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Interactive comment on "Nitrous oxide (N_2O) production in axenic *Chlorella vulgaris* cultures: evidence, putative pathways, and potential environmental impacts" *by* B. Guieysse et al.

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We fully acknowledge that the pathways discussed in this manuscript have not yet been fully validated. This hypothesis is however the most probable explanation (as explained below) and represents a valuable contribution to knowledge. This work aims to raise awareness and catalyse further research to validate/invalidate the hypothesis proposed. We invite other researchers to collaborate with us or demonstrate/invalidate our theory independently.

Genome analysis: We 'did not' verify rather than "could not" verify the potential via genome analysis: This correction is important to prevent bias in assessing our results.

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We initially did not undertake this analysis because of potential false positives and false negatives (Galperin and Koonin, Trends in Biotechnology, 2010, 28, 398-406). We can now confirm the genome of Chlorella variability harbours a gene with very high similarity to a NO-reductase encoding gene found in Chlamydomonas reinhardtii (see results below). We have also confirmed nitrite-dependant N2O emission in Chlamydomonas reinhardtii (unpublished data).

Algae versus Archaea: As in the case of genome analysis, we initially did not perform PCR analysis for archaea because of the potential false negatives (the gene is present but undetected) and false positives (the gene is detected but it does not have a significant impact) in the “much underexplored archaeal domain” since “the paucity of suitable molecular tools may hamper culture-independent investigations” (Rush A, 2013, Archaea, ID676450). There is for instance still debate around the true universality of universal primers (Gantner et al., 2011). Nevertheless, as suggested by the reviewer, we are now investigating the presence of key archaeal genes in our cultures and hope these results will be available by the time this discussion closes.

As can be seen from Figure 1, the addition of ammonium did not trigger N2O emissions form our cultures, which would have been expected in the case of AOA-emissions. In contrast, nitrite addition should not impact N2O generation by AOA based on the mechanisms reported by Vajrala et al. (2013). More importantly, there was simply not enough ammonium in the flasks (< 1 μmole) to sustain the levels of N2O production recorded via nitrification (9.66 μmoles after 30 hr).

Based on the yield reported by Löscher et al., (2012), the oxidation of 48.3 mmoles of ammonium would be required by archaea to sustain the N2O production recorded in the positive controls (9.66 μmoles after 30 hr), when only traces of ammonia are present in the medium (< 20 μM, J. Bacteriol. 2007, 189 (21) 7791-7798). Vajarla et al. (2013) confirmed this low inherent yield for AOA. We agree the quantitate comparison to a marine archaeon is not directly relevant but respectfully note this argument works both ways: there is currently no relevant literature supporting the hypothesis that

BGD
10, C4048–C4051, 2013

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archaea can cause N2O emissions in freshwater algae cultures.

We fully agree with the Reviewer that we cannot fully exclude the potential of archaeal N2O generation in our cultures. However, based on the impacts of photosynthesis repression and nitrite, the lack of response to ammonium, the magnitude of N2O emissions, and the current knowledge on NO and N2O biochemistries in photosynthetic eukaryotes (see paper) and archaea (Vajrala et al., 2013, PNAS), the rationale for an algal-mechanism is indeed very strong.

Antibiotics impact: The inhibitory effect of streptomycin takes a few days to become apparent and is only statistically significant after 5 days of exposure (on-tailed t-test, p = 0.05, the average biomass concentration was then 27% lower in the antibiotic-laden culture than in the control). A longer experiment showed biomass concentration was 81% lower in the antibiotic-laden culture than in the control after 7 days of exposure, and followed by a 4 fold increase in the specific rate of N2O emissions. As stated in the manuscript, the inhibitory effect of streptomycin on C. vulgaris has been demonstrated independently (Qian et al., 2010).

Relevance to fungi: The relevance to fungi can be discussed because evolutionary differences are not always correlated with differences in functional genes. Interestingly, the NO-reductase of Chlamydomonas reinhardtii has a high similarity with the NO-reductase of Fusarium oxysporum (NCBI). As explained in the paper, NOR-mediated NO reduction into N2O has been reported under aerobic conditions in denitrifying bacteria and fungi. We now provide evidence the Chlorella genus harbours a genome encoding an enzyme with high structural similarity to a fungal NO-reductase.

Nitrite concentration: The nitrite concentration of 12 mM is of course not relevant to commercial algae production. The impact of N2O emissions were however discussed based on the rates obtained under relevant conditions, when no nitrite was added externally: these particular results are therefore directly transferable.

Results from genome analysis: The protein sequence of Nitric Oxide Reductase

BGD
10, C4048–C4051, 2013

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from Chlamydomonas reinhardtii (NCBI accession: XP_001700272.1) was searched against the Chlorella variabilis NC64A genome assembly (Blanc et al 2010, Plant Cell) using tblastn. A significant hit was found on assembly scaffold CHLNCscaffold_7 (Expect = 1e-18) between positions 397077 to 397262 bp. Furthermore, blastp analysis enabled to identify a Chlorella variabilis hypothetical protein CHLNCDRAFT_51513 (accession: EFN56743.1) that is highly identical to the Chlamydomonas NO reductase (Expect = 1e-152; 57% identity). We gratefully thank Dr Chagne, Plant and Food Research New Zealand, for these results.

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