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Biogeosciences Discussions

Interactive comment on "Kinetics of N_2O production and reduction in a nitrate-contaminated aquifer inferred from laboratory incubation experiments" *by* D. Weymann et al.

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Manuscript BGD-2009-299, published in BGD 7, 503-543: Responses to comments of referee 2

On behalf of the co-authors I really appreciate the comments made by the referee. Generally, the referee identified three main weak aspects which have also been largely critized by reviewer 1. We will be able to overcome the mentioned problems by discussing additional data, e.g. concentrations of sulfate, pH and oxygen, which were not

shown in the submitted version of the manuscript.

The comments of the reviewer are repeated here and our responses are inserted after each comment. We numbered the comments according to our responses. Responses are marked with R (number).

1. Nitrate reduction to N2 is a rather complicate process consisting of several enzymatic steps which can be carried out by a single organism or by different organism in a microbial community network. The different steps (NO3- to NO2- to NO to N2O to N2) might be differentially regulated by the availability of electron donors, the oxygen concentrations, differences in enzyme activities etc. However, this is not discussed at all. The authors use the relationship between consumed nitrate and produced N2O/N2 to describe the overall nitrate reduction process, which is in my view an oversimplification of processes which actually occur.

R1: The reviews revealed this aspect as a crucial point. Referee 1 also addressed it as the first remark. Thus, we would like to express our respond here again: "We agree that nitrite as an intermediate compound often plays a key role in the denitrification reaction chain and it was taken into account by several modelling approaches (e.g. Almeida et al. 1997). However, nitrite did not accumulate during our incubation experiments and was not detectable in the samples which were simultaneously analyzed for nitrate. We attribute this phenomenon to the pH, which was typically between 4.0 and 5.5 throughout the entire incubation periods (and in a comparable range in the field). Van Cleemput (1998) stated that nitrite accumulation is favoured by high pH and high ammonium concentrations and - vice versa - is not stable in the presence of converse conditions (pH < 5.5-to-6.0). Furthermore, Konrad (2007) showed that nitrite must not necessarily accumulate during incubation of aquifer slurries. We add this important information to the "Material and Methods" section of the revised version by mentioning the nitrite analysis. Second, we introduce this result when Equation 3 is explained (see also Holtan-Hartwig et al., 2000) in order to clarify that no reaction limiting step between nitrate and N2O occurred and Equation 3 is an appropriate assumption as a

starting point for the underlying reaction kinetics."

NO as the precursor of N2O is a further possible intermediate in the denitrification reaction chain. However, Firestone and Davidson (1989) reported that very little, if any NO, was detected as a product of denitrifying cultures. For soils, several studies revealed that NO from soils is mainly produced by nitrification of NH4+ while N2O mainly originates from the denitrification of nitrate (Davidson et al., 1993; Skiba et al., 1997; Russow et al., 2008). Furthermore, lab incubation experiments based on a kinetic 15N isotope method did not reveal NO as a free intermediate in the denitrification pathway (Russow et al., 2000). This is attributed to the limitation of diffusion in soil environments with high water content, i.e. to conditions favouring denitrification. NO produced is therefore rapidly reduced to N2O before it can escape from the cells (Skiba et al., 1997). We conclude that diffusion limitation is even more valid for our laboratory approach with saturated aquifer slurries and that accumulation of NO was very unlikely. Thus, we argue that Equation 3 can be seen as an oversimplification from a theoretical point of view (despite it was also used in the studies of Holtan-Hartwig et al., 2000; 2002), but it is valid for our approach and the specific conditions. However, we enlarge the "reaction kinetics" section of the revised version and discuss the status of nitrite and nitric oxide before introducing Equation 3 in the way we did it here.

2. It is known for a long time that nitrate reduction rates (and also the reduction rates of the more reduced intermediates nitrite and NO) are usually controlled by the amount of oxygen present. The authors show no data for the oxygen concentrations within their microcosms and within the upper aquifer zones they investigated in situ. Therefore, it can not be ruled out that the low nitrate reduction rates the authors observed in their microcosms set up with sediment from the upper aquifer parts and observed in situ in the upper aquifer parts, are related to the oxygen content of the groundwater. The authors widely do not discuss whether oxygen might have influenced the denitrification rates observed in their experiments.

R2: We completely agree: oxygen as an important governing parameter has been

neglected until now. In the case of the multilevel wells (field data), oxygen was measured in the field during collecting the groundwater samples as well as pH and DOC concentrations. Thus, we decided to revise Figure 1 completely by including these parameters. Oxygen is indeed present in the near-surface groundwater (heterotrophic denitrification zone), concentrations mainly ranged between 2 and 6 mg L-1. We agree that this might contribute to lower nitrate reduction rates to a certain extent. Of course, these results are not only shown in the revised version but also discussed (first section of the discussion). In the case of the laboratory incubations, oxygen was monitored during the entire long-term experiment via GC analysis (thermal conductivity detector, measurements parallel to the measurements of N2O via ECD) to ensure that the approach can be denoted as (quasi) anaerobic. Determined oxygen concentrations were always found to be very low (not exceeding 2 % in the headspace gas, partly even not detectable). We will improve the manuscript by adding this information. We will also discuss the difference between lab and in situ conditions in the near-surface groundwater related to oxygen. However, we are strongly convinced that electron donor availability is by far the main reason for the different nitrate reduction rates in the different denitrification zones of the study area.

3. The authors assume that in aquifer material taken from the deeper parts of the aquifer, denitrification is mainly coupled to the oxidation of reduced sulphur species (autotrophic denitrification zone) as this process was shown to occur in the investigated aquifer in previous studies. However, at least in the microcosm experiments, this hypothesis was not supported by the experimental data, e.g. production of sulphate coupled to nitrate removal. For me, it remains unclear what have been the electron donors for nitrate reduction in the microcosms.

R3: Since formation of sulfate was to be expected in the samples with aquifer material from the "deeper" groundwater, we conducted sulfate analyses after the measurement of nitrate and nitrite in the water samples. We incorporated the results as sulfate production rates in the revised Table 2. Sulfate production rates are shown in

N-equivalents in order to enable a qualitative comparison with the denitrification rates. Please note that it was not possible to measure sulfate also in the microcosms with aquifer slurry from the near-surface groundwater (heterotrophic). The sample volumes after measuring the N species (NO3-, NO2-) were limited and an analysis based on back titration was not possible because of the very low sulfate concentrations (these low concentrations would require a substantial greater sample volume than it was possible to collect). This information is added to the title of the revised Table 2 and the respective results will generally be discussed.

4. Page 506, lines 1-3 Is it so? I guess in the absence of oxygen, N2O will be rapidly reduced to N2 by most denitrifyers. The crucial role of the presence of oxygen for N2O reduction should be highlighted. By the way, the authors should comment on the fact that substantial amounts of N2O can be produced in aquifers also by partial nitrification of ammonium.

R4: Of course, oxygen is a key parameter, but the process of N2O reduction is also controlled at least by (1) the availability of electron donors and (2) the pH (as you also mention in a comment below). For example, if (1) and (2) sufficiently inhibit denitrification or especially the N2O reductase, absence of oxygen must not necessarily result in complete N2O reduction. However, we will rephrase this part of the introduction with regard to the complexity relevant governing factors. Nitrification was identified as source for N2O for example in British limestone aquifers. We add this information and give a reference.

5. Page 507, lines1-2 As mentioned above, N2O concentrations might be strongly controlled in situ by the oxygen concentrations. This fact should be discussed.

R5: Done. We also refer to variable O2 concentrations in the near surface groundwater (field) vs. stable, controlled conditions in the lab.

6. Pages 507-509, chapter 2.1 I suggest moving most parts of this chapter in the introduction or discussion.

R6: We decided to follow the referee's suggestion and to restructure the manuscript. Since we feel that recent results obtained from denitrification investigations in the study area are a crucial requirement for the understanding of grouping the batch experiments, we now prefer to insert this section as main section following the introduction (→ section 2: "Denitrification zones and occurrence of N2O in the Fuhrberger Feld Aquifer"). We hope that this structure will better highlight all important recent key findings which we suppose to be a requirement for the understanding of the results we introduce in our study.

7. Page 510-511, chapter 2.3 I wonder how the authors removed the oxygen during the set up of the experiment. Where the aquifer material has been transferred to the transfusion bottles? I guess it was done in the laboratory under air atmosphere, as no glove-box was mentioned. Did the authors flush the K15NO3 solution with N2 gas to remove the oxygen? I can not believe that three cycles of evacuation and refilling with N2 were sufficient to adjust anoxic conditions in the microcosms. Line 26: 'oxygen-free K15NO3 test solution' – describe in detail how the oxygen was removed from the solution.

R7: Transfer of the aquifer material took place in the lab under air atmosphere. The K15NO3 solution was not flushed before. We argue that this was not necessary because we evacuated and flushed the samples anyway after closing the microcosms. Three cycles of evacuation and refilling with N2 were definitely sufficient to establish anoxid conditions in the microcosms (with our laboratory device). This was carefully pre-investigated in detail and controlled by measuring O2 via GC prior to the main lab incubations. Following the approach of Holtan-Hartwig et al. (2000), we started with eight cycles, but finally we discovered three cycles as sufficient to adjust anaerobic conditions. This was revealed by subsequent GC measurements after shaking the microcosms.We rephrase the text in order to clarify this aspect. Oxygen-free K15NO3 test solution was manufactured by flushing the solution in a closed (air-tight) 10 L glas tank with N2. The procedure is described in detail in the revised version.

8. Page 513, equation (3) Nitrate reduction to N2O is a rather complicate process consisting of several enzymatic steps. The different steps might be differentially regulated by the availability of electron donors, the oxygen concentrations, differences in enzyme activities etc. I wonder how you can generalize those steps by a single rate constant (k1). Please explain.

R8: Please see our R1.

9. Page 515, chapter 3.2 (i) The author's claim that the microorganisms thriving in the aquifer material sampled from deeper zones uses mainly reduced sulphur compounds for nitrate reduction (autotrophic nitrate reduction), since the in situ analyses indicate such a process (sulphate was produced in deeper aquifer zones). But why sulphate production was not analysed in the microcosm experiment to verify autotrophic denitrification? (ii) For the correct interpretation of the results, it would have been essential to analyse the oxygen content of the microcosms, at least at the beginning of the microcosm incubation. For me, it seems that nitrate reduction is inhibited in the microcosms made of aquifer material of the upper aquifer zones; and with increasing depth, the inhibition is reduced. Could that be due to the oxygen content of the microcosms?

R9: Please see our R2 ("oxygen aspect" and R3 ("sulfate aspect"), respectively. Oxygen concentrations were negligible in the microcosms made of aquifer material of the near-surface groundwater as well as in those of the deeper groundwater due to the same handling with regard to eliminate oxygen. Thus, oxygen is not a parameter which can explain different nitrate removal during the laboratory incubations.

10. Page 517, chapter 3.3 As a conclusion, no indications for ongoing autotrophic denitrifi- cation in the microcosms by the correlation analyses have been found?

R10: In lines 16-17 we identified a significant correlation between the denitrification rates and total sulfur for the autotrophic data set, but not for the heterotrophic. We highlight this result more precisely in context with the "new" sulfate production rates in the discussion section of the revised version in order to underline the different denitrifi-

cation types.

11. Page 518, line 8 ff. As stated above, nitrate reduction do not result directly in N2O.
R11: Corrected with regard to our argumentation in R1.

12. Page 519-520, discussion of heterotrophic denitrification (i) Oxygen is a key factor controlling several denitrification steps as well as N2O production/reduction rates. I wonder why the authors did not discuss whether oxygen could influence the in situ denitrification rates in the uppermost layers of the aquifer. Is there anything known about oxygen contents of the heterotrophic denitrification zone? (ii) The authors mention that the pH of the surface groundwater is lower than 5.5, which might inhibit denitrification. What pH is characteristic for deeper parts of the aquifer, e.g. the autotrophic denitrification zone? Is there a difference which might explain the different denitrification rates? (iii) The authors set up their microcosms by mixing aquifer material with K14NO3 solution. What has been the final pH of the microcosms? Is the pH comparable with pH values measured in situ (lower than 5.5)? Page 519-520, discussion of autotrophic denitrification I suggest discussing whether iron-dependent denitrification may contribute to the overall denitrification rate observed in the autotrophic aquifer zone.

R12: (i) Oxygen will be shown (revised Fig. 1) and discussed in the revised version. (ii) The pH will also be included. pH is similar in both denitrification zones and mainly <5.5, so there is no difference that might explain the different denitrification rates. (iii) pH measurements of the microcosms yielded similar results in comparison to the field data, with a tendency to slightly higher values for the heterotrophic microcosms. Results are given more precisely in the text of the revised version. Thus, we are able to show that pH found in the lab and in field is comparable. Iron-dependent autotrophic denitrification may also occur in the aquifer, but Fe was unfortunately not measured in the microcosms. However, we will highlight that there is the possibility that Fe2+ besides pyrite serves as an electron donor for autotrophic denitrification. However, as the qualitative comparison of the denitrification rates and the sufate production rates

revealed (revised table 2), autotrophic denitrification with pyrite dominates and ferrous iron can be assumed to be less important. This statement is included in the discussion of the revised version.

13. Page 521, lines 25-27 The authors do not show any data for pH and oxygen inside their microcosms, but state that both parameters were kept constant during incubation. How do they know?

R13: As discussed above, pH and oxygen were monitored during the incubations. The results are included and discussed in the revised version.

14. Page 523, lines 1-5 As stated above, sulphate was not analysed as indicator for sulphur-dependent denitrification taking place in the microcosms. How can the authors be sure that the denitrification process was mainly coupled to sulphur oxidation and not coupled to the oxidation of other substances, e.g. carbon compounds or ferrous iron?

R14: Please see our R12, second part, for explanation.

15. Page 524, line 1 As stated above, an analysis of sulphate during the microcosm incubation could have been resulted in experimental evidence for ongoing oxidation of reduced sulphur species linked to denitrification. Why was sulphate not analyzed?

R15: Please see our R3.

16. Page 524, lines 24-27 I agree that the availability of electron donors is important for any modelling approach concerning nitrate reduction. But I feel an improved approach might also incorporate kinetics of individual steps during denitrification, e.g. kinetics of nitrate reductase, nitrite reductase and NO reductase, and should consider inhibition effects caused by e.g. pH or oxygen.

R16: As explained above (R1) we argue that there is no evidence for occurrence of nitrtite and nitric oxide under the specific conditions of our laboratory approach. However, here we give a recommendation for an improved modelling approach. In the revised version we will thus emphasize that evolution of the intermediates nitrite and nitric ox-

ide as well as the respective kinetics should be generally evaluated. We also add that such a model should be flexible enough to include pH and O2 as governing factors.

17. Page 526, lines 15-22 The described differences between field and microcosm experiments might be due to different oxygen contents. This should be discussed here.

R17: Really important point we did not discuss until now. Differences exist for the heterotrophic zone (lab: quasi anaerob, field: presence of oxygen). The discussion will follow in the revised version.

18. Page 526, lines 24-28 I do not understand how (short-term?) aerobic conditions and physical disruption could alter the denitrifying community drastically. Most described heterotrophic denitrifyers are facultative aerobes and likely survive in the presence of oxygen.

R18: Perhaps we chose an expression which is not appropriate here: "alter the composition of the microbial communities" is surely too hypothetical or "direct". We decided the rephrase to "alter the conditions for the microbial communities". These conditions are described in the sentence before. Physical disruption can alter (improve) the bioavailability of particulate organic carbon. Presence of oxygen might induce a lag phase of microbial activity when the system returns to anaerobic conditions. Thus, of course denitrifiers will survive but their denitrifying activity might change under different conditions. This is specified in the revised version.

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