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Determination of Organic Nitrogen in the Aquatic Environment

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Determination of Organic Nitrogen in the Aquatic Environment

Juliana Antunes Galvão, Alexandre Matthiensen, and Marília Oetterer

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18.1 Introduction

18.1.1 Nitrogen in the Aquatic Environment

The main source of nitrogen is the air. When in water media, nitrogen can be divided into suspended and dissolved organic (from a living material) and inorganic (ammonia, nitrate, and nitrite) compounds. There is a huge variety of nitrogen organic compounds in the environment. Organic nitrogen (ON) can also enter systems as bodily wastes, discarded food material, or as components of cleaning agents. The nitrogen cycle further comprises the transformation of ON in inorganic compounds. The conversion of ON in ammonia (NH₃) and then in ammonium ion (NH₄⁺) indicates the onset of oxidation process, which will produce nitrite (NO₂⁻) and then nitrate (NO₃⁻), called the nitrification process. The importance of understanding the presence, and quantifying nitrogen in its various forms in water, refers to the consumption of dissolved oxygen required during the nitrification process and, mainly, in the proliferation of microalgae, whose nitrogen is a vital element in its growth.

Many studies have shown that the contribution of dissolved organic nitrogen (DON) in the aquatic environment can represent more than half the amount of nitrogen, about 60–69% (Bronk, 2002; Berman and Bronk, 2003; Natasha et al., 2008). A greater fraction of DON is bioavailable, providing much of
the nitrogen to oligotrophic systems (Seitzinger and Sanders, 1997). The amount of ON in water samples varies depending on the type of sample and place of origin. Usually the concentrations found are very low, in the order of ppm (part per million). In the aquatic environment, the various forms of ON may be natural (proteins, peptides, chlorophyll, and other organic compounds) and/or have its origins in animals and human activities (domestic and industrial effluents, fertilizers and animal excrement). In domestic sewage, nitrogen is predominantly from albuminous/organic form, followed by free NH$_3$/NH$_4^+$. The presence of nitrogen forms can be associated with the characteristics of the pollution sources in the sample. If the analysis of a polluted water sample from a river or lake demonstrates predominant nitrogen reduced forms (NH$_3$/NH$_4^+$), then the point of pollution is close; if nitrite and nitrate prevail (NO$_2^-$/NO$_3^-$), it means that the discharges of sewage are distant. In a natural river auto-depuration area the presence of ON may be distinguished in the zone of degradation, ammonia in the zone of active decomposition, nitrite in the recovery zone and nitrate in the zone with clean water. Although ON has been considered biologically unavailable, many studies have shown that it also causes impacts on water bodies and participates in the process of eutrophication (Hammer, 1993).

18.1.2 Importance of Nitrogen in Water Quality Control

Nitrogen compounds are macronutrients, and they participate in biological processes. When discharged into natural waters, together with phosphorus and other nutrients present in sewage, they cause environment enrichment, making it more fertile and allowing organism growth to a greater extent, especially microalgae, resulting in the process called eutrophication (Natasha et al., 2008). When the discharge of nutrients is very high, it may result in intense blooms of microalgae. The excessive growth of these populations can lead to environmental problems, compromising the use of water, seriously affecting the public water supply, or resulting in death to aquatic organisms by oxygen depletion from the decomposition of organic matter. The eutrophication control by reducing the inputs of nitrogen is compromised by the multitude of sources, some of them very difficult to be controlled, for example, the fixation of atmospheric nitrogen by phytoplankton.

In practice, the term ON is generally applied to the ON fraction remaining after the nitrate, nitrite, and ammonia/ammonium ion are subtracted. Many processes of sewage treatment employed today are not efficient for the removal of nutrients, and the sewages are quite diverse in organic compounds. Some industrial effluents have more restricted composition, with a differential effect on the ecosystem. The potential ON sources for the environment are already described in the literature, including wastewater, atmospheric deposition, agricultural inputs, combustion products coming from forest and urban areas (Cornell et al., 1995, 1998; Puckett, 1995), as well as by bacteria and phytoplankton (Hammer, 1993; Bronk and Gilbert, 1994; Cornell et al., 1995).

There is no particular target for ON concentration in fresh water; however, a guideline commonly used is 0.26 mg/L. According to the Environmental Pollution and Legislative Regulations (Law 48.1982 & Decree 8, of 1993) National Water Quality and Availability Management (NAWQAM) Project, jointly funded by the Government of Egypt, through the Ministry of Water Resources and Irrigation (MWRI), and Canada, through the Canadian International Development Agency (CIDA), the maximum limit of ON set is 1 mg/L in freshwater bodies (NAWQAM, 2003).

The analytical procedures described for determination of specific components of ON, such as urea, aliphatic and aromatic amines, amino acids, and nitrophenols usually involve enzymatic reactions (Hara et al., 1993; Mana and Spohn, 1996) or complex chromatographic separation processes (Ahel et al., 1992; Queiroz et al., 1992). The determination of ON fractions for monitoring and routine analysis purposes is often conducted using simple methods, using digestion in order to convert the organic fraction into their corresponding inorganic ones (Worsfold et al., 2008).

18.1.3 Presence of ON in Different Types of Water

Seawater contains about 0.5 ppm of nitrogen. This concentration is much smaller in surface area, about 0.1 ppb. Nitrogen concentrations in river waters vary widely, but they are about 0.25 ppm in general. In aerobic waters, nitrogen is mainly present as N$_2$ and NO$_3^-$ and, depending on environmental conditions,
can also occur as N₂O, NH₃, NH₄⁺, HNO₂, NO₂⁻, or HNO₃. Waters from coastal areas primarily contains elemental gaseous nitrogen (N₂). In general, in lakes and marine environments the present ON concentration is less than 1 mg N L⁻¹. In raw sewages, the ON concentrations are easily higher than 20 mg L⁻¹.

The importance of understanding and quantifying the presence of nitrogen in its various species in water is directly related to the consumption of dissolved oxygen required during the nitrification process, that is, “the capacity of the conversion of ammoniacal nitrogen (NH₃/NH₄⁺) to nitrite (NO₂⁻), and this to nitrate (NO₃⁻)” and, particularly, the proliferation of populations of microalgae which utilize nitrogen as a vital element for their growth. It is estimated that in oceanic and estuarine environments about 25–41% of the DIN (mainly ammonia and nitrite) used by the phytoplankton are transformed into DON (Bronk and Gilbert, 1994).

The low molecular weight DON fractions (<1 kDa) correspond to about 70–80% of the DON content in the marine environment (Benner et al., 1997; McCarthy, 1997; Curtis-Jackson et al., 2009). In oceanic environments, ≤14% of DON is at the molecular level, including monomers and small polymers such as urea, amino acids, amines, pyrimidines, nucleosides, and nucleotides (Nguyen and Harvey, 1997). The concentration of total Kjeldahl nitrogen (TKN) in rivers that are not influenced by excess of organic inputs range from 0.5 to 1.0 mg L⁻¹. DON concentrations in surface waters vary ranging from less than 0.1 to larger than 10 mg N L⁻³ (Wertherhoff and Mash, 2002).

18.2 Measurements of ON in Water Samples

18.2.1 Sample Collection and Preservation

The concentration of ON in water is influenced by biological activity and the balance between production and consumption of these compounds. Since low concentrations of ON are expected in natural waters, extra care must be taken on the preservation and integrity of the samples until the time of analysis, including concerns regarding external contaminants, to not interfere in the results.

Several options are available for sampling, depending on the location, fraction to be collected, type, or methodology to be employed. Some samples can be analyzed in the field, but for most of the determinations, they must be stored and preserved to be analyzed later in order to minimize possible losses and changes. External contamination may also occur by leaching of material from the bottle where the samples are stored. Usually the material for storage of samples is made up of glass or some type of polyethylene. For large amounts of sea water samples the storage material is made up of aluminum and stainless steel, as well as Niskin and Go-Flo bottles (Sharp et al., 2002).

Contaminations can be avoided by washing the bottles or flasks before use with diluted neutral detergent, rinsing with distilled water and subsequently washing with 1.2 M HCl, and complete washing repeatedly with distilled water. The flasks may also be dried in an oven at 450°C for 6 h to remove any organic residue (Badr et al., 2003; Worsfold et al., 2005). This procedure may be adopted for all material coming in contact with the sample. Niskin and Go-Flo bottles are used to minimize external contamination.

18.2.2 Sample Preparation

Filtration is one of the most commonly used methods for samples pretreatment, and may be performed before or after freezing the samples to separate organic compounds and eliminate biological activity that can interfere with and alter the balance between organic and inorganic nitrogen (Montluçon and Lee, 2001; Badr et al., 2003; Worsfold et al., 2005). It is essential that the sample be filtered immediately after sampling to avoid chemical changes in the compounds to be analyzed. Filter membranes composed of cellulose acetate and polycarbonate are recommended for separation of dissolved components in natural waters. They must be properly cleaned before use (VernonClark et al., 1995). For the removal of bacteria and phytoplankton, filters of 0.2 μm pore size are used (Worsfold et al., 2005).

The filtration should be preferably performed at low pressure (<20 kPa) to prevent cell lysis. For analysis of DON, filters of 0.7 μm pore diameter are normally used (Sharp et al., 2004), or another type of
membrane with diameters of 0.45 μm or less (Badr et al., 2003). For the analysis of dissolved amino acids, the literature indicates 0.2 μm as ideal membranes, and for combined amino acids, including proteins, filter membranes with 0.45 μm pore diameter are the most suitable ones (Yamashita and Tanoue, 2003). Ultrafiltration has also been applied for analysis of DON in water from lakes (Egeberg et al., 1999), rivers and estuaries (Maie et al., 2006), and marine environments (Powell et al., 2005).

Nanofiltration is a feasible pretreatment for DON measurements with selective NF membranes. Satisfactory DON measurements in synthetic water samples were obtained with optimized operating parameters and the effect of applied transmembrane pressure on DON measurements was negligible. The proposed NF pretreatment method performed similarly for DON measurement as the dialysis method for aqueous samples. At least 20 h of operating time and a large volume of deionized water can be saved, which is beneficial for laboratories undertaking DON analysis (Xu et al., 2010). The centrifugation method is also widely used as a pretreatment of water samples and sediments (Libby and Wheeler, 1994; Badr et al., 2003). This pretreatment is not recommended for water samples if the aim is to determine the presence of free amino acids. These samples should be subjected only to filtration as pretreatment (Fitzsimons et al., 2006).

Other separation methods have been reported in the literature based on molecule sizes, more particularly reverse osmosis and gel-permeation chromatography (GPC), but they have not been used for the separation of colloidal DON (Abbt-Braun and Frimmel, 1999). The use of solid-phase extraction (SPE) as a separation technique for DON is sparse (Lara et al., 1997).

Electrophoresis-based methods have been used for separation of protein nitrogen components from environmental samples (Schimitt-Kopplin et al., 2007). In marine and freshwater proteins are commonly isolated and identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Jones et al., 2004). Precipitation methods are also used as a pretreatment in order to concentrate and purify the samples. The literature describes the use of trichloroacetic acid (TCA) (Tanoue et al., 1996), methanol, chloroform, and water (Powell et al., 2005). Samples with large amounts of sediment and suspended organic matter can be stabilized by short periods of refrigeration (Montluçon and Lee, 2001). The immediate freezing of samples is the simplest method of preservation, ensuring the integrity of the samples for later analysis (Dore et al., 1996). Freezing can be used for samples with high concentrations of sediments and suspended organic matter for long periods, at temperatures ranging from −20°C to −70°C (Cowie and Hedges, 1992; McCarthy et al., 1997). When determining total dissolved nitrogen (TDN) the samples are usually acidified and stored in cold prior to the analytical process itself (Sharp et al., 1995).

Sample alterations due to flocculation can be prevented by homogenization by stirring intensively before analysis. Samples for dissolved free and combined amino acid analysis are generally stored at low temperatures (−20°C) in the dark (Keil and Kirchman, 1991). Pore water samples are treated with N₂ before being subjected to freezing (Pantoja and Lee, 2003).

### 18.3 Analytical Methods

The DON concentration in water samples cannot be quantified directly, but must be calculated by subtracting dissolved inorganic nitrogen (DIN) concentration from the total dissolved nitrogen (TDN) concentration. With the samples mineralized (pre-digested), the TDN can be determined, and the fraction of ON can be quantified by the difference:

\[
[\text{DON}] = [\text{TDN}] - [\text{DIN}]
\]

where DIN is the sum of nitrite, nitrate, and ammonia/ammonium ion nitrogen.

\[
[\text{DIN}] = [\text{N-NO}_2^-] + [\text{N-NO}_3^-] + [\text{N-NH}_4^+/\text{NO}_3^-]
\]

Because the analysis is done by subtraction of the inorganic compound concentrations, analytical errors can occur frequently (Vandenbruwane et al., 2007).
The first step for determining ON is the digestion of the samples to convert the organic compounds to inorganic, mainly nitrate, nitrite, and ammonia. The presence of other compounds such as NO$_3^-$ and N$_2$ are also described in the literature. The predigestion of the sample is the most time-consuming step in the process of ON analysis, and also is the one that has the largest percentage of errors in the whole analytical process. Efforts have been directed in order to develop automation in the process of solid sample digestion using flow injection analysis (FIA) techniques. The aim is to minimize one of the biggest barriers to this analysis, which is the time spent (Aoyagi et al., 1989).

### 18.3.1 Kjeldahl Method

Measurements of nitrogen species in water can be subdivided into two groups. The first corresponds to the dissolved inorganic forms where the analytical operation can be performed by raising the pH of the sample (usually using sodium hydroxide), and proceed the distillation of ammonia with recovery in acid pH. An increase in pH forces the conversion of dissolved ammonia and ammonium ions to ammonia gas, and the sample flask is placed in the heating mantle to cause detachment. After condensation, the distilled ammonia is collected in a boric acid solution flask. The distilled ammonia is finally quantified by titration with hydrochloric acid or sulfuric acid in the presence of a suitable color indicator. Adding the acid, the ammonia/boric acid solution collected tends to recover its original color.

A blank test should be carried out by distilling deionized water from the laboratory according to the same procedure. In the blank test noticeable color change should not occur in the boric acid solution. The solution used in the blank test is used as reference for identifying the titration endpoint. The results are expressed in mg L$^{-1}$ of N. The determination of nitrogen in water by titration is not suitable for concentrations below 1 mg L$^{-1}$.

The second nitrogen species group is the organic fraction. For the determination of dissolved and particulate ON, a prior chemical digestion of the nitrogenous compounds, which are converted into ammonia for the quantification of the TDN concentration is necessary. Based on this preliminary hot digestion using a solution of concentrated acid (usually sulfuric acid) for the decomposition of nitrogen in organic samples, the Danish chemist Johan Kjeldahl introduced the first method for analysis of nitrogen in 1883 (Figure 18.1). The end portion of the condenser is poured into a flask containing an acid receptor. The gaseous NH$_3$ evaporates and condenses in the receptor solution where it is converted again in NH$_3^+$. If the receptor solution is sulfuric acid it results in a new solution of ammonium sulfate; if the receptor solution is boric acid, it results in ammonia-borate complex [NH$_3^+$H$_2$BO$_3^-$]. This procedure takes approximately 1–2 h and leaves behind substances that could interfere with the analysis. A distillation rate of approximately 7.5 mL min$^{-1}$ is often cited in the accepted methodologies.

In short, the samples are digested by reacting concentrated sulfuric acid at high temperatures (360–380°C). Potassium sulfate is added to increase the boiling point, as well as copper (Rodrigues and Pasquini, 1991), mercury (MacKenzie and Young, 1975), titanium, selenium (Rodrigues and Pasquini, 1991) are added as catalytic agents. Improved efficiency is achieved using mercury as catalyst (Kirk, 1950) followed by selenium. The problem with these reagents is their high toxicity and the difficulty in dealing with toxic wastes generated facing today’s requirement of environmental agencies. Both catalysts have been replaced by copper, since its efficiency is also good and the environmental impact is smaller. Carrying only the distillation of ammonia and its titration the ammoniacal nitrogen present in the sample is measured. Making the digestion, distillation, and titration aims at the determination of organic (amines and amides) and inorganic nitrogen, which is called TDN or TKN (total Kjeldahl nitrogen). The name Kjeldahl also became the name of the type of balloon used in the digestion and distillation method of analysis (Tuschall and Brezonik, 1980).

The Kjeldahl method is the most widely used method for determination of ON. It is a standard method adopted by AWWA/APHA and the European Directive (Campins Falco et al., 2008). This method can be applied to samples containing high and low concentrations of ON. In case of very low concentrations large volumes of sample are required. Although this method is widely used for all kinds of water, it is time-consuming and the reagents used are toxic or considered environmentally aggressive.

Depending on the expected concentration of nitrogen in a sample, the method can be selected for macro- or micro-Kjeldahl variation. The term macro-Kjeldahl is applied when the sample volume is
between 25 mL (at concentrations of 50–100 mg N L\(^{-1}\)) to 500 mL (at concentrations of 1 mg N L\(^{-1}\) or less). The term micro-Kjeldahl is applied when the sample volume is between 5 mL (at concentrations of 40–400 mg N L\(^{-1}\)) and 50 mL (at concentrations between 4 and 40 mg L\(^{-1}\) or less). Basically, the difference between the macro- and micro-Kjeldahl methods is in the amount of sample and reagents used and the laboratory scale apparatus.

The Kjeldahl method was first developed for the determination of nitrogen content in biological samples, and certain aspects must be considered when applied to other matrices. The method is adapted for the determination of amino acids and primary amines, however, other ON compounds such as nitro derivatives, semi-carbazones, and some refractory tertiary amines cannot be converted into ammonium ions, resulting in errors when present in the sample. Although TKN is often called total nitrogen (TN), this does not convert nitrite and nitrate to ammonium ions. So, if TN of the samples must be quantified, further reduction conversion should be carried out for these anions to ammonium ions (Ginkel and Sinnaeve, 1980; Aoki et al., 1986).

Some procedures have been described in order to overcome the drawbacks of the method, particularly regarding the use of toxic reagents and the time spent in the determination process. Among them the use of hydrogen peroxide, allowing a shorter period of digestion and eliminating the use of metal catalysts (Ginkel and Sinnaeve, 1980). The improvement on the accuracy of the method is also described by avoiding the formation of metallic ions of ammonia.

Raimbault et al. (1999) adapted the Kjeldahl method for sea water samples analysis working on board, reporting 5% accuracy. However, the method is not suitable for areas impacted by human action (samples at concentrations \(\geq 700 \mu\text{mol N-NO}_3^-\text{L}^{-1}\)) due to nitrate interference in the determination of DON. The combination of sulfuric acid, hydrogen peroxide, and microwave radiation has been used for the determination of Kjeldahl nitrogen in food (Bermond and Ducauze, 1991; Feinberg et al., 1993). However, for water analysis its use is still scarce (Jassie and Kingston, 1988).
Besides titration, the ammonium ions derived from the Kjeldahl digestion can also be determined using colorimetric methods based on the reaction of Berthelot (Searle, 1984), in which ammonium ions react with phenol and hypochloride in alkaline media, resulting in the formation of indophenol blue, or by the Nessler method (Nessler, 1856), based on the reaction between ammonia and tetraiodomercuriate, with absorbance read at wavelengths between 400 and 425 nm. Still, it can be determined by potentiometer using selective electrodes for ammonium ions.

There are few references describing the use of FIA for determination of Kjeldahl nitrogen by using continuous digestion. Continuous digestion systems were described using a helicoidal thermostat at 300°C (Davidson et al., 1970). Metal catalysts are added to a mixture of sulfuric acid and hydrogen peroxide, and the resulting ammonium ions are analyzed using the Berthelot method. The digestion time is only 5 min, resulting in 70–100% recovery of the nitrogen compounds. This method has been used for analysis of TN in the water samples in the range of 0.01–0.5 mg N L⁻¹.

One of the most common problems in FIA-Kjeldahl digestion is clogging of the pipes by salt deposits in the digester and the low recovery of the compounds due to replacement of the catalyst salts (Davidson et al., 1970). To minimize these barriers for routine analysis, some authors have worked with semi-automatic systems, where the digestion is carried out discontinuously in a digester block while the determination of ammonium ions is performed using FIA techniques using electrodes sensitive to ammonia or ammonium ions (Lima et al., 1997), reaction of Nessler (Ginkel and Sinnaeve, 1980) or methods based on the reaction of Berthelot (Basson, 1982). Many devices aiming at interference reduction have been proposed to automate the distillation of ammonium ions prior to submission to FIA (McLeod, 1992a; McLeod, 1992b). Tests were also made using hydrogen sulfide coupled to photometer (Ginkel and Sinnaeve, 1980), potentiometer (Lima et al., 1997), and conductivimeter (Rodrigues and Pasquini, 1991). Microwave digestion has also been used for ON determination. The required analysis time and the amount of reagent are smaller, compared to traditional methods of digestion, and can be applied to different matrices (Burguera and Burguera, 1998).

Campins Falco et al. (2008) developed a microscale Kjeldahl nitrogen determination for environmental waters and showed that it was possible to reduce the size of the macro-Kjeldahl system and the sample volume without losses of sensitivity in the nitrogen Kjeldahl determination using a macro digester. The procedure proved to be faster, cheaper, easier to handle, more environmental friendly, and efficient and accurate in the application to real water samples at the same concentration levels as those obtained by macro Kjeldahl system.

### 18.3.2 Photo-Oxidation

The photo-oxidation method based on the oxidation of the water samples using hydrogen peroxide and ultraviolet (UV) was first proposed by Armstrong et al. (1966) for the determination of carbon, nitrogen, and organic phosphorus. When oxidants plus UV radiation are used ON and ammonium ions are converted into nitrates and nitrites by the Griess reaction (Sun et al., 2003), and are easily quantified by molecular spectroscopy.

Photooxidation process is quite complex. Reactions are influenced by several factors, such as pH, and may be potentiated by the presence of certain compounds. Some studies show that exposure to UV radiation may form a large number of potentially oxidizing molecules. This may explain the discrepancy of the analytical results under different operating conditions (Golimowski and Golimowska, 1996). The most important organic matter oxidants are the hydroxyl radicals, which can be generated directly from the reaction of hydrogen peroxide with low UV radiation. The most commonly used oxidants in UV mineralization during the quantification of total nitrogen, phosphorus, carbon, or quantification of metal ions are hydrogen peroxide and persulfate. In these cases, the UV radiation acts as catalyst for the oxidation reaction. When exposed to low UV radiation the hydrogen peroxide is decomposed to form hydroxyl radicals, starting a chain reaction involving the organic compounds present in the sample. Under experimental conditions 70% of the nitrogen found in the samples is in the form of nitrate, which under prolonged radiation can be decomposed to nitrite. This method has been applied to the analysis of ON in seawater within a range of 0.002–0.015 mg N L⁻¹.

Since the first publication of this methodology (Armstrong et al., 1966) some modifications have been established in order to improve its applicability and accuracy by changing the high-pressure lamp for
a medium-power mercury lamp (Amstrong and Tibbitts, 1968), working in semi-automatic methods (Henriksen, 1970), and using the digestion process in different steps (Gustafsson, 1984). Other authors have proposed a method for the determination of dissolved nitrogen in water by adjusting the pH with boric acid and reducing the time of digestion to 1.5–3 h, depending on the type of matrix to be examined, type of nitrogen found in the sample, and concentration of inorganic salts (Manny et al., 1971). Also, some authors use potassium persulfate as the oxidizing agent instead of hydrogen peroxide, because the UV radiation at 254 nm catalyzes this compound to sulfate and other reactive compounds such as oxygen and hydroxyl radicals, which in turn oxidizes organic compounds (Golimowski and Golimowska, 1996).

UV oxidation has also been performed along with FIA system (McKelvie et al., 1995) and semi-automated systems (Oleksy-Frenzel and Jekel, 1996), using reactors made of quartz or teflon coils. These systems reduce the digestion time to a few minutes (Lowry and Mancy, 1978). Many of the analytical system described in the literature do not allow the differentiation of organic from inorganic nitrogen, so results are often expressed as TDN. In analytical FIA systems, the nitrite formed in the sample after the oxidation by UV radiation is reduced to nitrate with hydrazine sulfate, and further analyzed using the Griess reaction. The system is designed as an analytical multiparameter and allows the analyst to select the compounds to be analyzed separately. It has good efficiency above 10 mg N L⁻¹ for all nitrogen compounds except for EDTA, barbituric, and aspartic acid, with reproducibility around 1.5–3%. The interference of the organic carbon in the determination of ON was also studied. Interference was not found when the ratio of carbon:nitrogen is up to 1:20 in the sample to be analyzed (McKelvie et al., 1995).

Nur and Nesuhi (2007) investigate the effect of metal ions on titanium dioxide (TiO₂)-mediated photocatalytic oxidation for the determination of dissolved ON compounds. A model using ethylenediaminetetra acetic acid to DON compounds was studied. At pHs 2, 5, 7, and 10, aqueous EDTA solutions were irradiated at 254 nm in the presence of Fe²⁺, Cu²⁺, Zn²⁺, Ni²⁺, or Co²⁺ ions. The sum of produced nitrate, nitrite, and ammonium ion concentrations gave the total oxidation recovery. At low pH, the photocatalytic oxidation recoveries of Fe-EDTA, Ni-EDTA, and Co-EDTA were significantly lower than the photocatalytic degradation of EDTA. The presence of free Fe²⁺, Ni²⁺, and Co²⁺ ions decreased the photocatalytic oxidation recovery. The NH₃/NO₃ ratio was higher for Cu-EDTA.

FIA multi-parameter systems can be used for determination of nitrogen, except ammonia, because there is the need for thermal treatment before submission to FIA. Comparative studies showed good correlation between photooxidation and Kjeldahl methods. Their reproducibility is higher, analyzing up to 30 samples day⁻¹, resulting in lower costs compared to Kjeldahl. A segmented system analysis based on UV oxidation after separation of the organic fraction using gel permeation chromatography was proposed by Oleksy-Frenzel and Jekel (1996), allowing the sequential determination of dissolved nitrogen and carbon.

### 18.3.3 High-Temperature Combustion

In high-temperature combustion (HTC) the determination of ON is achieved by oxidative pyrolysis at temperatures ranging from 650°C to 1100°C. At lower temperatures (650–900°C) it uses platinum as catalyst. During pyrolysis all forms of nitrogen are transformed into nitrogen monoxide (NO), and quantified by chemiluminescence (Clifford and McGaughey, 1982; Walsh, 1989). The effectiveness of this type of method depends on the experimental conditions, and the nitrogen compounds present and the matrix to be analyzed. The method is attractive because it allows to determine all forms of nitrogen, is extremely sensitive and, using automated equipment, it is possible to analyze a sample in 2–6 min. In these methods the rate of conversion of ON is variable, depending on the nature of the samples and the instrumental conditions, although it is less critical when compared to photo-oxidative methods which require greater control on the temperature and catalytic methods. The limit of detection may be below 1 μM (Badr et al., 2003).

HTC is normally used for freshwater, estuarine, and marine analysis, as well as pollutants from industrial sewage. Researchers have studied the oxidation rate of many nitrogen compounds and reported that the conversion rate of ON using platinum as catalyst ranged from 0 to 10 mg N L⁻¹ (Clifford and McGaughey, 1982). The analytical rate can be improved to values greater than 40 mg N L⁻¹ without interference of organic carbon, increasing the amount of oxygen as the carrier gas. Compared with the Kjeldahl method HTC shows excellent results for wastewaters.
Kaori et al. (2007) have studied the conversion efficiency of the HTC technique for total dissolved nitrogen analysis testing five different catalyst materials. The materials included four metallic catalyst and quartz beads. The slopes of the standard calibration graphs were lowest for TDN determination on the quartz beads. Using this catalyst, poor recoveries were obtained for a range of ON compounds. Among the metallic ones, 0.5% Pt-alumina material was an efficient catalyst for TDN analyses.

Daughton et al. (1985) studied the process of pyrolysis in 56 nitrogen compounds and reported conversion rates of 90–110%. When applied to industrial effluents the values acquired using HTC method were 10% higher compared to the Kjeldahl method, probably because certain compounds can be detected only by the HTC method. HTC is also suitable for low nitrogen concentrations (0.07–0.6 mg of TDN), consistently found in seawater samples. When the performance of this method is compared to the photolysis method, apparently no great differences in the results were found. However, 18–22 h of irradiation are required to mineralize the samples with the UV radiation (Walsh, 1989).

Although compared to other mineralization methods HTC results more effective, and the equipment used in this method is more sophisticated compared to the Kjeldahl UV oxidation. Rogora et al. (2006) did not find statistically significant differences between peroxodisulfate digestion and HTC methodologies when analyzing TDN in 800 freshwater samples. Bronk et al. (2000) have compared different methodologies: peroxodisulfate digestion, UV oxidation, and high-temperature catalytic combustion (HTCC) for the determination of TDN using representative model compounds of DON in natural waters, and the results were similar for the methods tested. Shi et al. (1996) have proposed the separation of the organic components from the sample by chromatographic system using supercritical fluid with CO_2 as the mobile phase, and the introduction of eluents in the pyrolysis furnace. The nitrogen compounds were transformed into ON and detected by chemiluminescence.

Some authors have proposed the determination of ON after reduction to N_2. After oxidative pyrolysis at high temperatures (850–950°C) in a quartz tube, the resulting nitrogen oxides are reduced to molecular nitrogen after undergoing 650°C oven temperature, catalyzed with the addition of metallic copper, and detected by conductivity. Following this same methodology other authors have tested the experimental conditions of oxidation, as well as its effects on the different chemical structures of the nitrogen compounds (Pietrogrande et al., 1993). Sharp et al. (2004) conducted instrumental comparisons using seawater samples from estuary, coast, and offshore. Combustion at 680°C with the use of catalysts was reported as the best results. Higher temperatures resulted in equipment damage and reduced analytical precision, perhaps due to sublimation of NaCl (Badr, 2005).

The HTC method has the same disadvantages observed in other methods such as peroxodisulfate digestion and UV oxidation. Once they are used to determine TDN, and DON is determined by the subtraction of fractions, it results in less accurate measurements when large amounts of DIN are present in the samples (Sharp et al., 2004). To fix this factor, Lee and Westerhoff (2005) used dialysis as the pretreatment in order to reduce the concentration of DIN, thus minimizing errors regarding the analysis of DON.

As frequently observed, some authors have proposed a mix of the existing methodologies. Daughton et al. (1985) have tested the combination of separation by chromatographic methods and gas diffusion, allied to the HTC methodology. First, the separation of polar and nonpolar nitrogen compounds by using reverse-phase C_{18} columns is performed. Polar compounds, particularly inorganic salts, are eluted from the column, while the less polar ones, as aromatic amines and N-heterocycles, are retained. The results of this operation underestimate ON because only the polar ON is actually measured. The separation of volatiles and nonvolatile compounds is performed by gas-diffusion membranes. The quantification of the nonvolatile polar-fraction ON compounds, together with the nonpolar nitrogen, provides a high estimate of the ON in a given sample, although certain organic substances are simultaneously polar and volatile, such as the aliphatic amines, and should be excluded from the calculation. This method also presents similar results when compared to Kjeldahl.

### 18.3.4 Alkaline Persulfate Oxidation

The first report of methodology using potassium persulfate to oxidize nitrogen compounds in water samples was described in 1969 (Koroleff, 1969). In this method of alkaline digestion (pH about 13) also controlled temperature (120°C) and high pressure (2 bar) were used. It is a faster and simpler method
when compared to Kjeldahl. In addition to natural water (Smart et al., 1981) this method is also used to seawater matrix samples with low nitrogen concentration (1 mg L\(^{-1}\)) (d’Elia et al., 1977). The release of oxygen provides optimal conditions for the oxidation of nitrogen compounds in the samples, resulting in nitrate formation. Then the nitrate can be quantified by several methods.

This method provides high recovery and a good reproducibility in a wide variety of nitrogenous compounds such as urea, EDTA, inorganic salts of ammonium ions, amino acids, including compounds capable of withstanding high temperatures (refractory compounds) that are not mineralized by the Kjeldahl method, such as nitropyridine compounds. However, as other methods using digestion, when alkaline persulfate is applied to compounds containing N--N bonds or NH--C groups, a low recovery is produced or even the complete oxidation of nitrate is prevented (Ebina et al., 1983).

FIA systems have been used for the determination of TN using microwave digestion. The sample, with the reagents, are irradiated and mineralized while digestion occurs (Dafner et al., 1999). The resulting nitrate is reduced to nitrite using hydrazinium sulfate, and the nitrite is analyzed by Shinn–Griess reaction. The linear range is between 0.3 and 20 mg N L\(^{-1}\), the limit of detection is 0.2 mg L\(^{-1}\), and the relative standard deviation of the method is 3% for samples containing 5 mg N L\(^{-1}\). The complete procedure of analysis (digestion and subsequent determination of nitrate and nitrite) takes approximately 2 min, obtaining a frequency of 45 samples h\(^{-1}\) (Cerdà et al., 1997). Other FIA methodologies have been described in which analytical times lesser than 5 min, high levels of mineralization, and selenium (Aoki et al., 1986) and platinum (Aoyagi et al., 1989; Goto et al., 1988) as catalysts were achieved. Egeberg et al. (1999) have used peroxodisulfate digestion and ultrafiltration to study the distribution of DON compounds in seawater.

### 18.3.5 Chromatography

Chromatography has been widespread used for analysis of various compounds, including the nitrogen compounds. For direct ON determination both gas and liquid chromatography have been used.

Gas chromatography (GC) has been used for the analysis of methylamines, largely present in the aquatic environment and used as substrate for algae and bacteria (Gibb et al., 1995). Reports from literature suggest that there are major problems concerning the adsorption in the solid-phase column causing problems during the analysis. The low reproducibility of the methodology, however, can be minimized by treating the column with KOH or NaOH (Dacosta et al., 1990). Most of the GC techniques available for ON compounds are facing free amines (FitSimons et al., 1997). Some efforts have been made for the development of analytical methodology for ON compounds using capillary GC. Different materials and the interaction of capillary GC with SPME techniques were tested. The method resulted faster and showed good reproducibility, but with limited application for samples in the presence of trace amounts of amines (Abalos et al., 1999).

Mass spectrometry (MS) coupled with GC is used routinely for the detection of organic molecules, but is of limited use to ON due to the low volatility of these molecules (Petritis et al., 2000).

High-performance liquid chromatography (HPLC) can be used for separating and detecting volatile and nonvolatile ON compounds. The samples are introduced in aqueous solution and the analytes are separated on a packed column using either isocratic or gradient elution. A range of HPLC detectors are available but most aquatic methods use fluorescence (FL) detection for maximum sensitivity the FL detectors are at least an order of magnitude more sensitive (fmol) than other detectors (Worsfold et al., 2008).

Free amino acids are usually analyzed by HPLC after a derivatization pretreatment using fluorescing reagent. Some reagents have been described in the literature, taking into account its stability and the presence of interfering substances in the sample, the most cited are ortho-phthalaldehyde (OPA) (Lindroth and Mopper, 1979), dimethylaminoazobenzene sulfonyl chloride (DABS-CI) (Stocchi et al., 1989) and 6-aminouquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Jorgensen and Jensen, 1997). Combined free amino acids can also be analyzed by HPLC, but cannot be quantified at the molecular level. The samples must be first hydrolyzed in order to break the peptide bonds (Jorgensen and Jensen, 1997).

Curtis-Jackson et al. (2009) developed a method for the extraction and analysis of low molecular weight (LMW) peptides in saline waters. The procedure involved an SPE pre-concentration step using a polystyrene-divinylbenzene sorbent, then elution and detection by liquid chromatography—electrospray.
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ionization–mass spectrometry (LC/ESI/MS). The procedure was tested in water samples containing 10 ng L\(^{-1}\) of peptides ranging from 188.2 to 1946.0 Da. The analytes were characterized using “top down” sequencing to confirm their structure. This method allows for the detection of peptides at the ng L\(^{-1}\) level and further pre-concentration is possible. The SPE step allowed determination of peptides from saline water, a matrix incompatible with direct LC/MS analysis. This method is recommended to DON characterization studies.

### 18.3.6 Gel Electrophoresis

This methodology is used for the characterization of DON with high molecular weight, such as proteins and peptides. In oceanic environments amino acids comprise the largest portion of DON compounds (Sharp et al. 1983), and this methodology has been extensively used for protein identification in aquatic systems (Jones et al., 2004; Yamada and Tanoue, 2006). The main electrophoresis gels used are SDS-PAGE and two-dimensional polyacrylamide (2D-PAGE) (Jones et al., 2004; Powell et al., 2005). Such methodology requires large amounts of samples, about 20–60 L which first need to be filtered and then subjected to ultrafiltration (glass micro-filter or 0.2 µm pore size filters). The molecular weight of the compounds removed during the ultrafiltration are around 10–14 kDa and the samples were reduced below a volume of 1 L. The limit of detection for SDS-PAGE and 2D-PAGE is about 10 pg (Suzuki et al., 1997). The total protein concentration of a given sample is previously subjected to gel electrophoresis to ensure standardization of gel preparation. This is done by the Lowry protein assay (Jones et al., 2004) or densitographically (Suzuki et al., 1997).

Proteins are visualized in both Coomassie Brilliant Blue staining or silver ions electrophoresis techniques, the latter being the most sensitive technique (0.1–1.0 ng) (Jones et al., 2004; Powell et al., 2005; Yamada and Tanoue, 2006). Coomassie Blue shows sensitivity of 1–10 ng. After the coloration the gels are scanned by a densitometer (Jones et al., 2004). Due to their molecular weight, peptides cannot be analyzed by this technique (Worsfold et al., 2008).

### 18.3.7 Review of Other Methodologies

#### 18.3.7.1 Nuclear Magnetic Resonance Spectrometry

This methodology has been employed for the analysis of complex organic structures such as humic acids in environmental samples (Kovac et al., 2002; Pant et al., 2002), but the vast majority of these studies have been conducted in solid state using cross polarization and magic angle spinning (CP-MAS) (Mao et al., 2002). Solid-state \(^{15}\)N has been used in order to detect amide-N in environmental samples (Worsfold et al., 2008). Gerard et al. (2010) analyzed \(^{15}\)N-DON, however, the analysis has been hindered by the lack of simple reliable and established methods. Three off-line techniques for measuring \(^{15}\)N signature of DON in the presence of inorganic N using persulfate digestion followed by microdiffusion were evaluated. The \(^{15}\)N-DON signature was calculated from the difference between total dissolved \(^{15}\)N (\(^{15}\)N-TDN) and inorganic \(^{15}\)N. The \(^{15}\)N recovery and the signature of DON, NH\(_3\) and NO\(_3\) in a series of inorganic N/DON mixtures (with a TDN concentration of 10 mg N L\(^{-1}\)) for three lab protocols were quantified. Phenylalanine was used as a model compound for DON. The best lab protocol determined the concentration of inorganic N and TDN prior to diffusion using improved spectrophotometric techniques. An accuracy of 88% for \(^{15}\)N-DON should be routinely possible; and the coefficient of variation was <2.9%. Hence, reliable \(^{15}\)N-DON values are obtained over a DON concentration range of 2.3–10 mg L\(^{-1}\). High levels of DON could influence the accuracy of \(^{15}\)N-NO\(_3\) mainly at DON:NO\(_3\) ratios above 0.4.

Urumu et al. (2008) presented a method for high sensitive isotopic analysis of particulate organic nitrogen (PON) in fresh water and sea water, for the purpose of determining the aquatic nitrogen fixation rate through the \(^{15}\)N\(_2\) tracer technique for samples that contain low abundance of organisms. The method is composed of the traditional oxidation/reduction methods, such as the oxidation of PON to nitrate (using persulfate), the reduction of NO\(_3\) to NO\(_2\) (using spongy cadmium), and further reduction of NO\(_2\) to N\(_2\)O (using sodium azide). Then nitrous oxide was purged from the water and trapped cryogenically with subsequent release into a gas chromatography column to analyze the stable nitrogen isotopic composition.
using continuous flow isotope ratio mass spectrometry (CF-IRMS) by simultaneously monitoring the NO$^+$ ion currents at masses 30, 31, and 32. This method can be used also to determine the nitrogen isotopic composition of ON in general, such as that of total dissolved nitrogen.

### 18.3.7.2 X-Ray Spectrometry

This methodology is used to analyze the chemical surface of a particular sample or material, not being usually applied to organic analysis, but may be useful in order to provide information about pyrolic, pyridinic, quaternary, and aromatic amines. The x-ray spectrometry combined with other methodologies have presented interesting results in the study of ON compounds (Patience et al., 1992).

### 18.4 Future Trends

When the subject is about to use and develop new methodologies, there is constant concern in the accuracy, reproducibility, and robustness of the method in question. There is a long way to go for statistical validation of the results from a particular new methodology. So far a definitive methodology with direct results of DON in environmental water samples has not yet been developed. Research efforts in this direction have to be prioritized, given the importance of this determination for the analysis of environmental factors. Worsfold et al. (2008) point out that improvements in the methodologies for the determination of DON are needed.

Another bottleneck of this type of determination is the preservation of samples prior to analysis. Worsfold et al. (2008) suggests the use of passive, diffusive sampling devices as sample preparation for analysis of DON, however, suitable protocols need to be developed. A major concern of scientists is the loss of the molecular fractions during traditional determinations. So, the efforts have been focused on developing better methodologies regarding the separation of their components and instrumentation, aiming at the characterization and identification of individual protein moieties.

Regarding the aquatic matrices, the wastewaters are the most problematic to be analyzed for DON, and LC-MS methods associated with appropriate pretreatments of the matrices are fundamental tools to be used (Pehlivanoglu-Mantas and Sedlak, 2006). Khan et al. (2009) have developed a methodology for wastewater evaluation of biodegradable dissolved organic nitrogen (BDON). The method adopts the approaches used in the biochemical oxygen demand to make it usable as a routine procedure at wastewater treatment plants. Sara et al. (2011) developed a model that can be used to simulate possible measures to reduce the nitrogen load and, after some modification and recalibration, it can be applied at other nine sites affected by N rich effluents. The study of modeling nitrogen in water is also very important.

Recently, micro sensors based on enzymatic reactions have been developed to measure the amount of protein in river water and effluents (Marrakchi et al., 2005). The combination of mass spectroscopy, immune precipitation, and purification techniques appears to be very promising for the study of enzymes of interest in the aquatic environment. Proteomic technologies have been successfully used in the environmental research area, for example, using techniques of electrophoresis in order to isolate marine proteins (Jones et al. 2004).

Regarding atomic emission new technologies, continuous flow sample introduction technique with a hydride generator system in conjunction with an inductively coupled emission spectrometer (ICP-AES-HG) was used for quantitative determination of organic bound nitrogen in aqueous samples. This method proved reliable and faster than the conventional and tedious Kjeldahl method, superior to the ICP-AES spray chamber method, and almost free from matrix interference, which is usually a critical factor in atomic emission spectroscopic techniques (Jaber et al., 2009).

Also, the development of microscale chemistry in laboratory is a new tendency, which brings reduced amounts of chemicals used, safe and easy making techniques, and miniature labware (Skimer, 1998). There is a trend to use microscale chemistry equipments to carry out the analysis because it is more environmental friendly and based on the green chemistry approach (Campsins Falco et al., 2008). However, few applications have been truly developed in the quantitative analytical field.

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