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# **The Speciation and Reactivity of Wastewater-Derived Organic Nitrogen**

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## Abstract

Nitrogen often is the limiting nutrient for the growth of algae and phytoplankton in estuaries and surface waters in California. To control cultural eutrophication (i.e., excessive growth of algae and plankton related to anthropogenic sources) regulatory agencies often focus on the control of point source discharges, including municipal wastewater effluent. Recent attempts to control cultural eutrophication in nitrogen-limited systems have focused on the simultaneous control of all forms of inorganic nitrogen with the underlying assumption that inorganic and organic nitrogen are equally bioavailable. To assess the validity of this assumption, algal growth bioassays were conducted using denitrified wastewater effluent samples that contained mainly organic nitrogen. The growth assays were performed with a species of algae (*Selenastrum Capricornutum*) that is commonly used for regulatory compliance monitoring. Results of the study indicate that the wastewater-derived dissolved organic nitrogen (DON) is not bioavailable to the algae in the absence of bacteria. However, approximately half of the wastewater-derived organic nitrogen was available to the algae in the presence of bacteria during a two-week incubation.

In conjunction with the experiments on bioavailability, the nature and properties of wastewater-derived organic nitrogen was characterized by measuring concentrations of free and combined amino acids and by subjecting wastewater effluent samples to ultrafiltration. Results of these experiments indicate that most of the wastewater-derived organic nitrogen was associated with unidentified compounds that are capable of passing through a 1 kDa ultrafilter.

Wastewater-derived organic nitrogen also plays an important role in the formation of the carcinogenic disinfection byproduct, N-nitrosodimethylamine (NDMA). NDMA is formed when wastewater effluent is disinfected with chlorine. It also can be formed when surface water or groundwater that has been impacted by wastewater effluent discharges undergoes disinfection in drinking water treatment plants. Recently, concerns associated with NDMA have caused great concern among utilities that practice indirect potable water reuse. Most of the attention to date has focused on the removal of NDMA from the treated wastewater effluent. However, the presence of NDMA precursors also could be a problem if the NDMA precursors in drinking sources are stable after they are discharged because they could result in NDMA formation during drinking water treatment. To assess the stability of NDMA precursors associated with wastewater-derived organic nitrogen, NDMA precursor concentrations were measured in effluent samples before and after incubation with bacteria under aerobic conditions. Results of the experiments indicate that the NDMA precursors are stable for at least 30 days.

These results suggest that wastewater-derived organic nitrogen consists of complex compounds that are not very reactive. However, under certain conditions, wastewater-derived organic nitrogen species can serve as a source of nutrients in nitrogen-limited systems and as disinfection byproduct precursors. The results of this research will be useful in the development of indirect potable water reuse systems and the design of watershed protection plans designed to protect aquatic ecosystems from the effects of cultural eutrophication.

## Introduction and Problem Statement

Nitrogen is one of the most important pollutants in municipal wastewater effluent. Depending on its form, nitrogen can threaten human health or damage the integrity of aquatic ecosystems. Nitrate, which is the predominant form of nitrogen in nitrified wastewater effluent, is one of the most prevalent drinking water pollutants in California. Nitrite, a form of nitrogen that typically accounts for less than 5% of the total inorganic nitrogen in wastewater effluents, also can be a problem in drinking water because it is more toxic to humans than nitrate. Ammonium, the predominant form of nitrogen in secondary wastewater effluent, is toxic to fish and aquatic organisms at concentrations typically present in wastewater effluent. All three forms of nitrogen can lead to cultural eutrophication in estuaries and in California's nitrogen-limited lakes.

Organic nitrogen is the fourth form of nitrogen present in wastewater effluent. Organic nitrogen often is ignored by scientists because it typically accounts for less than 10% of the total nitrogen in secondary wastewater effluent or surface water and is not as well understood as inorganic forms of nitrogen. However, recent developments in watershed management, indirect potable water reuse and improvements in analytical chemistry necessitate further study of the nature and behavior of wastewater-derived organic nitrogen. Several recent developments necessitating a reexamination of organic nitrogen are summarized below.

One reason why it is important to reexamine organic nitrogen in wastewater effluent is that it plays an increasingly important role in Total Maximum Daily Load (TMDL) regulations. To avoid complications associated with biogeochemical cycling of nitrogen, many TMDL plans are based on total nitrogen (*i.e.*, the sum of all inorganic and organic forms of nitrogen). This simplification implies that all forms of nitrogen contribute equally to cultural eutrophication or that all forms of nitrogen are readily interconverted. While this assumption probably is reasonable for inorganic forms of nitrogen, it is questionable for organic nitrogen. Despite the potential shortcomings of this simplifying assumption, scientists and regulators use it because organic nitrogen is believed to be relatively unimportant. However, after installation of nutrient removal systems at wastewater treatment plants, organic nitrogen can be very important. An extreme example of such a situation is provided by the TMDL for the Truckee River, which originates at Lake Tahoe and terminates at Pyramid Lake, NV. Since the Truckee Meadows Wastewater Treatment Plant (WWTP) installed a nitrification/denitrification system in 1988, organic nitrogen has accounted for approximately 85% of the total nitrogen discharged by point sources in the watershed (TMWRF 2001). Engineers and regulators now are faced with questions about the merits of installing advanced treatment systems (*e.g.*, reverse osmosis) to remove the organic nitrogen from the effluent or implementing control measures for non-point sources of inorganic nitrogen. Decisions about which approach will yield the most cost-effective control of cultural eutrophication depend upon the ability of organic nitrogen to stimulate the growth of algae and periphyton in the river.

Another reason to examine wastewater-derived organic nitrogen is that it serves as a precursor to toxic disinfection byproducts. In California, municipal wastewater effluent is disinfected prior to discharge or reuse. Despite the recent proliferation of ultraviolet disinfection systems, most wastewater treatment plants still use chlorine for disinfection. Furthermore, indirect potable water reuse projects (*i.e.*, the practice of employing wastewater effluent for groundwater recharge or augmentation of surface water flow) frequently employ chlorine disinfection. Chlorination of nitrogen-containing components of municipal wastewater effluent results in the formation of three major types of toxic byproducts: (1) organic chloramines, which are toxic to aquatic organisms and resistant to transformation by dechlorinating agents such as sodium bisulfite (*i.e.*, NaHSO<sub>3</sub>) (Jensen and Helz, 1998; Jameel and Helz, 1999); (2) trihalomethanes (THMs), which are carcinogens of concern in drinking water (Scully *et al.*, 1988a); (3) *N*-Nitrosodimethylamine (NDMA), which is an extremely potent carcinogen formed in conventional and advanced wastewater treatment plants (Mitch *et al.* 2003).

The formation of chloramines disinfection byproducts that present human health risks, such as THMs and NDMA may not present a problem if they are removed during transport in surface and groundwater systems. However, if the disinfection byproduct precursors are not removed prior to drinking water treatment, indirect potable reuse might still pose a human health risk.

Until recently, measurements of organic nitrogen in wastewater effluents were restricted to quantification of total organic nitrogen, which is measured after converting the organic nitrogen into an inorganic form that is readily measured. For example, organic nitrogen is measured in the Kjeldahl nitrogen test after conversion of organic nitrogen into NH<sub>3</sub> followed by distillation and subtraction of NH<sub>4</sub><sup>+</sup> present prior to the conversion step (APHA, 1998). Other, more sensitive approaches for quantifying organic nitrogen use persulfate digestion to convert organic nitrogen to NO<sub>3</sub><sup>-</sup> (APHA, 1998). New analytical methods, developed by biologists and marine chemists, have been adopted to identify specific organic nitrogen compounds in wastewater effluents. Using these techniques, scientists have characterized organic nitrogen in a limited number of wastewater effluent samples (Grohman *et al.* 1998; Confer *et al.*, 1995; Scully *et al.* 1988b; Burlison *et al.*, 1980). Combining measurements made on different wastewater effluent samples, it is possible to identify approximately 10% of the organic nitrogen species (Table 1). Other synthetic organic compounds account for less than 5% of the organic nitrogen (Grohman *et al.* 1998). It is likely that much of the remaining organic nitrogen species consists of polymeric species (*e.g.*, humic substances). However, additional research is needed to characterize the nature of the remaining organic nitrogen species.

**Table 1:** Typical concentrations of organic nitrogen species in secondary wastewater effluent.

<i>Nitrogen Species</i>	<i>Concentration Range (<math>\mu\text{M}</math>)<sup>1</sup></i>	<i>% of Total Organic Nitrogen</i>
Free Amino Acids	0.2-2.1	0.5%
Combined Amino Acids	3.9-19	5%
EDTA	0.1-15	3%
Dimethylamine	5.1	2%

<sup>1</sup> references: Free and combined amino acids: Burleson *et al.*, 1980; Confer *et al.*, 1995; Scully *et al.* 1988b; EDTA: Kari and Giger, 1996; Bedsworth and Sedlak, 1999; Dimethylamine: Hwang *et al.*, 1995.

## Objectives

To provide a better understanding of the role of organic nitrogen in current water quality issues, a research program was conducted to assess the composition of organic nitrogen in wastewater effluent and the bioavailability and the reactivity of those compounds in the aquatic environment. Specific objectives include:

- Characterization of organic nitrogen in municipal wastewater effluent;
- Assessment of the ability of organic nitrogen to cause growth in nitrogen-limited algae;
- Evaluation of the stability of wastewater-derived precursors of NDMA in the presence of bacteria.

Results of this research will provide scientists and engineers with a better understanding of the nature and behavior of wastewater-derived organic nitrogen. Ultimately, this understanding will provide a basis for protecting human health and aquatic ecosystems from problems related to organic nitrogen.

## Procedure

### Characterization of Wastewater-Derived Organic Nitrogen

To assess the composition of wastewater-derived organic nitrogen, effluent samples were collected from three municipal wastewater treatment plants. The Dublin-San Ramon wastewater treatment plant is located in Dublin San Ramon, CA and has an average dry weather flow of  $0.5 \text{ m}^3/\text{s}$  (11.5 MGD). The treatment plant is a secondary activated sludge plant where the treated effluent is chlorinated and dechlorinated before discharge into San Francisco Bay. The Truckee Meadows Water Reclamation (TMWRF) facility is a  $1.8 \text{ m}^3/\text{s}$  (40 MGD) treatment plant and is located in Reno, NV. The treatment plant consists of an activated sludge unit, and nitrification and denitrification tanks. The effluent in TMWRF is chlorinated and dechlorinated as well prior to the discharge into the Truckee River. The Mt. View wastewater treatment is a small wastewater treatment that treats  $0.1 \text{ m}^3/\text{s}$  (1.8 MGD) of sewage. The treatment plant has a nitrification unit in addition to a trickling filter for secondary treatment. The treated effluent is disinfected using an ultraviolet system and the effluent is discharged into the treatment wetlands for further treatment and for habitat restoration.

Wastewater effluent samples were collected in Teflon-lined polypropylene containers and was filtered either on site or was kept on ice until filtration within 24 hours. All samples were filtered through  $0.2 \mu\text{m}$  cartridge filters (Millipore) to remove particles and inhibit microbial transformation of organic nitrogen species. All analyses were performed within seven days of sample collection. Organic nitrogen species were measured using the analytical techniques described below:

Dissolved nitrogen concentrations were measured using the macro-Kjeldahl nitrogen test after conversion of organic nitrogen into  $\text{NH}_4^+$  followed by distillation and subtraction of  $\text{NH}_4^+$  present prior to the conversion step (4500-Norg B) (APHA, 1998). Organic nitrogen was measured as the difference between the total N measured after conversion of all the nitrogen forms to nitrate using a persulfate oxidation (method 4500-Total N; APHA, 1998). Ammonia was analyzed by the indophenol colorimetric method (method 4500-NH<sub>3</sub> B&E; APHA 1998), nitrite was analyzed with the diazonium colorimetric method (method 4500-NO<sub>2</sub><sup>-</sup>B; APHA, 1998) while nitrate was analyzed by reduction to nitrite and colorimetry (method 4500-NO<sub>3</sub><sup>-</sup>E; APHA, 1998) or ion chromatography.

Amino acid concentrations were measured by derivatization with ortho-phthaldialdehyde (OPA) followed by high-performance liquid chromatography (HPLC) and fluorescence (Confer et al., 1995). The derivatization reaction was performed with the HPLC autosampler immediately prior to sample injection to avoid artifacts associated with manually stopping the reaction with acetic acid. Also, acetonitrile was used as the organic solvent instead of methanol to prevent the precipitation of salts and clogging of the column.

Combined amino acid concentrations were determined by subtracting free amino acid concentrations from total amino acid concentration measured after

vapor-phase hydrolysis of the sample. The recoveries of individual amino acids from protein extracts ranged between 30-170%. Despite numerous attempts to identify steps where losses occurred recoveries could not be improved. No corrections were made to the concentrations using the recovery efficiencies. Primary amines also were measured with a Shimadzu RF-10Axl fluorescence detector after derivatization with OPA (Parsons *et al.*, 1984).

Samples were fractionated by molecular weight using an Amicon stirred ultrafiltration cell (Millipore Corp., Model 8050). The ultrafilters were employed in series and included filters with nominal molecular weight cutoffs of 30kDa, 10kDa, 3kDa and 1kDa. The stirred cell was placed on ice and stored in the dark during the ultrafiltration, which was conducted at 45 psi. The wastewater was placed on ice in a cooler while it was filtered through the ultrafilters at a maximum pressure of 45 psi.

### **Bioavailability of Wastewater-Derived Organic Nitrogen to Algae**

Assessment of the bioavailability of wastewater-derived organic nitrogen to algae was conducted using effluent from the Truckee Meadows Water Reclamation Facility, described in the previous section. This facility was chosen for the analysis because the plant's effluent usually contains very low concentrations of inorganic nitrogen species. Samples were collected after dechlorination in 10-L polyethylene containers and kept on ice for 24-48 hrs prior to initiation of the experiments. All samples were filtered through a 0.2- $\mu$ m cartridge filter (Whatman 75 AS) upon receipt.

In three sets of experiments, an indigenous Truckee River bacterial inoculum was used to assess the role of bacteria in the uptake of wastewater-derived DON. The inoculum was acquired from a small stream that received most of its flow from the Truckee Meadows WWTP approximately 3 km downstream of the outfall. Approximately 3 L of river water, collected on immediately before the initiation of the experiment was filtered through a 1- $\mu$ m glass-fiber filter (Type A/E, Pall Corp.) to remove large particles and algae prior to filtration through a 0.2- $\mu$ m membrane filter (Osmonics). The retentate on the 0.2- $\mu$ m filter was resuspended in 100 mL of 0.2  $\mu$ m-filtered river water and used as the natural inoculum.

The bioavailability of wastewater-derived organic nitrogen was determined with algal growth bioassays. *Selenastrum Capricornutum* was chosen as the algal species for the bioassay experiments because it is readily cultured in the laboratory and has been used as a standard test organism for eutrophication studies for over 30 years. The test alga was obtained from UTEX, at the University of Texas at Austin. During the experiments, all glassware, deionized water and nutrient solutions were autoclaved and the wastewater effluent was filter-sterilized using 0.2  $\mu$ m cartridge filters (Whatman 75 AS). To assess nitrogen bioavailability, nitrogen-limited algae were cultured by amending samples with nutrients according to the standard freshwater algae toxicity test protocol (APHA 1998), except for nitrate. Potassium phosphate ( $K_2HPO_4$ ) was added to the media to give a molar N/P ratio of 3. In the first two experiments, 5



mL of algal inoculum at logarithmic growth phase was added to a 400 mL sample. In the second experiment, 1 mL of bacterial inoculum also was added. 40 mL of the fractionated wastewater was used in the third experiment and the inocula volumes were decreased accordingly. All alga cultures were incubated on a shaker in triplicate 500 mL-Erlenmeyer flasks, at 20-22°C, with a 12-hour light/dark cycle, except for the MW-fractionated samples, which were kept in 125 mL-Erlenmeyer flasks and were shaken by hand every 12 hours. The growth of algae was monitored with *in vivo* Chlorophyll-a (chl-a) measurements with a fluorometer (TD-700, Turner Instruments) until the stationary growth phase was reached (i.e., approximately 2 weeks). The chl-a to biomass ratio may vary when algae are grown under different nutrient conditions. In these experiments, all of the nutrient concentrations excluding nitrogen were equal and the variation in the nitrogen concentration was relatively small. Therefore, it is assumed that the chl-a to biomass ratio is approximately constant and that chl-a is a reasonable surrogate for algal biomass.

Samples collected at different times after initiation of the experiments were analyzed for nitrogen and algal growth. Total dissolved nitrogen was measured with a modified version of the standard persulfate digestion method described in the previous section. Nitrate concentrations were measured by ion chromatography, after conversion of all the nitrogen forms to nitrate. Dissolved organic nitrogen (DON) was calculated as the difference between the total dissolved nitrogen and the sum of inorganic nitrogen species. In most cases, DON was measured three times during the algal growth experiments: before the beginning of the algal experiments ( $\text{DON}_{\text{initial}}$ ), in the middle ( $\text{DON}_{\text{middle}}$ ) and at the end ( $\text{DON}_{\text{end}}$ ).

### **NDMA Precursor Bioavailability**

The bioavailability of NDMA precursors was evaluated by adding a bacterial inoculum, collected from the mixed liquor of an activated sludge wastewater treatment plant, to wastewater effluent samples. 4-L wastewater effluent samples were collected prior to disinfection from the Mt. View, Whittier Narrows and Riverside municipal wastewater treatment plants. The Mt. View treatment plant was described in the previous sections. The Whittier Narrows WWTP, located in Los Angeles County, is a 0.66 m<sup>3</sup>/d (15 MGD) activated sludge treatment plant equipped with a nitrification/denitrification system. The Riverside WWTP is a 0.35 m<sup>3</sup>/d (8 MGD) activated sludge treatment plant.

After collection, the samples were filtered through a 0.2  $\mu\text{m}$  cartridge filter, placed in open-top glass containers covered with an-air permeable plastic barrier (Handi-Pads, Kendall Corporation) to prevent contamination of the sample by dust. Samples were inoculated with 3 mL of inoculum to achieve a volatile suspended solids concentration of approximately 3 mg/L. One set of sterilized controls was run for each treatment by adding 0.5 mM HgCl<sub>2</sub>. The absence of bacteria in the sterile controls was verified at the beginning and end of the experiments by monitoring re-growth in bacteria media.

After times ranging from 2 to 30 days, 450-mL samples were collected and analyzed for NDMA precursors according to the method described by Mitch *et al.* (2003). Briefly, the NDMA precursor method involves the addition of a relatively high dose (*i.e.*, 140 mg Cl<sub>2</sub>/L) of monochloramine to water samples. After 10 days, samples quenched by adding 5 mL of 20 mg/L ascorbic acid solution, extracted on Ambersob resin and analyzed for NDMA by gas chromatography/tandem mass spectrometry (GC/MS/MS) using the chemical ionization mode.

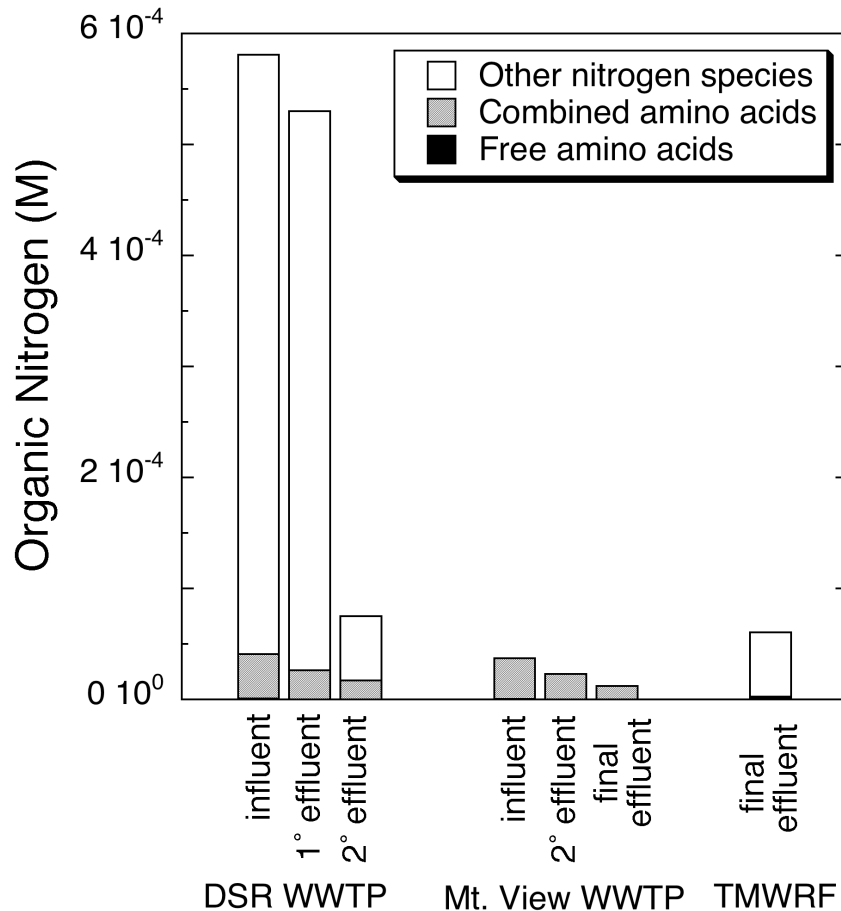
To assess the effect of chlorine disinfection on NDMA precursors, several experiments were conducted on samples that had been subjected to simulated chlorine disinfection. Free chlorine disinfection was accomplished by adding an initial concentration of 0.14 mM (10 mg Cl<sub>2</sub>/L) from a freshly prepared stock solution of NaOCl. Chloramine disinfection, with the same initial concentration, was accomplished using a monochloramine stock solution prepared by the method described by Mitch and Sedlak (2004). For both treatments, samples were dechlorinated after 1 hour using sodium bisulfite.

## Results

### Characterization of wastewater-derived organic nitrogen

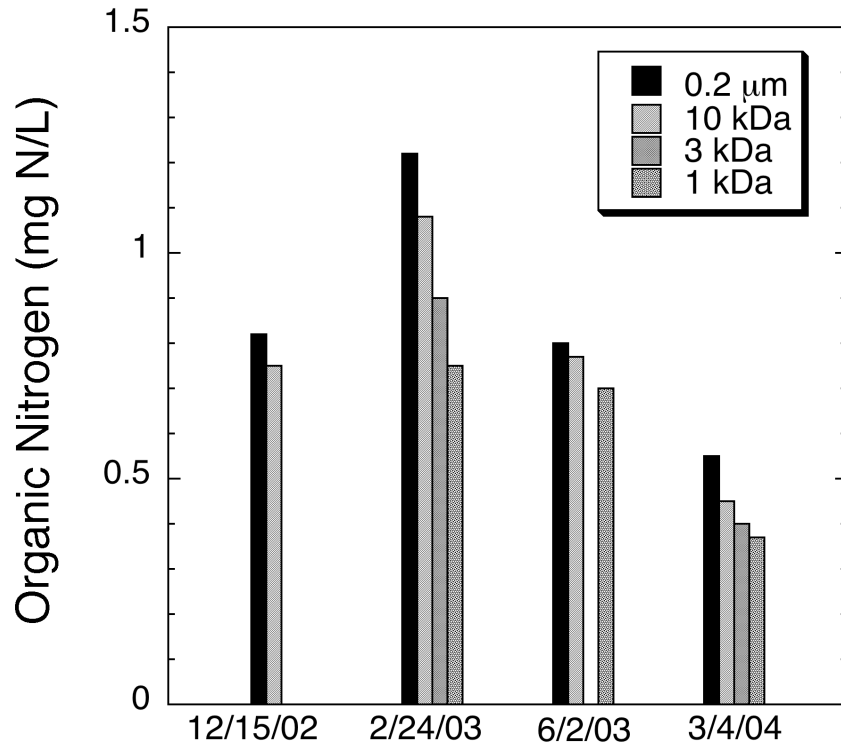
Analysis of wastewater effluent samples indicated that the combined amino acids accounted for a relatively small percentage of the overall concentrations of wastewater-derived organic nitrogen (Figure 1). At the Dublin-San Ramon (DSR) WWTP, combined amino acid concentrations, expressed on the basis of N, decreased from approximately 50  $\mu\text{M}$  in the plant influent to 20  $\mu\text{M}$  in the plant effluent. In the same samples, dissolved organic nitrogen concentrations decreased from approximately 580  $\mu\text{M}$  in the plant influent to 75  $\mu\text{M}$  in the plant effluent. Combined amino acids accounted for approximately 25% of the organic nitrogen in the effluent of the DSR WWTP. The concentrations of free amino acids in the effluent of the DSR WWTP accounted for less than 0.2% of the total organic nitrogen in all of the samples. Concentrations of free and combined amino acids measured in the wastewater influent, trickling filter effluent and final plant effluent at the Mt. View WWTP were comparable to those measured at the DSR WWTP. At the TMWRF plant, the concentrations of free amino acids and dissolved organic nitrogen were comparable to the concentrations detected in effluent samples from the other two treatment plants.

The concentrations of dissolved and total amino acids detected were slightly higher than those reported by previous researchers. For example, DCAA concentrations in wastewater effluent reported by previous researchers ranged between 1 and 19  $\mu\text{M}$  with most of the data falling in the range of 1 and 3  $\mu\text{M}$  (Scully et al., 1988; Confer et al., 1995; Grohmann *et al.*, 1998). It is possible that the lower values of combined amino acids reported by other researchers is related to incomplete hydrolysis of polypeptides during the hydrolysis or other analytical artifacts.



**Figure 1:** Concentrations of free amino acids, combined amino acids and dissolved organic nitrogen in wastewater. The concentrations of free amino acids in most samples were very small and do not show up in the figure. Dissolved organic nitrogen was not measured in samples from the Mt. View WWTP due to matrix interference. Combined amino acids were not measured in the sample from the TMWRF.

The measurements of free and combined amino acids indicate that most of the wastewater-derived organic nitrogen consists of species other than amino acids, proteins and polypeptides. To further characterize the unknown organic nitrogen species, wastewater effluent samples from the TMWRF were subjected to ultrafiltration (Figure 2). Analysis of samples collected on four different dates indicates that most of the wastewater-derived organic nitrogen consisted of species that can pass through a 1-kDa ultrafilter. If the organic nitrogen species were composed mainly of humic acids a greater extent of removal would have been expected during ultrafiltration. These findings are consistent with data reported by other researchers using complimentary techniques (Keller et al. 1978; Parkin and McCarty 1981).

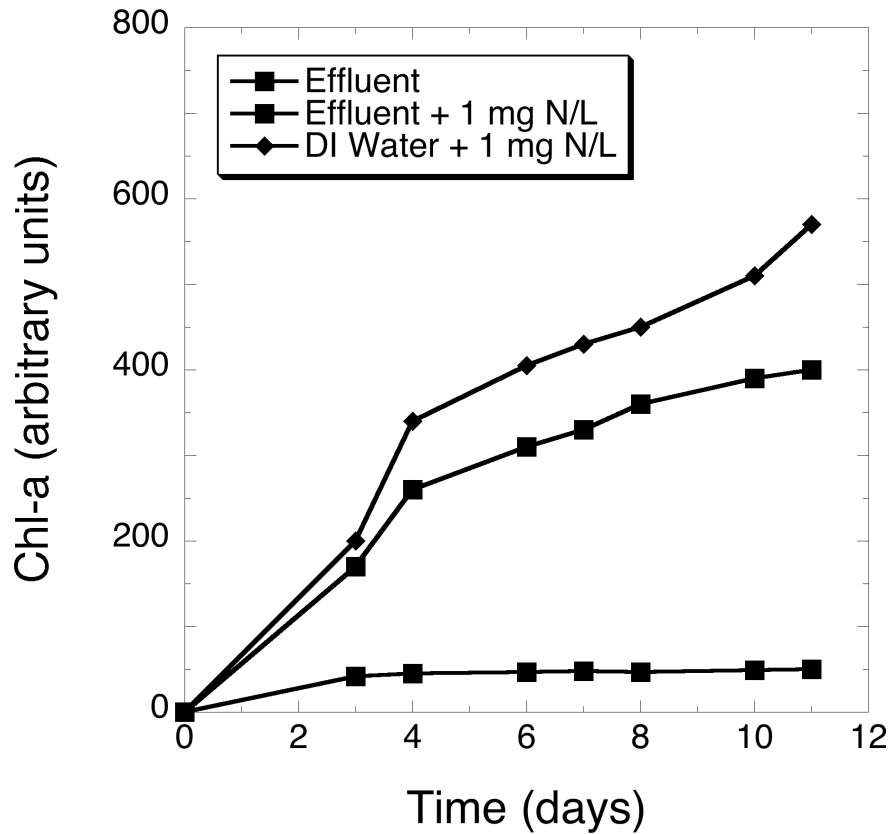


**Figure 2.** Organic nitrogen content of wastewater effluent samples from the TMWRF after passage through ultrafilters.

Results of these experiments and previous research support the hypothesis that wastewater-derived organic nitrogen consists of a suite of compounds other than polypeptides and humic substances. It is possible that the organic nitrogen species are partially metabolized biomolecules (e.g., cross-linked protein fragments) and other biological material that would not appear in the combined amino acid fraction.

### Bioavailability of Wastewater-Derived Organic Nitrogen to Algae

Algal growth experiments conducted in the absence of added bacteria indicated that most of the wastewater-derived DON was not bioavailable (Figure 3). During the 11-day incubation with DON a very small amount of chl-a production was observed in the wastewater effluent sample. When wastewater effluent was amended with 1 mg N/L nitrate (squares in Figure 1), chl-a production increased approximately as much as chl-a increased when the same concentration if nitrate was added to deionized water. The slightly smaller increase in algal growth in the nitrate-amended wastewater effluent could have been caused by antibacterial agents of wastewater origin, such as triclosan (Orvos et al. 2002) or some other phytotoxic compounds in the wastewater effluent (Yamane et al. 1984). Although the nitrate-amended treatments did not reach steady-state conditions with respect to chl-a at the end of the experiment, they did show significantly more chl-a production than the unamended treatment.



**Figure 3.** Production of chlorophyll a by algae incubated in wastewater effluent from TMWRF and deionized (DI) water. The sample contained 0.81 mg/L of organic nitrogen.

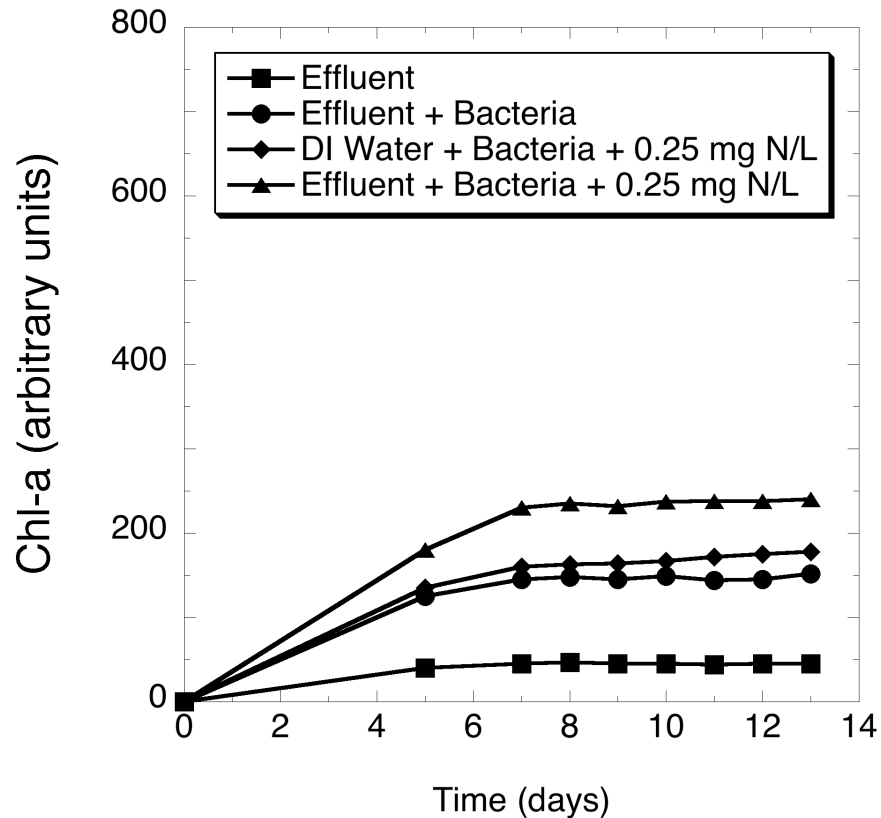
In both the unamended wastewater effluent and the nitrate-amended wastewater effluent, DON decreased by approximately 40% during the incubation (Table 2). For the unamended wastewater effluent, the decrease in DON was not accompanied by either an increase in inorganic nitrogen (data not shown). The most likely explanations for the decrease in DON are adsorption of DON to the glass walls of the container or bacterial uptake of DON, possibly after conversion into an inorganic form.

**Table 2:** Changes in dissolved organic nitrogen concentrations during algal growth experiments.

	DON <sub>init.</sub> ± s.d (mg N/L)	DON <sub>middle</sub> ± s.d (mg N/L)	DON <sub>end</sub> ± s.d (mg N/L)
Effluent	0.81 ± 0.03	0.66 ± 0.06	
Effluent + 1 mg N/L	0.81 ± 0.03	0.67 ± 0.07	0.51 ± 0.14
DI + 1 mg N/L	not measured	<0.1 ± 0.05	<0.1 ± 0.03
Effluent	0.62 ± 0.11	0.62 ± 0.04	0.62 ± 0.04
Effluent + bacteria	0.62 ± 0.11	0.61 ± 0.05	0.61 ± 0.06
Effluent + 0.25 mg N/L + bacteria	0.62 ± 0.11	0.50 ± 0.01	0.55 ± 0.01
DI + 0.25 mg N/L + bacteria	not measured	<0.1 ± 0.01	0.08 ± 0.01
Effluent + 0.4 mg N/L + bacteria	0.82 ± 0.09	not measured	0.61 ± 0.04
Effluent + bacteria	0.82 ± 0.09	not measured	0.55 ± 0.03
Effluent (<1kDa) + bacteria	0.27 ± 0.08	not measured	0.36 ± 0.07

By assuming algal growth per mole nitrogen was independent of the source of nitrogen, we estimated the chl-a produced per mole of nitrogen from the difference between the chl-a measured in the unamended and the nitrate-amended wastewater effluent. It should be noted that bacteria could decrease the amount of DON available to the algae, and hence increasing the amount of chl-a produced per mole of bioavailable DON. On the basis of this calculation, the slight increase in chl-a measured in the unamended wastewater effluent sample corresponds to a bioavailable nitrogen concentration of 0.13 mg/L N. However, if the contribution of the algal inoculum used in our experiments (~0.05 mgN/L) is taken into account, then only about 0.08 mg N/L of the 0.3 mg/L DON that disappeared during the incubation could be accounted for by chl-a production. It is possible that some of the missing DON was taken up by bacteria. However, a decrease of 0.22 mg N/L would have corresponded to a bacterial abundance of  $9 \times 10^7$ /mL (assuming 5.4 fg N/cell for the N content of a bacterial cell Keil and Kirchman, 1991) and such a high bacterial density was unlikely under these conditions. In any case, in the absence of added bacteria, wastewater-derived DON did not produce as much chl-a as it would have if DON was completely bioavailable.

Algal growth bioassays also were conducted with and without a bacterial inoculum. In these experiments chl-a production increased in the presence of bacteria (Figure 4). The chl-a production in the wastewater effluent inoculated with bacteria was slightly lower than that observed for bioassays performed in deionized water amended with 0.25 mg N/L nitrate. When wastewater effluent was amended with 0.25 mg N/L nitrate and inoculated with bacteria, chl-a production approximately doubled compared to wastewater amended with bacteria.



**Figure 4.** Production of chlorophyll a by algae incubated in wastewater effluent from TMWRF and deionized (DI) water with and without addition of bacterial inoculum.

The DON concentration in the wastewater effluent with no added nitrate increased slightly during the incubation, whereas the concentration of DON in both of the wastewater effluents inoculated with bacteria decreased slightly during the bioassay (Table 2), indicating the importance of bacteria in the cycling of the organic nitrogen. The slight increase in DON in the unamended wastewater (not statistically significant at  $p=0.05$ ) is inconsistent with the decrease in DON in wastewater effluent observed during the first set of experiments (Table 2). The differences between these two experiments could be attributable to variability in the composition of the DON. The inconsistency also could have been due to differences in the algal inoculum used in two experiments. Using the method for estimating bioavailability of DON described in the previous section, we calculate that approximately 56% of DON (*i.e.*, 0.34 mg N/L) in wastewater is available to the algae in the presence of bacteria. For this experiment, the estimated

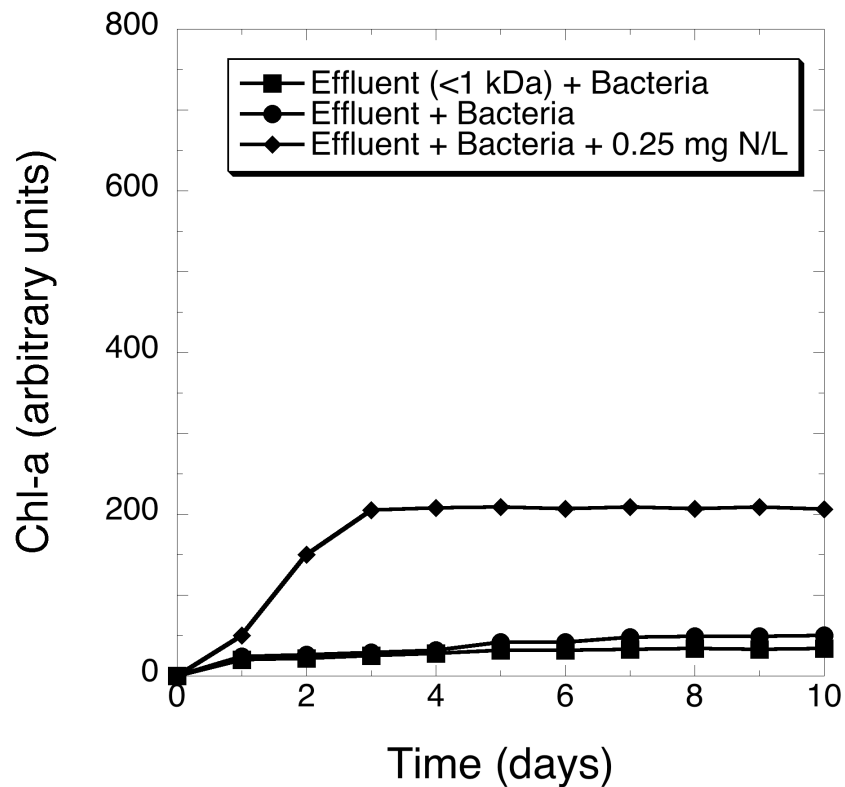


concentration of bioavailable DON in the absence of bacteria is also less than 0.10 mg N/L, which agrees with the calculation made for the data depicted in Figure 3.

The concentrations of inorganic nitrogen species were very small compared to the DON in the unspiked treatments, and they remained approximately constant during the experiments. The constant concentration of inorganic forms of nitrogen does not exclude the possible conversion of organic nitrogen into inorganic nitrogen species during the incubation; it is indeed very likely that in the treatments with bacteria, DON was converted into inorganic forms that were taken up by algae. Our results are in agreement with previous research indicating that the DON cycle in natural waters involves bacterial transformation of DON and subsequent uptake of mineral forms of nitrogen by algae (Stephanauskas et al. 1999; Carlsson et al. 1999). The observed bioavailability of wastewater-derived DON also is in agreement with the bioavailability of DON to bacteria (40-72%) measured in large rivers with possible wastewater discharges (Seitzinger and Sanders 1997).

To gain further insight into the nature of the bioavailable wastewater-derived organic nitrogen, the algal growth bioassays were repeated on molecular weight-fractionated wastewater, in the presence and absence of bacterial inoculum. When inoculated with bacteria, the chl-a production was nearly identical in the unfractionated wastewater effluent and in the 1kDa ultrafiltered wastewater effluent, indicating that the low molecular weight DON accounted for most of the bioavailable nitrogen (Figure 5), even though the  $DON_{initial}$  in the ultrafiltered wastewater was approximately 30% of the  $DON_{initial}$  in unfractionated wastewater (Table 1). The small increase in  $DON_{end}$  in the ultrafiltered sample is possibly due to the excretion of DON by algae. Using the approach for estimating bioavailable DON described previously, we estimate that approximately 25% of the total DON and 57% of the low MW fraction DON were bioavailable when inoculated with bacteria. Therefore, less than 1% of the DON in >1kDa fraction was bioavailable in the inoculated sample.

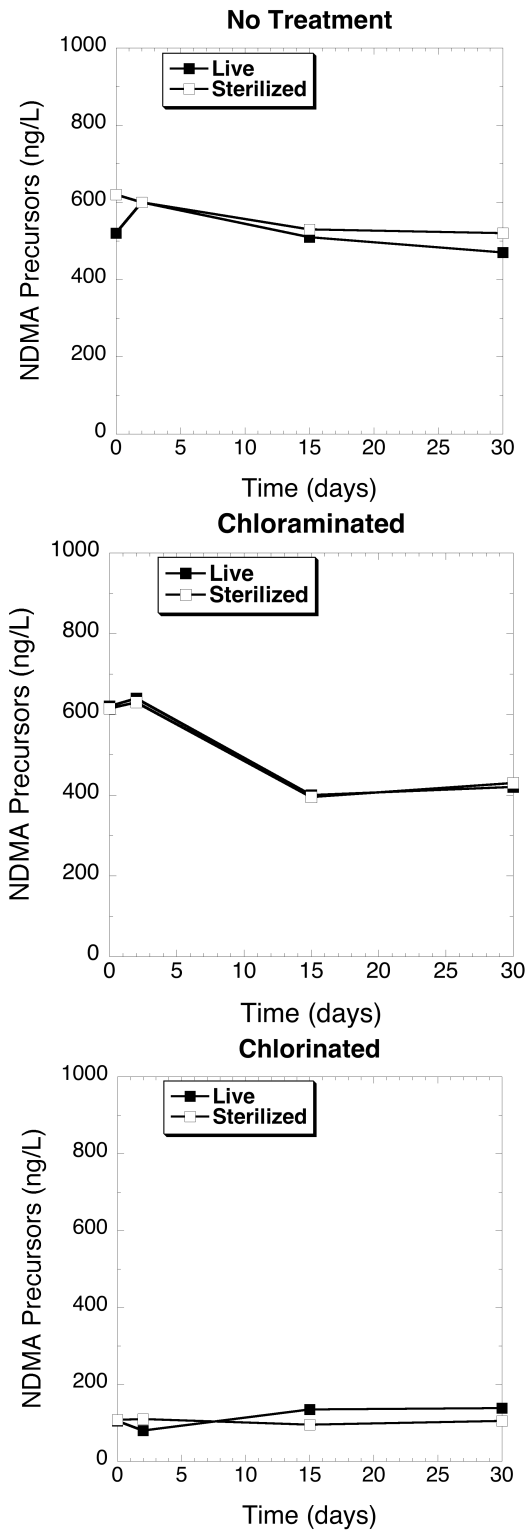
Previous research on DON uptake has suggested that only amino acids are readily directly bioavailable to algae (Anita et al. 1991). As indicated in the previous section, the concentration of free amino acids in wastewater effluent are very low (less than 0.01 mg N/L). Previous research also indicates that bacterial or algal extracellular enzymes present in the non-axenic cultures may hydrolyze DCAA into smaller molecules or liberate ammonium (Stepanauskas et al. 1999). Assuming 5% of the DON consisted of DCAA in the wastewater effluent, the DCAA in the effluent would be approximately 0.04 mg N/L. The sum of these two forms of bioavailable DON (*i.e.*, 0.05 mg N/L) is smaller than the apparent concentration of bioavailable DON observed in the experiments with bacteria addition (*i.e.*, approximately 0.3 mg N/L). Therefore, it is likely that other low molecular weight DON species were converted into bioavailable forms by bacteria during the bioassay.



**Figure 5.** Production of chlorophyll a by algae incubated in wastewater effluent from TMWRF before and after passage through a 1 kDa ultrafilter.

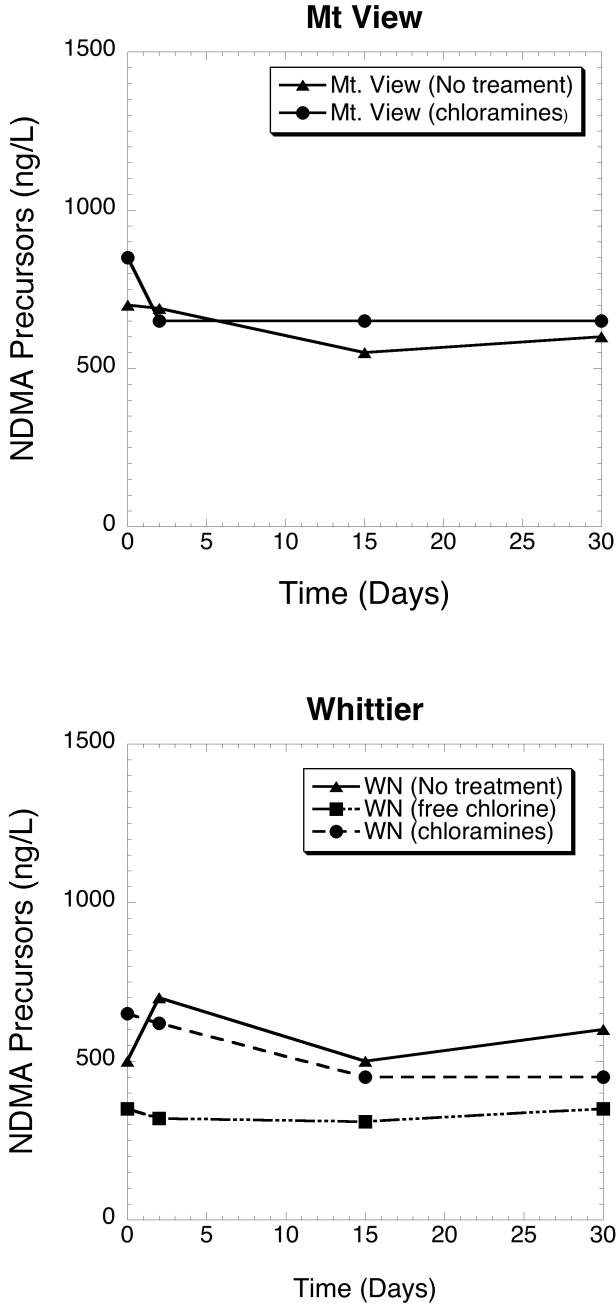
### NDMA Precursor Bioavailability

Experiments on the stability of NDMA precursors indicate that the NDMA precursors are extremely stable in the presence and absence of bacteria (Figure 6). In the experiment conducted with wastewater effluent that had not been subjected to any treatment prior to initiation of the incubation experiment, the NDMA precursor concentration decreased by less than 20% during the 30-day incubation with no apparent difference between the sterilized and live treatments. Decreases in NDMA precursor concentrations of approximately 30% were observed in the sample that was subjected to 1-hr chloramination prior to incubation. These samples also did not show a difference between the live and killed treatments. In the sample that was subjected to free chlorine disinfection prior to incubation, initial NDMA precursor concentrations were much lower than those observed in the other two treatments but no apparent decrease in concentration occurred during the incubation.



**Figure 6.** Effect of incubation of wastewater effluent on NDMA precursor concentrations in samples from the Riverside WWTP. Samples subjected to no treatment (top), chloramination (middle) or chlorination (bottom) prior to initiation of the incubation experiments.

The NDMA precursors also were stable during incubation in samples collected from the Mt. View and Whittier Narrows treatment plants (Figure 7). As was the case in the sample collected from the Riverside WWTP, the samples from the Whittier Narrows WWTP exhibited a lower concentration of NDMA precursors than the untreated or the chloraminated samples. However, for both samples, less than 20% of the NDMA precursors disappeared during the incubations.



**Figure 7:** Effect of incubation of wastewater effluent on NDMA precursor concentrations in samples collected from the Mt. View and Whittier Narrows WWTPs.

The similarity between the NDMA precursor incubation results in the live and killed samples suggests that the modest decrease in precursor concentrations observed in the untreated and chloraminated samples was not attributable to biotransformation. Possible explanations for these results include physical loss of precursors through adsorption on the glass walls of the container or loss through an abiotic process, such as hydrolysis. Furthermore, the stability of the NDMA precursors in samples from the three different sites indicates that NDMA the phenomenon is not limited to one facility.

As noted above, application of free chlorine to wastewater effluent samples results in a decrease in the concentration of NDMA precursors detected at the start of the experiment. The apparent decrease in NDMA precursor concentrations may be due to the addition of chlorine to an amine functional group in the wastewater effluent, which would result in the formation of a N-chloro amine. As indicated by laboratory studies with model NDMA precursors (Mitch and Sedlak, 2002) and studies of NDMA formation in full-scale water treatment plants (Wilczak et al. 2003) N-chloro compounds have a much smaller tendency to form NDMA than amines. The decrease in NDMA formation also could be due to other reactions of the NDMA precursors that also result in destruction of NDMA precursors.

The stability of NDMA precursors contrasts with the behavior of NDMA. Preliminary studies of NDMA biodegradation and NDMA transport in soil and groundwater indicate that NDMA is degraded rapidly by aerobic bacteria. Therefore, when wastewater effluent is applied to soil (e.g., during landscape irrigation) or groundwater (e.g., during soil aquifer treatment) the NDMA is likely to be removed readily while the NDMA precursors will persist.

Under the conditions typically employed in drinking water treatment plants that use chloramines for disinfection, the slope of the relationship between NDMA precursors, as measured by the method of Mitch et al. (2003), and NDMA formation during disinfection is approximately 0.3 (Gerecke and Sedlak 2003). Therefore, 500 ng/L of NDMA precursors (i.e., a typical concentration observed after the 30-day incubation) could result in the formation of approximately 170 ng/L of NDMA, which is more than an order of magnitude higher than the California Department of Health Services Action Level for NDMA (i.e., 10 ng/L).

The calculation described above does not consider the potential removal or retardation of NDMA precursors due to sorption on soil surfaces or the potential for removal of NDMA precursors during drinking water treatment. Therefore, additional research is needed to assess the partitioning of NDMA precursors in the aquatic environment.

## Conclusions

Wastewater derived-organic nitrogen species can be problematic to nitrogen-limited aquatic ecosystems. The research conducted as part of this project has demonstrated that a significant fraction of the wastewater-derived organic nitrogen species in denitrified effluent can stimulate the growth of nitrogen-limited algae if the algal cultures also contain bacteria that convert the organic species into bioavailable forms. These results indicate that it may be worthwhile to control the release of wastewater-derived organic nitrogen in nitrogen-limited ecosystems. At present, few cost-effective techniques are available for controlling the release of organic nitrogen. Therefore additional research is needed to assess methods for removing organic nitrogen from wastewater effluent.

Wastewater-derived organic nitrogen species also can serve as precursors for the formation of the carcinogenic disinfection byproduct, NDMA. Research conducted as part of this project showed that NDMA precursors are extremely resistant to biodegradation. Therefore, NDMA precursors may be released back into indirect potable reuse systems where they could result in the formation of unacceptable levels of NDMA during water treatment. Additional research is needed to assess the fate and transport of NDMA precursors to soil, sediment and filter media.

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