The following supplements accompany the article

Uptake of dissolved organic and inorganic nitrogen in microalgae-dominated sediment: comparing dark and light *in situ* and *ex situ* additions of ^{15}N

Philip M. Riekenberg*, Joanne M. Oakes, Bradley D. Eyre

*Corresponding author: phrieken@gmail.com

Marine Ecology Progress Series 571: 29–42 (2017)

Supplementary Data

Supplement 1:

Sample analysis

Sediment characterization

The surface layer (0-1 cm) was comprised of 3% coarse sand (500 µm – 1 mm), 25% medium sand (250-500 µm), 66% fine sand (125-250 µm), 5% very fine sand (63-125 µm), and 1% mud (<63 µm). Deeper sediment (1-3 cm) contained less medium sand (19%) and mud (0.5%), but more fine sand (73%).

Sediment KCl extraction

Sediments were freeze-dried, homogenized and extracted with 2 M KCl to remove adsorbed inorganic and organic N. Briefly, sediment for KCl-extraction (2 g dry weight) was placed in a centrifuge tube with 5 ml of 2 M KCl, shaken, and centrifuged (15 min, 9 *g*). The supernatant was removed and three times thereafter 5 ml of milli-Q was added before shaking, centrifugation (15 min, 9 *g*), and removal of the supernatant. The washed KCl-extracted pellet was then dried at 60°C and weighed into a tin capsule for analysis of $\delta^{15}N$ and %N using a Flash elemental analyzer coupled on-line to a Thermo Fisher Delta V Plus IRMS. Reproducibility of $\delta^{15}N$ values for samples with $\delta^{15}N$ enrichment < 100 ‰ was ± 0.2 ‰. Precision decreased with enrichment beyond 100 ‰.

N removed from control sediment during KCl extraction was calculated using concentration values from both raw and KCl extracted sediment as $N_{removed}$ = Raw – KCl, where Raw is the concentration of N (µmol/g) of untreated sediment and KCl is the concentration of N (µmol/g) in KCl extracted sediment. This represents a potential maximum estimate for porewater N as the recovered N includes both porewater N as well as N bound to the sediment.

HAA extraction

A subsample of sediment from each sample and aliquots of the DON treatment solution (DON 125 and DON 250) were analyzed for concentration and $\delta^{15}N$ of hydrolysable amino acids according to the method outlined by Veuger et al. (2005). Freeze dried sediment (7 g) was rinsed with 2M HCl, centrifuged (5min, 900*g*), and the supernatant discarded. This was repeated with milli-Q water 3×, and the sediment pellet and DON treatments were then hydrolyzed with 6 M HCl (110°C, 20 h). After hydrolysis, a standard spike of L-norleucine (2.5 mg ml^{-1}) was added and the sample shaken and centrifuged again with the supernatant being removed and retained. The pellet was then resuspended in 10 ml milli-Q water, centrifuged 2×, and the additional supernatant retained. The combined supernatant (25 ml) was

purified through cation exchange chromatography (Dowex 50WX8-100), amino acids derivatized with isopropanol and penta-fluoropropionic anhydride, and further purified via solvent extraction with chloroform. Concentrations and $\delta^{15}N$ of the derivatized amino acids were determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) on a HP 6890 GC interfaced via a Thermo Conflo III interfaced with a Thermo Delta V Plus IRMS.

Statistical evidence for sample independence

Brown-Forsythe homogeneity of variance (HOV) tests were performed to compare C biomass calculated from Chl-α between subplots within each treatment and treatments and found no statistically significant decrease in the variance between subplots and treatments ($DF_{1,25}$ F= 0.01 to 3.30, p= 0.08 to 0.89), or controls ($DF_{1,25}$ F=1.8 p=0.3). Brown-Forsythe HOV was also used to compare the TN removed via KCl extraction in the control cores ($DF_{2,6}$ F=0.9 p=0.5) and within treatments ($DF_{7,16}$ F=0.8 p=0.6) and found no statistically significant differences. Brown-Forsythe HOV was selected due to this analysis being more robust to differences in sample size than Levene's HOV. All in situ sediment samples incorporated 44 cm^3 of sediment (0-1 cm depth) that was homogenized before sample analysis for each individually labeled subplot. This sample size should sufficiently incorporate smaller-scale heterogeneity that exists in the microbial community within site sediments for the assessment of short-term uptake of ^{15}N. The carbon:Chl-α ratio may have varied between sampled sediments, but uncertainty introduced by this is unlikely to affect conclusions, as MPB biomass was comparable between all sediment sampled at the site using this method.

Preparation of and characterization of ^{15}N-labeled DON

^{15}N-labeled DON ($DO^{15}N$) was extracted from an axenic culture of the diatom *Thalassiosira pseudonana* (CSIRO, ANACC Culture CS-20). Diatoms were batch-cultured within 3 L Fernbach flasks containing 2 L of artificial seawater amended with F/2 growth medium (Guillard & Ryther 1962) and 20 mg 99% $^{15}NH_4Cl$ (Cambridge Isotope Laboratories NLM-467). Cells were harvested after 10 d via centrifugation (7 min, 900*g*) within 50 ml centrifuge tubes and the supernatant removed. Cells were re-suspended with DIN-free artificial seawater and centrifuged 3 times (7 min, 900*g*) to remove unincorporated extracellular DIN. Cells were then lysed in acidified milli-Q (5 drops 6N HCl in 10 ml) to remove inorganic carbon (necessary for an experiment which was not part of the current study). Cells were further lysed through freezing (24 h) and were then thawed, resuspended in milli-Q (additional 20 ml), sonicated (5 min), and centrifuged (7 min, 900*g*). The supernatant (30 ml) was filtered (precombusted 0.45 µm) to remove non-dissolved components, then passed through an ion retardation column (20 cm × 3 cm) containing 100 ml of resin (Bio-Rad AG 1-X8 50-100 mesh) prepared according to Hatch et al. (1957). The column removed charged ions, specifically intracellular NH_4^+ and NO_3^- released during cell lysis, and ions from the artificial seawater (predominately Na^+, Cl^-). This method is detailed in Bronk and Glibert (1991). Briefly, 30 ml of filtered algal supernatant was passed through the column, rinsed with 70 ml of milli-Q, with the eluent collected and concentrated via gentle boiling. Between samples, the column was regenerated with 100 ml of 1 M NaOH, then 2 L of milli-Q to flush removed ions and NaOH from the column. $\delta^{15}N$ values for the extracted $DO^{15}N$ were determined by drying an aliquot onto 1 cm glass fiber filters (Whatman GF/F). Triplicate samples were analyzed using a Flash elemental analyzer coupled on-line to a Thermo Fisher Delta V Plus isotope ratio mass spectrometer (IRMS). The $DO^{15}N$ had a ^{15}N atom percent value of 47.9 ± 0.2 and contained a small concentration of remaining DIN (0.6 µmol N L^{-1}), accounting for <0.02% of the ^{15}N present. DON (µM) was quantified via measurement of total dissolved nitrogen

through flow injection analysis using persulfate digestion (Valderrama 1981) and subsequent subtraction of DIN concentrations (Lachat 1994).

The small quantity of unincorporated ^{15}N-labeled DIN that remained in the DON treatment solution represented 0.02% of the nitrogen added within both DON additions. Incorporation of 100% of this contaminating DIN is unlikely, but could account for only <8% of the ^{15}N uptake observed in the highest DON treatment (DON-250) in slurry incubations. Contaminating DIN is therefore likely to have made a negligible contribution to ^{15}N uptake within the DON treatments.

Supplement 2:

Biomass calculations

During hydrolysis, some racemization of L-alanine (Ala) to D-Ala occurs, resulting in a D- : L-alanine ratio of 0.015-0.02 for algal cultures (Veuger et al. 2007) and 0.006 for dissolved free amino acids (Kaiser & Benner 2005). In the current study, uncorrected D/L-Ala ratios as low as 0.0062 were obtained, suggesting a low racemization rate for the study sediments. The lowest racemization value reported for liquid-phase hydrolysis-induced racemization of amino acids was therefore applied (0.006; Kaiser and Benner 2005). This corresponds with values obtained for racemization of L-Ala standard during hydrolysis under our laboratory conditions. Bacterial ratios of D/L-Ala content were assumed to be 0.05, which is at the lower end of the range reported within Veuger et al. (2007) (0.05 to 0.1). This assumes a negligible contribution of cyanobacteria and gram-positive bacteria, which have been shown to contribute a ratio closer to 0.1 due to thicker peptidoglycan layers, and is consistent with visual observations of few cyanobacteria and a low maximum D/L-Ala ratio (0.04).

Due to the considerable effect that racemization may have had on the relatively small amount of D-Ala obtained in this study, concentrations of D- and L-Ala were corrected prior to the calculation of bacterial contribution to ^{15}N uptake in order to more accurately account for original concentrations of D- and L- Ala using the equations of Kaiser and Benner (2005):

1. $L^*_0 = (L^* - D^* (0.6)) / 1 - 0.6$
2. $D^*_0 = D^* + L^* - L^*_0$

   Where L^*_0 and D^*_0 are the original concentrations of L- and D-Ala, and L^* and D^* are the measured concentrations of L- and D-Ala. Original concentrations of D- and L- Ala represent concentrations of both forms of alanine prior to hydrolysis induced racemization. The value of 0.6 represents the racemization factor associated with the free amino acid alanine during acid hydrolysis, as reported by Kaiser and Benner (2005).

   Biomass of HB, BMA, and total microbial biomass were calculated as described by Veuger et al. (2005) and the corresponding update (Veuger et al. 2007):

3. HB L-Ala = D^*_0-Ala × 20

   Where 20 represents the conversion factor used to account for excess ^{15}N present in D-Ala representing 5% of the total excess present within bacterial L-Ala. This conversion factor is based on culture analysis and literature values presented in (Veuger et al. 2005, Veuger et al. 2007).

4. HB contribution to microbial biomass (%) = (HB L-Ala / L^*_0-Ala) ×100
5. BMA contribution to microbial biomass (%) = 1 – HB contribution to microbial biomass
6. HB biomass = D^*_0-Ala × 400

Where the conversion factor 400 represents the % N in dry bacterial biomass (12%; Madigan et al. 2000), yielding a D-Ala content of 0.25% and a conversion factor of 400 × (Veuger et al. 2005).

7. BMA biomass = (HB biomass / (HB contribution to microbial biomass) × BMA contribution to microbial biomass
8. Total microbial biomass = HB biomass + BMA biomass

^{15}N uptake into HB, BMA, and total microbial biomass were calculated using equations 3-8, substituting excess ^{15}N values for L^*_0- and D^*_0-Ala in place of L^*_0- and D^*_0-Ala contents. To determine hourly uptake rates for intact sediment and slurry incubations, the excess ^{15}N within total microbial biomass was divided by 4 and 24 hours, respectively, based on the estimated time available for ^{15}N incorporation. For intact sediments, this was the 4 hours before tidal inundation and flushing occurred.

Investigation of error associated with individual parameters within this method indicated that a 1% change in the D/L- Ala ratio results in a 9.4% shift in bacterial biomass, a 5.3% shift in bacterial ^{15}N enrichment and a 16.7% shift in ^{15}N in microbial biomass. A 0.1% shift in the racemization factor resulted in a 1.9% shift in bacterial biomass, a 2.5% shift in bacterial enrichment, and a 0.01% shift in ^{15}N in microbial biomass.

Supplement 3:

Statistical analysis

For intact sediments, three-way ANOVAs determined the effect of treatment (DON-125, DON-250, DIN-250, DIN-500), light exposure (light or dark), and depth (0-1 cm, 1-2 cm, 2-3 cm) on total ^{15}N uptake into sediment organic matter (OM) and microbial nitrogen production over the 24 hour study. For total ^{15}N uptake into sediment OM, there was a significant interaction between treatment and depth, so three one-way ANOVAs were used to determine treatment effect. Data for one-way ANOVAs was transformed (ln(x+1)) to improve homogeneity of variance, due to statistically significant Levene's tests (Table S1). Where Levene's test was significant, alpha was reduced to $\alpha = 0.01$ to minimize the chance of falsely rejecting the null hypothesis.

Hourly ^{15}N uptake into sediment OM, HAAs, microbial biomass, bacterial contribution to ^{15}N uptake, and Chl-*a* was only determined for 0-1 cm. Due to the lack of a depth component, two-way ANOVAs were used to investigate the effect of light exposure and treatment on each of these parameters. Analyses were independently performed for treatment applications within each method of application (intact sediment and *ex situ* slurry) (Table S1). Significant two-way interactions were explored using one-way ANOVAs for each light exposure and treatment separately. Where ANOVAs indicated a significant difference ($p<0.01$), post hoc Tukey tests were used to test for significant differences ($\alpha=0.01$).

Differences in total and hourly ^{15}N uptake into sediment OM, HAAs, microbial biomass bacterial contribution, and microbial N production between intact sediment and slurry incubations were investigated using two-way ANOVAs, with factors of treatment and *in situ* or *ex situ* application (Table S2). One-way ANOVAs were used to explore significant interactions between treatment and intact sediment or *ex situ* slurry application. Where Levene's test was significant, alpha was reduced to $\alpha = 0.01$ to minimize the chance of falsely rejecting the null hypothesis. Where ANOVAs indicated a significant difference ($p<0.01$), post hoc Tukey tests were used to test for significant differences ($\alpha=0.01$). For microbial N production, a significant interaction was indicated between treatment and intact sediment/slurry, so two sample t-tests were used to explore the differences between application methods for each treatment. All

statistical tests comparing between intact sediment and *ex situ* slurry applications are listed in Table S2.

Supplement 4:

Downward transport of ^{15}N

Uptake of ^{15}N into 0-1 cm sediment OM (55.0 ± 11.1% of the applied ^{15}N within 24 h) was substantially lower than reported for laboratory incubations (e.g., 100%, Veuger and Middelburg 2007; 90%, Veuger et al. 2012), probably due to the loss of unincorporated and reworked ^{15}N via tidal flushing. Despite substantial uptake, the downward transport of ^{15}N within sediments was minimal (<13% below 1 cm after 24 h for all treatments, Fig. S1), with rates similar to those observed in laboratory incubations of sub-tidal sediments over the same period (Evrard et al. 2008) and far lower than observed in situ in sub-tidal sediments in the adjacent Brunswick Estuary (32% below 2 cm within 60 h; Eyre et al. 2016). This probably reflects the removal of ^{15}N from surface sediments via microbial re-working and tidal flushing, decreasing the opportunity for downward transport. This N loss from in situ sediment cannot be adequately addressed using ex situ incubations.

The bulk of the uptake that occurred within sediment within 0 to 3 cm occurred in the uppermost 0-1 cm layer, with 87% of the total ^{15}N recovered occurring within that layer (Fig. S1). Total N of intact sediments at each 1 cm depth increment was similar for control and ^{15}N-labeled intact sediment (0-1 cm depth: 14.8 ± 2.0 and 13.2 ± 1.1 µmol N g^{-1}, respectively; 1-2 cm: 8.8 ± 0.5 and 6.3 ± 0.4 µmol N g^{-1}; 2-3 cm: 8.2 ± 0.1 and 6.7 ± 0.7 µmol N g^{-1}). Within 0 to 1 and 1 to 2 cm depths, total uptake of ^{15}N into sediment OM showed significant differences between treatment applications (Table A.1 & A.2), with more DIN-500 being taken up than all other treatments in 0 to 1 cm and 1 to 2 cm depths and DIN-250 being taken up in a higher, but comparable rate to both DIN-500 and DON-250. No significant differences were observed between treatments in the 2 to 3 cm depth.

Supplement 5:

Supplemental figures and tables

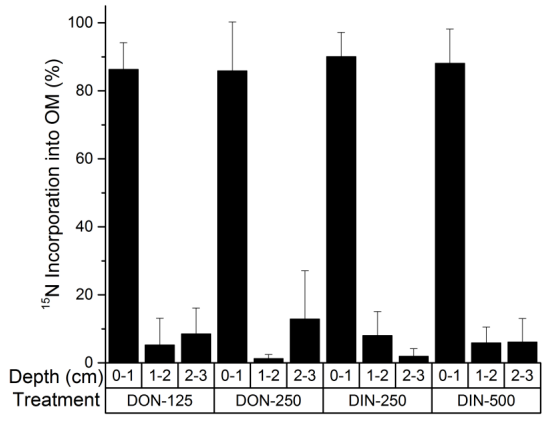

Figure S1: Percentage of excess ^{15}N incorporated into sediment organic matter within intact sediment for each treatment at depths 0-1 cm, 1-2 cm and 2-3 cm, 24 hours after ^{15}N application with tidal flushing occurring 4 hours after application (percentage ± SE).

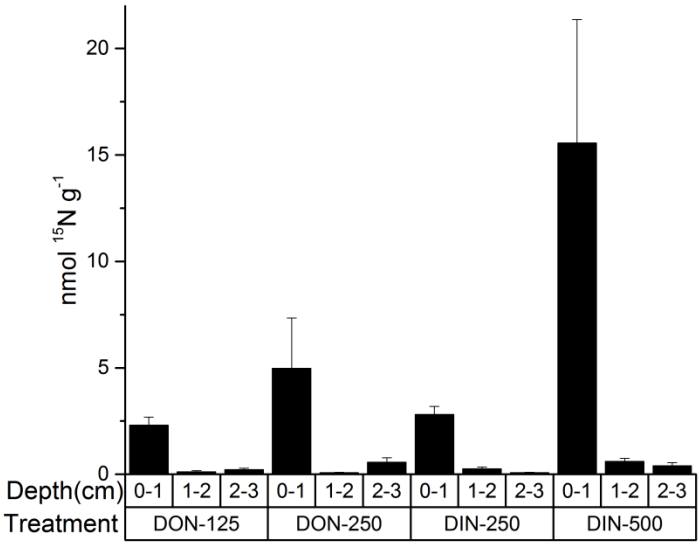

Figure S2: Total excess ^{15}N incorporated into organic matter in 0-1 cm, 1-2 cm, and 2-3 cm depths for intact sediments 24 hours after treatment applications (mean ± SE).

Table S1: Tests for treatment effects for each method of application. Bold text indicates significant differences at α=0.05.

Dependent Variable	Test Type	Depth	LN(x+1) Transform	(df, df_{error})	*F*	*p*	(df, df_{error})	Critical Value *F* or *T*	*p*	Tukeys	(df, df_{error})	*F*	*p*	(df, df_{error})	*F*	*p*	Interaction Parameters
Intact Sediments					**Dark/Light**			**Treatment**				**Depth**			**Interaction**		
Total uptake of ^{15}N in Bulk Sediment Organic Matter	3 Way	0 to 3 cm	Yes	1,48	0.4	0.537	3,48	3.6	0.021	A A A A	2,48	12.611	**<0.001**	6,48	73.5	**0.012**	Treatment × Depth
Total uptake of ^{15}N in Bulk Sediment Organic Matter	1 Way	0 to 1 cm	Yes				3,20	5.2	**0.008**	**A A A B**							
	1 Way	1 to 2 cm	Yes				3,20	7.5	**0.002**	**A A AB B**							
	1 Way	2 to 3 cm	Yes				3,20	2.2	0.115	A A A A							
Hourly ^{15}N uptake into Bulk Sediment Organic Matter	2 Way	0 to 1 cm	No	1,16	0.4	0.5	3,16	3.3	**0.049**	**A A A B**				3,16	0.1	0.960	
Hourly ^{15}N uptake into HAAs	2 Way	0 to 1 cm	No	1,5	0.4	0.578	3,5	28.6	**0.001**	**A A A B**				3,5	0.07	0.97	
Hourly ^{15}N uptake into Microbial Biomass	2 Way	0 to 1 cm	No	1,5	0.004	0.95	3,5	27.5	**0.002**	**A A A B**				3,5	0.03	0.99	
Bacterial Contribution to Biomass	2 Way	0 to 1 cm	No	1,5	0.25	0.634	3,5	1.8	0.267	A A A A				3,5	1.7	0.276	
Bacterial Contribution to ^{15}N uptake	2 Way	0 to 1 cm	No	1,5	14.6	**0.012**	3,5	37.3	**<0.001**	**A B B B**				3,5	2.7	0.15	
Microbial N production	2 Way	0 to 1 cm	No	1,5	0.1	0.8	3,5	6.3	**0.038**	**A A A B**				3,5	0.07	0.97	
Slurry Incubations																	
Total uptake of ^{15}N in Bulk Sediment Organic Matter	2 Way	0 to 1 cm	No	1,16	2.2	0.2	3,16	11.9	**<0.001**	**A A B B**				3,16	0.6	0.6	
Hourly ^{15}N uptake into Bulk Sediment Organic Matter	2 Way	0 to 1 cm	No	1,16	2.2	0.155	3,16	11.9	**<0.002**	**A A B B**				3,16	0.6	0.6	
Hourly ^{15}N uptake into HAAs	2 Way	0 to 1 cm	No	1,6	16.2	**<0.001**	3,6	16.2	**0.007**	**A A B C**				3,6	2.7	0.14	
Hourly ^{15}N uptake into Microbial Biomass	2 Way	0 to 1 cm	No	1,6	13.02	**0.011**	3,6	70.6	**<0.001**	**A A B C**				3,6	3.7	0.082	
Bacterial Contribution to Biomass	2 Way	0 to 1 cm	No	1,6	0.5	0.8	3,6	0.3	0.8	A A A A				3,6	0.4	0.8	
Bacterial Contribution to ^{15}N uptake	2 Way	0 to 1 cm	No											3,6	5.2	**0.041**	Dark/Light × Treatment
Bacterial Contribution to ^{15}N uptake	1 Way	0 to 1 cm	No				3,10	2.6	0.1	A A A A							
Bacterial Contribution to ^{15}N uptake	1 Way	0 to 1 cm	No	1,12	1.1	0.3											
Microbial N Production	2 Way	0 to 1 cm	No	1,6	0.4	0.6	3,6	6.9	**0.02**	**A A A B**				3,6	0.2	0.92	

Table S2: Statistical tests examining the effect of *in situ* and *ex situ* treatment application. Bold text indicates significant differences at α=0.05.

Dependant Variable	Test Type	LN(x+1) Transform	(df, df_{error})	Critical Value *F or T*	*p*	Tukeys	(df, df_{error})	*F*	*p*	Tukeys	(df, df_{error})	*F*	*p*	(df, df_{error})	*F*	*p*
Both			**Intact Sediment/Slurry**					**Treatment**				**Overall**			**Interaction**	
Total ^{15}N in Bulk Sediment Organic Matter	2 Way	No												3,40	11.5	**<0.001**
Total ^{15}N in Bulk Sediment Organic Matter	1 Way	No	1,46	30.4	**<0.001**	**A B**										
Hourly ^{15}N uptake into Bulk Sediment Organic Matter	2 Way	No												3,40	18.5	**<0.001**
Hourly ^{15}N uptake into Bulk Sediment Organic Matter	1 Way	No	1,46	25.2	**<0.001**	**A B**										
Hourly ^{15}N uptake into HAAs	2 Way	No												**3,19**	**6.3**	**0.004**
Hourly ^{15}N uptake into HAAs	1 Way	No	1,25	0.015	0.9	A A										
Hourly ^{15}N uptake into Microbial Biomass	2 Way	No	1,19	13.4	**0.002**	**A B**	3,19	73.5	**<0.001**	**A A B C**	7,19	33.5	**<0.001**	3,19	2.5	0.089
Bacterial Contribution to Biomass	2 Way	No	1,19	10.15	**0.005**	**A B**	3,19	0.8	0.5	A A A A	7,19	2.7	**0.041**	3,19	1.5	0.259
Bacterial Contribution to ^{15}N uptake	2 Way	No												3,19	11.4	**<0.001**
Bacterial Contribution to ^{15}N uptake	1 Way	No	1,25	0.003	0.96	A A										
Microbial N production	2 Way	No												3,19	11.2	**<0.001**
Microbial N production	T Test	No	4	-5.8	**0.005**	I < S	**DON 125**									
Microbial N production	T Test	No	4	-1.3	0.26	I = S	**DON 250**									
Microbial N production	T Test	No	4	-3.6	**0.034**	I < S	**DIN 250**									
Microbial N production	T Test	No	4	-3.1	**0.028**	I < S	**DIN 500**									

Supplemental References

Bronk DA, Glibert PM (1991) A ^{15}N tracer method for the measurement of dissolved organic nitrogen release by phytoplankton. Mar Ecol Prog Ser 77:171-182

Evrard V, Cook PLM, Veuger B, Huettel M, Middelburg JJ (2008) Tracing carbon and nitrogen incorporation and pathways in the microbial community of a photic subtidal sand. Aquat Microb Ecol 53:257-269

Eyre B, Oakes JM, Middelburg JJ (2016) Fate of microphytobenthos nitrogen in subtropical sediments: A ^{15}N pulse-chase study. Limnol Oceanogr

Guillard RR, Ryther JH (1962) Studies on marine plankton diatoms, I., *Cyclotella nana Hudstedt* and *Detonula confervacea*. Cleve Can J Microbiol 8:229-239

Hatch MJ, Dillon JA, Smith HB (1957) Preparation and use of snake-cage polyelectrolytes. Ind Eng Chem 49:1812-1819

Kaiser K, Benner R (2005) Hydrolysis-induced racemization of amino acids. Limnol Oceanogr-Meth 3:318-325

Lachat (1994) International methods list for the Quickchem automated ion analyzer. Lachat Instruments, Milwaukee, WI

Madigan MT, Martinko JM, Parker J (2000) Brock Biology of Microorganisms, Prentice-Hall

Valderrama J (1981) The simultaneous analysis of TP and TN in natural waters. Mar Chem 10:109-122

Veuger B, Middelburg JJ (2007) Incorporation of nitrogen from amino acids and urea by benthic microbes: Role of bacteria versus algae and coupled incorporation of carbon. Aquat Microb Ecol 48:35-46

Veuger B, Middelburg JJ, Boschker HTS, Houtekamer M (2005) Analysis of ^{15}N incorporation into D-alanine: A new method for tracing nitrogen uptake by bacteria. Limnol Oceanogr- Meth 3:230-240

Veuger B, Middelburg JJ, Boschker HTS, Houtekamer M (2007) Update of "Analysis of ^{15}N incorporation into D-alanine: A new method for tracing nitrogen uptake by bacteria" Limnol Oceanogr-Meth 5:192-194

Veuger B, van Oevelen D, Middelburg JJ (2012) Fate of microbial nitrogen, carbon, hydrolysable amino acids, monosaccharides, and fatty acids in sediment. Geochim Cosmochim Ac 83:217-233