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Development of an ATP luminescence-based method for assimilable organic carbon determination in reclaimed water

Guo-Qiang Li [a], Tong Yu [a], Qian-Yuan Wu [b], Yun Lu [a, *], Hong-Ying Hu [a, c, **]

[a] Environmental Simulation and Pollution Control State Key Joint Laboratory, State Environmental Protection Key Laboratory of Microorganism Application and Risk Control (SMARC), School of Environment, Tsinghua University, Beijing 100084, PR China
[b] Shenzhen Laboratory of Microorganism Application and Risk Control, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, PR China
[c] Shenzhen Environmental Science and New Energy Technology Engineering Laboratory, Tsinghua-Berkeley Shenzhen Institute, Shenzhen 518055, PR China

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ABSTRACT

Assimilable organic carbon (AOC) is an important indicator of the biological stability of reclaimed water. In this study, a new rapid and more stable method for AOC measurement in reclaimed water was proposed. Indigenous microbial culture from secondary effluent was used as the inoculum, and bacterial growth was determined by the quantity of adenosine triphosphate (ATP) in the form of luminescence instead of plate count. ATP luminescence had a high correlation with biogrowth both in pure acetate solutions and reclaimed waters. ATP luminescence analysis could be determined in 5 min. Three days of 10000 cells/mL inoculum incubated at 25 °C were enough for the bacteria to reach the stationary phase. The good correlations between ATP luminescence and the added acetate-C concentration illustrated the applicability of monitoring AOC level by luminescence method. And in reclaimed water samples, indigenous microbial culture produces the highest AOC results compared with the pure strains. This indicated that the integrity of indigenous microbial culture ensured the full utilization of matrix carbons, which demonstrated the advantage of indigenous microbial culture compared with the selected pure bacteria in the traditional AOC test. The average ATP content per cell of 3.95×10^{-10} nmol/cell was derived, and this value was stable in both the acetate solutions and reclaimed waters. Furthermore, the average yield coefficient of 1.5×10^{5} RLU/μg acetate-C (4.1×10^{-3} nmol ATP/μg acetate-C) was obtained from different indigenous cultures. Additionally, the indigenous microbial cultures from different secondary effluents would produce the similar AOC results for the same water sample, indicating the consistency of this assay. The ATP luminescence-AOC assay provides a faster, more stable and accurate approach for monitoring the biological stability of reclaimed waters.

1. Introduction

Water reuse is increasingly becoming a necessity because of freshwater scarcity caused by increasing populations and degradation of existing sources of water. However, water reuse could be limited by microbial growth because of the biodegradable organic matter (BOM) available in water. One of the main approaches to measure BOM is the assimilable organic carbon (AOC) assay. AOC is defined as a fraction of BOM that can be readily utilized by microorganisms for growth, which contains types of low molecular weight organic carbons such as amino acids, carboxylic acids, carbohydrates and oxalic acid (van der Kooij and Hijnen, 1984). In distribution systems, bacterial regrowth would cause the deterioration of water quality, biofilm formation and pipe corrosion. More importantly, the opportunistic pathogens (*Aeromonas*, *Legionella*, *Mycobacterium*, *and Pseudomonas*) supported by AOC occurred more frequently than indicator bacteria in reclaimed water (Jjemba et al., 2010), arousing public concern. Additionally, advanced treatment is required for water reuse. But some

Abbreviations: AOC, assimilable organic carbon; ATP, adenosine triphosphate; RLU, relative light unit; L_{max}, maximum ATP luminescence; AAO, anaerobic-anoxic oxic; MBR, membrane bio-reactor; TDC, total direct cell count; BOM, biodegradable organic matter; P17, *Pseudomonas fluorescens* P17; G3, *Pseudomonas saponiphila* G3; NOX, *Spirillum* NOX; DOC, dissolved organic carbon.

* Corresponding author. Room 724, School of Environment, Tsinghua University, Beijing, 10084, PR China.
** Corresponding author. Room 524, School of Environment, Tsinghua University, Beijing, 10084, PR China.
E-mail addresses: luyun@tsinghua.edu.cn (Y. Lu), hyhu@tsinghua.edu.cn (H.-Y. Hu).

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treatments, such as ozonation, UV/H_2O_2 and Fenton oxidation, would increase AOC (Bazri et al., 2012; Escobar and Randall, 2001; Li et al., 2013). However, AOC accounts for only 1.5–13.1% of total organic carbon in reclaimed waters (Shan et al., 2005; Thayanukul et al., 2013). Thus, it is very important to determine the concentration and the relative changes of AOC in reclaimed waters.

The AOC test was first described as a bioassay in drinking water by Kooij et al. (1982). Thereafter, researchers optimized the measurement procedures (Kaplan et al., 1993; van der Kooij and Hijnen, 1984). Generally, model bacteria *Pseudomonas fluorescens* strain P17 and *Spirillum* strain NOX were inoculated into the water samples. A long assay time of 6–12 days was required depending on the incubation temperature. The pure strain P17 was selected as an inoculum because of its versatility of nutrient utilization in drinking water (Kooij et al., 1982). However, strain P17 does not utilize low molecular weight carboxylic acids, therefore strain NOX which can grow on carboxylic acids and strain P17 were combined in the subsequent AOC test (van der Kooij and Hijnen, 1984). Recently, biopolymer-utilizing *Flavobatcrium johnsoniae* strain A3 was added in the AOC test because of its ability to utilize proteins and polysaccharides (van der Kooij et al., 2015). In reclaimed water, bioluminescence-based test using mutagenic P17 and NOX strains was developed as a faster method for AOC determination (Weinrich et al., 2009). Three new strains of *Stenotrophomonas* sp. ZJ2, *Pseudomonas saponiphila* G3 and *Enterobacter* sp. G6 were selected as the inoculum for AOC in reclaimed water because of their abilities to utilize polymers and carbohydrates (Zhao et al., 2013), which showed much higher growth potential than P17 and NOX. Hence, the use of pure bacterial cultures always presents a question of as to what extent the pure culture can mimic the real situation, in which most of the complicated carbon components can be utilized.

Hammes and Egli (2005) used indigenous microbial culture instead of pure test strains for AOC measurement in drinking water and the number of microbes was determined by flow cytometry. However, the flow cytometry is very expensive and often has high background for particles. The yield coefficient of indigenous microbial culture of 1×10^7 bacteria (μg of AOC)$^{-1}$ was deduced from the average carbon content of a bacteria cell and the percentage of carbon being assimilated. It was not verified in the acetate solutions. Further, considering the complicated matrix of organic matters in reclaimed water, whether the varied sources of indigenous microbial culture would produce a similar AOC result is not clear.

ATP-dependent luminescence analysis is viewed as a rapid method for the measurement of cell biomass, which serves as a useful quantitative indicator of the active bacteria in the environment. It was reported that the number of total bacteria had high correlation with the amount of ATP in the medium (Hammes et al., 2010; Karl, 1980; van der Kooij et al., 2015). Lechevallier et al. (1993) used ATP analysis to determine the growth of test strains P17 and NOX in AOC test. And Stanfield and Jago (1989) observed a good correlation between the ATP yields and the supplement of acetate in drinking water, indicating the ATP could be used as a measure of the AOC content of the water. Furthermore, ATP analysis is able to quantify an indigenous microbial culture accurately and quickly when a commercial ATP quantification kit was applied instead of the complicated ATP analysis in previous study (Lechevallier et al., 1993).

In this study, we described a rapid and stable AOC bioassay using indigenous microbial cultures from the secondary effluent as the inocula with bacterial growth determined by ATP assay. The conditions of the bioassay were optimized according to the bacterial growth characteristics. The yield coefficients of indigenous microbial cultures were determined in acetate solutions and compared with the Hammes and Egli (2005) recommendation. To provide insight into the effect of varied sources of microbial culture, the AOC of the same water sample incubated with different indigenous microbial culture was also investigated. Due to the application of the indigenous microbial culture a more accurate measurement of AOC would be obtained.

2. Materials and methods

2.1. Preparation of carbon-free glassware

The previous AOC test could detect the organic carbon as low as 10 μg/L (Kooij et al., 1982); therefore, all containers must be carbon free. 50 mL borosilicate bottles with frosted glass caps were prepared as described previously (Clesceri et al., 1998). Briefly, containers and caps were washed with tap water and then rinsed three times with ultrapure water. They were then soaked in 0.1 N HCl and treated in an ultrasonic apparatus for two hours before being rinsed three times with ultrapure water again. Vials were subsequently dried, capped with foil, and heated at 550 °C for 6 h in a muffle oven to oxidize any residual carbon.

2.2. Preparation of indigenous microbial culture

The preparation of the indigenous microbial culture was described by Hammes and Egli (2005). In this study, secondary effluents were sampled from wastewater treatment plants in Beijing, China. Water samples first were filtered through a 0.2 μm membrane (Millipore) to remove particulate matter. Then, 100 μL of unfiltered secondary effluent was inoculated to 40 mL filtrate and incubated in carbon-free vials at 25 °C for 10 days. The solution was subsequently centrifuged at 10000 rpm for 10 min to harvest all the cells, and then resuspended with sterile saline and then incubated at 25 °C for 4 more days to deplete all the residual organic carbon. This stock culture was stored at 4 °C for up to 2 months.

To compare with the traditional AOC test, pure strains were prepared. P17 (ATCC 49642) and NOX (ATCC 49643) were obtained from Liu et al. (2002). The culture of *Pseudomonas saponiphila* G3 (CGMCC 5814) was screened from reclaimed water (Zhao et al., 2013). The strains were stored at −80 °C in culture medium with 25% glycerol. They were recovered in a media of sodium acetate solution (2000 μg/L acetate-C) at 25 °C for 7 days prior to use. Cell concentrations of the stock solution were determined by plate count on R_2A agar (25 °C, 3–5 d).

2.3. ATP analysis: protocol optimization

The BacTiter-Glo™ Microbial Cell Viability Assay (G8231; Promega Corporation) and a high-sensitivity photon-counting luminometer (SpectraMax M5; Molecular Devices) were used for measuring the ATP luminescence (relative light unit, RLU). The mixed reagent was stored at −80 °C for one month and never thawed over three times. The protocol recommends a volume of reagent equal to the volume of cell culture present in each well (e.g., 100 μL reagent to 100 μL of sample for the 96-well plate format) and incubating the mixture at room temperature for 5 min (ensuring that the temperature of the sample and ATP reagent are constant). Large sample volume and reaction temperature were investigated in this protocol. The reagent volume was set to 50 μL and the sample volumes were 50, 100, and 150 μL, respectively. The maximum luminescence of the 150 μL sample was selected to investigate the influence of incubation temperatures (25–42 °C). From these results an optimized protocol was formulated for all the water samples.