

DIN vs. DON in Groundwater: Their Roles in Estuarine Eutrophication

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Abstract:

On Cape Cod, Ma., wastewater treatment plants and septic tanks release dissolved organic and inorganic nitrogen (DON and DIN) into groundwater which reaches estuaries. The relative importance of DON vs. DIN in causing eutrophication is not well understood. In this project I designed an experiment consisting of additions of estuarine water and groundwater with varying TDN load (30-1000 μM) and DIN:DON (0.5-5.2). I determined concentrations of NH_4^+ by phenol hypochlorite oxidation, $[\text{NO}_3^-]$ by cadmium-copper reduction to nitrite, TDN by persulfate oxidation, chlorophyll by fluorometry, and bacteria by flow cytometry. I conducted single and two factor ANOVA tests in Microsoft Excel. I expected rapid DIN uptake and chlorophyll production, which occurred in all incubations. [Chlorophyll] peaked rapidly with a corresponding $[\text{NO}_3^-]$ decline, followed by a peak after day 12 in high-DIN additions. I expected some DON uptake, which I did not observe. Bacterial production occurred in all incubations, with greater production in the light incubations than the dark incubations, suggesting that bacteria used phytoplankton release of DON as an N source. Overall, DIN was the greater contributor to eutrophication because NO_3^- uptake spurred phytoplankton production, which in turned spurred bacterial production.

Key Words and Phrases:

Eutrophication, dissolved inorganic nitrogen (DIN), dissolved organic nitrogen (DON), phytoplankton, chlorophyll, flow cytometry.

Introduction:

Dissolved nitrogen loading from groundwater to estuaries causes eutrophication. On Cape Cod, Ma., wastewater treatment plants and septic tanks release dissolved organic and inorganic nitrogen (DON and DIN) into groundwater which reaches estuaries through aquifers and seeps. Phytoplankton and bacteria take up the abundant nitrogen and their production skyrockets to unhealthy levels, causing toxic algal blooms, hypoxia/anoxia, and the death or migration of fish and shellfish populations (Valiela et al. 1992). What is not well understood, however, is the relative importance of DON vs. DIN in causing eutrophication (Antia et al. 1991). The purpose of this study is to determine, through experimental additions of groundwater to estuary water, which form of dissolved nitrogen leads to greater production of phytoplankton and bacteria over a three week incubation in a growth chamber, and thus which form of nitrogen comprises a greater threat of eutrophication when it reaches estuaries in the town of Falmouth on Cape Cod.

Why must the contributions of DIN and DON to phytoplankton and bacterial production be studied independently? DIN and DON, though both N-rich groups of compounds, are fundamentally different in composition. DIN, in the form of nitrate (NO_3^-

) and ammonium (NH_4^+), is simple, low-molecular weight N bioavailable to phytoplankton (Piehler et al. 2004). DON, in contrast, is bound up with carbon in complex, high-molecular weight compounds, most of which must be first decomposed by bacteria with enzymes before becoming bioavailable to phytoplankton. DON is a major portion of total dissolved nitrogen (TDN) in estuarine waters (Antia et al. 1991), yet it is unknown whether DON contributes significantly to phytoplankton production on a time-scale short enough to cause eutrophication in a tidally flushed estuary. Therefore, we can not assume that total TDN loads which have high DIN to DON ratios (>50%) threaten to cause eutrophication more than TDN loads with less DIN.

Scientists, therefore, need to study DIN and DON independently in order to understand the compounds' specific roles and importance in eutrophication. The problem arises when we imagine adding known concentrations of both N forms and measure chlorophyll and bacterial production over time, in a standard enrichment experiment. DIN can be isolated and added easily because of its simple composition, but DON cannot be similarly isolated and added because it is composed of many different compounds like proteins and DNA in various stages of decay from their living forms. Only 20-30% of DON in offshore seawater has been found readily identifiable as amino acids and other low-molecular weight compounds; the remainder is believed to be amorphous humus-like compounds low in N but very abundant (Antia et al. 1991). Assembling a cocktail of DON compounds, then, would be akin to assembling a puzzle with only one third of the pieces.

In this experiment I sought to circumvent the problem of DON composition by adding estuary water to groundwater with sources of widely differing [DON] and DON origins. The groundwater reaching West Falmouth Harbor (WFH) and Childs River (Ch. R.), estuaries in Falmouth, Ma., has long received wastewater-contaminated groundwater with TDN loads much higher than ambient loads (Ken Foreman, pers. comm.). WFH is in the plume of the Falmouth Wastewater Treatment Plant (FWTP), and receives wastewater from many septic tanks to boot. Ch. R. receives wastewater from septic tanks in several densely populated neighborhoods (Valiela et al. 1992). I selected Washburn Island State Park in Waquoit Bay, Ma. (WI) as a relatively pristine site that has no source of wastewater contamination in its groundwater.

I expected that the DIN:DON ratio would be highest in WFH (>50%), because the wastewater DON from FWTP would have traveled more than 2 mi. and degraded to DIN by the time it reached the estuary. I expected DIN:DON to be lower in Ch. R. (about 50%) because wastewater DON from septic tanks essentially reaches the estuary immediately from the multitude of homes adjacent to Ch. R. Lastly, I expected DIN:DON to be lowest in WI groundwater (<25%) because there is no DIN source other than DON decomposition on the undeveloped island. Furthermore, I expected the DON reaching WI groundwater to be partially decomposed plant material high in lignin and other non-labile N-containing compounds, while wastewater DON reaching WFH and Ch. R. groundwater would be comparatively labile.

In an experiment adding groundwater from the above sources to estuary water, what would be the response of the microorganisms? First, would DIN-rich groundwater cause rapid production by phytoplankton and bacteria? Second, how labile would wastewater DON be to the organisms? Would there be significant DON recycling to DIN by bacteria? Lastly, would phytoplankton grow with an addition of low DIN:DON groundwater?

I expected rapid uptake of NO_3^- and NH_4^+ , with corresponding rapid production of chlorophyll by phytoplankton. In the wastewater-contaminated additions from WFH and Ch. R., I expected greater DIN uptake and greater chlorophyll production than in the WI groundwater addition. In all sites, I expected some DON uptake and recycling to DIN, with corresponding bacterial production. DIN uptake, however, would greatly outweigh release of DIN from recycling, so [DIN] would steadily decrease. *Overall, I hypothesized that DIN would be the dominant contributor to eutrophication, represented by rapid bacterial and phytoplankton production, in my experimental groundwater additions.*

Methods:

Experimental Design. My experiment consisted of 4 incubations which were additions of 50/50 unfiltered WFH water column water with groundwater collected from WFH, and 12 incubations which were additions of 1 L of 93% filtered (*ca.* 0.7 μm)WFH water plus 1 L of 100% 0.7 μm filtered groundwater from WFH, Ch. R., and WI. In addition I had 2

controls in which I added unfiltered WFH water to deionized water, and 1 control in which I added 93% filtered WFH water to deionized water.

For each groundwater source I had two duplicate incubations in bottles kept in a 24-hr light growth chamber, and two duplicate incubations in the dark. For WFH groundwater I had a total of 4 incubations using the 93% filtered WFH and 100% filtered groundwater, and a total of 4 incubations using the unfiltered WFH water and groundwater. Incubations with groundwater additions plus incubations with deionized water totaled 19 incubations (see fig. 1).

Field Collection and Processing. On Monday, Nov. 12th, Rich McHorney and I collected ~10 L of groundwater from Ch. R. (near the permeable reactive barrier) and WI, combining ~2 L from each of 5 wellpoints and sampling between 0.5 and 1 m below the ground surface. On Tuesday, Nov. 13th, Will Longo and I collected ~12 L of groundwater combined from each of 5 wellpoints, and collected ~20 L of WFH water from a dock near the Eastern shore of the harbor (extreme landward edge of the harbor), about 0.5 m below the water surface. All groundwater wellpoints were between 1 and 5 m from the shore at high tide on both collection days. All water samples were kept refrigerated in the dark until I began incubations in the growth chamber. On Wednesday, Nov. 14th, I filtered 13 L of WFH water with Whatman 47 mm GF/F filters, added 1 L unfiltered WFH water, and mixed thoroughly. In all incubations I added NaCl to raise salinity to ~25 ‰, the salinity of water in WFH I had measured on Tuesday, Nov. 13th.

Growth Chamber Incubations, Sampling, and Storage. I took initial samples of WFH water and the 3 groundwater sources, as well as the 19 incubations, on Nov. 14th. I began incubations on Thursday, Nov. 15th in a growth chamber at 18°C and PAR ~1200. Light incubations were kept in 2.5 L, open-top bottles covered with plastic wrap. Dark incubations were also kept in covered bottles, placed inside cardboard boxes, and covered with black plastic (see fig. 2).

After initial samples, I sampled all incubations 8 subsequent times every 2-3 days with a final time point on Tuesday, Dec. 14th, 19.5 days after the first samples were taken. On Friday, Nov. 30th, my 6th time point after initial sampling, I decided to stir the bottles in order to resuspend particles that had grown over the 15-day incubation to that point, using a magnetic stirrer and stirrer bars while sampling each incubation bottle.

At each time point, I sampled all 19 incubations for 5 analyses at once, filling a 60 mL syringe with a 10 cm tube attachment. Chlorophyll and flow cytometry samples were transferred to fluorometry tubes and 20 ml plastic scintillation vials, respectively, without filtering. TDN/ NO_3^- and NH_4^+ samples were filtered with a Swinnex filter attachment using Whatman 25 mm GF/F filters, and transferred into 20 mL plastic scintillation vials. After sampling, flow cytometry samples were frozen at -80°C , TDN/ NO_3^- samples were frozen at -20°C , NH_4^+ samples were acidified with 5 N HCl and refrigerated at 2°C , and fluorometry samples were analyzed immediately.

Nitrogen Analysis. I determined $[\text{NH}_4^+]$ in all samples using the phenol hypochlorite method (Solorzano 1969). $[\text{NO}_3^-]$ was determined using cadmium-copper reduction to nitrite (Wood et al. 1967) and a Lachat Flow Injection Analyzer (FIA), thawing samples and analyzing immediately. Next, Rich McHorney and I determined [TDN] by persulfate oxidation (Solorzano and Sharp 1980) and Lachat FIA for time points 0 (initial), 4 (8.6 days elapsed) and 8 (19.4 days elapsed), thawing samples and analyzing immediately. Lastly, for time points 0, 4, and 8, I calculated $\text{DON} = \text{TDN} - (\text{NH}_4^+ + \text{NO}_3^-)$.

Productivity of Organisms. I determined chlorophyll abundance ($\mu\text{g/l}$) by fluorometric analysis (Parsons et al. 1984). Matthew Erickson and I determined bacterial concentrations of all samples by flow cytometry, using a Becton-Dickinson FACSCalibur™ flow cytometer. Into 0.5 mL of sample we micro-pipetted 30 μL of bead solution and 3 μL of Cyto-13 fluorescent dye. We plotted side scatter vs. DNA fluorescence, using previous research to determine gates in which to count bacteria (Matthew Erickson, pers. comm.) for the specific volume that passed by the laser (see fig. 13).

Statistical Analysis. Hugh Ducklow and I performed two-factor ANOVA tests in Microsoft Excel on final bacterial concentrations in light vs. dark incubations. We also performed single-factor, pairwise ANOVA tests on initial vs. final chlorophyll concentrations between each set of light incubations, WFH, Ch. R., and WI.

Results:

DIN:DON in Initial Water Sources. As expected, WI had the lowest TDN load of the three groundwater sources. WI also had the lowest DIN:DON of 0.5, about 33% DIN; I

had expected to measure almost nonexistent [DIN] as there was no wastewater NH_4^+ or NO_3^- source, but DIN was a significant portion of TDN (see fig. 4).

Interestingly, WFH water and groundwater had nearly identical DIN:DON (2.4 and 2.5, respectively) but WFH groundwater had a TDN load 4.5 times greater than the WFH water column sample (see fig. 4). The equal DIN:DON in the groundwater and water column, however, could merely be a coincidence founded on the timing of my sampling – DIN:DON in the water column during early summer when NO_3^- uptake by phytoplankton reaches its yearly peak could approach 0, while in the dormant late winter DIN:DON could be much higher than what I measured on Nov. 13th.

Ch. R. groundwater had by far the largest TDN load, DON load, NO_3^- load and DIN:DON (see fig. 4). [TDN] was 6.5 times greater than in WFH groundwater. The measured [DIN] of 855 μM in Ch. R. groundwater was all NO_3^- . DIN:DON of 5.2 was 2 times greater than that of the WFH water column and groundwater.

Ammonium. I found generally low concentrations of NH_4^+ in the initial water source samples and in the initial incubation samples. [NH_4^+] in the initial WFH water column sample was 6 μM ; groundwater [NH_4^+] was $>4 \mu\text{M}$ in all sites (see fig. 3). In the initial incubation samples, [NH_4^+] was $>4 \mu\text{M}$, suggesting that most of the NH_4^+ came from the WFH water (see fig. 5). Ch. R. light and dark incubations were the exception, beginning at $\sim 10 \mu\text{M}$. Interestingly, [NH_4^+] in Ch. R. groundwater was below the detectable limit – where did the extra NH_4^+ come from? Ch. R. light and dark [NH_4^+] remain around 10 μM through $t = 5.7 \text{ d}$, so the value for initial Ch. R. groundwater is suspect.

I had expected rapid NH_4^+ uptake by phytoplankton, but most initial concentrations were too low to measure large changes. There was, however, evidence for uptake in the Ch. R. light incubations after $t = 5.7 \text{ d}$; final [NH_4^+] was $9.3 \pm 1.4 \mu\text{M}$ less than initial [NH_4^+] (see fig. 5).

Nitrate. I determined initial [NO_3^-] in the incubations that ranged from approx. 10-400 μM depending on the groundwater source (see fig. 6). Of the light incubations, WI additions were lowest at 13 μM , while Ch. R. additions were highest at $406 \pm 64.6 \mu\text{M}$.

Final [NO_3^-] in WFH, WI and the light control were all close to 0, indicating phytoplankton uptake of nearly all available NO_3^- (see figs. 6 and 7b). In WFH total decrease in [NO_3^-] was approx. 60 μM . Declines in [NO_3^-] began roughly at 4 days of

incubation, though in WFH $[\text{NO}_3^-]$ did not approach 0 until approx. day 12 (see fig. 7b). In Ch. R. $[\text{NO}_3^-]$ remained astronomical, showing no trend of increase or decrease. The scale of $[\text{NO}_3^-]$ was so great during the entire incubation, however, that a decrease in $[\text{NO}_3^-]$ comparable to what I measured in the other additions could have gone unnoticed. In addition, variability between replicates at time points 2 and 3 (see fig. 7a) makes interpretation of $[\text{NO}_3^-]$ trends difficult at a time when $[\text{NO}_3^-]$ in the other incubations was dropping precipitously.

I observed no significant change in $[\text{NO}_3^-]$ in dark incubations from any site, suggesting that there was no NO_3^- uptake without phytoplankton production – bacteria alone probably did not take up NO_3^- in measurable amounts.

Chlorophyll. Corresponding to the decline in $[\text{NO}_3^-]$ underway on day 4, I observed a [chlorophyll] peak in light incubations at all sites except Ch. R. by day 4, showing that phytoplankton produced new chlorophyll very rapidly between time points 1 and 2 (see fig. 7c). I measured no such rise in [chlorophyll] at day 4 in Ch. R., at the same time as I observed no $[\text{NO}_3^-]$ decline.

[Chlorophyll] fell off by day 6, but there was a second peak in WFH beginning after day 12 accompanied by a peak in Ch. R. (see fig. 7c). This “second peak” could merely be the result of my use of magnetic stirring, as heavier phytoplankton could have settled and then been resuspended. It is interesting, however, that I observed no corresponding elevated [chlorophyll] in WI and control incubations – if there were settled algae resting at the bottom of the bottle which were then resuspended when I stirred them up, why did they not reappear in the fluorometer readings of WI and the control?

Visually, I recorded observations of green, spindly structures resting at the bottom of the Ch. R. and WFH incubation bottles by day 13, and no such obvious algal growth in the WI or control bottles. It was the presence of these algal structures that prompted me to stir the bottles.

Single-factor ANOVA tests showed that final [chlorophyll] was statistically greater in WFH than in WI ($p < 0.05$, see figs. 8 and 9), but final [chlorophyll] in Ch. R. could not be distinguished from that of either WFH or WI (see figs. 10 and 11), perhaps due to excessive variability in replicate measurements.

DON. I observed no significant differences in [DON] in initial vs. final samples, or between final light and final dark samples in any incubation (see fig. 12).

Bacteria. Bacteria grew from the initial to the final time point in all treatments and sites (see fig. 14). A two-factor ANOVA test revealed, however, that final [bacteria] was greater in the light incubations than in the darks for all sites ($p < 0.001$, see figs. 14 and 15). Overall, significantly more bacteria grew in the light than in the dark.

Discussion:

Of my initial groundwater sources, Ch. R. defied my expectations by containing far and away the highest TDN and DIN concentrations. TDN load in WFH was not only 6.5 times less, but had only half the DIN:DON ratio (see fig. 4). WI had a higher proportion of DIN than I had expected, but fell into the range of TDN that I envisioned in a pristine site. Overall I had a wide range of TDN and DIN:DON characteristics in my groundwaters, exactly as I had hoped.

The gargantuan NO_3^- load in Ch. R. is puzzling, though; I expected little conversion of DON in septic wastewater to DIN via biogeochemical processes as the septic tanks practically infiltrate directly into the estuary -- I reasoned that septic DON would have no time to undergo changes. Perhaps the well-appointed homes in the Ch. R. neighborhood have modern nitrifying septic systems which convert much of the particulate and dissolved organic N into NH_4^+ and bubble it in order to oxidize to NO_3^- .

I expected rapid DIN uptake in WFH and Ch. R., the polluted estuaries, with corresponding rapid chlorophyll production. $[\text{NO}_3^-]$ did decline in WFH, WI and control light incubations (see fig. 7a). I instead observed a rapid peak in chlorophyll in WFH, WI and the control, all at 1.5-2.5 $\mu\text{g/L}$, with no Ch. R. peak (see fig. 7c). Perhaps the Ch. R. incubations had unusually active communities of microbial grazers that exerted top-down control on the phytoplankton population. Whatever the cause of the absent chlorophyll production in Ch. R., there was a corresponding lack of NO_3^- and NH_4^+ uptake at time points 1 and 2.

Later in the 3-week incubation, after day 12, there was a second peak in WFH [chlorophyll] and a first peak in Ch. R. [chlorophyll] (see fig. 7c). Both high-TDN, high DIN:DON sites yielded chlorophyll production long after the incubation had begun,

while WI and the control did not. In WI and the control, virtually all NO_3^- had been exhausted by day 4, when I recorded peaks in [chlorophyll], whereas WFH $[\text{NO}_3^-]$ was still approx. $30 \mu\text{M}$ and Ch. R. $[\text{NO}_3^-]$ had not declined at all. After the first [chlorophyll] peak, WFH $[\text{NO}_3^-]$ continued to decline, and Ch. R. $[\text{NH}_4^+]$ declined as well. The incubations which were well-stocked with DIN continued to produce chlorophyll because phytoplankton still had a bioavailable N source.

As a side note, my experimental results have convinced me that change in [chlorophyll] is not a perfect analog for phytoplankton productivity. I measured $[\text{NO}_3^-]$ uptake of approx. $60 \mu\text{mol/L}$ in WFH and a peak [chlorophyll] of only $\sim \mu\text{g/L}$, far less than one would expect for the corresponding N uptake by phytoplankton (Hugh Ducklow, pers. comm.). Perhaps high light levels in the growth chamber (PAR ~ 1200) led phytoplankton to produce less chlorophyll than I would have seen under lower light conditions, because less chlorophyll would be sufficient. Conversely, in other experiments, phytoplankton might produce greater amounts of chlorophyll under experimental N-enrichment conditions than they would in natural conditions because they have an abundant source of material to produce the N-rich pigments. In addition, determination of chlorophyll by fluorometry could significantly underestimate the amount of chlorophyll as determined by the Lorenzen method (Lorenzen 1967) (Ken Foreman, pers. comm.).

Surprisingly, there was not statistically greater chlorophyll production in the phenomenally NO_3^- -rich Ch. R. addition than in WFH or even WI. $[\text{NO}_3^-]$ above $400 \mu\text{M}$ did not spur greater phytoplankton production than $15 \mu\text{M}$, whereas $[\text{NO}_3^-]$ of $60 \mu\text{M}$ in WFH did grow more chlorophyll than $15 \mu\text{M}$ in WI (see fig. 8). A possible explanation is that the pairing of WFH column water and WFH groundwater gave the particular community of phytoplankton species the maximum amount of DIN that the algae were accustomed to, and much higher $[\text{NO}_3^-]$ did not have any greater effect.

My expectation of observing some DON uptake in all incubations was refuted. There was no trend of decreasing [DON] in any incubation (see fig. 12). It is likely that the recycling of wastewater DON to DIN by bacteria happens on a longer time scale than the 3 week incubation in my experiment. This slow decomposition means that DON will be flushed out of estuaries like WFH and Waquoit Bay before being recycled to DIN and

spurring phytoplankton production. Perhaps in estuaries with much slower tidal turnover, such as Chesapeake Bay, DON could be retained long enough to be recycled and taken up by phytoplankton.

Bacterial production occurred in all incubations, but more bacteria grew in the light incubations than in the dark (see fig. 14). I had expected heterotrophic bacterial production to be linked to uptake of the groundwater DON, of which I found no evidence. Perhaps the bacterial production I witnessed was linked to a different source of DON than I had imagined, that of dead phytoplankton. With a C:N molar ratio at or around 6.6, phytoplankton-derived DON would be higher in N than wastewater DON and more palatable to decomposing bacteria.

Regarding my overall hypothesis that DIN would compose a greater threat of eutrophication than DON, as evinced in my experiment by higher microbial production, this expectation was supported but for a different reason than I imagined. High DIN:DON (Ch. R.) groundwater addition did not produce more chlorophyll or bacteria than low DIN:DON (WI) as I expected. Rather, DIN uptake in all light incubations caused phytoplankton production, which stimulated bacterial production by releasing a form of DON which was preferable to the bacteria. As I am constantly learning, experimental manipulation of one factor (groundwater source) to a complex ecosystem affects many components of that system (nutrients, phytoplankton, grazers, bacteria) and produces results that require an understanding of the system's initial structure and function. This project allowed me to apply ecosystems thinking to a simplified experimental ecosystem, and was a tremendous learning opportunity.

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