Application of Microbiological Monitoring Techniques at Biological Wastewater Treatment Plant in CSC

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China Steel Corporation (CSC) is an integrated steel plant and produces coke oven wastewater that is hard to be treated. The Biological Wastewater Treatment Plant (WWTP) processes the coke oven wastewater which has the highest COD in CSC. The main components treated in this WWTP are COD and ammonia. The WWTP has four basins which are denitrification, COD removal, and two nitrification basins and the process is Anoxi-Oxic-Oxic-Oxic. Water quality of the influent and effluent together with suspended solids (S.S) in the system are used to monitor the system. There is no technique to monitor the bioactivity of microorganisms in the system. Adenosine triphosphate (ATP) and Specific Oxygen Uptake Rate (SOUR) assays can be used to monitor the bioactivity of microorganisms from 2 different aspects. The results showed both values are positively correlated with nitrification function of the system. On the other hand, Fluorescence In-Situ Hybridization (FISH) assays is a molecular biological technique that can be used to investigate specific types of microorganisms in the system. The result showed FISH can be used to detect ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the system with proper genetic probe. Different microbiological monitoring techniques give information from different angles and are complementary to each other. The system can be controlled better with the more information we gather.

Keyword: Microbiological Monitoring, ATP, SOUR, FISH

1. INTRODUCTION

CSC uses biological treatment to degrade pollutants in coke oven wastewater. The major components removed in the Biological WWTP are COD and ammonia. Biological treatments rely on bacteria, nematodes, or other small organisms to break down organic wastes using cellular processes[1]. The whole system is often seen as a black box and it sometimes takes days for the operators to detect the treatment system is under abnormal condition. Once the system crashes, it normally takes weeks or even months to recover. The condition of the treatment system is currently determined by the water quality of effluent and the amount of biomass in the system. The operators would be able to control the system better and find the problems faster with the information related to the bioactivity of the microorganisms in the system.

The traditional tests for microorganisms have been developed since the late 19th century[2]. Many new techniques have been invented and expanded throughout the years which has increased the diversity of the microbiological toolkit. These techniques can help the experts and operators to make better and more rapid decisions on how to better control the process. Multiple biological monitoring techniques including culture test, microscopic examination, metabolic-based tests, molecular biology methods, particulate analysis, bioassays, respirometry, and chemical methods have been evaluated. Each of them can give differing information on microorganisms. Three of them were selected and on site applicability was tested.

ATP assay is a metabolic-base test and measures light produced from a luminescent reaction between ATP and a mixture of luciferin, luciferase, and magnesium[3]. The light output is measured by a luminometer and results can be obtained within minutes. ATP is involved in the energy transfer of all living cells and is considered a measure of cell viability. ATP is a short-lived molecule [4]. The ATP assay can only be used as an approximation of the amount of live cells in a solution. To identify changes in microbial quality, baseline conditions should be established and monitored regularly. Any fluctuations from the baseline may indicate a change of microbial concentration due to operational problems. The ATP assay was selected due to the requirement of the sample volume being small and the measurement time being short. Applicability assessment of ATP assay was tested at Biological WWTP in CSC and the sludge samples were taken from denitrification, COD removal, and nitrification basins. The cellular ATP concentration was

positively correlated with the nitrification function of the system.

The oxygen uptake rate (OUR) is one of the most important variables for estimating biological activity in the biological wastewater treatment process. Oxygen consumption rate assay was also conducted to determine the mixed liquor exposed to the contaminant, and was usually performed according to Standard Methods[5]. The OUR is proportional to the microorganism concentration and depends on the quality of the incoming wastewater. It is a measure for the quality of the activated sludge and may indicate the presence in the influent of sudden high loads of organic material (increase of OUR) or toxic elements (decrease of OUR)[6]. The Specific oxygen uptake rate (SOUR) is defined as the milligram of oxygen consumed per gram of volatile suspended solids (VSS) per hour and calculating by dividing the OUR by the MLVSS concentration. The oxygen consumption is directly tied to metabolism and provides direct information on the consumption and inhibition rate of biological degradation of chemical substrate. SOUR assay was selected due to the measurement time being short and can be easily performed on site. Applicability assessment of SOUR was tested at Biological WWTP in CSC and the sludge samples were taken from nitrification basins. The SOUR value was positively correlated with the nitrification function of the system.

Development of test methods based on genetic analysis dominates current research[7]. The most common ways include PCR (Polymerase Chain Reaction), FISH (Fluorescence In-Situ Hybridization), and MDM (Microbial Diagnostic Microarrays). The basic principle of genetic analysis is the detection of the presence of specific chains or sequences of genetic material. If the gene is unique to a certain organism, the presence of the organism can be identified by the genetic method. These methods are extremely useful to identify specific types of microorganisms. Fluorescence in situ hybridization (FISH) technique using oligonucleotide probes labeled with fluorochromes was described in 1989 by Edward F. DeLong[8]. This revolutionary approach enabled scientists to identify microorganisms in environmental samples rapidly. Identification of microorganisms with the FISH method can be performed with the availability of special molecular probes containing a sequence of the genetic material complementary to the known sequence of RNA or DNA. With the fluorescent molecule attached to the acid, the microorganisms become well visible under the fluorescence microscope. Applicability assessment of FISH was tested at Biological WWTP in CSC and the sludge samples were taken from nitrification basins. The FISH can be used to identify AOB and NOB groups in the sludge.

2. EXPERIMENTAL METHOD

2.1. Adenosine triphosphate (ATP) assay

Sludge from denitrification, COD removal, and nitrogen removal basins were collected. QG21W kit from Luminultra was used to measure the concentration of total ATP (tATP), dissolved ATP (dATP), and cellular ATP (cATP).

One ml of sludge sample was added to a 2 ml UltraLyse 30[21] Extraction Tube and incubated for at least 1 minute. The mixed sample was transferred into 8 ml UltraLute/Resin Tube and mixed well. 100 ul of mixed sample was collected and mixed with 300 ul of Luminase. Luminometer was used to measure the RLU_{tATP} value. On the other hand, 100 ul of sample was added into a 10 ml LumiSolve Tube and incubated for at least 1 minute. 100 ul of mixed sample was collected and mixed with 300 ul of Luminase. Luminometer was used to measure the RLU_{dATP} value. 2 drops of UltraChek1 was mixed with 300 ul of Luminase and luminometer was used to measure the RLU_{ATP1} value. The RLU value of UltraChek1 was used as standard calibration (ATP1) to convert RLU values into actual ATP concentration. tATP measures all ATP within the sample, including ATP from living cells in addition to ATP that has been released from dead cells. tATP(ng ATP/ml) = RLU_{tATP}/RLU_{ATP1} X11. dATP represents ATP within a sample that has been released from dead cells. dATP (ng ATP/ml) = RLUdATP/RLU_{ATP1} X101. cATP represents the amount of ATP contained within living cells and is direct indication of total living biomass quantity. cATP (ng ATP/ml) = tATP-dATP

2.2 Specific oxygen uptake rate (SOUR) assay

A modified SOUR method (1) was used. The sludge was collected and put in a sealed 500 ml serum bottle with a plugged in dissolved oxygen (DO) meter. The DO concentration was measured and recorded every 20 seconds until the value became zero or for a period of 10 minutes. The oxygen uptake rate was calculated and divided by the concentration of suspended solid.

A modified SOUR method (2) was used. Artificial wastewater was made using deionized water with 1,000 ppm $NaHCO_3$ and 75 ppm NH_4Cl. The air was pumped into the artificial wastewater for at least one hour. The sludge was collected and 100 ml of the sludge was put in the 500 ml serum bottled. 400 ml of artificial wastewater with saturated dissolved oxygen was put in the serum bottled with sludge. The serum bottle with DO meter was sealed. The DO concentration was measured and recorded every 20 seconds until the value became zero or for a period of 10 minutes. The oxygen uptake rate was calculated and divided by the concentration of suspended solid.

2.3 Fluorescence In-situ Hybridization (FISH)

The sludge was collected and the Nitri-vit kit (Vermicon Munich, Germany) was used for bacterial staining. The sludge was diluted with the wastewater to a 1:1 ratio. 30 ul of diluted sludge 1:1 was fixed with absolute ethanol for 5 minutes. Ten µl of sample was pipetted onto three slide wells ((+), (vit), and (-)) and the Nitri-Vit kit® was used to perform FISH staining. Once the staining was complete, the slides were analyzed using a Vit-adapted fluorescence microscope with a 100x oil immersion objective. The slides were analyzed under a LEICA florescent microscope using blue light, Cy3 filter, and green light, FITC filter. Due to the DNA probe in the dye, all viable cells illuminate red. The AOB groups illuminate red and the NOB groups illuminate green. The negative control well has no cell illumination. The amount of AOB and NOB in the sludge were determined by the Nitri-Vit kit® recommended evaluation formula. Each of the 20 visual fields was evaluated on a 0 to 5 scale then were average to give a final score for each sample.

3. RESULTS AND DISCUSSION

The flow chart of Biological WWTP in CSC is shown in Figure 1. The major functions of this WWTP are removing COD and ammonia. There are four basins which contain sludge (microorganisms) to degrade pollutants. The denitrification basin is anoxic and the COD removal basin and two nitrification basins are all aerobic.

3.1 ATP assay can be used to monitor the status of the bioreactor

ATP is a complex organic chemical that provides energy to drive many processes in living cells, such as nerve impulse propagation, muscle contraction, and chemical synthesis. Found in all forms of life, ATP is often referred to as the molecular unit of currency of intracellular energy transfer[9]. When consumed in metabolic processes, it converts either to adenosine diphosphate (ADP) or to adenosine monophosphate (AMP) [10]. ATP is a direct and interference-free indicator of total living biomass and is measured using the firefly luciferase assay. A sample containing ATP was introduced to a solution containing the enzyme Luciferase, which naturally occurs in the tails of fireflies, to produce light. The light is detected in a luminometer as Relative Light Units (RLU) and the ATP concentration can be determined.

The applicability assessment of the ATP monitoring technique was tested in denitrification, COD removal, and nitrification basins. The results from May 2018 to June 2019 showed that the cATP concentration in the denitrification basin was negatively correlated with the residual concentration of phenol. The residual concentration of phenol in the denitrification basin was used to be one of the indicators to evaluate the state of the denitrification basin. The cATP concentration in COD removal basin was positively correlated with the SCN^- removal ratio. The SCN^- removal ratio in COD removal basin was used to be one of the indicators to evaluate the status and the nitrification function of the system.

The results also showed the cATP concentration in both nitrification basins was similar and the correlation value was up to 0.95. The cATP concentration of the first nitrification basin could represent the bio-activity in both basins. The cATP concentration in nitrification basin was positively correlated with the ammonia removal ratio of the system (Figure 2). S.S. in the system and SCN^- removal ratio in COD removal basin used to be two key indicators to evaluate the nitrification function of the system. The correlation value of cATP concentration and nitrification ratio is 0.69, which is higher than the correlation value between S.S or SCN^- removal ratio and nitrification ratio (Table 1). In addition, the result showed cATP concentration in denitrification, COD removal, and nitrification basins need to be higher than 1,800, 2,000, and 3,000 ng/ml respectively to maintain the full function of the system.

Microorganisms need energy (ATP) to metabolize the pollutants. ATP monitoring provides another angle to evaluate the bio-activity of the system. In addition, the ATP assay takes less than 5 minutes which is shorter than the S.S. measurement.

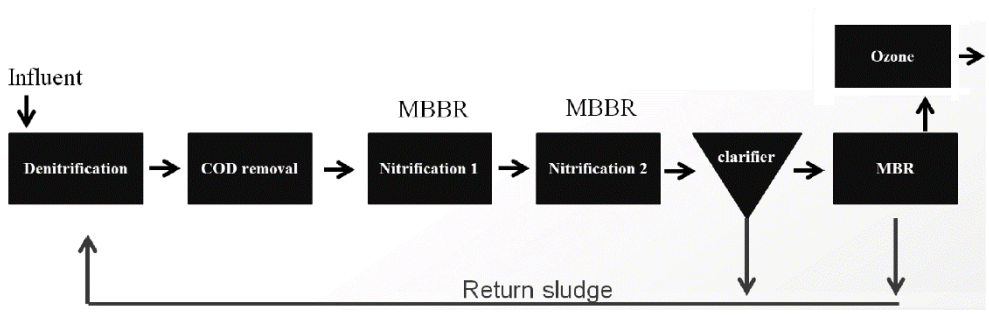


Fig.1. The flow chart of Biological WWTP in CSC.

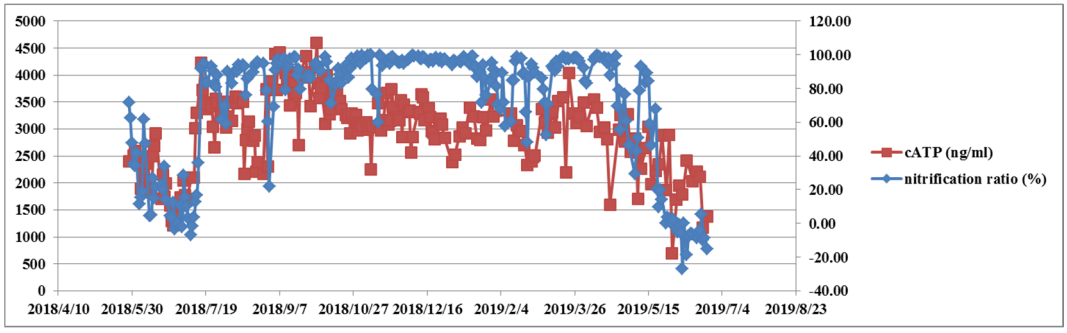


Fig.2. ATP concentration in nitrification basin was positively correlated with the nitrification ratio of the system.

Table 1 The correlation values between cATP, S.S., or SCN^- removal ratio and nitrification ratio of the system.

	Correlation value with ammonia removal ratio
cATP	0.69
S.S	0.27
SCN^-	0.53

3.2 Development of modified SOUR technique in Biological WWTP

The Specific Oxygen Uptake Rate (SOUR) is also known as the oxygen consumption or respiration rate. SOUR is defined as the milligram of oxygen consumed per gram of volatile suspended solids (VSS) per hour. This assay can rapidly measure organic load and biodegradability of influent. It can be the indication of the presence of toxic or inhibitory wastes.

The denitrification basin is anoxic so the SOUR assay could not be used. The applicability assessment of SOUR monitoring technique was tested in COD removal basin and 2 nitrification basins. The activity of the sludge in COD removal basin was found to be significantly high and the oxygen consumption was too fast to be measured. The DO was dropped dramatically in short period of time and the oxygen uptake rate (OUR) cannot be determined in time using the modified SOUR method (1).

The SOUR value was found to be positively correlated with nitrification ratio in nitrification basins when the system is under unstable circumstances using the modified SOUR method (1). The modified SOUR method (1) seemed can be one of the techniques to monitor the total bio-activity of sludge. However, it was found that there was no correlation between the SOUR value and nitrification ratio during the steady state. Similar phenomenon found in COD removal basin described above was also observed in the nitrification basins. The DO was consumed too fast and OUR value was unable to be determined in time. The modified SOUR method (2) was then developed and tested with sludge collected from 2 nitrification basins. The 5 month applicability assessment results showed that there was positive correlation between SOUR value and nitrification ratio in nitrification basins (Figure 3). The correlation values between the SOUR value using modified SOUR method (2) and nitrification ratio are higher than the values using the modified SOUR method (1) during the steady state (Table 2). The SOUR could be one of the parameter to monitor the bio-activity of the sludge and it could be done within 15 minutes. Currently, we are working on building an on-line SOUR monitoring machine.

3.3 FISH can identify AOB and NOB groups

FISH is a molecular cytogenetic technique that uses fluorescent probes binding to only parts of a nucleic acid sequence with a high degree of sequence complementarity. With test kit Nitri-VIT® by Vermicon, the nitrifying bacteria populations can easily be analyzed directly in wastewater samples. Both nitrifying bacteria groups, AOB and NOB, are differently stained and identified during one analysis. The results can be provided within 5 hours. Both ATP and SOUR are measuring the total bioactivity of microorganisms in the system. The FISH can give us the information regarding the growth and decline of particular functional groups of microorganisms.

The applicability assessment of FISH technique and Nitri-VIT kit was tested using the sludge collected from the Biological WWTP. The results showed that there are both AOB and NOB groups in the sludge (Figure 4). The amount of AOB and NOB in the sludge were quite equal determined by the Nitri-Vit kit® recommended evaluation formula.

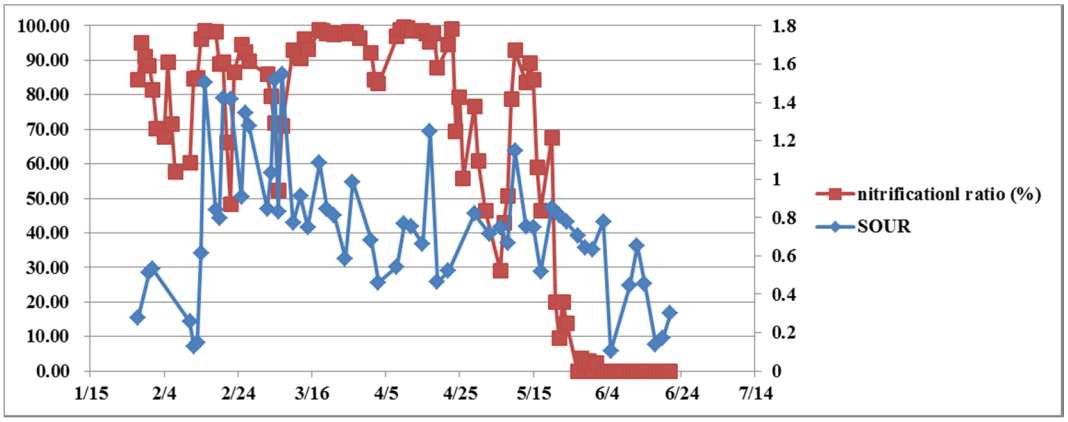
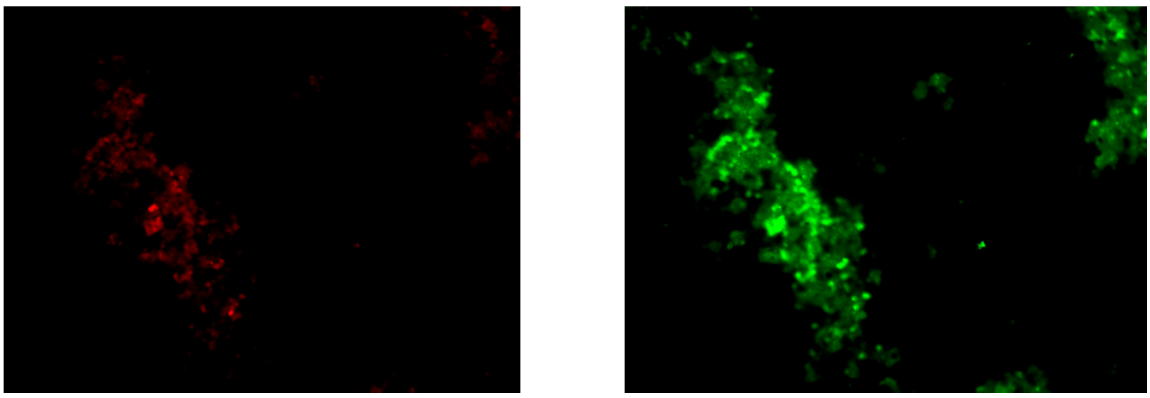


Fig.3. SOUR value in nitrification basin was positively correlated with the nitrification ratio of the system.

Table 2 The correlation values between the SOUR value from modified SOUR method (1) and (2) and nitrification ratio of the system.

	Correlation value with ammonia removal ratio
SOUR method (1)	0.01
SOUR method (2)	0.37



(a) (b)

Fig.4. AOB (A) and NOB (B) groups in the sludge were identified by FISH

4. CONCLUSIONS

Biological wastewater treatment process is used to treat coke oven wastewater in CSC. COD and ammonia are two major components needed to be removed using this process. The operators measure the water quality of the effluent and the suspended solids in the basin daily to monitor the condition of the system. The system was seen as a black box and there is no techniques used to assess the microorganisms in the system. In order to further dissect the system and help the operators to control the process better, microbiological evaluation techniques were investigated. Three techniques were selected and applicability assessment tests were performed on site.

ATP is referred to as the molecular unit of currency of intracellular energy transfer. ATP assays can give results in 5 minutes and is an indicator of bioactivity of microorganisms. Microorganisms of aerobic system consume oxygen to degrade pollutants in wastewater. The SOUR assays can give results in 15 minutes and is one of the most important variables for estimating bioactivity in biological wastewater treatment process. Both assays can give results in short period of time and is easy for operators to perform on site. These two assays can both used to determine the metabolic activity of a

microbial population. However, the fundamental of these two assays are different and give different aspects of information to operators to discover the problems of the bio-system in time.

FISH assays can be used to investigate specific types of microorganisms in the system. The Nitri-vit kit developed by Vermicon can be used to detect AOB and NOB groups in the bioreactor. FISH assay takes at least 5 hours to give results and people who perform the assays need high skills and experience in microscopy. It is not a good tool for the operators to perform on site, but it is a useful tool to trace the distribution of microorganisms in the system for long term tracking.

In conclusion, both ATP and SOUR assays give information about the total metabolism status of the system. On the other hand, the FISH assay identifies specific and functional groups of microorganisms in the system. Different techniques give information from different angles and are compatible to each other. The more information we have, the better we can control the system and find the problems sooner.

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