Lability of groundwater DON from pristine vs. anthropogenically influenced systems on Cape Cod, Massachusetts

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Abstract—Anthropogenic nitrogen loading to our estuaries has caused eutrophic conditions in many coastal regions. The contribution of DON as a stimulus of primary production is largely unknown. We hypothesize that the composition and lability of DON varies with the land use history of its source. We collected groundwater from Washburn Island, a pristine natural preserve, Green Pond, an impacted estuary, and the leach field of a standard septic system, filter-sterilized the water by high pressure filtration, then added an inoculum of estuarine bacteria. Over the course of 8 days, I measured the change in DIN and DON concentrations, bacterial abundance, and bacterial productivity. The septic system water contained nitrogen concentrations orders of magnitude higher than the other microcosms, and was the only incubation in which I observed net DON consumption (68% of the initial DON concentration). The highest bacterial growth rate was observed in the Green Pond incubation (1.45 mL^{-1} day^{-1}). The Green Pond microcosm also exhibited the highest levels of bacterial productivity (0.16 μmol C L^{-1} day^{-1}), and the highest productivity per cell. The Washburn Island groundwater supported the smallest, least productive microbial population; rates of DIN consumption were also highest in the Washburn incubation. The DON derived from the pristine source, therefore, was refractory in comparison to the anthropogenically-derived DON. Consequently, anthropogenic DON may be a substantial contributing factor to the diminishing health of our estuarine ecosystems.

Introduction

In marine ecosystems, plant, algal, and microbial production is generally limited by nitrogen (Vitousek and Howarth 1991). Anthropogenic sources of nitrogen, such as fertilizer, wastewater disposal, and the fossil fuel derived atmospheric deposition of nitrous oxides, have caused major increases of total nitrogen inputs to ecosystems (Galloway 2002). The resulting eutrophication can cause loss of habitat for benthic dwellers, regions of periodic hypoxia, and deplete the population of benthic organisms (Kroeger 1999). Early research in the causes of eutrophication focused largely on dissolved inorganic nitrogen (DIN) from anthropogenic sources (Committee on the Causes and Management of Coastal Eutrophication, et al. 2000). However, dissolved organic nitrogen (DON) is another source of nitrogen that could potentially stimulate primary production.

Little is known about the molecular structure of DON, or the extent to which it is labile. Sietzinger and Sanders (1997) have reported that up to 70% of the DON in rivers in the northeastern United States may be biologically available. Approximately 50% of the DON in northeastern rainwater may also be labile to estuarine bacteria (Seitzinger and Sanders 1999). Kroeger (1999) also reports that DON, on average, comprises approximately 60% of the nitrogen concentration in the groundwater at the seepage face of Green Pond in Falmouth, Massachusetts. The implication is that dissolved organic nitrogen could be a substantial portion of labile nitrogen flowing into coastal embayments.

We hypothesize that the composition and lability of DON varies with the land use history of its source. Therefore, we collected groundwater from three sites: a pristine nature reserve (Washburn Island), the perimeter of an impacted estuary (Green Pond), and the leach field of a standard septic system (specified in Title V of Massachusetts law). Groundwater is an effective gauge of the difference in DON among sites because it represents the land use history of the entire watershed feeding each estuary. Additionally, groundwater flow is the primary route carrying nutrients into the coastal zone of the Cape, where precipitation percolates rapidly through the sandy soil (Valiela et al 1992, 1997; Kroeger 1999). The lability of the DON in the groundwater of these three sites should differ proportionately with the extent of anthropogenic influence.

To measure the lability of DON, I conducted a series of assays measuring the change in nitrogen concentration, bacterial abundance, and bacterial productivity in the water over time. The consumption of DON by microbes is a critical process in the cycling of nitrogen through an ecosystem; bioavailable DON may be immobilized by estuarine microbes, stimulating the microbial food loop, or mineralized to DIN, stimulating further primary production (Jorgensen 1999). The site with the most labile DON should support the most productive and abundant microbial population. I also anticipate that DIN will be most readily mineralized in the water with the most labile dissolved organic matter.

Seitzinger and Sanders (2002) found that urban storm water runoff had a higher proportion of DON available to estuarine plankton than agricultural or forested sources. On this basis, I expect that the DON from the septic system leach field to be most labile for microbial utilization. Groundwater feeding Green Pond should reflect a watershed of forests, agricultural, and residential use (Kroeger 1999), and therefore contain intermediately labile DON relative to the septic system and Washburn groundwater. I expect the DON of Washburn groundwater to be mainly refractory, to not be readily consumed, and to support minimal microbial growth as a result of its origin from a forested watershed.

Methods

Site descriptions—Groundwater was collected in the late fall from three sites on Cape Cod, Massachusetts. The first was Washburn Island, a nature preserve located in Waquoit Bay, dominated by oak and pine forests. Our second site was Green Pond, a small coastal embayment in Falmouth, Massachusetts fed by the Backhus River. The estuary empties on the south shore of Cape Cod into Vineyard Sound. Green Pond separates two peninsulas that are densely populated, and is subject to nitrogen inputs from residential fertilizer use and septic tanks. Green Pond's watershed also contains agricultural land, cranberry bogs, and golf courses, and the estuary is contaminated by the Ashumet Valley wastewater plume that originates from the Massachusetts Military Reservation (Kroeger et al).

At both Washburn Island and Green Pond, we collected 28 L of groundwater (salinity less than 2 ppt) from 14 individual wellpoints. The fourteen wellpoints were distributed among three general regions at both sites (Fig. 1). Groundwater from each site was well mixed in the field to create a composite sample.

Our final site was the Massachusetts Alternative Septic System Testing Facility. The facility houses three standard Title V septic systems; lysimeters are located in the leach field of each system at depths of 1, 2, and 5 feet. Water was collected from lysimeters at all three depths in all three systems to create a composite sample of 28 L of leach field water.

Experimental setup—All water was filter-sterilized by high-pressure filtration through Whatman GF/F filters and 0.2 μm filters. Fifteen litres from each site was designated for bioassays to determine the DON lability, while the remaining water was used for molecular weight fractionation by ultrafiltration. The 15 L from each site were distributed equally across triplicate incubations, held in Nalgene 20 L carboys. Additionally, one carboy of 5 L of filter-sterilized deionized water served as a control.

To prevent nutrient limitation, I added 0.005 g of KNO_3 to the 5 L, or 10 μM N, in the control and the three incubations of Washburn groundwater. I did not enrich the Green Pond or Title V incubations with DIN; previously collected data indicated that both types of water would contain DIN in excess of the microbial uptake capacity of 10 μM (Kroeger 1999; Chiota 2003, unpub). Similarly, I added 0.0026 g of KH_2PO_4 to the 5 L, or 4 μM P, to the control, Washburn, and Green Pond incubations. The mean phosphate concentration of leach field water in a Title V septic system is approximately 25 μM P (Chiota 2003, unpub) therefore I did not enrich these incubations with phosphate. The ten carboys were kept at room temperature in the dark and stirred gently for eight days.

A bacterial inoculum was prepared by filtering estuarine water from Green Pond and Waquoit Bay through Whatman GF/F filters (0.7 μm nominal pore size), removing particulates and bacterial grazers. Equal volumes of the Green Pond and Waquoit Bay filtrate were combined and 250 mL of inoculum (5% of the microcosm volume) was added to each of the ten incubations. Samples were collected to measure ammonium, nitrate, total nitrogen, bacterial abundance, and bacterial productivity on the day of inoculation and 2, 4, 6, and 8 days following. Twenty millilitres were frozen in scintillation vials for nitrate analysis. Twenty millilitres were acidified with 5 N HCl in scintillation vials and refrigerated for ammonium analysis. Approximately 40 mL were frozen in 50 mL centrifuge tubes for total dissolved nitrogen analysis. A 9.5 mL sub-sample was fixed with 0.5 mL of glutaraldehyde to be used for bacterial cell counts. Finally, 4 mL were designated for measuring bacterial productivity by ^{14}C-leucine assimilation. The microbial assays were conducted on the day of sample collection; nutrient analysis was performed in duplicate at the end of the 8-day incubation period.

Analytical methods—I measured nitrate concentrations using a Lachat QuickChem 8000 flow injection analyzer, following a modification of the cadmium reduction method (Wood et al. 1967). The Green Pond samples were diluted 1:2, while the septic system samples were diluted 1:20 with deionized water. Ammonium concentrations were measured by a phenyl-hypochlorite technique modified from Solarzano (1969). I measured total dissolved nitrogen by a modification of the persulfate digestion method described by D'Elia et. al.(1977). A series of urea standards (5, 10, 50, and 100 µM) was included in the analysis to calculate the digestion efficiency. DON was calculated by subtracting the concentration of DIN from the measured TDN.

Direct bacterial counts were conducted by staining 2.0 mL of the glutaraldehyde-fixed sub-sample with 100 μL of 200 μg/mL 4,6-diamidino-2-phenylindole (DAPI) (Porter and Feig 1980). The cells were stained for five minutes before being mixed with sterile phosphate buffered saline (PBS) in a 15 mL Millipore tower and filtered through 0.22 μm pore size polycarbonate filter (Millipore GS) and a Whatman GF/F backing filter (<12 mmHg of pressure). I counted five fields of each slide with the 100x oil-immersion objective of a Zeiss epifluorescence microscope. I estimated the mean bacterial growth rates by fitting an exponential curve from the mean initial counts to the mean peak abundance in each microcosm type.

To estimate bacterial productivity, I placed 2 mL of each sample into two 15 mL centrifuge tubes. One tube, the initial, received 0.2 mL of 50% trichloroacetic acid (TCA). I then added 0.5 μL of 6 μM 14C-leucine (100 Ci/mmol) to each vial. After approximately 30 minutes, I added 0.2 mL 50% TCA to the second tube and refrigerated the samples. At the end of the eight-day incubation, I vacuum-filtered the initial and final solutions of each sample through a 0.22 μm Millipore nitrocellulose filter. The tubes were rinsed with cold PBS, and the filters were rinsed 3 times with cold 5% TCA to remove unassimilated ^{14}C-leucine. The filters were placed in scintillation vials, dried, and dissolved by 1.0 mL ethylene glycol monoethyl ether ("Cellusolve"). Ten millilitres of scintillation cocktail was added and the incorporated label was measured on a scintillation counter. I subtracted the disintegrations per minute (DPM) of the initial tubes from the final tubes for every sample, and then calculated bacterial productivity (μmol C L-1 day-1) from the corrected DPM.

Results

The concentrations of nitrogen varied substantially across the microcosms. In the deionized water of the control, Washburn groundwater, Green Pond groundwater, and the septic system leach field water (hereafter referred to as Title V), initial mean ammonium concentrations were less than or equal to 5 μM. Initial mean DIN concentrations in the control and Washburn microcosms were on the same order of magnitude, following their nitrate addition, as the Green Pond microcosm. The mean initial DIN concentrations in the Title V incubations was 1257 μM, two orders of magnitude greater than the other microcosms. Similarly, the Title V incubation contained initial mean DON concentrations an order of magnitude greater than any other microcosm (Table 1).

Over the 8-day time course, the concentration of total dissolved nitrogen (TDN) remained relatively constant. In the Title V microcosm, the mean DIN concentration increases as the mean DON concentration decreases over time, maintaining the TDN concentration around 1600 μM (Fig. 2). In the Green Pond incubation, the TDN increased linearly during days 4 through 8 as DON was produced, reaching a maximum N concentration of approximately 60 μM (Fig. 3). DON was also produced in the Washburn incubation, but the mean TDN concentration was maintained at a substantially lower level than in the Green Pond microcosm (approximately 20 μM) by the consumption of DIN (Fig. 4). In the control, I observed a slight increase in the TDN concentration in conjunction with DON production beginning after day 2 (Fig.5).

The mineralization of DIN was achieved only in the Title V microcosm; the mean net change of DIN in the Title V incubations was +333.8 μM. I observed the consumption of DIN in both the Washburn and Green Pond incubations, at concentrations of –5.5 and –2.2 μM respectively (Fig. 6). With the consumption of DIN, I also observed the net production of DON in the Washburn and Green Pond microcosms, at concentrations of +5.7 and +24.3 respectively. To complement the net production of DIN in the Title V incubation, I observed a net consumption of –312 μM DON in the Title V microcosm (Fig. 7). Fig. 8 is a conceptual diagram of the uptake, immobilization, and mineralization of DON by a microbial population.

During the 8-day time course, the dissolved organic matter of the different microcosms supported varying degrees of microbial growth and productivity. The mean peak bacterial abundance was lowest in the control incubation (1.5×10^6 cells/mL) (Fig. 9) and highest in the Title V incubation (1.2×10^7 cells/mL) (Fig. 10). The Washburn groundwater supported a microbial population that peaks at approximately 3.4×10^6 cells/mL (Fig. 9). The mean peak bacterial abundance in the Green Pond microcosm was approximately 1.1×10^7 cells/mL, which was reached on day 2 of the time course (Fig. 10, 11). Fig. 11 is a plot of the mean bacterial abundances of each microcosm over the 8-day incubation period. The data points are fitted by an exponential trend line from the initial DAPI counts to the peak abundance. Data points after the peak abundance was reached were not used in the growth rate analysis and are indicated by open symbols. The mean bacterial growth rates were estimated from the equations of the exponential trend line and are presented in Table 2. The Green Pond incubations supported the highest mean growth rate (1.45 mL^{-1} day^{-1}), followed by the Title V (0.95 mL^{-1} day^{-1}), Washburn (0.53 mL^{-1} day^{-1}), and the control incubation (0.27 mL^{-1} day^{-1}).

The rate of bacterial productivity generally paralleled the pattern of microbial growth in the microcosms (Fig. 9, 10). Green Pond groundwater supported the most productive bacterial population (0.15 μmol C L^{-1} day^{-1}); this level of mean peak productivity was sustained from day 2-4. The Washburn incubations had the lowest mean peak productivity measurements of 0.03 μmol C L^{-1} day^{-1}. Among the three microcosm treatments, the Green Pond groundwater had the highest mean bacterial productivity per cell for seven days of the time course, with a peak on day 4 of 1.8×10^{-8} μmol C L^{-1} day^{-1} cell^{-1} (Fig. 12). The Washburn and Title V microcosms had very similar values of mean productivity per cell throughout the eight-day timecourse.

Discussion

The nitrogen concentrations in the Title V incubations were orders of magnitude higher than in the other microcosms (Table 1), but DON comprised only 26% of the mean initial total nitrogen concentration of 1710 μM. Dissolved organic nitrogen comprised a much more substantial portion of the nitrogen load in the groundwater feeding Green Pond, approximately 60% of the TDN. In the Washburn incubations, the mean TDN was approximately one-half the TDN concentrations of Green Pond. The mean initial DON concentration of 0.5 μM is inconsistent with previously collected data (Foreman, unpub), but over the remainder of the time course the concentration of DON comprised approximately 40% of the TDN in the Washburn incubations. In the control, DON production began following day 2 of the time course (Fig. 5). However, since the concentration of DON produced in the control was small (<10 μM), I considered it to be negligible relative to the mean DON concentrations in the treatment microcosms.

I observed the net production of DON in the control, Washburn, and Green Pond incubations (Fig. 7). The DON produced in the Washburn incubation was on the same order of magnitude as in the control (approximately 6 μM). In Green Pond, however, there was a substantial concentration of DON produced (24.3 μM). Bacterial peptides, enzymes, and nucleic acids may account for this observed increase in DON concentration. During similar incubation experiments, Seitzinger and Sanders did not witness net in situ DON production, but recognized that DON production and consumption were simultaneous processes (1999). In contrast the other microcosms, approximately 312 μM DON was consumed in the Title V incubation (68% of the mean initial DON concentration). From the consumed DON, 334 μM DIN was mineralized, roughly maintaining the mean TDN concentration in the microcosm (Fig. 6).

The production or consumption of DIN over the time course could be explained by the C:N ratio of the dissolved organic matter which the bacteria consumed (Fig. 8). Bacteria take up carbon and nitrogen in the same ratio that comprises the dissolved organic matter they are consuming. A small percentage of the consumed organic matter will be assimilated, and C and N will be immobilized in a weight ratio of 5:1 (Vallino, personal communication). In the case that the weight C:N of the consumed DOM is 25:1, and the bacteria assimilate 20% of the consumed organic matter, the bacteria assimilate 5 units of carbon and 0.2 units of N. Assume 20% growth efficiency with respect to carbon, and 4 units of carbon are respired while 1 unit is immobilized. To maintain a weight ratio of 5:1, the bacteria will immobilize 0.2 units of N. In this scenario, DIN is not mineralized because all of the assimilated nitrogen is utilized for microbial nutrition. Maintaining the same assimilation and growth efficiencies, bacteria will mineralize DIN if the consumed DOM has a lower C:N than 25:1 (weight ratio). Conversely, if the DOM has a C:N weight ratio higher than 25:1, the microbe will consume DIN to maintain the standard bacterial C:N ratio of 5:1.

In the Green Pond incubations, the change in DIN concentration was only –2.2 μM. This small consumption of DIN suggests that the DOM consumed by the bacteria may have a C:N weight ratio near 25:1, a molar ratio of nearly 30:1. Hopkinson et al (1998) report that the DOM in groundwater from the Childs River, another impacted site in Falmouth, MA, has a C:N ratio of 34:1. Therefore, an estimated C:N ratio of 30:1 in Green Pond DOM is consistent with previously collected data.

The consumption of DIN in the Washburn incubation suggests that the nitrogen component of the consumed DOM was insufficient to maintain the bacterial C:N ratio of 5:1. The C:N of the consumed DOM in the Washburn groundwater, therefore, is likely higher than 30:1. Equally, the mineralization of DIN in the Title V incubations suggests that the consumed DOM from the septic leach field has a lower C:N ratio than the consumed DOM in the Green Pond groundwater.

Microbial growth has been positively correlated with an N/C ratio (Hopkinson 1998). Considering that the overall peak bacterial abundance was attained in the Title V incubation, this is further support for the claim that the consumed DOM in the Title V had the lowest C:N ratio among the three treatments. Alternatively, the highest growth rate was estimated to be in the Green Pond incubations. However, the exponential curve was fit to the peak abundance of each treatment, which was reached on day 2 in the Green Pond incubations. The Green Pond growth rate was therefore calculated from only 2 data points rather than from 3 points as used to calculate the Title V and Washburn growth rates (Table 2). It is slightly unusual that the peak abundances in the control, Washburn, and Title V incubations were reached beyond day 2 (Fig. 11). Previously conducted studies indicate that bacterial numbers peak at 2 days, after which grazing pressure from a reestablished protozoan population increases (Hopkinson et al. 1998). The peak in bacterial abundance on day 2 in the Green Pond incubation may reflect the onset of grazing pressure; alternatively, the rapid growth may have been spurred by the compatibility of the DOM's C:N ratio with the bacterial C:N weight ratio of 5:1.

The rapid growth rate in the Green Pond incubation is mirrored by a rapid increase in bacterial productivity (Fig. 10). Unlike the bacterial counts, however, the high productivity is maintained for several days. Bacterial productivity peaks in the Title V incubations on day 4, similarly mirroring the peak in bacterial abundance. Over the time course, the highest mean productivity per cell was calculated in the Green Pond microcosm (Fig. 12).

Based upon the high rates of bacterial growth, mean productivity, and productivity per cell, the Green Pond groundwater seems to contain the most labile DON. From the rate of mineralization, the consumption of 68% of the initial DON, and the high peak bacterial abundance in the Title V incubations, one could also cite the DON from the septic system leach field as the most labile. I believe that the bioavailability of the two types of DOM is relative to the organism utilizing the organic matter. Estuarine bacteria are adapted to consuming the DOM entering the estuary at the seepage face. In contrast, the DOM from the leach field has not undergone the usual transformations that occur in the vadose zone and aquifer before reaching the seepage face. The lag before the estuarine bacteria reach peak abundance and productivity in the Title V microcosm may be attributed to this difference. In any case, anthropogenically-derived DON is more labile for bacterial production than dissolved organic nitrogen originating from a pristine source. Consequently, large DON inputs may induce serious nuisance blooms and eutrophic conditions. To successfully manage the diminishing health of our coastal zones, we must consider the contribution of both organic and inorganic nitrogen to anthropogenic nutrient loads.

Acknowledements

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Appendices

FIGURES AND TABLES

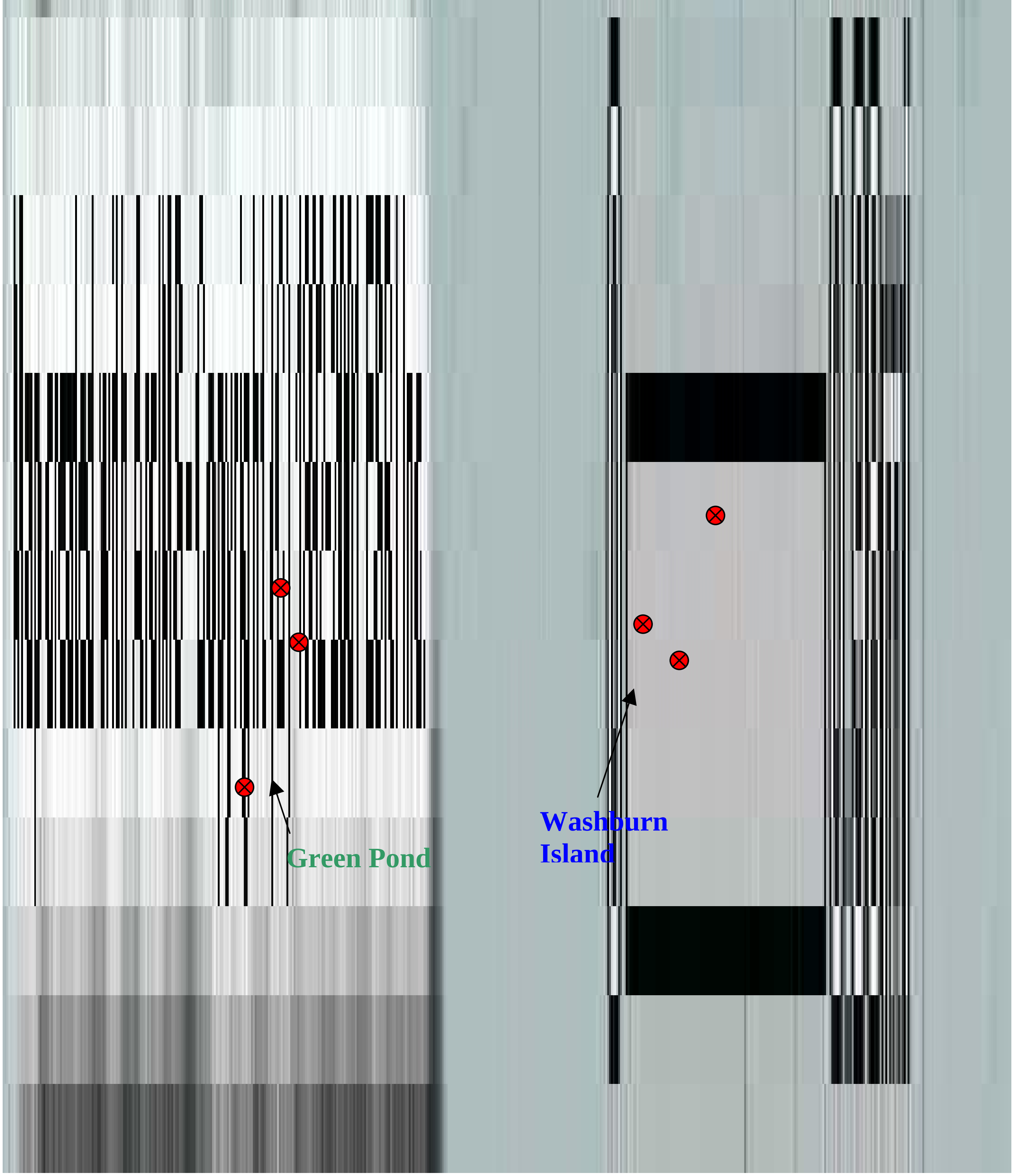
Green Pond
Washburn Island

Table 1. Mean initial concentrations of DIN and DON in each microcosm (after DIN addition to Control and Washburn water). The nitrogen concentration of water from the leach field of a Title V septic system is orders of magnitude higher than the pristine or moderately impacted sources.

	Initial Mean N Concentrations (μM)		
	NH_4^+	NO_3^-	DON
Control	0.5	9.7	0.0
Washburn	0.6	17.4	0.5
Green Pond	5.0	9.5	26.8
Title V	1.0	1256.4	452.7

Fig. 2. Change in mean total dissolved nitrogen (TDN) over time in the *Title V* microcosm. Note the change in scale of N concentrations. TDN peaks at approximately 2000 μM.

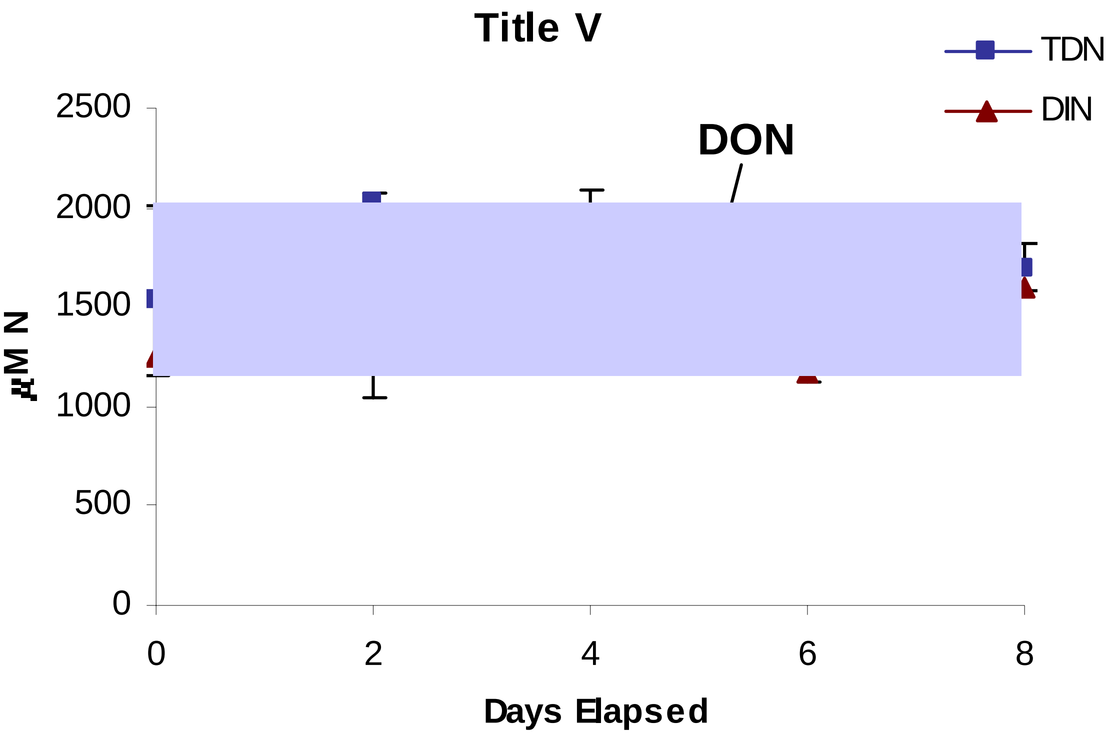

Fig. 3. Change in mean total dissolved nitrogen (TDN) over time in the *Green Pond* microcosm. TDN peaks at approximately 60 μM.

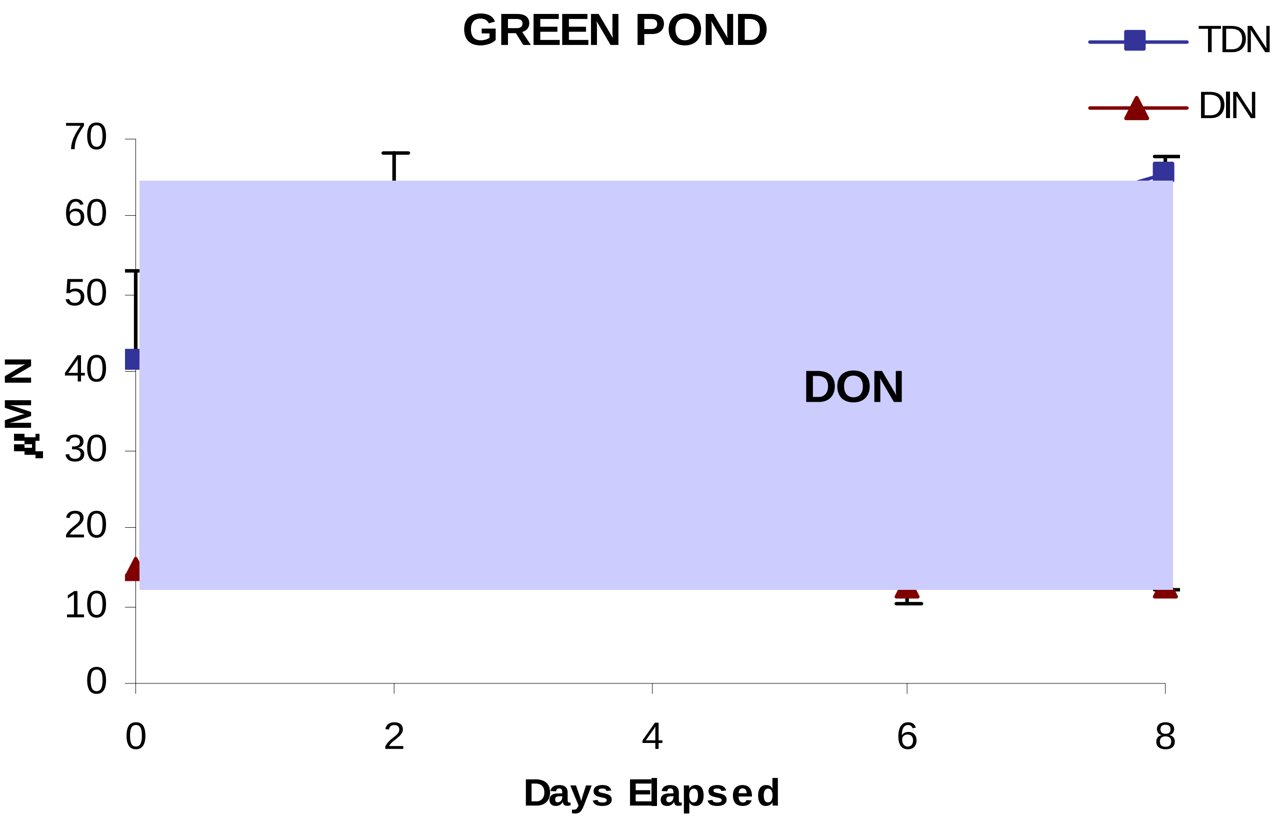

Fig. 4. Change in mean total dissolved nitrogen (TDN) over time in the *Washburn* microcosm. TDN peaks at approximately 32 μM.

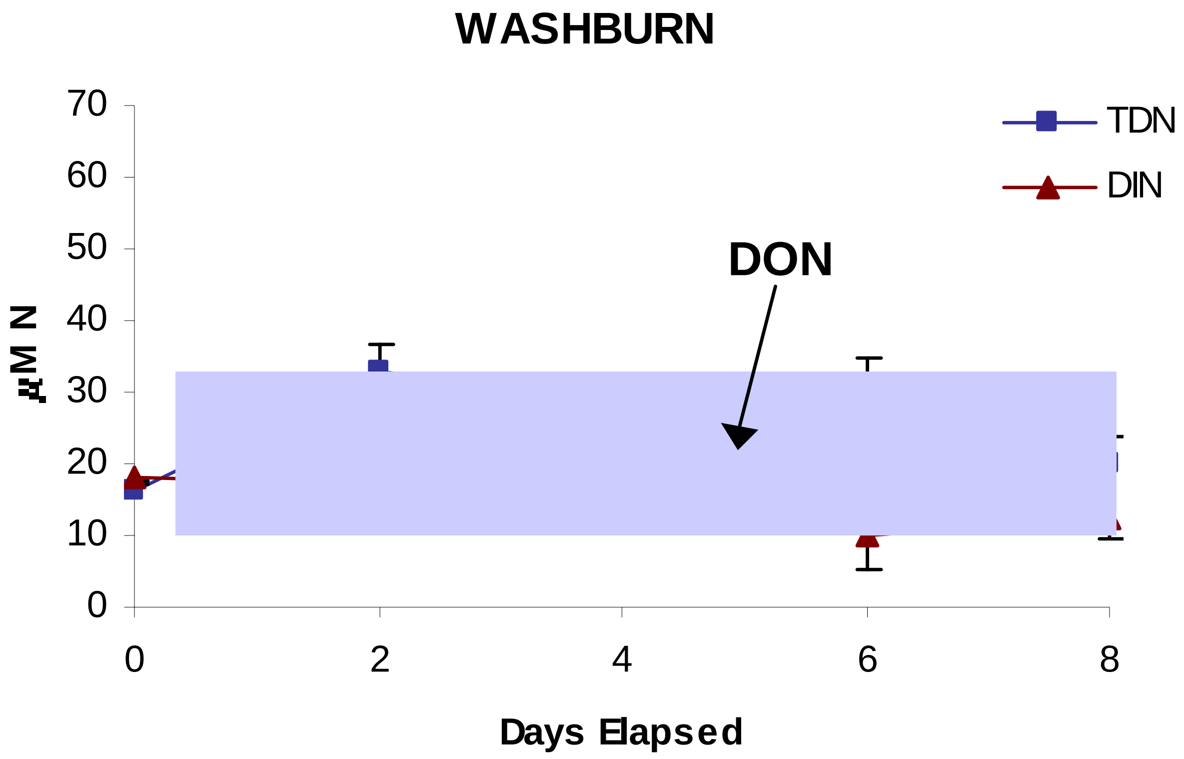

Fig. 5. Change in total dissolved nitrogen (TDN) over time in the *control* microcosm. DON production begins after day 2. TDN remains relatively constant at approximately 16 μM.

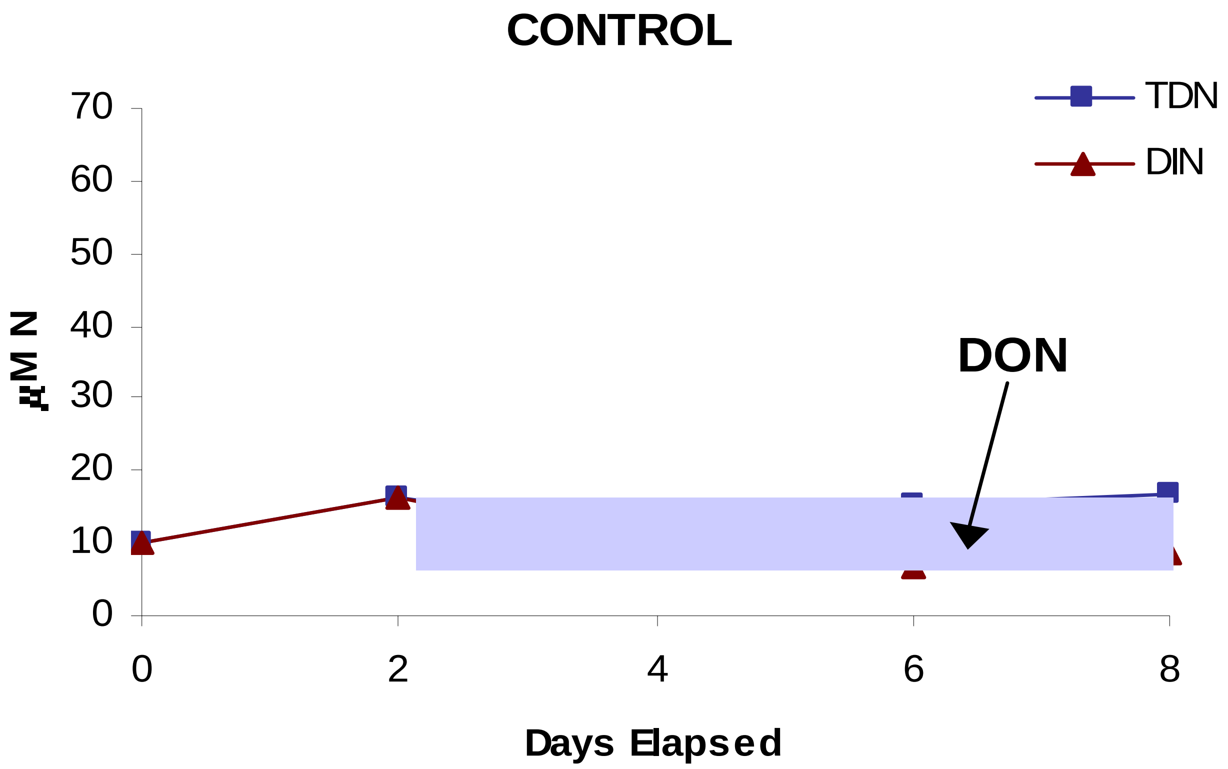

Fig. 6. Net change in mean DIN concentrations (final minus initial) of each microcosm. DIN concentrations increased only in the Title V leach water incubations.

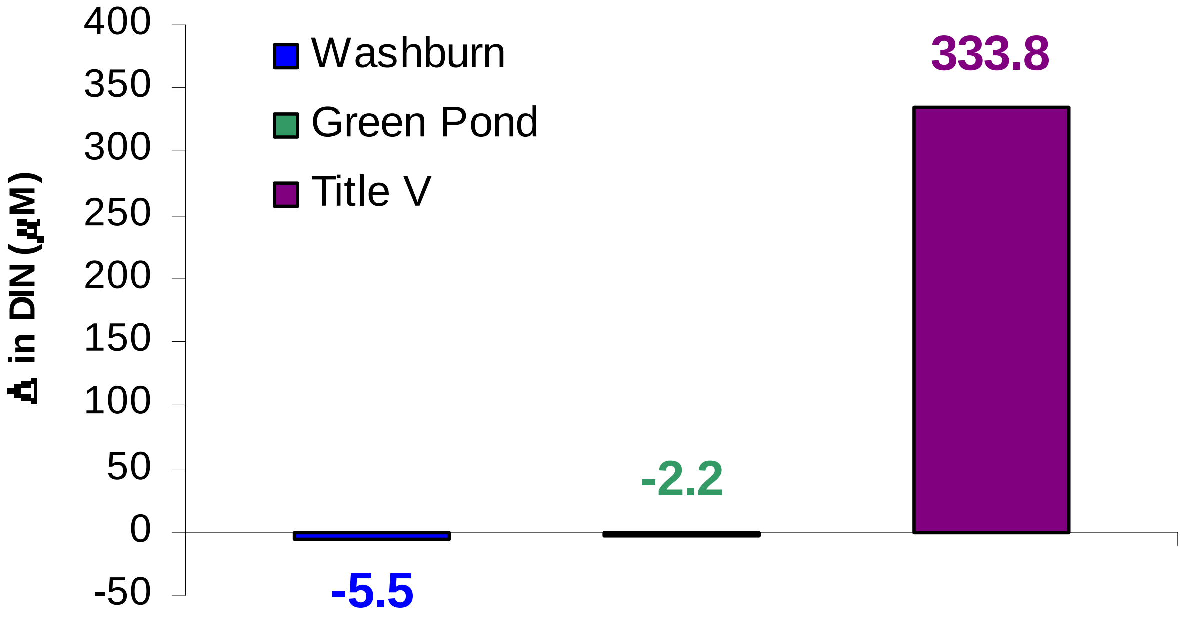

Fig. 7. Net change in mean DON concentrations (final minus initial) of each microcosm. DON was consumed only in the Title V leach water incubations.

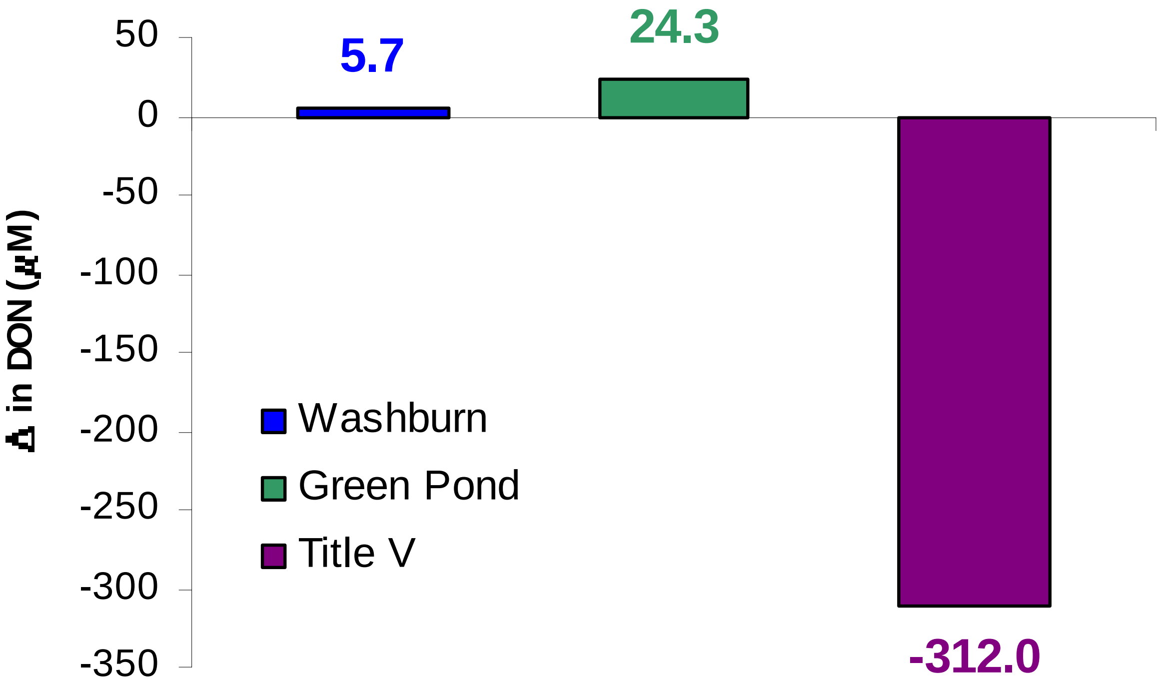

Fig. 8. Diagram of DON assimilation, immobilization, and mineralization to DIN by microbes. Dissolved organic matter is taken up by bacteria according to the C:N ratio of the DOM. It is assimilated and immobilized in approximately a 5:1 C:N weight ratio. The excess N is mineralized; the excess C is respired.

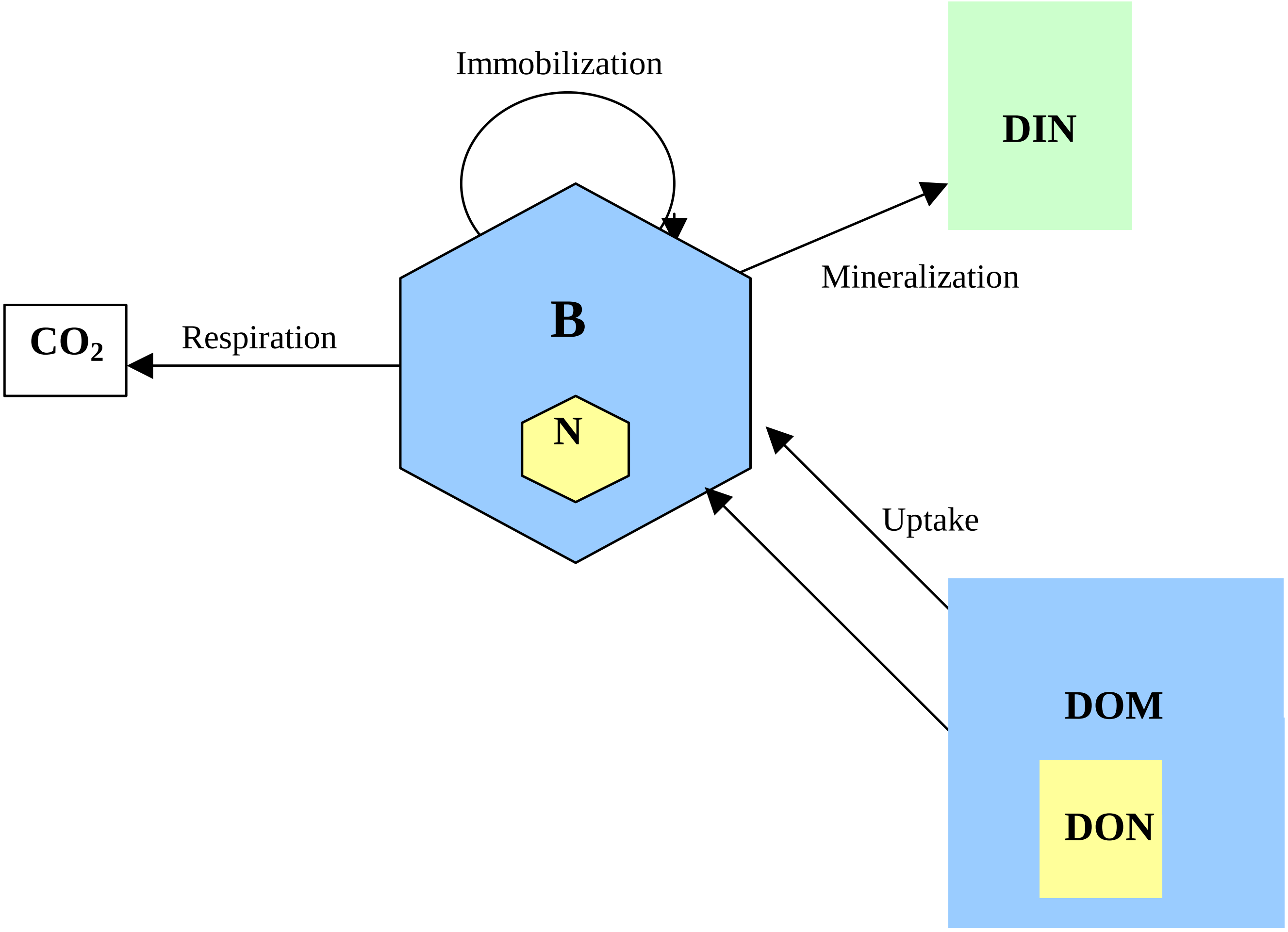

Fig. 9. Mean bacterial abundance (left axis) and productivity (right axis) in the *control* and *Washburn* microcosms during the course of the 8-day incubation.

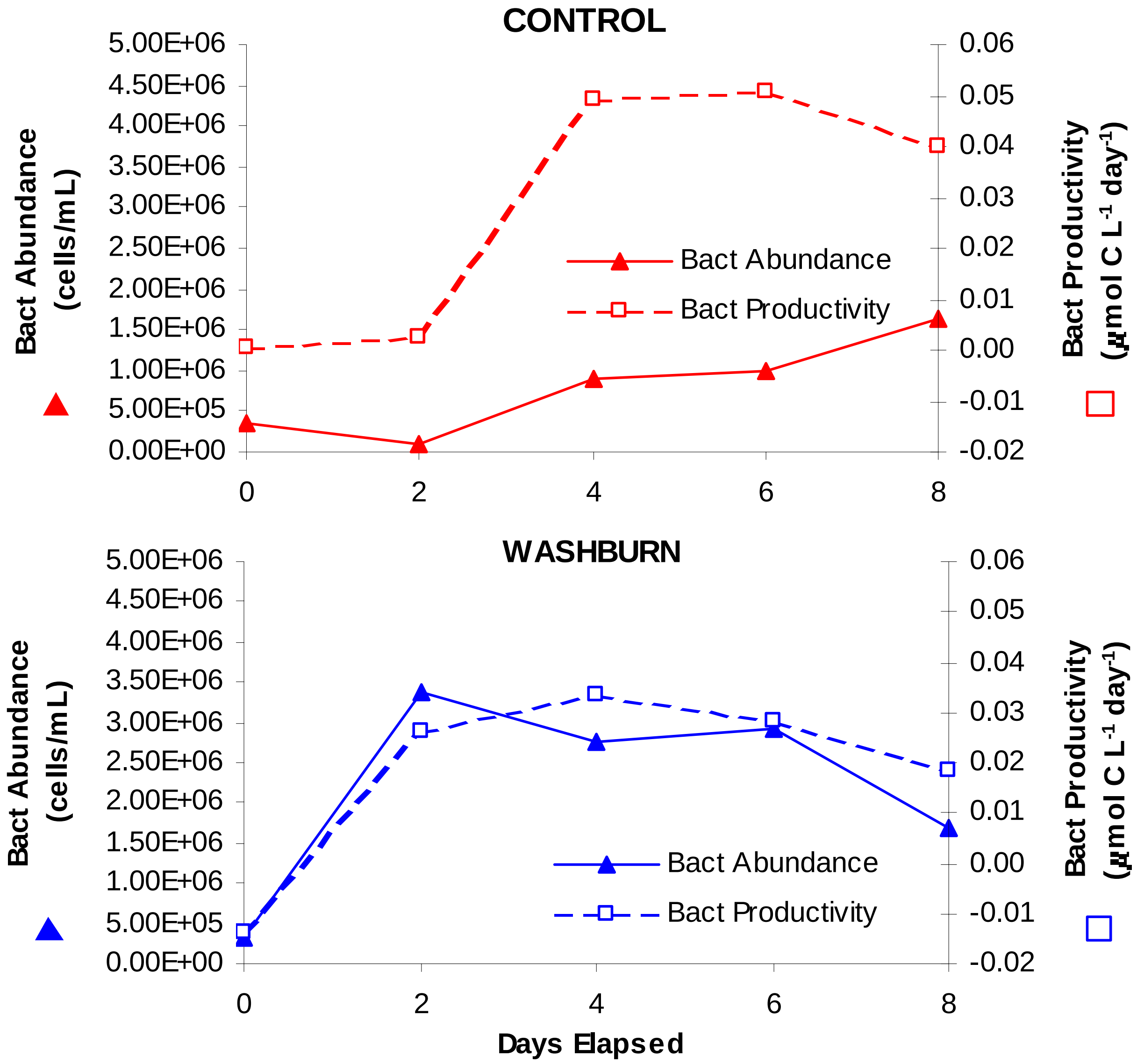

Fig. 10. Mean bacterial abundance (left axis) and productivity (right axis) in the *Green Pond* and *Title V* microcosms during the course of the 8-day incubation. Note the change in scale relative to Fig. 9.

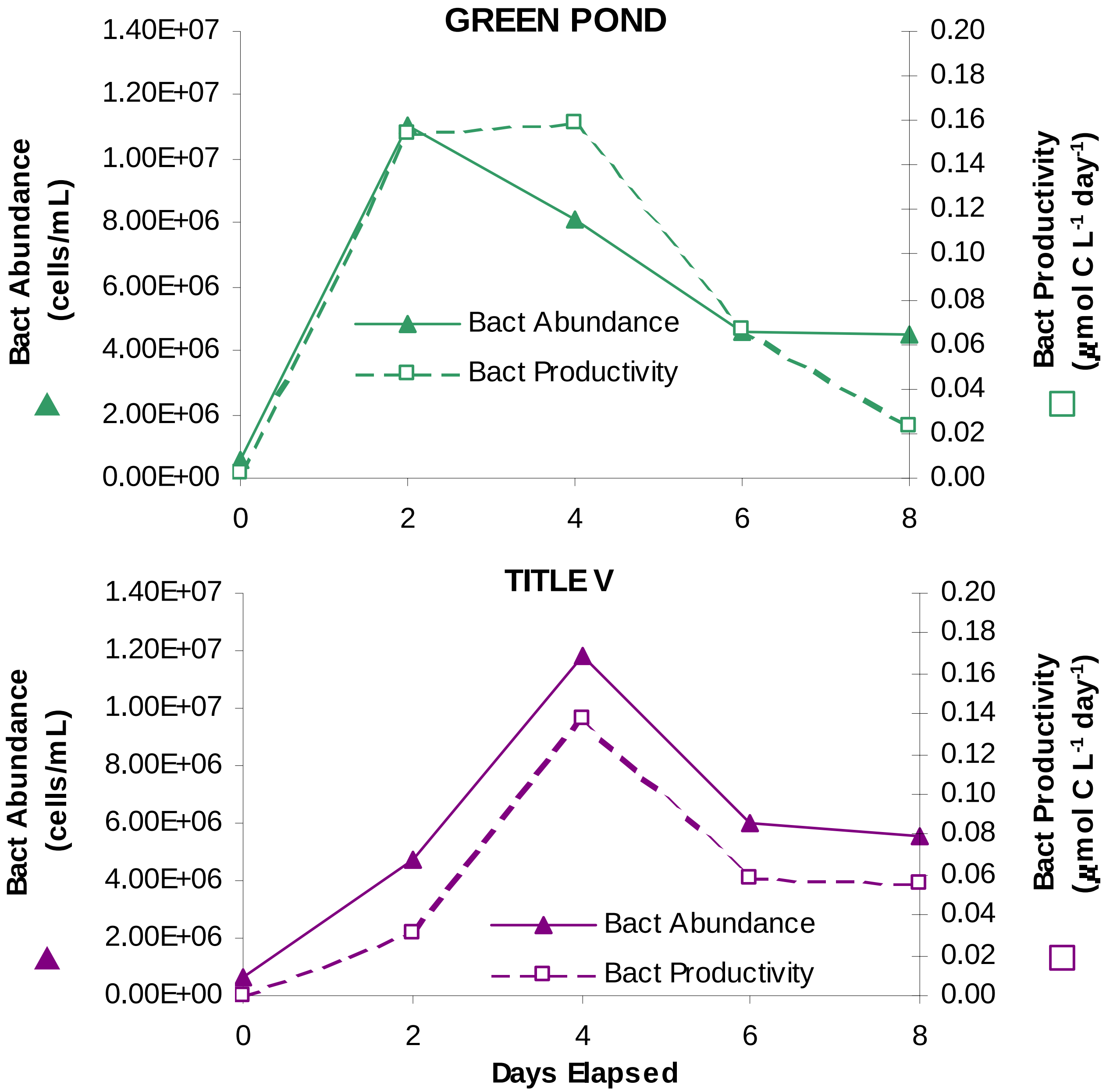

Fig. 11. Mean bacterial abundance in each microcosm during the 8-day incubations. An exponential curve was fit from the initial counts to the peak abundances. Open symbols indicate the counts following the peak abundance; these points were not included in the growth rate analysis.

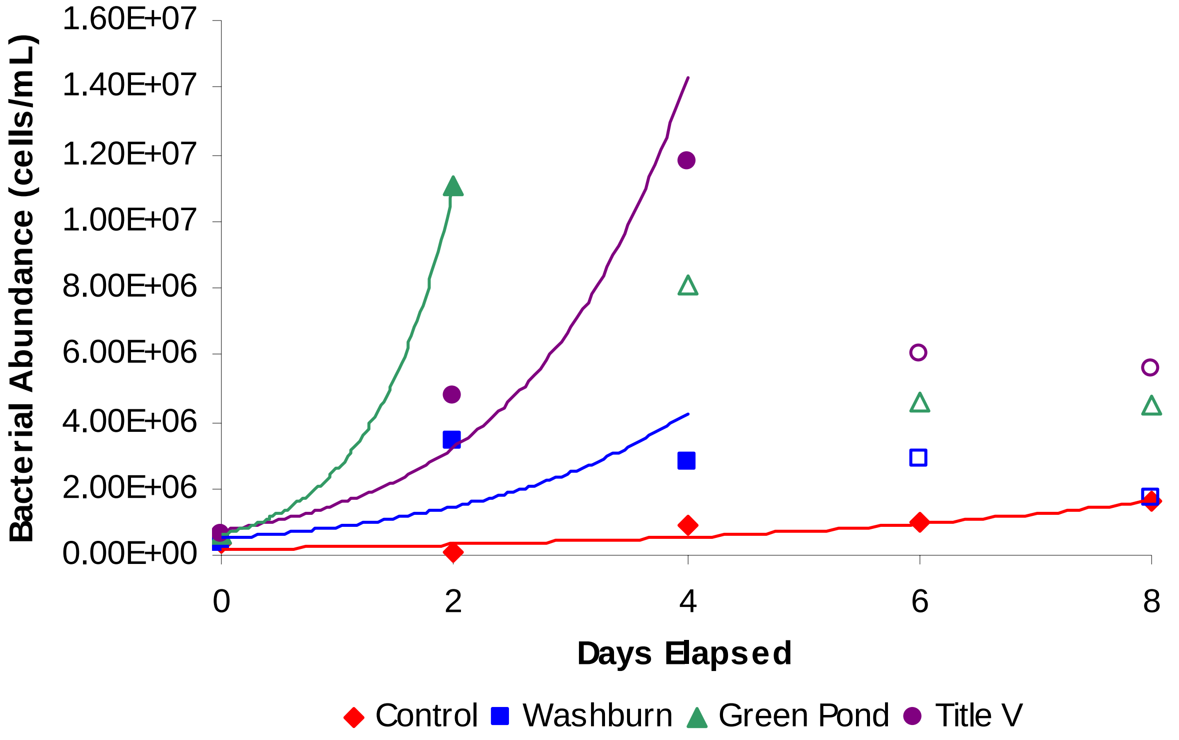

Table 2. Mean bacterial population growth rates in each microcosm. Green Pond groundwater supports the most rapid microbial growth.

Microcosm	**Mean Population Growth Rate (ml^{-1} day^{-1})**	**R^2 Value**
Control (DI)	0.27	0.95
Washburn	0.53	1.00
Green Pond	1.45	0.68
Title V	0.95	0.61

Fig. 12. Mean bacterial productivity per cell of each microcosm. Green Pond groundwater supports the most productive microbial population for approximately 7 days.