Nitrate reduction temperature responses

1   Similar temperature responses suggest future climate warming will not alter partitioning between

2   denitrification and anammox in temperate marine sediments

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4   Lindsay D. Brin[1*], Anne E. Giblin[2], and Jeremy J. Rich[3]

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6   [1]Canadian Rivers Institute, University of New Brunswick, 100 Tucker Park Road, Saint John,

7   New Brunswick E2L 4L5, Canada

8   [2]Ecosystems Center, Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts

9   02543, USA

10   [3]School of Marine Sciences, Darling Marine Center, University of Maine, Walpole, Maine 04573

11   *Corresponding author, email: lindsay.brin@gmail.com, phone: (506) 654-6872

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18　**Abstract**

19　Removal of biologically available nitrogen (N) by the microbially mediated processes

20　denitrification and anaerobic ammonium oxidation (anammox) affects ecosystem N availability.

21　Although few studies have examined temperature responses of denitrification and anammox,

22　previous work suggests that denitrification could become more important than anammox in

23　response to climate warming. To test this hypothesis, we determined whether temperature

24　responses of denitrification and anammox differed in shelf and estuarine sediments from coastal

25　Rhode Island over a seasonal cycle. The influence of temperature and organic C availability was

26　further assessed in a 12-week laboratory microcosm experiment. Temperature responses, as

27　characterized by thermal optima (T_{opt}) and apparent activation energy (E_a), were determined by

28　measuring potential rates of denitrification and anammox at 31 discrete temperatures ranging

29　from 3 to 59°C. With a few exceptions, T_{opt} and E_a of denitrification and anammox did not differ

30　in Rhode Island sediments over the seasonal cycle. In microcosm sediments, E_a was

31　somewhat lower for anammox compared to denitrification across all treatments. However,

32　T_{opt} did not differ between processes, and neither E_a nor T_{opt} changed with warming or carbon

33　addition. Thus, the two processes behaved similarly in terms of temperature response, and this

34　response was not influenced by warming. This led us to reject the hypothesis that anammox is

35　more cold-adapted than denitrification in our study system. Overall, our study suggests that

36　temperature responses of both processes can be accurately modeled for temperate regions in the

37　future using a single set of parameters, which are likely not to change over the next century as a

38　result of predicted climate warming. We further conclude that climate warming will not directly

39　alter the partitioning of N flow through anammox and denitrification.

40

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41   **<u>Introduction</u>**

42   Marine nitrogen (N) availability affects both regional and oceanic primary productivity as

43   well as regional susceptibility to eutrophication (Ryther & Dunstan, 1971; Perry & Eppley, 1981;

44   Diaz & Rosenberg, 2008). An important oceanic N sink is via microbially mediated N removal,

45   particularly in coastal and continental shelf sediments, which receive and remove 50-80 Tg N y^{-1}

46   from terrestrial and marine sources (Howarth $et\ al.$, 1996; Galloway $et\ al.$, 2004; Gruber &

47   Galloway, 2008). Benthic N removal occurs through denitrification and anaerobic ammonium

48   oxidation (anammox), both of which are anaerobic processes that reduce NO_3^- or NO_2^- to N_2.

49   While denitrification is primarily a heterotrophic process that uses NO_3^- to oxidize organic

50   carbon, anammox uses NO_2^- to oxidize NH_4^+ and is primarily autotrophic. However, anammox

51   depends on organic carbon mineralization indirectly as a source of NH_4^+. Both denitrification

52   and anammox are microbially mediated enzymatic processes that may respond differently to

53   changes in temperature (Dalsgaard & Thamdrup, 2002; Rysgaard $et\ al.$, 2004; Brin $et\ al.$, 2014).

54   As temperatures in coastal waters are predicted to continue to rise over the next century (Nixon

55    $et\ al.$, 2004; Christensen $et\ al.$, 2007), differences in temperature responses between processes

56   could alter the flux of N through denitrification versus anammox.

57   The temperature response of an enzymatic process can be described by its activation

58   energy (E_a), which reflects the increase in rate with increase in temperature (temperature

59   dependence), as well as its thermal optimum (T_{opt}), the temperature at which rates are maximal

60   (Arrhenius, 1915). In nature, the temperature response of a biogeochemical processes is

61   determined by the combined temperature response of the assemblage of organisms performing

62   the reactions in any given environment (Allen $et\ al.$, 2005; Hall $et\ al.$, 2008, 2010; Yvon-

63   Durocher $et\ al.$, 2014). Ecosystem level processes may display distinct temperature dependence,

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64   as has been demonstrated for photosynthesis and respiration (Yvon-Durocher *et al.*, 2010;

65   Demars *et al.*, 2011). For microbially mediated processes, changes in temperature responses

66   could reflect: 1) changes at the cellular level, through physiological acclimation by individual

67   microbial strains; or 2) changes at the microbial population level, through changes in abundance

68   of strains adapted to different temperatures (Angilletta Jr., 2009; Hall *et al.*, 2010; Crowther &

69   Bradford, 2013). However, rates or temperature responses may be more strongly limited by other

70   factors than temperature in the environment, such as substrate supply. Thus, in some cases there

71   may not be a strong selective advantage to adapt to changes in temperature (Hartley *et al.*, 2007,

72   2008; Crowther & Bradford, 2013).

73   The hypothesis that temperature may be a key driver of the relative importance of

74   denitrification and anammox as N loss pathways was provided by studies in permanently cold

75   sediments, which found that anammox was relatively more favored over denitrification at colder

76   temperatures (Dalsgaard & Thamdrup, 2002; Rysgaard *et al.*, 2004). More recent studies

77   examining seasonal patterns or temperature responses of anammox and denitrification rates in

78   marine sediments also support anammox being cold-adapted or hindered at higher temperatures

79   (Teixeira *et al.*, 2012; Brin *et al.*, 2014; Canion *et al.*, 2014a, 2014b). Besides temperature,

80   availability of organic C likely exerts a strong influence on the relative importance of anammox

81   and denitrification as N loss pathways, with organic C favoring denitrification over anammox

82   (Thamdrup & Dalsgaard, 2002; Engström *et al.*, 2005). As temperature also influences organic

83   matter decomposition rates and therefore organic C availability, the effects of temperature could

84   be mediated indirectly through changes in organic C availability rather than as a direct result of

85   inherent differences in enzyme kinetics between the anammox or denitrification pathway

86   (Isaksen & Jørgensen, 1996; Canion *et al.*, 2014a; Brin *et al.*, 2015).

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87   Despite indications that anammox and denitrification rates may respond differently to

88   temperature, this control has only been examined in a few studies (Dalsgaard & Thamdrup,

89   2002; Rysgaard *et al.*, 2004; Canion *et al.*, 2014a, 2014b). Furthermore, it is unknown whether

90   changes due to climate warming may alter not only rates but also the temperature dependence of

91   each process (King & Nedwell, 1984; Acuña *et al.*, 2008; Robador *et al.*, 2009; Perkins *et al.*,

92   2012). Differences in temperature dependence of each process over the range of temperatures

93   experienced *in situ* could alter the relative rates of each process, and thus its contribution to N_2

94   production (Holtan-Hartwig *et al.*, 2002). Furthermore, climate warming could have indirect

95   effects on temperature dependence by influencing organic C availability. This could occur if

96   warming alters the deposition of organic C to benthic sediments, e.g. via changes in spring

97   phytoplankton blooms in coastal ecosystems (Sommer & Lengfellner, 2008; Nixon *et al.*, 2009;

98   Lewandowska & Sommer, 2010), or the rate of consumption of sediment organic C (Alsterberg

99   *et al.*, 2012).

100   We have examined controls on anammox and denitrification in temperate marine

101   sediments previously by measuring potential rates in field collected samples over a seasonal

102   cycle and in a separate microcosm experiment (Brin *et al.*, 2014, 2015). In this paper, we report

103   new measurements on the temperature responses of anammox and denitrification rates in the

104   same sediments, to directly test the hypothesis that anammox and denitrification have different

105   temperature responses. We asked whether T_{opt} or E_a 1) vary between anammox and

106   denitrification, 2) vary by sampling site or season within each process, and 3) can be altered by

107   manipulations of temperature or organic C availability in a microcosm experiment.

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108   **Materials and Methods**

109   *Seasonal study*

110   To determine how temperature responses varied by site and season, two study sites were

111   sampled in coastal Rhode Island, USA: an inner continental shelf site, Rhode Island Sound

112   (RIS2) and an estuarine site, Providence River Estuary (PRE) (i.e., Heiss *et al.*, 2012; Brin *et al.*,

113   2014). These sites will be referred to as shelf and estuarine sites, respectively. The shelf site had

114   a water depth of 38 m, and bottom water temperatures were between 7 and 17ºC during sampling

115   dates. The estuarine site had a water depth of 5 m and greater seasonal temperature variation,

116   with measured bottom water temperatures between 3 and 22ºC across sampling dates. Sediments

117   at both sites were fine-grained, with a higher organic carbon content at the estuarine site (2.6%)

118   than the shelf site (0.8%) (NC2100 Elemental Analyzer).

119   The shelf site was sampled in January, June, July, and September 2011 and March 2012,

120   and the estuarine site was sampled in June and August 2011 and January 2012. At the shelf site,

121   PVC tubes were fastened to the inside of a box core that was deployed from the research vessel

122   to obtain intact sediment cores. At the estuarine site, intact cores were collected into PVC tubes

123   (10 cm inner diameter) using a pull corer. After collection, the cores were immediately

124   transported back to the laboratory at near-*in situ* temperature. Sediment cores were held in the

125   dark at *in situ* temperature under air-bubbled site water in aquaria. This approach was taken

126   because water columns at the sites were generally well mixed, indicating that bottom water was

127   near air saturation. O_2 microprofiles were measured in the cores 1-4 days after sample collection

128   to determine the O_2 penetration depth, as described previously (Brin *et al.*, 2014). Cores were

129   then removed from aquaria and a 1 cm depth layer of sediment just below the O_2 penetration

130   depth (<0.5 cm) was extruded from the core tube, sliced off, and collected for temperature

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131   response measurements. This depth interval was the focus of this study as it contained the NO_3^-

132   reducing layer, based on O_2 penetration depth and concentration of NO_3^- in porewater profiles

133   (Brin *et al.*, 2014). Sediment from 4-5 cores corresponding to any given site and sampling date

134   were pooled to obtain enough sediment to conduct temperature response measurements.

135

136   *Microcosm experiment*

137   A total of fifteen microcosms were set up and maintained as described previously, using

138   sediment collected at the shelf site in March 2012 (Brin *et al.*, 2015). Briefly, microcosms

139   consisted of sieved (1 mm) surface sediment (0-4 cm depth interval) layered approximately 4 cm

140   deep in glass pans, each placed in an aquarium containing 6 L of 0.2 μm-filtered Narragansett

141   Bay seawater (salinity 32), which was kept air saturated with aquarium pumps. Half of the

142   overlying water was replaced every two weeks to prevent buildup of nutrients or other

143   compounds. All microcosms were initially held at 4°C for 16 days, after which three microcosms

144   were destructively sampled, and potential rate experiments were conducted (t_0 experiments). The

145   microcosms were then exposed to temperature treatments by maintaining half of the microcosms

146   at 4°C and shifting the other half to 17°C. This temperature manipulation represents seasonal

147   minimum and maximum temperatures at the site (Emery & Uchupi, 1972; Brin *et al.*, 2014).

148   Carbon was added biweekly to half of the microcosms at either temperature in the form of

149   *Chlorella* algae, in the form of a suspension that was gently mixed into the top 1 cm of sediment

150   at a rate equivalent to 3.1 μmol C cm^{-2} d^{-1}, which is expected to maintain sediment labile C

151   availability (Brin *et al.*, 2015). This resulted in four treatments in a full factorial design, referred

152   to here as 4°C, 4°C+C, 17°C and 17°C+C, with three replicate aquaria in each treatment. O_2

153   consumption was increased by both carbon addition and temperature. O_2 penetration into the

154   sediment was at most 0.5 cm, with shallower penetration in sediments with greater O_2

155   consumption, indicating that added organic C reached anoxic layers in all the microcosms.

156   Treatments were maintained for 12 weeks, after which point the overlying water was aspirated

157   off and the contents of each pan were collected into a beaker for temperature response

158   measurements.

159

160   *Temperature responses of denitrification and anammox potential rates*

161   Sediment from a given site or microcosm replicate was homogenized in a beaker, and 1.5

162   mL of this sediment was transferred into replicate vials (5.9 mL, 93 replicate vials per site or 31

163   replicate vials per microcosm replicate) to conduct parallel incubations at different temperatures.

164   The headspace of the vials was made anoxic by purging the headspace with helium, and vials

165   were pre-incubated overnight at the associated *in situ* or experimental microcosm temperature to

166   remove ambient porewater NO_x^-. For the microcosm experiment, replicates were maintained

167   within the thermoblock, yielding 3 measurements of E_a for each treatment.

168   Temperature responses were measured using a thermal gradient incubator (thermoblock)

169   similar to Rysgaard et al. (2004). The thermoblock consisted of a 1.8 m long piece of aluminum

170   with a silicone rubber heater on one side, a Peltier cooler at the other, and 31 parallel rows of 3

171   holes (vial wells) along its length to fit the vials. This created a stable linear temperature gradient

172   with endpoints at 2.8 ± 0.7°C and 58.9 ± 0.8°C (mean ± s.d.), as determined by measurement of

173   temperatures in vial wells before and after experiments, as well as with temperature probes

174   embedded in the thermoblock during all incubations. The vials were transferred from their pre-

175   incubation temperature into the thermoblock for approximately 90 minutes to allow for complete

176   temperature equilibration of sediments. Potential rate measurements were commenced after the

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177   90 min equilibration period by adding 50 μL of $^{15}NO_3^- + {}^{14}NH_4^+$ (100 nmol N mL^{-1} sediment) to

178   the vials. After 5-50 min incubations in the presence of added $^{15}NO_3^- + {}^{14}NH_4^+$, all reactions were

179   completely stopped by adding 100 μL 7M $ZnCl_2$. The amount of ^{15}N-N_2 that accumulated in

180   vials during the incubation was used to determine rates of denitrification and anammox. Shorter

181   incubations were conducted for sediments with higher inherent rates, such as estuarine

182   sediments. Rates were plotted as a function of temperature in the thermoblock, which by

183   definition is referred to as a thermal profile in this study.

184   ^{15}N-N_2 production in the vials was measured with an isotope ratio mass

185   spectrophotometer (Isoprime CF-IRMS interfaced with Multiflow-Bio Unit) and rates were

186   calculated as described in Thamdrup and Dalsgaard (2002). By convention, the percent of N_2

187   production accounted for by anammox is abbreviated as *ra* (relative anammox), and calculated as

188   100 X (anammox) / (anammox + denitrification).

189   In addition to thermoblock experiments, parallel sets of potential rate measurements in

190   triplicate vials were run to serve as different controls, as follows. One set of vials received

191   unlabeled NO_3^- and NH_4^+ and was incubated at *in situ* temperature in the seasonal study, or 17°C

192   for the microcosm experiment, in order to assess NO_3^- concentrations remaining in the vials after

193   time intervals that were used in thermoblock incubation. This confirmed that NO_3^- was not

194   depleted during incubations. Three additional ^{15}N isotope additions were run for samples

195   collected on the different sampling dates at the estuarine and shelf sites. These incubations were

196   done at *in situ* temperature, in parallel to thermoblock incubations. One incubation received the

197   same $^{15}NO_3^- + {}^{14}NH_4^+$ addition as in the thermoblock incubation, with four equally spaced

198   measurement time points starting immediately after N addition, confirming linear production of

199    $^{29}N_2$ and $^{30}N_2$ during the incubation. An additional incubation received $^{15}NH_4^+ + {}^{14}NO_3^-$, and

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200   another received $^{15}NH_4^+$ alone, confirming the presence or absence of anammox and that N_2 was

201   not produced by some other process independent of NO_3^- reduction (Yang *et al.*, 2012). The rates

202   from incubations with added $^{15}NH_4^+ + ^{14}NO_3^-$ or $^{15}NH_4^+$ alone were reported previously (Brin *et*

203   *al.*, 2014), and those results are consistent with the relative rates of anammox reported in this

204   study. Vials with no added N were also included at the beginning of the experiment to correct for

205   any residual $^{14}NO_3^-$ that might have remained after the pre-incubation. The fraction of ^{15}N-

206   labelled NO_3^- in the incubations, accounting for the fraction of ^{15}N in added $^{15}NO_3^-$ (i.e., 0.99),

207   was >0.96 across all incubations.

208

209   *Statistical analysis*

210   Statistical analyses were conducted using R version 2.15.0 (R Development Core Team).

211   For all analyses, statistical tests were considered significant at the *p*<0.05 level.

212   To statistically define T_{opt}, a general additive model was fit to each profile using the R

213   package *mgcv* (Wood, 2006, 2011; Zuur *et al.*, 2009), using cubic regression splines and cross-

214   validation. Temperatures with modeled rates that fell within the 95% confidence interval of the

215   maximum rate were all considered to be T_{opt}. Therefore, the T_{opt} values reported below reflect

216   this statistically defined range. One exception was made for denitrification in PRE sediments in

217   January 2012, for which there was a double peak; both peaks were subject to this analysis and

218   considered to be part of T_{opt}. If the range in T_{opt} overlapped between any given comparison of

219   samples, we considered T_{opt} to be not significantly different. Whether relationships between T_{opt}

220   and temperature in the seasonal study or microcosm experiment were significant were

221   determined with linear regression (p <0.05).

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222   Temperature-rate relationships were examined with the linearized form of the Arrhenius

223   equation with a standardized temperature (Rysgaard *et al.*, 2004; Yvon-durocher *et al.*, 2010):

224   (1) $\ln[\text{Rate}(T)] = -E_a*(1/kT - 1/kT_c) + \ln[\text{Rate}(T_c)]$

225   where T_c is the standardized temperature of 15°C (Perkins *et al.*, 2012); $\ln[\text{Rate}(T_c)]$ is the

226   Arrhenius constant in the traditional derivation; E_a is the apparent activation energy for the

227   measured process; k is the Boltzmann constant ($8.62 * 10^{-5}$ eV K^{-1}); and T is the measurement

228   temperature in Kelvin. E_a is calculated as the negative slope of the linear regression through the

229   linear range of the thermal profile below T_{opt}. E_a values in eV and kJ mol^{-1} are presented here to

230   compare directly with previous work on both nitrogen cycling (kJ mol^{-1}) and ecosystem

231   respiration (eV). In the seasonal study, the standard error in E_a was estimated from regression

232   lines in Arrenhius plots, whereas in the microcosm experiment, standard error was determined

233   across microcosm replicates. Relationships between the linear intercept (rate at 15ºC) and *in situ*

234   temperature were assessed with linear regression.

235   We used similar linear mixed effects models using the function *lme* within the R package

236   *nlme* (Pinheiro *et al.*, 2016) to test for differences in E_a between processes, sampling sites, or

237   microcosm treatments (Zuur *et al.*, 2009). Three datasets were analyzed corresponding to the

238   seasonal study, microcosm experiment, or the combined data. For each analysis, models

239   included the following main effects: measurement temperature, site or treatment, process, and

240   interactions between temperature and both site/treatment and process. For each analysis, we used

241   Akaike information criterion (AIC) scores to compare three models with all main effects to

242   determine the random effects structure of the data: with no random effects; with random

243   intercepts; and with random slopes and intercepts. Random effects assessed variation at the level

244   of individual and distinct thermal profiles. As such, sampling date (for the seasonal study) and

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245   treatment replicate (for the microcosm study) were treated as random effects on the slope and

246   intercept. Comparisons between these three models indicated whether the random effects term

247   varied in slope (E_a) as well as intercept (magnitude of rates). We continued with the model with

248   the lowest AIC score to test for significance of main effects. For all microcosm analyses, models

249   with random slopes and intercepts had lowest AIC scores and were selected further analysis. E_a

250   was considered to vary significantly for main effects if their interaction with temperature was

251   significant. For example, to assess differences in E_a across sites, we assessed whether there was a

252   significant interaction between site and temperature, which would indicate that the relationship

253   of rate with temperature varied by site.

254   In the seasonal study, within each site and process, we further explored which sampling

255   dates contributed to differences in apparent activation energies (i.e., denitrification or anammox)

256   using a similar linear mixed modelling approach in which temperature was the sole main effect.

257   Select dates were removed to determine their effect on random effects structures.

258 **Results**

259 *Temperature responses by site and season*

260   Rates of denitrification and anammox increased with temperature up to 20-35°C, with

261 declining rates thereafter (Fig. 1a-d). There were strong seasonal differences in absolute rates

262 within a site (sampling date $p<0.001$), particularly for denitrification in shelf sediments, with the

263 lowest rates in January 2011 and highest rates in March 2012 (Fig. 1a). Potential denitrification

264 reached a higher maximum rate in estuarine (PRE) compared to shelf (RIS2) sediments, but the

265 range in maximum rates between the two sites overlapped, indicating strong potential for

266 denitrification at both sites during the sampling period (Fig. 1a vs. b). In shelf sediments,

267 potential anammox rates were 2-6 times lower than denitrification rates (Fig 1a vs. c). Rates

268 were not related to *in situ* temperatures for either site or process. In estuarine sediments,

269 anammox rates were undetectable or close to the detection limit (<1 nmol N h^{-1} mL^{-1} sediment)

270 (Fig. 1d). We therefore did not calculate T_{opt} and E_a values for anammox at the estuarine site.

271   The range in T_{opt} was 18-35°C for denitrification and 22-33°C for anammox (Table S1,

272 Fig. 2). T_{opt} overlapped for anammox and denitrification on each sampling date. There was no

273 relationship between T_{opt} and *in situ* temperature, nor was there a consistent pattern in T_{opt} across

274 sites or seasons. Within each site and process, T_{opt} overlapped for all sampling dates, with the

275 exception of denitrification in January 2011 in shelf sediments, which had a narrower profile and

276 higher T_{opt} than September and March (Fig. 2). The thermal profile for denitrification in

277 estuarine sediments in January 2012 had a double peak that bracketed those for other seasonal

278 measurements.

279   Apparent E_a values were between 0.40 and 0.63 eV (38.5 and 60.4 kJ mol^{-1}) for

280 denitrification in shelf sediments, 0.36 and 0.69 eV (34.3 and 66.9 kJ mol^{-1}) for anammox in

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281   shelf sediments, and 0.37 and 0.55 eV (35.8 and 53.0 kJ mol^{-1}) for denitrification in estuarine

282   sediments (Table S1, Fig. 1e-g). Apparent E_a did not differ significantly between sites for

283   denitrification nor between denitrification and anammox (linear mixed effects model, $p>0.05$).

284   The mixed model with the lowest AIC score included both random slope and intercept,

285   indicating that E_a differed across sampling dates. Differences in denitrification E_a by sampling

286   date were driven by high E_a at the shelf site in January 2011 and low Ea at the estuarine site in

287   June 2011, as models without random slopes became optimal when these dates were omitted.

288   Anammox E_a also differed by date in shelf sediments, driven by higher E_a values in July and

289   September 2011. However, in the full model, the variance was much greater for the intercept

290   (capacity; $d^2=0.40$) than for the slope (E_a; $d^2=0.0085$), indicating that differences among dates

291   were more dependent on overall capacity than temperature dependence.

292   Across all thermoblock measurements in shelf sediments, ra ranged from negligible to

293   62%. In 3 out of 5 sampling dates, there was no change in ra as a function of thermoblock

294   temperature across a range of 3-35°C (Fig. 3). However, in January 2011, ra was negatively

295   correlated with temperature ($p<0.001$, $R=-0.89$), decreasing from 62% at 3°C to 28% at 35°C.

296   This switch to a negative correlation was driven not by a change in anammox temperature

297   dependence or capacity across sampling dates, but by a change in the shape of the denitrification

298   thermal profile on this particular date (Fig. 1a, c). In contrast, in September 2011, ra was

299   positively correlated with temperature ($p=0.001$, $R=0.70$) (Fig. 3).

300

301   *Microcosm experiment*

302   Incubating microcosm sediments at 4°C without C addition for 12 weeks did not change

303   denitrification rates compared to t_0 measurements, while anammox rates decreased slightly,

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304   relative to the t_0 control (Fig. 4a, b). Contrary to expectations, denitrification rates decreased

305   significantly in 17°C treatments, with or without C, relative to t_0, as well as in the 4°C treatment

306   with C (Fig. 4a; linear mixed effects model with random slope and intercept, $p<0.001$).

307   Anammox rates showed a similar decrease with treatments as denitrification (Fig. 4b; linear

308   mixed effects model, $p<0.001$). T_{opt} overlapped for anammox and denitrification, as well as

309   across treatments for each process (Fig. 2, Table S1). Similarly, T_{opt} in the microcosm

310   experiment did not differ from the seasonal study, although ranges were more consistent in the

311   microcosm experiment (Fig. 2).

312   The sediment that was used in the microcosm experiment was from March 2012, when E_a

313   of anammox was the lowest across sampling dates (Table S1). This lower E_a was reflected in the

314   microcosm experiment, as E_a was significantly lower for anammox than denitrification (linear

315   mixed effects model, process x temperature interaction $p<0.001$). Apparent E_a values were

316   between 0.38 and 0.48 eV (36.5 and 46.4 kJ mol^{-1}) for denitrification and 0.20 and 0.32 eV (19.3

317   and 30.8 kJ mol^{-1}) for anammox. However, E_a was not significantly different between treatments

318   for either process (Table S1, Fig. 4c, d), and as with the seasonal study, variance was much

319   greater for the intercept (capacity; $d^2=0.026$) than for the slope (E_a; $d^2=0.0014$). Furthermore,

320   neither denitrification nor anammox E_a differed significantly between the microcosm experiment

321   and the seasonal study.

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322 **Discussion**

323   The denitrification T_{opt} values measured in this study (21 to 35°C) indicate a mesophilic

324 community of denitrifiers in temperate Rhode Island sediments. Given overlapping T_{opt} and

325 mostly similar apparent E_a values, there was no indication of a specifically cold- or warm-

326 adapted population of denitrifiers that developed seasonally, between sites, or in response to

327 experimentally manipulated temperatures. This indicates functionally equivalent denitrifier

328 populations in terms of temperature response, despite variation in rates (Fig. 1e-g, 4 c-d).

329 Furthermore, warmest *in situ* temperatures were within the range of T_{opt}, suggesting that

330 denitrifiers were reasonably well adapted to the annual temperature regime at the sites. Our

331 results agree with the general finding that denitrification rates display a mesophilic T_{opt} and

332 comparable E_a values in a broad range of sediments from temperate to Arctic systems (Dalsgaard

333 & Thamdrup, 2002; Rysgaard *et al.*, 2004; Canion *et al.*, 2014a, 2014b). This implies that

334 relatively large temperature changes from the Arctic to temperate regions do not cause

335 significantly different temperature responses for denitrification. In contrast, denitrification in

336 subtropical sediments has been shown to have distinctly higher T_{opt} and E_a values compared to

337 colder sediments (Canion *et al.*, 2014b). Thus, warmer climates may cause a change in the

338 temperature response of denitrification. However, the degree of warming needed to cause such a

339 change is probably greater than the 2-2.5ºC warming that is predicted to occur in our study

340 region over the next century (Meehl *et al.*, 2007; Taboada & Anadón, 2012; Mills *et al.*, 2013).

341   Previous studies have suggested that anammox bacteria are more cold-adapted than

342 denitrifiers, due to lower T_{opt} (9-18°C) or E_a in anammox bacteria, and measurements of higher

343 *ra* values at lower temperatures (Dalsgaard & Thamdrup, 2002; Rysgaard *et al.*, 2004; Canion *et*

344 *al.*, 2014a, 2014b). However, most of these studies have been conducted in permanently cold

345   marine sediments. The present study is one of the few that has been conducted in temperate

346   sediments (Canion et al., 2014b). We found that the range in T_{opt} values of denitrification and

347   anammox were not significantly different in the seasonal study or microcosm experiment (Fig. 2,

348   Table S1). E_a of anammox was significantly lower than E_a of denitrification in the microcosm

349   experiment, which appeared to be driven by initial values of E_a in the sediments used to set up

350   the microcosm experiment rather than any significant influence of experimental treatments. E_a

351   values of anammox and denitrification were not significantly different across the seasonal study,

352   indicating that there was not an overall consistent difference in E_a between the two processes.

353   Cumulatively, we conclude that overall populations of active anammox bacteria are not more

354   cold-adapted than denitrifiers in our study system. Similar to denitrification, the results do not

355   indicate consistent seasonal shifts in temperature responses of anammox. On the one sampling

356   date when *ra* did decrease with increasing temperature (January 2011), this was driven by a shift

357   in the temperature response of denitrification rather than anammox. The correlation between

358   *ra* and temperature across seasons that was previously noted (Brin et al., 2014) may therefore

359   have been due to other factors besides temperature that vary seasonally, rather than relatively

360   faster rates of denitrification compared to anammox at warmer temperatures. As anammox may

361   depend on denitrification for a source of NO_2^- (Trimmer et al., 2003; Risgaard-Petersen et al.,

362   2004; Meyer et al., 2005; Brin et al., 2014), similar temperature responses overall might reflect

363   the relationship between the two processes.

364   The capacity for denitrification, as reflected in thermal profiles and in the linear intercept

365   of Arrhenius plots (Fig. 1, 3), changed across sampling dates in the seasonal study as well as

366   with treatment in the microcosm study. These changes in magnitude could be associated with

367   changes in the abundance of denitrifier populations, the amount of enzyme being produced by

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368   the denitrifiers present, substrate availability, or a combination of factors. The lack of a

369   correlation between linear intercept and *in situ* temperature in the seasonal study suggests that

370   temperature effects may be indirect, and that potential rates are controlled by other factors in

371   addition to temperature. One potential control of denitrification rates in coastal and marine

372   sediments is organic C availability, with higher rates reflecting greater C availability (Dalsgaard

373   *et al.*, 2005; Brin *et al.*, 2014). Experiments with Arctic sediments demonstrated that addition of

374   organic acids (i.e., acetate, lactate) significantly increased sulfate reduction or denitrification

375   rates in thermoblock experiments (Isaksen & Jørgensen, 1996; Canion *et al.*, 2014a). Similarly,

376   we expected that organic C addition in our microcosm experiment would increase denitrification

377   rates relative to microcosms without C addition. Surprisingly, organic C addition did not yield

378   this result. The lack of response of denitrification rates in our microcosm experiment may have

379   been due to competition for NO_3^- with other processes, as potential dissimilatory nitrate

380   reduction to ammonium (DNRA) rates were stimulated by the organic C addition, while potential

381   denitrification rates were not (Brin *et al.*, 2015). The form of organic C added may also have had

382   an influence on this result, with regular additions of freeze-dried phytoplankton favoring DNRA

383   bacteria over denitrifiers.

384   The aim of this study was to determine how shifts in temperature and C availability

385   through seasonal changes or experimental manipulations influence the temperature responses of

386   anammox or denitrification. We found that temperature responses of anammox and

387   denitrification were more similar to each other than previously reported (Dalsgaard & Thamdrup,

388   2002; Rysgaard *et al.*, 2004; Canion *et al.*, 2014b), and both processes were characterized as

389   mesophilic instead of anammox being more cold-adapted than denitrification. Overall, our

390   results suggest that predicted warming in our study region over the next century will not act

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391   through direct temperature effects to decrease the contribution of anammox to N_2 production

392   relative to denitrification. In contrast, strong differences in absolute rates with season suggest

393   that factors other than temperature dependence are important regulators of relative rates of

394   anammox and denitrification.

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549   **Supporting information captions**

550   **Table S1** – Apparent activation energies (E_a) and thermal optima (T_{opt}) of denitrification and

551   anammox in shelf and estuarine sediments and the microcosm experiment. Asterisks denote

552   E_a values that differ significantly from others within the same site and process.

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553 **Figure captions**

554 **Figure 1**. Thermal profiles (a-d) and Arrhenius plots (e-h) of denitrification and anammox in

555 shelf and estuarine sediments. Panels are as follows: Denitrification in shelf (a, e) and estuarine

556 (b, f) sediments; anammox in the shelf (c, g) and estuarine (d, h) sediments. Curves in (a)

557 through (d) are general additive models fit to the data, and asterisks on the x-axis denote *in situ*

558 bottom water temperatures at the time of sampling. Lines in (e) through (h) are significant linear

559 regressions, the negative slopes of which are the activation energy (E_a).

560 **Figure 2**. Denitrification and anammox T_{opt} for all seasonal sampling dates and microcosm

561 treatments, and bottom water *in situ* or microcosm incubation temperature. Error bars denote T_{opt}

562 ranges.

563 **Figure 3.** Relative contribution of anammox to N_2 production (*ra*) in shelf sediments as a

564 function of incubation temperature.

565 **Figure 4.** Thermal profiles (a, b) and Arrhenius plots (c, d) of denitrification (a, c) and anammox

566 (b, d) in the microcosm experiment. Curves in (a) and (b) are general additive models fit to the

567 data. Lines in (c) and (d) are significant linear regressions, the negative slopes of which are the

568 activation energy (E_a).

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569   **Supporting information**

570   **Table S1** – Apparent activation energies (E_a) and thermal optima (T_{opt}) of denitrification and

571   anammox in shelf and estuarine sediments and the microcosm experiment. Asterisks denote

572   E_a values that differ significantly from others within the same site and process.

Site and process	Treatment or sampling date	Seasonal or microcosm temperature (°C)	Activation energy[#] (kJ mol^{-1})	Activation energy[#] (eV)	T_{opt} (°C)	T_{opt} range (°C)
Shelf denitrification	January 2011	6	60.4 ± 2.8*	0.63 ± 0.03*	35.0	33.1 – 35.0
	June 2011	11	43.7 ± 5.6	0.45 ± 0.06	27.3	19.8 – 33.0
	July 2011	16	50.5 ± 6.6	0.52 ± 0.07	27.5	23.8 – 31.3
	September 2011	17	38.5 ± 6.8	0.40 ± 0.07	23.7	18.2 – 27.4
	March 2012	7	43.7 ± 2.9	0.45 ± 0.03	25.4	21.8 – 27.2
Shelf anammox	January 2011	6	38.2 ± 4.1	0.40 ± 0.04	31.1	29.2 – 33.1
	June 2011	11	38.0 ± 3.0	0.39 ± 0.03	23.6	21.7 – 29.2
	July 2011	16	49.4 ± 3.6*	0.51 ± 0.04*	27.5	25.7 – 29.4
	September 2011	17	66.9 ± 12.3*	0.69 ± 0.13*	29.2	27.4 – 31.0
	March 2012	7	34.3 ± 2.6	0.36 ± 0.03	29.0	25.4 – 30.8
Estuary denitrification	June 2011	16	35.8 ± 2.1*	0.37 ± 0.02*	31.1	29.2 – 34.9
	August 2011	22	46.2 ± 4.1	0.48 ± 0.04	26.6	22.7 – 30.4
	January 2012	6	53.0 ± 5.3	0.55 ± 0.05	21.3	19.5 – 23.1
					33.9	23.1 – 37.5
Microcosm denitrification	t_0	4	41.2 ± 2.6	0.43 ± 0.03	24.5	22.7 – 26.3
	4°C	4	40.2 ± 4.1	0.42 ± 0.04	24.7	22.8 – 26.5
	4°C+C	4	36.5 ± 1.7	0.38 ± 0.02	22.8	21.0 – 26.5
	17°C	17	44.4 ± 0.4	0.46 ± 0.005	23.1	21.3 – 26.8
	17°C+C	17	46.4 ± 1.9	0.48 ± 0.02	23.1	21.3 – 26.8
Microcosm anammox	t_0	4	30.5 ± 12.1	0.32 ± 0.13	28.1	22.7 – 29.9
	4°C	4	30.8 ± 6.4	0.32 ± 0.07	28.4	24.7 – 32.0
	4°C+C	4	26.3 ± 4.2	0.27 ± 0.04	26.5	24.7 – 28.4
	17°C	17	19.3 ± 9.9	0.20 ± 0.10	26.8	NA[§] – 32.3
	17°C+C	17	21.5 ± 8.4	0.22 ± 0.09	26.8	21.3 – 30.4

573

574   [#]Ea is the negative of the mean slope ± s.e. of the regression line in Arrenhius plots

575   corresponding to shelf and estuarine sediments, while in the microcosm experiment, it

576   corresponds to the negative mean ± s.e. of E_a for three replicate aquaria.

577   [§]Not able to calculate lower limit as all rates below the maximum rate were within 95%

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578   confidence interval of maximum rate.