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Isolation and cultivation of microalgae select for low growth rate and tolerance to high pH

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ABSTRACT

Harmful microalgal blooms or red tides are often associated with high levels of pH. Similarly, species and strains of microalgae cultivated in the laboratory with enriched media experience recurrent events of high pH between dilutions with fresh medium. To study the potential for laboratory selection by high pH, we compared, under identical experimental conditions the upper pH tolerance limits for growth in addition to growth and production rates of 23 strains of the common bloom-forming dinoflagellate *Heterocapsa triquetra*. The strains had been cultivated in official culture centres from ca. 1 to 51 years (corresponding to 200–10,000 generations). Strains cultivated for less than 10 years had significantly lower mean and median upper pH tolerance limits for growth, and higher growth and production rates compared to strains cultivated for more than 20 years. The range and variation of upper pH tolerance limits were higher in the younger (<10 years) than in the older strains (>20 years). These results suggest selection of strains best adapted to tolerate or postpone/avoid events of high pH in the laboratory. Our data have implications for experimental studies of pH response and reaction norms in general of microalgae and the inclusion of species-specific data into ecosystem models.

1. Introduction

During microalgal blooms or red tides in eutrophic coastal and estuarine waters, the rate of photosynthetic incorporation of carbon into biomass often exceeds the supply of carbon (gaseous CO_2) into seawater from the atmosphere. This leads to an elevation of seawater pH (Hansen et al., 2007). Intensive photosynthetic production during blooms often causes the pH to increase and reach levels above 9.0. Such high pH levels are lethal for several microalgal and heterotrophic microzooplankton species (Goldman et al., 1982a; Hinga, 2002; Hansen, 2002; Pedersen and Hansen, 2003a,b). Some species of photosynthetic microalgae, especially oceanic forms from stable pH environments (ca. pH 8.1 ± 0.5) and large species (especially diatoms and large dinoflagellates) in coastal areas seem to be quite sensitive to elevated pH (over pH 8.5) (Lundholm et al., 2004; Søderberg and Hansen, 2007). On the other hand, bloom-forming or red-tide coastal species tolerate extremely high pH-levels in terms of seawater (above pH 9.0) (Hansen et al., 2007). Because microalgae have species-specific differences in their upper pH tolerance limits for growth, high levels of pH drive the species succession in controlled laboratory experiments and in natural communities (Goldman et al., 1982b; Hansen, 2002; Hinga, 2002; Pedersen and Hansen, 2003b). Species-specific differences are also considered to affect phytoplankton communities in responses to anthropogenic ocean acidification or lowered pH (Langer et al., 2006).

Our knowledge of species-specific growth responses to pH in microalgae is mostly based on generalizations from culture experiments using single cultivated strains (Hansen, 2002; Langer et al., 2006; Berge et al., 2010). Often such laboratory strains have been cultivated in the laboratory for several years, some even decades (Hansen et al., 2007; Riebesell et al., 2000, 2007; Iglesias-Rodriguez et al., 2008), and the results are assumed to reflect the natural pH response of the species. Species generalizations based on culture experiments implicitly assume that the measured properties of the laboratory cultivated strain reflect those of the natural population from which the cell was originally isolated and all strains of the particular species. However, large intraspecific variations in key ecophysiological traits are common within species and standing diversity in populations of microalgae is

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generally high (Brand, 1982; Gallagher, 1980; Wood and Leatham, 1992). This indicates a high potential for laboratory selection for strains best adapted to the cultivation environment.

Cultivated strains are usually established by single cell isolations, followed by asexual growth in enriched laboratory growth media. Standard seawater culturing media (e.g. K, F/2 and L1) spoils the growth of the cultures by containing very high concentrations of all necessary nutrients, vitamins and trace metals (Guillard and Hargraves, 1993; Hansen et al., 2007). Another important difference from the natural environment is the absence of mortality by grazing in the laboratory cultivation environment. In combination, these conditions allow photosynthetic production to become very high (Goldman et al., 1982a). Intense culture photosynthesis increases the pH of the medium until growth stops in the stationary phase. High pH represents a major cause of loss of species and strains in culture collections and is an important aspect of commercial large-scale biomass production (Goldman et al., 1982a). The time taken before the strains approaches the death phase depends on their growth rates and upper pH tolerance limits for growth (Hansen, 2002; Lundholm et al., 2004; Hansen et al., 2007). Recurrent high pH events between sub-culture re-inoculations (dilution) into fresh medium represent population bottlenecks and may present an important selective pressure, favouring strains with a high tolerance to elevated pH.

Heterocapsa triquetra commonly forms blooms (red tides) in coastal nutrient rich areas (Hansen et al., 2007), and populations often reach cell concentrations between 10,000 and 100.000 cells ml^{-1} (Hansen, 2002; Litaker et al., 2002). The species does not seem to produce toxins, but harmful effects on ecosystems are caused indirectly by its ability to form red tides and tolerate the high pH that may form under the buildup of high cell concentrations, which may be followed by oxygen depletion during bloom degradation (Hansen et al., 2007; Litaker et al., 2002). The growth of *H. triquetra* at elevated pH has been extensively studied in the laboratory using single cultivated strains. These studies suggest that this species cannot tolerate pH levels above pH 9.2–9.4 (Hansen, 2002; Hansen et al., 2007).

Two different types of experiments have been applied to study the upper pH tolerance limits for growth of phytoplankton in the laboratory: (1) constant pH experiments, where pH is kept constant by dilution and/or controlled gas-inflow and growth is measured over a range of pH levels and (2) pH drift experiments, where photosynthetic incorporation of carbon in closed batch or semi-continuous cultures (without air exchange), gradually elevates the pH in the medium, until the stationary phase is reached at the upper pH tolerance limit for growth (Hansen, 2002; Lundholm et al., 2004; Hansen et al., 2007). While keeping all other factors constant across a defined pH range, the advantage of the constant pH technique is the assurance that pH alone is the growth limiting factor. However, studies comparing the results of both techniques, report similar upper pH tolerance limits for growth between the two types of experiments (Lundholm et al., 2004; Hansen et al., 2007; Søderberg and Hansen, 2007).

We used pH drift experiments to investigate the potential for laboratory selection between strains in terms of adaptations to elevated pH. These experiments mimic closely a natural bloom situation (net carbon incorporation and elevation of pH) and the laboratory culture environment (recurrent high pH events). We compared the upper pH tolerance limits for growth and exponential growth- and production-rates of 23 strains of the common red-tide species *H. triquetra* that had been in culture for ca. 1–51 years. Experiments were performed with plenty of nutrients and high irradiance in closed experimental containers (no air-exchange). Our main aim was to test if upper pH tolerance limits and production rates differed between recently established strains and strains that had been cultivated for several decades.

2. Materials and methods

2.1. Strains and culture conditions

H. triquetra was chosen as a model red-tide species because it is easy to identify in the light microscope, easy to culture in the laboratory and available in culture collections. The included strains originated from culture collection centres around the world and from recently established cultures (Table 1). The culture age (length of cultivation period) of the strains from the official culture collection centres spanned from 4 to 51 years, while the recently established strains spanned 10–14 months in the laboratory (Table 1). No strains with a culture age of 10–20 years were obtained in our strain sampling, and we arbitrary designated strains with an age between 1 and 10 years as younger strains, and strains with an age in culture between 20 and 51 years as older strains.

For isolation and establishment of new strains, 65 ml seawater samples from three different populations (one seawater sample was taken from each population) (Table 1) were brought to the laboratory, and single cells (a total of 10–30 cells per population) of the dinoflagellate *H. triquetra* were pipetted into multidish wells (96 well plates), containing L1 medium (pH 8.0) without silicate (recipe obtained from the Scandinavian Culture Collection for Algae and Protozoa, SCCAP). The medium was based on sterile-filtered and autoclaved nutrient depleted seawater collected in the North-Sea. The multidishes were placed under an irradiance of ~50 μmol photons m^{-2} s^{-1}, in a 16/8 light:dark cycle in a temperature regulated room (15 °C), and sealed with laboratory plastic film to avoid evaporation. After ca. 4 weeks, the cells in wells with algal growth were re-inoculated into larger volumes (2 ml) in 24 well plates. After ca. 4 additional weeks, living strains were transferred into sterile polycarbonate bottles (65 ml) with ventilation cap containing 40 ml of L1 medium (pH = 8.0). The initial cell transfer from the natural water sample by transferring and washing of single cells in fresh medium (i.e. the isolation phase) was the most critical part of culture establishment. Of the 50 cells isolated in total, 25 cells survived to create early cultures (after 1 month), and of these almost all (22 cultures; 90% of early cultures) were successfully established into laboratory cultures after 2 months and survived until the experiment started.

For the next 8–12 months before experimentation, recently isolated strains and older strains obtained from culture collections (Table 1) were maintained under identical conditions. The temperature was 15 °C, irradiance 50 μmol photons m^{-2} s^{-1} (Cool white) in 16:8 light:dark cycle and salinity was 30. During the culture maintenance period cultures were re-inoculated into fresh medium (pH = 8.0) every 1–3 months, depending on growth rates. We randomly picked 12 of the recently established strains to be included in the pH drift experiments.

2.2. Experimental conditions

To compare upper pH tolerance limits for growth of the strains we measured cell concentration and pH as a function of time until stationary phase was reached under pH-drift conditions (Hansen, 2002; Lundholm et al., 2004; Hansen et al., 2007). From the growth curves of all strains we also estimated strain-specific exponential growth (d^{-1}) and production rates (μm^3 $cell^{-1}$ d^{-1}) under the experimental conditions provided. Experimental conditions differed from the culture maintenance conditions (see above), by a higher irradiance (150 μmol photons m^{-2} s^{-1}) and no air-exchange (closed bottles filled to capacity). Acclimation for the new light conditions lasted for >10 generations of exponential growth

Table 1
Strain identification, location, origin, and number of years maintained as clonal laboratory culture for strains of *Heterocapsa triquetra* used in the present study, including their acclimated growth rates, cell sizes, production rates and upper pH tolerance limits for growth. Numbers are observed means of the three replicate cultures including SEM (in brackets). Strains differed significantly for all properties (ANOVA, $p \ll 0.001$, df = 22). The range and overall average are included in the lower row. K numbers refer to accession number in the Scandinavian Culture Collection of Algae and Protozoa, Denmark. NA refers to not available due to loss in culture collection. KAC = Kalmar Algae Collection, Sweden; NIES = Microbial Culture Collection at National Institute for Environmental Studies, Japan; CCMP = Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA; Ply = Plymouth Algal Culture Collection, England. For strains established during this experiments, both author identification tag and identification number at SCCAP are provided. Strain HTMS0402 was kindly provided by Dr. Hae Jin Jeong.

Strain ID	Origin/ Location	Population/Seawater sample	Year of isolation	Cultivation period (years)	Growth rate (d^{-1})	Cell size (μm^3)	Production rate ($\mu m^3 d^{-1}$)	Upper pH tolerance limit
A4c, NA	Baltic	Copenhagen, Denmark	2007	0.9	0.80 (0.02)	1964 (66)	1578 (92)	9.26 (0.01)
A5c, K-1134	Baltic	Copenhagen, Denmark	2007	0.9	0.72 (0.05)	1688 (67)	1217 (84)	9.18 (0.01)
A2b, K-1131	Baltic	Copenhagen, Denmark	2007	0.9	0.62 (0.01)	1311 (69)	807 (74)	9.28 (0.01)
A4a, NA	Baltic	Copenhagen, Denmark	2007	0.9	0.56 (0.01)	1747 (79)	970 (76)	8.85 (0.01)
F2e, NA	Baltic	Gedser Harbour, Denmark	2007	1.0	0.66 (0.03)	1685 (73)	1115 (84)	9.26 (0.01)
F1f, NA	Baltic	Gedser Harbour, Denmark	2007	1.0	0.68 (0.01)	1621 (98)	1110 (116)	9.16 (0.01)
F1c, K-1125	Baltic	Gedser Harbour, Denmark	2007	1.0	0.78 (0.04)	1737 (71)	1353 (97)	9.24 (0.01)
F1a, K-1124	Baltic	Gedser Harbour, Denmark	2007	1.0	0.50 (0.05)	1588 (59)	793 (51)	9.17 (0.02)
2–1, K-1127	Baltic	Stege Harbour, Denmark	2007	1.2	0.62 (0.01)	1269 (74)	792 (80)	8.84 (0.01)
2–3, NA	Baltic	Stege Harbour, Denmark	2007	1.2	0.44 (0.04)	1701 (100)	748 (77)	8.72 (0.03)
2–5, NA	Baltic	Stege Harbour, Denmark	2007	1.2	0.58 (0.02)	1871 (91)	1076 (90)	9.12 (0.01)
2–6, NA	Baltic	Stege Harbour, Denmark	2007	1.2	0.55 (0.04)	1708 (85)	948 (82)	8.90 (0.01)
HTMS0402	Pacific	Masan Bay, Korea	2004	4	0.58 (0.01)	1898 (86)	1094 (86)	9.41 (0.01)
KAC 26	Baltic	Kalmarsund, Sweden	2000	8	0.53 (0.01)	1096 (100)	577 (91)	9.05 (0.01)
KAC 27	Baltic	Kalmarsund, Sweden	2000	8	0.31 (0.01)	1362 (77)	420 (41)	8.47 (0.02)
NC 98	Atlantic	West Coast, Norway	1998	10	0.60 (0.01)	1921 (82)	1157 (86)	9.40 (0.01)
K-0482	Atlantic	Kattegat, Denmark	1988	20	0.72 (0.03)	1419 (68)	1019 (84)	9.20 (0.01)
KAC 49	Atlantic	West Coast, Sweden	1986	22	0.68 (0.02)	1354 (78)	920 (47)	9.34 (0.01)
K-0447	Baltic	The Sound, Denmark	1984	24	0.45 (0.03)	1481 (65)	664 (51)	9.38 (0.01)
NIES 235	Pacific	Osaka Bay, Japan	1982	26	0.37 (0.02)	1989 (82)	731 (52)	9.23 (0.01)
NIES 7	Pacific	Harima-Nada, Japan	1981	27	0.49 (0.01)	1049 (67)	518 (57)	9.36 (0.01)
CCMP 449	Atlantic	Lawrebnce Estuary, Canada	1960	48	0.46 (0.01)	1445 (67)	663 (53)	9.39 (0.01)
PLY 169	Atlantic	Tamar Estuary, England	1957	51	0.18 (0.03)[a]	1702 (76)	322 (57)	9.30 (0.01)
Overall range					0.18–0.80	1049–1964	322–1578	8.47–9.41
Overall average					0.56 (0.04)	1592 (74)	895 (78)	9.15 (0.05)

[a] Growth of this strain in pH from 7.5 to 8.0 = 0.32 (0.02) d^{-1}.

(≈3 weeks). During this period, the flasks were diluted every 2–5 d with fresh medium at pH 8.0.

The pH in our medium was adjusted by adding 1 M HCl or NaOH and measured using a two-point calibration pH-meter (Radiometer, Copenhagen pHM-83 Autocal). The pH-meter was calibrated daily using IUPAC buffers 10.0 and 7.0. Total inorganic carbon (TCO_2) measurements were done using an infrared gas analyser (IRGA) by comparison with a 2 mM standard. The concentration of TCO_2 in the fresh medium at pH 7.5 and pH 8.0 was 1.8 and 2.0 mM, respectively.

After the acclimation period, 300 cells ml^{-1} were inoculated in 270 ml L1 medium (pH = 7.5; TCO_2 = 1.8 mM) in closed polycarbonate bottles that were filled to capacity. The experiment was done simultaneously for all strains. The replicate experimental culture flasks ($n = 3$) of all strains were allowed to grow for 4 additional days prior to the start of sampling. The sampling lasted up to 48 d and stopped when the strains had reached the stationary growth phase. Samples (5 ml) were fixed in Lugols acid (1% final concentration), and replaced by fresh medium at pH = 8.0 (TCO_2 = 2.0 mM). Sample volume corresponded to <2% of the experimental volume, and dilutions did not change the pH in the experimental bottles. We measured the TCO_2 in stationary phase at three different pH levels covering the range observed at stationary phase. At pH levels of 8.5, 9.0 and 9.4 in stationary phase, TCO_2 levels were 1.6 mM, 1.3 and 1.1 mM, respectively. The lower TCO_2 at high pH in the experimental flasks at stationary phase was a consequence of incorporation of carbon into biomass in the closed batch cultures.

Cell concentrations were counted manually using a Sedgewick-Rafter counting chamber and a compound microscope with a 10× objective. At least 300 cells were counted. The flasks were illuminated from below and their positions were changed randomly between samplings. This was done despite the fact that the light field experienced by the bottles was homogenous. pH in the experimental flasks was measured before samples for cell concentrations were withdrawn.

2.3. Estimation of growth and production rates and upper pH tolerance limits for growth

Exponential growth rates μ (d^{-1}) were calculated according to $\mu = \ln(X_{t_2} - X_{t_1})/t_2 - t_1$, where X_{t_2} and X_{t_1} is the cell concentration at end (t_2) and start (t_1) of the sampled interval, respectively. Successive growth rates (between daily samplings: t1–t0, t2–t1, t3–t2, etc.), in each replicate flask, were averaged over the exponential period represented by the linear part of the semi-logarithmic growth curve (cell concentration as a function of time) (Fig. 1). The end of the exponential growth phase was defined as the point where the growth rates of successively added sampling points changed significantly compared to the sampling time interval between day 0 and 4 (t-test, $p < 0.05$) (Fig. 1). For all strains the exponential period corresponded to balanced growth over at least 5 generations for all strains.

The upper pH tolerance for growth was estimated as the pH level during the stationary growth phase (Hansen, 2002; Lundholm et al., 2004). The stationary growth phase was determined as the time period between the first successive time interval where average growth rate of the three replicates was not significantly different from 0.0 d^{-1} (Fig. 1). This was determined using confidence intervals around the mean growth rate of the three replicates.

We estimated the cell size (biovolume $cell^{-1}$) of the different strains during the exponential growth phase. Cell dimensions (length and width) were measured by mixing an equal number of cells from three Lugol fixed samples per strain (taken at three

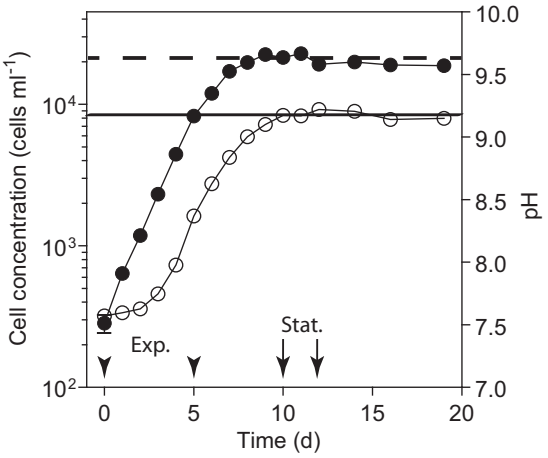

Fig. 1. Example of a pH drift curve (Strain K-0482) illustrating how exponential growth rates (arrow heads indicate time interval of exponential growth (Exp.)), cell concentration at stationary growth phase (dashed line) and upper pH tolerance limits for growth (solid line) were determined (arrows indicate time interval of stationary growth phase (Stat.)). Points represent means of three replicate cultures.

different points in the exponential growth period). The cells were allowed to settle for 24 h in 25 ml sedimentation chambers, before micrographs were taken. Settled cells were photographed using an inverted microscope with a 25× objective. The length and width of the first 60 cells encountered on enlarged micrographs were measured manually, using the ruler tool in Adobe Photoshop CS3. Pixels were converted to micrometers using a high precision object-micrometer. Cell size (μm^3) was calculated assuming two rotational cones according to Hillebrand et al. (1999). Production rates were estimated as the product of mean cell size (biovolume) and exponential growth rate.

2.4. Statistical analyses

We determined the upper pH limits for growth, growth rates, production rates and the number of days before the onset of death phase in all 69 experimental flasks (3 replicates per strain). Diagnostic raw residual plots of our data showed variance homogeneity and quantile–quantile plots of the raw residuals showed normally distributed errors for all properties measured. We therefore tested for differences between strains in growth rate, cell size, production rate and upper pH tolerance limits using one-way ANOVA tests (factor = strain). All statistical analyses were conducted with the statistical software R version 2.10.1 (R Development Core Team, 2010).

In order to examine effects of cultivation period and sampling location (origin) on growth and pH responses in general, we used hierarchical mixed effects models with both random effects and fixed effects (linear mixed effect model, LMEM) (Zuur et al., 2009). This was done to account for the potential inter-correlation in the phenotypic properties between strains originating from the same population/seawater sample (i.e. dependent samples). Random effects included were strains nested in populations/seawater samples, while fixed effects were length of cultivation period, age groups (younger vs. older strains) and location (origin). Diagnostic quantile–quantile plots showed that the predicted random effects were normally distributed for all phenotypic properties. *p*-Values for the fixed effects were calculated using Maximum Likelihood Ratio tests (ML, chi-square distribution) comparing the model that contained the fixed effect with the nested model without the fixed effect. The significance of interaction between origin and time in culture was tested similarly. The LMEM's were conducted using the R-package lme4 (Bates and Maechler, 2010).

3. Results

The growth curves and the parallel pH measurements for the strains of *H. triquetra* cultivated between 1–10 and 20–51 years were plotted separately (Figs. 2 and 3). Cell concentrations of all strains increased exponentially as a function of time until the cultures entered the stationary phase. None of the strains experienced a lag phase, and the growth rates were balanced during the linear parts of the growth curve from the first sampling points (Figs. 2A and 3A). However, the oldest strain showed an abnormal growth curve (insert in Fig. 3A). Initially, balanced exponential growth rate was 0.32 ± 0.02 in the pH range from 7.5 to 8.0, but slowed down to 0.18 ± 0.03, in the pH range from 8.0 to 9.2 (Fig. 3A and B, Table 1). Among all the strains, stationary phase occurred after a period of 6–41 d after the first sampling (following the initial 4 d of incubation) (Figs. 2 and 3). Cell concentration in stationary phase differed significantly between the strains (ANOVA, $p \ll 0.001$) and varied from 7380 to 35,550 cells ml^{-1} (Figs. 2 and 3A).

In parallel with cell concentration, pH increased as a function of time until the growth of the cultures slowed down and levelled off at significantly different pH levels in the stationary growth phase (ANOVA, $p \ll 0.001$) (Figs. 2 and 3B). The overall average upper pH tolerance for growth of all strains was 9.15 ± 0.05, but differed significantly between the strains and ranged from 8.47 to 9.41 (ANOVA, $p < 0.05$) (Table 1).

The overall average growth rate of all strains was 0.56 ± 0.04 d^{-1}, but differed significantly between the strains and ranged from 0.18 to 0.80 d^{-1} (Table 1). Cell size in terms of biovolume (μm^3 cell^{-1}) also differed significantly between the strains and ranged from 1049 to 1989 μm^3 around the overall average of 1592 ± 74 μm^3. Likewise, production rates (μm^3 cell^{-1} d^{-1}), calculated as the product of cell size (μm^3 cell^{-1}) and growth rates, differed significantly between the strains (ANOVA, $p < 0.05$) (Table 1). Production rates ranged from 322 to 1578 μm^3 cell^{-1} d^{-1}, around the overall average of 895 ± 78 μm^3 cell^{-1} d^{-1} (Table 1).

When accounting for the random variation due to strains nested in populations/seawater samples, sampling location or strain origin (factor with 3 levels) had no significant effect on growth rates, upper pH tolerance limits and production rates, and there was no significant interaction effect of strain origin/location and cultivation period (LMEM, ML, χ^2, $p > 0.05$) (Table 2). On the other hand, cultivation period as a numerical variable had significant effects on growth rates, upper pH tolerance limits and production rates (LMEM, ML, χ^2, $p < 0.05$), but not on cell size (LMEM, ML, χ^2, $p = 0.56$) (Table 2). In general, growth and production decreased as a function of years of cultivation (Fig. 4A), while upper pH tolerance limits for growth increased (Fig. 4B). Since a pH range above 8.0 is the common standard pH of cultivation media, we included the growth and production rates of the oldest strain (PLY 169) in this range in our analysis, but the same conclusions (significant *p*-values) were obtained when including the higher growth and production rates observed in the pH range 7.5–8.0 (see above).

From the growth curves we also estimated the number of days the strains grew under light and nutrient replete conditions before entering the stationary phase and followed by death phase. This was also significantly dependent on the cultivation period (LMEM, ML, χ^2, $p < 0.05$) (Table 2), and increased as a function of age in the laboratory (Fig. 4C).

Production rates were significantly higher and upper pH tolerance limits for growth were significantly lower in the group of the strains that had been cultivated for 1–10 years than in the group of strains that had been cultivated for 20–51 years (LMEM, ML, χ^2, $p < 0.05$) (Fig. 5A and B). Again, inclusion of the higher production rates observed <pH 8.0 of the oldest strain (PLY 169) in the dataset, did not change this conclusion (LMEM, ML, χ^2,

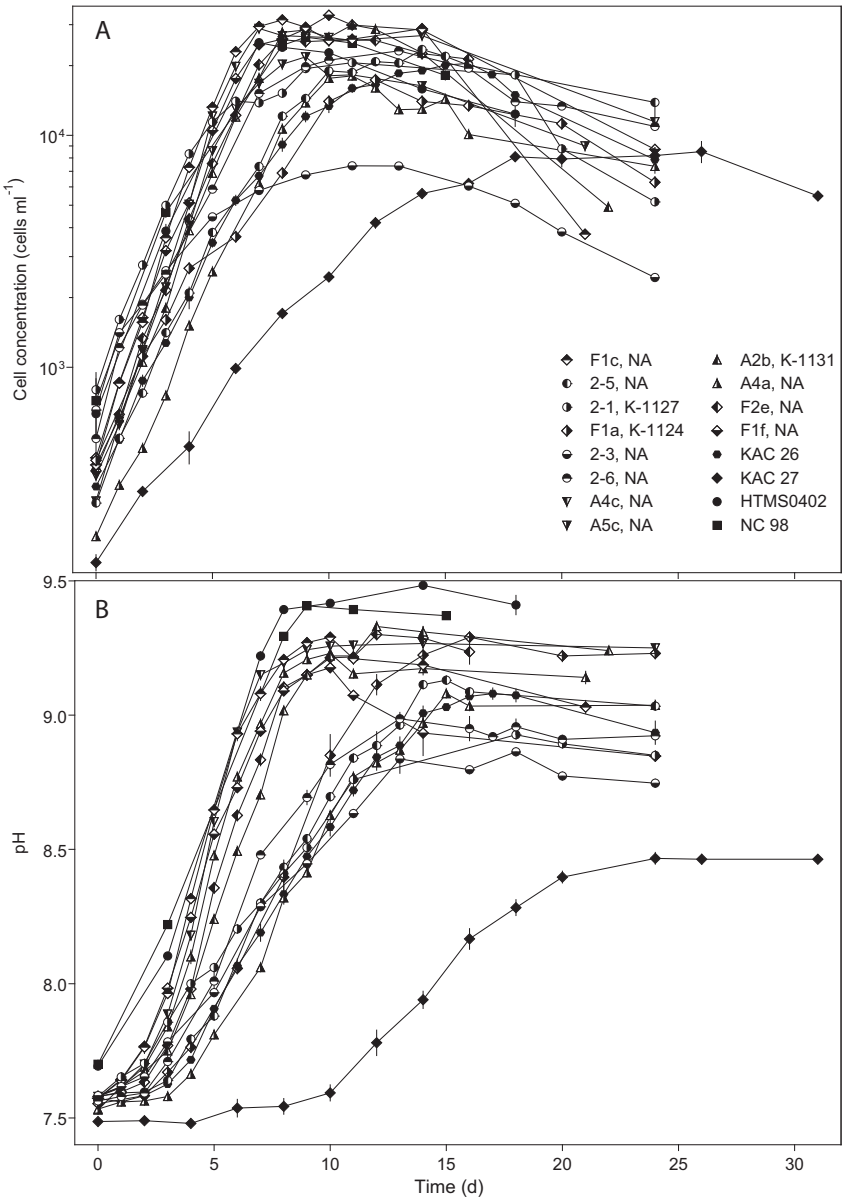

Fig. 2. pH drift growth curves for strains of *Heterocapsa triquetra* kept in culture for less than 10 years. Cell concentrations (A) and pH (B) as functions of time. Data points are means ± SEM of three replicate cultures.

$p < 0.05$). The largest range and variation (height of boxes in Fig. 5A and B represents the inter-quartile range) of strain specific upper pH tolerance limits and production rates were observed within the younger (1–10 years) compared to older strains (20–51 years) (Fig. 5A and B). Upper pH tolerance limits for growth ranged from 8.47 to 9.41 in the group of younger strains and from 9.20 to 9.39 in the group of older strains.

4. Discussion

4.1. Microalgal cultivation and laboratory selection of strains

In congruence with previous studies involving many strains of a single microalgal species we found large amounts of intraspecific variation and significant differences between the strains' ecophysiology (Gallagher, 1980; Brand, 1982; Wood and Leatham, 1992; Lakeman et al., 2009). Overall, intraspecific pH tolerance limits for growth ranged by nearly 1 pH unit, and growth rates ranged from ca. 0.2 to 0.8 d^{-1}. Intraspecific variation in growth rates of other species of similar magnitude have previously been reported (Wood and Leatham, 1992; Lakeman et al., 2009), but data on intraspecific variation in upper pH tolerance limits are lacking. Large amounts of intraspecific variation in pH response and growth rate, suggest that there exists a high potential for laboratory selection.

Events of elevated pH between re-inoculations or dilutions during cultivation of microalgae may be a major source of culture loss in culture collection centres. The combinations of a high upper pH tolerance limit for growth and/or a lower production rate will postpone the onset of stationary phase followed by death (Fig. 4) and increase the probability of long-term survival. The strain with the lowest upper pH limit observed (8.5), the 8-year-old KAC 27 had low growth- and production rates (0.3 d^{-1} and 420 μm^3 $cell^{-1}$ d^{-1}). This enabled it to survive for a long period before it eventually decreased in cell concentration (i.e. 24 d), and illustrates that a lower production rate alone, may be an adaptive advantage in the laboratory environment, allowing pH sensitive strains to survive cultivation. Nevertheless, in general, upper pH

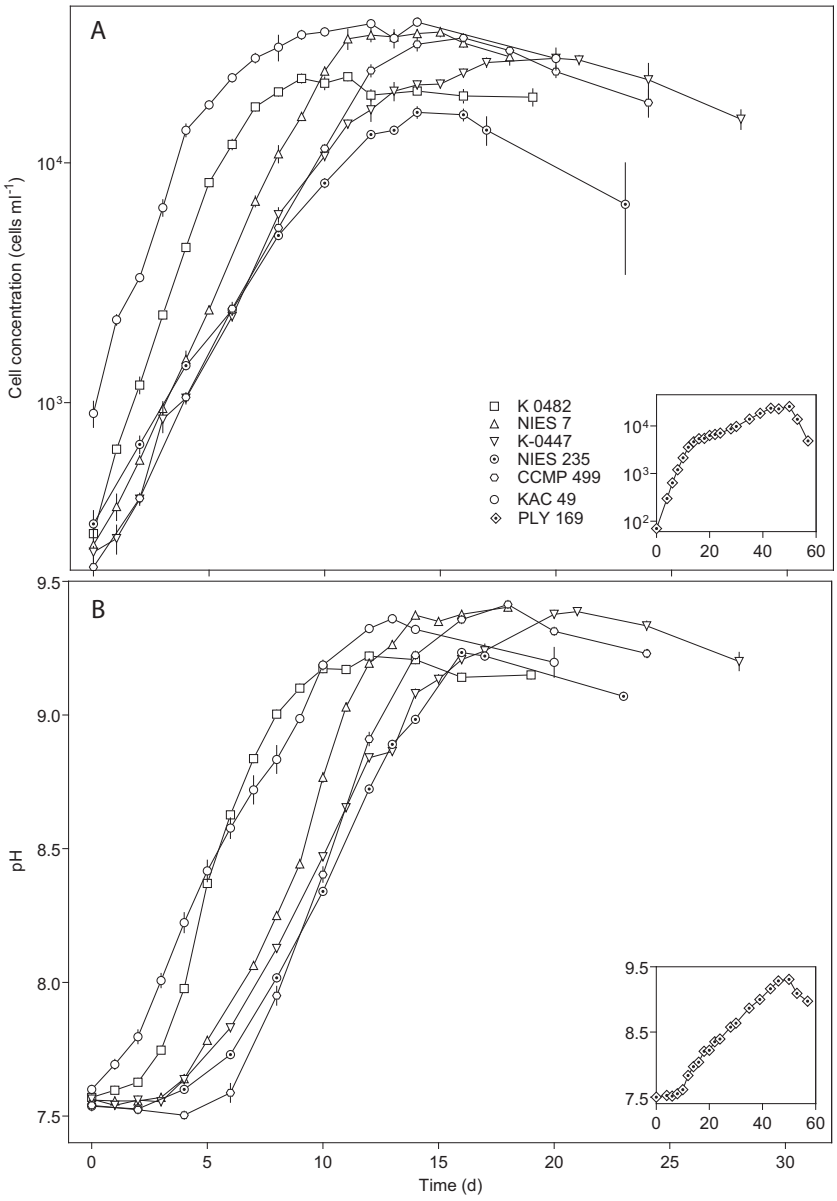

Fig. 3. pH drift growth curves for strains of *Heterocapsa triquetra* kept in culture for more than 20 years. Cell concentrations (A) and pH (B) as functions of time. Data points are means ± SEM of three replicate cultures. Inserts show results of PLY 169 on different x-axis.

tolerance limits were higher and production rates lower in the strains cultivated for more than 20 years compared to the strains cultivated for less than 10 years. Moreover, intraspecific variation and range of upper pH tolerance limits for growth were higher in the group of strains cultivated for less than ten years than in the group of strains cultivated for more than 20 years. Reduced trait variation is a common consequence of directional selection, also in microalgae (Lakeman et al., 2009).

Our comparison of intraspecific variation between the group of 15 younger strains, with the group of 8 older strains is based on unbalanced and dependent sampling. Twelve of the younger strains originated from 3 populations or seawater samples, while

Table 2
p-Values from the linear mixed effects model (LMEM) of the phenotypic properties as functions of age in laboratory (numerical variable), origin/location and the potential interaction between the two fixed effects. Strains nested in population/sample represented random effects.

Phenotypic property	Origin, O (df = 2)	Age in culture, A (df = 1)	Interaction term, O * A (df = 2)
Growth rate	0.076	0.006**	0.861
Cell size	0.869	0.597	0.016*
Production rate	0.088	0.006**	0.839
Upper pH limit for growth	0.094	0.041*	0.630
Number of days before death phase	0.160	0.003**	0.585

* Significant effect at $\alpha < 0.05$.
** Significant effect at $\alpha < 0.01$.

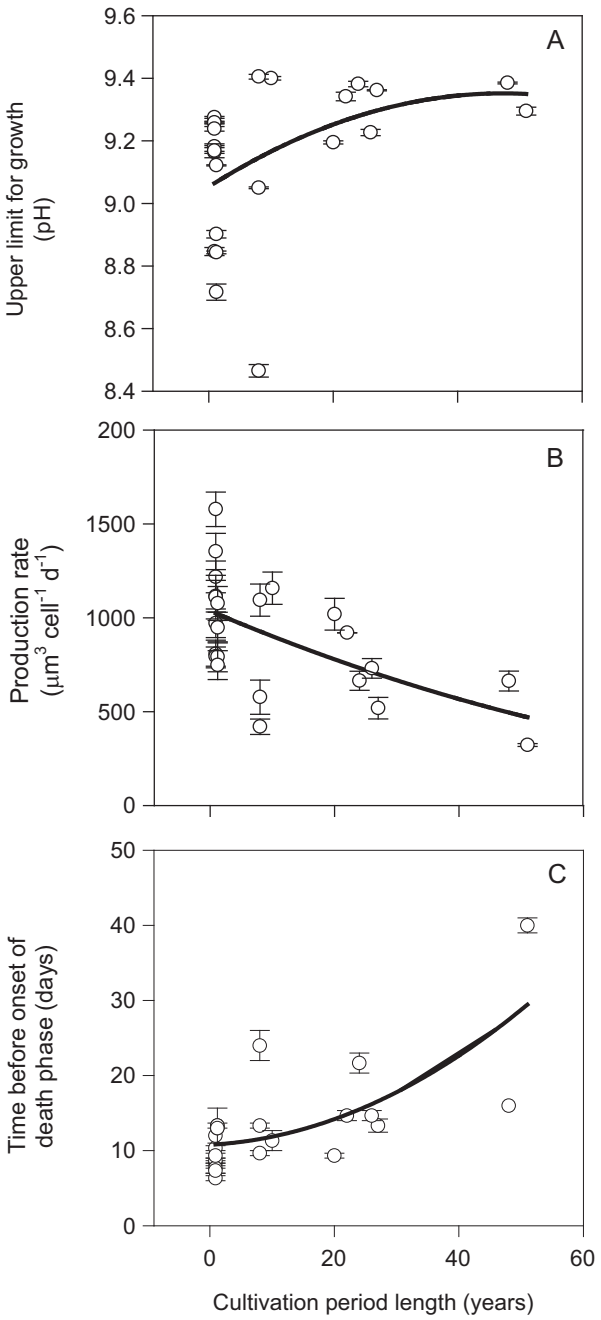

Fig. 4. Mean strain specific upper pH tolerance limits for growth (A), production rates during exponential phase (B) and the number of incubation days before the onset of the death phase (C) as functions of laboratory maintenance period. Data points are means of 3 replicates and bars represent SEM. Lines represent the general trend in the data as determined with non-parametric regression splines. The mixed linear models of the phenotypic property as functions of age (numerical variable) and strains nested in samples as random effects showed significant effects of laboratory culture age for all properties (Table 2; LMEM, ML, χ^2, $p < 0.05$, df = 1).

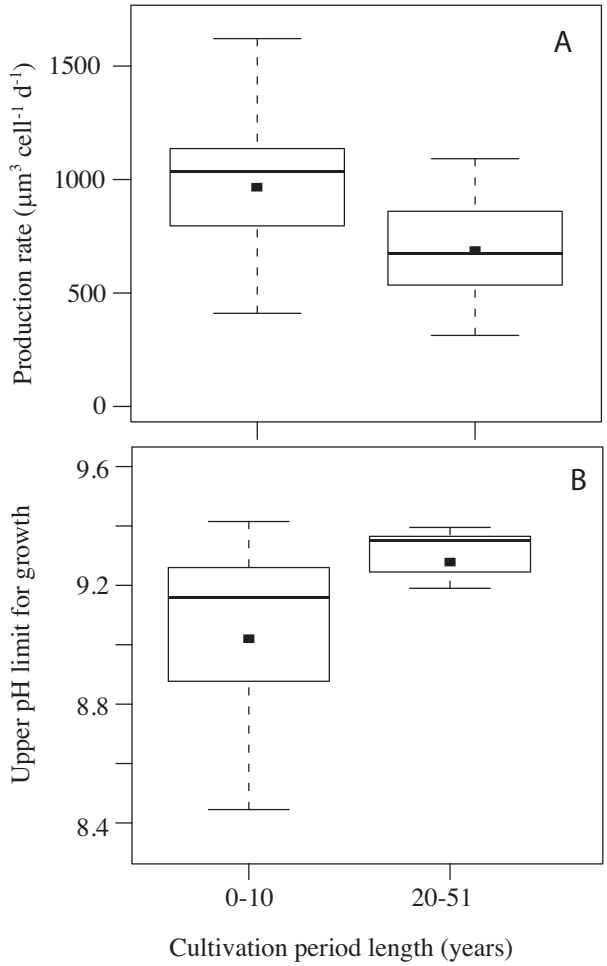

Fig. 5. Range and variation of strains grouped according to cultivation periods of 1–10 years and 20–51 years. Median (thick horizontal line) production rates (A) and upper pH tolerance limit for growth (B) of all strains in each of the two age groups. Variation visualized as inter-quartile range; upper and lower edges represent the upper and lower quartiles, respectively. Whiskers represent the maximum and minimum observation; i.e. the range. There was significantly lower mean upper pH limit for growth (LMEM, ML, $\chi^2 = 6.25$, $p = 0.012$, df = 1) and higher mean production rate (LMEM, ML, $\chi^2 = 4.12$, $p = 0.042$, df = 1) in the group of strains cultivated for 1–10 years compared to the older (20–51 years of cultivation) strains.

all the 8 older strains originated from 8 different populations/seawater samples of global origin (Table 1). Thus, with no selection, the older strains would be expected to contain higher levels of intraspecific variation than the group of recently established strains. However, range and variation (quantified as IQR) in upper pH tolerance was much larger in the group of new strains (Fig. 5B). Range in upper pH tolerance limits for growth in the group of new strains was twice as large as in the group of the 8 older strains of diverse origins. Variation was even higher in a group of only 4 strains from a single population/seawater sample (Table 1; Copenhagen population) than in the group of older strains. This suggests a major reduction in diversity due to laboratory selection. The recurrent high pH events of microalgal cultivation seem to present an important selection pressure towards higher upper pH tolerance limits for growth.

4.2. Acclimation and the use of pH drift cultures to derive upper pH tolerance limits

To be able to compare genetically determined ecophysiological traits, the cultures must be acclimatized properly to the experimental environment. This includes that strains or cultures are grown exponentially in a consistent set of conditions (e.g. temperature, salinity, light and nutrients) until the property of interest has stabilized (e.g. growth- or photosynthetic rate). This is normally considered to have happened within a few generations (Lakeman et al., 2009). In our study, acclimation was obtained by maintaining the strains under identical and stable environmental conditions for at least 8 months before the experiment started, only changing the light conditions for the last ca. 3 weeks and keeping the cultures in the exponential growth phase (10–25 generations) before the experiments started.

In terms of temperature, nutrients, salinity and light conditions the cultures must be considered well acclimatized, and the strain-specific growth and production rates measured represent genetically determined phenotypes. On the other hand acclimation to the initial pH level in the experimental cultures was rapid. We inoculated cells from cultures of a pH between 8.0 and 8.4 directly into medium of pH 7.5 at the start of the experiment. This rapid drop in pH at the start of the experiment, could potentially affect the growth curves. However, we observed no lag-phases in our cultures (Figs. 2 and 3), and balanced growth rates were achieved over the range from pH 7.5 to 9.0 (however, see PLY 169). Other reports of no lag-phase in growth curves of phytoplankton laboratory strains after sudden pH changes, support this trend (Berge et al., 2010; Ramos et al., 2010), and suggest that acclimation to pH changes is rapid (Chen and Durbin, 1994). In *Emiliania huxleyi*, acclimation to increased free aqueous CO_2 (CO_{2aq}) and lowered pH has recently been shown to occur within hours (Ramos et al., 2010). Rapid acclimation to pH changes supports the use of pH drift experiments to determine upper tolerance limits for growth, and has good implications for literature comparisons across different acclimation periods.

The cause of growth limitation in microalgae at high pH has been extensively studied, and pH drift experiments have been widely used to study upper pH tolerance limits for growth (e.g. Lundholm et al., 2004; Hansen et al., 2007; Søderberg and Hansen, 2007). These experiments assume that elevated pH is the cause for growth limitation at stationary phase (e.g. Hansen et al., 2007). Standard laboratory media for microalgal cultivation contains macro- and micronutrients in far excess, which theoretically could support a biomass several times larger than observed in stationary phase. Assuming the relationship between biovolume and cellular carbon presented by Menden-Deuer and Lessard (2000), and taking the overall average cell size from our study (i.e. 1574 μm^3 $cell^{-1}$), gives a crude estimate of average carbon content per *H. triquetra* cell of ca. 18 pmol C $cell^{-1}$. Multiplying carbon per cell with the number of cells in stationary growth phase (from 7380 to 35,500 cell ml^{-1}), and assuming the molar Redfield ratio between C:N:P (116:16:1) gives a range of cellular bound macronutrients of 20.1–96.8 $\mu mol\ N\ L^{-1}$ and 1.3–6.1 $\mu mol\ P\ L^{-1}$ in the stationary phases observed in this study. Since phosphorous is the nutrient in lowest supply in relation to the Redfield ratio (medium contains 882.4 $\mu mol\ N\ L^{-1}$ and 36.2, $\mu mol\ P\ L^{-1}$; thus molar N:P of medium = 24.4), our medium could support cell concentrations ca. 6–30 times higher than observed in stationary phase, if all phosphorous was exploited. Although, these considerations are only theoretical, the calculations suggest that it is very unlikely that the concentrations of carbon, phosphorous or nitrogen limited the growth and biomass production in our experiment.

The speciation of inorganic carbon (TCO_2) depends on the pH level, and microalgae seem to be unable to utilize CO_3^{2-}, which increases at high pH (Giordano et al., 2005). Thus, decreased available TCO_2 ($CO_{2aq} + HCO_3^-$) could potentially limit phytoplankton growth. At very high pH (>pH 9), CO_{2aq} is extremely low and the growth is based on HCO_3^-. It has been investigated whether growth of phytoplankton at high pH is mainly limited by lower available TCO_2 or by high pH alone (Hansen et al., 2007). *H. triquetra* has been found to be able to maintain maximum growth rates at very low TCO_2 levels (0.4–0.6 mM) at pH 8.2 and pH 9.0. In our study this is well below the TCO_2 levels measured over the range of pH during stationary phase (minimum 1.1 mM TCO_2 at pH 9.4). Also data from other red tide dinoflagellates, report that very low levels of TCO_2 are required to limit the growth at high pH (pH = 9, 0.5–1.0 mM in Hansen et al. (2007)). It therefore seems unlikely that low available TCO_2 should limit the growth rates in our experiment.

The availability of other nutrients than carbon is also affected at high pH. Phosphorous, the limiting macronutrient in our L1 medium, has been found to precipitate at very high pH levels (Olsen et al., 2006). However, the required pH levels for precipitation of phosphorous are far above the levels observed during stationary phase in our study, i.e. pH > 10.0. Thus, the most likely cause of growth limitation in our experiment seems to be directly related to the high pH itself.

4.3. Importance of pH during the different stages of microalgal cultivation

Three fundamentally different stages of phytoplankton cultivation may involve laboratory selection; i.e. single cell isolation, culture establishment and long-term maintenance (Lakeman et al., 2009). During single cell-isolation, small changes in temperature, nutrients, salinity and light conditions may provide an important selection pressure on the isolated genotypes (i.e. the standing diversity). In our collection of recently established strains, culture success rates of isolated cells were ca. 50%. This success rate compares well with previously published values in phytoplankton from 20 to 90% (Lakeman et al., 2009). A success rate less than 100% may indicate that selection has already occurred. However, in terms of high pH, selection is very unlikely to be important at the time of isolation, because the cells are most often isolated into fresh medium with a pH similar to that of the environment in which the cells were sampled. Moreover, due to the very low biomass and photosynthetic activity during the isolation process and early cell divisions and air-exchange, the pH in the medium is kept stable. Other chemical and physical differences and especially random effects of the physical treatment/damage of the cells are probably much more important during the initial process of isolation.

After the initial isolation process strains that are best adapted for longer-term survival in the laboratory, involving frequent events of high pH, will be favoured. Strains with the highest tolerance to high pH and/or low production rates will have the highest probability of survival (because of the delay of the onset of the stationary phase). At some point in time during long-term cultivation, i.e. decades or thousands of generations, an inevitable supply of mutations in an established culture may also cause directional shifts in traits through selection on the newly arisen intra-strain variation. It may be argued, that in the absence of biotic interactions such as grazing and competition, and due to the constancy of physical and chemical conditions in the laboratory environment the rate of phenotypic evolution during cultivation may be slow, because events of environmental changes are rare (Huey and Rosenzweig, 2009). However, pH is highly fluctuating, contrasting the stability of most environmental factors of laboratory cultivation, and may be especially important under long-term maintenance. Evidence exists showing that genetically determined changes in carbon utilization can take place in strains of microalgal cultures (see below) (Collins and Bell, 2004; Lakeman et al., 2009). More studies on the same strain at different times during cultivation are needed to further address this issue.

4.4. Growth at low pH/high CO_{2aq}

Ribulose-1-5-bisphosphate-carboxylase-oxygenase, RuBisCO, the primary enzyme catalyzing the initial step of photosynthesis is undersaturated with CO_{2aq} under present-day seawater levels (i.e. ca. 8.1). Yet, most red tide species have efficient carbon concentrating mechanisms (CCMs), enabling maximum photosynthetic rates under current conditions (Giordano et al., 2005). Growth enhancement at low pH/high CO_{2aq} is however, observed in some species that lack or have low CCM activity (Riebesell et al.,

1993). In our medium at pH 7.5, CO_{2aq} is 5–10 times higher than at standard cultivation levels of pH 8.0–8.5 (Berge et al., 2010). Except for the oldest strain included in our study (the 51 year old strain, PLY 169), all strains achieved balanced growth rates in the range of pH from 7.5 to their upper pH tolerance limits. However PLY 169 grew nearly twice as fast when pH was between 7.5 and 8.0 (i.e. increased CO_{2aq}) as compared to at higher pH (range 8.0–9.2) (Fig. 3A and B). This indicates that lowered pH/increased CO_{2aq} stimulates the growth rate of PLY 169. A possible explanation for this observation could be reduced CCM activity in this strain, due to phenotypic change by mutations. In cultivated strains, a lowering of the investment in CCMs would be an effective way to reduce growth rates, which seems to be adaptive for the laboratory condition. Genetically determined reduction of function of CCMs has been reported in the chlorophyte *Chlamydomonas* as a response to experiencing elevated CO_{2aq} over 1000 generations of growth (Collins and Bell, 2004). The oldest strain (PLY 169) in our study had been maintained for ca. 51 years, which corresponds to ca. 10,000 generations, suggesting sufficient time for the accumulation of mutations in the CCMs. Microalgal cultures have fast generation times (d) and huge populations sizes which both relates to high probability and frequency of rare, but beneficial mutations. Rapid heritably changes in physiological traits during only 100–1000 generations in cultured strains exist for several different asexually reproducing eukaryotic organisms (Lakeman et al., 2009; Loxdale and Lushai, 2003) and clonally reproducing organisms in general seems to possess dynamic and adaptive genomes (Lushai et al., 2003).

The fact that laboratory cultivation seems to select for growth responses to pH may also have relevance for studies and focus on the response of primary production to the present day ocean acidification due to human combustion (Beardall and Raven, 2004). The ocean experiences currently the most dramatic acidification event on earth over the last 300 Myr (Caldeira and Wickett, 2003), and species-specific differences are considered to affect phytoplankton communities in regard to lowered pH/increased CO_{2aq} (Langer et al., 2006). Using single cultivated strains to represent species, we recently tested the effect of lowered pH/increased CO_{2aq} over a pH range from 7.0 to 8.5, on growth rates of 7 species from the major groups of coastal marine phytoplankton (Berge et al., 2010). Our data corroborated well with other experimentally derived growth rates at lowered pH found in the literature, suggesting that phytoplankton species in general are tolerant to the projected pH levels expected by year 2100 (i.e. average pH 7.8 compared to present day level of 8.1). However, in the present study we show that growth response to pH may be particular sensitive to laboratory cultivation. This may indicate that experimentally derived data for microalgal species at different pH/CO_{2aq} levels are biased by laboratory selection, because they involve long-term cultivated strains. In a study of 5 strains of the calcifying coccoltithophorid *E. huxleyi*, significant intraspecific variation in growth responses to lower pH/increased CO_{2aq} was suggested to explain the contradictory results reported for this species (Langer et al., 2009). Future studies should consider laboratory selection and intraspecific variation more carefully when addressing responses to pH other environmental factors.

5. Implications

Microalgal monocultures allow us to experimentally study traits and environmental factors in isolation, under stable and highly controlled environments. They have been widely used to access knowledge on the ecophysiology of bloom forming and toxic microalgae and phytoplankton in general. However, as shown here, in terms of upper pH tolerance limits for growth and production rates, cultivated strains may have undergone significant laboratory selection. Therefore, the use of a single long-term cultivated laboratory culture seems to be inappropriate for species generalizations both in studies on pH responses and environmental reaction norms in general (due to the high level of intraspecific variation in growth rates). Our data reemphasize the importance in some studies of isolating and establishing several strains immediately prior to experimentation (the so-called "Brand's gold approach").

Another way to overcome the problem of long-term cultivation and laboratory selection for pH tolerance studies of coastal species seems to be the use of cultures based on germination of dormant resting-stages (cysts). Studying the coastal cyst-forming dinoflagellate *Penthapharsodinium dalei* under the same conditions and analyses used here, Ribeiro et al. (2011) found no effect of dormancy time on growth and production rates of vegetative cultures revived from 0, 40 and 100 year-old cysts (each age group with 6 examined strains. Far from all coastal microalgal species are known to produce resting-stages. Cryopreservation represents another alternative to avoid laboratory artefacts by long-term cultivation, but only a few species seem to survive such a treatment.

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