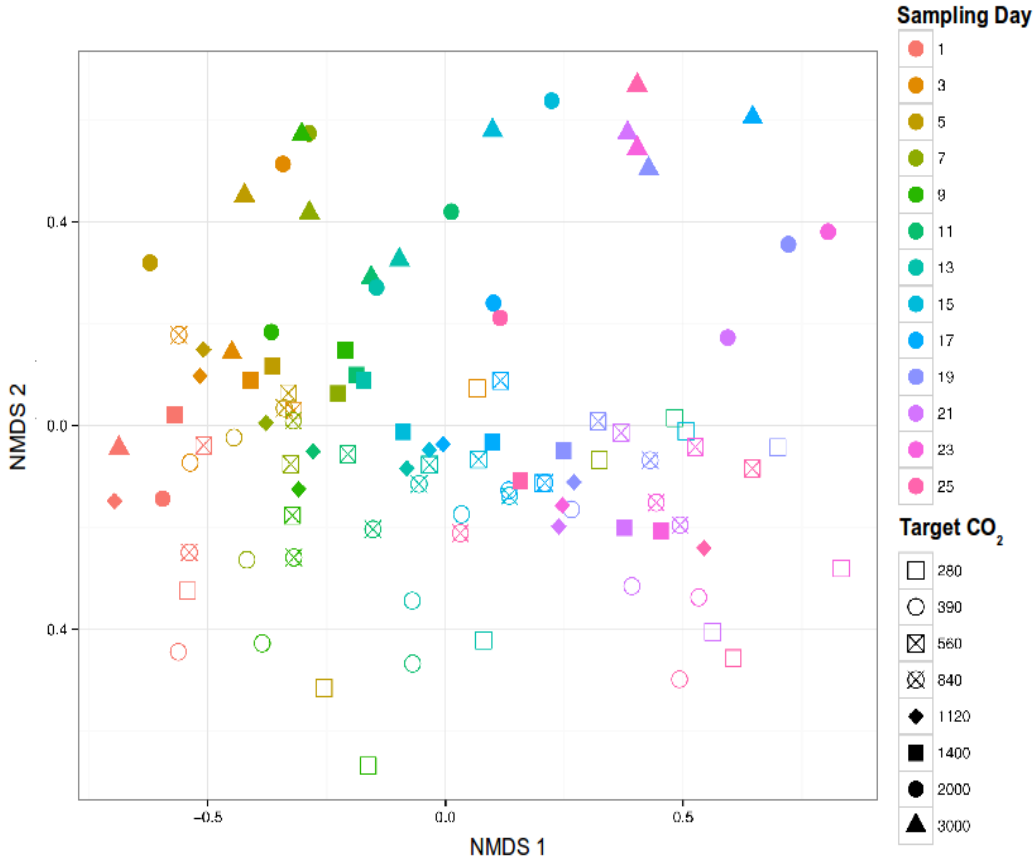

Figure S2. Non Metrical Multidimensional Scaling (NMDS) of the plankton community composition in terms of calculated biomass change through sampling days and CO_2 treatment. The NMDS 1 axis show that the phytoplankton communities strongly diverge through time, while the NMDS 2 axis show that the communities split between high (top) and low (bottom) CO_2 treatment levels. The ANOSIM analysis show a significant difference on community composition between days (ANOSIM statistic R: 0.41, p= 0.0002) while the analysis between CO2 treatments although significant was weaker (ANOSIM R: 0.17, p= 0.0002).

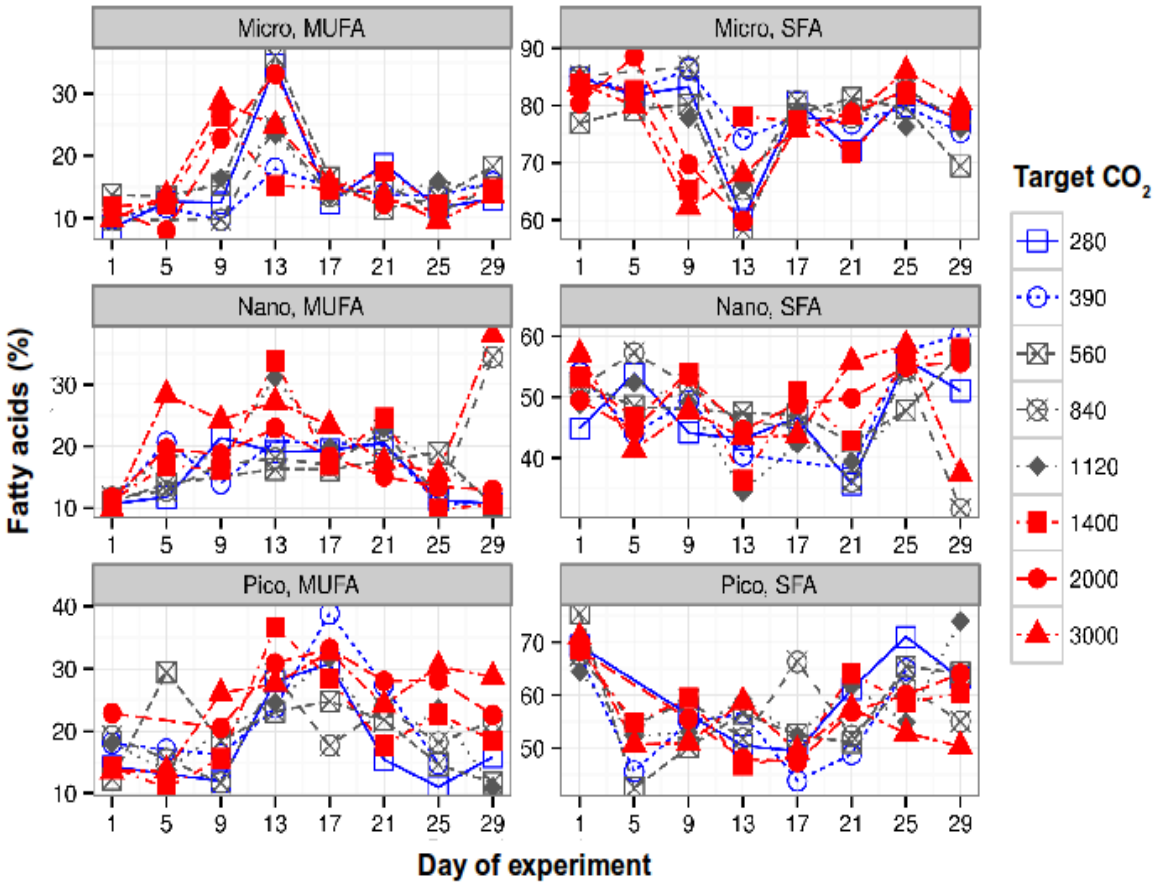

Figure S3. Relative MUFA and SFA content in Seston during the experiment divided in size fractions: 100-10 µm (Micro panel), 10-2.7 µm (Nano panel) and 2,7-0.3 µm (Pico panel) of the different CO_2 treatments.

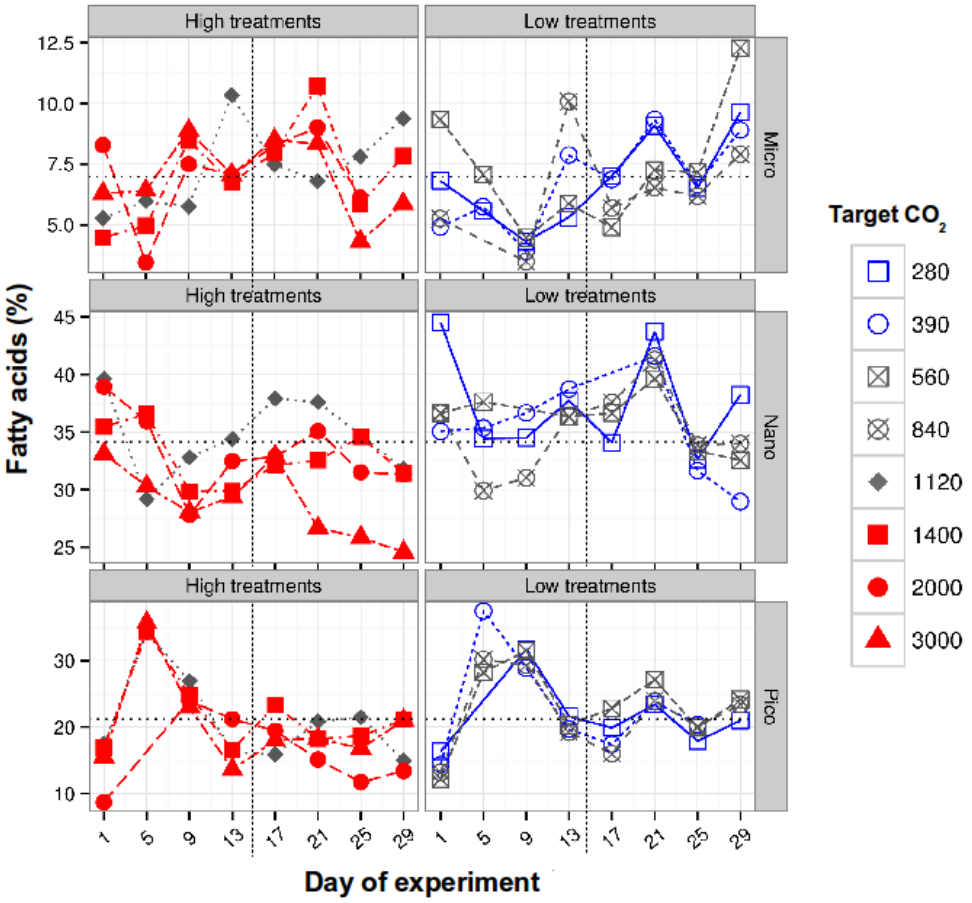

Figure S4. Relative PUFA content in Seston during the experiment divided in size fractions: 100-10 µm (Micro panel), 10-2.7 µm (Nano panel) and 2,7-0.3 µm (Pico panel) of the different CO_2 treatments. Horizontal dashed line indicates the position of the overall mean PUFA value. Vertical dashed line indicates nutrients addition at day 14.

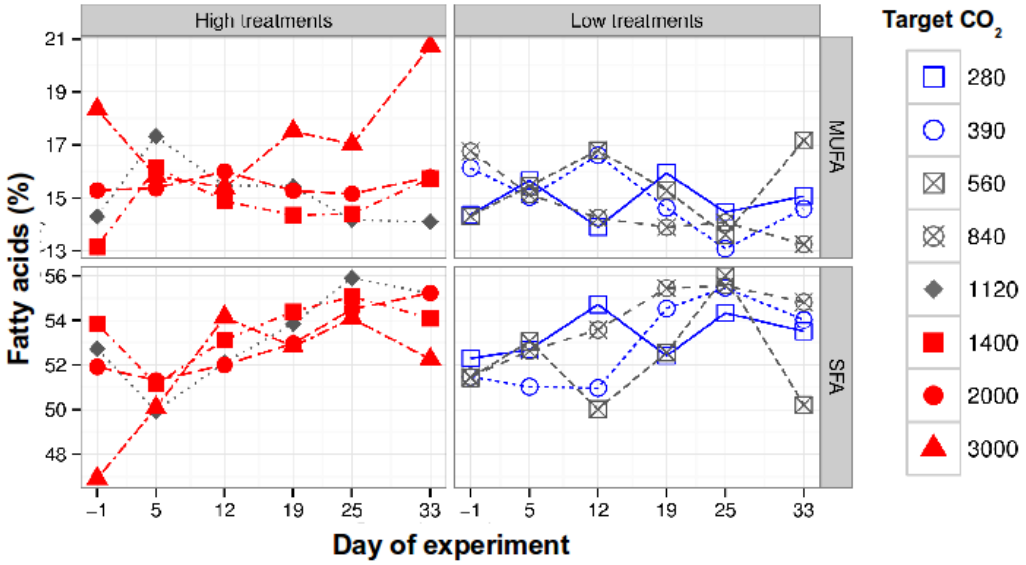

Figure S5. Relative MUFA and SFA content in the copepod *C. finmarchicus* on the different CO_2 treatments.

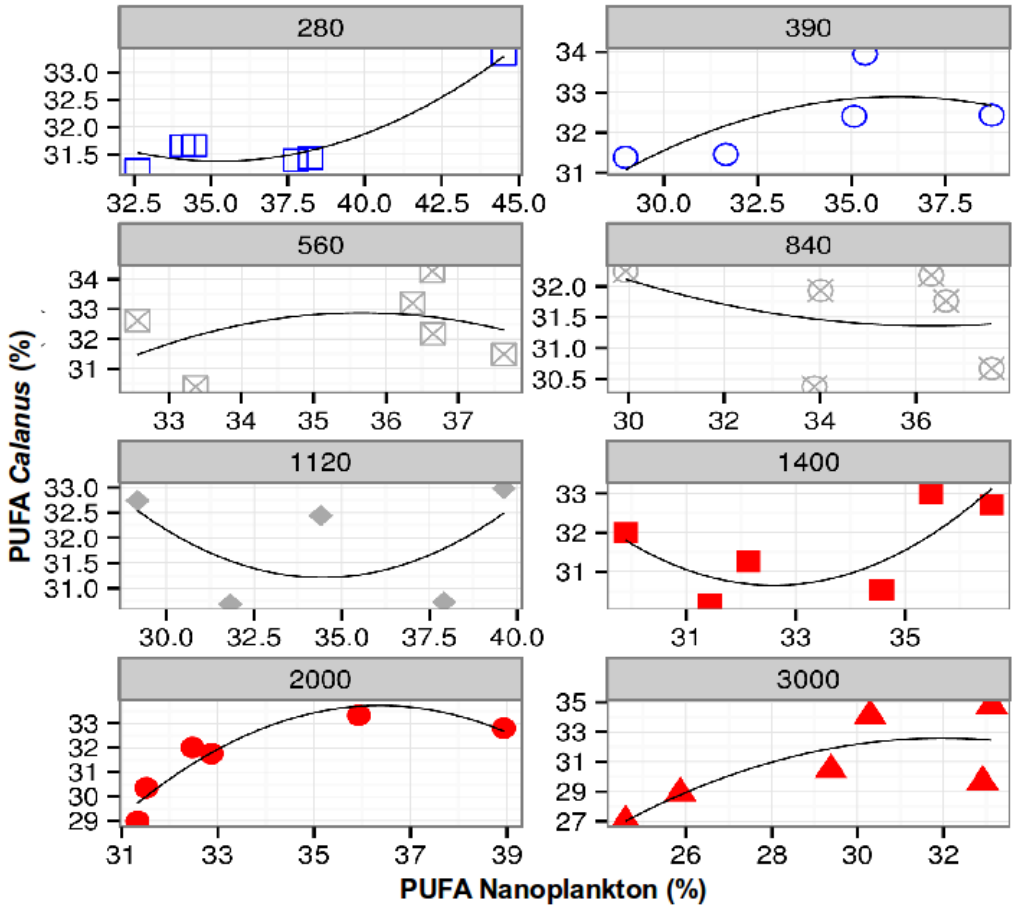

Figure S6. Relative PUFA content in *C. finmarchicus* versus relative PUFA content in the Nano-size fraction (10-2.7 µm) on the different CO_2 treatments. The correlation in the 2000 and 3000 µatm pCO_2 treatment is high although not significant (R^2= 0.48, p= 0.07 and R^2= 0.40, p= 0.10 respectively).

Table S1. Physical variables measured during the experiment when FA seston samples were taken. *Nutrients are given in µmol l^{-1}

Mesocosm	Target CO_2	Day	Temperature (°C)	pCO_2 (µatm)	pH (Free scale)	Nitrate*	Phosphate*	Silicate*	Ammonia*
M1	840	1	8.57	464.39	7.73	1.70	0.16	1.13	0.20
M1	840	5	9.47	891.40	7.50	0.49	0.08	0.80	0.05
M1	840	9	9.24	810.33	7.53	0.20	0.06	0.50	0.08
M1	840	13	9.84	745.51	7.56	0.20	0.07	0.45	0.13
M1	840	17	9.72	711.52	7.59	4.31	0.15	0.34	0.10
M1	840	21	9.68	623.95	7.64	1.90	0.04	0.04	0.07
M1	840	25	9.75	606.31	7.65	1.50	0.05	0.08	0.20
M1	840	29	NA	535.99	7.65	1.30	0.06	0.06	0.34
M3	1120	1	8.62	453.71	7.74	1.30	0.14	1.07	0.19
M3	1120	5	9.52	1168.60	7.41	0.39	0.06	0.77	0.08
M3	1120	9	9.25	1042.56	7.47	0.10	0.05	0.50	0.05
M3	1120	13	9.84	920.16	7.50	0.10	0.05	0.41	0.05
M3	1120	17	9.71	848.38	7.53	4.41	0.13	0.26	0.10
M3	1120	21	9.69	762.68	7.56	2.40	0.04	0.03	0.07
M3	1120	25	9.74	742.88	7.57	1.90	0.08	0.08	0.12
M3	1120	29	NA	621.29	7.58	1.80	0.07	0.06	0.36
M4	280	1	8.62	323.94	7.86	1.60	0.15	1.13	0.18
M4	280	5	9.52	300.03	7.89	0.59	0.08	0.80	0.12
M4	280	9	9.25	304.92	7.89	0.31	0.07	0.56	0.13
M4	280	13	9.85	319.20	7.88	0.10	0.06	0.52	0.16
M4	280	17	9.72	319.71	7.88	4.71	0.19	0.51	0.12
M4	280	21	9.69	299.58	7.90	2.50	0.05	0.18	0.10
M4	280	25	9.73	292.62	7.91	2.20	0.09	0.22	0.25
M4	280	29	NA	284.07	7.92	1.50	0.09	0.16	0.38
M5	1400	1	8.62	471.56	7.72	1.50	0.16	1.16	0.26
M5	1400	5	9.51	1324.86	7.33	0.49	0.08	0.80	0.05
M5	1400	9	9.28	1227.31	7.38	0.10	0.06	0.48	0.07
M5	1400	13	9.84	1101.05	7.43	0.10	0.04	0.35	0.10
M5	1400	17	9.72	967.45	7.48	4.31	0.12	0.16	0.12
M5	1400	21	9.69	854.84	7.52	3.20	0.06	0.02	0.19
M5	1400	25	9.74	826.70	7.54	2.00	0.07	0.07	0.20
M5	1400	29	NA	662.00	7.54	1.70	0.07	0.05	0.35
M6	390	1	8.62	373.88	7.81	1.50	0.17	1.11	0.22
M6	390	5	9.52	394.11	7.80	0.49	0.08	0.79	0.06
M6	390	9	9.26	392.27	7.81	0.20	0.07	0.62	0.11
M6	390	13	9.83	393.84	7.81	0.10	0.05	0.57	0.13
M6	390	17	9.72	390.08	7.81	4.31	0.13	0.43	0.14
M6	390	21	9.71	355.85	7.85	2.60	0.06	0.19	0.07
M6	390	25	9.73	353.47	7.85	1.80	0.04	0.20	0.23
M6	390	29	NA	334.87	7.84	1.40	0.04	0.13	0.24
M7	2000	1	8.62	454.67	7.74	1.30	0.15	1.07	0.21
M7	2000	5	9.51	2065.92	7.20	0.29	0.07	0.79	0.03
M7	2000	9	9.25	1746.04	7.25	0.10	0.05	0.51	0.05
M7	2000	13	9.83	1539.45	7.31	0.10	0.04	0.42	0.07
M7	2000	17	9.72	1260.96	7.38	4.31	0.13	0.24	0.03
M7	2000	21	9.71	1133.00	7.42	2.10	0.04	0.03	0.12
M7	2000	25	9.73	922.60	7.49	1.90	0.04	0.09	0.22
M7	2000	29	NA	753.07	7.50	1.50	0.04	0.02	0.23
M8	560	1	8.62	465.18	7.73	1.20	0.18	1.04	0.06
M8	560	5	9.53	594.55	7.65	0.39	0.07	0.79	0.03
M8	560	9	9.26	568.96	7.67	0.20	0.09	0.63	0.11
M8	560	13	9.85	570.57	7.67	0.10	0.07	0.55	0.11
M8	560	17	9.72	532.43	7.70	4.41	0.15	0.42	0.08
M8	560	21	9.72	484.37	7.73	2.60	0.05	0.12	0.03
M8	560	25	9.73	466.94	7.74	1.70	0.05	0.15	0.19
M8	560	29	NA	442.62	7.74	1.30	0.05	0.11	0.24

Mesocosm	Target CO_2	Day	Temperature (°C)	pCO_2 (µatm)	pH (Free scale)	Nitrate*	Phosphate*	Silicate*	Ammonia*
M9	3000	1	8.63	468.91	7.72	1.30	0.16	1.08	0.05
M9	3000	5	9.53	3056.76	7.05	0.29	0.08	0.85	0.01
M9	3000	9	9.27	2570.59	7.11	0.10	0.06	0.59	0.05
M9	3000	13	9.85	2095.81	7.19	0.10	0.04	0.50	0.08
M9	3000	17	9.72	1645.23	7.28	4.51	0.14	0.42	0.10
M9	3000	21	9.71	1541.59	7.31	3.50	0.06	0.26	0.09
M9	3000	25	9.72	1197.49	7.40	1.90	0.05	0.28	0.05
M9	3000	29	NA	932.22	7.35	1.00	0.05	0.16	0.01

4.5 Effect of ocean acidification on the structure and fatty acid composition of a natural plankton community in the Baltic Sea

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Abstract

Increasing atmospheric carbon dioxide (CO_2) is changing seawater chemistry towards reduced pH, which consequently affects various properties of oceanic organisms. Coastal and brackish water communities are expected to be less affected by ocean acidification (OA) as these communities are typically adapted to high fluctuations in CO_2 and pH. Here we investigate the response of a coastal brackish water plankton community to increasing CO_2 levels as projected for the coming decades and the end of this century in terms of community and biochemical (fatty acid, FA) composition. A Baltic Sea plankton community was enclosed in a set of off-shore mesocosms and subjected to a CO_2 gradient ranging from natural concentrations (240 µatm pCO_2) up to values projected for the year 2100 (1400 µatm pCO_2). We show that the phytoplankton community composition was very resilient to CO_2 and did not diverge between the treatments, while the seston FA composition was at least partially influenced by phosphate limitation in the mesocosms and showed no differences between the CO_2 treatments. These results suggest that CO_2 effects are dampened in coastal communities that already experience high natural fluctuations in pCO_2. However, although this coastal plankton community was tolerant to high pCO_2 levels, increase in hypoxia and CO_2 uptake by the ocean can aggravate acidification and may lead to pH changes outside the currently experienced range for coastal organisms.

Introduction

The steady increase of carbon dioxide (CO_2) since the beginning of the industrial era due to anthropogenic emission has caused a double in its atmospheric concentration (IPCC, 2013). The ocean has a large carbon sink capacity, and increasing atmospheric CO_2 absorbed by the ocean is changing the chemistry of the seawater, causing a decline in pH termed Ocean Acidification (OA) (IPCC, 2013). OA has been shown to affect various biological processes of diverse marine species (Doney et al., 2009; Kroeker et al., 2010). For instance OA can impact the biochemical and elemental composition of organisms (Sato et al., 2003; Torstensson et al., 2013), which can be later transferred to higher trophic levels (Rossoll et al., 2012); also can drive alterations in the community composition structure of primary producers (PP) (Hare et al., 2007; Biswas et al., 2011; Schulz et al., 2013). Strong CO_2-effects might be particularly significant in oceanic species that experience low natural fluctuations in pCO_2. In contrast, coastal and brackish-water environments encounter wide and frequent fluctuations in pCO_2 (Hinga, 2002) due to large fluxes of organic and inorganic carbon from river runoff and lower alkalinity and hence reduced buffer capacity (Melzner et al., 2013). Consequently, it can be expected that coastal and brackish communities are more tolerant to OA effects (Rossoll et al., 2013; Reusch, 2013) and deleterious CO_2 effects in terms of the biochemical makeup of PP and variations in community composition may be diminished.

Fatty acids (FA) are the main component of lipids in cell membranes. In particular polyunsaturated FA (PUFA) have important physiological roles in algae, which synthesize them high in amounts. Heterotrophs in higher trophic levels cannot synthesize certain FA *de novo*, especially PUFA, and have to acquired them from dietary sources (Arts et al., 2009). Diverse laboratory studies of monocultures showed that CO_2 alter the FA profile of individual algal species (Sato et al., 2003; Fiorini et al.,2010; Torstensson et al., 2013). A CO_2-driven change in algal food quality can be detrimental for grazers, as has been shown in a laboratory study under elevated CO_2 levels (Rossoll et al., 2012), where a strong decline of PUFA in a diatom grown at high CO_2 affected the FA composition of copepods grazing on them and severely impaired their development and egg production rates. Furthermore, increasing seawater CO_2 can modify phytoplankton community

composition by favoring certain taxa of primary producers (Graeme et al., 2005). In particular, small-sized cells benefit from high CO_2 (Hare et al., 2007; Biswas et al., 2011; Brussaard et al., 2013). This is ecologically relevant as taxonomic phytoplankton groups have contrasting FA profiles (Dunstan et al., 1992; Viso & Marty, 1993; Zhukova & Aizdaicher, 1995) and a change in community structure can affect higher trophic levels. For instance, a field study of two cladocerans having different phytoplankton composition as food source showed a decreased egg production, lipid reserves and body size/abundance when fed with algae from an acidic lake (Locke & Sprules, 2000).

The above observations suggest that changes in planktonic biochemical makeup and associated shifts in community composition of primary producers as a result of OA can affect the transfer of essential compounds to upper trophic levels. However, organisms and communities from coastal/brackish environments may show a high tolerance to elevated pCO_2 levels due to adaptation (Thomsen et al., 2010; Nielsen et al., 2010; Rossoll et al., 2013). In coastal/brackish systems variation in CO_2 are more frequent and severe due to river runoffs (Hinga, 2002), reduced buffer capacity (Feely et al., 2004), seasonal processes (Melzner et al., 2013) and upwelling of CO_2 enriched water (Feely et al., 2008), all of which leads to wider pH variation in coastal systems compared to the open ocean (Hinga, 2002). Laboratory studies have shown that algae subjected to long-term high CO_2 levels can restore its physiological optima through adaptive evolution (Lohbeck et al., 2012) and that coastal communities are resilient to OA-driven changes in community composition and biomass (Nielsen et al., 2010; Rossoll et al., 2013). Therefore, it can be expected that organisms in these areas are adapted to high CO_2 fluctuations, hampering any CO_2-driven effects previously observed in plankton communities (Locke & Sprules, 2000; Biswas et al., 2011).

The goal of the present study was to determine if an increase in CO_2 affects phytoplankton community composition and their FA makeup, and if any effects are transferred to grazers of a natural plankton community in a coastal/brackish environment. A set of off-shore mesocosm CO_2 perturbation experiments that enclosed a natural plankton assemblage of the Baltic Sea were used as experimental units. The CO_2 levels ranged from current to predicted values by the end of this century (IPCC,

2013). Algal FA were measured as a whole in the seston and in the copepods *Acartia tonsa* and *Eurytemora affinis*, respectively, which are dominant zooplankters in this ecosystem.

Material and Methods

Experimental set-up and CO_2 manipulation

Our study was conducted during an off-shore CO_2 mesocosm perturbation experiment at the Tvärminne Biological station in the Archipelago Sea in the northern Baltic between the Gulf of Bothnia, the Gulf of Finland and the Sea of Åland during late spring 2012 using nine enclosures with a length of 17 m containing ~50 m^3 of natural sea water. The mesocosm were set up and manipulated as described in detail by Riebesell et al. (2013). Carbon dioxide enrichment was achieved through the addition of CO_2-saturated seawater to seven out of nine mesocosm in five steps between day 1 and day 5 to achieve target values of 390, 540, 840, 1120 and 1400 µatm plus two controls unit with a natural concentration of 240 µatm. Samples for phytoplankton counts were taken every second day and for fatty acid every 4th day using a depth-integrated water sampler (Hydrobios, Kiel, Germany) covering the upper 15m of the water column; integrated zooplankton tow nets were taken every 7th day starting before the CO_2 manipulation (day -1).

Phytoplankton abundance and biomass calculation

Phytoplankton cell counts up to a cell size of 20µm were carried out from 50ml sample water, fixed with alkaline Lugol's iodine (1% final concentration) using the Utermöhl's (1958) method with an inverted microscope (ZEISS Axiovert 100). At 200 times magnification, cells larger than 12µm were counted on half of the chamber area, while smaller cells were counted at 400 times magnification on two radial strips; ciliates were counted in the entire chamber with a magnification of 50. Plankton were identified to genus or species level according to Tomas (1997), Hoppenrath et al. (2009), Kraberg et al. (2010) and von Quillfeldt (1996). Algae biovolumen was calculated according to geometric shapes and converted to cellular organic carbon using taxon-specific

conversion equations [for phytoplankton Menden-Deuer & Lessard (2000) and for ciliates Putt & Stoecker (1989)].

Fatty acids composition

For algal FA, 500 ml of seawater was filtered by using pre-combusted (450°C, 6 h) Whatman GF/F (~0.7 µm ø pore) filters. FA Individuals of the copepods *Acartia tonsa* and *Eurytemora affinis* (adult females) were sorted for FA measurement. All samples were immediately stored at -80°C until analysis. FA were measured by gas chromatography as fatty acid methyl esters (FAMEs) following Klein Breteler & Schouten (1999). 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, FAME C19:0 (Restek, Bad Homburg, Germany; c= 20.0 ng component^{-1}µl^{-1}) was added, and a C23:0 FA standard (c= 25.1 ng µl^{-1}) 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 (100-150 mbar), re-dissolved in Chloroform and transferred into a glass cocoon. The solvent was evaporated again (10-30 mbar), and esterification was performed over night using 200µl 1% H_2SO_4 (in CH_3OH) 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. FAME were analyzed by a Thermo GC Ultra gas chromatograph equipped with a non-polar column (RXI1-SIL-MS 0.32 µm, 30 m, company Restek) and Flame ionization detector. 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. A 1 µl aliquot of the sample was injected. The system was calibrated with a 37-component FAME-mix (Supelco, Germany) and chromatograms were analyzed using Chrom-Card Trace-Focus GC software (Breteler & Schouten, 1999) and the fatty acids were clustered according to their degree of saturation: Saturated (SFA), Monounsaturated (MUFA) and Polyunsaturated (PUFA).

Statistical analyses

To identify differences in thecalculated plankton biomass and relative content of FAs between the pCO_2 treatments, absolute deviations (AD) from the overall mean were calculated for each mesocosm for the different plankton taxa and FA groups (SFA, MUFA, PUFA). Therefore, the residuals (RE) were calculated by subtracting the arithmetic mean of all mesocosm observations per time-point from each mesocosm observation at that time-point according to Engel et al. (2013). The RE were analyzed by a nested mixed effects model (MEM) to determine the differences in taxa biomass (µgC/ml) and relative fatty acid content (%) between the CO_2 treatments (µatm CO_2) with the treatment level as nested random variable (random distribution of CO_2 treatments among the mesocosm). Average mesocosm CO_2 was calculated for the total duration of the sampling period (day 1 to 25 for seston and day -1 to 33 for zooplankton). The similarity in the structure of the plankton community between the treatments was analyzed by Non Metrical Multidimensional Scaling (NMDS) with Bray distance, auto-transformation and 3 dimensions (k=3). This analysis distributes the samples in an ordination space according to the biomass of the different taxa in the community along orthogonal principal components using non Euclidean distances for ordination space, which makes it more robust to the presence of zero values (Clarke, 1993). An analysis of similarity (ANOSIM) using a Bray-Curtis distance matrix and 5000 permutations was used to corroborate the NMDS results. ANOSIM is a non-parametric multivariate procedure that is widely used for testing whether or not groups are statistically different in respect to their relative similarities in community composition (Clarke, 1993). All statistical analyses were done using the R software environment 3.0.1 (R Development Core Team 2005).

Results

Plankton community composition

A seemingly poor plankton flora was present in the mesocosms, with a high abundance of either heterotrophic or mixotrophic species, which is a typical after spring bloom situation in the Baltic and occurs when the nutrients have been depleted. The plankton community between 4 and up to 20 µm in size showed a high microzooplankton biomass

during the entire experimental period, particularly the Choanoflagellate *Calliacantha natans* was the most abundant planktonic organisms in all mesocosms (Fig. 1). The initial algal community consisted of post-bloom species dominated by small-sized cells with Heterokontophyta and Dinophyta being the most abundant phytoplankton groups in all mesocosm throughout the experiment (Fig. 1). Phytoplankton biomass gradually increased until day 10 when a bloom started, it reached a peak around day 15 in all treatments and began to decay at around day 21 (Fig. 1).

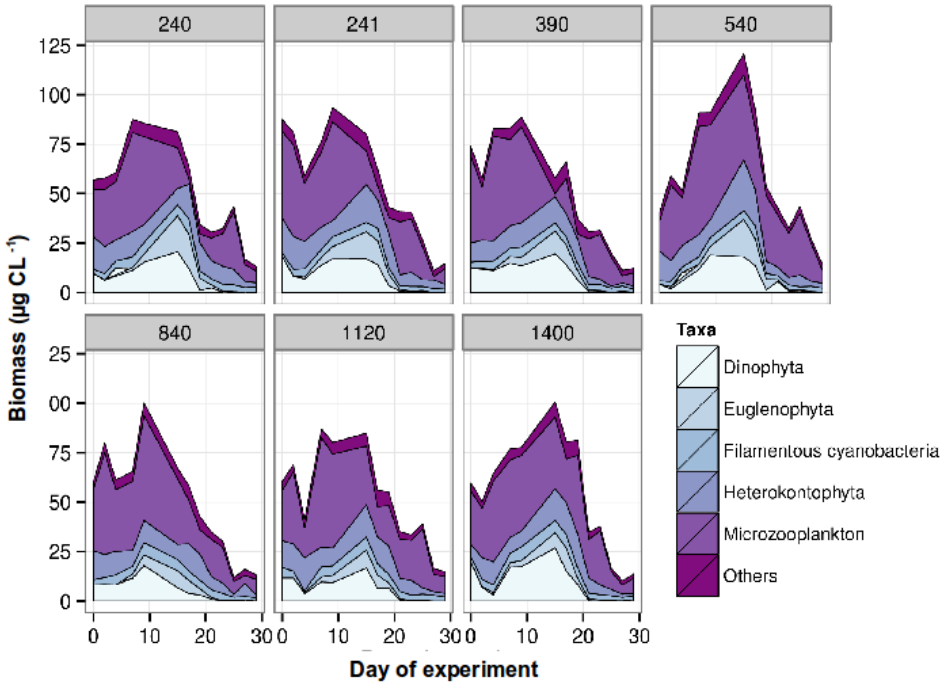

Figure 1. Calculated biomass after cell counts of the main plankton taxonomic groups in the different CO_2 treatment. Each treatment is labeled with its target CO_2 level (top).

An NMDS analysis of the entire phytoplankton community showed three distinctive phases in the community composition over time: 1) pre-bloom from day 0 to 10; 2) bloom from 11 to 21; and 3) post-bloom period from day 22 to 29 (Fig. 2). There was no clear difference between CO_2 treatments in the NMDS analysis (Fig. 2). The temporal variation in community composition was confirmed by ANOSIM ($R= 0.68$; $p<0.0001$) with no CO_2-related effect in the plankton community composition ($R= -0.01$; $p= 0.8$).

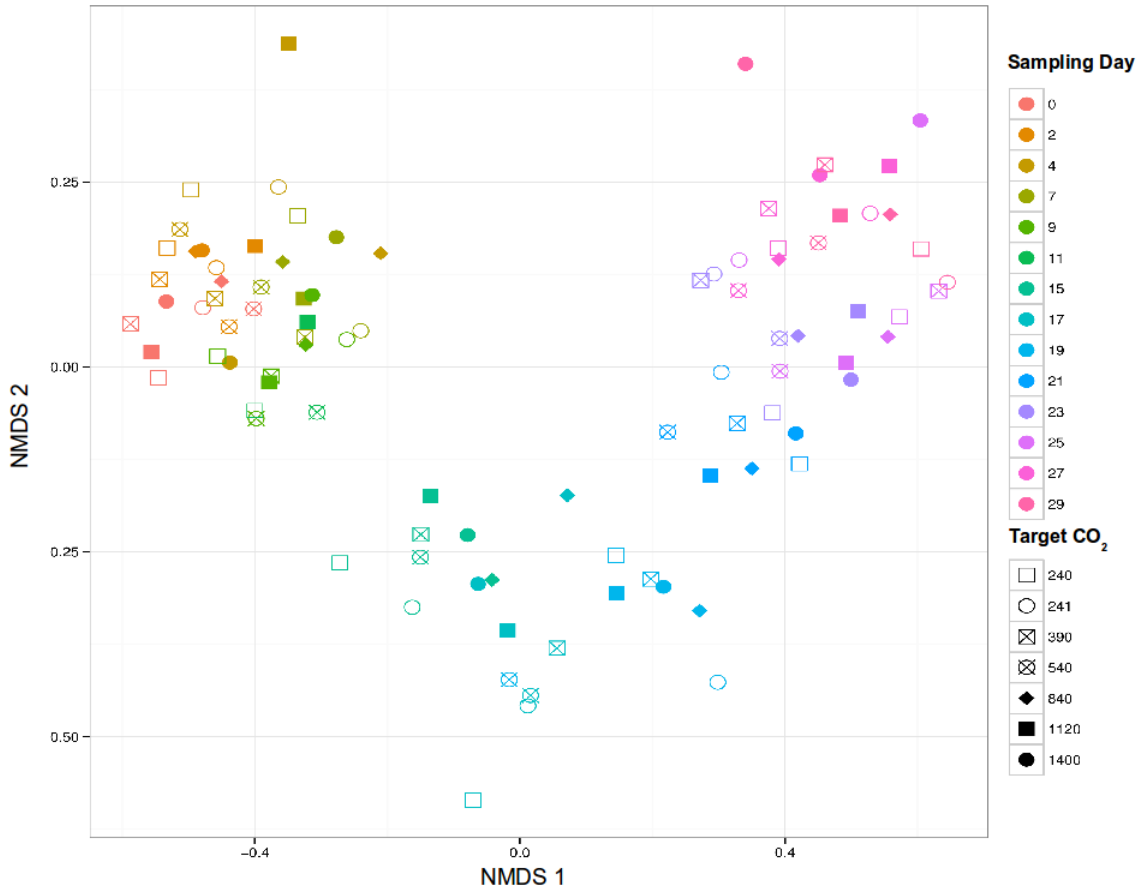

Figure 2. Non Metrical Multidimensional Scaling (NMDS) of the plankton community composition in terms of calculated biomass change through sampling days and CO_2 treatment. The NMDS 1 axis show that the phytoplankton communities strongly diverge through time (ANOSIM, R= 0.68; p<0.0001) while the NMDS 2 axis show that the communities don not differ with CO_2 treatment levels (ANOSIM, R= -0.01; p= 0.8).

Although the more abundant taxa did not show differences in abundance between the CO_2 treatments, the biomass of some of the less abundant groups was affected by CO_2 within the different phases. In phase 1 the nested mixed effects model analysis of the algal biomass showed that Chlorophyta decrease significantly towards high CO_2 levels (Fig. 3a) (MEM, F= 7.27, p= 0.01, df= 20). The same declining trend of Chlorophyta at high CO_2 was observed during the post bloom conditions (Fig. 3c) (MEM, F= 4.26, p= 0.05, df= 20). In phase 2 during the algae bloom microzooplankton, particularly Ciliophora showed a significant increase towards high CO_2 (Fig. 3b) (MEM, F= 16.38, p< 0.001, df= 18).

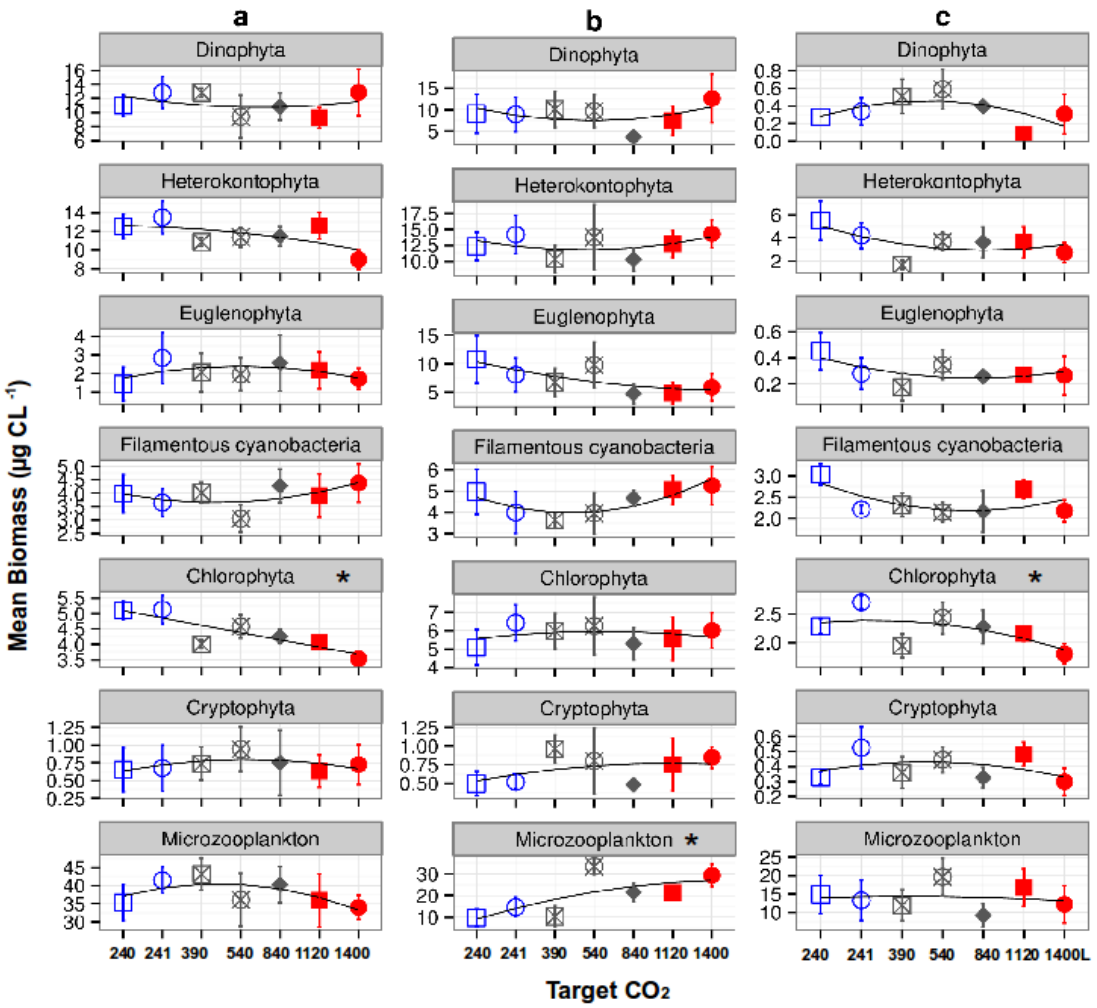

Figure 3. Mean of the calculated biomass of each plankton taxa between the days a) 0 to 10, b) 11 to 21, and c) 22 to 29 in the CO_2 gradient treatments. An * indicates statistical significance (MEM, p< 0.05). Blue, grey and red indicates low, intermediate and high CO_2 treatments respectively. The x-axis show the target CO_2, error bars show standard error (n=5 for a; n=4 for b; n= 4 for c).

Seston fatty acid composition

The PUFA represented on average ~26 ± 4%, MUFA ~22 ± 3% and SFA ~52 ± 4% of the total FA content in the seston over the entire experimental period. The FA composition of the seston showed that the relative PUFA content significantly decrease over time in all mesocosms (Linear regression, R^2= 0.52, t= -7.64, p<0.0001) (Fig. S1), while the MUFA and SFA increased, although the relation of both with time is weak (Linear regression, R^2= 0.12, t= 2.88, p= 0.005 and R^2= 0.15, t= 3.26, p= 0.001 respectively) (Fig. S1). However the analysis of the residuals of the overall mean PUFA data showed that the decrease is homogeneous in all mesocosm and did not show any significant difference among the CO_2 treatments (MEM, F= 0.0, p= 0.98, df= 45) (Fig. 4a). The MUFA and SFA

also did not show any significant change in abundance in relation with CO_2 (MEM, F= 0.0, p= 0.8, df= 45 and F= 0.06, p= 0.79, df= 45 respectively) (Fig. 4b,c). Nevertheless, PUFA showed a relation with phosphate in the mesocosms (quadratic fit, R^2= 0.49, t= -2.8, p= 0.001) (Fig. 5); while the phosphate showed a significant decrease over time (quadratic fit, R^2= 0.6, t= -6.9, p= 0.001), reaching a minimum concentration at the peak of the bloom with a slight increase afterwards (Fig. S2).

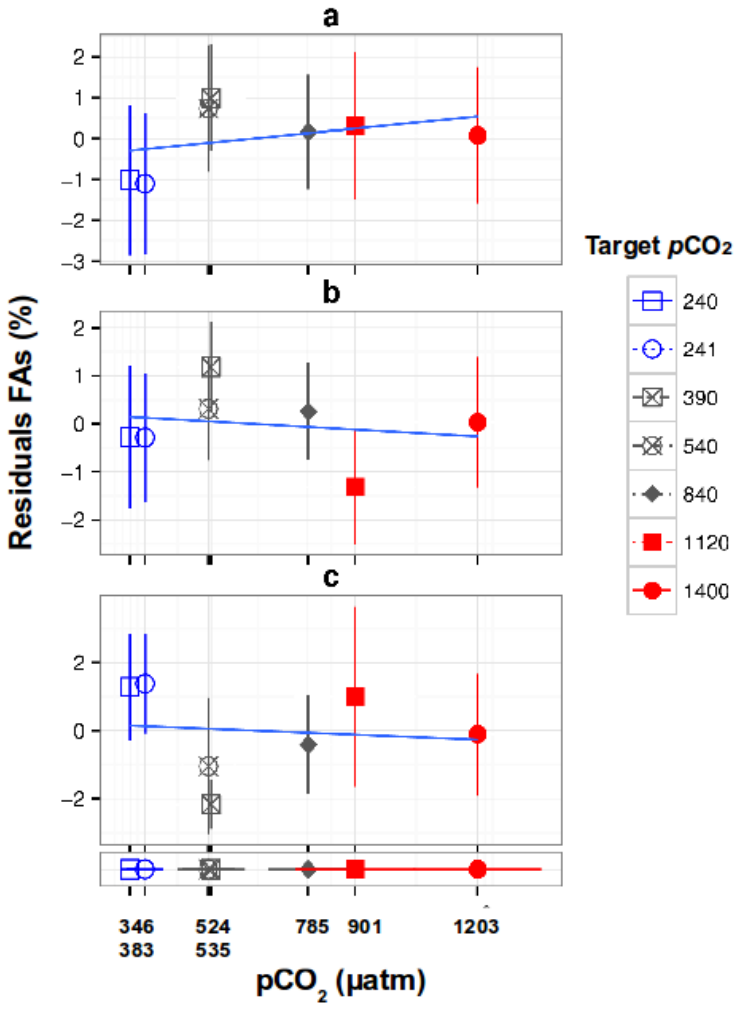

Figure 4. Residuals of the relative a) PUFA, b) MUFA, and c) SFA content in the seston under the CO_2 gradient treatments. The x-axis and bottom panel show the mean pCO_2 measured during the sampling period, bars shows standard error (MEM, p>0.05).

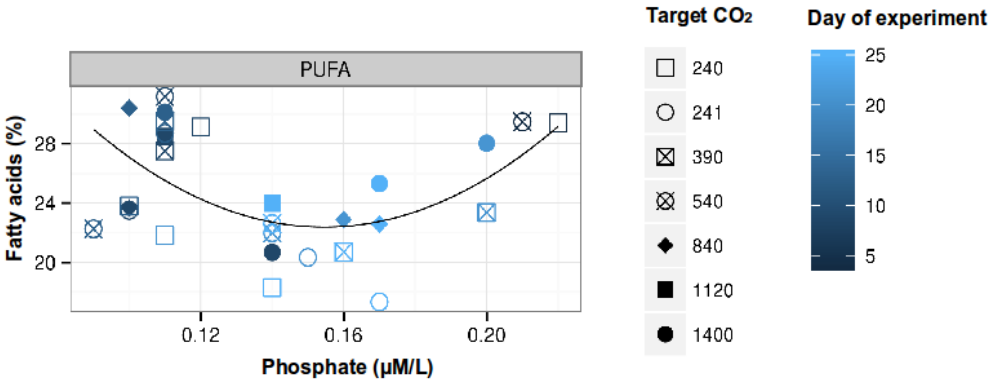

Figure 5. Relation between the relative seston PUFA content and phosphate across the CO_2 treatments (quadratic fit, R^2= 0.6, t= -6.9, p=0.001).

Copepods fatty acids

The overall PUFA content of the copepod *A. tonsa* represented ~12% (311 ± 175 ngFA/mg dry wt.) and in *E. affinis*a ~16% (433 ± 597 ngFA/mg dry wt.) of the total FA. The relative PUFA content in *A. tonsa* and *E. affinis* showed a significant decrease over time in all mesocosm (linear regression, R^2= 0.22, t=-3.288, p= 0.002 in *A. tonsa*; R^2= 0.47, t= -5.51, p<0.0001 in *E. affinis*), while MUFA and SFA increased in both species (Fig. S3). Furthermore, the relative FA group content in *E. affinis* varied over time following the changes in the seston FA, although this relation was weak (Fig. S4); while in *A. tonsa* this changes appeared only in the MUFA (Fig. S4). However the PUFA decrease was not related to CO_2 levels in any of the copepods (MEM, F= 0.62, p= 0.4374, df= 26 in *A. tonsa*; F= 0.13, p= 0.71, df= 26 in *E. affinis*) (Fig. 6).

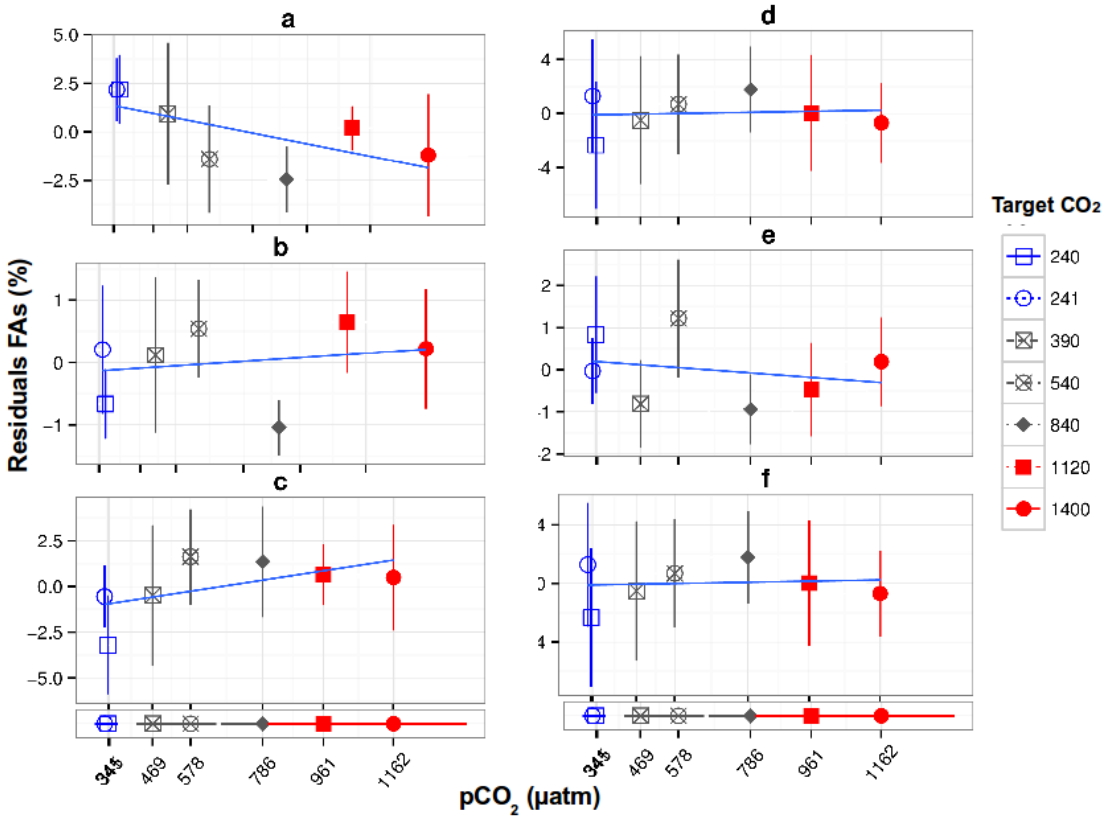

Figure 6. Residual of the relative PUFA (a, d), MUFA (b, e), and SFA (c, f) content in the copepod *Acartia tonsa* (a, b, c) and *Eurytemora affinis* (d, e, f) in the CO_2 gradient treatments. The x-axis and bottom panel show the mean pCO_2 measured during the sampling period from day 1-25, error bars show standard error (MEM, p>0.05).

Discussion

Community composition

The plankton community composition in the present experiment did change over time and showed little differences in relation to the different CO_2 treatments. The rather homogeneous change in community composition between the mesocosms mover time and the algae bloom can be attributed to the initial mixing of the water column during mesocosms setup, which carried nutrient rich bottom water to the upper layer. The observed absence of a strong CO_2 effect on the community composition in the present study is in line with the observations in the western Baltic Sea (Thomsen et al., 2010; Nielsen et al., 2010; Rossoll et al., 2013). In these studies the authors suggested that the plankton community is adapted to OA due to the recurrent large seasonal and daily variance of pH and CO_2 experienced by the communities in this productive low-salinity

region (Thomsen et al., 2010; Nielsen et al., 2010; Rossoll et al., 2013). Our study region, a coastal zone in the Archipelago Sea of the northern Baltic, is a brackish environment with low salinity (~5.7 ‰), a high fresh water runoff (~111 km^3 $year^{-1}$) (Savchuk, 2005) and a strong inter and intra seasonal pH variability sometimes reaching extreme values of 9.2 and 7.4 with an average of 8.1 (Brutemark et al., 2011). Therefore, it seems that the plankton community in our study area, which experiences high natural pH fluctuations, is composed of species and genotypes that are less pH/CO_2 sensitive (Nielsen et al., 2010; Lohbeck et al., 2012; Melzner et al., 2013; Rossoll et al., 2013) which allows them to cope with the CO_2 range applied in our experiment.

Chlorophytes were the only group that showed a significant response to CO_2 variation, although their contribution to total biomass was relatively low. Chlorophyta decreased at increasing CO_2 in our experiment, which is contrasting laboratory studies showing that several species benefit from high CO_2 and increase their growth rates (Tsuzuki et al., 1990; Yang & Gao, 2003). The decrease in our experiment could also be related to increasing grazing pressure from ciliates, which increased at high CO_2, which can however not be disentangled with the available data.

Seston FAs

The relative seston PUFA content showed a significant decrease over time in our study, which can be attributed to nutrient depletion in the mesocosm, particularly phosphate concentration, although the trend is not very clear and further analysis is required. Phosphorus is required for the production of PUFA-rich membrane phospholipids during cell division and growth (Guschina & Harwood, 2009). Nutrient limitation, which causes reduced cell division rates, result in a lower production of phospholipid and increased production of storage lipid, primarily triacylglycerols (TAG), which tend to be rich on SFA and MUFA, therefore the increase in TAG with nutrient limitation typically resulted in decreased proportions of PUFA in most algae (Guschina & Harwood, 2009). This goes in line with our observations in the mesocosms, where the relative seston PUFA content followed the phosphate concentration in the mesocosms. Under this perspective it is reasonable to expect that any CO_2 effect in algal PUFA will occur when cells are actively

growing since nutrient limitation (phosphorus) will have more profound consequences in the cell physiology that an excess of CO_2.

The absence of a PUFA response to CO_2 in our study contrast with a recent report of an Arctic plankton community showing an increase of PUFA at high CO_2 levels during part of an experiment with mesocosms where nutrients were added (Leu et al., 2013). This was attributed to a change in the plankton community composition due to a rise in abundance of dinoflagellates at high CO_2 (Leu et al., 2013). Although dinoflagellates were abundant during our study they showed similar amounts across the CO_2 treatments, and overall there were no significant differences in the plankton community composition among the mesocosms. The different responses between these experiments can be attributed to the specific plankton community composition and their related FA profiles alongside with phosphate limitation in our study. Species composition of a natural plankton assemblage determines its food quality properties as distinct algal taxonomic groups have different FA composition profiles (Dunstan et al., 1992; Viso & Marty, 1993; Zhukova & Aizdaicher, 1995). A CO_2-driven change in the community composition of the Arctic plankton community (Leu et al., 2013) promoted the presence of species rich in PUFA, while in our study the absence of a CO_2 response in taxa composition and the apparent influence of phosphate limitation in the algal FA composition resulted in a rather homogeneous PUFA concentration between treatments.

Copepod fatty acids

The PUFA concentration of the dominating copepod species, *A. tonsa* and *E. affinis* did not change between the different CO_2 treatments. However, the PUFA decrease in both copepods over the experimental period mimics the decline in the seston. This observation is consistent with other studies showing that copepods strongly rely on their diet as source of FA and that their composition, especially PUFA, mirrors the algal they graze on (Ishida et al., 1998; Caramujo et al., 2007; Rossoll et al., 2012).

A direct CO_2 effect in copepods is unlikely, as previous experiments have shown that some species, like *A. tonsa*, are rather insensitive to projected high CO_2 scenarios and up

to 5000 µatm CO_2 (Kurihara et al. 2004; Kurihara & Ishimatsu, 2008). Several studies have demonstrated that food quality of the available prey in terms of PUFA content affect egg production, hatching success and nauplii survival in copepods (Jónasdóttir, 1994; Caramujo et al., 2007; Jónasdóttir et al., 2009). Although indirect CO_2 deleterious effects through the diet of primary consumers have been reported in laboratory and field experiments (Rossoll et al., 2012; Locke & Sprules, 2000), the absence of a CO_2-driven change in the primary producers community composition and the homogeneous algal FA makeup due to phosphate limitation, counteracted-masked any noticeable CO_2-related effects in the algae FA profile that could have affected the copepods during our experiment.

Considering that the Baltic Sea is a coastal ocean with a natural frequent and wide pH variability (Omstedt et al., 2009), it can be expected that the effects of OA on plankton communities will be rather small within the range of predicted values for this century (Havenhand, 2012). A reduced OA effect in systems experiencing high CO_2 fluctuations is supported by our results and other studies using communities from the same region (Thomsen et al., 2010; Nielsen et al., 2010; Rossoll et al., 2013). However, in coastal upwelling areas undergoing an increase on hypoxia events, it is likely that elevated CO_2 values as presently experienced by coastal organisms and projected by the end of the century (Melzner et al., 2013; IPCC, 2013) will be more recurrent in the future (Feely et al., 2004), with the potential to affect various properties of plankton communities.

Nonetheless, it is clear that the plankton community response to OA and concomitant effects on its food quality for higher trophic levels will strongly depend on the sensitivity of primary producers and on how OA affects the specie composition of plankton assemblages (Leu et al., 2013; Rossoll et al., 2013). This result is important as any change in primary producers in terms of FA, particularly essential biomolecules such as PUFA, may scale up in food webs since FAs are incorporated into the lipids of larval fish (Fraser et al., 1989; Izquierdo et al., 2001). Considering that fish is a critical natural resource (FAO, 2010), deleterious OA effect on food quality can be a threat for human populations who rely on fisheries as an important food source (Sargent et al., 1997; Arts et al., 2001).

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Supplementary Information

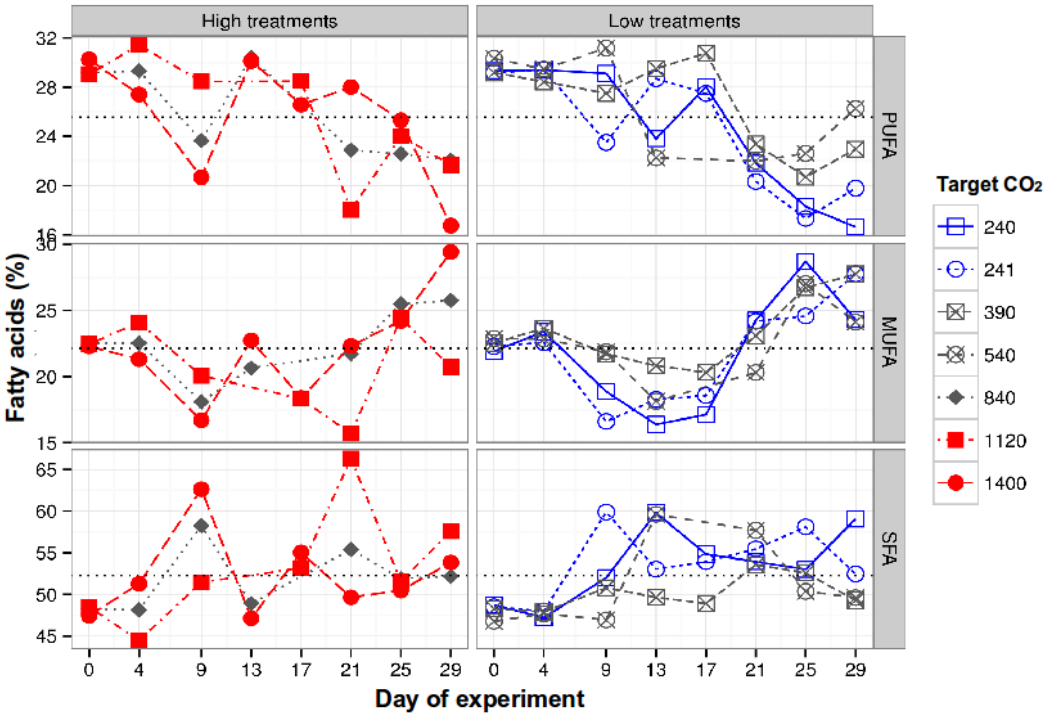

Figure S1. Relative seston PUFA, MUFA and SFA content over experimental duration across the CO_2 treatments. The PUFA of all CO_2 treatments showed a significant decrease over time (Linear regression, R^2= 0.52, t= -7.64, p<0.0001). The MUFA and SFA increase, although the relation of both with time is weak (Linear regression, R^2= 0.12, t= 2.88, p= 0.005 and R^2= 0.15, t= 3.26, p= 0.001 respectively) Horizontal dashed line indicates the position of the overall mean FA value.

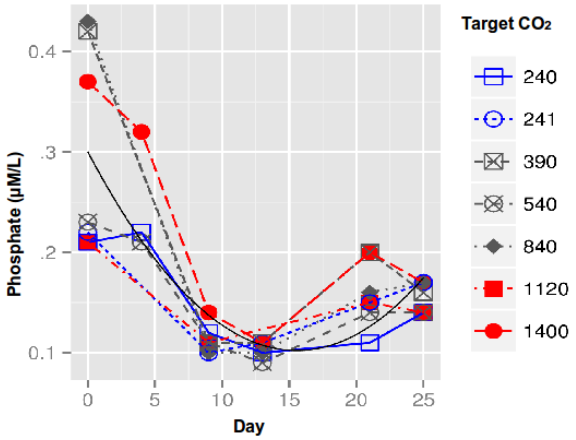

Figure S2. Phosphate content in the different mesocosm over time (Quadratic fit, R^2= 0.60, t= -6.9, p<0.0001).

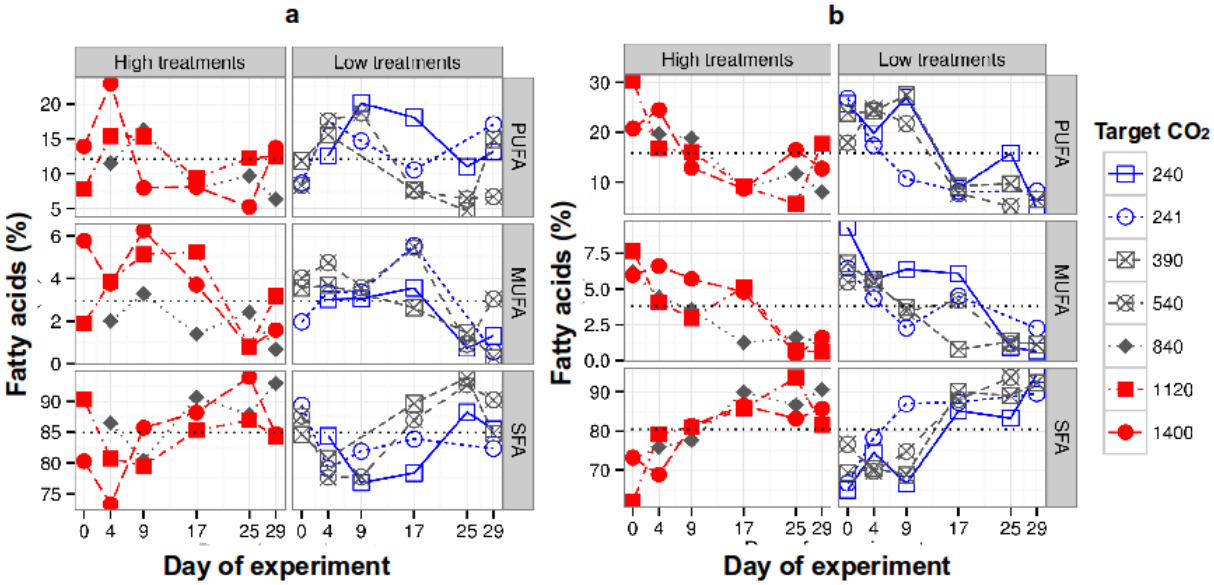

Figure S3. Relative PUFA, MUFA and SFA content of the copepod (a) *Acartia tonsa* and (b) *Eurytemora affinis* across the CO_2 treatments. The PUFA of all CO_2 treatments showed a significant decrease over time (Linear regression, R^2= 0.22, t= -3.288, p= 0.002 in *A. tonsa* and R^2= 0.47, t= -5.51, p<0.0001 in *E. affinis*). Horizontal dashed line indicates the position of the overall mean PUFA value.

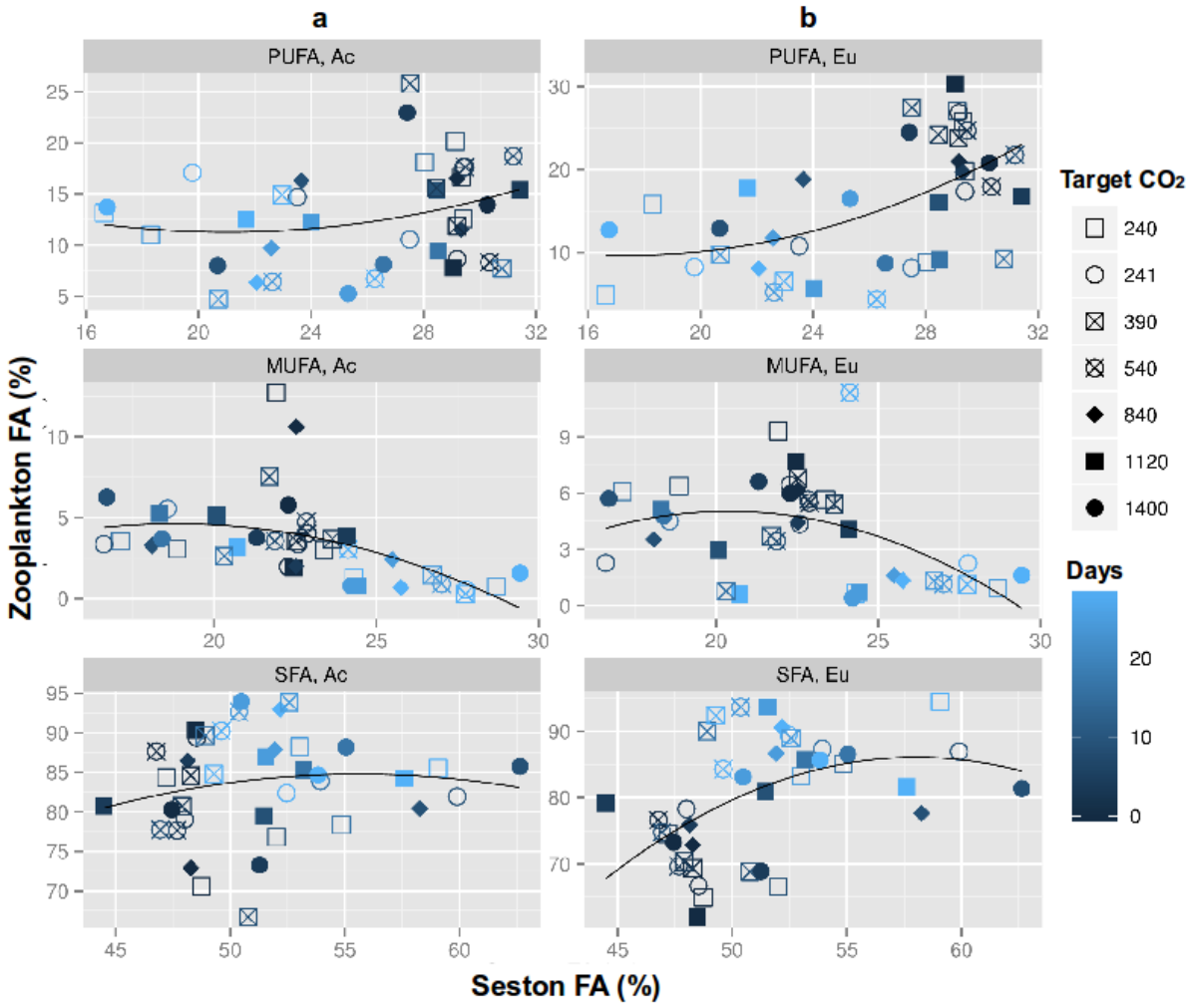

Figure S4. Relative PUFA, MUFA and SFA content of the copepods (a) *Acartia tonsa* and (b) *Eurytemora affinis* in relation to the respective seston FA across the CO_2 treatments. (Linea regression, *E. affinis*: R^2= 0.18, t= 2.818, p= 0.008, PUFA; R^2= 0.10, t= -2.37, p= 0.02, MUFA; R^2= 0.16, t= 2.91, p= 0.005, SFA; *A tonsa*: p= 0.2 PUFA; R^2= 0.18, t= -2.97, p= 0.005, MUFA; p= 0.5, SFA)

4.6 Effect of long-term high CO_2 exposure on the fatty acid composition of two key marine phytoplankton species

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Abstract

Rising atmospheric CO_2 levels and its uptake by the ocean, a process termed ocean acidification (OA), affects the macromolecular composition of a wide range of marine organisms. Of particular interest is the fatty acids (FA) composition of primary producers, because it critically determines their nutritious value for higher trophic levels. Several studies have shown that high CO_2 levels can affect the FA profile of marine algae and alter the content of polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acids in the cells. However, the majority of these studies are short-term experiments and do not account for potential long term metabolic regulation or adaptive evolution in the algae. Here we analyzed the FA composition in cultures of the coccolithophore *Emiliania huxleyi* and the diazotrophic cyanobacterium *Trichodesmium* sp. that had been long-term adapted (>1000 generations) to a range of different CO_2 conditions in laboratory selection experiments. Our results show an differentiate adaptive response to elevated CO_2 in both plankton species among FA. The different FA classes were similarly affected by CO_2 in both, but differed between FA groups. Adaptation to elevated CO_2 increased the FA content of all groups, especially SFA. In *E.*

huxleyi the PUFA content was reduced in high CO_2 adapted relative to control lines when subjected to high CO_2 conditions. *Trichodesmium* sp. had very low PUFA concentrations in general and we did not find any adaptive responses. Finally the MUFA content showed a moderate adaptive response in both. Our observations show that CO_2 has a strong influence in FA metabolism and that planktonic organisms can respond to this influence through adaptation. Thus, short-term effects on FA, affecting the nutritious value of marine phytoplankton for higher trophic levels may be mitigated by adaptive evolution.

Introduction

Ocean acidification (OA), caused by altered ocean carbon chemistry as a result of increasing uptake of anthropogenic carbon dioxide (CO_2) (IPCC, 2013) affects the biochemistry of a wide range of marine organisms in terms of elemental and macromolecular composition (Riebesell, 2004; Doney et al., 2009; Kroeker et al., 2010). Macromolecular energy storage and consumption in the form of lipids are a crucial process in all living organisms. Lipids consist mostly of hydrocarbon chains of different length and saturation (number of double bonds) known as fatty acids (FA). FA are generally classified in saturated (SFA, no double bonds), monounsaturated (MUFA, one double bond) and polyunsaturated (PUFA, with two or more double bonds). For heterotrophic organisms PUFA are particularly relevant as consumers are largely depend on their prey as source of these essential metabolites, which are produced in high amounts by primary producers (PP). The macromolecular status of prey algae in terms of FA quality and quantity, and its transfer to higher trophic levels is a crucial factor in the life cycle of marine zooplankton and fish (Izquierdo et al., 2001; Jónasdóttir et al., 2009). However, the amount of the diverse FA produced by algae is strongly affected by environmental conditions like temperature (Rousch et al., 2003), nutrients (Harrison et al., 1990; Reitan et al., 1994) and CO_2 (Tsuzuki et al., 1990; Sato et al., 2003).

The effect of OA on algal FA composition has gained increasing awareness in recent years. Laboratory experiments conducted in mono-specific cultures show that the effects of high CO_2 on algal FA, particularly in PUFA content, seems to be species

specific, ranging from positive (Hoshida et al., 2005; Fiorini et al., 2010) or neutral (Tsuzuki et al., 1990), to negative, with several studies showing a reduction in PUFA and a concomitant increase in SFA content under high CO_2 conditions (Riebesell et al., 2000; Rossoll et al., 2012; Torstensson et al., 2013). The mechanisms by which CO_2 affects algal FA are unresolved, although Sato et al. (2003) suggested that an elevate SFA level in cells grown at high-CO_2conditions may result in enhanced FA synthesis and accumulation. However, the majority of these studies were short-term experiments where an organism was exposed to high CO_2 conditions to determine its physiological response (Doney et al., 2009; Reusch, 2013). Short-term studies offer valuable insights into the tolerance of an organism to CO_2 related stress but do not account for the potential of evolutionary adaptation to the new environmental conditions that may affect metabolic regulation in the cell in an unforeseen way (Collins, 2011; Lohbeck et al., 2012). Understanding the potential for adaptation of planktonic FA composition to high CO_2 is important as changes in primary producer food quality will most likely be transferred to higher trophic levels, affecting egg production and development in consumers (Rossoll et al., 2012).

Recently Lohbeck et al. (2012) showed that high CO_2 adapted populations of the fast growing coccolithophore *Emiliania huxleyi* were able to partly restore calcification and growth rate through adaptive evolution. Considering that growth and FA synthesis for the production of cell membranes are associated processes in the cells, the observation Lohbeck et al. (2012) suggests that the CO_2 effects observed in the FA profile of several algae species (Fiorini et al., 2010; Rossoll et al. 2012; Torstensson et al., 2013) might be a temporary responses to CO_2 perturbations that may be mitigated in the long-term. Given that marine microalgae reproduce quickly and have large population sizes, they should be particularly prone to respond to ocean changes through adaptive evolution (Lohbeck et al., 2012; Collins et al., 2013), therefore we wanted to determine whether planktonic organisms are able to eventually restore its original FA profile through adaptation.

The objective of our study was to investigate potential long-term changes in composition of total and specific PUFA, MUFA and SFA in different phytoplankton species under high CO_2 conditions. To determine adaptive FA changes in response to

high-CO_2 we made use of two long-term CO_2 selection experiments with the ecologically relevant coccolithophore *E. huxleyi* (Lohbeck et al., 2012) and the diazotrophic cyanobacteria *Trichodesmium* sp. and assessed their FA profiles in a set of reciprocal assay experiments after >1000 generations under high CO_2-conditions.

Materials and Methods

Experimental set up

We analyzed fatty acid profiles of two phytoplankton species, the coccolithophore *Emiliania huxleyi* and the diazotrophic cyanobacteria *Trichodesmium* sp. cultured at low and high CO_2 levels for >1000 asexual generations. The test for adaptation involved the comparison of the FA profile of populations adapted to increased CO_2 conditions with those grown under ambient CO_2 and kept under the same laboratory conditions. The long-term pCO_2 treatments were: 400, 1100 and 2200 µatm (with 2200 as proof of principle) for *E. huxleyi*, and 380 and 750 µatm for *Trichodesmium* sp., respectively. The test to determine if any FA change in the long term cultures was permanent or transitory consisted of a set of reciprocal experiments where both, low and high CO_2 long term cultures, were transferred to a different CO_2 levels (Lohbeck et al., 2012). The transfer to the assay culture conditions were as follow: from 400 to 1100, 400 to 2200, 1100 to 400, 2200 to 400 for *E. huxleyi,* and 380 to 750, 750 to 380 for *Trichodesmium* sp. To account for physiological acclimation all CO_2 shift cultures were acclimatized for about 7 generations (5-7 days) before FA samples were collected. These reciprocal assay experiments were conducted under non-competitive exposure to CO_2 enrichment. All cultures were harvested at exponential growth before reaching nutrient limitation.

Culture conditions

The plankton organisms were cultured in artificial seawater media (ASW) prepared following the protocol by Kester et al. (1967) with a salinity of 35. The advantage of using ASW is the possibility of an easier carbonate system manipulation and the exclusion of possible contaminants present in seawater that could alter the physiological condition of the cultures. Nitrate and phosphate were added to concentrations of

minimum 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) along with 10 ml per liter of pre-filtered (Whatman® 0.2 µm Puradisc™ 25 AS filter) natural seawater to avoid micro-nutrient limitation. The algae were kept in a set of laboratory batch cultures under continuous rotation with an incident photon flux density of ~150µmol photons $m^{-2}s^{-1}$, on a 16:8 light:dark cycle, and a temperature of 15°C. The seawater carbonate system was set up by bicarbonate addition and subsequent aeration using a controlled CO_2-gas-mixing system. Carbonate chemistry was determined by dissolved inorganic carbon (DIC) and total alkalinity measurements. Average culture pCO_2 values were calculated from DIC and total alkalinity measurements and drawdown estimates.

To minimize CO_2 loss DIC samples were collected by filtering 10ml of water through a disposable sterile filter (Whatman® 0,2 µmPuradisc™ 25 AS filter) into 4ml vials air-tight sealed with a Teflon coated septa, wrapped in parafilm® and kept at 4°C until analysis according to the photometric method of Stoll et al. (2001). For alkalinity determination 500ml of water was filtered through pre-combusted (450°C, 6 h) glass-fiber filters (Whatman® GF/F, nominal pore size of 0.7µm) poisoned with mercury-chloride ($HgCl_2$) and kept at 4°C until analysis by potentiometric titration following the method of Dickson et al. (2003). A photometrical determination of NO_3^- plus NO_2^- and PO_4^{3-} was carried out by the method of Hansen & Korolef (1999). The pCO_2 in the treatments was determined by DIC, pH, alkalinity and nutrients measurements conducted as is described hereinafter and calculated with the program CO2sys software (Müller et al., 2010).

Fatty acid quantification

The FA were measured by gas chromatography as fatty acid methyl esters (FAME) following Klein-Breteler et al. (1999). 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, FAME C19:0 (Restek, Bad Homburg, Germany; c= 20.0 ng $component^{-1}\mu l^{-1}$) was added, and a C23:0 FA standard (c= 25.1 ng μl^{-1}) used as an esterification efficiency control (usually 80-85%). Water-soluble fractions were removed by washing with 2.25ml 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), re-dissolved in Chloroform and transferred into a glass cocoon. The solvent was evaporated again (10-30mbar), and esterification was performed over night using 200µl 1% H_2SO_4 (in CH_3OH) 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 non-polar column (RXI1-SIL-MS 0.32µm, 30m, company Restek) using a Flame ionization detector. 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. A 1µl aliquot of the sample was injected. The system was calibrated with a 37-component FAME-mix (Supelco, Germany) and chromatograms were analyzed using Chrom-Card Trace-Focus GC software (Klein-Breteler et al., 1999) and the fatty acids were clustered according to their degree of saturation: Saturated (SFA), Monounsaturated (MUFA) and Polyunsaturated (PUFA) and their relative concentration (%) calculated.

Statistical analysis

Statistical analysis consisted on two-sided Student's t-test or two-way ANOVA (when specified). Homogeneity of variance and normality of distribution were checked with a Fligner and a Shapiro test, respectively. Principal component analysis (PCA) was used to determine the similarity in individual FA composition of algae across the treatments within each FA group. To increase the resolution of the PCA analysis, a threshold of >10% in relative concentration of the individual FA within its own class was set for its inclusion it the analysis. All statistical analysis were done using the R software environment 3.0.1 (R Development Core Team 2005).

Results

Emiliania huxleyi

The total FA concentration in the long-term *E. huxleyi* selection cultures adapted to ocean acidification showed a significantly higher total FA content at 2200 µatm CO_2 treatment (+ ~26%) compared to the treatment at 400 µatm CO_2 (t-test= -2.46, df = 8, p = 0.04), while there was no difference between the 400 and 1100 µatm CO_2 treatments (Fig. 1a). In the reciprocal assay, intended to determine whether the above observed FA change in the long-term high CO_2 cultures was permanent or transitory, we compared long term 1100 and 2200 µatm CO_2 selected populations with those selected at 400 µatm CO_2, both subjected to ambient CO_2 assay conditions. The 400, 1100 and 2200 µatm CO_2 selected lines contained similar amounts of FA under ambient conditions (Fig. 1a). When comparing the long-term 400 µatm CO_2 selected populations with those selected at 1100 and 2200 µatm CO_2, both subjected to intermediate and high CO_2 assay conditions, the 400 µatm CO_2 selected lines showed no difference with the 1100 µatm CO_2 treatment, and an elevated FA concentration compared to the 2200 µatm CO_2 selected lines (+ ~19%), although this difference was not significant (Fig. 1a).

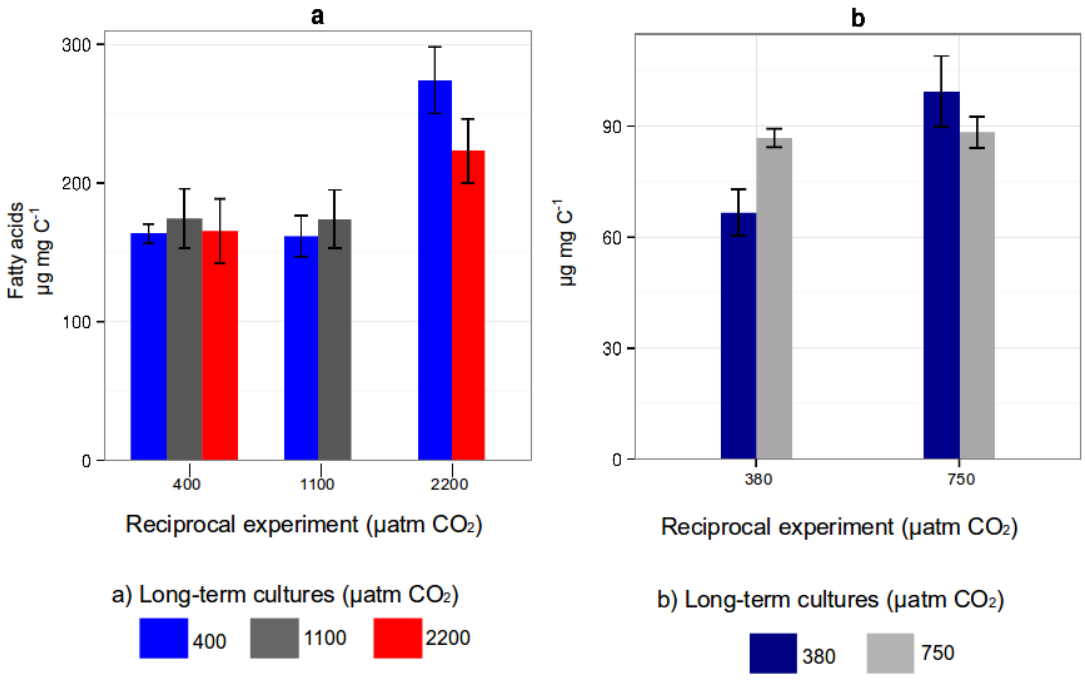

Figure 1. Total fatty acid content of the coccolithophore *Emiliania huxleyi* cultured under different CO_2 conditions after 1200 generations (a) and the cyanobacterium *Trichodesmium* sp. (b). Bars indicate standard error.

The analysis of the specific FA groups in the long-term cultures showed that PUFA and MUFA were significantly more abundant in the long-term 2200 µatm CO_2 selection treatment (+ ~44% PUFA and + ~35% MUFA) compared with long-term 400 µatm CO_2 (t-test= -3.23, df = 8, p = 0.01 for PUFA; t-test= -2.79, df = 8, p = 0.02 for MUFA) (Fig. 2a, b). The SFA, although more abundant in the 2200 µatm CO_2 treatment (+ ~20%), had no significant difference with the 400 µatm CO_2 treatments (Fig. 2c). The 400 and 1100 µatm CO_2 treatments did not show significant differences in any of the FA groups (Fig. 2).

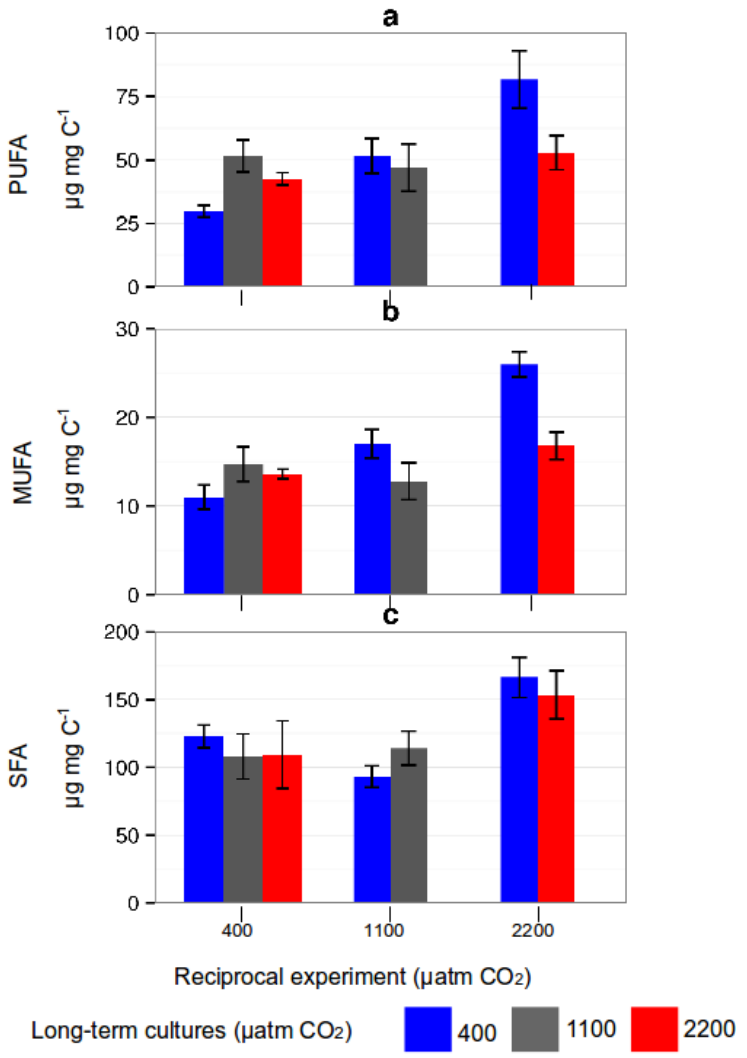

Figure 2. Polyunsaturated, PUFA (a), Monounsaturated, MUFA (b) and Saturated, SFA (c) fatty acid content of the coccolithophore *Emiliania huxleyi* cultured under different CO_2 conditions for >1000 generations. Bars show standard error.

The analysis of the specific FA groups in cells of the reciprocal experiment showed that the 1100 and 2200 µatm CO_2 treatments have a significantly higher PUFA content (ANOVA, F= 6.96, df= 2, p= 0.009) in relation with those selected at 400 µatm CO_2, when kept under ambient CO_2 levels (+ ~43% and + ~31% for the 1100 and 2200 µatm CO_2 treatments, respectively) (Fig. 2a), while the MUFA and SFA did not show any significant difference (Fig. 2b,c). When comparing the 400 µatm CO_2 treatments with those selected at 2200 µatm CO_2 with both subjected to high-CO_2 assay conditions, the PUFA and MUFA showed a significantly higher abundance in the 400 µatm CO_2 selected lines (+ ~ 35% for both) in relation to the 2200 µatm CO_2 lines (t test= -2.19, df = 8, p = 0.05 for PUFA; t-test= -4.44, df= 8, p= 0.002 for MUFA) (Fig. 2a, b) while the SFA did not show any significant difference (Fig. 2c).The 400 and 1100 µatm CO_2 treatments did not show differences when both were subjected to intermediate-CO_2 assay conditions (Fig. 2a, b, c).

The 22:6n3 Docosahexaenoic acid (DHA) represented about half of the PUFA in *E. huxleyi* and drove the trends observed in the total PUFA of the long-term and reciprocal experiment cultures of the algae (Fig. S1). Also, both the relative (Fig. S1a) and total (Fig. S1b) DHA content followed the same trends, which was common to all FA kinds in the experiment (data not shown).

The Principal component analysis (PCA) grouped together the cultures according to their similarity in FA composition. The PUFA in the long-term cultures had a similar composition and clustered together while the assay cultures formed a separate group; The differentiate grouping between both lines were mostly driven by the difference in their content of Eicosapentaenoic acid (EPA, 20:5n3), Docosapentaenoic acid (DPA, 22:5n3) and Eicosadienoic acid (20:2n6) (Fig. 3a). The MUFA showed similar groups as PUFA; the clustering was driven by Nervonic (24:1n9) and Myristoleic (14:1n5) acids (Fig. 3b). The SFA did not show a clear differentiation between the treatments, although showed that the high CO_2 cultures had a high dominance of Palmitic (16:0) and Stearic (18:0) acids (Fig. 3c). The loads of each group are given in Figure S2.

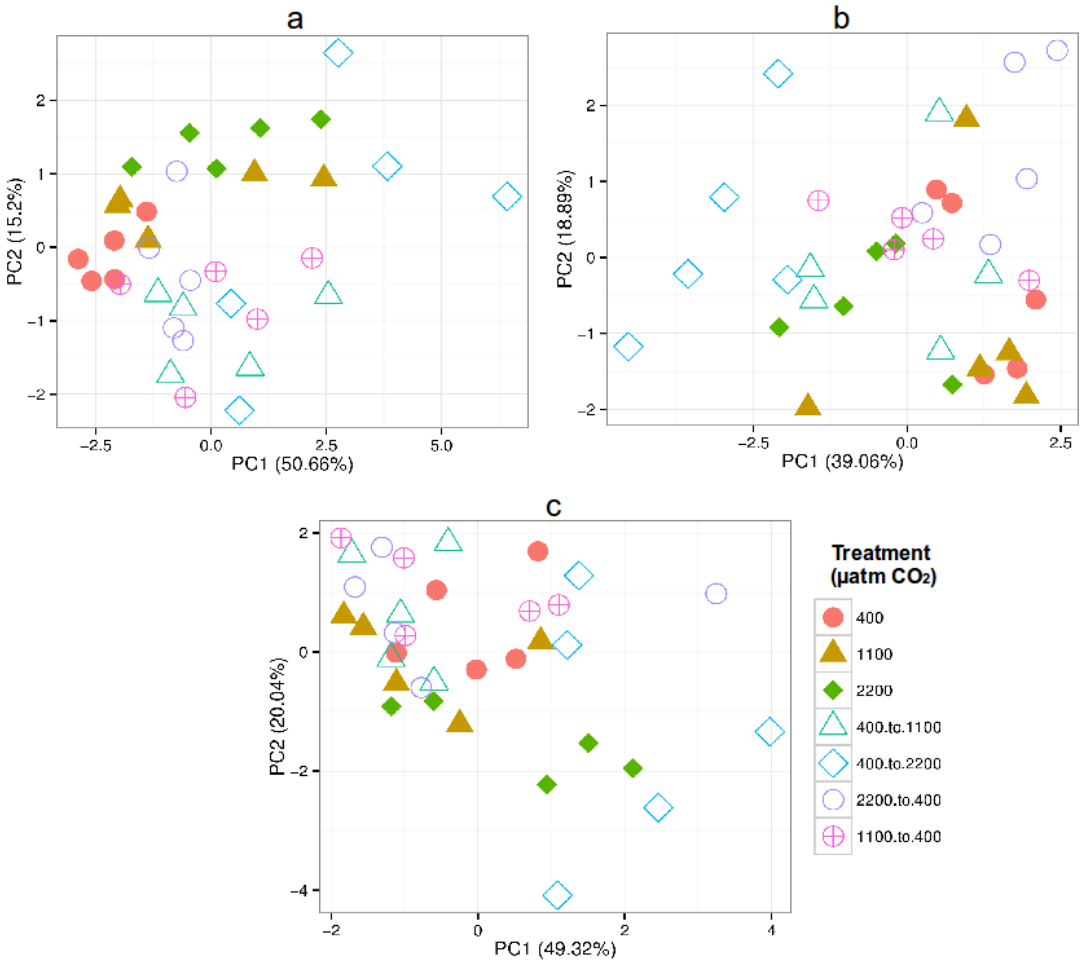

Figure 3. Principal component analysis [PCA] of Polyunsaturated, PUFA (a), Monounsaturated, MUFA (b) and Saturated, SFA (c) fatty acid content of the coccolithophore *Emiliania huxleyi* cultured under different CO_2 conditions for >1000 generations. The fill points show cultures >1000 generations in that CO_2 (long-term cultures), and hollow figures indicates culture transferred from their original long-term CO_2 conditions into a new CO_2 treatment (reciprocal experiment). The data used was in µgFA mgC^{-1}. FA >10% in abundance within each group were include in the analysis.

Trichodesmium sp.

The total FA content in the long-term *Trichodesmium* sp. cultures showed a significantly higher total FA content at 750 µatm CO_2 (+ ~25%) compared to the control started at the same time at 380 µatm CO_2 (t-test= -2.88, df= 10, p= 0.02) (Fig. 1b). The total FA content in the reciprocal experiment showed that the 750 µatm treatment contained significantly higher amounts of FA (+ ~23%) under ambient conditions in relation to those selected at 380 µatm CO_2 when both were keep to low-CO_2 levels (t-test= -3.00, df= 10, p= 0.01) (Fig. 1b). When comparing the 380 µatm CO_2 selected populations with

those selected at 750 µatm CO_2, both subjected to high CO_2 conditions, the low-CO_2 treatments did not differ from the long-term high-CO_2 selected lines (Fig. 1b).

The analysis of specific FA groups of the long-term cultures showed that PUFA, MUFA and SFA had a significantly higher concentration under the long-term 750 µatmCO_2 selection treatment in relation with the long-term 380 µatm CO_2 treatment (+ ~25%, + ~39% and + ~23% for PUFA, MUFA and SFA, respectively) (t-test= -2.56, df= 10, p= 0.03 for PUFA; t= -2.43, df= 10, p= 0.03 for MUFA; t= -2.41, df= 10, p= 0.04 for SFA) (Fig. 4a,b,c). The reciprocal experiment analysis of the PUFA showed no differences between the 750 and 380 µatm CO_2 treatments when both are kept under ambient CO_2 levels (Fig. 4a). However, the MUFA and SFA were significantly more abundant in the 750 µatm CO_2 in relation to the 380 µatmCO_2 treatment (+ ~46% and + ~24% for MUFA and SFA respectively) (t-test= -3.19, df= 10, p= 0.009 for MUFA; t = -2.35, df = 10, p= 0.04 for SFA) (Fig. 4b,c). Comparing the 380 µatm CO_2 treatment with those selected at 750 µatm CO_2 when subjected to high CO_2 assay conditions revealed that PUFA and SFA showed no difference (Fig. 4a,c) while MUFA were significantly more abundant in the 380 µatm CO_2 group (+ ~32%) (t-test= -2.53, df= 10, p= 0.03) (Fig. 4b).

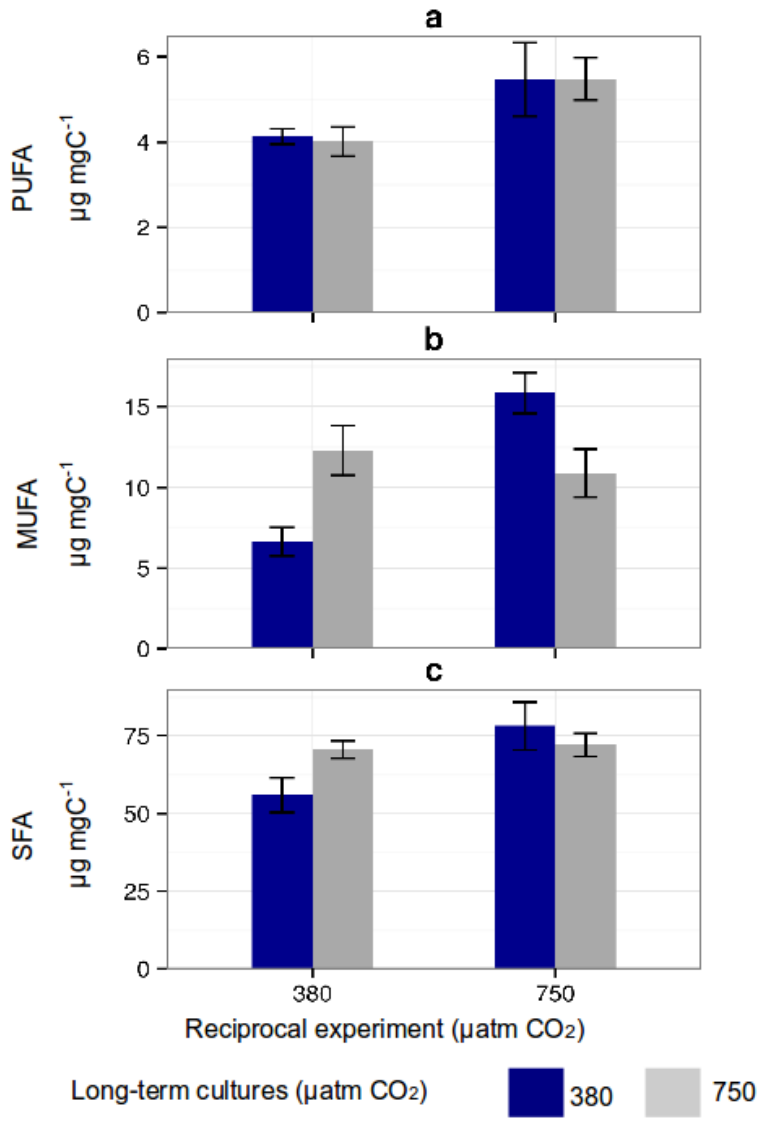

Figure 4. Polyunsaturated, PUFA (a), Monounsaturated, MUFA (b) and Saturated, SFA (c) fatty acid content of the cyanobacteria *Trichodesmium* sp. cultured under different CO_2 conditions after 1200 generations. Bars show standard error.

The PCA of individual FA in *Trichodesmium* sp. showed a clustering of the long-term and essay experiments in MUFA and SFA. The PUFA did not differentiate between treatments (Fig. 5a). The MUFA clustered by the Myristoleic (14:1n5), Vaccenic (18:1n7) and the odd Pentadecenoic (15:1) acids (Fig. 5b). The SFA did show some differentiation, due to Myristic (14:0) Capric (10:0), and the odd Undecylic (11:0) acids (Fig. 5c), although the last two had a small abundance. The loads of each group are given in Figure S3.

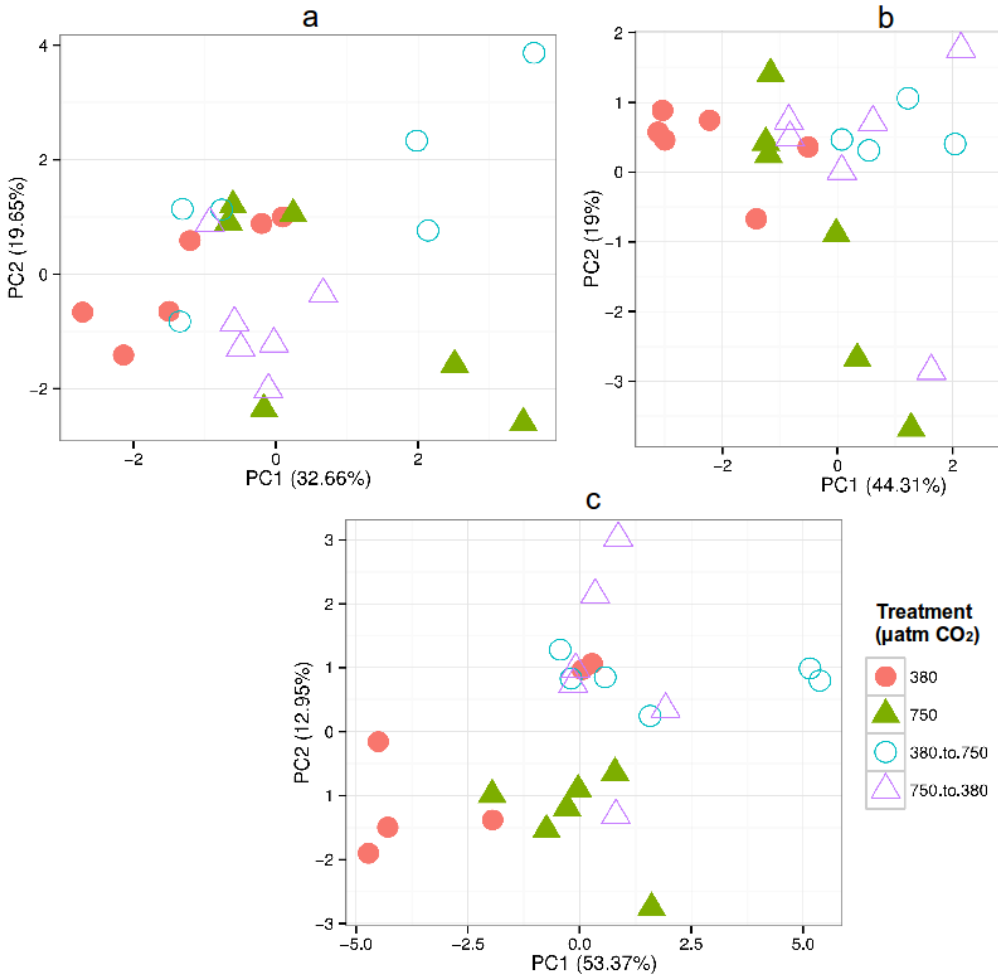

Figure 5. Principal component analysis [PCA] of Polyunsaturated, PUFA (a), Monounsaturated, MUFA (b) and Saturated, SFA (c) fatty acid content of the cyanobacteria *Trichodesmium* sp. cultured under different CO_2 conditions after 1200 generations. The fill points show cultures >1000 generations in that CO_2 (long-term cultures), and hollow figures indicates culture transferred from their original long-term CO_2 conditions into a new CO_2 treatment (reciprocal experiment). The data used was in µgFA mgC^{-1}. FA >10% in abundance within each group were include in the analysis.

Discussion

Our results showed that CO_2 can affect the FA composition in both *E. huxleyi* and *Trichodesmium* sp. However, the reactions to CO_2 of the different FA in the long-term cultures of both algae differ between groups and treatment levels (Table 1).

Table 1. Adaptation response of the two phytoplankton taxa. + Response only at extreme high CO_2; 0 physiological/immediate change; - no response.

FA class	*Emiliania*	*Trichodesmium*
Total FA	0	0
PUFA	+	-
MUFA	+	+
SFA	0	0

In *E. huxleyi*, the absence of a difference in total and group specific FA content between the 400 and 1100 µatm CO_2 long-term selected lines when both are subjected to intermediate-CO_2 assay conditions indicate that its FA profile is rather non-responsive to the CO_2 levels projected for the end of the century (IPCC, 2013). However, the significant differences between the 400 and 2200 µatm CO_2 lines indicates sensitivity to higher CO_2 conditions and that the threshold for a CO_2 effect in the FA profile of this algae lays between the intermediate and high CO_2 levels. Although the high-CO_2 treatment falls outside the range of projected oceanic CO_2 concentrations (IPCC, 2013), it is within the range of values occurring temporarily in coastal and upwelling areas and is likely to be a more recurrent condition in the future (Feely et al., 2004).

In long-term high CO_2 selected *E. huxleyi* lines the amount of total FA was significantly higher than in ambient CO_2 selected control lines. The analysis of the specific FA groups showed that this trend was primarily driven by SFA while PUFA and MUFA were significantly higher in the 400 µatm CO_2 lines when shifted to high-CO_2 levels in the reciprocal assay. This indicates different adaptive responses among the major FA groups in this algae specie. SFA are typically dominant components of neutral storage compounds such as triacylglycerol and wax ester (Pond & Harris, 1996), while MUFA and particularly PUFA are abundant in the polar lipids of *E. huxleyi*, which are functional

molecules with an important role in cellular metabolic processes (Bell & Pond, 1996). The high amount of MUFA and PUFA in the 400 µatm control lines subjected to high CO_2 conditions indicates that this is a physiological response to CO_2 stress. Therefore the comparatively significant lower amount of MUFA and PUFA observed in the long-term high-CO_2 adapted lines indicates are capable of regulate their FA metabolism more efficiently under elevated CO_2. This allows them to restore their FA profile towards pre-CO_2 conditions and in direction to the FA profile of cells under ambient CO_2 conditions. However, the MUFA and PUFA content and composition in long-term high-CO_2 adapted were only partly restored and did not reach the same levels as found in the long-term low-CO_2 control lines. This indicates that the experimental period (>1000 asexual generations) has not been sufficient to fully restore total FA content to values found under ambient CO_2. An overview of the adaptation response in *E. huxleyi* of the total and specific FA classes is given in Table 1.

In a recent study by Rokitta et al. (2012) a comparative microarray-based transcriptome profiling of *E. huxleyi* cultures kept under high CO_2 levels was used to screen for gene expression of activated cellular processes and showed that lipid-synthesizing machinery was induced at elevated CO_2. Rokitta et al. (2012) suggested that this change in carbon (C) metabolism is due to an alteration in gene expression, causing a reduction of calcification and the relocation of C towards the photosynthesis in the chloroplast, producing more glucose (organic carbon), which is subsequently stored as glucan and lipids. Our observation confirm that *E. huxleyi* accumulate lipids under high CO_2 exposure. Lohbeck et al. (2012) reported that calcification rates, albeit lower under increased CO_2 conditions in all cultures, were partly restored in high CO_2 adapted cultures (>500 asexual generations) compared with non-adapted cultures, indicating that there is some metabolic adaptation to relocate C back into calcification. Our results agree with this observation as the reduced allocation of C in MUFA and PUFA (and in SFA although not significant) after long-term high CO_2 exposure may imply that C is partly relocated back to calcification.

The C allocation towards photosynthesis in the chloroplast reported by Rokitta et al. (2012) indicates that the photosynthetic apparatus in *E. huxleyi* under high CO_2 levels

might be up-regulated, therefore producing more lipids and FA, which would explain the present observations. Our results agree with previous findings showing an increase of FA under short-term high CO_2 conditions in *E. huxleyi*, especially PUFA (Fiorini et al., 2010). However, it has also been reported a decrease of PUFA with increasing CO_2 levels in this specie (Riebesell et al., 2000). Contrasting physiological responses to high CO_2 where observed between different strains of this coccolithophore and have been attributed to a high genetic diversity of *E. huxleyi* (Langer et al., 2009). This highlights the necessity of further studies in the physiological response of marine microalgae to high CO_2 conditions.

In terms of food quality coccolithophores are generally considered good sources of PUFA (Volkman et al., 1981; Pond & Harris, 1996). The significant increase of FA observed in the present study under high CO_2 conditions improves even further the food quality of *E. huxleyi* for higher trophic levels. Such an improvement could compensate for the observed adverse effects of CO_2 on the FA profiles, particularly PUFA of other algal groups such as Chlorophyta (Tsuzuki et al., 1990; Sato et al., 2003) and Heterokontophyta (Rossoll et al., 2012; Torstensson et al., 2013). This enhanced food quality of *E. huxleyi*, when present in plankton assemblages, may probably hamper some of the negative consequences observed in the biological performance of consumers at higher trophic levels when feeding with algae negatively affected by CO_2 (Locke & Sprules, 2000; Rossoll et al., 2012).

In the long-term cultures of *Trichodesmium* sp. the amount of total FA increased significantly at 750 µatm CO_2 in comparison to the 380 µatm CO_2 treatments, similar to *E. huxleyi*. The specific FA groups showed that the trend in the total FA content is driven by SFA and PUFA while MUFA are higher in the 380 µatm CO_2 cultures when shifted to high-CO_2 levels in the assay experiment. Here we also observed different adaptive responses among FA groups. SFA form neutral storage compounds and PUFA represent a very small amount of the total FA in cyanobacteria (Carpenter et al., 1997). PUFA function within the cyanobacteria cell is not well understood, while MUFA are the functional FA molecules in the metabolism of the cell (Sato & Wada, 2009). As in *E. huxleyi*, a lower amount of MUFA in the long-term 750 µatm CO_2 relative to the 380

µatm CO_2 treatment under high CO_2 assay conditions indicates that the cellular metabolism of adapted cells is more efficiently regulated and back to pre-selection conditions. However, it is interesting that the SFA and PUFA seems to be "stuck" in higher levels in the long-term 750 µatmCO_2adapted populations when subjected to ambient CO_2 conditions, a correlated adaptive response that needs further investigation. The fact that MUFA, the functional FA molecules in the cell, are still at higher amounts in the long-term 750 µatm CO_2 treatment in comparison to the long-term 380 µatm CO_2 treatment indicates that the experimental period (>1000 generations) was not been sufficient to fully restore the cellular metabolism to levels found under ambient CO_2. An overview of the adaptation response in *Trichodesmium* sp. of the total and specific FA classes is given in Table 1.

The reason for the observed increase of FA in *Trichodesmium* sp. can be related, like in *E. huxleyi*, to carbon allocation. The majority of plankton species, including cyanobacteria, operate carbon concentrating mechanisms (CCM) (Price et al., 2008). The CCM is an energy consuming process whose function is to transport and accumulate inorganic C actively (HCO_3^- and CO_2) within the cell where the C pool is utilized to provide elevated CO_2 concentrations around the Rubisco enzyme, which fixes inorganic carbon to organic compounds (Price et al., 2008). In *Trichodesmium* sp. under high environmental CO_2 levels the CCM for the active uptake of HCO_3^- is down-regulated with only the CO_2 uptake system remaining active (Levitan et al., 2010). This may generate into a net metabolic energy saving and an improved C metabolism which can translate into increased production of organic compounds. The most prevalent storage compounds in cyanobacteria are carbohydrates, particularly glycogen, with other storage compounds as FA being less abundant (Beck et al., 2012). It has been reported in some cyanobacteria that high CO_2 conditions increased carbohydrate production up to cell saturations levels (Gordillo et al., 1999). Since both carbohydrates and FA have the same initial synthesis molecule, 3-Phosphoglycerate (3PG), which is the immediate product of CO_2 fixation by Rubisco and therefore an intermediate metabolic step between both glycogenesis and lipogenesis, there is the possibility that when the cyanobacteria cell reach carbohydrate saturation level, 3PG is diverted into FA

production, therefore increasing the accumulated amount within the cell. Nonetheless more detailed studies are needed to confirm this hypothesis.

In terms of food quality our results agree with previous studies showing that cyanobacteria are bad sources of essential FA (Patil et al., 2007) and *Trichodesmium* sp. is not an exception (Carpenter et al., 1997). The total amount of PUFA was about ~4%, and in spite of the significant PUFA increase under high-CO_2 conditions it was only a further ~2%, which is far below the PUFA, for instance, of *E. huxleyi* (~30%). Therefore such improvement would probably not make much of a difference in natural plankton assemblages in terms of food quality for higher trophic levels.

The mechanisms through which CO_2 affects algal FA remains unresolved, however, Sato et al. (2003) suggested that in green algae the higher saturation levels in cells grown at high-CO_2 level are at least partially due to enhanced SFA synthesis and a reduced MUFA and PUFA production. The observations by Rokitta et al. (2012) and the results presented in our study support an up-regulation of the FA synthesis as proposed by Sato et al. (2003) occurring also in coccolithophores and cyanobacteria. However, the increase MUFA and PUFA content in both plankton organisms used in our study at high CO_2 levels suggest that there is no determent on their production as hypothesized by Sato et al. (2003) in this two species.

It has been proposed that the increase of algal SFA content under high CO_2 levels results in more rigid cell membranes as a mechanism to better control of the internal cell pH, as a membrane built of short-chain FA is less fluid and permeable to CO_2 inflow (Rossoll et al., 2012). This would explain the increased amount of SFA observed in both planktonic organisms in the present experiment and the fact that SFA, unlike MUFA and PUFA, was not significantly down-regulated in the long-term high CO_2 cultures in neither of the two investigated species.

Our observations show that CO_2 has an strong influence in FA metabolism and that planktonic organisms can respond to this influence through adaptation. This indicates that the short-term CO_2 effects on FA observed in several algae (Riebesell et al., 2000;

Rossoll et al., 2012; Torstensson et al., 2013) might be temporal and may eventually be overcome through adaptive evolution given enough time. Our results also show that the CO_2 effects on FA are species specific and likely even strain specific (Riebesell et al., 2000; Fiorini et al., 2010; Rossoll et al., 2012; Torstensson et al., 2013), which challenges a more assertive prediction of the future ocean scenarios and how CO_2 can directly affect primary producers and the possible consequences for higher trophic levels (Locke & Sprules, 2000), highlighting the need of further research of cellular processes involved in FA synthesis and cell metabolism under changing pCO_2 and pH levels.

Acknowledgements

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Supplementary information

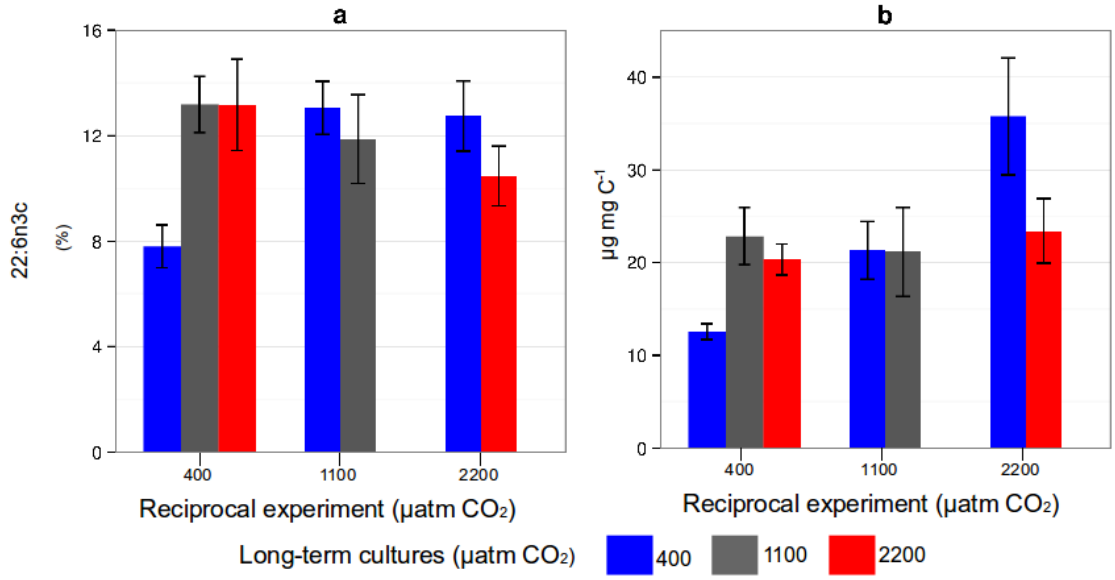

Figure S1. Relative (a) and absolute (b) 22:6n3 Docosahexaenoic acid (DHA) content of the coccolithophore *Emiliania huxleyi* cultured under different CO_2 conditions during 1200 generations. Bars show standard error.

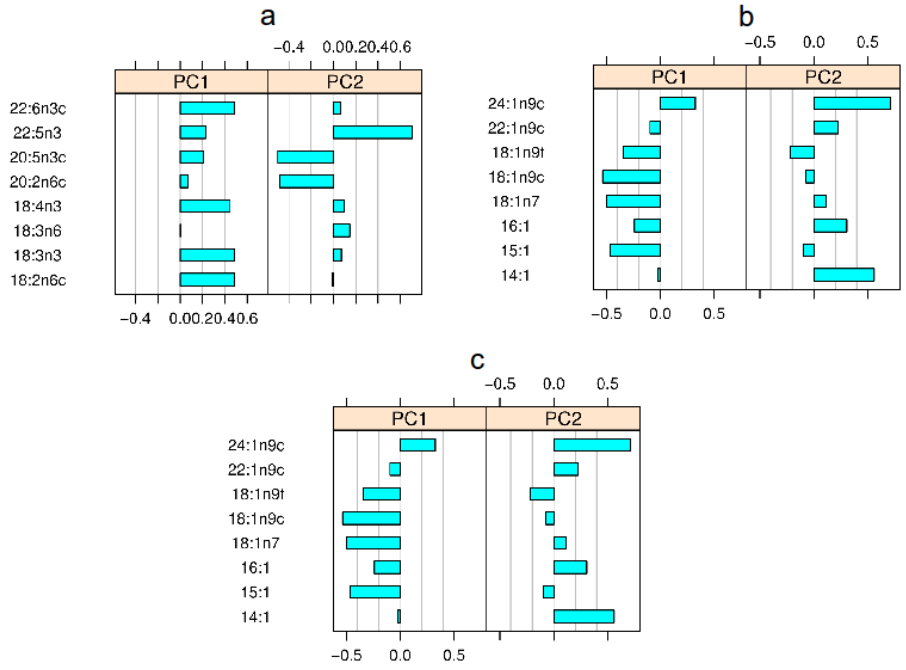

Figure S2. Axis loads of the Principal component analysis [PCA] of Polyunsaturated, PUFA (a), Monounsaturated, MUFA (b) and Saturated, SFA (c) fatty acid content of the coccolithophore *Emiliania huxleyi* cultured under different CO_2 conditions for >1000 generations. The data used was in µgFA mgC^{-1}. FA >10% in abundance within each group were include in the analysis.

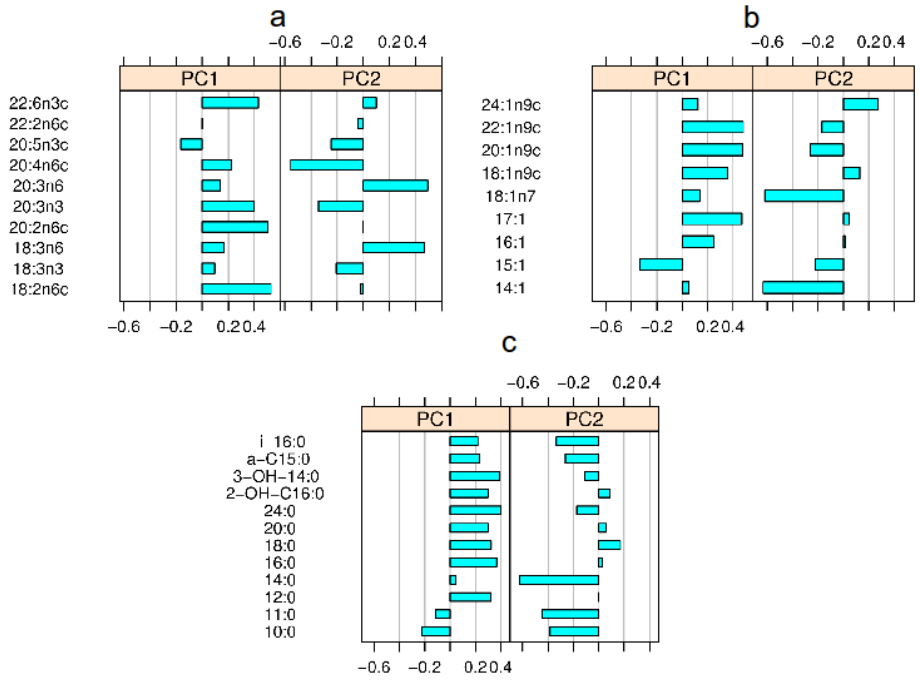

Figure 5. Axis loads of the Principal component analysis [PCA] of Polyunsaturated, PUFA (a), Monounsaturated, MUFA (b) and Saturated, SFA (c) fatty acid content of the cyanobacteria *Trichodesmium* sp. cultured under different CO_2 conditions after 1200 generations. The data used was in µgFA mgC^{-1}. FA >10% in abundance within each group were include in the analysis.

5. Synthesis

5.1 Overview

The individual studies of this thesis showed that CO_2 has the potential to influence the FA profile of primary producers at individual and community level. The algal FA response to CO_2 is, however, diverse and species specific. Nevertheless, our studies suggest that in the long term any algal FA CO_2 effect can be at least partially dampened through adaptive evolution.

Our experiments at multi-trophic level indicated that CO_2 effects on algae FA profile were transferred to primary consumers, which typically reflect the FA profile of their prey. The FA trophic transfer was both observed under laboratory conditions and in natural communities, indicating the potential for significant CO_2-driven bottom up effects in the production and trophic transfer of essentials biomolecules.

Furthermore, our experiments revealed that CO_2 affects the structure of plankton communities by favoring certain taxa, and that those CO_2-driven shifts in community composition at the base of the food web can affect the transference of essential FA to zooplankton grazers. However, the sensitivity of plankton seems to be reduced in communities that experience large natural CO_2 or pH fluctuations, suggesting that their ecological and physical environmental history affects the resilience to ocean acidification.

Below I discuss the perspectives of future ocean acidification scenarios in terms of the possible influence of CO_2 on algal FA synthesis, the potential effect of an altered algal FA profile on the life cycle of primary consumers, and the consequences of elevated CO_2 on the structure of plankton communities and how this may affect food quality for consumers in terms of FA composition of primary producers.

5.2 Algal fatty acids synthesis and CO_2

The response of the four algae species to high CO_2 conditions during exponential growth in the diatoms *Cylindrotheca fusiformis* and *Thalassiosira pseudonana*, the coccolithophore *Emiliania huxleyi* and the diazotrophic cyanobacteria *Trichodesmium* sp. differed in terms of their FA composition. The results of the different experiments included in the present work are summarized in Table 1.

Table 1. Summary of the effect of exposure to elevated CO_2 levels of total, polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acid content of four different algae. ↑ increase, ↓ decrease, — no change, nd= not determined. *mild, **moderate, ***strong based on effect size. Color refers to the CO_2 level: orange= ~750 µatm, red= 2200 µatm. All algae were exposed during ~7 generations to their indicated CO_2 level, $^{+}$exposed for >250 generations.

Specie	Total	PUFA	MUFA	SFA
Cylindrotheca fusiformis^{+}	nd	↓ *	—	↑*
Thalassiosira pseudonana	↓*	↓***	—	↑***
Emiliania huxleyi	↑**	↑**	↑*	↑**
Trichodesmium sp.	↑**	↑*	↑**	↑**

Our results show that SFA concentration increased in all of the algae at elevated CO_2 level; PUFA decreased in diatoms but increased in the coccolithophore and cyanobacteria along with MUFA in both of these groups. This contrasting response in FA composition indicates the occurrence of metabolic changes on how carbon is used within the cells. Most algae (also cyanobacteria) share a somewhat similar basic carbon metabolism mechanism as describe in section 3.4.1 (Section 3.4.1, Fig. 2) which consists of a) inorganic carbon uptake, b) carbon fixation, and c) synthesis of biomolecules.

In general terms the mechanism undelying the synthesis of biomolecules like FA, and carbon fixation is an analogous process among algae, and although each taxa has a particular set of enzymes for each step, in most cases they follow the same sequence as described in Box 1 (Radakovits et al., 2010; Mühlroth et al., 2013). Considering that FA synthesis is regulated by the availability of precursors and the amount of fixed carbon directed towards it (Fan et al., 2012; Valenzuela et al., 2012), the mechanisms of carbon

uptake in the different algal taxa examined in our experiments might be responsible for their contrasting response in FA composition at elevated CO_2. Under this perspective, a more detailed model of carbon metabolism mechanism in the different algae taxa is outlined below. Emphasis is given to the influences of elevated CO_2 on carbon uptake and its effect on FA synthesis.

5.2.1 Physiology of carbon uptake and FA synthesis

Algae actively accumulate dissolved inorganic carbon (DIC) (HCO_3^- and CO_2) to compensate the low affinity for CO_2 of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme in order to conduct an efficient photosynthesis (Reinfelder, 2011). Many algae acquired mechanisms for the active uptake of DIC known as carbon concentration mechanisms (CCM), which consist of a series of transport channels and several energy consuming enzymes known as carbonic anhydrases (CA) (Giordano et al., 2005). According to their structure the CCM are classified as biophysical C3, which is characteristic of cyanobacteria, coccolithophores, green algae and some diatoms, and a biochemical C4, which has been described in several diatoms (Reinfelder, 2011).

The biophysical cyanobacterial CCM of *Trichodesmium* sp. is based on active uptake of HCO_3^-/Na^+ transporter (Price et al., 2004) and a relative low uptake of CO_2 by other transporters (Shibata et al., 2001; Maeda et al., 2002), and deliver HCO_3^- to the cytosol (Giordano et al., 2005; Price et al., 2008) (Fig. 1a). The HCO_3^- then diffuses into compartments denominated the carboxysomes where the only cyanobacterial CA dehydrates the accumulated HCO_3^- into CO_2 for its fixation by the Rubisco enzyme. (Price et al., 2008). The coccolithophore *E. huxleyi* also possesses a biophysical CCM, which also involves active uptake of HCO_3^-/Na^+ by a transporter (AEL1) and nine putative CA, although their location in the cell is unknown (Bach et al.,2013). However, according to their position in the cell CA have different functions. External CA (eCA) dehydrates HCO_3^- to generate CO_2 at the cell surface to allow its uptake (Fig. 1b). Cytoplasmic CA (iCA) hydrates CO_2 to HCO_3^-, effectively trapping and accumulating it within the cytoplasma (Fig. 1b) (Spalding, 2008). Finally chloroplastic CA (cCA) provides a direct

supply of CO_2 to Rubisco from the dehydration of the accumulated HCO_3^- (Reinfelder, 2010). Unlike the other two algae, *T. pseudonana* posseses a biochemical eukaryotic CCM which consist of four putative CA (Crawfurd et al., 2011) for the uptake of CO_2 and HCO_3^- into the cell which, like in the coccolithophore, can be located in the periplasm and cytoplasm (Fig. 1c). However instead of being transported into the chloroplast, the accumulated HCO_3^- taken up from the medium is fixed together to phosphoenolpyruvate (PEP) into the four-carbon organic acid oxaloacetate (OAA) by the enzyme phosphoenolpyruvate carboxylase (PEPC) in the cytoplasm. Then the OAA is transported by a dedicated dicarboxylic acid transport (DA) system into the chloroplast where it is subsequently decarboxylated by a second enzyme, PEP carboxykinase (PCKase) (Reinfelder et al. 2000, 2004; McGinn & Morel, 2008) (Fig. 1c).

It can be expected that under elevated CO_2 the algal CCM are down-regulated as with increasing carbon dioxide concentration ($[CO_2]$) in the environment, the leakage of CO_2 is reduced and its diffusion gradient into the cell becomes at some point sufficient to saturate photosynthesis and maintain particulate organic carbon (POC) fixation and growth rates (Bach et al., 2013). However, experimental evidence has shown that this is only true for the biophysical C3 CCM in cyanobacteria and coccolithophores. In *Trichodesmium* sp. the active uptake of HCO_3^- is suppressed (Levitan et al., 2010) (Fig. 2a), in *E. huxleyi* the CCM is gradually down-regulated following the CO_2 concentration ($[CO_2]$) up to a basal level (Bach et al., 2013) (Fig. 2b). However, in *T. pseudonana* only one out of its four CA is down-regulated (Crawfurd et al., 2011), with cytoplasmic CA maintaining a low concentration of CO_2 in this compartment by continuously hydrating it to HCO_3^-, which is fixed into OAA by PEPC, causing its accumulation in the cytoplasm (McGinn & Morel, 2008; Granum et al., 2009) (Fig. 2c).

This elevated internal DIC pool promotes carbon fixation and growth in *Trichodesmium* sp. (Levitan et al., 2010), which imply an increase in the availability of precursors for FA synthesis and therefore FA content in the cells (Fig. 2a). A similar process as in the cyanobacteria was observed in *E. huxleyi*, although POC production does not show such a clear coupling to $[CO_2]$ (Bach et al., 2013) (Fig. 2b). In contrast *T. pseudonana*, in spite of an apparently elevated OAA content in the cell, the PCKase enzyme responsible of

splitting OAA in to PEP and HCO_3^- for its fixation by Rubisco, keep constant levels under high environmental CO_2 conditions (McGinn & Morel, 2008; Granum et al., 2009) (Fig. 2c). This implies that the production of FA is not promoted as there is no additional POC production, as was observed in our results.

The above mechanisms indicate that cyanobacteria and coccolithophore have an environmentally controlled carbon metabolism systems, while diatoms have an apparently genetic controlled system. In the case of *Cylindrotheca fusiformis* it is not clear which kind of carbon system it may have, however the similarity in FA shifts to the ones observed in *T. pseudonana* and the presence of the same CCM mechanism in several diatom species (Reinfelder, 2011) point towards a similar system, however this has to be confirmed by further research.

Box 1. Fatty acid metabolism in plankton as depicted in figures 1 and 2.

A) Carbon uptake (Green)
Algae posses mechanisms for the active uptake of CO_2 and HCO_3 known as carbon concentration mechanisms (CCM) which consist of a series of transport channels (black circles) and energy consuming enzymes known as carbonic anhydrases (CA). The CO_2 is then fixed into 3-phosphoglycerate (3PG) by the Rubisco enzyme and used in carbohydrate, protein or FA synthesis on the following steps.

B) Elongation of fatty acids (Cyan)
Algae have a type-II enzyme system catalyzing the synthesis of fatty acids which is composed of monofunctional proteins. Fatty acid biosynthesis involves five distinct enzymatic steps:

1) The first step, catalyzed by malonyl-CoA:ACP transacylase, transfers the malonyl group from CoA to acyl carrier protein (ACP) to form malonyl-ACP which serves as an immediate donor of the two-carbon acetyl units used in fatty acid elongation.
2) The condensing step, catalyzed by β-ketoacyl-ACP synthases, adds the two-carbon unit to the growing acyl-ACP.
3) The NADPH-dependent reduction step catalyzed by β-ketoacyl-ACP reductases yields β-hydroxyacyl-ACP.
4) The dehydration step catalyzed by β-hydroxyacyl-ACP dehydrases yielding trans-2-enoyl-ACP.
5) The last reductase step catalyzed by enoyl-ACP reductases which forms a saturated acyl-ACP serving, in turn, as the substrate for another condensation reaction. A repetitive series of reactions follows adding two-carbon units per cycle, until a saturated fatty acid of 16-18 carbons is formed.

C) Fatty acid desaturation (Purple)
Fatty acid biosynthesis systems have evolved to produce acyl chains approximately 16-18 carbon atoms long; longer fatty acids are produced in many organisms by specific systems (fatty acid elongases). In bacteria there is no specific mechanism for terminating acyl chain elongation; when acyl-ACP reaches 16 or 18 carbons it becomes a substrate for the acyltransferases that will attach the fatty acyl chain onto the glycerol backbone to produce phospholipids. In the plastid the most common synthesized glycolipids are: PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol (Fig. 1d).

Algae have a special class of enzymes that will terminate the acyl chain elongation by hydrolyzing the thioester bond of acyl-ACP thus releasing free fatty acids and ACP. This enzyme is known as fatty acyl-ACP thioesterase (FAT) and is localized in the plastid (Lykidis & Ivanova, 2008).

D) Lipid formation (Blue)
Free fatty acids produced by FAT exit the plastid and are re-esterified with CoA. The resulting acyl-CoAs are utilized for glycerolipid (triacylglycerol, TAG; Phospholipids) biosynthesis in the Smoot Endoplasmic Reticulum.

Figures based on Giordano et al. (2005); Price et al. (2008), McGinn & Morel (2008), Sato & Wada (2010), Radakovits et al. (2010), Huerlimann & Heimann (2012), Smith et al. (2012), Mühlroth et al. (2013) and Bach et al. (2013).

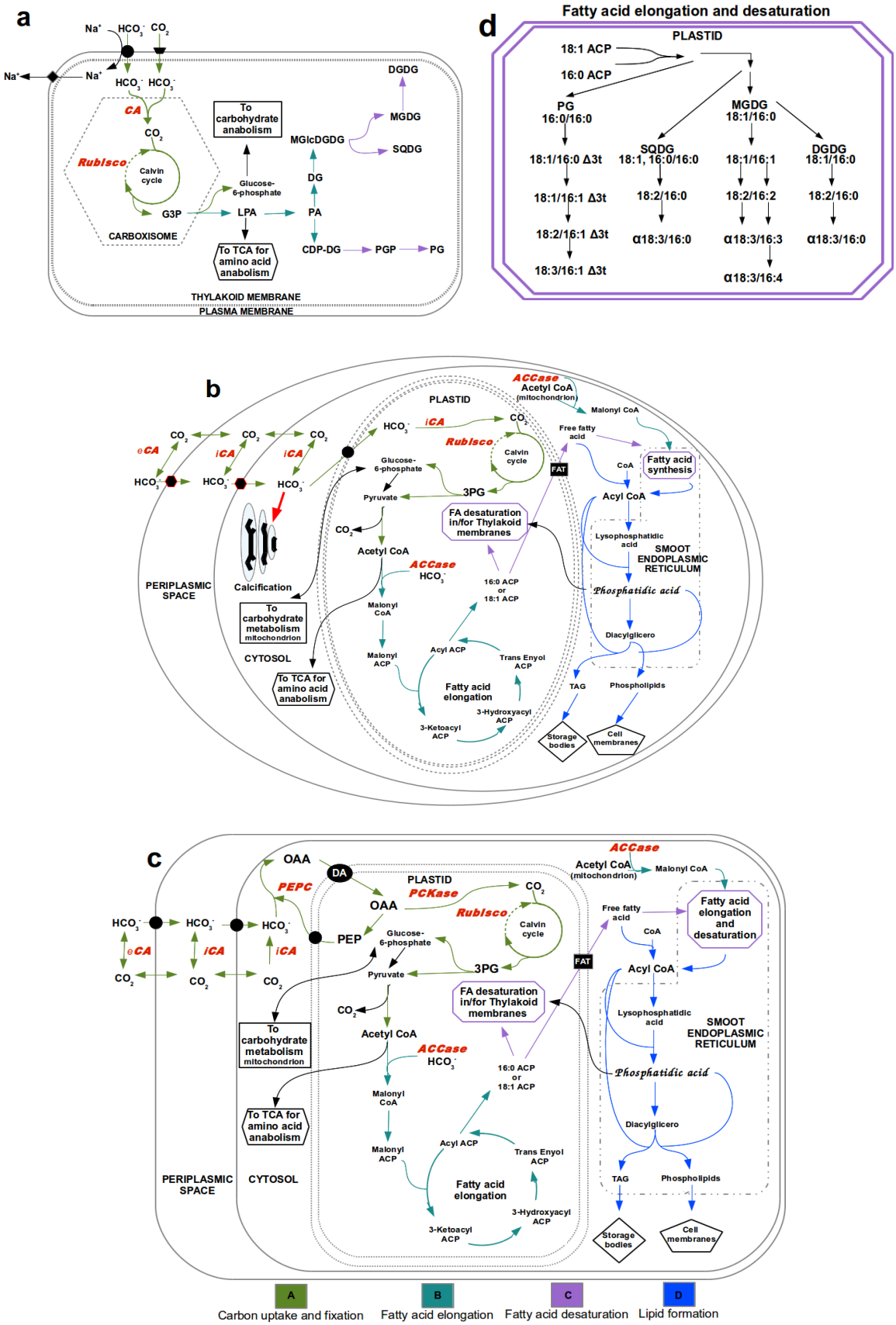

Figure 1. Fatty acid metabolism in cyanobacteria (a), coccolithophores (b) and diatoms (c) under low CO_2 conditions. d) Overview of glycerolipid elongation in algae (purple hexagon). Cyanobacteria depic the synthesis of their most common glycolipids. Details are given in Box 1.

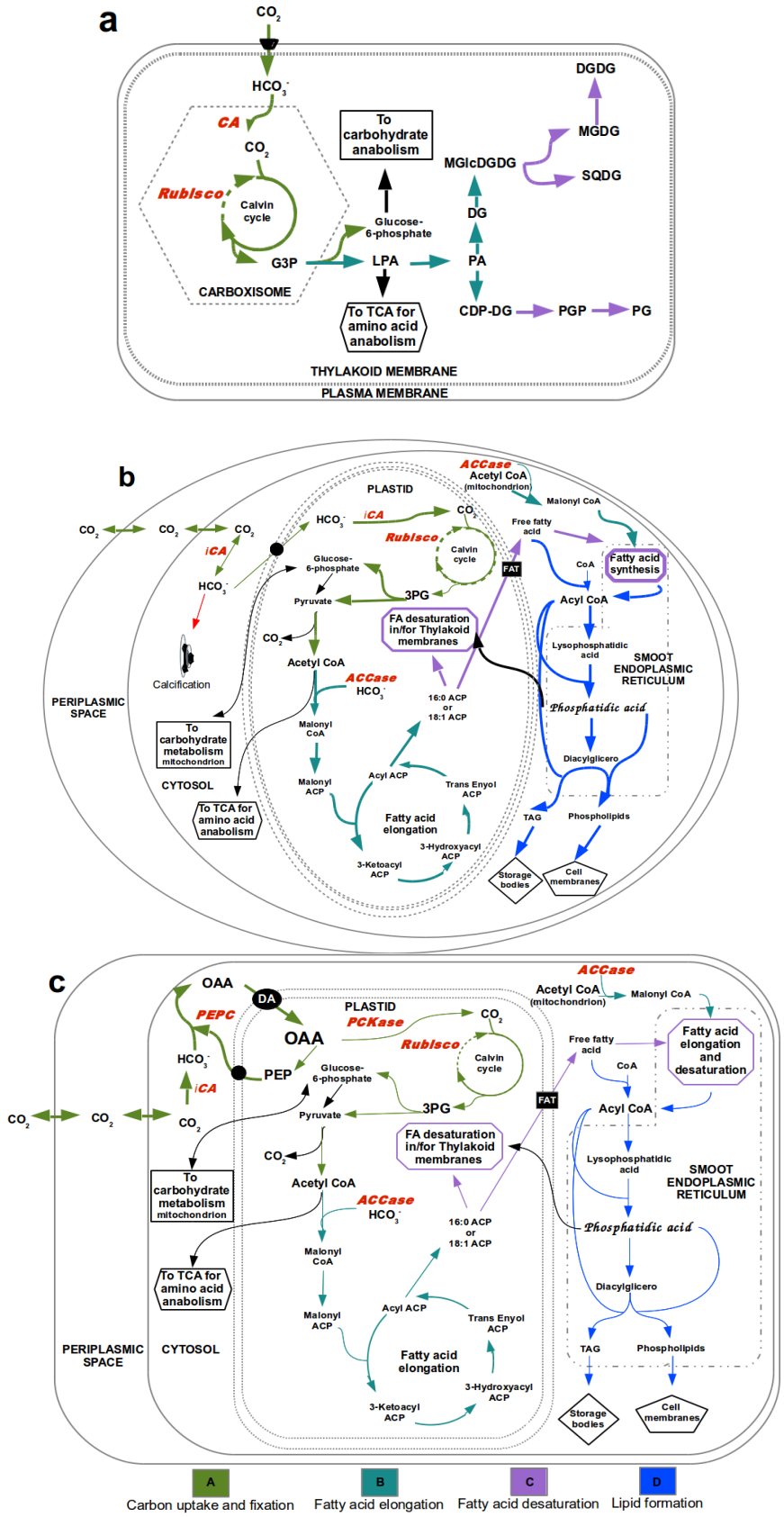

Figure 2. Fatty acid metabolism in cyanobacteria (a), coccolithophores (b) and diatoms (c) under elevated CO_2 conditions. A thick line indicates an up-regulated pathway. Cyanobacterya depic the synthesis of their most common glycolipids. Details are given in Box 1.

5.2.2 Algal FA composition and CO_2

As suggested in Article II, the increase of algal SFA content under high CO_2 levels in *T. pseudonana* may help in the production of more rigid cell membranes, as membranes with elevated SFA content are less permeable to CO_2 inflow. This can be a response mechanism to keep a balanced functioning of the CCM as the fixation of HCO_3^- into OAA is not spontaneous and therefore any additional CO_2 inflow into the cell may unbalance its internal pH (Lane et al., 1981). A similar mechanism may occur in cyanobacterium cells and coccolithophores under high CO_2 conditions to avoid a change in the intracellular pH which may severely affect their internal homeostasis. However, unlike a specific FA synthesis, all the FA machinery is up-regulated, including SFA synthesis (Fig. 2a,b).

5.2.3 CO_2 and adaptation of algal FA synthesis mechanisms

In evolutionary terms, the CO_2 influence in FA synthesis on the cyanobacteria and the coccolithophore can be overcome over the generations as the cells become capable of a better control of carbon fluxes and metabolism. On the contrary *T. pseudonana* do already have an efficiently regulated carbon metabolism, and have shown little to no CO_2 influence in their metabolism even after a long-term exposure to high CO_2 levels (Crawfurd et al., 2011).

In terms of evolution and food quality, as shown in Article V, the improvement in PUFA content in a coccolithophore, and at a lower degree in a cyanobacterium, are a transitory CO_2 effect; while the decrease in PUFA in the diatom, due to their efficiently regulated carbon metabolism, might be a constant response to future CO_2 levels. Therefore, it can be expected that species with a biophysical CCM will show a transitory positive food quality, while algae species with biochemical CCM will show a rather variable response in terms of their food quality and will depend on how their metabolism is regulated.

However, CO_2 is not the only factor influencing FA synthesis in algae, and there are many physical and chemical variables the oceanic environment (Fig. 3). Therefore, food quality of primary producers in the future ocean scenarios will depend on the interaction of the CO_2 with other environmental variables such as nutrients and temperature.

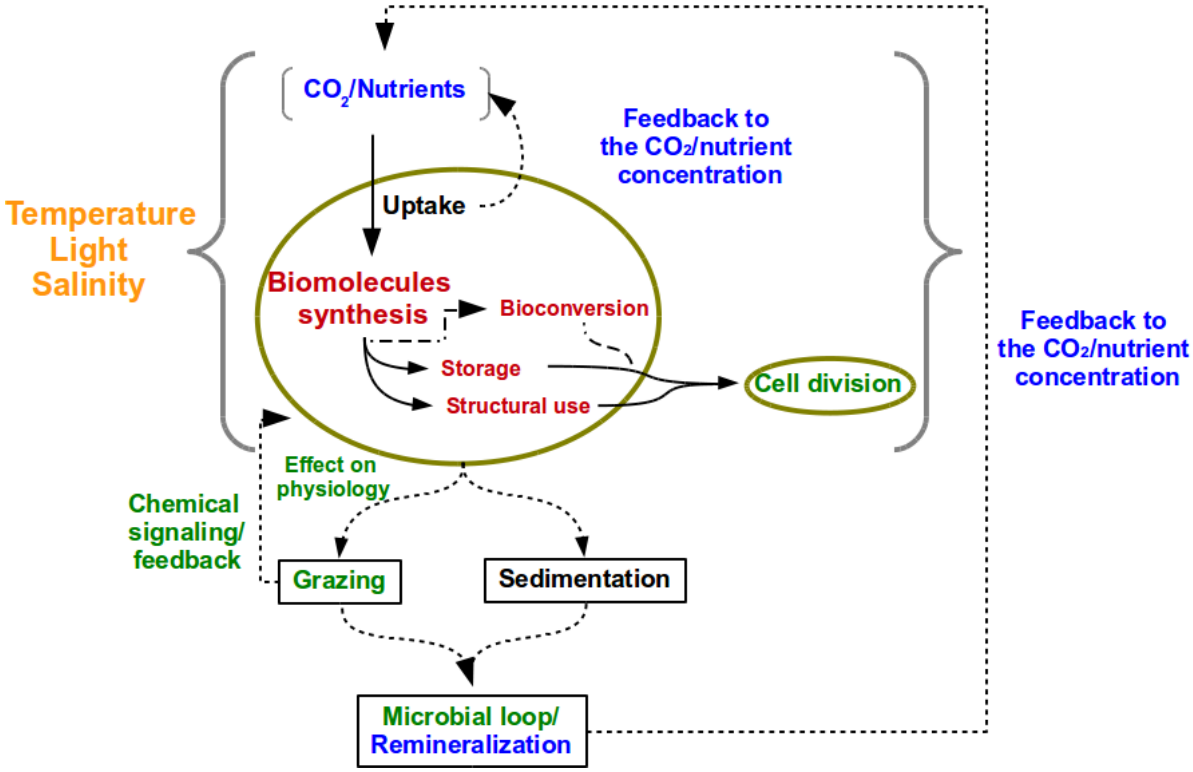

Figure 3. Schematic of the different physical (orange), chemical (blue) and biological (green) processes in the marine environment that can have a direct (CO_2, nutrients, temperature, light, salinity, cell division) or an indirect (Microbial loop/remineralization and grazing) influence on the composition and synthesis of biomolecules (red) in primary producers. Dotted connector indicates an exogenous influence. Dashed connectors indicate a putative path. The influence of each process can vary widely among algae.

5.3 Algal biomolecules and the life cycle of grazers

Any effect of elevated CO_2 on algae FA content can directly affect consumer organisms as these are essential components required by them. Our experiments showed that the FA profile of the copepod *Acartia tonsa* in a laboratory study (Article II) and *Calanus finmarchicus* in a mesocosm experiment (Article III) mirrored the FA profile of their algal prey. CO_2-driven changes in the food quality of their algae food source had negative consequences for the life cycle and reproduction of *A. tonsa* and decreased essential FA relative content in *C. finmarchicus,* consequently reducing the food quality of both copepods for higher trophic levels. The absence of a significant CO_2-related but strongly

phosphate-influenced effect in algal FA profile grazed by the copepods *A. tonsa* and *E. affinis* from a Baltic community (Article IV) is discussed in section 5.4.

Table 2. Change in total, polyunsaturated (PUFA), monounsaturated (MUFA) and saturated (SFA) fatty acid content in copepods fed with algae exposed to elevated CO_2 levels in relation to individuals fed with algae exposed to low CO_2 levels. ↑ increase, ↓ decrease, — no change. *mild, **moderate, ***strong. Color refers to the CO_2 level: yellow= ~750 µatm, orange= from ~700 to ~1200 µatm, red= 1700 µatm. [§]observations from a laboratory experiment, [+]observations from mesocosms.

Specie	Total	PUFA	MUFA	SFA
Acartia tonsa [§]	↓**	↓***	↓*	↑***
Calanus finmarchicus[+]	↓*	↓ *	—	↑ *
Calanus finmarchicus[+]	↓**	↓ **	↑**	↑*

Copepods are arguably the most abundant primary consumers in the world's ocean and are a crucial link for the transference of essential biomolecules between primary producers and higher tropic levels. There is mounting evidence of the importance of algal FA, and specially PUFA, in the life cycle of grazers as the fundamental building blocks of lipids for structural (i.e., phospholipids and sterols) and storage (i.e., triacylglycerols and wax esters) functions in higher trophic levels (Jónasdóttir, 1994; Müller-Navarra et al., 2006; Jónasdóttir et al., 2009; Kainz & Fisk, 2009; Chen et al., 2012) (Fig. 4). This goes in line with the observations in the present studies pointing that any negative effect in algae food quality can have serious consequences on the life cycle of grazers.

The requirement of essential FA by grazers and other biomolecules that cannot be synthesized by themselves implies that any deleterious CO_2-driven change in primary producer PUFA, even of a small magnitude, can have an "amplified impact" on the consumer, depending of its physiological requirements. For instance, the PUFA decrease of *A. tonsa* was 1.5 times higher compared to the PUFA decrease in their food source (Article II) and can be attributed to the physiology of the grazers itself. Essential biomolecules like FA, particularly PUFA are required throughout the life cycle of primary consumers, especially by spawning females for egg production (Jónasdóttir, 1994; Jónasdóttir et al., 2005; Brett et al., 2009) (Fig. 4). Such "amplified impacts" in

consumers will depend on species-specific dietary needs as different zooplankton organisms have shown contrasting FA requirements for egg production (Müller-Navarra, 2006; Wacker & Martin-Creuzburg, 2007; Abrusán et al., 2007).

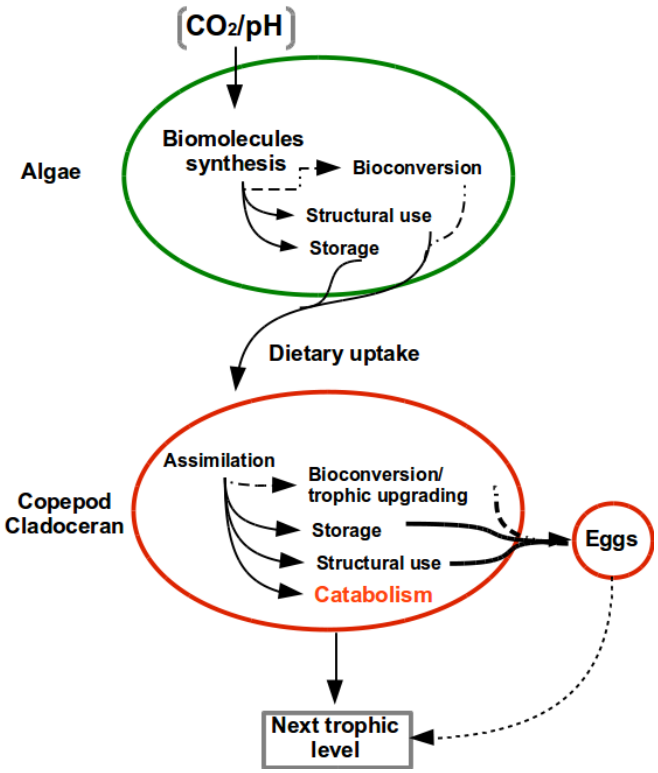

Figure 4. Schematic of the physiological processes of essential biomolecules through algae producers, which can be influenced by CO_2/pH, and transference to primary consumers (eg. copepods and cladocerans). The relative importance of each process varies widely with the biomolecule and organism in question. Although storage and structural use in the primary consumer are similar in secondary and higher level consumers, additional processes like reproduction (eg. egg production) and the catabolism of complex molecules, including essential biomolecules, imply an additional sink/losts in the consumer. Dashed connector indicates a putative path. Based on Kainz & Fisk (2009).

The decrease in PUFA observed in *C. finmarchicus* during the mesocosm experiment in a natural plankton assemblage matched their algae prey (Article III), and did not show an accentuated PUFA decrease compared to in the prey algae. This can be attributed to selective predation, which unlike to monocultures might mitigate indirect deleterious CO_2 effect in copepods (Rossoll et al., 2013). However, stage V copepodites were sampled for FA analysis during the experiment, therefore it can be expected that spawning adult females may show a comparatively poor FA profile under high CO_2 conditions due to investment in egg production.

Furthermore, considering that not all essential biomolecules are required in similar amounts by grazers (Brett et al., 2009; Kainz & Fisk, 2009) and that different algae taxa have diverse reactions to short term elevated CO_2 (Torstensson et al., 2010; Fiorini, 2010; Articles I, II, V), it is possible that under the same algal diet but contrasting CO_2 conditions different grazers species are favored. This has consequences for higher tropic levels as different fish species have also particular dietary requirements (Izquierdo et al., 2001) and a particular average prey size preference (Fig. 5) (Kainz et al., 2004). If the change in algal food quality is sustained in time or particularly deleterious for the dominant zooplankter species there is the possibility of a shift in the dominant taxa towards species with a lower PUFA physiological demand, for instance Tunicates. This zooplankton group is consider less nutritious for fish than crustaceans because of their high water content (Sommer & Stibor, 2002) (Fig. 5). Nevertheless, the trophic interactions in natural plankton communities are more complex and any indirect CO_2 effects on grazers in natural environments is more likely to be greatly regulated by the algal community composition, which is addressed below.

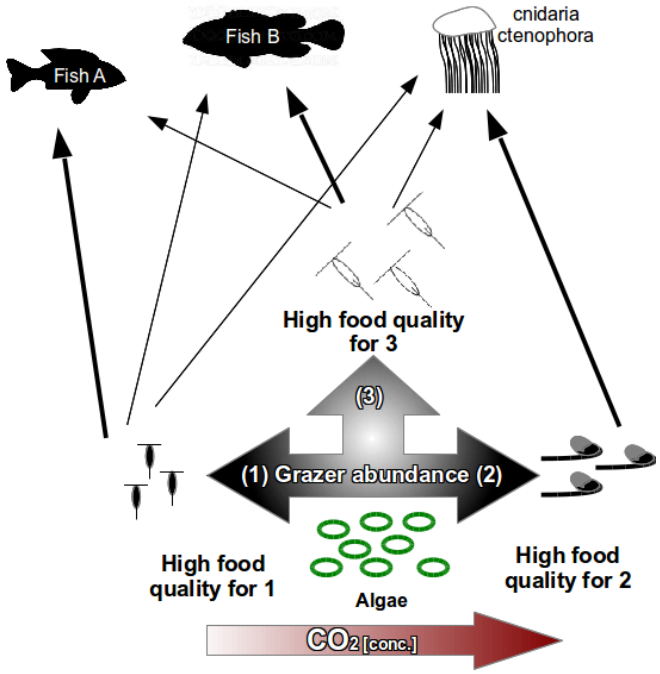

Figure 5. Illustration of the effect in food web structure of an algae whose food quality is affected by CO_2 in terms of its essential biomolecular composition, as for instance fatty acids (FA). The change in algal FA composition may benefit the grazer specie with physiological FA requirements that are better fulfilled by the algae. This could produce contrasting food web structures as higher trophic levels have also particular dietary requirements and average prey size. Thick arrows indicate preferred prey, thin arrows indicates lower preference. The position of the zooplankton taxa in the diagram is arbitrary in relation to the CO_2 concentration.

5.4 CO_2 and plankton community composition

The observations presented in this work show that the CO_2 influence in the plankton community composition depends on the environment. The North Sea plankton community, which is an oceanic environment, showed a CO_2-related change in community composition with picoeukaryotes becoming the more dominant taxa at elevated CO_2 conditions. In contrast, in the Baltic Sea, a brackish environment with high natural CO_2 fluctuations showed no significant variation among the dominant taxa in the community. A summary of the relative abundance of some of the plankton taxa is given in Table 3.

Table 3. Relative biomass abundance of several representative plankton taxa in a natural plankton assemblage during mesocosm experiments in a marine (North Sea) and a brackish (Baltic Sea) environment subjected to a CO_2 gradient. [ß]Baltic community CO_2 range: Low= 240-390; Middle= 540-840; High= 1120-1400 µatm CO_2 . [§]North sea community CO_2 range: Low= 280-390; Middle= 560-1120; High= 1400-3000 µatm CO_2. Direction of change in biomass in relation to CO_2 level: ↑ increase, ↓ decrease, — no change. Magnitude of change in biomass during the experiment: *mild, **moderate, ***strong. Color refers to abundance during the experiment: yellow= low, orange= intermediate, red= high. [+] North Sea community: picocyanobacteria, Baltic Sea community: filamentous non-diazotrophic cyanobacteria. nd= not detected, na= data not available.

	North Sea community[§]			Baltic Sea community[ß]		
Taxa	**Low**	**Middle**	**High**	**Low**	**Middle**	**High**
Picoeukaryotes	—	↑**	↑***	na	na	na
Chlorophyta	↑***	↓*	↓ *	↑***	↓*	↓*
Cyanobacteria[+]	↑*	↑**	↑***	↑*	—	↑**
Haptophyta	↑***	↓*	↓***	nd	nd	nd
Heterokontophyta	—	↓*	↓**	—	—	—
Dinophyta	↑*	—	—	—	—	—
Protozoa	—	↑*	—	↑*	↑*	↑*

The reason for the different CO_2 effects between the North and Baltic Sea plankton communities can be due to adaptation to the already natural high and variable CO_2 levels in the Baltic environment, which is in line with previous observation in the same

region (Nielsen et al., 2010; Rossoll et al., 2013). Additionally the phosphate depletion in the mesocosms in the Baltic experiment had a strong influence on the PUFA content of primary producers, overriding any CO_2 influence on them.

Some phytoplankton taxa had a similar response to CO_2 (Table 3). For instance cyanobacteria and specially picoeukaryotes thrived under high CO_2, while Chlorophyta and particularly Haptophyta decreased. Unfortunately the data of these first two taxa from the Baltic Sea experiment was not available at the moment of the present analysis, nevertheless the North Sea data showed strikingly similar traits to observations in tems of picoplankton abundance conducted in a similar mesocosm study in the Arctic (Brussaard et al., 2013).

As discussed in Article III, it can be expected that picoplankton in general have an advantage over larger cells when it comes to acquiring CO_2 and nutrients because of their large surface area per unit volume, which allows a higher supply of CO_2 at the cell surface, enhancing their photosynthesis and growth (Riebesell et al. 1993; Reinfelder, 2011). It has been estimated that for cells with radii smaller than approximately 10µm, CO_2 diffusion could support high specific carbon fixation rates at seawater CO_2 concentrations >10µM. At CO_2 concentrations from 3 to 20µM, diffusion-supported specific carbon fixation rates drop sharply as cell radius increases from 10µm to 20µm (Reinfelder, 2011). Therefore we can expect that in future ocean scenarios under elevated CO_2 concentrations picoplankton will be a dominant taxa due to this additional competitive advantage over larger-size cells, which has been observed in several mesocosm experiments where cyanobacteria and particularly picoeukaryotes dominated (Hare et al., 2007; Biswas et al., 2011; Brussaard et al., 2013). Furthermore, elevated temperatures may even enhance the dominance of picoplankton (Daufresne et al., 2009).

The reason why picoeukaryotes are often more abundant than picocyanobacteria can be attributed to their efficient CCM and Rubisco enzyme. The presence of an active CCM has been described in picoeukaryotic marine algae (Iglesias-Rodríguez et al., 1998). As discussed in section 5.3, eukaryotic organisms have an efficient CCM for carbon uptake

with several CA enzymes, while cyanobacteria posses only simple DIC transport systems (Giordano et al., 2005; Price et al., 2008; Reinfelder, 2011). Furthermore the cyanobacterial Rubisco has a lower CO_2 affinity than eukaryotic algae (Andersson & Backlund, 2008). A more efficient carbon metabolism would actually give an advantage to picoeukaryotes compared to cyanobacteria, particularly their comparatively more efficient Rubisco. Since high CO_2 levels would benefit both algae through diffusion and reduced leakage, the lower saturation threshold of the picoeukaryotic Rubisco (Andersson & Backlund, 2008) would improve its carbon fixation rates. Unfortunately, very little is known about carbon metabolism in picoeukaryotes and which species or taxa actually benefit from elevated CO_2 levels.

The decline of Haptophyta algae at elevated CO_2 is addressed in Article III, and can be attributed to their sensitivity to high CO_2 (Riebesell et al., 2000; Bach et al., 2011). The decrease of Chlorophyta is rather puzzling considering that there is no evidence of a negative physiological sensitivity of green algae to CO_2 with the exception of an increase in SFA content under high CO_2 levels (Tsuzuki et al., 1990). However, it may be associated to its displacement due to the competition of the picoplankton fraction.

At community level the dominance of small-sized plankton cells can strongly affect the species composition at higher trophic levels. High CO_2 conditions would produce a food web similar to ones in current oligotrophic oceanic systems as proposed by Sommer et al. (2002). Elevated CO_2 promotes growth and nutrient capture by picoplankton, producing a system where the average prey size is too small to be ingested by copepods (Kainz et al., 2004) and most of the primary production is channeled through the "microbial loop" (picoplankton – heterotrophic nanoflagellates – ciliates) with pelagic tunicates sporadically consuming a substantial proportion of the picoplanktonic primary production (Fig. 6). Copepods would feed on heterotrophic nanoflagellates and ciliates, thus occupying a food chain position between 3 and 4, which leads to a food chain position between 4 and 5 for zooplanktivorous fish (Sommer et al., 2002) (Fig. 6). This is important because food chain length affects trophic efficiency (defined as the proportion of biomass fixed by PP transferred to the top trophic level) (Vander Zanden & Fetzer, 2007). Furthermore, considering that only about 20% of essential

macromolecules like PUFA are incorporated into new biomass of organisms at the next trophic level (Gladyshev et al., 2011), any additional CO_2 related loss at the primary producer essential PUFA content, as show in Article III (~10%), could seriously impair transference efficiency between trophic levels (Fig. 6). This can have far reaching consequences in marine food webs since these essential macromolecules are sequentially incorporated into the lipids of larval fish (Fig. 6) (Fraser et al., 1989; Izquierdo et al., 2001) and might become a threat for human populations who rely on fisheries as an important food source (Sargent et al., 1997; Arts et al., 2001).

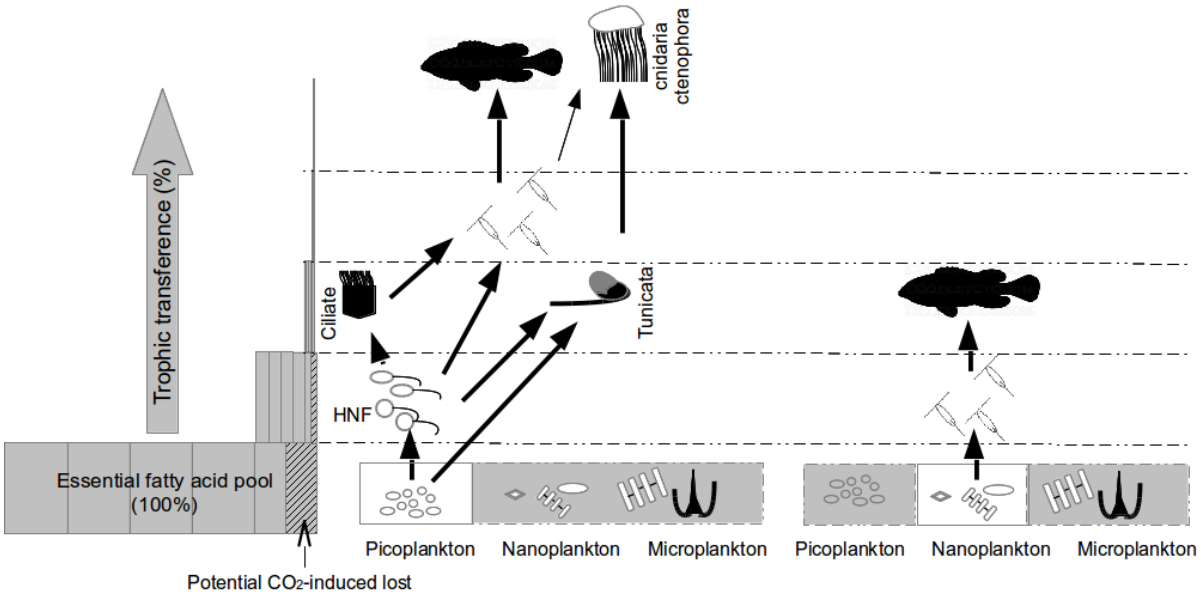

Figure 6. Main pathways of FA biomolecules flow to the primary carnivore trophic levels under elevated CO_2 which promotes picoplankton dominance in the primary producers level (left) compared to a pathway were nanoplankton and diatoms are dominant (right). Only 20% of the essential fatty acids are transferred from one trophic level to the next, therefore any additional step decreases considerably trophic FA transference from plankton to fish. This is depicted by the square on the left which represents the total (100% divided in 20% parts) essential FA of phytoplanktonic origin in the next successive trophic level. Additionally, the dashed area represents a potential 10% PUFA CO_2-related loss in the nano and picoplankton primary producers and reflected by the copepods PUFA as observed in a natural community (Article III). HNF stands for heterotrophic nanoflagellates. Based on Sommer et al. (2002).

5.5 Future research perspectives

The transfer of essential biomolecules within food webs is not completely understood under current ocean conditions and the perspective of OA and elevated temperatures adds to the uncertainty. Future research is needed at different levels in the food webs, for instance at specie level the effect of elevated CO_2 conditions in dominant plankton

taxa needs to be clarified; at single species specific interactions, the effects of a varying algal food quality have to be assessed for their influence on different zooplankton taxa. However, focus should be given to the origin and transfer of essential biomolecules up to fishes, and on assessing the sensitivity of food web structures to OA with emphasis on highly productive areas where some of the major fisheries are located due to their importance as an essential food resource (FAO, 2010).

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7. Eidesstattliche Erklärung

Hiermit bestätige ich, dass die vorliegende Arbeit mit dem Titel:

Primary producers and future ocean scenarios: Effect of environmental change on the biomolecular composition of phytoplankton and transference to higher trophic levels

von mir selbstständig angefertigt wurde.

Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt.

Dies ist mein einziges und bisher erstes Promotionsverfahren.

Ich habe keine als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.

Teile dieser Arbeit wurden als Manuskripte in wissenschaftlichen Fachzeitschriften veröffentlicht:

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Kiel, im April 2014 Jorge Rafael Bermúdez Monsalve