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Effects of experimental warming and carbon addition on nitrate reduction and respiration in coastal sediments

Lindsay D. Brin · Anne E. Giblin ·
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Abstract Climate change may have differing effects on microbial processes that control coastal N availability. We conducted a microcosm experiment to explore effects of warming and carbon availability on nitrate reduction pathways in marine sediments. Sieved continental shelf sediments were incubated for 12 weeks under aerated seawater amended with nitrate ($\sim$50 μM), at winter (4 °C) or summer (17 °C) temperatures, with or without biweekly particulate organic C additions. Treatments increased diffusive oxygen consumption as expected, with somewhat higher effects of C addition compared to warming. Combined warming and C addition had the strongest effect on nitrate flux across the sediment water interface, with a complete switch early in the experiment from influx to sustained efflux. Supporting this result, vial incubations with added ^{15}N-nitrate indicated that C addition stimulated potential rates of dissimilatory nitrate reduction to ammonium (DNRA), but not denitrification. Overall capacity for both denitrification and DNRA was reduced in warmed treatments, possibly reflecting C losses due to increased respiration with warming. Anammox potential rates were much lower than DNRA or denitrification, and were slightly negatively affected by warming or C addition. Overall, results indicate that warming and C addition increased ammonium production through remineralization and possibly DNRA. This stimulated nitrate production through nitrification, but without a comparable increase in nitrate consumption through denitrification. The response to C of potential DNRA rates over denitrification, along with a switch to nitrate efflux, raises the possibility that DNRA is an important and previously overlooked source of internal N cycling in shelf sediments.

Keywords Anammox · Climate change · Continental shelf · Denitrification · Dissimilatory nitrate reduction to ammonium · Warming

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Introduction

The amount of nitrogen (N) available in marine ecosystems is a determining factor for primary productivity at both local and global scales (Ryther and Dunstan 1971; Perry and Eppley 1981), and in

excess causes eutrophication, a serious problem in coastal areas (Diaz and Rosenberg 2008). Available N is dependent not only on ecosystem inputs, but also on the balance of microbially mediated N cycling processes that lead to N removal or recycling. N is removed from coastal systems by denitrification and anammox, which reduce nitrate (NO_3^-) or nitrite (NO_2^-) to gaseous N_2 (Canfield 2005; Thamdrup 2012). Denitrification in coastal and marine sediments has been estimated to be one of the largest sinks of N in the marine environment, and may remove up to half of the total N inputs into the ocean (Christensen 1994; Codispoti et al. 2001). Anammox appears to be less important, especially in sediments where denitrification rates are high, but its importance is still being constrained (Trimmer and Engström 2011). In contrast to denitrification and anammox, dissimilatory NO_3^- reduction to ammonium (DNRA) retains N in the system as ammonium (NH_4^+) (Joye and Anderson 2008). While there is increasing evidence that DNRA can be as relevant as denitrification to net N availability and to NO_3^- reduction in shallow systems (Giblin et al. 2013), its importance in shelf sediments has not been well studied.

Changing temperatures in coastal ecosystems could alter the partitioning between these processes and thus the balance between N removal and recycling, but little is known about the specific effects of increased temperatures over relevant time scales. Studies that link seasonally changing temperatures to DNRA and denitrification rates suggest that warming may influence the balance of N cycling (Jørgensen 1989; Kelly-Gerreyn et al. 2001; Gruca-Rokosz et al. 2009). Higher temperatures may increase sediment O_2 consumption and thus be linked to lower sediment redox potentials that favor DNRA (Giblin et al. 2013). Furthermore, DNRA has been found to be dominant in warmer tropical ecosystems, and negligible in cold, deep sediments (Dong et al. 2011; Crowe et al. 2012). If warming sediments have a similar effect as seasonally or spatially changing temperatures, climate change could increase the relative importance of DNRA to NO_3^- reduction.

Many of the impacts of temperature are likely to be mediated through its influence on C availability, which may be the ultimate driver of the balance of the N cycle (Tiedje et al. 1982; Christensen et al. 2000; Thamdrup and Dalsgaard 2002). Availability of organic C may influence denitrification and DNRA

more than anammox. Denitrification and DNRA can be directly coupled to organic C respiration, while anammox is primarily autotrophic (Thamdrup 2012). However, anammox can still be affected by variability in organic C through release of NH_4^+ and NO_2^- during heterotrophic remineralization (Trimmer et al. 2003; Babbin et al. 2014). Relationships between warming and sediment organic C availability could be manifested in different ways, particularly through changes in phytoplankton delivery or sediment respiration. Warming may change the timing and magnitude of spring phytoplankton blooms in coastal ecosystems (Sommer and Lengfellner 2008; Nixon et al. 2009; Lewandowska and Sommer 2010), potentially altering C availability in benthic sediments. Warming may also interact with C availability by increasing total benthic respiration, leading to a shortage of labile organic matter in sediments (Alsterberg et al. 2012).

Changes in organic C availability could alter rates of denitrification, anammox, and DNRA differently. Based on thermodynamic considerations, Tiedje et al. (1982) hypothesized that DNRA should be favored over denitrification by a high ratio of organic matter to NO_3^-. This hypothesis has been supported by culture experiments with a denitrifier and DNRA bacterium, and by measurements in estuarine and reservoir sediments with varied organic matter loading rates (Rehr and Klemme 1989; Christensen et al. 2000; Gruca-Rokosz et al. 2009; Gardner and McCarthy 2009). These studies suggest that DNRA is favored over denitrification under very high organic C loading rates, and therefore high respiration rates. However, for other types of sediments typical of coastal systems, experimental evidence showing the relationship between C availability and DNRA is more limited, as denitrification is also expected to increase with organic C (Giblin et al. 2013). A recent modeling study showed that anammox was favored under lowest organic C to NO_3^- ratios, denitrification at intermediate ratios, and DNRA at the highest ratios (Algar and Vallino 2014). However, experimental evidence for this hypothesized pattern is currently limited.

We conducted a microcosm experiment to investigate the effect of increased temperature on sediment NO_3^- reducing processes in continental shelf sediments, and determine whether effects are mediated by organic C availability. Building on previous studies that have used microcosms or mesocosms to examine

N cycling in sediments (Jensen et al. 1994; Fulweiler et al. 2008; Neubacher et al. 2013), our microcosm experiment simultaneously tested the influence of warming and C addition on all three processes currently known to respire NO_3^- or NO_2^-. We hypothesized that warming alone would increase sediment respiration rates and eventually decrease organic C availability. This would in turn lead to decreased denitrification potential rates, and possibly decreased DNRA. Anammox might be favored under conditions with lower C availability, but not if there was a direct negative effect of warming (Dalsgaard and Thamdrup 2002). As C was added at a modest rate comparable to in situ deposition rates, we hypothesized that it would favor denitrification relative to DNRA, leading to increased net consumption of inorganic N.

Methods

Sampling

On March 14, 2012, sediment was collected from a continental shelf site in Rhode Island Sound (RIS2). This site was chosen as it was previously shown to exhibit appreciable potential rates of anammox and denitrification (Brin et al. 2014). RIS2 has a water column depth of 38 m. The water column is typically well mixed during winter, suggesting that O_2 was likely near air saturation. At the time of collection, bottom water was 4 °C and NO_3^- concentration was 0.3 μM. During a 2-year period when this experiment was conducted, bottom water temperature ranged from 3 to 17 °C (Brin et al. 2014), and NO_3^- concentration was 0.3–13 μM (Hardison, Giblin and Rich, unpublished).

Sediments were collected using a box core, and the top 4 cm of sediment were collected into coolers, covered with bottom water at in situ temperature, and brought back to the laboratory. Sediment was held in the dark at 4 °C, with aquarium bubblers used to keep the overlying water oxic.

Microcosm experiment

Fifteen microcosms were set up 4 days later, with all processing done at 4 °C. A microcosm consisted of sieved sediment (1 mm) layered into a glass pan to a depth of ~ 4 cm (20.9 × 11.1 cm). The pan was placed in an aquarium containing 6 L of 0.2 μM-filtered Narragansett Bay seawater (salinity 32), which was kept air saturated with aquarium pumps, in the dark. There were three replicate microcosms in each experimental treatment, as described below. To decrease potential NO_3^- limitation in the experiment, we adjusted the NO_3^- concentration in the aquarium water to 50 μM. We monitored NO_3^- concentration weekly in the microcosms. In cases where there was net consumption, we adjusted the concentration back up to 50 μM. Every other week, half of the overlying water in each microcosm was refreshed with initial seawater as a precaution against buildup of potential inhibitors or any other toxin. Afterwards, the NO_3^- concentration was again adjusted back up to 50 μM. There was never a smell of hydrogen sulfide, ammonia, or volatile fatty acids coming from the microcosms.

All microcosms received an initial pre-incubation at 4 °C for 16 days, after which three microcosms were sampled destructively, and potential rate experiments were conducted (t_0 experiments). Half of the remaining microcosms were kept at 4 °C, and the other half were warmed to 17 °C, reflecting winter low and summer high temperatures at the collection site. Sediment temperatures were monitored using iButtons (Dallas Semiconductor Corp) for the duration of the experiment (12 weeks). Temperatures were maintained by placing microcosms in a 4 °C cold room or at 17 °C in a large temperature-controlled water bath. Every 2 weeks, half of the microcosms at each temperature received additions of C in the form of *Chlorella* algae, resulting in four treatments (4 °C, 4 °C + C, 17 °C, and 17 °C + C) (Fig. 1). Before addition, *Chlorella* was leached to remove soluble C as follows: for each microcosm, 240 mg of *Chlorella vulgaris* powder (Jarrow Formulas, 100 % pure Yaeyama Chlorella) was placed in a plastic centrifuge tube with deionized water (45 mL), and tubes were shaken and allowed to sit overnight. The next day, tubes were centrifuged ($3,000 \times g$ for 3 min) and the supernatant was poured off, resulting in *Chlorella* pellets with a C:N ratio of 5.3. *Chlorella* pellets were resuspended in filter sterilized seawater (15mL), and suspensions were evenly added directly to the top of the sediment in each microcosm after gently removing the overlying water. The algae was mixed in by gently stirring the top 1 cm of sediment with a spatula, and then overlying microcosm water was added back. C
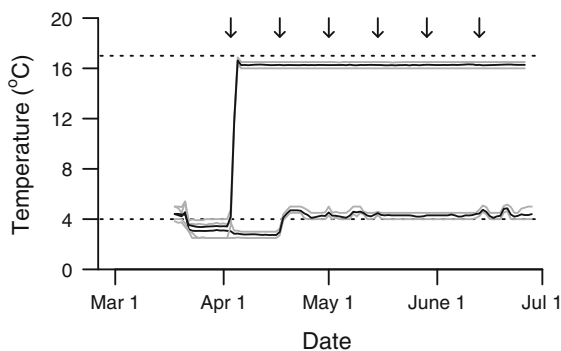
Fig. 1 Timeline of experimental treatments, and temperatures for each treatment. *Dashed lines* indicate 4 and 17 °C. *Dark lines* indicate means of iButton measurements for each temperature treatment, and *light lines* indicate minimum and maximum iButton measurements, both of which deviated slightly from 4 or 17 °C. *Arrows* indicate dates of *Chlorella* addition

was added at a rate equivalent to 3.1 µmol C cm^{-2} day^{-1}. This rate was chosen as it is somewhat higher than diffusive O$_2$ consumption measured at the site (approximately 1.5 µmol cm^{-2} day^{-1}) (Brin et al. 2014), and was therefore expected to maintain labile C stocks. Microcosms without C additions had the same treatment except using seawater without *Chlorella*.

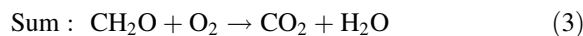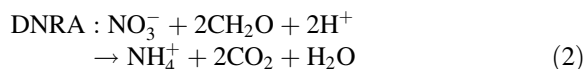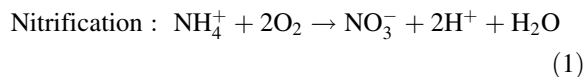**Oxygen microprofiles, sediment C and N, and inorganic N measurements**

To determine O$_2$ consumption rates, O$_2$ microprofiles were measured with a microelectrode (OX100, Unisense) in 4 microcosms just prior to initiating treatments (t_0), in 17 °C microcosms in week 1, and in all microcosms in weeks 5 and 11. Details about profiling methods and rate estimates were described previously (Brin et al. 2014).

Total sediment C and N were measured on sediments harvested for rate measurements, at (t_0) or after 12 weeks, using a CE Instruments NC2100 Elemental Analyzer (CE Elantech, Lakewood, NJ).

Overlying water samples were collected and frozen about three times a week to measure concentrations of NO$_3^-$, NO$_2^-$, and NH$_4^+$. NO$_3^-$ + NO$_2^-$ were measured colorimetrically using spongy cadmium (Jones 1984), and NO$_2^-$ was measured without Cd reduction, either by hand or on a Westco autoanalyzer (SmartChem 200, Westco Instruments, Brookfield, Connecticut). Pore water NH$_4^+$ was measured using the phenol/

hypochlorite method (Koroleff 1983), by hand or on a Westco autoanalyzer. Net fluxes of DIN over the sediment–water interface were calculated before t_0 and during weeks 1, 3, 5, 7, 9, and 11 as the change in aquarium water nutrient concentration over a 3- or 4-day period during which no water column NO$_3^-$ adjustments were made.

To further assess nitrification and N losses during later stages of the experiment, we used O$_2$ consumption rates from week 11 to calculate nitrification, assuming that (1) O$_2$ was used for aerobic remineralization and nitrification of all NH$_4^+$ produced by remineralization, as there was little to no NH$_4^+$ flux from the sediments throughout the experiment, (2) organic matter composition followed Redfield stoichiometry, and (3) all heterotrophic respiration was accounted for by O$_2$ consumption. In total, this would mean that 138 mol O$_2$ were consumed by every 16 mol NO$_3^-$ produced, i.e., 106 mol O$_2$ for remineralization of C and 32 mol O$_2$ for nitrification of the 16 mol of NH$_4^+$ produced (Paulmier et al. 2009). The ratio of NO$_3^-$ produced to O$_2$ consumed is the same whether or not DNRA occurs, because coupled nitrification-DNRA causes internal cycling of N, and the net reaction is simply aerobic respiration, rather than a change in available NO$_3^-$:

$$\text{Nitrification}: \ NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \tag{1}$$

$$\text{DNRA}: \ NO_3^- + 2CH_2O + 2H^+ \rightarrow NH_4^+ + 2CO_2 + H_2O \tag{2}$$

$$\text{Sum}: \ CH_2O + O_2 \rightarrow CO_2 + H_2O \tag{3}$$

This is analogous to the internal recycling of oxidants and reductants involved in Fe, Mn and SO$_4^{2-}$ reduction (Canfield et al. 1993). Total N loss in the microcosms, assumed to be removal mainly by denitrification, was calculated by adding NO$_3^-$ influx to NO$_3^-$ production by calculated nitrification. Results were compared to similar calculations using week 5 fluxes.

Potential rate measurements

Potential rate measurements of denitrification, anammox, and DNRA were conducted at the beginning of the experiment, just before the treatments were initiated (t_0), and then 12 weeks later at the end of

the experiment. Pans of sediment were removed from aquarium and the overlying water was aspirated off. The sediment was homogenized into a beaker and then 1.5 mL of sediment was dispensed into 5.9 mL Exetainer vials (LABCO, UK), which were flushed with helium and held overnight at the same temperature treatment that they were incubated at during the 12-week experiment (4 or 17 °C). This pre-incubation was conducted to remove any residual NO_3^-, as described previously (Brin et al. 2014). After the overnight pre-incubation, half of the vials continued to be incubated at the same temperature, while the other half of vials were shifted to the opposite 12-week temperature treatment for 1 h (i.e., 4–17 or 17–4 °C). This was enough time for all the sediment in the vial to reach the new temperature, at which point potential rate measurements were initiated. Thus, potential rates were measured at 4 and 17 °C for both 12-week temperature treatments. This allowed us to distinguish whether there was an effect due to factors such as physiological acclimation or microbial population shifts during the 12-week experiment, rather than simply a direct kinetic effect on reaction rates due to increasing or decreasing measurement temperatures.

To initiate potential rate measurements, $^{15}NO_3^- + {^{14}NH_4^+}$ (100 nmol N mL^{-1} sediment) was added to the vials, and production of $^{29}N_2$ and $^{30}N_2$ was measured after a 15-min incubation, at which point biological activity was halted with addition of 100 μL 7 M ZnCl$_2$. The produced ^{15}N-N_2 was measured with an isotope ratio mass spectrophotometer (Isoprime Continuous Flow-IRMS interfaced with Multiflow-Bio Unit). Calculation of denitrification and anammox rates was conducted following Brin et al. (2014), using equations described by Thamdrup and Dalsgaard (2002). DNRA rates were measured by determining $^{15}NH_4^+$ production over the same time interval, correcting for significant background $^{15}NH_4^+$ production, which occurred in samples that were killed immediately at the start of time course incubations. A small background correction was also needed for anammox, but none was necessary for denitrification. DNRA was measured following Holmes et al. (1998) by desorbing NH_4^+ in NaCl solution (1 M) and then diffusing it onto acidified Teflon-wrapped GF-C filters. The ^{15}N-NH_4^+ on the filters was measured using a Europa ANCA-SL elemental analyzer–gas chromatograph preparation system attached to a continuous-flow Europa 20–20 stable isotope ratio

mass spectrometer, as described in Koop-Jakobsen and Giblin (2010).

The presence of DNRA could lead to an overestimate of denitrification compared to anammox in potential rate incubations due to conversion of $^{15}NO_3^-$ into $^{15}NH_4^+$ and its subsequent conversion to $^{30}N_2$ by anammox (i.e., $^{15}NO_2^- + {^{15}NH_4^+} = {^{30}N_2}$) (Kartal et al. 2007). To address this, we calculated the potential magnitude of this effect, and determined that it would have been negligible, due to the large background of added $^{14}NH_4^+$ and fairly low anammox rates. In this calculation, we assumed an average at.% ^{15}N composition of $^{15}NH_4^+$ from linear DNRA rates during the 15 min incubation. Given initial additions of 100 nmol $^{14}NH_4^+$ mL^{-1} sediment, $^{15}NH_4^+$ from DNRA comprised 0–4.9 % of total NH_4^+, causing a 0–5.1 % underestimate of potential anammox and a 0–0.7 % overestimate of denitrification. These are conservative estimates, as the actual pool of $^{14}NH_4^+$ was likely higher due to exchangeable NH_4^+, decreasing the effect of $^{15}NH_4^+$. Because of the insignificance of this effect, we did not correct for this in our reported rates.

Statistical analysis

Statistical analyses were conducted using R version 2.15.0 (R Development Core Team). Two-way or three-way ANOVAs were conducted to test for treatment differences for various factors (O$_2$ consumption, potential rates, sediment C and N), depending on how often a particular measurement was conducted during the 12-week experiment. We conducted two-way ANOVA for treatment effects on total organic C and N and potential rate data, with the factors of 12-week temperature treatment and C addition treatment, and an interaction between the two. In cases where more time points were taken during the course of the experiment (i.e., O$_2$ consumption rates and inorganic N fluxes), we conducted three-way ANOVA, with the factors of 12-week temperature treatment, C addition treatment, week (as a continuous variable), and all interactions as factors. In all cases, residuals were tested for normality and homogeneity of variance. In general, the data met the assumptions of ANOVA. Linear regression was used to examine relationships between potential rates and diffusive O$_2$ consumption, both measured at the

12-week incubation temperature. Statistical tests were considered significant at $p < 0.05$.

Results

Diffusive O_2 consumption

Complete O_2 consumption always occurred within the top 0.5 cm of the sediment, indicating, as expected, that an oxic/anoxic interface was established in the microcosm sediments at a similar depth to in situ (Brin et al. 2014). O_2 consumption increased significantly as a result of C addition and warm temperature treatment over the 12-week experiment (Fig. 2). There was no significant interactive effect between C addition and 12-week temperature treatment and no effect of the week in which the measurements were conducted (Three-way ANOVA for weeks 5 and 11: 12-week temperature treatment, $F = 14.3$, $p = 0.002$; C treatment, $F = 60.7$, $p < 0.001$; Week, $F = 0.6$, $p = 0.46$). C had a stronger effect than temperature based on the F statistic. Week was also not a factor when only the 17 °C data were analyzed in a two-way ANOVA, for weeks 1, 5, and 11, with C treatment and week as factors (Two-way ANOVA: C treatment, $F = 75.0$, $p < 0.001$; Week, $F = 4.3$, $p = 0.06$). Thus, native pools of organic C were sufficient to maintain O_2 consumption rates in cores that lacked C addition during the course of the 12-week experiment.

Sediment C and N

At the end of the experiment, +C treatments had higher total organic C (0.691 ± 0.057 %, mean $\pm$ SD) than treatments without C added (0.589 ± 0.052 %) ($F = 8.82$, $p = 0.02$) (Fig. 3). The level of

C buildup in sediments receiving C additions (~ 0.1 %) was as expected based on the cumulative amount of C that we added. Temperature did not influence total organic C, as indicated by the lack of difference between microcosms subject to different temperature treatments but similar C addition treatments. Sediment percent N was also higher in +C treatments (0.092 ± 0.008 %) compared to treatments without C added (0.074 ± 0.007 %) ($F = 12.81$, $p = 0.007$). There was no difference in total sediment C:N among treatments (Table 1) (12-week
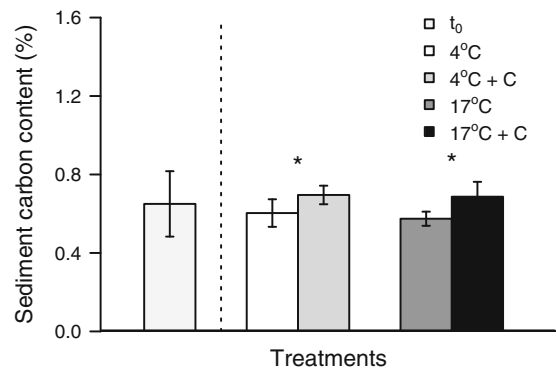

Fig. 3 Sediment organic C in microcosm sediments exposed to warming and organic C additions. Mean $\pm$ SD are plotted for each treatment ($n = 3$). *Asterisks* indicate significant differences as assessed by 2-way ANOVA with temperature and C addition as factors (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Table 1 Total C:N ratio for all treatments, from measurements of total sediment C and N	Treatment	C:N
	t_0	7.3 ± 1.1
	4 °C	8.0 ± 0.3
	4 °C + C	7.5 ± 0.3
	17 °C	7.9 ± 0.9
	17 °C + C	7.6 ± 0.2

Fig. 2 O_2 consumption rates, calculated from sediment microprofiles measured during the experiment. Mean $\pm$ SD are plotted for each treatment ($n = 3$). Temperature and C addition are significant, but week is not, in a 2-way ANOVA analysis including weeks 5 and 11
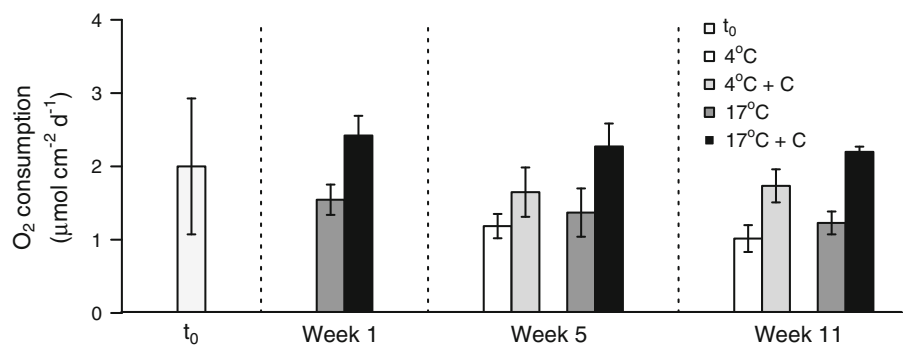
temperature treatment, $F = 0.039$, $p = 0.74$; C treatment, $F = 1.79$, $p = 0.22$).

Microcosm nutrient fluxes

At the beginning of the experiment, all microcosm treatments consumed NO_3^- at similar rates (Fig. 4). Microcosms held at 4 °C continued to have the greatest net NO_3^- influx throughout the rest of the experiment. In the other treatments, there was a shift in NO_3^- flux, ranging from reduced influx in 4 °C + C and 17 °C to a complete reversal from influx to efflux in 17 °C + C (Fig. 4). Treatment differences were evident by week 3 and remained consistent for the rest of the experiment. These results were reflected in significant effects for all main factors in the three-way ANOVA, as well as the C addition by week interaction (12-week temperature treatment, $F = 71.31$, $p < 0.001$; C treatment, $F = 45.7$, $p < 0.001$; Week, $F = 10.41$, $p = 0.002$; C × Week interaction, $F = 16.05$, $p < 0.001$). Treatment differences in NO_2^- or NH_4^+ were not as dramatic as for NO_3^-, but differences were evident during a similar time frame to when NO_3^- flux shifted to efflux for the 17 °C + C treatment. In particular, there was production of NO_2^- and NH_4^+ efflux in week 1 in the 17 °C + C treatment that preceded the change in NO_3^-, and a similar efflux NH_4^+ in week 3 in the 4 °C + C treatment (NO_2^-: 12-week temperature treatment, $F = 1.35$, $p = 0.25$; C treatment, $F = 4.94$, $p = 0.03$; Week, $F = 5.71$, $p = 0.02$; Temperature x

Week interaction, $F = 6.3$, $p = 0.01$; Temperature x C x Week interaction, $F = 5.99$, $p = 0.02$) (NH_4^+: no significant main effects or interactions). After week 3, NO_2^- and NH_4^+ fluxes were usually around zero, with some negligible treatment differences.

In situ nitrification, calculated from O_2 consumption rates from week 11, was comparable to net NO_3^- influx in the 4 °C treatments (Table 2). Nitrification was higher in the other microcosm treatments, whereas NO_3^- flux into the sediments decreased. Calculated rates of denitrification were comparable among treatments (Table 2). Conclusions were the same whether the calculation was based on week 5 or 11 fluxes.

Denitrification, anammox and DNRA potential rates

In t_0 sediments, and for all treatments at the end of the experiment, denitrification potential rates were higher when measured at 17 °C than when measured at 4 °C for the same treatment. However, regardless of whether denitrification potential rates were measured 4 or at 17 °C, rates were lower in sediments that had been subjected to the 17 °C warming treatment than in sediments held at 4 °C (At the 4 °C measurement temperature: $F = 115.03$, $p < 0.001$; at the 17 °C measurement temperature, $F = 12.56$, $p = 0.008$). C addition had no influence on this warming effect (Fig. 5a).

Fig. 4 Fluxes of **a** NO_3^-, **b** NO_2^- and **c** NH_4^+ across the sediment water interface in microcosms exposed to warming and organic C addition. Mean ± SD are plotted for each treatment ($n = 3$). Negative fluxes indicate influx
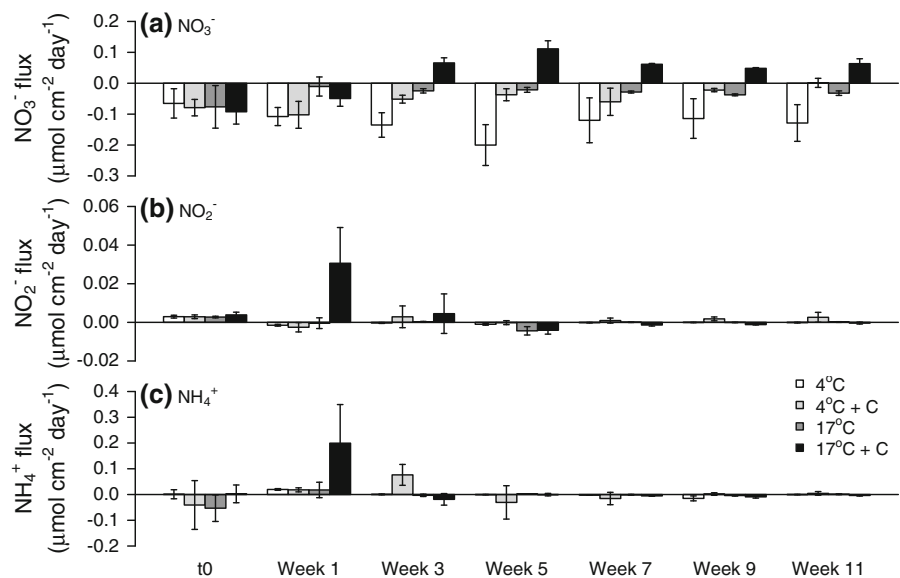
Table 2 Nitrification and denitrification in sediment microcosms, calculated from measured NO_3^- flux and stoichiometric conversion of organic C remineralization based on measured O_2 consumption

Treatment	NO_3^- flux (μmol N cm^{-2} day^{-1})	Diffusive O_2 flux (μmol O_2 cm^{-2} day^{-1})	Calculated remineralization (μmol C cm^{-2} day^{-1})	Calculated nitrification (μmol N cm^{-2} day^{-1})	Calculated N loss (denitrification) (μmol N cm^{-2} day^{-1})
4°	−0.129 ± 0.059	1.016 ± 0.183	0.780 ± 0.141	0.118 ± 0.021	0.246 ± 0.079
4° + C	0.001 ± 0.015	1.734 ± 0.226	1.332 ± 0.173	0.201 ± 0.026	0.200 ± 0.012
17°	−0.032 ± 0.007	1.229 ± 0.156	0.944 ± 0.120	0.142 ± 0.018	0.175 ± 0.024
17° + C	0.063 ± 0.016	2.198 ± 0.071	1.689 ± 0.055	0.255 ± 0.008	0.192 ± 0.009

Nitrification was calculated from O_2 consumption assuming that for every 138 mol of O_2 consumed, 16 mol of NO_3^- was produced. Denitrification was calculated as nitrification minus NO_3^- flux (negative values denote influx). Mean ± SD are shown for each treatment ($n = 3$)

Fig. 5 Potential rates of **a** denitrification, **b** anammox and **c** DNRA in sediments from microcosms harvested before initiation of treatments (t_0) and from all experimental treatments at the end of the experiment. For each treatment, potential rates were measured at 4 °C and at 17 °C. Mean ± SD are plotted for each treatment ($n = 3$). *Asterisks* indicate significant differences as assessed by 2-way ANOVA analysis conducted for assays at each measurement temperature, with 12-week temperature treatment and C addition treatment as factors (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)
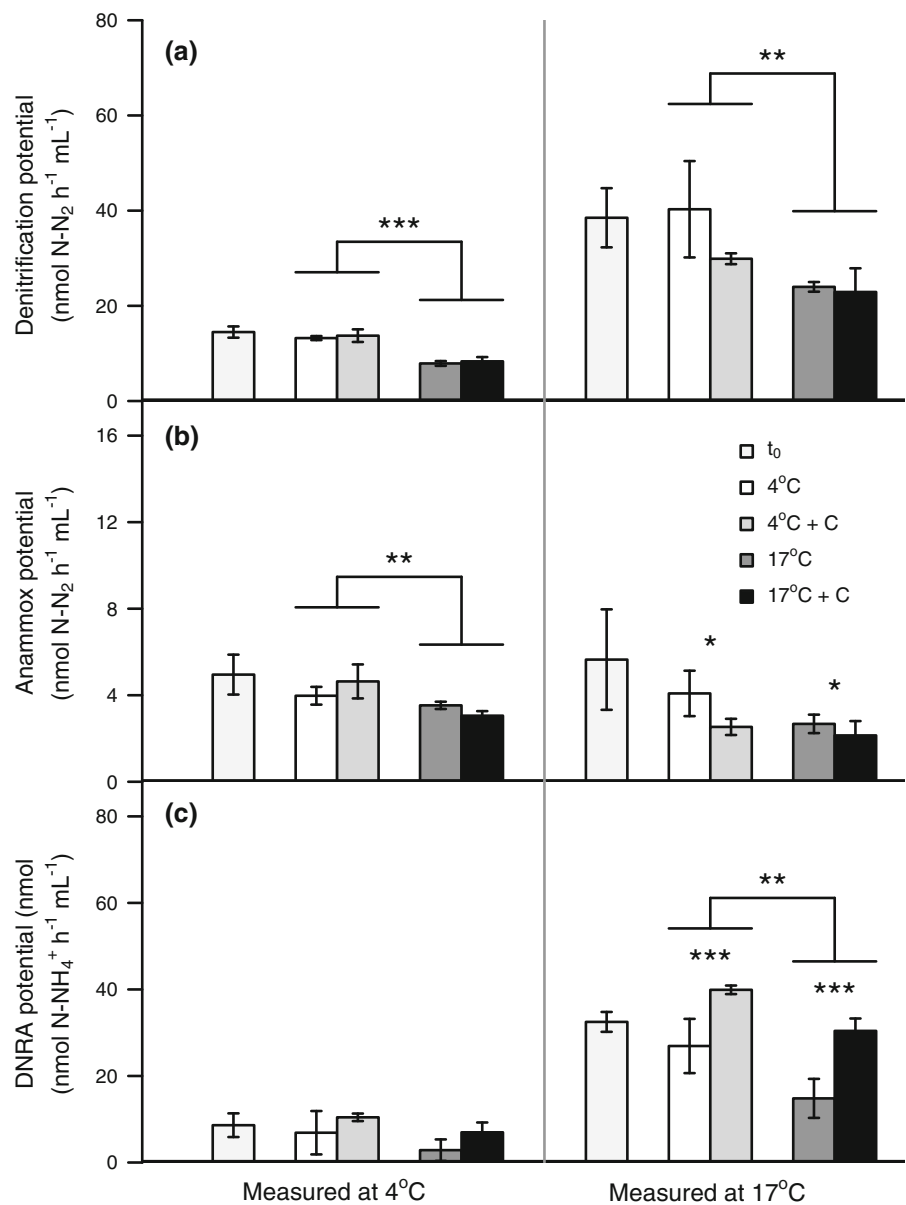
Potential anammox rates were 7–46 % of denitrification rates and 7–31 % of total N loss for all treatments at both measurement temperatures, with relatively higher importance when measured at lower temperatures. After 12 weeks, potential anammox for all treatments measured at 4 °C was 26.3 ± 3.4 % of total N loss, while measurements at 17 °C were 9.6 ± 2.2 %. In 4C microcosms, without warming or carbon addition, anammox was 23.1 ± 1.6 % of total N loss when measured at the treatment temperature of 4 °C. Anammox rates showed slight but statistically significant treatment effects depending on whether they were measured at low or high temperature (Fig. 5b). When measured at the higher temperature, C additions decreased anammox slightly ($F = 6.98$, $p = 0.03$), whereas warming decreased anammox slightly at the lower measurement temperature ($F = 14.39$, $p = 0.005$).

Potential DNRA rates were of a similar order to denitrification. Similarly to denitrification, DNRA rates within a treatment were higher when measured at 17 °C than at 4 °C. However, DNRA rates were significantly lower in warmed treatments when measured at 17 °C (Fig. 5c) ($F = 20.35$, $p = 0.002$). At this same measurement temperature, DNRA rates were also significantly higher in +C treatments ($F = 35.64$, $p < 0.001$). There were no interactive effects of C addition and 12-week temperature treatment for any measured process at either high or low measurement temperature. Potential DNRA rates correlated positively with O_2 consumption, with measurements of both at temperatures corresponding to 12-week temperature treatments ($p = 0.004$, adj $r^2 = 0.53$), whereas denitrification and anammox rates did not (Fig. 6).

Warming for 12 weeks did not change the relative importance of DNRA or denitrification to total NO_3^- reduction (i.e., DNRA or Denitrification/Total NO_3^- reduction). However, C addition decreased relative denitrification rates and increased relative DNRA rates. This effect was measured at the higher measurement temperature (17 °C) (Relative denitrification, $F = 15.84$, $p = 0.004$; relative DNRA, $F = 15.99$, $p = 0.004$), as well as at measurement temperatures corresponding to the 12-week temperature treatments (Relative denitrification, $F = 6.89$, $p = 0.03$; relative DNRA, $F = 5.85$, $p = 0.04$).
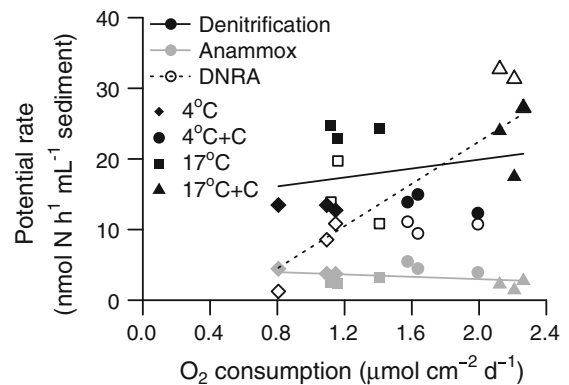

Fig. 6 Relationship between sediment O_2 consumption measured in week 11 and potential rates of denitrification, anammox, and DNRA, measured at the same temperature as O_2 consumption, i.e., 12-week temperature treatment. Each symbol represents an individual microcosm. Symbol shape indicates treatment: 4 °C *diamonds*, 4 °C + C *circles,* 17 °C *squares,* 17 °C + C *triangles*. Symbol color indicates process: denitrification *black*; anammox *gray*; DNRA *open symbols*. Lines show linear regressions (denitrification, *solid black line*: $r^2 = -0.01$, $p = 0.381$; anammox, *gray line*: $r^2 = 0.04$, $p = 0.25$; DNRA, *dotted black line*: $r^2 = 0.53$, $p = 0.004$). Note that only the regression line for DNRA is statistically significant, and that one data point for 17 °C + C DNRA (*open triangle*) is mostly obscured by a 17 °C + C denitrification point (*black triangle*)

Discussion

Climate change may alter benthic N cycling in coastal ecosystems, either through direct temperature effects or through indirect effects mediated by changes in C availability. These indirect effects may be due to changes in sediment remineralization rates or to shifts in phytoplankton blooms and organic matter delivery to the benthos (Sommer and Lengfellner 2008; Nixon et al. 2009; Lewandowska and Sommer 2010), which may maintain C availability at times when it would otherwise be depleted by respiration, such as between spring and fall phytoplankton blooms. Impending and already occurring changes have prompted microcosm studies that explore the effects of multiple stressors on benthic N cycling, but few have examined the effects of temperature in conjunction with other factors in coastal systems (Fitch and Crowe 2011; Alsterberg et al. 2012). Most of the knowledge of the effect of temperature and organic C on NO_3^- reduction comes

from seasonal studies and comparisons of rates with changing environmental factors measured in the field (Dalsgaard et al. 2005; Trimmer and Engström 2011; Giblin et al. 2013). The present study extends knowledge of these responses by examining both warming and organic C addition in a mechanistic laboratory microcosm experiment (Jensen et al. 1993; Fulweiler et al. 2008; Neubacher et al. 2013). Although our temperature manipulation does not simulate gradual temperature increases expected due to future climate change, it demonstrates that multiple factors can elucidate potential changes in N cycling that may not be observed in response to varying a single factor, and that indirect effects of warming may have stronger effects than direct effects. In particular, the combination of both warming and C addition was required to reverse the net flux of NO_3^- over the sediment–water interface (Fig. 4). However, contrary to expectations, warming did not influence the relative importance of DNRA as a NO_3^- reduction pathway (Ogilvie et al. 1997; Kelly-Gerreyn et al. 2001). Instead, DNRA increased in response to C addition, supporting the hypothesis that warming effects observed in previous studies of benthic sediments may have been mediated by changes in availability of organic C (King and Nedwell 1984; Brin et al. 2014).

O_2 consumption and microcosm sediment C

Oxygen consumption reflects overall sediment reactivity as driven by organic C availability. Our biweekly C additions were at the low end of the range in values reported for primary production in the Middle Atlantic Bight (de Haas et al. 2002). O_2 consumption rates in microcosms with and without C additions agreed well with rates measured over 2 years in intact sediment cores from the study site, which are typical of near shore continental shelf sediments in general (Glud 2008; Brin et al. 2014). We acknowledge that the timing and nature of our C additions did not mimic in situ variability. However, O_2 consumption rates suggested that our level of C addition was within the range encountered in situ.

Sediment O_2 consumption was significantly greater in microcosms with added C than in sediments that did not receive C additions, demonstrating that treatments were effective in increasing sediment organic C availability (Fig. 2). Increased O_2 consumption due to C additions at either temperature indicated that

respiration rates were stimulated by organic C inputs, even at winter temperatures. Differences in O_2 consumption rates between sediments with and without C additions (i.e., ~ 1 µmol cm^{-2} day^{-1}) were about one-third the rate of C addition, suggesting an equivalent fraction of the added C was rapidly mineralized. This enabled a detectable difference in sediment organic C in sediments that received C additions compared to those without added C (Fig. 3).

Warming also increased O_2 consumption, after accounting for differences due to C addition (Fig. 2). This suggests that eventually, given enough time, O_2 consumption rates would begin to decline in the 17 °C microcosms, although we were not able to detect a statistically significant decline after 11 weeks of treatment. This lack of decline in warmed treatments without C additions indicates a relatively constant supply of native organic C fueling respiration. Regardless of the lack of a rate decline over time, greater O_2 consumption in 17 °C treatments than in 4 °C treatments indicate that more organic C was being consumed at the warmer temperature. This may have led to decreased labile C that was available for heterotrophic NO_3^- reduction during potential rate incubations at the end of the experiment, as has been observed previously in microcosm sediments in response to experimental warming (Alsterberg et al. 2011).

Nutrient fluxes in microcosms

Measuring net fluxes of inorganic N across the sediment–water interface allowed us to examine treatment effects on sediment N cycling throughout the experiment. It is noteworthy that there was a strong influx of NO_3^- into sediments at 4 °C without C additions for the duration of the experiment (Fig. 3). This influx of NO_3^- with little to no associated NH_4^+ or NO_2^- efflux indicated a net loss of N from the system. Potential rate measurements of anammox and denitrification suggested that this N loss could be largely attributed to denitrification, with anammox responsible for approximately 23.1 %. This partitioning is slightly lower than that predicted assuming remineralization of organic C by denitrification with a C/N ratio of Redfield (6.6), producing NH_4^+ and equivalent NO_3^- that was consumed by anammox (28 %) (Trimmer and Engstrom 2011; Babbin et al. 2014). This comparison neither indicates nor refutes the

presence of DNRA; although DNRA could affect the relative proportion of NO_2^- and NH_4^+ from remineralization available for denitrification and anammox, this effect may be countered by subsequent nitrification. Consistent NO_3^- consumption in sediments at 4 °C without C addition throughout the experiment suggests capacity for significant N loss during winter months, even in the absence of any new organic C inputs. This agrees with comparable N_2 fluxes during winter and other seasons at the site (Heiss et al. 2012).

Warming and C addition individually decreased the net influx of NO_3^- over the sediment–water interface. However, the most striking result occurred under both warming and C addition, as there was complete reversal from net NO_3^- consumption to production early on in the experiment, which was preceded by a transient pulse of NH_4^+ production (Fig. 4). The delayed pulse of NH_4^+ production in the 4 °C + C treatment as compared to the 17 °C + C treatment could indicate a temperature effect on the rate of heterotrophic response to organic C additions. Furthermore, no significant NH_4^+ effluxes occurred after these pulses, indicating that from that point forward, NH_4^+ produced in sediments was completely consumed by nitrification and possibly heterotrophic uptake.

The result of reduced NO_3^- consumption was at first puzzling, as we expected C addition at this level to stimulate denitrification and increase NO_3^- consumption. However, the switch could have been due to increased remineralization of added organic C, releasing NH_4^+, and subsequent nitrification of this NH_4^+ to NO_3^-, without any change in N loss. Based on Redfield conversion of O_2 consumption rates, nitrification in 4 °C treatments was comparable to net NO_3^- influx, thereby making the estimate of N loss about double that of measured NO_3^- influx (Table 2). In contrast, in the 17 °C + C treatments, in which increased O_2 consumption indicated increased nitrification, nitrification supplied the majority of the NO_3^- used to support sediment NO_3^- reduction processes (Table 2). These calculations suggest that in situ denitrification rates did not change across experimental treatments, despite increases in nitrification by both warming and C addition, with the net effect of positive N efflux from 17 °C + C microcosm sediments. Although this mass balance can be explained solely in terms of nitrification and denitrification, it is still counter-intuitive that denitrification was not stimulated by C addition. A possible further explanation is provided by invoking results from potential rate experiments, which indicated that DNRA rates were increased with warming and C addition, but denitrification rates were not. This may have led to a disproportionate increase in internal N recycling over N removal in sediments that received C additions, i.e., DNRA was stimulated when denitrification was not.

Our stiochiometric calculations should be viewed with caution in light of our simplifying assumptions, i.e. (1) that the remineralized organic C had a constant Redfield C:N ratio, an assumption supported by past research (e.g., Burdige 1991), (2) that all of the NH_4^+ released through heterotrophic respiration was completely nitrified, an assumption supported by the very little overall NH_4^+ release from the sediments, and (3) that all heterotrophic respiration was accounted for by O_2 consumption. However, heterotrophic denitrification is not balanced by re-oxidation of end products with O_2 (Canfield et al. 1993), and so NH_4^+ released through this pathway would not be included in the calculation, causing nitrification to be underestimated. Additionally, if there were a NH_4^+ sink other than nitrification, such as microbial assimilation, the ratio of O_2 consumption to NO_3^- production would be higher than the assumed 138:16, and our calculated nitrification and denitrification rates would be overestimates.

Relatively few studies have examined the effects of experimental warming and organic C addition on DIN fluxes from coastal sediments, and those that do exist have found different results from our study. Warming or organic C addition increased remineralization but usually with a stimulation of denitrification as well (Caffrey et al. 1993; Fitch and Crowe 2011; Alsterberg et al. 2012), which we did not observe. A potential explanation for the difference is the presence of DNRA and cycling of N between DNRA and nitrification in our experimental sediments (Burgin and Hamilton 2007). This link has been quantified in a moist tropical forest soil, in which DNRA accounted for about 35 % of NO_3^- production by nitrification (Templer et al. 2008), and a positive relationship between DNRA and nitrification was predicted in a modeling study of a coastal freshwater sediment (Canavan et al. 2007). Several other studies of estuarine or marine sediments have noted links between DNRA and nitrification, in terms of DNRA outcompeting denitrification as a sink for NO_3^-

produced by nitrification (Tobias et al. 2001), in situ nitrification providing $^{14}NO_3^-$ for DNRA in $^{15}NO_3^-$ addition experiments (Gardner and McCarthy 2009), and nitrification preventing NH_4^+ accumulation when DNRA rates were high (Jäntti and Hietanen 2012). To our knowledge, however, this link has not been carefully studied in aquatic sediments.

Denitrification, anammox and DNRA potential rates

Potential rate measurements in homogenized sediments are often criticized for generally overstimulating rates compared to in situ, although this is not always the case (Laverman et al. 2006; Behrendt et al. 2013). To assess this, we converted O_2 consumption- and NO_3 flux-based denitrification rates in Table 2 to volumetric rates assuming a depth interval of NO_3^- reduction of 0.2 cm, which was similar to the depth interval of O_2 penetration. The depth interval of NO_3^- reduction could theoretically vary with both temperature and C addition, but for simplicity, we used a constant 0.2 cm depth interval for all treatments. Converted rates of N loss from Table 2 ranged from 33 to 68 nmol N h^{-1} mL^{-1} sediment, which were similar to or somewhat higher than measured potential rates of denitrification (Fig. 5).

Perhaps more concerning is the potential for slurry incubations to overstimulate specific NO_3^- reduction processes compared to others, with denitrification being favored over anammox, or DNRA over denitrification (Revsbech et al. 2006; Trimmer et al. 2006; Behrendt et al. 2013). Explanations for this are not currently certain, but could relate to more denitrifiers or DNRA bacteria being exposed to favorable conditions in slurries. Overstimulation of denitrification relative to anammox is generally observed in shallower sediments (Trimmer and Engström 2011). Although we cannot completely rule out overstimulation of denitrification over anammox in our potential rate incubations, our anammox rates as a percent of denitrification were typical for shelf sediments measured by slurries or intact cores (Trimmer and Engström 2011). Slurry incubations have also been found to overstimulate DNRA relative to denitrification by disrupting natural gradients and increasing NO_3^- availability for DNRA bacteria (Behrendt et al. 2013). DNRA bacteria may reside deeper than denitrifiers in intact sediments, and so have less access to

NO_3^- in sediments in which NO_3^- is typically supplied by diffusion rather than advection. It is not known whether this overstimulation occurred in our experiment. However, taken together with DIN fluxes, potential rate measurements support the hypothesis that DNRA became a more important process in the microcosms due to warming and C additions.

DNRA is typically associated with sediments with high organic matter availability and O_2 consumption rates (MacFarlane and Herbert 1984; Tomaszek and Gruca-Rokosz 2007; Gardner and McCarthy 2009; Nizzoli et al. 2010). We also found a relationship between potential DNRA rates and O_2 consumption in our experiment (Fig. 6). Furthermore, C addition increased the relative importance of DNRA versus denitrification in NO_3^- reduction. This agrees with theoretical considerations and chemostat experiments that indicate that DNRA is favored over denitrification in sediments with a greater relative supply of organic C compared to NO_3^- (Tiedje 1988; Kraft et al. 2014; Algar and Vallino 2014).

This link between C and DNRA could also occur via sulfur cycling. Addition of C could stimulate sulfate reduction and flux of hydrogen sulfide into the NO_3^- reducing layer. Sulfide has been shown to inhibit the last step in denitrification, while not adversely influencing DNRA, and the presence of DNRA has been positively correlated with the presence of sulfide in marine sediments (Brunet and Garcia-Gil 1996; An and Gardner 2002). However, there is also evidence that sulfide may favor denitrification over DNRA, depending on NO_3^- availability, or have no strong influence on the competition between the two processes (Dong et al. 2011; Kraft et al. 2014). Furthermore, nitrification is also inhibited by sulfide (Joye and Hollibaugh 1995), but our organic C addition increased, not decreased, nitrification rates based on stoichiometric calculations. This suggests that if sulfide production was stimulated, it was too low to have any negative influence on nitrification.

Stimulation of DNRA over denitrification due solely to the level of C added in our experiment would be surprising, as DNRA is generally favored in sediments with substantially higher O_2 consumption rates than we measured in our study (Christensen et al. 2000; Revsbech et al. 2006). Perhaps differences in the chemical composition of added compared to native C was a factor in the tradeoff between reduction of NO_3^-

to NH_4^+ or N_2 (Akunna et al. 1993; Bonin et al. 1999; Gardner and McCarthy 2009). Gardner and McCarthy (2009) found that DNRA was higher than expected based on O_2 consumption alone during a cyanobacterial depositional event in shallow tropical sediments, suggesting that composition of organic C may have been a factor. In our experiment, the effects of warming on potential rates and O_2 consumption may similarly suggest the influence of chemical composition of organic C. Both denitrification and DNRA potentials decreased in 12-week warmed treatments relative to cool treatments measured at the same temperature (Fig. 5), with a more consistent result for denitrification than DNRA. We attribute this decrease to increased respiration and thus C depletion in warmed compared to winter 12-week temperature treatments, as reflected by O_2 consumption rates. C additions increased potential rates of DNRA, potentially by mitigating C limitation, but had no effect on denitrification. This may suggest that denitrifying bacteria in our experiment were better adapted to utilize native organic C compared to added organic C. In contrast, DNRA bacteria appeared to show a response to either type of C.

Conclusions

Effects of increasing coastal water temperatures on benthic N cycling processes may be mediated primarily by changes in sediment organic C availability. These effects are generally examined by correlation of rates with environmental factors that change in situ (Dalsgaard et al. 2005; Trimmer and Engström 2011), and few microcosm studies have examined the effects of warming in conjunction with other factors (Fitch and Crowe 2011; Alsterberg et al. 2012). By examining both warming and organic C addition in a mechanistic laboratory microcosm experiment, this study provided new insights about potential controls on relationships between NO_3^- reduction processes and shifts from N removal to recycling. We demonstrated that temperature and changes in organic C, alone or in concert, could affect NO_3^- reduction processes and the net balance of benthic N cycling. In particular, nutrient and O_2 fluxes indicated that warming and C addition increased the relative importance of N recycling over removal. This change was driven by increased NH_4^+ production by remineralization, which increased nitrification but not N loss, shifting net NO_3^- fluxes towards efflux over the sediment–water interface. Potential rate measurements of microcosm sediments indicated significant capacity for DNRA, which increased due to C addition and at the higher measurement temperature. Although results from potential rate measurements must be viewed with caution, stimulation of DNRA but not denitrification in microcosm sediments is consistent with a switch towards internal N recycling and NO_3^- efflux. This suggests stimulation of N cycling between nitrification and DNRA, a coupling that has not been well studied in aquatic sediments.

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