Clara FUCHSMAN
Lewis and Clark Astrobiology Travel Grant
June 2006

Expedition to the mid-Proterozoic: Understanding the N cycle in the Black Sea suboxic zone

Reports rec'd 6/14/10

Project Report

The mid-Proterozoic ocean (1.8-1.2 Ga) could have resembled the modern stratified Black Sea with an oxygenated layer, a suboxic layer, and a sulfidic layer. Processes occurring in the mid-Proterozoic ocean may have affected the atmosphere. For example in suboxic waters, both heterotrophic denitrification and the anammox process produce nitrogen gas. However, heterotrophic denitrification (Organic matter + $2NO_3^- \rightarrow N_2$) proceeds through a nitrous oxide intermediate while the anammox process ($NO_2^- + NH_4^+ \rightarrow N_2$) does not. We hope that by studying the nitrogen cycle in the Black Sea, we will be able to predict which mechanism was dominant in the mid-Proterozoic and thus whether nitrous oxide was an important greenhouse gas at that time.

The counter point to nitrogen gas production is nitrogen fixation, which produces ammonium from nitrogen gas. This process is important because algae need fixed nitrogen (ammonium and nitrate) to undergo photosynthesis. Almost all bacteria and archaea need ammonium or nitrate as well. We need to understand how nitrogen fixation and nitrogen gas production are linked in regions with low oxygen. Does the consumption of fixed nitrogen by anammox and denitrification limit productivity in surface waters? Or does nitrogen fixation increase to balance the cycle? I hoped to obtain clues for both these topics.

In our Black Sea group, we combine molecular biology with in situ natural stable nitrogen isotopes. Natural stable isotopes record microbial activity in the water column while molecular biology tools indicate which bacteria are present. To address questions mentioned above, I obtained natural stable isotopes and concentrations for nitrogen gas, nitrate, and suspended particulate matter and sinking particulate material from sediment traps. We examined bacterial DNA and RNA. I also did experiments using reactants with 100% 15N as a tracer for nitrogen fixation, denitrification and anammox. Samples were obtained at 44.46 N 37.76 E (see Figure 1).

Traveling through Russia to obtain these samples was an adventure. Though I was hoping to go on a Turkish Black Sea cruise in the summer of 2007, the cruise did not occur. Instead I went on a Black Sea cruise on the RV AKBAHABT out of Glenedzhik Russia. When I reached Glenedzhik, it turned out that the rules (or at least the enforcement of the rules) had changed. Now foreigners were not allowed on the Russian ships at all. I had to teach my Russian colleagues how to obtain samples for me. We did this by pretending to take samples with the ship still at the dock. Not being able to take my own samples was particularly stressful as even a small amount of air contamination could destroy the gas samples. Thankfully, my Russian colleagues did an excellent job, and exhausted themselves doing my work as well as their own. They obtained the samples, and I processed them in the chemical lab on land afterwards. The chemical lab was an old house in which lab benches had been installed. However, the house did not have good ventilation or an available hood. The deep Black Sea has potentially toxic amounts of hydrogen sulfide. To deal with this, I processed samples outside on the balcony. Returning the samples to Seattle was also a challenge. Due to my type of VISA, I was required to fly in and out of Moscow even though it was a 36 hour train ride to Glenedzhik. Though not a problem prior to the cruise, this long trip did cause difficulties returning to Moscow with frozen samples. Dry ice was not available in the Glenedzhik area and normal ice would not last 36 hours in the heat. Luckily, a Russian colleagues managed to talk the ladies in the dining car into putting the frozen samples in their fridge.

Our lab is interested in the importance of N_2 fixation in the Black Sea. Experimentally, I found evidence for low levels of nitrogen fixation in the suboxic zone. This rate of 0.7 μmol N/m^2-d was almost identical to those found by McCarthy et al. (2007) *Estuarine Coastal and Shelf Science* **74**: 493-514 in 2001 that greatly surprised the community. My lab mate John Kirkpatrick has been looking for nitrogen fixation genes in the RNA and DNA collected on this cruise. Looking in the suboxic zone at DNA of NifH, a gene used in nitrogen fixation, John found genes belonging to Green sulfur bacteria *Chlorobium phaeobacteroides*. *Chlorobium* photosynthesize using extremely low levels of light and hydrogen sulfide (Mankse et al., 2005). John also found NifH genes belonging to methane oxidizing bacteria. It is at first surprising that N_2 fixation would occur in the Black Sea suboxic zone because it takes a lot of energy to undergo N_2 fixation, and N_2 fixation is typically linked to photosynthetic organisms in surface waters. However, nitrate and ammonium concentrations are quite low in the lower suboxic zone in the Black Sea (see red box in Figure 2). Methane is available to methane oxidizing bacteria in the same depth range where nitrate and ammonium concentrations are reduced (Figure 2C). Using bacterial 16S rRNA TRFLP, a community fingerprinting technique, I found that *Chlorobium phaeobacteroides* cells are present in the depth range where nitrate and ammonium concentrations are reduced. Since both Green sulfur bacteria and methane oxidizing bacteria are operating where nitrate and ammonium concentrations are quite low, they may use N_2 fixation as a source of nitrogen needed for growth.

John also looked at nitrogen fixation genes used in the surface of the Black Sea. He did not find nitrogen fixation genes for cyanobacteria—the usual oxygenic photosynthesizers undergoing nitrogen fixation in the ocean. Instead he found nitrogen fixation genes belonging to heterotrophic organisms. I examined suspended particulate organic matter in the surface waters. Suspended particulate organic matter includes algae, bacteria and small pieces of detritus. Depleted suspended PON (-0.2 to 2.2‰) in the surface waters (euphotic zone) indicated that nitrogen fixation was likely occurring there. However, a sediment trap sample under the euphotic zone was much less depleted (4‰). Indicating that though biomass (suspended PON) was created by N_2 fixation, this organic matter was preferentially not directly transferred to the suboxic zone and deeper layers. This difference between suspended and sinking organic matter could be because single small cells are less likely to sink that clumps of large non-N_2 fixing algae such as diatoms. A lot of work remains to be done on the importance of N_2 fixation in the Black Sea, both at the surface and in the suboxic zone, and its implications for the mid-Proterozoic.

We would like to understand the N_2 production pathways in the Black Sea. More and more the scientific community is linking denitrification to sinking aggregates and high organic matter conditions. This is important for the mid-Proterozoic as denitrification has the greenhouse gas N_2O as an intermediate and anammox does not. We hoped that on this cruise we would catch the spring bloom, a high organic matter event. However, May 2007 was particularly low in organic matter. Experiments with labeled 15N-NO_3^-/NO_2^- indicated that anammox not denitrification was the dominant N_2 producing mechanism at this time. Older flux data from the Black Sea had indicated that during some cruises, more N_2 gas was produced in the suboxic zone than nitrate and ammonium were consumed, implying that organic matter was a source of N atoms to N_2 gas (Fuchsman et al., 2008). I hypothesized that anammox, which uses ammonium as a reactant, was incorporating organic N molecules that were similar to ammonium into N_2. I did an experiment on this cruise; I had grown cyanobacteria on 15N labeled ammonium and then killed and cleaned them so they became 15N labeled organic matter. I then added this labeled organic matter to Black Sea suboxic seawater and measured the N_2 gas produced. I expected the label to be transferred to the N_2 gas, but it did not happen, at least in 36 hours. Fixed N consumption and N_2 production were not imbalanced in samples measured on this cruise. Anammox was the primary N_2 production pathway at this time, so anammox appears not to incorporate organic N into N_2 gas. When re-examining the older data, it appears that the imbalance between nitrate and ammonium consumption and N_2 gas production only happens in years where we have evidence for denitrification. Thus we now hypothesize that denitrification is responsible for the transfer of N atoms from organic matter to N_2 gas. When this happens, more N_2 (or N_2O) can be produced.

Could heterotrophic denitrification have produced enough N_2O to warm the Earth during the mid-Proterozoic? Predictions are that productivity would have been reduced during that time (Anbar and Knoll [2002] *Science* **297**: 1137-1142). As heterotrophic denitrification appears to be linked to high organic matter concentrations (Engstrom et al. [2005] *Geochemica et Cosmochimica Acta* **69**: 2057-2065; Fuchsman et al. [2008] *Marine Chemistry* **111**: 90-105; Ward et al. [2008] *Deep-Sea Research I* **55**:1672-1683), I think it unlikely that it was a dominant N_2 production process during the mid-Proterozoic. The anammox process would be more likely to be favored under low organic matter conditions. On the May 2007 cruise I found low levels of autotrophic denitrification ($H_2S + 2NO_3^- \rightarrow N_2$) below the suboxic zone. This process, which produced N_2O as an intermediate, is not very important in the Black Sea because nitrate and sulfide do not often co-exist there. However, it is an important process in the Baltic Sea (Hannig et al. [2007] *Limnology and Oceanography* **52**: 1336-1345), another suboxic and sulfidic ocean basin. I think autotrophic denitrification should be more closely examined to predict its importance as a N_2O production pathway during the mid-Proterozoic.

Figures

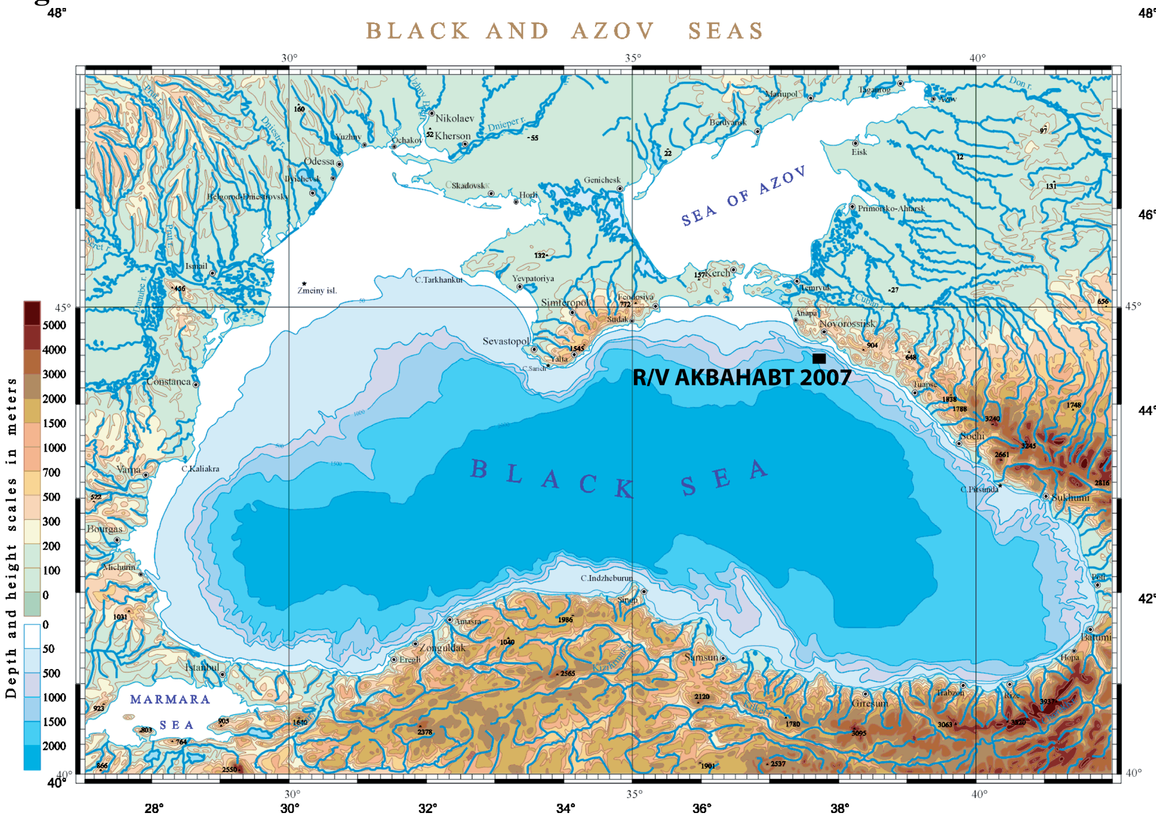


Figure 1. A map of the Black Sea, indicating the sampling station visiting in May 2007 (black square). The map was made by the Remote Sensing Department, Marine Hydrophysical Institute, Ukraine. The shade of blue indicates the depth of the bottom of the Black Sea.

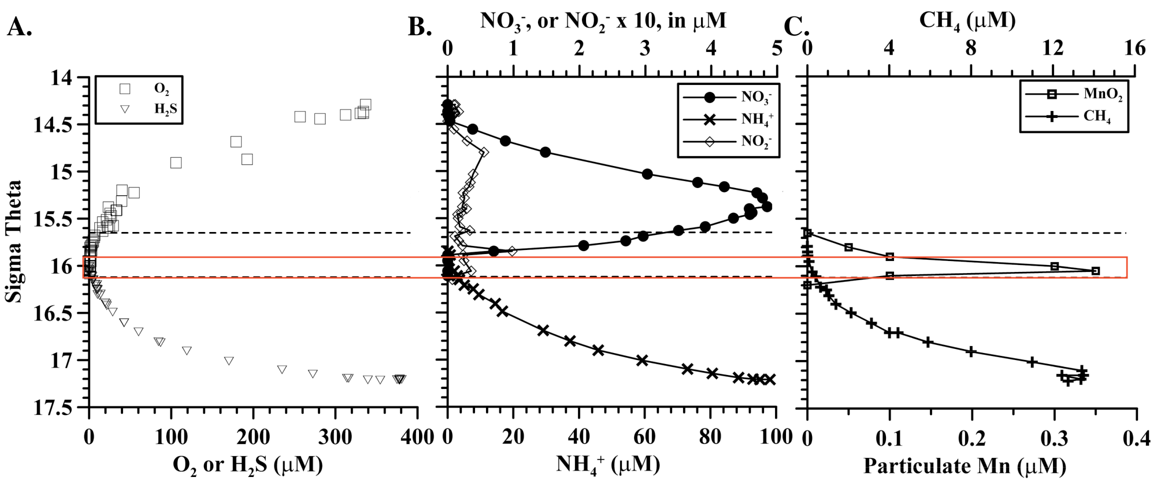
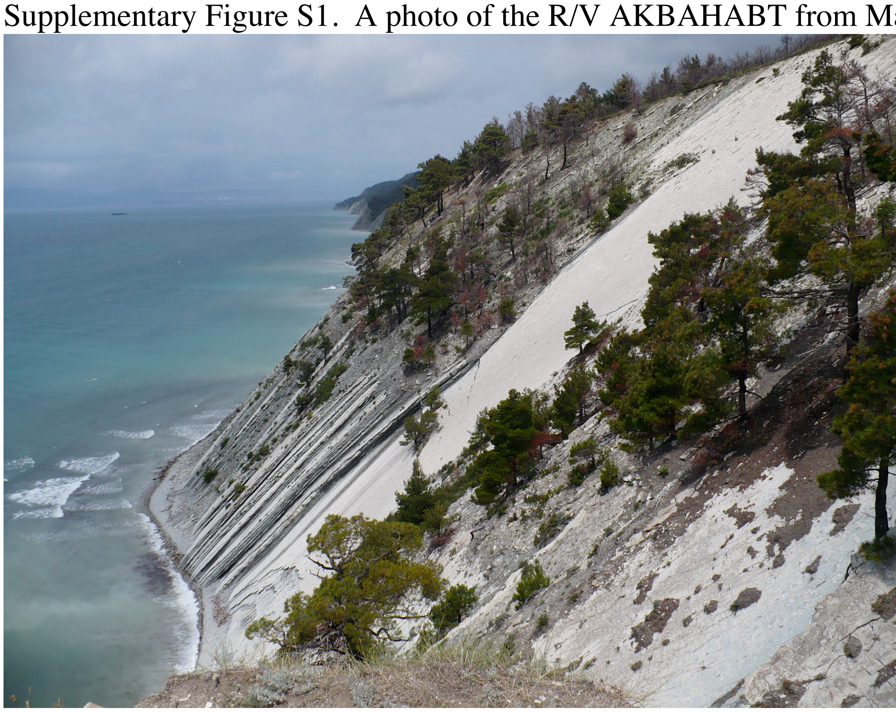


Figure 2. Typical chemical concentration profiles for the Black Sea versus density. The top of the y-axis is the surface of the Black Sea and the bottom of the y-axis is the bottom of the Black Sea. A) Oxygen (squares) and sulfide (triangles). The suboxic zone is indicated by the dotted line. B) Fixed nitrogen: Nitrate (circles), nitrite

(diamonds), and ammonium (x) C) Methate (crosses) and particulate manganese (Konovalov et al. [2003] *Limnology and Oceanography* **48**: 2369-2376) (bold squares). The red rectangle indicates the depth range where nitrate, ammonium and nitrite are all at extremely low concentrations, making N_2 fixation a favorable process. Values are from the Western Gyre sampled on the R/V Endeavor in 2005 (except manganese).

Supplementary Figure S1. A photo of the R/V AKBAHABT from May 2007.


Supplementary Figure S2. A photo of the Black Sea coast near Glenedzhik.