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Microbial uptake dynamics of choline and glycine betaine in coastal seawater

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Abstract

Choline and glycine betaine (GBT) are utilized as osmolytes to counteract osmotic stress, but also constitute important nutrient sources for many marine microbes. Bacterial catabolism of these substrates can then lead to the production of climate active trace gases such as methylamine and methane. Using radiotracers, we investigated prokaryotic choline/GBT uptake and determined biotic and abiotic factors driving these processes in the Western English Channel, UK. Kinetic uptake parameters indicated high affinity (nM range) for both osmolytes and showed a seasonal pattern for choline uptake. Generalized linear modeling of uptake parameters suggested a significant influence of sea surface temperature and salinity on prokaryotic uptake of both osmolytes. The presence of diatoms significantly influenced prokaryotic choline/GBT uptake dynamics. Choline uptake was further related to the occurrence of *Phaeocystis* spp., which were highly abundant in the phytoplankton community during spring, and dinoflagellates abundance during summer. While Rhodobacteraceae were the most important bacterial drivers for prokaryotic choline uptake, prokaryotic GBT uptake was associated with various groups such as SAR11 (Pelagibacterales) and Gammaproteobacteria, suggesting a wider capacity for GBT catabolism than previously recognized. Furthermore, using a newly developed approach we determined the first available data for dissolved GBT concentrations in seawater and found both osmolytes to be at the sub-nanomolar range. Together, this study improves our understanding of the biogeochemical cycling of these environmentally important osmolytes and highlights how their cycles may be affected by a changing climate.

Marine organisms accumulate organic solutes (osmolytes) within their cells in order to maintain favorable osmotic pressure (Yancey 2005). These include, but are not limited to, glycine betaine (GBT), choline, and dimethylsulfoniopropionate (DMSP). Noteworthy, the cation choline potently alleviates osmotic stress in some bacteria (Csonka and Hanson 1991 and references within), but also acts as a precursor to the zwitterionic GBT. Choline can be oxidized to GBT which accumulates inside cells (Abee et al. 1990; Kiene 1998). As environmental conditions change, for example, fluctuations in osmolarity or changes in sea surface temperature, osmolytes are then released into the environment and serve as important sources of nutrients for marine microorganisms. Microbial metabolism of the sulfur-containing DMSP has received much attention over the past few decades because it is the precursor of the climate-active gas dimethyl sulfide (DMS) (Charlson et al. 1987; Quinn and Bates 2011). However, there is an increasing body of evidence suggesting that the degradation of nitrogen-containing osmolytes (N-osmolytes) may also contribute to the release of climate-active gases, for example, methylated amines which are important for aerosol nucleation in the marine atmosphere (Almeida et al. 2013; Schobesberger et al. 2013). Furthermore, it is known that N-osmolytes can be readily utilized by the marine microbial community to produce the greenhouse gas methane (Welsh 2000; Jameson et al. 2019; Jones et al. 2019).

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Additional Supporting Information may be found in the online version of this article.

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Methanogenic Archaea capable of methanogenesis directly from GBT and choline have also been recently isolated (reviewed by Kurth et al. 2020). Hence, there is a pressing need to better understand the GBT and choline cycle in marine waters.

Unfortunately, microbial uptake and catabolism of nitrogen-containing osmolytes (e.g., GBT, choline) in oxygenated waters is poorly understood (King 1988; Mausz and Chen 2019). This is somewhat surprising given that not only are N-osmolytes ubiquitous in marine organisms, but also their standing concentrations in surface marine waters and in phytoplankton particles are comparable to, if not greater than, that of DMSP and DMS (Keller et al. 2004; Airs and Archer 2010; Beale and Airs 2016). Despite their abundance, while detectable in phytoplankton particles (Airs and Archer 2010; Beale and Airs 2016), no reliable method exists to directly measure in situ GBT and choline concentrations in seawater. So far, only an enzymatic assay exploiting the fact that choline can be oxidized via choline oxidase to produce H_2O_2 provides an indirect means to detect choline and puts its concentration in the 0–45 nM range for coastal seawater (Roulier et al. 1990).

N-osmolytes are key components of the oceanic labile, and thus rapidly recycled, dissolved organic matter (DOM) pool. Their cycling in the oxygenated water column generates key nutrients for microbes like the *Roseobacter* and SAR11 (Pelagibacterales) clades, thereby contributing to the global biogeochemical cycling of essential elements (Lidbury et al. 2015; Noell and Giovannoni 2019). We and others have shown that cosmopolitan marine heterotrophic bacteria can grow on choline/GBT as sole carbon and nitrogen sources (Sun et al. 2011; Lidbury et al. 2015), thereby enabling them to better adapt to oligotrophic marine surface waters. Recent estimates using cultures of SAR11 bacteria have shown that SAR11 clade isolates have high-affinity membrane transporters with an affinity for GBT of approximately 1 nM (Noell and Giovannoni 2019). This agrees with early radiotracer experiments using ^{14}C-GBT, showing that once released, GBT is turned over rapidly (a few hours) in coastal seawater, indicating that free-living marine bacteria in surface waters serve as a major sink for GBT (Kiene et al. 1998).

In this study, we set out to determine the uptake of choline and GBT by coastal marine microbes, particularly the free-living fraction among prokaryotes, using ^{14}C-radioisotopes in order to better understand the seasonality and key environmental drivers for N-osmolyte uptake. Using amplicon sequencing of 16S rRNA genes, flow-cytometry and microscopy, we determined the key microbial and phytoplankton groups that are strongly associated with choline and GBT dynamics in surface coastal waters. Furthermore, we present a new assay which enabled us to generate the first estimates of standing concentrations of dissolved N-osmolytes in seawater.

Materials and methods

Sample collection

Seawater samples used for this study were sampled between April 2015 and April 2017 at the long-term monitoring Station L4 (50° 15.00′N, 4° 13.02′W) of the Western Channel Observatory (https://www.westernchannelobservatory.org.uk). Station L4 is located approximately 10 km off the coast of Plymouth, UK (Fig. 1a). Together with the Gulf of Maine

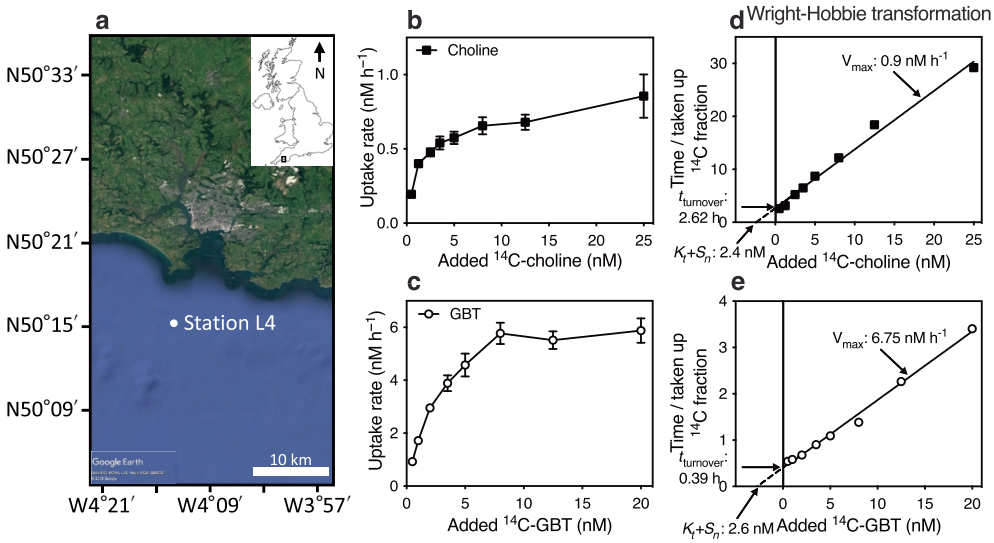

Fig. 1. Prokaryotic uptake of choline and glycine betaine (GBT). (**a**) Location of sampling Station L4 of the Western Channel Observatory, UK. (**b**,**c**) Representative kinetic uptake curves for (**b**) choline, (**c**) GBT, and (**d**,**e**): Linearization of the corresponding uptake data for (**d**) choline, and (**e**) GBT using Wright–Hobbie transformation (Wright and Hobbie 1966). Arrows mark the intersects with the *x*- and *y*-axes (equaling $K_t + S_n$ and $t_{turnover}$, respectively) and the lines slope which corresponds to the inverse of V_{max}. Results present the mean of biological triplicates; error bars denote standard deviation. Figure (**a**) was produced using Google Maps.

(Keller et al. 2004), Station L4 represents one of only two sites where intracellular concentrations of choline and GBT from phytoplankton are available (Airs and Archer 2010; Beale and Airs 2016). Water was collected from 2 m depth using a rosette of 10-L Niskin bottles attached to a conductivity, temperature, and depth analyzer. For both uptake experiments and community analysis, two aliquots of 10 L seawater were immediately transferred into acid-rinsed 10 L carboys (polypropylene, Nalgene). During transit to the lab (approximately 4 h after sampling), sample integrity was preserved by storing the carboys in the dark to prevent light-mediated reactions and inside a run-through water basin to maintain sea surface temperature.

Choline and GBT uptake kinetics

In the lab seawater was immediately transferred to a temperature-controlled room maintained at in situ temperature and stored in the dark. To determine uptake kinetics, samples were processed as described by Kiene (1998) and Kiene and Hoffmann Williams (1998), which are detailed in the Supporting Information. Approximately 5 L seawater were gently gravity filtered through GF/C glass fiber filters (nominal retention, > 1.2 μm, Whatman) to remove phytoplankton. Seawater was stored for 20–24 h prior to further processing to allow degradation of DMSP released by phytoplankton, a known competitive inhibitor of GBT uptake (Kiene et al. 1998). Eight different concentrations of [methyl-^{14}C-]choline (55.2 mCi mmol^{-1}, Perkin Elmer) or [methyl-^{14}C-]GBT (38.5 mCi mmol^{-1}, Moravek Inc. via Hartmann Analytic) ranging from 0.5 to 25 nM (choline) or 0.5 to 20 nM (GBT) were added to the samples in triplicate and short incubations of 20 min (choline) or 10 min (GBT) were conducted to ensure compound uptake with only negligible CO_2 production. After incubation, cells were collected by filtration onto 0.2-μm pore size Supor filters (⌀ 25 mm, Pall Corporation) and radioactivity measured using a liquid scintillation counter (Tri-Carb 2910TR, Perkin Elmer). Kinetic uptake parameters (V_{max}: maximum uptake velocity, $K_t + S_n$: with K_t being the half-saturation constant, also referred to as the transport constant [Wright and Hobbie 1966], and S_n being the natural [endogenous] substrate concentration, and $t_{turnover}$: turnover time) were determined using linear transformation according to Wright and Hobbie (1966) (Fig. 1b–e). Using Wright-Hobbie plots, the slope of the linearized graph equals $1/V_{max}$, the x-intercept represents $K_t + S_n$, and the y-intercept represents $t_{turnover}$.

Uptake of ^{14}C-choline/GBT into particulate cell biomass and oxidation to $^{14}CO_2$

The same seawater used for determining uptake kinetics as described above was also used for determining choline/GBT uptake into particulate cell biomass and oxidation to $^{14}CO_2$. These experiments were conducted from February 2016 to April 2017. Aliquots of 23 mL prefiltered seawater were transferred to 50-mL centrifuge tubes (SARSTEDT), spiked with 10 nM ^{14}C-choline or ^{14}C-GBT, incubated in the dark at in situ temperature and incubations terminated in triplicate after 0.15, 1, 2.5, 4, 7, 10, 12, and 24 h. For particulate cell biomass, 20 mL samples were processed as described above. Total oxidation of ^{14}C-choline or ^{14}C-GBT to $^{14}CO_2$ was analyzed in analytical triplicate from the same tube as described by Dixon et al. (2011) and described in more detail in the Supporting Information.

Estimation of the natural substrate concentration (S_n) of choline/GBT

Currently, due to the sub-nanomolar predicted concentrations and the complexity of an analytical extraction technique, there is no method available to directly determine S_n for choline or GBT (i.e., concentrations of dissolved choline/GBT in seawater). We thus developed an approach which was theoretically laid out by Wright and Hobbie (1966). We diluted the natural seawater sample 1 : 1 (v : v) with seawater derived from the Sargasso Sea (Sigma-Aldrich) which is supposedly free of the substrate of interest (i.e., choline/GBT). We then performed uptake kinetics of undiluted and diluted samples to determine ($K_t + S_n$) in the undiluted sample and, since K_t is a constant, ($K_t + S_n/2$) in the diluted sample. S_n can be calculated by solving the equations with these two variables. To further validate this approach, we (i) used a dilution of 1 : 3 (v : v) of natural seawater from Station L4 : choline/GBT-free seawater from Sigma-Aldrich, resulting in the equation ($K_t + S_n/3$) and (ii) spiked diluted samples with a known concentration (A) of choline or GBT (10 or 5 nM) resulting in the equation ($K_t + S_n/2 + A$). Stock solutions of choline and GBT used for spiking were quantified on a cation-exchange ion chromatograph (881 Compact IC pro, Metrohm) supplied with a Metrosep C 4 guard and a Metrosep C 40250/4.0 separation column, and a conductivity detector (all Metrohm) to confirm their concentration.

Environmental parameters and plankton community analysis of samples from Station L4

Seawater from Station L4 is collected weekly (weather permitting), and environmental parameters and community abundance data were available for all dates when uptake experiments were performed. Chlorophyll *a* (Chl *a*) was measured by Turner fluorometry according to Welschmeyer (1994). Nutrient concentrations were analyzed as described by Woodward and Rees (2001) with the following limits of detection: nitrate, inorganic phosphate (PO_4^{3-}), and silicate 0.02 μmol L^{-1}, nitrite 0.01 μmol L^{-1}, and ammonium 0.05 μmol L^{-1}.

Other environmental parameters such as sea surface temperature, salinity, depth, and photosynthetically active radiation were recorded by sensors mounted on the rosette bottle sampler. Phytoplankton and microzooplankton species were counted by light microscopy in samples collected from 10 m depth as described in Widdicombe et al. (2010). Species were grouped into functional groups including diatoms, dinoflagellates,

coccolithophores, colorless dinoflagellates, zooflagellates, and ciliates. In addition, data at species level were also used for multivariate statistics (see below). Bacterioplankton (bacteria with high and low nucleic acid content, *Synechococcus*) as well as some phytoplankton groups (*Phaeocystis*, cryptophytes, picoeukaryotes and nano-eukaryotes) were determined by flow cytometry (Tarran and Bruun 2015).

DNA extraction, 16S rRNA gene library preparation, and amplicon sequencing

Triplicate samples of 1 L seawater were filtered through 0.2-μm pore size Sterivex filters (Durapore, Millipore), snap-frozen in liquid nitrogen and stored at −80°C prior to DNA extraction following a published protocol (Neufeld et al. 2007). 16S rRNA gene amplicon libraries were produced following recommendations from Illumina's Metagenomic Sequencing Library Preparation guide and detailed in the Supporting Information using the 515F-Y/926R primer set (5′-GTGYCAGCMGCCGCGGTAA, 5′-CCGYCAATTYMTTTRAGTTT) for better amplification of marine groups (Parada et al. 2016). Sequencing of 16S rRNA gene amplicon libraries was performed on an Illumina MiSeq platform at the University of Warwick Genomics Facility using 2 × 300 bp paired end chemistry. The detailed bioinformatics pipeline used is presented in the Supporting Information. Bacterial community data were either summarized at the family level or all ribosomal sequence variants (RSVs) were used for statistical analysis.

Statistical analyses

To determine statistical support for a seasonal pattern, uptake parameters $K_t + S_n$, V_{max}, and $t_{turnover}$ were grouped into seasons (spring: March–May, summer: June–August, autumn: September–November, and winter: December–February). We then used GraphPad Prism version 8.4.3 to test for normality selecting a Shapiro–Wilk's test due to the low number of autumn samples for choline and winter samples for both osmolytes ($n = 6$ and $n = 3$, respectively). Each parameter was tested for differences between seasons using a Kruskal–Wallis test followed by Dunn's multiple comparison test.

Uptake parameters ($K_t + S_n$ and V_{max} only, since $t_{turnover}$ is not independent of the other two) were analyzed by interpretive multivariate statistics to explore which parameters (environmental factors or community abundance) had the highest influence. The number of environmental factors used as predictors in the multivariate statistical analysis was reduced based on a correlation analysis performed in GraphPad Prism to select only independent predictors, and parameters with a high correlation (> 0.6) were replaced with others explaining most of the variance (Supporting Information Fig. S1). Salinity and PO_4^{3-} were excluded from this selection procedure, and nitrite, nitrate, and ammonium were combined in a predictor as total nitrogen (N).

Multivariate statistics involved generalized linear models (GLM, Supporting Information Table S1) to predict uptake parameters from environmental factors, canonical correlation analysis of principal coordinates (CAP_{CCorA}) (Anderson and Willis 2003) followed by multiple regression analysis to determine correlations between the plankton community and uptake, and distance-based redundancy analysis (db-RDA) to investigate relationships between environmental factors and the community as detailed in the Supporting Information.

Data availability

Kinetic parameters for prokaryotic choline or GBT uptake, uptake rates into particulate cell biomass and oxidation to CO_2, nutrient data, and phytoplankton, microzooplankton, and bacterioplankton taxonomy and abundance data from microscopy counts and flow cytometry are available from the British Oceanographic Data Centre (https://www.bodc.ac.uk/) (Tarran and May 2019; Mausz et al. 2021; Widdicombe and Harbour 2021; Woodward and Harris 2021). 16S rRNA gene amplicon sequencing reads were submitted to the Sequence Read Archive of the National Center for Biotechnology Information (BioProject accession number PRJNA808436).

Results

Choline and GBT uptake kinetics and seasonality

We performed a 2-yr seasonal study (April 2015–April 2017) of prokaryotic uptake of choline and GBT from Station L4 of the Western Channel Observatory, UK. We observed Michaelis–Menten-like uptake kinetics for both substrates, suggesting that these transporters likely possess a single-substrate binding site (reviewed by Diallinas 2014) (Fig. 1b,c). Wright and Hobbie transformation (Wright and Hobbie 1966) allowed determination of kinetic parameters of prokaryotic uptake (Fig. 1d,e), $K_t + S_n$, with K_t representing the transport constant and S_n representing the natural substrate concentration in seawater, and V_{max} representing the maximum uptake velocity. $K_t + S_n$ values for choline ranged between 1.9 and 5.3 nM with lowest values observed in August and October 2015 and during the spring bloom in 2016 (Fig. 2a). V_{max} (the "uptake rate") ranged between 0.2 and 3.4 nM h^{-1} with the largest value coinciding with the shortest turnover time ($t_{turnover}$) for S_n of 0.54 ± 0.14 h determined for August 2015 (Fig. 2b,c). These values are similar to choline kinetic parameters reported from coastal waters of Mobile Bay (Kiene 1998). For GBT, $K_t + S_n$ ranged from 1.4 to 17.2 nM (November 2015) with values < 5 nM observed during most of spring, summer and early autumn (Fig. 2d), indicating that the transport system has a high affinity for GBT. V_{max} values ranged between 0.6 and 40.8 nM h^{-1} and GBT was mostly taken up in less than 2 h with $t_{turnover}$ ranging from 0.19 to 6.2 h (Fig. 2e,f).

Next, we analyzed the prokaryotic uptake kinetic parameters covering roughly 2 yr for apparent seasonal patterns using

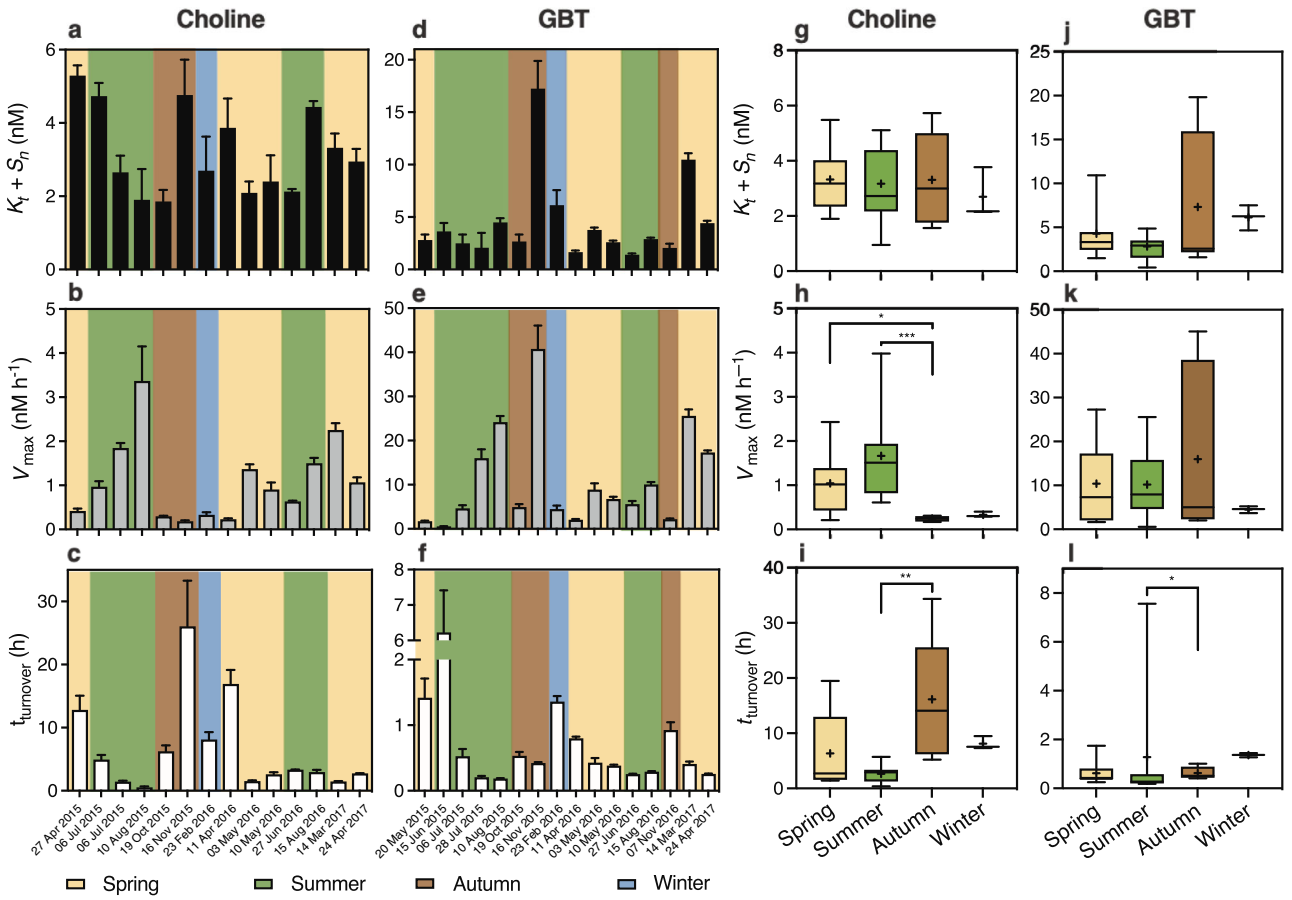

Fig. 2. Uptake kinetic parameters and analysis of seasonal differences of choline and glycine betaine (GBT) uptake in prokaryotic fractions of surface seawater from Station L4, UK, on selected dates between spring 2015 and spring 2017. Uptake kinetic parameters for (**a**–**c**) choline and (**d**–**f**) GBT were determined from kinetic curves as shown in Fig. 1. (**a**,**d**) Half-saturation constant, also referred to as transport constant, and natural substrate concentration, $K_t + S_n$; (**b**,**e**) Maximum uptake velocity, V_{max}; (**c**,**f**) turnover time, $t_{turnover}$. Results present the mean of biological triplicates; error bars denote standard deviation. Box–Whisker plots of uptake kinetic parameters (**g**,**j**) $K_t + S_n$, (**h**,**k**) V_{max}, and (**i**, **l**) $t_{turnover}$ for (**g**–**i**) choline and (**j**–**l**) GBT per season. A "+" indicates the mean, whiskers reach from minimum to maximum value. Colors mark different seasons with spring corresponding to March–May, summer June–August, autumn September–November, and winter December–February. Asterisks indicate significant differences between seasons as determined by Dunn's multiple comparison test with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0005$.

a non-parametric Kruskal–Wallis test for non-normal distributed data. For $K_t + S_n$, there was no statistical support for seasonal differences for either choline or GBT (Kruskal–Wallis test p-value 0.8954 and 0.1178, respectively) (Fig. 2g,j). In contrast, V_{max} significantly differed between seasons for choline (Kruskal–Wallis test p-value 0.0003) with uptake rates in spring and summer exceeding those of autumn (Dunn's multiple comparisons test, adjusted p-value 0.0271 and 0.0004, respectively) (Fig. 2h), while no significant seasonality was found for GBT (Kruskal-Wallis test p-value 0.7997) (Fig. 2k). $t_{turnover}$ showed a seasonal pattern for both choline and GBT (Kruskal–Wallis test p-value 0.0044 and 0.0106) with most profound differences between summer and autumn samplings (Dunn's multiple comparisons test, adjusted p-value choline: 0.0064, GBT: 0.0247) (Fig. 2i,l).

Environmental conditions and description of plankton communities

Environmental parameters at Station L4 on days when samples for prokaryotic choline and GBT uptake analysis were taken are summarized in Supporting Information Table S2. The coastal Station L4 is prone to salinity fluctuations and other riverine influences due to its location near the mouth of the River Tamar (Fig. 1a), which is reflected in the physicochemical conditions. Salinity ranged from 34.78 to 35.31 PSU and was mostly high on sampling dates in 2015, while in 2016 values were low in winter and spring increasing toward summer and autumn. Sea surface temperature (SST) followed a seasonal trend being lowest in the winter and first spring sample (< 10°C) and reaching its maximum of ~ 16°C in the August summer samples (Supporting Information Table S2).

Salinity and SST were highly correlated (0.67) (Supporting Information Fig. S1). Levels of the macronutrients nitrite, nitrate, and phosphorus decreased to near the level of detection (nanomolar range) from spring to summer sampling dates, increasing again toward autumn and winter (Supporting Information Table S2), thereby following typical trends observed at this coastal station (Smyth et al. 2010). Since we hypothesized some of these physicochemical factors might influence prokaryotic choline or GBT uptake, we needed to reduce the number of variables tested. Therefore, we determined which environmental parameters were independent using correlation analysis (Supporting Information Fig. S1), and selected SST, salinity, total nitrogen (N, combining nitrite, nitrate, and ammonium), PO_4^{3-}, particulate organic carbon (POC) and nitrogen (PON), and Chl *a* for further analysis.

Seasonal trends in physicochemical conditions typically initiate a succession of phytoplankton groups at Station L4 leading to a mixed community during most of the spring and summer (Fig. 3) (Widdicombe et al. 2010). We observed two successive Chl *a* maxima (Supporting Information Table S2), the first during late spring (May 2015 and 2016) associated with the presence of *Phaeocystis* spp. and to a lesser extent zooflagellates (Fig. 3d, Supporting Information Fig. S2a). The Chl *a* maximum during summer coincided with high abundances of pico-eukaryotes and nano-eukaryotes, diatoms, dinoflagellates, ciliates, colorless dinoflagellates, and *Synechococcus* sp. (Fig. 3a–c,g–i; Supporting Information Fig. S2b). Coccolithophores, especially *Emiliania huxleyi*, were most abundant during early spring, autumn, and winter months with highest numbers in the October 2015 sample similar to cryptophytes, which also remained abundant during most of the spring and summer of 2016 (Fig. 3e,f). A total of 189 species of phytoplankton and microzooplankton were observed over the 17 dates sampled for either prokaryotic choline or GBT uptake.

Bacteria with high and low nucleic acid content showed similar patterns in abundance with maxima in August and October 2015 and (early) summer 2016 (Supporting Information Fig. S2c,d). Community analysis using 16S rRNA gene

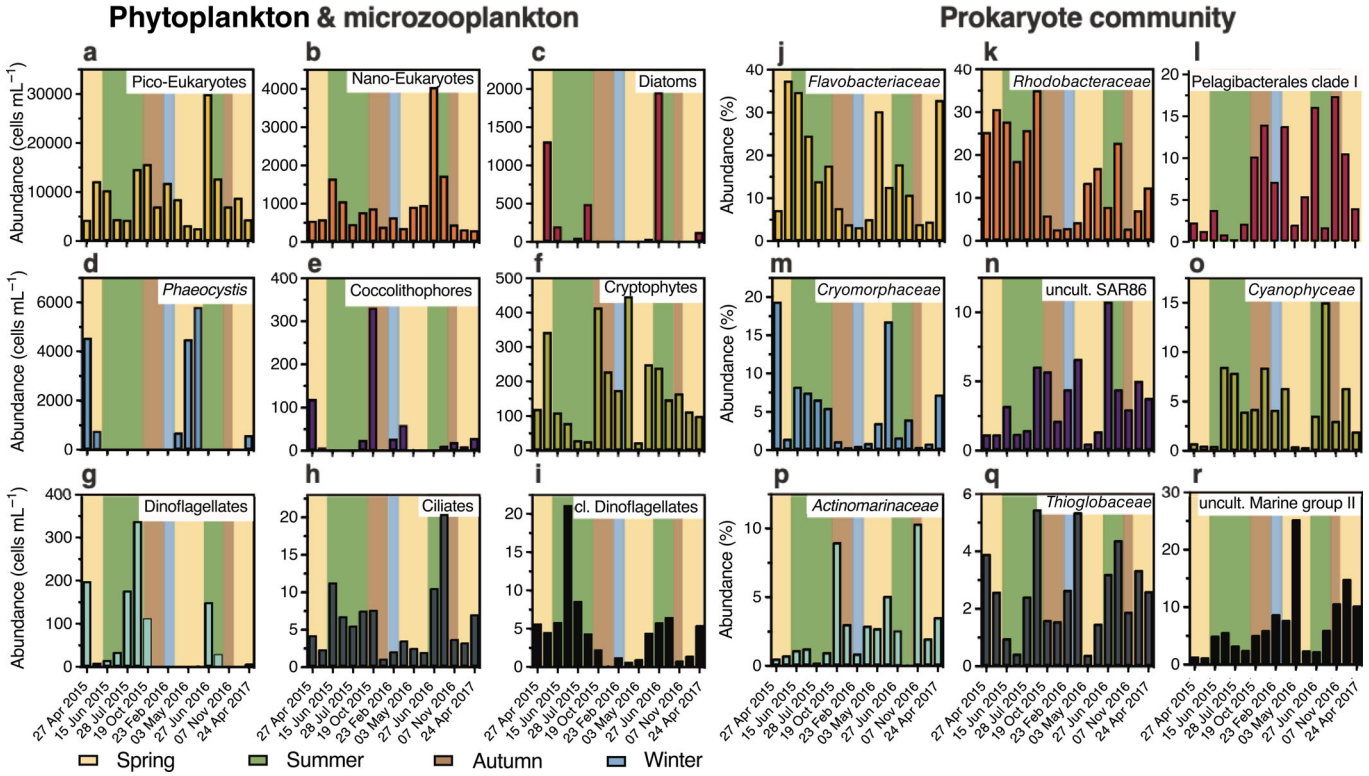

Fig. 3. Abundance of functional groups of phytoplankton and microzooplankton and relative abundance of prokaryotes in surface seawater at Station L4, UK, on selected dates between spring 2015 and spring 2017. (**a**–**i**) Phytoplankton and microzooplankton: (**a**) pico-eukaryotes, (**b**) nano-eukaryotes, (**c**) diatoms, (**d**) *Phaeocystis* spp., (**e**) coccolithophores, (**f**) cryptophytes, (**g**) dinoflagellates, (**h**) ciliates, and (**i**) colorless (cl.) dinoflagellates. Pico-eukaryotes, nano-eukaryotes, cryptophytes, and *Phaeocystis* spp. were determined by flow cytometry, the remaining functional groups by light microscopy counts. (**j**–**r**) Relative abundances of the main representatives of the (**j**–**q**) bacterial and (**r**) archaeal community are presented at family level: (**j**) Flavobacteriaceae, (**k**) Rhodobacteraceae, (**l**) Pelagibacterales clade I, (**m**) Cryomorphaceae, (**n**) uncultured SAR86, (**o**) Cyanophyceae, (**p**) Actinomarinaceae, (**q**) Thioglobaceae, and (**r**) Archaea of the uncultured marine group II. Dates represent time points when choline and GBT uptake assays were performed. Colors represent seasons as defined in Fig. 2.

amplicon sequencing led to the detection of 1975 bacterial and archaeal RSVs from 203 different families (Supporting Information Table S3) with relative abundances of the most numerous families displayed in Fig. 3j–r. The bacterial community present at Station L4 is known to show strong and repeating seasonal dynamics dominated by Alphaproteobacteria of the Rhodobacterales and SAR11 clade (Gilbert et al. 2012; Sargeant et al. 2016). In our samples the two dominant families, Flavobacteriaceae and Rhodobacteriaceae, showed similar seasonal patterns peaking in late spring and summer samples (~ 20–35% of the total community in 2015, ~ 10–20% in 2016) and declining toward autumn and winter (Fig. 3j,k). In contrast, Pelagibacterales (SAR11) clade I bacteria dominated in samples from autumn, winter and early spring reaching ~ 10–17% of the total community (Fig. 3l). Cryomorphaceae mostly occurred in spring (> 15%) and remained abundant during summer 2015 contributing > 5% of the total bacterial community (Fig. 3m). The uncultured marine Group II constituted the most abundant Archaean family accounting for > 5% of the community in autumn to mid spring samples (Fig. 3r).

Biotic and abiotic drivers of choline/GBT uptake

We used three independent approaches of multivariate statistical analysis to determine the key abiotic and biotic parameters which may have affected choline or GBT uptake by the microbial community. (i) GLMs were used to explore the influence of environmental factors (SST, salinity, N, PO_4^{3-}, POC, PON, and Chl *a*). (ii) Using canonical correlation analysis of principal coordinates (CAP_{CCorA}) we preselected potential key players among the eukaryotic and prokaryotic communities, then tested them for a significant contribution to uptake by multiple regression analysis. (iii) We applied db-RDA to investigate relationships between environmental factors and the surface seawater plankton communities sampled for prokaryotic choline/GBT uptake.

Among the selected environmental factors, SST and salinity underwent seasonal or repeating patterns at Station L4 (Smyth et al. 2010) and appeared to be the strongest drivers influencing choline (V_{max}) and GBT (V_{max} and $K_t + S_n$) uptake (Tables 1, 2). No significant relationship between the $K_t + S_n$ of choline and environmental factors was observed during this sampling period (*F*-statistic: 1.23, *p*-value: 0.4080, 6 degrees of freedom [df]), despite it being more than likely that dissolved concentrations of osmolytes are driven by environmental factors, particularly salinity (or SST), which induce osmotic stress. In contrast, SST, salinity, PON, Chl *a*, and the interaction of SST and PON significantly structured V_{max} of prokaryotic choline uptake (*F*-statistic: 46.1, *p*-value: <0.0001, df = 8) (Table 1). For GBT uptake, N and PO_4^{3-} determined $K_t + S_n$ in addition to SST and salinity (*F*-statistic: 6.4, *p*-value: 0.0065, df = 11), and N, PO_4^{3-}, Chl *a*, and the interaction of PON and Chl *a* determined V_{max} (*F*-statistic: 10.5, *p*-value: 0.0018, df = 8) (Table 2). Interestingly, while N was positively linked to GBT uptake, PO_4^{3-} showed a negative estimated coefficient in GLM analysis (Table 2). Model predictions of kinetic uptake parameters are visualized in Supporting Information Fig. S3.

Next, we sought to reveal which members of the plankton community correlated with kinetic parameters of prokaryotic choline and GBT uptake using correlation coefficients obtained by CAP_{CCorA} (Supporting Information Figs. S4, S5). Despite CAP_{CCorA} analyses showing no significant correlation between community abundances and uptake parameters at the level of plankton groups or prokaryote family (Supporting Information Table S4), these analyses allowed the selection of particular community members (Supporting Information Figs. S4, S5) tested for a significant influence on prokaryotic uptake by subsequent multiple regression analysis (Supporting Information Tables S5, S6). Only *Synechococcus* and high nucleic acid-containing bacteria significantly affected prokaryotic choline uptake (inv. $K_t + S_n$), while none of the functional groups structured GBT uptake (Supporting Information Table S5; Fig. S4a,b). Species whose abundance significantly correlated with kinetic parameters of prokaryotic choline uptake belonged to various phytoplankton and microzooplankton groups such as *Phaeocystis*, which coincided with the 2016 spring bloom, multiple diatoms, the coccolithophore *Gephyrocapsa* sp., dinoflagellates (e.g., *Gonyaulax* spp., *Gymnodinium*), colorless dinoflagellates (*Katodinium* or *Noctiluca scintillans*), and ciliates (Supporting Information Table S5). In contrast, GBT uptake seemed only affected by the occurrence of diatoms, most prominently *Thalassiosira* spp. known to produce high concentrations of GBT in culture (Durham et al. 2019; Spielmeyer et al. 2011), colorless dinoflagellates (*Diplopsalis*, *Katodinium*), and ciliates of the genus *Strombidium* (*p*-value: 0.0004) (Supporting Information Table S5). Since phytoplankton were removed for prokaryotic uptake experiments, interactions between them and prokaryotes are most likely indirect, for example, via the release of N-osmolytes.

Several bacterial groups, particularly Rhodobacteraceae appeared to play a critical role for choline uptake and significantly affected V_{max} (Supporting Information Table S6; Fig. S5a). Cultured representatives of Rhodobacteraceae are known to catabolize choline degradation as a sole carbon, nitrogen, and energy source (Lidbury et al. 2015). Interestingly, the OM43 clade, a known group of methylotrophic marine bacteria (Giovannoni et al. 2008), was strongly associated with choline uptake. In contrast, Alphaproteobacteria such as members of the Pelagibacterales (SAR11) Clade I bacteria, and known for GBT catabolism (Noell and Giovannoni 2019), significantly affected prokaryotic GBT uptake (Supporting Information Table S6). In addition, several Gammaproteobacteria also appeared to significantly affect GBT uptake (Supporting Information Table S6), suggesting that their potential in GBT metabolism might have been previously overlooked.

Table 1. Generalized linear regression model (GLM) statistics for choline uptake.

Parameter	GLM			
			Model statistics	
$K_t + S_n$*	Model type	Distr.	*F*-test	*p*-value
	Linear	Normal	1.23	0.4080
	Env. factor	**Est. coeff.**	***t*-statistic**	***p*-value**
	Intercept	−39.1050	−0.1596	0.8784
	SST	−0.0482	−0.1633	0.8757
	Sal	1.2446	0.1762	0.8659
	N	−0.3023	−0.2845	0.7856
	PO_4^{3-}	4.4082	0.2702	0.7961
	POC	−0.0032	−0.2654	0.7996
	PON	0.0803	0.8724	0.4165
	Chl *a*	−1.8283	−2.3816	0.0546

Parameter	GLM				Optimized GLM			
			Model statistics				Model statistics	
V_{max}	Model type	Distr.	*F*-test	*p*-value	Model type	Distr.	*F*-test	*p*-value
	Linear	Normal	17.00	**0.0015**	Interaction	Normal	46.10	**<0.0001**
	Env. Factor	**Est. coeff.**	***t*-statistic**	***p*-value**	**Env. Factor**	**Est. coeff.**	***t*-statistic**	***p*-value**
	Intercept	265.9300	4.1193	**0.0062**	Intercept	339.7300	9.4433	**<0.0001**
	SST	0.2401	3.0893	**0.0214**	SST	0.7047	5.1751	**0.0008**
	Sal	−7.6634	−4.1189	**0.0062**	Sal	−9.9610	−9.2949	**<0.0001**
	N	0.0449	0.1604	0.8778	PON	0.2901	4.0740	**0.0036**
	PO_4^{3-}	−2.3603	−0.5491	0.6028	Chl *a*	0.5449	4.1151	**0.0034**
	POC	−0.0006	−0.1799	0.8632	SST : PON	−0.0159	−3.1986	**0.0126**
	PON	0.0609	2.5103	**0.0459**				
	Chl *a*	0.4012	1.9835	0.0945				

Values in bold indicate significance.
Chl *a*, chlorophyll *a*; coeff., coefficients; Distr., distribution; Env., environmental; Est., estimated; K_t, transport constant; N, nitrite + nitrate + ammonium; PO_4^{3-}, inorganic phosphate; POC, particulate organic carbon; PON, particulate organic nitrogen; Sal, salinity; S_n, natural substrate concentration; SST, sea surface temperature; V_{max}, maximum uptake velocity.
*No optimized model could be found.

Finally, we used db-RDA to investigate whether environmental factors structured the abundance of communities. The statistical parameters of these analyses are summarized in Supporting Information Table S7. Environmental factors significantly affected prokaryote communities both at the family or RSV level on dates sampled for choline (*p*-value: 0.0065, and 0.0105, respectively) and GBT uptake (*p*-values: 0.0001) (Supporting Information Table S7), suggesting that uptake of choline/GBT is strongly influenced by bacterial abundance.

Environmental factors explained ~ 57% of the variability of functional groups of the phytoplankton and microzooplankton community, but only ~ 35% of the variability of plankton species by the first two axes constituting a SST gradient and a nutrient gradient (Supporting Information Fig. S6). For prokaryotes at the family level (Supporting Information Fig. S7a,c), nutrients, salinity, and Chl *a* (as a proxy for biomass) structured the first axis, while the second axis was best described by a SST gradient. SST exerted strong effects on prokaryote families irrespective of the N-osmolyte sampled: Rhodobacteraceae, Haliaceae, and Cyanophyceae correlated positively and SAR11 Clades I and II, Cryomorphaceae, and uncultured Marinimicrobia negatively (Supporting Information Tables S8, S9). This complements previous reports (Gilbert et al. 2012; Sargeant et al. 2016) which showed that Rhodobacterales dominated surface waters of Station L4 during warmer water conditions, while SAR11 was highly abundant in winter. Interestingly, the nutrient gradient of the first axis seemed to also separate sampling dates: samples taken in late spring and

Table 2. Generalized linear regression model (GLM) statistics for glycine betaine (GBT) uptake.

	GLM				Optimized GLM			
Parameter			Model statistics				Model statistics	
$K_t + S_n$	Model type	Distr.	*F*-test	*p*-value	Model type	Distr.	*F*-test	*p*-value
	Linear	Inv. Gaussian	3.75	**0.0419**	Linear	Inv. Gaussian	6.40	**0.0065**
	Env. Factor	**Est. coeff.**	***t*-statistic**	***p*-value**	**Env. Factor**	**Est. coeff.**	***t*-statistic**	***p*-value**
	Intercept	−23.9860	−1.7951	0.1104	Intercept	−31.4770	−2.6262	**0.0236**
	SST	−0.0432	−2.8870	**0.0203**	SST	−0.0407	−2.9009	**0.0144**
	Sal	0.7063	1.8385	0.1033	Sal	0.9153	2.6481	**0.0227**
	N	0.0787	1.5623	0.1568	N	0.1157	2.5302	**0.0280**
	PO_4^{3-}	−1.6368	−2.2916	0.0511	PO_4^{3-}	−1.9602	−2.9970	**0.0121**
	POC	−0.0007	−1.2065	0.2621				
	PON	0.0013	0.2223	0.8297				
	Chl *a*	−0.0008	−0.0135	0.9896				
			Model statistics				Model statistics	
V_{max}	Model type	Distr.	*F*-test	*p*-value	Model type	Distr.	*F*-test	*p*-value
	Linear	Gamma	5.92	**0.0114**	Interaction	Gamma	10.50	**0.0018**
	Env. factor	**Est. coeff.**	***t*-statistic**	***p*-value**	**Env. factor**	**Est. coeff.**	***t*-statistic**	***p*-value**
	Intercept	−95.8560	−3.2963	**0.0109**	Intercept	−101.3800	−5.0105	**0.0010**
	SST	−0.1273	−4.0725	**0.0036**	SST	−0.1493	−5.5177	**0.0006**
	Sal	2.7830	3.3168	**0.0106**	Sal	2.9585	5.0443	**0.0010**
	N	0.3309	3.1695	**0.0132**	N	0.3414	4.7756	**0.0014**
	PO_4^{3-}	−5.0745	−3.4389	**0.0088**	PO_4^{3-}	−5.5424	−5.2387	**0.0008**
	POC	−0.0001	−0.1819	0.8612	PON	−0.0082	−1.6530	0.1369
	PON	0.0026	0.3752	0.7172	Chl *a*	−0.3071	−2.6875	**0.0276**
	Chl *a*	−0.0621	−0.9291	0.3800	PON : Chl *a*	0.0074	2.3519	**0.0465**

Values in bold indicate significance. Abbreviations as in Table 1.

summer correlated with salinity, POC, PON, and Chl *a*, while autumn, winter, and early spring samples aligned with N and PO_4^{3-} (Supporting Information Fig. S7). The db-RDAs suggest that SST, salinity, and a combination of N and PO_4^{3-} together strongly influenced both plankton and prokaryote community structure, which also affected choline and GBT uptake kinetics by the Station L4 microbial community.

Choline and GBT oxidation by seawater microbial community overtime

In addition to analyzing choline and GBT uptake kinetics and biotic and abiotic drivers, we also determined their uptake into particulate cell biomass and total oxidation to CO_2 over 24 h (Supporting Information Table S10). In general, uptake into particulate cell biomass increased linearly over the first 4 h and mostly < 5% of substrate was oxidized to $^{14}CO_2$ within 1 h (except for GBT oxidation in August 2016 and April 2017). This supported our selection of short incubations (10–20 min) for determining uptake kinetics. Interestingly, we observed seasonal variations in the percentage of labeled substrates taken up by the prokaryote community, with lower uptake in the autumn and winter. GBT was generally taken up faster compared to choline (Supporting Information Table S10). Only about 20% of choline/GBT was oxidized to $^{14}CO_2$ in 24 h in spring samples in 2016, suggesting that these compounds may be taken up primarily for osmolyte function (Supporting Information Table S10). In the summer, however, both uptake activity and further oxidation to $^{14}CO_2$ were greatly enhanced, agreeing with the aforementioned GLM analyses showing that SST is a key determinant for choline/GBT catabolism.

Estimation of dissolved choline and GBT concentration in natural seawater (S_n)

We used the approach theoretically outlined by Wright and Hobbie (1966) to determine differences in uptake kinetics for undiluted and diluted seawater to estimate S_n. Calculated concentrations were in the low nanomolar range (choline: undetectable to ~ 0.8 nM, GBT: undetectable to 1.5 nM) (Table 3). To verify our approach, we performed uptake kinetics of samples spiked with a known concentration of choline/GBT and carried out threefold dilution experiments at three

Table 3. Estimation of the natural substrate concentration (S_n) of choline or glycine betaine (GBT) in natural seawater from Station L4, Plymouth, UK.

	Choline			GBT		
Sample	$K_t + S_n$ (nM)	K_t (nM)	S_n (nM)	$K_t + S_n$ (nM)	K_t (nM)	S_n (nM)
11 April 16	3.9 ± 0.8	3.1 ± 1.2	0.8 ± 1.7	1.7 ± 0.2	1.7 ± 0.2	0.0
03 May 16	2.1 ± 0.3	2.1 ± 0.3	0.0	3.8 ± 0.2	3.8 ± 0.2	0.0
10 May 16	2.4 ± 0.7	1.9 ± 0.6	0.5 ± 1.2	2.6 ± 0.2	2.0 ± 1.0	0.6 ± 1.0
27 June 16	2.1 ± 0.1	1.4 ± 0.5	0.8 ± 0.5	1.4 ± 0.1	1.4 ± 0.1	0.0
15 August 16	4.4 ± 0.2	3.8 ± 0.9	0.7 ± 0.9	2.9 ± 0.1	2.0 ± 0.7	1.0 ± 0.7
24 April 17	3.0 ± 0.3	3.0 ± 0.3	0.0	4.4 ± 0.2	nd	nd
14 March 17	3.3 ± 0.4	3.3 ± 0.4	0.0	10.5 ± 0.6	10.1 ± 2.6	0.4 ± 2.8
22 May 17	2.7 ± 0.4	2.7 ± 0.4	0.0	4.0 ± 0.5	2.5 ± 0.4	1.5 ± 0.8

K_t, transport constant; nd, not determined.

sampling dates (Supporting Information Table S11). In general, calculated S_n values largely agreed with substrate concentrations that were spiked into those samples. Although this newly established bioassay warrants further development to improve its accuracy, our data provide the first estimate of dissolved GBT in seawater, suggesting that the standing concentrations of choline/GBT are in the sub-nanomolar range in seawater at Station L4.

Discussion

In this study, we carried out the first seasonal measurements of choline/GBT uptake kinetics in coastal seawater. Our data showed that bacteria possessed high-affinity uptake systems (low nM) for these N-osmolytes throughout the 2-yr study period (2015–2017), with kinetic parameters ($K_t + S_n$, V_{max}, $t_{turnover}$) largely in agreement with the few uptake values available from previous studies in temperate waters (Kiene 1998; Kiene and Hoffmann Williams 1998). Previous choline uptake affinity values from estuarine waters in Mobile Bay (Gulf of Mexico) were 2.9 and 1.7 nM for samples taken in December 1996 and September 1997, respectively (Kiene 1998). Similarly, estuarine and shelf waters off the US east coast had GBT affinity values ranging between 1.2 to 49 nM (Kiene and Hoffmann Williams 1998). These earlier data as well as the measurements we present here suggest that low standing concentrations of these osmolytes in surface seawater necessitate high-affinity transport for uptake and subsequent catabolism of these substrates. Indeed, the half saturation constant for the SAR11 GBT transporter is ~ 1 nM (Noell and Giovannoni 2019).

Our radiotracer uptake approach, besides providing uptake kinetics, also allowed an approximation of the in situ standing concentration of these solutes, that is, S_n, albeit values cannot be separated from the value of the transport constant, K_t (i.e., values are $K_t + S_n$). Nevertheless, uptake kinetic data derived from bacterial communities have previously been used to estimate the boundaries of choline concentrations (from the $K_t + S_n$ value) in Arctic sea-ice brines to be 50–100 nM (Firth et al. 2016), values far above the sub-nanomolar concentrations reported here. This large difference is potentially related to release from sea-ice diatoms, which have high intracellular choline and GBT concentrations (Torstensson et al. 2019), following strong temperature-driven osmolarity fluctuations in the brines (Firth et al. 2016). Our data showed high prokaryotic uptake with rapid turnover resulting in low S_n irrespective of release rates from phytoplankton at an osmolarity much lower than in sea-ice brines. In contrast to those boundary estimates by Firth et al. (2016), actually measuring S_n for choline/GBT is a challenge for oceanographers and the only reported concentrations for choline in coastal seawater (0–45 nM) were quantified from an indirect measurement of hydrogen peroxide formation from choline oxidation (Roulier et al. 1990). Hence, the approach we developed using dilution bioassays to directly determine S_n according to Wright and Hobbie (1966) and validated by spiking with substrate of known concentration should constitute a direct approach with theoretically more accurate results for S_n. The high error in the present data set though, indicates that further methodological improvement is warranted. However, this approach has the potential to provide a useful and sensitive tool for quantifying sub-nanomolar concentrations of solutes in natural seawater. The data we report here represent the first known data set for in situ concentrations of dissolved GBT in seawater.

GLMs showed a significant influence of several environmental factors on prokaryotic uptake of these osmolytes, particularly SST and salinity. The influence of salinity is perhaps not surprising, since it induces osmotic stress and GBT is a well-known compatible solute for marine organisms (Yancey et al. 1982). Previous radiotracer studies already showed that increasing salinity resulted in accumulation of intracellular choline/GBT and favored the conversion of choline to GBT, which was subsequently retained by cells as an osmolyte (Kiene 1998; Kiene and Hoffmann Williams 1998; Firth et al. 2016), while a salinity decrease facilitated respiration of

choline to $^{14}CO_2$ (Firth et al. 2016). It is also known that the optimum temperature for GBT uptake is near to the in situ temperature (Kiene and Hoffmann Williams 1998), supporting our finding that SST significantly influenced microbial choline and GBT uptake at Station L4. In addition, the nutrients nitrogen (in the forms of nitrite, nitrate and ammonium) as well as PO_4^{3-} significantly influenced prokaryotic uptake of GBT based on our GLM analysis, although apparently with opposite effects (positive for N, negative for PO_4^{3-}). Indeed, it is well known that GBT can be utilized as a carbon and nitrogen source by marine bacteria (Welsh 2000), which includes widely distributed bacterial taxa such as Rhodobacterales and Pelagibacterales that are abundant at Station L4 (Sun et al. 2011; Gilbert et al. 2012; Lidbury et al. 2015; Sargeant et al. 2016). Accordingly, our db-RDA showed that SST and macronutrients (N, PO_4^{3-}) were the main drivers for community composition and hence also determined microbial choline and GBT uptake kinetics. Overall, our data support the hypothesis of Kiene and co-workers (Kiene et al. 1998), that the extent osmolytes are retained or catabolized depends on the level of stress either in the form of salinity, nutrient availability, or temperature that a microorganism is exposed to.

The significant correlation of several phytoplankton and microzooplankton groups with microbial uptake of choline and GBT, as found by our multivariate analysis, may seem obvious considering that many marine organisms accumulate and actively produce choline/GBT for osmoprotection (Yancey 2005; Kageyama et al. 2018). Nevertheless, current knowledge of N-osmolyte production in plankton groups is limited and lies beyond the perspective of this study. Only a few cultivated groups have ever been tested for their particulate GBT concentration (Keller et al. 1999; Spielmeyer et al. 2011) and reports for choline are limited to sea-ice diatoms (Torstensson et al. 2019). Both choline and GBT have been found at low fmol cell^{-1} concentrations in sea-ice diatoms (Torstensson et al. 2019) and intracellular GBT has been reported from a couple of diatom cultures (Spielmeyer et al. 2011), supporting the affiliation of diatoms to prokaryotic uptake of N-osmolytes. However, in the few available studies intracellular osmolyte concentrations depend on culture growth phase (Keller et al. 1999) and are influenced by culture conditions, increasing with higher temperature while decreasing at elevated carbon dioxide (Spielmeyer and Pohnert 2012). This could hence explain the differences between reported concentrations and associations observed here. It can further be expected that the phytoplankton and microzooplankton intracellular choline/GBT pool is not immediately available for microbial uptake, because of a delayed release from cells to the DOM pool depending on, for example, cell degradation, sloppy feeding, or viral lysis. Thus, an alternative explanation for the observed correlation pattern might lie in a recent study suggesting that cross-feeding of ammonium between Rhodobacteraceae bacteria and their associated diatoms constitutes a widespread metabolic interaction (Zecher et al. 2020) explaining their co-occurrence. To support this hypothesis, further research would be required, though.

Analysis of correlations between prokaryote community dynamics and choline/GBT uptake offers interesting insights into the cycling of these osmolytes. Choline uptake seemed to be solely driven by the presence of Rhodobacteraceae, which dominate the bacterial community at Station L4 during the spring and summer months (Gilbert et al. 2012; Sargeant et al. 2016). Accordingly, we also observed seasonality in choline uptake parameters (V_{max}, $t_{turnover}$), which were most explicit between spring/summer and autumn samples. Thus, choline uptake might be structured by seasonal dynamics of those bacteria catabolizing choline, a trait prevalent in marine *Roseobacter* clade bacteria (Lidbury et al. 2015). While *Roseobacter* genera are also known to degrade GBT (Lidbury et al. 2015), prokaryotic GBT uptake correlated with other groups instead, among them Gammaproteobacteria and SAR11 (Pelagibacterales) clade I. Pelagibacterales oxidize GBT to CO_2 (Sun et al. 2011). In addition, GBT can be catabolized via GBT demethylation by a GBT demethylase (GbcAB) (Wargo et al. 2008). The presence of multiple bacterial catabolic pathways might explain the observed correlation between several members of the prokaryote community and GBT uptake. If multiple taxa are able to take up and catabolize GBT, this might have implications for seasonality. With different GBT consumer populations present in different seasons, prokaryotic GBT uptake would be less prone to seasonal patterns. Indeed, we found no seasonal pattern of GBT uptake parameters $K_t + S_n$ and V_{max}. Accordingly, while Pelagibacterales apparently dominate at Sta. L4 during the winter, the abundance of Gammaproteobacteria, which showed multiple correlations with prokaryotic GBT uptake, varied throughout the year, independent of temperature and day length (see also Gilbert et al. 2012). Our data thus indicate the existence of yet more overlooked key players in marine GBT uptake and degradation such as the Gammaproteobacteria.

In summary, our data suggest that SST, salinity, and PON function as the main drivers of choline uptake which was largely driven by Rhodobacteraceae and, like their abundance, showed seasonal variation. Rhodobacteraceae are known for their interaction with phytoplankton, especially diatoms (Zecher et al. 2020). Similarly, prokaryotic GBT uptake was influenced mainly by SST, salinity, and macronutrients (nitrogen, PO_4^{3-}). Critically, our data suggest that prokaryotic choline/GBT uptake provides an effective alternative nutrient source for prokaryotes in marine systems and the ability for choline/GBT uptake by marine microbes might be more widespread than previously thought. Given that we are at a tipping point in the climate change crisis (e.g., sea surface warming, expansion of gyres and nutrient depletion in surface waters), more research into the fate of choline/GBT is required, especially since their degradation leads to the formation of climate active trace gases such as methylamine and methane.

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Conflict of interest

None declared.

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