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Automated determination of nitrate plus nitrite in aqueous samples with flow injection analysis using vanadium (III) chloride as reductant

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ABSTRACT

Determination of nitrate in aqueous samples is an important analytical objective for environmental monitoring and assessment. Here we report the first automatic flow injection analysis (FIA) of nitrate (plus nitrite) using VCl_3 as reductant instead of the well-known but toxic cadmium column for reducing nitrate to nitrite. The reduced nitrate plus the nitrite originally present in the sample react with the Griess reagent (sulfanilamide and N-1-naphthylethylenediamine dihydrochloride) under acidic condition. The resulting pink azo dye can be detected at 540 nm. The Griess reagent and VCl_3 are used as a single mixed reagent solution to simplify the system. The various parameters of the FIA procedure including reagent composition, temperature, volume of the injection loop, and flow rate were carefully investigated and optimized via univariate experimental design. Under the optimized conditions, the linear range and detection limit of this method are 0–100 μM (R^2=0.9995) and 0.1 μM, respectively. The targeted analytical range can be easily extended to higher concentrations by selecting alternative detection wavelengths or increasing flow rate. The FIA system provides a sample throughput of 20 h^{-1}, which is much higher than that of previously reported manual methods based on the same chemistry. National reference solutions and different kinds of aqueous samples were analyzed with our method as well as the cadmium column reduction method. The results from our method agree well with both the certified value and the results from the cadmium column reduction method (no significant difference with P=0.95). The spiked recovery varies from 89% to 108% for samples with different matrices, showing insignificant matrix interference in this method.

1. Introduction

Nitrate (NO_3^-) is one of the most active nitrogen species involved in the global nitrogen cycle. Nitrate is an essential nutrient that is often found depleted in open, oligotrophic regions of the ocean [1]. On the other hand, nitrate levels are typically high in areas with considerable human inhabitation and disturbances, resulting in acidification and eutrophication of aquatic ecosystems, and endangerment of aquatic life [2 and references therein]. Nitrate concentration is regulated in drinking water sources, primarily because excess levels may cause methemoglobinemia, or "blue baby" disease, and other potential health problems including cancer, disruption of thyroid function, birth defects, and developmental disorders in children [3, 4]. Monitoring and assessment of nitrate content in various aquatic ecosystems (e.g. river, lake, aquifers, estuary and coastal sea) as well as in drinking water sources are particularly important for both researchers and managers. Therefore, it is an essential and significant research direction to develop sensitive, accurate and automated analytical methods to determine nitrate in aqueous samples.

There are several analytical methods available for the determination of nitrate and nitrite (NO_2^-) which have been reviewed in the previous literatures (e.g. [5, 6 and references therein]). One of the most popular methods used for the determination of nitrate is based on the reduction of nitrate to nitrite using a particular procedure (*vide infra*). The reduced nitrate plus the nitrite originally present in the sample is then measured by the classic Griess assay [5]. The Griess assay is based on the diazotization reaction of nitrite with sulfanilamide (SAM) and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic conditions. The resulting pink azo dye is monitored at 540 nm to indicate the nitrate concentration. The Griess reaction has the advantages of easier operation, lower cost and higher sensitivity compared with other nitrite determination methods [5]. As the Griess assay has already been comprehensively optimized, there is limited room for further improvement. Thus, many of the recent research studies have focused on the nitrate reduction approaches using copper-coated cadmium column [7], zinc column [8],

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hydrazine with copper catalyst [9], UV photo-induced device [10], etc. Some of these reduction methods have been applied for the analysis of environmental samples, but they have several shortcomings such as potential metal toxicity, variations in reduction efficiency with different sample matrices, issues due to presence of air bubbles, time-consuming activation procedures, and interference from ions and organic matter.

Vanadium (III) chloride (VCl_3) was initially used for reducing nitrite and nitrate to nitric oxide at 80–90 °C, and the nitric oxide product was detected by chemiluminescence [11]. To avoid the requirement of sophisticated chemiluminescence equipment, Miranda et al. [12] combined Griess assay and VCl_3 reduction for the spectrophotometric determination of nitrate in biological samples at room temperature. Since then, researchers have modified and applied this method in different matrices, such as aqueous samples, biological fluids, as well as milk and milk powder [13–19]. However, to the best of our knowledge, automatic flow analysis of nitrate using VCl_3 as reductant has not been reported before.

The aims of this research are to: (1) optimize a flow injection analysis (FIA) system for the determination of nitrate plus nitrite using VCl_3 as the reducing agent instead of toxic cadmium; (2) evaluate the stability of the single reagent solution containing both VCl_3 and Griess reagent; (3) evaluate the reproducibility and reliability of the optimized method by analyzing several different water samples and reference materials, and comparing results with other previously reported method. Our results indicate that this newly developed automated FIA method for nitrate determination is relatively sensitive and fast across a wide analytical range, and appears well suited for laboratory study as well as for potential on-line environmental monitoring applications.

2. Experimental

2.1. Reagents

All chemicals used in the study were reagent grade or better, purchased from Sinopharm Chemical Reagent Co., China (www.reagent.com.cn) and used without further purification. Pure water (18.2 MΩ cm) was obtained from a Millipore Purification System (www.Millipore.com) and used for preparing solutions. Nitrite and nitrate stock solutions of 100 mM were prepared by dissolving pre-dried solid $NaNO_2$ and KNO_3 in pure water, respectively. Working solutions were prepared by stepwise dilution of these stock solutions with pure water. The SAM solution of 10 g/L was prepared by dissolving SAM in 10% HCl (v/v) solution, and the NED solution of 1 g/L was prepared by dissolving NED in pure water. The VCl_3 reduction solution of 2% (m/v) was prepared by dissolving VCl_3 solid in 50% HCl (v/v) solution. It took about 1 h for the solid to be fully dissolved with agitation. The combined reagent solution was prepared by mixing the solutions of SAM, NED, and VCl_3 with water at a volume ratio of 1:1:1:7, which involved a 10-fold dilution of the individual reagent stock solutions. All the standard and reagent solutions were stored at 4 °C in a refrigerator while not in use. The cadmium reduction method was used for method comparison, and has been described in detail elsewhere [20]. Artificial seawater, prepared with solid NaCl and $MgSO_4$ according to a national standard [21], was used as a matrix for salt-effect experiments. Reagent bottles were sealed with air inlet clean-up devices which consisted of a syringe filled with acidic silica gel, as described previously [20, 22].

National reference solutions (GSBZ50008-88), produced by the Ministry of Environmental Protection of China and sealed in ampoules, were purchased from the website of standard reference materials (www.bzwz.com). Different types of bottled water were purchased from the local market. Water samples from river, lake, rain and tap were collected, filtered through a 0.45 μm nitrocellulose membrane, stored in a cooler at 4 °C during transportation, and kept frozen in the laboratory until analysis. The samples were analyzed within 3 days of collection.

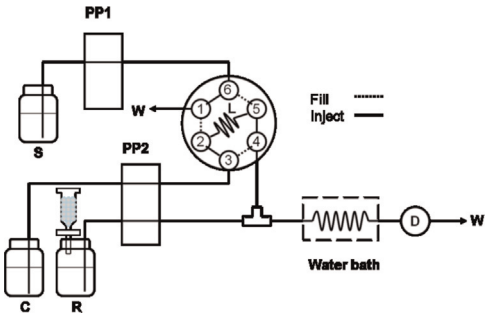

Fig. 1. Schematic diagram of the FIA system. S, sample; C, carrier (ultra-pure water); R, mixed reagent of SAM, NED and VCl_3; PP1/2, peristaltic pump 1/2; W, waste; L, sample loop; D, detector.

2.2. Experimental setup of FIA

The FIA experiments were conducted using a laboratory-made universal flow analysis system, which consisted of two dual-channel peristaltic pumps (www.lgpump.com.cn), one 6-port two-position injection valve (www.vici.com) and one 8-position selection valve (www.vici.com, not in use in this study). The system was controlled by specially designed hardware and Labview software (www.ni.com). A more detailed description of the system is not possible at this time, due to its patent pending status. The operation of the system is depicted in Fig. 1, and described briefly below (the abbreviations used in Fig. 1 were bracketed). During the "Fill" position of the 6-port injector valve, samples (S) were injected through the valve with pump 1 (PP1) and filled into injection loop (L). When the valve was switched to "Inject" position, the injected samples were pushed onwards from the loop by the carrier solution (C, at flow rate of 0.8 mL/min) and mixed with reagent (R, at flow rate of 0.2 mL/min) via a "T" connector, which were propelled by pump 2 (PP2). In the knotted mixing coil (MC) immersed in water bath of 60 °C, nitrate was reduced by VCl_3 and reacted with Griess reagent to form a pink dye, which was monitored in the detection system (D) and the waste (W) was collected in a waste bottle. The detection system consisted of a "U" shaped flow cell of 1-cm path length, a laboratory-made cell holder, a white LED fixed as light source, a USB 2000 miniature fibre optic CCD spectrometer (www.oceanoptics.com) as detector, and one QP600-2-SR fibre optic (www.oceanoptics.com) connecting the cell holder and spectrophotometer. Light absorption was monitored at 540 nm and 437 nm for detection and for correction of light intensity variation. Detailed data processing procedures are described elsewhere [23].

3. Results and discussion

3.1. Optimization of the analytical system

The effects of various parameters such as reagent composition, temperature, volume of the injection loop, and flow rate on the determination process were investigated and optimized via univariate experimental design. It should be noted that the length of the MC was chosen as 350 cm without optimization, merely for the ease of immersing the knotted coil in the water bath. A 20 μM nitrate standard solution was used throughout the parameter-

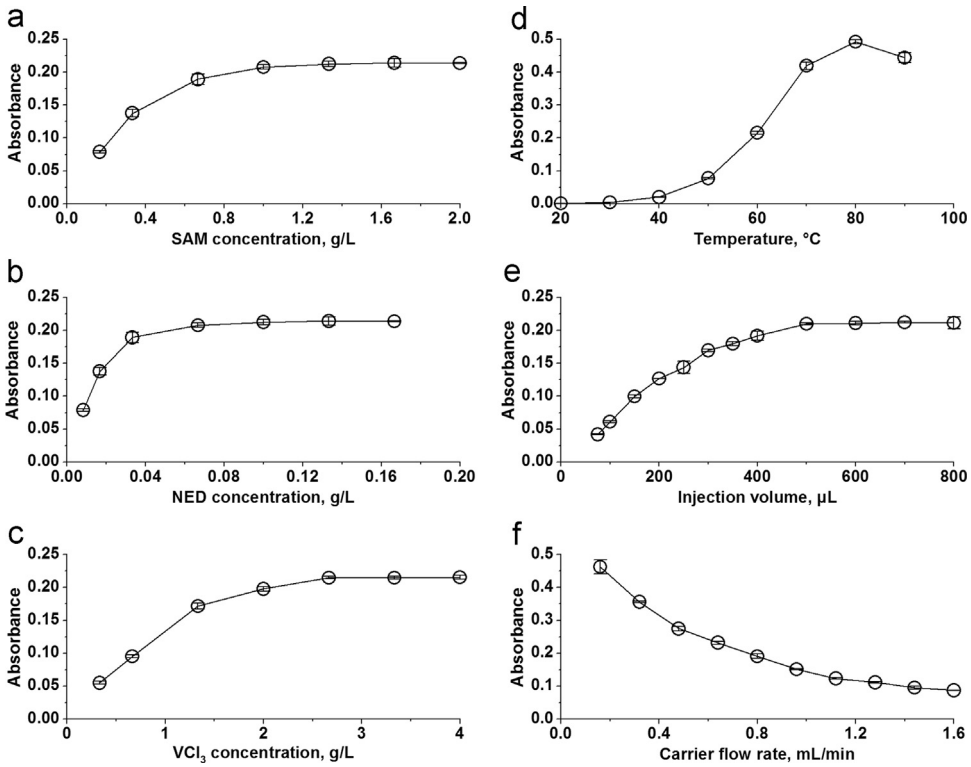

Fig. 2. (a) Effect of VCl_3 concentration (0.33–4.0 g/L) on absorbance with SAM concentration=3.3 g/L, NED concentration=0.33 g/L, temperature=60 °C, injection volume=500 µL, and flow rate=0.8 mL/min. (b) Effect of SAM concentration (0.17–2.0 g/L) on absorbance with VCl_3 concentration=2.0 g/L, NED concentration=0.33 g/L, temperature=60 °C, injection volume=500 µL, and flow rate=0.8 mL/min. (c) Effect of NED concentration (0.008–0.17 g/L) on absorbance with VCl_3 concentration=2.0 g/L, SAM concentration=1.0 g/L, temperature=60 °C, injection volume=500 µL, and flow rate=0.8 mL/min. (d) Effect of temperature (20–90 °C) on absorbance with VCl_3 concentration=2.0 g/L, SAM concentration=1.0 g/L, NED concentration=0.10 g/L, injection volume=500 µL, and flow rate=0.8 mL/min. (e) Effect of injection volume (75–800 µL) on absorbance with VCl_3 concentration=2.0 g/L, SAM concentration=1.0 g/L, NED concentration=0.10 g/L, temperature=60 °C, injection volume=500 µL, and flow rate=0.8 mL/min. (f) Effect of carrier flow rate (0.16–1.6 mL/min) on absorbance with VCl_3 concentration=2.0 g/L, SAM concentration=1.0 g/L, NED concentration=0.10 g/L, temperature=60 °C, and injection volume=500 µL.

optimization procedures. Each sample was quantified at least three times, and the results were shown as an average ± the standard deviation (SD, $n \geq 3$). The criteria for the optimization are based on the balance of sensitivity, sample throughput and analytical range.

Mixing the Griess reagent and reductant into one single solution simplifies the procedure and is also considered to minimize the NO formation [13, 18]. Therefore, a series of mixed reagent solutions containing different concentrations of VCl_3, SAM and NED were prepared and evaluated. The effects of reagent concentrations on the measurement procedure are shown in Fig. 2(a–c). It can be seen that the absorbance increases as the reagent concentration increases up to a certain value, showing the reagents were enough for the reaction (at least at the tested range). Thereafter, the signal remains stable until the upper range of the tested concentrations. Based on this data, optimal concentrations of 2.0 g/L of VCl_3, 1.0 g/L of SAM and 0.10 g/L of NED were chosen for the subsequent experiments. It should be noted higher reagent concentrations might be needed for analyzing samples with higher nitrate concentration.

Temperature has a significant influence on the kinetics of nitrate reduction by VCl_3. The reduction reaction can be completed within minutes or several hours or even longer, depending on the reaction temperature [17]. Under the tested temperature from 20 to 70 °C, the absorbance signal has an exponential growth. The increase in absorbance slows at 80 °C and the absorbance even drops a little bit at 90 °C (Fig. 2(d)). This is consistent with previously published results, as higher temperatures accelerate the reduction rate, but also cause the nitrate to be over reduced to NO [17]. Reaction temperature of 60 °C was chosen based on the balance of sensitivity, analytical range and avoidance of air bubble formation at higher temperature. However, the kinetics of this reduction under different temperature have not been comprehensively evaluated, which needs further study in the future.

As the final absorbance signal is controlled by the amount of nitrate in the reagent–sample mixture, the injection volume of the sample was varied between 75 and 800 µL to determine the optimized value. Signal increases were observed for injection volumes up to 500 µL and remained stable afterwards as shown in Fig. 2(e). Thus, the injection volume was maintained at 500 µL for all subsequent measurements to reduce the sample consumption (e.g. for sediment pore water sample analysis).

As the length of the MC is fixed, flow rate will control the reaction time; thus flow rate is a critical parameter for this time-consuming reaction. As illustrated in Fig. 2(f), the absorbance steadily decreased as the carrier flow rate increased from 0.16 to 1.6 mL/min. A flow rate of 0.8 mL/min was selected by considering both sensitivity and sample throughput. When a wide analytical range is needed for particular applications (e.g. waste water or polluted area), it is convenient to just speed up the flow rate without changing any hardware and reagents.

3.2. *Analytical figures of merit*

Under the optimized conditions shown in Table 1, a typical detector signal output and calibration curve was obtained over the concentration range of 0–100 µM (Fig. 3). Sample throughput for the optimized conditions was found to be 20 h^{-1} at a flow rate of 0.8 mL/min. The detection limit of 0.1 µM, was calculated as 3 times the SD for measurement of low-concentration samples

Table 1
Recommended analytical parameters.

Parameters	Range of tested values	Selected value
VCl_3 concentration, g/L	0.33–4.0	2.0
SAM concentration, g/L	0.17–2.0	1.0
NED concentration, g/L	0.0083–0.17	0.10
Temperature, °C	20–90	60
Injection volume, μL	75–800	500
Flow rate, mL/min	0.16–1.6	0.80

(2.5 μM, $n=9$) divided by the slope of the calibration curve. The relative standard deviation (RSD) for repetitive determinations of 2.5 and 20 μM nitrate solutions was 4.7% ($n=9$) and 2.0% ($n=11$), respectively. The RSDs for determination at other concentrations ranged from 0.2% to 8.7% ($n=3$–5) during the entire experimental period for all the data, and most of the data (82.5%) has RSD less than 5% indicating good reproducibility. The slopes of calibration curves prepared at different concentrations were found to vary within ±5% deviation, but it is recommended to measure a quality control sample once every 20 samples are analyzed, and to calibrate the system after changing the pump tubing due to possible changes in the flow rate.

3.3. Stability of reagent

The long term goal of this study is to apply the FIA-based method for on-line nitrate monitoring in a river or other fresh water bodies, thus it is essential to evaluate the long-term stability of the reagent. Two aliquots of mixed reagent solution were prepared according to the optimized result. One aliquot was stored in the dark at room temperature and the other was stored at 4 °C in a refrigerator. The reagents were used for measuring standard curves on alternate days. The criteria of reagent stability are that the sensitivity should not vary more than 5% on different days. As shown in Fig. 4, the slopes of the standard curve remain stable over 13 days for the reagent stored at 4 °C and 9 days for the reagent stored at room temperature. These reagents can be used for a further 4 weeks but the sensitivity drops to about 75% (for reagent stored at 4 °C) and 50% (for reagent stored at room temperature) respectively, compared with the freshly prepared reagent (data not shown). Though it has been reported that the mixed reagent is stable for at least one month at room temperature [18], it is recommended to re-check the efficiency of the reagent after one or two weeks, depending on the storage conditions. Furthermore, purging the reagent solution with N_2 to remove dissolved oxygen and tight sealing might be helpful to extend the reagent shelf life [13].

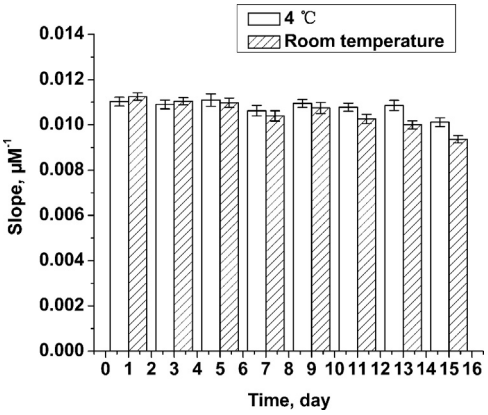

Fig. 4. Stability study of the reagent kept at 4 °C and room temperature.

3.4. Influence of salinity

The experiments described above were conducted using pure water as the carrier, and all the samples were also prepared in pure water. Although the potential application of this method is for freshwater analysis, it is still necessary to evaluate the effect of salinity on the determination, and thereby extend the scope of this work to include seawater analysis and marine research applications. Samples of different salinity were prepared by diluting low nitrate artificial seawater with pure water. The effect of salinity on the determination was conducted by measuring five different concentrations (0–80 μM) of nitrate to get calibration curves for a given salinity sample. The results show a decrease in sensitivity with increasing salinity of the sample. The slopes of the calibration curve can be fitted as a function of salinity, as shown below:

$$S_X = S_{DIW} - (4.28 \times 10^{-4})X + (5.96 \times 10^{-6})X^2, \quad R^2 = 0.9772, \quad n = 8$$

where S_X is the slope of calibration curve made in water at salinity of X; S_{DIW} is the slope of calibration curve made in pure water; X is the salinity of sample. This equation is necessary when the method is applied to seawater analysis.

3.5. Recovery

To evaluate the degree of interference from the matrix, recovery tests were conducted using several different water samples. As shown in Table 2, the recovery varies from 89% to 108%,

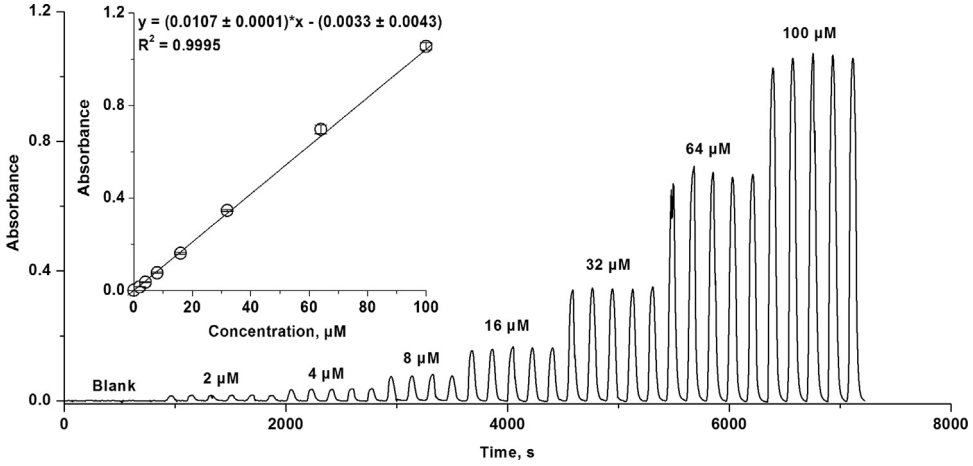

Fig. 3. Typical system response under the optimized parameters and calibration curves (inset). Error bars are ± S.D. for $n=4$–6.

Table 2
Summary of nitrate concentration and recoveries in different aqueous samples ($n=3$).

Sample	Added, μM	Found, μM	Added, μM	Found, μM	Recovery, %
Bottled purified water	0	2.08	20	20.51	92.2
Bottled mineralized water	0	5.60	20	25.16	97.8
Bottled natural mineral water 1	0	33.27	80	112.71	99.3
Bottled natural mineral water 2	0	42.09	80	123.02	101.2
Bottled natural spring water	0	14.10	50	64.67	101.1
Tap water	0	32.27	80	108.43	95.2
Lake water	0	40.28	80	111.29	88.8
Rain water	0	26.52	60	83.86	95.6
River water 1	0	36.37	80	122.77	108.0
River water 2	0	35.39	80	121.04	107.1

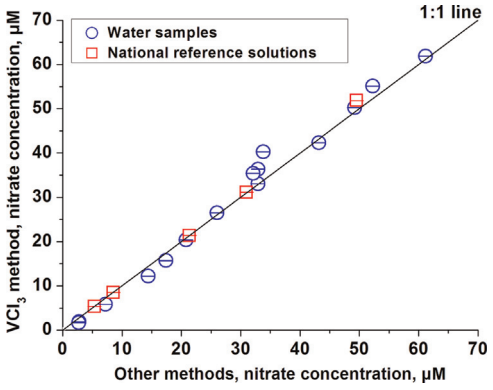

Fig. 5. Comparison of the proposed method and other method for the determination of nitrate plus nitrite in different water samples and national reference solutions.

indicating little matrix interference in the determination of nitrate using this method.

3.6. Validation of the method

Two separate approaches were used to validate this method: (1) National reference solution (GSBZ50008-88) was analyzed and there was no significant difference observed between the obtained results and the label values ($t_{stat}=1.02<t_{critical,}$ $P=0.95$). (2) A comparison experiment was carried out between this FIA method and the Cd-reduction method, which is commonly used in nutrient analysis research. Several different types of water samples were analyzed using both methods. The measured nitrate plus nitrite concentrations ranged from 2 to 62 μM in different matrices. There was no significant difference found between the results from these two methods ($t_{stat}=1.05<t_{critical,}$ $P=0.95$). All the comparison data are plotted in Fig. 5, showing the high level of accuracy of this method.

4. Conclusions

The combination of VCl_3 reduction and FIA provides a sensitive, accurate, simple, and robust approach for the determination of nitrate in aqueous samples. Compared with other methods based on the same chemistry using manual operation, the protocol developed in this study allows easy operation, good reproducibility and high sample throughput for the analysis. The experimental setup can also be easily modified for on-line environmental monitoring of nitrate in aqueous samples, and we plan to report the long term application of this method in a reservoir in the future.

Acknowledgements

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