Rapid quantification of polyhydroxyalkanoates (PHA) concentration in activated sludge with the fluorescent dye Nile blue A
M. Oshiki, H. Satoh and T. Mino

ABSTRACT
The present study was conducted (1) to develop a rapid quantification method of polyhydroxyalkanoates (PHA) concentration in activated sludge by Nile blue A staining and fluorescence measurement and (2) to perform on-line monitoring of PHA concentrations in activated sludge. Activated sludge samples collected from laboratory scale sequencing batch reactors and full-scale wastewater treatment plants were stained with Nile blue A and their fluorescence intensities were determined. There was a high correlation ($R^2 > 0.97$) between the fluorescence intensities of Nile blue A and PHA concentrations in activated sludge determined by gas chromatography. The Nile blue A staining and fluorescence measurement method allows us to determine PHA concentrations in activated sludge within only five minutes and up to 96 samples can be measured at once by using microplate reader. On-line monitoring of PHA concentrations in activated sludge was achieved by using a fluorometer equipped with a flow cell and the time point at which PHA concentration in activated sludge reached the maximum level could be identified. In addition, we examined the influence of pH, floc size and co-existing chemicals in activated sludge suspension on the fluorescence intensities of Nile blue A.

Key words | activated sludge, fluorometric quantification, Nile blue A, polyhydroxyalkanoates

INTRODUCTION
Polyhydroxyalkanoates (PHA) are polymers of hydroxylated fatty acids that are synthesized by different prokaryotic microorganisms as intercellular carbon storage material (Anderson & Dawes 1990). The production of PHAs by microorganisms has been intensively studied as one of the potential alternatives to petrochemical plastic materials since PHAs have the nature of biodegradable thermoplastic produced from organic wastes. While pure cultures such as *Cupriavidus necator*, formally known as *Ralstonia eutropha* are widely employed for the production of PHA, there are researchers who work on PHA production by mixed culture systems (Satoh et al. 1998; Lemos et al. 2004) because of the following potential advantages; no need of sterilization, which leads to lower initial and operating cost, and higher chances to make use of organic wastes as a cheaper carbon source. Recently, PHA production capability using a mixed culture system has been intensively improved (Serafim et al. 2004; Johnson et al. 2009). Johnson et al. (2009) achieved a PHA content of 89% in dry cell weight with an average PHA accumulation rate of 1.2 g g$^{-1}$ h$^{-1}$ by using the microorganisms in activated sludge, which had similar or superior PHA production capability to the system using pure cultures. It is now recognized that cultivation conditions such as pH, sludge retention time (SRT), substrate concentration (Chua et al. 2005) and C/N ratio (Wang et al. 2006) have a large impact on the PHA production capability of activated sludge. Thus, large sets of batch experiments are required to screen the optimal cultivation condition for the higher PHA production capability. A rapid and high-throughput PHA quantification method is essential for this purpose.

The concentration of PHA in activated sludge has been determined by gas chromatography (Comeau et al. 1988; Oehmen et al. 2005) or ion chromatography (Hesselmann et al. 1999). These conventional methods are, however, composed of time-consuming procedures and required more
than 1 day or 1.5 h for the measurement of gas chromatography or ion chromatography, respectively. On the other hand, Page & Tenove (1996) proposed a PHA quantification method using Nile blue A. Nile blue A is known as a specific fluorescent dye for PHA granules (Ostle & Holt 1982) and PHA granules emit strong fluorescence when they are bound with Nile blue A. Page and Tenove stained PHA-accumulating cells of *Azotobacter vinelandii* and *Cupriavidus necator* with Nile blue A, measured fluorescence intensity and confirmed a high correlation between fluorescence intensity and PHA concentration ($R^2 > 0.95$). This Nile blue A staining and fluorescence measurement method has the potential to be a rapid PHA quantification method for activated sludge samples since the measurement procedure is just composed of Nile blue A staining for 10 min and subsequent fluorescence measurement. However, Page & Tenove (1996) also pointed out that the slope and Y-intercept of the standard curve was quite different between the two microbial species, which cast doubt on the application of the Nile blue A staining and fluorescence measurement method to mixed cultures such as activated sludge.

The aims of the present study were (1) to develop a rapid quantification method of PHA concentration in activated sludge by Nile blue A staining and fluorescence measurement and (2) to perform on-line monitoring of PHA concentrations in activated sludge. For the first purpose, activated sludge samples were stained with Nile blue A and the fluorescence intensities were determined. The PHA concentrations in those activated sludge samples were determined by gas chromatography and the correlation between the fluorescence intensities and PHA concentrations was examined. For the on-line monitoring of PHA concentrations in activated sludge, we employed a fluorometer equipped with a flow cell and the fluorescence intensities of Nile blue A were monitored after the addition of acetate. In addition, the influences of pH, floc size and co-existing chemicals in activated sludge suspension on the fluorescence intensity of Nile blue A were examined.

### MATERIALS AND METHODS

#### Activated sludge samples

The activated sludge samples used in the present study are listed in Table 1 and were collected from laboratory scale sequencing batch reactors (SBRs) or full-scale wastewater treatment plants (WWTPs). Two SBRs, SBR-I and SBR-II, were operated in the laboratory. Activated sludge obtained from a domestic wastewater treatment plant was used for seeding of SBR-I and SBR-II. Both SBR-I and SBR-II were operated at room temperature (22 ± 2°C), and the pH was not controlled. The working volume of SBR-I was 10 L and operated in 6 hour cycles: 12 min feeding, 288 min aerobic reaction, 30 min settling, and 30 min effluent discharge. In the feeding phase, the SBR-I was fed 5 L influent containing: acetate 150 mg C L$^{-1}$, CaCl$_2$·2H$_2$O 8.8 mg L$^{-1}$, MgCl$_2$·6H$_2$O 90.8 mg L$^{-1}$, KCl 42.0 mg L$^{-1}$, NH$_4$Cl 17.6 mg L$^{-1}$, (NH$_4$)$_2$SO$_4$ 21.6 mg L$^{-1}$, K$_2$HPO$_4$ 18.0 mg L$^{-1}$, and KH$_2$PO$_4$ 14.0 mg L$^{-1}$. The hydraulic retention time was 12 h, and the SRT was 12.5 days. The SRT was controlled by removing sludge suspension once per day (during aerobic reaction) 0.8 L from the reactor using a plastic syringe. The working volume of SBR-II was 12 L and operated in 24 hour cycles: 5 min feeding.

#### Table 1: Activated sludge samples used in the present study

<table>
<thead>
<tr>
<th>Source</th>
<th>Sample name</th>
<th>Carbon source</th>
<th>Source</th>
<th>Sample name</th>
<th>Carbon source</th>
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<tbody>
<tr>
<td>Laboratory scale SBR</td>
<td>S-I</td>
<td>Acetate</td>
<td>A WWTP</td>
<td>A</td>
<td>Acetate</td>
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<td></td>
<td></td>
<td>Propionate</td>
<td>A2</td>
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<td>Acetate</td>
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<tr>
<td>Laboratory scale SBR</td>
<td>S-II</td>
<td>Acetate</td>
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<td>Propionate</td>
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1,400 min aerobic reaction, 30 min settling, and 5 min effluent discharge. In the feeding phase, the SBR-II was fed 10 L influent containing: skim milk (Morinaga milk industry, Tokyo, Japan) 200 mg L\(^{-1}\) and yeast extract 200 mg L\(^{-1}\). The control of SRT and the discharge of excess sludge were not performed on SBR-II. The hydraulic retention time was 29 h. The SBR-I and SBR-II were operated for two weeks, and the activated sludge sample was taken at the end of the aerobic phase. The activated sludge samples taken from SBR-I and SBR-II are described as S-I and S-II hereafter.

Activated sludge samples were also collected from the end of the aeration tank in three municipal WWTPs, A, B and C WWTP, operated in fully aerobic mode. As for A and B WWTP, activated sludge samples were collected weekly during April to May 2008, which were referred to as A1 to A5 and B1 to B4, respectively. As for C WWTP, the activated sludge sample was taken on November 2010 and referred to as C.

### Batch experiment

Activated sludge was put in a 1-L glass beaker and incubated after the addition of acetate or propionate as shown in Table 1. Prior to the incubation, mixed liquor suspended solids (MLSS) of activated sludge samples were set to around 500 mg L\(^{-1}\) solids (MLSS) of activated sludge samples were set to

<table>
<thead>
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<th>Sample</th>
<th>Operation</th>
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<tr>
<td>A1</td>
<td>Addition of acetate or propionate</td>
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<tr>
<td>A2</td>
<td>Incubation</td>
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<tr>
<td>A3</td>
<td>Monitoring</td>
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<tr>
<td>A4</td>
<td>PHA Concentration</td>
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<tr>
<td>A5</td>
<td>Analysis</td>
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</table>

The acetate or propionate concentrations were monitored during the batch experiment by ion chromatograph DX-AQ1110 equipped with an AS-9HC column (Dionex, Osaka, Japan). Supplemental acetate or propionate was fed at a final concentration of 600 mg C L\(^{-1}\) when the concentration was below 50 mg C L\(^{-1}\).

Samples for Nile blue A staining or the determination of PHA concentrations by gas chromatography were taken at the end of incubation except for S-I, S-II and A1. As for S-I, S-II and A1, activated sludge suspension was collected at 0, 1, 2, 3, 4, 6, 8 and 12 h after the addition of carbon source to collect the activated sludge suspension containing different amounts of PHA. For Nile blue A staining, 1 mL of sludge suspension was dispensed into 1.5 mL plastic centrifuge tubes and stored at –80 °C. For the determination of PHA concentrations by gas chromatography, ten mL of sludge suspension was dispensed into glass test tubes (Nichidenrika glass, Hyogo, Japan), centrifuged, and the sludge pellet was stored at –20 °C.

### Nile blue A staining and fluorescence measurement

One hundred microlitre of activated sludge suspension was dispensed into a 96-well microplate (Black microwell SI, Thermo Fisher Scientific, Roskilde, Denmark). Equal volume of 0.02% (w/v) Nile blue A (Sigma-Aldrich Japan K.K., Tokyo, Japan) aqueous solution was dispensed into each well and mixed with 8-channel micropipette (Eppendorf, Tokyo, Japan). Nile blue A staining was performed at room temperature (18–25 °C) for 5 min and then the fluorescence intensity was determined by a fluorescent microplate reader MTP-601 (Corona electric, Ibaraki, Japan) at excitation and emission wavelengths of 490 and 590 nm, respectively. All the activated sludge samples were analyzed in fourfold.

### Determination of PHA concentration by gas chromatography

PHA concentrations in activated sludge were determined after methanolytic degradation as per the procedures stated by Oshiki et al. (2008). Briefly, an activated sludge sample was lyophilized and methanolyzed, and the monomeric unit of PHA was determined by a gas chromatograph Shimadzu GC2010/FID (Shimadzu, Kyoto, Japan) equipped with an Inert Cap1 capillary column (GL science, Tokyo, Japan). Sodium 3-hydroxybutyrate (3HB, Tokyo Chemical Industry, Tokyo, Japan) and the copolymer composed of 81% of 3HB and 19% of 3-hydroxyvalerate (3HV) (Sigma-Aldrich Japan K.K., Tokyo, Japan) were used as the standard for 3HB and 3HV unit in PHA, respectively.

### On-line monitoring of PHA concentrations in activated sludge

The on-line monitoring system shown in Figure 1 was employed and the fluorescence intensities of Nile blue A were monitored. Sludge C was diluted with tap water to MLSS concentration of around 500 mg L\(^{-1}\) and put in a 1-L glass beaker. After the addition of acetate at the final concentration of 600 mg C L\(^{-1}\), Sludge C was incubated with air supplied by an air pump. The peristaltic pump equipped with PharMed® BPT tube (inner diameter: 3 mm, Saint-Gobain Performance Plastics, OH, USA) continuously collected the activated sludge suspension from the beaker...
and 0.02% (w/v) Nile blue A aqueous solution in the glass bottle simultaneously at the flow rate of 0.5 mL min⁻¹ each. For this purpose, a plastic tube Y fitting (inner diameter: 3 mm, AS ONE, Osaka, Japan) was set on the tube line. The length of pump tube between the peristaltic pump and fluorometer was set to 0.5 m, which allowed us to stain the biomass with Nile blue A in the pump tube for more than 3 min. The flow cell was fabricated by putting the silicone rubber cap on a 3 mL plastic cuvette (Bio-rad, Tokyo, Japan) and the inside was continuously mixed with a small magnetic stirring bar (6 × φ 4 mm, AS ONE, Osaka, Japan) at 300 rpm. The fluorescence intensity in the flow cell was monitored by versafluor (Bio-Rad, Tokyo, Japan) at excitation and emission wavelengths of 490 and 590 nm, respectively, and manually recorded every 5 min. Fluorescence measurement was performed with the following conditions; gain: medium, AVRG: 1 sec and range: 00000. This experiment was performed at room temperature (20°C) and the control of pH and temperature was not performed.

Figure 1 | On-line monitoring system for the fluorescence measurement of Nile blue A.

The following chemicals were added into the suspension of activated sludge A₁ separately; 1) peptone; 0.05 and 0.5% (w/v), 2) yeast extract; 0.05 and 0.5% (w/v), 3) beef extract; 0.05 and 0.5% (w/v), 4) acetic acid; 0.05 and 0.5% (w/v), 5) propionate; 0.05 and 0.5% (w/v), 6) olive oil; 0.05% (v/v), 7) *Escherichia coli* (JCM1649); 0.09 and 0.44% (dry-w/v), 8) silt; 0.05 and 0.5% (w/v), 9) mixture of glucose and sucrose; 0.05 and 0.5% (each, w/v), 10) Tris-HCl; 0.06% and 0.6% (w/v), 11) ethylenediaminetetraacetate (EDTA); 0.19 and 1.9% (w/v), 12) sodium dodecyl sulfate (SDS); 0.05 and 0.5% (w/v), 13) phosphate; 1 and 10 mmol/L, 14) ammonia; 1 and 10 mmol/L, 15) nitrate; 1 and 10 mmol/L and 16) nitrite; 1 and 10 mmol/L. All the concentrations are the concentrations in the mixture after adding Nile blue A solution. Nile blue A staining and fluorescence measurement using the microplate reader were performed as per the procedures stated above.

**RESULTS AND DISCUSSION**

**PHA accumulation during the batch experiments**

All the activated sludge samples accumulated PHA with the consumption of acetate or propionate. PHA concentrations in activated sludge determined by gas chromatography ranged from 0.3 to 94.3 mg C L⁻¹. When acetate was fed as the carbon source, the fraction of the 3HB unit accounted for above 95% in accumulated PHA. On the other hand, the fraction of 3HV unit accounted for more than 70% in accumulated PHA when the propionate was fed.

**Correlation between the fluorescence intensities of Nile blue A and PHA concentrations in activated sludge**

The correlation between the fluorescence intensities of Nile blue A and PHA concentrations determined by gas chromatography is shown in Figure 2. A linear relationship was observed, with the slope 0.0493 (95% confidence interval, 0.0484 to 0.0502), the intercept 0.462 (95% confidence interval, 0.304 to 0.62), and the correlation coefficient 0.972. This outcome indicates that PHA concentration in activated sludge can be quantified based on the fluorescence intensity of Nile blue A even when (1) the source of the activated sludge sample and (2) the monomeric composition of PHA are different.

There was a possibility that autofluorescence derived from mineral crystals or microorganisms in activated sludge interferes with the fluorescence measurement of...
Nile blue A. However, the fluorescence intensities of Nile blue A prior to the batch experiment were almost the same in the activated sludge samples examined. In addition, the significant increase of fluorescence intensities corresponded to the increase of PHA concentration, as shown in Figure 2. These observations indicated that the influence of autofluorescence was minor at the fluorescence measurement of Nile blue A.

The measurement procedures developed in the present study were based on the one originally proposed by Page & Tenove (1996) but differ in the following two points; (1) Nile blue A staining was performed at ambient temperature (18–25°C) instead of 55°C and (2) the staining period was shortened from 10 to 3 min. We confirmed that these modifications did not give a significant difference (p < 0.05, Student t-test) in the fluorescence intensity of Nile blue A. The protocol of NBA staining and fluorescence measurement method developed in the present study is composed of the following procedures (See details in the section on Materials and Methods); (1) mix equal volume of activated sludge suspension and 0.02% Nile blue A aqueous solution, (2) incubate at ambient temperature (18–25°C) for 3 min, and (3) measure the fluorescence intensity of Nile blue A.

The concentration of PHA in activated sludge has been determined by gas chromatography or ion chromatography, while those conventional methods are not high-throughput techniques and do not allow us to deal with a large number of samples at once. For instance, the measurement procedures of gas chromatography are composed of (1) lyophilization (overnight), (2) digestion with methanol and sulfuric acids (>2–20 h), (3) mixing with chloroform and collection of the chloroform phase (1 h) and (4) measurement with gas chromatograph (>20 min per sample). As for ion chromatography, (1) digestion with propanol and sulfuric acids (>1 h), (2) separation and collection of aqueous phase (15 min) and (3) measurement with ion chromatograph (>30 min per sample) are required. The Nile blue A staining and fluorescence measurement method developed in the present study enables us to finish the analysis from sample preparation to the measurement of fluorescence intensity within only 5 min and up to 96 samples can be measured at once by using a microplate reader. PHA production capability of activated sludge depends on the incubation conditions such as pH, sludge retention time (SRT), substrate concentration (Chua et al. 2003) and C/N ratio (Wang et al. 2006). Thus, large sets of batch experiment and subsequent PHA quantification are required to screen the optimal incubation condition of activated sludge for the higher PHA production capability. For this purpose, our developed rapid and high-throughput PHA quantification method using Nile blue A is valuable. Another advantage of the Nile blue A staining and fluorescence measurement method is the safety. This method does not require handling the solvents (chloroforms, methanol or propanol) and acids (sulfuric acids), which has been used at the PHA quantification by gas chromatography or ion chromatography.

**On-line monitoring of fluorescence intensities of Nile blue A during PHA accumulation**

As shown in Figure 3, the fluorescence intensity of Nile blue A in Sludge C rapidly increased within 10 min after the addition of acetate and then linearly increased until 100 min. The fluorescence intensity reached a steady level after 100 min while acetate was still available (>100 mg C L⁻¹).

The on-line monitoring method for PHA concentrations in activated sludge has not been developed so far. The system shown in Figure 1 enabled us to monitor the PHA concentration in activated sludge and we could identify the time point where PHA concentration in Sludge C reached the maximum level. In Figure 3, the fluorescence intensity of Nile blue A was saturated after 100 min, which indicated that PHA concentration in Sludge C had reached the maximum level because there was a close relationship between PHA concentrations and fluorescence intensities of Nile blue A, as shown in Figure 2. In order to achieve economic PHA production, the reactor operation should be halted when PHA concentration in biomass reached the maximum level. For this purpose, our developed on-line monitoring system shown in Figure 1 is valuable. Furthermore, this
method should be useful to study the metabolism of PHA accumulation and consumption by polyphosphate-accumulating organisms or glycogen-accumulating organisms which are responsible for biological phosphorus removal.

On the other hand, the following two points should be considered prior to the application of Nile blue A staining and the fluorescence measurement method. Firstly, the determination of the monomeric composition of PHA is difficult by the Nile blue A staining and fluorescence measurement method. The biodegradability (Ikejima et al. 1998), crystallinity and brittleness (Anderson & Dawes 1990) will be altered due to the change of monomeric composition in PHA and thus their monomeric composition has to be determined when the produced PHA is used as a source of biodegradable plastics. The Nile blue A staining and fluorescence measurement method should be used as (1) a screening method to examine the optimal incubation condition of activated sludge for PHA production or (2) non-line monitoring method of PHA concentration to reach at the maximum level. The spectrum analysis of Nile blue A • fluorescence intensity is the fluorescence intensity of Nile blue A at the concentration of 0.5%, while such an increase was never observed at the concentration of 0.05%. Therefore, dilution of co-existing chemicals is an effective way to decrease the influence of co-existing chemicals on the fluorescence intensity of Nile blue A.

**CONCLUSION**

The close relationship between PHA concentrations in activated sludge and the fluorescence intensities of Nile blue A was confirmed by using the activated sludge samples taken from laboratory scale SBRs and full-scale WWTPs. The difference of pH gave less than 10% difference of fluorescence intensity of Nile blue A in the range of pH 6 to 9. The difference of floc size (300 µm or 20 µm) also gave less than 10% difference of fluorescence intensity of Nile blue A. The following co-existing chemicals gave less than 10% difference of fluorescence intensity of Nile blue A; yeast extract (at a final concentration of 0.05%), beef extract (0.05%), propionate (0.05%), silt (0.05%), peptone (0.5%), acetate (0.5%), mixture of glucose and sucrose (0.5%), Tris-HCl (0.06%), Escherichia coli (0.44%), phosphate (10 mmol/L), ammonia (10 mmol/L), nitrite (10 mmol/L) and nitrate (10 mmol/L). On the other hand, the fluorescence intensity was higher than 110% when the following chemicals were added; yeast extract (0.5%), beef extract (0.5%), propionate (0.5%), olive oil (0.05%) and EDTA (1.9%). The fluorescence intensity was lower than 90% when the following chemicals were added; silt (0.5%), Tris-HCl (0.6%) and SDS (0.05%).

PHA production by using organic wastes such as food wastes (Wong et al. 2000) or palm oil mills effluent (Loo et al. 2005) has the potential to reduce its production cost. In those processes, it is expected that the influent contain a variety of organic or inorganic matter which potentially affect the fluorescence intensity of Nile blue A. In addition, there was a possibility that pH and floc size affect the fluorescence intensity of Nile blue A and these influences have not been examined so far. Therefore, the influence of pH, floc size and co-existing chemicals on the fluorescence intensity of Nile blue A was examined in the present study. Some chemicals decreased or increased the fluorescence intensity of Nile blue A, which causes the underestimation or overestimation of PHA concentration in activated sludge. Those influences, however, can be reduced by the washing or dilution of the activated sludge sample. For instance, co-existing yeast extract caused the increase of fluorescence intensity of Nile blue A at the concentration of 0.5%, while such an increase was never observed at the concentration of 0.05%. Therefore, dilution of co-existing chemicals is an effective way to decrease the influence of co-existing chemicals on the fluorescence intensity of Nile blue A.

**Interference of pH, floc size and co-existing chemicals**

The difference of pH gave less than 10% difference of fluorescence intensity of Nile blue A in the range of pH 6 to 9. The difference of floc size (300 µm or 20 µm) also gave less than 10% difference of fluorescence intensity of Nile blue A. The following co-existing chemicals gave less than 10% difference of fluorescence intensity of Nile blue A; yeast extract (at a final concentration of 0.05%), beef extract (0.05%), propionate (0.05%), silt (0.05%), peptone (0.5%), acetate (0.5%), mixture of glucose and sucrose (0.5%), Tris-HCl (0.06%), Escherichia coli (0.44%), phosphate (10 mmol/L), ammonia (10 mmol/L), nitrite (10 mmol/L) and nitrate (10 mmol/L). On the other hand, the fluorescence intensity was higher than 110% when the following chemicals were added; yeast extract (0.5%), beef extract (0.5%), propionate (0.5%), olive oil (0.05%) and EDTA (1.9%). The fluorescence intensity was lower than 90% when the following chemicals were added; silt (0.5%), Tris-HCl (0.6%) and SDS (0.05%).

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**CONCLUSION**

The close relationship between PHA concentrations in activated sludge and the fluorescence intensities of Nile blue A was confirmed by using the activated sludge samples taken from laboratory scale SBRs and full-scale WWTPs. The

![Figure 3](https://iwaponline.com/wst/article-pdf/64/3/747/444351/747.pdf)
Nile blue A staining and fluorescence measurement method allows us to determine PHA concentration in activated sludge within only 5 min without the handling of hazardous reagents. This method could be applied for the on-line monitoring of fluorescence intensity of Nile blue A in activated sludge, which should be a powerful tool that aids the economic PHA production using the microorganisms in activated sludge as a PHA producer.

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