NITROGEN REMOVAL BY MICROBIAL-MEDIATED PROCESSES UNDER HYPOXIC CONDITIONS IN LAKE ERIE

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1   Problem and Research Objectives

Lake Erie is facing a critical problem: eutrophication is no longer effectively controlled by solely limiting P loading. In the past, it was shown that P availability limited algal growth, particularly blooms of cyanobacteria (blue-green algae) in Lake Erie. This was evident with the low N: P ratio in lake waters and diminished phytoplankton blooms following declines in P-loading mandated by the Great Lakes Water Quality Agreement (GLWQA 1978, 1987). However, large scale phytoplankton blooms have recurred in Lake Erie in recent years with increasing frequency and extent. Given that P-centered management is still enforced and local P-loading has not significantly changed in Lake Erie (Dolan and McGunagle, 2005) (USEPA Great Lakes Monitoring, http://www.epa.gov/glnpo/monitoring/limnology), the unexpected return of undesirable phytoplankton blooms clearly indicates that the nutrient status of Lake Erie may no longer be solely P-limited. The cause of this shift is not clear, but it is clear that to control eutrophication, new management strategies must be developed. Phytoplankton of Lake Erie being co-limited by both P and N have been repeatedly reported or implicated (Wilhelm et al., 2003) (Guildford et al., 2005) (Hill et al., 2006) (Moon and Carrick, 2007) (North et al., 2007). The indicated limitation of N to phytoplankton may be attributed to an unexpected decrease of N in Lake Erie. The N concentration in Lake Erie has dropped from 0.26 mg/L in 2005 to 0.18 mg/L in 2008 (USEPA Great Lakes Monitoring, http://www.epa.gov/glnpo/monitoring/limnology). This is exceptional to a generally increased pattern observed in the other four Great Lakes and is extremely surprising. These multiple lines of evidence suggesting N becoming co-imitated with P, clearly indicate that N is actively removed through internal processes within Lake Erie ecosystems.

Two processes leading to N removal are currently known: denitrification and anaerobic ammonium oxidation (anammox). Both reactions convert reactive nitrogen species to inert dinitrogen gas (N_2), which is only available to nitrogen fixers. Anammox and denitrification are performed exclusively under anaerobic conditions by microorganisms. Suitable low-oxygen conditions are commonly found in Lake Erie, especially in the central and western basins, with increasing frequency, duration and extent (Edwards et al., 2005). Anammox and denitrification, however, follow distinct reaction routes to produce N_2, meaning that factors that influence their importance and rates are undoubtedly different. Despite the potential importance to ecosystem function,

knowledge on denitrification and anammox activity and bacteria in Lake Erie or the Great Lakes in general is extremely limited.

Our proposal aims to **study denitrification and anammox, the two processes that may lead to the lost of fixed nitrogen in Lake Erie.** Effective management of Lake Erie and the other Great Lakes requires knowledge on the nutrient status and its consequences. This project responds to these two needs. Specifically, we will provide data to advance our understanding of the current nutrient status, which has apparently shifted from historic P limitation to more P and N co-limitation. We evaluated whether or not microbially-mediated N loss could drive this shift and identify responsive microorganisms. In addition to the growth and structure of phytoplankton community, N dynamics affect the formation and diminishment of hypoxia through close interactions between anammox, nitrification and denitrification. Therefore, acquired data from this project would also aid management decisions to address hypoxia in Lake Erie water. Research on freshwater anammox is limited and virtually absent in Lake Erie. This project attempted to fill this gap and expand our understanding on N dynamics in the Great Lakes and other freshwater systems.

2 Methodology

Sample Collection and Processing.

Samples were taken from three sites in Lake Erie along a transect from Sandusky bay to the central basin on Sept 13th, 2010 (Figure 1), one in the Sandusky Bay (SB), one in the Sandusky Sub-Basin (SS) and one in the Central Basin (CB). At each site, three individual samples were collected each from the sediment and its overlaying water. Sediment samples were collected using a sediment grab sampler and subsurface layer (2-4 cm) were collected into whirl-pak bags, obvious air was squeezed out when seal the bags. Sediment overlaying water samples were collected by direct pumping water using a peristaltic pump. Samples were further processed differently according based on their use.

For nutrient analysis, one liter of water was filtered through GF/F filter and collected in autoclaved media bottles before immediately stored on ice or at 4°C. For bacterial cell counting, 1.8 ml whole water was mixed with 0.2 ml freshly made paraformaldehyde (final concentration 2% wt/vol). For DNA-based molecular analyses, 500 ml of whole water was filtered through 0.2 μm-pore-size membrane filters. Cells collected on the filters were transferred into a 15 ml sterilized Eppendorff tube and stored on ice. For ^{15}N-incubation analysis, 250 ml of whole water was collected in acid-washed BOD glass bottles with 2- to 3-fold overflow. When capping the glass bottles, care was taken to avoid head space or bubbles in the glass bottles.

All samples were stored on ice on site and transported (2-hr) back to the lab and processed immediately.

Anammox and denitrification potential measurements.

^{15}N tracer incubation analysis was performed to measure anammox and denitrification potentials, following a procedure developed previously (Kuypers et al., 2005) with minor modifications. Briefly, 250 ml of whole water samples were mixed individually with three isotopic 5 µmol of Na^{15}NO$_3$, 2.5 mol of ^{15}NH$_4$Cl or 5 µmol Na^{15}NO$_3$ and 2.5 mol of ^{14}NH$_4$Cl. The amended water was used to fill up a set of exetainer tubes and flushed for 15 min with helium. One ml of water was taken from the tubes at the end of the flush and replaced with 1 ml of helium. All tubes were incubated in the dark at in situ temperature for a total 7 days with the sampling time at 0, 48 h and 7 days of incubation. At each sampling time, 9 exetainer tubes (3 each from the three kinds of amendments) were sacrificed by replacing 1 ml of water with 1 ml of helium gas followed by ZnCl$_2$ treatment to stop the total biological activity. Sacrificed sampled tubes were stored at 4°C until headspace gas analysis. Sediment samples were made to slurry by mixing with the same volume of sediment overlying water and then processed the same as the water samples. Production of ^{15}N^{15}N from denitrification and ^{15}N^{14}N from anammox in the head space was measured by by gas chromatography isotope ratio mass spectrum (GC-IR-MS) at the UC Davis Stable Isotope Facility.

DNA extraction and gene analyses.

DNA was extracted from sediment and water samples using MoBio PowerSoil DNA extraction kits (MoBio Laboratory Inc., Carlsbad CA). Anammox-specific 16S rRNA genes were amplified using a sequential PCR protocol described previously (Tal et al., 2005). Briefly, *Planctomycetales*-specific 16S rRNA genes were amplified from extracted DNA using Pla46 forward primer (Neef et al., 1998) and universal bacterial reverse primer (Delong et al., 1989) (Britschgi and Giovannoni, 1991). The PCR amplicons then served as templates for the second anammox specific PCR using Pla46 forward and Amx1240 reverse primers (Britschgi and Giovannoni, 1991). Anammox-specific 16S rRNA genes were also amplified using a second set of primers Brod541F/1260R (Junier et al., 2010).

Gene amplicons were used to construct clone libraries. Clones with inserts of correct PCR amplicons were sequenced for phylogenetic identification. General primers for 16S rRNA genes were also amplified by Fam-labeled 27F and 1522R primers (Delong et al., 1989) and subjected to terminal restriction length polymorphism (T-RFLP).

Denitrifying-specific *nosZ* genes were quantitatively PCR amplified using a primer set described previously (Scala and Kerkhof, 1998). To access the diversity of denitrifying bacteria, *nosZ* genes were also subjected to quantitative PCR analysis (qPCR).

Clone library sequencing and T-RFLP analyses were outsourced to the Plant-Microbe Genetics Facility at the Ohio State University.

Nutrient analysis

To describe the physical, chemical and biological properties of the abiotic factors at the time of sampling, a series of variables were measured following standard methods. Temperature, dissolved oxygen concentration, and conductivity were measured using a Hydrolab H_2O multi-datasonde at the time of sampling. Concentrations of N compounds were measured following standard flow injection protocols (APHA, 1999). Nitrate/nitrite concentrations were determined by the cadmium reduction method using Lachat 8000 QuikChem Analyzer and measured directly using an Ion Chromatography Dionex system. NH_4^+ was measured fluorometrically (Holmes et al., 1999). Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) were measured using a Shimadzu TOC/TN analyzer by combustion oxidation/infrared detection and combustion chemilluminescence detection methods, respectively. Soluble reactive phosphorus (SRP) was determined following the standard colorimetric molybdenum blue method using flow injection protocols (APHA, 1999). Bacterial abundance was measured by flow cytometric analysis using 1μm-diameter yellow-green beads as the internal standard.

Figure 1. Sampling sites of this study. Map generated by Google Earth.

Figure 2. Depth profile of temperature and dissolved oxygen concentration at sampling sites.
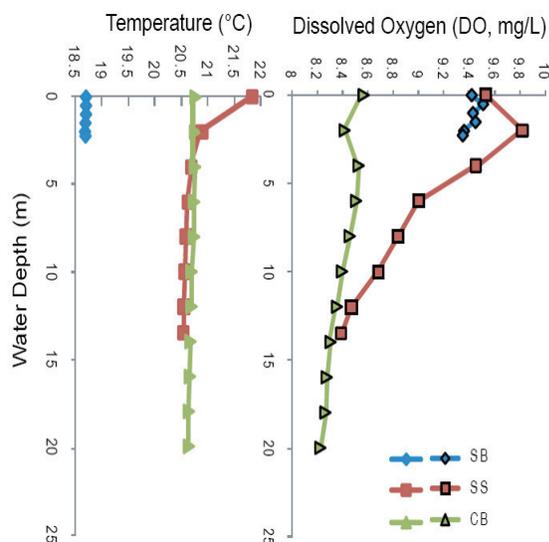
Statistical Analysis

A suite of statistic analyses were performed to identify which biotic and/or abiotic factors may affect N_2 production using a software package that has been specifically designed for community ecology and environmental science (Primer v5, Plymouth Marine Laboratory, Plymouth, United Kingdom). A similarity matrix of bacterial 16S rRNA gene T-RFLP data was calculated based on Bray-Curtis similarity. Nonmetric multiple displacement analysis (MDS) of T-RFLP pattern was performed within the Primer 5 software package.

3   Principle Findings and Significance

Table 1. Average measurements of bacterial cell abundance and nutrient supplies in the sediment overlaying water samples. Standard deviation for each parameter measured was less than 10% of the average values.

Sample	Bact. Abun. (x10^6 cells/ml)	Nitrate (μmol/L)	Ammonium (μmol/L)	TOC (mg/L)	TON (μg /L)
SB-BW	0.42	19.50	7.66	3.57	0.36
SS-BW	0.13	17.81	8.57	2.43	0.23
CB-BW	0.13	18.14	8.37	2.47	0.23

Table 2. Anammox-specific 16S rRNA genes analysis. –w was to label sediment overlaying water samples; -s was to label sediment samples.

Genes	Presence/Absence (+/-)					
	SB-w	SB-s	SS-w	SS-s	CB-w	CB-s
PLA-46F/1037R, nested Amx 368F/820R	-	+	+	+	+	+
Brod541F/1260R	-	-	-	+	-	+

The bottom water at all three sampling sites were well oxygenated, although DO concentration was the lowest at CB (Figure 2). The depth profiles of temperature and dissolved oxygen (DO) also indicated that the water in SB and CB were well mixed, while the water at SS had slight stratification.

Bacterial cell number was the highest in the SB site, which was in accordance with its high organic and inorganic nutrient supply. Bacterial community structure of the sediment and sediment overlaying water samples at the three sites were measured based on 16S rRNA genes using a community fingerprinting method, namely T-RFLP. MDS analysis of T-RFLP data grouped all sediment samples together and away for those of the sediment overlaying water samples, indicating distinct bacterial community structures between these sample types (Figure 3). Sediment samples had more T-RF than the water samples, indicating a higher diversity. The distribution pattern also indicated that sediment overlaying water samples from the three sites were composted

Figure 3. MDS ordination of microbial community structure based on T-RFLP analysis of 16S rRNA genes.
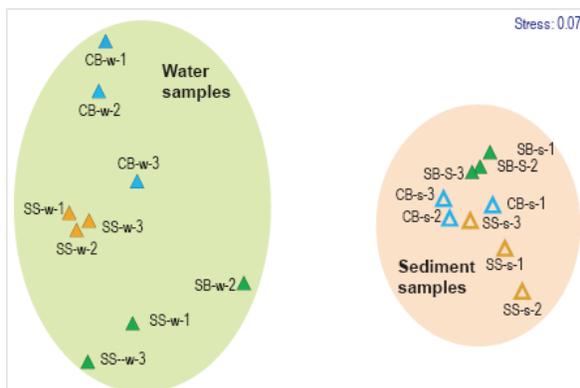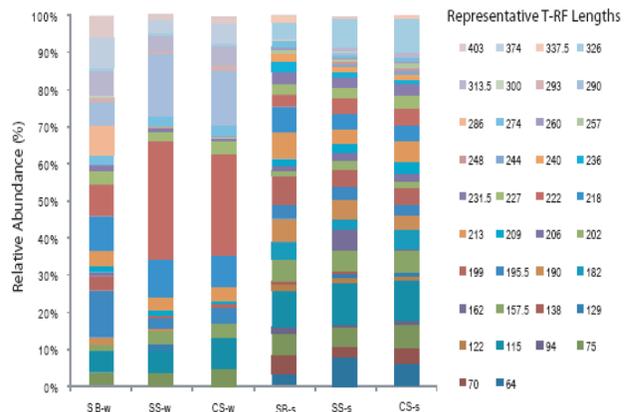
Figure 4. T-RF of 16S rRNA genes in the sediment and their overlaying water.

differently. The taxa associated with T-RFs of 222 bp and 290 bp accounted for over 27% and 15% for sediment overlaying water at the SS and CB sites, respectively, but were only 8% and 6% in the SB sites, respectively (Figure 4). Community structures in the sediment samples, on the other hand, were quite similar to each other. Further statistical analyses, such as ANOSIM, were planned to identify the taxa that contributed significantly to the grouping patter of the T-RFLP data.

Figure 5. Abundance of nosZ genes in samples.

The denitrifying and anammox potential were measured for the bottom water and sediment samples using both genetic and biogeochemical methods. Diagnostic genes for denitrification, i.e.., *nosZ* genes, and anammox, i.e., anammox-specific 16S rRNA genes, were analyzed for the sediment and overlaying water samples (Table 2). Denitrifiers have varying functional capability, only those that carry *nosZ* genes are capable of reduce nitrogen to dinitrogen gas. The *nosZ* genes were amplified from all sediment and their overlaying water samples. But their copy numbers in the original samples were different. Water samples in general have very low copies of the *nosZ* genes, which were all significantly lower than their corresponding sediment samples (Figure 5). The process of anammox is less studied than denitrification. Functional genes of anammox have been identified recently in an anammox bacterial strain, however, its ubiquity among anammox bacteria was less clear. Studies have indicated that anammox capability were restricted within a distinct subset of *Planctomycetales.* Currently, genetic identification of anammox is mainly using 16S rRNA gene primers to target this subset. In this study, anammox 16S rRNA genes were analyzed by direct and nested PCR of primer sets developed previously (Junier et al., 2010). Except for the sediment overlaying water samples in SB, anammox 16S rRNA genes were amplified from all the other sediment and overlying water samples by both or either of the approaches. The gene amplicons were further cloned and prepared for upcoming sequencing analysis.

Dinitrogen production potentials in the sediment overlaying water samples through anammox and denitrification were measured using the ^{15}N incubation assay (Figure 6). Anammox and denitrification produce N$_2$ gas through distinct pathways. The two atoms of N are both from oxidized N (Nitrate/nitrite), while, for anammox, one atom is nitrate/nitrite and the other is from ammonium. Trace amount of ^{15}N-nitrate and/or ^{15}N-ammonium were added to the samples and the production of ^{30}N through denitrification and ^{29}N through anammox were measured following different duration of incubation. Consumption of total nitrate, ammonium and DOC were also tracked. Our results showed positive anammox potential in the sediment overlaying water of the SB and CB sites, but not the SS site. At the mean time, denitrification potential was measured in the SS and CB sites, but not the SB site. Overall, N$_2$ production the sites was attributed mostly to denitrification in the sites within Lake Erie (i.e., SS and CB) and to anammox in the site within the Sandusky Bay.
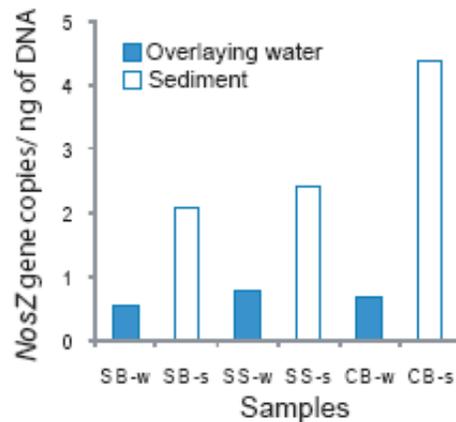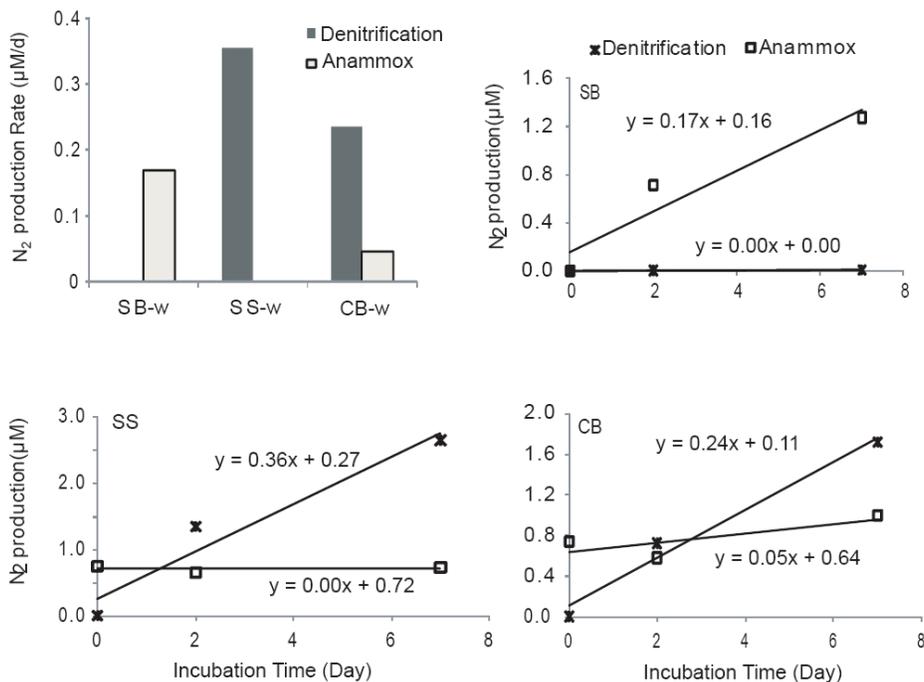
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Figure 6. Total N₂ production during ¹⁵N incubation experiment and calculated potential N₂ production rate through denitrification and anammox processes.

Our study represented one of the first investigations on anammox and their relative importance in nitrogen loss through production of N_2 in Lake Erie and the Laurentian Great Lakes in general. Anammox potential was measured in the sediment overlaying water samples at the CB site, where the oxygen was the lowest among the three sites. To a larger extent, anammox potential was also measured at SB within the Sandusky Bay, a small coastal water reservoir semi-isolated from the main body of the Lake Erie. This was somewhat surprising since the SB site was well oxygenated throughout the water column. However, the site is also known for its eutrophic condition, and cyanobacterial blooms are frequently developed throughout the summer. We hypothesize that during cyanobacteria blooms, microzones of hypoxia can be developed to incubate anammox bacteria. Further investigation is needed to test this hypothesis and verify our finding in this project.

5 Publication citations

Dissertations

Lu, Xinxin, (in progress), The Relative Importance of Denitrification and Anammox in Freshwater Lakes and Coastal Marine Environments, Ph.D. Department of Biological Sciences, Kent State University, Kent OH

Conference Proceedings

Bade, D, Feb 2011, Should we care about nitrogen in Lake Erie, Ohio State University, *invited talk*.

Lu, Xinxin; Darren Bade, Laura Leff, Robert Heath and Xiaozhen Mou, 2011, Denitrification Is More Important Than Anammox In Microbially Mediated N Removal In Lake Erie, International Association for Great Lakes Research (IAGLR) 54th Annual Conference on Great Lakes Research, Duluth, Minnesota (*Abstract accepted*).

Heath, Robert, Xiaozhen Mou, Laura Leff, Darren Bade, Curtis Clevinger and Xinxin Lu, 2011, Nitrification, denitrification, and anammox in the central basin of Lake Erie, 21st Biennial Conference of the Coastal and Estuarine Research Federation (CERF 2011), Daytona Beach, FL (*Abstract Submitted*).

5   Student Supported

Lu, Xinxin, PhD, Kent State University, Major: Ecology

6   Awards or Achievements

None.

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