Developing a field microbial biodegradation activity monitoring method based on the detection of adenosine phosphate (ATP, ADP, and AMP) for petroleum-contaminated groundwater

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ABSTRACT

As the focus of microbial degradation kinetics research, microbial activity detection is essential for the study of microbial degradation of contaminated groundwater. In this paper, luciferase assay was used to determine the microbial activity, and a rapid quantitative method for the determination of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) was developed. The experiment on microorganism of petroleum-contaminated groundwater was compared with ultraviolet and visible spectrophotometry (UV spectrophotometry) and plate count. Then, the practicability of the method for microbial degradation of petroleum-contaminated groundwater was verified. The results were as follows: the method had a detection range of 0.025–50 ppb, and each test took 10–15 s. The RSD of ATP, ADP, and AMP were 10.03, 2.84, and 6.57, respectively, and the R^2 values of the standard curve were all >0.99, the method was accurate and precise; the correlation coefficients of UV spectrophotometry were all >0.99, and the correlation coefficients of plate count were all >0.98, the P-values were both <0.001. The results showed that the luciferase assay method developed in this study was convenient, fast, reliable, and economical.

Keywords: Microbial activity; Adenosine phosphate; Luciferase assay; Petroleum-contaminated groundwater

1. Introduction

Microbial activity is a manifestation of microbial metabolic capacity, and in the case of pollution, it represents the capacity of microbial degradation [1]. Microbial degradation is the main way to remove pollution from groundwater [2], and even the only way to completely remove harmless organic pollutants from groundwater [3]. Therefore, the detection of microbial activity is of great significance for the degradation of contaminated groundwater, especially for the assessment of real degradation capacity.

At present, laboratory test methods for microbial activity detection include ultraviolet and visible spectrophotometry (UV spectrophotometry) [4], plate count [5], and respiration measurement [6]. However, because the microbial activity is susceptible to environmental conditions such as temperature, humidity, nutrients, pH, and oxygen content, etc. [7], the current storage of organic groundwater sample generally requires temperatures below 4°C to ensure the loss such as volatile organic compounds [8]. The microbial activity under these conditions is almost completely inactivated [9], and the activity determined by laboratory test such as UV spectrophotometry, plate count, and respiration measurement cannot reflect the on-site metabolic capacity, that is, the microbial degradation capacity cannot be presented. Therefore, a method for on-site, rapid, and accurate detection of microbial activity needed to achieve real microbial degradation capacity assessment.

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It is generally accepted that adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) is the most effective indicator of microbial activity [10]. In recent years, with the advancement of detection technology and instruments, the bioluminescence-luciferase assay has been developed. The instrument with a market price of 10–30 thousand RMB has a sample detection time of 10–15 s, and the minimum concentration of ATP is detected to be as low as 10^{-11} mol/L [11], and the linear relationship between ATP and microbial activity is excellent in the detection range [12], which is appropriate for microbial activity research. At present, this technology has been widely used in microbiology such as the fields of food, sewage, drinking water, etc. [13–15], The ATP index was also detected in groundwater samples, but it was presented as a numerical index for microorganism [16,17]. In recent years, luciferase assay of ATP + AMP [18], ATP + ADP + AMP [19] has also been mature but has not been used in contaminated groundwater research for the microbial activity detection. Based on the on-site quantitative monitoring of ATP – ADP – AMP, it may even provide a possibility to reveal the conversion mechanism of on-site microbial adenosine phosphate, which has been a bottleneck that plagues microbial dynamics monitoring.

In view of this, this study took petroleum-contaminated groundwater microbes as the research object to optimize the combination of existing rapid detection products, verify the accuracy of the detection system, determine the maximum and minimum detection range and standard curves and to establish a concentration conversion system as well as to conduct experiments of microbes of petroleum-contaminated groundwater. Compared with UV spectrophotometry and plate count, the paper verified the practicability of the method for the study of microbial degradation of petroleum-contaminated groundwater.

2. Materials and methods

2.1. Instruments and consumables

In this study, the SystemSURE plus ATP fluorescence detector manufactured by Hygiena (USA) was used to adopt the ATP value of samples. The matching disposable swab was the special water sample swab produced by Wuhan Scithera Microbiological Technologies Co., Ltd [20]. The luciferase used in the swab had good thermal stability and can be stored for 1 y at 2°C–8°C. It is compatible with the portable ATP bioluminescence detectors, which had a minimum of 1×10^{-9} mol/L (~5.0×10^{-5} ppm) ATP, the system was named as 1A system.

The ADP and AMP were measured using a PD-30 fluorescence detector manufactured by KIKKOMAN (Noda, Japan). ATP + AMP was measured by a LuciPac Pen-AQUA water sample special swab, and ATP + ADP + AMP were measured by the LuciPac A3 Water special swab, which were also produced by KIKKOMAN (Noda, Japan). The instrument and two types of swabs were named as 2A system and 3A system, respectively. ATP single standard can be tested by 1A, 2A, 3A system, AMP single standard, and ATP + AMP mixed standard can be tested by 2A, 3A system, ADP single standard, and ATP + ADP + AMP mixed standard can only be tested by 3A system.

The ATP, ADP, and AMP standard samples and other reagents used in the experiment were all produced by J&K Scientific Ltd., (Beijing, China). Please refer to the specification for the operation methods and precautions of the above instruments.

2.2. Design of detection technology

2.2.1. Detection system accuracy verification

The specific method was to collect a certain concentration of ATP, ADP, and AMP standard samples, using three systems to observe at regular intervals whether the instrument and swab meet certain laws, and how was the repeatability of the reference standard with the same concentration.

2.2.2. Determination of the minimum/maximum detection range

The measuring range of SystemSURE plus ATP fluorescence detector was from 0 to 9,999 relative light units (RLU). The PD-30 fluorescence detector had a minimum range of 0 RLU and no maximum range. Therefore, the available minimum/maximum detection range for the two devices need to be determined experimentally.

Three systems of 1A, 2A, and 3A were employed. The specific scheme was to prepare ATP, ADP, and AMP standard samples with the minimum/maximum concentrations of the detection range (5.0×10^{-6} ppm to 5.0×10^{-2} ppm) according to specifications. After the three systems were tested, the concentration of the standard samples was adjusted according to the test results until the minimum/maximum detection range of the test system was determined.

2.2.3. Standard curve establishment

The minimum/maximum detection range of the three systems were determined. Different concentrations of ATP, ADP, and AMP standard samples were added within the range of the assay to establish standard curves for different swabs. The standard solution was 1,000 ppm, prepared with sterile ultrapure water, dispensed into a 2.0 mL centrifuge tube, stored at –20°C, and used a new tube each time. The shelf life of the standard sample was 1 month.

The standard curve was prepared by stepwise dilution of 1,000 ppm standard solution. 100 μL of the upper concentration standard solution was added to 900 μL of sterile ultrapure water, then stepwise diluted to 100 ppb, and diluted the 100 ppb sample separately to 50, 25, and 10 ppb. On the basis of these three concentrations, it continued to dilute until the minimum range of detection was reached. In order to prevent the degradation of ATP which may lead to inaccurate test data, it is better to dilute a concentration and quickly detect, then do the next dilution and detection.

2.2.4. Construction of ATP, ADP, and AMP concentration conversion system

Due to the restriction of the principle, the 1A system can only detect the ATP concentration in the sample. 2A system could test the value of ATP + AMP, 3A system could measure the value of ATP + ADP + AMP. It had to establish the

concentration conversion system of ATP, ADP, and AMP to obtain the real concentration value, respectively, based on the standard curves of ATP, ADP, and AMP single standards and mixed standards as well as the relationship between each standard curve.

2.2.5. Simulation of actual sample testing

Because it was difficult to determine the initial value of the samples of the contaminated site, the actual samples in this simulation were related to the microbial flora isolated and cultured from the petroleum-contaminated groundwater [21], and the bacterial flora were inoculated into the enriched combination culture media for 2–4 d: K_2HPO_4, 1.0 g, KH_2PO_4, 1.0 g, $MgSO_4·7H_2O$, 0.5 g, NH_4NO_3, 1.0 g, soluble starch 10.0 g, $CaCl_2$, 0.02 g, $FeCl_3$, sucrose 2 g, petroleum 0.5%, water 1,000 mL, The pH was 7.0, and the temperature was under 121°C to sterilize for 30 min. After mixing, the cultured bacterial solution was taken 20 mL, and then centrifuged for 5 min at 4,000 rpm/min, the supernatant was discarded, and the microorganisms were collected. After resuspended in 0.85% sterile saline, the relevant experiments were conducted. Comparing the OD600 value determined by UV spectrophotometry and the number of strains obtained by the plate count, it was determined whether the adenosine phosphate indicator test for simulating the actual sample correlated with the number of strains measured by other methods, thereby demonstrating the pyrolysis of the strain and verified that ATP, ADP, and AMP tests were in line with the requirements of the application. Among them, the plate count was determined by Chinese national relevant standards [22–24].

3. Results and analysis

3.1. Verification of detection system accuracy

Three 150 μL ATP standard samples with different concentrations were, respectively, added to the 1A system, 2A system and 3A system, and the same swabs were measured every 2 min. Curve fitting measured by time and RLU value showed that the test results and time of all concentration values showed a cubic polynomial relationship, and R^2 was all >0.95, the three detection systems consisting of the fluorescence detectors and the fluorescent swabs showed the high accuracy. The specific data were shown in Table 1.

3.2. Determination of the minimum/maximum detection range

As for preparing the ATP, ADP, and AMP standard samples of the maximum/minimum limit concentration of

the detection range (5.0×10^{-6} ppm to 5.0×10^{-2} ppm) and the mix of ATP + AMP, ATP + AMP + ADP, they were tested separately and the concentration of the standard were adjusted according to the test results until the minimum/maximum detection range of the detection system was determined. The results were as follows:

It can be seen from the table that the minimum detection ranges of the 1A, 2A, and 3A systems all were 0.025 ppb for the ATP standard. The maximum detection range of the 1A system was 50 ppb due to the range of the system value. The maximum detection range of the 2A and 3A systems were both 100 ppb. For AMP standard samples, it can only be determined by 2A, 3A systems with a minimum range of 0.025 ppb and a maximum range of 50 ppb. In terms of the ADP standard sample, it can only be determined by the 3A system, with a minimum detection range of 0.025 ppb and a maximum detection range of 50 ppb. For ATP + AMP mix, the minimum range of detection for 2A, 3A systems both were 0.025 ppb with a maximum range of 50 ppb. For the ATP + ADP + AMP mix, the minimum range of detection was 0.025 ppb and the maximum range of detection was 25 ppb. In terms of the RSD of ATP, ADP, and AMP detection, it was selected as the mean values of the maximum and minimum detection range were obtained, and the RSD of the three were 10.03, 2.84, and 6.57, respectively.

3.3. Establishment of standard curve

The minimum/maximum detection range for the three systems was determined in the last section. Within the range of the detection, different concentrations of ATP, ADP, and AMP standards can be added to establish the standard curves for different swabs. The standard curves were prepared by stepwise dilution method. The initial concentration was 1,000 ppm, the standard curves were divided into single standards and mixed standards. The single standards included ATP, ADP, and AMP while the mixed standards contained ATP + AMP, ATP + ADP + AMP. The specific values of the ATP, ADP, and AMP indicators of each sample also needed to be calculated by using the above single standard and mixed standard curves to obtain the specific values of the three indicators of adenosine phosphate.

The experimentally determined ATP standard curves was shown in Table 2. The test data listed in the table was the mean of three tests at the same concentration:

It can be seen from Table 3, for the ATP single standard, the standard curves measured by the 2A and 3A systems were more consistent, and the R^2 values were higher. The 1A

Table 1
System accuracy verification of ATP fluorescence method

System	Concentration (ppb)	0	2	4	6	8	10	12	R^2
1A	20	4,420	1,064	734	640	608	590	586	0.953
2A	15	6,662	2,556	1,666	1,376	1,270	1,212	1,186	0.979
3A	10	5,846	1,830	1,300	1,142	1,082	1,060	1,048	0.962
1A	8	4,900	1,698	1,114	890	816	770	746	0.974
2A	5	2,974	996	658	536	478	440	418	0.971
3A	2	1,218	572	362	272	222	188	162	0.991

Table 2
Determination of the minimum/maximum detection range

System detection range	Concentration (ppb)	RLU	Average (RLU)	Standard Deviation (SD)	Relative SD (RSD)
1Aminimum-ATP	0.025	6/5/4	5.00	1.0	20.0
1Amaximum-ATP	50	9,999/9,998/9,989	9,993.5	5.51	0.06
2Aminimum-ATP	0.025	7/6/5	6.0	1.00	16.67
2Amaximum-ATP	100	25,074/23,264/23,076	23,170	1,103.29	4.76
3Aminimum-ATP	0.025	7/6/7	6.67	0.58	8.66
3Amaximum-ATP	100	22,196/22,642/21,962	22,302	345.46	1.55
2Aminimum-AMP	0.025	15/14/12	13.67	1.53	11.18
2Amaximum-AMP	50	20,214/20,178/19,521	19,971	390.13	1.95
3Aminimum-AMP	0.025	16/14/13	14.33	1.53	10.66
3Amaximum-AMP	50	20,832/19,948/19,928	20,236	516.25	2.55
3Aminimum-ADP	0.025	11/10/10	10.33	0.58	5.59
3Amaximum-ADP	50	9,792/9,781/9,799	9,790	9.07	0.09
2Aminimum-ATP + AMP	0.025	24/23/22	23.0	1.0	4.35
2Amaximum-ATP + AMP	50	34,137/36,524/32,682	34,603	1,939.75	5.61
3Aminimum-ATP + AMP	0.025	23/21/24	22.67	1.53	6.74
3Amaximum-ATP + AMP	50	33,839/34,558/35,072	34,815	619.33	1.78
3Aminimum-ATP + AMP + ADP	0.025	35/34/31	33.33	2.08	6.24
3Amaximum-ATP + AMP + ADP	25	24,681/24,251/24,651	24,451	240.07	0.98

Table 3
Standard curves of ATP

Concentration (ppb)	1A system/RLU	2A system/RLU	3A system/RLU
0.025	5.0	6	6.67
0.05	25.17	17.33	17.33
0.25	45.33	47.67	45.33
0.50	129.33	176.67	185.33
2.50	487.80	535.33	526.33
5.00	981.40	984.00	1,002.33
10.00	2,179.33	2,390.00	2,395.00
25.00	4,570.75	5,571.667	5,575.00
50.00	9,998.00	12,235.67	11,133.33
100.00	/*	22,772.33	23,369.33
Regression equation	$y = 0.0051x + 0.023$	$y = 0.0043x - 0.04$	$y = 0.0043x + 0.28$
R^2	0.9978	0.9987	0.9994

/*, beyond detection range.

system had a maximum detection range of 50.0 ppb due to the restriction of the instrument display.

The experimentally determined ADP and AMP standard curves were shown in Table 4. The test data listed in the table were the average of three tests at the same concentration.

As shown in Table 4, for the AMP single standard, the standard curves of the 2A and 3A systems were more consistent. As for the ADP single standard, a standard curve with a higher R^2 value was also obtained, and the measurement range was the same as that of the AMP.

At the same time, the AMP single standard test was also carried out by 1A system. The results were mostly 0, the average values of the high concentrations at 25.0 and 50.0 ppb,

respectively, were 12 and 18 RLU, which was lower than the minimum detection range of the 1A system. In terms of the ADP single standard, the 1A and 2A systems were used for test verification. The points tested in the marking range all were 0 RLU, which proved that the test of standard curve of AMP and ADP single standard tested by AMP and ADP single standard preparation and the 2A and 3A systems was more accurate.

The standard curve of the experimentally determined ATP + AMP mixed standard was shown in Table 5.

It can be seen from Table 5 that the standard curves of the 2A and 3A systems were more consistent for the ATP + AMP mixed standard. The ATP + AMP mixed standard was verified

Table 4
Standard curves of AMP, ADP

Concentration (ppb)	2A system-AMP/RLU	3A system-AMP/RLU	3A system-ADP/RLU
0.025	13.67	14.33	10.33
0.05	26.67	42.00	28.00
0.25	91.00	105.67	47.00
0.50	183.67	204.67	102.00
2.50	964.00	965.00	511.00
5.00	2,002.67	2,036.33	917.00
10.00	4,306.00	4,013.67	2,040.00
25.00	9,801.50	9,636.50	4,974.00
50.00	19,971.00	20,236.00	9,790.00
Regression equation	$y = 0.0025x - 0.028$	$y = 0.0025x + 0.083$	$y = 0.0051x - 0.039$
R^2	0.9996	0.9995	0.9998

Table 5
Standard curves of ATP + AMP

Concentration (ppb)	2A system-ATP + AMP/RLU	3A system-ATP + AMP/RLU	1A system-ATP verification/RLU
0.025	23.0	22.67	3.0
0.05	42.33	42.00	16.67
0.25	166.67	164.67	35.67
0.50	297.67	341.33	125.0
2.50	1,524.33	1,469.67	527.67
5.00	3,579.33	3,322.33	1,175.33
10.00	7,036.00	7,664.67	2,677.33
25.00	16,947.00	17,747.50	5,197.23
50.00	34,603.00	34,815.00	9,998.0
Regression equation	$y = 0.0014x + 0.051$	$y = 0.0014x - 0.025$	$y = 0.0050x - 0.51$
R^2	0.9998	0.9994	0.9957

by the 1A system. Since the 1A system can only test ATP, the verification result can be compared with the standard curve of the ATP single standard tested by the 1A system. After comparison, the ATP single standard curve tested by 1A system was $y = 0.0051x + 0.023$ ($R^2 = 0.9978$), and the standard curve of the ATP + AMP mixed standard tested by the 1A system was $y = 0.0050x - 0.51$ ($R^2 = 0.9957$), these two curves were basically same. This verification proved the accuracy of the 1A system for the ATP single standard curve test and also verified that the 1A system did not respond to the AMP single standard.

As Table 6 shown, for the ATP + ADP + AMP mixed standard, it only can be tested by the 3A system. Meanwhile, the ATP + ADP + AMP mixed standard was verified by the 2A system and 1A system.

The verification results showed that the standard curve of the 2A system verification was $y = 0.0014x + 0.032$ ($R^2 = 0.9982$), and the standard curve for the ATP + AMP mixed standard was $y = 0.0014x + 0.051$ ($R^2 = 0.9998$), two standards curves were basically same and ADP did not interfere with the 2A system. The standard curve of the 1A system for ATP + ADP + AMP mixed standard was $y = 0.0050x - 0.35$ ($R^2 = 0.9989$). Compared with the other two standard curves, the ATP single standard curve of the 1A system test was $y = 0.0051x + 0.023$

($R^2 = 0.9978$), the standard curve of ATP + AMP mixed standard tested by 1A system was $y = 0.0050x - 0.51$ ($R^2 = 0.9957$), these three curves were basically same, indicating that the 1A system was more repeatable than the ATP standard curve test and ADP, as well as AMP, did not interfere with the 1A test system.

3.4. Construction of ATP, ADP, and AMP concentration conversion system

As for the test of an actual sample, since the content of ATP, ADP, and AMP, that is, the concentration value (ppb), was unknown, it was necessary to use three systems to obtain the specific RLU test values of three adenosine phosphate indicators first, then use the standard curves to calculate the real concentration. The obtain of concentration value of the ATP was relatively simple, it can be directly converted from the ATP standard curve.

If the ppb concentration value of AMP is to be obtained, the ppb concentration value of ATP should be converted to the RLU value via the ATP standard curve of 2A system, the RLU measured value of AMP was obtained after the measured value of ATP + AMP of the 2A system minus the RLU measured value of the 2A system ATP, and the

ppb concentration value of AMP was obtained by converting the standard curve of the AMP single standard of 2A system.

Since the standard curve of the ATP + AMP mixed standard was basically same for the 2A and 3A systems. The RLU measured value of ADP can be obtained by the measured value of the 3A system minus the measured value of the ATP + AMP of the 2A system. Furthermore, the ppb concentration value of ADP can be obtained according to the converting standard curve of the ADP single standard by the 3A system. Since the minimum detection range of each standard curve was 0.025 ppb, when each converted concentration value was lower than 0.025 ppb, it was counted as 0.

To verify the above conversion process, the ATP single standard plus the AMP single standard of the 2A system was compared to the measured value of the ATP + AMP mixed standard of the 2A system. For the same reason, it was critical to verify whether the measured value of the ATP + AMP mixed

standard plus the ADP single standard of the 3A system was consistent with the measured value of the ATP + ADP + AMP mixed standard of the 3A system.

For the 2A system, the comparison of two standard curves were shown in Fig. 1.

It can be seen from Fig. 1 that, as for the 2A system, the standard curve composed of the ATP + AMP mixed standard test value was basically same as the standard curve composed of the ATP single standard + AMP single standard, so the concentration value of the AMP can be converted by the above conversion system.

In terms of the 3A system, the comparison of the two standard curves were as follows:

It can be seen from Fig. 2, as for the 3A system, the standard curve composed of the ATP + ADP + AMP mixed standard test value was basically same as the standard curve composed of the ATP + AMP mixed standard + ADP single standard, thus, the concentration value of the ADP can be converted via above conversion system.

Table 6
Standard curves of ATP + ADP + AMP

| Concentration (ppb)	3A system-ATP + ADP + AMP/RLU	2A system-ATP + AMP verification/RLU	1A system-ATP verification/RLU
0.025	33.33	14.00	/*
0.05	54.00	42.67	/*
0.25	177.33	128.33	39.00
0.50	542.67	456.00	125.00
2.50	1,995.00	1,307.67	527.67
5.00	4,748.67	2,988.00	1,186.00
10.00	10,489.00	8,087.00	2,290.00
25.00	24,451.00	16,947.00	5,197.33
50.00	/*	34,603.00	9,993.5
Regression equation	$y = 0.0014x + 0.057$	$y = 0.0014x + 0.033$	$y = 0.0050x - 0.35$
R^2	0.9985	0.9982	0.9989

/*, beyond detection range.
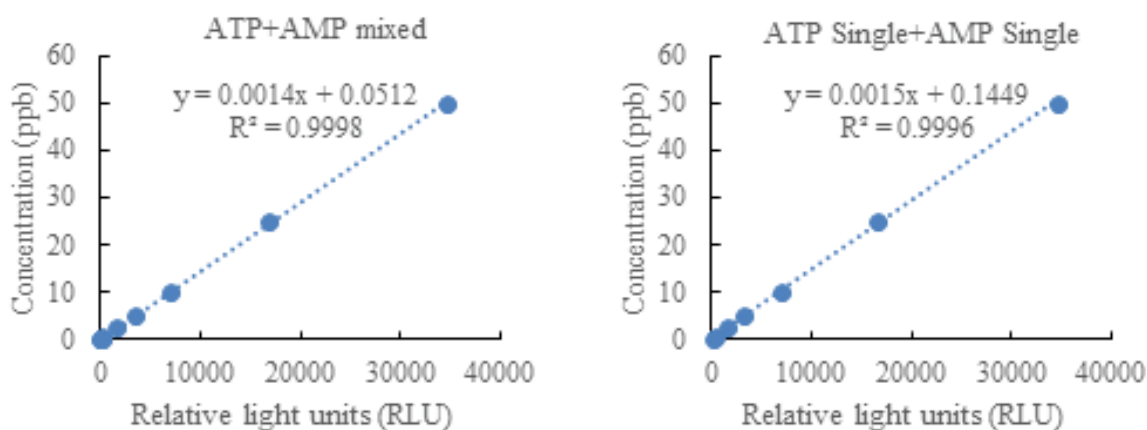
Fig. 1. Comparison of standard curves using 2A system.

3.5. Simulation of the test of actual sample

The simulated actual sample was also determined by comparing the OD600 value measured by UV spectrophotometry with the number of strains obtained by the plate count and determined whether the adenosine phosphate indicator test of the actual sample has a correlation with the number of strains measured by other methods. Thereby, the pyrolysis of strains and the testing of adenosine phosphate indicators such as ATP, ADP, and AMP all met the requirements of the application. The unit of adenosine phosphates index test results such as ATP, ADP, and AMP was ppb, which was a concentration value obtained by converting the measured value RLU according to ATP, ADP, and AMP concentration conversion systems. The specific results were shown in Table 7.

From the data in Table 7, the relationship among OD600, plate count as well as ATP, ADP, and AMP was presented as follows:

As can be seen from Fig. 3, OD600 had a linear relationship with ATP, ADP, and AMP, and the R^2 values were all >0.99, and the *P*-value calculated by SPSS was lower than 0.001. It was indicated that the adenosine phosphate indicators ATP, ADP, and AMP can correspond well to the bacterial concentration determined by UV spectrophotometry.

Fig. 4 demonstrated that the total number of bacteria measured by the plate count was linear with ATP, ADP, and AMP, and the R^2 values were all >0.98 and the *P*-value was <0.001. It showed that the adenosine phosphate indicators ATP, ADP, AMP, and the total number of bacteria measured by the plate count can also correspond well. The above results indicated that the strains pyrolysis of the simulated test and the ATP, ADP, and AMP tests all met the requirements of the application.

4. Discussion

High-performance liquid chromatography [25] can also measure ATP, ADP, and AMP [26] simultaneously. Since the energy metabolite is a polar and hydrophilic compound, it is essential to select an appropriate chromatographic column and pretreatment of samples according to its properties, and the minimum detection range of the method is four orders of magnitude higher than the introduced luciferase assay, and pretreatment of samples may cause changes in adenosine phosphate indicators [27,28]. Petroleum substances may also interfere with the detection of adenosine phosphate indicators. The luciferase assay established in this paper was fast, simple, and low-cost, and was more appropriate for the application of adenosine phosphate indicators in petroleum-contaminated sites.

It has been reported that there are disturbances of ATP detection from somatic cells and free ATP in surface water [29], but since groundwater is less likely to exist in animals and plants than surface water, somatic cells rarely have interference; free ATP is released by dead microbial cells [30], it may exist in the environment of groundwater. It has been reported that in surface water, the proportion of free ATP
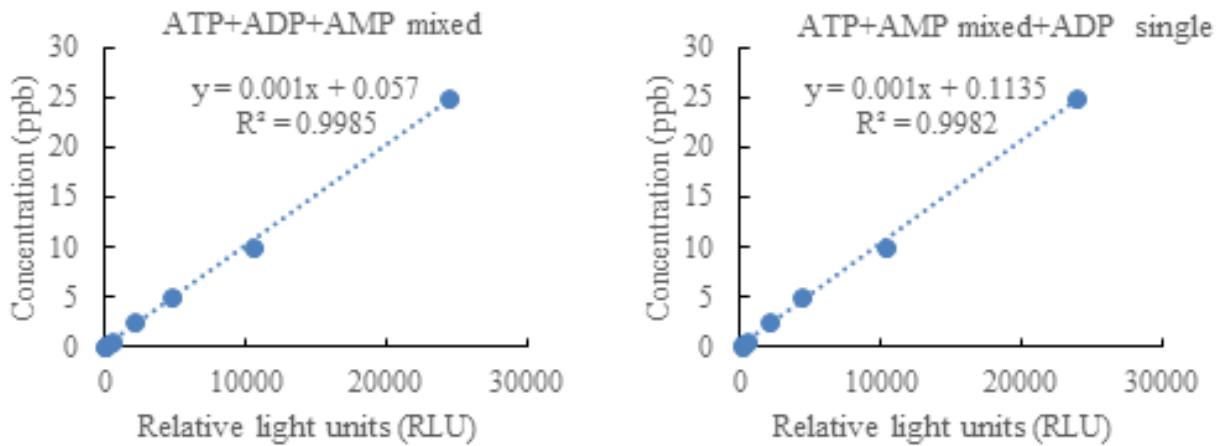

Fig. 2. Comparison of standard curves using 3A system.

Table 7
Adenosine phosphate indicators test result of actual sample energy

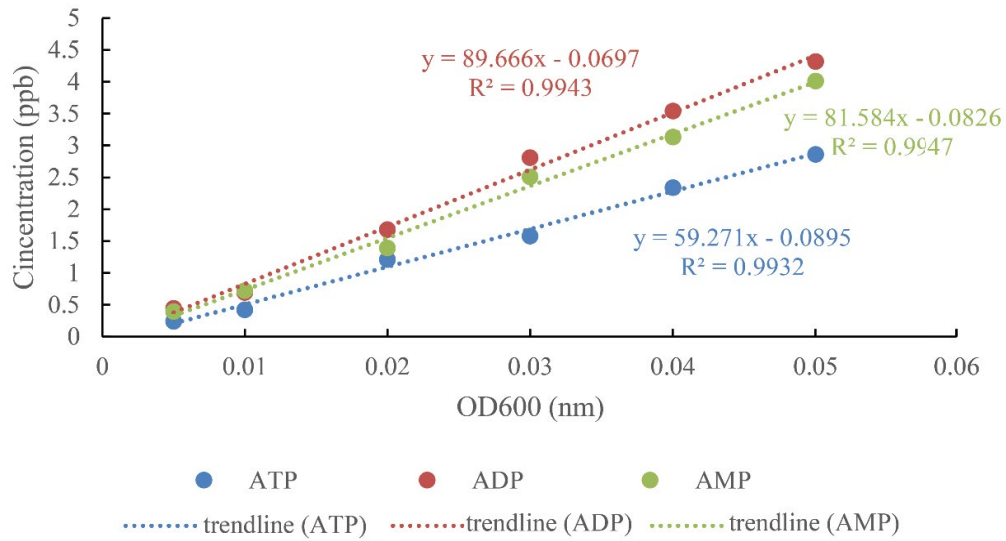| OD600 (nm)	Plate counting (10^6 cfu/L)	ATP (ppb)	ADP (ppb)	AMP (ppb)
0.005	5.7	0.24	0.44	0.39
0.01	10.1	0.42	0.69	0.72
0.02	18.9	1.21	1.68	1.39
0.03	28.8	1.58	2.81	2.51
0.04	39.2	2.34	3.54	3.13
0.05	51.6	2.86	4.32	4.01

Fig. 3. Relationship curves of OD600 and ATP, ADP, and AMP.
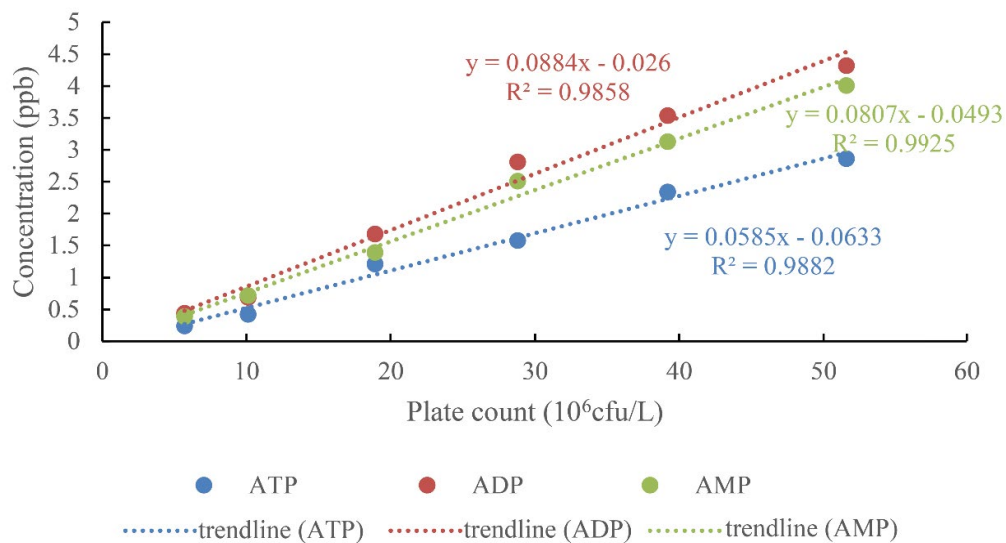

Fig. 4. Relationship curves of plate count and ATP, ADP, and AMP.

is about 1%–5% of total ATP. In the groundwater environment [31], the microorganisms are relatively stable and the replacement is slower. Therefore, the free ATP released by the dead microorganisms should be less than the surface water. However, its degree of interference with groundwater microbial activity detection remains to be studied.

In general, the content of ATP in single cells of bacteria is 1×10^{-18} mol/L [32], which is 5.07×10^{-11} ppb. The minimum detection ranges of 1A, 2A, and 3A systems used in this study are all 0.025 ppb, which can detect the ATP in 5×10^8 cells/L of microorganisms. It was reported that the number of respiring bacteria was $0.55–4.9 \times 10^8$ cells/L in pure groundwater from sandy and gravelly deposits, and the proportion of respiring bacteria was 0.66%–7.4% of the total bacteria [33].

So the number of total bacteria in that pure groundwater is more than 5×10^8 cells/L. Because the size of microbial cells in groundwater is different and the bacteria alive in different hydrogeological conditions, the minimum detection limit of ATP of microorganisms can only be tested on-site. Since the groundwater microorganisms researched in this study exist in the petroleum-contaminated environment, the electrons required for their growth are sufficient for the receptors. Therefore, the minimum detection ranges of the three systems used by the luciferase assay can meet the requirements of field detection.

In order to obtain uniform initial conditions of a comparison test of the simulated petroleum-contaminated groundwater microbial activity, the groundwater microbial

samples collected from the petroleum-contaminated place were enriched by the culture method, and the laboratory conditions were optimized. It should be noted that the actual sample also contained non-cultivable microorganisms when detecting the groundwater samples in the field [34], so the data may be different from the luciferase assay determined in this paper, and the detection might be influenced by field conditions and sampling methods. It is recommended to avoid the swab, the detection equipment contact to the medium containing the microbial active substance such as the human body and saliva during the detection. In the process of groundwater sampling and well flushing, the ATP measurement in this research can be combined with the water quality indexes test such as DO, EC, pH, ORP, water temperature, to monitor the progress of well washing progress [35–39]. When the ATP and other indicators are stable, indicating that the well flushing is finished, at this time, the indicators of ATP, ADP, and AMP could be measured simultaneously. We suggest that more than three parallel samples could be tested for each groundwater sample to reduce errors, the mean value could be selected as the microbial activity values of the actual samples.

5. Conclusion

The luciferase assay established in this study can simultaneously detect the concentration of ATP, ADP, and AMP rapidly. The Standard samples of ATP, ADP, and AMP with a volume of 150 μL, the minimum detection range of 0.025 ppb and the maximum detection range of 50 ppb were obtained, which met the detection range of groundwater microbial activity. The standard curves of ATP, ADP, and AMP were established, respectively, and the R^2 values were all >0.99. The method adopted in this paper was accurate and precise. On this basis, the concentration conversion system of the three were established, and the comparison experiment of microbial activity detection of petroleum-contaminated groundwater was carried out. It was shown that the correlation coefficient between this technique and UV spectrophotometry as well as plate count were >0.99 and 0.98, respectively, and the *P*-value was <0.001. The method developed in this study tends to be more convenient, faster, more reliable, and more economical than the existing methods.

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