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Characterization and growth response to temperature and salinity of psychrophilic, halotolerant *Chlamydomonas* sp. ARC isolated from Chukchi Sea ice

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ABSTRACT: Sea ice provides a habitat for a diverse community of microorganisms, which comprise a substantial portion of primary production in ice-covered seas. Organisms immured in sea ice have to withstand strong changes in temperature and salinity. We report on the growth rate response to salinity and temperature of the chlorophyte *Chlamydomonas* sp. ARC, isolated from land-fast sea ice in the Chukchi Sea, Alaska. We found it to be a euryhaline psychrophile capable of growth at temperatures as low as –5°C and at salinities from 2.5 to 100‰. The maximum growth rate of 0.41 d^{-1} (±0.027) was found at 5°C and a salinity of 30‰. The salinity growth range of this organism indicates that it is well adapted to the variable salinity environment associated with brine channels in sea ice, as well as the hypotonic conditions associated with melting ice. Based upon morphology and molecular phylogenetic reconstructions using the 18S ribosomal ribonucleic acid (rRNA) gene and the ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, this Arctic *Chlamydomonas* falls into a distinct clade containing other as yet unassigned psychrophilic *Chlamydomonas* strains isolated from Arctic and Antarctic environments, pointing to a bi-polar distribution of this clade. It is also very closely related to the brackish water mesophile *Chlamydomonas kuwadae* Gerloff, and is capable of growth above the psychrophilic range in low-salinity medium, indicating that it may represent an intermediate between mesophilic and psychrophilic lifestyles.

KEY WORDS: *Chlamydomonas* · *Chlorophyta* · Arctic sea ice · Chukchi Sea · Psychrophile · Euryhaline · Phylogeny

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INTRODUCTION

Sea ice covers 3 to 6% of the Earth's surface, making it one of the planet's largest single biomes, with a maximal extent of 16 million km^2 in the northern hemisphere and 19 million km^2 in the southern hemisphere (Comiso 2003). Sea ice provides a diverse habitat for a wide variety of photo- and heterotrophic organisms, which can attach themselves to or become immured in the ice substrate (Horner 1985, Lizotte 2003). In seasonally ice-covered seas, ice algae generally dominate primary production during the ice-covered season, and contribute significantly to annual primary production (reviewed in Arrigo 2003).

Sea ice is a habitat characterized by strong changes in salinity. Initially, when seawater freezes, constituent ions are concentrated into so-called brine channels and pockets, which may reach salinities of up to 225‰ (Krembs et al. 2000). These brines may also differ from the original seawater in ionic composition due to differential precipitation of salts (Assur 1958). As sea ice ages, bulk salinity tends to decrease because of brine drainage. When ice warms in the spring, the brine pockets connect and become channels, flushing even more salt out of the ice (Eicken 2003). Eventually, when the low-salinity ice melts, it creates a layer of fresher water, exposing the organisms from melting ice to hypotonic conditions, indicating that, in addition to cold

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temperatures, ice algae growing in the brine channels need to be tolerant of widely varying salinities.

In physiological studies testing the adaptation of Antarctic ice algae to low temperature and high salinity, Bartsch (1989) found that cell division was observed at temperatures as cold as –5.5°C and a salinity of 90‰. Aletsee & Jahnke (1992) reported growth of the marine diatoms *Nitzschia frigida* Grunow and *Thalassiosira antarctica* Comber at temperatures as low as –8 and –6°C and at salinities of 145 and 109‰, respectively. In general, sea-ice diatoms are often euryhaline and thrive in conditions from 10 to 60‰ (Grant & Horner 1976).

Diatoms have been the focus of most studies on sea-ice algae, because they are considered the most abundant phototrophs in sea ice (Horner 1985, Lizotte 2003). Green algae are usually a relatively minor component, but the chlorophyte *Chlamydomonas* spp. has been found repeatedly in Arctic sea ice (see 'Discussion'). *Chlamydomonas* species may play a disproportionably large role in the initial colonization of sea ice. Weissenberger (1998) found *Chlamydomonas* sp. to dominate newly formed ice for the first 3 mo in a tank experiment inoculated with an enrichment culture from sea-ice brine. Despite their ubiquity in Arctic sea ice, most research into psychrophilic green algae has focused on snow algae (e.g. Hoham et al. 2002) or Antarctic strains (Morgan et al. 1998, Liu et al. 2006).

We report for the first time on the growth response of a strain of *Chlamydomonas* sp. isolated from Arctic land-fast sea ice to variations in both salinity and temperature, encompassing nearly the entire growth range of this organism. We also report on the major pigments present in this strain. Furthermore, we use morphology and phylogenetic reconstructions based on the 18S ribosomal ribonucleic acid (rRNA) gene and the ribulose 1,5-bisphosphate carboxylase/ oxygenase large subunit (*rbcL*) gene to determine its relationship to psychrophilic chlorophytes described from other cold habitats.

MATERIALS AND METHODS

Isolation and culture conditions. *Chlamydomonas* sp. ARC was isolated from an enrichment culture, where it was growing in close affiliation with a diatom later identified as *Melosira arctica* Dickie var. *krembsii* (Kaczmarska & Jahn 2006). Cold-adapted algae were enriched from a bottom sample of a 1.5 m sediment-free Chukchi Sea ice core that was collected in June 2001, from a snow-free location offshore of Barrow, Alaska (71° 20′ N, 156° 40′ W). The bottom 10 cm was melted at 0°C in pre-filtered seawater at a volume ratio of 3:1 to avoid osmotic shock. Aliquots in 12 ml glass vials were incubated at temperatures ranging from –5 to –20°C in light starting at 50 µmol photons m^{-2} s^{-1} and decreasing to 0 µmol photons m^{-2} s^{-1} in 4 steps over the course of a week. After 1 wk in the dark, light was brought back up to 50 µmol photons m^{-2} s^{-1} in 4 steps over a week, and cultures were incubated at 4°C for 2 more weeks to allow autotrophic organisms to grow. A sample of the enrichment was sent to Arizona State University (ASU), where both *M. arctica* and *Chlamydomonas* sp. strain ARC were isolated after plating the enrichment culture on marine agar plates. Both strains are currently in culture in S. Neuer's laboratory at ASU.

Growth rate experiments. Cultures of *Chlamydomonas* sp. ARC were grown in artificial seawater medium with salinity adjusted to vary between 2.5 and 150‰, to approximate the wide range of salinities that may be encountered by sea-ice organisms. The artificial seawater medium was prepared with Instant Ocean salt mixtures (Aquarium Systems) and enriched with NO_3^- and PO_4^{3-}, to a final concentration of 500 µM nitrate and 35 µM phosphate, and supplemented with trace metals and vitamins (Neuer 1992). Media were autoclaved for 10 min at 121°C. Batch cultures were grown in 250 ml Erlenmeyer flasks, under continuous white light at a photon flux of 11 to 13 µmol photons m^{-2} s^{-1} (measured with a LICOR-250 light meter). This light level was chosen to simulate light conditions at the bottom of sea ice in spring, which, depending upon temperature, snow cover and immured sediment and biota, can be <1% of the incident light (Arrigo 2003). We determined growth rates of cultures of *Chlamydomonas* sp. for a range of salinity and temperature conditions, with salinity varying from 10 to 90‰ and temperature from –10 to 20°C. Cultures were grown at salinities outside of this range in preliminary experiments to determine the nominal salinity range. Cultures at temperatures between 0 and 20°C were maintained in triplicate in a Shel-Labs Low Temperature/B.O.D. Incubator Model 2005 (Sheldon Manufacturing). For –5 and –10°C experiments, a modified Sears Coldspot freezer compartment was used with photon flux averaging 11 µmol photons m^{-2} s^{-1}. Due to space limitations only 1 replicate per salinity level could be grown at –5°C, and the experiment was repeated 3 times. For experiments conducted at –10°C, cultures were inoculated into small glass vials containing 6 ml of 30‰ medium. At each of the 4 weekly samplings, one of the experimental vials was thawed and sampled. After 4 wk, live cells were no longer observed and the experiment was discontinued.

Growth rates were determined over the course of 2 to 4 wk using cell counts obtained by epifluorescence microscopy. Samples of 0.5 to 10 ml were vacuum filtered onto 0.22 µm polycarbonate membrane filters,

using autoclaved seawater medium to dilute samples of <3 ml to ensure even distribution of cells. The volume filtered was adjusted to give a statistically significant number of cells (30 to 300) when counting 10 to 30 fields. The filters were embedded in immersion oil, mounted on slides, and stored at –15°C until analysis. The slides were examined under blue light excitation using a Zeiss Axio A.1, with a 100× objective. Cells displaying red auto-fluorescence were counted as live cells; cells with no chlorophyll fluorescence were not counted. The natural logarithm of cell number (N) was plotted against time (t), and the instantaneous growth rate (μ, d^{-1}) was calculated for the duration of exponential growth by the formula:

$$\mu = \text{Ln}(N_t N_0^{-1})t^{-1}$$

Light and electron microscopy. Differential interference contrast (DIC) photomicrographs of wet mounts from mid-exponential phase cultures were taken with an Olympus C7070 digital camera (Olympus). Cell dimensions were measured in pixels, and converted to micrometers by calibrating pixel size with a micrometer slide.

For electron microscopy, a mid-exponential phase culture grown at 5°C and 30‰ was fixed in 3% (v/v) glutaraldehyde in 30‰ seawater (Sigma), the cells were pelleted in a centrifuge and embedded in 1% (w/v) ion-agar. Secondary fixation was 2 h with 1.5% (w/v) osmium tetroxide in seawater, and stained en bloc with 0.5% (w/v) uranyl acetate. Agar blocks were then dehydrated in acetone and infiltrated with Spurr's resin in 20% increments. Cured blocks were thin sectioned at 70 to 80 nm with a Leica Ultracut-R microtome and viewed at 80 kV using a Philips-FEI CM12S microscope.

Molecular analysis. DNA was extracted from *Chlamydomonas* sp. ARC, *C. raudensis* Ettl UWO 241, and *Dunaliella tertiolecta* Butcher CCMP 1320 using a plant DNA extraction kit (MoBio). An ~1800 bp region of the nuclear 18S rRNA gene and a ~1400 bp fragment of the *rbcL* gene were amplified using the polymerase chain reaction (PCR) with primer sets EUK A/B, SN1/2, and *rbcL* F/R (Table 1) in separate reactions. PCR products were purified using a QIAquik PCR cleanup kit (QUIAGEN Sciences). For PCR of the 18s rRNA gene, the thermocycler was programmed with a 5 min hot start at 94°C, followed by 1 min at 80°C and 30 cycles of 95, 60, and 72°C for 1 min each, with a 9 min final elongation at 72°C. For the *rbcL* gene amplification, the annealing temperature was lowered to 45°C. DNA extracts and PCR products were detected and quantified on standard agarose gels.

Amplified regions were sequenced commercially using all 6 primers. DNA sequences were aligned using ClustalW in MEGA 3.1 (Kumar et al. 2004) to generate a combined 18S rRNA sequence of 1689 bp length and a *rbcL* sequence of 1349 bp length. The 18S rRNA sequences of other cultured organisms (Table 2) were selected on the basis of having high similarity to the 18S rRNA gene of *Chlamydomonas* sp. ARC, using the basic local alignment search tool (BLAST) to search GenBank. Sequences from other organisms were included to produce phylogenetic trees with an overall topology similar to the *Chlamydomonas* phylogeny shown in Pröschold et al. (2001). The phylogenetic tree based on the *rbcL* gene sequences included the 10 organisms in the 18S phylogenetic tree that also had *rbcL* sequences available in GenBank. In addition, we included *C. kuwadae* because of its close match to *Chlamydomonas* sp. ARC.

Phylogenetic trees of 18S and *rbcL* sequences were inferred by maximum parsimony (MP) and minimum evolution (ME) methods using MEGA 3.1 (Kumar et al. 2004). MP phylogenies were inferred using the close-neighbor-interchange algorithm with search level 2 (Nei & Kumar 2000), with gaps and absent ends of short sequences treated as missing data. Initial trees were made by random addition (100 reps). ME phylogenies were inferred using the Kimura 2-parameter model with Gamma shape parameters of 0.61 and 1.67 for 18S and *rbcL*, respectively, taken from Liu et al. (2006). Gaps were deleted pairwise. Confidence of

Table 1. Oligonucleotide primers used for PCR amplification and sequencing. Sequences of fragments generated by EUK A and EUK B were aligned with sequences of fragments generated by SN1 and SN2, to give a total of 1689 usable bases

Primer	Sequence	Binding site (positions)
SN1	AATTCCAGCTCCAATAGC	*Saccharomyces cerevisiae*, 18S, 580–597
SN2	AAAGAGCTCTCAATCTG	*Saccharomyces cerevisiae*, 18S, 1252–1236
EUK A[a]	AACCTGGTTGATCCTGCCAGT	*Saccharomyces cerevisiae*, 18S, 1–21
EUK B[a]	TGATCCTTCTGCAGGTTCACCTAC	*Saccharomyces cerevisiae*, 18S, 1795–1772
rbcL (F)[b]	ATGGTTCCACAAACAGAAAC	*Chlorella ellipsoidea*, *rbcL*, 1–20
rbcL (R)[b]	TTGTCAATAGTATCAAATTC	*Chlorella ellipsoidea*, *rbcL*, 1421–1402

[a]Medlin et al. (1988)
[b]Nozaki et al. (1995)

Table 2. Strain name, accession number, and habitat information of the organisms included in the phylogenetic trees (Figs. 3 & 4). Sequences listed as unpublished may be part of manuscripts in preparation. SAG: Sammlung von Algenkulturen der Universität Göttingen; CCMP: Provasoli-Guillard National Center for Culture of Marine Phytoplankton; UTEX: Culture Collection of Algae at the University of Texas at Austin; CGC CC: *Chlamydomonas* Genetics Center Culture Collection; MBIC: Marine Biotechnology Institute Culture Collection; NIES: National Institute for Environmental studies

Strain	Accession no. 18S	Accession no. *rbcL*	Habitat, location of isolation	Source
Chlamydomonas sp. ARC	EF537906	EF537908	First year sea ice, Barrow, AK, USA	Present paper
Carteria crucifera NIES-421		D63431	Kept in freshwater, 20°C[a]	Nozaki et al. (1995)
Chlamydomonas kuwadae NIES-968		AB084334	Lake Nojiri, Japan, kept in freshwater, 20°C[a]	Nozaki et al. (2002)
Chlamydomonas monadina SAG 31.72	U57694		Yellowwood fishponds, Bloomington, IN, USA[a]	Buchheim et al. (1996)
Chlamydomonas nivalis UTEX LB1969	U57696		Snow, Cascade Mountains[a]	Buchheim et al. (1996)
Chlamydomonas pulsatilla CCCryo 038-99	AF514404		Snow, Spitsbergen, Norway, near shore[b]	Leya (2004)
Chlamydomonas pulsatilla UTEX 410/ CCAP 11/44	DQ009748	AB007322	Marine, Tvärminne, Finland, kept in freshwater medium at UTEX[a]	18S: (unpubl.); *rbcL*: (unpubl.)
Chlamydomonas raudensis CCMP 1619, UWO 241	AJ781313	DQ196177	Lake Bonney, Antarctica[b]	Pocock et al. (2004)
Chlamydomonas reginae SAG 17.89	DQ009749		Marine sand in the intertidal, Roscoff, France[a]	Unpubl.
Chlamydomonas reinhardtii 18S: CGC CC-1952 *rbcL*: CGC CC-125	AY665727	J01399	CC-1952: MN, USA[b] CC-125: MA, USA[a]	18S: (unpubl.); *rbcL*: Dron et al. (1982)
Chlamydomonas sp. Antarctic 2E9	AB001374		Not reported	Unpubl.
Chlamydomonas sp. CCCryo 002b-99	AF514398		Snow, Spitsbergen, Norway, near shore[b]	Leya (2004)
Chlamydomonas sp. CCMP 233	DQ009753		Pismo Beach, CA, USA[a]	Unpubl.
Chlamydomonas sp. ICE-W	AY731083	AY731087	Antarctic sea ice[b]	Liu et al. (2006)
Chlamydomonas sp. ICE-L	AY731082	AY731086	Antarctic sea ice[b]	Liu et al. (2006)
Chlamydomonas sp. MBIC 10592	AB058371		Seawater, Kii Peninsula, Japan[a]	Unpubl., MBIC
Chlamydomonas subcaudata SAG 12.87	AJ781310		Freshwater, Hokkaido, Japan[b]	Pocock et al. (2004)
Chlamydomonas uva-maris SAG 19.89	DQ009757		Brackish water, Norfolk, England[a]	Unpubl.
Chloromonas nivalis CU563D		AF517088	Snow, Santa Catalina Mountains, AZ, USA[b]	Hoham et al. (2002)
Chloromonas rubrifilum (*C. clathrata*) SAG 3.85	AJ410455		Pond under ice, Dannenberg, Germany[a]	Pröschold et al. (2001)
Chloromonas subdivisa SAG 67.72	AF517096	AF517080	Alpine pond[b]	Hoham et al. (2002)
Dunaliella tertiolecta CCMP 1302		DQ313205	Baja California, Mexico[a]	Unpubl.
Dunaliella tertiolecta CCMP 1320	EF537907		Unknown[a]	Present paper
Pseudoscourfieldia marina K-0017	AJ132619		Marine, Oslofjord, Norway, 15°C[a]	Unpubl.

[a]Information from culture collection holding strain
[b]Based upon reference given

branching was inferred by bootstraps for all 4 trees, using 500 pseudoreplicates.

Pigment extraction and analysis. Cells from 75 ml of a mid-exponential growth phase culture grown at 5°C, 30‰, and a photon flux of 12 µmol photons m^{-2} s^{-1} were pelleted by centrifugation, and pigments were extracted in 5 ml of ice-cold 100% acetone for 1 h. Cellular debris was removed by centrifugation, and the supernatant was filtered through a 0.22 µm polycarbonate filter, concentrated by evaporation on ice with a stream of N_2 gas, and stored at –40°C until analysis. The pigments in the extract were separated by HPLC by injecting 50 µl of the extract into a Spherisorb S10 ODS2 (10 mm by 250 mm) semi-prep analytical column filled with reverse-phase silica gel (Waters) and run on a variable solvent gradient of water, methanol, and acetone at a pressure of approximately 38 bar (Soule et al. 2007). Pigments were detected continuously using an online photodiode array detector in the 405 to 665 nm range, identified by spectra, and quantified using published extinction coefficients (Köst 1988, Rowan 1989).

RESULTS

Cell morphology

We found *Chlamydomonas* sp. ARC to be a biflagellated unicellular chlorophyte, with cells 8 to 14 µm long and 5 to 10 µm wide (Fig. 1). Average cell diameter (±SD) at 0°C and 30‰ was 8.4 µm (±1.2), increasing to 10.6 µm (±1.6) and 9.3 µm (±0.90) at 10 and 90‰, respectively. At 10°C and 10‰, cell diameter decreased to 9.5 (±1.1). Analysis of variance (ANOVA) indicated that differences in cell diameter between the 0°C/30‰ treatment and the other treatments listed above were statistically significant ($p < 0.05$). Flagella were 1 to 1.5 times the length of the cell and emerged from a papilla that is round from one angle and flat from another (Fig. 1B–D, G). It had a single, parietal, cup-shaped chloroplast that filled the posterior half of the cell and was cupped around a slightly anterior nucleus (Fig. 1A,B,E). The cells displayed 2 contractile vacuoles when grown at 10‰ (Fig. 1B) and 20‰, but, at 30‰, all that remained was the tubular spongiome complexes (Fig. 1A,D). Cells contained a round to slightly oblong pyrenoid, located in the basal portion of the chloroplast. The pyrenoid was surrounded by thick starch plates and penetrated by thylakoid membranes (Fig. 1E), occasionally giving it a very faint striped appearance in the light microscope (Fig. 1A). Iodine staining revealed visibly stained starch only around the pyrenoid. Cells also had a single oblong eyespot approximately 1 to 1.5 µm long located in the middle third of the cell between the thylakoid membranes and the cell wall (Fig. 1A,F,H). During asexual reproduction, sporangia developed with 4 zoospores (Fig. 1E) that were subsequently released. Flagella were absent during mitosis in the sporangia, but formed before the cells were released (Fig. 1D–F). The cells in the sporangia were surrounded by an extracellular matrix and sometimes clumped into aggregates of >50 cells. Sexual reproduction is unknown. Cells in nutrient-depleted cultures of up to 6 mo old did not form resting stages. Attempts to induce gamete production by 1 wk N-starvation followed by 48 h of darkness, as is known from *Chlamydomonas reinhardtii* (M. Sommerfeld, ASU, pers. comm.), were unsuccessful. The culture may have been monoclonal, accounting for the lack of gamete production.

Growth response to salinity and temperature

Chlamydomonas sp. ARC is a psychrophile with highest instantaneous growth rates of 0.41 d^{-1} (± 0.03) attained in nutrient-replete medium at 5°C and a salinity of 30‰ (Fig. 2, Table 3). The organism displayed tolerance to salinities from 10 to 75‰, at temperatures between 0 and 10°C, with growth rates >0.2 d^{-1} (>50% of growth rate maximum), indicating the euryhaline nature of the strain at these temperatures. Growth rates in 10‰ medium remained close to maximum from 0 to 20°C. Growth was still observed at 2.5 and 100‰, but not at 150‰ (data not shown). At 20°C, the salinity range was severely restricted, with growth in all 3 replicates at 10‰, in only 1 of 3 replicates at 20‰, and no growth at ≥30‰. Salinity and temperature showed clear interactive effects; high salinities decreased the maximum temperature at which the organism could grow, and low salinities (10 and 20‰) enabled faster growth at warmer temperatures.

At –5°C, growth was inconsistent, with only 1 replicate growing at each salinity between 30 and 90‰. The –5°C growth experiments froze only intermittently at 30‰. Of these 30‰ liquid samples, temperature excursions from –5 to –7°C induced ice formation, but no detrimental effects on cell morphology were detected microscopically. Motile cells were observed in the culture immediately following melting. Cultures grown at –5°C and salinities of 50, 75, and 90‰ remained liquid throughout the experiment. Cells survived –20°C with a corresponding brine salinity of 260‰ (Assur 1958) in the dark, but failed to grow at temperatures of –10°C.

18S rRNA sequence identity and phylogeny

PCR amplification using primer set EUK A/B yielded a ~1800 bp fragment. The sequence from *Chlamy-*

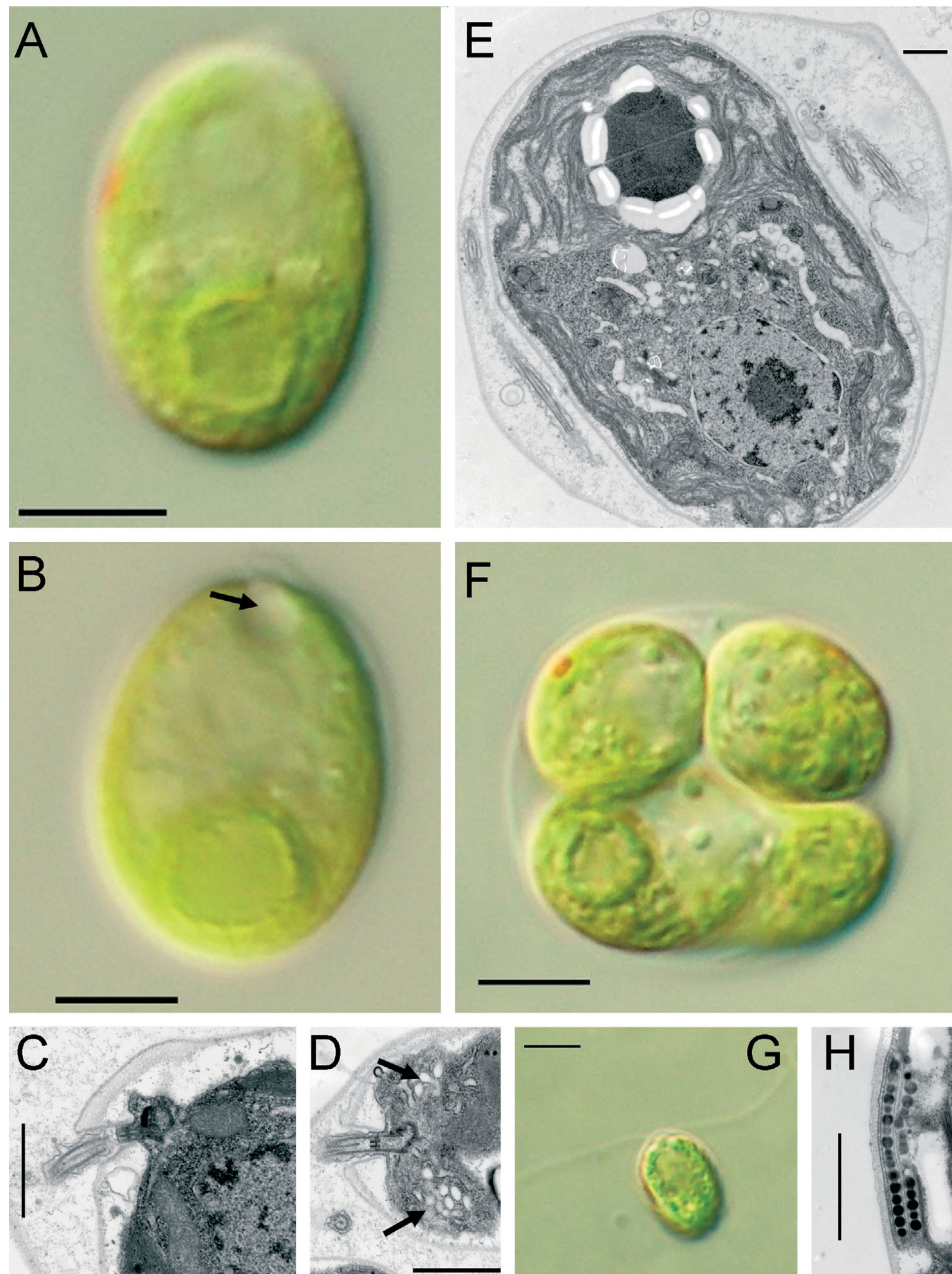

Fig. 1. *Chlamydomonas* sp. ARC. (A,B,F,G) Light microscope and (C,D,E,H) transmission electron micrographs of (A) unicell grown at 5°C and 30‰; (B) cell grown at 5°C and 10‰, showing contractile vacuole (arrow); (C) papilla and flagellar base; (D) zoospore prior to release, showing spongiome (arrows) and flagellar base; (E) zoospore prior to release, showing nucleus and thylakoid membranes penetrating the pyrenoid; (F) 4 cells after mitosis (note the common wall); (G) cell fixed in glutaraldehyde, showing 2 flagella; and (H) eyespot with double layer of pigment globules. Scale bars = 5 µm (A,B,F,G) and 1 µm (C,D,E,H)

domonas raudensis UWO 241 was used as a control for sequence accuracy and resulted in a sequence identical to the one in GenBank. The partial sequence (1689 bp) of the 18S rRNA gene allowed us to establish the phylogenetic relationship of *Chlamydomonas* sp. ARC to other strains of the genus *Chlamydomonas* (Fig. 3). We found no perfect match, but phylogenetically, the strain falls within a tight clade formed by psychrophilic isolates from both poles, with strain CCCryo 002b-99 being the closest relative with a sequence similarity of 99.7% (difference of 4 bases out of 1689). Only 1 isolate has been assigned formally to a known species, strain CCCryo 038-99, which is named in GenBank as *C. pulsatilla*. This listing is incongruent with

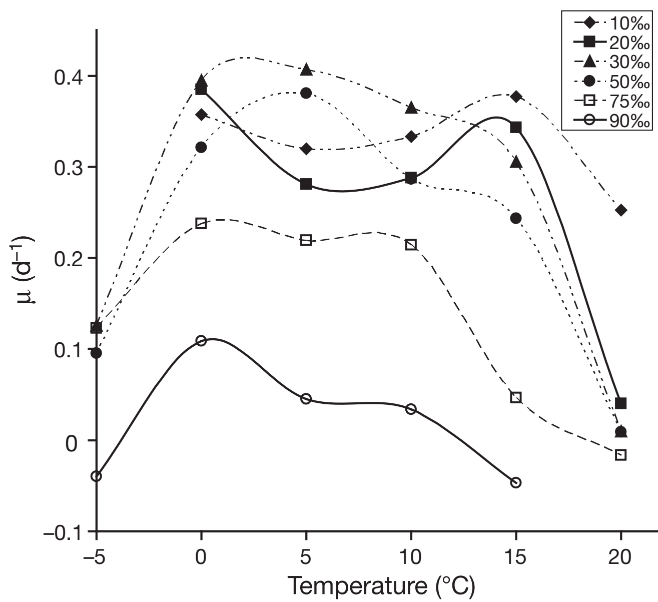

Fig. 2. *Chlamydomonas* sp. ARC. Growth rate (μ) as a function of temperature at different salinities. Results are from of a total of 11 individual experiments. Growth rates at 10 and 20‰ were obtained after the completion of the other 4 salinities

our phylogenetic analysis (Fig. 3) as *C. pulsatilla* CCCryo 038-99 has a similarity of only 96.4% to the strain *C. pulsatilla* Wollenweber UTEX 410, which has been in culture since 1950. *Chlamydomonas* sp. ARC falls into the Monadina subclade (Pröschold et al. 2001), and is part of a sister group to the lineage to which *C. monadina* Stein and *Chloromonas subdivisa* (Pascher et Jahoda) Gerloff et Ettl belong (Fig. 3). Furthermore, since *Chlamydomonas* sp. ARC is closely allied with *Chlamydomonas* sp. Antarctic 2E9 and *Chloromonas subdivisa*, it is basal to Clade A from Hoham et al. (2002), which contains a large number of psychrophilic *Chloromonas* strains.

PCR amplification of the *rbcL* gene yielded a ~1450 bp fragment, which, when sequenced and aligned, gave a 1349 bp alignment. There were no perfect matches, but *Chlamydomonas kuwadae* Gerloff NIES 968 resulted in the closest match, with a difference of 4 bp, all of which were silent mutations. Consequently, in a phylogenetic tree generated by the MP method, *Chlamydomonas* sp. ARC was most closely related to *C. kuwadae*, with *Chlamydomonas* spp. ICE-W and ICE-L forming a sister group (Fig. 4).

Pigments

HPLC of lipid soluble pigments showed the presence of chorophylls *a* and *b* (chls *a* and *b*), as well as lutein, neoxanthin, lycopene and γ-carotene among the carotenoids. Chl *b* was present at a molar ratio of 444 mmol chl *b* mol^{-1} chl *a*. Lutein was the most abundant carotenoid (669 mmol mol^{-1} chl *a*), and neoxanthin was present at 179 mmol mol^{-1} chl *a*. Lycopene was present at a ratio of 44 mmol mol^{-1} chl *a*, and γ-carotene was present at 36 mmol mol^{-1} chl *a*. β-carotene, if present, was below the detection level of approximately 25 mmol mol^{-1} chl *a*.

DISCUSSION

We have demonstrated that *Chlamydomonas* sp. strain ARC is a psychrophile, displaying euryhaline characteristics at low temperature. *Chlamydomonas* sp. ARC is well adapted to the variable salinity typical of a sea-ice habitat and Arctic estuarine and coastal environments. Similar to sea-ice diatom taxa studied by Grant & Horner (1976), our isolate is euryhaline, with a growth range from 2.5 to 100‰, and grows at >75% of its maximum growth rate at salinities of 10 to 50‰. There is an interactive effect of temperature and salinity upon growth; in cultures grown at 10, 30, and 90‰, the maximum temperatures at which we detected growth were 20, 15, and 10°C, respectively. Furthermore, low temperatures seemed to mitigate the effects of high salinity; at 90‰ and 10°C, the organism barely grew, but at 90‰ and 0°C, the growth rate was

Table 3. *Chlamydomonas* sp. ARC. Mean instantaneous growth rates (μ ± SD; n = 3), as a function of medium salinity and temperature

	10‰	20‰	30‰	50‰	75‰	90‰
–5°C			0.13[a]	0.096[a]	0.12[a]	–0.04[a]
0°C	0.36 ± 0.028	0.39 ± 0.065	0.40 ± 0.025	0.32 ± 0.024	0.24 ± 0.024	0.14 ± 0.020
5°C	0.32 ± 0.0052	0.28 ± 0.0084	0.41 ± 0.027	0.38 ± 0.020	0.22 ± 0.032	0.05 ± 0.032
10°C	0.33 ± 0.0085	0.29 ± 0.051	0.37 ± 0.080	0.29 ± 0.078	0.21 ± 0.066	0.03 ± 0.026
15°C	0.38 ± 0.012	0.34 ± 0.019	0.31 ± 0.026	0.24 ± 0.017	0.05 ± 0.043	–0.05 ± 0.047
20°C	0.25 ± 0.062	0.04 ± 0.051[b]	0.01 ± 0.025	0.01 ± 0.0019	–0.02 ± 0.0048	

[a]Values from a single experiment. An attempt to repeat the experiment resulted in no growth for all salinity treatments
[b]Of the 3 trials, growth rates ranged from 0.098 to 0.002

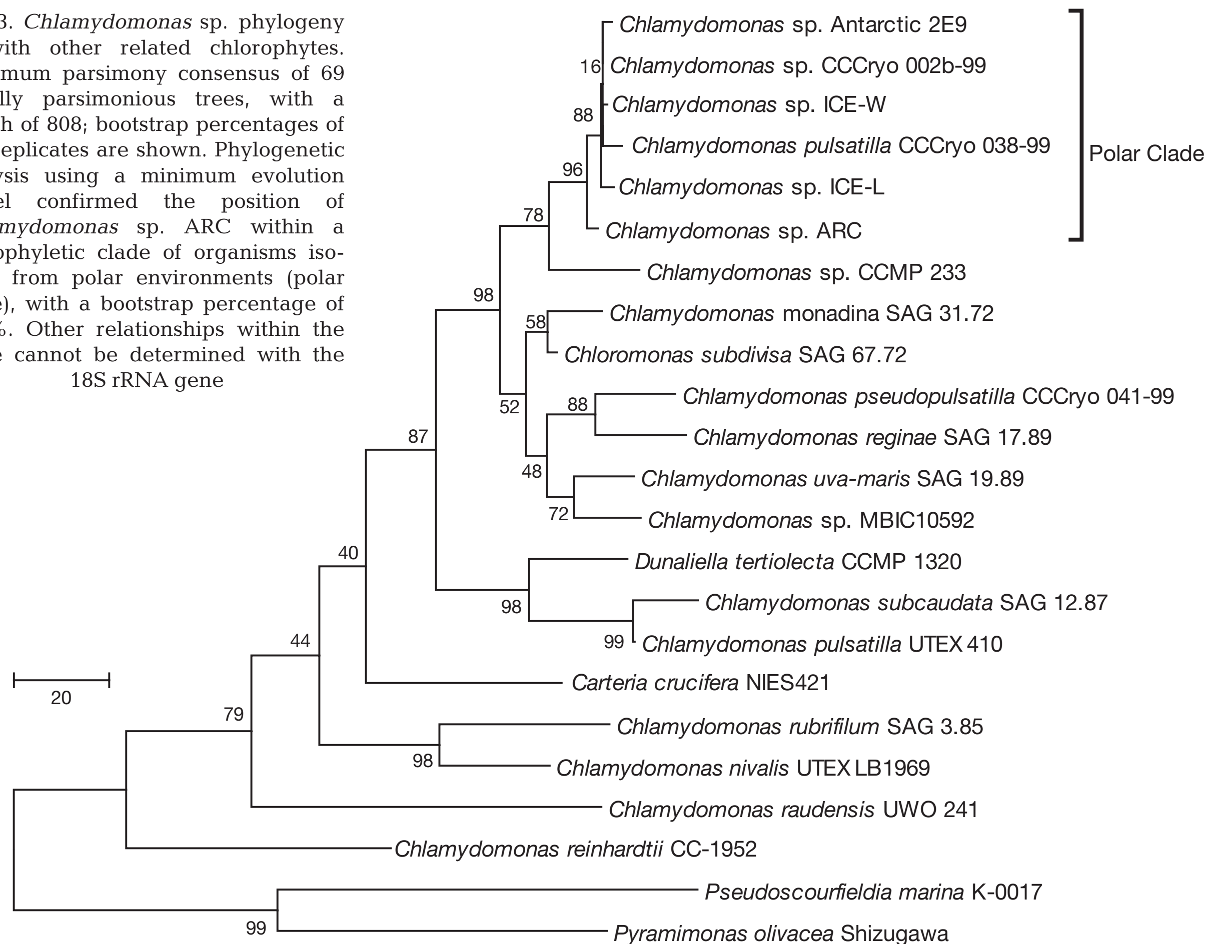

Fig. 3. *Chlamydomonas* sp. phylogeny of with other related chlorophytes. Maximum parsimony consensus of 69 equally parsimonious trees, with a length of 808; bootstrap percentages of 500 replicates are shown. Phylogenetic analysis using a minimum evolution model confirmed the position of *Chlamydomonas* sp. ARC within a monophyletic clade of organisms isolated from polar environments (polar clade), with a bootstrap percentage of 100%. Other relationships within the clade cannot be determined with the 18S rRNA gene

0.14 d^{-1} (±0.020). This agrees with findings reported by Aletsee & Jahnke (1992) that *Thalassiosira antarctica* cultured in 73‰ medium would not grow at 6°C, but grew at –4 and –6°C. The combined effect of temperature and salinity on growth rate has implications for understanding the physiological adaptations required for psychrophily in sea ice. In part due to the interactive effects of salinity and temperature, *Clamydomonas* sp. ARC blurs the line between a psychrophilic and psychrotolerant organism. By Morita's (1975) definition, a psychrophile is an organism that has an optimum growth temperature below 15°C, a minimum temperature for growth at or below 0°C, and a maximum temperature for growth below 20°C. A psychrotolerant organism, on the other hand, has a maximum growth rate above 15°C and is capable of growth at 5°C. At marine and sea-ice salinities, our strain is clearly a psychrophile, but at 10‰, it displays a growth pattern more consistent with a psychrotolerant organism.

The pigment analysis indicated an abundance of lutein and a curious absence of β-carotene and its derivatives zeaxanthin and antheraxanthin, pigments that are nearly always found in Chlorophyceae (Rowan 1989). Sea ice, particularly when under snow cover, attenuates light very quickly and lowers the need for photoprotective pigments. However, low temperature stress has been found to stimulate responses similar to high irradiance such as increased photoprotective pigments and decreased light-harvesting capability (Maxwell et al. 1994). Thus, it is interesting to find high levels of lutein, which can function as both a photoprotective pigment and a low-efficiency antenna pigment as part of light-harvesting Complex II, but no detectable zeaxanthin or antheraxanthin. These latter pigments are primarily photoprotective and increase lutein's non-photochemical quenching ability (Demmig-Adams et al. 1999). Lutein was present at higher concentrations than in the shade-adapted sub-ice alga *Chlamydomonas raudensis* UWO 241 grown at 20 µmol photons m^{-2} s^{-1} and 8°C (282 ± 5.2 mmol mol^{-1} chl *a*) (Pocock et al. 2004), but was comparable to the mesophile *C. reinhardtii* grown at 150 µmol photons m^{-2} s^{-1} and 16°C (747 ± 105 mmol mol^{-1} chl *a*) (Morgan et al. 1998). However,

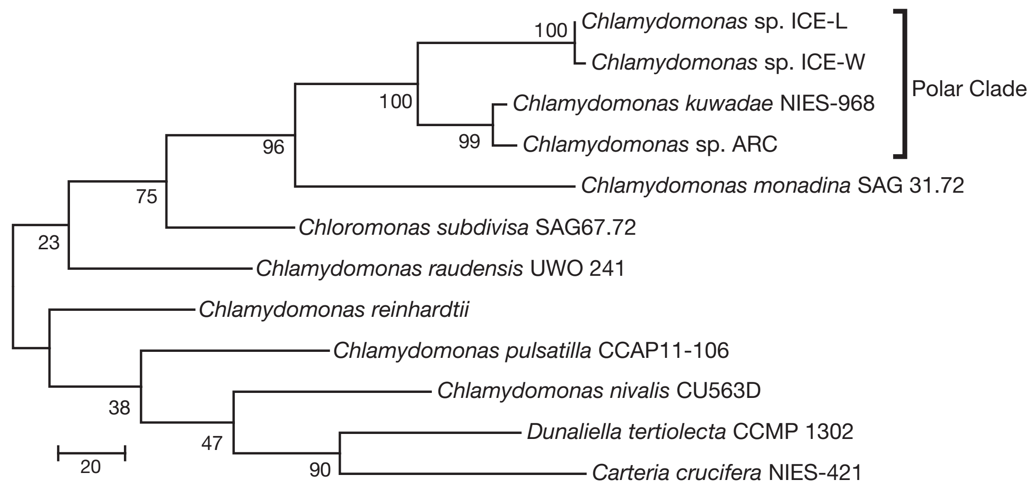

Fig. 4. Maximum parsimony consensus of the 2 most parsimonious *rbcL* phylogenies, with a length of 766. Bootstrap percentages of 500 replicates are shown, indicating strong support of the relationship between *Chlamydomonas* sp. ARC and the other members of the Monadina clade (Pröschold et al. 2001)

because we determined the pigment composition only under 1 set of conditions (5°C, 30‰, 12 µmol photons m^{-2} s^{-1}), it is difficult to ascertain the true function of these pigments in *Chlamydomonas* sp. ARC.

Chlamydomonas sp. ARC has an 18S rRNA sequence that is only slightly different from 5 other strains that form a clade of 6 closely related psychrophiles, which we have dubbed the 'polar clade', including the Antarctic marine strains *Chlamydomonas* sp. Antarctic 2E9 and *Chlamydomonas* spp. ICE-W and ICE-L, which were isolated from an ice floe near Antarctica (Liu et al. 2006). It is noteworthy that these 6 very closely related strains have been sequenced in 4 separate studies, and that 3 strains are Arctic and the other 3 are Antarctic (see Table 2), with no clear divergence between them. Also, The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (http://ccmp.bigelow.org/) lists 7 unidentified and unsequenced *Chlamydomonas* spp. isolated from Arctic sea ice. This suggests a widespread group of psychrophilic marine *Chlamydomonas* that has not yet been described in any detail.

Compared to 18S rRNA sequences, fewer strains of *Chlamydomonas* have had their *rbcL* genes sequenced. Based upon the available data, *Chlamydomonas* sp. ARC matched most closely the brackish water mesophile *C. kuwadae* Gerloff NIES-968 (Ettl 1976). The next 2 closest matches were the Antarctic strains *Chlamydomonas* ICE-W and ICE-L. This helps confirm the position of *Chlamydomonas* sp. ARC basal to the other 5 psychrophiles, and suggests that perhaps it represents an evolutionary intermediary between a mesophile and a psychrophile. However, the growth range of *C. kuwadae* Gerloff NIES-968, which is kept in freshwater medium at 20°C in the culture collection (see Table 2), has not been reported, leaving the option that this strain is capable of a psychrophilic lifestyle. In any case, psychrophily seems to be a rather transient trait among some chlorophytes (Hoham et al. 2002). There is at least one other example of a psychrophilic and mesophilic strain of the same species, namely *C. raudensis* Ettl (UWO 241) isolated from an ice-covered saline lake in Antarctica (Pocock et al. 2004, Szyszka et al. 2007). Thus, we hypothesize that this seemingly psychrophilic clade of *Chlamydomonas* simply exhibits a great amount of flexibility with respect to adaptation to a wide range of environmental conditions.

In terms of its morphology, *Chlamydomonas* sp. ARC does not match any of the reported species descriptions of *Chlamydomonas* shown in the phylogenetic trees in Figs. 3 & 4. *C. monadina* Stein has a semispherical or horseshoe-shaped pyrenoid; cells of *C. monadina* Stein are also about twice as large, and inhabit freshwaters (Ettl 1976). *C. uva-maris* Butcher has an ellipsoid, striped pyrenoid, and, while some striping is seen on the pyrenoid of *Chlamydomonas* sp. ARC, it is quite faint and difficult to see using a light microscope. In cells of *C. uva-maris* Butcher, the striping is caused by the starch granules surrounding the pyrenoid (Ettl 1976), while in *Chlamydomonas* sp. ARC, the striping seems to be mostly due to penetrations by the thylakoid membrane. *C. kuwadae* Gerloff is slightly different from *Chlamydomonas* sp. ARC morphologically, with a more horizontal and ellipsoid pyrenoid and a thinner basal portion of the chloroplast than *Chlamydomonas* sp. ARC. *C. kuwadae* Gerloff can also have 4 or 8 daughter cells, while only 4 were

observed in *Chlamydomonas* sp. ARC. The morphological description of *Chlamydomonas* sp. ICE-L matches *Chlamydomonas* sp. ARC more closely, although it is slightly larger, with a smaller papilla.

Chlamydomonas sp. ARC cells grown at 10 and 20‰ have 2 contractile vacuoles, but, at 30‰, the vacuoles break down into tubular complexes (spongiomes). Hellebust et al. (1989) showed that in *C. pulsatilla* Wollenweber, contractile vacuoles are present in freshwater, but only spongiomes are left above salinities of 10‰. Spongiomes seem to assist in the rapid formation of contractile vacuoles, which expel excess water from the cell, maintaining proper turgor pressure under hypotonic conditions. This likely makes them important for the survival of *Chlamydomonas* sp. ARC under the low-salinity conditions associated with melting sea ice. A tough cell wall enables microorganisms to better withstand hypoosmotic shock (Hellebust 1985), which is suggested to be a reason why diatoms are so abundant in sea ice (Arrigo & Thomas 2004). However, for rapidly growing organisms, the metabolic costs of operating contractile vacuoles are much lower than those of synthesizing a stronger organic cell wall (Raven 1982), which suggests a strategy for fast-growing opportunistic chlorophytes in decaying sea ice.

This research provides insight into the succession of microbial communities in areas seasonally covered by sea ice. High concentrations of *Chlamydomonas* sp. and other phototrophic flagellates (Weissenberger 1998) in newly formed sea ice suggest that *Chlamydomonas* sp. ARC could be an important early colonizer of sea ice. As the season progresses, sea-ice communities develop, with diatoms dominating the bottom of the ice and phototrophic dinoflagellates and chrysophytes occupying the upper portion of the ice (Stoecker et al. 1998). In spring and summer, a stable layer of low-salinity and low-density meltwater forms under melting sea ice and provides a transient habitat capable of supporting ephemeral blooms of green algae such as *Pyramimonas* sp. (Gradinger 1996) and potentially *Chlamydomonas* sp. ARC. *Chlamydomonas* sp. ARC was isolated from a bottom segment of an ice core, yet it could also occur in higher portions of the ice or in under-ice meltwater lenses. Our research has shown that its euryhaline and psychrophilic/psychrotolerant nature enables it to survive winter sea-ice conditions and that it can rapidly multiply in melt water and the water column in spring, suggesting a ubiquitous occurrence of this species in polar seas.

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