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Growth rates of three geographically separated strains of the ichthyotoxic *Prymnesium parvum* (Prymnesiophyceae) in response to six different pH levels

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ABSTRACT

Continued anthropogenic carbon emissions are expected to cause a decline in global average pH of the oceans to a projected value of 7.8 by the end of the century. Understanding how harmful algal bloom (HAB) species will respond to lowered pH levels will be important when predicting future HAB events and their ecological consequences. In this study, we examined how manipulated pH levels affected the growth rate of three strains of *Prymnesium parvum* from North America, Denmark and Japan. Triplicate strains were grown under pH conditions ranging from 6.6 to 9.1 to simulate plausible future levels. Different tolerances were evident for all strains. Significantly higher growth rates were observed at pH 6.6–8.1 compared to growth rates at pH 8.6–9.1 and a lower pH limit was not observed. The Japanese strain (NIES-1017) had the highest maximum growth rate of 0.39 divisions day^{-1} at pH 6.6 but a low tolerance (0.22 divisions day^{-1}) to high levels (pH 9.1) with growth declining markedly after pH 7.6. The Danish (SCCAP K-0081) and North American (UTEX LB 2797) strains had maximum growth rates of 0.26 and 0.35 divisions day^{-1}, respectively between pH 6.6–8.1. Compared to the other two strains the Danish strain had a statistically lower growth rate across all pH treatments. Strain differences were either attributed to their provenance or the length of time the strain had been in culture.

1. Introduction

Prymnesium parvum is an ichthyotoxic and mixtotrophic haptophyte that causes dense blooms worldwide resulting in devastating effects to aquaculture, native fish and shellfish populations (Blossom et al., 2014 and references therein). Due to its euryhaline nature *P. parvum* resides in fully marine to brackish inland aquatic systems. Toxicity is associated with a collection of compounds known as prymnesins. At least 16 different prymnesins subdivided in original, B– and C-types were recently characterised by Rasmussen et al. (2016). Exposure to *P. parvum* toxins can cause death in gill-breathing organisms by cytotoxic, haemolytic, neurotoxic and ichyotoxic effects (Manning and La Claire, 2010). Several factors have been shown to affect *P. parvum* and the potency of prymnesins including salinity, growth phase, stoichiometric imbalances, water hardness and pH (Manning and La Claire, 2010; Roelke et al., 2016).

Since the industrial revolution atmospheric concentration of CO_2 has increased by ≈ 69% from a pre-industrial level of 280 (Doney et al., 2009) to 404 ppm in October 2017 (NOAA global Greenhouse Gas Reference Network).This increase has been somewhat alleviated by the world oceans acting as a reservoir for CO_2. The oceans have absorbed approx. 30% of anthropogenic carbon emissions over the last 250 years (Stocker et al., 2013). However, oceanic uptake has consequences for marine systems, e.g. altering chemical balances and ocean carbon cycle (Das and Mangwani, 2015; Doney et al., 2009). Hence, understanding the resulting impacts of increased CO_2 concentrations on marine organisms has become a most pressing issue. Increasing CO_2 concentrations can influence phytoplankton productivity in two ways. Firstly, the direct impacts on photosynthesis and secondly the impacts caused by the elevated CO_2 on pH and the equilibrium between CO_2, bicarbonate and carbonate species which alters dissolved inorganic carbon (DIC) in seawater. While DIC can enhance photosynthesis and growth, the carbonic acid raises hydrogen ion (H^+) concentrations while lowering the carbonate ion (CO_3^{-2}) concentration. The acidity of the oceans is determined by the concentration of H^+ ions, which causes a decrease in oceanic pH

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(Doney et al., 2009). Since pre-industrial times, pH of the oceans has fallen from an average of pH 8.2 to 8.1. This is lower than it has been for hundreds of millennia and the rate of change is more than 100 times greater than at any other time in recent history (Raven et al., 2005). Future projections state that this value will fall to ≈7.8 by the end of the century. This decline in pH has been synonymous with altered chemical balances and is now referred to as ocean acidification (OA) (Raven et al., 2005).

While most attention has focused on how elevated CO_2 concentrations are likely to impact global climate, less attention has been placed on how elevated concentrations are also likely to have effects on the growth, physiology and biochemistry of plants and algae (Ziska et al., 2009). Physiological responses may be observed at the ecosystem level through changes in competitive interactions, dominant species and the overall community structure (Riebesell, 2004).

Some coastal systems and estuaries are already experiencing the effects of anthropogenic acidification. As many HABs occur in these regions we need to assess how HAB species will respond to changing pH levels. The effects of lowered pH and OA have already been studied for a number of HAB species including (dinoflagellates) *Alexandrium catenella* (Fu et al., 2012), *A. minutum* (Flores-Moya et al., 2012, Hwang & Lu 2000), *Karlodinium veneficum* (Fu et al., 2010), *Prorocentrum donghaiense* (Huang et al., 2005) and *P. minimum* (Fu et al., 2008); (diatoms) *Nitzschia navis-varingica* (Lundholm et al., 2004; Trimborn et al., 2008), *Pseudo-nitzschia seriata*, *P. multiseries* (Lundholm et al., 2004, Sun et al., 2011) and *P. fraudulenta* (Tatters et al., 2012); (raphidophytes) *Heterosigma akashiwo*, *Chattonella subsalsa* (Fu et al., 2008, 2012) and *C. marina* (Liu et al. 2015) and (prymnesiophytes) *Phaeocystis globosa* (Chen and Gao, 2011; Hoogstraten et al., 2012; Wang et al., 2010) and *Prymnesium parvum* (Berge et al., 2010). Common for most of these studies was the use of a single strain to predict the response to lowered pH.

In this study, we examined the effects of pH on three strains of *P. parvum,* representing a wide biogeographical distribution. We assessed how pH affected growth rates by culturing strains over both higher and lower values compared to current environmental conditions.

2. Material and methods

2.1. Cultures

Three *Prymnesium parvum* cultures were selected to provide a worldwide distribution (Table 1). Cultures were maintained in L1 medium at 15 °C under a light intensity of 110–130 μmol photons $m^{-2}\,s^{-1}$ with a light:dark cycle of 16:8 h. Light intensities were measured with a spherical PAR sensor (ULM-500, Walz, Germany). The Danish strain was grown at a salinity of 10 and the Japanese and N. American strains at a salinity of 30.

2.2. Calculating cell abundances using RFU

Prior to experimentation each strain was grown in 50 ml Nunc flasks in batch mode and in triplicate. Every second day an aliquot (2 ml) was removed from each flask and the raw fluorescence (RFU) was measured using a Turner Trilogy laboratory fluorometer (Turner Designs Inc., USA). A further aliquot (1.2 ml) was removed and preserved with Lugol's iodine solution (1% final concentration) and enumerated using a 1 ml Sedgewick-Rafter counting chamber using an Olympus CH microscope. The raw fluorescence was then plotted against cell number estimates to test for a linear regression. The best-fit R^2 was used to calculate cell number estimates from the pH experiments. A separate equation was used for each strain.

2.3. pH experiment

Non-axenic cultures of the three isolates were grown in 50 ml Nunc flasks in batch mode and in triplicate. Experiments were carried out at 15 °C (± 1 °C) under a light intensity of 110–130 μmol photons $m^{-2}\,s^{-1}$. The inoculum cell densities for all experiments were ≈100 cells ml^{-1}. Media was adjusted to six different pH levels: 6.6, 7.1, 7.6, 8.1, 8.6 and 9.1. The pH of the culture media was measured using a Jenway 3510 pH meter (Cole-Parmer, UK). pH was held constant by adjusting every second day with either a few drops of medium that had been bubbled with CO_2 (pH 5) or medium with 0.1 M NaOH (pH 10). pH fluctuations and adjustments during the 12-day experiment are shown in Fig. 1.

2.4. Growth rates

Cell abundances were estimated every second day using fluorometry. Aliquots (2 ml) were removed from each flask and the raw fluorescence (RFU) measured using a Turner Triology fluormeter. Cell numbers were estimated using the regression plots described above (data not shown). The duration of the exponential growth phase was determined by calculating the maximum achievable R^2 when fitting straight lines to logged plots of cell density. At least six data points were used for the calculations of the growth rate. The maximum growth rate was calculated for each replicate and the mean was based on the three replicates. The maximum growth rate (equation (1) was defined as the number of cell divisions day^{-1} and calculated as:

$$\mu = \ln(Nt_2/Nt_1)/(t_2 - t_1), \quad (1)$$

where Nt_2 and Nt_1 were the cell abundances at the start (t_1) and end (t_2), respectively.

2.5. Statistical analysis

Statistical procedures were carried out in Minitab statistical software. After testing for normality, a one-way ANOVA was used on log-transformed data. $P < 0.05$ was considered significant and variability was measured by standard error of the mean (SEM).

3. Results

3.1. Calculating cell abundances using RFU

When RFU measurements were plotted against cell counts a highly significant linear relationship was observed for all strains

Table 1
Information on strains of *Prymnesium parvum* used in the present study, including culture collection, year of isolation, country and place of collection.

Strain no.	Year of Isolation	Country of Origin	Place of collection
SCCAP K-0081	1985	Denmark	Flade sø, Thy
NIES-1017	1997	Japan	Jogashima Miura, Misaki Kanagawa
UTEX LB 2797	2001	N. America	Texas Colorado River

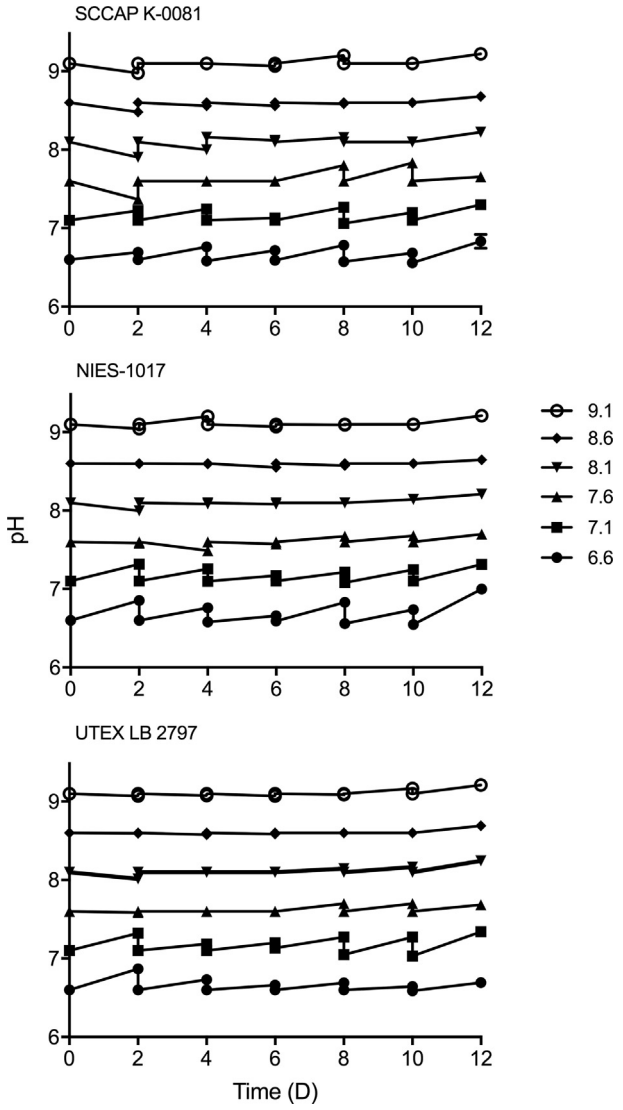

Fig. 1. pH levels and adjustments during the 12-day experiment.

(SCCAP K-0081: $y = 3.0652 \times (R^2 = 0.97)$; NIES-1017: $y = 7.1609 \times (R^2 = 0.99)$, UTEX LB 2797: $y = 9.7359 \times (R^2 = 0.92)$). These equations were used to calculate cell abundances in the pH experiments.

3.2. Growth rates

The concentration of cells increased exponentially and no lag phase was observed for any of the strains (data not shown). The average maximum growth rate varied between strains at all pH levels ($p < 0.01$) (Fig. 2). At pH levels 6.6–7.6 the Japanese strain (NIES-1017) had significantly higher ($p < 0.01$) growth rates (0.37–0.39 divisions day^{-1}) compared to the other two strains (0.24–0.35 divisions day^{-1}). At pH levels 8.1–9.1 the strain from N. America (UTEX LB 2797) had significantly higher ($p < 0.01$) growth rates (0.3–0.35 divisions day^{-1}) compared to the Danish and Japanese strains (0.18–0.32 divisions day^{-1}).

No lower pH limit was observed for any of the stains. The Danish strain (SCCAP K-0081) reached its highest maximum growth rate of 0.26 divisions day^{-1} at pH 8.1. Above this value the growth rate began to decline reaching 0.23 division day^{-1} at pH 8.6. This reduction was however not significant. At pH 9.1 the growth rate

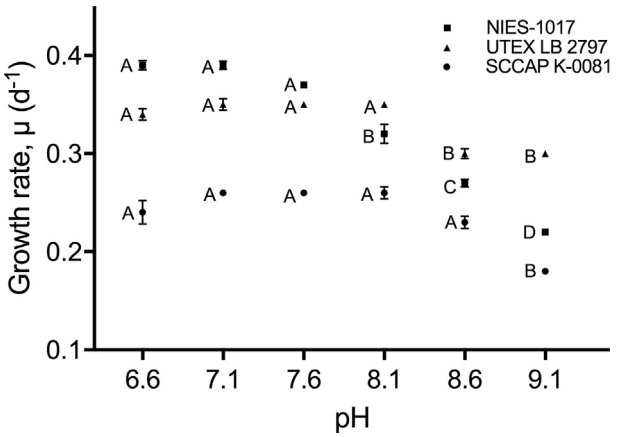

Fig. 2. Maximum growth rates (d^{-1}) of the three *Prymnesium* strains at six different pH levels (Japanese strain = NIES-1017, squares; North American strain = UTEX LB 2797, triangles; Danish strain = SCCAP K-0081, circles). Error bars represent ± SEM. The letters (A–D) show significant differences among pH treatments within strains (one-way ANOVA, $p < 0.01$).

had significantly declined to a minimum value of 0.18 divisions day^{-1} ($p < 0.01$). Over the entire range of pH values the average growth rate declined by ≈ 31%. The Japanese strain NIES-1017 had the highest maximum growth rate of any of the strains (0.39 divisions day^{-1}) but the lowest tolerance to high pH levels, with a ≈44% decrease in growth rate. The growth rate began to decline after pH 7.6 and quickly dropped to a minimum of 0.22 divisions day^{-1} at pH 9.1 ($p < 0.01$). The Texan strain UTEX LB 2797 had the most stable growth rate, which only declined by ≈ 14% over the range of pH vales. Between pH 6.6–8.1 the growth rate was stable at 0.35 divisions day^{-1}, after which the growth rate declined to 0.30 divisions day^{-1} between pH 8.6 and 9.1 ($p < 0.01$).

4. Discussion

4.1. Strain differences to manipulated pH levels

When comparing growth rates between the three strains examined here, strain specific responses to pH were evident. All strains had growth rates ranging from 0.24 to 0.39 day^{-1} at pH 6.6. This is at a level far lower than the pH value of 7.8 predicted for oceans by the end of the century (Orr et al., 2005). The Danish strain (SCCAP K-0081) had a consistently lower growth rate compared to the strains from N. America or Japan. The Japanese strain (N-1017) was the most sensitive to high pH and grew optimally below pH 7.6. The Scandinavian and N. American strains grew optimally until pH values reached 8.1. Similar broad tolerances to manipulated pH levels have also been observed in other marine phytoplankton (see table 2 in Berge et al., 2010). In a study by Pancic et al. (2015) resilience to pH changes due to phenotypic plasticity was also shown for 6 Greenlandic strains of the pennate diatom *Fragilariopsis cylindrus*. Berge et al. (2010) included a strain of *Prymnesium parvum* (SCCAP K-0623) in their pH manipulation study. Unfortunately, origin and year of isolation was not available to them. Growth rates were similar to those obtained here, thus confirming that yet another *P. parvum* strain will continue to grow despite of ocean acidification.

4.2. Differences linked to provenance and culture age

Strain differences can often be linked to the provenance, as well as the genetic variability within a species. Certain optima can be

traced back to their native environmental conditions and past adaptations (evolutionary history). However, the consistently lower growth rate of the Scandinavian strain could also be due to the length of time that it has been in culture. More than thirty years has passed since this strain was established and therefore a reduced growth rate may have evolved to avoid a high pH (Berge et al., 2012). Furthermore, the strain grew optimally at pH 8.1, which is also the pH of the medium in which it is cultured.

4.3. Future bloom events of *P. parvum* and their impact

Growth of *Prymnesium parvum* appears to be highly resilient to low pH. So, despite a predicted decline of ocean pH we will likely continue to experience bloom events of *P. parvum*. The numbers or magnitudes of these blooms may not be the only aspect of *P. parvum* growth impacted by OA. *P. parvum* blooms often have lasting repercussions on food webs due to their diverse array of toxic compounds altering the structure of phytoplankton communities (Prosser et al., 2012).

A number of studies have concluded that due to prymnesins being acid-base labile compounds the different modes of prymnesin toxicity will be restricted to different pH ranges, with hemolysis and ichyotoxcity being highest under certain pH conditions. Shilo and Rosenberger (1960) observed that haemolytic activity of prymnesins occurred as low as pH 5, but this activity declined with an increase in pH. Valenti et al. (2010) observed that the ichthyotoxic effect declined below pH 8. The same pH threshold was also observed by Grover et al. (2010) and Ulitzur and Shilo (1964). Similarly, McLaughline (1958) described a reduction in exposure time associated with mortality in fish species of *Lebistes* and *Gambusia* when cells of *P. parvum* were exposed to elevated pH conditions. As toxin production is directly linked to autotrophic metabolism in most HAB species, changes in CO_2 availability will have an effect on toxin production.

Contrary, Prosser et al. (2012) observed that when *P. parvum* was grown in experimental enclosures at low (pH 7) and intermediate levels (pH 7.5) maximum cell densities were significantly impacted. These authors suggested that the reduction in toxicity and thus the allelochemical activity at low pH levels rendered *P. parvum* unable to supress the growth of competing organisms. In losing this ability *P. parvum* would be unable to form blooms. More studies are needed to fully understand to potential impact of *P. parvum* blooms at ecosystem level.

4.4. Will long-term studies uncover genetic adaptations to altered pH?

Marine organisms are currently experiencing changes in OA at rates faster than they have encountered in their recent evolutionary history (Raven et al., 2005). Genotypic variation complimented with beneficial mutations in response to declining pH values maybe an advantage when adapting to new pH levels. If different species/strains evolve and adapt to lowered pH at different rates, this may alter the community composition (i.e. microbial diversity).

In this study, we did not consider long-term genetic adaptation of strains from the same or different populations. In a few long-term studies that do address pH and adaptations in marine and freshwater microalgae very few documented genetic changes. For example, Collins and Bell (2004) examined the ability of a freshwater chlorophyte *Chlamydomonas*to to adapt to new pH conditions. No genetic adaptations were observed despite its ability to adapt to CO_2 conditions three times current levels. Crawfurd et al. (2011) and Low-Décarie et al. (2013) also failed to detect evolutionary responses to altered CO_2 concentrations when cultures of marine and freshwater diatoms and freshwater cyanobacteria and chlorophytes were maintained for 100's to 1000's of generations. The exception to this was the study by Flores-Moya et al. (2012) who documented an increased growth rate resulting from genetic adaptation in two strains of *Alexandrium minutum* maintained at an alternate pH (7.5) and temperature (25 °C) for 180 and 250 generations, respectively. To further our understanding of physiological responses and long-term genetic adaptations we will need to explore differences in gene expression levels (transcriptome) when growing multiple microalgal strains under manipulated pH levels for several thousand generations (Fu et al., 2012). Then we may strive to predict how reduced pH will affect marine ecosystem structure and functioning in the future. Yet, changes are likely to be highly complex and non-linear with alternations varying seasonally and geographically with pH being just one factor determining algal physiology, growth, toxin production and community structure.

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