Study on the use of NADH fluorescence measurements for monitoring wastewater treatment systems

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Abstract

Fluorescence measurement of intracellular nicotinamide adenine dinucleotide (NADH) provides information about the physiological response of microbes towards changing conditions in their environment and has been suggested to be useful for the control of wastewater treatment plants. In this study, the practical usefulness of such measurements was evaluated from batch experiments with a commercially available NADH sensor in a bench scale reactor. The sensor was linear in the NADH concentration, robust, almost maintenance free, and hardly sensitive to floc size distribution. Measured fluorescence intensity proved to depend strongly on the concentration of active heterotrophic biomass. The NADH level was supposed to be dependent on the ratio of electron donor/electron acceptor availability inside the cells; however, neither acetate nor ammonium addition was reflected by the measurement signal.

A jump wise NADH signal change was observed at complete oxygen or nitrate depletion as also reflected by bends in the redox curve. In the near zero concentration ranges of oxygen and nitrate (0.1–0.5 mg/l) the signal changes only slightly in the opposite direction to the redox trend.

Keywords: Fluorescence measurements; Monitoring; NADH; Redox; Wastewater

1. Introduction

Robust and almost maintenance-free fluorescence measurement devices are available nowadays to be used for monitoring wastewater treatment plants. These measurements are related to microbial activity, as was discovered in 1957 by Duysens and Amesz [1]. They showed that bakers yeast suspension exhibit fluorescence when exposed to UV light and that the fluorescence spectrum resembles that of NADH. Harrison and Chance [2] showed that when a culture is exposed to fluorescence light at a wavelength of 340 nm, fluorescence measured at 460 nm is proportional to its NAD(P)H content.

NADH/NAD\textsuperscript{+} plays a key role in the electron transfer from electron donor to electron acceptor inside living cells. It is generally assumed that the NADH/NAD\textsuperscript{+} ratio reflects the balance between the internal electron donor and electron acceptor supply with respect to the catabolic redox reactions [3]. A surplus of electron donor (or shortage of electron acceptor) leads to a high NADH/NAD\textsuperscript{+} ratio, whereas a relative shortage of electron donor (or a surplus of electron acceptor) is reflected in a low NADH/NAD\textsuperscript{+} ratio. The normal situation under substrate limitation is thus a low NADH/NAD\textsuperscript{+} ratio [4]. At short time scales the summed amount of NAD\textsuperscript{+} and NADH is constant so that a changing NADH/NAD\textsuperscript{+} ratio is reflected by a change in the NADH concentration which is expected to give a variation in the fluorescence measurement.
Information on practical applications of fluorescence measurements in wastewater treatment plants is still scarce. On-line fluorescence measurements of NADH was evaluated for its potential contribution to the control strategy for evaluation and optimisation of a glucose-fed digester [5] and as indicator of stability in a glucose-fed anaerobic methanogenic digester [6]. But anaerobic fermentation, due to absence of oxidative phosphorylation, has very different NADH response as aerobic or anoxic processes.

The fluorescence technique was used to detect the transition from anoxic to anaerobic conditions [7,8] and to identify the moment of nitrate depletion [9]. Especially Nørgaard et al. [10] suggested using the NADH sensor to control the simultaneous nitrification/denitrification process at very low oxygen concentration (0.4 mgO₂/l). Due to floc internal gradients, the ratio between oxic and anoxic activity is influenced by the dissolved oxygen (DO) concentration. This ratio is supposedly correlated to the fluorescence measurement, the latter could sustain oxygen control for optimising total nitrogen removal.

Other papers in the literature did not make an in depth analysis of the value of the NADH signal in activated sludge systems and accepted that the signal could be used for what it was proposed for or had been evaluated on in pure culture experiments. Therefore, in this study the measurement technique was evaluated and several potential problems on using the NADH probe at full scale will be highlighted.

In this paper both the responses of heterotrophs (acetate as electron donor) and autotrophs (ammonia as electron donor) in activated sludge from a full scale wastewater treatment plant were examined as well as the effect of presence/shortage of electron acceptor (oxygen and nitrate) on NADH fluorescence was studied.

2. Methods and procedures

Measurements were conducted in a 20 l plastic, opaque, vessel that was equipped with a fluorescence measurement device (Biobalance, Denmark), an air diffuser, Ingold electrodes for oxygen, pH and redox, and a stirrer with variable speed (marine blade impeller) (Fig. 1). The dissolved oxygen concentration was automatically controlled by varying the airflow rate (0–50 l/min). The pH was controlled within the range around 7 (6.5–7.5), the effect of pH on the fluorescence measurement has been corrected by a calibration procedure. To enhance anoxic conditions, nitrogen could be sparged through the vessel or through the partially covered headspace. The fluorescence sensor emits UV light at a wavelength of 340 nm through a quartz window and monitors fluorescence at 460 nm through the same window. Measured fluorescence was converted to a 4–20 mA signal. In this paper, we use arbitrary ‘fluorescence units’ (fu) with a scale of 0–1000, linear proportional to the 4–20 mA range. The sensor window was for the forthcoming measurements positioned at 10 cm below the water surface so that no wall effects could occur. Concentrated sludge (67 g DW/l) was taken from the filter press for excess sludge of a nutrient removing wastewater treatment plant and diluted in the vessel before the experiments with 20 l tap water resulting in 4 g DW/l. Experiments were performed at room temperature (17–20°C). Samples for determining nitrate were centrifuged to stop denitrification. The supernatant was stored at 4°C before analysis with a Dr. Lange LCK 339 test kit (spectrophotometric UV-test after reaction to 4-nitro-2,6-dimethylphenol). Samples taken from the vessel with a wide tube (5 mm opening) and transferred to a Petri dish were subject to a visual inspection in order to evaluate the floc size distribution.

3. Results and discussion

3.1. Evaluation of measurement techniques

3.1.1. Influence of floc size distribution

For practical use it is important to know whether flocs are completely penetrated by the excitation light and emitted fluorescence light and therefore whether the sensor value is not influenced by the floc size distribution. As it is difficult to directly measure the light penetration depth, the average floc size distribution was influenced by varying the stirrer speed between 50 and 550 rpm. To eliminate the influence of differences in oxygen gradients inside the flocs, the sludge was not aerated. Samples revealed that the flocs were considerably smaller in the sample taken at the highest speed. Results of two experiments are shown in Fig. 2. If the stirrer speed was increased from 50 to 550 rpm, the NADH signal tends to decrease almost 7% (curve I). However, when the vessel was covered and nitrogen was blown over the surface, the decrease over this interval
reduces to 1–2% only (curve II). Enhanced oxygen transfer via the liquid surface at higher stirrer speeds likely caused the decreasing trend of curve I. Especially in small scale equipment with a relatively high surface to volume ratio such effects should not be neglected. Since there was hardly any direct influence of the stirrer speed on the fluorescence signal, we expect that under practical circumstances a varying floc size distribution will influence the signal only due to a changing NADH level resulting from electron donor/acceptor gradient changes inside the flocs.

3.1.2. Biomass and sludge concentrations

If light scattering is negligible, the NADH concentration, hence the measured fluorescence, was supposed to be linearly proportional to the amount of active biomass per unit volume at constant cell external conditions. This was verified by adding compressed baker’s yeast in steps to tap water buffered with phosphate at pH 6.6. No substrate was added, and the liquid was not aerated. As can be seen in Fig. 3 (● symbol), a linear relation was obtained between yeast concentration and fluorescence up to almost 2 g yeast DW/l. When under similar conditions sludge was added in steps only to tap water without yeast, the signal value levels off at higher sludge concentrations ( ■ symbol). At near zero concentrations, the slopes for yeast and sludge are similar. After 12 days the relation between NADH signal and sludge concentration was determined again by diluting the sludge suspension ( ◇ symbol, lower sludge curve). This was done to exclude potential adaptation effects when fresh sludge was used. Essentially the same relation was found. An explanation for the levelling-off might be the increased light scattering due to (inert) particles present in the sludge. An increased particle concentration reduces the fraction of excitation light reaching bacteria in the observable sensor region, and reduces the amount of fluorescence light received by the sensor as well. This would imply that for a given amount of light distorting particles the signal should still be linear in the concentration of microbes, which was verified by adding yeast to 4 g/l sludge (● symbol, upper curve). This experiment was done after acetate additions to the sludge, so that the initial sludge concentration yielded a higher level of fluorescence than in the batch for determining the effect of the sludge concentration ( ■ and ◇ symbols, lower two curves). The signal now increases linearly indeed with the concentration of the added yeast, with a smaller slope than the one obtained for yeast in tap water. This supports the assumption that the presence of light scattering particles reduces the measurement sensitivity. The NADH signal is linear in the amount of active (heterotrophic) biomass at a given sludge concentration. This is a measure for potential heterotrophic activity that may vary at a constant level of MLVSS. Due to the diminished sensitivity of the NADH sensor at higher sludge concentrations, the sensor signal will be quite insensitive to small sludge concentration variations under practical conditions, which is an advantage for signal interpretation.

3.2. Electron donor supply

3.2.1. Ammonia

Nitrification contributes significantly to the respiration in activated sludge processes. Since NADH is not involved in ammonium oxidation it can be expected that the NADH-fluorescence signal is insensitive towards nitrification. Aerated activated sludge was supplied with a pulse of ammonium (to give 25 mg NH₄-N/l) the
respiration rate increased strongly, but the fluorescence signal did not significantly respond, showing that the measurement is, as expected, insensitive for nitrification (Fig. 4). This is a complicating factor if the NADH signal is used to control aeration in nitrifying activated sludge systems.

3.2.2. Acetate

The effect of acetate supply was studied. It would be expected that with increasing addition rates of a COD source the NADH concentration in the cells would increase. The addition rate was increased stepwise from 0.07 to 0.7 g COD/g sludge/day and decreased stepwise afterwards (Fig. 5). The oxygen concentration was automatically controlled at 25% of the air saturation value by adjusting the airflow rate. The increasing sludge activity with increasing acetate load was therefore apparent from the increase in airflow rate. At first glance, the fluorescence level remained constant with increasing acetate load, and decreased slightly when the load was stepwise lowered. However, over a much longer period of time of 67 h (not represented here), the fluorescence level of the aerated, (endogenous) sludge, showed a decreasing trend, indicated by the dotted line. Compared to this trend, the fluorescence level increased slightly with increased acetate load and converged to this trend when the load was decreased. The response variation for a 10-fold increase in acetate load was however hardly visible and for practical applications with more signal disturbances due to process noise, negligible.

Several other experiments (not presented here) performed with pulse or continuous acetate additions confirmed that the acetate load is hardly detected by the NADH sensor.

3.3. Electron acceptor supply

3.3.1. Oxygen

The availability of electron acceptor is supposed to have the opposite effect of supply of electron donor: a decrease in the NADH signal. This was evaluated by addition of a pulse of acetate to activated sludge. The oxygen concentration decreased immediately (Fig. 6) indicating increased biomass activity. The acetate pulse was as such not reflected by the fluorescence signal. Five minutes after the acetate pulse the dissolved oxygen reached a very low value of 0–1% (<0.1 mg/l), while the aeration was maintained at a constant rate. The redox signal started to decrease continuously and the NADH signal rose slowly with 20 units. Half an hour later, oxygen could not be detected anymore. The redox curve
showed a bend on this transition to ‘anaerobic’ conditions, and started to decrease more pronouncedly. The ‘anaerobic’ conditions occurred despite the continuous aeration. The redox ‘knee’ was clearly visible in the NADH signal, clearly the NADH signal can be used to control aeration to a certain extent when aeration at extremely low DO was needed.

In conclusion, under aerobic conditions, the fluorescence level was only sensitive to oxygen at near zero concentrations. As long as minor amounts of oxygen are present, the NADH signal changes opposite to the redox curve. When oxygen was depleted completely, fluorescence reached a maximum value, probably indicating a state of minimum conversion rate, while the redox value decreased further. At these transition points, the NADH signal clearly represents the respiration state of the micro-organisms, whereas the absolute redox value has no correlation with that. However, expected changes in the respiration rate due to oxygen limitation in the range of 0.1–0.5 mg O2/l are not observed with the sensor. This and the absence of NADH in nitrifying bacteria make the electrode less suitable for use in control of simultaneous nitrification/denitrification systems.

### 3.3.2. Nitrate

In a further experiment to evaluate the addition of electron acceptor, nitrate was added to give 16 mg NO3-N/l (Fig. 7). Nitrogen was blown through the vessel instead of air to guarantee anoxic conditions. The fluorescence level was not influenced by this transition, probably due to the endogenous or starvation conditions for the sludge. Therefore, the nitrogen flow rate was further increased to enhance the anoxic conditions, this change was clear from the bend in the redox curve but this again did not influence the NADH signal. Nitrate was consumed at an endogenous constant rate (2 mg NO3-N/l/h). When acetate was added, the denitrification rate doubled but again the acetate pulse as such could not be directly observed from the NADH curve. The NADH signal rose more pronouncedly than before, which was probably a response to the faster decreasing cell external redox value. For a yield of 0.7 g COD biomass per g NO3-N, the biomass increase was estimated at 11 mg/l which was in the order of 0.4% of the initial biomass concentration and corresponds to less than 1 fluorescence unit. The slightly up-going trend observed in the NADH signal can therefore not be explained from biomass growth. As with oxygen, the trend resembled the trend in the redox curve, in opposite direction. Later a sudden transition in the redox state was observed. Probably nitrite (not measured) was exhausted at this point as well, and we observe a transition to anaerobic conditions (similar to [9]). When aeration was switched on, the fluorescence level decreased to the level before the redox transition. The anaerobic-oxic transition was however less pronounced than in the previous experiment with abundant acetate.
The fluorescence response under anoxic conditions was similar to aerobic conditions: (1) an increase in electron donor availability was not registered as such and (2) clear changes in the NADH level occur only when the electron acceptor was exhausted. Before this point, the NADH signal changes only weakly with the cell external redox value.

3.4. Biomass activity

Regarding the above observations on the influence of biomass concentration on the measured NADH level (Fig. 3), it can quite safely be stated that the strong, steady increase in fluorescence under aerated conditions was due to heterotrophic growth.

The long-term effect of continuous acetate consumption is shown in Fig. 8. Sixty grams of acetate (3 g/l) were provided under anoxic conditions. At \(t = 0\) aeration was switched on. Some regions can be distinguished. In the first 20 h the NADH signal increased while, when the readily available substrate was depleted (indicated by the reduced air demand at 23.3 h), the fluorescence curve further flattened. Note that growth was oxygen limited before this point, so that the increase in biomass was linear related to the oxygen transfer rate. At the moment the oxygen concentration became measurable, the NADH curve exhibited an expected downward jump. Later the oxygen controller turned off the aeration, while the oxygen concentration rose due to oxygen transfer via the liquid–air interface, indicating almost zero respiration. The fluorescence level remained hereafter more or less constant.

The observations could be the result of biomass production, we assumed a yield of 0.5 g biomass-COD/g acetate-COD [11], linearity between the fluorescence signal and the heterotrophic biomass concentration, and complete consumption of the added acetate. The COD value for acetate is 1.07 g COD/g HAc. The increase of fluorescence signal in this period with 200 fu then corresponds to the production of \((0.5 \times 1.07 \times 60) = 32.1\) g COD biomass, or 1.6 g COD/l. Using a COD value per g dry weight biomass of 1.37 g COD/g DW biomass [12], the biomass accumulation is estimated to be \((1.6/1.37) = 1.2\) g DW/l. The linearity of the fluorescence signal with biomass concentration for a given amount of sludge implies that the initial fluorescence level of 500 fu should correspond to \((300/200 \times 1.2) = 3\) g biomass DW/l. This would imply that the sludge initially consists for \((3/4.2) = 75\%\) of heterotrophic biomass. Although this value seems somewhat high, the estimations are in the right order of magnitude.

Using these numbers, an average specific growth rate of \(0.3\) d\(^{-1}\) is calculated for the period 0–23.3 h. The growth rate of heterotrophs is usually reported in the range of 4–6 d\(^{-1}\). Growth was here clearly oxygen limited as also shown by the linear increase in NADH and active biomass.

4. Conclusions

Fluorescence measurements at 460 nm at an emission wavelength of 340 nm provide a measure for the NADH content inside active micro-organisms and can be used for monitoring (potential) sludge activity in waste water treatment. The floc size distribution does not influence the signal response. The NADH signal is linear in the amount of active (heterotrophic) biomass at a given sludge concentration. Although the measurements are sensitive to changing redox conditions, variations are small, and changes in cell activity are only registered at near zero oxygen or nitrate concentration, coinciding with bends in the redox curve. Decreased respiration activity at low oxygen concentrations (0–0.5 mg/l) was
however not reflected in changed fluorescence. Also, nitrification and changing organic load with electron donor (acetate) are not detected. Several potential problems on using the NADH probe at full scale have been highlighted. As an example the sensor seems to be of limited value for improving oxygen control in a nitrifying activated sludge plant and controlling simultaneous nitrification/denitrification proved not to be as straightforward as suggested in the literature.

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References