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# Evidence for psychrophiles outnumbering psychrotolerant marine bacteria in the springtime coastal Arctic

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*Abstract*

During a springtime study of pelagic microbial activities in the coastal Alaskan Arctic (Chukchi Sea, June 1998), a selective serial dilution technique was used to estimate the relative abundance of cold-loving (psychrophilic) and cold-tolerant (psychrotolerant or psychrotrophic) pelagic bacteria at four stations representing different stages of an algal bloom. Psychrophiles were numerically dominant, regardless of the organic nutrient concentration of their source waters (51–101 $\mu$mol $L^{-1}$ total organic carbon; 2–17 $\mu$mol $L^{-1}$ total organic nitrogen). At all stations, lower temperature incubations generated significantly higher most probable number (MPN) estimates. Further, these psychrophiles were unable to acclimate to 20°C over the course of a 3-month incubation. Our pelagic experiments are the first of their kind reported from the Western Arctic, where surface waters are dominated by the Pacific Ocean inflow, and suggest that psychrophiles can dominate even in waters only recently subjected to perennially cold conditions, independent of nutrient status or bloom condition.

Active microorganisms living in low-temperature environments are comprised primarily of two groups: psychrophilic and psychrotolerant (sometimes called psychrotrophic) organisms. According to a widely accepted definition proposed by Morita (1975), psychrophiles have optimum growth at ≤15°C, with a maximum growth temperature <20°C and a minimum growth temperature ≤0°C (see recent reviews by Russell 1990; Gounot 1991; Karl 1993 and reference therein, especially earlier reviews of Baross and Morita 1978 and Morita 1966, 1975). Despite living at these low temperatures, psychrophiles often have metabolic rates comparable with those displayed by microorganisms adapted to more moderate temperatures. Psychrotolerant organisms are typically considered able to grow near freezing but with optimum growth >20°C (Russell 1990), although some argue for a continuum of adaptation (Gerday et al. 2000).

The two main environmental factors thought to control competition between psychrophilic and psychrotolerant groups are nutrient availability and temperature (Sieburth 1967; Herbert 1986; Deming 2002). In culture, temperature and nutrients were found to determine the outcome of competition between a psychrophilic and a psychrotolerant species (Harder and Veldkamp 1971). The psychrophilic species out-competed a psychrotolerant species at −2°C irrespective of nutrient levels. The opposite response was observed at 16°C. Between 4°C and 10°C, nutrient levels determined the outcome, with a psychrophile dominating under high-nutrient conditions and a psychrotolerant species under low-nutrient levels. Psychrophiles also outgrew mesophiles when grown together at 10°C (Morita and Burton 1970). It would appear from data such as these that psychrophiles should dominate in perennially subzero waters (Harder and Veldkamp 1971). The presence of psychrotolerant microorganisms in perennially cold waters, however, implies that these organisms are able to coexist with psychrophiles. Further, most studies concerning the relative abundance of the two groups have found that psychrotolerant bacteria are the dominant group (Leduc and Ferroni 1979; McMeekin 1988; DeLille 1992). In their recent review of many years of experiments from a large suite of Arctic and Antarctic environments, Helmke and Weyland (2004) summarize that although sea ice and polar sediments are regularly found to be dominated by psychrophiles, their dominance in polar surface waters is only rarely measured, depending on hydrographic conditions and nutrient availability. In addition to polar areas, the cold water of the deep sea is another environment where psychrophiles may be expected to have a significant role.

The primary goal of our research was to identify the relative abundance of psychrophiles versus psychrotolerant microorganisms in a coastal, pelagic Arctic ecosystem. As these Arctic waters are cold year-round, psychrophiles were hypothesized to dominate the community, and the proportion of psychrotolerant organisms was expected to change according to increasing organic nutrient conditions along different stages of a phytoplankton bloom. Yager et al. (2001) describe the bloom progression in detail. Stages of a phytoplankton bloom were differentiated using a variety of

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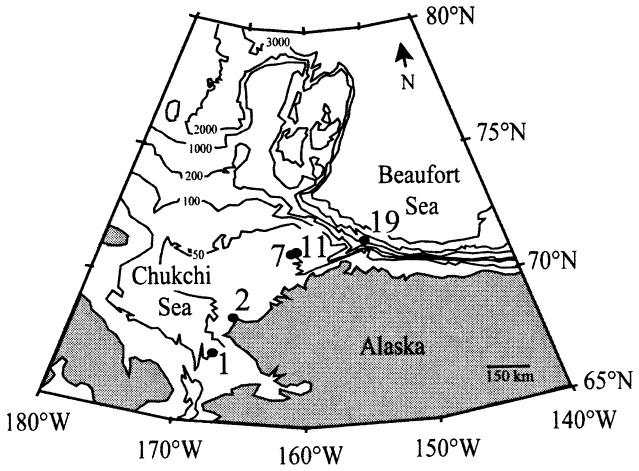


Fig. 1. Bathymetric map of the Chukchi Sea, with station numbers labeled adjacent to sampling locations. Depth contours are in meters. The time sequence of sampling points is as follows: Stations 1 and 2 at the beginning of the expedition (4 and 9 June 1998), Stations 7 and 11 within 5 d of each other midcruise (13 and 18 June 1998), and Station 19 later in the expedition (24 June 1998).

methods including nutrient concentrations, chlorophyll *a*, total organic carbon, and particulate organic carbon. Total organic carbon ranged from 51 to 101 $\mu$mol $L^{-1}$ over the course of the bloom; total organic nitrogen ranged from 2 to 17 $\mu$mol $L^{-1}$ (Yager et al. 2001). Stations were defined as prebloom, early bloom, late bloom, and postbloom. The coastal Arctic experiences dramatic changes in productivity over the late spring and early summer once the light levels increase. If in situ organic substrate levels influence competition between the two groups in this ecosystem, changes in their relative abundance should occur through the succession of these algal blooms.

## Methods

Fieldwork took place in June 1998 aboard a U.S. Coast Guard Cutter *Polar Sea* expedition to the Chukchi and Beaufort Seas off the coast of Northwest Alaska (Arctic West Section [AWS 1998], 69–73°N, 166–154°W). Seawater samples were collected with a Niskin bottle from a depth of 10 m at four stations (numbered 2, 7, 11, and 19; Fig. 1) as described previously (Yager et al. 2001). For this region, annual mean sea surface (0–10 m) temperatures have a maximum of 1.92°C (with a maximum standard error of 1.08°C), and seasonal mean temperatures (May–September) can range as high as 3.86°C (±0.85°C; NOAA-CIRES/Climate Diagnostics Center). All stations were under heavy (>80%) ice cover, and the in situ water temperature was always between −1°C and −2°C. Snow cover at our stations varied, and likewise the light regime and the extent of both sea-ice algae and phytoplankton changed across the stations (Yager et al. 2001). Subsamples were collected aseptically from the Niskin bottle into sterile 1-L glass or polypropylene bottles and kept cold in a seawater-ice bath until further processing, which began within 30 min of arrival on deck.

Selective serial dilutions were carried out to determine the presence and relative abundance of psychrophilic and psychrotolerant groups within the microbial community (Ferroni and Kaminski 1980; Gow and Mills 1984; Button et al. 1993). Theoretically, the last tube with growth in a dilution series represents the most numerically abundant organism that can grow under the specified incubation conditions. Seawater treatments consisted of three sets of quadruplicate serial dilutions inoculated into media made from seawater collected at Station 1 (Fig. 1; <100 $\mu$mol $L^{-1}$ total organic carbon; Knap et al. 1996) and gently filter-sterilized (0.22 $\mu$m) three times. Twenty-seven–milliliter aliquots of media were dispensed into sterile 50-mL centrifuge tubes using a sterile Cornwall syringe (Fisher Scientific). Again, care was taken to handle samples aseptically and keep them cold at all times. At sea, media aliquots were stored in a dark 2°C incubator until transferred to a −1°C seawater-ice bath several hours before inoculation.

An eight-step serial dilution-to-extinction scheme was designed assuming a typical pelagic bacterial abundance of $1 \times 10^9$ $L^{-1}$. The first step of each series was a 1:28 dilution made by adding 1 mL of sample to 27 mL of chilled media and then mixing by vortexer. Seven successive decadal dilutions (1:10) were made by adding 3 mL of each previous step into 27 mL of media. Between each dilution step, the pipette tip was changed and the sample tube vortexed. For each dilution step, three sets of four replicates were made. A set of four replicates was incubated at either −1°C ± 0.5°C in a seawater-ice bath, 11°C ± 0.5°C in a temperature-controlled recirculating water bath, or 20°C ± 3°C in a room-temperature water bath. Samples were transferred from −1°C to the warmer temperatures directly, without acclimatization. Seawater temperature inside the tubes equilibrated to the warmer incubators within 24 h. Uninoculated media was also incubated to check for contamination.

Growth patterns between these different temperatures help identify the thermal characteristics of the dominant microorganisms. Temperatures for selective serial dilutions were chosen as follows: −1°C was near the in situ temperature, 11°C was shown to have the highest growth rate potential within the psychrophilic range for other Arctic marine microorganisms (Yager unpubl. data), and growth observed at 20°C is due to the presence of psychrotolerant types. The difference in growth between 20°C and either −1°C or 11°C identifies the presence of psychrophiles.

After a 3-month incubation, all tubes were marked positive or negative for growth. As turbidity could not be observed visually in the oligotrophic media, growth was determined with epifluorescence microscopy by filtering 3 mL of sample and using a 0.1% acridine orange stain (Aldrich 15-855-0; Hobbie et al. 1977). The mounted slide was scanned for fluorescent cells (at least 20 fields observed); if no cells were seen, the tube was scored negative for growth. Based on the volume of sample filtered and the geometry of the microscope, the limit of detection (one cell observed in 20 fields) is about 2000 cells $mL^{-1}$. Tubes scored in this manner were either clearly positive or negative. A positively scored tube typically had more than 30 cells per field (or at least $6 \times 10^4$ cells $mL^{-1}$). Cell morphology in the most diluted step that scored positive was noted.

When a series did not dilute to extinction (when growth

Table 1. logMPN values for serial dilutions calculated using the BAM spreadsheet (Garthright and Blodgett 2003). Results are presented for different temperature incubation treatments at four stations in the Arctic, June 1998. The number of series used in the analysis (after eliminating those series with growth in the terminal tube) is in parentheses to the right of the value. Ninety-five percent confidence intervals are in parentheses under log MPN values. Bloom stage is represented in parentheses following station number (see Yager et al. 2001).

| | Station 19 (prebloom) | Station 7 (early bloom) | Station 11 (late bloom) | Station 2 (postbloom) |
|---|---|---|---|---|
| 1°C | 5.10 (4) | 5.88 (1) | 4.36 (3) | 5.10 (2) |
| | (4.66–5.53) | (5.02–6.74) | (3.75–4.97) | (4.47–5.72) |
| 11°C | 4.78 (2) | 5.05 (3) | NA (0) | 4.36 (2) |
| | (3.98–5.57) | (4.55–5.54) | | (3.62–5.10) |
| 20°C | 3.52 (4) | 3.30 (4) | 2.19 (4) | 2.74 (4) |
| | (3.15–3.89) | (2.93–3.66) | (1.74–2.65) | (2.20–3.28) |

was observed in the final dilution), it was omitted from analysis. The remaining scores were entered into the U.S. Food and Drug Administration's (FDA) Bacteriological Analytical Manual (BAM) spreadsheet for calculating most probable number (MPN; Garthright and Blodgett 2003; spreadsheet available at http://www.cfsan.fda.gov/~ebam/bam-a2.html). The BAM analysis calculates a 95% confidence limit on the logMPN. When 95% confidence intervals do not overlap between two treatments, they are determined to be statistically different at $p < 0.05$. Note that the BAM analysis is reported to have a tendency to overestimate abundance. Although this is problematic with respect to absolute values, this tendency should pose no difficulty for comparative studies such as this research.

## Results and discussion

Using this method, psychrophiles showed clear numerical dominance throughout the springtime pelagic Arctic, regardless of the changing status of the algal bloom and nutrient concentrations. When compared across the different incubation temperatures, all stations had significantly ($p < 0.05$) greater growth for psychrophiles at −1°C and 11°C than psychrotolerant bacteria at 20°C (Table 1; Fig. 2). Therefore, these psychrophiles were not able to grow at higher temperatures when transferred relatively quickly (samples warmed from sub-zero to room temperature within about 24 hours). Results further suggest that the numerically dominant organisms, once warmed to 20°C, were unable to adapt advantageously over a 3-month period. These overall results concur with a separate short-term experiment from the same expedition, which noted higher maximum uptake velocity ($V_{max}$) for amino acids at temperatures below 15°C, when compared with $V_{max}$ at 20°C at all stations (Connelly unpubl. data). Likewise, these results are consistent with previous research that found some microbial communities in polar waters to be functionally dominated by psychrophiles (Gillespie et al. 1976; Yager and Deming 1999).

Efforts to quantify relative abundance with culturing techniques can be hard to interpret due to the unknown selectivity of the isolation methods and media used (Gow and Mills

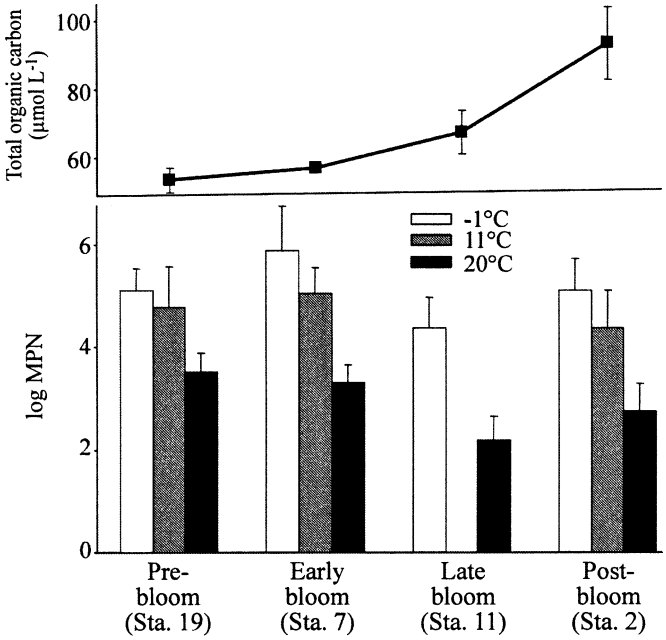


Fig. 2. Average logMPN and total organic carbon (from Yager et al. 2001) for each station through a bloom sequence. All samples were taken at a water depth of 10 m. Ninety-five percent confidence intervals are indicated by error bars in the figure. Stages of the bloom sequence are as follows: Station 19 (prebloom), Stations 7 and 11 (bloom), and Station 4 (postbloom). Because of growth in all four terminal tubes, there is no logMPN data from Station 11 at 11°C.

1984; Button et al. 1993). In most cases, when isolating bacteria from marine environments the type of media determines which bacteria are cultured (Tan et al. 1996). Dilution-to-extinction using unamended, filter-sterilized seawater is probably the most appropriate method for cultivation of numerically dominant bacteria (Ishida and Kadota 1981; Button et al. 1993; Schut et al. 1993). Use of oligotrophic media may explain why our results differ from those of similar experiments that use relatively nutrient-rich media. Filter-sterilized seawater, however, most closely represents in situ oligotrophic nutrient conditions and therefore tends to allow growth and isolation of the most abundant cells, rather than the most cultivatable (Button et al. 1993).

For nearly all cases (10 of 11), the average MPN of cultivable bacteria at a given temperature was below the direct count and in those cases gave percent cultivable values ranging from <1% to 53% (Table 2). These percentage cultivable values are relatively high in the lower temperature incubations when compared with typical temperate seawater samples (e.g., Jannasch and Jones 1959), although the method of cultivation we used tends to produce higher percentages from oligotrophic seawater cultures (Button et al. 1993). The percentages are generally lower, however, than results reported for Arctic sea ice (Junge et al. 2002), but are similar to results reported for a cold seawater environment in the Antarctic (Helmke and Weyland 1995). The one group where the MPN exceeded the direct count was at Station 7 at −1°C. In this case, three out of four series were omitted from analysis because growth was observed in their terminal tube, so

Table 2. Direct counts (Yager et al. 2001) and percent cultivable bacteria for different temperature incubation treatments at four stations in the Arctic, June 1998. The percent cultivable bacteria was determined by dividing MPN values by the direct count. Ninety-five percent confidence intervals for percent cultivable are based on propagation of errors from both MPN and direct counts. Values in bold note where average MPN value is below the direct count measured. Bloom stage is represented in parentheses following station location.

| | Station 19 (prebloom) | Station 7 (early bloom) | Station 11 (late bloom) | Station 2 (postbloom) |
|---|---|---|---|---|
| Direct counts ($10^8$ $L^{-1}$) | 4.1 | 2.1 | 2.8 | 9.4 |
| % Cultivable | | | | |
| −1°C | **30**% (4) | 360% (1) | **8%** (3) | **13%** (2) |
| | (11–84) | (50–2600%) | (2–33%) | (3–55%) |
| 11°C | **14**% (2) | **53%** (3) | NA (0) | **2%** (2) |
| | (2–90) | (17–170%) | | (<1–13%) |
| 20°C | <**1**% (4) | <**1%** (4) | <**1%** (4) | <**1%** (4) |
| | (<1–2%) | (<1–2%) | (<1–16%) | (<1–<1%) |

the MPN was calculated from the growth pattern observed in only one series. Still, results from 11°C (based on triplicate series) support the conclusion of numerical dominance of psychrophiles at Station 7. Since the BAM spreadsheet is known to statistically overestimate MPN values, however, little can be made of the percentage cultivable results except that they seem reasonable and reflect well on the method overall.

One important question resulting from this research was why bacteria samples did not dilute to extinction in some instances and whether these results influence our conclusions. According to in situ bacterial abundance counts made on subsamples taken from the same Niskin bottles (ranging from 0.20 to 0.94 × $10^9$ $L^{-1}$; Yager et al. 2001), the eight-step serial dilution should have been adequate to dilute all samples to extinction. In some cases (15 out of 48), however, the community did not dilute to extinction and growth was observed in the terminal tube. These series, all from either −1°C and 11°C, were omitted from further analysis (Table 1). In one case, (Station 11, 11°C), all four series showed growth in the terminal tube so we have no MPN value for that treatment. In addition, microscopic examination of these terminal tubes did not always reveal a single morphology. Possible explanations for these results may be (1) internal contamination from incomplete sterilization of media; (2) external contamination during sample handling; or (3) sample heterogeneity, primarily due to high concentrations of organic particles. A low number of very small bacteria could have made it through the filter-sterilization process and then grown up over the three months. In that case, however, one would expect the uninoculated controls to be positive for growth, which was not found. We would also then expect all of the tubes to show contamination, which did not occur. More than two thirds of the series showed no growth in the terminal tube. Further, only incubations at −1°C and 11°C showed the non–dilution-to-extinction pattern, whereas samples incubated at 20°C did not. External contamination during sample processing would likely favor nonpsychrophilic bacteria with more or equal growth at 20°C than either −1°C and 11°C, which did not occur. Sample heterogeneity would have had to occur despite vigorous mixing between dilution steps.

It appears most likely that the presence of particles, as discussed below, influenced the dilution experiment. Particles could have influenced dilution results by (1) affecting the homogeneity of the dilutions and (2) serving as important sources of organic carbon for bacteria. Although we do not have particle counts for these samples (only estimates of particulate organic carbon, which ranged from 15 to 25 $\mu$mol $L^{-1}$; Yager et al. 2001), the total number of particles was almost certainly less than the total number of bacteria and should not have caused a problem at the high dilutions. The act of vortexing each tube, however, likely sheared any larger particles and broke them into smaller and smaller pieces with each dilution step. To be conservative, dilution series where growth was observed in the final dilution were omitted from our analysis. Although this increased our standard error and eliminated one treatment from analysis, psychrophiles still showed clear dominance at all four stations.

We found that the numerical dominance of psychrophiles contrasts with results from similar environments that show psychrotolerant organisms dominating the water column (McMeekin 1988; Russell 1990; Karl 1993), even in places where the overlying sea ice is dominated by psychrophiles (Helmke and Weyland 1995). One explanation put forth for the greater abundance of psychrotolerant bacteria in the Arctic coastal waters is the temperature history of the different water masses investigated. Karl (1993) suggested that greater seasonal variation in seawater temperature might place different selection pressures on the microorganisms in the North Atlantic as opposed to the Southern Ocean. Helmke and Weyland (2004) report that they found psychrophiles dominant in the polar surface waters exiting the Arctic Ocean through Fram Strait; these waters have a polar residence time of 10–25 years (Becker and Bjork 1996). The very recent source water for the Chukchi Shelf, where we sampled, is primarily Bering Sea water (Weingartner et al. 1998), which experiences seasonal mean temperatures (May–September; 0–50 m) as high as 9.85°C (±3.0°C; NOAA-CIRES/Climate Diagnostics Center). Our pelagic experiments suggest, therefore, that psychrophiles can dominate even in waters only recently subjected to perennially cold conditions (Chukchi Sea summer surface waters, ≤5°C; NOAA-CIRES/Climate Diagnostics Center). Some input from the East Siberian Shelf does occur via the Siberian Coastal Current (Weingartner et al. 1999), and these waters, along with potential riverine inputs to the area, may have been perennially colder for a longer time. Nevertheless, our

data do not fit with the idea that psychrophiles only dominate in surface water that has been cold for a long time.

Rather than thinking about the temperature history of free-living bacteria in the surface water, perhaps a different source (or seed population) of the bacteria should be considered. A sea ice core collected from the same region (von Quillfeldt et al. 2003) exhibited a highly diverse algal community that included Arctic and non-Arctic pelagic species, benthic species, and aquatic species; presumably, the bacteria in the ice are from equally diverse sources. Recent research in the Arctic has shown sea ice to be dominated by psychrophilic bacteria (Junge et al. 2002) with as much as 94% of the bacterial activity associated with particles in the ice. Attached bacteria can directly utilize particulate organic matter (POM) as an energy source with hydrolytic enzymes (Smith et al. 1992). When the ice melts, the particles and their attached cells would presumably seed the water column. Thus, our finding psychrophiles in the water column may illustrate a tight linkage between sea ice and pelagic communities. Brinkmeyer et al. (2003) found bacterial communities in another perennially cold environment to be affected by temperature and having strong associations with surfaces such as microalgae and particles. Diver and remotely operated vehicle (ROV) observations of the particles in the water column during AWS98 strongly suggest that most were ice algal in origin (*Melosira arctica*; Ambrose et al. 2001). The pelagic samples at Station 7 were likely affected further by the large amount of ''dirty ice'' (sediment-rich sea ice likely formed in the shallow waters of Kotzebue Sound, in contact with the benthos, and exported offshore by winds; W. B. Tucker pers. comm.) observed during sampling. Although particle-associated bacteria in this region account for only about 20% of the total abundance, a large fraction (65–100%) of the active cells is associated with particles (Yager et al. 2001). The importance of particle-associated microbial activity in this region may explain why our results differ from other regions.

Although there are times in the polar spring and summer when nutrients become readily available for microorganisms, the most common condition in these permanently cold environments is probably low concentrations of organic nutrients. As with psychrophiles, there are indications that for oligotrophs, survival strategies and definitions of optimality may be fundamentally different from those we consider standard (see reviews by Morgan and Dow 1986; Fry 1990; Morita 1997). These two types of ''extreme'' environments and the constraints on organisms associated with them probably come together in polar areas, much like they do in the deep sea. The common link, as described by temperature and nutrient availability, between the polar oceans and the deep sea may be reflected in the thermal tolerance and feeding strategies of the organisms living in these environments. The numerical dominance of psychrophiles despite low organic nutrients in the surface waters in this study (Fig. 2) indicates that the prevalence of psychrophiles in the deep sea may not be limited to local pockets with high nutrients as previously suggested (Helmke and Weyland 2004). Because most of the ocean is cold, psychrophiles may play a significant, yet currently unrealized, role in the cycling of organic matter in much of the world's oceans. Our results suggest that we need to further explore the occurrence of psychrophily in polar waters as well as in deeper waters below the thermocline.

Results from the selective-temperature dilution to extinction experiments support the hypothesis that psychrophiles were numerically dominant in this western region of the coastal pelagic Arctic, regardless of algal bloom stage and ambient organic nutrient levels. These results contrast with the currently accepted idea that psychrophiles are able to grow with high concentrations of organic substrate present, but are not likely to be numerically dominant in pelagic environments like the Arctic Ocean. In this case study, competition between psychrophilic and psychrotolerant groups favored psychrophilic bacteria capable of existing in the low organic ($<100$ $\mu$mol $L^{-1}$ total organic carbon) waters throughout a bloom progression. Our pelagic experiments are the first of their type reported from the Western Arctic, where surface waters are dominated by the Pacific Ocean inflow, and suggest that psychrophiles can dominate even in waters only recently subjected to perennially cold conditions. These results suggest that psychrophilic bacteria may have an important, largely overlooked role in the cycling of organic matter for export to depth during the Arctic growing season.

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