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Total Nitrogen (TN) Analysis by Combination of Nitrate Biosensor and Electrochemical Nitrification Reactor

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A biosensor based on nitrate reductase (NaR) can detect nitrate at the level from 50 to 250 mM in one minute but is limited to the nitrate analysis. The social requirement for real time analysis of total nitrogen (TN) contained in water has been increased. However, no system for the real time analysis of TN has been developed. To analyze TN with the nitrate biosensor, we developed an electrochemical nitrification reactor, by which organic nitrogen compounds (organic Ns), ammonium can be oxidized to nitrate. Nitrification could be reached up to 98% of ammonium and 80% of organic Ns. In this study, the real time TN analyzer applicable to both laboratory and field was accomplished by combination of the nitrate biosensor and the electrochemical nitrification reactor.

Key words : Nitrate biosensor, nitrate reductase, electrochemical nitrification, total nitrogen analysis, real time analysis system

1. Introduction

Most countries have a strict guideline of nitrate content in drinking water, natural water (stream, river and lake, etc.), and effluent water discharged from wastewater treatment system. The quantitative analysis of various factors for determination of water contamination degree has been depending upon physicochemical systems, which have been mainly developed and improved for the laboratory application.[1,2] Recently, a portable system for real time monitoring of flowing water such as stream and river has been required.[3,4] Real time analysis of flowing water is required to protect the source of water supply. The biosensor based on enzyme is the most proper system for the real time analysis because of basically portable, rapid analysis and no interferences by other contaminants.[5-12] The nitrate reductase (NaR) can selectively catalyze nitrate reduction to nitrite coupled to oxidation of artificial electron donor such as methyl viologen (MV) or benzyl viologen instead of NADH.[13-14] This is an advantageous property of the NaR to be applied to the biosensor.

Meanwhile, nitrification of organic Ns, ammonium and nitrite is required to analyze total nitrogen (TN) with nitrate biosensor. According to the standard method, combined treatment of heating (autoclave) and chemical oxidant have been used to nitrify nitrite, ammonium and organic Ns.[15] It is a limiting factor to develop a portable nitrification system. A portable nitrification reactor has to be structurally simple and operated with battery. In this study we developed a portable nitrification reactor for ammonium and organic Ns based on electrochemical redox reactions and accomplished a real time TN analysis system by combination of the nitrate biosensor and the electrochemical nitrification reactor.

2. Experimental Section

2.1. Nitrate reductase

A nitrate reductase (NaR) was purchased from NECi (Item no, YNaR-1, The Nitrate Elimination Co. Inc.,

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Michigan, USA, www.nitrate.com).

2.2. Cyclovoltammetry

The cyclovoltammetry was carried out with a cyclovoltammetric potentiostat (BAS model 50W, USA) by employing of Ag/AgCl reference electrode, glassy carbon working electrode (3 mm diameter) and platinum wire counter electrode (0.5 mm diameter and 40 mm length) under anaerobic Ar atmosphere. Reaction mixture was basically containing MV, sulfite, MOPS buffer, NaR and different concentration of nitrate. Scan range was adjusted from -300 mV to -1000 mV vs. Ag/AgCl and scan ratio was adjusted to 25 mV/s.

2.3. Composition of sensor strip and sensing mechanism

A sensor strip was prepared by screen printing of carbon paste for working (WE) and counter electrode (CE) and AgCl/Ag for reference electrode (RE) as shown in Fig. 1(A). Ag bottom was previously immobilized on plastic base by electroplating method to make Ag/AgCl reference electrode. Methyl viologen (MV_{red}) electrochemically reduced functions as a reducing power instead of NADH, from which the electrons can drive to nitrate through the redox reaction of FAD, Heme-Fe and Mo-protein. The potential difference between MV_{red} and FAD, FAD and Heme-Fe, Heme-Fe and Mo-protein, and Mo-protein and nitrate is about 250 mV, 60 mV, 140 mV and 430 mV, respectively, which are all enough to produce negative free energy ($\Delta G = -nFE$, F: Faraday constant, E: redox potential difference between redox couples) for spontaneous redox reaction as shown in Fig. 1(B).[16] Consequently, electrochemical reduction of MV and enzymatic nitrate reduction to nitrite is spontaneous coupling reaction, in which the current consumption may be proportional to the nitrate concentration.

2.4. Immobilization of NaR on sensor strip

NaR was immobilized on working electrode by polymer entrapment with polyvinyl alcohol (PVA) as a binder. Methyl viologen (MV) and MOPS were co-immobilized with NaR as an electron mediator and buffer, respectively. 10 μL of enzyme mixture containing MV, MOPS buffer, 1% polyvinyl alcohol and NaR was dropped on working electrode of sensor strip and

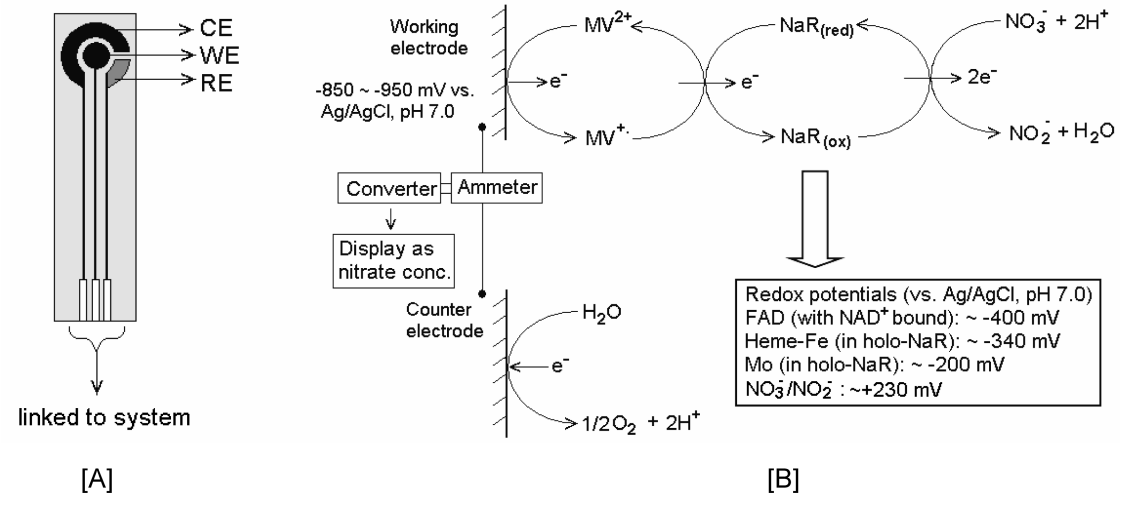

Fig. 1. Structure of disposable sensor strip (A) composed of working electrode (WE), counter electrode (CE) and reference electrode (RE), and mechanism of enzymatic reduction of nitrate to nitrite by NaR (B). 25 mU NaR is co-immobilized with 1mM methyl viologen, 50 mM MOPS, 1 mM sulfite on SPCE by entrapment using 1% poly vinyl alcohol, which is the biosensor strip. The nitrate and reduced methylviologen (MV^+) functions as an electron acceptor and an electron donor, respectively. The oxidized methylviologen (MV^{2+}) is electrochemically reduced and reoxidized coupling with reduction of nitrate to nitrite. The redox reaction of methylviologen is proportional to the nitrate concentration.

then dried at room temperature (~25°C). The dried NaR on sensor strip was stored in container tightly sealed. Sulfite is an oxygen scavenger but can not be immobilized to electrode because it is spontaneously oxidized to sulfate under aerobic condition. Instead 2 μl of 15 mM fresh sulfite solution was dropped on sensor strip before 30 μl of sample was loaded on sensor strip.

2.5. NaR assay and stability

Activity of NaR immobilized on the sensor strip was continuously analyzed during storage for 42 days at the intervals of 7 days. After 30 μl of sample containing nitrate was loaded, the current consumed coupled to nitrate reduction to nitrite was measured for 30 seconds, which was automatically converted to coulombic value by microprocessor equipped in nitrate biosensor system. Stability of NaR was estimated by difference of coulombic consumption measured at the appointed times.

2.6. Nitrification reactor

A reactor was separated into two parts dilution vessel (10~20 ml) and reaction vessel (1 ml), in which two titanium (8 × 8 mm) sheets were horizontally set up at bottom and zenith, and eight platinum plates (3 × 5 mm) were vertically set up as shown in Fig. 2. Combination of titanium and platinum is proper for nitrification of ammonium and combination of platinum and platinum is proper for nitrification of organic Ns. Anode and cathode were exchanged at the intervals of 15 seconds. The electric voltage charged to the electrode and reaction time was optimized by using relationship between nitrification efficiency and concentration of nitrogen compounds. Ammonium chloride and glycine was used as ammonium standard and a representative material of organic Ns, respectively.

2.7. Analysis

Nitrate, nitrite and ammonium were analyzed by ion chromatography (IC, Dionex DX-500, USA) equipped with anion column (IonPac, Dionex AS14A). TN was analyzed by spectrophotometric method with HACH system (DR2500, USA). Especially, the nitrate contained in samples obtained by the electrochemical nitrification was analyzed by IC, HACH system and the biosensor.

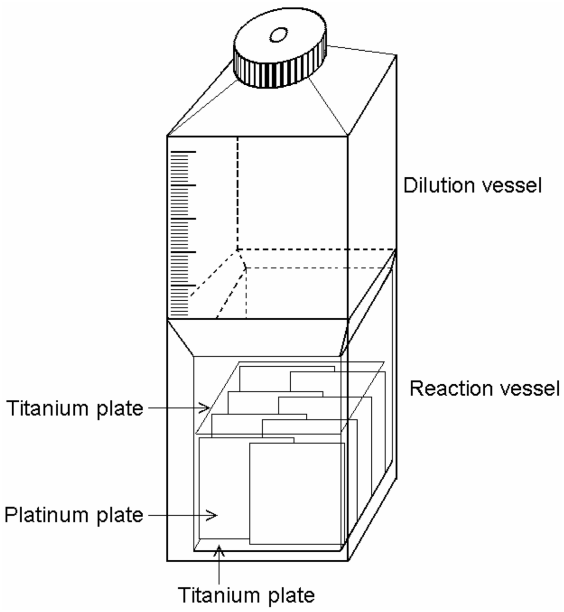

Fig. 2. Schematic structure of reactor for electrochemical nitrification of ammonium and organic nitrogen, wherein four pairs of Pt electrodes are facing each other and disposed vertically, and one pair of titanium electrode is facing each other and disposed horizontally. Area of each Pt plate is 0.15 cm^2, area of Ti plate is 0.64 cm^2. The electrode combination of Ti (upper and lower) and Pt (total) is used for nitrification of ammonium and Pt (left side) and Pt (right side) is used for nitrification of organic nitrogen compounds. The anode and cathode were exchanged at intervals of 15 seconds.

3. RESULTS and DISCUSSION

3.1. Optimization of biosensor ingredients

MV, sulfite and MOPS is an electron mediator, an oxygen scavenger and a buffer, respectively, however each ingredient may interfere the series redox reaction in Fig. 1B. To minimize interference and maximize functions of the ingredients, optimal concentration was electrochemically determined exclusive of NaR. As shown in Fig. 3(A), the current consumed in coupling with MV reduction was greatly increased in the reaction condition with MV more than 2 mM. The high concentration of MV may be required to get sufficient

reducing power for enzymatic redox reaction but may consume more current than optimum. Theoretically, 1 mM MV is enough as the reducing power for reduction of nitrate to nitrite at the level of 500 μM, which is higher than detection limit. Meanwhile, the influence of sulfite (Fig. 3B) or MOPS (Fig. 3C) on current consumption was similar each other independent of the concentrations. On the basis of the results, lowest concentration of sulfite and MOPS was chosen. 1 mM sulfite and 50 mM MOPS are enough for oxygen scavenging and buffering function because oxygen solubility is 0.315 mM in water and proton generated from nitrate reduction to nitrite is maximum 0.3 mM (Fig. 4). For optimization of NaR concentration, reaction mixture containing 1 mM MV, 1 mM sulfite, 50 mM MOPS and different concentration of NaR was immobilized to sensor strip. As shown in Fig. 3D, current consumed per mU NaR was relatively higher in the range from 5 to 15 mU but relatively lower in the range from 25 to 50 mU. On the basis of current consumption per mU, 15 mU NaR may be a proper concentration however 25 mU NaR is chosen allowing for the activity decrease in proportion to the storage time or by other factors (Fig. 6).

3.2. Estimation of biosensor strip

NaR selectively catalyzes nitrate reduction to nitrite in coupling with electrochemical redox reaction of MV (Fig. 1B). The NaR activity was electrochemically estimated by analysis of correlation between nitrate concentration and current consumption. The cyclovoltammetric potentiostat is suitable to estimate coupling redox reaction between two electron carriers.[17-19] As shown in Fig. 4(A), the current (upper peaks) consumed in coupling redox reaction between MV and nitrate catalyzed by NaR was linearly increased, which was plotted for standard regression analysis. As shown in Fig. 4(B), the current consumption was statistically correlated with nitrate concentration from 50 to 200 μM. On the basis of this result, we measured nitrate concentration with complete sensor strip composed of 1 mM MV, 1mM sulfite, 50 mM MOPS buffer and 25 mU

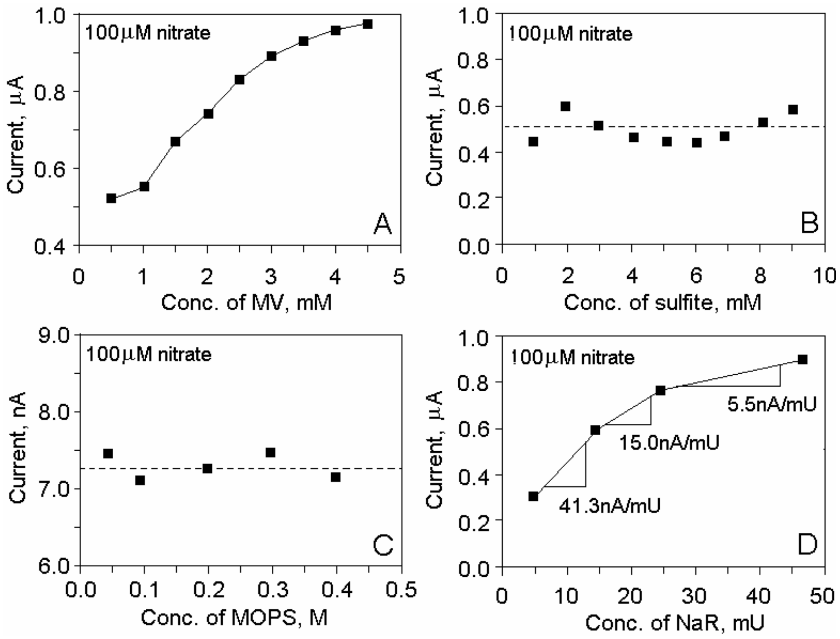

Fig. 3. Optimization of MV concentration (A), sulfite concentration (B), MOPS concentration (C) and NaR unit (D) for application to sensor strip. The optimum concentration of MV, sulfite and MOPS was 1 mM, 1mM and 50 mM, respectively, which was determined by the minimum concentration minimally influencing on the current consumption. The optimum unit of NaR was 25 mU, which is a critical point between minimal value and maximal value of current consumed per mU NaR.

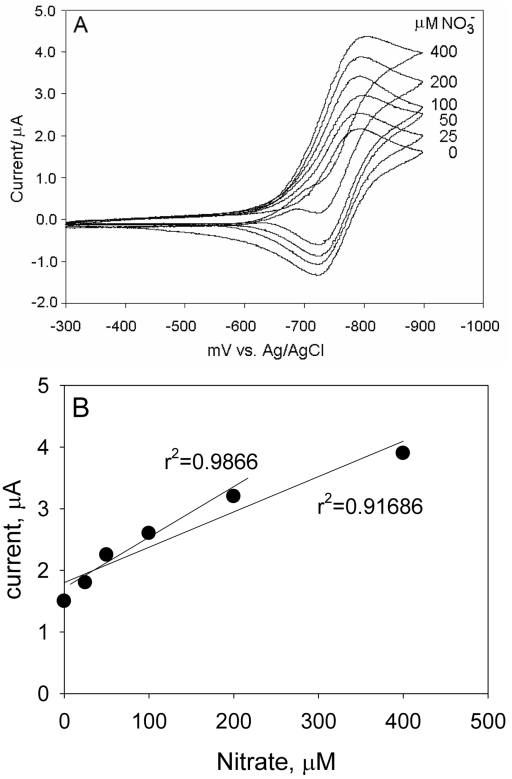

Fig. 4. Cyclic voltammograms measured with a glassy carbon electrode, platinum counter electrode and Ag/AgCl reference electrode following the introduction of the electrode into a solution containing 50 mM MOPS (pH 7.0), 1 mM MV, 25 mU NaR and different concentration of NO_3^- from 0 to 400 M under anaerobic Ar atmosphere (A), and correlation between current consumed coupled to reduction of different nitrate concentrations (B).

NaR. As shown in Fig 5, the current consumed by the redox reaction of sensor strip was accord with nitrate concentration at the level of 0.997. This is statistically reasonable value to analyze nitrate in the range of concentration from 50 to 250 μM.

3.3. Stability of NaR

Function of biosensor is dependent upon enzyme stability immobilized on sensor strip. According to the information served by NECi, NaR activity is stable in both dried and wet condition even at room temperature. According to the information, activity of NaR immobilized on sensor strip was tested during storage for 42 days. The coulombic value measured by the nitrate

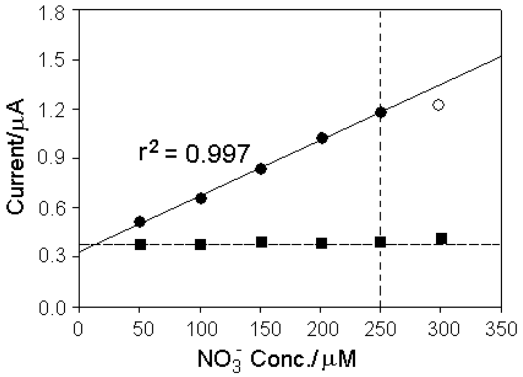

Fig. 5. Real application of sensor strip to nitrate measurement. The sensor strip was composed of 1 mM MV, 1 mM sulfite, 50 mM MOPS buffer and 25 mU NaR (●), which were left at room temperature without humidity for 42 days before used. NaR denatured by autoclave was used for base line under complete same condition with the normal test (■).

biosensor was decreased in proportional to storage time as shown in Fig. 6. The coulombic value measured with 42 days old sensor strip was minimum 80% of initial value and was not influenced by storage temperature. Theoretically, the residual activity of 42 days old sensor strip was 20 mU, which correspond to catalytic activity capable of producing maximum 3.853 mA/min or 64 μA/sec in coupling to nitrate reduction to nitrite on biosensor.[20] A precision device can detect the current

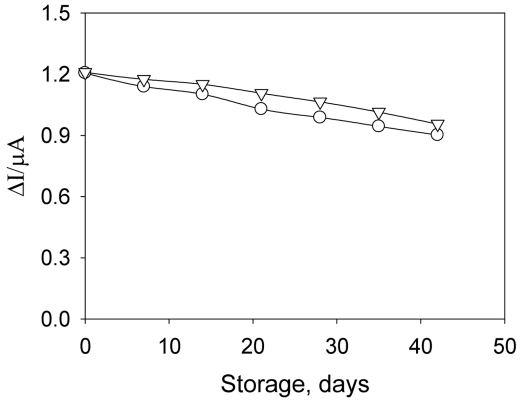

Fig. 6. Activity decrease of NaR immobilized on sensor strip during storage. NaR immobilized on working electrode by entrapment with PVA was dried and stored at room temperature (○) or in refrigerator (▽) for 42 days. The current value was an average of results obtained from eleven times measurement.

variation at the level of nA/sec. These results show that the NaR purchased from NECi is a proper for biosensor and can be stable for at least 50 days under dried condition.

3.4. Nitrification of ammonium and organic N

According to the standard method,[15] the TN contained in water can be spectrophotometrically analyzed at 220 nm after heating treatment at 120°C for 30 min under strong oxidation condition.[15] However, this is not useful to analyze complex organic N such as amino acid, nucleic acid and peptide because the complex organic Ns are difficult to be completely nitrified by heating treatment at 120°C. This is the reason why Kjeldahl method is performed with the strong oxidant at extremely high temperature (max. 370°C) under alkaline condition.[21] An electrochemical reactor was designed based on the Kjeldahl method, by which organic carbons can be oxidized to carbon dioxide. The electrodes are thought to function as catalyst and substitute for oxidant and heating treatment used in Kjeldahl method. Exactly, however, how the ammonium and organic Ns are oxidized to nitrate is not known. We only show here that in the presence of electrodes ammonium and organic N are readily oxidized to nitrate. This electrochemical mechanism needs to be resolved in the future. For optimization of nitrification efficiency, various treatment time and voltage were applied to reaction system. Optimum treatment time and electric potential are determined as 15 min and 15 volt, and nitrification efficiency of ammonium was 1.3 times higher than that of organic Ns as shown in Fig. 7 and 8. On the basis of the optimized condition, ammonium, organic N and wastewater was examined with the electrochemical nitrification reactor.

3.5. Application of biosensor to real samples

Generally, nitrate has been analyzed by ion chromatography or spectrophotometric colorization reaction (HACH, DR2500, USA). Both methods have been extensively employed for water analysis in the environmental area however ion chromatography is more precise than the spectrophotometric method. We

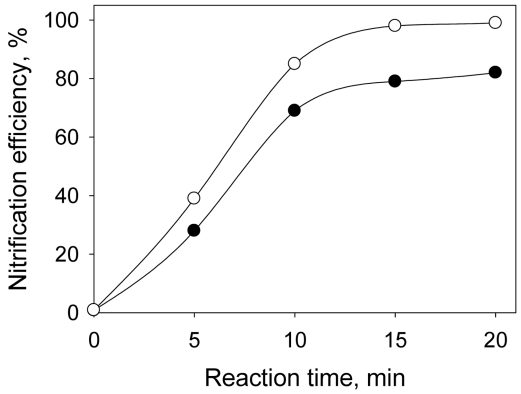

Fig. 7. Nitrification efficiency of ammonium (○) and organic nitrogen (●) in the electrochemical reactor according to the reaction time. The electric potential charged to the reactor was fixed to 15 volt. Electric pulse was produced by interchange of anode and cathode at intervals of 30 sec.

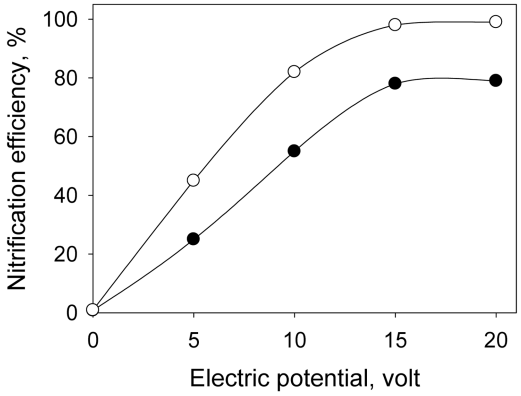

Fig. 8. Nitrification efficiency of ammonium (○) and organic nitrogen (●) in the electrochemical reactor according to the electric potential difference for 20 min.

compared the results obtained by the nitrate biosensor with those obtained by ion chromatography and spectrophotometric method. As shown in Table 1, results analyzed by the nitrate biosensor are more close to those by ion chromatography. This shows that the nitrate biosensor is a proper system as a real time analyzer capable of utilizing in the field and can substitute for ion chromatography and spectrophotometric method in even laboratory. The biosensor system also can be utilized for TN analysis by combination with a nitrification reactor of ammonium or organic Ns.

Table 1. Comparison of nitrate concentration determined by ion chromatography, spectrophotometry and nitrate biosensor (unit, μM).

Methods / Samples	Ion Chromatography	Spectrophotometry	Nitrate sensor*
Han river	107.9	100.0	100.4
Efflux water from wastewater treatment system	126.4	109.3	123.1
Tap water	126.4	100.0	106.3

* An average of 40 times determinations

Table 2. The nitrification efficiencies of ammonium, organic N and wastewater by the electrochemical nitrification reactor. TN contained in samples was determined using HACH (DR 2500, USA) system, which was compared with that measured by combination of the nitrate biosensor and the electrochemical nitrification reactor.

Samples	TN measured by HACH (mg/L)	[a]Nitrate measured by IC (mg/L)	Nitrifica-tion efficiency (%)	[b]Nitrate measured by BS (mg/L)	Deviation (a-b)/a
Ammonium ion	49.2	47.8	97.2	48.1	0.0062
[c]Organic N	95.8	77.7	81.1	79.1	0.018
Reactant from anaerobic digester	94.0	94.5	100	94.5	0
[d]Wastewater 1 before treatment	63.1	59.9	94.9	61.4	0.025
[e]Wastewater 2 before treatment	59.5	56.9	100	57.7	0.014
Reactant from aerobic reactor	71.8	68.2	94.8	66.3	0.028

a. Samples were prepared by electrochemically nitrification.
b. Samples were prepared by electrochemical nitrification
c. Glycine
d, e. Samples were obtained from same place at different time

3.6. TN analysis by combination of biosensor and nitrification reactor

Different samples were electrochemically nitrified then were analyzed with the nitrate biosensor (BS) and ion chromatography (IC). TN was analyzed with HACH system without nitrification. As shown in Table 2, the nitrification efficiency of all samples except organic N was from ~95% to 100% but organic N was ~80%. Most of inorganic Ns contained in natural water or wastewater is ammonium and nitrate while organic Ns are greatly different according to the wastewater sources. The organic Ns such as amino acid, nucleic acid, peptides or soluble proteins may be wasted or discharged from meat, fish, soybean, milk and wheat processing factories but rapidly dissimilated or assimilated by hetetotrophic bacterial consortia living in water.[21-24] According to the data base reported from Ministry of Environment of Korea (http://www.me.go.kr/user/db/db index.html), mean value of TN, nitrate and ammonium content in 486 natural streams located in south Korea were below 15 mg/L,10 mg/L, 1 mg/L, respectively. The ratios of TN to sum of ammonium and nitrate of the streams were mostly from 0.15 to 0.4. Theoretically, after the stream water was treated with the electrochemical nitrification reactor, the residual organic N is from 0.04 to 0.1, which corresponds to the technical error in spectrophotometric analysis system. Accordingly, the deviation occurred from the relatively low nitrification efficiency of organic Ns can be solved by using a statistic relationship between accumulative data that have been reported and analytical data obtained by combination system of the nitrate biosensor and the nitrification reactor.

3.7. Summary and conclusion

The advantageous point of biosensor is to be portable, precise and rapid but the weak point is to be limited to nitrate analysis of water. The combination of the nitrate biosensor and the electrochemical nitrification reactor can make application scope of the biosensor

expanded to TN analysis. The combination of biosensor and electrochemical nitrification reactor is useful to analyze nitrate and TN in both laboratory and field. Ammonium content in water can be presumed by using nitrate and TN concentration because most of inorganic Ns in water are nitrate and ammonium, and TN is sum of nitrate, ammonium and organic Ns. At the present, we are developing a new reactor to improve the nitrification efficiency of organic N, and a functional electrode to separately nitrify organic Ns and ammonium.

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