ESTABLISHING A RESEARCH AGENDA FOR ASSESSING THE BIOAVAILABILITY OF WASTEWATER TREATMENT PLANT-DERIVED EFFLUENT ORGANIC NITROGEN IN TREATMENT SYSTEMS AND RECEIVING WATERS

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I. INTRODUCTION

Overview

This report summarizes the outcomes and recommendations from a two day workshop on wastewater treatment plant-derived effluent organic nitrogen (EON) that occurred in Baltimore, Maryland on September 26 and 27, 2007. The targeted outcomes from this workshop were to develop a prioritized research strategy for:

- implementing a reliable protocol(s) to determine the bioavailability of EON in receiving waters, and
- understanding how upstream treatment technologies influence the generation or removal of this bioavailable organic nitrogen fraction.

The participants, identified in Table 1, were a highly multidisciplinary mix of wastewater utility personnel, wastewater design engineers, watershed modelers, regulatory and government personnel, natural systems researchers, and wastewater engineering researchers. This mix of participants successfully articulated a research plan for EON that identifies the research needs in the treatment plant as well as downstream of the treatment plant (in the watershed).

The workshop was co-sponsored through a collaborative effort between the Scientific and Technical Advisory Committee (STAC) to the Chesapeake Bay Program and the Water Environment Research Foundation's (WERF's) Nutrient Challenge program.

Table 1. Workshop Participants.

Nitrogen Removal & Wastewater Design	***Organic Nitrogen Bioavailability Researchers***	***Nitrogen Biogeochemical Cycling Researchers***
James Barnard, Black and Veatch Nancy Love, Virginia Tech* JB Neethling, HDR Vikram Pattarkine, Brinjac Engineering* Amit Pramanik, WERF* Cliff Randall, Virginia Tech Tom Saddick, CH2M Hill David Stensel, Univ. Washington* Bev Stinson, Metcalf & Eddy	Debbie Bronk, College of William and Mary, Virginia Institute of Marine Science (VIMS)* April Gu, Northeastern University Eakalak Khan, North Dakota State Univ. Margaret Mulholland, Old Dominion Univ.* Krishna Pagilla, Illinois Institute of Technology David Sedlak, Univ California-Berkeley Robert Sharp, Manhattan College	Walter Boynton, Chesapeake Biological Laboratory Jack Brookshire, Princeton University Elizabeth Canuel, College of William and Mary, VIMS Sujay Kaushal, Chesapeake Biological Laboratory Leigh McCallister, Virginia Commonwealth Univ. Hans Paerl, Univ North Carolina
Regulatory and Government	***Modeling***	***Industry/Utility***
Rich Batiuk, EPA Chesapeake Bay Program Office Dave Clark, HDR, Regulatory Liaison Steve Luckman, Maryland Department of the Environment Mark Smith, EPA Region 3 Tonya Spanyo, Metropolitan Washington Council of Governments (MWCOG)* Kyle Winter or Allan Brockenbrough, VA DEQ Phil Zahreddine, EPA, Office of Water and Wastewater/Municipal Technology Branch+ Ning Zhao, EPA Chesapeake Bay Program Office	Dom DiToro, Univ. of Delaware Lewis Linker, EPA	Jeannette Brown, Stamford, CT Randal Gray, Truckee, Nevada Bernard Kiernan, Philip Morris Sudhir Murthy, DC WASA Jim Pletl, Hampton Roads Sanitation District Dipankar Sen, Santa Clara Valley Water District, California Keith Bailey, Smithfield Foods Dave Waltrip, Hampton Roads Sanitation District

* Represents planning committee members.

Some participants represent more than one category but are placed in their primary category

Rationale for the Workshop

The United States Environmental Protection Agency (EPA) requested guidance from the Scientific and Technical Advisory Committee (STAC) of the Chesapeake Bay Program regarding the bioavailability of organic nitrogen (ON) released through wastewater treatment plant effluents (effluent organic nitrogen or EON) and the appropriateness of a proposed assay for assessing its bioavailability. According to Virginia law (see below), dischargers can argue cases before a nutrient control board to increase their discharge allowances or caps based on their assessment of EON bioavailability. A facility in Virginia employed a bioassay in an attempt to demonstrate that a large fraction of their EON was biologically unavailable. In the short term, EPA requested guidance on: 1) whether EON is bioavailable in the proximate and ultimate receiving waters, and 2) whether the assay employed by the Virginia facility is appropriate for assessing EON bioavailability. In the longer term, the EPA has sought guidance on developing appropriate assays of EON bioavailability. In response to this request STAC created a sub-committee to formulate a report with the requested guidance for the short-term. Subsequently, members of this STAC sub-committee along with representatives from the Water Environment Research Foundation (WERF) teamed up to develop a workshop aimed at uniting distinct stakeholder communities to address the longer-term goal of developing appropriate bioassays that can be used by the regulated community to allow them to meet the demands of EPA’s water quality criteria.

In many estuarine systems, freshwater end members tend toward phosphorous (P) limitation and marine end members tend toward nitrogen (N) limitation (e.g. Doering et al. 1995; Fisher et al. 1999). Most wastewater facilities discharge to fresh water. Therefore, decades of research and technological advances have been implemented to reduce P loads to receiving waters. Treatment to reduce P loads from wastewater treatment plants and the detergent ban in the mid-1980’s have been a major success story nationwide. However, these successes have not improved the quality of estuarine systems, such as the Chesapeake Bay, because success is limited to the proximate receiving waters. The Chesapeake Bay, other estuarine systems, and the marine environment in general are more often N limited (Boynton et al. 1995; Howarth et al. 1996; Kemp et al. 2005). Consequently, P reductions in wastewater have “moved the problem downstream.” This has been documented in a number of cases including the Neuse and Potomac Rivers (Paerl 1995) where P reductions were implemented without concomitant N reductions. In fact, reduced P inputs resulted in enhanced downstream N transport. Even in systems where discharges are to freshwater, material ultimately is transported downstream where it can enter the estuarine and marine environment where its reactivity changes and where N-limited organisms are adapted to using a broad spectrum of N compounds including organic N. Furthermore, unlike P, total N loads have increased since WWII as a result of increased use of N fertilizers (Howarth et al. 2002). In the Chesapeake Bay region, human activity has resulted in a 6 to 8-fold increase in N loading (Boynton et al. 1995), an increase that is typical of the region (Howarth et al. 1996).

In addition to the amount of N or P added to an estuary (e.g., loading), there are substantial differences in how N and P are cycled along the length of an estuary. Because freshwaters are often P-limited, P introduced at the head of an estuary may be rapidly removed by phytoplankton resulting in increased algal growth in the freshwater end members. In contrast, N delivered to freshwater systems is likely to move downstream until it reaches the N-limited estuarine portion of the watershed where it can result in excess algal production in more saline waters. An excellent example of this is the Neuse River estuary in NC; when P loadings were reduced during the mid-1980's, the chlorophyll maximum moved down-estuary from the P-limited freshwater end member to the more N-limited saline end member where nuisance phytoplankton blooms are now a regular feature (Paerl et al. 2004). As we alter nutrient loads to manage water quality, we need to determine the relative contribution of N versus P loading to water quality degradation in the upper versus lower estuary; we need a dual nutrient management strategy. In short, the spatial and temporal extent of downstream N limitation may be highly dependent on upstream nutrient management (Paerl et al. 2004).

In 2005, 370 million pounds of N were introduced into the Chesapeake Bay, more than twice the restoration target of 175 million pounds (Chesapeake Bay Program 2006). Although wastewater effluent from point sources represents only about 28% of the N load into the Bay (Kemp et al. 2005), effluents from wastewater treatment are the primary N load in many freshwater tributaries (e.g., Potomac, Rappahannock). Furthermore, controlling N at point sources (such as wastewater effluents) is logistically easier than controlling inputs from more diffuse sources, such as agriculture and atmospheric deposition. Accordingly, to ameliorate N pollution (and its effects) in the Bay, the Chesapeake Bay 2000 agreement mandated 48% reductions in N loads from point sources to the Bay and its tributaries (based on 1990 input levels). This agreement has resulted in increasingly stringent effluent discharge limits for wastewater utilities discharging into the Chesapeake Bay watershed; down to as low as 3 mg/L total N by January 1, 2011.

The capital cost to achieve this level of treatment by point sources discharging into Chesapeake Bay is estimated to be several billion dollars (Nutrient Reduction Technology Cost Task Force, 2002**).** Furthermore, the impact of implementing effluent guidelines down to 3 mg/L increases the cost of compliance substantially. The Nutrient Reduction Technology Cost Taskforce estimated that the capital cost to achieve effluent N levels of 5 mg/L at a 10 million gallon per day (MGD) plant that was not previously performing biological N removal was around $4.9 million. At the same plant, to implement limit of technology (LOT) treatment to achieve an effluent total N guideline of 3 mg/L would cost $9.6 million in capital costs. Operational costs also double for this scenario. Clearly, the economic impact of implementing LOT treatment levels is substantial. Consequently, the regulated community is unconvinced that reduction beyond that currently realized using conventional methods will provide substantial environmental benefits relative to the costs incurred given the uncertainty over whether all of the effluent N is bioavailable and therefore harmful to the Bay.

The regulated community has initiated an effort to determine, and discount from their total N loads, the fraction of total N in their effluent that is considered recalcitrant (Biological Nutrient Removal Boundary Conditions Workshop, Washington DC, March 2006; International Water Association/Water Environment Federation Nutrient Removal 2007, Baltimore, MD, March 2007). Much of the organic fraction of N in wastewater effluents has been considered to be recalcitrant. By extension, based on in-plant microbial processes, an argument has been made that this fraction is nonbiodegradable or bio-unavailable in the environment (Murthy et al. 2006). In concert with the perception that a fraction of EON may be inert, and therefore not harmful, dischargers are applying to regulatory agencies to amend their nutrient discharge allowances to exclude recalcitrant N. Indeed, a new Virginia regulation includes a provision that allows dischargers to argue for an increased discharge cap if they can demonstrate that nitrogen in their effluent is not bioavailable (9 VAC25-820). In order to safely apply this regulatory tool, it is necessary first to identify appropriate methods to assess the bioavailability of EON not just to treatment plant microbes but also within a watershed such as the Chesapeake Bay (STAC 2007) or any other N sensitive estuarine system around the world that contains a diverse microbial community. To be appropriate, any method that is developed must be applicable to not only the proximate receiving waters (typically freshwater), but also to the estuarine and marine systems downstream. Furthermore, it must be sensitive to changing environmental conditions along the length of the estuarine gradient. Finally, it must consider the impact of those changing conditions (salinity, changes in microbiota, generation of photodegradation products) on the overall bioavailability of EON.

At the same time, the ability of current LOT treatment plants to address the problem of bioavailable EON must be considered. Assays focused on assessing the fate of organic nitrogen in treatment processes over the time frame of the treatment technology used should be considered “technology-based assays” (Murthy, pers. comm.) while assays focused on assessing point source EON bioavailability in the receiving waters can be considered “water quality-based assays”. A technology-based biodegradability assay is needed to determine the effect of treatment process factors and wastewater influent characteristics that impact what is finally released from the plant as EON. The nature of this assay may be very different from what is needed for a water quality-based assay assessing impact in the environment. Furthermore, information generated by the two different assays should advise each other. For instance, if the water quality-based assay identifies a fraction of EON from a given treatment plant (and, therefore, a given treatment technology) that is bioavailable somewhere along the freshwater to saltwater continuum, this material should be characterized to determine what makes it bioavailable. Subsequent assessment of where that type of organic nitrogen might be degradable within a plant (through the technology-based assay) *or generated* within treatment plants gives design engineers and operators key information toward understanding how their plant is contributing to removing bioavailable EON, and a pathway to finding a workable and realistic solution within the confines of LOT capability.

Definitions and Acronyms

The organic nitrogen constituents of interest are shown below in Figure 1. The influent organic nitrogen (iON) equals the sum of the influent particulate organic nitrogen (iPON) and influent DON (IDON). The influent DON consists of biodegradable (biDON) and non-biodegradable or recalcitrant (riDON). The main organic nitrogen component of interest in the wastewater treatment (WWT) process is the dissolved organic nitrogen (DON), because most of the iPON will either be captured in solids removal processes or converted to DON. The DON in WWT processes is referred to as treatment process DON (tDON) and it consists of a biodegradable component (btDON) and a non-biodegradable component (rtDON). The organic nitrogen in the WWT process effluent is referred to as effluent organic nitrogen (EON) and this also consists of particulate (EPON) and dissolved organic nitrogen (EDON). The particulate portion is defined by the effluent filtration pore size, with 0.45 μm commonly used for this application. The organic nitrogen in the filtrate is defined as dissolved but it may also contain some colloidal organic nitrogen. Of interest for the EDON is what portion is available for microbial growth (i.e, bioavailable –denoted bEDON) and what portion is not available or recalcitrant (rEDON) in the environment. The difference between btDON and bEDON is that btDON should be related to bacterial activity in the WWT process while the bEDON is should be related to the activities of microbes (both bacteria and algae) in receiving waters. These acronyms and their relationships are summarized in Figure 1 and defined below.

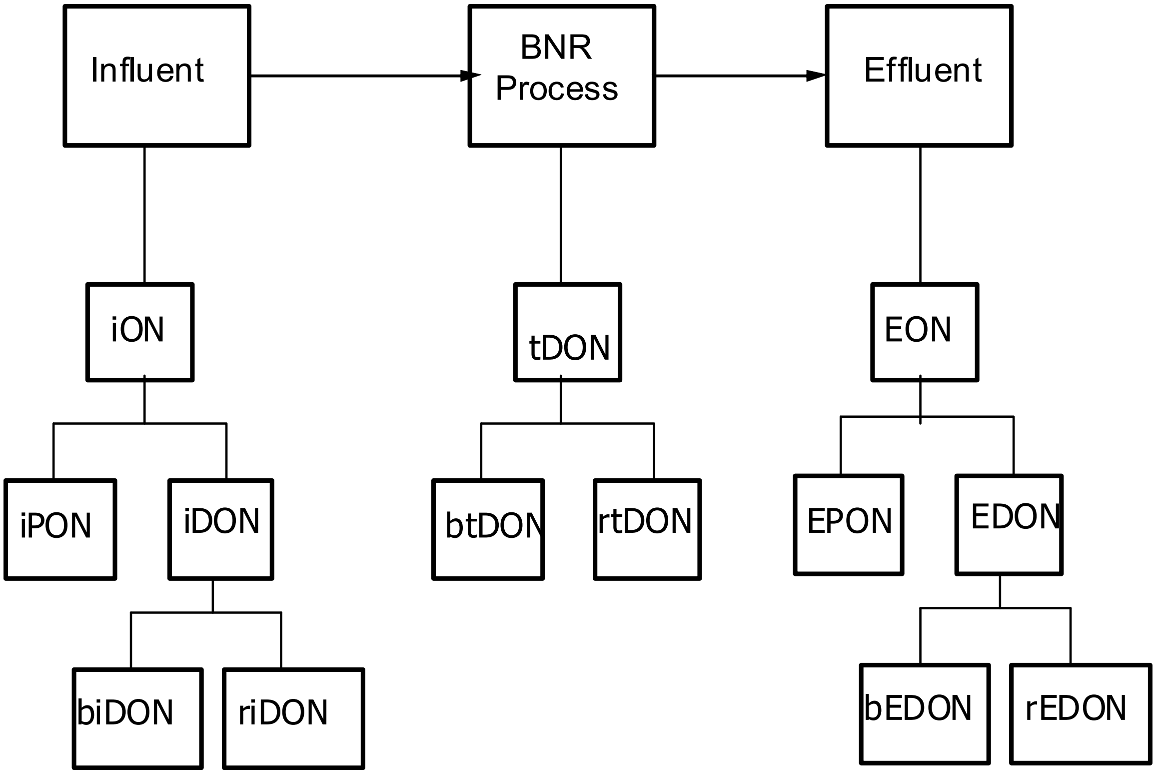


Figure 1. Organic nitrogen components of interest in WWT processes and receiving surface waters.

NH_3-N	Total ammonia-nitrogen: includes both free ammonia (NH_3) and ionized ammonium (NH_4^+)
NO_2-N	Nitrite-nitrogen
NO_3-N	Nitrate-nitrogen
TIN	Total inorganic nitrogen: sum of NO_2-N, NO_3-N, and NH_3-N.
TKN	Total Kjeldhal nitrogen: measures sum of organic nitrogen and NH_3-N
TN	Total nitrogen: Sum of inorganic and organic nitrogen as N
ON	Organic nitrogen; nitrogen contained in organic compounds (i.e. amino acids, peptides, and protein) and can be in dissolved form or contained in particulate material
DON	Dissolved organic nitrogen: organic nitrogen measured in the filtrate of a sample (influent, mixed liquor or effluent) following filtration
PON	Particulate organic nitrogen: organic nitrogen contained in wastewater solids or biomass.
iON	Influent organic nitrogen
iPON	Influent particulate organic nitrogen
iDON	Influent dissolved organic nitrogen
biDON	Biodegradable influent dissolved organic nitrogen
riDON	Non-biodegradable influent dissolved organic nitrogen
tDON	Dissolved organic nitrogen in the BNR treatment system
btDON	Biodegradable dissolved organic nitrogen in the BNR treatment system
rtDON	Non-biodegradable dissolved organic nitrogen in the BNR treatment system

EON	Effluent organic nitrogen: the sum of DON and PON in wastewater treatment plant effluent
EPON	Effluent particulate organic nitrogen
EDON	Effluent dissolved organic nitrogen
bEDON	Bioavailable EDON is effluent dissolved organic nitrogen that can be used in surface waters due to bacteria activity and algae uptake of nitrogen
rEDON	Recalcitrant EDON is effluent dissolved organic nitrogen that is resistant to biological transformation and uptake by microbes (algae and bacteria) in surface waters.
BNR	Biological nutrient removal: includes biological process designs for nitrogen and phosphorus removal.
SRT	Solids retention time: average time in days that solids are in the activated sludge system. It can be based on aerobic volume only or total volume.

II. FATE AND TRANSPORT OF ORGANIC N IN AQUATIC SYSTEMS

The largest pool of fixed nitrogen (N) in most aquatic systems is DON (Bronk 2002). This is true even in oligotrophic environments (i.e. nutrient poor) where primary production is limited by available N. The persistence of DON in areas believed to be N-limited led to the traditional view that DON is largely refractory and therefore unimportant to microbial N nutrition in the environment. It was also widely believed that what DON was used was taken up by bacteria over relatively long scales. More recent research, however, has shown that even highly refractory compounds can be a source of bioavailable N to plankton as well as a vehicle to transport N through estuarine systems. Recent findings with new approaches also indicate that DON fuels a significant amount of autotrophic production (Berman and Bronk 2003; Mulholland and Lomas 2008). There is a wealth of data in the limnology and oceanography literature that can inform the discussion of EON bioavailability. As a broad overview, here we review the composition of DON in marine and aquatic systems, what we have learned about its lability, and conclude with why the issue is so important.

Is organic N Labile in Natural Waters?

Based on research to date it is safe to say that at least some fraction of organic N in marine and aquatic systems is labile. Although most DON in aquatic systems is uncharacterized, some similarities between the components of the naturally occurring DON pool and organic N in effluent suggest that the same could be true for EDON. The important question then becomes – what percentage of EDON is labile (bEDON), or more importantly, refractory (rEDON)?

Organic N Composition in Natural Waters

In the ocean, the DON pool is generally treated like a “black box”, the composition of which is unknown but is expected to change over small space and time scales. One approach that has been used to characterize DON is size fractionation (e.g. Benner et al. 1992, Aluwihare et al. 1997, McCarthy et al. 1996, reviewed in Benner, 2002). Using an ultrafilter with a 1000 Dalton cutoff a number of researchers have collected sufficient high molecular weight (HMW) material for analysis. These investigations show that amide-linked N comprises the largest fraction of HMW DON (92%) with the remaining 8% consisting of amines (Aluwihare et al. 2005). In some estuarine and coastal systems, however, humics can contribute a significant fraction of measured DON (e.g. Alberts and Takács 1999). For example, in the Savannah and Altamaha estuaries of coastal Georgia humics contributed an average of 70% of the DON pool over a three-year period (Bronk et al., unpublished data).

Another approach to characterizing DON is based on lability. In this sense, the largest fraction within the DON box likely includes the truly refractory components that persist in the environment for months to hundreds of years (reviewed in Bronk 2002 and Bronk et al. 2007). Using terminology from the dissolved organic carbon (DOC) literature, a second fraction of the pool can be described as semi-labile (Carlson and Ducklow 1995). This fraction likely includes compounds such as proteins, dissolved combined amino acids (DCAA), and amino polysaccharides, which turnover on annual time scales. Mixed in with the refractory background, however, is highly labile DON; highly labile moieties including urea, dissolved free amino acids (DFAA), nucleic acids (reviewed in Bronk 2002), and peptides (Mulholland and Lee, in press). These labile compounds turnover on timescales of minutes to hours for amino acids (Fuhrman 1987) and peptides (Mulholland and Lee, in press), to days for urea (Bronk et al. 1998) and DNA (Jørgensen et al. 1993).

The bulk of research on DON availability has focused on the labile fraction. Recent work, however, has also shown that even HMW compounds such as humic substances, considered to be highly refractory, can be a source of N (i.e. See et al. 2006). Humics are operationally defined as DOM that adheres to a macroporous resin (i.e. XAD-8 or DAX-8; Peuravuori et al. 2002) at a pH of 2 (Aiken 1988). They can be further categorized into: 1) fulvic acids, which tend to be smaller (500-2000 Daltons) and are soluble in water at any pH, 2) humic acids, which are larger (2000-5000 Daltons or larger) and precipitate from solution at pH lower than 2 (Thurman et al. 1982), and 3) humins, which are insoluble at any pH.

Natural humic substances, isolated by XAD extraction, have been shown to contain 0.5 to 6% N (Rashid, 1985; Thurman, 1985; Hedges and Hare, 1987). Amino acids, amino sugars, ammonium (NH_4^+), and nucleic acid bases comprise 46 to 53% of the N associated with humic acids and 45 to 59% of fulvic acids (Schnitzer, 1985) with the remaining approximately 50% of humic-N unidentified (Carlsson and Granéli, 1993). Previous work indicates that the C to N (C:N) ratio of aquatic humic substances, isolated with XAD resin, ranges from 18 to 30:1 for humic acids and 45 to 55:1 for fulvic acids, but can vary considerably (Thurman, 1985; See, 2003; See and Bronk, 2005). The C:N of humic substances isolated with macroporous resins, however, may not reflect the C:N ratios of humic substances *in situ*. During the isolation procedure humic substances are acidified to a pH of 2, thus bombarding the solution with free protons. These free protons can bump off loosely associated amino groups such that humics isolated using resins have a C:N ratio higher than humics in natural waters (See and Bronk, 2005).

Bioavailability of Organic N in Natural Waters

The unknown composition of the bulk aquatic DON pool makes determining its bioavailability difficult. Bulk DON uptake by microorganisms has been examined using a bioassay approach (Berg et al. 2003; Stepanauskas et al. 1999a, b; Wiegner et al. 2006) as well as by synthesizing ^{15}N-labeled DON (Bronk and Glibert 1993, Bronk et al. 2004). Isotopic tracers are currently available for only a small fraction of the pool. As a result, bioassay approaches have been used to monitor the decrease in DON concentrations over time. One difficulty with the bioassay approach is it requires the ability to measure relatively small concentration changes in a large pool. Bioassays only measure net flux within a pool, such that even large DON uptake rates could be immeasurable if rates of DON regeneration or production are also high. Despite these drawbacks, a number of studies have used dark bioassays in aquatic systems to measure heterotrophic bacterial utilization of DON. In general, this work suggests that 12 to 72% of the DON pool is bioavailable on the order of days to weeks (reviewed in Bronk 2002). However, it should be noted that phytoplankton can also take up DON during the dark (see Mulholland and Lomas 2008)

In another study, water samples were collected from rivers and estuaries differentially impacted by anthropogenic modification (Wiegner et al. 2006). Dark bioassays were performed with a single bacterial inoculum to compare DON and DOC lability across a range of systems that varied in their amount of forest cover. As much as 40% of the DON was consumed over a 6 day incubation and up to 80% of the total N utilized by the inoculum was organic in form. These results show that classifying all DON as refractory underestimates the bioavailability of this pool in the marine environment.

The refractory nature of humic substances has also recently been challenged, and accumulating evidence indicates that coastal phytoplankton may have the ability to take up humic-N, either directly or after remineralization (Carlsson et al. 1995, 1999). More recently, the uptake of laboratory-produced ^{15}N-labeled humic compounds by the > 0.7 μm size fraction has been observed in both riverine and coastal ecosystems (Bronk et al., unpubl. data), humic substances have been implicated as a potential source of C and N to the toxic dinoflagellate *Alexandrium catenella* (Doblin et al. 2000), and growth of another toxic dinoflagellate *Alexandrium tamarense* was shown to increase when exposed to humic substances (Gagnon et al. 2005). Uptake of humic-N into phytoplankton biomass was also measured directly using ^{15}N-labeled humic substances produced in the laboratory (See and Bronk 2005). In this study, non-axenic cultures of 17 recently isolated estuarine and coastal phytoplankton strains took up ^{15}N-labeled humic-N (See et al. 2006), however, high rates of humic-N uptake were not sustained over long periods of time, suggesting that only a finite pool of labile N is associated with these compounds (See et al. 2006). No uptake of ^{15}N-labeled humic-N was detected in two axenic cultures suggesting that in at least these two cultures, bacterial remineralization was required to make the humic-N bioavailable.

Factors that impact the fate of organic N

DON bioavailability in estuarine and marine systems has received a lot of attention recently; see reviews in Antia et al. (1991), Bronk (2002), Bronk and Flynn (2006), and Bronk et al. (2007). In contrast, our knowledge of DON bioavailability in freshwaters is still in its infancy (deBruyn and Rasmussen 2002, Pellerin et al. 2006), largely because freshwaters are generally P limited. Overall, the lability of natural dissolved organic matter (DOM) appears to vary across aquatic ecosystems with higher lability in lakes and marine systems and lower lability in river systems (del Giorgio and Davis 2003). Another recent study found that anthropogenically-derived DON was more bioavailable than forest-derived DON (Seitzinger et al. 2002).

Salinity. Changes in salinity are known to alter the bioavailability of DOM and to affect photochemical reactions (McCallister et al. 2005, See 2003, See and Bronk 2005, Minor et al. 2006). In addition, the microbial community (bacteria and phytoplankton) changes along the estuarine gradient (Crump et al. 2004, Marshall et al. 2005), which will affect nutrient processing and ecosystem functions (see below). Salinity can also result in conformational changes that can influence both the abiotic and biotic reactivity of DOM, including humic substances (Baalousha et al. 2006). Salinity effects are important to consider when discussing EON bioavailability because the salinity increases along the length of the estuarine transit of a waste stream, and salt influences the behavior, conformation, and reactivity of DOM as it moves through an estuary (Baalousha et al. 2006).

Salinity may also affect the transport of N associated with organic compounds. Recent studies show that humic substances are capable of adsorbing NH_4^+ from surrounding waters to cation binding sites located on the humic structure (See and Bronk 2005). The adsorption of NH_4^+ to humic substances makes them a potentially important shuttle for transporting N that is produced upriver to the estuary and coastal ocean. As the humic materials move downriver, encountering higher salinities, the salt ions can displace the loosely bound amino groups on the humic structure, releasing them into the environment. In laboratory experiments with humics isolated from three different rivers, concentrations of free NH_4^+ increased in solutions with humics when the salinity of the surrounding water increased; the release of NH_4^+ was rapid and reproducible (See 2003, See and Bronk 2005). The question is whether EON, which contains humic substances, operates in a fashion analogous to the humic shuttle. If NH_4^+ binds to EON within the treatment plant, it may not be removed by the coupled nitrification/ denitrification process. Similarly, when reduced forms of N are released from the plant as EON, ammonified or loosely associated amino groups may dissociate from the EON as it is transported into water with higher salinities; in effect, resulting in an EON shuttle.

Light. Recent findings in freshwater and marine systems indicate that photochemical processes can effect the release of labile nitrogen (N) moieties from DOM (reviewed in Bronk 2002). Bushaw et al. (1996) demonstrate that DON from a freshwater pond is a source of labile N for microbial processes, but only after the DON is irradiated with sunlight and that wavelengths in the ultraviolet (UV) region (280 - 400 nm) produce these compounds from DOM sources most efficiently. This photochemical reactivity can alter the bioavailability of DON. However, photochemical reactions can affect the lability of organic material along estuarine gradients (Bushaw et al. 1996, Minor et al. 2006) and readily convert refractory DON to labile forms. A recent paper shows that biologically recalcitrant DOM can be converted into bioavailable forms via photochemical reactions and subsequently stimulate N-limited microbial food webs (Vähätalo and Järvinen 2007). Additionally, previous work has shown that NO_2^- and NH_4^+ can be released from DON photochemically (e.g. Kieber et al. 1999, Koopmans and Bronk 2002). This release may explain why bacterial growth efficiency, bacterial nutrient demand, and bacterial biomass and respiration rates are influenced by light (McCallister et al. 2005). Previous studies of EON bioavailability confined their work to dark reactions using technology-based assays (Murthy et al. 2006).

Plankton community composition. The microbial community present in a given environment will also likely impact what organic compounds are bioavailable. Various bacteria and phytoplankton species have different transport and enzyme systems that allow them to take up a range of N substrates (see Berges and Mulholland 2008, Mulholland and Lomas 2008). The composition of DOM is known to be affected by bacterial growth and bacteria alter the composition of the DOM (e.g. Hopkinson et al. 1998). In the case of phytoplankton, we now know that algal uptake of components of the DON pool, such as dissolved free amino acids (DFAA), can be significant in aquatic environments (e.g. Bronk and Glibert 1993; Mulholland et al. 2002, 2003; Berman and Bronk 2003; Bronk et al. 2007). In addition, a variety of other identifiable DON forms can be used as N sources by algae including dipeptides (Mulholland and Lee, in press), urea (Bronk et al. 1998, Lomas et al. 2002), dissolved combined AA (DCAA)

(Jørgensen and Jensen 1997), peptidoglycan (Jørgensen et al. 2003), and cyanate (Palenik et al. 2003). Further, humic-bound N, which is also found in effluent, can be used by phytoplankton as an N source (See et al. 2006) and bacterial reactions can degrade other DON compounds making them available for uptake by algae (e.g. Berg and Jørgensen 2006). In addition to direct uptake of specific DON compounds, there are a variety of extracellular enzymatic systems used by microbes (including algae) to convert HMW DON into LMW labile organic forms (e.g. Palenik and Morel 1990; Pantoja and Lee 1994, 1999; Pantoja et al. 1997; Mulholland et al. 1998, 2002, 2003; Berg et al. 2002; Stoecker and Gustafson 2003; Mulholland and Lee, in press).

Importance of Determining the Lability of Organic N and its Ultimate Fate

In a review of DON in rivers, Seitzinger and Sanders (1997) estimate that 14 to 90% of the total N in a suite of rivers around the world is organic. This DON represents a large source of N to the coastal zone that is currently ignored in some N loading budgets. This is especially troubling when one considers that effluent from even the most efficient wastewater treatment plants contain approximately 285 μM N with roughly two thirds of the discharged N being organic in form (Pehlivanoglu and Sedlak 2006). Some individuals argue that EON should not be included in N discharge budgets based on the traditional view that DON is not bioavailable and therefore will not contribute to eutrophication. The brief review of recent studies above suggests that this traditional view is incorrect. Collectively, data from bioassays and tracer approaches suggest that bioavailable DON can be utilized within estuaries with water residence times on the order of weeks to months. In systems where residence times are shorter, riverine DON will pass through the estuary and be a source of bioavailable N to coastal waters. Results from studies with individual organic compounds indicate that some fractions of DON have much quicker turnover times and consequently contribute to plankton nutrition even in systems with very short residence times. It is becoming increasingly evident that a significant fraction of DON is bioavailable and contributes to coastal eutrophication and, as such, should be included in N loading budgets. The challenge will be to determine what fraction is biologically available.

Although research on DIN and DON uptake by phytoplankton and bacteria has been fairly extensive, relatively little is known about how these two groups compete for limiting N resources and the time scales of the competition (see Mulholland and Lomas 2008). This is an important issue because it will ultimately determine the ecological effects of releasing the material into the environment. In estuarine and coastal ecosystems, the relative use of organic N (or EON) by autotrophs versus heterotrophs will potentially affect plankton community composition, energy transfer to higher trophic levels, and benthic-pelagic coupling. If DON (or EON) is primarily used by phytoplankton it is more likely to make it into higher trophic levels, including, for example, commercially important fish. Phytoplankton also generate oxygen during growth and sequester CO_2^-, an important consideration when discussing global change issues. If its ultimate fate is bacterial uptake than the N and C is less likely to make it into higher trophic levels. Bacteria release CO_2^- and take up oxygen, thus potentially generating or exacerbating the environmental problem of hypoxia or anoxia. Finally if the organic compounds are not used by phytoplankton or bacteria in a time period less than the residence time of the water in a given area that the ultimate fate is advection – either down river, down estuary, or out to sea. Clearly, the type of N entering coastal and estuarine waters can play a significant role in altering plankton community structure, but may also affect broader scale processes determining overall ecosystem health.

III. EON Components in Wastewater Treatment Processes

Nitrogen Components in Wastewater Treatment Plant Effluents

The wastewater treatment plants where EON exists as a significant fraction of the total effluent nitrogen are biological nitrogen removal (BNR) facilities. Table 2 shows the effluent nitrogen constituents that contribute to the effluent TN concentration from a BNR treatment process, and the BNR process mechanism and factors that affect the respective effluent concentration. Note that key process design parameters that affect the ability to achieve minimal effluent TN concentrations (LOT performance) from BNR systems are longer solids retention times (SRTs), carbon addition for NO_3-N and NO_2-N removal, and enhanced effluent solids removal by membrane separation or filtration. Other factors may be the impact of variable loadings due to seasonal or wet weather conditions and the impact of in-plant recycle streams such as nitrogen-rich centrate return.

Table 2. BNR effluent nitrogen constituents and process removal mechanisms.

Nitrogen constituent	Process removal mechanisms	Known factors affecting ability to reach minimum concentrations
NH_3-N	Nitrification	Temperature, pH, dissolved oxygen, SRT
NO_2-N	Oxidation to NH_3-N Denitrification	Temperature, pH, dissolved oxygen, SRT Temperature, SRT, carbon source, anoxic detention time
NO_3-N	Denitrification	Temperature, carbon source, anoxic detention time
EDON	Hydrolysis and ammonification	Temperature, SRT
EPON	Clarification, filtration or membrane separation	Liquid-solids separation process design

Filter pore size is used to define EDON, iDON and tDON

The DON concentration measured for influent, treatment process or effluent samples will depend on the filter pore size used to separate particulate and colloidal solids from a sample. The common filter size for “dissolved constituents” is 0.45 µm and has been used to define EDON in many studies. In bioassays aimed at determining the biodegradable DON by bacteria in wastewater treatment processes (btDON) (Khan 2007) and on the bioavailable EDON for freshwater algae consumption (Pehlivanoglu and Sedlak 2004), a 0.20-0.22 µm filter size has been used. A 0.45 µm filter size has also been used to quantify EDON. An unquantified fraction of the total colloidal organic nitrogen passes through 0.45 µm filters and possibly through a 0.20 µm filter and ends up as part of the EDON. The only way to separate this from the truly dissolved fraction is with ultrafiltration, and to date those studies have not been done.

The data in Table 3 were presented by Pagilla (2007) and show the effect of filtration pore size on the organic nitrogen concentration for effluents from a number of wastewater treatment facilities. For some plants the effluent colloidal organic nitrogen contained in the so-called DON fraction can be significant. There is also the possibility of colloidal organic nitrogen in filtrate from 0.10 µm filtration.

Table 3. EDON measurements (mg/L) as a function of filter pore size (Pagilla 2007)

	Filter pore size		
WWTP	**1.2 m**	**0.45 m**	**0.10 m**
Stickney	2.9	1.7	1.6
Hinsdale	4.2	3.6	3.6
Elmhurst	2.1	2.0	2.0
Gdynia	3.4	2.4	1.5
Gdansk	1.9	1.3	0.4
Elblag	5.0	2.7	2.0
Slupsk	1.6	1.6	1.0

What fraction of the effluent TN is EDON?

Figure 2 illustrates effluent TN concentrations possible from a BNR LOT system and the relative contributions of the nitrogen constituents. In this case the EDON concentration is assumed to be 1.0 mg/L. The effluent TN concentration may range from 2.0 to 4.0 mg/L, depending on the ability to minimize the NO_3-N and NH_3-N concentrations and maximize effluent suspended solids removal. For BNR LOT processes filtration or membrane separation would be used, so the EPON contribution would be negligible or minimal. No single minimum TN concentration value can be projected for all facilities as the effluent value is affected by influent flow and strength variations, equipment malfunctions, recycle streams, process design, and plant operations.

The figure shows that the EDON concentration can account for 25 to 50% of the effluent TN concentration and thus is very significant for systems needing to reach minimum TN concentrations. For applications with an effluent TN concentration goal of less than 10 mg/L (typical value for water reuse applications), the EDON concentration is not as great of a concern.

Typical EDON concentrations in BNR processes

Table 4 summarizes EDON values from various BNR facilities and shows EDON concentrations ranging from 0.10 to 2.80 mg/L. Figure 3 shows a composite summary of the data. The 50 and 90 percentile values are 1.2 and 2.1 mg/L, respectively. There is a wide range of observed EDON concentrations observed from BNR processes, and it appears that in some cases the EDON can be at a high enough concentration to make it impossible to meet an effluent TN concentration goal of 3.0 mg/L. Furthermore Pagilla (2007) (Table 4) indicated that about 65% of 188 facilities in Maryland and Virginia had EDON concentrations at 1.0 mg/L or less. The reasons for the higher EDON concentrations are not known at this time.

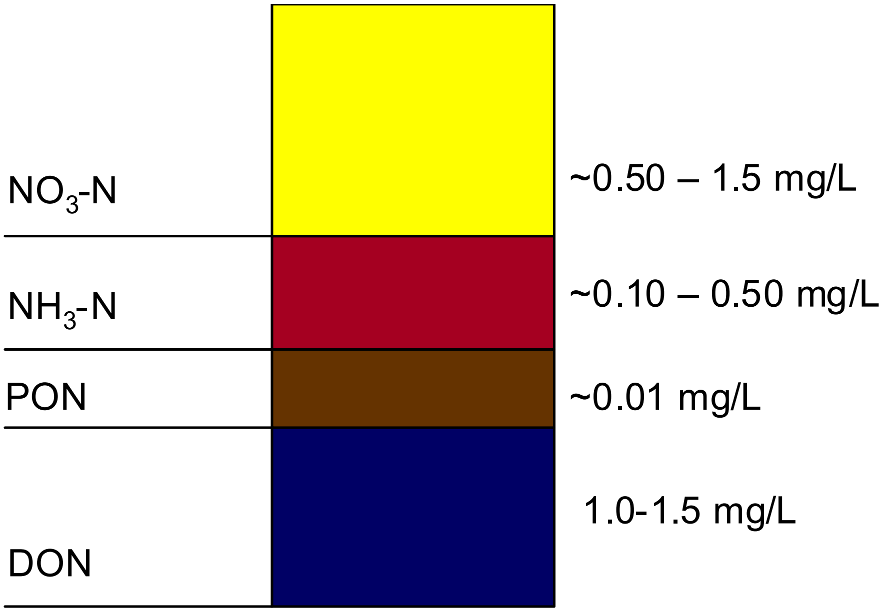


Figure 2. BNR effluent TN concentration possible and amount from nitrogen constituents

What is the composition of EDON?

Sedlak and Pehlivanoglu (2007) evaluated the molecular weight distribution of EDON and hypothesized that the HMW fraction (MW greater than 1 kDa) was not biologically available. The composition of this fraction has not been determined but is expected to be made up of larger molecular weight humic substances. Of the lower molecular weight compounds that may be bioavailable, only about a third have been identified as free and combined amino acids and ethylenediaminetetraacetic acid (EDTA). Other N-containing compounds in BNR effluents may include N-containing pesticides, pharmaceuticals, and other trace organics.

What are possible sources of DON in BNR facility influent or in the treatment process?

DON originates in domestic wastewater influent as urea (60-80% of domestic influent TKN), amino acids, proteins, aliphatic N compounds and synthetic compounds, such as EDTA. DON may also be produced and released or altered during biological wastewater treatment processes, including sludge digestion, due to cell metabolism processes that excrete biomolecules, cell decay and cell lysis. Humic organic substances may be present in some drinking water supplies and therefore contribute to the wastewater DON. Little is known about industrial wastewater compounds that may contribute to DON in combined municipal-industrial wastewater treatment plants. Thus, EDON may consist of influent recalcitrant DON, DON produced through or altered by microbial activity in the BNR process, and biodegradable DON that remains in the effluent.

Table 4. Summary of effluent dissolved organic nitrogen values reported.

Plant Location	EDON mg/L	Percentile %	Reference
Gordonsville, VA	2.80	97	Pagilla (2007)
Daytona Beach, Fl, Bethune	2.46	94	Jimenez et al. (2007a)
Back River WWTP	2.24	91	Parkin and McCarty (1981)
New Smyrna, Fl	2.10	88	Jimenez et al. (2007a)
Daytona Beach, Fl	2.00	85	Jimenez et al. (2007a)
City of Bradenton, Fl	2.00	82	Jimenez et al. (2007a)
JEA Black Fords, Fl	1.88	79	Jimenez et al. (2007a)
City of Palmetto, Fl	1.80	76	Jimenez et al. (2007a)
Stamford, CT	1.70	74	Sharp and Brown (2007)
Orange County, Fl, Eastern	1.55	71	Jimenez et al. (2007a)
Fort Meyers, Fl, Central	1.50	68	Jimenez et al. (2007a)
TMWRF, NV	1.50	65	Pagilla (2007)
Palo Alto, CA (2)	1.50	62	Randtke and Mccarty (1977)
Homestead, Fl	1.40	59	Jimenez et al. (2007a)
Lynn Haven, Fl	1.40	56	Jimenez et al. (2007a)
Bayou Marcus, Fl	1.37	53	Jimenez et al. (2007a)
City of Tarpon Springs, Fl	1.20	50	Jimenez et al. (2007a)
City of Clearwater, Fl	1.20	47	Jimenez et al. (2007a)
City of Largo, Fl	1.20	44	Jimenez et al. (2007a)
Chesapeake Beach, MD	1.20	41	Pagilla (2007)
Blue Plains, D.C.	1.20	38	Pagilla (2007)
City of Dunedin, Fl	1.18	35	Jimenez et al. (2007a)
Truckee Meadows, NV	1.00	32	Sedlak and Pehlivanoglu. (2007)
Titusville, Fl	0.95	29	Jimenez et al. (2007a)
Fort Meyers, Fl, south	0.94	26	Jimenez et al. (2007a)
Piscatway, MD	0.90	24	Pagilla (2007)
Palo Alto, CA	0.90	21	Randtke and McCarty (1977)
Orlando, Fl	0.88	18	Jimenez et al. (2007a)
Tampa, Florida	0.73	15	Jimenez et al. (2007b)
Alexandria, VA	0.70	12	O'Shaughnessy et al. (2006)
Boone WWTP, VA	0.69	9	Wikramanayake et al. (2007)
Fort Meyers, Fl	0.60	6	Jimenez et al. (2007a)
Upper Potomac R., MD	0.10	3	Pagilla (2007)

* DON in Jimenez et al. (2007a) reference estimated from effluent TN and TIN concentrations

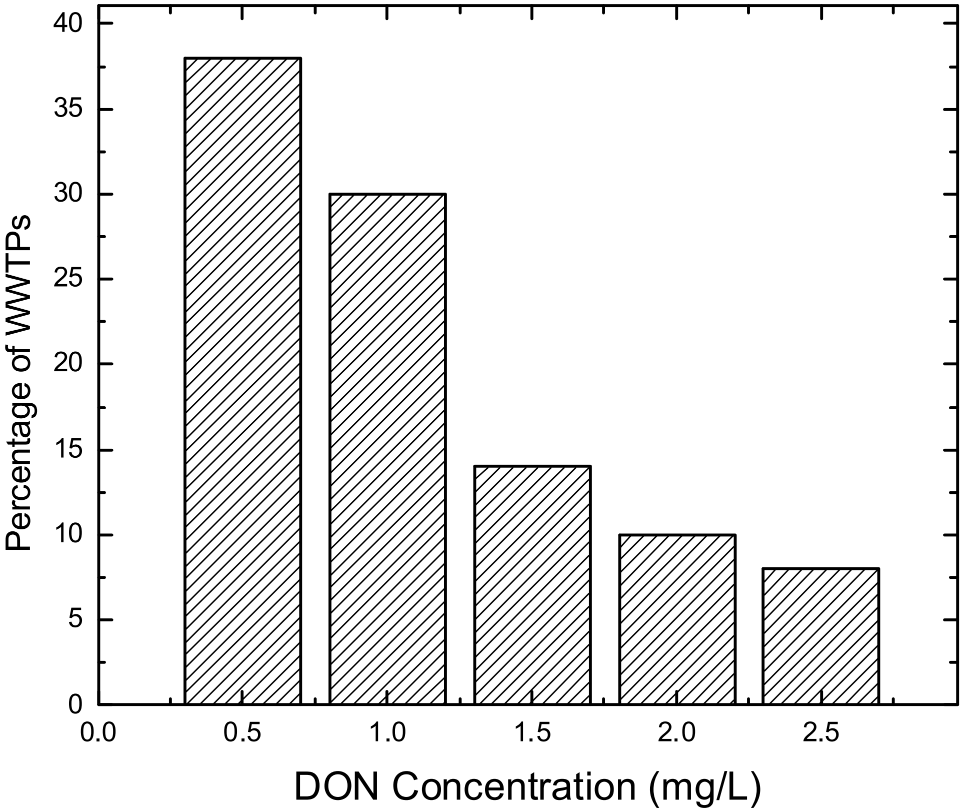


Figure 3. Summary of EDON concentration (0.45 m filtration) from 188 Maryland and Virginia wastewater treatment plants (Pagilla 2007).

IV FATE OF DON IN BIOLOGICAL WASTEWATER TREATMENT

In early work by Parkin and McCarty (1981), the composition and fate of DON at the Palo Alto, CA wastewater treatment plant was studied. The average EDON concentration was 1.5 mg/L. They claimed that 52% of it was recalcitrant from influent wastewater sources, 20% was produced from biomass endogenous decay in the activated sludge process, 15% was in equilibrium between that sorbed to biomass and the liquid and about 13% could be further degraded. However, they noted that while increasing the activated sludge SRT could further degrade influent DON, DON could also be added via biomass endogenous respiration thereby negating any positive effect. Based on the balance between consumption and production of DON, they claimed that the optimal operating point leading to a minimal EDON concentration after influent DON biodegradation and microbial DON release was at an SRT of 6-10 days. A number of important concepts regarding the fate of DON in wastewater treatment were revealed in this work: 1) some portion of the influent DON was not bioavailable, 2) increasing the system SRT could minimize the biodegradable DON concentration, and 3) increasing the SRT could increase the non-biodegradable DON concentration due to contributions from biomass endogenous decay.

Determining the fraction of influent DON that is biodegradable is a subject of current research. Work reported by Khan (2007) suggested that 40-60% of influent DON is biodegradable. This is in the range of that given by Parkin and McCarty (1981) above. The relative effectiveness of different biological treatment process technologies on degrading influent or biomass-derived organic nitrogen has not been studied.

V CONTROLLING AND MINIMIZING EDON FROM BNR FACILITIES

The design and operating conditions that can minimize EDON concentrations in BNR facilities is also a current research topic. One issue is whether the optimal SRT required to achieve minimal EDON concentration is compatible with the SRT needed to maximize inorganic nitrogen removal efficiency. The impact of DON in recycle streams from aerobic or anaerobic digestion and dewatering needs to be further evaluated.

Of further interest is identifying process technologies that can be used to achieve effective EDON removal from a BNR process effluent. Randtke and McCarty (1977) evaluated physical-chemical processes for EDON removal in the Palo Alto, CA effluent. The EDON concentration in bench scale tests with the Palo Alto facility effluent was 1.3 mg/L. For chemical treatment, the removal efficiencies were 33% with lime, 28% with 200-300 mg/L alum, and 40% with 200-300 mg/L ferric chloride. These are very high coagulant doses that are unlikely to be practical. Removal efficiencies were lower for cation and anion exchange (less than 13%). About 71% of the EDON was removed with activated carbon adsorption.

Generally, HMW EDON constituents are considered to be non-biodegradable or recalcitrant (rEDON). Other removal methods for rEDON constituents would be very expensive, requiring either chemical oxidation processes or reverse osmosis. The chemical oxidation processes would need to be followed by a biological treatment step to biodegrade the oxidation products.

VI FATE AND EFFECT OF EDON IN SURFACE WATERS

In general, the fate and effect of EDON in surface waters is not currently known. The potential impact of bEDON on surface waters was discussed in section II. Whether EON is more or less reactive than naturally-derived organic nitrogen is not yet known. Based on what we know about EDON, however, the following can be stated. Hydrolysis and deamination of EDON can produce inorganic and organic forms of N that can be taken up by estuarine microbes, including algae (see above). Further, many microbes can hydrolyze large compounds extracellularly prior to their uptake (Pehlivanoglu and Sedlak 2004, Mulholland and Lee in press, see also above). There is less known about the availability of nitrogen in HMW humic substances; however, in general, it is considered less bioavailable by some and has been termed recalcitrant EDON (rEDON), even though some environmental studies suggest that at least portions of this pool are bioavailable (see above).

Key to this debate is defining the fraction of EON that is recalcitrant. rEDON is that portion of effluent DON that is considered not available for algal or bacterial growth over time scales of days to weeks. During this timeframe, discharged EON may move through fresh water or both fresh water and more saline waters, depending upon the residence time in particular segments of an estuary. Salinity may play a key role in the bioavailability of at least a portion of the EON pool. At this time, it is not known if the specific type of humic compounds and possibly other HMW nitrogen compounds present in BNR effluents are bioavailable in saline environments.

VII IMPACT OF rEDON ON MEETING REGULATED EFFLUENT TN CONCENTRATIONS

Just as there is a wide range of EDON concentrations observed at BNR facilities (e.g. Table 3), it is not possible to generalize regarding the fraction of the EDON that is rEDON at all treatment plants. Using a freshwater bioassay procedure that included algae and bacteria, and effluents with low final TN concentrations, the fraction of EDON available for algae growth over a 14-day incubation period was 56% (Pehlivanoglu and Sedlak, 2004) and 18 to 61% (Urgun-Demirtas et al. 2007). Based on these observations, the potential fraction of rEDON in EDON from BNR facilities may be 40-80%. A similar analysis has not been done for treatment plants that discharge into watersheds that are significantly estuarine, which constitutes all of the treatment plants in the Chesapeake Bay region and many others located near coasts.

There is great interest in determining the effect of rEDON on the cost and ability to meet stringent effluent TN concentration permit values. Here, we provide a simple estimate of that cost considering typical values currently available from the research that has been done to date. For eutrophication-impaired surface waters, a common regulated effluent TN concentration value is 3.0 mg/L. Assuming that the EDON concentration is 1.0 mg/L, and that 50% is available for algae growth, the rEDON accounts for 0.50 mg/L of the effluent TN concentration. This is a significant concentration and affects the operational and design challenge for TIN removal. If the rEDON contribution is not included in the permit effluent TN concentration, the plant allowable effluent TIN concentration could be increased to 2.5 mg/L from the 2.0 mg/L concentration in this example; a reduction of 0.50 mg/L in the amount of NO_3-N that must be removed.

The impacts of removing 0.50 mg/L of NO_3-N are increased operating cost for carbon addition and increased carbon dioxide emissions that contribute to greenhouse gases. Therefore, if this nitrate did not need to be removed because 0.5 mg/L of the EDON is found to be recalcitrant, the annual savings can be estimated (see Table 5 for different plant sizes). The calculation assumes a methanol dose of 3.2 mg methanol per mg of NO_3-N removed and a methanol cost of $0.20/lb. For a 100 Mgal/d facility, the methanol cost savings is about $97,000 per year and for a 5 Mgal/d facility it is about $5,000 per year. If a nutrient trading program is in place, the value of selling the rEDON as a credit can increase significantly beyond the estimated values in Table 5.

Table 5. Annual reduction in operating cost if 0.50 mg/L NO_3-N is not removed from the effluent to compensate for an rEDON concentration of 0.50 mg/L for a plant with an effluent TN concentration goal of 3.0 mg/L.

Flowrate, Mgal/d	5	10	20	100
Annual Methanol Cost	$4,900	$9,700	$19,000	$97,000

VIII BIOASSAYS FOR MEASURING DON

At present, there is no consensus as to the appropriate way to determine bEDON or rEDON using bioassays. Two possible approaches are outlined below.

Goals of Different DON Bioassays

Bioassays are done to determine the biodegradability or bioavailability of DON. The recalcitrant DON in the wastewater influent (determined through technology-based assays) and in the rEDON (determined through water quality based assays) is of major interest from both a wastewater treatment perspective and a regulatory perspective. The type of bioassay employed depends on the ultimate goal of the test. For in-plant issues the test goals may include: 1) determining what portion of iDON is not subject to biotreatment or is recalcitrant (riDON), 2) what portion of the EDON from the treatment process may be biodegradable and thus removed with longer treatment time in the BNR process, and 3) what amount of recalcitrant DON may be in recycle streams to the treatment process. All of these goals involve the BNR treatment process and the biodegradability of DON by bacteria within the treatment plant. Therefore, the bioassay procedure should incorporate biomass from the BNR process being assessed. This approach is referred to as a "***technology-based bioassay***" because it assesses the biodegradability of DON during the treatment process **within** the plant (Awobamise et al., 2007).

On the other hand, to evaluate the impact of EDON in wastewater treatment effluents on the environment (the goal of the CBP and regulatory agencies), the bioassay goal is to determine the fraction of the EDON that is recalcitrant (rEDON) in receiving waters and thus will not contribute to eutrophication. In this case, the bioassay needs to account for the independent and combined effects of light, salinity, and microbial (bacteria and algae) community structure on the bioavailability of EDON in the environment. This bioassay is a "***water quality-based bioassay***." The recalcitrant fraction is determined by measuring the EDON that remains in a bioassay after exposure indiginous conditions experienced as effluent is transported from proximate to ultimate receiving waters. The time period of this bioassay has to be long enough and conditions appropriate to allow evaluation of bEDON as EDON transits through the system and experiences natural or simulated changes in the environment. However, bioassays cannot be so long as to allow steady state internal recycling of EDON within the bioassay to mask changes that might occur in the environment.

The Technology-Based DON Bioassay Protocol

Khan (2007) used a technology-based assessment protocol (Table 6) to determine if activated sludge biomass could further biodegrade EDON in wastewater plant effluent samples The outcome from this test can be used to determine if treatment plant biomass can further degrade the EON if given more time than was provided through the treatment process. The test is in its early stages of development and application, so that future modifications to the protocol are possible. The test is done with 300 mL BOD bottles and follows changes in dissolved oxygen (DO) concentration to thus also determine the BOD satisfied in the sample over time. The test also requires DON measurements at time intervals. The bEDON concentration is the difference between the initial EDON concentration and that at time t. Because the method is a technology-based bioassay that looks at the potential for BNR mixed liquor to further biodegrade EDON if the process retention time were to be extended, it is appropriate to conduct the assays in the dark because photosynthetic metabolisms do not routinely occur in activated sludge treatment. This bioassay may be used to evaluate the impact of various BNR process designs on minimizing bEDON, the contribution and impact of recycle flows, and the potential for increasing the system SRT to further reduce the bEDON concentration.

Table 6. Biodegradable (bEDON) bioassay protocol[a] (Awobamise et al. 2007) (300 mL BOD bottles)

Test Components	Procedure	Comments
Sample preparation	Use filtrate from 0.22 μm glass fiber filtration	Effluent filtrate or primary effluent?
	Saturate DO by aeration or shaking	
	Add 2 mL inoculum	Inoculum is mixed liquor from the same treatment plant at 240 mg/L
Seed control	Add 2 mL inoculum to distilled water	
Test bottle incubation	Unmixed and at 20^{0}C	In the dark
	5-20+days	For ultimate bEDON, the time is not yet known
	Check and adjust DO periodically	Time intervals may be 0, 5, 10, 20 days or more*
DON measurements	Measure DON at sample time intervals	Time intervals may be at 0, 5, 10, 20 days or more[b]

[a] Although this is listed as a bEDON method, unfiltered samples can be used to determine the bEON

[b]- Awobamise et al. (2007) found most bEDON to be gone by 20-30 days

A First-Generation Water Quality-Based DON Bioassay Protocol

A surface water quality-based assessment protocol under consideration is summarized in Table 7 below. It was first applied to measure bEDON by Pehlivanoglu and Sedlak (2004) and later by Urgun-Demirtas et al. (2007) for a number of BNR effluents. In both cases, more EDON was consumed when bacteria were present in the test with algae versus algae alone, indicating a synergistic relationship between algae and bacteria, consistent with Bronk's results regarding humic-N (see above). The test uses a freshwater alga, thereby limiting its application to BNR plants that discharge into exclusively freshwater watersheds. Modifications to the protocol are needed to determine the bEDON (DON lost) or rEDON (DON retained) for treatment plants located in watersheds that discharge into freshwater estuarine end-members or estuarine watersheds (Mulholland et al. 2007). The bEDON consumed by the algae is estimated by measuring the conversion of bEDON into plant (chlorophyll *a*) biomass relative to control incubations. The test protocol is in its early stages of development and application, and future modifications are possible.

The value of this water quality-based assessment method is not presently fully understood due to the use of a single, non-indigenous lab-cultivated freshwater alga and activated sludge biomass that may not be indicative of biomass found in surface waters. Furthermore, application of the method is limited to treatment plants contained entirely in freshwater watersheds. A benefit of this method is that it is relatively easy to standardize and implement. If results from this method are found to correlate in a predictable way with more complex bioassays that use indigenous microbiota, then it could be valuable as an indicator.

Important Factors for a Revised Surface Water Quality-Based DON Bioassay Protocol

Key parameters that appear to affect the bioavailability of EDON by microbes include the salinity and pH of the water receiving EDON. It appears that nitrogen-containing humic substances are more bioavailable in saline water versus fresh water. The sorption of ammonium on humic material is also affected by salinity and ammonium is likely to desorb in higher salinity waters (see above). Further, organic material undergoes conformational changes as a result of exposure to saline waters (Canuel, pers. comm., see above). In addition to physical and chemical interactions of nitrogen species due to water chemistry, it is known that populations of bacteria and algae species present in aquatic systems have particular salinity tolerances. These variations in population dynamics across a receiving stream watershed are not captured in the previously mentioned protocols that employ organisms that are oligohaline or have a limited range of environmental tolerances that do not span the entire estuarine continuum. Therefore, the ideal water quality-based assessment protocol should consider the receiving water physical characteristics and microbial diversity. This complicates the development of a simple protocol as few organisms span the entire estuarine continuum.

Another factor not addressed in the protocols presented above is whether the microbes responsible for the uptake or conversion of EDON to nitrogen forms that may be bioavailable for algae require additional carbon sources or other nutrient elements (e.g. P, trace metals, or vitamins) to maintain their activity during the incubation periods used in the assays. Evidence from previous studies on natural (not effluent) DON bioavailability in surface waters suggests that long assay times may not be necessary and, in fact, may be detrimental to effective interpretation of results. Del Giorgio and Davis (2003) concluded that the only portion of any bioassay that can be compared to *in situ* metabolic rates is the initial stage, when the pool of labile ON and the physiological state of organisms stills reflect *in situ* conditions. Additionally, bacteria can modify DOM, making it resistant to further degradation (Ogawa *et al.* 2001; Keil and Kirchman 1991). The net effect of long bioassays is simply to cycle N among dissolved and particulate pools in a closed system where there is tight coupling of N reactions. Thus, long incubation times under closed-bottle conditions likely reflect steady state N recycling rather than true bioavailability of the initial starting material. Appropriate incubation times that allow EON bioavailability or recalcitrance to be assessed in bioassays needs to be determined and is likely to be system-specific.

Table 7. A water quality-based assessment protocol for determining rEDON using 500-mL sample flasks (Pehlivanoglu and Sedlak 2004)

Test Components	Procedure	Comments
Sample preparation	1. Chlorinated effluent samples dechlorinated with sulfur dioxide 2. Use filtrate from 0.20 µm glass fiber filtration and fractionate with ultrafilters down to 1 kDa MW. 3. Distilled water and EDON samples spiked with 1 mg/L NO_3-N were run in parallel	
Bacteria inocula	1. Filter 3L of surface water first with 1 µm glass fiber filter 2. Filter 1 µm filtrate through 0.20 µm membrane filter 3. Suspend retentate of 0.20 µm membrane filter in 100 mL of 0.20 µm filtered surface water 4. Add 1 mL of bacteria suspension to 400 mL sample	Biomass is obtained from surface water samples
Algae inocula	1. A lab-cultivated freshwater algal species, *Selanastrum Capricornutum*, was used 2. Algae cultured per freshwater algae toxicity test protocol (APHA, 1998), amended with nutrients except nitrate. K_2HPO_4 added to media to give N/P molar ratio of 3.0. 3. 5 mL of algal suspension at logarithmic growth phase added to 400 mL sample	
Test flask incubation	1. In shaker at 20-22^{0}C 2. 12 hr light/dark cycle	
Algal growth	Monitor with vivo chlorophyll- α measurements using fluorometer until stationary growth phase reached	Stationary growth was found in about 14 days
DON measurements	Measure DON at sample time intervals	

The dissolved inorganic nitrogen (DIN) content of the sample may also affect the accuracy of bioassay protocols that involve use of algae and rely upon measuring chlorophyll *a* (plant biomass) production. High ratios of effluent DIN (EDIN) to EDON will result in very high levels of chlorophyll *a* production from DIN relative to DON. It can be difficult to accurately quantify the amount of plant biomass due to EDON versus DIN when there is a high background concentration of DIN. Similarly, assessing changes in bacterial biomass suffer the same limitations as they can also take up DIN and DON to support growth. To overcome this, DIN must be removed from or reduced in samples while retaining the DON, which is not a trivial feat.

IX THE REGULATING COMMUNITY

Rich Batiuk, Associate Director for Science, Chesapeake Bay Program Office, U.S. Environmental Protection Agency Region 3, represented the EPA's Chesapeake Bay Program at the workshop. He pointed out that people are proportional to nutrients and that the populations of the Chesapeake Bay and most coastal watersheds are growing rapidly around the world and putting pressure on existing infrastructure and nutrient removal technologies. Nutrient discharge reduction goals in the Chesapeake Bay are based on 1990 levels; however, the population in the watershed has grown substantially and wastewater treatment not only needs to accommodate the initial reductions but also the growth in population pressure that has increased the treatable wastestream. This challenge has been addressed by improving technologies for DIN removal and BNR processes. However, we are at a tipping point because as populations increase, loads increase but allocations decrease. Thus, even current limit of technology (LOT) plants are being challenged.

Mr. Batiuk detailed the approach taken by the Chesapeake Bay 2000 Agreement in setting goals and allocating loads on a watershed specific basis. He pointed out that in 9 VAC 25-820-10 General VA NPDES Watershed Regulation for Total Nitrogen and Phosphorus Discharges and Nutrient Trading in the Chesapeake Bay Watershed in Virginia Effective November 1, 2006, it is stated that, "unless otherwise noted, entire nitrogen and phosphorus waste load allocations assigned to the permitted facilities are considered to be bioavailable to organisms in the receiving stream. On a case-by-case basis, a discharger may demonstrate to the satisfaction of the board that a portion of the nutrient load is not bioavailable; this demonstration shall not be based on the ability of the nutrient to resist degradation at the wastewater treatment plant, but instead, on the ability of the nutrient to resist degradation within a natural environment for the amount of time that it is expected to remain in the Bay watershed. This demonstration shall also be consistent with the assumptions and methods used to derive the allocations through the Chesapeake Bay models. In these cases, the board may limit the permitted discharge to the bioavailable portion of the assigned waste load allocation." Thus, for regulatory purposes, the main driver is an assessment of the bioavailability of N in the environment. Until an appropriate assay is developed, all N must be assumed to be bioavailable and therefore counted as part of the permitted discharge. The state regulatory representative from Virginia also pointed out that some standardization of such an assay would be desirable. He further pointed out that the regulatory goals laid out in the Chesapeake Bay Agreement would require an assay to demonstrate bioavailability in the environment, a water quality-based assay. Technology-based assays are useful if the goal is to change the waste load allocation and the plant is already doing the best that it can.

The regulated community has used LOT technologies to achieve significant N removal. However, as population grows, total volume/mass of treatable N grows and increasingly, final effluents are dominated by organic N, which is difficult to treat using current LOT. Because this N is deemed untreatable based on current LOT processes and unreactive to treatment plant microbes, it has been called recalcitrant. However, the microbial community in a treatment plant is highly selected to promote certain metabolic processes. In nature, the microbial community (including algae) is completely different from that in a treatment plant. So, what is deemed recalcitrant in a plant setting may be entirely bioavailable in the natural environment. Further, after discharge, EON is transported downstream where it may encounter salinity (e.g, the Chesapeake Bay system) where it becomes bioavailable (see above).

Concerns in the regulating community are aimed at failures to achieve water quality goals even after 20 years of targeted efforts. Because of these failures, more stringent regulations will come into effect and a more careful evaluation of nutrient inputs and their bioavailability must be undertaken in order to determine why previous targets for nutrient reduction have failed to improve water quality. The role of the regulatory community is to advise research and the regulated community so that treatment plant technologies are developed that will result in the desired outcome, improvements in water quality in the environment. The needs of the regulatory community are in-plant technologies that remove effluent constituents that negatively affect the proximate and ultimate receiving waters.

There appears to be a major disconnect between the regulated and regulating communities. This may be due to conflicting definitions rather than conflicting goals. The regulated community defines nitrogen pools and bioavailability within the treatment plant and in association with treatment plant microorganisms, while the regulating community defines nitrogen pools and bioavailability in the environment where different biotic and abiotic factors come into play. This is important because the regulatory driver relies on monitoring of receiving waters, and is concerned with biological endpoints measured in the environment. In contrasts, dischargers trying maximize in-plant biological N removal. Conflicting definitions of bioavailability and just what is inert and where, has resulted from studies employing technology-based assays of the ecosystem living inside the plant, versus studies based on the water quality-based assays trying to determine impacts to the environment.

In order to better develop reasonable but effective nutrient removal strategies, environmental biogeochemists specializing in organic nitrogen cycling need to team with process engineers to: 1) develop an effective assay for determining environmental bioavailability of EON that can advise regulators, 2) identify components of the EON pool that are immediately or become bioavailable in the environment, and 3) develop processes that can remove these components from treated effluents. Because the composition of EON varies between wastestreams and the different types of processes they undergo during treatment, we know little about the reactivity, bioavailability and fate of organic nitrogen in the environment. This needs to be the first task so that the resulting information can feed back to advise in-plant removal processes.

X RESEARCH NEEDS

As regulations require further nutrient reductions from dischargers to protect impaired natural waters from eutrophication, the relative importance of EDON in final effluents has increased and represents a new challenge in the area of biological nutrient removal. Initial efforts to measure EDON, its bioavailability to aquatic microbes (including bacteria and algae), and bioavailability to treatment plant microbes in order understand potential impacts of EDON in the environment and the effectiveness of EDON removal during BNR treatment processes, has led a heightened awareness that more research on this topic needs to be done. The research needs identified during this workshop are summarized here by topic area and as identified during the workshop.

1 Bioassay Protocol to Determine Impacts of rEDON in Fresh and Salt Water

It has been suggested that not all of the EDON from BNR treatment facilities may be bioavailable to natural microbial communities in aquatic systems and that the rEDON fraction may vary for different receiving waters. Thus, regulators need a means to monitor plant effluent quality to assure that their goals for limiting the effect of nitrogen N discharges on eutrophication are being met while dischargers need to be able to implement effective nutrient removal at reasonable cost. One possible approach to setting nutrient discharge allowances would be to permit an effluent "effective" TN concentration that is equal to the measured effluent TN concentration minus the measured rEDON concentration. To do this, there must be an effective, accurate, and adaptable assessment of rEDON.

A rEDON bioassay must provide a measurement of recalcitrant EDON that would indeed be inert in the receiving water over exposure conditions during transport that are deemed consistent with the proximate and ultimate receiving waters. Any viable bioassay protocol for rEDON must be accepted by the environmental engineering and aquatic science professions, as well as the utilities and regulators. To achieve the goal of a viable rEDON assay, research is needed to understand factors that influence the outcome of the assay under environmental conditions (e.g. salinity, the microbial community used, etc.), and the variability they introduce into the bioassay results. It may be that protocols need to be specific for the discharge environment and that one set of assay conditions may be appropriate for dischargers who are wholly contained within freshwater watersheds versus another set of conditions would be applied to those dischargers contained within estuarine watersheds (discharges in the latter may discharge locally into a freshwater receiving body that flows to the estuarine; therefore, the estuarine test condition is relevant even though the immediate receiving water condition is freshwater). It is important that we understand how the transport and degradation of EDON in natural waters occurs along a salinity gradient in order to model the effect of point discharged nitrogen on proximate and downstream eutrophication. Research is needed to determine if the bioavailability of EDON and the composition of rEDON changes along salinity gradients to improve models describing the impact of discharged N in the environment. This topic will be investigated under an on-going National Science Foundation-sponsored research grant (PI: D. Bronk; co-PI's: N. G. Love, M. R. Mulholland, E. Canuel, and P. Hatcher).

Table 8 presents research issues that should be addressed in order to develop an acceptable rEDON bioassay or collection of bioassays.

Table 8. Research needs for rEDON bioassay test protocol

Test Parameter	Research Issue	Comment
Definition of filter pore size distributions needed to fractionate DON	Sample filter pore size to define dissolved portion	There may be a significant amount of colloidal organic nitrogen between 0.45 and 1.2 µm filter pore size, and below 0.45 µm.
pH control	Buffer addition and appropriate pH particularly in freshwater	Should the test alkalinity be similar to that of the receiving water?
Incubation time	What is the appropriate time period for bioassays that measure rEDON?	If the test is too long, N cycling will reach steady state within the bottle and will compromise interpretation of the results.
Incubation temperature	Is 20^0C test condition satisfactory for predicting rEDON concentrations in receiving water?	Should temperature in receiving waters be simulated.
Light intensity and diurnal variability in rate processes	Should bioassays be conducted in the light, dark, or both to assess rEDON?	Many algal processes are linked with the daily rhythm of photosynthesis.
Bacteria seed source	Can it be from wastewater plant or must it be from receiving water?	To test the fate of rEDON in the environment (freshwater or estuarine), seed would be obtained from receiving waters.
Need for carbon addition	Is a carbon source needed to maintain activity of bacteria needed for effective EDON hydrolysis and transformation? Would carbon addition reduce necessary test incubation time?	This might complicate interpretation of results because of C associated with EDON.
Effect of total inorganic concentration in test sample	A sample preparation method must be developed to reduce the sample TIN concentration so that an acceptable portion of the test sample microbial production is from EDON	Removal of inorganic N has been problematic in the past.
Algae seed type and source	Is *Selenastrum capriconutum* satisfactory for the fresh water rEDON protocol? What is the effect of collecting and using different algal seed sources along the fresh water to saline water gradient? Is there an acceptable standard pure or mixed culture that can be used?	There is currently no euryhaline test organism that could be used at all salinities and most aquatic algae are currently uncultured.
Algae growth condition prior to sample inoculation	Is the exponential growth condition the preferred physiological state for test organisms? What should the N source and N:P ratios be for cultivating or acclimating the algal test organism?	Nutrient prehistory is crucial for determining algal uptake capabilities.
Water quality conditions within bioassay	What is the appropriate solvent to use during the bioassay, and how does it differ for freshwater versus estuarine situations? Should the solvent composition change over time or with different bottles as part of the procedure?	It is expected that a salinity gradient influences amino bioavailability for some organic N compounds.
QA/QC methods	What EDON compound(s) could be used to test and demonstrate the accuracy of the bioassay? What other QA/QC methods should be employed in protocol?	This is crucial for the end goal of protecting the environment from excess N inputs.

2 Bioassay Protocol to Determine Influent Wastewater (IDON) Biodegradability (bIDON)

Protocols for determining the bioavailability of influent and effluent ON are currently unavailable and, until recently, there has been no effort to fill this gap in our ability to effectively regulate this fraction of the total N discharge from wastewater treatment plants. In addition to the need to assess the impact of EDON in the environment, there is a significant need to understand how constituents of influents into treatment plants and their relative reactivity, contribute to the final composition of EDON. It is currently not known how the plant design and operation, recycle streams, and influent organic nitrogen characteristics differentially affect EDON concentrations and composition. A method is needed to characterize the organic nitrogen in wastewater influents as well as effluents. It is particularly important to characterize any riDON and to determine if rEDON concentrations are related to the riDON (especially if it comes from controllable sources, such as industrial wastewater inputs, reject water recycle streams, and/or additives in the water supply). A biDON bioassay would use biomass from the treatment plant being evaluated to assess the capacity of that biomass to transform the organic nitrogen in the plant's influent waste stream. Because bacteria can also produce organic nitrogen, tests assessing organic N concentrations as endpoints can be misinterpreted because while the organic nitrogen in influent ON can be taken up or degraded simultaneous production of organic nitrogen as a consequence of metabolism can confound interpretation of net changes in DON concentrations. Therefore, it is envisioned that any protocol developed would include an assessment technique that differentiates in a general way the nature of the organic matter in the bioassay over time; whether it was produced during the bioassay or was preexisting in the influent ON.

3 Bioassay Protocol to Determine if Further Wastewater Treatment Will Eliminate bEDON

The bEDON bioassay protocol may be less complex and than the rEDON bioassay; however, the methods give extremely different information. The research needs for further development of the bEDON bioassay method and for establishing an accepted protocol are summarized here:

- What is the contribution of colloidal matter to the bEDON? Filter pore sizes should be selected to allow for evaluating the bEDON of colloidal matter versus truly dissolved EDON. Colloidal matter would not necessarily be removed by the treatment facility or by effluent filtration.
- Should bottle conditions be altered to reflect metabolic conditions experienced during the treatment process (in the plant)? If supplemental readily biodegradable carbon is added to shorten the test time, how will that affect the measured bEDON concentration? How much and how often should it be added?
- What known DON standards could be used to gauge the precision of the bEDON test in order to establish a quality assurance protocol?

4 Removal and Production of bEDON and rEDON in a BNR Treatment Process

Research is needed to determine which design and operating conditions in a BNR facility affect the effluent bEDON and rEDON concentrations? Key questions for this research are:

- Is there an optimal SRT for which the bEDON is minimized by balancing degradation of bEDON against bEDON production from in-plant microbes?

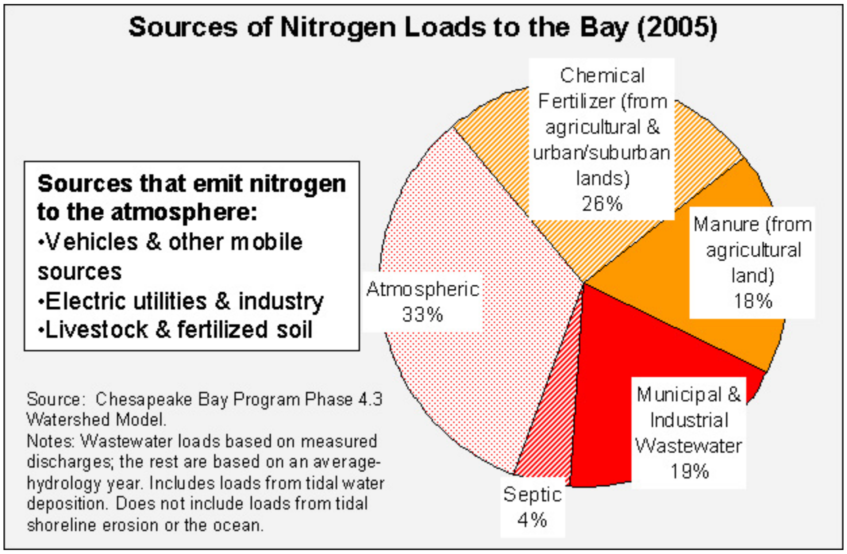
- If SRT is increased to decrease bEDON, will it cause a concomitant increase in rEDON? Can changes in the fraction of bEDON and rEDON be assayed simply as changes in the relative proportion of HMW EDON?
- What is the amount of bEDON and rEDON in recycle streams, including anaerobic sludge digestion and aerobic sludge digestion?
- Is there an effect of the BNR design and configuration (anaerobic and anoxic contact) on concentrations of rEDON and bEDON?
- Is the bEDON and rEDON removal efficiency different for membrane and granular media filtration processes?
- What are promising tertiary processes for bEDON and rEDON removal?

5 Non-Bioassay Methods to Characterize rEDON

Research is needed to characterize the rEDON measured using any bioassay protocol. Previous work suggests that rEDON is primarily HMW humic material that also contains amide compounds and synthetic organics such as EDTA. If suitable progress can be made to characterize rEDON, it may be possible to develop methods to measure key indicator compounds in lieu of conducting complex and time consuming bioassays to assess rEDON.

Treatment Plants to Consider for Partnership in Conducting Future Research

There are more than 300 wastewater treatment facilities discharging over 1.5 billion gallons per day of treated effluent from almost 75% of the approximately 16 million people living in the 64,000 square mile Chesapeake Bay watershed. Wastewater entering treatment plants and treated wastewater leaving the treatment plant contains highly variable nutrient (nitrogen and phosphorus) concentrations resulting in variable loading to aquatic ecosystems. Of the total nutrient load to the Chesapeake Bay watershed, agriculture contributes the largest proportion of the total nitrogen load (42%), followed by atmospheric deposition of N (33%), and finally wastewater facilities (19%).



Source: Chesapeake Bay Program Phase 4.3 Watershed Model.
Notes: Wastewater loads based on measured discharges; the rest are based on an average-hydrology year. Includes loads from tidal water deposition. Does not include loads from tidal shoreline erosion or the ocean.

Reference: http://www.chesapeakebay.net/status/status_dev.cfm?SID=126&SUBJECTAREA=INDICATORS

The largest number of wastewater treatment facilities in the Chesapeake Bay watershed is in Pennsylvania (123), followed by Virginia (81), Maryland (65), New York (22), West Virginia (9), Delaware (3), and the District of Columbia (1) [source: Chesapeake Bay Foundation, October 29, 2003. Sewage Treatment Plants: The Chesapeake Bay Watershed's Second Largest Source of Nitrogen Pollution]. Some of these plants are owned and operated by utilities who are also Subscribers of the Water Environment Research Foundation (WERF). These facilities range in size and effluent load to the Bay area, as well as spatial location and potential impact to water quality (with respect to nitrogen). They include, but are not limited to:

- Alexandria Sanitation Authority, VA
- Arlington County, VA
- DCWASA's (DC Water and Sewer Authority) Blue Plains Advanced Wastewater Treatment Facility, DC
- Howard County, MD
- Fairfax County, VA
- Hampton Roads Sanitation District, VA
- Hanover County, VA
- Henrico County, VA
- Hopewell Regional Wastewater Treatment Facility, VA
- Loudon County Sanitation District, VA
- Lynchburg Regional WWTP, VA
- Prince William County Service Authority, VA
- City of Richmond, VA
- Rivanna Water & Sewer Authority, VA
- Philadelphia Water Department, PA
- Prince William County Service Authority, VA
- WSSC (Washington Suburban Sanitary Commission), MD – with several plants on the DC metropolitan area

Several of these subscribers are actively involved in WERF research and/or have expressed interest in participating in additional water quality research forums. It is suggested that any future research include the following utilities that have different wastewater treatment capacity and configurations and which are also spatially distributed throughout the Bay area:

- Alexandria Sanitation Authority, VA
- DCWASA's Blue Plains Advanced Wastewater Treatment Facility, DC
- Howard County, MD
- Loudon County Sanitation District, VA
- Prince William County Service Authority, VA
- WSSC (Washington Suburban Sanitary Commission), MD
- City of Richmond, VA
- Hampton Roads Sanitation District, VA

WERF's targeted collaborative research (TCR) program provides opportunities for their subscribers and others to share and leverage resources (funding, test sites, laboratory, intellectual, etc.). WERF also has an extensive ongoing research program on their "Nutrient Removal

Challenge” and it is suggested that studies or activities proposed on the dissolved organic nitrogen issue be coordinated with this organization.

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