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USE OF EXOGENOUS GLYCINE BETAINE AND ITS PRECURSOR CHOLINE AS OSMOPROTECTANTS IN ANTARCTIC SEA-ICE DIATOMS[1]

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Wide salinity ranges experienced during the seasonal freeze and melt of sea ice likely constrain many biological processes. Microorganisms generally protect against fluctuating salinities through the uptake, production, and release of compatible solutes. Little is known, however, about the use or fate of glycine betaine (GBT hereafter), one of the most common compatible solutes, in sea-ice diatoms confronted with shifts in salinity. We quantified intracellular concentrations and used [^{14}C]-labeled compounds to track the uptake and fate of the nitrogen-containing osmolyte GBT and its precursor choline in three Antarctic sea-ice diatoms *Nitzschia lecointei*, *Navicula* cf. *perminuta*, and *Fragilariopsis cylindrus* at −1°C. Experiments show that these diatoms have effective transporters for GBT, but take up lesser amounts of choline. Neither compound was respired. Uptake of GBT protected cells against hyperosmotic shock and corresponded with reduced production of extracellular polysaccharides in *N. lecointei* cells, which released 85% of the retained GBT following hypoosmotic shock. The ability of sea-ice diatoms to rapidly scavenge and release compatible solutes is likely an important strategy for survival during steep fluctuations in salinity. The release and recycling of compatible solutes may play an important role in algal–bacterial interactions and nitrogen cycling within the semi-enclosed brines of sea ice.

Key index words: **Antarctica; choline; compatible solutes; diatoms; glycine betaine; osmolytes; salinity; sea ice**

***Abbreviations*: DMS, dimethyl sulfide; DMSP, dimethylsulfoniopropionate; EPS, extracellular polysaccharide substances; ESAW, enriched seawater, artificial water; GBT, glycine betaine; K_m, half saturation constant; LSC, liquid scintillation counter; pEPS, particulate EPS; V_{max}, maximal uptake rate; μ, specific growth rate**

Sea ice is an important polar ecosystem, providing an early spring food source for higher trophic levels and seeding pelagic phytoplankton blooms under favorable conditions (Horner and Schrader 1982, Lizotte 2001, Riaux-Gobin et al. 2011, Galindo et al. 2014). The algae and bacteria that inhabit sea ice also play important roles in the biogeochemistry of polar regions (e.g., DMS production and CO_2 cycling; Stefels et al. 2007, Fransson et al. 2013, Vancoppenolle et al. 2013). In the ice-covered waters of the Southern Ocean, for example, sea-ice algae may contribute up to 25% of the annual primary production (Arrigo and Thomas 2004), providing a critical food source for juvenile and sub-adult krill (Kottmeier and Sullivan 1987, O'Brien 1987). Within the brine networks of sea ice, 20–30% of this algal primary production is cycled through heterotrophic bacteria, creating complex microbial interactions within the ice (Staley and Gosink 1999, Stewart and Fritsen 2004, Deming and Collins 2017). The major primary producers in this microbial ecosystem are sea-ice diatoms (Arrigo and Thomas 2004). These extremophile microalgae are capable of thriving within brine networks of sea ice, reaching chl *a* concentrations in excess of 600 mg · m^{-3} in bottom ice blooms (Palmisano and Sullivan 1983), despite the variations in physicochemical properties that characterize this environment. Temperatures in Antarctic sea ice, for example, range from 0°C (in melting ice) to −15°C in winter ice (Helmke and Weyland 1995), with corresponding shifts in brine salinity from below seawater salinity to 175 within the semi-enclosed porous space of the ice (Cox and Weeks 1983). The fluctuations in temperature and brine salinity within Arctic sea ice can be more extreme (Ewert and Deming 2014).

The seasonal progression of sea ice exposes microorganisms associated with the ice to fluctuations and extremes in temperature, salinity, light, and nutrients (Thomas and Dieckmann 2002, Ewert and Deming 2014). Recent work with field samples indicates that, among the properties of sea ice, brine salinity may be the main driver of the diversity and community structure of bacteria in both Antarctic pack ice (Torstensson et al. 2015a) and Arctic fjord ice (Maccario 2015, Firth et al. 2016), favoring those organisms with strong osmoregulation strategies. Sea-ice algae generally grow and are photosynthetically active across a wide range of salinities, from 5 to 70 (Grant and Horner 1976, Lyon et al. 2016, Torstensson et al. 2018), with the implicat-

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ion that they, too, engage in active osmoregulation favoring their survival in sea-ice brine (Krell et al. 2007, Lyon et al. 2016). Plants and microorganisms in general accumulate a wide range of organic compatible solutes that can serve numerous functions in the cell, but are mainly known to act as osmolytes maintaining positive turgor pressure and stabilizing protein structure in water-stressed environments (Yancey et al. 1982). Polar marine diatoms produce some major sulfur- and nitrogen-containing compatible solutes that include, respectively, DMSP and isethionic acid (Lyon et al. 2011, Boroujerdi et al. 2012) and proline and GBT (Krell et al. 2007, Boroujerdi et al. 2012). Many studies have examined the role of DMSP in algal assemblages, including in sea ice where the algal use of DMSP as an osmo- and cryoprotectant is well known (Kirst et al. 1991, Lyon et al. 2016). High ratios of DMSP to chl *a* are often associated with communities dominated by *Phaeocystis* spp. (Kirst et al. 1991), but sea-ice diatoms can also be important producers of DMSP under certain environmental conditions (Levasseur et al. 1994, DiTullio et al. 1998, Trevena et al. 2000). The quaternary ammonium compound GBT, a small zwitterionic molecule, is one of the most common compatible solutes on the planet, synthesized by microorganisms from most taxonomic groups (Welsh 2000). These compounds may also be released into the sea-ice brine as organisms adjust to changes in their environment (Firth et al. 2016), which makes them readily available for scavenging by other organisms. However, the synthesis, regulation, and ultimate fate of GBT in marine eukaryotic microorganisms are still poorly understood; the transport of exogenous GBT by sea-ice diatoms and the possible consequences to sea-ice microbial ecology have not been examined, to our knowledge.

In planktonic microorganisms, compatible solutes can accumulate to relatively high cellular concentrations; for example, concentrations of GBT can reach well above 0.1 M in mesophilic phytoplankton species (Dickson and Kirst 1987, Spielmeyer and Pohnert 2012). Both marine and estuarine bacterioplankton and phytoplankton are known to scavenge significant amounts of exogenous choline and GBT as compatible solutes from their temperate surface-water environments (Kiene 1998, Kiene and Williams 1998, Vila-Costa et al. 2006), a strategy also recently documented for choline in sea-ice bacteria exposed to salinity shifts at low temperature (Firth et al. 2016). Although reports of compatible solute uptake rates in diatoms are scarce, DMSP uptake rates have been estimated at 0.1 fmol $\cdot$ cell^{-1} $\cdot$ h^{-1} in the estuarine benthic diatom *Cylindrotheca closterium* during a salinity upshift from 11 to 33 at 25°C (when the medium was supplemented with 50 μM exogenous DMSP; Van Bergeijk et al. 2003). Whether sea-ice diatoms also take up or release choline or GBT in response to the salinity fluctuations of their much colder, semi-enclosed environments are unexplored. Often given the high biomass of diatoms in sea ice, their use of compatible solutes and release of intracellular pools during a salinity downshift (sea-ice melting) could have significant consequences for sea-ice algal physiology, microbial ecology, and nitrogen biogeochemistry. Some bacteria are well known to tolerate osmotic shock by uptake and/or synthesis of GBT (Boch et al. 1994, Nyyssölä et al. 2000, Firth et al. 2016), while recent work in sea ice suggests that GBT is metabolized as a nitrogen-containing substrate or else released when salinity shifts to more favorable levels (Collins and Deming 2013, Firth et al. 2016). Released compatible solutes may be an important source of osmolytes for other sea-ice microorganisms, as well as play a role in nitrogen cycling in sea ice. Hence, the ability to synthesize, transport, and metabolize compatible solutes could provide an important competitive advantage in sea ice, helping to explain the diversity and functions of the microorganisms that dominate in this habitat.

The uptake, production, catabolism, and release of compatible solutes by dense communities of sea-ice diatoms could have significant implications for nitrogen biogeochemistry in sea ice, particularly as the extent, thickness, and physicochemical properties of sea ice are changing. The overall goal of this study was to examine the hypothesis that sea-ice diatoms use exogenous GBT and its precursor choline to survive the wide range of salinities (17–55 in the present study) that diatoms can experience in Antarctic sea ice (Torstensson et al. 2018). We investigated the uptake rates and metabolism of exogenous GBT and choline in relation to endogenous concentrations, in the three Antarctic sea-ice diatoms *Nitzschia lecointei*, *Navicula* cf. *perminuta,* and *Fragilariopsis cylindrus*. Additional experiments with *N. lecointei* were performed to quantify release of choline and GBT and production of EPS, and to test how darkness may affect the metabolism of compatible solutes. The results may help us to understand sea-ice algal acclimation to changes in salinity, interactions between algae and bacteria, and biogeochemical cycling in this climate-sensitive environment.

MATERIALS AND METHODS

Culture conditions. Axenic cultures of the sea-ice diatoms *Nitzschia lecointei* and *Navicula* cf. *perminuta*, previously isolated from Antarctic bottom sea ice in 2011 (Torstensson et al. 2013, Aguirre et al. 2018), and *Fragilariopsis cylindrus* strain CCMP1102, purchased from NCMA Bigelow and known to flourish in bottom sea ice (Mock et al. 2017), were established according to Appendix S1 in the Supporting Information. Unless otherwise specified, all experiments and incubations were performed at −1°C and under a photosynthetic photon fluence rate of 45 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} over the waveband 400–700 nm (16:8 light:dark cycle), provided by 19W blue spectrum-enriched light emitting diode lights (Feit Electric, Pico Riviera, CA, USA).

Uptake kinetics. Substrate saturation curves were performed for [^{14}C]choline and [^{14}C]GBT in duplicate to determine uptake kinetics and to select the optimal concentration of substrate for use in uptake experiments. Diatom cells were centrifuged (1,000 *g* for 10 min) and washed three times in sterile F/2 medium (Guillard 1975) prepared from artificial seawater (salinity 31) following the salt composition of ESAW (Harrison et al. 1980). Cells were re-suspended and inoculated (~10^4 cells · mL^{-1} for *Nitzschia lecointei* and *Navicula* cf. *perminuta*, ~10^5 cells · mL^{-1} for *Fragilariopsis cylindrus*) into 10 mL sterile, pre-chilled F/2 medium (salinity 31), supplemented with either [methyl-^{14}C]choline (52.0 mCi · mmol^{-1}; Perkin Elmer, Boston, MA, USA) or [methyl-^{14}C]GBT (55.0 mCi · mmol^{-1}; American Radiolabeled Chemicals, St. Louis, MO, USA) in the range of 25–400 nM. A higher initial cell concentration of *F. cylindrus* was chosen to compensate for reduced biomass given its smaller size compared to *N. lecointei* and *N.* cf. *perminuta*. All incubations were performed for ~1 h in acid-washed and sterile 50 mL glass serum bottles. One sample of each substrate concentration was fixed in 2% formaldehyde (final concentration) prior to the incubation to serve as killed control. Incubations were terminated by filtering the cells onto 0.2 µm Supor filters (Pall Corporation, Ann Arbor, MI, USA). The filters were rinsed three times with filtered, isohaline NaCl solution, and placed in 5 mL Ecolume scintillation cocktail (MP Biochemicals, Santa Ana, CA, USA). The amount of [^{14}C] uptake by the cells was determined by the [^{14}C] activity on the filters measured using a Packard LSC (Packard Instrument, La Grange, IL, USA). Uptake kinetic data were fitted using the Michaelis–Menten model, eq. (1):

$$Y = V_{\max}\left(\frac{X}{K_m + X}\right) \quad (1)$$

where Y is the uptake rate (fmol · cell^{-1} h^{-1}), $V_{\max}$ the maximal uptake rate (fmol · cell^{-1} · h^{-1}), X the substrate concentration (nM) and K_m the half saturation constant (nM).

Time-course experiments. The fate of [^{14}C]choline and [^{14}C]GBT at three different salinities was measured in the three diatoms during 6 d incubations following Firth et al. (2016). Transported [^{14}C]choline and [^{14}C]GBT could be either retained intracellularly as compatible solutes, respired to inorganic carbon or incorporated into macromolecules. Diatom cultures were washed three times in sterile F/2 medium via centrifugation (1,000*g* for 10 min) and inoculated (~10^4 cells · mL^{-1} for *Nitzschia lecointei* and *Navicula* cf. *perminuta*, ~10^5 cells · mL^{-1} for *Fragilariopsis cylindrus*) in acid-washed and sterile 50 mL glass serum bottles, containing 10 mL sterile F/2 medium of salinity 17, 31, and 55, and supplemented with 100 nM [^{14}C]choline or 200 nM [^{14}C]GBT (final concentrations). A salinity of 31 was used in the control (unchanged) treatment, whereas salinities of 17 and 55 simulate shifts that microalgae experience in summer Antarctic pack ice (Torstensson et al. 2018) and that fall within the species' growth ranges. Choline and GBT concentrations were chosen based on uptake kinetics data (Fig. 1). For the *N.* cf. *perminuta* and *F. cylindrus* experiments, the [^{14}C]GBT was diluted to 25% with unlabeled GBT. The three salt concentrations were prepared using the same salt ratios as in ESAW, with added F/2 nutrients. Diatoms were incubated with the substrates for 1–140 h, thus providing data inclusive of the initial shock to the cells and their subsequent acclimation over time. Independent triplicate incubation bottles were used for each time point and discarded thereafter (no pseudoreplication over time).

After terminating the incubation by filtration onto 0.2 µm Supor filters (as described above), total uptake was measured on the filters after they were rinsed three times with filtered, isohaline NaCl solution, and placed in 5 mL of Ecolume scintillation cocktail. Respiration of [^{14}C]choline and [^{14}C]GBT to [^{14}C]inorganic carbon was measured in separate serum bottles by acidifying the sample with 0.4 mL 2 M H_2SO_4, and capturing the de-gassed CO_2 with a wick soaked in phenylethylamine (0.2 mL) in a sealed bottle (after >4 h of shaking at ~175 rpm at room temperature). To separate the incorporation into macromolecules from intracellular pools (Baross et al. 1975), that is, from [^{14}C]choline and [^{14}C]GBT retention, the acidified samples were filtered onto 0.2 µm Supor filters and rinsed three times with filtered, isohaline NaCl solution. Sample acidification lysed cells leaving larger molecules (macromolecules) captured on the filter and smaller molecules, including compatible solutes, in the filtrate. The wicks and filters were placed in 5 mL Ecolume scintillation cocktail; all samples were counted using a Packard LSC. Retention was calculated by subtracting the [^{14}C] activity of the acidified sample (macromolecules) and respiration from

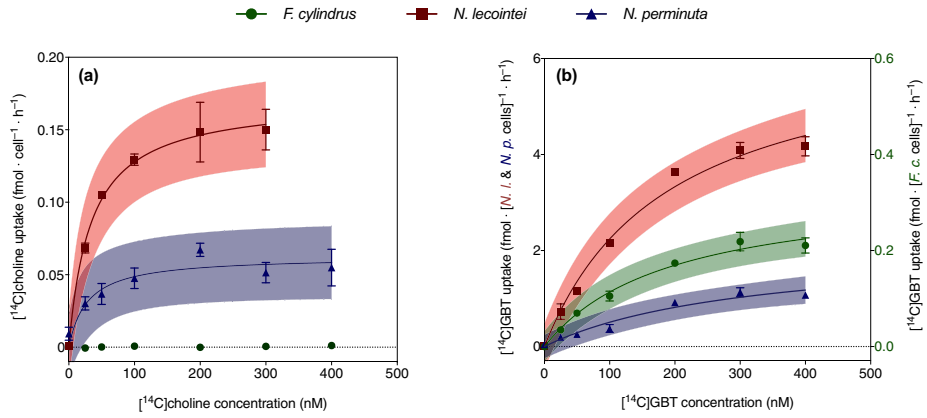

FIG. 1. Uptake kinetics of [^{14}C]choline (a) and [^{14}C]GBT (b) in axenic sea-ice diatom cultures of *Fragilariopsis cylindrus* (circles), *Nitzschia lecointei* (squares), and *Navicula* cf. *perminuta* (triangles). Note that [^{14}C]GBT data for *F. cylindrus* are plotted on the right *y*-axis in (b). Data are fitted using the Michaelis–Menten equation, and the color-corresponding envelopes represent 95% prediction intervals of the fitted line. Kinetic data ($V_{\max}$ and K_m) are presented in Table 1. Error bars indicate the standard error of the mean (n = 3). Note the different scales in (a) and (b). [Color figure can be viewed at wileyonlinelibrary.com]

the total uptake. Incubations were performed in triplicate, with an additional sample fixed in 2% formaldehyde (final concentration) prior to the incubation to serve as killed control.

Parallel incubations with and without unlabeled choline and GBT were performed in quadruplicate for cell counting. All cell counts were fixed in 2% formaldehyde (final concentration) and counted under a light microscope, using a Palmer-Maloney counting chamber. Specific growth rate (μ) was calculated from the logarithmic period of growth using eq. (2):

$$\mu = \frac{\ln D_x - \ln D_0}{t_x - t_0} \quad (2)$$

where D_x is the cell concentration at day x, D_0 is the cell concentration at day 0, t_x is the time in days at day x, and t_0 is the time in days at day 0.

For *Nitzschia lecointei*, the remaining cells were collected on GF/F filters and frozen at −20°C until processed for analysis of pEPS using the phenol-sulfuric acid assay (DuBois et al. 1956), quantifying pEPS as glucose equivalents according to Krembs et al. (2011).

Dark uptake. To test the influence of light on the use of [^{14}C]choline and [^{14}C]GBT, an additional dark experiment with *Nitzschia lecointei* was run in similar fashion as the time-course experiments. Independent triplicate samples, including killed controls, were incubated 68 h under identical conditions as described previously, but in complete darkness. At the end of the incubation period, total uptake, respiration, and incorporation into macromolecules were measured, and retention was calculated as described above.

Release of compatible solutes in response to rapid salinity shifts. An additional experiment was set up to measure the amount of released [^{14}C] activity at different salinity conditions. *Nitzschia lecointei* cells were washed three times in sterile F/2 medium (1,000*g* for 10 min), inoculated (~10^4 cells · mL^{-1}) into pre-chilled sterile F/2 medium prepared from ESAW, salinity 31, and supplemented with 100 nM [^{14}C]choline or 200 nM [^{14}C]GBT. Aliquots of 1.8 mL were distributed into microcentrifuge tubes in triplicate and incubated for 3 d. A separate sample for each treatment and time point was fixed in 2% formaldehyde (final concentration) to serve as killed control. Diatom cells were then centrifuged and washed twice in F/2 medium, salinity 31, followed by a third wash in either salinity 17, 31, or 55 and incubated for 68 h. This period was chosen because maximum uptake occurred after 68 h in the time-course experiments, and the levels of macromolecular incorporation were measurable. Incubations were terminated by centrifugation (1,500*g* for 15 min) of both pre- and post-acidified (72 μL of 2 M H_2SO_4) cells. Supernatant was transferred into 5 mL of Ecolume scintillation cocktail to measure released [^{14}C]-choline and [^{14}C]GBT. Pellets were washed three times and transferred into 5 mL Ecolume scintillation cocktail to measure total uptake and macromolecular incorporation. All samples were counted using a Packard LSC.

Intracellular quantification of choline and GBT. Intracellular (endogenous) concentrations of choline and GBT were quantified in a separate experiment performed under identical PAR radiation and temperature conditions as the time-course experiments, with no addition of choline or GBT. Cells were grown in F/2 medium prepared from ESAW, salinity 31, in 35 mL acid-washed glass test tubes. Cells were counted, and biovolume estimated, under a light microscope according to Hillebrand et al. (1999). After 6 d, cells were collected on PTFE filters and stored at −80°C until extraction. Osmolytes were extracted using a modified Bligh–Dyer extraction (Bligh and Dyer 1959, Canelas et al. 2009) resulting in an aqueous and organic fraction as previously described (Boysen et al. 2018). GBT and choline were quantified in the aqueous fraction by standard addition into a representative sample matrix and analyzed by liquid chromatography–mass spectrometry using a Waters Acquity I-Class UPLC fitted with a SeQuant ZIC-pHILIC column and coupled to a Waters Xevo TQ-S triple quadrupole with electrospray ionization; separation and detection as in Boysen et al. (2018).

Statistical analyses. Time-course experiments and growth data were analyzed with two-way ANOVA, and the remaining experimental data with one-way ANOVA using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Tukey's post hoc test was used to explore significant relationships between all treatments. To reduce the number of multiple comparisons over time, Dunnett's method for multiple comparisons was used for time-course experiments, using normal salinity (31) as control group for the upshift (55) and downshift (17) treatments. A probability level of ≤0.05 was used for statistical significance.

RESULTS

Uptake kinetics. Uptake data indicated that *Nitzschia lecointei* and *Nitzschia* cf. *perminuta* followed Michaelis–Menten kinetics for [^{14}C]choline (Fig. 1a, Table 1), whereas *Fragilariopsis cylindrus* did not take up detectable levels of exogenous [^{14}C]choline (Fig. 1a). Maximum rate of uptake in *N. lecointei* was more than twice the rate observed in *N.* cf. *perminuta*, even though both species had similar K_m for choline of ~30 nM, and similar growth rates and cell size.

Uptake of [^{14}C]GBT followed Michaelis–Menten kinetics in all three species (Fig. 1b, Table 1). V_{max} for [^{14}C]GBT was higher in *Nitzschia lecointei* and *N.* cf. *perminuta* than in *Fragilariopsis cylindrus* (by 19- and 6.3-fold, respectively; Table 1). It was also higher than the V_{max} for [^{14}C]choline in *N. lecointei* and *N.* cf. *perminuta* (38- and 34-fold, respectively; Table 1). The affinity for GBT, however, was lower than for choline in *N. lecointei* and *N.* cf. *perminuta*, as indicated by the higher K_m for GBT by 7- and 11-fold, respectively.

For further experiments, we used 100 nM [^{14}C]-choline and 200 nM [^{14}C]GBT (Table 1, Fig. 1). Based on K_m values, these concentrations represented ~75% saturation for choline, and ~50% for GBT (except for *Navicula* cf. *perminuta*, where uncertainty in K_m was high). Use of under-saturating concentrations was a compromise between uncertainties in K_m and substrate cost.

Time-course experiments. As expected, diatoms (*Nitzschia lecointei* cells) incubated at the lowest salinity (salinity 17), when the need for compatible solutes was reduced, had the lowest uptake of both choline and GBT (Fig. 2). This reduced uptake could not be fully attributed to slower cell growth, however, even though the macromolecular incorporation of choline was directly related to growth rate during the experiment (Fig. S1 in the Supporting Information). Total choline uptake did not vary significantly between salinities 31 and 55, nor did it significantly reduce the exogenous pool at these

TABLE 1. Uptake kinetic parameters for [^{14}C]choline and [^{14}C]GBT in three axenic sea-ice diatom cultures. Data show mean ± SD (n = 3) of the kinetic parameters, as estimated using the Michaelis–Menten equation.

	[^{14}C]choline		[^{14}C]GBT	
Diatom species	V_{max} (fmol · cell^{-1} · h^{-1})	K_m (nM)	V_{max} (fmol · cell^{-1} · h^{-1})	K_m (nM)
Fragilariopsis cylindrus	0.000	0.0	0.33 ± 0.03	193 ± 42
Nitzschia lecointei	0.171 ± 0.010	33.8 ± 7.3	6.47 ± 0.48	189 ± 31
Navicula cf. *perminuta*	0.062 ± 0.006	28.4 ± 14	2.10 ± 0.45	315 ± 122

higher salinities, which remained at >90% initial concentration over the course of the experiment (Fig. S2 in the Supporting Information).

In contrast, total uptake of GBT increased with increasing salinity treatment (Fig. 2, d–f), a pattern that did not match growth rate (Fig. 3a). Cellular content reached a maximum after 68 h, decreasing at later time points, likely due to depletion of GBT from the media, which was more pronounced at salinity 55 (Fig. S2). When scaled to time at a lower degree of depletion (33%, after 20 h), total uptake rate of GBT at salinity 31 was 0.36 ± 0.008 fmol · cell^{-1} · h^{-1}, an order of magnitude lower than the rates at saturating concentration (Figs. 1 and 2b). The reduction in GBT concentration (Fig. S1) cannot fully explain the reduced uptake rate.

Respiration of [^{14}C]choline or [^{14}C]GBT was not detectable in any of the species at any salinity. For [^{14}C]choline retention in *Nitzschia lecointei*, the interaction between salinity treatment and time was significant (two-way ANOVA, $F_{6,24}$ = 41.1, P < 0.0001, Fig. 2, a–c). A post hoc test revealed that cells in salinity 17 always had lower retention of

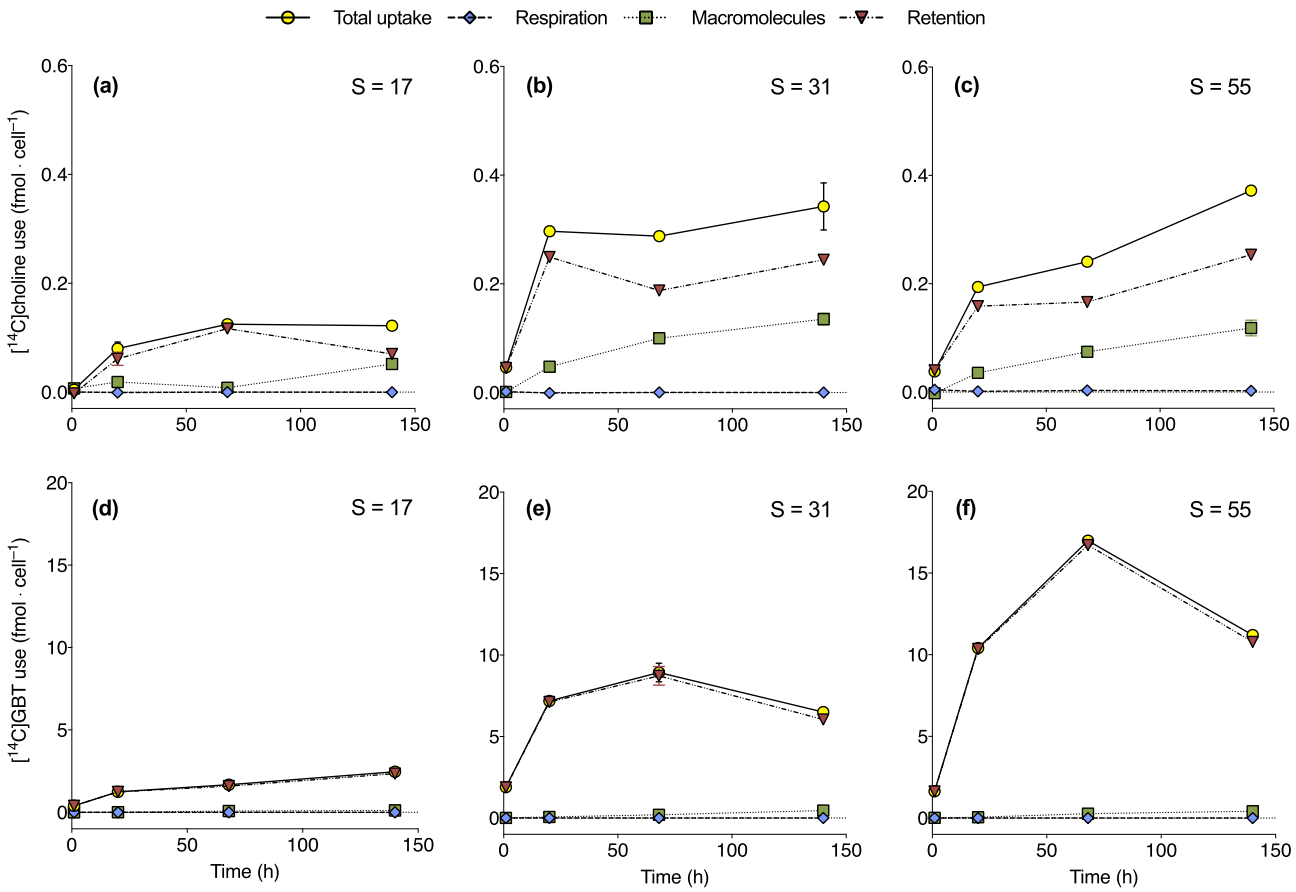

FIG. 2. Cell-specific time-course [^{14}C]choline (a–c) and [^{14}C]GBT (d–f) uptake experiments in axenic cultures of the Antarctic sea-ice diatom *Nitzschia lecointei*. Cells were treated with a rapid salinity (S) downshift (salinity 17, a and d), neutral (unchanged) condition (salinity 31, b and e), or upshift (salinity 55, c and f). During the experiment, the use of [^{14}C]choline (100 nM final concentration) and [^{14}C]GBT (200 nM final concentration) was measured as total uptake, respiration, macromolecule incorporation, and retention. Note the different scales for [^{14}C]choline (a–c) and [^{14}C]GBT (d–f). Error bars show standard error of the mean when wider than the symbol size (n = 3). [Color figure can be viewed at wileyonlinelibrary.com]

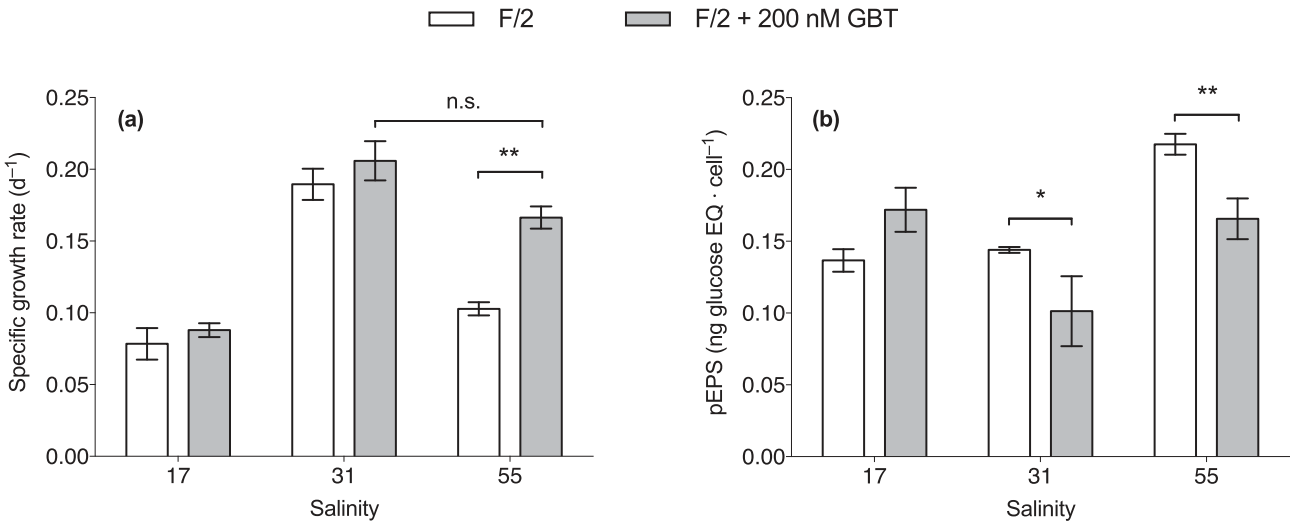

FIG. 3. Specific growth rate (a) and concentration of particulate extracellular polysaccharides (pEPS) (b) in the Antarctic sea-ice diatom *Nitzschia lecointei* growing axenically during 6 days of logarithmic growth at different salinities in F/2 with and without the addition of 200 nM GBT. Growth rate was significantly enhanced by the addition of GBT in salinity 55 (a), and restored to control (salinity 31) values ($P = 0.074$; n.s. indicates not significantly different). Concentrations of pEPS were significantly higher in samples without the addition of GBT in salinity 31 and 55 (b). Asterisks denote statistical significance, where * ≤0.05 and ** ≤0.01 (Tukey's test). Error bars show standard error of the mean ($n = 4$).

[^{14}C]choline than in the control salinity ($P > 0.0001$, Dunnett's test), though the relative amount of retention was high due to limited macromolecular incorporation. After 20 h of incubation, retention was significantly lower at salinity 55 compared to 31 (Dunnett's test, $P < 0.0001$). For the remaining time points, there were no significant differences between salinity 31 and 55 (Dunnett's test, $P > 0.05$). For [^{14}C]GBT, retention was significantly higher in salinity 55 compared to 31 at all incubation time points (Dunnett's test, $P < 0.0001$), except after 1 h (Dunnett's test, $P = 0.661$). Retention was significantly lower in salinity 17 compared to the control salinity during all time points (Dunnett's test, $P < 0.0001$).

For macromolecular incorporation of [^{14}C]-choline in *Nitzschia lecointei* cells, the interaction between salinity treatment and time was significant (two-way ANOVA, $F_{6,23} = 10.0$, $P < 0.0001$, Fig. 2, d–f). Neither salinity upshift nor downshift had an effect on macromolecular incorporation after 1 h. However, for the remaining time points, incorporation was significantly lower in salinity 17 compared to the control group (Dunnett's test, $P < 0.03$). After 140 h, the average proportions of macromolecular incorporation of [^{14}C]choline were 42 ± 3%, 36 ± 6%, and 32 ± 6% for salinity 17, 31, and 55, respectively. In contrast to choline, almost all GBT was retained as compatible solute with relatively small proportions of total uptake converted into macromolecules, regardless of salinity treatment. The only significant difference between macromolecular incorporation of [^{14}C]GBT and salinity treatments occurred at the last time point, where incorporation was lower in salinity 17 than salinity 31 (Dunnett's test, $P < 0.0001$). Only a limited amount of the total uptake of [^{14}C]GBT was incorporated into macromolecules after 140 h of incubation: 4.6 ± 8.3%, 7.0 ± 0.5%, and 3.8 ± 0.7% in salinity 17, 31, and 55, respectively.

Nitzschia cf. *perminuta* demonstrated a similar strategy as *Nitzschia lecointei* for the use of [^{14}C]choline and [^{14}C]GBT over time (Fig. S3 in the Supporting Information and Fig. 2, a–c). Although uptake of choline was higher in *N.* cf. *perminuta* than *N. lecointei*, it was low compared to GBT uptake by both species. Up to 36.5% of the total uptake of choline was incorporated into macromolecules at salinity 31. Higher salinity did not stimulate choline uptake or retention, but did increase GBT uptake and retention significantly, with the majority of the GBT retained intracellularly. *Fragilariopsis cylindrus* took up significant amounts of [^{14}C]GBT but no [^{14}C]-choline (Fig. S4 in the Supporting Information). Again, GBT uptake was initially stimulated by the higher salinity treatment. Due to higher cell concentration in the experiments with *F. cylindrus* than with the other species, the exogenous GBT was rapidly depleted (Fig. S2), and relative cellular content of [^{14}C]GBT decreased in salinity 31 and 55 after 20 h because the cells were growing.

Growth and EPS production during time-course experiments. Both growth rate and pEPS concentration were affected by salinity treatment and GBT addition during the time-course experiments with *Nitzschia lecointei*. The interaction between salinity treatment and GBT addition on cell growth was significant (two-way ANOVA, $F_{2,18} = 4.9$, $P = 0.02$; Fig. 3a).

Without GBT, cells grew more slowly at salinity 17 and 55 compared to 31 (Tukey's test, $P < 0.0001$). Growth rates at salinity 17 and 31 were not affected by the addition of GBT (Tukey's test, $P = 0.98$ and $P = 0.82$, respectively), but the growth rate at salinity 55 was significantly higher when the medium was supplemented with GBT (Tukey's test, $P = 0.0018$), reaching those observed at salinity 31, with and without GBT (Tukey's test, $P = 0.074$ and $P = 0.52$, respectively). A significant interaction between salinity treatment and GBT addition on pEPS concentration was also detected (two-way ANOVA, $F_{2,15} = 6.6$, $P = 0.0088$; Fig. 3b). Concentrations of pEPS were significantly lower in samples with GBT at salinity 31 and 55 (Tukey's test, $P = 0.032$ and $P = 0.0073$, respectively), though GBT had no effect at salinity 17 (Tukey's test, $P = 0.089$).

Dark uptake. The fate of both [^{14}C]choline and [^{14}C]GBT in *Nitzschia lecointei* was significantly affected by light, though different patterns were observed. Retention of [^{14}C]choline in the dark increased across all salinities, whereas no difference was observed in the light at salinities 31 and 55. Macromolecule incorporation decreased with salinity in the dark, whereas it increased in the light. For GBT, retention was similar in both the light and the dark, whereas the dark showed little to no incorporation into macromolecules compared to light across all salinities.

There was a significant effect of treatment salinity on retention of [^{14}C]choline in darkness (one-way ANOVA, $F_{2,6} = 23.3$, $P < 0.003$; Fig. 4a). Retention differed significantly in all treatments (Tukey's test, $P < 0.04$; Fig. 4a), and was higher with higher salinity treatment in the dark. Macromolecular incorporation of [^{14}C]choline in darkness was also affected by salinity (one-way ANOVA, $F_{2,6} = 8.58$, $P = 0.017$; Fig. 4b). Macromolecular incorporation of [^{14}C]-choline was significantly higher in salinity 17 compared to 55 (Tukey's test, $P = 0.015$; Fig. 4b). This trend is opposite to that observed in the light, where macromolecular incorporation in salinity 31 and 55 was higher compared to salinity 17 (Fig. 4b), which was similar to the time-course experiments (Fig. 2, a–c). Retention of [^{14}C]GBT in *Nitzschia lecointei* showed a similar pattern in darkness as in light, that is, significantly higher retention at higher salinity treatments (Tukey's test, $P < 0.001$; Fig. 4c). However, macromolecular incorporation was lower in darkness than in the light control and in previous experiments, and was not significantly affected by salinity in darkness (one-way ANOVA, $F_{2,6} = 2.09$, $P = 0.205$; Fig. 4d). No [^{14}C]choline or [^{14}C]GBT was respired in darkness.

Release of compatible solutes in response to rapid salinity shifts. The fate of [^{14}C]choline and [^{14}C]GBT, taken up by cells that experienced a shift in salinity from 31 to either 17, 31, or 55, is shown in Figure 5 for *Nitzschia lecointei*. Relative release and relative retention of [^{14}C]choline were significantly affected by salinity treatment (one-way ANOVA, $F_{2,6} = 73.7$ and 15.1, $P < 0.0001$ and 0.0045, respectively; Fig. 5a). Cells treated with salinity 17 had a significantly higher proportion of release and lower retention of [^{14}C]choline compared to the other treatments (Tukey's test, $P < 0.0001$ and $P < 0.02$, respectively). There was no significant difference in macromolecular incorporation between the treatments (one-way ANOVA, $F_{2,6} = 0.77$, $P = 0.503$).

Differences between the salinity treatments for [^{14}C]GBT retention, release, and macromolecular incorporation were significant (one-way ANOVA, $F_{2,6} = 342$, 230 and 13.9, $P < 0.0001$, $P < 0.0001$, and $P < 0.0056$, respectively; Fig 4b). Retention differed significantly in all treatments (Tukey's test, $P < 0.05$), with higher retention in the high salinity treatment. Release was significantly higher at salinity 17 compared to 31 and 55 (Tukey's test, $P < 0.0001$), but not significantly different between 31 and 55 (Tukey's test, $P = 0.608$). Cells treated with salinity 55 incorporated less [^{14}C]GBT into macromolecules compared to 17 and 31 (Tukey's test, $P = 0.011$ and $P = 0.0079$, respectively).

Intracellular quantification of choline and GBT. Intracellular concentrations of endogenous choline and GBT after 140 h of incubation at salinity 31 are presented in Table 2. On average, the three sea-ice diatoms contained 1.2 fmol choline · cell^{-1} and 3.2 fmol GBT · cell^{-1}, which are equivalent to 15 and 40 mM of choline and GBT, respectively, when scaled to cell volume.

DISCUSSION

Our study provides insight on the synthesis, uptake, and fate of the nitrogen-containing compatible solute GBT and its precursor choline in Antarctic sea-ice diatoms. All of our results indicate that GBT is readily scavenged from the environment and used as an effective compatible solute in sea-ice diatoms, alleviating cells from osmotic stress and, where tested, reducing the production of EPS, an alternative response to osmotic stress (Krembs and Deming 2008). GBT was primarily utilized as a compatible solute, with cells responding rapidly to changes in salinity through uptake or release of GBT. Choline, however, was taken up much more slowly, by one to two orders of magnitude, than GBT in two of the tested species, and not at all in *Fragilariopsis cylindrus*. A relatively large proportion (up to 42% of total uptake) of the ^{14}C from exogenous choline in *Nitzschia lecointei* and *Navicula* cf. *perminuta* was incorporated into macromolecules, as compared to GBT (up to 7%), which we hypothesize could be related to the biosynthesis of phospholipids, such as phosphatidylcholine and GBT lipids (Kates and Volcani 1996). All three species lacked the capacity to respire the ^{14}C-methyl group of choline and GBT. GBT, like many other organic compatible solutes (Keller 1989, Ruffert et al. 1997,

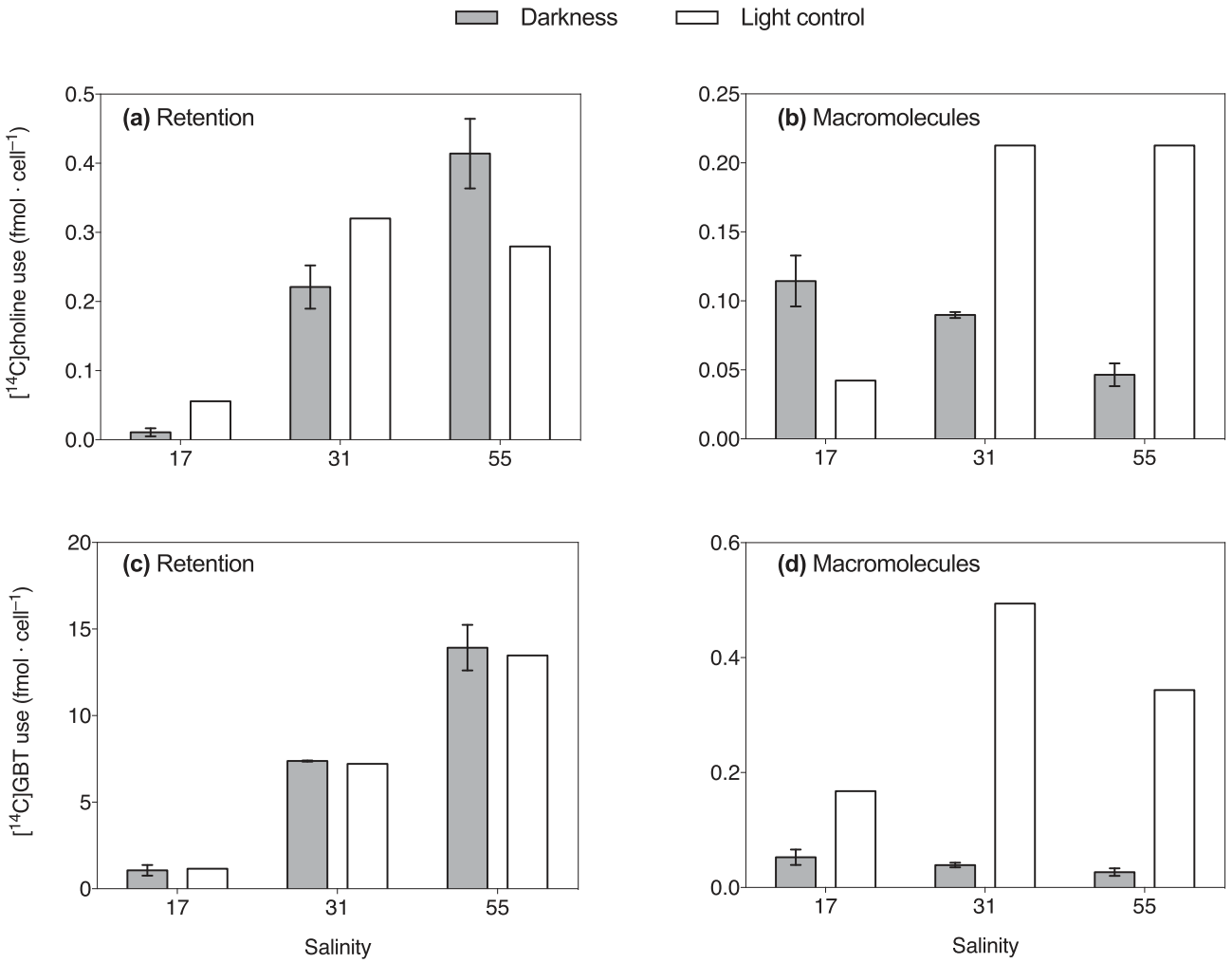

FIG. 4. Use of the exogenous radiolabeled metabolites [^{14}C]choline (a and b) and [^{14}C]GBT (c and d) in dark incubations with axenic cultures of the Antarctic sea-ice diatom *Nitzschia lecointei*. Retention (a and c) and macromolecular incorporation (b and d) were measured after 68 h incubations with 100 and 200 nM of choline and GBT, respectively. Error bars for the darkness treatment show standard error of the mean ($n = 3$). The control under light conditions (45 μmol photons · m^{-2} · s^{-1} PAR radiation, 16:8 light:dark cycle) was not replicated, but matches the replicated values in the time-course experiments.

Booth and Louis 1999, Firth et al. 2016), was released from cells when the environmental salinity decreased. The release of GBT by diatoms in sea-ice brine pockets has several ecological and biogeochemical implications, as discussed below.

The saturation and K_m levels we measured for GBT and choline in our experiments with Antarctic sea-ice diatoms (Table 1) are similar to those reported for choline in Arctic sea-ice brine assemblages at −1°C (Firth et al. 2016), but they are high, by at least 10-fold, compared to those for pelagic microbial communities in temperate coastal and estuarine areas (Kiene 1998, Kiene and Williams 1998). High K_m values in sea-ice microorganisms are consistent with adaptation to a semi-enclosed environment containing high concentrations of choline and GBT (further addressed below). The rates of GBT uptake in two of our diatoms, *Nitzschia lecointei* and *Navicula* cf. *perminuta*, are also higher, by more than an order of magnitude, than those reported for DMSP uptake in the mesophilic diatom *Cylindrotheca closterium* at 25°C (Van Bergeijk et al. 2003), and similar to those in *Thalassiosira weissflogii* at 14°C (Spielmeyer et al. 2011). These similar and even much higher rates are remarkable, considering that our experiments were performed at −1°C. High saturation levels at low temperature suggest effective transport systems for choline and GBT in sea-ice diatoms (with the exception of choline in *Fragilariopsis cylindrus* which transported only GBT). The accumulation of exogenous choline and GBT in the diatoms reached levels similar to or even higher than those synthesized when no exogenous sources were provided (Fig. 2, Figs. S3 and S4, Table 2), which further supports the importance of exogenous compatible solute uptake as an effective strategy for survival in sea-ice brine.

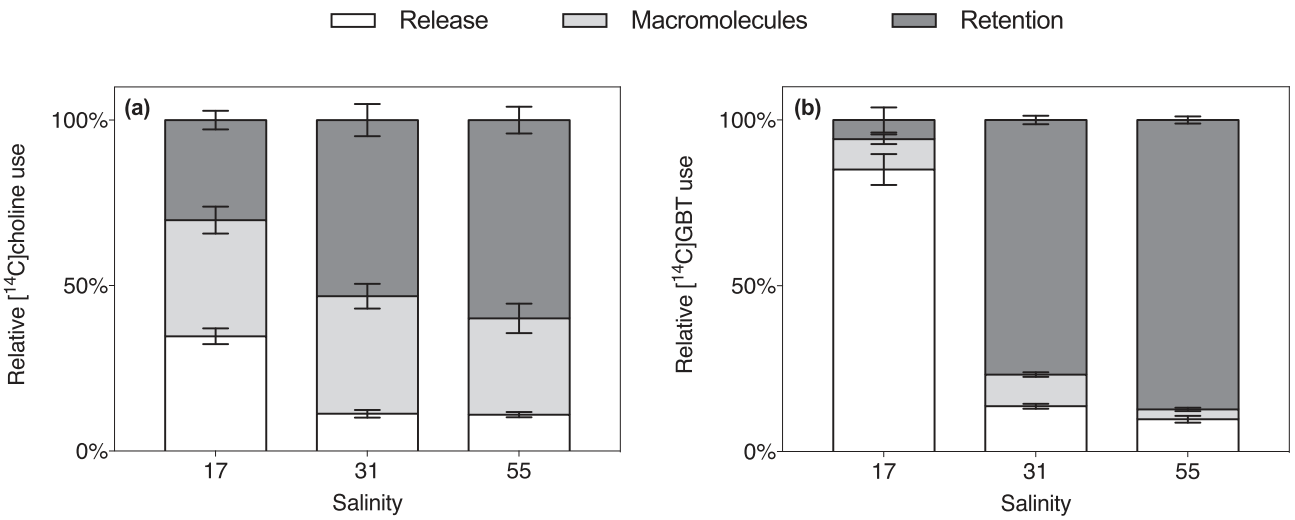

FIG. 5. Relative use of the exogenous radiolabeled metabolites [^{14}C]choline (a) and [^{14}C]GBT (b) in axenic cultures of the Antarctic sea-ice diatom *Nitzschia lecointei*. Cells had been growing with 100 and 200 nM of choline and GBT, respectively, before treatment with a rapid salinity downshift (salinity 17), normal condition (salinity 31), or upshift (salinity 55). After 68 h in the respective salinity, ^{14}C activity was measured in the supernatant to estimate the release of compatible solutes, and in the cells to estimate macromolecular incorporation and retention. Error bars show standard error of the mean (n = 3).

TABLE 2. Intracellular concentrations of choline and GBT in axenic cultures of three sea-ice diatoms after 140 h incubation at salinity 31. Data show mean ± SD (n = 2).

	Choline		GBT	
Diatom species	fmol · cell^{-1}	mM	fmol · cell^{-1}	mM
Fragilariopsis cylindrus	0.07 ± 0.002	3.3 ± 0.1	1.16 ± 0.06	53.1 ± 2.7
Nitzschia lecointei	0.68 ± 0.089	3.6 ± 0.5	6.00 ± 0.38	31.7 ± 2.0
Navicula cf. *perminuta*	2.95 ± 0.551	39.1 ± 7.3	2.58 ± 0.38	34.3 ± 5.1

Although no direct analytical quantification of dissolved choline or GBT concentration (or method to obtain one) is available for sea-ice brine to our knowledge, choline concentrations have been estimated at 50–100 nM in early winter Arctic sea-ice brines, based on uptake kinetics of bacterial assemblages (Firth et al. 2016). Here, we provide intracellular concentrations of three sea-ice algal taxa that can be used with our experimental results to calculate potential sources and levels of dissolved choline and GBT in sea-ice brine. The sea-ice diatoms of this study contained on average 1.2 fmol choline · cell^{-1} and 3.2 fmol GBT · cell^{-1}, and *Nitzschia lecointei* released up to 35% choline and 85% GBT upon a salinity downshift. Algal cell concentrations often reach 10^8–10^9 cells · L^{-1} in bottom sea ice (Arrigo 2017) and the microalgal community is often dominated by the genera in our study. From this information, we estimate that in sea-ice brine channels dissolved concentrations of these compounds can reach and exceed our experimental concentrations (100 nM choline, 200 nM GBT), as well as measured K_m values (~30 nM for choline, ~230 nM for GBT), solely due to release of endogenous compounds from diatoms during salinity shifts. Although these estimates are based on several assumptions, including that upscaling from culture conditions for three species with large interspecies variability is representative of environmental conditions, we expect compatible solute concentrations in sea-ice brines to be high and variable, due to the relatively high microbial biomass, cell lysis, and possibly sloppy grazing of that biomass, and both passive diffusion and active transport across membranes during fluctuating conditions. These factors, added to the characteristic physical partitioning of solutes into brines during sea-ice formation, make it likely that concentrations are considerably higher in brine pockets and channels compared to seawater (as is the case for dissolved organic matter in general; Thomas et al. 2001). We conclude that sea-ice diatoms have evolved to adapt quickly to large salinity fluctuations with the uptake of exogenous compatible solutes.

Heterotrophic bacterial degradation of organic matter released from algae is a key step in many marine biogeochemical cycles. As GBT accumulates to concentrations >30 mM in *Nitzschia lecointei* at salinity 31, it alone contributes to ~1%–2% of the total cellular carbon and nitrogen content (Torstensson

et al. 2015b). The release of diatom-derived metabolites, generally believed to play an important role in marine microbial food webs (Durham et al. 2015, Firth et al. 2016), can therefore contribute to the pool of organic matter in systems like sea ice with high algal biomass. The three species of sea-ice diatoms we examined did not respire choline or GBT, but respiration of these solutes does occur in natural systems containing an abundance of heterotrophic bacteria (Kiene 1998), including sea ice (Firth et al. 2016). Here, we have shown that *Nitzschia lecointei* released a large amount of GBT when salinity was rapidly downshifted, a process that would make such metabolites readily available for heterotrophic bacteria in natural sea-ice systems. In turn, bacterial remineralization of the metabolites would yield CO_2 and potentially ammonium (Firth et al. 2016) for reuse by diatoms during photosynthesis and growth. The recycling of metabolites can be expected to generate complex algal–bacterial interactions, which are among the least well-studied phenomena in sea ice.

In this study, we also observed that EPS production in *Nitzschia lecointei* decreased when the medium was supplemented with an exogenous source of GBT at salinity 31 and 55 (though not at salinity 17; Fig. 3). These findings suggest that 200 nM of exogenous GBT is sufficient to relieve the cells from the osmotic stress of such a hypersalinity shock, and that in the absence of GBT the production of EPS can serve as an alternative protective mechanism against high salinity (Deming and Young 2017). For *Fragilariopsis cylindrus*, a diatom we observed to have less versatility in the uptake of solutes (GBT but not choline) than the other species, more EPS may be needed for effective osmoprotection. In a previous study of EPS as cryoprotectant, without considering the use of compatible solutes, Aslam et al. (2012) reported copious production of EPS by *F. cylindrus* when grown at salinity 52 (compared to 34). They highlighted the need to resolve how EPS production may be integrated with other diatom survival strategies in sea ice, which our work with *N. lecointei* advances. Alternating the uptake and release of compatible solutes in the semi-enclosed environment of sea-ice brine pockets may represent a more rapid, sensitive, and efficient strategy for coping with the fluctuating salinities inherent to sea ice (Ewert and Deming 2014) than the production of EPS, despite the other critical roles for EPS in diatom survival (Krembs et al. 2002, 2011, Aslam et al. 2012, Deming and Young 2017). Given that GBT was both taken up and released rapidly by diatoms in this study, brine pockets in sea ice could act as external pools of compatible solutes. Availability of extracellular compatible solutes in a brine pocket, or an EPS-algal biofilm within the pocket (Deming and Young 2017), may facilitate rapid survival responses of diatoms to salinity shifts. Other members of the microbial community may then use this pool for osmoprotection, energy, or macromolecular incorporation, in analogy to sharing the pool of EPS in sea-ice brines for cryoprotection regardless of which organisms produced the EPS (Collins et al. 2008, Krembs et al. 2011), leading to complex microbial interactions (Durham et al. 2015).

Although the membrane transport systems for GBT were very able to import large quantities of this compound very quickly, only limited amounts of exogenous choline were utilized by *Nitzschia lecointei* and *Navicula* cf. *perminuta*, and no uptake was observed in *Fragilariopsis cylindrus*, even though endogenous choline was detected in all three species. Choline uptake was not stimulated by the exposure to higher salinity compared to the control, suggesting that its uptake and conversion to GBT is not as effective an osmoregulatory strategy in sea-ice diatoms as in sea-ice bacteria (Firth et al. 2016). The genome of *F. cylindrus* has eight genes (Fracy1_184258, 188063, 192148, 193695, 207649, 211423, 226251, and 268660) annotated for choline transporter-like proteins (Mock et al. 2017), but our results suggest that either these genes were not expressed in our experiments or the proteins are not functional as choline transporters from medium into cell, much like the choline transporter-like proteins in the yeast *Saccharomyces cerevisiae* which are not considered to function in that role (Zufferey et al. 2004). *Fragilariopsis cylindrus* also has the genes coding for choline dehydrogenase and betaine-aldehyde dehydrogenase (Mock et al. 2017), suggesting the presence of a two-step GBT production pathway similar to that in higher plants (Sakamoto and Murata 2002). The presence of these genes suggests that *F. cylindrus* can convert choline into GBT, even though it is unable to scavenge exogenous choline.

The uptake rate of GBT in *Fragilariopsis cylindrus* was approximately an order of magnitude lower than in *Nitzschia lecointei* and *Navicula* cf. *perminuta*. The smaller cell volume of *F. cylindrus*, or its possible dependence on alternative compatible solutes, may explain the slower uptake. In a previous physiological study, *F. cylindrus* was shown to increase the intracellular pool of DMSP by 85% following a gradual upward salinity shift to 70 (Lyon et al. 2011). Other studies, however, have shown that the major compatible solutes in *F. cylindrus* are isethionic acid, proline, and GBT, all of which are present in much higher relative concentrations than DMSP (Krell et al. 2007, Boroujerdi et al. 2012). In our study, intracellular GBT concentrations at normal seawater salinity (Table 2) exceeded the values previously reported for DMSP in *F. cylindrus* (Lyon et al. 2011, 2016) by ~3.5 times. Species-specific uses of different compatible solutes, and their environmental controls, could have many important biogeochemical and ecological consequences.

Although temperature and salinity vary spatially and temporally in sea ice, very little is known about the function of GBT in sea-ice microbial communities

or in cold environments in general, even though GBT is the most commonly used compatible solute across taxa in more moderate environments (Welsh 2000). Here, we have shown that sea-ice diatoms have effective transport systems for osmoregulation and specifically for GBT, as well as the potential to reuse and recycle (through associated heterotrophic bacteria) compatible solutes within the semi-enclosed brine network of their subzero habitat. Previous studies of osmoregulation in psychrophilic diatoms have mainly addressed sulfur-containing DMSP as the compatible solute of interest, due to its role as a precursor to the climatically active trace gas DMS (Stefels et al. 2007). We emphasize that its nitrogen-containing analog, GBT, plays important roles in both the microbial ecology and nitrogen cycling of sea ice through rapid uptake, production, and release by diatoms in response to changes in salinity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Positive correlation between macromolecular incorporation of [^{14}C]choline and growth rate in *Nitzschia lecointei*. Cells were treated with a rapid salinity downshift (salinity 17), neutral (unchanged) condition (salinity 31), or upshift (salinity 55) and incubated for 140 h. Error bars show standard error of mean (n = 3).

Figure S2. Remaining relative amounts of [^{14}C]choline (a–c) and [^{14}C]GBT (d–f) in the growth medium during time-course experiments with *Nitzschia lecointei* (a and d), *Navicula perminuta* (b and e), and *Fragilariopsis cylindrus* (c and f). Initial concentrations were 100 and 200 nM [^{14}C]choline and [^{14}C]GBT, respectively (10 mL incubations). Cells were treated with a rapid salinity downshift (salinity 17), neutral (unchanged) condition (salinity 31), or upshift (salinity 55). Error bars show standard error of mean when wider than the symbol size (n = 3).

Figure S3. Time-course [^{14}C]choline (a–c) and [^{14}C]GBT (d–f) uptake experiments in axenic cultures of the Antarctic sea-ice diatom *Navicula perminuta*. Cells were treated with a rapid salinity downshift (salinity 17, a and d), neutral (unchanged) condition (salinity 31, b and e), or upshift (salinity 55, c and f). During the experiment, the use of [^{14}C]choline and [^{14}C]GBT was measured as total uptake, respiration, macromolecule incorporation, and retention as compatible solute. Error bars show standard error of mean when wider than the symbol size (n = 3).

Figure S4. Time-course [^{14}C]GBT uptake experiment in axenic cultures of the Antarctic sea-ice diatom *Fragilariopsis cylindrus*. Cells were treated with a rapid salinity downshift (salinity 17), neutral (unchanged) condition (salinity 31), or upshift (salinity 55). During the experiment, the use of [^{14}C]GBT was measured as total uptake, respiration, macromolecule incorporation, and retention as compatible solute. Error bars show standard error of mean when wider than the symbol size (n = 3).

Appendix S1. Method description for preparation and verification of axenic diatom strains.