Quantification of glycine betaine, choline and trimethylamine *N*-oxide in seawater particulates: minimisation of seawater associated ion suppression.

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Abstract:

A liquid chromatography/mass spectrometry (LC/MS, electrospray ionisation) method has been developed for the quantification of nitrogenous osmolytes (N-osmolytes) in the particulate fraction of natural water samples. Full method validation demonstrates the validity of the method for measuring glycine betaine (GBT), choline and trimethylamine *N*-oxide (TMAO) in particulates from seawater. Limits of detection were calculated as 3.5, 1.2 and 5.9 pg injected onto column (equivalent to 1.5, 0.6 and 3.9 nanomoles per litre) for GBT, choline and TMAO respectively. Precision of the method was typically 3% for both GBT and choline and 6% for TMAO. Collection of the particulate fraction of natural samples was achieved via in-line filtration. Resulting chromatography and method sensitivity was assessed and compared for the use of both glass fibre and polycarbonate filters during sample collection. Ion suppression was shown to be a significant cause of reduced instrument response to N-osmolytes and was associated with the presence of seawater in the sample matrix.

Keywords:

Glycine Betaine

Choline

Trimethylamine N-oxide

Nitrogenous osmolytes

Seawater particulate analysis

Liquid chromatography mass spectrometry

1. Introduction:

Glycine betaine (GBT), trimethylamine N-oxide (TMAO) and choline are nitrogen-containing osmolytes (N-osmolytes) that are widely used by organisms in the marine environment to maintain favourable osmotic tension and positive turgor [1, 2]. However, other roles for N-osmolytes are beginning to be elucidated. For example, TMAO and GBT interact with photosystem I [3]. Increased recovery rates of photosystem II (PSII) have been observed in a cyanobacterium engineered to accumulate glycine betaine in the cytoplasm [4]. TMAO also stabilizes the folded state of proteins [5]. Furthermore, GBT has been shown to act as a chemoattractant in the marine microbial food web [6].

Knowledge of the distribution of nitrogenous osmolytes among marine phytoplankton is limited to two studies [7, 8] and discrepancies exist between them. For example, Keller et al. [7] did not detect GBT in *Prorocentrum minimum*, but Spielmeyer et al. [8] found *Prorocentrum minimum* to contain the highest levels of GBT of the cultures studied. This could be due to different strains used for the two studies, different culture conditions, or methodological differences. Culture conditions have since been found to affect nitrogenous osmolyte concentrations; the production of GBT by two diatoms and a strain of *E. huxleyi* increased under both elevated temperature and carbon dioxide (CO_2) [9]. Once released from phytoplankton cells, for example by viral lysis, nitrogenous osmolytes become part of the dissolved organic nitrogen pool and are therefore an attractive substrate for marine bacteria [10]. The capacity for choline catabolism is widespread in marine heterotrophs of the marine Roseobacter clade (MRC, [11]), and model organisms of the MRC can grow on choline and GBT as a sole carbon source [11] resulting in remineralisation of osmolyte nitrogen to ammonia. Similarly, MRC have been shown to use TMAO as an energy

source which also resulted in ammonia production [12], and the capacity for TMAO binding in MRC is thought to be widespread [13]. Members of the Pelagibacterales bacteria (SAR11 clade) also have the capacity to degrade TMAO [14]. Marine or estuarine methanogens can also grow on nitrogenous osmolytes [15, 16, 17] indicating a link between quaternary amines and biological methane production in marine environments. Furthermore, marine metagenomic data-mining indicates the presence of genes encoding the production of trimethylamine from quaternary amines in the open ocean [18], providing a possible route and marine biogenic source of atmospheric amines [19], recently discovered to be important for new particle formation [20, 21].

Despite their potential importance in the marine nitrogen cycle, particularly as a substrate for bacteria, and as potential precursors of climate-active compounds, little is known about the standing concentrations of GBT, choline and TMAO in seawater. Choline and GBT can be measured using HPLC with UV detection [22], but the method has limited sensitivity for application to natural samples. LC/MS gives much improved sensitivity for GBT and choline [23], and is a promising approach for all three analytes. Ion chromatography has been used to measure TMAO [24] in aerosol, but the sensitivity of this method is not suitable for application to seawater. TMAO has been measured previously in seawater samples off the Antarctic Peninsula following enzymatic conversion to trimethylamine [25] where it was found to be highest in surface waters, reaching 77 nM [26]. A chromatography method for choline, TMAO and glycine betaine extracted from tissues of marine fish using ion exchange chromatography has been reported previously [27], but is complex due to the use of sequential columns, and has been used to fractionate extracts for subsequent radioactive tracer determination, rather than being directly applied to quantitative analysis in seawater. A range of osmolytes from different matrices have been determined using an LC/MS approach, including mammalian serum [28, 29, 30] and coral tissues [31], but limits of detection in animal tissues and fluids are not sensitive enough for the expected concentrations in seawater [23]. Here, we present an LC/MS for the simultaneous determination of Choline, GBT and TMAO in seawater particulates.

2. Materials and methods:

2.1 Chemicals:

All glassware was acid-rinsed before use with 10% hydrochloric acid (purchased from Sigma Aldrich) followed by MilliQ water. Betaine hydrochloride and choline dihydrogen citrate were purchased from Sigma Aldrich. Trimethylamine N-Oxide.$2H_2O$ was obtained from Fluka. Deuterated GBT (d_{11}-GBT), used as an internal standard (ISTD), was sourced from Cambridge Isotope Laboratories Inc.. Methanol (LC/MS grade), chloroform (HPLC grade), Acetonitrile (HPLC grade), formic acid (LC/MS additive) and ammonium acetate (LC/MS grade) were purchased from Fisher Scientific.

2.2 Preparation of standards

Stock standard solutions of d_{11}-GBT (ISTD) GBT, choline and TMAO were prepared in glass volumetric flasks by weighing aliquots of the solid reference materials and diluting in methanol:chloroform:water (12:5:1). Typical stock standard concentration was 0.5millimoles per litre (mM). When not in use, standards were kept in the fridge (<4^{o}C). When required, stock solutions were allowed to warm to room temperature before serial dilution was performed to generate working standards over the required concentration range.

2.3 Sample collection & extraction:

Seawater samples were routinely collected from Station L4, 10km from the Plymouth coast in the Western English Channel (http://www.westernchannelobservatory.org.uk/). Surface seawater (typically 2-5m depth) was collected aboard the *RV Plymouth Quest* in Niskin bottles attached to a rosette sampler. Seawater was transferred to a 10 L Nalgene sample bottle via Tygon tubing and transported back to the laboratory. Both the Nalgene sample bottle and Tygon tubing were pre-rinsed with seawater prior to use. The Tygon tubing was stored in 10% hydrochloric acid (HCl) when not in use, and rinsed thoroughly with MilliQ water before

sampling. Transfer time back to Plymouth Marine Laboratory after sampling was typically 2 hours.

Approximately 4L of the surface seawater sample was transferred to an acid-rinsed glass beaker through a nylon mesh (pore size 200 μm to remove zooplankton), and stirred gently to homogenise cell distribution via a magnetic stirring plate. Aliquots of seawater (typically 5-100 mL) were removed via a plastic syringe and filtered through an in-line polycarbonate filter (Nucleopore; 47mm, 0.2μm). Before use, filters were soaked in 100% methanol (LC/MS grade) for 2 hours, after which, they were rinsed in clean methanol and allowed to dry at room temperature. After filtration, the residual seawater left on the filter was minimised by blotting the underside on laboratory absorbent paper. The filter was then immersed immediately in 1.5 mL of methanol:chloroform:water (12:5:1) in a 50 mL Sarsdedt© tube. Internal standard (10μl) was added to yield a final concentration of 10 picograms per microliter (pg μL^{-1}) d_{11}-GBT. Samples were briefly vortexed and left to soak for 1 hour. Samples were then re-vortexed and the solvent transferred to an Eppendorf tube for clarification by centrifugation (4 min at 13,000rpm). Finally, the supernatant was transferred via Pasteur pipette to an autosampler vial for LC/MS analysis.

2.4 LC/MS conditions & optimisation:

The LC/MS system comprised an Agilent 1200 High Pressure Liquid Chromatograph (HPLC) incorporating a degasser (G1379B), binary pump (G1367B), temperature-controlled autosampler (G1367B), and thermostatted column compartment (G1316A). The HPLC was coupled to an Agilent 6330 ion trap mass spectrometer via an Electrospray ionisation (ESI) source operated in positive ion mode.

For separation of the analytes a Discovery HS F5 column (150 x 2.1mm, 3μm particles) was used in combination with a guard column (HS F5 Supelguard) both supplied by Sigma Aldrich. The column temperature was maintained at 60 ^{o}C during analysis.

Mobile phase composition comprised (A) 0.15% formic acid in water containing a final concentration of 10mM ammonium acetate and (B) 100% methanol (LC/MS grade) in the ratio 80:20 (A:B), run isocratically at a flow rate of 0.35mL min^{-1} for 6 minutes, with a 20µl injection volume. After use, the column was stored in 100 % acetonitrile and was routinely cleaned according to the manufacturer's instructions.

The LC/MS settings were as follows: nebuliser gas 55psi; drying gas 12 L min^{-1}; vapouriser temperature 350°C. Once protonated, GBT was detected at *m/z* 118, choline at *m/z* 104 and TMAO at *m/z* 76. Notably under the conditions used, TMAO also formed a dimer, detected at *m/z* 151). Deuterated GBT (d_{11}-GBT) used as an internal standard was detected at *m/z* 129 (Figure 1). For extracted ion chromatograms, a 0.5 amu mass window was applied around the respective target ion.

For tuning the detector, a solution of all 4 analytes at a concentration of approximately 1 µM was infused into the LC flow at 5 µL min^{-1} via a syringe pump, just prior to the MS source. The ion optics were tuned for each compound, and optimum settings were typically: capillary -2000 V; skimmer 15 V; capillary exit 79.2 V; octopole 1 DC 6.58 V; octopole 2 DC 0.63 V.

2.5 Calibration:

Instrument calibration was performed on the same day as sample analysis. Standards were freshly prepared in 12:5:1 methanol:chloroform:water from stock solutions, which were found to be stable at 4 °C for at least 4 weeks. Five mixed working standards were typically prepared containing GBT (6, 9, 28, 60 and 600 nM), choline (3, 5, 14, 30, 300 nM) and TMAO (8, 14, 40, 80 and 800 nM). In addition, check standards containing approximately 150nM of each analyte were injected after every 3 samples analysed to demonstrate continued system performance throughout the analytical sequence. Deuterated GBT (d_{11}-GBT) was spiked into all standards and samples as an internal standard (10pg $µl^{-1}$) and the calibration curves plotted as concentration verses the peak area ratio (analyte:ISTD, Figure 2).

3. Results and discussion

3.1 Assessment

This method has been developed and optimised specifically for the co-analysis of GBT, choline and TMAO in the particulate fraction of seawater samples. The efficiency, validity and the suitability of the method to accurately quantify particulate N-osmolytes in natural samples has been investigated, and particular attention has been paid to reducing ion suppression associated with a seawater matrix.

To demonstrate the linearity of the LC/MS system over a wide concentration range, 12 mixed standards containing GBT, choline and TMAO were prepared (0.005-1.3 μM for GBT, 0.003-0.3 μM for choline and 0.006-1.6 μM for TMAO). Duplicate injections of each standard were performed, and the resultant plots of standard concentration versus peak area ratio (analyte peak area/internal standard peak area) plotted with $R^2 > 0.99$ for all three compounds (Figure 2). Natural particulate N-osmolyte concentrations are not expected to exceed these calibrations.

The system showed no carryover between injections, even following high concentration standards. We used an injection programme recommended by Agilent to minimise carryover [32].

Precision of the method and hence its' consistency, was determined by calculating the intraday and interday coefficients of variation (C.V. %) for GBT, choline and TMAO.

The intraday C.V. % was calculated from measurements of two standard solutions at different concentrations, injected six times consecutively. Values of precision ranged between 2-3 % for GBT, were 3 % for choline and 6 % for TMAO.

The interday variance was assessed by injecting a standard, prepared at the same concentration, 20 times over the course of 1 month (5 separate days). We found the reproducibility of this method over the month to be 6 % for both GBT and choline and 8 % for TMAO.

The limit of detection (y_D) for the three analytes extracted by this method was calculated according to

$$y_D = \mu_b + K_D \sigma_b \quad \text{equation.1}$$

and defines the smallest signal response that can be reliably distinguished from the baseline noise of the instrument [33]. Where μ_b is the population mean, K_D is 3 (relating to the fact that sample signal must be 3 times the baseline noise to be classified as a 'positive' result), and σ_b represents the population standard deviation.

Baseline peak widths for GBT, choline and TMAO were determined from three standards at different analyte concentrations and subsequently averaged. Ten separate sections of baseline noise were then integrated on 3 different standard injections spanning the widths previously determined for each analyte. This resulted in 10 peak area responses for baseline noise which were subsequently averaged to give the population mean (μ_b) and standard deviation (σ_b). These values were used with equation1 to generate a limit of detection (y_D) which was then converted to an analyte concentration using calibration curves. The limit of detection for GBT, choline and TMAO using this method was 3.5, 1.2 and 5.9 pg/injection (1.5, 0.6 and 3.9nM) respectively. This is an improvement on the sensitivity of GBT and choline detection reported in Airs & Archer [23]. The TMAO LOD is similar to that reported in Gibb and Hatton [26](2nM).

To demonstrate a sample blank, a clean, pre-rinsed filter (no sea water) was extracted in the same manner as sample filters. Internal standard was always present with the correct peak area and no GBT, choline or TMAO was detected. This highlights that there is no contamination from the extraction procedure and that both the extraction solvents and the LC/MS system are clean. Results obtained from sample extractions with concentrations >LOD are therefore assumed to be positive signals for N-osmolytes contained within the particulate fraction of natural samples.

To avoid unwanted or unknown analyte deterioration, which would adversely affect the peak area ratio, standard stability was assessed. Stock solutions of the 4 analytes were prepared and

subsequently used to produce a working standard (a 100 times dilution of stocks) which was made fresh on each test day. The stock solutions were analysed 17 times over the course of 50 days following initial production and the response of the analyte and internal standard used to calculate the peak area ratio in each instance. For GBT a mean peak area ratio (standard deviation) of 0.9(0.05) was observed; for choline 0.5(0.04) and 0.1(0.004) for TMAO. This generated a coefficient of variation (C.V.) of 5%, 8% and 7% respectively, similar to our interday precision data. Stock solutions were therefore freshly prepared on a monthly basis.

3.2 Application to natural samples

Surface sea water was collected from coastal Station L4, in order to test the methods applicability for marine samples.

Previous work shows that the filtration technique employed to separate the particulate material from bulk sea water can have a pronounced effect on the osmolyte concentration observed [23, 34]. Significant differences in concentrations derived from gravity versus vacuum filtration are reported, especially for choline, likely due to cell breakage and subsequent loss of osmolyte to the dissolved phase. Thus, vacuum filtration was not employed in the development of this extraction procedure. Instead, an in-line filter, designed to minimise sample contact with laboratory air was employed, thereby reducing cell damage via desiccation.

After sample filtration, filters were transferred directly into extraction solvent and were left to soak for 1 hour for osmolyte extraction. A comparison with filters allowed to soak in extraction solvent overnight (in the dark and at $<4^{o}C$) was made in case 1 hour was not sufficient for this process. The comparison tests were carried out using polycarbonate filters and 50mL aliquots of L4 surface sea water.

For GBT, the results between same day and overnight extraction showed no significant difference at the 95% confidence level (n=3) indicating that one hour in extraction solvent is

sufficient for GBT abstraction from particulate material and that storage overnight does not affect the stability of GBT in solution. However, for choline, only 1 of triplicate samples showed a positive result after overnight extraction, but all three were positive after 1 hour. Further investigation by increasing the number of samples stored overnight (n=15) showed that choline was not detected in 80% of the samples suggesting that choline was not stable in the extraction matrix over a period of approximately 18 hours. For TMAO, the average concentration of triplicate samples extracted overnight was the same as those extracted for 1 hour. However the standard deviation for the data from the overnight extractions was higher at 6nM compared to 0.3nM for the 1 hour extracted samples, suggesting increased variability in the samples extracted for longer. Tests showed that stock standard solutions of all three osmolytes were stable when stored in the fridge for periods of up to 51 days (see above). Therefore, either biological or chemical processes linked to the sample matrix may be altering the choline and TMAO content during overnight extraction. The latter is more probable as the extraction solvent is likely to prevent biological processes from remaining active following filtration. A possible chemical reaction may be adduct formation with other available ions in the solution matrix thereby altering the mass of the desired osmolyte so that they are no longer detected at m/z 76 (TMAO) and 104 (Choline).

Ion suppression is commonly observed during LC/MS analysis of components extracted from a seawater matrix [34]. To explore the potential ion suppression of GBT, choline and TMAO with this method, a series of 6 standards with different proportions of filtered sea water from 0-8 % were analysed. The final concentration of each standard was kept identical. The signal response for these standards with increasing amounts of sea water in their matrix showed a striking effect on analyte response (Figure 3A). As the proportion of seawater in the standards increased, a drop in signal response for all analytes and deterioration of peak shape was observed (Figure 3A). Additionally, the signal response of d_{11}-GBT in these standards was inversely related to the percentage of seawater in each matrix (Figure 3B; P<0.001, students t-test, 95% confidence level). A similar significant relationship was observed with GBT (P<0.001).

Standards with a seawater content of ≥ 1.5% showed significant reductions in sensitivity. At 3% seawater, neither choline nor TMAO could be integrated due to complete deterioration of peak shape. This is in contrast to the work of Spielmeyer et al. [35] who report improved chromatography in saline matrices when using a hydrophilic interaction liquid chromatography (HILIC) column to measure DMSP in algal cultures.

To further investigate the effect of ion suppression, local seawater was sampled using four sets of filters, chosen for their differing seawater retention: polycarbonate (47 and 25 mm) and glass fibre filters (GF/F, 47 and 25 mm) filters. Before use, each filter type was assessed to determine its seawater retention capacity. Briefly, filters were weighed, soaked in filtered seawater for 5 min, held in the air for 10 seconds, and re-weighed to calculate the volume of seawater retained. Glass fibre filters held considerably more seawater than polycarbonate filters (Table 1).

Equal volumes of fresh seawater were passed through each filter type through an in-line cartridge, before transferring the filter to extraction solvent and adding internal standard solution. After extraction, the extracts were analysed by LC/MS. The response of ISTD was affected drastically by the filter type, and hence the proportion of seawater contained in the extract (Figure 4). Increasing the diameter of the GF/F filters from 25 to 47mm caused the response of d_{11}-GBT to decrease by 90%. The same test with polycarbonate filters saw a drop of only 13%. Direct comparison shows that the d_{11}-GBT response from using GF/F filters was reduced by 54 and 95% compared to the response obtained using PC filters, for 25 and 47 mm filters respectively.

To further demonstrate the ion suppression effect that was caused by the proportion of seawater in sample extracts, 10 mL aliquots of fresh local seawater were filtered through 47 mm GF/F filters. Three filters were extracted in 5, 7, 10, 12 and 15mL extraction solvent. The response of the ISTD observed was 3 times higher in the largest volume extract compared to the smallest, despite being present at the same concentration. However, the response of the d_{11}-GBT in the 15mL extraction solvent (which contained the smallest proportion of seawater) achieved

only 49% of the response from a standard solution containing the same concentration of internal standard and no filter or sea water. Figure 5 shows the relationship between the increasing volume of extraction solvent used (and hence the decreasing percentage of sea water in the matrix) and the observed increase in response of internal standard due to reduced ion suppression.

Residual seawater residing on the filter following sample filtration can be minimised by blotting the underside on laboratory absorbent paper. However, even if the seawater retained by a 47mm GF/F filter was halved by blotting, 31mL extraction solvent would need to be added to the filter in order to maintain 1.5% sea water and hence retain signal response (Figure 3A). This value increases to 93mL extraction solvent to reduce the proportion to 0.5% sea water. Increasing the extraction solvent volume by these amounts would significantly reduce the sensitivity of the method or would require a lengthy evaporation step to be incorporated.

Although polycarbonate filters retain much less seawater than GF/Fs (Table 1), a disadvantage is that they were found to provide another, direct source of contamination which also caused ion suppression of the target analytes. During extended analytical sample runs (>10 sample injections involving the use of PC filters) ions at *m/z* 177.0, 213.9 and 222.9 gradually began to increase, of which the latter dominated (Figure 6). The polycarbonate filters were found to be the source of these contaminating ions. The elution of *m/z* 222.9 (from 1.4-1.9 minutes) spanned the retention time of both d_{11}-GBT and GBT (1.6mins; Figure 6) and dominated the mass spectrum causing ion suppression of the target analytes. The mobile phase ((A) 0.15% formic acid in water containing a final concentration of 10mM ammonium acetate and (B) 100% methanol (LC/MS grade) in the ratio 80:20 (A:B)) was not suitable to elute the contaminating components quickly. After the first injection of an extract that had been in contact with a PC membrane, the components were found to elute (and therefore suppress the analyte signal) after a consistent number of injections (24-26). Methanol (100%) was found to efficiently remove these ions from the LC system. Therefore after a set of 6 sample injections a methanol

311 wash programme was employed to prevent these ions from interfering with subsequent
312 analyses. This comprised a 25 minute run starting and ending with normal mobile phase
313 conditions (0.15% formic acid in milliQ + 10mM ammonium acetate:methanol, 80:20) but
314 maintaining 100% methanol for 15 minutes in-between. During this period the MS source was
315 diverted to waste to minimise source contamination. Furthermore, polycarbonate filters
316 themselves were prewashed in 100% methanol for 2 hours prior to use (see Methods). After
317 this period they were rinsed in clean methanol and left to dry at room temperature. Figure 6
318 shows the reduction in the intensity of *m/z* 222.9 ion between extractions of unwashed and
319 washed polycarbonate filters. Use of the methanol wash programme and pre-washing the
320 polycarbonate membranes before use prevented suppression by the contaminating ions (Figure
321 6B).

322 The analytical method was mostly developed using seawater samples collected from Station L4.
323 On the days where adverse weather conditions prevented travel to L4, sea water was collected
324 by hand from Millbay Marina, close to Plymouth Marine Laboratory. An LC/MS chromatogram
325 generated from particulate extraction of both Marina and Station L4 seawater (50mL) shows
326 clear peaks for GBT, choline and TMAO at the expected retention times (Figure 7A and B and
327 Figure 1 respectively). Particulate N-osmolyte concentrations are likely to be subject to large
328 variability which may be dependent on location and/or season. Further work is required to
329 determine whether these compounds have a seasonal signal and if they do, which
330 environmental variables are likely to be driving particulate concentrations.

331

332 ***4. Conclusions***

333 Accurate analytical determination of N-osmolytes is critical to understanding their contribution
334 to the marine nitrogen cycle and their role as potential precursors of climate-active compounds.
335 The sensitivity of this method at the low nanomolar range permits its use for studies into the
336 cycling of N-osmolytes in the marine environment. Low limits of detection for these compounds

means that subtle changes to concentrations can be measured. Furthermore, the wide linearity range achieved enables easy adaption to low and high N-osmolyte concentrations, and reduction of sample volume below 50 mL which may be important for fragile cells [36]. The extraction procedure is simple, relatively fast and is convenient for consecutive sample filtrations, thereby maximising the number of samples that can be processed daily. The lack of derivatisation or chemical transformation steps in this analytical procedure reduces both lengthy analysis times and possible analyte loss. Additionally, the stability in retention time and reproducibility of the standards over time suggests that the column is robust and well-suited to this application providing continued confidence in the sample data generated.

Ion suppression has been shown to be detrimental to both data quality and method sensitivity. The presence of sea water in the extraction matrix was a direct cause of ion suppression that significantly increased the detection limit of this method. For this reason polycarbonate filters are recommended for use with seawater samples due to their decreased water retention which maintains a low seawater to extraction solvent ratio. Polycarbonate filters should be washed in methanol to prevent co-extraction of contaminating components.

The sensitivity of this technique holds promise for quantification of N-osmolytes extracted from the dissolved phase of bulk seawater which is important for understanding turnover rates of these compounds. The sensitivity of the technique may also permit determination of N-osmolyte concentrations in natural populations of phytoplankton sorted by flow cytometry. Such information would contribute to modelling studies designed to determine the main drivers of N-osmolyte fluctuations in the marine environment enabling their inclusion into ecosystem models such as ERSEM.

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Table 1: Seawater retention of glass fibre filters (GF/F) compared to polycarbonate (PC) filters

Filter material	Filter size (mm)	Mass of water retained (g)	Volume of water retained (mL)
GF/F	47	0.95	0.93
GF/F	25	0.22	0.22
Polycarbonate	47	0.11	0.11
Polycarbonate	25	0.015	0.015

n=3 for each filter type/size. Density of seawater used to calculate volume = 1.02 g cm^{-3}. Where GF/F represents glass microfiber filters of grade GF/F.

Table 2. N-osmolyte concentrations (nmol/L) in marina verses coastal seawater

	Millbay Marina nmol/L filtered sample (Sept' 2015)	Station L4 nmol/L filtered sample (Feb' 2016)
GBT	9.2 (±0.2)	0.9
Choline	0.5	0.2
TMAO	6.9	Not Detected

Sample volume filtered in both cases was 50 mL. Method was as described in the text. Result in nmol/L is essentially the concentration of N-osmolytes extracted from cells contained within 1L sample.

Figure Captions:

Figure 1: Typical extracted ion chromatograms from the LC/MS analysis of a standard solution containing (A) d11-GBT as an internal standard (m/z 129), (B) GBT (m/z 118), (C) choline (m/z 104), (D) TMAO (m/z 76) and (E) TMAO dimer (m/z 151).

Figure 2: Linearity for (A) GBT over the range 0.007-1.3μM; (B) Choline over range 0.003-0.25μM and (C) TMAO over 0.008-1.6μM. Standards all run in duplicate. Error bars denote ±1 standard deviation.

Figure 3: Effect of sea water on analyte response for (A) GBT standards of the same concentration (0.07μM) but with increasing proportions of sea water in the matrix (0-8% sea water) and (B) for d_{11}-GBT showing a significant negative relationship ($P<0.001$) between peak area response and increasing percentage of sea water in the standard solution. Please refer to text for LC/MS conditions.

Figure 4: Response of d_{11}-GBT internal standard (ISTD) in extracts of particulates from seawater collected on glass fibre (GF/F) filters (25 and 47mm) and polycarbonate (PC) filters (25 and 47mm).

Figure 5: Relationship between increasing extraction volume (hence decreasing proportion sea water in sample matrix) and increasing internal standard (ISTD) response. Tests were carried out with 47mm GF/F filters, 10mL seawater filtered. Error bars denote 1 standard deviation.

Figure 6: Unwashed versus methanol washed polycarbonate, 47mm filters. (A) Extracted ion chromatogram (EIC) showing typical response of *m/z* 223 which extracted from PC filters and was observed at intensities as high as $1x10^8$, and (B) resultant full mass spectrum. (C) EIC of a

539 typical *m/z* 223 response following extraction of a methanol washed polycarbonate filter
540 (intensity was reduced to $1x10^6$), and (D) resultant full mass spectrum.

541

542 Figure 7. Extracted ion chromatograms showing N-osmolytes in the particulate phase of marina
543 seawater (A) and seawater sampled from Station L4 (B). Internal standard d_{11}-GBT at *m/z* 129,
544 GBT at *m/z* 118, choline at *m/z* 104, TMAO at *m/z* 76 and TMAO dimer at *m/z* 151. Samples are
545 representative of particulate N-osmolytes from 50mL of surface marina and coastal seawater
546 collected on a 47mm, 0.2µm, pre-rinsed polycarbonate filter and extracted as per the method
547 detailed in main text. NB., no TMAO or TMAO dimer was detected in the sample collected at
548 Station L4 (B).