Plankton in an acidifying ocean: from individual responses to community changes and the potential of adaptation

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„In der lebendigen Natur geschieht nichts, was nicht in der Verbindung mit dem Ganzen steht.“

- Johann Wolfgang von Goethe -

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Summary

Ocean acidification has strong direct (decreased seawater pH) and indirect (altered food regimes) effects on the performance of marine organisms, their trophic interactions and, consequently, whole ecosystems. Therefore, the aim of this thesis was to investigate potential direct and indirect effects of elevated pCO_2 on marine planktonic organisms and their community composition, with a main emphasise on copepods and their adaptational potential.

The development of high throughput sequencing technologies has provided scientists with an efficient tool to assess the biodiversity of marine communities, particularly with the recent advances in community barcoding technologies using universal primers. Another aim of this thesis was therefore, to test whether community barcoding is suitable for the assessment of marine planktonic communities, and allows the detection of compositional changes, which would probably remain unnoticed using classical morphological approaches. Research results are presented in four chapters (CHAPTER I to IV), each of which is representing a manuscript for scientific publication.

Additional stressors such as ocean warming, eutrophication, the threat of invasive species and parasite infections might accelerate or dampen the effects of ocean acidification on marine taxa, emphasizing the importance of community level, multi-stressor experiments. The traceability and taxonomic classification of parasitic species based on morphological characteristics is particularly difficult and often insufficient. Thus, little is known about their impacts on food web interactions and ecosystem functions. This applies especially to marine oomycetes, which are predominantly parasites of marine diatoms, crustaceans and nematodes. In this study, (CHAPTER I) a primer pair (gene region cytochrome-c-oxidase subunit II) already successfully used for phylogenetic investigations of marine and terrestrial oomycetes, was tested to be suitable for community barcoding on a high throughput sequencing platform. The Illumina sequencing of a plankton sample, taken in the Brudenell River (Canada), revealed six distinct phylotypes, reflecting the results of a comparable community barcoding study based on the 16S gene region. However, not all operational taxonomical units could be assigned to corresponding reference

sequences, indicating the incompleteness of present genetic databases and the lack of knowledge about marine oomycete biodiversity.

In the KOSMOS (Kiel Off-Shore Mesocosms for Future Ocean Simulations) 2013 experiment, carried out in the Gullmar Fjord, Skagerrak (58° 15' 9 N, 11° 28' 7 E), the response of different trophic levels to elevated pCO_2 concentrations during a winter-to-summer plankton succession was investigated over 113 days. Using community barcoding, the diversity of a broad spectrum of the biocoenosis including all trophic levels (producers, consumers, decomposers) was assessed and the effects of ocean acidification on the coastal plankton community composition investigated (CHAPTER II). Based on the community barcoding data we found no significant effect of elevated CO_2 on the relative abundance or compilation of the coastal planktonic taxa. Observed shifts in the planktonic community composition were predominantly caused by seasonal changes in temperature and availability of nutrients.

As the large-scale community experiment did not allow to discriminate between direct (decreased seawater pH) and indirect (altered food regimes) pCO_2 effects on zooplankton, an additional short-term laboratory experiment was conducted. Therein, a heterotrophic dinoflagellate and a calanoid copepod were cultured at low and high pCO_2 concentrations and fed with phytoplankton grown at the same pCO_2 levels (CHAPTER III). While direct effects of high pCO_2 exposure were negligible, the ingestion of algae cultured under high pCO_2 conditions, significantly decreased the growth and developmental rate of both grazers. Furthermore, copepods fed algae, cultured under high pCO_2, showed an increased respiration rate, probably a reaction to excrete excess carbon ingested with food.

To investigate the combined, direct and indirect, effects of elevated pCO_2 on a calanoid copepod and its adaptability, a 3 ½ years long selection line study was designed (CHAPTER IV). In reciprocal transplant experiments the performance of the low and high CO_2 selection line copepods were compared analysing their body stoichiometry and different fitness parameter. The reduced algae food quality (higher carbon to nutrient ratio) in combination with the high seawater pCO_2 significantly altered copepods' stoichiometry and consequently their developmental rates independent from the prior CO_2 treatment in the selection lines. However, copepods

from both selection lines showed a high plasticity in the regulation of their elemental body composition and became more homeostatic after one year. Most likely due to the lack of a strong selective pressure, no beneficial acclimatization or adaption of the high CO_2 selection line copepods (e.g. an increased developmental rate or egg production under high CO_2 conditions compared to the controls) was observed. After 3 years, despite their increased homeostasis, high CO_2 selection line copepods had a, in relation to their body length, higher carbon and nitrogen body content, indicating that under nutrient repletion copepods' body mass might increase in high pCO_2 environments. The results suggest that ocean acidification might have fundamental long-term effects on marine copepods and consequently for ecosystem structures and functions.

Overall, the results showed that mesozooplankton communities are hardly directly affected by ocean acidification, but the laboratory studies indicated that indirect ocean acidification effects due to a changed nutritional status of the primary producer can significantly impact mesozooplankton development and, in the long-term, their physiology. However, results from laboratory studies with single species and / or artificial predator-prey combinations cannot simply be translated to the community level, where e.g. complex food web interactions might accelerate or mitigate the effects of ocean acidification. Generally, the sensitivity of marine organisms to elevated pCO_2 conditions very much depends on their environmental prehistory. Since coastal plankton communities already experience pronounced fluctuations in seawater pH, caused by e.g. upwelling events of CO_2-rich deep-water masses, they are probably acclimatised or adapted to higher pCO_2 conditions, reducing their sensitivity to future ocean acidification conditions.

The results of the thesis highlight two main objectives for future ocean acidification and climate change research. First, the importance to perform, long-term, near natural, multi-stressor, mesocosm community experiments during different seasons (nutritional situations), to get a possibly comprehensive picture of the effects of climate and environmental changes on marine communities and ecosystems. Second, as the response of marine communities is shaped by their taxa compilation and environmental prehistory, future climate change research should intensify the monitoring of relevant seawater parameters (e.g. temperature, pCO_2, nutrients and light penetration) and biodiversity assessments, to substantially improve the

predictability of how future oceans might look like. Thereby, with little restrictions, community barcoding offers a promising tool for the assessment of the marine biodiversity and thereby for the detection of e.g. pCO_2 induced changes in community compositions, during short-term observations and large-scale monitoring project.

Zusammenfassung

Die Versauerung der Ozeane hat starke direkte (erniedrigter pH-Wert) und indirekte (geänderte Nahrungsqualität) Auswirkungen auf marine Organismen, ihre trophischen Interaktionen und schlussendlich auf gesamte Ökosysteme. Ziel dieser Arbeit war es daher, potentielle direkte und indirekte Auswirkungen von erhöhtem pCO_2 auf marine Planktonorganismen und die Zusammensetzung ihrer Lebensgemeinschaften zu untersuchen sowie das Anpassungspotenzial von Copepoden zu ermitteln. Die Entwicklung von Hochdurchsatz-Sequenzierungstechnologien insbesondere „community barcoding“ bieten Wissenschaftlern eine effiziente Methode zur Erfassung mariner Lebensgemeinschaften. Es war daher auch Aufgabe dieser Arbeit zu testen, ob „community barcoding“ für die Erfassung mariner Planktongemeinschaften geeignet ist und dazu genutzt werden kann Veränderungen in der Artenzusammensetzung aufzudecken, welche mit klassischen morphologischen Methoden wahrscheinlich unbemerkt bleiben würden. Die Forschungsergebnisse sind in vier Kapitel (KAPITEL I bis IV) gegliedert, wobei jedes ein Manuskript für eine wissenschaftliche Publikation repräsentiert.

Zusätzliche Stressfaktoren wie die Ozeanerwärmung, Eutrophierung und die Bedrohung durch invasive Arten sowie Parasiteninfektionen könnten die Auswirkungen der Ozeanversauerung auf marine Organismen beschleunigen oder abmildern. Daher sind besonders solche Experimente wichtig, die mehrere Umweltstressoren kombinieren und auf der Ebene von Lebensgemeinschaften durchgeführt werden. Die Erfassung und taxonomische Einordnung parasitärer Arten anhand morphologischer Merkmale ist sehr zeitintensiv und liefert oft nur eine unzureichende taxonomische Auflösung. Daher ist nur wenig über ihre Rolle in Nahrungsnetzen und über ihre Einflüsse auf Ökosystemfunktionen bekannt. Dies gilt insbesondere für marine oomyceten, welche vorwiegend als Parasiten von marinen Kieselalgen, Krebsen und Nematoden leben. In dieser Arbeit (KAPITEL I) wurde getestet, ob ein Primerpaar (für die Genregion der Cytochrome-c-Oxidase Untereinheit II), das bereits erfolgreich für phylogenetische Untersuchungen mariner und terrestrischer oomyceten verwendet wurde, auch für „community barcoding“ auf einer Hochdurchsatz-Sequenzierungsplattform genutzt werden kann. Die Illumina-

Sequenzierung einer Planktonprobe aus dem Brudenell River (Kanada) ergab sechs verschiedene Phylotypen und reflektiert damit die Ergebnisse einer vergleichbaren, auf der 16S Genregion basierenden, „community barcoding“ Studie. Die Tatsache, dass nicht alle operativen taxonomischen Einheiten entsprechenden Referenzsequenzen zugeordnet werden konnten, zeigt die Unvollständigkeit vorhandener genetischer Datenbanken und das noch lückenhafte Wissen über die Diversität mariner oomyceten.

In dem im Jahr 2013 im Gullmarsfjord, Skagerrak (58° 15’ 9 N, 11° 28’ 7 E) durchgeführten KOSMOS (Kiel Off-Shore Mesocosms for Future Ocean Simulations) Experiment wurden die Reaktionen verschiedener trophischer Ebenen marinen Planktons auf erhöhtes pCO_2 während einer Winter-Sommer-Sukzession über einen Zeitraum von 113 Tagen untersucht. Unter Verwendung von „community barcoding“ wurde die Diversität eines breiten Spektrums der Biozönose einschließlich aller trophischer Ebenen (Produzenten, Konsumenten, Zersetzter) und die Auswirkungen der simulierten Ozeanversauerung auf die Zusammensetzung der Küstenplanktongemeinschaft untersucht (KAPITEL II). Basierend auf den „community barcoding“ Daten fanden wir keinen signifikanten CO_2 Effekt bezüglich der relativen Häufigkeiten einzelner Arten oder der Zusammensetzung der Planktongemeinschaft. Die Artenzusammensetzung wurde hauptsächlich durch saisonale Temperaturveränderungen und wechselnde Nährstoffverfügbarkeiten bestimmt.

Da während des Mesokosmen-Experimentes keine Unterscheidung zwischen direkten (erniedrigter pH-Wert) und indirekten (geänderte Nahrungsqualität) pCO_2 Auswirkungen auf Zooplankton möglich war, wurde ein zusätzliches Laborexperiment durchgeführt. Dabei wurde ein heterotropher Dinoflagellat und ein Copepod jeweils unter niedrig und hoch pCO_2 Bedingungen gehältert und mit Phytoplankton gefüttert, welches bei den entsprechend gleichen pCO_2 Konzentrationen kultiviert wurde (KAPITEL III). Während die direkten pCO_2 Effekte vernachlässigbar waren, verringerte die Aufnahme von Algen, die unter hohen pCO_2 Bedingungen kultiviert wurden, das Wachstum und die Entwicklungsrate beider Konsumenten signifikant. Darüber hinaus war die Atmung von Copepoden, die Algen gefressen hatten, welche unter hohem pCO_2 kultiviert

wurden, erhöht. Wahrscheinlich diente diese Reaktion dazu, überschüssigen Kohlenstoff, der mit der Nahrung aufgenommen wurde, auszuscheiden.

Um die direkten und indirekten Auswirkungen einer erhöhten pCO_2 Konzentration auf einen calanoiden Copepoden und seine Anpassungsfähigkeit zu untersuchen, wurde über einen Zeitraum von 3 ½ Jahren eine Selektionsstudie durchgeführt (KAPITEL IV). In reziproken Transplantationsexperimenten wurde durch die Analyse der Körperstöchiometrie sowie verschiedener Fitnessparameter die Leistungsfähigkeit der Copepoden aus den Niedrig- und Hoch-CO_2-Selektionslinien miteinander verglichen. Die Hälterung unter erhöhten pCO_2 Bedingungen und die damit verbundene schlechtere Futterqualität der Algen (erhöhtes Kohlenstoff : Nährstoff-Verhältnis) veränderte die Stöchiometrie der Copepoden und verlangsamte ihre Entwicklung unabhängig von den vorherigen CO_2 Bedingungen in den Selektionslinien. Copepoden aus beiden Selektionslinien zeigten eine hohe Plastizität in der Regulierung ihrer Körperstöchiometrie, was sich nach einem Jahr in einer erhöhten Homöostase zeigte. Während der Selektionsstudie wurde keine vorteilhafte Akklimatisierung oder Anpassung der Copepoden aus den Hoch-CO_2-Selektionslinien an die erhöhten CO_2 Bedingungen (z.B. eine im Vergleich zu den Niedrig-CO_2-Selektionslinien schnellere Entwicklung oder höhere Eiproduktion unter Hoch-CO_2-Bedingungen) beobachtet, was wahrscheinlich an einem zu geringen Selektionsdruck lag. Trotz der gesteigerten Homöostase wiesen die Copepoden der Hoch-CO_2-Selektionslinien nach 3 Jahren einen im Verhältnis zu ihrer Körperlänge erhöhten Kohlenstoff- und Stickstoffgehalt auf. Dies deutet an, dass in Habitaten mit einer erhöhten pCO_2 Konzentration die Körpermasse von Copepoden zunehmen könnte. Die Ergebnisse des Selektionsexperimentes zeigen, dass die Versauerung der Ozeane langfristige Auswirkungen auf marine Copepoden und damit auf Ökosystemstrukturen und -funktionen haben könnte.

Insgesamt lässt sich sagen, dass die direkten Auswirkungen der Ozeanversauerung auf marines Mesozooplankton gering sind. Die Laborstudien haben allerdings gezeigt, dass eine erhöhte pCO_2 Konzentration indirekt durch eine veränderte Stöchiometrie der Primärproduzenten die Entwicklungsgeschwindigkeit von Copepoden verlangsamen und über einen längeren Zeitraum ihre Physiologie beeinflussen kann. Allerdings können Ergebnisse aus Laborexperimenten mit

einzelnen Arten und künstlichen Räuber-Beutetier-Kombinationen nicht einfach auf die Gemeinschaftsebene übertragen werden, wo z.B. komplexe Interaktionen im Nahrungsnetz die Auswirkungen der Ozeanversauerung beschleunigen oder abmildern könnten. Im Allgemeinen hängt die pCO_2 Empfindlichkeit von marinen Organismen sehr stark von ihrer Umweltgeschichte und den erlebten pCO_2 Bedingungen ab. Planktongemeinschaften in Küstennähe, welche starken pH-Wert Schwankungen durch z.B., den Aufstieg von CO_2-reichem Tiefenwasser ausgesetzt sind, haben sich wahrscheinlich bereits akklimatisiert oder an höhere pCO_2 Bedingungen angepasst, wodurch sie gegenüber zukünftigen Ozeanversauerungszuständen weniger empfindlich sind.

Basierend auf den Ergebnissen dieser Arbeit ergeben sich zwei Hauptziele für künftige Forschungsprojekte im Bereich Ozeanversauerung und Klimawandel. Um ein besseres Bild von den Auswirkungen der Klima- und Umweltveränderungen auf marine Lebensgemeinschaften und Ökosysteme zu erhalten, sollte ein Fokus auf Mesokosmos-Experimenten liegen, welche mehrere Umweltstressoren kombinieren und möglichst lange Zeiträume umfassen. Diese sollten weiterhin zu verschiedenen Jahreszeiten (Ernährungssituationen) und in möglichst vielen verschieden Habitaten durchgeführt werden. Da die Reaktion von marinen Lebensgemeinschaften auf Umweltveränderungen substantiell durch ihre Artenzusammensetzung und Umweltvorgeschichte geprägt ist, sollten des Weiteren die Überwachung relevanter Meerwasserparameter (z.B. Temperatur, pCO_2, Nährstoffgehalt und Lichtdurchflutung) und die Erfassung der marinen Biodiversität intensiviert werden, um bessere Vorhersagen treffen zu können wie die Weltmeere in Zukunft aussehen könnten. „community barcoding“ bietet mit wenigen Einschränkungen eine schnelle und effiziente Methode zur Erfassung mariner Lebensgemeinschaften und kann sowohl in Kurzzeitexperimenten als auch in großangelegten Monitoringprojekten für die Untersuchung von z.B., durch pCO_2 ausgelöste Veränderungen in der Artenzusammensetzung von Lebensgemeinschaften, genutzt werden.

Abbreviations

%	Percent
~	Approximately
°C	Degree Celsius
µatm	Microatmosphere
µg	Microgram
µL	Microliter
µmol	Micromole
ANOVA	Analysis of variance
ASW	Artificial seawater
BIOACID	Biological Impacts of Ocean ACIDification
BLAST	Basic Local Alignment Search Tool
BMBF	Bundesministerium für Bildung und Forschung
bp	Base pair
BSA	Bovine serum albumin
C	Carbon
$CaCO_3$	Calcium carbonate
CASY	Cell Counter and Analyzer System
CCA	Canonical correlation analysis
chl *a*	Chlorophyll a
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ions
COI	Cytochrome-c-oxidase subunit I
*cox*1	Cytochrome-c-oxidase subunit I
*cox*2	Cytochrome-c-oxidase subunit II
CTD	Conductivity-Temperature-Depth
d	Day
DIN	Dissolved inorganic nutrients
DNA	Deoxyribonucleic acid
dNTP	2´-Desoxyribonucleosid-5´-triphosphate
DOM	Dissolved organic matter
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
EPSAG	Experimental Phycology and Culture Collection of Algae at the University of Göttingen
Fig	Figure
g	Gram
h	Hour
H^+	Hydrogen ions
H_2CO_3	Carbonic acid
H_2SO_4	Sulfuric acid
HCO_3^-	Bicarbonate
Het	Heterotrophic
HPLC	High-performance liquid chromatography
HTS	High-throughput sequencing
i.a.	Inter alia
i.e.	Id est (Latin), that is to say
ID	Identity
ind	Individual
IPCC	Intergovernmental Panel on Climate Change
IWS	Integrating Water Sampler
kg	Kilogramme
KOSMOS	Kiel Off-Shore Mesocosms for Future Ocean Simulations
L	Litre
m	Meter
M	Molar
m^2	Square meter
MEGAN	MEtaGenome ANalyzer
$MgCl_2$	Magnesium chloride
min	Minute

mL ..... Millilitre
mM ..... Millimolar
n ..... Number of measurements, replicates
N ..... Nitrogen
NaCl ..... Sodium chloride
NaH_2PO_4 ..... Sodium dihydrogen phosphate
$NaNO_3$ ..... Sodium nitrate
NCBI ..... National Center for Biotechnology Information
ng ..... Nanogram
NGS ..... Next generation sequencing
NH_4^+ ..... Ammonium
nMDS ..... Nonmetric multidimensional scaling
NO_2^- ..... Nitrite
NO_3^- ..... Nitrate
nt ..... Nucleotide
OA ..... Ocean acidification
OTU ..... Operational taxonomical unit
p ..... P-value, statistical significance
P ..... Phosphorus
PCA ..... Principal component analysis
pCO_2 ..... Partial pressure of carbon dioxide
PCR ..... Polymerase chain reaction
pH ..... Potentia hydrogenii
PO_4^{3-} ..... Phosphate
POM ..... Particulate organic matter
psu ..... Practical Salinity Units
RDA ..... Redundancy analyses
rDNA ..... Ribosomal DNA
RNase ..... Ribonuclease
SD ..... Standard deviation
SDS ..... Sodium dodecyl sulfate
SiO_4^{3-} ..... Dissolved silicate
SL ..... Selection line
sp. ..... Species (singular)
spp. ..... Species (plural)
t ..... Time point
T ..... Treatment
TA ..... Total alkalinity
TAQ ..... Thermus aquaticus
temp ..... Temperature
Tris ..... Trishydroxymethylaminomethane
Tris-HCl ..... Tris hydrochloride

Introduction

Ocean acidification

The oceans cover over two-thirds of the Earth's surface and play an essential role in global biogeochemical cycles and climate regulation. Furthermore, they contribute enormously to the planet's biodiversity and provide a livelihood for million humans. Despite their importance for human life, marine ecosystems face many anthropogenic induced threats like pollution (Gworek et al. 2016; Li et al. 2016), overfishing (Coll et al. 2008; Srinivasan et al. 2010), warming (Levitus et al. 2005) and acidification (Raven et al. 2005; Reid et al. 2009). Since the industrial revolution, the concentration of carbon dioxide (CO_2) in the atmosphere has increased from about 280 µatm to 380 µatm which represent an increase unprecedented in the last 800 000 years (IPCC 2014). Atmospheric CO_2 is mainly produced through the combustion of fossil fuels i.e., coal, petroleum, and natural gas (Le Quere et al. 2009), whereby parts of the released CO_2 dissolves in the oceans. The CO_2 reacts with seawater to form carbonic acid (H_2CO_3) which further dissociates to bicarbonate (HCO_3^-) and hydrogen ions (H^+). In a second, pH-dependent reaction, bicarbonate ions dissociate to carbonate (CO_3^{2-}) and hydrogen ions. As a result, seawater pH decreases with increasing pCO_2 and free carbonate ions protonate and form bicarbonate (Fig. 1).

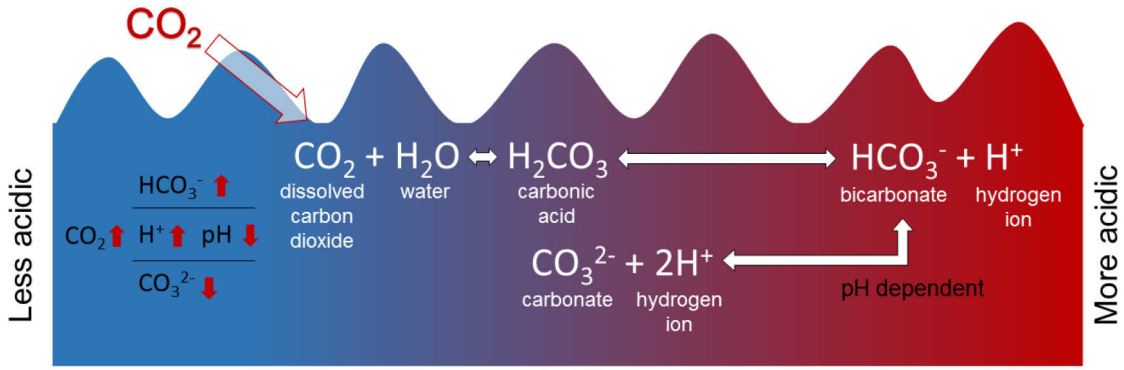

Fig. 1 Ocean acidification process

(after: https://www.oceanacidification.de/ocean-acidification/?lang=en).

Therefore, ocean acidification (OA) not only decreases seawater pH, but also changes marine carbonate chemistry. Over the last 200 years, the oceans have taken

up around 25 - 50% of anthropogenic CO_2 emissions (Sabine et al. 2004; Feely et al. 2009), resulting in a pH decrease in surface waters of ~ 0.1 units since pre-industrial times. Based on the IPCC worst case scenario, atmospheric CO_2 concentrations are predicted to reach levels exceeding 1000 µatm by the end of this century, which could lead to a further decrease in seawater pH by up to 0.3 - 0.5 units (Caldeira and Wickett 2003). Since the CO_2 solubility is higher in cold compared to warm waters, most rapid changes in ocean chemistry will occur in high latitude areas (Orr et al. 2005; Fabry et al. 2009).

Marine pelagic food webs

The foundation of the sea's food chain is largely invisible. Masses of phytoplankton organisms populate the sunlit upper ocean layer. Those photosynthetic organisms play a major ecological role, as they provide more than 99% of the organic matter used by marine food webs (Field et al. 1998). Converting dissolved CO_2 to organic carbon, they account for around 40% of the total global primary productivity (Falkowski 1994). Furthermore, it is considered that at least half of the oxygen in the atmosphere is produced by phytoplanktonic primary produces (Suthers and Rissik 2009). For phytoplankton growth nitrate (NO_3^-), phosphate (PO_4^{3-}) and, for diatoms, silicate (SiO_4^{3-}) are the most important macronutrients. If nutrients are not limited, phytoplankton cells take up dissolved forms of carbon (C), nitrogen (N) and phosphorus (P) in an atomic ratio of 106C:16N:1P (Redfield ratio; (Redfield 1934)). In areas of low phytoplankton productivity, most of phytoplankton growth is sustained through recycled nutrients. The primary organisms involved in the recycling activities of the microbial loop (Fig.2; (Azam et al. 1983)) are water-column bacteria, heterotrophic flagellates and ciliates.

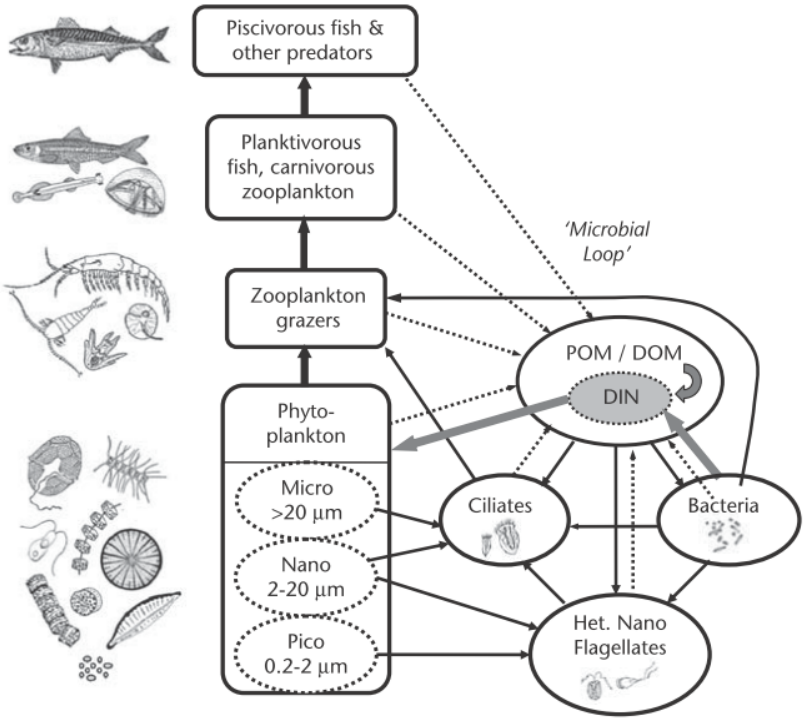

Fig. 2 Simplified pelagic food web including the classical food chain (left side) and the microbial loop (right side). Arrows represent trophic pathways. The flow of particulate and dissolved organic matter (POM, DOM), in excretory products and dead organisms (dashed arrows), and the flow of dissolved inorganic nutrients (DIN) back to the phytoplankton. Het. = Heterotrophic (after Suthers and Rissik 2009).

Those organisms break down organic molecules contained in particulate organic matter (POM) and in dissolved organic matter (DOM) derived from living cells, faecal pellets, dead and decomposing bodies. Decomposers convert organic matter to dissolved inorganic nutrients (DIN), such as NO_2^-, NO_3^-, PO_4^{3-} and SiO_4^{3-}, which are then available for rapid uptake by phytoplankton. Pelagic bacteria are also consumed by e.g. microzooplankton organisms like ciliates and nano-flagellates, which are in turn food sources for larger zooplankton organisms. Most of the marine mesozooplankton biomass is made up by grazers like copepods (Verity and Smetacek 1996). They mainly feed on different microzooplankton organisms and nano-heterotrophs with a cell size of around 5 - 100 µm, as well as larger diatoms. Smaller phytoplankton, although part of their natural food, are consumed in lower proportions (Frost 1972). Energy produced by smaller autotrophs (< 2 µm) is therefore mostly not directly transferred to higher trophic levels but indirectly due to

the grazing of mesozooplankton on intermediate consumers like ciliates and heterotrophic flagellates (Calbet and Landry 1999). Fish, large invertebrate zooplankton and mammals represent the top predators in marine food webs.

Direct and indirect effects of OA on marine biota

OA is expected to impact marine environments in various ways (Riebesell et al. 2009; Kroeker et al. 2010; Mostofa et al. 2016), whereby most of all the rapid changes in ocean chemistry are likely to have substantial direct and indirect effects on marine organisms and their habitats (Fabry et al. 2008). In contrast to direct effects which are caused by an increased CO_2 concentration and acidity, indirect effects are driven by changes in the availability or composition of nutrients. The decrease in both pH and availability of free carbonate ions affects above all calcifying organisms including i.a. various algae, crustaceans, echinoderms and molluscs (Feely et al. 2004; Fabry et al. 2008). Their ability to build up calcium shells and skeleton structures is directly affected by seawater CO_2 chemistry (Riebesell et al. 2000; Orr et al. 2005; Enochs et al. 2016; Kwiatkowski et al. 2016), whereby the sensitivity of organisms is depending on the mineral forms (e.g. aragonite, calcite) of calcium carbonate ($CaCO_3$) they use to build up their shells or skeletons (Kroeker et al. 2010). Furthermore, plankton organisms might be directly impacted by hypercapnia, the acidification of body fluids. As the CO_2 concentration in seawater increases, dissolved CO_2 diffuses more easily across body surfaces and equilibrates in both intra- and extracellular spaces. In the body, the CO_2 reacts, as in seawater, with internal body fluids, causing H^+ ions to increase and the pH to decrease. Hypercapnia can suppress metabolic processes (Michaelidis et al. 2005; Pörtner 2008) and interfere with acid-base homeostasis (Miles et al. 2007), resulting in decreased growth rates and reproductive success and increased mortality (Yamada and Ikeda 1999). Thereby, due to their smaller volume to surface ratios, microzooplankton are likely to be more affected by hypercapnia than larger mesozooplankton (Hinga 2002). In contrast, elevated CO_2 levels can also have positive effects on marine organisms. Various studies dealing with eukaryotic phytoplankton suggested that increased carbon availability due to OA could also have fertilizing effects on primary production (Riebesell et al. 2007; Low-Décarie et al. 2014; Liu et al. 2017). As described before, mesozooplankton organisms play a key role in marine food webs, as they transfer energy captured by primary producers

to higher trophic levels. Since copepods (Fig. 3) typically form a significant proportion of the marine mesozooplankton (Verity and Smetacek 1996), any impact of OA on their growth, development or overall survival, could have far reaching consequences for marine food webs and trophic interactions. Although adult copepods are hardly directly affected by lower pH, there is evidence that early life stages are much more sensitive to decreased pH or changes in seawater carbonate chemistry (Ross et al. 2011; Cripps et al. 2014b). Elevated pCO_2 concentrations also influence the stoichiometry (higher C to nutrient ratios) of autotrophs, which is more pronounced in areas of low nutrient supply (Urabe et al. 2003; Verschoor et al. 2013; Meunier et al. 2016). As heterotrophs are more restricted in their elemental composition compared to autotrophs, stoichiometric discrepancies between predator and prey can diminish consumer growth, fecundity and survival (Sterner and Elser 2002; Bukovinszky et al. 2012). Therefore, copepods can be indirectly effected by OA due to changes in the elemental composition of their food sources (Cripps et al. 2016). Correlations between the decreases in the developmental rate and reproduction of copepods and the intake of CO_2 manipulated algae were demonstrated in various laboratory experiments (Rossoll et al. 2012; Schoo et al. 2013; Meunier et al. 2016).

In general organisms at higher latitudes are likely to be more impacted due to the higher solubility of CO_2 in cold waters (Andersson et al. 2008; Cummings et al. 2011). At small regional and temporal scales seawater pH and carbonate chemistry can strongly fluctuate due to an increased uptake of C during bloom events (Riebesell et al. 2007), stratification and / or upwelling events of CO_2 - rich deep-water (Feely et al. 2008; Hofmann et al. 2011). Especially, in coastal environments the pH can fluctuate seasonally and even daily by up to one or more pH units. Therefore, open ocean plankton communities are considered more vulnerable to OA, as species living in coastal waters that need to be pH-tolerant to cope with the relatively large natural pH fluctuations (Nielsen et al. 2010; Nielsen et al. 2012; Baumann et al. 2015; Wahl et al. 2015)

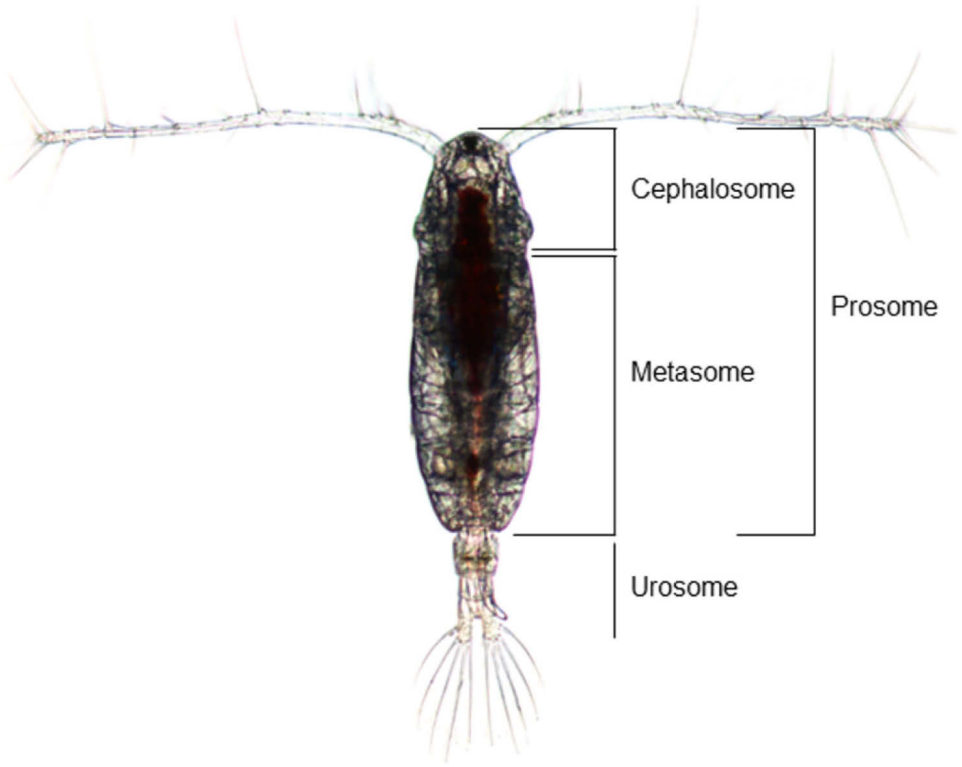

Fig. 3 ***Acartia tonsa*** **female** (after: https://alchetron.com/Acartia).

Physiological acclimatisation and genetic adaptation

The physiological performance of a species determines its tolerance to variations and shifts in the environment. As climate or other conditions change, organisms initially respond based on physiological acclimatisation and / or genetic adaptation shaped by their evolutionary history (Somero 2012; Kelly and Hofmann 2013). However, new environmental conditions might be physiologically intolerable, promoting migration, change phenology, or even result in the death and local extinction if genetic adaptation is not possible (Parmesan 2006). Different to physiological acclimatisation, genetic adaptation only occurs at population level. If one genotype contributes more offspring to the next generation than others, this changes the mean population fitness. In other words, the superior genotype is positively selected, becomes more abundant in the population with time, and might displace other genotypes after several generations. Thus, evolution is a process where natural selection results in an increased population fitness. Physiological acclimatisation on the other hand describes processes in which individuals adjust to different

environmental conditions (Garland and Kelly 2006). Such phenotypic plasticity is usually defined as the ability of one genotype to produce different phenotypes when exposed to various environments (Scheiner 1993) Thereby, the range of phenotypes that a given genotype can produce represent its reaction norm (Garland and Kelly 2006). Different to phenotypic plasticity (the extent to which the phenotype is changed in dependency of the environment), phenotypic buffering describes how broad the spectrum of environmental conditions is under which a genotype can maintain its fitness (Reusch 2014). Both, phenotypic plasticity and buffering can alleviate the impacts of environmental changes and help populations to persist in the face of climate change (Chevin et al. 2010). Individual phenotypic plasticity in the handling of acidified conditions could reduce directional selection and may enable populations to persist in the course of environmental change without a loss of genetic diversity (Fig. 4). Additionally, phenotypic plasticity could be itself a trait subject to natural selection, and such selection might also act to decrease selection pressure of OA.

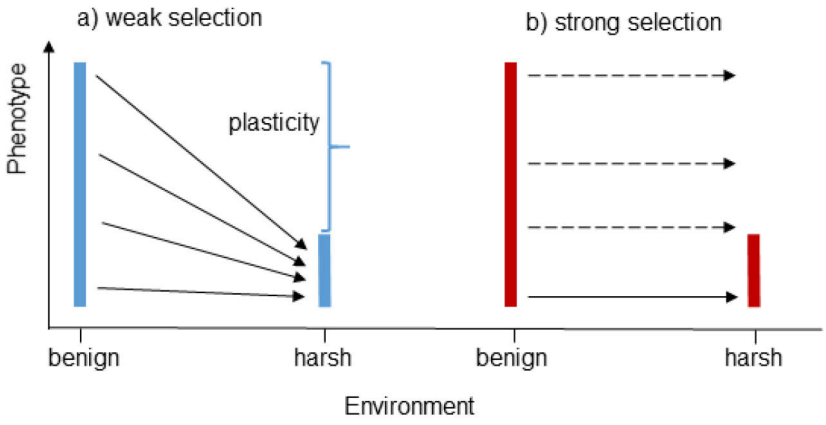

Fig. 4 Phenotypic plasticity might weaken selective pressure during environmental change. a) Individuals from the blue population can change their phenotype so that all genetic information is retained during environmental changes. b) ¾ of the genetic diversity is lost in the red population due to lack of phenotypic plasticity (dashed horizontal arrows).

As an extension of phenotypic plasticity, transgenerational effects like epigenetic inheritance can even decouple phenotypic from genetic change over multiple generations and allow populations to respond to environmental changes even in the absence of genetic variation (Bonduriansky et al. 2012). By, for example, the

transmission of nutritional, somatic, cytoplasmatic or epigenetic material, parents can significantly affect the performance (survival and fitness) of their offspring (Bonduriansky and Day 2009, Marshall and Morgan 2011, Klironomos, Berg et al. 2013).

Even the smallest change of a single species will influence species' interactions within the food web regarding e.g. competition, grazing, predation and disease dynamics. In combination with invasion and extinction of species, these processes can alter whole community structures or even result in the development of novel ecosystems (Matthews et al. 2011; Doney et al. 2012). Therefore, it is tremendously important to consider the adaptation potential, including physiological acclimatisation and genetic adaptation, of species when investigating the effects of OA.

Investigating the effects of OA on marine plankton communities

Experimental approaches

Laboratory experiments with single species as well as bi- or tri-trophic approaches are well suited to investigate physiological thresholds and biological mechanisms. However, those experiments cannot answer the question how planktonic organisms respond in their natural environments. Under natural conditions, effects of OA, detected in laboratory studies, might be dampened or accelerated by the presence of competitive and synergistic trophic interactions, may causing extreme changes in the community structure and functioning of marine ecosystems (Rossoll et al. 2013). To predict how OA might impact and change future marine ecosystems, investigations on a community level including multiple species are needed (Riebesell and Gattuso 2014). Additionally, long exposure to high pCO_2 conditions (over several weeks) can either trigger negative effects on the survival of marine invertebrates (Langenbuch and Portner 2004; Shirayama and Thornton 2005; Kurihara and Ishimatsu 2008) or allow species to acclimatise or even adapt to the new environmental conditions (Munday et al. 2013; Sunday et al. 2014; Thor and Dupont 2015). Besides laboratory set-ups, where the effects of OA can be investigated under controlled conditions, long-term studies including various species over several generations should be performed, to get a profound knowledge of the effects of OA on marine ecosystems. Besides laboratory studies, mesocosm experiments are a suitable tool to study the

impacts of OA on marine ecosystems including natural marine communities at their usual habitats (Riebesell et al. 2008).

Community barcoding

Up to now detections of possible changes in the community composition of planktonic organisms were mainly based on optical (microscopic and flow cytometry) investigations, whereby the actual diversity of species might be underestimated, especially in organism groups with little morphological differences. Marine planktonic taxa (phyto-, bacterio- and zooplankton) are highly diverse and discrimination by eye at genus and species level is time consuming and requires high specialized taxonomic expertise. Additionally, there is a potentially high diversity of cryptic species as well as larval stages, which are not captured by morphological investigations.

The dramatic progress in molecular biological methods over the last ~ 60 years represent one of the most exciting stories of scientific development (Moss 2010). The drastic development started with the description of the DNA double helix (Franklin and Gosling 1953; Watson and Crick 1953) and was followed by the development of the polymerase chain reaction (Mullis et al. 1994) and DNA sequencing (Sanger et al. 1977) up until the modern high-throughput sequencing (HTS) technologies, that are available today. In recent years, 454 pyrosequencing and Illumina sequencing were the most frequently used next generation sequencing (NGS) techniques. Platforms like Illumina MiSeq enable the processing of a large number of samples in parallel. Around five million paired end sequence reads with a length of 300 base pairs (bp) can be obtained during a single run. Molecular biological techniques like community barcoding, which combines DNA barcoding and HTS, can provide accurate and high-resolution taxonomic data for species identification and provides unprecedented insights into the composition of communities (Pochon et al. 2013; Wood et al. 2013; Lejzerowicz et al. 2015; Zaiko et al. 2015).

However, there are general challenges associated with the use of community barcoding for ecosystem assessments. The success of those studies mainly depends on the quality and availability of reference sequences in molecular data bases, which are still incomplete. For a steady improvement of those databases good cooperation between taxonomists and molecular biologists is therefore crucial. Furthermore, sampled organisms have vastly different biomasses and occur in different amounts

within a sample, thus small or very scarce species might be overlooked due to a low number of resulting sequence reads (Carew et al. 2013). Another technical challenge is preferential annealing of universal primers in some species over others which might severely bias results (Elbrecht and Leese 2015). Furthermore, data processing in bioinformatic pipelines can be challenging, requiring specialized bioinformatic expertise at different stages during data processing, such as the assembly of paired-end reads, chimera removal, correction of sequencing errors, and the clustering of obtained sequences into operational taxonomic units (OTUs), which can subsequently be used for estimations of species diversity and composition. Furthermore, pipeline specifications depend on peculiarities of taxonomic markers, sequencing platform (e.g. Illumina) and chemistry, as well as experimental needs, such as requirements for sample multiplexing (sample identification based on unique sequence tags). Therefore, even existing pipelines must be adjusted to the applied experimental design and research question. Nevertheless, community barcoding rapidly gains importance in ecology including marine community assessments (Porco et al. 2013; Hirai et al. 2015; Lejzerowicz et al. 2015; Zaiko et al. 2015; Endo et al. 2016).

Estimations of the global biodiversity suggest that there are 0.7 - 1 million marine species, and that most undescribed taxa are small or very small, meio- and microbiota (Appeltans et al. 2012). During the two and a half year TARA expedition, high quality and standardised genetic (total DNA / RNA), morphological, and physico-chemical samples from 210 stations across the world oceans were collected. The molecular biological analysis of 334 plankton samples collected during the TARA expedition revealed that most of the eukaryotic plankton biodiversity is made up by heterotrophic protists, especially those known to be parasites or symbiotic hosts (de Vargas et al. 2015). Based on the genetic data it was even possible to identify interactions between grazers, primary producers, viruses, and (mainly parasitic) symbionts (Lima-Mendez et al. 2015). As parasites and mutualistic symbionts increase the connectivity and complexity of pelagic food webs they significantly contribute to its stability (Lafferty et al. 2006). Therefore, to allow a comprehensive understanding of the impacts of e.g. OA on marine biocoenoses, heterotrophic protists, like parasites should more intensively be considered in ecosystem analyses.

Research aims & chapter outline

The ongoing acidification process of the oceans is predicted to have consequences for many marine biota and could potentially alter marine plankton communities and thereby ecosystem functions. As an additional environmental stressor, like ocean warming and eutrophication, parasitism may enhance or mitigate the effects of OA on marine planktonic organisms and should be particularly considered in ecosystem analyses. To be able to detect already little, pCO_2 induced, changes in the composition of species, plankton communities need to be recorded as accurately as possible. Morphological assessments of marine communities are very labour-intensive and require high taxonomic expertise. Especially, the identification of marine parasites based on morphological characteristics is difficult, as some species are even only visible after dissecting their host. Furthermore, small pico-, nano- and microplankton organisms can easily be overlooked and a clear taxonomic determination of larval stages and cryptic species is not possible. Prior studies (Pochon et al. 2013; Wood et al. 2013; Lejzerowicz et al. 2015; Zaiko et al. 2015) have shown that, the combination of DNA barcoding and next generation sequencing (community barcoding) can provide accurate and high-resolution taxonomic data.

The main objectives of this thesis were:

- to advance the molecular biological detection of marine oomycetes,
- to investigate the effects of simulated OA on the composition of a coastal plankton community during a near natural mesocosm experiment using community barcoding,
- to determine potential direct and indirect effects of elevated pCO_2 on two zooplankton species: *Oxyrrhis marina* and *Acartia tonsa*,
- to examine the ability of the calanoid copepod *A. tonsa* to acclimatise and adapt to the combined effects of elevated pCO_2 and an entailed food quality reduction.

CHAPTER I

Assessment of marine oomycetes using community barcoding

Saprophytic living oomycetes play an important role in the decomposition and recycling of decaying matter. As oomycetes zoospores significantly contribute to the pool of heterotrophic flagellates in the plankton of streams, estuaries and marine habitats (Sime-Ngando et al. 2011) they serve as an important food source for zooplankton organisms (Buck et al. 2011). Parasitic living oomycetes are common in marine environments and well known to infect several planktonic (Drebes 1966; Sparrow 1969; Gotelli 1971) and benthic diatoms (Scholz et al. 2014; Scholz et al. 2016b) as well as macroalgal, seagrass species (Sekimoto et al. 2008a; Sekimoto et al. 2008b; Govers et al. 2016) and nematodes (Beakes et al. 2012). Diatom pathogens are potentially of great ecological importance as they might play a role in the breakdown of plankton blooms (Thines et al. 2015). As marine oomycetes are highly diverse and with morphological methods hard to classify they are, however, little studied so far. In CHAPTER I we used community barcoding, to identify marine oomycetes directly from a mixed plankton water sample. This preliminary study was carried out to verify, if the cytochrome-c-oxidase subunit II (*cox*2) barcode primers, introduced by Choi et al. (2015) as to be well suited for phylogenetic studies of oomycetes, can also be used on HTS platforms. In the subsequent mesocosm experiment, the tested *cox*2 primers should than be used in a community barcoding approach to investigate the potential effects of OA on the diversity of marine oomycetes.

Publication

Langer JAF, Sharma R, Nam B, Beorsma M, Schwenk K, Thines M **Marine oomycetes: community barcoding results from an early winter plankton bloom (2012) in the Brudenell River (Prince Edward Island).** *Mycological progress* DOI: in review

The sample was taken by participants of the Prince Edward Island Mussel Monitoring Program (Smith and Ramsay 2012) and send to the Biodiversity and Climate Research Centre, Germany. Bora Nam did the laboratory work and the Illumina MiSeq sequencing was carried out at Eurofins Genomics (Ebersberg, Germany). The bioinformatic data processing was performed by Rahul Sharma.

Further data processing, calculation of phytogenic trees, and writing were done by Julia Langer under the guidance of Marco Thines. All authors contributed to the final manuscript.

The Illumina sequencing of the investigated plankton sample, revealed six distinct phylotypes, reflecting mean oomycete diversity in a marine plankton sample. The implemented community barcoding pipeline provides a reliable application for the identification of marine oomycetes and would also be suitable for future barcoding programs.

CHAPTER II

Coastal plankton communities under elevated CO_2

In CHAPTER II, the results from the KOSMOS (Kiel Off-Shore Mesocosms for Future Ocean Simulations; Fig. 5) 2013 large-scale mesocosm study in the Gullmar Fjord, Skagerrak (58° 15' 9 N, 11° 28' 7 E), are presented. In the long-term mesocosm experiment, the response of different trophic levels to elevated pCO_2 concentrations during a winter-to-summer plankton succession was investigated over 113 days. Thereby, we focused on the assessment of the diversity of a broad spectrum of the biocoenosis including all trophic levels (producers, consumers, decomposers). In prior experiments the assessment of possible changes in the community composition of planktonic organisms, due to the elevated pCO_2 conditions, were mainly based on optical (microscopic and flow cytometry) investigations. Thereby, the actual diversity of species may be underestimated, especially in organism groups with small morphological differences. In CHAPTER II we used community barcoding to investigate potential pCO_2 induced shifts in the plankton community compositions. We choose a molecular genetic approach as we aimed for a possibly high resolution of the present plankton community, even including genetic changes within species.

Publication

Langer JAF, Sharma R, Schmidt SI, Bahrdt S, Horn HG, Algueró-Muñiz M, Nam B, Achterberg EP., Riebesell U, Boersma M, Thines M, Schwenk K (2017) **Community barcoding reveals little effect of ocean acidification on the composition of coastal plankton communities: evidence from a long-term mesocosm study in the Gullmar Fjord, Skagerrak.** *PloS one* DOI: 10.1371/journal.pone.0175808

This study was performed in the framework of a mesocosm experiment carried out under the project administration of Ulf Riebesell. Sampling on side was accomplished by Julia Langer, Henriette Horn and María Algueró-Muñiz. DNA isolations and PCRs were carried out by Julia Langer and supported by Sebastian Bahrdt. Barcode PCRs were done by Bora Nam. The Illumina MiSeq sequencing was performed at Eurofins Genomics (Ebersberg, Germany) and the bioinformatic data processing was done by Rahul Sharma under the guidance of Marco Thines.

Environmental data were kindly provided by Henriette Horn, María Algueró-Muñiz, Eric Achterberg, Lennart Bach and Leif Anderson. Development of the statistical analyses was done in cooperation with Susanne Schmidt. Data evaluation and manuscript writing was carried out by Julia Langer under the guidance of Klaus Schwenk and Maarten Boersma. All authors contributed to the final manuscript.

Based on the community barcoding data elevated CO_2 had no significant effect on the relative abundance or composition of coastal planktonic taxa in the mesocosms. Observed shifts in the planktonic community composition were mainly related to seasonal changes in temperature and nutrients. Despite the successful pilot study based on the plankton sample from the Brudenell River, unresolved problems occurred during the data processing of the *cox*2 sequences, which made an evaluation impossible. Hence, those data were not considered in the corresponding publication.

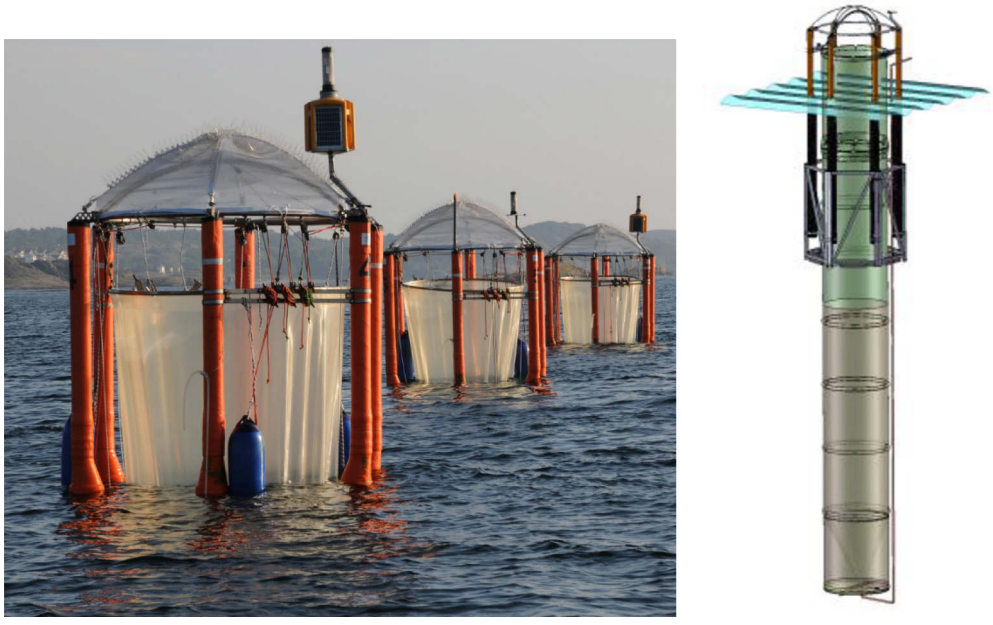

Fig. 5 KOSMOS outdoor mesocosms in the Gullmar Fjord, Sweden, and a schematic drawing including the underwater part (Photo: Maike Nicolai; drawing: Riebesell et al. (2013)).

CHAPTER III

Direct and indirect effects of pCO_2 on zooplankton

OA may affect marine organisms either directly due to changes in seawater pH or indirectly via trophic pathways. In CHAPTER III, the effects of elevated pCO_2 on zooplankton (micro- and mesozooplankton) were investigated in more detail using a laboratory set-up with two model organisms. Thereby, the laboratory experiment was especially designed to discriminate between direct OA effects due to an increased water pH and indirect effects caused by food quality changes. During the experiment the individual effects of short-term exposure to high and low seawater pCO_2, and different qualities of the alga *Rhodomonas salina* on the growth and development of two zooplankton species: *O. marina* and *A. tonsa*, were investigated. Although microzooplankton are an essential component in planktonic ecosystems, this is the first study directly investigating the sensitivity of a microzooplankton species to hypercapnia.

Publication

Meunier CL, Algueró-Muñiz M, Horn HG, Lange JAF, Boersma M (2016) **Direct and indirect effects of near-future pCO_2 levels on zooplankton dynamics.** *Marine & Freshwater Research* DOI: 10.1071/MF15296

The experiment was setup and carried out by Julia Langer, Henriette Horn, Maria Algueró-Muñiz and Cedric Meunier. The determination of the developmental rate of *A. tonsa* was done by Julia Langer. All other laboratory analyses were carried out in equal parts by the authors. Final data evaluation and preparation of the first draft was done by Cedric Meunier. Julia Langer, Henriette Horn and Maria Algueró-Muñiz equally contributed to the final manuscript.

Results show that consumers are hardly directly affected by a lowered seawater pH. However, stoichiometric discrepancies between the primary producers, cultured under high pCO_2 conditions, and the needs of the consumers, resulted in a significant reduced growth and developmental rate of the micro- and mesozooplankton species, respectively. Lower growth rates of zooplankton can lead to lack of food at the next trophic level, which in turn may reduce the fitness of these consumers. Furthermore, stoichiometric discrepancies can travel up the food chain and affect higher trophic levels.

CHAPTER IV

Long-term selection experiment

Although evolutionary responses are expected during persisting environmental change, most studies only investigated the effects on one generation. Little is known about the evolutionary potential of populations to counter direct or indirect effects of elevated pCO_2. In CHAPTER IV, this study aimed to investigate the impacts of elevated pCO_2 on the fitness of the cosmopolitan calanoid copepod *A. tonsa* and its adaptability to the combined effects of elevated seawater pCO_2 and an associated food quality reduction.

Publication

Langer JAF, Meunier CL, Ecker U, Horn HG, Schwenk K and Boersma M (2018) **On the evolutionary potential of the calanoid copepod *Acartia tonsa* towards ocean acidification: insights from a long-term laboratory selection study.** *Marine Ecology Progress Series* DOI: in review

The long-term selection experiment was setup by Julia Langer. Further maintenance was performed by Julia Langer, Toshia Schmithüsen, Saskia Ohse, Ursula Ecker, Maria Algueró-Muñiz, Rebekka Schüller, Joon Sudhir Kumar, Julia Haafke, Thomas Lesniowski and the students Anna Sophie Brandes, Gerald Leichtweiß and Svenja Schliefke (volunteers doing an ecological year). The Transplant experiments in 2014 and 2015 were carried out by Julia Langer. In 2016 the transplant experiment was performed by Cedric Meunier and in 2017 by Julia Haafke. Laboratory work and analyses of sequencing data were done by Julia Langer. Statistical analyses, evaluation and writing was performed by Julia Langer under the guidance of Maarten Boersma and Klaus Schwenk. All authors contributed to the final manuscript.

The elevated seawater pCO_2 concentration in combination with the dietary conditions in the high CO_2 treatment significantly altered the stoichiometry of the copepods and reduced their developmental rates. Although *A. tonsa* showed a high plasticity level in the regulation of its body stoichiometry, we found evidence that the cultivation under elevated pCO_2 and the ingestion of high CO_2 algae, with a high energy content (high C : nutrient ratio), for several years, might lead to an increased lipid storage and reduced body length.

Contribution of the PhD candidate in percentage of the total work load
(up to 100% for each of the following categories):

Manuscript I

Experimental concept and design:	5%
Experimental work and/or acquisition of (experimental) data:	0%
Data analysis and interpretation:	70%
Preparation of Figures and Tables:	100%
Drafting of the manuscript:	95%

Manuscript II

Experimental concept and design:	70%
Experimental work and/or acquisition of (experimental) data:	80%
Data analysis and interpretation:	80%
Preparation of Figures and Tables:	100%
Drafting of the manuscript:	90%

Manuscript III

Experimental concept and design:	10%
Experimental work and/or acquisition of (experimental) data:	35%
Data analysis and interpretation:	25%
Preparation of Figures and Tables:	0%
Drafting of the manuscript:	3%

Manuscript IV

Experimental concept and design:	90%
Experimental work and/or acquisition of (experimental) data:	50%
Data analysis and interpretation:	95%
Preparation of Figures and Tables:	100%
Drafting of the manuscript:	90%

CHAPTER I

Marine oomycetes: community barcoding results from an early winter plankton bloom (2012) in the Brudenell River (Prince Edward Island)

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Submitted to:
Mycological Progress

Abstract

Marine oomycetes are highly diverse, globally distributed and play key roles in marine food webs as decomposers, food source, and parasites. As species identification based on morphological characteristics and molecular genetic analyses of specimens is labour-intensive, knowledge about oomycetes remains limited and is mostly restricted to pathogenic species of economically important crops. As a result, despite their potential importance in the world's ocean ecosystems, marine oomycetes are comparatively little studied. Here, we tested if the primer pair cox2F_Hud and cox2-RC4, which is already well established for phylogenetic investigations of oomycetes, can also be used on a high throughput sequencing platform in a community barcoding approach. The Illumina sequencing of the investigated plankton sample, taken in the Brudenell River, revealed six distinct oomycete phylotypes. The fact that the identified phylotypes could not unambiguously be assigned to available reference sequences from online databases reflects that marine oomycetes are still understudied. The community barcoding pipeline implemented here provides a fast and accurate tool for the identification of marine oomycetes and might also be suitable for future barcoding studies.

Introduction

Oomycetes are a diverse group of fungus-like eukaryotic microorganisms occurring in terrestrial, limnic and marine habitats worldwide. They are classified within the kingdom Straminipila together with diatoms, golden-brown and brown algae (Baldauf 2003). Oomycetes species are none-photosynthetic, and exhibit either a saprophytic or pathogenic lifestyle (Grenville-Briggs and van West 2005; Thines 2014). Saprotrophic oomycetes absorb their food from the dead organic substrates they colonise, thereby playing an important role in the decomposition and recycling of decaying matter. By the colonization of plant debris, they change the nutritional content of the substrate. Furthermore, host penetration and germination of the oomycetes increase the palatability of the organic material for detritivores (Marano et al. 2016). Besides, oomycete zoospores substantially contribute to the pool of heterotrophic flagellates in the plankton of marine habitats (Sime-Ngando et al. 2011). Thereby, zoospores serve as an additional source of essential compounds, such as polyunsaturated fatty acids (PUFAs) and sterols for zooplankton organisms (Marano et al. 2016).

As pathogens, oomycetes can invade living plants, insects, crustaceans, fish, other vertebrate animals, and various microorganisms. As such, they cause diseases in various plants and algae (Tyler 2009; Grenville-Briggs et al. 2011) or severe losses in aquaculture and fisheries. Representatives of the oomycetes are common in marine environments and well known to infect several planktonic (Drebes 1966; Sparrow 1969; Gotelli 1971; Hanic et al. 2009) and benthic diatoms (Scholz et al. 2014; Scholz et al. 2016b) as well as macroalgal, seagrass species (Sekimoto et al. 2008a; Sekimoto et al. 2008b; Govers et al. 2016), and nematodes (Beakes et al. 2012). Diatom pathogens are potentially of great ecological importance as they might play a role in the breakdown of plankton blooms (Thines et al. 2015). Although parasitism is a frequent strategy in most natural habitats, infectious disease agents were only recently considered in food web analyses (Lafferty et al. 2008). Thompson et al. (2005) showed that parasites have the potential to uniquely alter marine food web topology, especially in terms of chain length and the proportions of top, intermediate and basal species. Furthermore, parasites and pathogens might also influence food-web stability and energy flow and should therefore receive more attention in future investigations of marine food webs.

As the most basal lineages of the oomycota are predominantly marine parasites (Beakes et al. 2012), oomycetes have most likely evolved in marine environments (Thines 2014). However, of the roughly 2000 reported oomycetes species so far, only about 60 have been described from marine environments (Nigrelli and Thines 2013). The identification of marine oomycetes using morphological methods, is labour-intensive and time-consuming. Furthermore, species determination by eye is challenging due a limited amount of available morphological characters (Bennett et al. 2017). Several studies using molecular biological methods, have already revealed that morphological investigations have largely underestimated the diversity of terrestrial, limnic and marine oomycetes (Bala et al. 2010; Hulvey et al. 2010; Man in 't Veld et al. 2011; Singer et al. 2016; Thines and Choi 2016). Furthermore, their impact on marine ecosystems and / or prevalence on marine biotas remain virtually unknown.

Community barcoding (DNA barcoding in combination with high throughput sequencing (HTS)) could provide a fast and accurate method to identify marine oomycete species with high taxonomic resolution directly from a mixed plankton water sample. In the TARA project, this technique was already successfully used to explore patterns of the global photic-zone eukaryotic plankton biodiversity, including oomycota (de Vargas et al. 2015). Also utilizing community barcoding Singer et al. (2016) detected highly diverse oomycete communities in peat bog micro-habitats. However, both studies were based on the analysis of the V9 region of the nuclear gene that encodes 18S rRNA, which on the one hand allows the detection of a broad range of eukaryotic taxa but on the other hand shows a poor resolution on the species level. The most common gene region currently used for the identification of oomycetes to the genus or species level is the internal transcribed spacer (ITS) region of rDNA (Robideau et al. 2011). However, due to the lack of functional constrains on this untranslated gene region, alignments of ITS sequences can be hampered by large amounts of insertions and deletions, which complicate species classification. Additionally, differences in alleles or among the multiple copies of the ITS, can even lead to the appearance of indels within a single oomycete strain, as observed by Kageyama et al. (2007). Furthermore, the resolution of ITS is insufficient to discriminate between species in some groups (Choi et al. 2015b).

Based on the study of Choi et al. (2015) the *cox*2 locus otherwise seems to be an appropriate gene region to serve as a universal barcode for the investigation of

oomycetes, according to the PCR efficiency and in terms of the intra- and interspecific divergence. Furthermore, the *cox*2 locus was already widely used in phylogenetic studies of various oomycete species, wherefore the amount of available reference sequences, especially for terrestrial species, is accordingly high (Choi et al. (2015) and references therein). The aim of this study was to test if the *cox*2 barcode primers which were applied in the study of Choi et al. (2015) are also suitable for HTS platforms and therefore for community barcoding of oomycetes.

Methods

The sample analysed in this study was taken in November 2012 in the Brudenell River, Prince Edward Island (PEI). Sampling took place within the framework of the PEI Mussel Monitoring Program (Smith and Ramsay 2012) which was carried out from September 4th to December 13th. For the sampling, an Apstein net (20 µm mesh size, 1 m long, Hydro-Bios) was drawn through the water for 100 m. The concentrated plankton sample was rinsed into a 100 mL bottle and filled up with 90% pure ethanol.

DNA isolation and PCR

DNA isolation was performed using the innuPREP Plant DNA Kit from Analytic Jena AG (Jena, Germany), following the instructions of the manual for gDNA isolation from plant material with Lysis Solution SLS. From the 100 mL bottle ~ 150 mg sample material were transferred in a 2 mL tube and homogenized for 5 min at 25 Hz with the Mixer Mill MM 200 from Retsch (Germany). Deferred to the Kit manual, lysis was carried out for 60 min and for the final DNA elution we used 50 µl elution buffer. Until further use, the isolated DNA and the remaining part of the plankton sample were stored at -20 °C and -80°C, respectively.

For the community barcoding the gene region *cox*2 was amplified using the primer pair cox2F_Hud (5`- GGCAAATGGGTTTTCAAGATCC - 3`) / cox2-RC4 (5`- ATTWAYNCCACAAATTTCRCTACATTG - 3`) (Hudspeth et al. 2003; Choi et al. 2015). Amplification was carried out in a total volume of 25 µL using ~ 10 ng DNA. The reaction mix contained 1 x Phusion HF buffer, 0.2 mM dNTPs, 0.5 µM each primer and 0.02 units Phusion polymerase. PCR conditions were 2 min at 95 °C, followed by 35 cycles of 20 s at 95 °C, 30 s at 50 °C, and 1 min at 72 °C, and a final

elongation for 6 min at 72 °C. 228 ng μl^{-1} PCR product was send to Eurofins Genomics (Ebersberg, Germany) for HTS on an Illumina MiSeq platform using the paired end (2 x 300 bp) option. Sequence reads are deposited in the European Nucleotide Archive (PRJEB25385).

Illumina data processing

The Illumina adapter and primer sequences of the raw sequence pairs were trimmed using the Trimmomatic (Bolger et al. 2014) software. Quality filtration was performed using a window size of 5 bp with an average phred quality score of 25 and a read length cutoff of 220 bp. Furthermore, sequences including ambiguous bases (Ns) were deleted and sequences were again filtered by keeping a minimum quality phred score of 3 per base using the FastQFS tool (Sharma and Thines 2015). Afterwards, related forward and reverse reads were concatenated. Then the primer sequences were trimmed and all reads were oriented in 5` - 3` direction. The prediction of operational taxonomical units (OTUs) was carried out with the USEARCH v7 software (Edgar 2010). For this, both a de-novo and a reference-based chimera filters were applied. The minimum cluster size was three reads using an identity cutoff of 99%.

Phylogenetic analyses

To separate the oomycetes OTUs from other eukaryotic OTUs we firstly aligned the representative OTU sequences with the NCBI nucleotide database (nt) using the BLASTn (Altschul et al. 1990) algorithm and further analysed the result with MEGAN (Huson et al. 2007). All OTU sequences which were assigned to oomycetes were extracted and used for the subsequent phylogenetic analyses. For this, we generated a MUSCEL alignment with default settings (Edgar 2004) using MEGA 7.0.26 (Tamura et al. 2011), including the obtained oomycete OTUs and reference sequences from the NCBI database, focussing on marine samples. Sequences related to amplification artefacts (non-homologous amplification) were manually detected based on their lack of homology to the target regions and deleted from the set. Afterwards sequences were trimmed to the same length. The subsequent calculation and evaluation of the phylogenetic trees were also performed in MEGA 7.0.26. Minimum evolution (ME) inference was calculated with 1000 bootstrap replicates

using the Tamura-Nei substitution model and default settings. Maximum-likelihood (ML) inference was calculated, with 1000 bootstrap replicates using the Tamura-Nei substitution model with 4 Gamma categories.

Results

Illumina data processing

The Illumina MiSeq sequencing resulted in about nine million paired-end sequence reads. The raw reads were processed for Illumina adapter and primer sequences, and filtered in terms of their sequencing quality and sequenced read length. Around one half (50.9%) of the reads passed these data processing and filtering steps. Afterwards the oligomer and primer sequences were clipped off, and all sequences were oriented in 5' to 3' direction, which resulted in 39,041 sequences reads. In the final clustering step, we obtained 216 OTUs. (Table 1). In the MEGAN analysis 172 OTUs could be assigned to corresponding reference sequences encompassing Straminipila, Rhodophyta, Chlorophyta and Streptophyta. In total, 33 OTUs (19%) assigned to different oomycete lineages. After homology checking 3 OTUs resulting from off-target amplification were manually removed.

Table 1 Steps of the bioinformatic pipeline, including the programs used, decreasing sequence reads, and OTU numbers.

Pipeline step	Program	File	Reads	OTUs
Raw sequence data		forward	9,163,244	
		reverse	9,163,244	
1. Illumina adapter trimming	Trimmomatic	forward	4,662,906	
2. Quality filtering	FastQFS	reverse	4,662,906	
3. De-multiplexing				
4. Barcode and primer trimming		cox2	39,041	
5. Reorient reads 5' – 3'				
6. Similarity clustering (initial clusters)	USEARCH v7	cox2		689
7. OTU clusters with > 3 reads	USEARCH v7	cox2		216

Phylogenetic analyses

The final sequence alignment included 30 oomycete OTUs and 37 reference sequences of ~ 240 bp length. Both phylogenetic analyses, ME and ML, yielded highly similar topologies and we did not observe strongly supported inconsistencies. Hence, the bootstrap values from the ML inference were included in the Figure of the ME tree (Fig 1). In the phylogenetic analyses, the 30 oomycete OTUs clustered together in four distinct groups and two single OTUs, representing six different oomycete phylotypes. Two groups, comprising three and four OTUs each, were assigned to the basal oomycete orders Haliphthorales and Olpidiopsidales. The groups with 12 and nine OTUs were allocated in the Pythiaceae s.lat. and Peronosporaceae s.lat. family, respectively. The two single OTUs were assigned within the Saprolegniomycetes, next to the genera *Pythiopsis* and *Saprolegnia*.

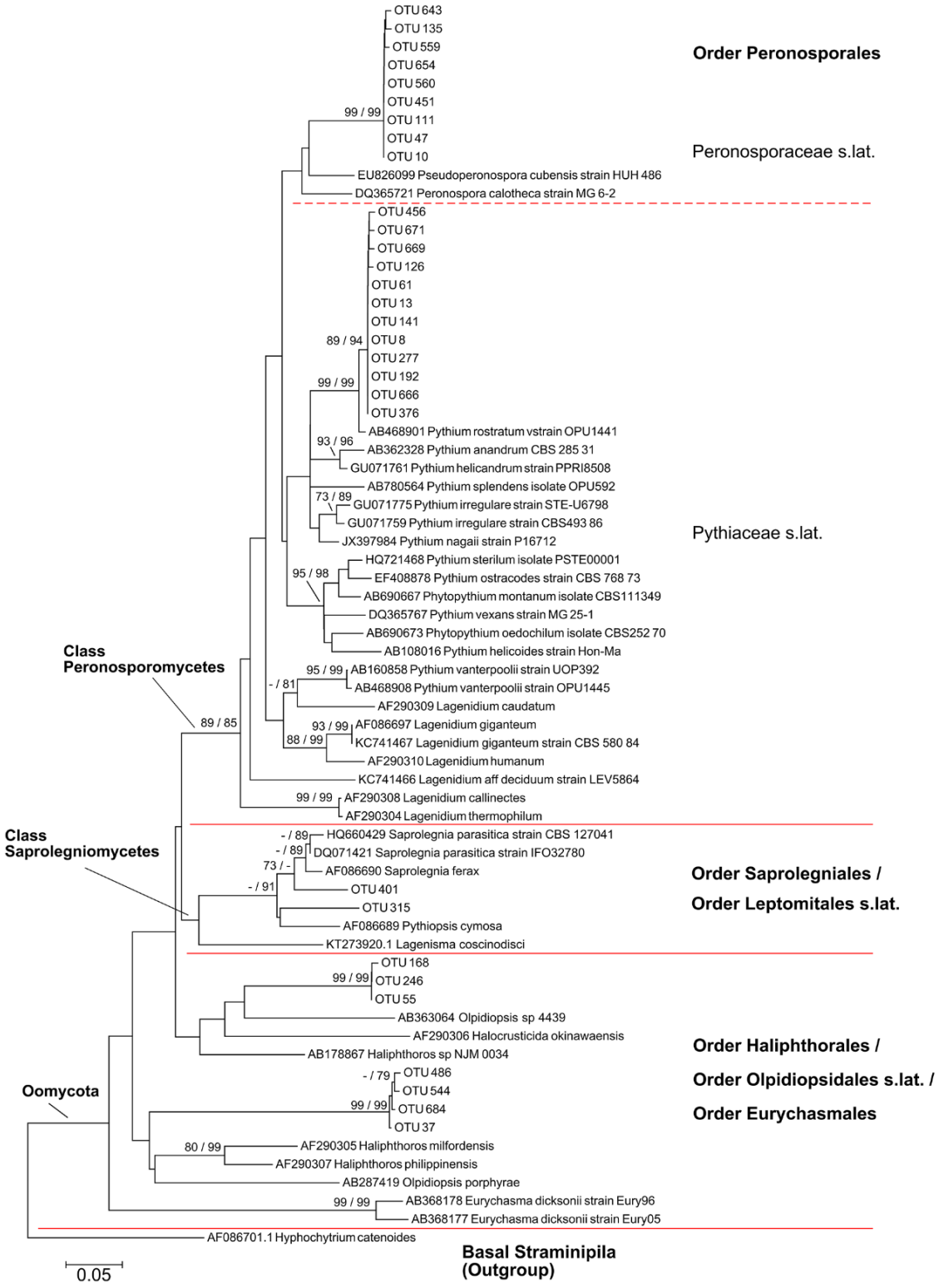

Fig. 1 Minimum Evolution phylogenetic tree inferred with MEGA 7.0.26 from the *cox*2 gene region. Numbers on branches denote bootstrap values equal to or larger than 70% from 1000 replicates, from Minimum Evolution and Maximum Likelihood analyses, at the first and second position, respectively. Numbers next to the specific names of the reference sequences represent the NCBI accession numbers. The scale bar indicates the number of substitutions per site.

Discussion

Method

The analyses of the community barcoding data resulted in a total number of 216 OTUs. The filtering steps applied in the bioinformatic pipeline resulted in a high confident set of OTUs. During the MEGAN analyses 81% of the OTUs (172) could be assigned to reference sequences on the class level. The remaining OTUs could not be assigned, likely because corresponding *cox*2 reference sequences are still missing in the reference database.

At first glance, community barcoding seems to have revealed a rather low oomycete diversity (6 phylotypes) in the investigated plankton community of the Brudenell River. However, compared to the globally observed diversity of marine oomycetes in the TARA project, which encompasses 69 phylotypes from 334 size fractionated plankton samples (de Vargas et al. 2015), the magnitude of around six highly distinct phylotypes appears to represent a normal oomycetes diversity in a marine plankton sample. In conclusion, community barcoding using the *cox*2 gene region with the primer pair cox2F_Hud and cox2-RC4 seems to reliably reflect the oomycete diversity present at the sampling site.

Phylogenetic assignment

The phylum oomycota represents the largest group within the Straminipila, including marine, freshwater, and terrestrial species (Dick 2001b; Dick 2001a). The base of the oomycota is formed by four early-diverging orders: Eurychasmales, Haptoglossales, Olpidiopsidales s.lat., and Haliphthorales. Most of the early-diverging genera, are marine organisms that are mainly parasites of seaweeds, diatoms, nematodes and crustaceans (Thines and Kamoun 2010). Members of the later-diverging monophyletic classes Saprolegniomycetes and Peronosporomycetes mainly occur in freshwater and terrestrial ecosystems. The class Saprolegniomycetes is split into the orders Atkinsiellales, Leptomitales s. lat. and Saprolegniales. The Peronosporomycetes comprises the orders Rhipidiales, Albuginales, and Peronosporales s. lat. (Beakes et al. 2014).

In our phylogenetic analyses, the two largest OTU groups assigned to the order Peronosporales. Although the *cox*2 locus resolved the terminal nodes of the phylogenetic trees for the most parts, deeper nodes towards the backbone did not

show a high resolution. Therefore, the assignment of the two OTU groups to a specific genus could not be exactly determined in case of one OTU affiliated with the highly diverse downy mildews, while the other was a member of the genus *Pythium*, closely related to *P. rostratum*. The OTUs of the phylotype clustering with the downy mildews probably represent a terrestrial species, with conidia having been blown into the water by wind. One known and abundant terrestrial oomycete on the PEI is *Peronospora cakiles* (Savile 1957) a parasites of the plant *Cakile endentula* (Bigelow) Hook. (Maun et al. 1990) growing in salty places like coasts, dunes and salt pans. Unfortunately, there is no *cox*2 reference sequence available for *P. cakiles,* yet. Two of the six phylotypes (represented by one OTU, each) were located within the Saprolegniaceae, with unclear genus affinity. The two remaining phylotypes were assigned to the basal oomycetes, one to the order Olpidiopsidales s.lat. and the other showing some affinity to the Haliphthorales. Those early diverging orders are both marine and, without exception, parasites (Beakes et al. 2012). During the PEI Mussel Monitoring Program a dramatic decline in the population of the marine diatom *Pseudo-nitzschia* was observed. Hanic et al. (2009) identified one oomycete parasite infecting the diatom on-site, which was recently described as a new genus and species, *Miracula helgolandica* (Buaya et al. 2017). It seems possible that the phylotype with the long branch and affinities to *Haliphthoros* represents this species, but unfortunately, no *cox*2 sequence is available for this enigmatic species. The other early-diverging phylotype is affiliated with *Olpidiopsis* s.lat., a genus that also includes diatom parasites (Sparrow 1960). Recently, Buaya et al. (2017) have described *O. drebesii* from a marine diatom. However, also for this species, no *cox*2 sequence is available, yet, so that it remains unclear, if the OTUs associated with *Olpidiopsis* can be assigned to that species. However, with the steady growth of available sequences for the *cox*2 barcoding locus (Choi et al. 2015), and the fast pace at which new oomycetes from the marine environment are being described (Hulvey et al. 2010; Bennett et al. 2017; Bennett and Thines 2017) it seems likely that in the near future, a species-level assignment will be possible using *cox*2-based community sequencing.

Although marine oomycetes have attracted renewed interest during the last decade little is known about their significance in ecosystem functioning (Strittmatter et al. 2008). There are reports of oomycete infections in marine primary producers (Grahame 1976; Wetsteyn and Peperzak 1991; Hanic et al. 2009; Scholz et al. 2014)

however, it is unclear whether these play a role in the breakdown of phytoplankton blooms. Saprophytic and pathogenic oomycetes might have important impacts on marine ecosystem functioning including energy fluxes and overall stability (Lafferty et al. 2006; Wood et al. 2007). Besides, there is evidence that oomycetes are likely to play a significant and similarly nuanced role in the biology and abundance of their hosts (Strittmatter et al. 2008). Wilce et al. (1982) suggested that an *Eurychasma dicksonii* infection of the sessile filamentous brown alga *Pylaiella littoralis* might have a positive influence on its distribution range, as during the release of zoospores from the sporangia algal filaments are fragmented and further distributed. Recent investigations place the origin of oomycete-plant associations in the Devonian, or earlier (Taylor et al. 2006), when lycophytes, ferns, seed ferns and gymnosperms were hosts of early oomycete species (Selosse et al. 2015). Since then, oomycetes have shaped our biosphere and probably will also take on a major role in the future times of climate change. The technique presented here seems to be well suited for the detection of marine oomycetes in a mixed plankton sample. As a fast and reliable method for species identification it can lay the foundation for future studies, enhancing and enlarging our view of marine oomycetes and their influence on marine ecosystems.

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CHAPTER II

Community barcoding reveals little effect of ocean acidification on the composition of coastal plankton communities: evidence from a long-term mesocosm study in the Gullmar Fjord, Skagerrak

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Abstract

The acidification of the oceans could potentially alter marine plankton communities with consequences for ecosystem functioning. While several studies have investigated effects of ocean acidifications on communities using traditional methods, few have used genetic analyses. Here, we use community barcoding to assess the impact of ocean acidification on the composition of a coastal plankton community in a large scale, *in situ*, long-term mesocosm experiment. High-throughput sequencing resulted in the identification of a wide range of planktonic taxa (Alveolata, Cryptophyta, Haptophyceae, Fungi, Metazoa, Hydrozoa, Rhizaria, Straminipila, Chlorophyta). Analyses based on predicted operational taxonomical units as well as taxonomical compositions revealed no differences between communities in high CO_2 mesocosms (~ 760 µatm) and those exposed to present-day CO_2 conditions. Observed shifts in the planktonic community composition were mainly related to seasonal changes in temperature and nutrients. Furthermore, based on our investigations, the elevated CO_2 did not affect the intraspecific diversity of the calanoid copepod *Pseudocalanus acuspes*. However, accompanying studies found temporary effects of raised CO_2 on single taxa. Picoeukaryotic phytoplankton profited from high CO_2 during times of nutrient limitation, leading to higher abundances. Furthermore, towards the end of the experiment dinoflagellate abundance was significantly higher under elevated CO_2 conditions. However, those differences in taxa compilations between the CO_2 treatments could only be observed in a specific period of the experiment. Based on our genetic investigations, no compositional long-term shifts of the plankton communities exposed to elevated CO_2 conditions were observed. Thus, we conclude that the composition of planktonic communities, especially those in coastal areas, remain rather unaffected by increased CO_2.

Introduction

Enhanced atmospheric CO_2 concentrations result in an increase in surface ocean pCO_2 with a subsequent decrease in pH. Several studies have investigated the effects of ocean acidification (OA) on marine biota under laboratory and field conditions. Generally, OA has been shown to negatively affect survival, calcification, growth and reproduction of a range of organisms (Kroeker et al. 2010). However, there is significant variation among marine species in their sensitivity to elevated CO_2. Direct and indirect effects of OA on single species, which retard development (Schoo et al. 2013), reduce reproduction (Kurihara et al. 2004; Rossoll et al. 2012) and survival (Cripps et al. 2014b), may also alter community structures (Doney et al. 2012) and thereby impact the food web (Fabry et al. 2008; Peck et al. 2015). Investigations during large-scale mesocosm field studies in Espegrend (Bergen, Norway) and Kongsfjorden (Ny-Ålesund, Svalbard) showed an overall resilience of plankton communities (bacteria, phytoplankton, micro- and mesozooplankton) towards enhanced CO_2 concentrations, whereby observed biological responses were largely depending on temperature and nutrient availability (Riebesell et al. 2008; Aberle et al. 2013; Niehoff et al. 2013; Silyakova et al. 2013; Sperling et al. 2013).

Up to now possible changes in the community composition of planktonic organisms were mainly based on optical (microscopic and flow cytometry) investigations, where the actual diversity of species may be underestimated, especially in organism groups with little morphological differences. Marine planktonic taxa (phyto-, bacterio- and zooplankton) are highly diverse and the differentiation at genus and species level is time consuming and requires highly specialized taxonomic expertise. Additionally, there is a potentially high diversity of cryptic species as well as larval stages, which are not captured by morphological investigations. New molecular techniques show rapid advancements, with promising tools for species identification. Especially the combination of DNA barcoding and next generation sequencing (community barcoding) can provide accurate and high-resolution taxonomic data (Pochon et al. 2013; Wood et al. 2013; Lejzerowicz et al. 2015; Zaiko et al. 2015).

In the long-term mesocosm experiment in Kristineberg (west coast of Sweden), which was part of the BIOACID II project, the response of different trophic levels to elevated CO_2 concentrations during a winter-to-summer plankton succession over 113 days was investigated (also see other publications within this collection). Within

that framework our present study focused on the assessment of the diversity of a broad spectrum of the biocoenosis including all trophic levels (producers, consumers, decomposers).

DNA barcoding in combination with high-throughput sequencing (HTS) was utilized to investigate potential CO_2 induced shifts in the plankton community compositions. We hypothesized that OA may induce taxonomic shifts in the planktonic community composition, although undetectable using morphological techniques, they might be revealed using genetic methods.

Methods

Experimental design

In Gullmar Fjord, Sweden (58° 15' 9 N, 11° 28' 7 E) ten pelagic mesocosms reaching to 19 m water depth were deployed. All of them enclosed roughly 50 m^3 of seawater and contained all organisms present in the fjord which were < 1 mm at the time the mesocosms were closed (note, however, that larger species were included in the study later on). Organisms grew under *in situ* temperature and close to *in situ* light levels. Five of the mesocosms were manipulated with CO_2 enriched water (~ 760 µatm), the others remained unchanged to serve as controls (~ 380 µatm). Due to net outgassing, the CO_2 concentration decreased in the high CO_2 mesocosms and needed to be re-adjusted repeatedly. However, the ambient and high CO_2 treatment remained different throughout the experiment (Fig 1). As some taxa appearing later in the seasonal succession might not have been present in the water column when the mesocosms were closed, we added 22 L of water from the fjord every fourth day to each mesocosm. Additional to smaller plankton, we also added herring eggs (*Clupea harengus*) and green sea-urchin gastrula stages (*Strongylocentrotus droebrachiensis*) to the mesocosms. Adult herring, caught in the Oslo fjord were taken over from a local fisherman. In the laboratory eggs from the dead herrings were stripped off and fertilized. Afterwards, eggs were stuck on plastic plates and placed in the middle of each mesocosm at 3 m depth from day 48 until peak hatching on day 63. Around day 71 after they reached the yolk-sac stage herring larvae presumably fed on copepod nauplii and ciliates. With growing size, they switched to lager prey. The species used in this study is not endangered and animal welfare was assured according to the ethical permission (number 332-2012). Sacrificed specimens were anaesthetized in

advance with MS-222, to reduce stress to a minimum (for further details see Sswat et al. (2018)). Green sea-urchin larvae were cultured in the laboratory following Dorey et al. (Dorey et al. 2013). When they reached the swimming gastrula stage they were gently added to each mesocosm on day 56 (for further details see Dupont et al., PloS collection). CTD (Conductivity-Temperature-Depth) casts were taken every second day between 11 am and 3 pm to monitor *inter alia* the development of salinity, temperature and pH in the mesocosms and fjord. Depth-integrated water samples were taken every second day using an Integrating Water Sampler (IWS, Hydro-Bios, Kiel, Germany) which sampled a total volume of 5 L evenly distributed over the depth range from 0 - 18 m. From this water among other properties, concentrations of nitrate (NO_3^-), nitrite (NO_2^-), dissolved silicate (SiO_4^{3-}), ammonium (NH_4^+), and phosphate (PO_4^{3-}) were measured using standard auto-analyser (Seal Analytical QuAAtro) techniques according to Murphy and Riley (1962) (Murphy and Riley 1962), Hansen and Grasshoff (1983) (Hansen 2007) and Holmes et al. (1999) (Holmes et al. 1999), in addition to nanomolar nutrient techniques (Patey et al. 2008) for periods with depleted nutrient levels. In addition, pigment analyses (Bach et al. 2016) and microzooplankton microscopy (Horn et al. 2016) were performed. Mesozooplankton samples were taken with an Apstein net (55 µm mesh size, 1 m long, Hydro-Bios) from 17 m depth to the top of each mesocosm (Algueró-Muñiz et al. 2017a). The chlorophyll *a* concentrations measured via pigment analysis indicated two bloom events which occurred simultaneously in the ten mesocosms (Fig 1). Further details on mesocosm setup and performance as well as CO_2 manipulation can be found in the overview paper of the PloS collection (Bach et al. 2016).

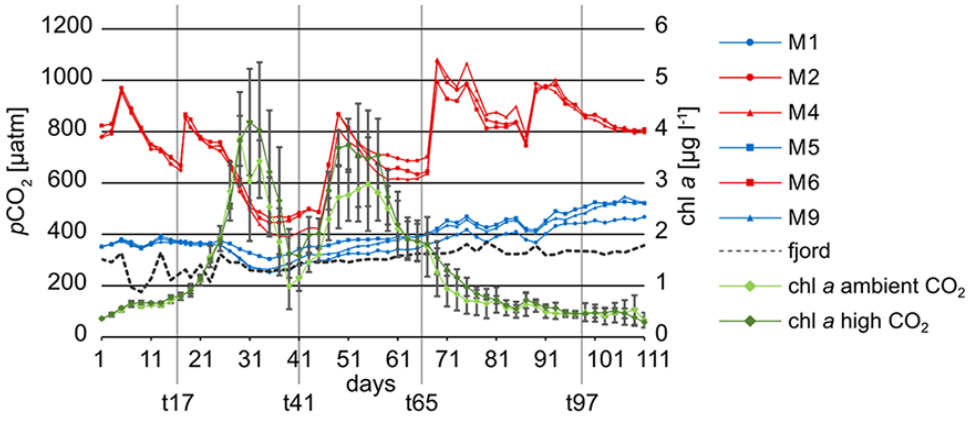

Fig. 1 pCO_2 and chlorophyll *a* concentration in mesocosms over the experimental period. High CO_2 mesocosms (red lines); ambient CO_2 (blue lines); fjord (black dashed line); mean chlorophyll *a* concentration in the selected ambient CO_2 mesocosms (light green line); mean chlorophyll *a* concentration in the selected high CO_2 mesocosms (dark green line); x axis: experimental days; left y axis: pCO_2 concentrations; right y axis: mean chlorophyll *a* concentrations. Data pooled from three ambient and three high CO_2 mesocosms, error bars = SD.

Plankton samples were taken every 8th day from each mesocosm plus the surrounding fjord. For the mesoplankton (> 200 µm) samples the content of the mesozooplankton net hauls was prescreened with a 200 µm sieve and fixed with 90% pure ethanol in 100 mL bottles. To sample small-sized plankton including pico-, nano- and microplankton (> 0.45 µm and < 200 µm), 500 mL of water sampled with the IWS was sieved with a 200 µm mesh to remove large phyto- and zooplankton. The sieved water was subsequently filtered onto a nylon filter (0.45 µm pore size, 25 mm diameter, Whatman®) and fixed with 99.9% pure ethanol in 2 mL tubes.

For the community barcoding, filter and net samples from three randomly selected ambient (M1, M5, M9) and high (M2, M4, M6) CO_2 mesocosms, and the fjord, from four time points (t17, t41, t65, t97), were used (in total 28 samples). In this study we focused on long-term effects of OA on the composition of plankton communities, as sample size for the HTS was financially limited. Therefore, we selected the time points to be equally distributed over the experimental period, before and after the bloom events.

DNA isolation

For DNA isolation we used a modified version of a phenol/chloroform method (McKinney et al. 1995). To extract the DNA of the mesoplankton the sample bottles were gently homogenized, 2 mL sample were transferred into a 2 mL tube and ethanol was removed with a pipette. This procedure was repeated until 0.25 µL (maximum number of organisms per isolation tube) of the tube was filled with organisms. In total, 10% (10 mL) of each mesoplankton net sample was used for DNA isolation. Samples were dried at 40°C to completely remove the ethanol. Nylon filter carrying pico-, nano- and microplankton were placed in a petri dish, cut in small pieces and dried at 40°C. The filter pieces were put in 2 mL tubes with three metal beads (3 mm) and frozen overnight at -80°C. The frozen filter pieces were tissue-lysed once for 50 s at a frequency of 20 Hz (TissueLyser II, Qiagen) to crush phytoplankton cells. For cell lysis 800 µL lyse buffer (50 mM Tris-HCl pH 8, 200 mM NaCl, 0.2 mM EDTA, 0.5% SDS, 0.1 mg mL^{-1} proteinase K, 0.25 mg mL^{-1} glycogen) was added. Subsequently samples were vortexed, centrifuged and incubated at 37°C overnight with gentle mixing. Then 4 µL RNase (100 mg mL^{-1}) was added followed by another incubation at 37°C for 15 min. The buffer and RNase volume was doubled for the mesoplankton samples. Afterwards the lysate was transferred into new 2 mL tubes whereby the mesoplankton solution was divided into two tubes. Filter and organism leftovers were not transferred. Then 804 µL phenol/chloroform/isoamyl alcohol (25:24:1) were added. These samples were vortexed and centrifuged at 13000 g for two min. Subsequently the liquid supernatant was pipetted into a new 2 mL tube followed by another addition of 4 µL RNase and an incubation at 37 °C for 15 min with gentle mixing. Then the phenol/chloroform addition was repeated and the liquid supernatant was again transferred into new 2 mL tubes (maximum 400 µL). Afterwards, 36 µL sodium acetate solution (3 M) and 900 µL pure ethanol (98%) were added, the samples were vortexed and centrifuged at 8000 g for 10 min. The liquid above the DNA pellets was removed and pellets from the same sample were pooled and washed twice with 500 µL pure ethanol (70%). Then the pellets were dried at 40 °C and resuspended in 100 µL 1x TE buffer (10 mM Tris, 1 mM EDTA). Until further use the isolated DNA was stored at -20 °C.

Polymerase chain reaction (PCR)

We analysed a part (V4 and V5) of the small subunit rDNA (18S) to cover a potentially wide range of marine planktonic taxa in the community barcoding (Hadziavdic et al. 2014). Furthermore, we sequenced the more variable gene region cytochrome-c-oxidase subunit I (*cox*1) to achieve a sufficient resolution for species identification. The gene regions 18S and *cox*1 were amplified using the primer pairs 554f (5`-AAGTCTGGTGCCAGCAGCCGC-3`) / 1282r (5`-TCACTCCACCAACTAAGAAGGGC-3`) and LCO1490_t1 (5`-TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGA-3`) / HCO2198_t1 (5`- CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAA-3`), respectively (Messing 1983; Folmer et al. 1994; Huys et al. 2006). Amplification was carried out in a total volume of 20 µL (*cox*1) and 25 µL (18S) using ~ 10 ng DNA. The reaction mix contained 2.5 (*cox*1), 1.25 (18S), mM $MgCl_2$, 1 x PCR-buffer, 0.2 (*cox*1), 0.25 (18S) mM dNTPs, 0.2 (*cox*1), 0.3 (18S), 0.4 µM each primer and 0.5 (*cox*1), 0.1 (18S) units TAQ polymerase. Additionally, 0.1 µg μL^{-1} BSA were added for the 18S PCRs. PCR conditions for *cox*1 were 2 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 45 °C, and 1 min 30 s at 72 °C, and a final elongation for 7 min at 72 °C. For 18S, the PCR conditions were 2 min at 95 °C, followed by 34 cycles of 30 s at 95 °C, 1 min at 56 ° C, and 1 min 30 s at 72 °C, and a final elongation for 10 min at 72 °C. PCR-products were purified using the ISOLATEII PCR and Gel Kit from Bioline. Clean PCR-products were used as templates for a barcode PCR in which the DNA fragments of each sample were tagged with a specific barcode to enable the identification after the multiplexed sequencing. Therefore, primers were labeled with tags of 6-7 bps. Amplifications were carried out in a total volume of 25 µL using ca. 10 ng PCR product as template. The reaction mix contained 1 x Phusion® High Fidelity buffer, 0.2 mM dNTP's, 1 µM each primer, and 0.02 units Phusion® High Fidelity polymerase. PCR conditions for *cox*1 were 2 min at 98 °C, followed by 27 cycles of 20 s at 98 °C, 30 s at 45 °C, and 1 min at 72 °C, and a final elongation for 6 min at 72 °C. For 18S the PCR conditions did not change - only the number of cycles was reduced to 27. Amplification success was 99% and was controlled with gel electrophoresis. PCR of the isolated DNA from the net sample of mesocosm six (t97) yielded no amplicon for the *cox*1 gene region.

After purification, 68.57 and 76.80 ng DNA $sample^{-1}$ from the 18S and *cox*1 PCR products were pooled, respectively. The amplicon pool was sequenced on an

Illumina MiSeq platform using the paired end (2 x 300 bp) option at Eurofins Genomics (Ebersberg, Germany). Sequence reads are deposited in the European Nucleotide Archive (PRJEB15126).

Prior to the preparations for the community barcoding we set up a reference database, containing 18S and *cox*1 sequences, obtained by standard Sanger sequencing, of single individuals of the most abundant taxa from the mesocosms. PCR conditions and primer were equal to those described above.

Illumina data processing

The Illumina adapter and primer sequences of the raw sequence pairs were trimmed by using the Trimmomatic (Bolger et al. 2014) software. Quality filtration was performed using a window size of 5 bp with an average phred quality score of 25 and a read length cutoff of 220 bp. Furthermore, sequences including ambiguous bases (Ns) were deleted and sequences were again filtered by keeping a minimum quality phred score of 3 per base using the FastQFS tool (Sharma and Thines 2015). Afterwards, based on the barcode sequence, each read was renamed after the corresponding sample ID and related forward and reverse reads were concatenated. Then the barcode and primer sequences were trimmed and all reads were oriented in 5` - 3` direction. The prediction of operational taxonomical units (OTUs) was carried out with the USEARCH v7 software (Edgar 2010). Thereby both a *de-novo* and a reference-based (reference sequences for each primer i.e., 18S and *cox1* were downloaded from the NCBI database) chimera filters were applied. The minimum cluster size was three reads with an identity cutoff of 99%. To further de-multiplex sequence data, the 18S and *cox1* reads were re-assembled with a minimum identity of 99% using the program Geneious® version 7.0.4 (Kearse et al. 2012). Consensus sequences of clustered OTU sequences were generated on the strength of base majority. Afterwards, sequences of new and not further assembled OTUs were aligned and trimmed to the same length. Sequences related to amplification artifacts were manually detected based on their lack of homology to the target regions and deleted from the set. The resulting set of high-confidence OTUs were mapped by the processed reads, only those OTUs were kept which had a mapping support of at least three reads. This filtered set of OTUs was then used for the community analysis and abundance tables were generated keeping only those OTUs which occurred in a minimum of 2 samples (S1 (18S), S2 (*cox*1)). OTU distribution among net and filter

samples were calculated in Microsoft Excel 2016. For species identification OTU representative sequences were aligned with the NCBI nucleotide database (nt) using the BLASTn (Altschul et al. 1990) algorithm. For this, OTUs were separated according to their sample ID. Those from the same time point and CO_2 treatment were processed together. Further taxonomical classification of the OTUs was carried out by using the MEGAN program (Huson et al. 2007). Thereby, we kept only those taxa for which the pairwise identity between the forward and/or reverse part of the assigned OTU sequences and the NCBI reference sequence was 100%. If an OTU assigned to different taxa with equal pairwise identities, we used the next higher taxonomical level. Resulting taxa and their number of assigned OTUs were put together in a table for further community composition analyses.

Statistics

Data analyses were performed using the vegan package in R software (Oksanen et al. 2016). For the statistical analyses we used two Hellinger transformed (Rao 1995) OTU datasets of the 18S gene region. The first data set consisted of the raw OTU abundance data, i.e. the number of sequence reads from the HTS. The second data set were the presence-absence-transformed OTU abundance data (as suggested by Borcard, Gillet (Borcard et al. 2011)). In contrast to the presence-absence-transformed data which only reflects the OTU composition, the number of HTS reads per OTU provide additional information on the relative abundance of species, since the amount of sequence reads is positively correlated with the available amount of genetic material. Although the amount of sequence reads per OTU does not reflect real biomasses or abundances of the planktonic taxa on-site, these values can serve as a proxy for relative abundances or biomasses of taxa and can be compared between mesocosms and/or time points. The data set consisting of raw sequence reads per OTU is referred to as "sequence read" data subsequently. Statistical analyses were performed with environmental data which were $log10(x + 1)$ transformed, to approximate normality, and standardized to bring all of the variables into proportion with one another (*decostand* with the option "*standardize"*). For all statistical analyses, a probability value of $p < 0.05$ was considered significant. To test for significant differences in the OTU composition and/or in the amount of assigned HTS reads per OTU between the size fractions (filter and net samples), permutational multivariate analyses of variance (*adonis*) were performed, based on the Bray-Curtis

distance matrix of the 18S presence-absence-transformed and sequence read data. To investigate whether there were significant differences in the OTU composition of the plankton samples between the ambient and high CO_2 treatment over the experimental period we performed an *adonis*, based on Bray-Curtis dissimilarity matrices of the 18S presence-absence-transformed and sequence read data. OTU composition changes within and between mesocosms and fjord system, were visualized in nonmetric multidimensional scaling (nMDS) ordinations.

The development of the environmental conditions over the experimental period was investigated with a principal component analysis (PCA). For the PCA we considered temperature, salinity and chlorophyll *a* as measured via pigment analysis using HPLC, as well as the concentrations of different nutrients (NO_3^- / NO_2^-, PO_4^{3-}, SiO_4^{3-}, NH_4^+) and the abundances of micro- and mesozooplankton. Here, these data were only included as covariates, detailed interpretations and discussions are presented elsewhere (Bach et al. 2016). In this study, counts of the most abundant microzooplankton group (ciliates) and the most abundant mesozooplankton species (all stages of *Pseudocalanus acuspes*) were considered in the corresponding taxa abundance variables. Chlorophyll *a* measurement in the fjord for day 41 was missing, therefore this data point was extrapolated by averaging the measurements from days 33, 35, 37, 39, 43, and 45. Due to a deviating sampling schedule, ciliate counts from day 103 instead of day 97 were used. Furthermore, biomass calculations of the microzooplankton, which included also different dinoflagellate species, were included in the PCA analysis.

Since absence represents a statistical information in presence-absence-transformed data sets, redundancy analyses (RDA) is preferred over canonical correspondence analysis (Dray et al. 2003). Additionally, for short gradients as found in the present study, where species abundance or frequency is a linear function, canonical correlation analysis (CCA) is an inappropriate model (Palmer 1993). Therefore, OTU compositions of the plankton (pico-, nano-, micro- and mesoplankton) in the mesocosms in dependence of environmental variables were investigated via RDA (Legendre and Legendre 2012) on the Hellinger-transformed sequence read and presence-absence-transformed data. The stepwise model builder based on permutation tests (*ordistep*) was used to determine the set of significant explanatory environmental variables for the final RDA.

As already mentioned for the sequence read data, the number of assigned OTUs to certain taxa, identified by the BLAST searches and MEGAN analyses, do also not necessarily reflect species abundances and/or biomasses on-site. However, they can be used to test whether the composition of the planktonic taxa was different between the CO_2 treatments and/or over the time. Therefore, we performed an *adonis* based on the number of assigned OTUs to certain taxa. To investigate potential differences in the diversity of the detected taxa between the high and ambient CO_2 treatment and/or over the experimental period, we calculated taxa diversity indices ($H' = -\sum_{i=l}^{R}(p_i \ln p_i) * (-1)$) (Shannon 1948), where p_i is the proportion of OTUs belonging to the i^{th} taxa in the subset. Furthermore, OTU evenness ($J` = \frac{H'}{\ln(S)}$) (Pielou 1966) (S = total number of detected taxa in the dataset), was calculated to detect imbalances in the number of assigned OTUs among taxa.

We additionally investigated whether elevated CO_2 affected the intraspecific genetic diversity of *Pseudocalanus* spp. (later identified as *P. acuspes*), which was the most abundant copepod throughout the experiment. Therefore, we performed an *adonis* based on presence-absence-transformed and sequence read data, which exclusively included OTUs from this species.

Results

Illumina data processing

The Illumina MiSeq sequencing resulted in about five million paired-end sequence reads. The raw reads were processed for Illumina adapter and primer sequences, and filtered in terms of their sequencing quality and sequenced read length. In total, 32.6% of the reads passed the data processing and filtering steps. This filtered set of reads was then further processed to assign sample information (18S and *cox*1) on the basis of their unique oligomer and primer sequence combinations. After categorizing the filtered reads into samples, the oligomer and primer sequences were clipped off, and all sequences were oriented in 5' to 3' direction. Using this sample assignment step 653,789 and 3,417 sequences were assigned as 18S and *cox*1 sequences, respectively. Assigned sequences were then clustered into OTUs, which resulted in 7,734 and 96 OTUs for 18S and *cox*1 samples, respectively. After the re-assembly, alignment clean up and mapping, the final 18S and *cox*1 abundance tables contained a high confident set of 740 (Table S1) and 31 (Table S2) OTUs, respectively. Steps

of the bioinformatics pipeline, including the programs used, and the numbers of obtained sequence reads and OTUs, can be found in the supplementary data (Table S3). Although the utilized *cox*1 primers were already successfully used for NGS metabarcoding (Raupach et al. 2015), here only an insufficient number of sequence reads was obtained. Thus, further analyses were based on the 18S OTU abundance table only. 18S OTUs originated mainly (40.8 ± 3.3%) from filter samples representing the small size fraction including pico-, nano- and microplankton. Only 11.2 ± 1.2% corresponded to the mesoplankton size fraction of the net samples. Furthermore, there was a substantial overlap (47.9 ± 2.1%) between both fractions.

Community composition and succession (OTUs)

The overall OTU composition between the size fractions was significantly different for the 18S OTU presence-absence-transformed and sequence read matrices respectively, although ~ 50% of the OTUs were present in both filter and net samples (*adonis*: $R^2_{0/1} = 0.30$, $p_{0/1} < 0.05$; $R^2_{reads} = 0.21$, $p_{reads} = 0.01$). Thus, filter and net samples were analysed separately in the following. Analyses based on the 18S OTU abundance tables revealed no significant differences in the OTU compositions between the ambient and high CO_2 treatments neither for the presence-absence-transformed ($R^2_{filter\_0/1} = 0.01$, $p_{filter\_0/1} = 0.96$; $R^2_{net\_0/1} = 0.04$, $p_{net\_0/1} = 0.27$) nor for the sequence read data ($R^2_{filter\_reads} = 0.012$, $p_{filter\_reads} = 0.924$; $R^2_{net\_reads} = 0.054$; $p_{net\_reads} = 0.091$), but a significant change occurred over time ($R^2_{filter\_0/1} = 0.29$, $R^2_{net\_0/1} = 0.28$; $R^2_{filter\_reads} = 0.309$, $R^2_{net\_reads} = 0.272$; $p < 0.001$). Especially at the first and second time point the mesocosms exhibited similar OTU compositions which can be seen also in the nMDS ordinations where they are situated close to each other (Fig 2). With progressing time, distances among mesocosms within the ordination increased, revealing differences in the OTU compositions between the enclosures at the end of the experiment. In the nMDS ordination mesocosms were however not separated according to their CO_2 treatment. The amount of sequence reads per OTU and the OTU composition of the 18S mesoplankton size fraction was significantly different between the mesocosm and fjord samples over the experimental period ($R^2_{net\_0/1} = 0.11$; $R^2_{net\_reads;}$ $p < 0.05$). However, no such differences were observed for the smaller plankton groups from the filter samples ($R^2_{filter\_0/1} = 0.08$, $p = 0.06$; $R^2_{filter\_reads} = 0.079$, $p_{filter\_reads} = 0.09$).

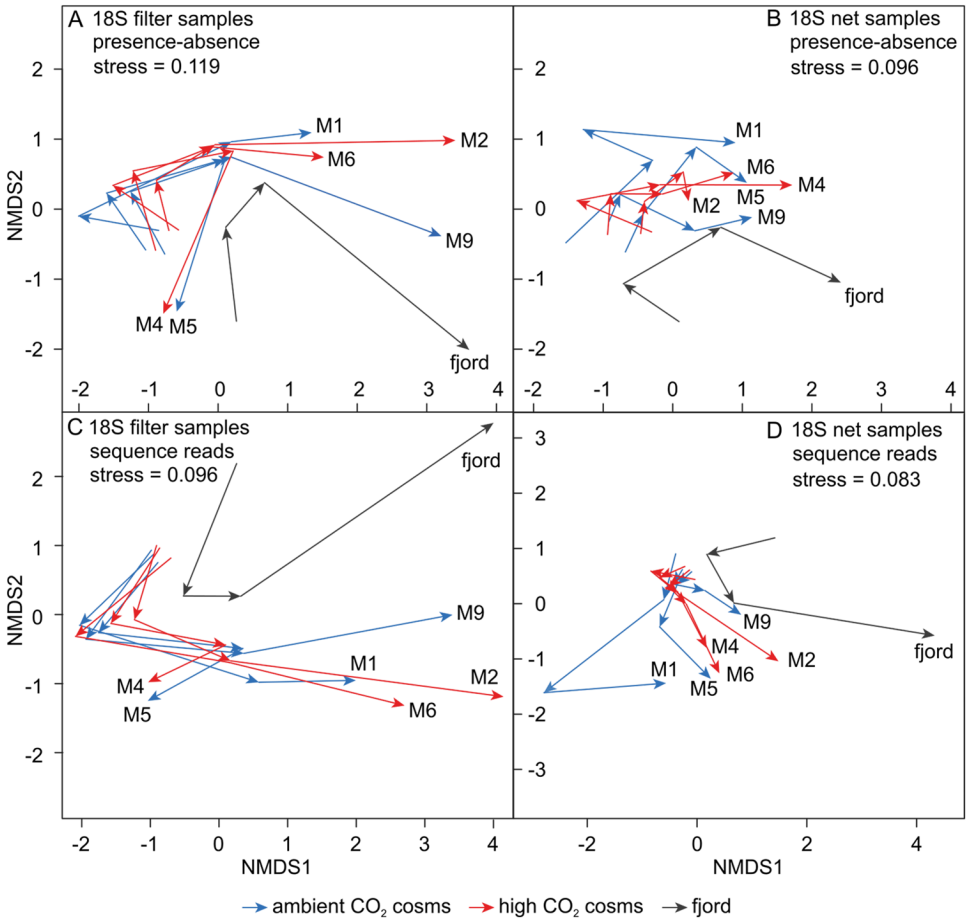

Fig. 2 nMDS, filter (left) and net samples (right), based on the 18S presence-absence-transformed (top row) and sequence read (bottom row) data. Mesocosms are indicated by their number (M1, M2, M4, M5, M6, M9); in each subplot, the beginning of the first arrow for each mesocosm represents the first time point (t17); the second (t41), third (t65) and fourth (t91) time points are represented by the subsequent arrowheads, respectively; ambient CO_2 mesocosms (blue arrows); high CO_2 mesocosms (red arrows); fjord (grey arrows).

Environmental conditions differed only slightly between the investigated mesocosms (M1, M2, M4, M5, M6, M9) but showed a significant temporal development (Fig 3). The first sampling point was characterized by high nutrient (NO_3^- / NO_2^-: 6.69 ± 0.12, PO_4^{3-}: 0.69 ± 0.00, SiO_4^3: 7.47 ± 0.04 [µmol L^{-1}]) concentrations. Then two phytoplankton blooms followed, represented by increased chlorophyll *a* values (t41: 1363.7 ± 409.4, t65: 1858.4 ± 379.6 [ng L^{-1}]), which were probably triggered by increasing water temperatures (t17: 2.19 ± 0.03, t41: 4.66 ± 0.01, t65: 8.11 ± 0.04 [°C]), resulting in the associated increase in micro- and mesozooplankton abundances (t65 microzooplankton: 6113 ± 2113, t65 mesozooplankton: 102 ± 18

[ind L^{-1}]) and a simultaneous decrease in nutrients. The end of the experiment was characterized by nutrient depletion (NO_3^- / NO_2^- and PO_4^{3-} concentrations were below the detection limit; SiO_4^3: 0.03 ± 0.05 [µmol L^{-1}]), maximum water temperature (t97: 14.28 ± 0.03 [°C]), decreased mesozooplankton abundance (t97: 11 ± 2 [ind L^{-1}]) and slightly increased microzooplankton biomass (t65: 33.9 ± 14.3 [µg C^{-1}]). Ammonium concentration in the mesocosms stayed rather low during the experiment (t17 to t97: 0.08 ± 0.07 [µmol L^{-1}]) and salinity increased slightly (t17: 29.22 ± 0.13, t97: 29.35 ± 0.11 [psu]). Values for all sampling days and mesocosms can be found in the overview paper (Bach et al. 2016).

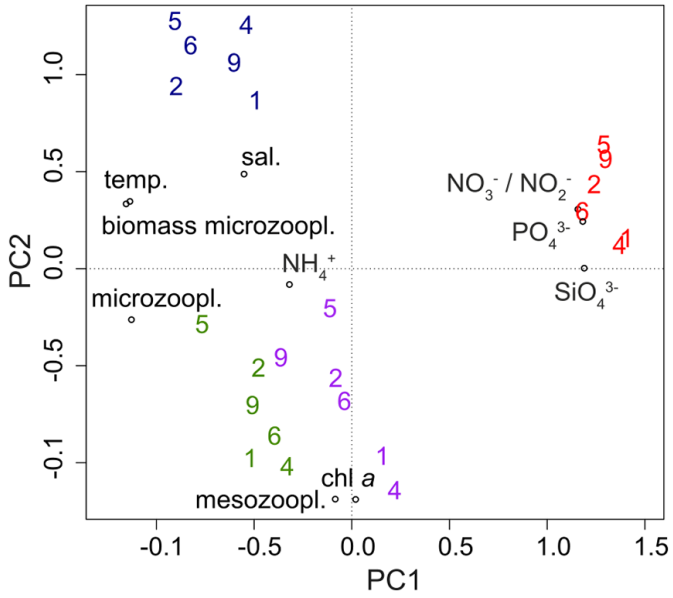

Fig. 3 PCA of measured environmental variables and CO_2 concentrations in the mesocosms. Mesocosms are indicated by their number; high CO_2 (2, 4, 6); ambient CO_2 (1, 5, 9); the time points are indicated by color: t17 = red, t41 = purple, t65 = green and t97 = blue; environmental variables are represented by circles, with NO_3^- / NO_2^- = nitrate and nitrite [µmol L^{-1}]; NH_4^+ = ammonium [µmol L^{-1}]; PO_4^{3-} = phosphate [µmol L^{-1}]; SiO_4^{3-} = silicate [µmol L^{-1}]; sal. = salinity [psu]; temp. = temperature [°C]; chl *a* = chlorophyll *a* [ng L^{-1}]; mesozoopl. = mesozooplankton [ind L^{-1}]; microzoopl. = microzooplankton [ind L^{-1}]; biomass microzoopl. = biomass microzooplankton [µg C L^{-1}].

Shifts in the OTU compositions of the 18S plankton matched the changes of environmental conditions over time as we determined based on the presence-absence-transformed and sequence read datasets (Fig 4). The OTU composition of the plankton was mainly shaped by nutrient and temperature development. After the first bloom (t41), the OTU composition (presence-absence-transformed data) of the

small-sized plankton (filter samples) was additionally characterized by a high abundance of mesozooplankton. Based on the 18S sequence read data, variation among the plankton samples after the first bloom (t41) were characterized by the increased chlorophyll *a* concentration. The mesocosms were not separated according to their CO_2 treatment in the RDA ordination (ambient: M1, M5, M9; high: M2, M4, M6) indicating no CO_2 induced significant differences in the OTU composition or number of assigned HTS reads per OTU.

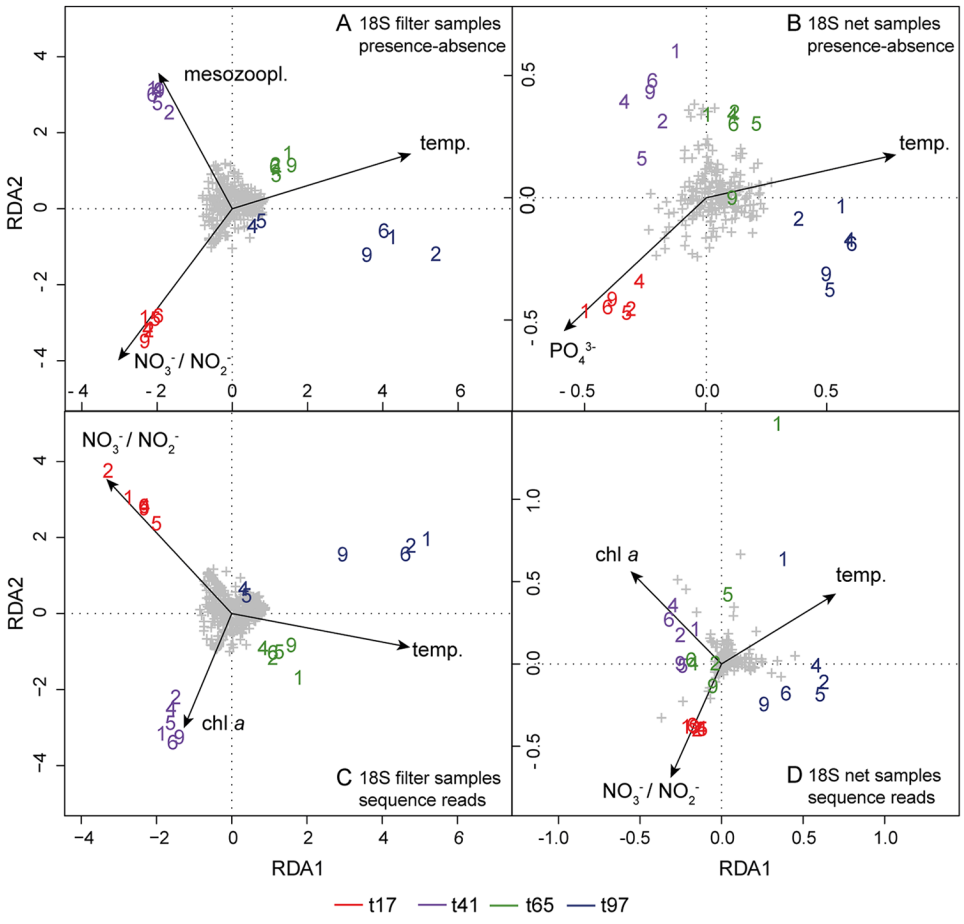

Fig. 4 RDA of OTU compositions from filter (left) and net samples (right) of the mesocosms in dependence of ecological variables, based on the 18S presence-absence-transformed (top row) and sequence read (bottom row) data. Mesocosms are indicated by their number; high CO_2 (2, 4, 6); ambient CO_2 (1, 5, 9); OTUs are represented by grey plus signs; time points are indicated by color: t17 = red, t41 = purple, t65 = green and t97 = blue; explanatory variables are displayed as vectors, with NO_3^- / NO_2^- = nitrate and nitrite [µmol L^{-1}]; PO_4^{3-} = phosphate [µmol L^{-1}]; temp. = temperature [°C]; chl *a* = chlorophyll *a* [ng L^{-1}]; mesozoopl. = mesozooplankton [ind L^{-1}]. Only significant ecological variables were retained for the respective RDA plot; thus, variables shown vary for the four RDA plots.

Plankton community composition and succession (Taxa)

Comparing the taxonomic composition recorded with the 18S marker, in the high (M2, M4, M6) and ambient (M1, M5, M9) CO_2 mesocosms, there were no significant differences (R^2 = 0.02; *adonis*; $p > 0.05$). However, the plankton community composition was significantly changing over time (R^2 = 0.61; *adonis*; $p < 0.01$). The BLAST searches and MEGAN analyses of the 18S and *cox*1 OTUs resulted in the identification of 30 (± 5) and 10 (± 3) taxa per treatment and time point from different hierarchal levels, respectively. 18S OTUs assigned to various planktonic taxa within the groups Ciliophora, Dinophyceae, Amoebozoa, Fungi, Haptophyceae, Cryptophyta, Deuterostomia, Copepoda, Hydrozoa, Rhizaria, Straminipila and Chlorophyta covering all trophic levels (Fig 5). OTU evenness for the investigated 18S community was 0.57 (± 0.04), indicating the presence of dominating species in terms of biomass. From the 18S OTUs nearly half (45.1% ± 3.1) assigned to *Pseudocalanus* spp. (later identified as *P. acuspes*) which was also the most abundant copepod throughout the experiment - with a decrease towards the end of the experiment (based on mesozooplankton counts, Algueró-Muñiz et al. (2017a)). With the exception of copepods (54.9% ± 2.2), OTUs assigned mainly to green algae (9.3% ± 1.6), diatoms (9.2% ± 1.2) and other Straminipila (12.2% ± 3.3) at day 17 and 41. With time ciliate (t97: 13.0% ± 0.1) and dinoflagellate (t97: 6.0% ± 0.4) diversity increased. During most of the experimental period (t17, t41, t65) the recorded taxa diversity based on the 18S marker was quite similar among the mesocosms (Taxa diversity index: 2.2 ± 0.07). At the last time point (t97) taxa diversity increased (Taxa diversity index: 2.5 ± 0.03) and we detected two additional copepod species (*Calanus helgolandicus*, *Temora longicornis*), two Ciliophora (*Askenasia* sp., *Acineta flava*) and four Dinophyceae (*Scippsiella* sp., *Protoperidinum bipes*, *Protoperidinium pellucidum*, Lophodiniales) taxa as well as three other taxa (*Oikopleura dioica*, Gnathostomata, Amoebozoa) belonging to various groups.

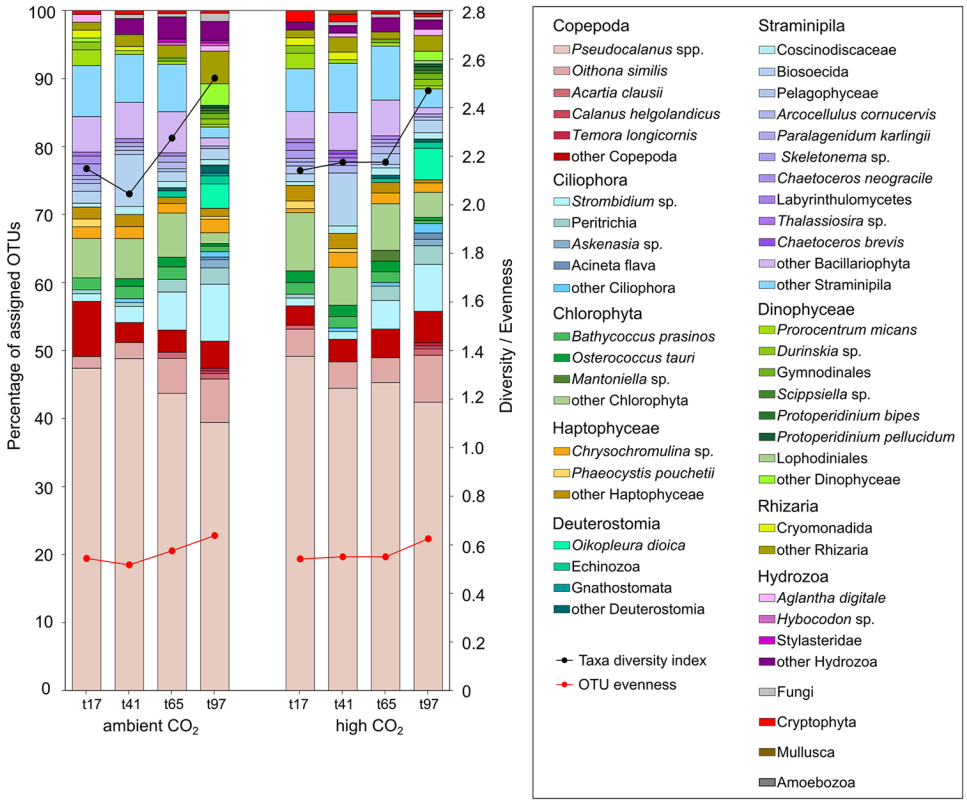

Fig. 5 Taxonomic composition in the ambient (left) and high (right) CO_2 mesocosms based on the BLAST searches and MEGAN analyses of the 18S sequences. Ambient CO_2 mesocosms (M1, M5, M9), high CO_2 mesocosms (M2, M4, M6). Time points (t17, t41, t65, t97). The left y axis shows the percentage of assigned OTUs per taxon. The right y axis shows taxa diversity index (black line) and OTU evenness (red line). Data pooled from *N = 3* replicates.

All taxonomical groups detected in the surrounding fjord were also found in the mesocosms, although in slightly deviating diversities. Especially Bivalvia and Cirripedia were less prominent in the mesocosms. Only 31 OTUs were obtained from the *cox*1 gene region, and MEGAN analyses resulted only in few identifiable taxa. With decreasing proportion OTUs mainly assigned to Proteobacteria (t17 to t65: 69.6% ± 10.2, t97: 37.5% ± 12.5). Beside that the *cox*1 BLAST search and MEGAN analysis delivered hits for *Pseudocalanus acuspes*, *Bathycoccus prasinos* and *Hybocodon prolifer* which supports species identification based on the 18S gene region.

***Pseudocalanus acuspes* – species identification & intraspecific diversity**

BLAST searches of the 18S sequences from the community barcoding and our reference database, delivered *P. elongates* as the best hit (100% pairwise identity over 187 bp and 685 bp, respectively). In contrast to that, BLAST searches of *cox*1 sequences (141 individuals) from our reference database exclusively revealed hits for *P. acuspes*. Therefore, we aligned (Geneious® version 7.0.4) the NCBI 18S reference sequence of *P. acuspes* (GenBank: KF991205.1, 419 bp) with our own 18S reference sequences (~ 675 bp), which were proven to belong to *P. acuspes* by Sanger sequencing of the *cox*1 gene region of the same individuals. The alignment (89% pairwise identity over 214 bp) suggested that the sequence fragments represent different parts of the 18S gene region, what prevented an appropriate species identification using BLAST. Another investigation in the Gullmar Fjord based on species-specific PCR of 100 individuals, also resulted in the identification of *P. acuspes* exclusively (Thor and Oliva 2015). Thus, based on these findings, we consider *P. acuspes* as the only present *Pseudocalanus* species throughout the experiment.

Investigations of the intraspecific genetic diversity revealed no differences between the CO_2 treatments or sampling days, except for mesocosm two (M2). There were no CO_2 induced differences in the OTU composition (presence-absence-transformed data, *adonis*: $R^2 = 0.008$, $p = 0.924$) nor in the OTU frequencies (sequence read data, *adonis*: $R^2 = 0.010$, $p = 0.991$) of the investigated *P. acuspes* populations. However, in M2 the OTU frequency and composition and therefore the haplotype compilation changed significantly in the course of the experimental period (presence-absence-transformed, *adonis*: $R^2 = 0.297$, $p < 0.05$; sequence read data, $R^2 = 0.308$, $p < 0.05$).

Discussion

The combination of DNA barcoding and high throughput sequencing (HTS) applied here revealed no CO_2 induced compositional shifts in the investigated coastal plankton communities. Changes in relative taxa abundances and taxa compilations were mainly observed along the seasonal succession, which was driven by temperature and nutrient availability.

Experimental setup

Ambient and high CO_2 treatments remained different with respect to pCO_2 throughout the whole experiment, although CO_2 concentrations were fluctuating in the mesocosms, due to outgassing and CO_2 uptake by algae followed by additions of CO_2 enriched water to compensate for CO_2 losses. Starting with similar biogeochemical variables and plankton communities, variation among mesocosm replicates increased over time (Fig 2). Initial subtle differences between the enclosures intensified over the succession. Therefore, the detection of possible CO_2 effects became more difficult with time, because they potentially occurred at different days in the replicates. Although increasing variation among replicates is a common complication of long-term studies in natural systems, it will be a major task to resolve this problem for future studies to avoid an underestimation of the implications of increasing CO_2.

The mesocosms included the majority of the natural plankton community, all taxa found in the surrounding fjord were also detected in the enclosures. However, the OTU composition of the mesoplankton was significantly different between the mesocosms and the fjord at the investigated time points (Fig 2). This indicates either a displacement in species succession or differences in species abundances. Some species were underrepresented in the mesocosms compared to the fjord e.g. Bivalvia and Cirripedia. As mesocosm walls were regularly cleaned, substrate-bound or sessile taxa probably did not find an appropriate environment. Although blooms in the fjord and the mesocosms developed in parallel, they were more intense in the enclosures (Bach et al. 2016). This suggests a similar succession, but differences in species composition and abundance among the mesocosms and the fjord.

Community barcoding

The analyses of the community barcoding data resulted in a total number of 771 OTUs. This indicated a high sequencing depth and thereby a high coverage of the species (and intraspecific genetic variation) diversity present in the mesocosms and in the surrounding fjord. The plankton communities in the three ambient (M1, M5, M9) and high CO_2 (M2, M4, M6) mesocosms were represented by 375 ± 66 OTUs per time point. During the MEGAN analyses around one half of the OTUs (198 ± 33) could be assigned to reference sequences. The other half could not be assigned,

probably because corresponding 18S reference sequences are missing in the database yet. Furthermore, multiple OTUs were assigned to the same taxa, since some OTUs represent haplotypes of the same species. Overall, based on the 18S and *cox*1 sequences we could identify 19 species, 9 genera, 16 higher taxonomical groups i.e. 44 different taxa. The success of studies such as the present depends very much on the quality of the available molecular data bases. For a steady improvement of those databases good cooperation between taxonomists and molecular biologists is therefore crucial.

Despite the high taxonomic resolution based on OTUs, metabarcoding data cannot be used to make predictions about species abundances and / or biomasses on-site, due to preferential annealing of universal primers in some species over others during PCRs and HTS (Elbrecht and Leese 2015). Therefore, relative abundances and diversity indices, based on HTS reads and the number of OTUs assigned to certain taxa, respectively, are not directly comparable to those determined using count abundance data. However, comparisons among the enclosures and time points are feasible because amplification and sequence success between taxa remains constant.

Based on the number of assigned OTUs to a certain taxon, predictions about the intraspecific diversity is only possible to a limited extent. For investigations of the intraspecific diversity a sufficient sample size is crucial, because up to a certain point the observed intraspecific variation is positively correlated with the number of investigated individuals. Therefore, the percentages of assigned OTUs shown in Fig 5 do not reflect the actual level of intraspecific variation, since it is unlikely that a representative sample size could be reached for all taxa detected with the community barcoding. *P. acuspes,* however, was the most abundant copepod (based on mesozooplankton counts, Algueró-Muñiz et al. (2017a)) throughout the experiment and with 1,351 (± 561) assigned sequence reads per sample also the best covered taxa in the HTS of the 18S gene region. We observed no effect of elevated CO_2 on the genetic intraspecific diversity of the investigated *P. acuspes* populations. Furthermore, in all investigated mesocosms (M1, M4, M5, M6, M9) except M2 the OTU, and thus the haplotype compilations of the *P. acuspes* populations, were similar at the sampling days 17, 41, 65, and 97. The temporal change in the OTU composition of the *P. acuspes* population of M2 was probably due to a decline in the number of detected OTUs (~ 40 less) on the filter sample on day 97. Since the intraspecific diversity decrease was only detected in one of the three replicates and

we further cannot exclude a potential methodological flaw this finding should not be overinterpreted.

The relatively high number of 18S OTUs (174) assigned to *P. acuspes*, might indicate that several cryptic lineages co-occur in a species complex. Although such a high intraspecific variation was not observed with our *cox*1 reference sequences (141 individuals), the actual genetic variation could have been underestimated due to the relatively low number of investigated individuals. The community barcoding of the *cox*1 gene region delivered only one OTU assigned to *P. acuspes* (containing 14 sequences), i.e. not a representative sample of the population. Thus, to resolve the *Pseudocalanus* species complex, further investigations will be necessary.

The OTU distribution among the filter and net samples was quite different. A higher proportion of the OTUs originated from filter samples. However, this is not surprising since species diversity is in general higher in small organisms (here microplankton) compared to large ones (here mesoplankton) and taxa larger than 1 mm were excluded. Around 50% of the OTUs corresponded to both size fractions (filter and net samples), because eggs, larvae and nauplii of mesozooplankton as well as smaller cells of large diatoms (e.g. Coscinodiscaceae) are also captured on the filter samples. Furthermore, pico-, nano- and microplankton were ingested by larger planktonic taxa or just attached to them and will be detected along with the grazer/predator.

CO_2 effects on the plankton community composition and food web interactions

The composition of plankton communities investigated in this mesocosm study was not affected by increased CO_2 levels. At the analysed time points (t17, t41, t65, t97) no significant differences between the CO_2 treatments could be observed. Yet, there were significant compositional changes over time (Fig 2), which were primarily induced by changing temperature and decreasing nutrient availability over the course of the succession (Fig 4). Additionally, at day 41, the 18S OTU composition (presence-absence-transformed data) of the small-sized plankton communities (pico-, nano- and microplankton) also seemed to be driven by mesozooplankton abundances, i.e. rather by top-down effects from predators than by bottom-up effects from food sources (Fig 4, top row). Löder et al. (2011) found copepods to be less important

phytoplankton grazers, but important top-down regulators of microzooplankton, especially with decreasing quality of phytoplankton food due to nutrient limitation during blooms (Löder et al. 2011). Furthermore, many copepods are known to be size-selective feeders, preferring a size class between 11 and 87 µm (Frost 1972). During the bloom events the phytoplankton community was mainly made up by picoeukaryotes (< 2 µm) and *Coscinodiscus* sp. (> 200 µm), a giant diatom. Therefore, the vast majority of the phytoplankton present did not represent a suitable food source for copepods, due to the unfavorable cell size. Thus, copepods probably intensively grazed on microzooplankton organisms (Horn et al. 2016), most likely ciliates and dinoflagellates. This is corroborated by the 18S mesoplankton OTU composition (presence-absence-transformed data) obviously not being related to phytoplankton densities (chlorophyll *a*) in the RDA analysis (Fig 4, top row). On day 97 towards the end of the experiment more OTUs assigned to ciliate and dinoflagellate species (Fig 5), after the top-down control by mesoplankton subsided due to decreasing copepod abundances. Considering the amount of assigned HTS reads per OTU in the RDA the predator-prey food web interaction was probably masked, because the high numbers of sequence reads, which correspond to OTUs representing the main contributing taxa of the blooms, (Chlorophyta, Rhizaria and Stramenopila) carried more weight in the analysis then the actual composition of the OTUs. Therefore, in the RDA, variation between plankton samples at day 41 were rather explained by the chlorophyll *a* concentration than by the abundance of mesozooplankton. The introduced herring larvae potentially controlled both micro- and mesozooplankton abundances. In their early life stages from day ~ 64 to 74 they were probably feeding first on small-sized microzooplankton e.g. ciliates and subsequently on larger microzooplankton organisms and nauplii stages of mesozooplankton taxa. Towards the end of the experiment they most likely started to feed on adult mesozooplankton organisms. As *P. acuspes* was the dominant copepod species it was probably mostly consumed by the herring larvae. Decreasing densities of *P. acuspes* towards the end of the experiment, thereby also facilitated the development of other copepod species (Fig 5) due to reduced competition.

As part of this mesocosm experiment other studies found temporal effects of high CO_2 on planktonic taxa. Dinoflagellates were more abundant in the high CO_2 mesocosms towards the end of the experimental period (Horn et al. 2016), and the phytoplankton community structure was significantly different between the CO_2

treatments during the second bloom period, with a higher abundance of picophytoplankton (Bach et al. 2016). Lischka et al. (2015) also observed temporal effects of elevated CO_2 on a microzooplankton community whereby *inter alia* a shift towards smaller taxa with increasing CO_2 was detected (Lischka et al. 2015). The second bloom was fueled by recycled nutrients, thus resources for plankton growth needed to by delivered by the food web, thereby the limitation of inorganic nutrients seemed to indirectly enhance CO_2 effects on planktonic organisms. The food web becoming rather bottom-up driven is supported by a higher Proteobacteria diversity (number of assigned OTUs based on the *cox*1 gene region) in the period after the blooms (t41, t65). After decomposition and remineralization of the particulate organic matter, Proteobacteria diversity decreased towards day 97. Dependencies between nutrient availability and impacts of elevated CO_2 on marine organisms were also demonstrated in previous studies (Li et al. 2012; Rouco et al. 2013; Hofmann et al. 2015a; Sala et al. 2015). Therefore, future studies investigating the impacts of OA on marine biota should focus on nutrient limited systems.

Study hypothesis

Based on the results of this study our hypothesis that DNA barcoding in combination with HTS can unravel previously hidden (based on morphological analyses) CO_2 sensitivities of plankton communities cannot be confirmed. We focused on the investigation of potential long-term effects of CO_2 on planktonic taxa compilations, because the number of samples for the HTS was limited (financial constrains). Thereby, samples from the distinct bloom events were not considered for the community barcoding. Thus, we cannot assess whether the CO_2 induced increase in the abundance of picoeukaryotic phytoplankton (Bach et al. 2016), which was observed during the second bloom, could have been detected with community barcoding. Although our genetic approach allowed a detailed investigation of the majority of the planktonic communities (down to haplotype level), the technique is not appropriate to detect slight changes in species abundances and stage specific effects, as it has been observed for some dinoflagellate (Horn et al. 2016) and copepod species (Algueró-Muñiz et al. 2017a), respectively. However, community barcoding data delivers additional information about the genetic diversity of a community and the presence of potentially cryptic or even unknown species. Thus, for future studies we suggest to use community barcoding as applied here, not as a

standalone technique but as a tool to complement classical morphological investigations.

Conclusion

Based on the community barcoding data elevated CO_2 had no significant effect on the relative abundance or compilation of coastal planktonic taxa during a winter-to-summer succession in the mesocosms investigated in this field experiment. The resilience of coastal plankton communities (bacteria, phytoplankton, micro- and mesozooplankton) towards future OA conditions was also demonstrated in earlier large scale mesocosm field and indoor studies (Kim et al. 2006; Riebesell et al. 2008; Aberle et al. 2013; Niehoff et al. 2013; Silyakova et al. 2013; Sperling et al. 2013; Horn et al. 2015). In agreement with those results, we assume that low sensitivities for high CO_2 conditions are common for coastal plankton communities which are exposed to pronounced natural fluctuations in seawater pH.

Supplementary data

Table S1 and S2 are only digitally available

Table S3 Pipeline steps

Pipeline step	Program	File	Reads	OTUs
Raw sequence data		forward reverse	4,987,876 4,987,876	
1. Illumina adapter trimming and filtering	Trimmomatic	forward reverse	1,630,884 1,630,884	
2. Quality filtering	FastQFS	forward reverse	1,630,807 1,630,807	
3. De-multiplexing 4. Barcode and primer trimming 5. Reorient reads 5' – 3'		18S *cox*1	653,789 3,417	
6. Similarity clustering	USEARCH v7	18S *cox*1		7,734 96
7. Re-assemble	Geneious® 7.0.4	18S *cox*1		1,037 39
8. Alignment	Geneious® 7.0.4	18S *cox*1		851 36
9. Mapping	USEARCH v7	18S *cox*1		826 35
10. OTU abundance table	Microsoft Excel 2016)	18S *cox*1		740 31

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CHAPTER III

Direct and indirect effects of near-future pCO_2 levels on zooplankton dynamics

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Abstract

Ocean acidification has direct physiological effects on organisms by, for example, dissolving the calcium carbonate structures of calcifying species. However, non-calcifiers may also be affected by changes in seawater chemistry. To disentangle the direct and indirect effects of ocean acidification on zooplankton growth, we carried out a study with two model organisms. We investigated the individual effect of short term exposure to (1) high and low seawater pCO_2 and (2) different phytoplankton qualities as a result of different CO_2 incubations on the growth of a heterotrophic dinoflagellate and a copepod species. It has been previously observed that higher CO_2 concentrations can decrease phytoplankton food quality in terms of carbon : nutrient ratios. We therefore expected both seawater pCO_2 (pH) and phytoplankton quality to result in a decrease of zooplankton growth. Although we expected lowest growth rates for all zooplankters under high seawater pCO_2 and low algal quality, we found that direct pH effects on consumers seem to be of lesser importance than the associated decrease in algal quality. The decrease of primary producers' quality under high pCO_2 conditions negatively affected zooplankton growth, which may lead to lower availability of food for the next trophic level and thus potentially affect the recruitment of higher trophic levels.

Introduction

Human industrial activities have increased atmospheric carbon dioxide (CO_2) concentrations which have now reached values of over 400 ppm on average (Tans and Keeling 2013), the highest level for millions of years (Royer 2006; Pagani et al. 2011). About 25% of the CO_2 enters the oceans which can act as carbon (C) sinks (Canadell et al. 2007). As a result, the carbonate chemistry of oceans has changed, especially in the upper 100 meters where ocean acidification has a major influence (Doney et al. 2009).

Ocean acidification negatively affects a number of organisms directly by, for example, dissolving the calcium carbonate structures of calcifying species (Orr et al. 2005). However, marine organisms, such as micro- and mesozooplankton, may be subjected to other adverse effects. Lower seawater pH resulting from increasing pCO_2 could directly affect the physiology of both phyto- and zooplankton by changing intracellular pH, membrane potentials, and enzyme activities (Nielsen et al. 2010). This acidification of body fluids is known as hypercapnia (Fabry et al. 2008). When CO_2 levels increase in seawater, dissolved CO_2 more readily diffuses across body surfaces and equilibrates in both intra- and extracellular spaces. As in seawater, CO_2 reacts with internal body fluids causing H^+ ions to increase and pH to decrease. Hypercapnia can cause the suppression of metabolic processes (Michaelidis et al. 2005; Pörtner 2008) and disrupt acid-base homeostasis (Miles et al. 2007), thus decreasing growth rate and reproductive success and increasing mortality of marine organisms (Yamada and Ikeda 1999). Smaller organisms are likely to be more affected by changes in seawater chemistry than larger ones as a result of the differences in volume to surface ratios and future conditions will increase variations in pH at the cell surface (Flynn et al. 2012). However, despite their ecological importance, only few studies have focused on the impact of ocean acidification on microzooplankton so far, and, to our knowledge, their sensitivity to hypercapnia has never been investigated. Microzooplankton are an essential component in planktonic ecosystems. Indeed, they often comprise the major predatory group in microbial food webs (Sherr and Sherr 2002), and microzooplankters form a trophic link between pico-, nano- and microplankton on the one hand and higher trophic levels, such as copepods, on the other hand (Sommer et al. 2005). Although there is indication that microzooplankton are sensitive to elevated pH (Hinga 2002; Pedersen and Hansen

2003), to our knowledge no studies have directly tested the effects of CO_2-induced lowering of the pH on marine microzooplankton. The few existing experimental studies were carried out using natural plankton communities and observed no effects of a high pCO_2 / low pH on microzooplankton, independent on whether the systems were fixed pH, or whether pH was allowed to drift as it would in reality (Suffrian et al. 2008; Rose et al. 2009; Aberle et al. 2013). Ocean acidification does not only reduce the global base pH but also influences smaller-scale fluctuations. For example, ocean acidification may influence spatial and seasonal variations by modulating seawater alkalinisation during intense C-fixation associated with phytoplankton blooms (Flynn et al. 2012). Having said this, as the knowledge on effects of ocean acidification on microzooplankton is so limited we decided to focus on effects of fixed changes in pCO_2 only. However, it is generally difficult to observe physiological effects in natural community experiments whereas smaller and more controlled microcosm studies are more helpful to understand physiological responses such as microzooplankton sensitivity to hypercapnia. Further, most studies measuring copepod physiological performance at lower seawater pH observed that copepods are relatively tolerant to hypercapnia (Mayor et al. 2012; McConville et al. 2013). Yet, recent studies observed strong negative effects of near-future ocean acidification levels on copepods (Lewis et al. 2013; Thor and Dupont 2015), and others suggest that inappropriate experimental designs might have underestimated the actual direct effect of ocean acidification on copepods, and potentially microzooplankton (Cripps et al. 2014a; Cripps et al. 2014b) Since micro- and mesozooplankton play different roles in the pelagic ecosystem, it is important to determine whether those two groups are differently affected by high pCO_2.

Apart from direct acidification effects, the increasing C availability in the marine environment will likely change primary productivity and the quality of phytoplankton as food for higher trophic levels (Low-Décarie et al. 2014). As primary producers reflect the nutrient composition of their surrounding medium, they are expected to show higher C : nutrient ratios as CO_2 availability increases (Burkhardt et al. 1999; Urabe et al. 2003; van de Waal et al. 2010). Further, algae with high C : nutrient ratios are known to often be food of inferior quality for herbivorous consumers since there is a larger difference between resource chemical composition and consumer metabolic requirements (Sterner and Elser 2002). Recent results indicate negative indirect effects of ocean acidification on copepods caused

by a decline in prey quality when grown under high pCO_2 (Rossoll et al. 2012; Schoo et al. 2013). In the light of the predicted increase of pCO_2 and the observed trend of decreasing nitrogen (N) and phosphorus (P) inputs to coastal areas (Grizzetti et al. 2012), the potential for an excess of C and a concurrent nutrient limitation at the base of the food web is considerably heightened. Although evidence is increasing that the growth rate of zooplankters decreases with increasing CO_2 availability to the algae (Olson and Kawaguchi 2011; Rossoll et al. 2012; Schoo et al. 2013), it remains unclear whether direct or indirect effects on consumer fitness play the more prominent role. Indeed, only one study investigated the direct and indirect effects of acidification on zooplankton growth and reproduction (Rossoll et al. 2012). This study concluded that high CO_2 availability decreases copepods fitness mostly indirectly. Further, Melzner et al. (2011) showed that the effects of high CO_2 may be less pronounced when there is enough food available, as the energy needed to deal with the unhealthy environment is available. If energy is the limiting compound in the study of Melzner et al. (2011), at low food concentrations higher algal carbon : nutrient ratios might benefit herbivores since algae grown under high CO_2 conditions are more energy rich as they often contain more lipids (Rossoll et al. 2012). The generality of this result remains unclear, as trophic upgrading and differential algae sensitivity in terms of growth rate and stoichiometry to pCO_2 may compensate for low food quality. For instance, Isari et al. (2015) recently observed that increased pCO_2 does not affect the stoichiometric quality of the phytoplankton species *Heterocapsa* sp. and, logically, did not alter performances of copepods feeding on that prey. Further, Klein Breteler et al. (1999) showed that inadequate algal food could be biochemically upgraded by protozoans to high quality food for copepods. Hence, protozoan herbivores might dampen the negative effect of acidification on algal food quality through trophic upgrading. In fact, Caron & Hutchins (Caron and Hutchins 2013) identified lack of data on the effects of ocean acidification on microzooplankton as one of the major knowledge gaps.

To disentangle the direct and indirect effects of ocean acidification on both microzooplankton and mesozooplankton, we carried out a study with model organisms. We investigated the individual effect of short term exposure to (1) high and low seawater pCO_2 and (2) different qualities of the alga *Rhodomonas salina* on the growth and development of two model zooplankton species *Oxyrrhis marina* (Montagnes et al. 2011) and *Acartia tonsa* (Mauchline 1998). The different algal

qualities were obtained by growing *R. salina* in high and low seawater pCO_2 relative to current scenarios. We hypothesize that a significant interaction of seawater pCO_2 and phytoplankton quality should affect zooplankton growth and that lowest growth rates for both micro- and mesozooplankters should be observed under high pCO_2 seawater and low algal quality.

Material and methods

To test whether planktonic herbivores growth is affected by seawater pCO_2 or by algal quality differences caused by seawater pCO_2 (direct versus indirect effect), we conducted a laboratory experiment with nauplius and copepodite stages of the copepod *A. tonsa* as well as with the heterotrophic dinoflagellate *O. marina*. The zooplankton species were cultured under high and low pCO_2 and were fed with two algal qualities in full factorial design, i.e. four treatments. The different algal qualities were obtained by growing the model organism *R. salina* in high and low seawater pCO_2 relative to current scenarios. Using model organisms such as *O. marina* and *R.* salina entails limitations regarding the extent to which experimental results can be interpreted. Nevertheless, due to its high growth rate and to the reproducibility of nutrient treatments, *R. salina* is a useful model organism when studying the importance of phytoplankton food quality for zooplankton. Further, Davidson et al. (2011) made a critical assessment of the advantages and disadvantages of using *O. marina* as a model organism. Their study supports the use of this dinoflagellate in experimental studies since its feeding mode and predator:prey size ratio are comparable to most protozoa. Both *O. marina* and *R. salina* are planktonic, not benthic, and in this sense, they are appropriate model organisms for planktonic processes.

Phytoplankton

R. salina (Wislouch) Hill et Wetherbee was kept in f/2 medium prepared with 0.2µm filtered seawater (Guillard and Ryther 1962). *R. salina* was grown in continuous chemostat cultures maintained at steady state. The phytoplankton cultures were constantly aerated with a mixture of air stripped of CO_2 by soda lime and pure CO_2 adjusted to 200 and 800 µatm (Rho 200 and Rho 800) to represent pre-industrial and predicted future scenarios. The pre-defined pCO_2 level was achieved following

Schoo et al. (2013). A sensor (HTK Hamburg) continuously monitored the pCO_2 of the gas mixture distributed to the algal cultures and automatically adjusted the CO_2 content and flow rate. Preliminary experiments showed that the pCO_2 of the growth medium was in equilibrium with the target level of the gas pCO_2 within 1h of aeration. The algal chemostat cultures (5 L) were stirred continuously and kept at 18°C under a 16h:8h light:dark regime (185 µmol m^{-2} s^{-1}). Subsamples from the surplus culture collected in an overflow container were taken daily to measure the cell density of the cultures with a CASY cell counter (SCHÄRFE SYSTEMS, Reutlingen, Germany) as well as the algal stoichiometry (see procedure below).

To feed the zooplankton populations, two new *R. salina* batch cultures were created daily at concentrations of 0.5 * 10^{-6} cells L^{-1} to ensure that, within each treatment, zooplankters were feeding on the same algal quality for the duration of the experiment. We needed to prepare batch cultures since the volume of algae needed to feed the zooplankton populations was too important to be taken from the chemostats. The phytoplankton cultures were constantly aerated with air at pCO_2 of 200 and 800 µatm (Rho 200 and Rho 800). It is important to note that this study was conducted using a controlled system with fixed pH which might induce different responses than in the field where pH drifts with phytoplankton growth (Flynn et al. 2015). The algae were cultivated in 1 L batch cultures in f/2 medium at 18ºC under a 16h:8h light:dark regime (185µmol m^{-2} s^{-1}). After three days of growth, an aliquot of each *R. salina* culture was filtered onto pre-combusted Whatman GF/F filters. The particulate C and N content of *R. salina* was measured with a Vario Micro Cube/CN-analyser (Elementar). Particulate P was analysed as orthophosphate after acidic oxidative hydrolysis with 5% H_2SO_4 (Grasshoff et al. 1999).

Microzooplankton

O. marina Dujardin was obtained from the Göttingen culture collection (Strain B21.89) and fed *R. salina* at 18ºC under a dim continuous light regime (50 µmol m^{-2} s^{-1}). Prior to the experiment, the *O. marina* culture was starved for 1 week in order to eradicate any effects of preculture conditions. This culture was then split into 28 separate cultures (four treatments, seven replicates) which were all diluted to a start concentration of 20,000 cells mL^{-1} with CO_2 preconditioned artificial, sterile and nutrient-free seawater (Aqua Marin) at a salinity of 32. Cell concentrations of the cultures were determined using a CASY particle counter (SCHÄRFE SYSTEMS,

Reutlingen, Germany). The *O. marina* cultures were gently aerated with a mixture of air and pure CO_2 adjusted to 200 (Water 200) and 800 µatm (Water 800). As for phytoplankton cultures, a sensor continuously monitored the pCO_2 of the gas mixture distributed to the algal cultures and automatically adjusted the CO_2 content and flow rate. Pre-experiments indicated that gentle bubbling does not affect *O. marina* growth rate. The cultures had pH of 8.3 (±0.06) and 7.8 (±0.05) when aerated with 200 and 800 pCO_2, respectively. The total alkalinity of the CO_2 preconditioned artificial seawater was 3300 µmol L^{-1} for the 200 µatm and 3197 µmol L^{-1} for the 800 µatm pCO_2 treatment. The pH was measured with a ProLab 3000 pH meter with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A170MFDIN-N). TA was estimated from open-cell duplicate potentiometric titration and calculation with modified Gran plots (Bradshaw et al. 1981), using a TitroLine alpha plus titrator with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A120MF-DIN-N). The carbonate system was calculated from TA, pH, temperature and salinity using CO2SYS (Lewis and Wallace 1998), the pCO_2 values obtained were 292 and 911 ppm for the 200 and 800 treatments, respectively. To calculate the carbonate system, we used the equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987). Each of the two pCO_2 culture sets (Water 200 and Water 800) was fed *ad libitum* with 35 algal cells per *O. marina* daily during four days with either Rho 200 or Rho 800 (Rho 200-Water 200, Rho 200-Water 800, Rho 800-Water 200, Rho 800-Water 800). The quantity of food given daily was sufficient to prevent food quantity limitation and was adjusted at the last feeding day to minimize the amount of remaining algal cells after 24h. After four days of growth, the cell concentration of each *O. marina* culture was determined by CASY counting and the relative growth rate of each culture was calculated.

Mesozooplankton

Eggs of the calanoid copepod *A. tonsa* were produced in 200-litre cylindrical tanks, where the animals were cultivated at 18ºC at a 16:8 light:dark cycle. Copepods were fed with *R. salina*. Eggs were siphoned from the bottom of the tanks daily and stored in seawater at 4ºC for later use. The stored eggs were incubated in fresh seawater at 18ºC for hatching. Since hatching peaks between 24h and 36h of incubation, we collected the nauplii hatched during this period to minimize age differences between individuals. Nauplii were placed at 18ºC under a dim 16:8 light:dark regime in the

same four treatments above-described for microzooplankton and fed daily for 12 days. The cultures were put in 1 L glass containers at densities of 1,000 individuals L^{-1} in seven replicates per treatment. Copepods were fed *ad libitum* with 20,000 algal cells per copepod and all experimental animals were washed daily over a sieve (75 µm mesh size) to separate them from any algae before being introduced to fresh CO_2 preconditioned artificial seawater prior to feeding in order to minimise changes in the nutrient composition of the algae by waste products of the animals. The developmental rate of the juvenile copepods was determined after 12 days. The developmental stages of the copepods were determined for at least 100 individual animals from subsamples at the end of the experiment. For the calculation of the developmental rates, all naupliar larvae were assigned to stage 6, the last naupliar stage before transition to the copepodite stages. The number of individuals per stage was divided by the number of days of growth, and the developmental rate per treatment was then calculated by dividing this sum by the number of individuals counted. We also tested the copepods reaction to the treatments by measuring the respiration rates of stage 4 nauplii and stage 3 copepodites. Copepods respiration rates were measured following the procedure described by Schoo et al. (2013). Respiration rates were determined with a microsensor oxygen metre (PreSens Precision Sensing, Germany) equipped with oxygen microoptodes. Approximately 100 nauplii and 75 copepodites were sampled from the incubation containers at day 5 and 10 and were washed over a sieve (75 µm mesh size) to separate them from any algae before being introduced into the 5 mL incubation vessel. Oxygen air saturation values were below 80% at the end of the 1h measurements. Bacterial respiration rates were measured as a control treatment at the same time and the measured bacterial respiration rates were deduced from the total respiration rates of the copepod measurements. The animals were collected and counted after the incubation to determine the precise number of animals in each vessel, permitting an accurate calculation of respiration rates per individual animal. Respiration rates were calculated by linear regression of oxygen concentration over time. Technical issues unfortunately prevented us from measuring the respiration for *O. marina*. It is not possible to separate *O. marina* from *R. salina* due to small size differences between the two species and pre-experiments showed that using a control with algae only generates too large standard deviations.

Results

The population density and the stoichiometry of the algae grown in the continuous chemostat cultures were affected by the exposure to the different pCO_2 during growth (Fig. 1). The phytoplankton cultures had significantly higher cell densities when grown under elevated pCO_2 (Fig. 1A, Repeated Measures ANOVA, $F_{2,19} = 44.46$, $p < 0.01$). Both C : N (Fig. 1B, Repeated Measures ANOVA, $F_{2,19} = 44.46$, $p < 0.01$) and C : P ratios (Fig. 1C, Repeated Measures ANOVA, $F_{2,18} = 159.57$, $p < 0.01$) were significantly higher in *R. salina* cultures reared under elevated pCO_2.

We cultured zooplankton in low and high pCO_2 seawater and fed them algae grown in batch cultures under low and high pCO_2. The CO_2 treatments significantly affected algal C content; Rho 800 was 30% richer in C than Rho 200 (Tab. 1, t-test $p < 0.05$). This difference in C content resulted in lower C : N (10.1 ± 3.1) and C : P ratios in Rho 200 (294 ± 24) than in Rho 800 (14.7 ± 0.9 and 396 ± 31; t-test $p < 0.05$). Further, the CO_2 treatments did not affect the N and P content and the N : P ratio of *R. salina*. Although one may argue that the change in algal C : N : P reflects a short-term response caused by the small duration of adaptation, our chemostat culture indicates that such changes persist over generations (Schoo et al. 2013).

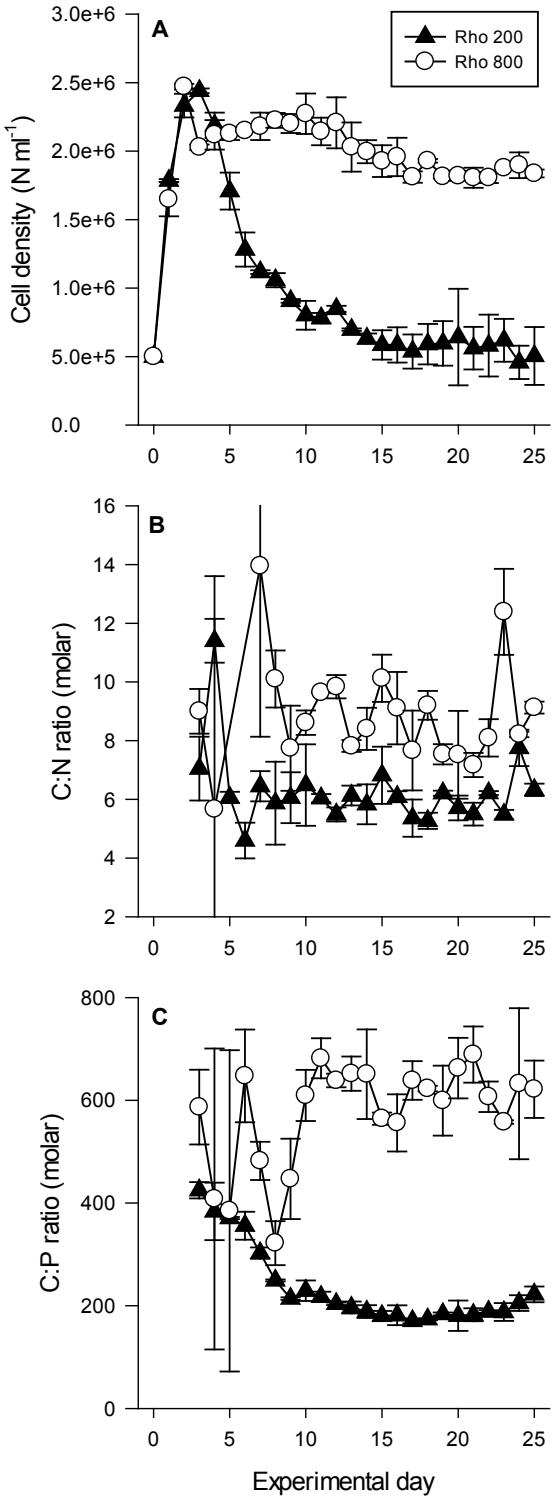

Fig. 1 *R. salina* (A) cell densities, (B) C : N ratios, and (C) C : P ratios grown in chemostats under different pCO_2. Data presented are means and standard deviations of three replicates.

Table 1 Mean carbon, nitrogen, phosphorus cell content (pg cell^{-1}) and C : N : P of *R. salina* used to feed the zooplankton cultures. Numbers in brackets are standard deviations of five replicates and stars indicate significant differences (n = 5; FG = 8; $p < 0.05$).

	Rho 200	**Rho 800**
C (pg cell^{-1})	57.2 (1.1)*	79.5 (0.6)*
N (pg cell^{-1})	6.2 (1.1)	6.2 (0.4)
P (pg cell^{-1})	0.57 (0.01)	0.53 (0.03)
CN (molar)	10.1 (3.1)*	14.7 (0.9)*
CP (molar)	294 (24)*	396 (31)*
NP (molar)	22.6 (3.4)	26.3 (3.8)
Growth rate (d^{-1})	0.42 (0.04)	0.43 (0.03)
ESD (µm)	9.46 (0.08)	9.81 (0.12)

We observed that the growth rate of *O. marina* was significantly affected by the food quality treatment (Fig. 2, two-way ANOVA, $p < 0.05$), while seawater pCO_2 did not alter the dinoflagellate growth. *O. marina* growth rate was 40% lower when feeding on the C-rich Rho 800 (0.18 d^{-1}) than when feeding on Rho 200 (0.3 d^{-1}). Further, although we expected to observe the lowest growth rate under high pCO_2 seawater and low algal quality, the ANOVA interaction effects between seawater pCO_2 and algal quality did not significantly affect the growth of *O. marina* (two-way ANOVA $p > 0.05$). Similarly, only algal quality had an effect on the development of *A. tonsa* (Fig. 3). While seawater pCO_2 did not affect *A. tonsa* development, Copepod populations feeding on C-rich Rho 800 contained a lower percentage of the older C6 and C5 copepodite stages at the end of the 12 days growth experiment than those feeding on Rho 200 (Fig. 3A). Consequently, *A. tonsa* developmental rates were influenced by algal quality and we observed a significant development decrease in the Rho 800 treatment compared to the Rho 200 treatment (Fig. 3B, two-way ANOVA, $p < 0.05$); while seawater pCO_2 had no significant effect.

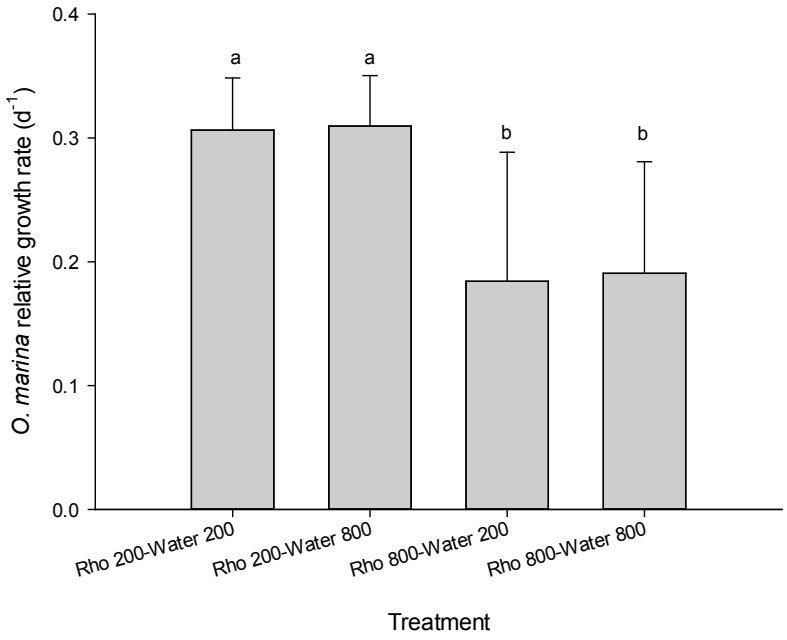

Fig. 2 Growth rates of *O. marina* reared under different pCO_2 and fed with different pCO_2 *R. salina*. Data presented are means and standard deviations of seven replicates. Statistically significant differences ($p<0.05$) are indicated by letters.

The different food qualities also led to differences in copepod respiration rates. Copepods feeding on C-rich Rho 800 had significantly higher respiration rates than those feeding on Rho 200 (Fig. 4, two-way ANOVA, Tukey's honest significant difference posthoc test, $p < 0.01$); while no effect of seawater pCO_2 could be identified. Further, although we expected the combination of high pCO_2 seawater and low algal quality to have a strong effect, the ANOVA interaction effects between seawater pCO_2 and algal quality did not significantly affect the development and respiration of *A. tonsa* (two-way ANOVA $p > 0.05$).

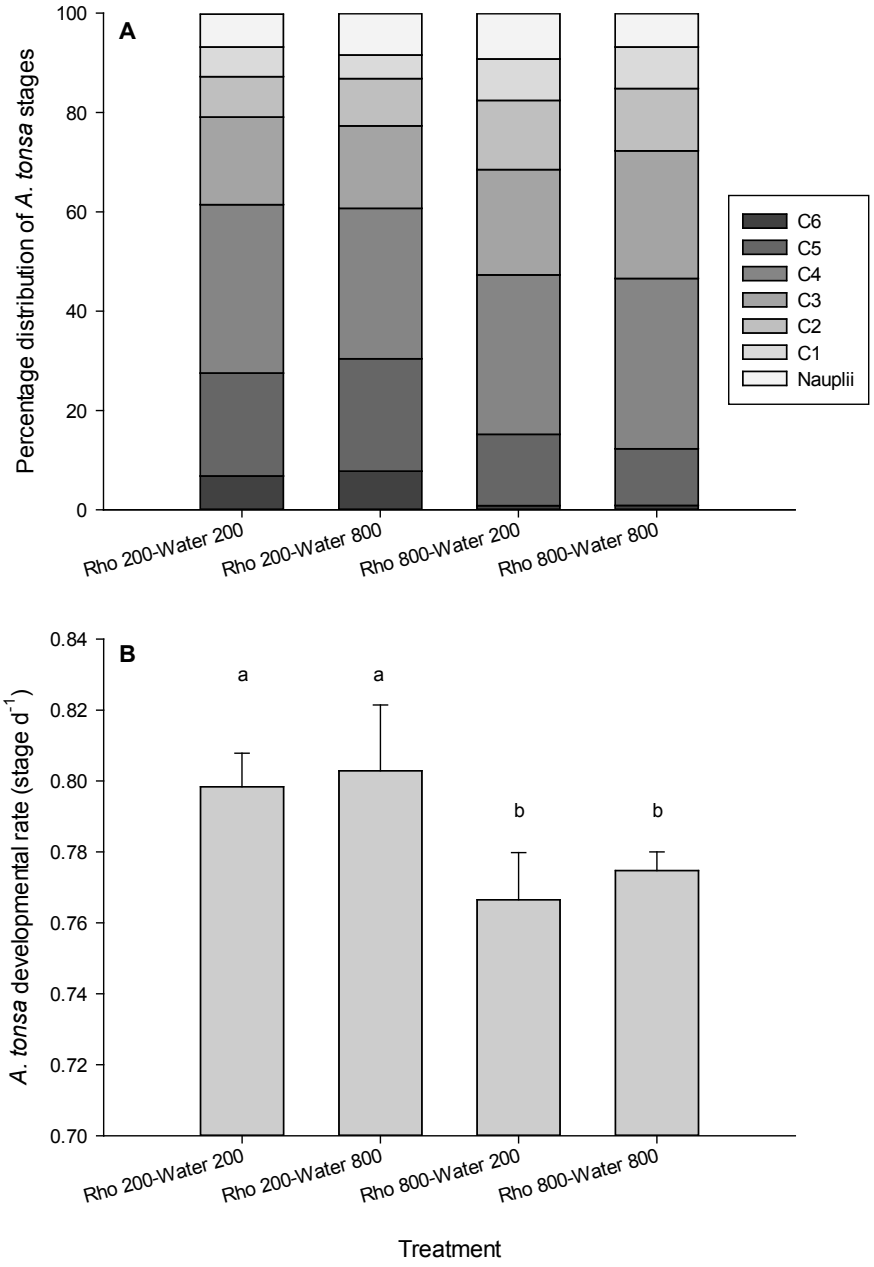

Fig. 3 (A) Percentage distribution and (B) developmental rates of *A. tonsa* development stages reared under different pCO_2 and fed with different pCO_2 *R. salina*. Data presented are means and standard deviations of seven replicates. Statistically significant differences ($p<0.05$) are indicated by letters.

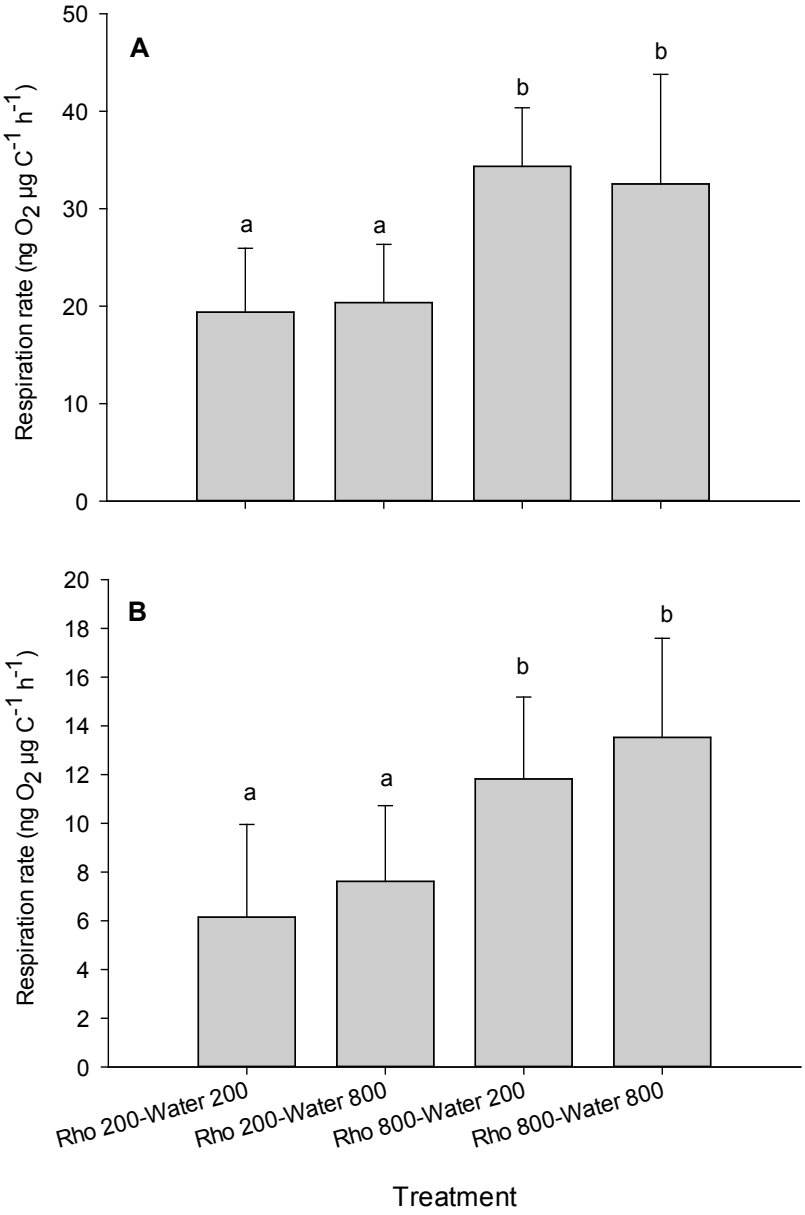

Fig. 4 Respiration rates of (A) *A. tonsa* nauplii and (B) copepodites reared under different pCO_2 and fed with different pCO_2 *R. salina*. Data presented are means and standard deviations of seven replicates. Statistically significant differences ($p<0.01$) are indicated by letters.

Discussion

Although it has previously been suggested that smaller organisms should be more affected by ocean acidification (Flynn et al. 2012), we found no direct effect of seawater pCO_2 on the dinoflagellate and copepod species we studied. This indicates that zooplankton might already be resistant to hypercapnia. Due to environmental variability (*e.g.*, upwelling, rock pools), diapause at depth, many zooplankton (including larval stages) already face pH levels much lower than those predicted for surface waters in the coming century (Olson and Kawaguchi 2011). Thus, predicted changes in surface seawater pH may be small relative to the range of pH zooplankton experience during their lifespan. These organisms could already be well adapted to seawater pH variations and potential effects of hypercapnia.

Our study, however, shows that the primary producer used in this study increased its cellular carbon content when cultured under elevated pCO_2. Both higher C fixation and increased growth rate under high pCO_2 could result in increased C : nutrient ratios. Culturing *R. salina* under different pCO_2 at identical dilution rates (i.e. growth rates) in chemostats yielded different C : nutrient ratios (Fig.1, see also Schoo et al. 2013). This indicates that elemental stoichiometric differences are caused by higher C fixation rather than by higher growth rates under high *p*CO2. This change in algal biochemical composition, and therefore quality, decreased the growth of the dinoflagellate *O. marina* as well as the development of the copepod *A. tonsa*. Thus, not only copepodites (as shown by Schoo et al. 2013) but also nauplii and microzooplankton react with decreasing growth with increasing CO_2 availability to the algae. Altogether, the growth rate and development of microzooplankton and mesozooplankton decrease at higher pCO_2, coupled with the suppression of reproductive scope identified by other studies (Cripps et al. 2014a) have clear potential to damage population growth dynamics.

To our knowledge, none of the previous studies investigated the direct and indirect effects of near-future pCO_2 levels on zooplankton. Only Rossoll et al. (2012) conducted a full factorial experiment testing direct and indirect high pCO_2 effects on copepods. However, their work suffers from the fact that copepods did not contain any long chain fatty acids in acid treatments although they were provided by the algae (at decreased amounts). This points to an unexplained lack of feeding rather than an effect of food quality. Our results therefore bring important new knowledge

on the impact of ocean acidification on copepods. Further, our study contradicts the conclusions of the few existing experimental studies on microzooplankton which found no effects of increased pCO_2 (Suffrian et al. 2008; Rose et al. 2009; Aberle et al. 2013). However, the major focus of those studies was on the direct effects of increased pCO_2, which, as we identified here, does not impact microzooplankton. Although we expected that the direct effect of high CO_2 would be most pronounced when the grazers were feeding on low algal quality, the interaction between these two treatments did not alter zooplankton performances. This could be the result of an elevated energy expenditure enabled by higher algal energy content and should result in higher grazers' respiration rates. However, nauplii and copepodites respiration was only increased by low algal quality. As previously described by Schoo et al. (2013), we suggest that increased respiration rates represented a physiological response to excrete the excess C obtained from prey grown in high pCO_2 conditions, rather than a stress response to deal with low pH.

In this study, we found that direct pCO_2 effects on consumers seem to be of lesser importance than the associated decrease in algal quality. Several studies have investigated the direct effects of ocean acidification on zooplankton. While elevated pCO_2 does not seem to affect adult copepods, hatching rates are negatively affected by very high pCO_2 (Kurihara et al. 2004; Mayor et al. 2007; Cripps et al. 2014b). Further, the decrease of primary producers' quality under high pCO_2 conditions negatively affects zooplankton production and growth. However, the generality of this result remains uncertain, as community level dampening, such as species richness and complex trophic interactions, may compensate for low food quality (Rossoll et al. 2013). Indeed, the CO_2 effect in the one alga – one copepod species food chain in the study by Rossoll et al. (2012) vanished when the same zooplankton species fed on a semi-natural food mixture in mesocosms (Rossoll et al. 2013). Nevertheless, lower growth rates of zooplankton, as shown in this study, may lead to lower availability of food for the next trophic level and thus potentially affect the recruitment of higher trophic levels. Furthermore, quality effects have also been shown to travel up the food chains (Malzahn et al. 2007), and decreased algal quality may affect higher trophic levels as well.

Acknowledgments

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CHAPTER IV

On the evolutionary potential of the calanoid copepod *Acartia tonsa* towards ocean acidification: insights from a long-term laboratory selection study

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Abstract

The ongoing acidification process of the oceans is likely to have consequences for many marine biota. Although evolutionary responses are expected during persisting environmental change, little is known about the adaptability of copepods. Therefore, we set up a 3 ½ years long selection study, culturing Acartia tonsa populations in water treated with 200 and 800 µatm pCO_2, feeding them with algae grown in f/2 medium under 200 µatm pCO_2 and in f/2 -N-P medium under 800 µatm pCO_2. After three reciprocal transplant experiments we measured copepods' developmental rates, carbon to nutrient ratios, egg production and resulting hatching rates. Under high CO_2 conditions, stoichiometric discrepancies between the requirements of A. tonsa and its food resulted in a significantly decreased developmental rate independent from the selective history. After one year, these discrepancies appeared alleviated by an increased homeostasis of the copepods, indicating a high body stoichiometry regulation plasticity. Egg production and the overall hatching success was unaffected by the experimental conditions, however, results indicated a premature hatching of eggs from females with a high CO_2 selective history. Potentially due to the lack of a strong selective pressure, we did not detect any beneficial evolutionary adaptations of the copepods cultured under high CO_2 conditions. Towards the end of the experiment, copepods cultured under elevated pCO_2 and fed with high CO_2 algae (high energy, low nutrients), showed an increased body mass and decreased prosoma length. Such physiological changes could have profound long-term consequences for marine copepods, food web interactions, and ultimately ecosystem structures and functions.

Introduction

Since the industrial revolution, oceanic uptake of CO_2 from the atmosphere has resulted in a decrease of the ocean surface water pH by more than 0.1 units, representing the most rapid change in carbonate chemistry experienced in the past 20 million years (Caldeira and Wickett 2003; Pörtner et al. 2014). This decrease in both pH and the availability of free carbonate ions (CO_3^{2-}) affects above all calcifying organisms such as coccolithophores, foraminifera, echinoderms, molluscs and corals, as their ability to build up calcium shells and skeleton structures is directly affected by seawater carbonate chemistry (Riebesell et al. 2000; Langdon et al. 2003; Feely et al. 2004; Orr et al. 2005; Raven et al. 2005; Kleypas et al. 2006; Fabry et al. 2008). Direct effects on non-calcifying zooplankton taxa such as cnidaria (Attrill et al. 2007; Lesniowski et al. 2015) and crustaceans (Wittmann and Pörtner 2013) are rarer. Most experiments on adult copepods, for example, indicated low sensitivities to increased pCO_2 (decreased seawater pH) conditions (Kurihara and Ishimatsu 2008; Mayor et al. 2012; McConville et al. 2013). Only under pCO_2 pressures above 5000 µatm (which are unlikely to be reached in most parts of the oceans by the end of the century (Caldeira and Wickett 2003)), developmental rates, egg production, hatching rates and survival of copepods were negatively affected (Kurihara et al. 2004; Mayor et al. 2007; Niehoff et al. 2013; Hildebrandt et al. 2014; Pedersen et al. 2014). However, younger developmental stages seem to be more vulnerable to direct effects of increased seawater pCO_2 than older ones. Fitzer et al. (2012) observed a negative effect of increased pCO_2 (~ 600 µatm) on the nauplii production in *Tispe battagliai*. Furthermore, Cripps et al. (2014b) detected lethal effects on *Acartia tonsa* nauplii already under 1000 µatm pCO_2 concentrations. Contrary, in another experiment the developmental rate and egg production of *A. tonsa* was not affected by increased pCO_2 (~ 800 µatm) (Meunier et al. 2016). Conducted studies indicate that copepods' responses to ocean acidification (OA) are very much species- and stage-specific, with adult stages to be less sensitive.

Besides direct effects due to a lower seawater pH, increased CO_2 levels can also have indirect effects on herbivores through changes in stoichiometry (higher carbon (C) to nutrient ratios) and fatty acid composition of primary producers (Urabe et al. 2003; Rossoll et al. 2012; Verschoor et al. 2013; Meunier et al. 2016), which may be especially pronounced under nutrient limitation during e.g. phytoplankton bloom

periods (Bach et al. 2016). Since heterotrophs are more restricted in their elemental composition than autotrophs, stoichiometric discrepancies between predator and prey can diminish consumer growth, fecundity and survival (Sterner and Elser 2002; Bukovinszky et al. 2012). Indeed in previous studies, the copepod *Acartia tonsa* showed a decreased developmental rate (by nearly 50%) and reproduction (from 34 to 5 eggs female^{-1} day^{-1}) when fed algae cultured under high pCO_2 (~ 800 µatm) conditions (Rossoll et al. 2012; Schoo et al. 2013; Meunier et al. 2016).

Confronted with a changing environment, organisms can *inter alia* react with geographic range shifts, physiological acclimatisation and / or genetic adaptations (Kelly and Hofmann 2013). Studies on species with short generation times, for example on the coccolithophore *Emiliania huxleyi* (Lohbeck et al. 2012) and the microalgae *Chlamydomonas* (Collins and Bell 2004), revealed evolutionary adaptations to OA after 500 and 1000 generations, respectively. However, as most temperate copepod species have generation times of approximately 15 to 30 days (Peterson 2001) and are elaborate to culture, real-time investigations on acclimatisation and / or adaptation to environmental change are scarce. In a laboratory experiment Colin and Dam (2004) exposed the copepod *Acartia hudsonica* to a diet containing the toxic dinoflagellate *Alexandrium fundyense.* After three generations, copepods acclimatised or adapted to the food conditions, reflected by an improved ingestion and egg production of the adult females. Thus, although there is evidence that some species can adapt to altered environmental conditions within a short time scale, organisms with longer generation periods such as copepods often show phenotypic plasticity to compensate unfavourable changes in their environment (Dam 2013; Thor and Dupont 2015). This kind of flexibility allows species to persist in the face of climate change and gives populations time to adapt to new environmental settings (Chevin et al. 2010). Additional, as an extension of phenotypic plasticity, transgenerational effects such as epigenetic inheritance are observed among organisms. By, for example, the transmission of nutritional, somatic, cytoplasmatic or epigenetic material, parents can significantly affect the performance (survival and fitness) of the following generation (Bonduriansky and Day 2009). Transgenerational carry-over effects of marine organisms exposed to OA were for example found in fishes (Munday 2014), oysters (Parker et al. 2012), sea urchins (Dupont et al. 2013) and copepods (Vehmaa et al. 2012; Cripps et al. 2014a; Thor and Dupont 2015). In the study of Thor and Dupont (2015), populations of the

copepod *Pseudocalanus acuspes* were cultured for two generations under 400, 900 and 1550 µatm pCO_2. Reciprocal transplant experiments showed that the investigated fecundity decrease under higher pCO_2 conditions was reversible and thus an expression of phenotypic plasticity. Furthermore, due to transgenerational effects the reduction in fecundity of the offspring was lower if the parents were cultured in a high pCO_2 environment. Further, based on their investigations of the effects of simulated OA on *Acartia* sp. during a mesocosm experiment in the Baltic, Vehmaa et al. (2016) suggested maternal effects as important components defining phenotypic buffering of copepods against OA. However, as the mesocosm experiment only lasted 45 days, potential long-term effects remained unknown. Although adaptive responses of various marine taxa to OA have been observed in several studies, long-term studies covering several generations are still lacking.

Evolutionary processes can affect inter- and intraspecific interactions within the food web and, at a larger scale, alter the marine ecosystem (Thompson 1998; Matthews et al. 2011). In the context of stoichiometry, rapid evolution can affect the elemental balances (C, N, P) of organisms, thereby not only influencing population and community dynamics but also ecosystem functions such as nutrient cycling (Declerck et al. 2015; Yamamichi et al. 2015). As copepods (zooplankton) are indispensable to the functioning of pelagic food webs and contribute to many ecosystem services (Bron et al. 2011), their reaction to stressors is particularly relevant. As a cosmopolitan primary consumer *A. tonsa* plays a key role in marine communities, transferring energy captured by phytoplankton to higher trophic levels. Changes in the performance (e.g. reproduction, development) or physiology (e.g. body stoichiometry) of such a primary consumer could have far reaching consequences for ecosystem structures and functions.

In our long-term selection study, we investigated the combined direct (lower water pH) and indirect (changed food quality) effects of elevated pCO_2 on laboratory populations of the calanoid copepod *A. tonsa* and its potential to acclimatise and / or adapt to increased pCO_2 conditions accompanied with a reduced food quality. We set up a 3 ½ years long selection study, culturing *A. tonsa* populations in water aerated with 200 and 800 µatm pCO_2 concentration, feeding them with algae, grown in f/2 medium under 200 µatm pCO_2 and in f/2 -N-P medium under 800 µatm pCO_2. We hypothesised that the high CO_2 selection line (SL) populations should adapt to the high CO_2 conditions of increased seawater pCO_2 and associated food quality

reduction, leading to an increased fitness under high CO_2 conditions compared to the low CO_2 SL copepods. To detect possible adaptations of the high CO_2 SL populations, we carried out two different reciprocal transplant experiments, which were repeated at an interval of around one year. In those transplant experiments, we investigated the performance of the high and low CO_2 SLs in their original and experimental CO_2 treatment, comparing several fitness parameters (developmental rate, egg production and hatching rate) and their elemental body stoichiometry. Furthermore, we investigated possible changes in the body size of *A. tonsa* by measuring the prosoma length of adult females throughout the long-term experiment. To exclude effects of genetic drift (e.g. bottleneck effects) that could reduce the genetic diversity and thus the physiological and genetic adaptability of our experimental populations, we regularly reviewed the genetic diversity of the selection lines by analysing the neutral gene region cytochrome-c-oxidase I (COI).

Methods

Long-term CO_2 selection experiment

In September 2013, the long-term CO_2 SLs of *A. tonsa* were started using a random selection of eggs from our copepod cultures already present in the laboratory to avoid running the risk of different initial conditions between the selection lines and replicates. The CO_2 SLs ran 41 months, which represents around 80 generations based on the investigations of Peterson (2001). Figure 1 shows a schematic illustration of the experimental setup and time line.

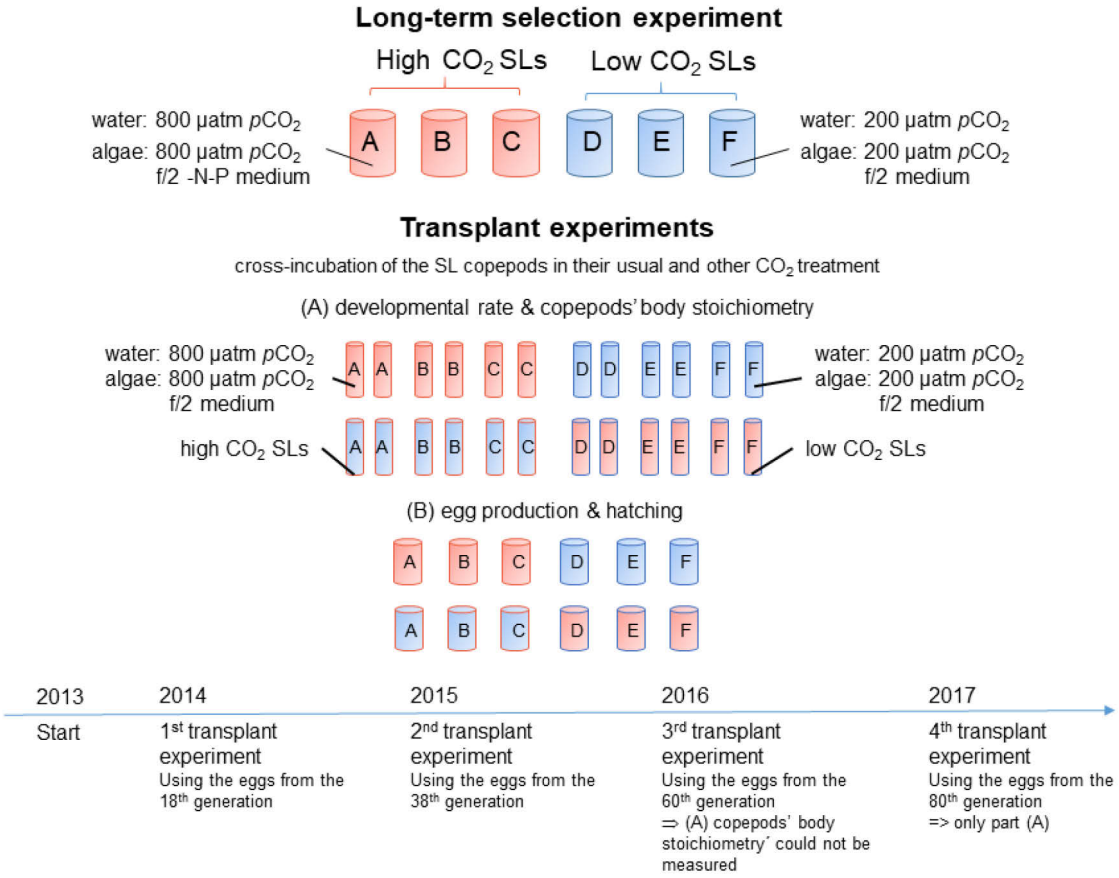

Fig. 1 Schematic illustration of the experimental setups and overall time line.

Long-term CO_2 selection experiment – *A. tonsa* selection lines

The copepods were cultured in artificial sterile and nutrient-free seawater (ASW; Aquamarin, Söll GmbH, Hof, Germany) at a salinity of 32, in 100 L plastic tanks, at 18°C. Since *A. tonsa* is photosensitive (Stearns and Forward 1984), copepods were kept in darkness. The water of the SL tanks was exchanged as required (~ every second month). The copepod densities were not managed, and subject to natural fluctuations, varying between 150 and 300 individuals L^{-1}. For the cultivation of *A. tonsa*, densities ranging from 100 to 600 adults L^{-1}, still allow a steady egg production and high egg viability (Jepsen et al. 2007). The experiment consisted of two pCO_2 SLs with three replicates each. Nominal target pCO_2 values used to fumigate the seawater in the SL tanks were 800 µatm (tank A, B, C) and 200 µatm (tank D, E, F). With a stable inflow of the desired CO_2/air mixture (aeration) and a plastic cover on the water surface to avoid outgassing a stable pH-level was assured. The CO_2 content and flow rate of the gas/air mixture was continuously monitored by

a sensor (HTK Hamburg) and automatically adjusted. The water pH of the SL tanks was controlled after cleaning activities or maintenance work on the gas mixing system using a ProLab 3000 pH meter with an IoLine pH combination electrode with temperature sensor (type IL-pHT-A170MFDIN-N). From 2015 on, we additionally started a weekly documented pH measurement of the SL tanks (Fig. 2).

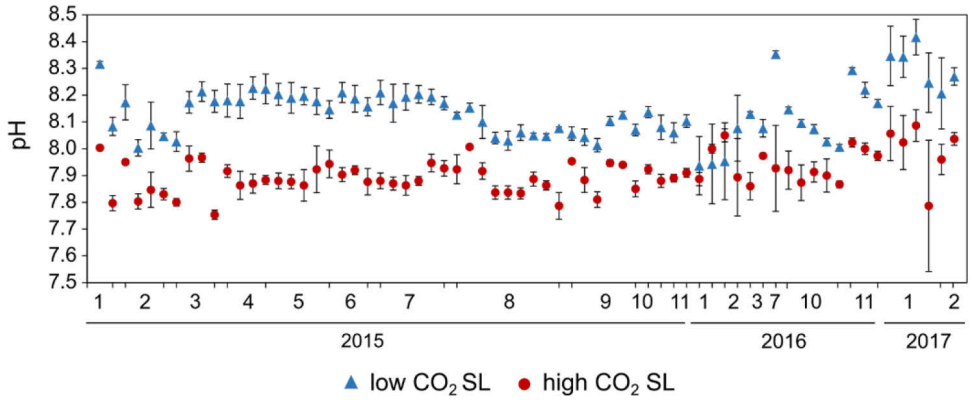

Fig. 2 Mean pH of the water in the selection line (SL) tanks. x axis: time points, numbers represent the month; y axis: mean pH; legend: CO_2 treatments of the SLs, (high CO_2) food and water manipulated with 800 µatm CO_2, (low CO_2) food and water manipulated with 200 µatm**.** Error bar = SD, data pooled from the three SL replicates (n = 3).

We aimed to lower our control treatment from today's pCO_2 level (400 µatm) to preindustrial conditions (200 µatm), to possibly maximise the difference between the experimental CO_2 treatments without exceeding the pCO_2 concentrations predicted for the end of the century (Intergovernmental Panel on Climate 2014). However, subsequent total alkalinity measurements illustrated, that the intended pCO_2 of 200 µatm could not be reached and instead slightly varied around 400 µatm pCO_2. To control the target pCO_2 values at the start of the long-term selection experiment we also measured the total alkalinity (TA) of the water from each SL tank. The SL tanks D, E and F aerated with 200 µatm pCO_2 had a TA (and pH) of 3102 µmol kg^{-1} ASW^{-1} (8.09), 2962 µmol kg^{-1} ASW^{-1} (8.06) and 3060 µmol kg^{-1} ASW^{-1} (8.12), respectively. For the SL tanks A, B and C aerated with 800 µatm pCO_2 a TA (and pH) of 3041 µmol kg^{-1} ASW^{-1} (7.87), 3106 µmol kg^{-1} ASW^{-1} (7.86) and 3082 µmol kg^{-1} ASW^{-1} (7.49) was measured, respectively. TA was determined by open-cell duplicate potentiometric titration and calculation with modified Gran plots (Bradshaw et al. 1981), using a TitroLine alpha plus titrator with an IoLine pH

combination electrode with temperature sensor (type IL-pHT-A120MF-DIN-N). Measured values were corrected using certified reference material (CRM, Batch No. 104, Scripps Institution of Oceanography, USA). For the calculation of the final pCO_2 the online program CO2calc version 1.3.0 (Robbins et al. 2010) was used, utilizing the dissociation constants of carbonic acid of Mehrbach et al. (1973) and Hansson (1973), refitted by Dickson and Millero (1987), and the dissociation constants for H_2SO_4 from Dickson (1990). The back calculated pCO_2 concentrations of the low CO_2 SL tanks (D, E, F) were 495, 512 and 450 µatm pCO_2 and of the high CO_2 SL tanks (A, B, C) 871, 913 and 838 µatm pCO_2, respectively. Additional alkalinity and pH control measurements of the water from one low CO_2 SL tank (D) and one high CO_2 SL tank (A) in 2016 resulted in a TA (and pH) of 2826 µmol kg^{-1} $water^{-1}$ (8.13) and 3093 µmol kg^{-1} $water^{-1}$ (7.86), respectively. The back calculated pCO_2 concentrations were 403 µatm for the low and 909 µatm for the high pCO_2 treatment. As the CO_2 content and flow rate of the gas/air mixture introduced in the SL tanks was continuously monitored by a sensor and automatically adjusted, there was no reason for additional TA measurements. Additionally, a change in the pCO_2 inflow would have led to a change in the water pH, which was measured regularly.

To measure copepods' prosoma length and to investigate possible genetic changes, we took two samples from each tank every second week, by pulling a small sieve through the water column (collecting ~ 50 individuals). For the genetic analyses and prosoma length measurements individuals were preserved in 96% ethanol and sodium tetraborate buffered formaldehyde (4%), respectively. For the determination of possible prosoma length differences due to the CO_2 treatments ~ 20 adult females were taken from formaldehyde samples, rinsed with tap water (2–3 min) and measured under a stereo microscope (SZX16) using the CellSens Dimension software version 1.6 (Olympus). Prosoma lengths were measured from 19 time points.

To analyse the genetic variability of the *A. tonsa* start population and to observe possible changes in the haplotype frequencies over the experimental period, the marker region COI was analysed. The DNA of five individuals per SL tank (A-F) from five time points (t1: 11.10.2013 A-F; t2: 06.12.2013 D-F, 08.01.2014 A-C; t3: 17.03.2014 A-F, t4: 15.04.2015 A-F, t5: 01.06.16 A-F) were isolated (ISOLATE II Genomic DNA Kit, Bioline) and amplified. The master mix consisted of 2.5 mM $MgCl_2$, 1 mM 10x PCR buffer, 0.2 mM dNTPs, 0.2 mM each primer (HCO2198_t1

(5`-CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAA-3`), LCO1490_t1 (5`-TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGA-3`) (Messing 1983; Folmer et al. 1994)) and 0.5 mM taq polymerase in a total volume of 20 µL with 1 µL template DNA. The amplification conditions for the PCR reaction were 2 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 45 °C and 1 min 30 s at 72 °C and a final elongation for 7 min at 72 °C. The amplification success was controlled via agarose gel electrophoresis. Positive PCR products of the predicted length and purity were sequenced. Single sequences were edited and aligned in Geneious 7.0.4 and used to calculate a haplotype network (median joining) in PopART 1.7. Statistics of DNA polymorphism were done in DnaSP version 5.10.01. Additionally, we calculated the frequency of each haplotype for the ambient and high CO_2 treatment at the different time points throughout the experimental period.

To ensure the persistence of the long-term experiment, eggs were collected every month and stored at 4°C. In case of the death of a SL population, those eggs were used to restart the specific replicate. Over the whole experiment, we only needed to restart the 200 µatm pCO_2 SL tanks from the collected eggs once, after one year.

Long-term CO_2 selection experiment - food cultures

Two 5 L chemostat cultures of the algae *Pyrenomonas salina* (EPSAG: Experimental Phycology and Culture Collection of Algae at the University of Göttingen, Germany) were grown under a 16h:8h light:dark regime (185 µmol m^{-2} s^{-1}) and stirred continuously. Chemostat cultures were continuously supplied with f/2 medium (Guillard and Ryther 1962), at a rate of 500 mL per day, resulting in a dilatation rate of 10% d^{-1}. Once per month we cleaned the chemostats and setup new algae cultures. For the preparation of the f/2 medium we used sterile filtered sea water (CA, pore size 0.2 µm, Sartorius) as it would have been an excessively effort to always keep a sufficient amount of ASW in stock. The chemostat cultures were aerated with the same 800 µatm or 200 µatm pCO_2 air as the copepod tanks. To prepare the food for the low CO_2 SL copepods, 500 mL of the 200 µatm pCO_2 chemostat culture were mixed with 2.5 L f/2 medium. For the high CO_2 SL copepods, 500 mL of the 800 µatm pCO_2 algae culture were mixed with 2.5 L f/2 -N-P medium (f/2 recipe without NaH_2PO_4 and 80% less $NaNO_3$). All algae mixtures were aerated with 800 µatm or 200 µatm pCO_2 and were grown for three days. After this time, algae entered the stationary phase (indicated by a yellowish colour) and were used as food.

SL copepods were fed *ad libitum* with 1 L of the corresponding algae culture, every day. Based on a mean density of the algae food culture of 14 x 10^5 cell mL^{-1} (calculated based on the density measurements of the algae food cultures during the transplant experiments) and a mean copepod density of 25,000 individuals per tank, ~ 350 mL algae culture would have been sufficient to reach a food supply of 20,000 cells ind^{-1}, which is recommended for *A. tonsa* in the literature (Marcus and Wilcox 2007).

We specifically chose to manipulate the medium of the high CO_2 SL food algae and thereby accelerating their stoichiometric change for several reasons. As algae take up nutrients from the surrounding water, a less intense stoichiometric change would have made it necessary to clean the tanks daily to assure constant food qualities (algae stoichiometry). Obviously, this would not have been possible during the entire long-term experiment. Therefore, it was necessary to exacerbate the nutrient stoichiometry of the high CO_2 food algae to ensure that the differences in food qualities between the CO_2 SL tanks persisted. Furthermore, as many studies on copepods have suggested that the indirect effects of OA are more important than the direct ones (Rossoll et al. 2012; Schoo et al. 2013; Verschoor et al. 2013; Meunier et al. 2016), especially under realistic scenarios, it was essential to make sure that food quality changes were included in the selection experiment. We specifically chose this approach, as we were not interested in a distinction between direct and indirect effects of increased pCO_2 on copepods, but rather in the combined effect.

Transplant experiments

Based on the long-term selection experiment we carried out two different short-term reciprocal transplant experiments (A, B) which were repeated three to four times at an interval of at least one year to investigate possible adaptations of the SLs to the CO_2 treatments. For reasons of time, a denser implementation of transplant experiments was not possible. With transplant experiment (A) we investigated the developmental rate and the elemental body composition of the copepods (C : N; C : P). The egg production and hatching rate, as further fitness parameters were measured in transplant experiment (B). The transplant experiments were carried out using the eggs produced by the copepods of the long-term selection experiment of roughly the 18^{th} generation in May 2014, the 38^{th} generation in April 2015 and the 60^{th} generation in April 2016. Due to a high nauplii mortality during the transplant

experiments in 2016, we repeated transplant experiment (A) in January 2017 using the eggs of the 80th copepod generation. All transplant experiments consisted of 4 treatments with 3 replicates and two additional replicates within the SLs. Thereby the two CO_2 SLs (high CO_2 A, B, C; low CO_2 D, E, F) were cross incubated under their usual and other CO_2 condition and fed with the corresponding CO_2 manipulated algae (Fig. 1).

Transplant experiments - food cultures

For the transplant experiments, we set up *P. salina* stock cultures grown in f/2 medium under ambient aeration. To ensure that the copepods were fed with equal algal stoichiometric qualities every day, precultures were prepared daily from the stock culture at a start concentration of 10 x 10^5 cells mL^{-1}. After one day, the precultures were used to prepare the food cultures started at concentrations of 7.5 x 10^5 cell mL^{-1} and aerated with either 200 µatm or 800 µatm pCO_2. After approximately five days, algae entered the stationary phase (indicated by a yellowish colour) and were used as food. During the transplant experiments we took filter samples of the food algae (at least every 2nd day), which were analysed after the experiment to investigate the C : N and C : P ratios. For the C and N measurements, 4 x 10^6 cells *P. salina* were filtered onto precombusted GF/F filters (Whatman) and analysed using a vario MICRO cube CHN analyzer (Elementar). The P content of the algae was measured photometrically (Hansen and Koroleff 2007). The cell concentrations were determined with a CASY cell counter (Schärfe System CASY Cell Counter and Analyzer System).

Transplant experiment (A) – *A. tonsa* cultures

In transplant experiment (A), the developmental rate of the nauplii was investigated. Eggs of the long-term SLs were collected at one day and incubated for two days in ASW at 18°C for hatching. Afterwards, 1500 nauplii were transferred into 1 L Schott incubation bottles, filled with ASW and connected to the CO_2 aeration system. One half of each SL was gently aerated with 200 µatm and the other half with 800 µatm pCO_2 (24 bottles in total). In correspondence with recommendations from literature (Marcus and Wilcox 2007), copepods were fed with 20,000 cells ind^{-1} day^{-1}, ensuring a sufficient food supply. For space reasons incubation bottles and food cultures needed to be placed in the same room. Therefore, we covered the incubation bottles

with a light-diminishing foil, to avoid further algae growth and to reduce the stress for the light sensitive copepods. To minimise changes in the nutrient composition of the algae by animal waste products, the water was exchanged with CO_2 preconditioned (overnight) ASW every day before feeding. For the transplant experiment in 2017 we used 3000 nauplii and 2 L incubation beakers. Furthermore, the incubation beakers were not directly aerated with CO_2. Instead, to further minimize the disturbance and mortality of the copepods, we aerated the headspace of the incubation beakers with the appropriate CO_2 concentrations.

To control the target pCO_2 of the preconditioned ASW, total alkalinity (TA) and pH were measured during the first transplant experiment in 2014. The ASW aerated with 200 µatm pCO_2 had a TA (and pH) of 2958 µmol kg^{-1} ASW^{-1} (8.43), for the water preconditioned with 800 µatm pCO_2 a TA (and pH) of 2951 µmol kg^{-1} ASW^{-1} (8.00) was measured. The back calculated pCO_2 concentration of the 200 µatm and 800 µatm pCO_2 treatments of the ASW were 180.98 and 615.23 µatm pCO_2, respectively. The mean pH of the ASW within the incubation bottles during the transplant experiments in 2015, 2016 and 2017 did not differ from the ones in 2014 and were 8.59 (±0.16) for the 200 µatm and 8.17 (±0.14) for the 800 µatm pCO_2 treatment (data pooled from n = 18 measurements; 4 per CO_2 treatment and year). Further TA measurements in 2015, 2016 or 2017 were not done because there was no reason to assume that the pCO_2 pressure in the incubation bottles of the both CO_2 treatments could have been significantly different from 2014, as copepods were incubated in ASW and pH values did not differ.

After ten days of incubation the stage distribution of the copepods was investigated. The individuals from the incubation bottles were fixed in acidic Lugol's solution and the developmental stage of at least 60 individuals per incubation bottle was determined. The arithmetic mean of the counted stages of each incubation bottle was calculated, averaged over the replicates, and divided by the number of incubation days to obtain the developmental rate. Furthermore, in 2014, 2015 and 2017, the C, N, P content of the copepods from each incubation bottle was measured and the C to nutrient ratios were calculated. Measurements were done with the same device as used for the algae samples. The exact number of analysed individuals can be found in the raw data, which is available in the PANGAEA repository. In 2016 a measurement of the copepods' body stoichiometry was not possible due to too low individual numbers after the transplant experiment.

Transplant experiment (B) – *A. tonsa* cultures

To investigate possible adaptations of the SL individuals to the CO_2 treatments in terms of egg production and hatching success, another transplant experiment (B) was carried out in 2014, 2015 and 2016. Eggs from the different CO_2 SLs, taken at the same day as for transplant experiment (A), were separately placed in 5 L beakers, filled with ASW, for hatching and development (6 beakers). To prevent shock reactions, copepods were kept in those beakers for 14 days, at 18°C, without light and gentle aeration until they reached adulthood. During this time, copepods were fed *ad libitum* with *P. salina*, cultured in f/2 medium. Afterwards the transplant experiment was carried out for four days. Each culture was split and each half was poured into a 5 L beaker with ASW and covered with a light-diminishing foil (12 beakers in total). The subsequent CO_2 treatment, water exchange and feeding procedures were equivalent to transplant experiment (A). After the incubation 18 females from each beaker were picked and separately placed into 6-well-plates containing ASW and ~ 20,000 cells *P. salina*. After 24 h we counted the number of produced eggs and removed the females from the wells to avoid cannibalism (Boersma et al. 2014). The number of hatched nauplii was counted after 24 h and 48 h.

Statistical analyses

All statistical analyses were carried out using the program STATISTICA version 9.1 (© StatSoft, Inc). In the analyses each high (A, B, C) and each low CO_2 (D, E, F) SL replicate as well as additional replicates within SLs were pooled. For the evaluation of the C, N, P and prosoma length measurements, we calculated whisker plots to identify outliers and extreme values. Values outside the 1.5 upper and lower interquartile range, the calibration range or negative values were not considered.

Results

Long-term CO_2 selection experiment – *A. tonsa* selection lines

Over the experimental period the mean pH in the low CO_2 SLs was 8.14 ±0.11 and in the high CO_2 SLs 7.91 ±0.09 (Fig. 2).

The prosoma length of the CO_2 SL copepods changed significantly over the experimental period (Repeated Measurement ANOVA using prosoma length as

dependent and SL as independent factor; SL and SL*time: $p < 0.001$; time: $p = 0.285$). Until August 2016 the prosoma length between the SL copepods was similar, with a mean length of 867.44 µm (± 17.95) and 855.73 µm (± 17.95) of the low and high SL copepods, respectively (Fig. 3). However, in the last half year of the experiment the prosoma length of the SL females started to differ, with a decreased prosoma length of the high CO_2 SL females (low CO_2 SLs: 887.37 µm ± 10.29, high CO_2 SLs: 812.23 µm ± 14.03; data pooled from the last three time points, Fig. 3).

The sequencing of the COI gene region of the DNA from the 150 individuals resulted in an alignment including 129 high quality sequences of 442 bp length. The genetic analyses identified four polymorphic segregating sites resulting in two main and two less frequent substitution haplotypes (Fig. 4). Over three years experiencing different CO_2 conditions there were no significant differences or changes in the composition and /or frequency of the haplotypes among the CO_2 SLs (two-way ANOVA using haplotype as dependent and date and SL as independent factors; date, SL and combined effects: $p > 0.05$).

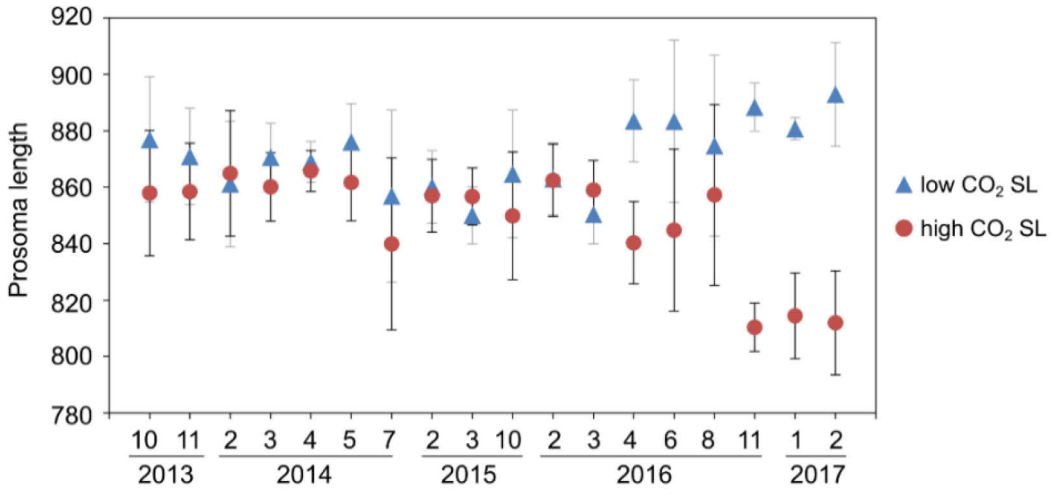

Fig. 3 Prosoma length differences of *A. tonsa* between the selection lines (SLs) during the long-term experiment. x axis: time points, numbers indicate the month; y axis: mean prosoma length of 6 to 49 adult females [µm]; legend: CO_2 treatments of the SLs, (high CO_2) food and water manipulated with 800 µatm CO_2, (low CO_2) food and water manipulated with 200 µatm. Error bar = SD, data pooled from the three SL replicates ($n = 3$) with $n = 30$ (± 14) measurements SL^{-1}.

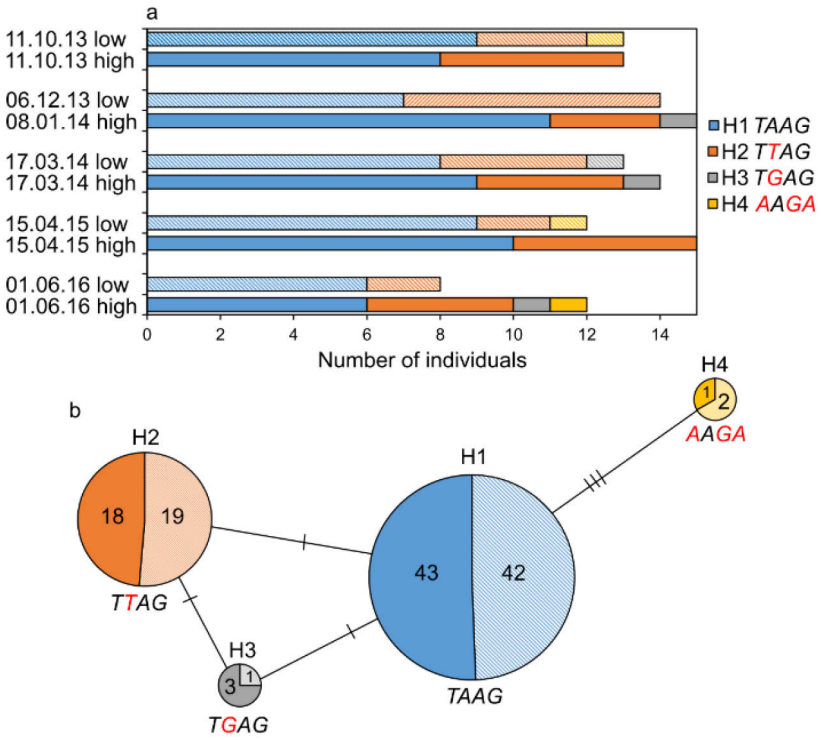

Fig. 4 (a) Haplotype frequencies of *A. tonsa* individuals from the CO_2 SL tanks based on COI sequences. x axis: number of individuals per haplotype (haplotype frequencies); y axis: time points and CO_2 treatments of the SLs, high = food and water manipulated with 800 µatm CO_2, low = food and water manipulated with 200 µatm; legend: haplotypes (H1, H2, H3, H4; bases varying between the subsitution sites among the haplotypes are written in red). (b) Haplotype network of *A. tonsa* individuals from the CO_2 SL tanks based on COI sequences. Haplotype names are given in text above the pies (H1, H2, H3, H4), number of substitutions between haplotypes are indicated by the number of dashes between the pies, numbers on pies represent the number of associated individuals.

Transplant experiments

Developmental rate of *A. tonsa*

In all transplant experiments except the one in 2016, the developmental rate of the copepods in the high CO_2 treatment was significantly reduced independent from the prior experienced CO_2 SL conditions (Fig. 5 and Fig. 6, two-way ANOVA using developmental rate day^{-1} as dependent and SL and treatment as independent factors; treatment: $p < 0.001$ (2014), $p < 0.05$ (2015), $p = 0.089$ (2016), $p < 0.05$ (2017); SL and treatment*SL: $p > 0.05$). After ten days of incubation, 60 - 75% of the copepods which developed under low CO_2 conditions reached the late copepodite stages C4 - C6, whereas under high CO_2 conditions only 5 - 7% (2014), 41 - 49% (2015),

44 - 55% (2016) and 44 – 48% (2017) of the copepods reached those stages (Fig. 6). Furthermore, from 2015 on copepods of both SLs developed significantly faster under high CO_2 conditions compared to 2014 (Fig. 5 and Fig. 6, one-way ANOVA using developmental rate day^{-1} as dependent and year as independent factor; $p < 0.05$). Over the years, we did not observe differences in the developmental rate between the high and low CO_2 SL individuals, nor did we observe significant interactions between the CO_2 treatment and CO_2 SL conditions.

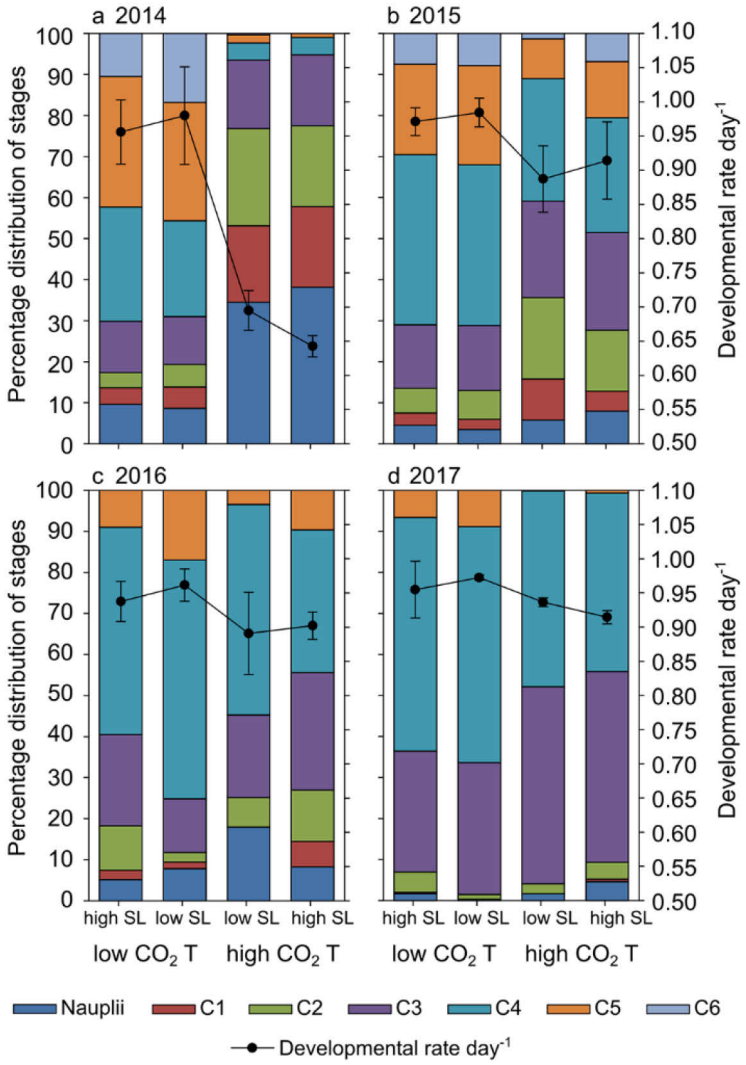

Fig. 5 Stage distribution of *A. tonsa* measured after the transplant experiments in (a) 2014, (b) 2015, (c) 2016 and (d) 2017. x axis: transplant combinations, (SL) selection line, (T) treatment, (high CO_2) food and water manipulated with 800 µatm CO_2, (low CO_2) food and water manipulated with 200 µatm CO_2; left y axis: percentage of the respective stages; legend: (Nauplii) nauplii stages N1 – N6, (C1, C2, C3, C4, C5, C6) copepodite stages; right y axis: developmental rate day^{-1}. Error bar = SD, data pooled from the three SL replicates (n = 3).

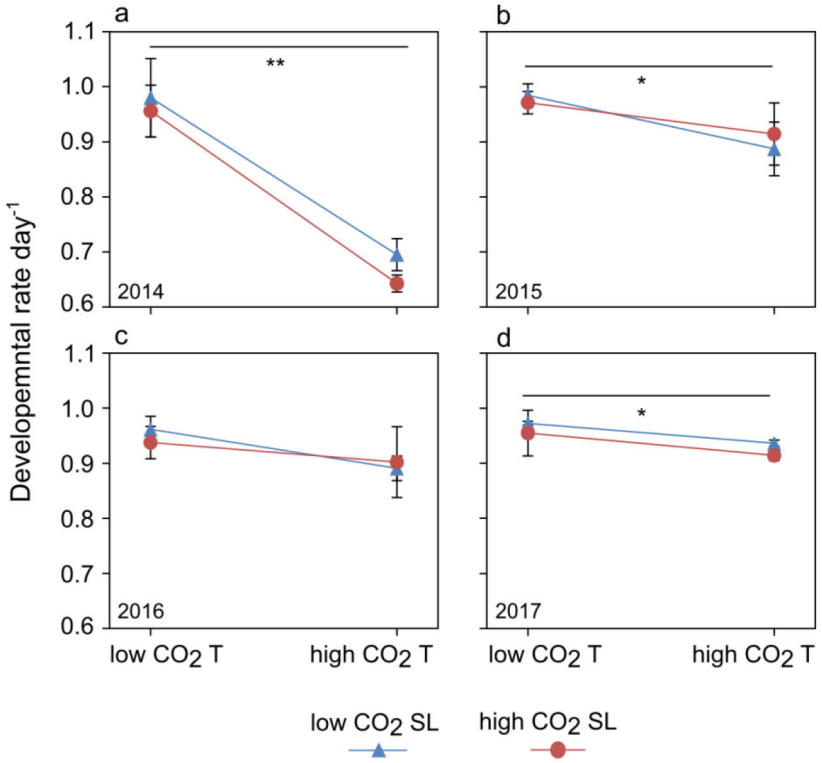

Fig. 6 Developmental rate of *A. tonsa* during the transplant experiments in (a) 2014, (b) 2015, (c) 2016 and (d) 2017. x axis: CO_2 treatments of the transplant experiments (T) treatment, (high CO_2) food and water manipulated with 800 µatm CO_2, (low CO_2) food and water manipulated with 200 µatm CO_2; y axis: developmental rate day^{-1}; legend: CO_2 treatments of the selection lines (SLs), (high CO_2) food and water manipulated with 800 µatm CO_2, (low CO_2) food and water manipulated with 200 µatm. Asterisks indicate significant differences (two-way ANOVA using developmental rate day^{-1} as dependent and SL and T as independent factors (**) = $p < 0.001$, (*) = $p < 0.05$), error bar = SD, data pooled from the three SL replicates ($n = 3$).

Cell and body stoichiometry of *P. salina* and *A. tonsa*

The C content, and consequently the C : N and C : P ratios of the algae, were significantly higher under high CO_2 conditions compared to the low CO_2 conditions (Table 1). Furthermore, the high CO_2 algae cultures contained significantly less N compared to the low CO_2 cultures. In the transplant experiment in 2014, the C : N and C : P ratios as well as the C content of the copepods from the high CO_2 treatment were significantly higher compared to those from the low CO_2 treatment, independent from the prior experienced CO_2 SL conditions (Table 2 and Fig. 7). In 2015, copepods from the high CO_2 treatment had again a significantly higher C : N ratio compared to the copepods, from the low CO_2 treatment. The C : P ratio and C

content of the copepods were, however, similar between CO_2 treatments. Furthermore, as in 2014, we observed no differences between the SLs.

Table 1 Stoichiometric differences of the algae food cultures between the CO_2 treatments in 2014, 2015 and 2016.

C to nutrient ratios			
	200	**800**	***p***
C : N 2014	9.16 (1.39)	12.61 (1.09)	**< 0.001**
C : N 2015	6.08 (1.59)	14.69 (3.84)	**< 0.001**
C : N 2016	6.84 (0.44)	14.94 (0.67)	**< 0.001**
C : N 2017	9.85 (0.69)	15.46 (1.02)	**< 0.001**
C : P 2014	220.7 (41.63)	267.48 (51.65)	**< 0.05**
C : P 2015	196.65 (79.99)	309.55 (153.49)	**< 0.001**
C : P 2016	351.88 (33.70)	476.06 (27.23)	0.231
C : P 2017	246.97 (47.26)	414.63 (59.37)	**< 0.001**
C, N, P [pg cell^{-1}]			
C 2014	67.2 (9.6)	84.6 (8.0)	**< 0.001**
C 2015	55.5 (7.6)	88.8 (8.8)	**< 0.001**
C 2016	41.26 (2.8)	67.2 (5.5)	**< 0.001**
C 2017	69.31 (7.32)	99.51 (10.18)	**< 0.001**
N 2014	8.6 (0.9)	7.8 (0.7)	**< 0.05**
N 2015	10.1 (1.9)	7.4 (1.5)	**< 0.001**
N 2016	6.1 (0.5)	5.1 (0.7)	0.647
N 2017	8.21 (0.6)	7.5 (0.4)	**< 0.05**
P 2014	0.79 (0.06)	0.80 (0.13)	0.594
P 2015	0.72 (0.26)	0.78 (0.30)	0.963
P 2016	0.30 (0.02)	0.34 (0.13)	0.630
P 2017	0.74 (0.10)	0.63 (0.10)	0.089

p-values are given for treatment (T) effects (students-t-test using C, N, P concentrations and C to nutrient ratios as independent variables). Standard deviations are given in brackets, data pooled from $n = 12$ (2014), $n = 22$ (2015), $n = 5$ (2016) and $n = 7$ (2017) measurements.

In 2017, the C and N content of the copepods as well as the resulting ratio were significantly different between the CO_2 treatments, with a higher C and N content of the copepods (both SLs) from the high CO_2 treatment (Table 2 and Fig. 8). Thereby, the C and N content was further increased in those individuals having a high CO_2 selective history (Table 2 and Fig. 7 - 8). Furthermore, the increased C content of the high CO_2 SL individuals also affected their C : P ratios, which were significantly

higher in both CO_2 treatments compared to the control SLs (Table 2 and Fig 7). It was further noticeable that in 2015 and 2017 the C : N ratios of the copepods (both SLs) from the high CO_2 treatment were significantly lower compared to 2014 (students-t-test with C : N ratio as independent variable, $p < 0.001$; Table 2 and Fig. 7). In 2016, the body stoichiometry of the copepods could not be measured because there were not enough individuals left after the transplant experiment.

Table 2 Body stoichiometry of *A. tonsa* measured after the transplant experiments in 2014, 2015 and 2017.

C to nutrient ratios (molar)							
	200 SL 800T	**800 SL 800T**	**200 SL 200 T**	**800 SL 200T**	***p* SL**	***p* T**	***p* SL*T**
C : N 2014	9.14 (0.79)	8.69 (1.09)	5.57 (0.36)	5.99 (0.74)	0.970	**< 0.001**	0.235
C : N 2015	5.66 (0.61)	5.65 (0.44)	4.84 (0.38)	5.05 (0.37)	0.447	**< 0.001**	0.412
C : N 2017	6.87 (0.34)	7.02 (0.25)	6.29 (0.41)	6.19 (0.42)	0.885	**< 0.001**	0.477
C : P 2014	195.26 (52.22)	212.57 (80.74)	153.64 (43.93)	117.20 (54.77)	0.887	**< 0.05**	0.152
C : P 2015	143.02 (59.93)	144.89 (57.48)	147.60 (62.14)	116.47 (63.62)	0.350	0.446	0.292
C : P 2017	169.93 (43.90)	279.40 (55.07)	127.76 (46.81)	153.66 (43.28)	**< 0.05**	**< 0.05**	0.081
C, N, P [µg ind^{-1}]							
C 2014	3.47 (0.86)	4.10 (0.72)	2.43 (0.71)	2.04 (0.46)	0.714	**< 0.001**	0.119
C 2015	2.01 (1.34)	1.85 (1.09)	1.67 (1.02)	1.51 (0.83)	0.960	0.493	0.578
C 2017	3.46 (0.34)	4.86 (0.67)	1.96 (0.22)	2.89 (0.88)	**< 0.001**	**< 0.001**	0.402
N 2014	0.44 (0.11)	0.55 (0.07)	0.51 (0.14)	0.40 (0.09)	0.965	0.375	**< 0.05**
N 2015	0.42 (0.27)	0.37 (0.21)	0.46 (0.30)	0.39 (0.25)	0.421	0.680	0.946
N 2017	0.59 (0.04)	0.81 (0.09)	0.37 (0.06)	0.54 (0.14)	**< 0.001**	**< 0.001**	0.613
P 2014	0.04 (0.11)	0.05 (0.01)	0.04 (0.01)	0.05 (0.02)	0.397	0.754	0.804
P 2015	0.03 (0.02)	0.02 (0.01)	0.02 (0.01)	0.03 (0.01)	0.705	0.137	**< 0.05**
P 2017	0.06 (0.02)	0.05 (0.01)	0.04 (0.02)	0.05 (0.02)	0.978	0.420	0.194

CO_2 treatments of the selection lines (SLs) and during the transplant experiments (T), (800) food and water manipulated with 800 µatm CO_2, (200) food and water manipulated with 200 µatm; *p*-values are given for treatment (T) selection line (SL) and combined (SL*T) effects (two-way ANOVA using SL and treatment as independent factors and C, N, P concentrations and C to nutrient ratios as dependent factors); standard deviations are given in brackets; data pooled form $n = 6$ (2014), $n = 15$ (2015) and $n = 6$ (2017) measurements.

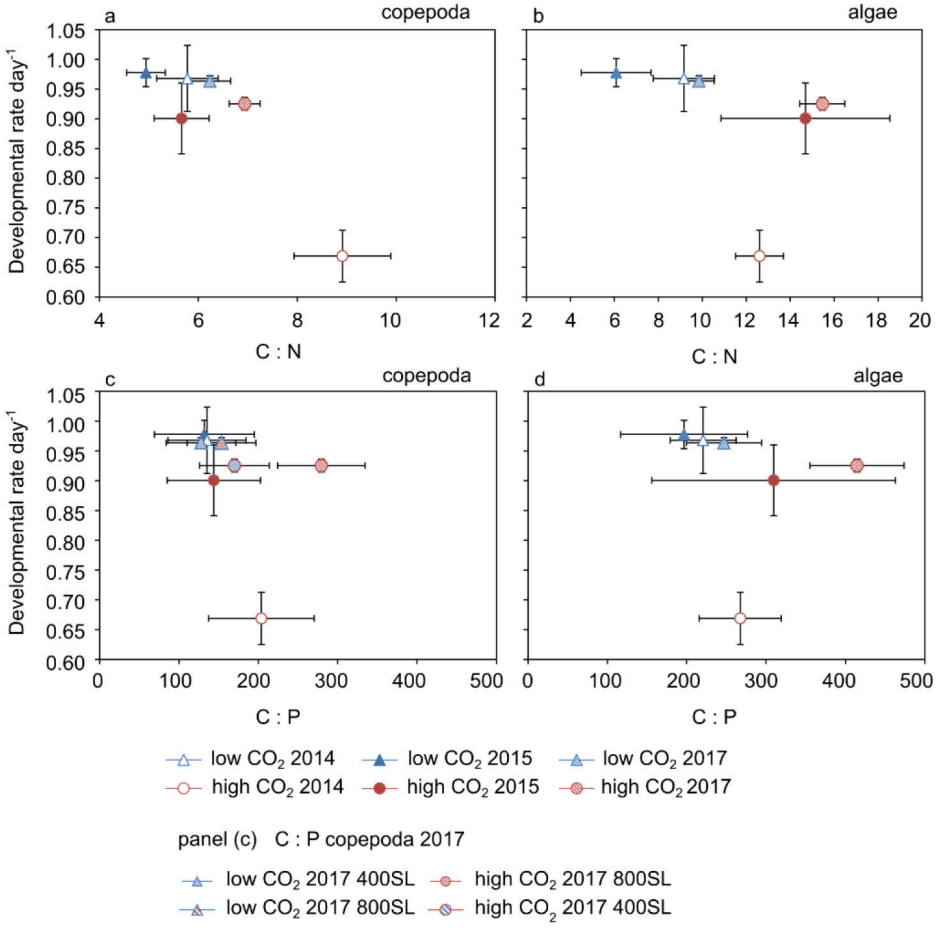

Fig. 7 Developmental rate of *A. tonsa* during the transplant experiments in 2014, 2015 and 2017 in dependency of the C : N and C : P ratios of (a, c) the copepods and (b, d) the food algae. x axis: C : N and C : P ratios; y axis: developmental rate day^{-1}; legend: CO_2 treatments, (high CO_2) food and water manipulated with 800 µatm CO_2, (low CO_2) food and water manipulated with 200 µatm CO_2; open, filled and shaded signs represent values from 2014, 2015 and 2017, respectively. Note, panel c: additional differentiation between the 400 and 800 pCO_2 SL copepods in 2017, due to significant different C : P ratios in the high CO_2 treatment. Error bar = SD, developmental rate: data pooled from the three SL replicates (n = 3); C : N ratios of the copepods: data pooled from n = 6 (2014), n = 15 (2015) and n = 6 (2017) measurements; C : P ratios of the copepods: data pooled from n = 6 (2014), n = 15 (2015), n = 3 (2017 high CO_2 SLs) and n = 3 (2017 low CO_2 SLs) measurements; C : N and C : P ratios of the algae: data pooled from n = 12 (2014), n = 22 (2015), n = 5 (2016) and n = 7 (2017) measurements.

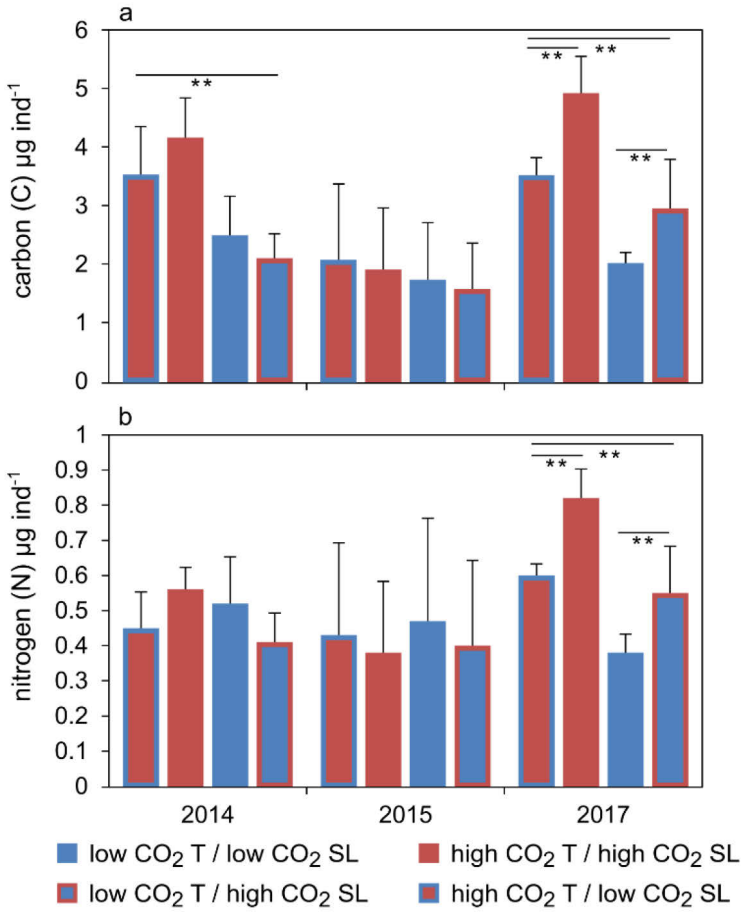

Fig. 8 Carbon (a) and nitrogen (b) content of *A. tonsa* measured after the transplant experiments in the years 2014, 2015 and 2017. x axis: years; (a) y axis: carbon (C) content [µg ind^{-1}]; (b) y axis: nitrogen (N) content [µg ind^{-1}]; legend: red bar = high CO_2 treatment (T) (food and water manipulated with 800 µatm CO_2), blue bar = low CO_2 treatment (food and water manipulated with 200 µatm CO_2), red frame = high CO_2 selection lines (SL), blue frame = low CO_2 SLs. Asterisks indicate significant differences between the SLs and treatments (two-way ANOVA using C and N concentrations as dependent factors and SL and treatment as independent factors (**) = $p < 0.001$), error bar = SD, data pooled form $n = 6$ (2014), $n = 15$ (2015) and $n = 6$ (2017) measurements.

Egg production and hatching success of *A. tonsa* females and eggs

The number of eggs produced in 2014 (14 ± 6 eggs female^{-1} day^{-1}; $n = 216$) and 2015 (7 ± 5 eggs female^{-1} day^{-1}; $n = 214$) did not significantly differ between the two CO_2 SLs and treatments (two-way ANOVA using eggs female^{-1} day^{-1} as dependent and SL and treatment as independent factors; SL, treatment and combined: $p > 0.05$). In 2016, we observed a similar egg production (8 ± 6 eggs female^{-1} day^{-1}; $n = 73$)

between the SL tanks B, C, D, E and F (n = 17, 12, 18, 12 and 14, respectively) under high CO_2 conditions (students-t-test with egg production as independent variable, $p > 0.05$). As the egg production of females from SL tank A (zero eggs, n = 18) was significantly different to the other high CO_2 SL replicates B (n = 17) and C (n = 12) (students-t-test with eggs female^{-1} day^{-1} as independent variable, $p < 0.001$), it was not considered for the calculation of the egg production in the high CO_2 treatment. In the low CO_2 treatment egg production was very low in all SL tanks (1 ± 1 eggs female^{-1} day^{-1}; n = 80). In 2014 and 2015 the percentage of hatched nauplii in the first 24 h was significantly different between the CO_2 SLs independent from the CO_2 treatment (Fig. 9, two-way ANOVA using the number of hatched nauplii after 24 h as dependent and SL and treatment as independent factors; SL: $p < 0.001$, treatment and combined: $p > 0.05$), with a higher hatching rate of the eggs from females with a high CO_2 selective history. In 2014, 76% (±27) of the eggs from females with a high CO_2 selective history and 55% (± 25) of the eggs from low CO_2 SL females hatched during the first 24 h. After 48 h, 90% (± 1) of the eggs from both SLs were hatched. In 2015, the percentage of hatched nauplii after the first 24 h differed only by 8% between the eggs from high and low CO_2 SL females. In 2016, we observed no significant differences between the SLs and CO_2 treatments (Fig. 9, two-way ANOVA; SL, treatment and combined: $p > 0.05$).

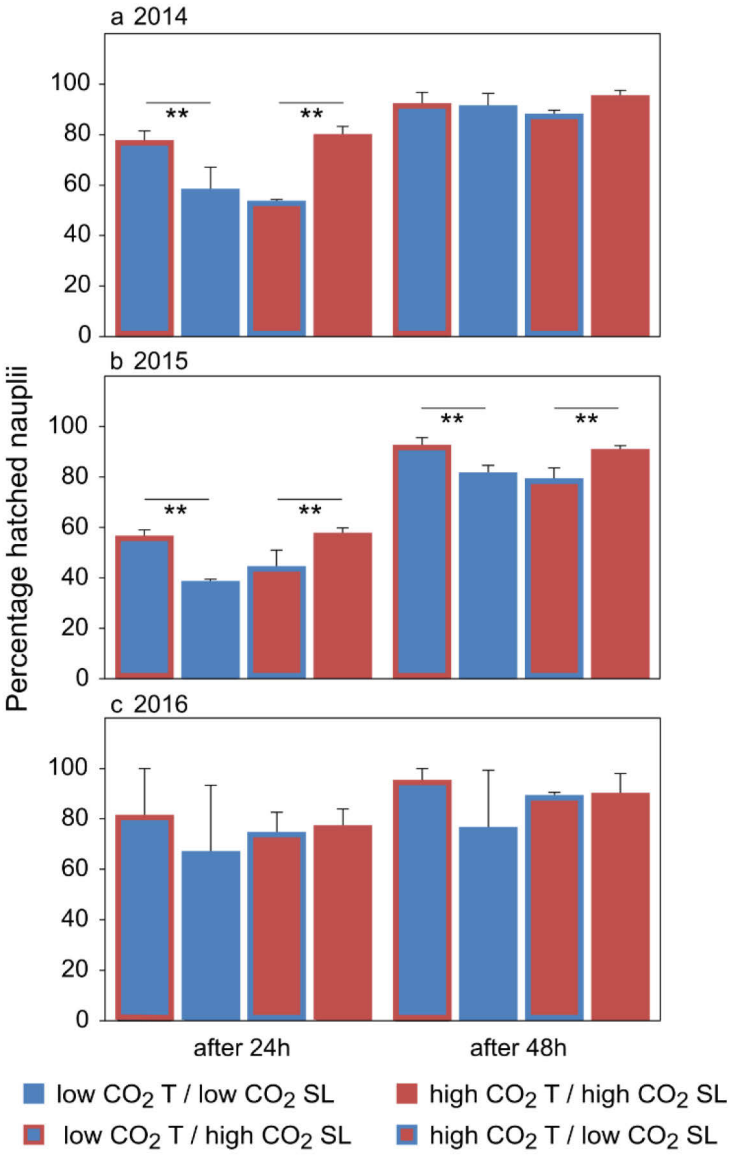

Fig. 9 Percentage of hatched nauplii measured after the transplant experiments in (a) 2014, (b) 2015 and (c) 2016. x axis: counting times of hatched nauplii, (after 24h) all nauplii hatched after 24 h, (after 48h) all nauplii hatched after 48 h; y axis: percentage of hatched nauplii; legend: red bar = high CO_2 treatment (T) (food and water manipulated with 800 µatm CO_2), blue bar = low CO_2 treatment (food and water manipulated with 200 µatm CO_2), red frame = high CO_2 selection lines (SL), blue frame = low CO_2 SLs. Asterisks indicate significant differences between the SL (two-way ANOVA using percentage of hatched nauplii as dependent and SL and treatment as independent factors (**) = $p < 0.001$, error bar = SD, data pooled form $n = 54$ (2014), $n = 48$ (2015: 200 SL 200 T), $n = 52$ (2015: 200 SL 800 T), $n = 51$ (2015: 800 SL 200 and 800 T), $n = 20$ (2016: 200 SL 200 T), $n = 37$ (2016: 200 SL 800 T), $n = 24$ (2016: 800 SL 800 T), $n = 6$ (2016: 800 SL 200 T), measurements.

Data availability

The datasets generated and analysed during the current study are available in the PANGAEA data repository (https://doi.org/10.1594/PANGAEA.879370).

Discussion

Within the 3 ½ years of the long-term selection experiment, we did not observe any clear adaptive advantages of the high CO_2 SL copepods under high CO_2 conditions (e.g. an increased developmental rate or egg production under high CO_2 conditions compared to the control SLs). The elevated seawater pCO_2 and changed food conditions in combination with the otherwise favourable and constant laboratory conditions were probably not harsh enough to trigger evolutionary adaptations in the high CO_2 SLs. As will be discussed below, phenotypic buffering and transgenerational effects probably further reduced the selective pressure on the high CO_2 SL populations. Nevertheless, our results demonstrated that an increased seawater pCO_2 and entailed food quality reduction can significantly alter copepods' stoichiometry, and over a long-term also effects physical characteristics such as body length and mass (indicated by a higher C and N content of the copepods in relation to body length), which probably could have consequences for the copepod itself and ecosystem functions. However, to clarify the extent to which changes in the physique (ratio between body length and mass) of *A. tonsa* will affect the copepod and consequently ecosystem functions (positively or negatively), further studies will be necessary.

Experimental setup

To possibly maximise the difference between the experimental CO_2 treatments without exceeding realistic end-of-the century pCO_2 concentrations, we aimed to lower our control treatment from today's pCO_2 level (400 µatm) to preindustrial conditions (200 µatm). TA measurements, performed at the beginning of the experiment and in 2016, however, illustrated that a pCO_2 concentration of 200 µatm was not reached in the low CO_2 SL tanks (D, E, F). The amount of the introduced gas/air mixture was probably not high enough to compensate for the respiratory activities in the tank. However, an increase in the flowrate was not possible as the resulting water turbulences would have injured the copepods. The target pCO_2

concentration of 200 µatm could only be reached during the transplant experiments, as the incubation bottles were 100 fold smaller compared to the SL tanks and included fewer copepods L^{-1}.

Although modest fluctuations in the experimental parameters are almost inevitable when carrying out an extensive life history experiment over several years, the pCO_2 treatments (represented by the water pH) of the high and low CO_2 SLs remained different over the whole experimental period. However, as it was the aim of the study to investigate possible long-term effects of simulated OA (combining direct and indirect effects) on *A. tonsa*, an exclusion of short-term fluctuations in the experimental parameters was not crucial for the experimental success.

Reducing the nutrient amount in the growth medium of the food algae supplied to the high CO_2 SL tanks was necessary to accelerate the stoichiometry change of the food algae and thereby ascertain a stable food quality in the high CO_2 SL tanks. However, since the differences in algae stoichiometry between the SL treatments cannot solely be attributed to differences in the pCO_2 concentrations we cannot state to have only studied the effects of increased pCO_2 on *A. tonsa*. However, manipulating only the pCO_2 concentrations in the SL tanks would have yielded less significant results, but most importantly, would have potentially underestimated the response of *A. tonsa* to future OA conditions. As it was the purpose of our experiment to investigate if *A. tonsa* may acclimatise and / or adapt to increased seawater pCO_2 and the associated food quality reduction, it was substantial to ascertain stable and pronounced differences in the food algae supplied to the SL copepods.

Genetic diversity of the *A. tonsa* SL populations

Based on the COI gene region, the genetic diversity of our laboratory population was comparable to natural *A. tonsa* populations investigated in other studies which observed less than 2% variation among haplotypes of one lineage and an average of four substitution haplotypes per lineage (Caudill and Bucklin 2004; Costa et al. 2014). Although haplotype frequencies were fluctuating over the experimental time there was no indication for genetic drift (e.g. bottleneck effects), proving stable and disturbance free maintenance conditions. However, as more sensitive analytical methods like microsatellite analyses might have revealed changes in the genetic diversity, which were not detected based on the analyses of the COI gene region, we cannot completely rule out changes in the genetic diversity within the selection lines.

Additionally, it is possible that the initial genetic variation at the COI locus was already too low to detect potential changes in haplotype compositions or frequencies.

Effects of elevated CO_2 on the stoichiometry and development of *A. tonsa*

Supporting the results of preceding studies (Riebesell et al. 2007; Schoo et al. 2013; Verschoor et al. 2013), we observed an increased cellular C content of the primary producer (here: *P. salina)* when cultured under elevated CO_2 conditions. As a consequence the C : N and C : P ratio of the high CO_2 algae increased. Differences in the nutrient content of the algae between the four years were probably due to differences in the nutrient uptake of the algae, potentially caused by slightly different growth rates and light conditions. As grazers have rather defined metabolic requirements, stoichiometric discrepancies between the prey and needs of the consumer can considerably impact its fitness (Sterner and Elser 2002; Bukovinszky et al. 2012; Schoo et al. 2013; Meunier et al. 2016). Many grazers, including copepods, can adjust their elemental body composition to a certain extent by, for example, the selective retention of scarce nutrients and / or the excretion of excess elements (Hall 2009). In our study, however, the C : N ratio and the C : P ratio as well as the C content (except in 2015) of the analysed *A. tonsa* individuals reared under high seawater pCO_2 conditions and fed with high CO_2 algae were significantly increased, and we did not detect any difference between the SLs until 2017. In 2014 and 2017, the significant difference in the C : N and C : P ratios between the CO_2 treatments were due to a significantly higher C content of the copepods (both SLs). As in 2015 the C contents of both SLs were not different between the CO_2 treatments, the significantly different C : N ratio was merely a joint effect of a slightly lower N and a slightly higher C content of the copepods under high CO_2 conditions. Under conditions in which C is present in excess, other essential nutrients (N and P) might be limited, with probably far-reaching physiological consequences for the consumer. In fact, in each of our transplant experiments, we observed a substantially reduced developmental rate of the nauplii reared under high seawater pCO_2 conditions getting high CO_2 algae. Beyond that, developmental rates of the nauplii were always similar between the SLs and thus independent from the preceding CO_2 experiences of the copepods.

Unexpectedly, we observed an enhanced regulation of the elemental body composition of both SL populations from 2015 on - they became both more

homeostatic throughout the long-term experiment. In 2015 after 38 generations, the C : N ratio of the copepods (both SLs) increased by just 15% and after 82 generations in 2017 by just 11% when reared under high seawater pCO_2 conditions and fed with high CO_2 algae. Simultaneously to the elevated homeostasis we observed a significantly improved juvenile development under high CO_2 conditions (Fig. 7), suggesting a correlation between the elemental body composition of *A. tonsa* and its developmental rate. During the transplant experiments in 2015, 2016 and 2017, the high and low CO_2 SL individuals developed significantly faster in the high CO_2 treatment compared to 2014 (one-way ANOVA using developmental rate day^{-1} as dependent and year as independent factor; $p < 0.05$). In a previous experiment, carried out with eggs from the same laboratory cultures, which were used to start the long-term experiment, and an equal algal food quality, Meunier et al. (2016) observed similar developmental rates as in our study in 2014. Therefore, it is highly unlikely that the observed developmental rates in the high CO_2 treatment in 2014 were just artefacts. Nevertheless, we do not have a concrete explanation for the elevated homeostasis of both SL populations, although it is conceivable that the very constant experimental conditions played a relevant role. However, we can say that the regulation of the elemental body composition of *A. tonsa* seemed to be rather flexible and independent from the formerly experienced CO_2 concentration. Although low CO_2 SL individuals were not exposed to high CO_2 conditions before the transplant experiments, low CO_2 SL copepods could reach a similar level of homeostasis as copepods with a high CO_2 selective history, which suggest a high plasticity level of *A. tonsa* in the regulation of its elemental body composition.

Long-term effects of elevated CO_2 on the phenotype of *A. tonsa*

There are three potential physiological solutions for herbivores to deal with excess C. (1) A reduced assimilation across gut walls. (2) Assimilated excess C can be stored as C-rich compounds like lipids and (3), assimilated C can be disposed by respiration or extracellular release of organic substances (Sterner and Hessen 1994). Our results indicate that *A. tonsa* can use different strategies to deal with excess C. In 2015, the C content of the copepods (both SLs), measured after the transplant experiment, was not increased when reared under high seawater pCO_2 conditions and fed high CO_2 algae, suggesting an elevated excretion of C. In a previous study, Schoo et al. (2013) also showed that *A. tonsa* released surplus C through excretion products (mainly

observed in adults) and a higher respiration rate (mainly observed in nauplii), when fed high CO_2 algae. As observed after the first transplant experiment in 2014, in 2017, the C content of both SLs was significantly higher under high CO_2 conditions compared to the low CO_2 treatment (Table 2 and Fig. 8). However, different from 2014, the N content of the copepods also significantly increased in the high CO_2 treatment (Table 2 and Fig. 8). Therefore, we assume that *A. tonsa* individuals retained more N to compensate for their elevated C content, which is also no atypical reaction. Likewise, investigations of Miller and Glibert (1998) of the N excretion of *A. tonsa* during a mesocosm study showed a high plasticity level of the copepod to regulate the amount of retained and released N, depending on its physiological and nutritional status.

Although in our study *A. tonsa* seemed to have a high phenotypic buffering capacity to deal with the impaired food quality under high CO_2 conditions, the detrimental elemental composition of their food probably had extensive physical long-term consequences for the copepod. Over the period of the long-term experiment, the mean female prosoma lengths slightly fluctuated, however not exceeding the range observed in other studies at 18°C (Holste et al. 2004; Holste and Peck 2005). Approximately three years after the start of the experiment we observed that *A. tonsa* individuals which were cultured under high CO_2 conditions became significantly smaller compared to the ones from the low CO_2 SL tanks (Repeated Measurement ANOVA using prosoma length as dependent and SL as independent factor; SL and SL*time: $p < 0.001$; time: $p = 0.285$; Fig. 3). Furthermore, measurements performed after the transplant experiment in 2017, showed a significantly increased C and N content of the high CO_2 SL individuals compared to the low CO_2 SL copepods independently from the CO_2 treatment (Table 2 and Fig. 8). This suggests that the body mass of the high CO_2 SL individuals that grazed on high C (energy) algae increased, even though they became smaller. Although there is usually a positive correlation between body length and body mass (C and N ind^{-1}) especially in the growth phase of copepods, this relationship is weak among adults (Kankaala and Johansson 1986; Durbin et al. 1992). Therefore, it is likely that adult copepods gain body mass although they became smaller at the same time. In our study, *A. tonsa* individuals probably stored excess C partly in the form of lipids as adipose tissue, resulting in the increased body mass. Generally, when food is sufficiently available herbivores store lipids as an energy reserve for times of low food supply and for

reproduction (Richard et al. 2006). However, a large lipid load can also result from an unbalanced diet (Sterner and Hessen 1994), as it was the case for the high CO_2 SLs (excess C and N, P in deficiency). During a mesocosm experiment (lasting 45 days) conducted at the south-western-coast of Finland (Baltic), Vehmaa et al. (2016) also found negative effects of elevated CO_2 on the prosoma length of *Acartia* sp. that were strongest in the highest CO_2 treatment at 1650 µatm pCO_2. Besides the pCO_2 concentration, the amount of total particulate carbon (reflecting food supply) significantly affected the prosoma length of the copepods. Differently to the mesocosm experiment, food was not limiting during our long-term selection experiment, therefore the observed effects on the prosoma length should have primarily been caused by the elevated pCO_2 conditions and associated algae food quality, not quantity.

Effects of elevated CO_2 on the reproduction of *A. tonsa*

Although extensive research has been conducted on the effects of food concentration on the egg production of *A. tonsa* (Roman 1991; Kleppel et al. 1998b; Teixeira et al. 2010; Zhang et al. 2015), surprisingly little is known about the effects of food quality (other than using different food species) on egg production. The few experimental studies dealing with the indirect effects of OA (changed nutritional quality of the food) on the reproduction of *A. tonsa* indicate a connection between the fatty acid composition of the prey and the egg production of the copepod, with a lower production rate under a high CO_2 nutrition (Kleppel et al. 1998a; Hazzard and Kleppel 2003; Rossoll et al. 2012). Since those studies comprised only one copepod generation, so far nothing is known about long-term dietary effects on the reproductive output of *A. tonsa*. During our experiment, copepod egg production (number of eggs female^{-1}) was not impacted by the combination of increased seawater pCO_2 and changed algal food quality. Measurements performed after the transplant experiments in 2014 and 2015, showed no substantial differences between the CO_2 SLs and treatments according to the number of eggs female^{-1}. However, our results indicated that the nutrition of the high CO_2 SLs affected females' egg production in terms of egg size and / or nutrient content. In 2014 and 2015 we observed that eggs from high CO_2 SL females hatched significantly earlier compared to the eggs from low CO_2 SL females. Guisande and Harris (1995) found a positive correlation between the size, protein, carbohydrate and lipid content of eggs from

Calanus helgolandicus females and the amount of available food. Thus, the nutritional status of a female can directly affect egg size. Especially the availability of N (protein) is known to effect egg production in copepods (Checkley 1980; Kiørboe 1989). Therefore, it is very likely that due to the detrimental elemental body composition, the high CO_2 SL females had fewer nutrients available for physiological processes like development and egg production, wherefore the production of smaller or nutrient-poor eggs would be beneficial to save resources and to avoid a decrease of reproductive power by lower egg numbers female^{-1} (no differences between the SLs). The reproductive output (number of eggs female^{-1}) of a population is important as there is a positive correlation between the number of offspring and the genetic diversity and thus the fitness of a population (Reed and Frankham 2003). Steele and Steele (1975) already showed that the time for the embryonic development is positively correlated with the egg size. Therefore, a reduced egg size or nutrient amount within the eggs could have shortened the time for embryonic development, which could have resulted in the earlier hatching we observed. The earlier hatching of eggs from high CO_2 SL females occurred independently of the CO_2 treatment experienced during the transplant experiment. So even under optimal environmental conditions females with a high CO_2 selective history seemed to invest less in the production of eggs. This pattern suggests epigenetic inheritance, whereby the quality of produced eggs was determined by the environmental conditions of the previous generations. It is known that transgenerational effects can even decouple phenotypic from genetic change over multiple generations and allow populations to respond to environmental changes even in the absence of genetic variation (Bonduriansky et al. 2012). If the expression of the here observed phenotype is solely due to transgenerational effects or has already been genetically assimilated remains, however, unclear. In 2016, we did not detect differences in the hatching between the eggs from the high and low CO_2 SLs neither under low nor under high CO_2 conditions. The normalization of the hatching pattern was probably a result of the improvements in the regulation of the elemental body composition. However, we cannot exclude that significant differences between the CO_2 SLs or treatments might have been obscured by the increased variation among replicates.

Effects of stoichiometric phenotypic plasticity on ecosystem functions

Eco-evolutionary dynamics posits that both directions of effects, from ecology to evolution and vice versa, are substantial in their influence (Schoener 2011), and, in some cases, may result in eco-evolutionary feedback loops (Post and Palkovacs 2009). It is well established that the product of evolution by natural selection (e.g. species richness and trait variability) can alter ecosystem functions (Matthews et al. 2011), but little is known whether phenotypic plasticity has the strength to influence ecosystem services (Fischer et al. 2014). Within one year after the start of the long-term experiment, we observed an increased regulation of the elemental body composition of the *A. tonsa* SLs. Since grazers like copepods preferentially retain nutrients limiting their growth (N and P) and release those supplied in excess (C), they can impact the amounts and ratio at which nutrients are recycled (Hall 2009). Additionally, copepods must get rid of surplus C when nutrients are limited. Excess C released by excretion of dissolved organic carbon, defecation and respiration can then intensify microbial growth. Thereby, low nutrient content of detritus typically means lower and slower decomposition and hence sequestration of C (Hessen et al. 2004). However, experimental studies (Olsen et al. 1986; Goldman et al. 1987) showed that simultaneously nutrient regeneration rates are constrained when nutrient elements are decreased relative to C in food substrates, which then again accelerate nutrient limitation in autotrophs. Therefore, the regulation of the elemental body composition of consumer species and the associated release of e.g. excess C or an increased retention of nutrients (here: N) under elevated CO_2 conditions, especially in nutrient depleted water, could negatively feedback on consumers' food sources and a priori the consumer itself. Consequently, stoichiometric changes in the range of phenotypic plasticity can substantially affect C and nutrient fluxes within the food web and alter ecosystem functions.

Conclusion

Our results showed that *A. tonsa* can be substantially affected by simulated OA, both immediately and in the long-term. In the first place, most likely due to the dietary conditions in the high CO_2 treatment, copepods' elemental body composition changed, and their developmental rate decreased. Although *A. tonsa* showed a high plasticity level in the regulation of its body stoichiometry, we found evidence that the

ingestion of high CO_2 algae with a high energy content (high C : nutrient ratio) for several years can result in a reduced body length accompanied by an increased lipid storage, as indicated by the increased C and N content of the high CO_2 SL copepods in both CO_2 treatments in 2017. However, we do not know whether these were phenotypic or genotypic changes, as we did not test the reversibility of these processes. Besides possible corresponding implications for the copepod itself, such changes in the phenotype and nutritional composition can have substantial consequences for trophic interactions in the food web.

Several studies on various species (oysters, sea urchins, mussels, and phytoplankton) predict that complex adaptations to OA are likely over longer time scales (Sunday et al. 2011; Parker et al. 2012; Reusch and Boyd 2013). Probably due to an insufficient selection pressure, we did not detect any SL specific adaptations to the CO_2 conditions in the observed traits (body stoichiometry, developmental rate, egg production and hatching rate) during our long-term CO_2 selection study. Additional, phenotypic buffering and transgenerational effects, as observed in *A. tonsa* in the regulation of its elemental body composition and egg production, probably further reduced the selective pressure on the high CO_2 SL populations. It cannot be ruled out that the effects on the prosoma length and body mass of the high CO_2 SL copepods, observed towards the end of this study, elevated the selective pressure, which would have possibly lead to specific acclimatisation and / or genetic adaptation in the future. Under naturally fluctuating conditions with changing food availability and differences in nutrient supply, indirect effects of elevated CO_2 on *A. tonsa* populations might be even worse and adaptions even more challenging. Especially, stoichiometric regulations may not occur because metabolic costs of the regulation processes would probably outweigh the advantages (Meunier et al. 2014). Furthermore, in natural field populations, many traits are under selection and an effective response to one stressor may limit the response to another, which hampers adaptations when multiple environmental changes occur (Hofmann and Todgham 2010; Collins et al. 2014). Thus, laboratory experiments possibly underestimate the intensity of constraints on acclimatisation and evolutionary responses to environmental changes (Collins et al. 2014). To be able to make fundamental predictions on how OA and potentially associated stoichiometry changes of primary producers will influence food web interactions, further investigations under natural

field conditions are needed, including multiple producer and consumer species as well as fluctuations in abiotic and biotic factors.

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General discussion

The uptake of anthropogenic CO_2 by the world's oceans induces fundamental changes in seawater chemistry, with far reaching consequences for marine ecosystems. Investigations presented in this thesis showed that copepods are hardly directly affected by lowered seawater pH (CHAPTER III) and revealed low sensitivities of coastal plankton communities to the combined direct and indirect effects of OA (CHAPTER II). In addition, observations made during the long-term selection experiment suggest that there is a high potential for acclimatisation and adaptation of copepods to increased pCO_2 conditions, but also, that a long-term exposure to future acidification states may alter copepods' physical characteristics (CHAPTER IV). Furthermore, this study demonstrated the applicability of the HTS technique community barcoding for the assessment of marine plankton communities, including oomycetes, and illustrated remaining methodological weaknesses (CHAPTER I and II).

In the following, findings of this study will be discussed in a broader context, including recent literature on the effects of OA on plankton communities and their acclimatisation and adaptation responses. Additionally, possibilities offered by new molecular biological techniques for investigation of the effects of OA on marine communities, like community barcoding, will be presented. Finally, an outlook on areas where future research efforts should be made will be given.

Effects of OA on copepods

Calanoid copepods account for up to 80% of the zooplankton biomass (Longhurst 1985) and play a key role in marine pelagic ecosystems, transferring energy captured by primary producers to higher trophic levels (Runge 1988; Calbet and Saiz 2005). Copepods are indispensable to the functioning of the entire pelagic ecosystem and contribute to many ecosystem services (Bron et al. 2011) e.g. the carbon transport from the surface to the deep sea (e.g. Schnack-Schiel and Isla 2005). Generally, crustaceans have been considered as less sensitive to end of the century pCO_2 scenarios (Wittmann and Pörtner 2013), however, copepods' responses to OA seem to be complex. Reactions differ among (Zhang et al. 2011) and within species (Drillet et al. 2008), i.a. depending on their ecological history (Vargas et al. 2017), as

well as between developmental stages, with early life stages being more vulnerable (Cripps et al. 2014b; Pedersen et al. 2014; Wang et al. 2018). In accordance with earlier studies (Mayor et al. 2012; McConville et al. 2013), results from the short-term laboratory experiment (CHAPTER III) support the observation that copepods (here *A. tonsa*) are hardly directly affected by increased pCO_2 concentrations (e.g. hypercapnia). As many zooplankton (including larval stages) already face pH levels much lower than those predicted for surface waters in the coming century (Meunier et al. 2016), copepods are potentially already adapted to variations in seawater pH and the effects of hypercapnia.

However, indirect effects of OA via altered food regimes might be a more serious threat to copepod species. Rising pCO_2, for instance, was shown to rise elemental C : N and C : P ratios (Tortell 2000; Urabe et al. 2003; Riebesell et al. 2007; van de Waal et al. 2010; Schoo et al. 2013; Verschoor et al. 2013; Eggers et al. 2014) in experimental studies using single species and phytoplankton communities. Furthermore, Rossoll et al. (2012) demonstrated that elevated pCO_2 (~ 750 µatm) significantly changes the fatty acid composition of the diatom *Thalassiosira pseudonana*, with a decline in both, total fatty acids and the ratio between long-chain polyunsaturated to saturated fatty acids. In contrast to that, Isari et al. (2015) found no effect of elevated pCO_2 on the dinoflagellate *Heterocapsa* sp., neither on the elemental ratio nor on the fatty acid composition. The fact that different studies yielded different results supports the assumption that organisms' responses to increased pCO_2 are highly species specific. In the short- and long-term laboratory experiments in this study (CHAPTER III and IV), culturing of the algae *R. salina* and *P. salina* under elevated pCO_2 (800 µatm) concentrations significantly increased the C content and thus the C : N and C : P elemental ratios. Besides fatty acids (Glencross 2009), N and P, as essential building blocks for proteins and RNA, play important roles in the construction of organisms. Thus, these elements mutually regulate the pace of protein synthesis and the associated growth rate (Elser et al. 2003; Hessen and Anderson 2008). Since heterotrophs are more restricted in their elemental composition compared to autotrophs, stoichiometric discrepancies between predator and prey can have far reaching consequences for the fitness of the consumer, diminishing its growth, fecundity and survival (Sterner and Elser 2002; Bukovinszky et al. 2012). Generally, many grazers including copepods can adjust their elemental body composition to a certain extent by e.g. selective retention of scarce nutrients or

the excretion of excess elements (Hall 2009). In this study (CHAPTER III and IV) however, the ingestion of algae, reared under elevated pCO_2, significantly reduced the developmental rate of the copepods. Additionally, measurements of copepods' elemental body composition (CHAPTER IV), revealed a significantly increased C content of copepods cultured under elevated pCO_2 conditions, grazing on high CO_2 algae. Hence, copepods could most likely not compensate the stoichiometric discrepancy between their food and their somatic needs for a balanced growth. Contradictory to a study of Rossoll et al. (2012), in which the egg production of *A. tonsa* females, fed algae reared under high CO_2 concentrations, significantly decreased from 34 to 5 eggs female^{-1} day^{-1}, no detrimental effects on the egg production of *A tonsa* were observed during the long-term selection experiment in this study (CHAPTER IV). However, since egg production was only measured after several months, copepods were probably already acclimatised to the elevated pCO_2 concentration and associated food condition, which will be discussed in more detail later.

As many studies investigating the effects of OA on marine organisms were performed with single species and / or artificial predator-prey combinations, obtained results cannot simply be extrapolated to the community or ecosystem level. For instance, it was shown that food quality effects can travel up the food web (Malzahn et al. 2007) and might affect higher trophic levels (e.g. fish) as well. However, under natural conditions, copepods probably compensate the deficiencies in the food quality by selective feeding, choosing those prey organisms most closely matching their metabolic needs. Indeed, copepod species actively choose specific food based on taxonomic differences of the prey (Gentsch et al. 2009; Saage et al. 2009) Gentsch et al. 2009), prey size (Paffenhöfer 1988), prey nutrient composition (Cowles et al. 1988) and in relation to their own developmental stages (Berggreen et al. 1988; Mauchline 1998). Besides, in nature, copepods' responses to OA are additionally significantly influenced by abiotic factors like temperature and light, by the diversity and abundance of potential prey organisms and their quality (nutrient composition) (Cripps et al. 2016; Algueró-Muñiz et al. 2017a; Lischka et al. 2017), as well as by the presence of predators and / or other harmful organisms e.g. parasites (Lafferty et al. 2006; Scholz et al. 2016a).

Effects of OA on plankton communities

Considering the constrains of laboratory experiments, large-scale *in situ* mesocosm experiments were carried out in recent years, as they provide a powerful tool to investigate the impact of elevated pCO_2 concentrations on marine communities under near-natural conditions (Riebesell et al. 2010).

Since organisms living at higher latitudes are expected to be more impacted by OA, due to the higher solubility of CO_2 in cold waters, mesocosm experiments were until recently preferably performed in northern areas. Mesocosm field studies conducted in Espegrend (Bergen, Norway) and Kongsfjorden (Ny-Ålesund, Svalbard, Norway) revealed an overall resilience of the present plankton communities (bacteria, phytoplankton, micro- and mesozooplankton) towards elevated pCO_2 conditions, whereby observed biological responses were mainly depending on temperature and nutrient availability (Riebesell et al. 2008; Aberle et al. 2013; Niehoff et al. 2013; Schulz et al. 2013; Silyakova et al. 2013; Sperling et al. 2013). Since both mesocosm experiments lasted only for around 30 days, the time span was probably not sufficient to detect changes in slowly developing artic communities, raising not only the need for longer lasting mesocosm studies but also the need of experiments at lower latitudes, where most taxa have shorter generation times (Niehoff et al. 2013).

The Gullmar Fjord KOSMOS 2013 study was the first study to last 113 days, encompassing a whole winter-to-summer plankton succession with two phytoplankton bloom phases. Overall, the results presented in CHAPTER II and from other accompanied studies revealed no persistent pCO_2 induced compositional shifts in the investigated coastal plankton communities. As observed during the prior mesocosm studies in Espegrend and Kongsfjorden, changes in relative taxa abundances and taxa compilations were mainly driven by temperature and nutrient availability (CHAPTER II). However, during the second bloom phase, some studies observed pCO_2 induced fertilizing effects on photosynthetic organisms, leading to an increased biomass of autotrophic phytoplankton (Bach et al. 2016) and associated micro- and mesozooplankton grazers (Horn et al. 2016; Algueró-Muñiz et al. 2017a; Taucher et al. 2017), which probably benefited from the increased food availability. In contrast to the first bloom, which was fuelled by inorganic nutrients that were initially available from the winter upwelling, the second bloom was mainly driven by remineralized nutrients in lower concentration. Resources for plankton growth needed to be delivered by the food web, whereby the limitation of inorganic nutrients

seemed to indirectly enhance pCO_2 effects on planktonic organisms. Theoretically, one would expect that larger phytoplankton species benefit more from increased pCO_2, because they are more diffusion-limited due to their lower surface to volume ratio (Flynn et al. 2012). Contradictory, during the Gullmar Fjord mesocosm experiment especially very small picophytoplankton species (0.2 - 2 µm) showed a pronounced positive response to the increased pCO_2 conditions during several succession stages (Bach et al. 2017). In the study of Bach et al. (2017) different mechanisms are presented to explain the advantages of small-sized phytoplankton under OA when inorganic nutrients are limiting. Firstly, they are more efficient in the acquisition of nutrients than larger species, due to their higher surface to volume ratio (Pasciak and Gavis 1974). Secondly, they possibly benefit from an increase of extracellular enzymes involved in organic matter remineralization under low pH. However, this phenomenon was so far only reported from auto- and heterotrophic bacteria (Grossart et al. 2006; Maas et al. 2013; Piontek et al. 2013; Endres et al. 2014). In a third mechanism, mixotrophic picoeukaryotes graze on bacteria as an alternative nutrient source, thereby additionally weakening them as nutrient competitors (Thingstad et al. 1996). Results from the community barcoding performed in this study are in line with this theory as they indicated decreasing Proteobacteria abundances over the experimental period (CHAPTER II). Furthermore, an under high pCO_2 conditions significantly increased abundance ratio of picophytoplankton to heterotrophic bacteria during the second bloom phase observed by (Bach et al. 2017), support this hypothesis.

Other mesocosm studies further revealed that nutrient conditions play an important role in the response of plankton communities to OA (Alvarez-Fernandez et al. 2018), with more intense pCO_2 effects under limiting inorganic nutrient concentrations (Paul et al. 2015; Sala et al. 2015; Spilling et al. 2016b). As mentioned before, investigations accompanied with the Gullmar Fjord KOSMOS 2013 experiment showed, that a CO_2-enhanced increase in autotrophs abundances can travel up the food chain, whereby heterotrophic consumers (Horn et al. 2016; Algueró-Muñiz et al. 2017a; Taucher et al. 2017) and higher trophic levels (Sswat et al. 2018) benefit from an increased availability of prey. A positive indirect pCO_2 effect on the food supply of zooplankton was also observed during a 45-days-long mesocosm experiment conducted in the Baltic, Storfjärden (southwest coast of Finland) in 2012. An interesting aspect of this experiment was the pronounced role of the microbial loop in

the study area (Uitto et al. 1997; Koski et al. 1999), which substantially shaped biological processes in the post bloom phase of the mesocosm experiment. Here, most of the secondary production was fuelled by carbon channelled from the microbial loop to higher trophic levels (Paul et al. 2015). After an initial phytoplankton bloom, which was of similar strength among CO_2 treatments, carbon was mainly channelled into sinking material flux and higher trophic levels, instead of being accumulated in the DOC pool, which was probably mediated by increased zooplankton grazing. In the further course of the post bloom phase, the zooplankton community in the high CO_2 mesocosms was dominated by the cladoceran *Bosmina* sp. and the mixtrophic ciliate *Myrionecta rubra*. These species seemed to benefit twice from increased pCO_2 concentrations, directly through their autotrophy and indirectly by an increased occurrence of potential prey organisms (i.e. picoeukaryotes). *Bosmina* species are filter feeders consuming algae and protozoans, therefore their reproduction was probably further stimulated by an increased food availability in the form of bacteria (Lischka et al. 2017). Thereby, the direct feeding of cladocerans on bacteria, which was evident in a reduced bacterial production (Crawfurd et al. 2017), enhanced the microbial loop and stimulated carbon transfer to higher trophic levels. As observed in earlier mesocosm experiments (Bach et al. 2017), elevated pCO_2 concentrations increased phytoplankton productivity, positively effecting higher trophic levels e.g. the calanoid copepod *Eurytemora affinis* (Almén et al. 2016). Under the nutrient depleted conditions, the picophytoplankton benefited most from the increased pCO_2 conditions (Hornick et al. 2017). As size is a key parameter for sinking speed, picoplankton organisms are very inefficient in transporting carbon out of the euphotic layer (Michaels and Silver 1988). Thus, a shift towards smaller size classes might result in a reduced carbon export and sequestration (Spilling et al. 2016a). Additionally, a persistent shift to smaller phytoplankton size classes might create a gap between prey size and primary consumer's needs, with negative impacts on population size and subsequently on the recruitment of secondary consumers and higher trophic levels. In line with the Gullmar Fjord results (CHAPTER II), the investigated plankton community in the Baltic appeared to be quite resistant towards future OA conditions as well. Again, this response was probably a result of the ecological prehistory of the present biocenosis. The northern Baltic Sea is characterised by a low salinity (~ 5.7 ‰) and, as many other coastal areas, by large

inter- and intra-seasonal pH variations, sometimes reaching extreme values of 9.2 and 7.4 (Brutemark et al. 2011). Thus, the on-site plankton community is probably composed by species and genotypes that are less sensitive to increased pCO_2 (reduced pH) (Nielsen et al. 2010; Lohbeck et al. 2012; Rossoll et al. 2013; Almén et al. 2014).

Observations of the zooplankton succession made during another mesocosm study conducted in 2014, in Gando Bay, on the west coast of Gran Canaria (oligotrophic conditions), showed, as observed during the Gullmar Fjord mesocosm study, a positive, however delayed response of the zooplankton community to the phytoplankton bloom under high pCO_2 conditions (Algueró-Muñiz et al. 2017b). Those changes in the phenology of species could cause mismatches between trophic levels and functional groups, with consequences for the entire ecosystem (Edwards and Richardson 2004).

All in all, past mesocosm experiments revealed that responses of marine plankton communities to OA (increase pCO_2) are highly complex, depending on various factors. While this is certainly only the tip of the iceberg, some factors shaping the effects of increased pCO_2 concentrations on marine plankton communities were revealed during the past years. Besides nutrient conditions, temperature and season, the taxa composition of the plankton community as well as their adaptive history seem to be essential factors. This raises the questions if observed responses of marine taxa to increased pCO_2 concentrations will either persist for longer periods covering multiple generations and growth cycles, or if organisms acclimatise and adapt to the changed environmental condition. However, prolonging mesocosm experiments is not trivial, due to e.g. wall effects and artificial water mixing conditions, as there is an inherent danger of driving the community away from the natural world over time.

Acclimatisation and adaptation

The main determinant of species' tolerance to environmental changes is their physiological performance. If conditions change, organisms initially respond based on physiological and behavioural adjustments that have been shaped by their ecological prehistory (Doney et al. 2012). New environmental conditions may be physiologically tolerable, allowing acclimatisation and adaptation, or may be intolerable, causing migration, change in phenology or death and local extinction (Parmesan 2006). Each of these responses of single species affect overall food web

interactions in a variety of ways, with potentially dramatic implications for ecosystem structure (e.g. community composition) and function. Mesocosm experiments revealed that zooplankton living in costal upwelling regions are potentially acclimatised or adapted to extremes in seawater chemistry, and therefore rather resistant against the effects of future OA conditions (Pansch et al. 2014). However, local adaptation to future OA conditions may not be a ubiquitous process in marine environments. Instead, there might be large variations among species in their evolutionary responses. For example, Lucey et al. (2016) showed that for the calcifying polychaete *Simplaria* sp., inhabiting naturally elevated pCO_2 volcanic vent areas, neither local adaptation nor phenotypic plasticity are likely to be sufficient to buffer the negative impacts of future OA. Among copepods, local adaptation to the environmental parameters temperature, oxygen concentration and salinity was described for *Tigriopus californicus* (Kelly et al. 2012), *A. tonsa* (Decker et al. 2003) and *Eurytemora affinis* (Lee et al. 2007), respectively. It was further suggested that the evolutionary history of marine populations greatly influences the adaptability of organisms to future OA conditions (Hofmann et al. 2011). Accordingly, organisms living in coastal areas characterised by strong variations in e.g. pH, oxygen content and temperature (Feely et al. 2008; Melzner et al. 2013), do not necessarily have a higher potential for acclimatisation or adaptation compared to organisms form temperate zones. Strong directional selection for resistance to e.g. low pH might reduce the total genetic variation of a population and thus the potential to acclimatise or adapt to alterations in other environmental parameters associated with climate change as ocean warming (Gaitan-Espitia et al. 2017). Likewise, phenotypic plasticity followed by genetic assimilation (selective pressure on a specific phenotype) could reduce a populations' genetic diversity. However, seasonal fluctuations in environmental parameters might result in the maintenance of alternatively adaptive phenotypes (higher genetic diversity) (Pespeni et al. 2013) or favour plastic phenotypes with large tolerance to pH variations (Futuyma and Moreno 1988). All in all, evolutionary responses of marine populations to future OA are likely to be very specific and shaped by the experienced environmental history.

To study the adaptive potential of organisms, one can measure the standing genetic variation in climate-sensitive traits, infer past adaptation from comparisons across time and space, as it was done in the studies describe above, or conduct real time evolution experiments (Sunday et al. 2014). Only the latter approach is a direct test

of adaptive evolution. Investigation of the adaptability of key species in marine food webs may enable the identification of the thresholds (rate and magnitude) of environmental change, beyond which evolution might not be effective to save marine organisms from extinction. Due to their rather long generation times of 15 - 30 days (Peterson 2001), investigations of real-time evolution in copepods experiencing future OA conditions are scarce. The long-term selection experiment presented in CHAPTER IV was the first one investigating the effects of simulated OA on the calanoid copepod *A. tonsa* over a period of 3 ½ years. At the first instance, the elevated seawater pCO_2 and impaired quality of the food algae (increased carbon to nutrient ratio), cultured under high pCO_2 concentrations, increased the C to nutrient (N, P) elemental body ratio and decreased the developmental rate of the copepod. However, under constant laboratory conditions, *A. tonsa* showed a high plasticity in the regulation of its body stoichiometry, indicated by an increased homeostasis and associated increased developmental rate of the copepods from both SLs under high CO_2 conditions, within one year. Organisms with longer generation periods often show phenotypic plasticity and buffering to compensate unfavourable changes in their environment (Dam 2013; Thor and Dupont 2015). If environmental change persists over the life time of organisms, transgenerational acclimation comes to effect. By, for example, nutritional, somatic, cytoplasmic or epigenetic transfer, parents can significantly affect the performance (survival and fitness) of their offspring (Bonduriansky and Day 2009; Marshall and Morgan 2011; Klironomos et al. 2013). Maternal effects on offspring can be a significant factor influencing population dynamics of copepods (Kahan et al. 1988) and other marine taxa (Fowler 2005; Räsänen and Kruuk 2007; Munday 2014). During the long-term experiment (CHAPTER IV) copepods' development was significantly reduced under simulated OA conditions, however, the number of produced eggs per female was not affected by the increased water pCO_2 combined with the poor food quality of the algae cultured under elevated pCO_2 concentrations. Under nutrient depleted conditions due to the changed stoichiometry of the food algae (high C, less nutrients), high CO_2 SL females optioned for quantity rather than quality, according reproduction, and invested less resources in the production of each egg. Consequently, those eggs were probably smaller or contained less yolk. Thus, eggs from females with a high CO_2 selective history hatched earlier compared to those from control SL females, independent from the experienced CO_2 treatment during the transplant experiments.

Hence, there was a potential trade-off between copepods' developmental rate and its reproductive output as well as between the number of produced eggs and their quality. Already Darwin (1859) referred to trade-offs as compensation and stated that "in order to spend on one side, nature is forced to economise on the other side." The results of the conducted laboratory experiments (CHAPTER III and IV) with *A. tonsa* in this study are in accordance with Fitzer et al. (2012), who observed similar responses of *Tispe battagliai* when exposed to OA-induced stress. They suggested that copepods preferentially reallocate resources to maintain their reproductive output at the expense of somatic growth and cuticle composition.

Although many laboratory experiments revealed a high potential of organisms to acclimatise and adapt to OA associated environmental changes e.g. lowered pH and altered food regimes, it is still unclear how organisms will respond under natural conditions. Physiological acclimatisation (in the form of phenotypic plasticity) and evolutionary adaptation processes, affect inter- and intraspecific interactions within the food web and, at a larger scale, alter the marine ecosystem (Thompson 1998; Matthews et al. 2011). Changes in the way organisms regulate their elemental ratios or changes in their stoichiometry, as observed during the long-term selection experiment (CHAPTER IV), could significantly influence population and community dynamics as well as ecosystem functions such as nutrient cycling (Yamamichi et al. 2015). A higher relative assimilation or retention of limiting elements might further distort the elemental ratios of primary producers (Hall 2009) and potentially reinforce existing stoichiometric selection pressures on herbivores (eco-evolutionary feedback) (Pelletier et al. 2009). In this study, *A. tonsa* exhibited a high plasticity in the regulation of its body stoichiometry, however, the one-sided ingestion of algae, cultured under high pCO_2 conditions, with a high energy content (high C : nutrient ratio), resulted in an increased body mass and reduced body length of the copepods (CHAPTER IV). In nature, besides potential food web implications, such physiological alterations could directly affect copepods' survivability by a reduced swimming speed and consequently lowered ability for predator avoidance (Strickler 1975; Sullivan et al. 1983; Burdick et al. 2007).

Whatever the case, observed impacts of OA on single species cannot simply be transferred to the community level as effects might be accelerated or compensated at the ecosystem level by additional environmental stressors, species richness, and trophic interactions. Indeed, experimental evidence suggests that adaptation to

environmental stress of phytoplankton (Collins et al. 2014; Eggers et al. 2014), bacteria (Lawrence et al. 2012) and zooplankton (Declerck et al. 2015; Spilling et al. 2016a) might follow different evolutionary paths in monocultures than in the presence of competitors. Therefore, to investigate the adaptive potential of species to environmental changes, selection studies should be performed in an environment as close as possible to natural conditions (Bach et al. 2018). Although mesocosm studies enable the investigation of the effects of future OA conditions on a community level under near natural conditions, these studies are limited technically by their massive maintenance effort and systematically by the inevitable progressive deviation of the enclosed biomes from natural patterns. However, to detect signals of climate change associated processes like OA from substantial natural variability, observation over multidecadal time spans are needed (Doney et al. 2012). Since marine communities at naturally-acidified sites are already adapted to the specific habitat conditions, those places are often used as OA analogues to investigate the effects of acidification on the present biocoenosis, either along pCO_2 gradients (Raulf et al. 2015) or between acidified and control sites (Hassenrück et al. 2015; Hofmann et al. 2015b). Furthermore, those sites can be used to investigate acclimatisation and adaptation patterns, e.g. by reciprocally transplanting species from ambient (i.e. high pCO_2) to low pCO_2 habitats (Lucey et al. 2016). A vulnerable point of such studies is the characterization of the naturally-acidified sites, which is often limited to OA-related variables, omitting other environmental variables that might interact with the effects of OA (Hassenruck et al. 2016).

Metagenomics

Marine environments are experiencing rapid changes in important processes and parameters like temperature, light penetration, nutrient availability, and OA. Resulting changes in the physiological condition of species, their ecological functioning and biogeographical distribution as well as their abundance will inevitable alter marine ecosystems. How species acclimatise and / or adapt to environmental changes, and how physiological or genetic adaptations of species might change food web interactions and ecosystem structures and functions, can be examined by various ways, whereby community barcoding seems to be a convenient and powerful tool. As presented in CHAPTER I and II, community barcoding approaches analyse DNA obtained from environmental samples and reflect the

biodiversity of entire plankton communities (de Vargas et al. 2015), with the advantage of detecting also the "hidden species diversity" (Lindeque et al. 2013). In this study, community barcoding was used, in a mesocosm experiment, to asses OA induced changes in the composition of a coastal plankton community (CHAPTER II). With the analysis of the 18S gene region, a broad range of the present biodiversity was captured, including phyto- and zooplankton of different size classes as well as fungi and prokaryotes. The results demonstrated that the composition of the investigated coastal plankton community is rather resistant to future OA condition. Over the experimental period, plankton community compositions were rather shaped by changes in light, temperature and nutrient availability, following the natural winter-to-summer succession. Despite the high taxonomic resolution, community barcoding data cannot be used to make predictions about species abundances and / or biomasses on-site. Preferential annealing of universal primers in some species over others hinder species abundance assessments of this PCR-based approach (Elbrecht and Leese 2015). For reliable biomass estimates, or even rough estimates, a PCR-free approach would be needed. However, to date no such approach is available (Zhou et al. 2013).

Recent research revealed that the ecological prehistory and taxonomical composition of marine plankton communities greatly influence their ability to acclimatise or adapt to future OA conditions. The lack of environmental data regarding seawater chemistry (e.g. pH / pCO_2 variability) and geographic distribution of species are critical research gaps for studies dealing with the projection of organismal responses to OA (Ducklow et al. 2009). Several studies and this thesis (CHAPTER I and II) demonstrated the suitability of community barcoding for biodiversity assessments of marine zooplankton (Lindeque et al. 2013; Pearman and Irigoien 2015), bacteria (Herlemann et al. 2011; Gilbert et al. 2012) and protozoa (Massana et al. 2015) communities as well as for long-term plankton monitoring projects (Abad et al. 2016) including e.g. microalgae (Visco et al. 2015). Additionally, community barcoding was successfully used for global ocean biodiversity analyses (Bik et al. 2012; de Vargas et al. 2015). Compared to classical microscopic examinations, a genetic approach like community barcoding allows for an accurate and fast assessment of extreme small (pico-, nano- and microplankton), rare and cryptic species. The approach is especially advantageous for the detection and investigation of marine parasites (CHAPTER I), which are virtually impossible to detect by eye as

they often inhabit their hosts. Community barcoding is a powerful technique with excellent possibilities for use in plankton assessment and monitoring as well as for an early detection of e.g. OA induced plankton diversity shifts including the immigration of new non-indigenous species (Abad et al. 2016). However, study success highly depends on the quality of public databases. Still, some organism groups, e.g. marine oomycetes, have none or only very few publicly available reference sequences. Thus, the current incompleteness of databases is one of the main drawbacks for community barcoding studies. For an improvement of those databases, fruitful cooperation between taxonomists and molecular biologists is needed.

Nonetheless, new population genomic approaches offer various research possibilities including i.a. the detection of impacts of selection and local adaptation (Stapley et al. 2010). Thereby, non-neutral markers which are targeted by selection can be used to reveal adaptation of populations to local environmental conditions across the distribution range of a species (Whitehead 2012). Furthermore, evolutionary drivers, like introgressive hybridization and hitch-hiking (Smith and Haigh 1974), which lead genomic traits away from neutrality (Bucklin et al. 2018), can indicate ongoing adaptation processes. The continuing development of sequencing technologies and decreasing costs might soon allow the frequent sequencing of whole genomes. The so obtained data would be a valuable resource for various population genetic approaches, including the detection of loci under selection and the investigation of genomic bases of adaptations of marine plankton (Bucklin et al. 2018).

Conclusion and future perspectives

Aims of this thesis were the investigation of the potential direct (lowered water pH) and indirect (changed food regime) effects of elevated seawater pCO_2 on *A. tonsa* (CHAPTER III) and the investigation of its adaptability to future OA conditions (CHAPTER IV). Furthermore, as a technique providing high resolution taxonomic data, community barcoding was used to detect changes in the composition of a coastal plankton community exposed to future OA conditions (CHAPTER II). Due to their cumbersome detection and taxonomical classification, marine parasites, although playing an important role in marine food webs, are often not considered in marine ecosystem analyses. Therefore, it was another goal of this study to advance the molecular biological detection of marine oomycetes, which are predominantly parasites of various marine taxa (CHAPTER I).

In conclusion, the effects of OA on marine biota were very species and developmental stage specific. Adult stages of the calanoid copepod *A. tonsa* seemed to be rather resistant towards direct effects of elevated pCO_2, while naupliar stages were more sensitive. Contrary to that, *A. tonsa* is indirectly impacted by OA, due to changes in the elemental composition of their prey. Stoichiometric discrepancies between predator and prey significantly diminished consumer growth. The ingestions of low quality food (unfavourable ratio between energy (C) and nutrients (N, P)) potentially resulted in a trade-off between copepods' developmental rate and reproductive output, which was in favour of the latter. However, during the long-term selection experiment, *A. tonsa* showed, independent from its selective history, a high plasticity in the regulation of its body stoichiometry and became more homeostatic, suggesting a high physiological adaptability to OA associated environmental changes.

Results from laboratory experiments on single species and / or artificial predator-prey combinations cannot simply be translated to the community level, because species richness and complex trophic interactions might mitigate or accelerate the potential direct and indirect effects of OA. Mesocosm experiments, which enable the investigation of the effects of OA on plankton communities under near natural conditions (Riebesell et al. 2013), revealed that coastal plankton communities were rather resistant towards future acidification states (Nielsen et al. 2010; Lohbeck et al.

2012; Nielsen et al. 2012; Rossoll et al. 2013; Almén et al. 2014; Baumann et al. 2015). Furthermore, a pCO_2 induced fertilisation effect on photosynthetic organisms was observed, whereby small picophytoplankton seemed to benefit most, especially under nutrient depletion (Bach et al. 2017). An increased food supply can in turn have advantageous effects on the recruitment of micro- and mesozooplankton grazers as well as higher trophic levels (Horn et al. 2016; Algueró-Muñiz et al. 2017a; Taucher et al. 2017; Sswat et al. 2018). Possible disadvantages for secondary consumers, due to a poor food quality of primary producers, might be alleviated in complex food webs by compensatory feeding (e.g. Cowles et al. (1988)). Summing up, the research of the past years revealed potential winners and losers of the ongoing OA process. A majority of marine organisms including several phyto- and zooplankton species tolerate an increase in pCO_2 and even benefit from it, while others, especially calcifying taxa like mussels and corals, are more vulnerable. Although it seems that OA is not the greatest threat for marine organisms, in combination with other environmental stressors, it will likely cause significant shifts in the community composition of marine species, and the resulting ecosystem might not be what people want.

The complexity of entire ecosystems cannot be incorporated into any feasible experimental set-up. Even in large-scale mesocosm experiments it is not possible to include all regional occurring species. For instance, big predators (exceeding a length of ~ 1 mm) need to be excluded, as they would otherwise eat up most of the enclosed zooplankton community in a short time. Furthermore, the running time of mesocosm experiments is limited, due to a progressing deviation of the enclosed system from real nature. This is especially problematic, as, although possible to take place over short timescales, physical acclimatisation and adaptation processes of e.g. marine copepods to OA, are often superimposed by responses to other varying environmental factors. Sometimes even multidecadal time spans are needed to separate climate change signals from substantial natural variability (Doney et al. 2012). Besides, the environmental prehistory and species composition of marine plankton communities significantly determine their sensitivity or resilience towards OA and associated environmental changes. Therefore, future research efforts should include intensive long-term observations (monitoring projects) at different sites, regularly assessing the present biodiversity and relevant seawater parameters, e.g. temperature, pH, pCO_2, light penetration and inorganic nutrient concentrations. If

time series data contain information on a sufficient number of generations across different environmental conditions, they would be extreme valuable in predicting how future oceans might look like (Attrill et al. 2007; Wiltshire et al. 2010). Furthermore, OA does not occur in isolation but in combination with other environmental stressors like ocean warming, eutrophication, invasive species, parasitism and pollution. Again, positive or negative correlations between the effects of OA and further environmental stressors could influence the magnitude at which marine biota and consequently ecosystems might be impacted (Breitburg et al. 2015). In conclusion, objectives for future research will be to continue assessing numerous reactions of marine communities and ecosystems to a multitude of stressors. Therefore, besides implementation of additional monitoring projects, the performance of long-term, near natural, multi-stressor, mesocosm community experiments during different seasons (nutritional situations) and at different geographical sites is recommended, to get a comprehensive picture of how marine communities respond to future acidification states and to uncover respond patterns that can be generalized. Furthermore, investigations at naturally-acidified sites are endorsed to improve understanding about acclimatisation and adaptation processes in marine communities.

The development of HTS techniques offered a fast and logistically convenient way to assess marine communities in environmental samples. However, sequencing techniques like community barcoding only provide relative but not absolute species abundance data. Although usually more time-consuming compared to sequencing, quantitative methods, such as classical microscopy-based techniques, should be used to confirm patterns in relative sequence abundance. To elucidate OA induced changes in marine communities, especially short time alterations in the abundance of a few species, a combined approach of screening and targeted techniques for future research studies is suggested until molecular techniques have been further improved. However, for long-term observations of marine biocoenoses, which imply enormous sample numbers and potentially cover wide areas, community barcoding can provide a fast, accurate and reliable tool.

The German philosopher Johann Wolfgang von Goethe said “In living nature nothing happens which is unconnected to the whole”. The consequences, of the ongoing OA and other climate change-related threats as well as anthropogenic influences for

marine ecosystems are to date impossible to forecast and, at best, quasi-predictable. Nevertheless, the effects of the ongoing environmental changes will not be restricted to specific areas but will affect the whole biosphere. No matter how many experiments will be performed and how much data will be collected, all that will not save our environment. We know what is needed in the first place – the global CO_2 emissions must be substantially reduced.

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Publications

2017 Algueró-Muñiz M, Alvarez-Fernandes S, Thor P, Bach LT, Esposito M, Horn HG, Ecker U, **Langer JAF**, Taucher J, Malzahn AM, Riebesell U, Boersma M (2017) Ocean acidification effects on mesozooplankton community development: results from a long-term mesocosm experiment. PloS one DOI: 0.1371/journal.pone.0175851

2016 **Lange JAF**, Sharma R, Schmidt SI, Bahrdt S, Horn HG, Algueró-Muñiz M, Nam B, Achterberg EP, Boersma M, Thines M, Schwenk K (2016). Community barcoding reveals little effect of ocean acidification on the composition of coastal plankton communities: evidence from a long-term mesocosm study in the Gullmar Fjord, Skagerrak. PloS one DOI: 10.1371/journal.pone.0175808

2016 Meunier CL, Algueró-Muñiz M, Horn HG, **Lange JAF**, Boersma M (2016). Direct and indirect effects of near-future pCO_2 levels on zooplankton dynamics. Marine and Freshwater Research: DOI: 10.1071/MF15296

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Langer JAF, Meunier CL, Ecker U, Horn HG, Schwenk K and Boersma M (2018) On the evolutionary potential of the calanoid copepod *Acartia tonsa* towards ocean acidification: insights from a long-term laboratory selection study. *Submitted*.

Langer JAF, Sharma R, Nam B, Beorsma M, Schwenk K, Thines M. Marine oomycetes: community barcoding results from an early winter plankton bloom (2012) in the Brudenell River (Prince Edward Island). *Submitted.*

Conference presentations

2015 **Langer JAF**, Sharma R, Schmidt S, Nam B, Thines M, Schwenk K, Boersma M.: Effects of ocean acidification on zooplankton: Metabarcoding results from a mesocosm experiment. 50th European Marine Biology Symposium, Germany

2015 **Langer JAF**, Sharma R, Schmidt S, Nam B, Thines M, Schwenk K, Boersma M.: Effects of ocean acidification on zooplankton: Metabarcoding results from a long-term mesocosm experiment in the North Sea. KOSMOS 2013 Data Workshop, Kiel, Germany

2015 **Langer JAF**, Sharma R, Schmidt S, Nam B, Thines M, Schwenk K, Boersma M.: Effects of ocean acidification on zooplankton: Metabarcoding results from a long-term mesocosm experiment in the North Sea. ASLO Aquatic Science Meeting, Spain

2014 **Langer JAF**, Röder N, Boersma M, Schwenk K.: Mesozooplankton's adaptations to ocean acidification: an overview. AWI PhD Days, Helgoland, Germany

2013 **Langer JAF**, Schwenk K, Boersma M.: Presentation about the plans for the genetic analyses of the taken meso- and microzooplankton samples during the KOSMOS experiment in Kristineberg. KOSMOS 2013 Data Workshop, Kiel, Germany

Poster

2015 **Langer JAF**, Sharma R, Schmidt S, Nam B, Thines M, Schwenk K, Boersma M.: Metabarcoding results from a long-term mesocosm experiment in the North Sea. BIOACID Phase II Final Meeting, Kiel, Germany

2014 **Langer JAF**, Boersma M, Schwenk K.: Evolutionary response to ocean acidification. BIOACID Phase II Meeting, Kiel, Germany

2013 **Langer JAF**, Boersma M, Schwenk K.: Can mesozooplankton organisms adapt to the changing chemical composition and degradation in food quality caused by ocean acidification? BIOACID Phase II Meeting, Warnemünde, Germany

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Research stays

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Education

Studies

2006 - 2012 Biology of Organisms at the University of Osnabrück

2012 Master of Science
Master Thesis: "Determination of microsatellite-markers for first phylogeography and population genetic studies of selected East-Atlantic populations of *Stygocapitella subterranea* (Parergodrilidae, Annelida)"

2009 Bachelor of Science

Bachelor Thesis: “Faunistic investigation and distribution ranges of selected taxa of marine meiofauna in the area of the “Königshafen” on the North Sea island of Sylt and the adjacent areas”

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

"Plankton in an acidifying ocean: from individual responses to community changes and the potential of adaptation"

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

Ort, Datum

Julia Anna Franziska Langer