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Denitrification temperature dependence in remote, cold and N-poor lake sediments

Carlos Palacin-Lizarbe^{1,2} (orcid.org/0000-0002-1572-6053), Lluís Camarero² (orcid.org/0000-0003-4271-8988) , and Jordi Catalan^{1,3} (orcid.org/0000-0002-2934-4013) 

¹ CREAM, Campus UAB, Edifici C, E-08193 Cerdanyola del Vallès, Spain, ² Center for Advanced Studies of Blanes, (CEAB–CSIC), C/ d'accés a la Cala St. Francesc 14, E-17300 Blanes, Girona, Spain, ³ CSIC, E-08193 Cerdanyola del Vallès, Spain

Correspondence to: C. Palacin-Lizarbe (cpalacli7@gmail.com)

Key Points:

- Denitrification rates in mountain lakes are both nitrate and temperature limited.
- The apparent activation energy (E_a , kJ mol⁻¹) for denitrification depends on nitrate (μM) as $E_a = 46 + 419 [\text{NO}_3^-]^{-1}$.
- The impact of nitrogen emission reductions on the adjustment of the nitrogen cycle may be enhanced by climate warming.

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Abstract

The reservoir size and pathway rates of the nitrogen (N) cycle have been deeply modified by the human enhancement of N fixation, atmospheric emissions, and climate warming. Denitrification (DEN) transforms nitrate into nitrogenous gas and thus removes reactive nitrogen (N_r) back to the atmospheric reservoir. There is still a rather limited knowledge of the denitrification rates and their temperature dependence across ecosystems; particularly, for the abundant cold and N-poor freshwater systems (e.g., Arctic and mountain lakes). We experimentally investigated the denitrification rates of mountain lake sediments by manipulating nitrate concentration and temperature on field collected cores. DEN rates were nitrate limited in field conditions and showed a large potential for an immediate DEN increase with both warming and higher N_r load. The estimated activation energy (E_a) for denitrification at nitrate saturation was $46 \pm 7 \text{ kJ mol}^{-1}$ ($Q_{10} 1.7 \pm 0.4$). The apparent E_a increased with nitrate (μM) limitation as $E_a = 46 + 419 [\text{NO}_3^-]^{-1}$. Accordingly, we suggest that climate warming may have a synergistic effect with N emission reduction to readjusting the N cycle. Changes of nitrate availability might be more relevant than direct temperature effects on denitrification.

1. Introduction

The anthropogenic alteration of the nitrogen (N) cycle is one of the most challenging problems for the Earth system [Rockstrom et al., 2009]. Human activity has at least doubled the levels of reactive nitrogen (N_r) available to the biosphere, largely as a result of the industrial N fixation for fertilizer productions and the burning of fossil fuels [Erisman et al., 2011]. The global N cycle is still evaluated with high uncertainty. A few flux estimates are quantified with less than $\pm 20\%$ error and many have uncertainties of $\pm 50\%$ and larger [Gruber and Galloway, 2008]. The transient situation of the planet with many factors that influence the N cycle changing simultaneously, demands a deeper understanding of the factors controlling the rates of the N cycle pathways [Baron et al., 2013; Greaver et al., 2016]. The temperature dependence of the rates of the distinct pathways is of particular interest for evaluating potential synergistic effects of climate warming and nitrogen emissions on N global cycle.

Denitrification (DEN) is the microbial activity by which nitrogenous oxides, mainly nitrate and nitrite, are reduced to dinitrogen gasses, N_2O and N_2 [Tiedje, 1988]. DEN is the primary process removing N_r from the biosphere [Seitzinger et al., 2006]. The DEN dynamics is typically episodic; driven by the fluctuating coexistence of primary resources, favorable conditions, and the microbial agents. Consequently, DEN rates are difficult to measure, model and upscale. Existing methods are problematic for different reasons [Groffman et al., 2006]. Much of the challenges arise from the fact that small areas (hot spots) and brief periods (hot moments) account for a high percentage of the denitrification activity both in terrestrial and aquatic ecosystems [Parkin, 1987; McClain et al., 2003; Groffman et al., 2009]. A substantial proportion of denitrification occurs in the upper part of the sediments, where necessary resources (e.g. N-oxides, fresh organic matter) meet anaerobic conditions. Lakes have been identified as the aquatic ecosystems with the highest seasonal and site variation in DEN rates [Piña-Ochoa and Alvarez-Cobelas, 2006], although part of the observed variability could be due to the methods applied. Particularly, the alteration of the integrity of the samples markedly affects the DEN rate (r_d) measured. Higher values in slurries are obtained compared to undisturbed core sediments [Ambus, 1993]. The former cannot be considered representing *in situ* rates. They only provide relative measures for comparisons, if applied in the same way. However, if the target is to integrate DEN rates in the evaluation of the nitrogen cycle, there is an urgent demand of

measurements in conditions as close as possible to the natural ones to reduce the uncertainty of the estimates and provide the elements for a reliable upscaling of the measurements [Galloway, 2004; Gruber and Galloway, 2008].

The temperature dependence of an enzymatic process can be described by its activation energy (E_a), which reflects the increase in the rate with temperature [Arrhenius, 1915] when there is no resource limitation. However, the biogeochemical E_a includes also temperature effects on molecular kinetics, physiological acclimation by microbial strains and microbial assemblage changes [Hall *et al.*, 2010; Crowther and Bradford, 2013]. In natural conditions, the E_a values reflect a multi-step process and thus can vary with the substrate availability [Brezonik, 1994] and may depend on the particular assemblage of organisms performing the biogeochemical reaction [Hall *et al.*, 2008]. Each of these DEN control levels has a longer characteristic time, from instantaneous to a few days.

In lakes, sediment DEN rates at timescales below a few days are more likely to be constrained by the substrate supply than temperature fluctuations. Nitrate declines by the same DEN activity and their supply depend on other microbial activities (e.g., nitrification) and physical transport. When measuring DEN E_a , experimentally, some environmental conditions may depart easily from those *in situ*, either because of the use of an unrealistic temperature range [Boulétreau *et al.*, 2012], very high nitrate addition [Holmes *et al.*, 1996] or both. Alternatively, some observational approaches to estimate DEN E_a use activities (r_d) measured at periods of the year with contrasting temperature [Bachand and Horne, 2000; Sheibley *et al.*, 2003]. In this case, the substrate availability may change but also the assemblage of microorganisms present.

Consequently, there are a number of aspects to consider in an assessment of the biogeochemical DEN E_a for a certain type of ecosystems (Fig. 1). They include 1) the general methodological approach; fundamentally, whether the approach is experimental, controlling the temperature change, or observational, using spatial or temporal natural temperature variation. 2) How the substrates (i.e., organic carbon, nitrogen) are considered; particularly, whether they are artificially saturated or maintained within natural conditions. And, 3) which are the biogeochemical processes that could be a source of added uncertainty in the assessment provided their competition for nitrate or nitrite or their release of nitrous oxide.

There are few studies of the DEN E_a in oligotrophic systems [Holmes *et al.*, 1996] and most are marine [Rysgaard *et al.*, 2004; Canion *et al.*, 2014a]. In the current situation of global change, the case of remote ecosystems, those that are mostly influenced by atmospheric processes rather than direct human action in the watershed, are of particular interest. Many of these sites (e.g., alpine and subarctic regions) have, are or would experience increased N_r deposition [Holtgrieve *et al.*, 2011] and warming [Smol, 2012]. Therefore, they are key sites for studying the interaction between temperature and the N cycle [Catalan *et al.*, 2013].

The increase of N_r deposition is eventually reflected in the stream and lake loads depending on the degree of N saturation in the soil and vegetation [Stoddard, 1994]. Long-term sustained high N_r deposition results in a watershed quasi-steady-state so that N_r deposition and streams show similar temporal trends and fluctuations. One may expect that lakes would follow streams mid-term temporal patterns. However, recently, Camarero and Catalan [2012] have found an opposite trend during the last decades in the Pyrenees between lakes and streams. The latter follows the still N_r increasing tendency in the deposition, but lakes show a decline. The authors have attributed the opposed trends to an increase in lake productivity related to a higher phosphorus deposition. However, they were not able to evaluate whether an enhancement of denitrification could be an alternative explanation due to the lack of empirical information. The

difficulty of the measuring DEN rates at low N_r concentrations may justify why remote systems have been overlooked with a few exceptions [McCrackin and Elser, 2010; 2012; Vila-Costa et al., 2016; Castellano-Hinojosa et al., 2017]. Indeed, it has been only one attempt to estimate DEN E_a in these systems [Myrstener et al., 2016]. Consequently with this gap in knowledge, the aim of this study was to assess the DEN temperature dependence (E_a) in remote, cold and relatively N-poor lakes. From the several options above introduced (Fig. 1), our approach was thought as one that could be useful for an evaluation of the actual *in situ* rates in the area studied, the potential upscaling of DEN estimations over large sets of oligotrophic lakes, and projections of future scenarios of air temperature and nitrate deposition. Therefore, DEN measurements were conducted using intact core sediments, the acetylene inhibition method combined with sensors for nitrous oxide and experimental control of temperature and nitrate availability within the natural range found in these lakes. For complementarity, we compiled the existing data on DEN temperature dependence in aquatic ecosystems across the literature and evaluated the results according to the framework introduced in Fig. 1, emphasizing the likely reasons for the large variation in the estimations and, if so, the discrepancies with our results.

2. Materials and Methods

2.1. General methodological approach

Three lakes with contrasting morphology and carbon flow characteristics (Table 1) were sampled to consider potential different microbial communities. In each sampling date, we collected five core sediments in the field, which were immediately transported to the lab to control for temperature and nitrate availability within the ranges that can be found in these mountain lakes. We used sensors for nitrous oxide combined with the acetylene inhibition method and anoxic conditions to minimize the disturbance of the sediment structure. For each core, we performed sequential estimations of the DEN rates (r_d) at several temperature values and nitrate concentrations. This procedure reduces the noise that the sediment spatial heterogeneity can introduce in the estimations but may introduce autocorrelation effects. To overcome the latter constraint, we did not follow the same experimental sequence in each trial, so we could statistically evaluate the autocorrelation influence and distinguish it from other sources of variation (e.g., nitrate concentration, lake, sensors). Although the experiment was planned for the same number of cores per lake (5), finally, we also included in the data set two preliminary tests - performed using only a part of the nitrate gradient in some Lake Redon cores - as they fitted in the general results obtained and thus increased the statistical robustness of the final model.

2.2. Sampling and experimental design

The selected three lakes are representative of the lake district of the Pyrenees (Table 1). They cover a broad range of maximum depth (9-73 m) and seasonal thermal variability [Catalan et al., 2002]. The experimental temperature (5-15°C) and the nitrate added levels (7-14-28 μM) covered the natural variability in the region and possible future scenarios. A total of 25 sediment cores (methacrylate, \varnothing 6.35 cm) were assessed (15 from Redon, 5 from Plan and 5 from Llong, Table S1). They were collected with a gravity corer [Glew, 1991] at midday around the deepest point of each lake (Table 1). Only undisturbed cores with clear overlying water and interface were used. The experimental setup included an incubation chamber that ensured dark conditions and controlled temperature ($\pm 1^\circ\text{C}$) (Fig. 2). Nitrate was measured at the beginning of the incubations (Table S1). As a precautionary action, glucose was added in excess (1.5 g L^{-1}) to

avoid carbon limitation [Vila-Costa *et al.*, 2016] despite that some previous tests did not show conclusive evidence of such limitation. DEN measurements at different nitrate concentrations and temperatures started the next morning and were conducted sequentially, commonly: Step 1: First 7 μM nitrate addition at 5°C (0-12h); 2: 15°C (12-24h); 3: Second 7 μM nitrate addition at 15°C (24-36h); 4: 5°C (36-48h). 5: 14 μM nitrate addition at 5°C (48-60h); and 6: 15°C (60-72h).

2.3. Denitrification measurement

DEN measurements were performed using the acetylene inhibition method combined with sensors for nitrous oxide (N_2O). Anoxia, first, and acetylene inhibition, after, were achieved by bubbling N_2 and C_2H_2 sequentially, respectively, during 10 minutes in the water phase of the core before each DEN measurement. Acetylene inhibits the reduction of N_2O to N_2 [Balderston *et al.*, 1976; Yoshinari and Knowles, 1976]. The accumulated N_2O was measured using a modified Clark electrode probe (N_2O -R microsensor, Unisense A/S, Denmark) (detection limit = 0.1 μM), in the water phase. A gentle magnet stirring was applied to avoid stratification but without resuspension of the sediment. Readings were taken every 5 minutes via a picoammeter logged to a laptop. The response of the electrochemical sensor is linear in the range of 0–1.2 mM [Andersen *et al.*, 2001]. The instrument was kept polarized during all the measurement period. It was calibrated at each temperature using a calibration chamber (CAL300, Unisense A/S, Denmark), zero gas water (Milli-Q) and a freshly prepared $\sim 50 \mu\text{M}$ N_2O solution. The latter was obtained adding a certain volume of N_2O saturated water [Weiss and Price, 1980] to the zero gas water following manufacturer's instructions as described in Foley *et al.* [2010].

2.4. Water and sediment characterization

Immediately after collection, we measured the temperature (°C) of water overlying the sediment core (Table 1). For chemical analyses, water samples were filtered through a pre-combusted (4 h at 450°C) GF/F glass fiber filter. Nitrate was determined by capillary electrophoresis using a Quanta 4000 (Waters) instrument. Ammonium and nitrite were determined by colorimetric methods in a segmented-flow autoanalyzer (AA3HR, Seal), using the Berthelot reaction for ammonium (Bran+Luebbe method G-171-96) and the Griess reaction for nitrite (Bran+Luebbe method G-173-96). Dissolved organic carbon (DOC) was measured by catalytic combustion to CO_2 and detection by IR spectroscopy in a TOC5000 (Shimadzu) analyzer. The water column of the lakes sampled show a circumneutral pH (~ 7) [Vila-Costa *et al.*, 2014].

After DEN measurements, the surface sediment was sliced (2 cm) and freeze-dried for 72 h. Around 5 mg of the dried sample was encapsulated together with a catalyst (V_2O_5) in tin capsules for the determination of C and N using a C-H-N-S (Carlo-Erba) analyzer. The dry weight percentage of organic matter content in the sediments was determined by loss on ignition (LOI) following Heiri *et al.* [2001]. In all cases, the samples were equilibrated to room temperature in a desiccator before weighing them. The median grain size of the sediment was determined by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd, UK). Freeze dried sediment was rehydrated in distilled water and introduced into the sample dispersion unit (Hydro 2000 G, Malvern Instruments Ltd, UK) adding hexametaphosphate and sonicating to avoid aggregates. Laser obscuration was between 10-20 % and the measuring range between 0.02 and 2000 μm .

2.5. Numerical methods

Denitrification activity rates (r_d) ($N=107$) were calculated by linear regression from the sequential readings of the N_2O sensors ($r = 0.89 \pm 0.02$ (mean \pm standard error), with an average of 66 point measurements). Raw r_d values in $\mu M N_2O \text{ core}^{-1} \text{ h}^{-1}$ were transformed to $\mu \text{mol } N_2O \text{ m}^{-2} \text{ h}^{-1}$ using the inner core section area. The apparent activation energy (E_a ; kJ mol^{-1}) of the denitrification process was estimated according to the Arrhenius equation: $\frac{r_{dj}}{r_{di}} = \exp \left[\frac{E_a}{R} \left(\frac{1}{T_i} - \frac{1}{T_j} \right) \right]$ where R is the gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$); T , the absolute temperature ($^{\circ}\text{K}$); and r_d , the denitrification activity rate. The subscripts (i, j) indicate two different thermal conditions. Statistics were conducted using R version 3.3.0 (R Development Core Team 2016). Linear mixed-effects models were performed using the *lme* and *lmer* functions within the *nlme* and *lme4* R packages, respectively [Pinheiro et al., 2007; Bates et al., 2015]. Functions *ANOVA* of the R core package *stats* (R Development Core Team 2016), *AICc* (Akaike Information Criterion for a small sample size) of the package *AICcmodavg* [Mazerolle, 2016] and fixed, global explained variance and *r.squaredGLMM* of *MuMIn* [Barton, 2016] were used to select the best fitting model.

2.6. Compiled data of DEN E_a in aquatic ecosystems

For comparison, we compiled data about the denitrification temperature dependence in aquatic ecosystems across literature (Table S2). For each study, the apparent activation energy (E_a ; kJ mol^{-1}) of the denitrification process was estimated by the Arrhenius equation above or by the slope of an Arrhenius plot of $\ln(r_d)$ as a function of T^{-1} when more than two temperature data were available.

3. Results

3.1. Denitrification temperature dependence in mountain lakes sediments

The denitrification activity rates (r_d) measured ranged from 0.5 to 60.5 $\mu \text{mol } N_2O \text{ m}^{-2} \text{ h}^{-1}$ (Table S1). The rates increased with the experimental temperature, and nitrate addition levels (Fig. 3a). Rates (mean \pm standard error) for 7, 14 and 28 μM nitrate added were at 5°C 2.2 \pm 0.3, 4.6 \pm 0.5, 11.3 \pm 1.2 $\mu \text{mol } N_2O \text{ m}^{-2} \text{ h}^{-1}$, respectively, and at 15°C 6.6 \pm 0.8, 12.7 \pm 0.9, 27.0 \pm 2.9 $\mu \text{mol } N_2O \text{ m}^{-2} \text{ h}^{-1}$, respectively.

From the measured rates, the apparent activation energy was estimated for each nitrate level (Table S1, average $E_a = 67 \pm 4 \text{ kJ mol}^{-1}$). DEN r_d at 5°C had a larger influence on the E_a values than those at 15°C as shown by a significant negative correlation ($r = -0.51$, $p = 0.0001$) between E_a and $\ln(r_d)$ at 5°C and not significant at 15°C.

E_a values and their variation declined when the nitrate added increased (Fig. 3b). E_a negatively correlated ($r = -0.33$, $p = 0.01$) with the initial experimental nitrate (water phase plus added) concentrations. E_a at nitrate saturation was estimated by fitting a linear relationship between the inverse of nitrate concentration and E_a (model 0 in Table 2). In this model, the intercept indicates the value of E_a when the influence of nitrate concentration tends to zero.

The variation of the rates measured under the same conditions of temperature and nitrate concentration was markedly high. Therefore, we investigated whether the estimation of the E_a dependency on nitrate concentration could be improved by taking into account other

experimental issues and the lake idiosyncrasy (Table 2). We developed alternative mixed regression models including different factors in the random part and maintaining the inverse of nitrate concentration as the only factor in the fixed part (see supporting information for details). The alternative models took into account: the five sensor performance; the autocorrelation intrinsic to subsequent experimental additions; the nitrate addition level; the order of the addition level (not all the experiments followed the complete sequence from 7 to 28 $\mu\text{M NO}_3^-$) and the lake of the core. The only models that significantly improved the initial model 1 were those accounting for the sensor effects (model 3 and 4 in Table 2). They showed the lower AICc values and explained more variance (ANOVA p-values of 0.047 and 0.008 for model 3 and 4, respectively). When we considered core, lake, autocorrelation or nitrate addition features did not improve the E_a estimation. We eventually selected model 4 as the best estimation because it is simpler than model 3. Consequently, the denitrification E_a at nitrate saturation is estimated to be $46 \pm 7 \text{ kJ mol}^{-1}$ (i.e., $Q_{10} = 1.7 \pm 0.4$) and the apparent E_a to vary according to nitrate concentration as:

$$E_a = 46 + 419 [\text{NO}_3^-]^{-1} \quad (1)$$

3.2. Comparison with other aquatic ecosystems

We identified a total of 21 previous studies (Table S2) to compare our results with estimations from other sites and methods. They included lakes [*Cavari and Phelps, 1977; Messer and Brezonik, 1984; Myrstener et al., 2016*], ponds [*Veraart et al., 2011*], streams [*Holmes et al., 1996; Boulétreau et al., 2012*], rivers [*Pfenning and McMahon, 1997; Pattinson et al., 1998; Silvennoinen et al., 2008*], denitrification beds (i.e., carbon supply to promote denitrification in eutrophic rivers, [*Cameron and Schipper, 2010; Warneke et al., 2011*]), hyporheic [*Sheibley et al., 2003*] and riparian [*Ambus, 1993*] zones, groundwaters [*Jørgensen et al., 2009*], swamps [*Westermann and Ahring, 1987*], wetlands [*King and Nedwell, 1984*], estuaries [*Brin et al., 2017*] and marine environments [*Rysgaard et al., 2004; Canion et al., 2014b; Canion et al., 2014a; Kraft et al., 2014; Brin et al., 2017*]. There is a large scattering in the E_a values estimated. However, the most similar sites to those in our study [*Rysgaard et al., 2004; Myrstener et al., 2016*] were also from remote, cold and N-poor areas, and showed similar E_a values plotted against the inverse of nitrate (Fig. 4).

4. Discussion

4.1. Denitrification rates, nitrate, and temperature

The denitrification rates (r_d) obtained were similar to the few other measurements in mountain lake sediments [*McCrackin and Elser, 2012; Vila-Costa et al., 2016*]. The values are in the low range of freshwater sediments [*Seitzinger, 1988; Piña-Ochoa and Alvarez-Cobelas, 2006*] as expected from cold and oligotrophic environments. However, despite the apparent harsh conditions, the denitrification activity intensifies with increasing nitrate and temperature without becoming saturated (Fig. 3a) within the range of values currently found in the Pyrenees [*Camarero and Catalan, 2012*]. Therefore, the current denitrification potential of these systems can respond to warming or increased N_r deposition (or watershed loading) without any time lag. It could be argued that the experimental sequential procedure of 72h incubations could facilitate an enrichment of denitrifiers in our experiment. We used the shortest time to obtain reliable measurements of the activity rate without disturbing the sediment interface. An alternative

experimental design, based on pseudo-replicates of several cores from the same site incubated at the various levels of nitrate and temperature, would notably increase the sample-related heterogeneity due to the patchy nature of any sediment. We think that the cold and oligotrophic conditions of the studied system prevent any significant enrichment in denitrifiers during the experimental development.

In the compiled DEN E_a studies, there are variable values for similar NO_3^- enrichments (Fig. 4), which may be attributed to changing assemblages of denitrifiers. A case of a latitudinal gradient in coastal marine sediments, with higher values for subtropical locations (121 and 100 kJ mol^{-1}) [Canion *et al.*, 2014b]. A case of cultures with a different nitrate reducing dominant species, *Pseudomonas sp.* or *Vibrio sp.*, with 98 and 60 kJ mol^{-1} E_a values, respectively, isolated at 10 and 25°C from a salt-marsh sediment [King and Nedwell, 1984]. And cases of seasonal variability, with E_a ranges of 70 to 76, 36 to 53 and 38 to 60 kJ mol^{-1} in swamp, estuary and continental shelf sediments from temperate ecosystems, respectively [Westermann and Ahring, 1987; Brin *et al.*, 2017]. Beyond the sequential experimental issue, different denitrifier assemblages between lakes could also be a source of variation in our data. However, models including the lake site as a source of random variation did not improve the fitting (Table 2). Only the use of molecular techniques to characterize the microbial assemblages (i.e., 16S rRNA) will settle discussions about this issue and clarify the relative influence of physicochemical and biological constraints.

To our knowledge, only another study measured DEN activities experimentally controlling both temperature and nitrate gradients close to the *in situ* conditions [Pattinson *et al.*, 1998]. In this study, E_a values also declined with increasing nitrate. Provided the difference of about three orders of magnitude in nitrate concentrations between the two studies, one has to conclude that DEN saturation by nitrate is achieved at different concentrations in eutrophic and oligotrophic ecosystems. Even so, the range of E_a estimated in the eutrophic experiment fell within the 95% confidence limit of our model (Fig. 4).

The higher E_a values (155-179 kJ mol^{-1}) have been found in eutrophic ponds [Veraart *et al.*, 2011]. In this case, there was not an experimental forcing of the anoxia, so the authors attribute the high effect of warming on denitrification rates to a decline in the oxygen interference due to a synergic effect of lowering both the oxygen solubility and the production/respiration ratio when the temperature increases. Boulêtreau *et al.* 2012 also found a high E_a (137 kJ mol^{-1}): they were using a wide, and high experimental temperature range (1-40°C) compared to the *in situ* temperature (7.2±1.7 °C) of their sites. The same experimental set, indicate a lower E_a (43 kJ mol^{-1}) when calculated only for a narrower temperature range (1-12°C) closer to that *in situ* (Table S2). The lowest E_a values (<20 kJ mol^{-1}) were found by Cameron and Schipper [2010].

Coupled nitrification could also interfere the DEN experiments if ammonium levels are high. Sheibley *et al.* [2003] performed the only study of DEN E_a at markedly low nitrate concentrations without any addition. Their E_a estimates depart from our model (Fig. 4). In that case, there was an intense nitrification, also highly dependent on temperature and ammonium concentrations (22 μM), and DEN E_a was estimated using activity rates at different seasons as a surrogate for temperature control.

In our compiled data, including our study, we did not find any significant correlation between DEN E_a values and any statistical temperature descriptor (T max, T min, T mean, T *in situ* or annual T mean, Table S2). Canion *et al.* [2014b] found a higher DEN E_a value for warmer (subtropical) than colder (temperate or polar) environments. They suggested an adaptation of denitrifiers to *in situ* temperature, supported by a previous study in the polar region [Canion *et*

al., 2013]. Although not universally, there are trade-offs between genetic adaptation to low and high temperature [Bennett and Lenski, 2007]. In a salt-march study, culture isolates at 10, and 25°C from the same sediment sample resulted in different nitrate-reducing dominant species, *Pseudomonas sp.* and *Vibrio sp.*, and showed different E_a (98 and 60 kJ mol⁻¹, respectively). The highest E_a was in the culture isolated at the closer temperature to *in situ* (15°C) [King and Nedwell, 1984]. Recurrently, DEN E_a values are higher when obtained from temperatures around *in situ*. In the literature data, a mean increase of 42±11% (±se) is achieved when E_a is calculated with a narrow temperature range close to the *in situ* one compared with the result using the complete temperature range of the experiment (Table S2). We found a similar increase (51%) when the temperature range was reduced to values close to the *in situ* temperature in the samples of Lake Redon (Table S2). Consequently, we highlight the convenience of measuring DEN temperature dependence as close as possible to *in situ* conditions of temperature.

In the current context of results (Fig. 4), it seems necessary to recommend experiments following a common procedure including an experimental nitrate gradient and temperatures no more than 15 °C beyond the *in situ* values. The spatial or temporal distribution of the samples should not be a surrogate for these gradients.

4.2. Carbon limitation, nitrate supply and competing process

There were two aspects with potential influence on DEN temperature dependence (Fig. 1) that we did not explicitly consider, carbon limitation and competing processes. We did not expect a denitrification limitation by carbon in the Pyrenean mountain lakes. There is a higher ratio of primary production to respiration in both the water column and the surface sediments, resulting in an elevated fresh carbon stock for bacterial activity [Camarero *et al.*, 1999]. All in all, our experimental measurements were made with the addition of glucose. In fact, one can expect a C availability influence on the denitrification-temperature dependence mostly in warm and eutrophic (nitrate-rich) aquatic ecosystems. The lowest DEN E_a values (<20 kJ mol⁻¹) in the compiled data were found by Cameron and Schipper [2010] in an extended 10-months incubation experiment. The low E_a values could be due to a C-limitation in the warmer treatments using labile C sources (green waste, maize cobs, and wheat straw). C deficiency could also cause the low E_a (26 kJ mol⁻¹) assessed in a *Pseudomonas aeruginosa* culture isolated from Lake Kinneret as there was no addition of any C source [Cavari and Phelps, 1977; Gal *et al.*, 2003].

In the method that we applied, the experimental assumption is that nitrification is not acting because of the induced anoxic conditions and the inhibition of the ammonium monooxygenase by acetylene [Hynes and Knowles, 1978]. Consequently, sources of NO₃⁻ supply variation restrict to diffusive transport and uptake by alternative biogeochemical pathways. Nitrate diffusion aspects appear to have had no significant influence in our experiment as core and lake factors, which implicitly account for differences in sediment particle size, did not improve the models (Table 2).

Nitrate uptake, anammox and dissimilatory nitrate reduction to ammonium (DNRA) are the biogeochemical processes that can compete with DEN for nitrate. We may assume that nitrate assimilation should not be relevant because of the high abundance of ammonium - the preferred N source [Luque-Almagro *et al.*, 2011] - and the common low rates of nitrogen uptake in dark conditions [Lorenzen *et al.*, 1998].

Anammox competes with DEN for nitrite. To our knowledge, there is no evidence of the dominance of anammox or DEN depending on the nitrate concentration. Anammox seems more

sensitive to nitrate fluctuations than DEN [Rysgaard *et al.*, 2004]. The highest anammox activity respect to DEN has been found at 5°C in Arctic marine sediments [Rysgaard *et al.*, 2004]. Canion *et al.* [2014a] found similar results in Arctic fjord sediments with anammox bacteria being more specialized for psychrophilic activity than denitrification. Recently, Brin *et al.* [2017] in a warmer habitat, temperate marine sediments, did not find differences in temperature responses for the two processes.

The ratio of electron acceptor (i.e., NO_3^-) to electron donor (i.e., organic C) is the most frequently mentioned partitioning factor between DEN and DNRA [Tiedje *et al.*, 1982]. DNRA is the dominant pathway under nitrate-limited conditions, while DEN is the favored pathway under nitrate-replete conditions [Smith *et al.*, 1982; King and Nedwell, 1985; 1987; Herbert and Nedwell, 1990; Laverman *et al.*, 2006; Dong *et al.*, 2009; Mania *et al.*, 2014; Nogaro and Burgin, 2014]. Slightly more energy is obtained per mol of NO_3^- by DNRA than by DEN [Strohm *et al.*, 2007] and, additionally, DNRA consumes more electrons (8 vs. 5) during the reduction of NO_3^- to NH_4^+ [Burgin and Hamilton, 2007]. Low NO_3^- and high organic C availability can thus create more favorable conditions for DNRA than DEN [MacFarlane and Herbert, 1982; Tiedje *et al.*, 1982]. The *in situ* C/N ratios of the sediment (Table 1) were always higher than 10 in our samples, in the range of values more favorable to DNRA. A C/N ratio for an equal contribution of the two processes of nitrate reduction is *c.* 7.5 [Yoon *et al.*, 2015]). In all our experimental treatments, the DOC/nitrate ratio was above 100 (a/a), thus with similar conditions favoring DNRA as in field conditions.

Some studies have also shown a dominance of DNRA over DEN at higher temperatures [Ogilvie *et al.*, 1997; Yoon *et al.*, 2015]. This dominance could be temporal during summer periods [King and Nedwell, 1984; Jørgensen, 1989] or spatial as occurs in some warm tropical ecosystems [Dong *et al.*, 2011]. Nonetheless, this apparent temperature effect may mask the true influence of the co-occurring higher reducing conditions and lower nitrate concentrations at higher temperatures that eventually determine a low ratio of electron acceptor to electron donor [Jørgensen, 1989; Gardner *et al.*, 2006; Gardner and McCarthy, 2009; Gross-Wittke *et al.*, 2010; Nizzoli *et al.*, 2010; Zhu-Barker *et al.*, 2015]. The only two studies with data of temperature dependence for the two nitrate reduction processes are at a high nitrate concentration (1 mM). Kraft *et al.* [2014] shows E_a values of 22 and 40 kJ mol^{-1} , for DEN and DNRA respectively, in the complete experimental range of temperatures (10 to 30°C), and 26 and 79 kJ mol^{-1} , respectively, in a narrow segment of temperature (10 to 15°C) – based on E_a values calculated from Fig. S8A. Yoon *et al.* [2015] investigated the switch between the two processes in a single microbial model, *Shewanella loihica*, a species capable of performing the two pathways. They found a dominance of DNRA at warmer temperatures, with DEN showing a decline from 21 to 30 °C and a null activity at 37°C. Temperature does not appear to be an issue in our experiment concerning DNRA and DEN partition. Using slurry incubations overestimate DNRA by enhancing nitrate availability to deeper layers of the sediments, where ammonifiers dominate over denitrifiers [Behrendt *et al.*, 2013]. This procedure could have affected other DEN estimates in mountain lakes [Vila-Costa *et al.*, 2016] but their results do not differ markedly from our ones.

The generation of N_2O due to abiotic processes could let to an overestimation of the denitrification activity. At the current stage of knowledge, it is hard to infer any contribution of chemical processes to the overall NO and N_2O production [Schreiber *et al.*, 2012]. There are two major abiotic N_2O production pathways. The NH_2OH decomposition to N_2O at circumneutral pH is favored by high Mn (IV), temperature and salinity, and low organic carbon. The

chemodenitrification of NO and NO_2^- to N_2O is favored by high pH, low O_2 and solid Fe (III) or Cu (II) catalysts [Zhu-Barker *et al.*, 2015]. Few of these favorable conditions are present in the studied lakes (e.g., the granitic nature of the bedrock in the studied lakes confers low levels of iron to the sediments [Catalan *et al.*, 2014]). However, these processes may be relevant in watersheds of metamorphic rocks rich in metals, which are common in some parts of the Pyrenees and other mountain ranges [Catalan *et al.*, 1993].

We can conclude that DNRA, at nitrate limiting conditions and high temperature, and anammox, at low temperature and C/N, are the most like processes influencing DEN yield in mountain lakes. Future experiments on the temperature dependence of DEN, Anammox, and DNRA across gradients of nitrate, C/N and temperature are necessary to clarify these interactions.

5. Conclusions

There is still a limited knowledge about denitrification rates and their temperature dependence in general and, particularly, for cold and N-poor systems, despite that the latter cover a high percentage of the continental aquatic ecosystems. Our study shows that the low rates of denitrification observed are not nitrate saturated and the system can respond to warming and increased N_r loadings, either from deposition or the watershed. The case-by-case estimation of DEN E_a present much variability, but there is a robust statistical behavior that can be applied to modeling, upscaling and as a benchmark for actual measurements. Three main conclusions derive from our results:

1. Under nitrate saturation conditions (e.g., $> 100\mu\text{M}$) a DEN E_a significantly different from 46 kJ mol^{-1} (e.g., > 67 or < 31) would suggest that there is another factor interfering (e.g., C quality or quantity limitation; or very distinct microbial assemblage related to other features of the system).
2. Below saturation and not at extremely low nitrate levels ($> 3\mu\text{M}$), equation (1) can be applied for modeling the temperature influence on DEN rates, accounting for nitrate levels.
3. In the natural environments of remote areas, *in situ* nitrate values are still far from DEN saturation. Currently, this feature leads to very high apparent DEN E_a values but this does not mean that with warming higher DEN rates could be sustained. In case of temperature increase, a short transitory period of high DEN would lead to a rapid depletion of nitrate unless nitrate supply rates would proportionally increase. Therefore, in a warmer scenario, variation in denitrification rates will continue mostly depending on nitrate supply processes that include proximal (e. g., sediment-related nitrification), local (e. g., N_r leaching from soils) and regional (e. g., atmospheric N_r deposition) components [Wallenstein *et al.*, 2006].

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Table 1. Study sites location and characteristics.

Lake	Redon	Plan	Llong
Latitude (N)	42.64208	42.62248	42.57431
Longitude (E)	0.77951	0.9307	0.95063
Altitude (m a.s.l.)	2235	2188	2000
Area (ha)	24	5	7
Maximum depth, (m)	73	9	12
Temperature ^a (°C)	4	5	3
NO ₃ ⁻ ^a (μM)	5 (4-6)	1 (1-2)	8 (7-9)
NO ₂ ⁻ ^a (μM)	0.17 (0.13-0.21)	0.05 (0.05-0.05)	0.15 (0.13-0.18)
NH ₄ ⁺ ^a (μM)	9 (6-14)	3 (2-5)	25 (19-31)
DOC ^a (mg L ⁻¹)	52 (2-88)	74 (6-99)	15 (2-70)
LOI ^b (%)	25 (18-35)	44 (40-48)	27 (24-31)
Carbon ^b (% dry weight)	12 (10-18)	20 (17-24)	13 (11-14)
Nitrogen ^b (% dry weight)	1.2 (0.9-2.1)	1.9 (1.6-2.3)	1.2 (1.0-1.5)
Sediment grain size (median - μm) ^b	252 (166-351)	333 (205-465)	174 (130-223)

^a Characteristics of the water overlying the sediment. Ice-free season average, minimum, and maximum values. ^b Characteristics of the surface sediment (0-2 cm layer): Loss on ignition (LOI), as a proxy of organic matter (carbonates < 2%, not shown).

Table 2. Alternative regression models relating the DEN E_a (kJ mol^{-1}) to the inverse of the nitrate concentration ($[\text{NO}_3^-]^{-1}$) (μM) in the overlying water of the lake sediments.

Regression model	Random part	Formula	E_a at nitrate saturation (Intercept \pm se)	p-value	Coefficient \pm se	p-value	AICc	Fixed R^2	Global R^2
0	Lm model, no random part	mod0=lme($E_a \sim [\text{NO}_3^-]^{-1}$)	50 \pm 8	<0.00001	315 \pm 141	0.0303	481	0.09	0.09
1	GLS model, no random part	mod1=glms($E_a \sim [\text{NO}_3^-]^{-1}$)	50 \pm 8	<0.00001	315 \pm 141	0.0303	465	0.09	0.09
2	GLS model, no random part	mod2=glms($E_a \sim [\text{NO}_3^-]^{-1}$, correlation=corAR1(form= \sim Add phase Core))	50 \pm 8	<0.00001	324 \pm 139	0.0239	467	0.09	0.09
3	Sensor effect	mod3=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 1+ $[\text{NO}_3^-]^{-1}$ Sensor)	44 \pm 8	<0.00001	451 \pm 227	0.0529	464	0.15	0.47
4	Sensor effect (just slope)	mod4=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 0+ $[\text{NO}_3^-]^{-1}$ Sensor)	46 \pm 7	<0.00001	419 \pm 175	0.0207	460	0.13	0.42
5	Sensor (with nested core effect)	mod5=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 1+ $[\text{NO}_3^-]^{-1}$ Sensor/Core)	45 \pm 8	<0.00001	438 \pm 228	0.0666	473	0.14	0.49
6	Core effect	mod6=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 1+ $[\text{NO}_3^-]^{-1}$ Core)	48 \pm 9	<0.00001	365 \pm 177	0.0505	469	0.11	0.34
7	Core effect (just slope)	mod7=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 0+ $[\text{NO}_3^-]^{-1}$ Core)	49 \pm 8	<0.00001	339 \pm 153	0.0368	465	0.1	0.29
8	Nitrate added effect	mod8=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 1+ $[\text{NO}_3^-]^{-1}$ Nitrate added)	42 \pm 15	0.0068	495 \pm 298	0.1038	472	0.18	0.3
9	Nitrate addition phase effect	mod9=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 1+ $[\text{NO}_3^-]^{-1}$ Add phase)	48 \pm 10	<0.00001	366 \pm 200	0.0732	472	0.11	0.15
10	Lake effect	mod10=lme($E_a \sim [\text{NO}_3^-]^{-1}$, random= \sim 1+ $[\text{NO}_3^-]^{-1}$ Lake)	50 \pm 8	<0.00001	315 \pm 141	0.0307	472	0.09	0.09

All the regression models have the same fixed part, that is the inverse of the nitrate concentration ($[\text{NO}_3^-]^{-1}$). Thus the models differ in the random part. The coefficient is the slope of the model and shows the influence of the inverse of nitrate concentration in the E_a . AICc is the second-order Akaike's information criterion for a small sample size [Mazerolle, 2016]. Fixed R^2 represents the variance explained by the fixed factor ($[\text{NO}_3^-]^{-1}$), and global R^2 represents the total variance explained by both fixed and random factors (i.e., the entire model) [Bartoň, 2016]. Model 2 takes into account the temporal autocorrelation. Models 3, 4 and 5 take into account the sensor (#1, #2, #3, #4 or #5) effect, thus correcting for differences in sensor performance. Models 5 (nested in the sensor), 6 and 7 consider the core (sample) effect. Model 8 takes into account the three nitrate enrichment levels, and model 9 the addition order (first, second and third). Model 10 takes into account the lake effect. Models with more than one factor crossed in the random part (not shown) were also built with the *lmer* function within the lme4 R package [Bates et al., 2015], these models did not improve model 1, 3 or 4 ($p > 0.05$) in ANOVAs and showed higher AICc values). Abbreviations: lm (linear model), gls (linear model fitted using generalized least squares), lme (linear mixed-effects model) and se (standard error).

Figure 1. Main processes and aspects that affect the assessment of the denitrification temperature dependence.

Figure 2. Experimental setup. The incubation chamber ensured dark and controlled temperature ($\pm 1^\circ\text{C}$) conditions. Five intact lake sediment cores could be processed at once using their respective N_2O sensors.

Figure 3. (a) Denitrification rates (r_d) at the two experimental temperatures and the three nitrate enrichments. Note the natural logarithm scale in Y-axis. (b) Apparent activation energy (E_a) at the three nitrate enrichments. The number of observations of DEN E_a was 12, 20 and 19, respectively for each level of nitrate added (7, 14 and 28 μM). Note that the actual experimental nitrate concentrations in each enrichment class varied according to the initial field concentration (Table S1).

Figure 4. Denitrification temperature dependence (E_a) against the inverse of the nitrate concentration in compiled data from aquatic ecosystems. The solid circles are data from this study. The small circles are the E_a values resulting from each experiment ($N = 51$) and the large circles correspond to the average E_a values for each of the three nitrate enrichment levels. The black dotted line indicates the model $E_a = 46 + 419 [\text{NO}_3^-]^{-1}$. The thin dotted lines indicate the 95% confidence intervals (see model 4 in Table 2). Note that X- and Y-axis are on a \log_{10} scale.

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Methodological approach

Experimental control vs. observational
(seasonal or spatial) inference?

Fixed anoxic conditions?

Substrate influence

Electron acceptor (e.g. nitrate)?

Electron donor (e.g. organic C)?

Denitrification
temperature
dependence
analysis (DEN E_a)

Parameter domain

Resource saturation vs. natural gradient?

Temperature range?

Confounding processes

DNRA nitrate competition?

Anammox nitrite competition?

Abiotic N_2O production?



