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Emiliania huxleyi shows identical responses to elevated pCO_2 in TA and DIC manipulations

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ABSTRACT

With respect to their sensitivity to ocean acidification, calcifiers such as the coccolithophore *Emiliania huxleyi* have received special attention, as the process of calcification seems to be particularly sensitive to changes in the marine carbonate system. For *E. huxleyi*, apparently conflicting results regarding its sensitivity to ocean acidification have been published (Iglesias-Rodriguez et al., 2008a; Riebesell et al., 2000). As possible causes for discrepancies, intra-specific variability and different effects of CO_2 manipulation methods, i.e. the manipulation of total alkalinity (TA) or total dissolved inorganic carbon (DIC), have been discussed. While Langer et al. (2009) demonstrate a high degree of intra-specific variability between strains of *E. huxleyi,* the question whether different CO_2 manipulation methods influence the cellular responses has not been resolved yet. In this study, closed TA as well as open and closed DIC manipulation methods were compared with respect to *E. huxleyi's* CO_2-dependence in growth rate, POC- and PIC-production. The differences in the carbonate chemistry between TA and DIC manipulations were shown not to cause any differences in response patterns, while the latter differed between open and closed DIC manipulation. The two strains investigated showed different sensitivities to acidification of seawater, RCC1256 being more negatively affected in growth rates and PIC production than NZEH.

1. Introduction

Since the industrial revolution, anthropogenic activities such as the burning of fossil fuels or changes in land use have increased atmospheric pCO_2 values from about 280 μatm to 385 μatm (Lüthi et al., 2008; Tans, 2009). About one third of the emitted CO_2 has already been taken up by the oceans, leading to increased DIC concentrations in surface waters (Wolf-Gladrow et al., 1999). The subsequent changes in speciation, such as increased CO_2 concentrations $[CO_2]$ and decreased CO_3^{2-} concentrations $[CO_3^{2-}]$, lead to decreasing oceanic pH values (Broecker et al., 1971). This process, commonly referred to as ocean acidification, has diverse effects on marine organisms, communities and ecosystems (e.g. Bijma et al., 1999; Kleypas et al., 1999; Raven et al., 2005; Tortell et al., 2002).

There have been several studies investigating ocean acidification effects on phytoplankton on the single species and community level (for review see Fabry et al., 2008; Rost et al., 2008). CO_2 perturbation experiments are the prime tools to mimic future CO_2 scenarios and to study organism responses. These experiments can be conducted by i) equilibrating with air of a certain pCO_2, ii) the addition of $NaHCO_3$, Na_2CO_3, or iii) the addition of a strong acid or base (Riebesell et al., 2010). In any of these perturbations, the seawater carbonate system will react by increasing or decreasing the relative proportions of the carbonate species or the DIC concentration according to its new equilibrium state. The most common perturbation methods, leading to very similar speciation with regard to pH, $[CO_2]$, $[CO_3^{2-}]$ and Ω_{Ca} (calcite saturation state), are the manipulation of dissolved inorganic carbon (DIC) by aeration with a certain pCO_2 (while keeping total alkalinity (TA) constant) and manipulation of the TA by the addition of HCl or NaOH (while DIC stays constant). DIC manipulations reflect current changes in the marine carbonate chemistry. Even though TA perturbations differ regarding the quantity manipulated, they mimic the carbonate speciation as occurring during ocean acidification quite closely (Schulz et al., 2009).

With regard to climate change and its effects on the world's oceans, calcifying organisms are of major importance. Coccolithophores are considered to account for a significant fraction of the pelagic biogenic carbonate precipitation (Baumann et al., 2004; Milliman, 1993) and are mainly responsible for creating and maintaining the oceans vertical gradient in total alkalinity (Wolf-Gladrow et al., 1999). This group of marine calcifying phytoplankton has received special attention within the framework of ocean acidification research as they were shown to exhibit distinct sensitivity to elevated pCO_2 values (Fabry et al., 2008; Rost et al., 2008). Riebesell et al. (2000) reported a reduction in calcification in the most prominent coccolithophore *Emiliania huxleyi* under future CO_2 scenarios. Since then, several studies have confirmed the sensitivity of this species to acidification (Delille et al., 2005; Feng

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et al., 2008; Langer et al., 2009; Sciandra et al., 2003). These findings have recently been challenged by Iglesias-Rodriguez et al. (2008a), who observed enhanced calcification under elevated pCO_2 in *E. huxleyi*. The authors attributed these striking differences to the application of different manipulation methods. As the TA manipulation used in Riebesell et al. (2000) do not mimic future scenarios as closely as DIC manipulations, Iglesias-Rodriguez et al. (2008a, 2008b) claimed that their results represent more realistic responses of *E. huxleyi* to ocean acidification.

As two different strains were used in these studies (PLYB92/11 in Riebesell et al., 2000; NZEH in Iglesias-Rodriguez et al., 2008a), intra-specific variability between *E. huxleyi* strains may have also caused differences in the response patterns. Intra-specific variability has been shown to lead to different responses for four strains of *E. huxleyi* (Langer et al., 2009). The strains used in Langer et al. (2009), however, neither included the one used by Riebesell et al. (2000) nor the one used by Iglesias-Rodriguez et al. (2008a). Therefore, the findings by Langer et al. (2009) are suggestive but not unambiguous with regard to the discrepancy between Riebesell et al. (2000) and Iglesias-Rodriguez et al. (2008a). Shi et al. (2009) compared responses of one *E. huxleyi* strain (NZEH) in growth, POC and PIC quota and production from closed TA and open DIC manipulations. Even though the cells were responding differently in the two manipulations, it remains unclear whether this was due to differences in the carbonate chemistry or mechanical effects of gas bubbling, occurring in the open DIC manipulation only.

As the reasons for differences between Riebesell et al. (2000) and Iglesias-Rodriguez et al. (2008a) are still unresolved, the aim of this study was to compare the effects of different CO_2 manipulation methods. To this end, the responses of two strains of *E. huxleyi* to changing carbonate chemistry were investigated in three different manipulation approaches. First, ecophysiological responses to TA (as applied by Riebesell et al., 2000) and DIC manipulations (as applied by Iglesias-Rodriguez et al., 2000a) were compared. Additionally, to further investigate the differences between TA and open DIC manipulations as found by Shi et al. (2009), the effect of mechanical perturbation was examined by comparing closed pre-equilibrated with open continuously aerated DIC manipulated incubations.

2. Material and methods

2.1. Cultures and media preparation

Monoclonal cultures of two strains of the coccolithophore *E. huxleyi* (NZEH / PLY M219, isolated near New Zealand, supplied by the Plymouth Culture Collection, http://www.mba.ac.uk/culturecollection.php; RCC1256, isolated near Iceland, supplied by the Roscoff Culture Collection, http://www.sb-roscoff.fr/Phyto/RCC) were grown in 0.1 μm sterile-filtered North Sea seawater. The salinity was 32.38 (Guildline Autosal 8400B salinometer, Ontario, Canada).

The seawater was enriched with vitamins and trace metals according to f/2 media (Guillard and Ryther, 1962; except for iron which was added in a concentration of 1.94 μmol L^{-1} $FeCl_3$). Seawater was also enriched with nitrate (NO_3^-) and phosphate (HPO_4^{2-}) to yield concentrations of 100 and 6 μmol L^{-1}, respectively. Nutrient concentrations were measured colorimetrically using a continuous flow analyzer (Evolution III, Alliance Instruments, Salzburg, Austria).

Dilute-batch cultures were grown in 2 L borosilicate bottles at 15 ± 0.2 °C. Daylight lamps (Lumilux De Luxe T8, Osram, München, Germany) provided light intensities of 170 ± 15 μmol photons m^{-2} s^{-1} as measured with a Li-Cor datalogger (Li-Cor, Lincoln, USA) equipped with a 4π-sensor (Walz, Effeltrich, Germany). A light: dark cycle of 16:8 h was applied and all samples were taken between 6 and 10 hours after the beginning of the light phase.

In order to keep cultures in exponential growth phase and to prevent significant changes in carbonate chemistry as well as attrition of nutrients in the media, cultures were diluted regularly (cell densities never exceeded 72,000 cells mL^{-1}). Cultures were kept at experimental temperatures, light intensities and cell densities for at least two weeks, followed by another week being pre-acclimated to experimental pCO_2 levels (5–7 generations).

2.2. CO_2 perturbation experiments

Different CO_2 manipulation methods (closed TA, closed and open DIC manipulation) were applied to test ecophysiological responses to different CO_2 concentrations. In the alkalinity manipulations, carbonate chemistry was adjusted by addition of calculated amounts of HCl or NaOH (1 N Titrisol, Merck, Darmstadt, Germany) to seawater for which DIC concentrations were known. The manipulated media were stored in 2 L borosilicate bottles, which were sealed immediately with Teflon-lined screw caps without head space to avoid CO_2 exchange with the atmosphere.

DIC manipulations and incubations were conducted in 2 L borosilicate bottles equipped with glass frits for aeration. The media were sparged continuously with humidified, 0.2 μm-filtered air of different partial pressures of CO_2 (180, 380, 750 and 1000 μatm). Gas flow rates were 130 ± 10 mL min^{-1}. Gas mixtures were generated using a custom-made gas flow controller. CO_2-free air (<1 ppmv CO_2; Dominick Hunter, Willich, Germany) was mixed with pure CO_2 (Air Liquide Deutschland, Düsseldorf, Germany) by a mass flow controller based system (CGM 2000 MCZ Umwelttechnik, Bad Nauheim, Germany). The CO_2 concentration was regularly controlled with a non-dispersive infrared analyzer system (LI6252, LI-COR Biosciences, Bad Homburg, Germany) calibrated with CO_2-free air and purchased gas mixtures of 150 ± 10 and 1000 ± 20 ppmv CO_2 (Air Liquide Deutschland, Düsseldorf, Germany). Experiments were started after 48 h of aeration in order to ensure equilibration. Bottles of the closed DIC treatments were sealed without head space with Teflon-lined screw caps. A roller table was used to keep the cells in suspension. Bottles of the open DIC treatments (only applied to strain NZEH) were sparged continuously with the respective gases over the duration of the experiment. Sedimentation of cells was minimised by aeration and shaking of bottles twice a day.

2.3. Determination of carbonate chemistry

Samples for TA measurements were 0.6 μm-filtered and stored in 150 mL borosilicate bottles at 3 °C. TA was determined by duplicate potentiometric titrations (Brewer et al., 1986) using a TitroLine alpha plus autosampler (Schott Instruments, Mainz, Germany), and calculation from linear Gran plots (Gran, 1952). Certified Reference Materials (CRMs, Batch No. 54) supplied by A. Dickson (Scripps Institution of Oceanography, USA) were used to correct the measurements. The average reproducibility was ± 5 μmol kg^{-1} (n = 10).

DIC samples were filtered through 0.2 μm cellulose-acetate syringe-filters and stored head-space free in 5 mL gas-tight borosilicate bottles at 3 °C. DIC was measured colorimetrically in triplicates with a QuaAAtro autoanalyzer (Seal Analytical, Mequon, USA) with an average reproducibility of ± 5 μmol kg^{-1} (n = 20). CRMs (Batch No. 54) were used to correct the measurements. Shifts in DIC concentrations due to CO_2 exchange were prevented by opening the storage vials less than one minute prior to each measurement.

Seawater pH was determined potentiometrically on the NBS scale using a glass electrode/reference electrode cell (Schott Instruments, Mainz, Germany), which included a temperature sensor and was two-point calibrated with NBS buffers prior to every set of measurements. Average repeatability was found to be ± 0.02 pH units (n = 30).

Calculations of the carbonate system were based on measurements of DIC, pH, temperature, salinity und nutrient concentrations. They were performed with the programme CO_2sys (Pierrot et al., 2006). The dissociation constants of carbonic acid of Mehrbach et al.

(1973), refit by Dickson and Millero (1987) and the dissociation constants for H_2SO_4 of Dickson (1990) were used for calculations.

2.4. Ecophysiological responses

Cell densities were determined using a Coulter Multisizer III (Beckman-Coulter, Fullerton, USA). Growth rates (μ) were calculated as:

$$\mu = (\ln c_1 - \ln c_0)\,\Delta t^{-1} \quad (1)$$

where c_0 and c_1 are the initial and the final cell counts in cells mL^{-1}, respectively, and Δt is the time between both counts in days.

For analysis of total particulate carbon (TPC) and particulate organic carbon (POC), cells were filtered onto precombusted (15 h, 500 °C) glass fibre filters (GF/F 0.6 µm nominal pore size; Whatman, Maidstone, UK) within 7 to 9 h after onset of light phase. Filters were stored at −20 °C and dried for at least 12 h at 60 °C prior to sample preparation. Analysis was performed using an Automated Nitrogen Carbon Analyser mass spectrometer system (ANCA-SL 20–20, SerCon Ltd., Crewe, UK). Contents of TPC and POC were corrected for blank measurements and normalised to filtered volume and cell densities of culture media to yield cellular quotas. Particulate inorganic carbon contents (PIC) were calculated as the difference in carbon content between TPC and POC. The production of particulate organic carbon (P_{POC}) and the production of particulate inorganic carbon (P_{PIC}) were calculated as:

$$P_{POC} = \mu\left(\text{POC cell}^{-1}\right) \quad (2)$$

$$P_{PIC} = \mu\left(\text{PIC cell}^{-1}\right) \quad (3)$$

where the production rates are given in pg POC $cell^{-1}$ d^{-1} and pg PIC $cell^{-1}$ d^{-1}, respectively.

2.5. Statistics

Significant responses were determined by analysis of covariances of the trend in response variables over the pCO_2 range tested, which were based on univariate general linear models performed with SPSS 11 (IBM, Chicago, USA). The null hypothesis ('no difference in responses') was rejected if the respective p-value was <0.05.

3. Results

3.1. Carbonate chemistry

In the different manipulation methods, the final pCO_2 values ranged between 153 and 1283 µatm (Table 1). In the DIC manipulation (i.e. aeration of the media with air of a certain pCO_2), TA stayed quasi-constant at values of 2325 ± 27 µmol kg^{-1} while DIC varied between 1922 ± 17 µmol kg^{-1} at the lowest and 2184 ± 26 µmol kg^{-1} at the highest pCO_2 treatment. In the TA manipulation (i.e. addition of HCl or NaOH), DIC stayed quasi-constant at values of 2080 ± 21 µmol kg^{-1} while TA varied between 2541 ± 64 µmol kg^{-1} at the lowest and 2198 ± 41 µmol kg^{-1} at the highest pCO_2 treatment. Lowest calcite saturation state was 1.33. DIC and TA depletion by the cells over the course of the experiment were on average 3% and 4% for TA and DIC, respectively.

3.2. Ecophysiological responses to carbonate chemistry manipulations

Both *E. huxleyi* strains used in this study responded in growth rate and the production of POC and PIC to changes in pCO_2. The response patterns found did not differ noteworthy between TA and closed DIC manipulation (Table 2). Differences in response patterns were, however, observed between the two DIC manipulations (open vs. closed). In the following, responses in growth rates, PIC and POC quota and production are described for the two strains RCC1256 and NZEH.

The growth rates of strain RCC1256 showed virtually the same response to increasing pCO_2 in DIC and TA manipulations (Table 2, Fig. 1a). In both manipulations, growth rates stayed constant at about 1.2 d^{-1} up to a pCO_2 value of ~800 µatm and declined statistically significant (i.e. $p<0.05$) beyond 800 µatm to values of about 1.1 d^{-1} ($F=18.02$, $p<0.001$). With respect to POC quota and POC production, RCC1256 responded differently to closed DIC and TA manipulation ($F=11.27$, $p=0.004$ and $F=5.97$, $p=0.025$, respectively). With respect to increasing pCO_2 levels, POC quota (Table 2, Fig. 1c) did not change in the closed DIC treatment, while a statistically insignificant increase (~30%) was observed in the TA manipulations. While POC production (Table 2, Fig. 1d) did not change substantially in the TA manipulations, a slight yet statistically insignificant decrease (~10%) was observed in the closed DIC manipulations.

With regard to PIC quota and production, no differences were found between the two manipulation methods. PIC quota (Table 2, Fig. 1e) decreased significantly by about 30% over the pCO_2 range

Table 1
Measured and calculated carbonate chemistry parameters of the media at 15 °C at the end of all experiments ($n=3$). Following Hoppe et al. (2010), DIC and pH were used as input parameters for calculations. Numbers in bold characters denote the manipulated parameter.

Strain	Experiment	Measured			Calculated				
		TA [µmol kg^{-1}]	DIC [µmol kg^{-1}]	pH (NBS)	pCO_2 [µatm]	CO_2 [µmol kg^{-1}]	HCO_3^- [µmol kg^{-1}]	CO_3^{2-} [µmol kg^{-1}]	Ω_{Ca}
RCC1256	TA manipulation closed	**2508 ± 82**	2114 ± 10	8.32 ± 0.03	288 ± 20	11 ± 1	1882 ± 19	221 ± 11	5.38 ± 0.26
		2299 ± 18	2097 ± 9	7.97 ± 0.05	672 ± 89	26 ± 3	1967 ± 17	104 ± 11	2.54 ± 0.27
		2173 ± 17	2063 ± 7	7.82 ± 0.01	946 ± 10	36 ± 0	1955 ± 6	73 ± 1	1.76 ± 0.03
		2160 ± 7	2082 ± 6	7.72 ± 0.01	1206 ± 30	46 ± 1	1978 ± 6	58 ± 2	1.42 ± 0.04
	DIC manipulation closed	2349 ± 7	**1940 ± 11**	8.44 ± 0.01	191 ± 3	7 ± 0	1670 ± 11	263 ± 3	6.38 ± 0.07
		2344 ± 8	**2058 ± 5**	8.20 ± 0.02	379 ± 14	14 ± 1	1876 ± 2	167 ± 6	4.06 ± 0.14
		2316 ± 7	**2123 ± 7**	7.98 ± 0.01	656 ± 10	25 ± 0	1989 ± 7	108 ± 1	2.64 ± 0.03
		2342 ± 13	**2156 ± 8**	7.88 ± 0.01	846 ± 24	32 ± 1	2036 ± 8	88 ± 2	2.14 ± 0.05
NZEH	TA manipulation closed	**2573 ± 19**	2089 ± 6	8.40 ± 0.01	232 ± 3	9 ± 0	1823 ± 5	257 ± 3	6.26 ± 0.08
		2366 ± 15	2070 ± 13	8.21 ± 0.03	369 ± 27	14 ± 1	1883 ± 20	173 ± 9	4.21 ± 0.21
		2257 ± 1	2046 ± 8	7.95 ± 0.03	680 ± 45	26 ± 2	1923 ± 4	98 ± 7	2.38 ± 0.16
		2236 ± 1	2081 ± 7	7.73 ± 0.06	1175 ± 157	45 ± 6	1976 ± 5	60 ± 9	1.47 ± 0.21
	DIC manipulation closed	2312 ± 17	**1912 ± 12**	8.14 ± 0.01	404 ± 7	15 ± 0	1759 ± 8	138 ± 4	3.34 ± 0.09
		2276 ± 14	**2025 ± 30**	7.95 ± 0.02	673 ± 31	26 ± 1	1903 ± 30	97 ± 3	2.35 ± 0.07
		2295 ± 3	**2118 ± 2**	7.82 ± 0.03	957 ± 57	36 ± 2	2006 ± 4	76 ± 4	1.84 ± 0.10
		2311 ± 3	**2184 ± 21**	7.79 ± 0.01	1066 ± 28	40 ± 1	2071 ± 20	72 ± 2	1.76 ± 0.04
	DIC manipulation open	2351 ± 8	**1913 ± 12**	8.51 ± 0.01	157 ± 3	6 ± 0	1610 ± 13	297 ± 3	7.23 ± 0.07
		2322 ± 7	**2051 ± 2**	8.24 ± 0.02	336 ± 18	13 ± 1	1854 ± 7	184 ± 8	4.47 ± 0.20
		2361 ± 3	**2211 ± 2**	7.86 ± 0.02	909 ± 45	35 ± 2	2090 ± 2	86 ± 4	2.10 ± 0.10

Table 2
Ecophysiological responses of *E. huxleyi* strains RCC1256 and NZEH to changing pCO_2 as found under TA, open DIC and closed DIC manipulations. a: significant ($p<0.05$) responses to pCO_2; b: significant ($p<0.05$) differences between TA and closed DIC manipulation; c: significant ($p<0.05$) differences between open and closed DIC manipulation.

Strain	Experiment	pCO_2	μ [d^{-1}]	POC [pg $cell^{-1}$]	P_{POC} [pg $cell^{-1}$ d^{-1}]	PIC [pg $cell^{-1}$]	P_{PIC} [pg $cell^{-1}$ d^{-1}]	PIC : POC
			a	*b*	*b*	*a*	*a*	*a, b*
RCC1256	TA manipulation	288	1.19 ± 0.03	10.35 ± 0.19	12.36 ± 0.22	10.19 ± 1.09	12.14 ± 0.85	0.98 ± 0.09
	closed	672	1.17 ± 0.03	12.39 ± 0.30	14.76 ± 0.18	8.86 ± 0.21	10.55 ± 0.38	0.72 ± 0.03
		946	1.10 ± 0.04	11.29 ± 0.71	12.42 ± 0.29	6.72 ± 0.00	7.19 ± 0.00	0.57 ± 0.00
		1206	1.06 ± 0.02	13.07 ± 0.93	13.82 ± 0.89	7.17 ± 0.42	7.59 ± 0.41	0.55 ± 0.06
			a	*b*	*b*	*a*	*a*	*a*
	DIC manipulation	191	1.17 ± 0.01	13.22 ± 1.10	15.41 ± 1.26	10.25 ± 0.30	11.95 ± 0.40	0.78 ± 0.08
	closed	379	1.17 ± 0.03	12.16 ± 0.18	14.27 ± 0.54	9.95 ± 0.60	11.68 ± 0.86	0.82 ± 0.05
		656	1.18 ± 0.04	11.93 ± 0.96	14.08 ± 1.22	8.48 ± 0.30	10.02 ± 0.64	0.71 ± 0.05
		846	1.10 ± 0.05	12.18 ± 0.97	13.46 ± 1.65	8.39 ± 0.99	9.28 ± 1.45	0.69 ± 0.03
				a		*a, b*	a	a
NZEH	TA manipulation	232	1.21 ± 0.07	9.90 ± 1.52	11.89 ± 1.19	10.97 ± 1.97	13.35 ± 3.16	1.15 ± 0.37
	closed	369	1.22 ± 0.04	10.35 ± 0.69	12.60 ± 1.18	10.78 ± 0.76	13.13 ± 1.30	1.04 ± 0.01
		680	1.22 ± 0.06	11.26 ± 0.88	13.67 ± 0.88	9.98 ± 0.77	12.11 ± 0.71	0.89 ± 0.01
		1175	1.18 ± 0.03	10.97 ± 0.41	12.93 ± 0.19	9.34 ± 0.68	10.99 ± 0.54	0.85 ± 0.03
			c	*a, c*	*a, c*	*a, b, c*	*a, c*	*a*
	DIC manipulation	404	1.22 ± 0.02	11.51 ± 0.29	14.03 ± 0.38	13.44 ± 2.88	14.42 ± 1.51	1.17 ± 0.28
	closed	673	1.25 ± 0.02	12.30 ± 0.20	15.33 ± 0.13	11.01 ± 0.93	13.74 ± 1.41	0.90 ± 0.09
		957	1.19 ± 0.04	12.22 ± 0.44	14.55 ± 0.80	10.71 ± 0.32	12.75 ± 0.74	0.88 ± 0.02
		1066	1.16 ± 0.06	13.35 ± 0.79	15.46 ± 1.66	10.28 ± 0.47	11.09 ± 0.96	0.77 ± 0.06
			c	*a, c*	*a, c*	*a, c*	*a, c*	*a*
	DIC manipulation	157	0.99 ± 0.12	8.57 ± 1.28	8.41 ± 0.87	8.92 ± 1.71	8.71 ± 0.83	1.04 ± 0.06
	open	336	1.08 ± 0.02	8.96 ± 0.46	9.68 ± 0.57	9.49 ± 0.51	10.25 ± 0.61	1.06 ± 0.10
		909	1.09 ± 0.04	9.73 ± 0.40	10.56 ± 0.48	7.37 ± 0.61	8.01 ± 0.79	0.76 ± 0.09

tested in both manipulation methods applied ($F=47.18$, $p<0.001$). Furthermore, PIC production (Table 2; Fig. 1f) decreased significantly in both manipulation methods by about 35–40% between 200 and 1200 μatm pCO_2 ($F=58.30$, $p<0.001$). The PIC:POC ratios (Table 2, Fig. 1b) decreased significantly with increasing pCO_2 by approximately 40% and 20% in TA and DIC manipulations, respectively ($F=43.21$, $p<0.001$). Absolute values and slopes differed significantly between the two kinds of manipulations ($F=11.17$, $p=0.004$).

The strain NZEH did not show any growth rate response to changes in pCO_2, neither in the TA manipulations nor in the closed DIC manipulations (Table 2, Fig. 2a). Average growth rate was 1.21 ± 0.03 d^{-1}. In the open DIC manipulations, however, growth rates were about 10–14% lower (leading to significant differences between the two DIC manipulations; $F=18.53$, $p<0.001$). In these manipulations, cells could not be held in suspension and sedimentation occurred below the gas frits. The POC quotas (Table 2, Fig. 2c) differed between the three manipulation methods (highest quota was found in the closed DIC manipulation and lowest in the open DIC manipulations, which significantly differed from the other treatments; $F=10.08$, $p=0.006$). The overall trend in all manipulations was a small, yet significant increase of about 10% in the range between 160 and 1200 μatm ($F=9.57$, $p=0.006$). POC production (Table 2, Fig. 2d) stayed more or less constant in TA manipulations, while in the closed DIC manipulations, POC production increased by about 10% below 600 μatm ($F=8.59$, $p=0.009$) and remained unaffected above this pCO_2 level. The pattern found in the open DIC manipulation significantly differed from those of the closed manipulations ($F=31.41$, $p<0.001$), with increased values of about 25% ($F=8.59$, $p=0.009$).

The PIC quotas (Table 2, Fig. 2e) differed between the manipulation methods ($F=6.09$, $p=0.023$), but showed the same significant trend of slightly decreasing values ($F=11.24$, $p=0.009$). PIC production (Table 2, Fig. 2f) decreased by about 30% with increasing pCO_2 ($F=9.63$, $p=0.007$) in both TA and closed DIC manipulations, while an optimum curve with maximum values at ~340 μatm was observed in the case of open DIC manipulations ($F=24.12$, $p<0.001$). The PIC:POC ratio (Table 2, Fig. 2b) was generally higher than that of RCC1256 and was found to decrease significantly from about 1.1 at low to 0.8 at high pCO_2 in all manipulation methods (15–20%; $F=14.04$, $p=0.001$).

4. Discussion

Differences in carbonate chemistry speciation between DIC and TA manipulation have been argued to be the reason for different ecophysiological responses of *E. huxleyi* to changing pCO_2 (Iglesias-Rodriguez et al., 2008a, 2008b). Other authors held differences in experimental protocols (Riebesell et al., 2008) or the intraspecific variability between *E. huxleyi* strains (Langer et al., 2009) responsible for these differences. Shi et al. (2009) found strain NZEH responding differently to TA manipulation and open DIC manipulation and argued that these differences are likely to be due to the mechanical effect of bubbling rather than to differences in carbon speciation.

4.1. Comparison of different manipulation methods

This study was able to show that the differences in the carbonate chemistry of the two manipulation methods (changing DIC with constant TA vs. changing TA with constant DIC) had no substantially different effect on the overall ecophysiology of two strains of *E. huxleyi* (Table 2, Figs. 1 and 2). Compared to the different responses between open and closed DIC manipulations, in which the carbonate chemistry did not differ, the differences between TA and DIC manipulation are negligible and probably due to variability in the organisms overall sensitivity.

The differences in the response patterns between open and closed DIC manipulations were probably caused by the mechanical effect of bubbling and/or the effect of sedimentation of cells in the open DIC manipulations. In the closed DIC manipulations cells were always kept in suspension by steady and gentle rotation of the culture flasks. In the open DIC manipulations, the aeration process established areas of low currents, where cells could sediment. In such cell congregations, changes in nutrient and light availability as well as carbonate chemistry may occur and influence the physiology of *E. huxleyi*. Differences in ecophysiological responses to increasing pCO_2 in this treatment could, however, also be caused by a sensitivity of *E. huxleyi* to stress imposed by bubbling (cf. Merchuk, 1991). The latter explanation was put forward by Shi et al. (2009), who reported reverse trends in POC and PIC production in TA and open DIC manipulations.

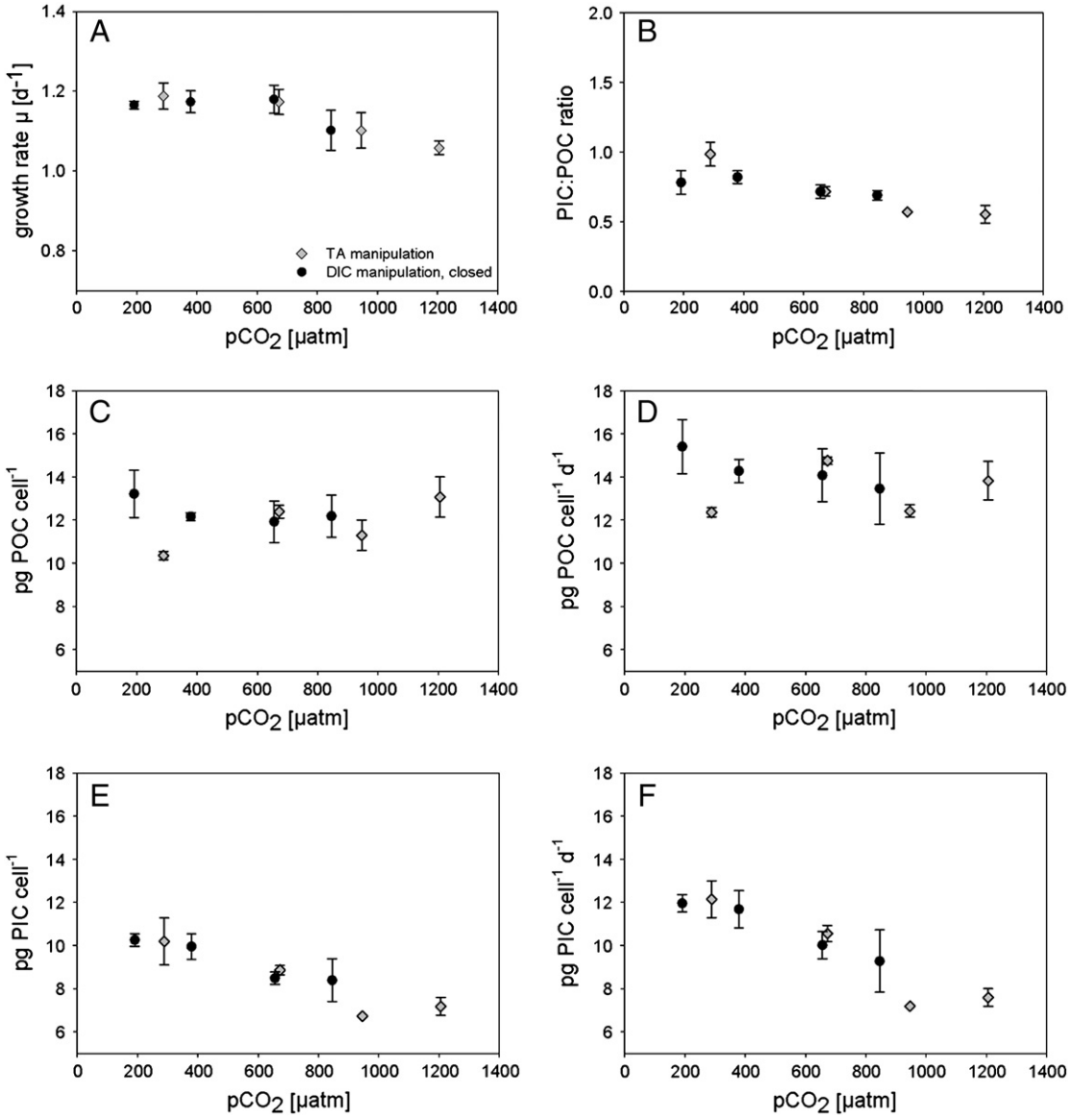

Fig. 1. Ecophysiological parameters of *E. huxleyi* strain RCC 1256 in response to changes in pCO_2 as observed in TA manipulation (grey symbols) and closed DIC manipulations (black symbols): A growth rates, B PIC:POC ratio, C POC quota, D POC production, E PIC quota, F PIC production.

The finding that differences in carbonate chemistry between DIC and TA manipulations do not cause changes in the ecophysiological response patterns is in agreement with other observations. Studies on coral recruitment and growth (Cohen and McCorkle, 2009; Schneider and Erez, 2006) obtained similar responses irrespective of the pCO_2 manipulation method applied. Our findings are in line with theoretical considerations raised by other authors (Hurd et al., 2009; Schulz et al., 2009), who argued that over the pCO_2 range tested, the physiological important components of the carbonate system ($[H^+]$, CO_3^{2-}, HCO_3^- as well as the calcite saturation state) do not differ significantly between the two manipulation methods.

4.2. Strain-specific responses of RCC1256 and NZEH

Strain-specific responses to increasing pCO_2 levels in *E. huxleyi* have been shown by Langer et al. (2009). In the following, the results for the two strains used in this study will be discussed with regard to the comparability to other findings on the same strains.

The sensitivity of strain RCC1256 to changes in carbonate chemistry seems to differ between studies. While Langer et al. (2009) found growth rates to decrease by 35% between 200 and 900 μatm, a decline of less than 10% was found in this study. It has to be noted, however, that the carbonate system in Langer et al. (2009) was calculated from TA and DIC, which can lead to an underestimation of pCO_2 (Hoppe et al., 2010). If the pCO_2 values are corrected for the discrepancies related to that combination of input parameters, as found for this particular set and quality of measurements, the same changes in growth rates, POC and PIC production spread over a wider pCO_2 range from 200 to 1200 μatm. Differences in growth rate responses of RCC1256 in the two studies now appear less pronounced. Both PIC quota and production significantly decreased with increasing pCO_2 in this study (Table 2; Fig. 1e; 1f), while in Langer et al. (2009) only production was found to decrease. In case of POC quota and production of RCC1256, no responses to changing pCO_2 levels were found in this study, while Langer et al. (2009) reported increasing POC quota and an optimum curve with a maximum at a (corrected) pCO_2 of about 800 μatm in case of production. The PIC:POC ratio declined with increasing pCO_2 in both studies. Whether the mentioned differences in responses between this study and Langer et al. (2009) are due to the fact that different light intensities and temperatures

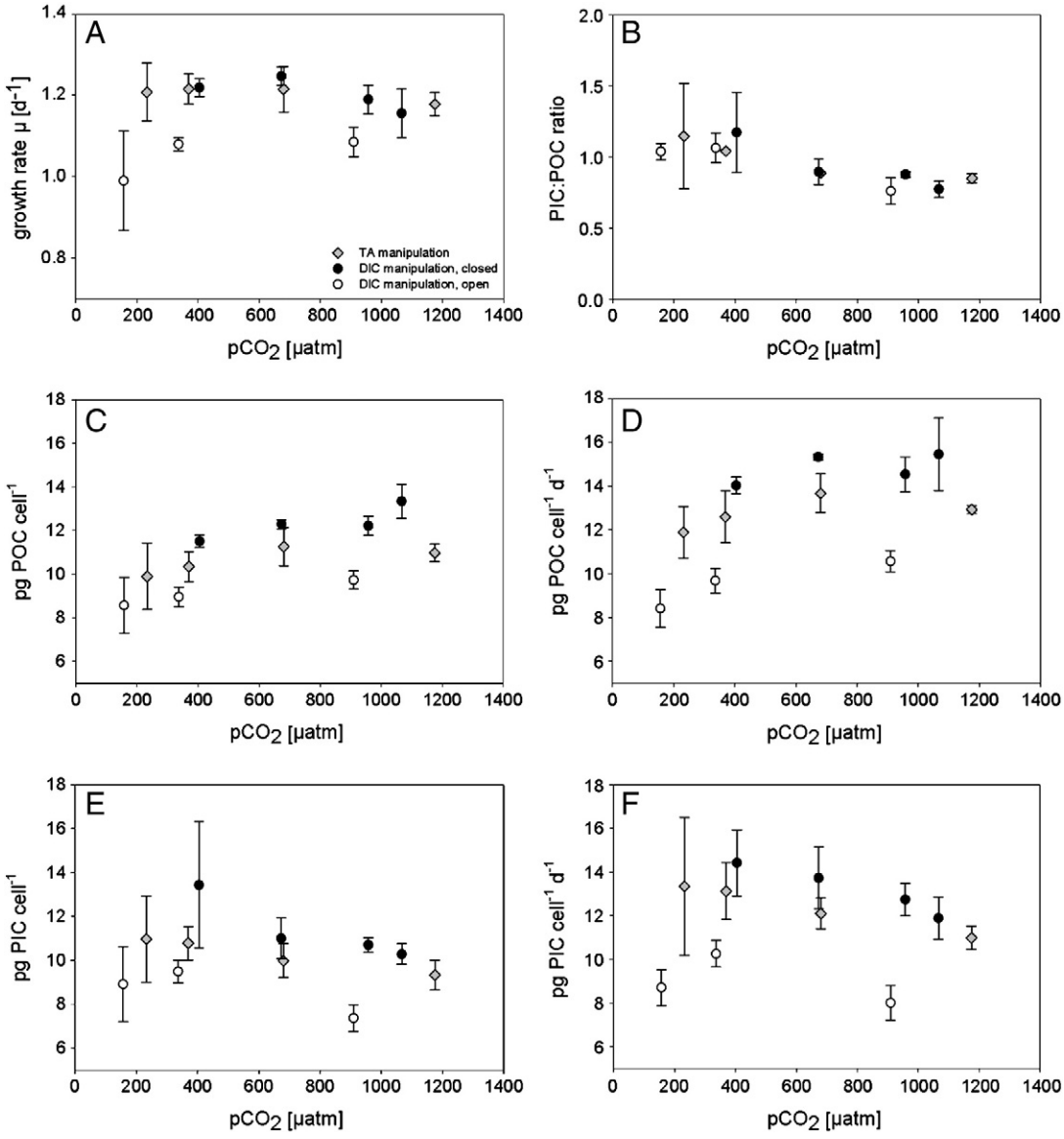

Fig. 2. Ecophysiological parameters of *E. huxleyi* strain NZEH in response to changes in pCO_2 as observed in TA manipulation (grey symbols), closed DIC manipulations (black symbols) and open DIC manipulations (white symbols): A growth rates, B PIC:POC ratio, C POC quota, D POC production, E PIC quota, F PIC production.

were used or simply reflect the natural variability in sensitivity of a single strain remains to be tested. Even if the latter is the case, major trends such as a decline or enhancement in physiological performance seem to be persistent in *E. huxleyi*.

The strain NZEH responded with slightly increased POC and slightly decreased PIC production under elevated pCO_2, while its growth rates remained constant over the pCO_2 range tested (Table 2, Fig. 2). These responses strikingly contradict the responses reported by Iglesias-Rodriguez et al. (2008a), who found the POC and PIC production of NZEH to increase by 150% and 80%, respectively, between pCO_2 values ranging from 280 to 750 μatm. This particular study, however, has been under debate because of shortcomings of its experimental protocol (Iglesias-Rodriguez et al., 2008b; Riebesell et al., 2008). As argued by Riebesell et al. (2008), the high cell densities of the pre-acclimations (up to 500.000 cells mL^{-1}) could have led to major changes in nutrient availability and carbonate chemistry. Furthermore, the actual experiments were run for 1 to 2 generations only. Even though physiological acclimation to changes in carbonate chemistry seems to be rather fast for this species (Barcelos e Ramos et al., 2009), a large proportion of the harvested biomass at the end of the experiment (25–50%) was probably produced prior to controlled experimental conditions and therefore cannot be interpreted as responses to the defined changes in pCO_2 levels. Further indications for the transfer of substantial biomass into the experiment from non-controlled pre-acclimations are the high numbers of detached coccoliths (Iglesias-Rodriguez et al., 2008a). Up to 80 detached coccoliths per cell, as observed in the high pCO_2 treatment (see their Table 1), are a strong indication for nutrient limitation having occurred. Another study on the same strain (Shi et al., 2009) also found PIC and POC production to increase with increasing pCO_2, even though to a lesser extent of about 36% and 69%, respectively. In Shi et al. (2009), cells were also not pre-acclimated, which could explain the agreement with Iglesias-Rodriguez et al. (2008a).

If these fundamental differences in the experimental approach were not the cause for the contradicting results, one would need to assume that the strains ecophysiological responses changed due to natural selection during long-term cultivation. Evolution in the lab was shown for bacterial cultures (Imhof and Schlotterer, 2001), a process that can be especially important when the number of transferred cells is low. Even though long-term cultivation of

phytoplankton strains is likely to put selection pressure on the population and to change its physiology compared to the ancestors in the field, it seems unlikely that similar cultivation conditions in culture collections lead to contrary ecophysiological responses as discussed above. The observation that absolute PIC and POC quotas and production vary between experiments, despite similar growth conditions, questions the reproducibility in terms of absolute values and numerically defined trends in ecophysiological studies. The general response patterns (i.e. decreasing growth rates at high pCO_2 values), however, seem to remain valid for a single strain and highlight the advantage of semi-quantitative or qualitative analysis when assessing the sensitivity of *E. huxleyi* (Fig. 3; cf. Fabry, 2008).

4.3. General response patterns of E. huxleyi

A noteworthy degree of intraspecific variability has been reported for *E. huxleyi* with regard to morphology, physiology and genetics (Iglesias-Rodríguez et al., 2006; Paasche, 2002; Young, 1994). Since Langer et al. (2009) published their study on the responses of four strains of *E. huxleyi* to changing carbonate chemistry, it is known that this also holds true for its responses to ocean acidification.

Over the last decade, several studies investigated responses to ocean acidification of eight different strains of *E. huxleyi* (Fig. 3). Most of the strains' growth rates were not influenced by increasing pCO_2. Furthermore, in those cases where changes have been observed (Iglesias-Rodriguez et al., 2008a; RCC1256 in: Langer et al., 2009; RCC1256 in this study), growth rates always declined. The latter is a strong hint that these cultures have been highly stressed, as *E. huxleyi* is usually capable to keep cell division rates constant over a wide range of conditions (Rost and Riebesell, 2004). More diverse responses have been found for the photosynthetic response in *E. huxleyi*. While in four experiments POC production (Feng et al., 2008; Langer et al., 2009; this study) did not change with increasing pCO_2, in four cases POC production increased (Iglesias-Rodriguez et al., 2008a; Riebesell et al., 2000; Shi et al., 2009; this study), a decrease was found in one study (Sciandra et al., 2003), and in two cases, strains showed optimum curves as response patterns (Langer et al., 2009). In summary, the CO_2-dependent changes in photosynthesis are highly variable and seem to differ between strains. With respect to PIC production, most of the studies found declining rates with increasing pCO_2, while in two studies a strain increased its PIC production rate (Iglesias-Rodriguez et al., 2008a; Shi et al., 2009) and one strain was found to be insensitive (Langer et al., 2009).

The comparison of morpho- (A, B and R; cf. Young et al., 2003) and ecotypes (oceanic vs. coastal strains) of all studies published so far does not reveal any group-specific patterns in the sensitivity to ocean acidification (Fig. 3; cf. Langer et al., 2009). With regard to the variable response patterns of different *E. huxleyi* clones (this study, Langer et al., 2009), it is at present merely possible to propose that this variability has a genetic basis, without defining its nature (Iglesias-Rodríguez et al., 2006; Langer et al., 2009). Taking all available data into account and considering some problems in the experimental setup of Iglesias-Rodriguez et al. (2008a) and Shi et al. (2009), the calcification process of *E. huxleyi* can be regarded as, although to a variable degree, sensitive to ocean acidification. It has to be kept in mind, however, that as long as the function and the ecological as well as evolutionary implications of coccolithophore calcification are unknown, the consequences of changes in calcification rates cannot be predicted with confidence. One fundamental question in this context is whether the process or the product of calcification is beneficial for the cell.

4.4. Implications for biogeochemical cycles

Similar trends were observed in several single-strain culture experiments, mesocosm experiments and field studies (e.g. Delille et al., 2005; Langer et al., 2009; Riebesell et al., 2000). Despite uncertainties in absolute quota and numerically defined trends obtained by these different ecophysiological studies, the same patterns of constant or increasing POC production and constant or declining PIC production were found in the majority of all studies mentioned. The predicted changes in coccolithophore carbon fixation and calcification have implications for future biogeochemical cycles, even though carbon fluxes also depend on other factors such as altered cellular elemental ratios and floristic shifts. Consequently, Earth system models critically depend on the input of ecophysiological responses of key functional groups such as marine calcifiers.

Based on the comparison of the data shown above, representative responses in form of absolute quota and trends with a certain slope appear oversimplified. Instead, uncertainties in the magnitude of

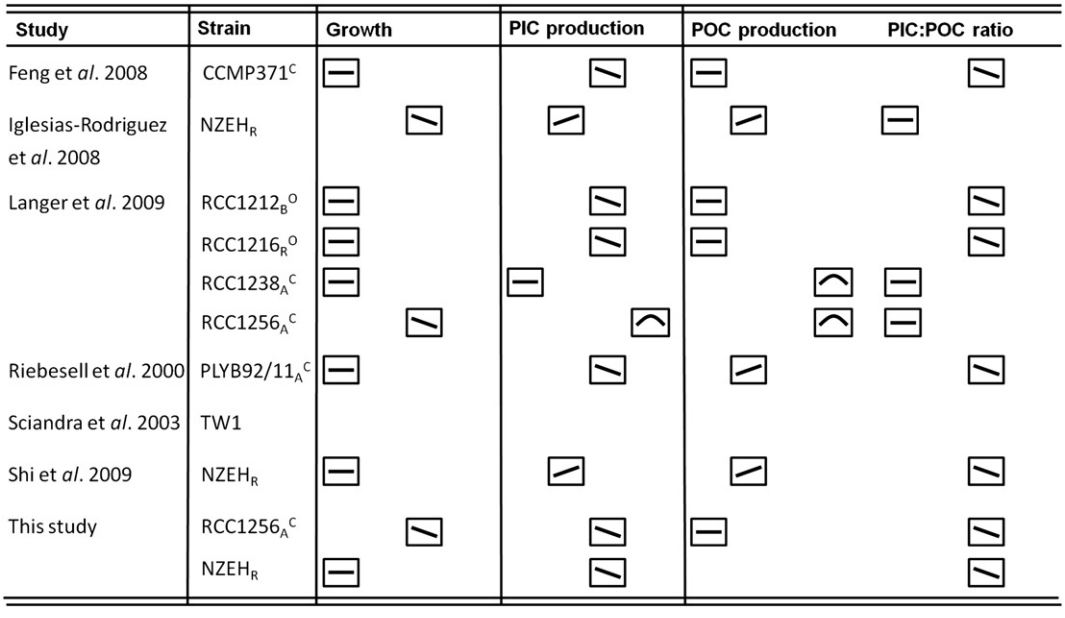

Fig. 3. Response patterns of *E. huxleyi* growth rates, POC production, PIC production and PIC:POC ratios as found in different studies. Subscript characters denote morphotype (A, B, R); superscript characters denote ecotype (C: coastal and O: oceanic). Modified after Fabry, 2008.

calcification responses to ocean acidification remain and have to be accounted for in global carbon models. As the parameterisation of calcification in global carbon cycle models is based on the results of experiments (Ridgwell et al., 2009), also the model predictions on future impacts of anthropogenic CO_2 emissions depend on the reproducibility and transferability of laboratory studies. As modellers base their parameterizations on different ecophysiological studies (e.g. Gehlen et al., 2007; Hofmann and Schellnhuber, 2009), there is no agreement in the estimates for the amount of anthropogenic CO_2 taken up by the ocean due to the reduction in coccolithophore calcification. Applying a "Eppely curve" behaviour (Eppely, 1972) to the available data on biogenic calcification (cf. Fig. 3), Ridgwell et al. (2009) suggested a progressively decreasing net community calcification with increasing CO_2 to be used for the parameterisation of carbon cycle models. Further investigations of inter- and intraspecific response-variability as well as community and ecosystem responses to changing seawater carbonate chemistry will help understanding and predicting the future fate of calcifying phytoplankton.

5. Conclusions

Differences between TA and DIC manipulations do not cause differences in the ecophysiological responses of *E. huxleyi* to changing pCO_2 levels. Other differences in the experimental protocol (e.g. continuous bubbling *vs.* pre-bubbled), however, can lead to changes in growth rates and other ecophysiological parameters. Although strain-specific differences and overall trends were confirmed, the CO_2- sensitivity within single strains of *E. huxleyi* seems to vary over time. This favours the analysis of experimental data in a semi-quantitative way, defining trends rather than numerical relationships. After comparing the ecophysiological responses of all *E. huxleyi* strains described in the literature, this species can be regarded as moderately sensitive to ocean acidification.

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