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*Effect of temperature on growth
and production of YTXs and
lytic compounds by
Protoceratium reticulatum from
Greenland*

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No data can be taken out of this work without prior approval of the authors.

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Abstract

Harmful algal blooms, known to produce potent toxins that may affect the ecosystem, human activities and health, have increased in frequency and intensity worldwide in the past decades. Numerous processes involved in Global Change; are amplified in the Arctic, but little is known about species specific response of arctic dinoflagellates. The aim of this work was to test the effect of temperature on growth, and production of Yessotoxins and lytic compounds by *Protoceratium reticulatum* isolated from Greenland. Six clonal isolates, the first isolates of *P. reticulatum* available from arctic waters, were characterised by morphology and sequencing and were shown to produce both yessotoxins (YTXs) and lytic compounds, which is the first report of lytic activity for *P. reticulatum*. Growth was strongly affected by temperature with a maximum growth rate at 15°C, with significant but slow growth at 1°C and cell death at 25°C. Generally, these results suggested an adaptation of *P. reticulatum* to temperate waters. Temperature had no major effect on YTXs cell quota or lytic activity. Nevertheless, both parameters were affected by the growth phase with a significant increase at stationary phase. A comparison of all six isolates at a fixed temperature of 10°C showed high variability between the isolates for all three physiological parameters tested. Growth rate varied from 0.06 to 0.19 days⁻¹ and YTXs from 0.1 to 24.1 pg YTXs cell⁻¹. The all six isolates performed lytic activity at stationary phase, however only four displayed measurable lytic activity at stationary phase.

Summary

Phytoplankton serves as the base of the marine food web and is essential for ecosystem function and for human exploitation of seafood resources. However, microscopic planktonic algal blooms might have negative effects on the ecosystem, human activities and health by the production of toxins and/or other bioactive metabolites negatively affecting competitors and grazers. The frequency and intensity of these blooms is increasing worldwide in the past decades and several forces and processes such as rising temperature, eutrophication, changes in salinity among many others, may be driving this situation. These forces and processes are highly amplified in the Arctic Ocean by various feedbacks. A number of toxic algal species have been described to occur in the Arctic area, but almost nothing is known how these species may react on global change in terms of growth and toxin production. The aim of this work was to test the effect of the temperature on growth, Yessotoxins (YTXs) and lytic compounds production by *Protoceratium reticulatum* isolated from the Arctic. A total of seven clonal isolates were set up from Greenland coastal waters. Morphologic and molecular analysis confirmed that the isolates from Greenland are the first isolates of *P. reticulatum* from arctic waters. One isolate (PR3) was used to test temperature effect at six different temperatures (1, 5, 10, 15, 20 and 25 °C). In addition, six isolates were grown at 10°C to test variability between isolates. Growth rate was measured during exponential growth by consecutive cell counts. YTXs were analyzed by LC-MS/MS and lytic activity by short-term (24 h) bioassay experiment with *Rodomonas salina* as target organism. Growth rate was strongly affected by temperature with a maximum growth at 15°C. A temperature of 25 °C caused cell death. The six isolates were identified as YTXs producers in a range of 0.1 to 24.1 pg YTXs cell⁻¹. This work is the first report of lytic activity performed by *P. reticulatum*. The all six isolates showed lytic activity at stationary phase; however no lytic activity could be measured at lower cell densities during exponential phase for the isolates PR5 and PR6. High variability was obvious between the six isolates for the three physiological parameters analyzed. Temperature did not have a major effect on the YTXs cell quota and the lytic activity; nevertheless both parameters are affected by the growth phase increasing from exponential to stationary phase. The growth response over a wide range of temperatures generally suggested an adaptation to temperate waters. The high intra-specific variability underlined the importance of using different isolates in laboratory studies on the physiology of phytoplanktonic organisms.

1. Introduction

Microscopic planktonic algae are critical food for zooplankton, filter-feeding bivalve shellfish (clams, oysters, mussels and scallops) as well as the larvae of important crustaceans and finfish. In most cases algal blooms are therefore beneficial for aquaculture and wild fisheries operations. However, in some situations algal blooms may have negative effects such as economical losses in aquaculture, tourism or fisheries, or may negatively impact human health or the environment by massive killing of fish, birds or mammals. All these cases are collectively known as harmful algal blooms (HABs) (Hallegraff, 1993). In addition to the production of compounds accumulating in the food web and being toxic to humans, many HABs genera, such as, *Karenia*, *Alexandrium* or *Fragilidium*, have been identified as allelochemical compound producers (Kubanek et al. 2005; Tillmann et al. 2007, 2008a). Both, toxins and allelochemical compounds may differ in target organism, effect, and mode of action along different groups of microalgae (Ilanora et al. 2011). The frequency and intensity of HABs and phytoplankton community shifts towards toxic species have increased worldwide. Several forces might drive these changes such as ocean acidification, global warming, and shifts in availability, ratios, and speciation of nutrients, changing exposure to solar irradiance, and altered salinity all have the potential to profoundly affect the growth and toxicity of these phytoplankton species (Fu et al. 2012). All these factors and processes, as many others, are likely amplified in the Arctic by various feedbacks, including ice and snow melting that decreases surface albedo, and atmospheric stability that traps temperature anomalies near the surface. Consequently, the temperature is increasing at a rate of two to three times that of the global average temperature in the past 150 year (Wassmann et al. 2011).

A number of harmful eukaryotes in the past have been recorded in the marine phytoplankton and sympagic communities across the entire Arctic regions. The most frequently recorded taxa of harmful algae throughout the Arctic regions, assessed by Poulin et al. (2011) were the domoic acid producing planktonic diatoms *Pseudo-nitzschia delicatissima*, *P. pungens* and *P. seriata*, the dinoflagellates *Alexandrium tamarense*, *Dinophysis acuminata*, *D. acuta*, *D. norvegica*, *Gonyaulax spinifera*, *Protoceratium reticulatum* (syn: *Gonyaulax grindleyi*) and the prymnesiophyte *Phaeocystis pouchetii*.

P. reticulatum is a dinoflagellate (Dinophyceae), one of the most important groups in marine plankton. It has been reported all around the World and was classified by Okolodkov (2005) as an arctic-boreal-tropical species. Cells of *P. reticulatum* are of medium size (28-43 µm long, 25-35 µm wide) with a polyhedral shape and with a very strongly reticulated theca (Dodge 1989). As many other species of the order Gonyaulacales, *P. reticulatum* is photosynthetic and able to grow in standard medium for dinoflagellates; nevertheless, there is some evidence reported by Jacobson and

Anderson (1996) of mixotrophy. *P. reticulatum* is known for being a producer of Yessotoxins (YTXs), what was first confirmed in cultures from New Zealand by Satake et al. (1997).

YTXs, a group of disulfated polyether toxin were first isolated from Japanese scallops by Murata et al. (1987). Historically YTXs have been considered among the toxin responsible for diarrhetic shellfish poisoning (DSP), due to the fact that YTXs are extracted together with the DSP toxins, okadaic acid (OA) and the dinophysistoxins (DTXs) when they co-occur in shellfish (Botana 2008). Nevertheless, YTXs do not cause diarrhetic effects when it has been tested in mice (Ogino et al. 1997; Aune et al. 2002), and YTXs toxicity in humans remain uncertain because no human intoxication has been reported. However, effects of YTXs in mice health seem to be more serious when YTXs are supplied by intraperitoneal injection than orally. There are reports on damages in cardiac muscle (Aune et al., 2002), liver and pancreas (Terao et al. 1990) and neuronal tissue (Franchini et al. 2004) by intraperitoneal YTXs supply in mice. Accordingly, YTXs should be considered as a potential risk for human health with a lethal dose (LD₅₀) between 80 and 750µg/Kg (Paz et al. 2008). Besides the possible toxic effects of YTXs, it has been suggested that YTXs may act as a defence against grazers as the feeding ratio of copepods was significantly reduced in the presence of YTXs (Makino et al. 2008). A number of studies have reported the presence of YTX as the most dominant toxin and other minor YTX analogues in *P. reticulatum* cultures (Satake et al. 1997; Ciminiello et al. 2003; Paz et al. 2004, 2007, 2013; Röder et al. 2011, 2012). According to the review of Paz et al. (2008), 36 natural derivatives from YTX have been identified and characterized either being produced by dinoflagellates or by shellfish metabolism. YTXs have been found in bivalves and phytoplankton samples in northern Adriatic Sea (Ciminiello et al. 1997; Draisci et al. 1999), Norwegian coastal waters (Aasen et al. 2005), U.S.A. western coastal waters (Howard et al. 2008), northern Chile (Alvarez et al. 2011), among several other locations around the world reviewed by Paz et al. (2008).

Apart from these well studied phycotoxins and their negative effects for human health, there probably are many more novel roles and more yet undescribed bioactive compounds in the plankton (Ivanova et al. 2011).

Historically, allelopathy was used for chemically mediated effects between plants, including microorganism. Recently it has been re-defined by Rice (1984) as: “any direct or indirect harmful or beneficial effect by one plant (microorganisms included) on another through the production of chemical compounds that escape into the environment”. Nevertheless, it is truly difficult to define “plants” and “animals” within the unicellular planktonic organisms, due to the rather widespread motility via flagella, mixotrophy and kleptochloroplasty (Flynn et al. 2013). Thus, we will use allelochemistry defined as “chemically mediated interactions between unicellular

organisms in general, including competitors, grazers and prey” (Tillmann et al. 2008b). Traditionally, changes in phytoplankton succession have been explained by shifts in physicochemical factors (Fistarol 2004). However, there is increasing evidence, that inter-specific interactions in the plankton play a major role in succession, food web structure and bloom development (Ivanova et al. 2011; Legrand et al. 2003; Tillmann et al. 2008b). Most of these metabolites are present in very low concentrations; both in the producer organisms and in the surrounding water mass, what makes a severe challenge for their detection, characterization and quantification. Furthermore, their activity may be subject to synergistic interactions with other natural and anthropogenic environmental toxicants that can cause harmful effects. Chemical stressors as ocean acidification and climate change can also alter the production and degradation of these products. This is particularly important in pristine ecosystems such as polar areas (Ivanova et al. 2011).

Thus, it seems important to understand present events such as, algal blooms and shifts in phytoplankton communities and how they will be affected by future changes. Therefore it is needed to gain baseline knowledge on how environmental parameters may affect physiological aspects of planktonic key species.

The aim of this work was to examine the effect of temperature on the growth and production of YTXs and bioactive secondary metabolites by different clonal isolates of *P. reticulatum* from Greenland coastal waters.

2. Materials and Methods

2.1. Isolation and identification of P. reticulatum

A total of seven clonal isolates of *P. reticulatum* were collected from plankton samples in western Greenland coastal waters (Fig. 1.), during a cruise on the research vessel “Maria S. Merian” in August 2012. Vertical nets tows were performed at each station through the upper 30 m of the water column with 20- μ m-mesh Nitex plankton net. Single cells of *P. reticulatum* were isolated on board from live net tow concentrates under a stereomicroscope (M5A, Wild, Heerbrugg, Switzerland) by micropipette. Single cells were transferred into individual wells of 96-well tissue culture plates (TPP, Trasadingen, Switzerland) containing 250 μ L of K medium (Keller et al., 1987) prepared from 0.2 μ m sterile-filtered natural Antarctic seawater diluted with seawater from the sampling location at a ratio of 1:10. Plates were incubated at 10°C in a controlled environment growth chamber (Model MIR 252, Sanyo Biomedical, Wood Dale, USA). After 3 to 4 weeks, unialgal isolates were transferred to 24-well tissue culture plates, each well containing 2 mL of 1/10 K medium (Keller et al., 1989), supplemented with selenite (Dahl et al., 1989), prepared from sterile-filtered (VacuCap 0.2 μ m Pall Life Science) natural Antarctic seawater with salinity of 34. Exponentially growing isolates were finally used as inoculum for batch cultures in 65 mL polystyrene cell culture flasks

and were maintained thereafter at 10° C under a photon flux density of 30-50 $\mu\text{mol s}^{-1} \text{m}^{-2}$ 16:8 h light:dark photocycle in a temperature-controlled walk-in growth chamber. The Isolation and first identification was carried out by Dr. Urban Tillmann. One of the isolates was lost after a first sampling for DNA (see below). The remaining six isolates were morphologically identified by light and fluorescence microscopy of calcofluor-stained samples. For cell size measurements Lugol fixed (1% final concentration) cells of the clonal isolate PR3 were collected from the temperature growth experiment (see below) at both exponential and stationary phase. To determine cell size, fixed cells were viewed under an inverted microscope (Zeiss Axiovert 200M, Zeiss, Göttingen, Germany) and photographed at 640x magnification with a digital camera (Axiovert, Zeiss, Germany). Measurements were taken using the analysis tool of the Axiovision software (Zeiss, Germany). A total of at least 30 cells were measured in length and width of the three replicate cultures. Characterization of thecal ornamentation, number and arrangement of cellulosic plates, was carried out with epifluorescence after applying a few drops of a 1 mg L⁻¹ solution of Fluorescent Brightener 28 (Sigma-Aldrich). Additionally, the plate pattern was analysed using an electron microscope (FEI Quanta FEG 200, Eindhoven, Netherlands). Therefore, a sample of strain PR3 from an exponentially growing culture was fixed with Lugol (1% final concentration), collected on polycarbonate filter (5 μm pore size, Millipore), dehydrated, mounted on stubs and sputter-coated as described in Tillmann et al. (2012).

All seven clonal Isolates of *P. reticulatum* were grown and harvested during exponential phase by centrifugation in 50 mL conical tubes. DNA was extracted from cell pellets using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and how it has been described before by Töbe et al. (2013). The D1/D2 hypervariable region of the large sub-unit (LSU) of the ribosomal operon from the resultant DNA was amplified by PCR with the primers D1R (forward) and D2C (reverse) (Scholin et al. 1994) and, PCR chemistry and cycling conditions as in Töbe et al. (2013). Products of PCR were purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and used in Sanger sequencing with the same primers as in PCR with a BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Darmstadt, Germany). Products of the sequencing reactions were cleaned up with a DyeEx 2.0 Spin Kit (Qiagen), and then read on an ABI 3130XL (Applied Biosystems) a capillary sequencer. Primer sequences were removed from contigs after assembly of forward and reverse sequences in Geneious Pro 5.4.4 (Biomatters Ltd., Auckland, New Zealand). A BLAST search was conducted with the sequences of the clonal isolates from Greenland and the resultant D1/D2 region LSU sequences of other *P. reticulatum* isolates were retrieved from GenBank. Sequences of *P. reticulatum* and a set of other sequences were aligned in MAFFT v7.017 using a plugin for Geneious Pro. The additional sequences corresponded to those gonyaulacoid dinoflagellates used in the phylogenetic analysis by Howard et al. (2009) and a set of sequences representing the five ribotypes of the *A. tamarensis* species complex. A *Prorocentrum minimum*

sequence was included as out-group. The resultant alignment was restricted to the length of the sequences of the clonal isolates of *P. reticulatum* from Greenland and the best fitting model of nucleotide substitution was determined according to scores of the Akaike Information Criterion in jModeltest 0.1.1 (Guindon and Gascuel 2003, Posada 2008). A Maximum Likelihood (ML) tree was constructed using the GTR+G model of nucleotide substitution in PhyML (Guindon and Gascuel 2003) via a plugin in Geneious Pro. Reliability of tree topology was estimated by 200 bootstrap replicates.

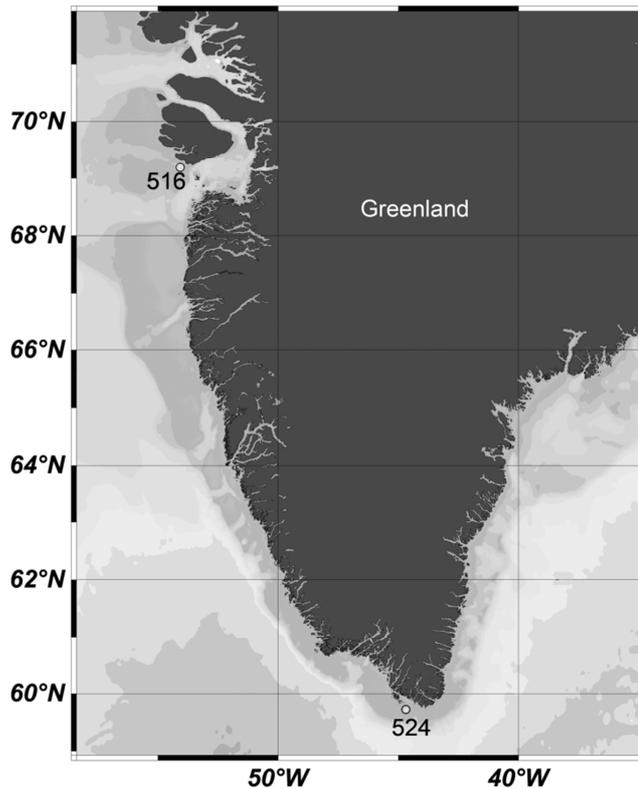


Fig. 1. Map representing two of the stations performed during the cruise on the research vessel “Maria S. Merian” in August of 2012, where the clonal isolates of *P. reticulatum* were isolated (stations 516 and 524).

2.2. Temperature experiments

In order to estimate the growth temperature range of arctic *Protoceratium reticulatum*, one of the isolates (PR3) was chosen for detailed growth experiments at six temperatures (1, 5, 10, 15, 20 and 25 °C \pm 1°C). Cultures were pre-adapted gradually by steps of five degrees to the respective experimental temperature before start of the experiment. Therefore, the culture of PR3, which routinely was kept at 10°C, was first split and moved also to 5°C and 15°C. After a couple of doubling, these cultures again were split and moved from here to 1°C and 20°C respectively, and with the same schedule finally also to 25°. Culture used to inoculate the experiment were thus kept at the experimental temperature for at least five generations (at the lowest

temperatures due to the slow growth) to at least ten generations at the higher temperatures.

At each temperature, triplicate cultures were set up in 250 ml tissue flasks in 1/10 K-medium at an initial concentration of 300-500 cells mL⁻¹ at photon flux density (PFD) of 80-90 μmol s⁻¹ m⁻² 16:8 h light:dark photocycle. Cultures were sampled for cell counts in intervals of two to five days, depending of the growth rate. Previous to each sub-sampling, culture flasks were gently shaken by hand for a homogenous cell distribution. Cell density was measured by cell counting in a sedimentation chambers with an inverted microscope (Zeiss Axiovert 40C, Gottingen, Germany) of samples fixed with Lugol's solution (1% final concentration). Depending of the cell concentration the volume fixed for cell counting varied from 0.1 to 1 mL, so that the total number of cells counted was always >300. Specific growth rate (μ, day⁻¹) was calculated separately for each replicate by exponential regression of cell density over time for a defined period of exponential growth. Growth curves for each clonal isolate were plotted from mean cell density of the three replicates. For yessotoxin quantification and lysis-bioassay experiments, samples were taken in the exponential phase at a cell density of about 2000 cells mL⁻¹ and in stationary phase, when three consecutive cell counts indicated no further increase of cell density. For YTXs quantification, 3 x 50 mL of each culture replicate in exponential phase, and 3 x 15 mL in stationary phase, were centrifuged at 4000 rpm for 15 minutes at 4°C using 50 and 15 mL centrifuge tubes, respectively. Algal pellets were transferred to 2 mL microcentrifuge tubes and stored at -20°C until analysis. Samples for the lysis bioassay were processed as described below.

2.3. Isolates variability experiments

In addition, all six available isolates were grown at 10°C to test the variability among all of them. The cultures were maintained in standard conditions as described for the temperature experiments. Cell density measured and sampling for YTXs analysis and lytic activity were carried out following the same protocol described before.

2.4. Extraction of yessotoxins and measurement by LC-MS/MS

Pellets (in technical duplicates for each replicate culture and sampling) were extracted with 0.5 mL of methanol (MeOH) using a FastPrep® FP120 Cell Disrupter for 45 s at 6.5 m s⁻¹ by reciprocal shaking, afterwards the samples were centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to a 2 mL Eppendorf tube with a spin filter with a pore diameter of 0.45 mm and centrifuged at 10,000 rpm for 30 s. Samples were stored at -20°C in autosampler vials until measuring by LC-MS/MS. The LC-MS/MS measurements were carried out as described by Krock et al. (2008).

Measurements were carried out on a triple quadruple mass spectrometer (API 4000 QTrap, ABI-Sciex) with turbo spray ionization in negative ion BDS C8 column (50 x 2

mm, 3 μm , 120A) at a flow rate of 0.3 mL min^{-1} using an elution gradient with two eluents (A: water and B: 95% acetonitrile/methanol (1:2 v/v) and 5% water, both eluents containing 2.0 mM ammonium formate and 50 mM formic acid). Initial composition was 40% B with a linear gradient to 100% B at 0 min isocratic 100% B until 15 min, then returning to initial conditions. YTXs were identified by comparing retention times and MS3 spectra at sample and a reference standard (IMB-NRC, Halifax, Canada). Compounds with masses between 1141.6 and 1061.1 Da were identified as YTXs. Relative abundances are based on peak area comparisons, identical response factors for all transitions are assumed. Results are expressed by the mean of the technical duplicates (pg YTXs cell^{-1}); the mean variation coefficient of all duplicate analysis was estimated as 13.9 %.

2.5. Quantification of lytic effects

Allelochemical effect of *P. reticulatum* was quantified with a short-term (24 h) lysis bioassay with the cryptophyte *Rhodomonas salina* as target organism. *R. salina* (strain KAC 30 from the Kalmar culture collection) was maintained in 100 mL Erlenmeyer flask at $15^{\circ}\text{C}\pm 1^{\circ}\text{C}$ in K-medium as described before, at a photon flux density (PFD) of $30 \mu\text{mol s}^{-1} \text{ m}^{-2}$ 16:8 h light:dark photoperiod. Counting the number of intact *R. salina* cells (normal cell shape still visible) after 24 h incubation with *P. reticulatum* samples enabled quantification of lytic effect. In order to quantitatively compare the lytic potency, dose-response curves covering a varying number of different *P. reticulatum* cell concentrations were performed.

Bioassays were performed as follows: cell concentration of a stock culture of *R. salina* was determined by microscope cell count and an aliquot was subsequently diluted to a concentration of $4.0 \times 10^5 \text{ mL}^{-1}$. Each set of experimental vials containing 3.9 mL of different dilutions of *P. reticulatum* (each dilution performed in duplicates) was inoculated with 0.1 mL of *R. salina* (final cell concentration 1.0×10^3) to start the experiment. Three replicates of 3.9 mL filtered seawater were taken as control. Vials were incubated at the respective temperature in absence of light to prevent growth. After 24 h, samples were gently shaken and a subsample of 0.5 mL was fixed with 1% Lugol's solution in a sedimentation chamber and cell density of both *R. salina* intact cells and *P. reticulatum* were determined by invert microscope counts. For *P. reticulatum* whole chambers were counted, while for *R. salina* three sub-areas corresponding to approximately 600 cells in the controls were counted.

Bioassays for PR3 grown at different temperatures were performed using a series of 10 dilutions (from undiluted culture to about 20 cells mL^{-1}). In these experiments, final concentrations of intact *R. salina* cells followed a sigmoidal declining pattern when plotted against log-transformed *P. reticulatum* cell concentrations. In order to estimate the EC_{50} concentrations (i.e. *P. reticulatum* cell concentration yielding a 50% decline in

R. salina cell concentration), data points were fit to the following non-linear model procedure in Statistica®:

$$N_{\text{final}} = \frac{N_{\text{control}}}{(1 + ((x/EC_{50})^h))}$$

where N_{final} is the final *R. salina* cell concentration, N_{control} is the final *R. salina* cell concentration in control samples (1/10 K-medium inoculated with 1.0×10^3 cells of *R. salina*) x is the log-transformed *P. reticulatum* concentration and EC_{50} and h are fit parameters. In order to visually compare all curves, (which are slightly different in *R. salina* control concentration), plots were normalized by setting the control as 100%. For the comparison of the six clonal isolates a full set of dilutions could not be performed. Here three (exponential phase) or four (stationary phase), different dilutions were tested, which at least allowed a rough comparison against the isolate PR3. Therefore the curve fitted for the full dose response curve of PR3 was shifted by eye to match the data points of the respective isolate and to roughly evaluate the EC_{50} concentration.

An initial bioassay experiment was performed to quantify and compare lytic activity of whole culture, of cell free supernatant and of cell extract. Therefore, a culture of isolate PR3 was grown at 15°C at the standard culture conditions described above and sampled at a cell density of 4205 cells mL⁻¹. To differentiate between intra-and-extracellular lytic activity cells and medium were separated by centrifugation of 100 mL of the culture at 4000 rpm for 15 minutes at 4°C using 50 mL centrifuge tubes. Subsequently a whole set of 10 different dilutions ranging from 4100 cells mL⁻¹ to 130 cells mL⁻¹ (each as duplicates) was prepared for both the whole cell culture and the cell-free supernatant. The cell pellets were combined and re-suspended in 1 mL of 1/10 K-medium. Algal extract was prepared as described by Eschbach et al. (2001): the cell suspension was sonicated with a Sonoplus HD70 disintegrator equipped with a MS73 sonotrode (Bandelin Electronics, Berlin, Germany) using the following settings: 50% pulse cycle, 70% amplitude for 1 min on ice. During the whole preparation procedure, algal extract was kept on ice. The lytic activity of the algal extract was tested simultaneous with the whole cell and supernatant with a set of dilutions

2.6. Statistics

Statistical comparisons of the growth rate, intracellular YTXs concentration and lytic activity of *P. reticulatum* at different temperatures were compared by analysis of the variance (ANOVA) and post-hoc Tukey's HSD tests. Likewise, significant differences between the six clonal isolates from the Arctic available were tested by ANOVA and post-hoc Tukey's HSD test as well.

3. Results

3.1. Morphologic and molecular characterization of *P. reticulatum*

P. reticulatum cells from the Arctic were slightly longer than wide, showing a size of about 22-31 μm in width and 25-35 μm in length (29.6 ± 3.0 μm in length and 26.2 ± 2.7 μm width; $n=90$ measurements at exponential phase). The cingulum was displaced about one width without overlapping; it was located slightly above the median line and was deeply incised (Fig. 2-A) as has been reported before by Dodge (1987), Hansen et al. (1997) and Röder et al. (2012). The cells had a very ornamented theca with numerous pores in the cellulosic plates (Fig. 2). On the first apical plate a large ventral pore was visible (arrow in Fig.2-B). The epithelial plate's tabulation as identified by electron microscopy (Fig. 3) was a pore plate (po), three apical plates (3'), one intercalary plate (1a) and six precingular plates (6''), - as has been defined before by Hansen et al. (1997). For the arctic isolate PR3 we observed some variability in the epithelial tabulation since for some cells the intercalary plate 1a seems to be in contact to the pore plate (Fig.3 D), whereas for other cells a clear separation between the both plates was visible. The pore plate was narrow and elongated as it was described by Hansen et al. (1997) and Röder et al. (2012). The hypothecal tabulation was posterior sulcal plate (Sp), five postscingular plates (5''') and two antapical plates (2''''') (Fig. 3-C).

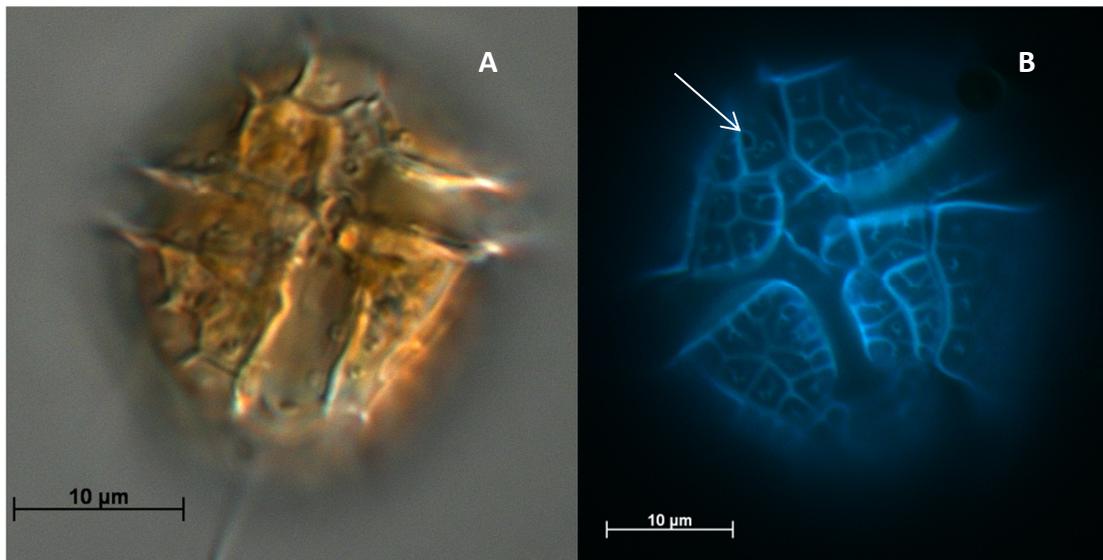


Fig. 2. Light microscopy picture (A) and fluorescence microscope picture (B) of *P. reticulatum* (isolate PR3). Ventral view showing the highly reticulated theca and the characteristic cingulum. Arrow indicating the ventral pore.

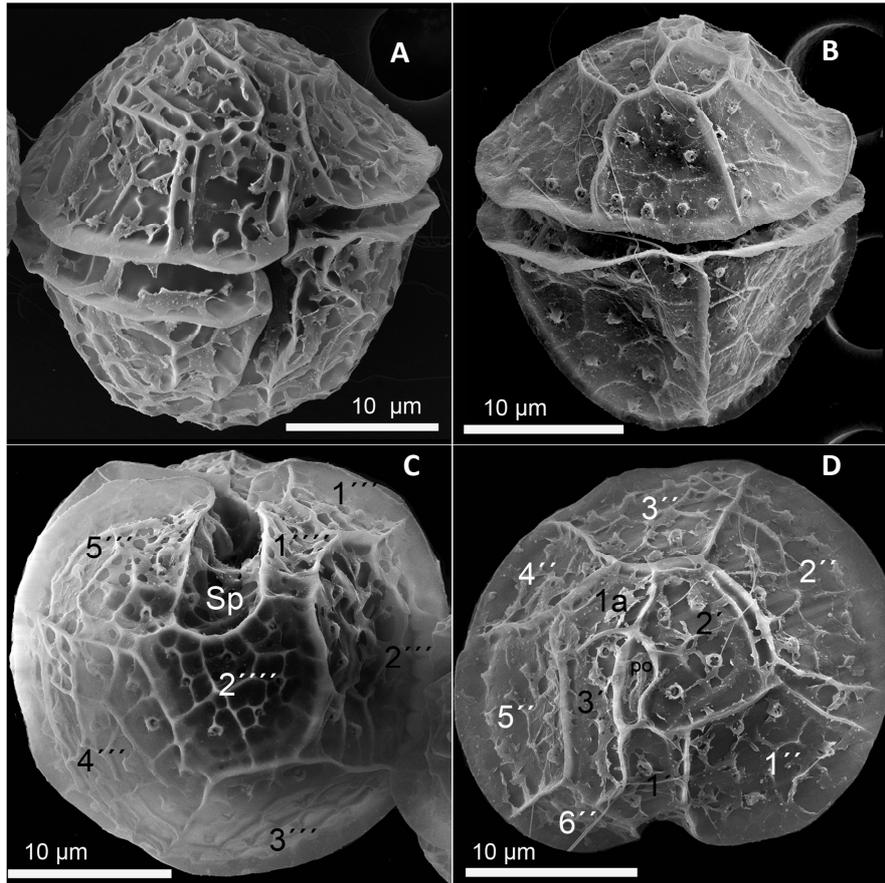


Fig. 3. *P. reticulatum* isolate PR3. Electron microscopy pictures, ventral view (A), dorsal view (B), antapical view (C) and apical view (D).

The maximum likelihood (ML) analysis showed that all seven clonal isolates of *P. reticulatum* from Greenland were identical to most other sequences of *P. reticulatum* that were included in the multiple sequence alignment. In the present ML tree, all *P. reticulatum* were part of a monophyletic clade that subdivides into two well supported, divergent sub-clades. The sequences of the isolates used in this study fell in the same clade as the majority of sequences of *P. reticulatum* and only three isolates of *P. reticulatum* (isolates from U.S.A.) comprised the second sub-clade within *P. reticulatum* (Fig. 4.).

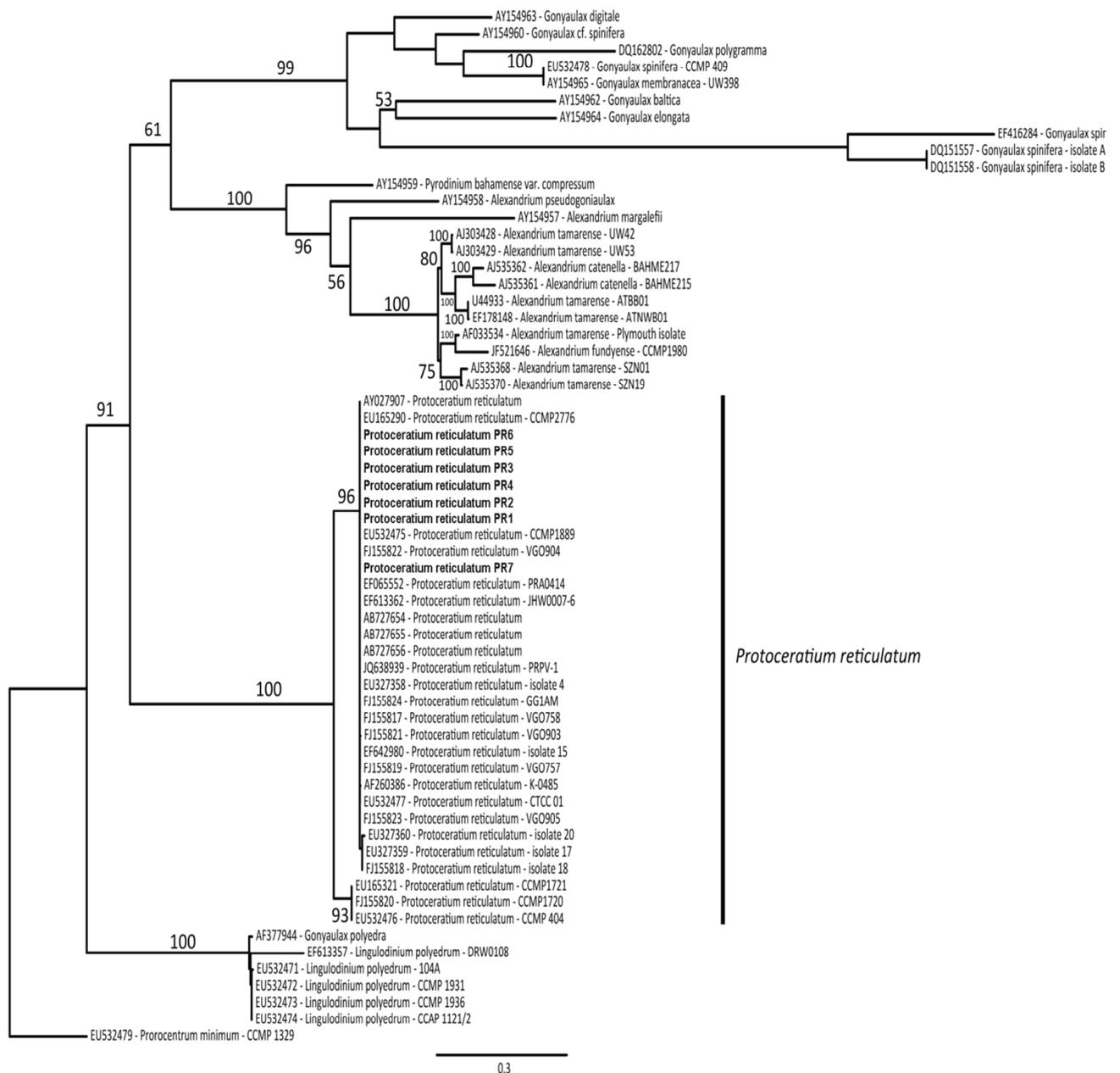


Fig. 4. Maximum Likelihood tree of the hypervariable region of the LSU 28S rDNA with branch support values derived from 200 bootstrap replicates. The scale bar shows average number of substitutions per site. All the clonal isolates from Greenland (bold) fall in the same clade as the majority of sequences of the two divergent clades formed by strains of *P. reticulatum* in this analysis.

3.2. Growth of *P. reticulatum*

Growth of isolate PR3 at different temperatures

P. reticulatum performed exponential growth at all the experimental temperatures except, for the cultures maintained at 25°C where no growth was performed, *P. reticulatum* cells cultured at 25°C (Fig. 5-F) died in all the cases after two weeks of inoculation. Cultures maintained at 1°C reached stationary phase after 56 days of

incubation, showing a slightly linear growth during the first weeks (Fig. 5-A). Meanwhile the cultures incubated at both, 15°C (Fig. 5-D) and 20°C (Fig. 5-E) achieved stationary phase after eleven days. The maximum cell density at stationary phase were around 6 to 10×10^3 cells mL⁻¹ with the highest values reached at 15°C ($\approx 9.8 \times 10^3$ cells mL⁻¹) and the lowest at 5°C, which, after 49 days of incubation, reached $\approx 6 \times 10^3$ cells mL⁻¹. Cultures at 10°C after 14 days of exponential growth, reached stationary phase with a cellular density of $\approx 4.8 \times 10^3$ cells mL⁻¹, however here a gradual deviation from the highest exponential growth before reaching the stationary phase was observed. The maximum cell concentration at 10°C was measured after 30 days ($\approx 7.8 \times 10^3$ cells mL⁻¹) (Fig. 5-C).

The exponential growth rate increase with temperature reaching the maximum at 15°C (0.27 ± 0.01 d⁻¹) closely followed by the growth performance at 20°C (0.25 ± 0.01 d⁻¹). The lowest growth rate was shown at 1°C (0.05 ± 0.00 d⁻¹) (Fig. 7.). Growth rate was significantly different ($P > 0.05$) between the all treatments.

Intra-species variability of growth

The all six clonal isolates available displayed exponential growth at 10°C and the cell yield at stationary phase ranged from $\approx 5 \times 10^3$ cells mL⁻¹ (PR1 and PR5; Fig. 6-A and E respectively) to $\approx 7.8 \times 10^3$ cells mL⁻¹ (PR3; Fig. 6-C). Variable cell concentration was measured during the stationary phase of the isolate PR2 (Fig. X B) with a range of 6.8×10^3 x mL⁻¹ to 7.7×10^3 mL⁻¹ after 28 days. No variation in cell concentration at stationary phase was measured for the cultures of PR4 (Fig. 6-D) and PR6 (Fig. 6-F), which reached a maximum cell yield of $\approx 6.4 \times 10^3$ cells mL⁻¹ after 27 days and 40 days respectively.

The six clonal isolates cultured at 10°C exhibited a high variability in terms of exponential growth rate. The range of growth rate was from 0.06 ± 0.00 d⁻¹ to 0.19 ± 0.00 d⁻¹ displayed by PR5 and PR3 respectively (Fig. 8.). ANOVA and post-hoc Tukey's HSD tests revealed significant differences ($P < 0.05$) between the all isolates, with the exception of the pair of PR1 and- PR6.

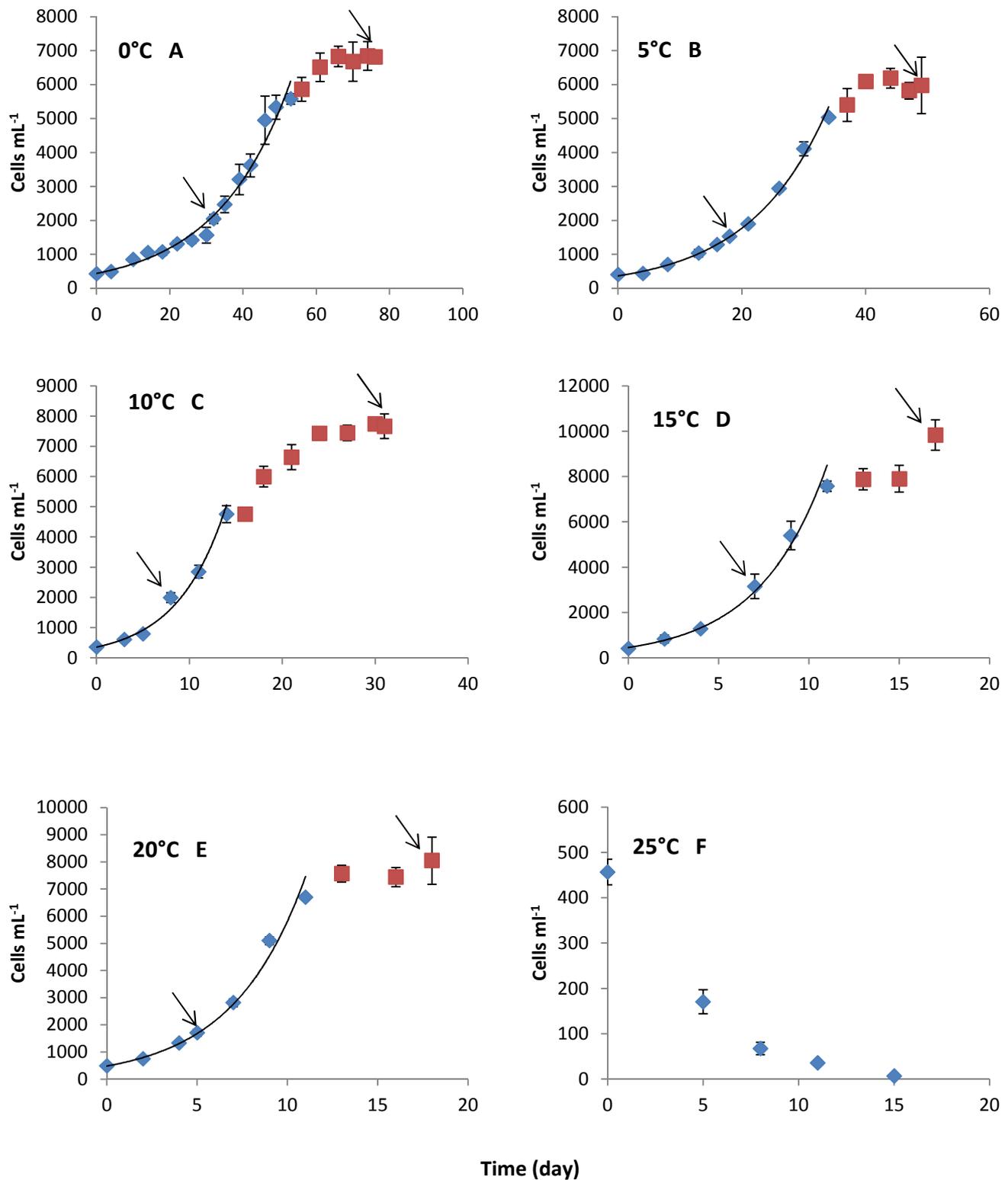


Fig. 5. Growth curves of the clonal isolate PR3 of *P. reticulatum* cultured at different temperatures. Data points represent mean cell counts of three replicates cultures (± SD). Values from time points indicated by blue dots were used to calculate exponential growth rate (see Material & Methods). Arrows indicate sampling points for YTXs and lytic activity. Notice the different axes according to the growth and the cell concentration for each temperature.

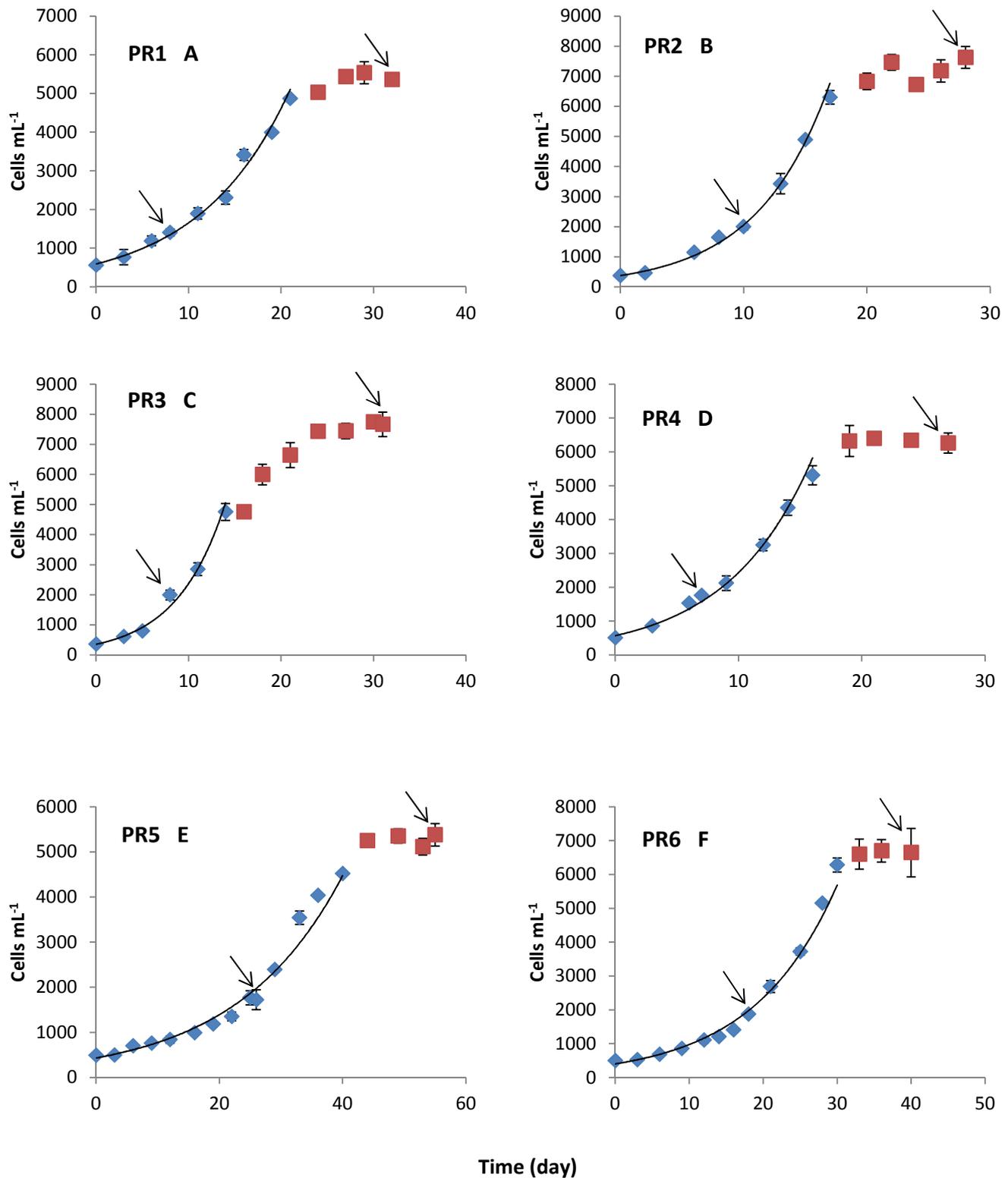


Fig. 6. Growth curves of the six clonal isolates of *P. reticulatum* cultured at 10°C. Data points represent mean cell counts of three replicates cultures (\pm SD). Values from time points indicated by blue dots were used to calculate exponential growth rate (see Material & Methods). Arrows indicate sampling points for YTXs and lytic activity. Notice the different axes according to the growth and the cell concentration for each temperature.

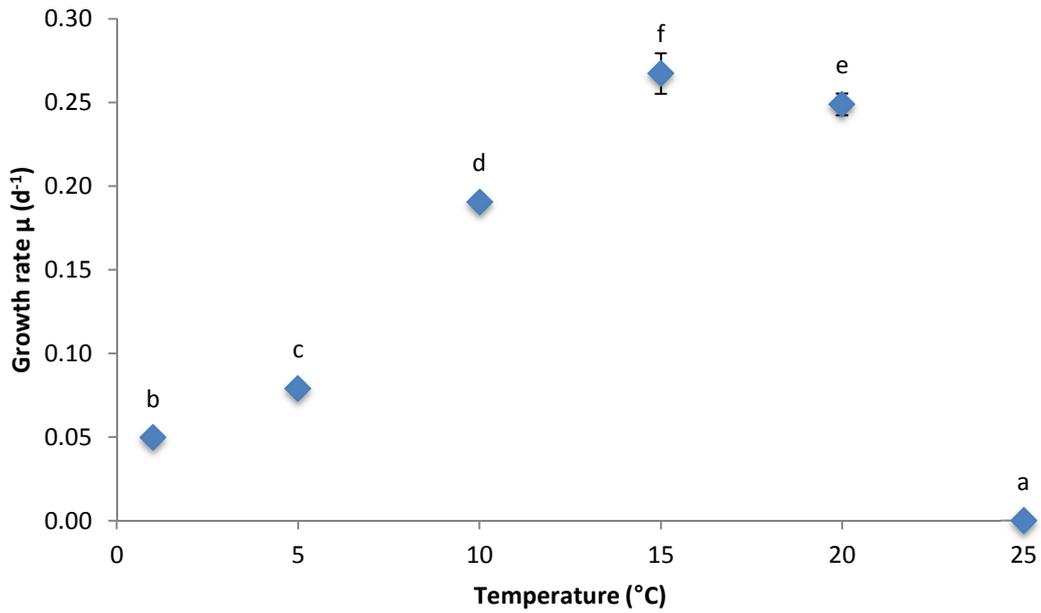


Fig. 7. Growth rate μ (d^{-1}) of *P. reticulatum* (clonal isolate PR3) as a function of experimental temperatures. Data points represent mean growth rate calculation of three replicates cultures (\pm SD). Different letters indicate significant differences between treatments ($P < 0.05$).

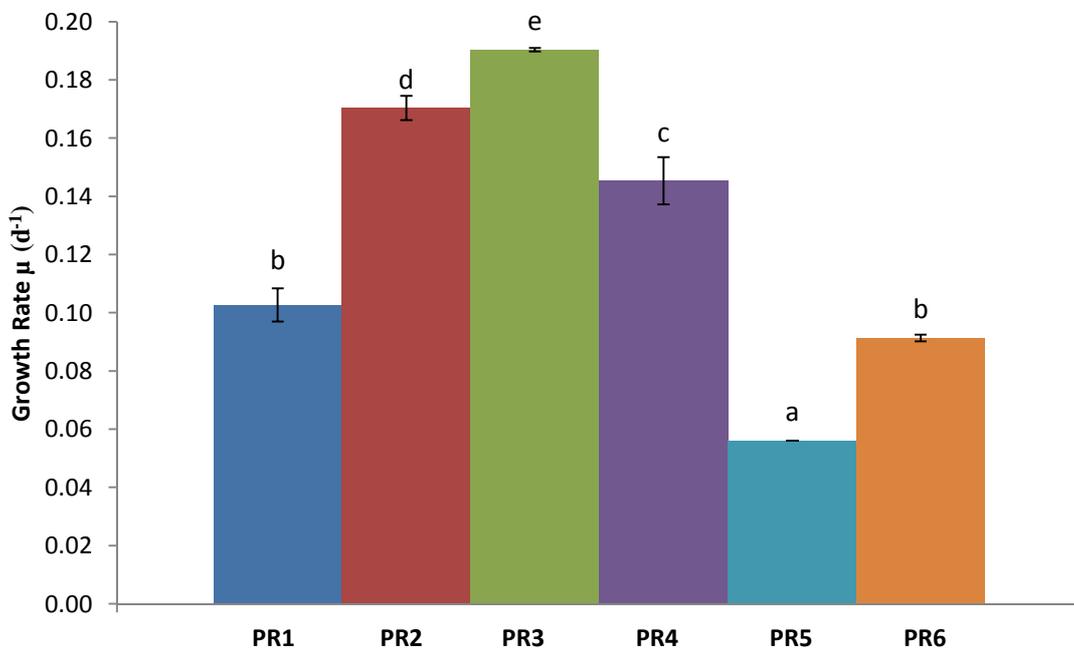


Fig. 8. Growth rate (d^{-1}) comparison of the six clonal isolates of *P. reticulatum* at 10°C. Bars show mean growth rate calculate of three replicate cultures with \pm SD. Different letters indicate significant differences between treatments ($P < 0.05$).

3.3. *P. reticulatum* YTXs cell quotas

YTXs were measured along the cultures in both experimental set ups. *P. reticulatum* produced YTXs in both exponential and stationary phase for the all isolates and under the six temperatures tested. Mass spectrometry analysis showed that the major compound produced by all isolates and along the experimental temperatures was yessotoxin; however other compounds were detected as minor components (< 1%). The range of mono- and di-anions and of their daughter ions in MS/MS fractions that those compounds were detected suggested that could be YTXs congeners according to the YTXs analogues characterizations reported by Miles et al. (2005). Further studies are necessary to identify and confirm those compounds as YTXs congers.

YTXs of isolate PR3 at different temperatures

The YTXs cell quotas for exponential and stationary phase of *P. reticulatum* for the six experimental temperatures are plotted in Fig. 9. The range of intracellular YTXs was from 8.7 ± 1.3 to 19.2 ± 3.8 pg cell⁻¹ performed at 10°C and 1°C respectively at the exponential phase. For the stationary phase an increase in the YTXs cell quota was measured at all the experimental temperatures except at 1°C, where the cultures displayed the lowest cell quota of 8.6 ± 1.1 pg YTXs cell⁻¹. The maximum YTXs cell quota was measured at stationary phase of 20°C cultures (27.5 ± 3.5 pg YTXs cell⁻¹). YTXs concentration measured at 5°C, 10°C and 15°C for stationary phase have not shown significant differences with the concentration of the cultures at 20°C.

Intra-specific variability of YTXs cell quota

The six isolates displayed a very high variability in terms of YTXs cell quota (Fig. 10.). At exponential phase the maximum YTXs intracellular concentration was found for the isolate PR3 (8.7 ± 1.3 pg YTXs cell⁻¹), closely followed by PR1 (7.9 ± 1.7 pg YTXs cell⁻¹) and PR4 (6.4 ± 0.8 pg YTXs cell⁻¹); no significant differences ($P < 0.005$) were measured between these three. In contrast, the clonal isolate PR6 displayed the lowest cell quota (0.1 ± 0.0 pg YTXs cell⁻¹). Compared to the exponential phase, a two-to-four fold-increase was measured at stationary phase for the intracellular YTXs concentration. The YTX cell quota at stationary phase ranged from 24.1 ± 2.0 to 0.4 ± 0.1 pg YTXs cell⁻¹ displayed by the clonal isolate PR1 and PR6, respectively. At stationary phase, ANOVA and Post-Hoc analysis indicate two homogenous groups without significant differences ($P \geq 0.05$), i.e. the isolates PR1, PR2, PR3 and PR4, and the isolates PR5 and PR6 (Fig. 10-B.).

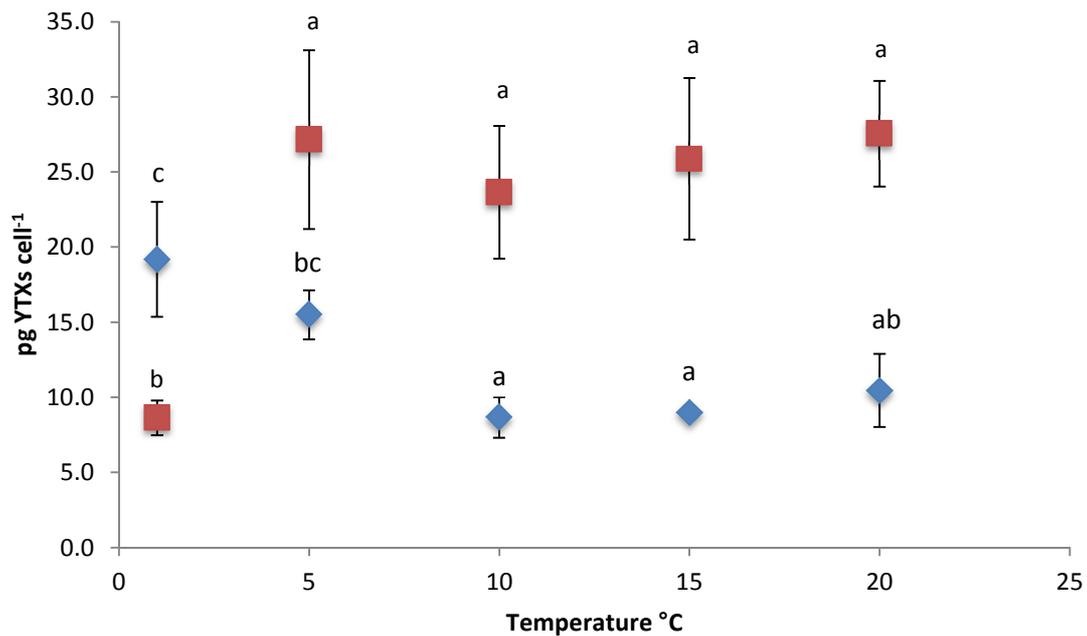


Fig. 9. *P. reticulatum*, strain PR3, yessotoxin cell quota as a function of different growth temperature. Dots in blue represent YTXs cell quota at exponential phase, and red dots show the cell quota at stationary phase. Dots represent mean values of three replicate cultures (\pm SD). Different letters indicate significant differences ($P < 0.05$) between treatments.

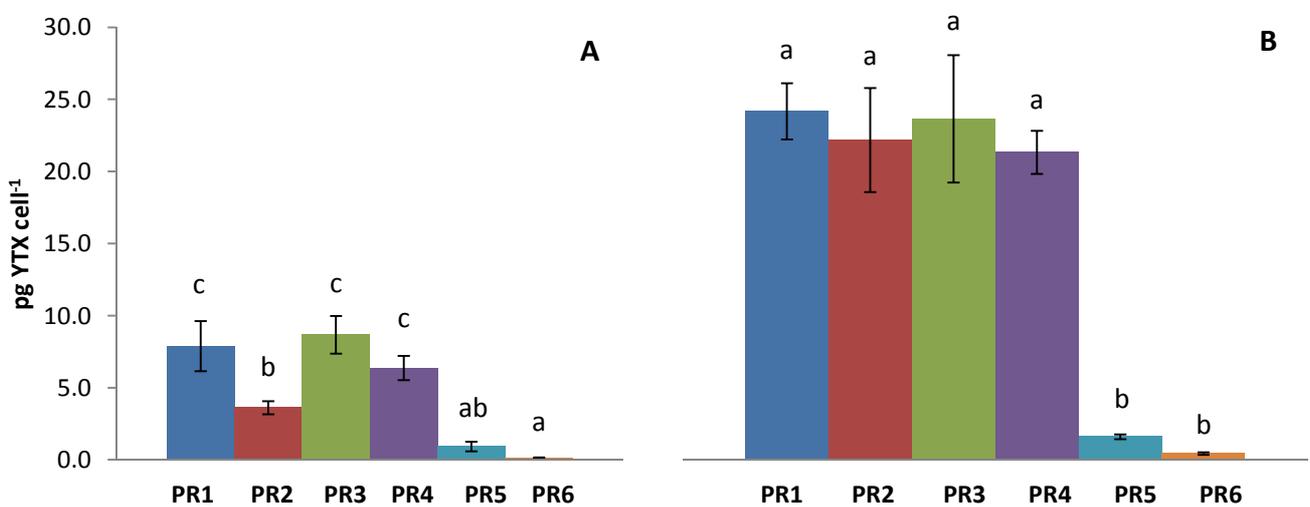


Fig. 10. YTXs intracellular concentration of the six clonal isolates of *P. reticulatum* at 10°C for both, exponential (A) and stationary phase (B). Bars represent mean data of three replicates cultures (\pm SD). Different letters indicate significant differences between clonal isolates ($P < 0.05$).

3.4. Lytic Activity

An initial experiment was performed to quantify lytic activity of the isolate PR3 for whole culture, cell-free supernatant and cell extract (Fig. 11.). The lowest EC_{50} (838 cells mL^{-1} , i.e the highest lytic activity) was displayed with the whole culture, followed by the bioassay performed adding cell-free supernatant, where a decrease about 50 % of the lytic activity was measured (1889 cell mL^{-1}). No lytic activity was observed in the bioassay performed using algal extract from cell pellets, despite the much higher cellular doses applied.

Lytic activity of the isolate PR3 at different temperatures

Dose-response curves for the isolate PR3 of *P. reticulatum* showing lytic activity on *R. salina* at the experimental temperatures for both exponential and stationary are shown in the Fig. 12. The clonal isolate PR3 caused cell lysis of the cryptophyte *R. salina* in the bioassays along the five experimental temperatures tested at both, exponential and stationary phase. EC_{50} at exponential phase ranged from 621 ± 93 to 933 ± 66 cells mL^{-1} at $1^{\circ}C$ and $5^{\circ}C$ respectively (Fig. 13.). No significant differences ($P < 0.05$) were found between the experimental temperatures, with the exception of the cultures grown at $1^{\circ}C$ which showed significant differences with the cultures performed at 5, 10 and $15^{\circ}C$. Compared to lytic activity at exponential phase, the EC_{50} at Stationary phase show an increase in the lytic activity for almost all experimental temperatures except for the cultures maintained at $1^{\circ}C$, for which a decrease in the lytic activity was measured. The lowest EC_{50} was measured at $10^{\circ}C$ (307 ± 83 cells mL^{-1}), but no significant differences ($P < 0.05$) were found between the results at 5, 15 or $20^{\circ}C$.

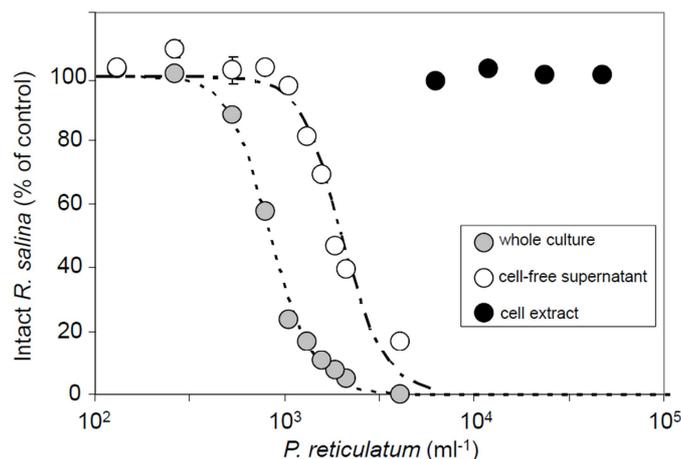


Fig. 11. Dose-response curves describing the lytic activity of three different culture fractions (whole culture, cell-free supernatant and algal cell extract). Data points represent the concentration of *R. salina* after 24 h incubation (as percent of control) as a function of log-transformed *P. reticulatum* concentration. Each data point represent a mean ($n = 2$), and error bars indicate the range of the two technical replicates. Lines represent a nonlinear, sigmoidal curve fit.

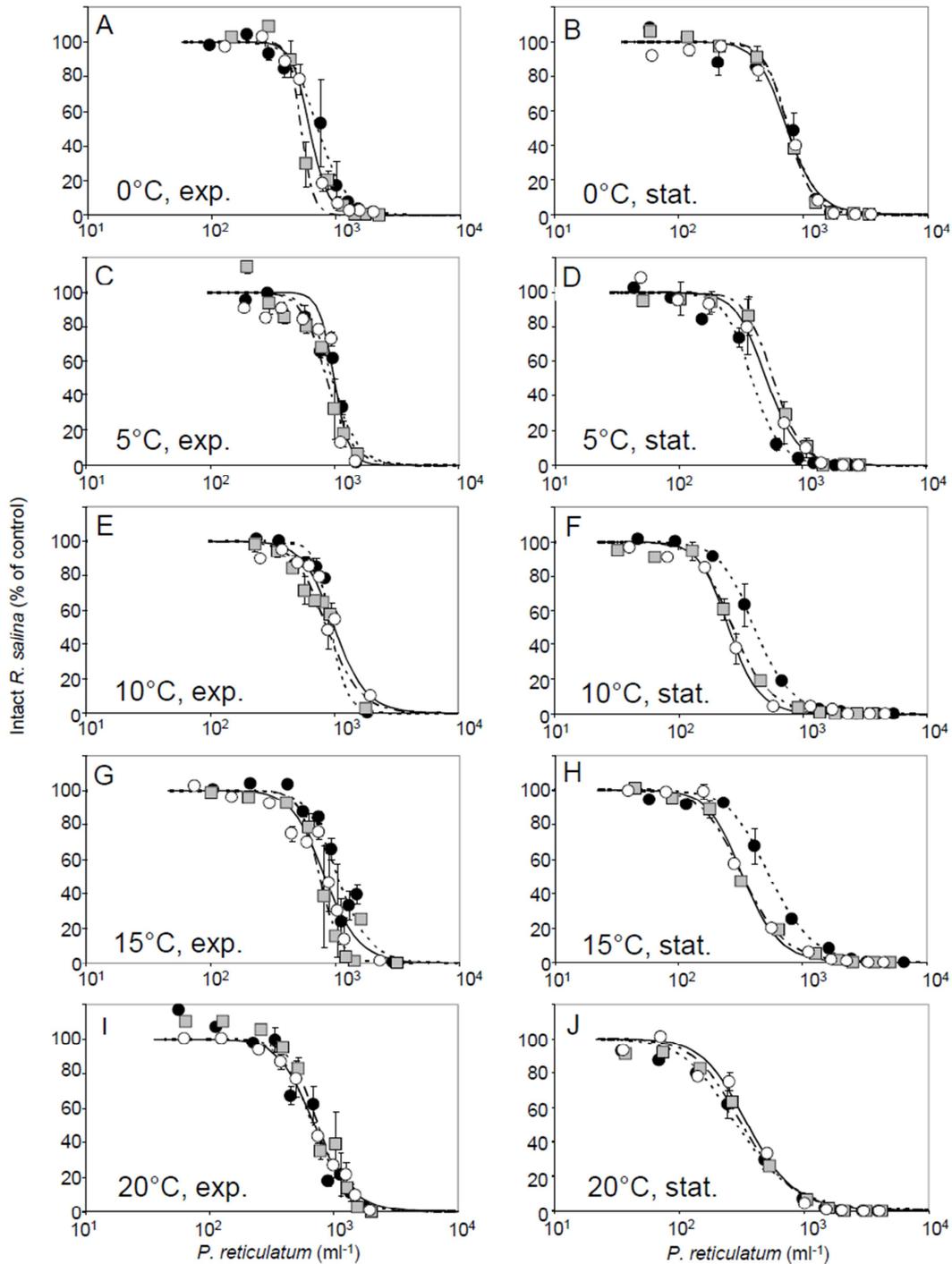


Fig. 12. Dose-response curves describing lytic capacity at exponential (left panel) and stationary phase (right panel) at the five experimental temperatures of the isolate PR3 of *P. reticulatum* as quantified with *R. salina* bioassay. Each graph shows the concentration of *R. salina* after 24 h incubation (as percent of control) as a function of log-transformed *P. reticulatum* concentration of three replicates cultures (white circles, black circles and grey squares). Each data point represent the mean ($n = 2$), and error bars indicate the range of the two technical replicates. Lines represent a nonlinear, sigmoidal curve fit.

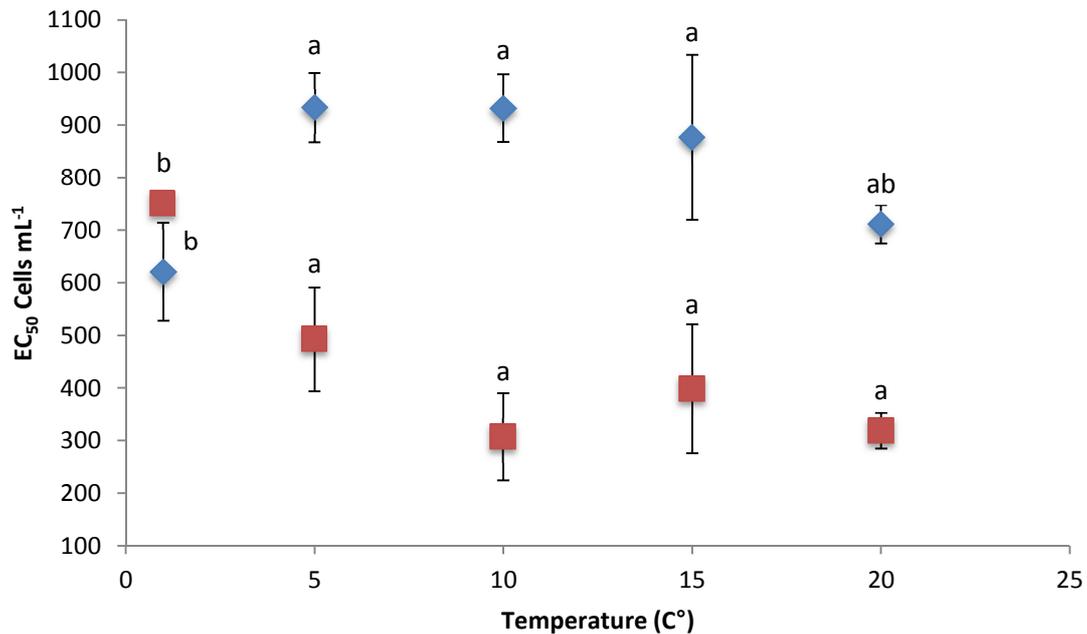


Fig. 13. EC₅₀ at exponential (blue dots) and stationary (red dots) at the six experimental temperatures. Data points represent mean values of three replicate cultures (\pm SD). Different letters indicate significant differences ($P < 0.05$) between treatments.

Intra-species variability of lytic activity

The six clonal isolates were compared based on three-point (exponential phase) or four-point (stationary phase) dilution bioassays (Fig. 14.). EC₅₀ estimates based on visually fitting a sigmoidal curve to the data point are summarized in Table. 1. High variability in terms of lytic activity was obvious between the six isolates of *P. reticulatum*. For the exponential phase sampling four of the six clonal isolates of *P. reticulatum* showed lytic activity, whereas the isolates PR5 and PR6 did not display any measurable lytic activity. Nevertheless, for these two isolates weak lytic activity was observed at stationary phase with EC₅₀ values of about 7.9×10^3 cells mL⁻¹ and 16×10^3 cells mL⁻¹ respectively. Both, PR1 and PR4 displayed a slightly higher lytic activity than PR3 at exponential phase, however during the stationary phase no clear differences between the three of them were observed. PR2 for both phases, exponential and stationary, performed a weaker lytic activity than PR3, although EC₅₀ values were closer to PR3 than PR5 and PR6.

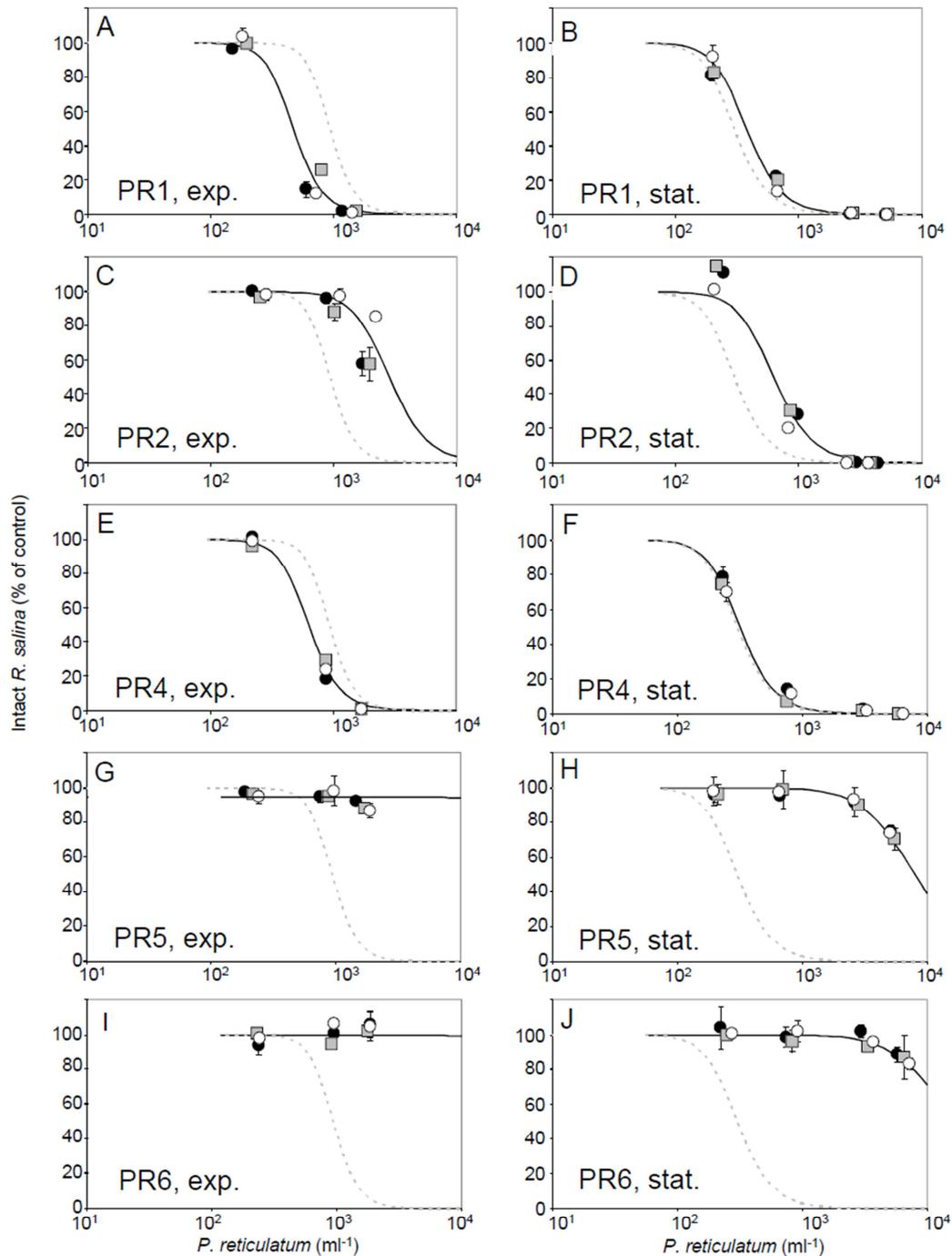


Fig. 14. Dose-response curves describing lytic capacity at exponential (left panel) and stationary phase (right panel) of the five clonal isolates (PR1, PR2, PR4, PR5 and PR6) of *P. reticulatum* as quantified with the *R. salina* bioassay (only three or four dilutions see section “Materials and methods”). Each graph shows the concentration of *R. salina* after 24 h incubation (as percent of control) as a function of log-transformed *P. reticulatum* concentration of three replicates cultures (white circles, black circles and grey squares). Each data point represent the mean ($n = 2$), and error bars indicate the range of the two technical replicates. Dotted line represents the sigmoidal curve fit for PR3 10° (see Fig. X) for comparison. Black lines are based on visually fitting the dotted line curve to be representing the data points.

Table 1. Resume of the lytic activity comparison of the five isolates with the isolate PR3 of *P. reticulatum*. Data of PR3 correspond to the EC₅₀ calculated before (see above); values for the other isolates are approximate values of EC₅₀ estimated from the curves in the Fig. 14. obtained by eye fitting.

Exponential Phase Lytic Activity					
PR1	PR2	PR3	PR4	PR5	PR6
470	2800	932±65	640	no lysis	no lysis
Stationary Phase Lytic Activity					
PR1	PR2	PR3	PR4	PR5	PR6
380	640	307±83	320	7900	16000

4. Discussion

Several studies have reported the presence either of *P. reticulatum* or YTXs in mussels in the Arctic Ocean (reviewed by Okolodkov. 2005, Vershinin et al. 2006, Poulin et al. 2011). However the six clonal isolates of *P. reticulatum*, as verified by detailed morphologic and phylogenetic analysis are the first isolates of this species from arctic waters. All The clonal isolates have been identified as YTXs producers. In addition, all the isolates have been shown to produce extracellular lytic compounds; which, makes this work the first report of allelochemical activity by *P. reticulatum*.

Morphologic analysis showed that size of the isolate PR3 (29.6±3.0 µm in length and 26.2±2.7 µm width) correspond to the general description (25-35 µm wide, 28-43 µm long and about 40 µm length and 35 µm width) given by Dodge (1989) and by Hansen et al. (1997), respectively, although some of the cells of PR3 were slightly smaller. We observed some variability in the position of the intercalary plate 1a. This agrees with the observations of Hansen et al. (1997), that nearly 50% of the cells analysed from a natural sample showed contact between 1a and the pore plate, indicating that this variability in the position of plate 1a and a consequential different plate pattern of either three or four apical plates (i.e. plates with contact to the pore plate) seem to be an inherent feature of the species.

Growth rate is a very significant parameter in population dynamics and phytoplankton ecology as it integrates numerous biochemical processes to yield a single resultant “output”, usually defined in terms of changes in cell number or biomass (Tillmann et al. 2009). Due to this, growth rate is considered an important measure to quantify the effect of any environmental factor. It is known that temperature is significantly influencing phytoplankton dynamics and microalgae geographical distribution (Guerrini et al. 2007). According to this and to the fact that is the first time that *P. reticulatum* isolates from the Arctic are available, a wide range of temperatures were tested to establish a base line of the physiology of this species. The cultures of *P. reticulatum* isolated from Greenland displayed a maximum growth at 15°C closely

followed by the cultures at 20°C (Fig. 7.). These results, in addition with the very low growth rate calculated from the cultures at 1°C and 5°C suggest better adaptation to temperate waters than cold waters. This concurs with the review published by Okolodkov in 2005; despite that *P. reticulatum* shows a worldwide distribution, the majority of reports about this species correspond to temperate areas. Additionally, several *P. reticulatum* blooms have been reported from temperate areas, such as, bloom in Flødevigen (Norway) in 2001 where the water temperature reminded between 13°C and 16°C (Aasen et al. 2005); Koike et al. (2006) reported a *P. reticulatum* bloom in Northern Japan where upper layer (0-5 m) temperature ranged from 14°C to 17°C. During February in 2007 a bloom of this species was detected in northern Chile with a water temperature oscillation of 17-21°C (Álvarez et al. 2011). Furthermore to these in situ evidences, few studies have tested the temperature effect on growth of *P. reticulatum* isolates from different locations, such as the Adriatic Sea (Guerrini et al. 2007), the North Sea (Röder et al. 2012) and from NW Spain (Paz et al. 2006). Growth rate comparison between studies is always a complex matter, because this parameter is affected and driven by inherent factors (genetics) and external conditions (environmental factors). Nevertheless, the results published in the three studies mentioned before support the conclusion of a temperate adaptation of *P. reticulatum*. Both, isolates from the North Sea and from the Adriatic Sea displayed a maximum growth rate at 15°C and a decrease in growth rate was measured a higher temperatures; however the adriatic isolates, which showed a decrease in growth rate above 20°C, still were able to grow at 26°C indicating a better adaptation to warmer waters than the clonal isolates used in the present work. In addition the temperature showed a positive effect on growth rate between 15-23°C for the isolate from NW Spain (Paz et al. 2006). A general optimum growth *P. reticulatum* in temperate conditions would, explains the absence of *P. reticulatum* blooms reported in warm waters, even though, the species regularly has been found in the tropics. Even though the arctic isolate PR3 shows is fastest growth at 15°C it is important to note that this isolate still is actively growing at 1°C, albeit with a low rate corresponding to a doubling time of about 10 days. Unfortunately, there are no growth rate data available for other temperate isolates of *Protoceratium* at such low temperature available, which would allow for a more detailed evaluation of the temperature adaptation of arctic isolates. In any case, with a doubling time of about 10 to 7 days for the temperature range of 1-5°C is seems unlikely that *P. reticulatum* in arctic areas will be able to form dense blooms, given the short duration of the growth season in the arctic and prevailing low water temperature. In Disco bay, Greenland, close to the stations where the Greenland strains had been isolated, summer (June-August) surface water (0-50 m) temperature ranged between -1.5 °C and 10.3 °C for the period between 1924-2010 (Hansen et al. 2012). The warmest water was recorded during late August where, temperature of the upper water may be in the range between 5 and 10°C. If we assume a maximum period of four weeks for such high summer temperatures (no exact data where reported by

Hansen et al. 2012) and assuming a mean of doubling time of 5 days, the resulting 4 generations (neglecting all natural loss processes) seem to be not enough to expect dense late summer blooms, However, these considerations largely depend on the assumed initial concentration which have to be highlighted as an important feature for bloom formation of dinoflagellates (Klais et al. 2011). Initial concentrations of dinoflagellates are in many cases strongly depended on the presence and the hatching intensity of the benthic cysts. *Protoceratium reticulatum* is known as a benthic cyst forming species (Marret and Zonneveld 2003) but nothing is known about cyst beds and cyst germination of Greenland *Protoceratium* populations.

It is known that natural populations of Dinoflagellates show a high genetic variation in terms of growth rate (Brand 1981; Costas 1990). The results of comparing growth rate of all available isolates of *P. reticulatum* from Greenland (Fig. 8.) followed the previous findings of both studies, displaying an important intraspecific variation. The growth rate for the isolates used in this study, all of which were determined under exactly the same environmental conditions, ranged from $0.06 \pm 0.00 \text{ d}^{-1}$ to $0.19 \pm 0.00 \text{ d}^{-1}$ displayed by PR5 and PR3, which in terms of doubling time is about ten days and three days respectively. Notwithstanding, it has to be kept in mind that growth rate for the strain have been estimated for nutrient replete condition and at a fixed light intensity. These conditions may vary in the field so the growth rate of *P. reticulatum* in the phytoplankton community may very well deviate from the ones reported here.

From our experiments it seems clear that temperature has no a major effect on the intracellular YTXs concentration of *P. reticulatum* (Fig. 9.). In contrast to the findings of Paz et al. (2006) and Guerrini et al. (2007), a higher YTXs concentration was not measured at warmer temperatures, but our data agree with the results published by Röder et al. (2012) for North Sea isolates of *P. reticulatum*, where likewise no increase of intracellular YTXs concentration was measured with the increase of temperature. A consistent increase in YTXs cell quota of PR3 was detected at stationary phase, which concurs with the results obtained by the three studies mentioned before; in addition, the other isolates tested showed a two to five fold increase at stationary phase in the intracellular YTXs concentration (Fig. 10.) as well. This increase may be driven by the stop of the cell division at stationary phase. Nevertheless, cells in stationary phase may partly retain metabolic activity during this phase what allows an accumulation of second metabolites in the cell. Despite that Fig. 9. does not show a major influence of temperature on the YTXs cell quota of *P. reticulatum*, there is a trend of slightly higher YTXs cell quotas at exponential phase for the lower temperatures tested. Plotting cell quota at exponential phase and the growth rate of the clonal isolate PR3 together over the experimental temperatures (Fig. 15), it becomes clear that the intracellular YTXs concentration slightly increase at lower growth rates. As absence of cell division and accumulation of second metabolites in the cell may explain the higher concentration during the stationary phase, the same principle might explain the higher concentration

at 5°C and especially at 1°C. Growth rate here is rather slow and thus a small difference between growth rate and production rate of a secondary metabolite might lead to a similar accumulation effect. The YTXs cell quota for the six isolates measured in this study ranged from 0.1 to 24.1 pg YTXs cell⁻¹, which is in the range of intracellular YTXs published before by Rhodes et al. (2006), Guerrini et al. (2007), Röder et al. (2012) and Paz et al. (2007, 2013), where two isolates from unknown origin (no YTXs detected for one of the isolate and 8.3 pg YTXs cell⁻¹ for the other one), one isolate from the Adriatic Sea (≈16-23 pg YTXs cell⁻¹), one isolate from The North Sea (≈4-12 pg YTXs cell⁻¹), four isolates from Spain (1.8-18.7 pg YTXs cell⁻¹) and three isolates from the United States (no YTXs were detected) and three isolates from NW Spain (≈6 to 16 pg YTXs cell⁻¹) were used respectively. On the light of these data it is obvious that the range of YTX cell quota displayed by different *P. reticulatum* isolates is quite wide. Additionally, some isolates or strains have been identified as non-toxicogenic, however that might depend on the method and the limit of detection used by each study; i.e. the isolate PR6 used in this work showed a YTXs cell quota between 0.1 and 0.4 which might be below or very close to the detection limits of some methods, for instance the detection limit of the method used by Rhodes et al. (2006) was 0.1 pg YTXs cell⁻¹.

Many studies have proved a high variability for various traits among different strains or isolates of microalgae species and also for dinoflagellates such as, species of *Alexandrium spp.* (Maranda et al. 1985; Orlova et al. 2007; Tillmann et al. 2009), *Prorocentrum micans*, *Gonyaulax excavata* and *Scrippsiella trochoidea* (Costas, 1990). This is important to keep in mind when discussing interspecific difference (differences between species) for any ecophysiological trait, as interspecific differences can only be evaluated when the range of intraspecific variability for a given species is known. It is thus important to include, if possible, multiple strains or isolates in research to advance understanding about toxicogenic algal species (Bukholder and Glibert 2006). The current study thus included a comparison of six clonal isolates to evaluate the intraspecific variability of *P. reticulatum*.

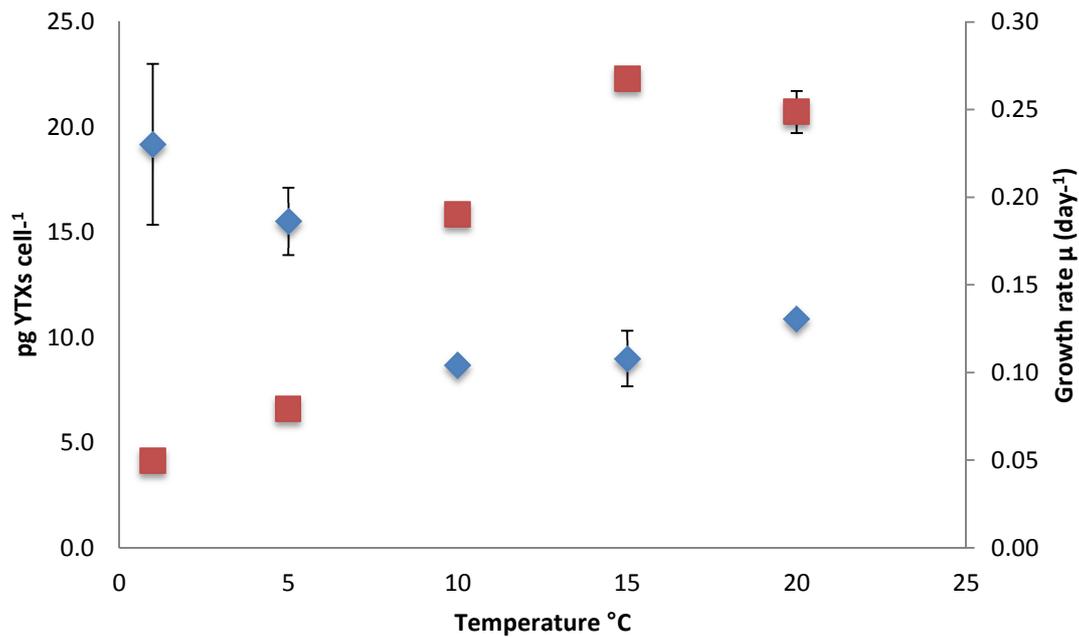


Fig. 15. YTXs cell quota of *P. reticulatum* at exponential phase versus temperature (blue diamonds) and growth rate at experimental temperatures (red squares). Data points represent mean values of three replicate cultures (\pm SD).

In addition to the presence of yessotoxins, it has been shown here for the first time that arctic *P. reticulatum* displayed lytic activity, and so it has to be discussed wither YTX, the phycotoxin produced by these dinoflagellate species, and compounds involved in lytic activity are from different nature. For related toxic dinoflagellates is clearly had been shown lytic activity was unrelated to the presence of know neurotoxic phycotoxins. This has been shown for *Karenia brevis* (Kubanek et al. 2005), where the neurotoxin Brevetoxin was shown not be responsible for negative effect on other plankton algae, for *A. tamarensis*, whose lytic activity was unrelated to the PSP toxins (Tillmann and John 2002), and for *A. ostenfeldii*, whose spirolide toxins likewise were shown not to be responsible for lysis other cells (Tillmann et al. 2007). For *Protoceratium*, our data also provide strong evidence that yessotoxins are not involved in lytic activity. It is known that the major part of the phycotoxin produced by dinoflagellates remain in the cells, as has been shown for the PSP toxins of *Alexandrium tamarensis* (Lefebvre et al. 2008). This has also been shown for *P. reticulatum* by Paz et al. (2006, 2013), Guerrini et al. (2007) and Röder et al. (2012), where the intracellular YTXs accounted for 60 to 90 % of the total YTXs measured. As clearly shown in Fig. 11., compounds involved in lytic activity of *P. reticulatum* are present in the cell-free supernatant, whereas algal extract did not cause any measurable lysis in *R. salina*, and that clearly indicate that compounds other than yessotoxin are responsible for lytic activity.

Allelochemical activity in marine phytoplankton is an emerging field, and numerous reports have published negative effect in protist interactions such as inhibition of growth or photosynthesis or even death of competitors or grazers (Cembella 2003; Legrand et al. 2003; Ianora et al. 2011). Allelochemical effects are well documented for a number of dinoflagellate species, e.g. for the genus *Karenia* (Kubanek et al. 2005), for the genus *Fragillidium* (Tillmann et al. 2008a), or for the genus *Alexandrium*, lytic activity has been reported for several species in several studies (Hattenrath and Dobler 2011); Tillmann and John 2002; Tillmann et al. 2008, 2009; Tillmann and Hansen 2009). Remaining in this work line, the present study is the first report of lytic activity of the dinoflagellate *P. reticulatum*. Additionally, it is the first time that lytic activity production by phytoplanktonic organism is tested at different temperatures. As it was the case for YTXs cell quota, the most significant differences in lytic activity were observed between exponential and stationary phase, where for almost all the temperatures a two to three fold decrease of EC_{50} (corresponding to an increase in lytic activity) was recorded. This performance was also measured at 10° for the all isolates of *P. reticulatum* (Fig. 14. and Table. 1.). A general increase in lytic activity at stationary phase may be explained by the accumulation mentioned before for the increase in YTXs cell quota at stationary phase, so that when the cell division stop the production of second metabolites, may at least partially, remain active, which result in an accumulation of lytic compounds in the medium. Since this is the first work that presented and quantified allelochemical activity of *P. reticulatum*, a comparison of our lytic activity result can only be made with data from other close related species of dinoflagellates, mostly the well-studied species of the genus *Alexandrium*. Lytic activity here had been tested with a number of different target species, including both autotrophs and heterotrophs (Tillmann and John 2002, Tillmann et al. 2008 and Tillmann and Hansen 2009). From these studies it became clear that, the sensitivity among different target may vary considerably, but the underlying reasons for that (e.g different target cell concentrations in a bioassay, different target cell size or differences in the presence/concentration of membrane targets molecules) are poorly understood. Thus, only lytic activity in terms of EC_{50} data published using *R. salina* as a target species will be used for a direct comparison of lytic activity of arctic *P. reticulatum* with literature data. EC_{50} Data published for *Alexandrium tamarense* were in the range of 100 cells ml⁻¹ (Zhu and Tillmann 2012) and thus considerably lower (higher lytic activity) compared to our data for *P. reticulatum*. These authors also reported an increase in lytic activity at stationary phase with EC_{50} values at exponential phase of 120 cells mL⁻¹ and an EC_{50} at stationary phase EC_{50} of 80 cells mL⁻¹ (Zhu and Tillmann 2012). In addition, a similar behaviour has been reported for *A. minutum* by Yang et al (2011) where values of EC_{50} at early exponential phase were about 2000 cells mL⁻¹, about in the same range we determined for the *P. reticulatum* isolates, and increase to about 200 cells mL⁻¹ at stationary phase. Moreover, EC_{50} values at stationary phase have been reported for *A. ostentfeldii* (209-252 cells mL⁻¹), *A. catenella*

(571-736 cells mL⁻¹ and *A. minutum* (472-579 cells mL⁻¹) by Tillmann et al. (2008). It is clear that *A. ostensfeldii* showed slightly higher lytic activity and that *A. cantenella* and *A. minutum* varied into the ranged presented here for *P. reticulatum* at stationary phase (Table. 1).

When comparing EC₅₀ of PR3 at exponential phase as a function of temperature (Fig. 16.) it seems clear that temperature has no major effect on the lytic activity. An exception was the cultures maintained at 1°C, where the EC₅₀ measured at exponential phase was significantly lower. The slow growth rate performed by the cultures at 1°C (0.05 day⁻¹) has an effect comparable to the stationary phase, causing a slightly higher accumulation of lytic compounds in the medium even during active growth.

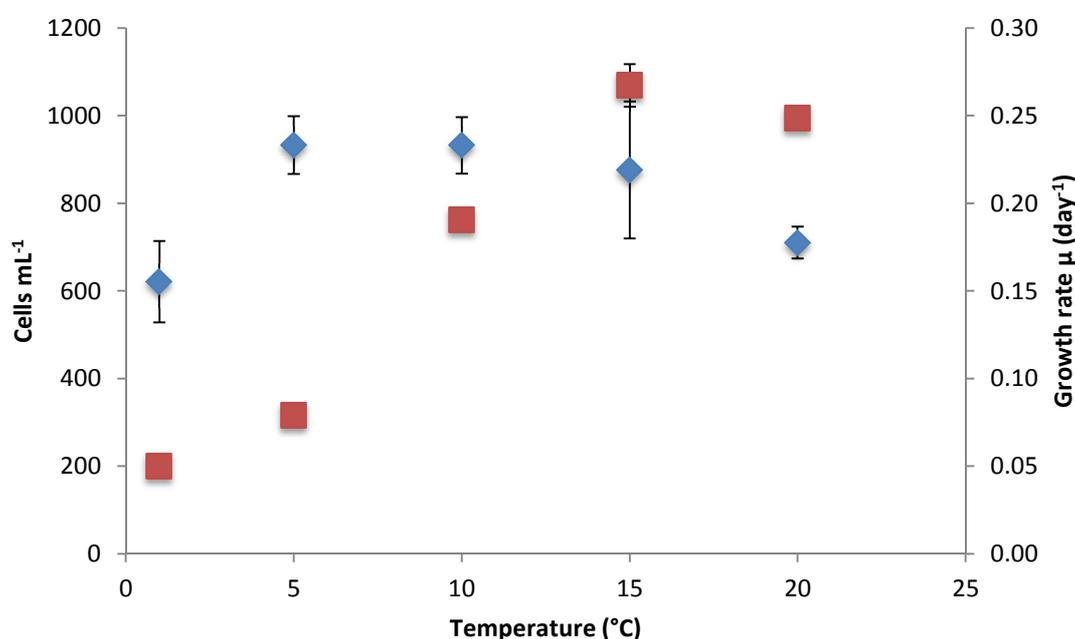


Fig. 16. EC₅₀ of *P. reticulatum* at exponential phase over temperature (blue dots) and growth rate at experimental temperatures (red dots). Data points represent mean values of three replicate cultures (±SD).

The very high variability among the isolates of *P. reticulatum* in terms of lytic potency (Table. 1.) agrees with the results published by Tillmann and John (2002) in a study between different biogeographical population of *Alexandrium* and with the results published by Tillmann et al. (2008, 2009) for *A. tamarense* isolates from the same origin.

Although the ecological role of allelochemical compounds remind under discussion, it nevertheless seem to gives a clear advantage to the pallelochemical-producing species against competitors by either reducing grazing rate or by negatively affecting growth rate (even causing complete cell lysis) of potential competitors for nutrients

acquisition. Thus, allelochemical activity could play an important role in dinoflagellate bloom dynamics and species successions in phytoplankton communities. When testing the lytic activity of different species of the genus *Alexandrium* and the related dinoflagellate *F. subglobosum* on autotrophs and heterotrophs targets (Tillmann et al. 2008) it was concluded that the lytic potency was more pronounced against ciliate and flagellate predators than against competitors such as diatoms; reducing grazing may allow dinoflagellates, which are known for their lower growth rate compared to other phytoplanktonic organisms such as diatoms, to out-compete and gain dominance over fast growing species.

A potential influence of bacteria in the lytic activity remains under discussion. In my experiments I used xenic cultures, i.e they contained accompanying bacteria. Obtaining axenic cultures of dinoflagellates is very difficult, and currently no axenic cultures of *P. reticulatum* are available and thus it is difficult to completely rule out that bacteria contribute to the production of lytic compounds. However, for lytic activity of *Alexandrium* it has been shown that a drastic reduction of bacteria by antibiotics treatment did not lead to any reduction in lytic activity, making an involvement of bacteria unlikely (Tillmann et al. 2008). Moreover, Suikkanen et al. (2005) have tested if the allelopathic effects caused by three species of cyanobacteria could have been caused by heterotrophic bacteria present in the cultures. Results published from that report showed that associated heterotrophic bacteria had no negative effect in any of the target organisms used. High variability in lytic activity was observed during the clonal isolates comparison (Fig. 14. and Table. 1.). Assuming a similar bacteria community associated with all the isolates of *P. reticulatum*, this also may be used as indirect evidence to rule out bacteria as producers of the lytic activity. However, specific attempt to get axenic *P. reticulatum* cultures are desired to unambiguously confirm that the dinoflagellate cells are the lytic compound producers.

5. Conclusion

In this work we have confirmed and established the first clonal cultures of the dinoflagellate *P. reticulatum* from arctic waters. As well we here report the first confirmation and quantification of allelochemical activity for this species. The growth response over a wide range of temperatures with high growth rates at 15° and 20°C generally suggest an adaptation to temperate waters. Nevertheless, cultures grew significantly at low temperatures (1-5° C), but the corresponding doubling times of about 10 to 7 days are rather long and, together with the short summer growth period in the Arctic, seem to not support the idea of *P. reticulatum* as an arctic bloom forming specie. Temperature has not shown a major effect on YTXs cell quota and EC₅₀ of *P. reticulatum*; on the other hand both parameters are affected by the growth phase showing an increased about two to four times from exponential to stationary phase for the all six isolates. A high variability was measured between the six clonal isolates for

the three physiological parameters tested, and thus the importance of using several different isolates in physiologic studies is highlighted.

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