Nitrous Oxide Production in Oxygen Minimum Zones

Thesis Research Proposal

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Abstract

The biological carbon pump regulates the earth's climate by cycling carbon dioxide between the atmosphere and oceans. In regions where the pump is particularly active, planktonic microbes can consume all available dissolved oxygen, resulting in oxygen minimum zones where denitrification becomes the predominant pathway to oxidize organic carbon. This process creates nitrous oxide, a greenhouse gas with 300 times the global warming potential of carbon dioxide, as a byproduct. Nitrous oxide production in oxygen minimum zones has important consequences for determining changes in global warming potential, especially in regions where the biological carbon pump is especially active. Here, we propose a method of using a representative cohort of microbes in a laboratory microcosm environment to understand the relationship between nitrous oxide production and increased organic carbon export via a stimulated biological carbon pump. Sterilized seawater will be inoculated with microbes captured from the Gulf of Maine. Organic carbon and microbial communities will be added in the form of copepod fecal pellets. Changes in nitrate and nitrite levels will be recorded, indicating the presence of denitrification, and used to calculate the relative production of nitrous oxide in response to organic carbon addition. These data will indicate the microbes' ability to anaerobically digest organic carbon and create nitrous oxide. This research informs the efficacy and risks of proposed anthropogenic manipulation of the biological carbon pump as a method of carbon capture and sequestration, which is still being tested as a means of meeting IPCC climate change mitigation goals. Experiments like this, which determine the production of nitrous oxide in response to such manipulation, will indicate whether this method has the potential to offer meaningful reductions in global warming risks.

Plain Language Summary

Increasing environmental stresses from climate change are forcing scientists to examine ways to actively remove greenhouse gases from Earth's atmosphere. Carbon dioxide (CO_2), the most abundant greenhouse gas responsible for the majority of global warming, is considered the easiest gas to remove from the atmosphere because of its central role in many natural processes. Possible pathways to remove atmospheric CO_2 include afforestation and reforestation, bioenergy and carbon capture, enhanced weathering of minerals, and enhancement of the ocean's ability to absorb CO_2. Here, I propose investigating the feasibility of a newly proposed method of stimulating the capture and storage of CO_2 by adding dilute amounts of clay minerals to the ocean's surface.

Because this technique could also lead to an increased release of nitrous oxide, a greenhouse gas more powerful than CO_2, careful examination of the benefits and risks is required. Using laboratory experiments, I will determine the amount of nitrous oxide produced when this clay addition approach is used in the ocean. This research will determine the suitability of this greenhouse gas capture method for large-scale testing and evaluate its potential to impact global climate conditions.

Introduction

Nitrous oxide (N_2O) is an especially potent global warming agent. N_2O's century-long atmospheric residence time and elevated global warming potential in comparison to other greenhouse gases like carbon dioxide (CO_2) and methane present challenges to meeting the climate stabilization goals outlined in IPCC reports (IPCC, 2022). Despite N_2O's warming capabilities, plans to limit anthropogenic warming often focus on limiting the more abundant CO_2, either by elimination of CO_2 sources such as fossil fuels or by carbon capture and storage (CCS) methods to remove atmospheric CO_2 for thousands of years (Lomax et al., 2015; Longman et al., 2020; Smith et al., 2019). At their core, CCS projects alter Earth's atmosphere to reduce warming caused by anthropogenic greenhouse gas emissions. Due to proposed global impact, any CCS method inherently carries with it substantial risks if it underperforms or fails to achieve its goals (McLaren, 2020). While modeling climate changes has inherent uncertainty, modeling the effects of a heretofore untested global scale project to mitigate climate change is a venture into even less predictable territory (Gasser et al., 2015; Peters et al., 2013). The success or failure of these projects can be determined by their net impact on global warming potential, which is itself a function of their scale of CO_2 capture, energy requirements and greenhouse gases produced in their implementation (Smith et al., 2016). The process of calculating these effects is necessarily complex and thorough, but their demonstrable necessity in meeting climate mitigation goals demands that any method with the potential for globally meaningful carbon capture be investigated (Rueda et al., 2021).

Due to their scale, technological ease of implementation, and lower energy requirements, nature based CCS solutions that leverage natural sources and sinks of greenhouse gas to mitigate global warming potential have emerged as a favorable approach to CCS (Seddon et al., 2020). Terrestrial nature based CCS methods compete with human needs for living space and agriculture but are relatively easy to study, leading to a better understanding of their efficacy than non-terrestrial methods. (Smith et al., 2019). In contrast, oceanic CCS methods have less competition for space, but the challenges posed in studying them have led to a less clear image of their scale of carbon capture, longevity of storage, and ecological and economic tradeoffs resulting from their implementation (Williamson et al., 2012).

Proposed marine CCS methods are diverse and include a portfolio of approaches that reflect the complexity of ocean-atmosphere dynamics. Ocean alkalinity enhancement chemistry increases atmospheric CO_2 drawdown by providing a buffer to increase seawater's abilities to store dissolved CO_2; ocean fertilization promotes phytoplankton growth by providing limiting nutrients to the photic surface waters, and clay mineral addition enhances the efficacy of organic carbon export following phytoplankton blooms (Hartmann et al., 2023; Longman et al., 2020, Sharma et al., in review). The clay mineral addition method proposed by Sharma et al. takes advantage of the increased settling velocities of larger particles, promoting the aggregation of organic carbon into larger, faster sinking particles and thereby increasing the flux of particulate organic carbon (POC) from the epipelagic zone towards the seafloor.

The settling of organic carbon is one step of the biological carbon pump (BCP) pathway, a biogeochemical cycle that processes 56 Gt of atmospheric carbon annually (Falkowski et al., 1998; Pennington et al., 2006). However, less than 1% of this atmospheric carbon is effectively sequestered by the BCP (Figure 1); the majority is remineralized back to CO_2 by biological activity (Hedges & Keil, 1995; Honjo et al., 2008; Xie et al., 2019). In regions of very abundant organic carbon, this biological activity, specifically microbial digestion of POC, consumes dissolved oxygen (DO) and leads to the creation of low-oxygen environments (Bange et al., 2005; Bianchi et al., 2018). In these conditions, microbes abandon aerobic respiration in favor of denitrification, chemically reducing nitrate (NO_3^-) to nitrite (NO_2^-) to serve as the terminal electron acceptor for digesting POC (Morrison et al., 1999; Naqvi, 1994; Ward et al., 2009; Wright et al., 2012). When DO concentrations within particles or the water column as a whole reach 20 μM, denitrification becomes the dominant method of respiration. In a fully deoxygenated region of the water column, known as the oxygen minimum zone (OMZ), DO concentrations are often less than 5μM (Paulmier & Ruiz-Pino, 2009). In spite of this adaptability to suboxic environments, oxidation of POC by denitrification is only about 80% as effective as aerobic oxidation (Glud et al., 2015). Because of this reduced rate of digestion, OMZs tend to act as net carbon sinks, with higher POC sequestration rates than oxygen rich waters (Devol & Hartnett, 2001; Stukel et al., 2023). While this increases the potential carbon capture in POC rich environments, denitrification lessens the efficacy of these regions as carbon sinks (Figure 2) (Codispoti et al., 2001; Wilson et al., 2014).

In-situ measurements of isotope-tagged N_2O production indicate up a 6 to 27% decrease in greenhouse gas warming mitigation achieved by carbon capture in coastal biomes, suggesting that further investigation of this warming balance is warranted, especially in biomes that will be manipulated for CCS purposes (Wan et al., 2023). Because clay mineral addition increases the flux of export carbon, I expect this method to increase atmospheric release of $N_2O$where this method is employed. **Because of this relationship, investigation of N_2O production in response to clay mineral stimulated export of organic carbon is critical to understanding the risks of this proposed CCS strategy (Figure 3).**

N_2O yielded by denitrification has been studied in aquatic environments, but not in the context of a stimulated biological pump. In estuaries that undergo water column stratification and high nutrient input, the mole ratio of N_2O produced to NO_3 reduced is between 0.1% and 0.3% (Seitzinger, 1988). Because N_2O yield varies with DO concentration; the suppression of the *nos* (nitrous oxide reductase) gene expression by low levels of oxygen has been proposed as a mechanism for increased N_2O yield at very low concentrations of DO. Suppression of *nos* prevents reduction of N_2O to N_2. In a suboxic environment where denitrification is the predominant respiratory pathway, the production of N_2O without a mechanism for its reduction to N_2 results in increased release of N_2O (Beaulieu et al., 2011). Oceanic OMZs, as would be expected to occur in regions of high POC export, match these conditions. This research addresses one of the most serious antagonistic side effects of this proposed CCS strategy.

Here, I propose studying the rate of N_2O production by inoculating low oxygen seawater with a microbial cohort and particulate organic carbon to simulate the oxygen minimum zone created by a highly active biological carbon pump, and recording the changes in seawater chemistry. Measurement of DO will indicate the consumption of oxygen and development of OMZ-like conditions by the microbial cohort, while measuring NO_3^- and NO_2^- will provide evidence of ongoing denitrification activity by that cohort. From the reduction of NO_3^- to NO_2^-, production of N_2O can be inferred. Additionally, transcriptomics can be used to evaluate the composition and activity of the microbiomes in experimental conditions, further elucidating the fate of POC exported via clay mineral addition and providing greater understanding of the efficacy of this method of warming mitigation. If evidence denitrification is observed, attempts to directly measure the production of N_2O by electron capture detection gas chromatography may be attempted with the help of collaborators. **I hypothesize that regardless of initial microbiome composition, introduction of POC to seawater will lead to the production of OMZ conditions and the generation of N_2O via denitrification.**

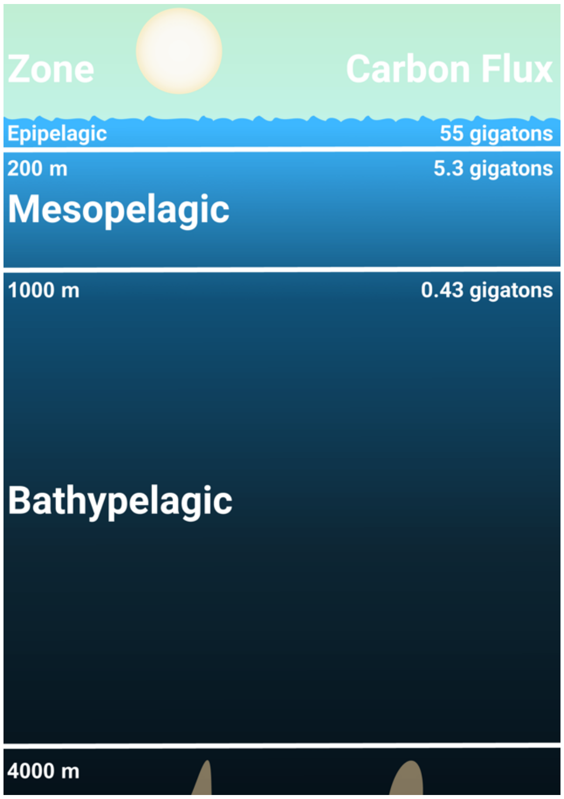

Figure 1: Global rates of carbon flux in the biological carbon pump. Data from Pennington et al. and 2006 Honjo et al. 2008. Graphic adapted from wikipedia user DieBuche 2010.

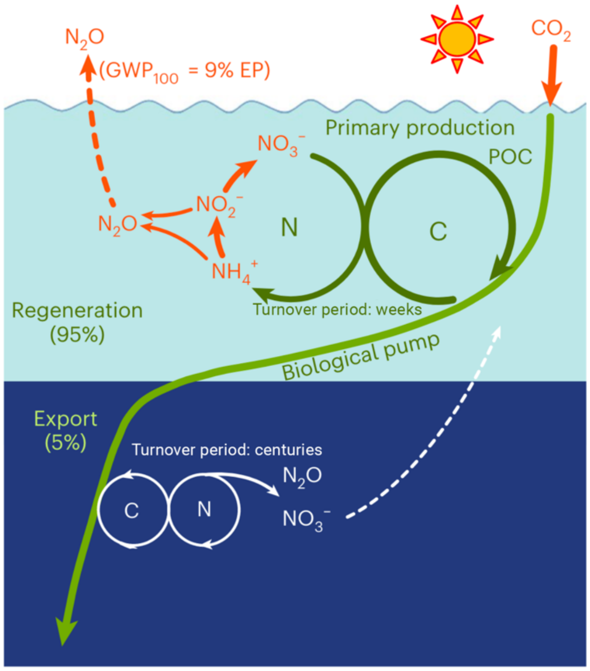

Figure 2: Proposed linkage between CO_2 removal and N_2O production by the biological carbon pump. Adapted from Wan et al. 2023.

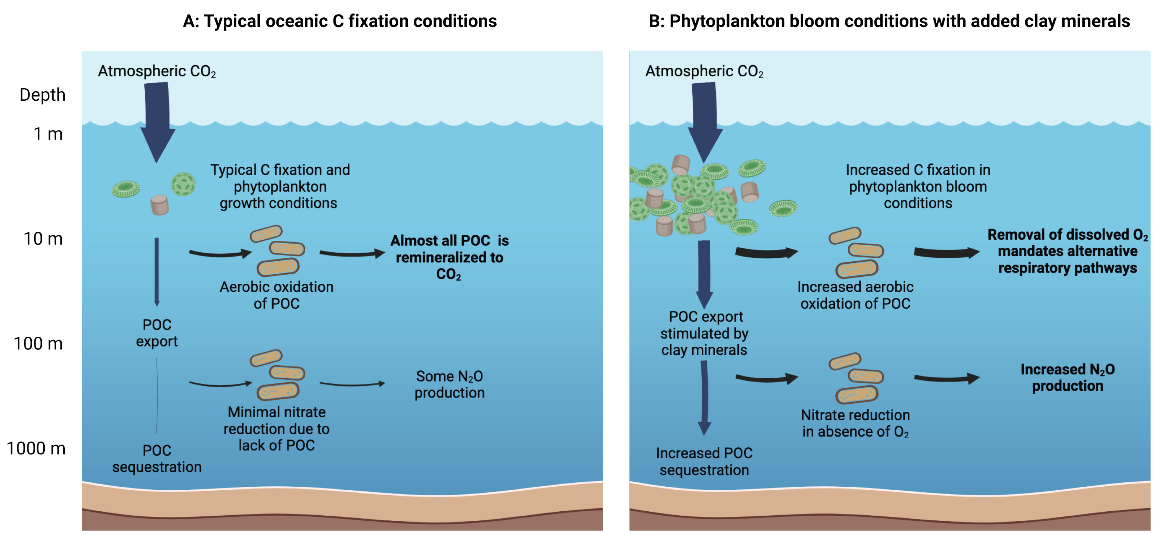

Figure 3: Demonstration of typical oceanic carbon fixation vs. phytoplankton bloom conditions and effects of clay minerals on the biological carbon pump. In Panel A, low phyoplankton density and fixation of atmospheric CO2 to organic carbon (POC) permits aerobic microbial oxidation of POC to consume almost all POC in the water column, leading to very high remineralization of POC to CO2 and very little deposition of POC as sediment. In a phytoplankton bloom scenario (Panel B), increased fixation of CO2 to POC and subsequent addition of clay minerals exports so much POC to the mesopelagic that despite microbial consumption of all available dissolved O2, POC remains and continues to sink towards the seafloor. In this scenaro, microbes switch to a nitrate-reducing metabolic pathway to continue digesting POC, leading to an increased production of N2O via denitrification and nitrifier denitrification. In this figure, arrow width is represents flux values of processes; wider arrows meaing greater flux. Images produced using BioRender.

Proposed Methods

The proposed method will evaluate the evolution of the geochemical conditions in seawater over time, simulating the mesopelagic zone of the ocean as particulate organic carbon sinks through it.

All containers are sterilized by triple washing with 10% HCl, and then triple rinsing with MilliQ water. Containers are stored filled with MilliQ water until ready for use.

Seawater was gathered from the Gulf of Maine and filtered (0.2 µm) to remove particulate matter, bacteria, archaea, and other planktons, and stored in sterile 20L HDPE jerry cans at 4°C until needed for this experiment. We deoxygenated the seawater by bubbling Ar gas at 283 L min^{-1} while simmering and stirring at 400 rpm, which decreased DO levels to approximately 25 µM to simulate the upper range of DO concentrations found in the OMZ. One hour of bubbling per liter of seawater was determined to sufficiently deoxygenate seawater in this fashion.

After deoxygenation, seawater is transferred to an anaerobic glovebox (120 to 140 ppm O2, Coy Lab Products) where it is aliquoted it into sterile 1L Nalgene HDPE bottles.

POC and microbial cohorts are introduced by adding *Calanus finmarchicus* fecal pellets to the sterile suboxic seawater. *Calanus finmarchicus* are a pelagic copepod, wild-caught in the Gulf of Maine and subsequently reared in the laboratory on a diet of *Rhodomonas*. Fecal pellets are collected from the laboratory aquaria, counted by hand and added to 50 mL centrifuge tubes which contain sterile suboxic seawater. Although counting and separation of fecal pellets does not occur in a low-oxygen enviroment, the centrifuge tubes are capped imediately and taken to the glovebox within half an hour.

The suspension of *Calanus finmarchicus* fecal pellets is added to each treatment bottle. After inoculation with fecal pellets, bottles are incubated inside the glovebox. Sample bottles are incubated at ambient room temperature (approximately 22 °C). Initial proposed sampling times are indicated in Table 1 and are based on observed sinking rates of *Calanus* fecal pellets, simulating approximately 1000 meters of sinking over 5 days. Later experiments may have an adjusted sampling schedule or duration based on results of preliminary experiments (Desai, personal communication).

At each sample point, samples are extracted from the bottle via pipette. Samples are aliquoted from the treatment bottles inside the anaerobic glovebox to prevent exposure to atmospheric oxygen. DO is measured using a modified Winkler titration method (Kenna, 2006). NO_3^- and NO_2^-

are measured using a Shimadzu UV-1900i and the spectrophotometric method described in Grasshoff (Hansen & Koroleff, 1999; Sharma et al., 2023). Pending indicators of activity, the active microbiome will be evaluated using RNA-seq sampling, to facilitate this samples will by aliquoting 500 mL of solution, filtering it with 0.2 µM GF filters, resuspending in extraction buffer, and storing at -80°C until further RNA isolation and sequencing are possible (Koch et al., 2018; Rajpathak et al., 2018). Additionally, gas headspace sampling may be employed for direct measurement of N_2O pending the development of collaborator partnerships.

Changes in the NO_3^- and NO_2^- concentrations will indicate ongoing denitrification by the microbial community present in *Calanus* fecal pellets, and evidence that the combined source of POC and microbes provided by stimulating the biological carbon pump presents a pathway to N_2O release to the atmosphere.

Table 1: Sampling timeline for N_2O production measurement

Time (hours)	0	24	48	96	144
Dissolved O_2	✓	✓	✓	✓	✓
NO_3^-	✓	✓	✓	✓	✓
NO_2^-	✓	✓	✓	✓	✓
Microbiome					✓

Preliminary Results

Table 2: Preliminary results of fecal pellet inoculation experiments.

Time (hours)	NO_2 - control	NO_2 – fecal pellet	DO - control	DO – fecal pellet
0	.002 µM	.002 µM	22 µM	22 µM
24			33 µM	37 µM
48	0.016 µM	0.064 µM		

When added to phytoplankton blooms, clay minerals induce the formation of particulate flocs rich in organic matter (Sharma et al, in review). The bacterial composition of these flocs has been evaluated and two dominant clades (*Rhodobacteraceae* and SAR11) identified. Despite the identification of these clades in laboratory systems, the likelihood that a microbial cohort is responsible for nitrogen cycling in the open ocean informed the decision to conduct experiments using a cohort cultured from a wild population (Gilly et al., 2013; Wright et al., 2012). Experiments with *Calanus finmarchicus* fecal pellets have already demonstrated the development of OMZ conditions and the presence of denitrifying bacteria.

Anticipated Results

Result 1: Decrease in dissolved oxygen concentration.

I expect that microbial activity will result in the consumption of residual DO in experimental treatments. A decrease in DO redox potential will indicate a preference for NO_3^- reduction, and therefore suggests a pathway for N_2O production in regions where clay mineral addition might be employed for CCS.

Result 2: Reduction of NO_3^- to NO_2^-, and the associated production of N_2O.

I expect that experimental treatments will demonstrate the reduction of NO_3^- to NO_2^- in suboxic systems, as expected when denitrification dominates microbial respiratory pathways. The presence of NO_2^- is indicative of the production of N_2O, and if observed, incentivizes working with collaborators to directly measure N_2O rather than inferring it via proxy, potentially using isotope tagging (Morrison et al., 1999; Nicholls et al., 2007). Additionally, evidence of NO_3^- reduction raises the possibility of an experiment that combines clay mineral flocculation experiments with N_2O production experiments to further quantify the relationship between these two processes.

Result 3: Identification of active denitrifying microbiomes in the *Calanus* gut.

I expect RNA-seq analysis to reveal that microbes living in the *Calanus* gut and fecal pellets are capable of NO_3^- reduction and denitrification, and identify the community composition in this environment.

Result 4: Fate of POC.

I expect that this experiment will enable quantification of POC degradation as a function of time, modeling the remineralization rate of fecal pellet sourced POC as it is exported towards the seafloor.

Expected Significance

Coupling N_2O production to clay mineral stimulated organic carbon export establishes a baseline effective rate of this method of climate warming mitigation. These data will guide further research evaluating the benefits and risks of clay mineral addition, including more detailed analysis of net greenhouse gas balance, identification of suitable locations to employ this CCS strategy, and identification of timing with other ecosystem events such as plankton blooms or diel migration (Kiko & Hauss, 2019).

Regional microbiomes are essential to understanding the dynamics of this process. While the composition of a microbial community can be expected to change in response to carbon and nutrient levels, this research enables greater understanding of the specificity of the microbiome response to these changing conditions (Muratore et al., 2023).

This research establishes methods for future research of suboxic marine environments. As the Earth's oceans warm in a changing climate, it is predicted that OMZs will grow in volume and NO_3^- reduction will become a more common respiratory pathway (Gilly et al., 2013; Keeling et al., 2010; Wright et al., 2012). Developing laboratory methods to study OMZs and their role in global greenhouse gas budgets builds a platform to answer questions about the future of these changing ecosystems. Despite their well-documented presence in natural settings, few laboratory simulations are designed specifically to mimic OMZ conditions. The protocols developed here contribute to researchers investigating these phenomena and serve as a springboard to develop more realistic laboratory approximations in the future.

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