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# FAILURE IN APPLICABILITY OF INT IN DEHYDROGENASE ACTIVITY ANALYSIS WHILE PRESENCE OF HIGH NITRATE CONCENTRATION

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Abstract
**The research was based on a determination of dehydrogenase activity (DA) of activated sludge operating under high ammonium load conditions. The dehydrogenase activity analysis was made using an INT assay where the tetrazolium salt (eg. INT) serves as an artificial acceptor of protons from respiration chain. In spite the widespread use of the test several analytical problems have appeared. One of the failures was a precipitation reaction between the tetrazolium salt INT and nitrates. In addition, there were problems with the determination of optimal concentrations of INT and the optimal incubation time and a decrease in INT formazan production over time.**

Streszczenie
**Przedstawione doświadczanie obejmuje oznaczanie aktywności dehydrogenaz osadu czynnego pracującego w warunkach znacznego obciążenia ładunkiem azotu amonowego. Do oznaczania aktywności dehydrogenaz użyto INT, sól tetrazolową stanowiącą sztuczny akceptor protonów w łańcuchu oddechowym. Mimo powszechnej stosowalności tego testu podczas oznaczeń napotkano na szereg problemów analitycznych. Zaobserwowano zachodzenie reakcji strącania pomiędzy solą tetrazolową INT a grupą azotanową. Ponadto wystąpiły problemy z wyznaczeniem optymalnego stężenia INT oraz optymalnego czasu inkubacji, a także stwierdzono ubytek w czasie powstałego formazanu INT.**

Keywords: **Dehydrogenase activity; Nitrifying activated sludge; INT.**

## 1. INTRODUCTION

Determination of dehydrogenase activity (DA) by applicability of tetrazolium salts (TTC, MTT, INT) has been found easy and relatively simple. Tetrazolium salt penetrates into the bacterial cell and become an artificial acceptor of electrons (and protons) transported by a dehydrogenases inside the electron transport chain (ETC). Acceptance of hydrogen atom by the tetrazolium salt molecules results in a formation of tetrazolium salt formazan (Fig. 1) that is soluble in organic solvents. The solution of the formazan in an organic solvent has a colour whose intensity can be measured. Determination of the amount of formazan formed per unit of time and per unit of bacterial mass lets a direct reflection of the intensity of biochemical processes in the cell [1, 2]. INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) is one of the commonly used tetrazolium salt whose main advantage is high sensitivity for dehydrogenase activity determination.

Literature reports indicate widespread use of INT in the studies of activated sludge [3, 4], soil [5, 6, 7] and biofilms [8]. Based on the results obtained by means of this method, researchers confirm the impact of

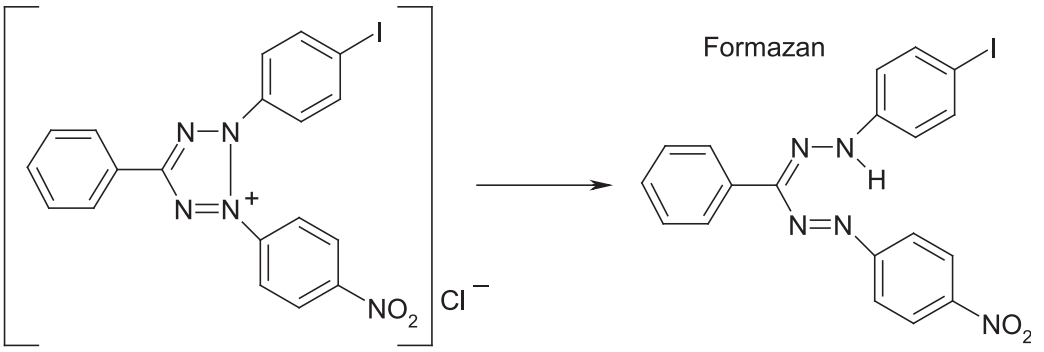


**Figure 1.**
**INT formazan production**

changes in pH and temperature, the influence of a type and concentration of available substrate and oxygen concentration on the microorganisms activity [9, 3, 4, 5, 7]. Dehydrogenase activity performed with the use of INT correlated with the amount of protein and extracellular polymers secreted by bacteria [8, 10], the number of active bacteria [10] (Ragusa et al., 2004), or sludge age [11]. INT is also used in the microscopic analyzes, to determine the quantity of active biomass [11, 12, 13].

Dehydrogenase activity in nitrifying systems has been studied mainly in the biofilm-based wastewater treatment systems (total nitrogen concentrations in these studies did not exceed 100 mg N/L) [8, 11]. The authors noted the relationship between dehydrogenase activity and the age of bacterial cells and the quality of the porous structure of the biofilm [11].

The aim of the study was to determine the dynamics of dehydrogenase activity in the activated sludge originating from reactors operating under a significant ammonium load and its relation with oxygen uptake rate (OUR) and intensity of intermediate products production. The purpose of this publication is to show the difficulties encountered when conducting experiments based on the determination of dehydrogenase activity using INT as an artificial proton acceptor.

## 2. MATERIALS AND METHODS

Dehydrogenase activity was examined in activated sludge taken from two laboratory membrane bioreactors (MBR A and MBR B). Activated sludge from MBR A had sludge retention time (SRT) of 12 days and was exposed to the ammonium load of 0.05-0.35 $gNH_4$-N/dgVSS. SRT of activated sludge

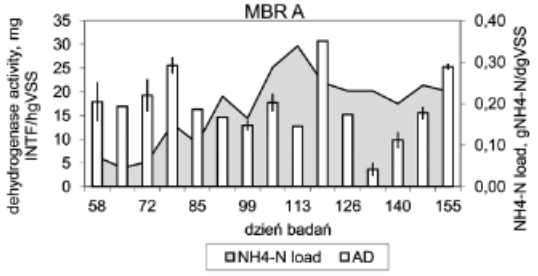


**Figure 2.**
**Dehydrogenase activity of activated sludge from MBR A**

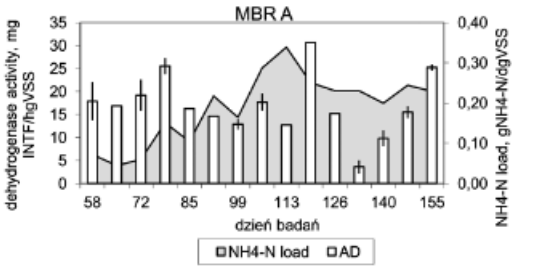


**Figure 3.**
**Dehydrogenase activity of activated sludge from MBR B**

from the MBR B was 32 days, a load of ammonium load was gradually increased between 0.01-0.1 $gNH_4$-N/dgVSS.

Dehydrogenase activity was performed with INT test according to [7]. Activated sludge samples were incubated in the presence of INT at its optimum concentration at room temperature in the dark for 15-40 minutes. The optimal incubation time was determined at the beginning of the study. After the incubation period, samples were centrifuged and the supernatant was removed. Then methanol was added

in order to interrupt the enzymatic reaction and to extract the formed INT formazan (INTF). After vigorous mixing the samples were again centrifuged to separate the liquid from activated sludge. The concentration of INTF in the supernatant was determined based on the absorbance measured at a wavelength of 490 nm and compared with calibration curve (analytical standard: INTF solution in methanol, Sigma-Aldrich). Dehydrogenase activity was presented as the amount of INTF formed per unit of time (linear increase of formazan concentration) and unit of VSS.

The research covers a period of about 3 months. During this time, the sludge of MBR A and MBR B was adapted to higher ammonium load and chosen SRT. Optimum concentration of INT was determined once a month. Dehydrogenase activity assay was made once a week in each activated sludge in four replications.

The VSS were calculated as the difference between a mass of sample dried at 105°C for two hours (SS) and mass of its ash (burnt at 550°C).

Performance of the nitrification process was monitored by influent and effluent analysis for nitrogen according to the following procedures: ammonium determined colorimetrically with Nessler reagent according to PN-C-04576-4:1994; nitrite-colorimetrically with alpha-naphthylamine reagent [14]; and nitrate – colorimetrically with dimethylphenol reagent according to ISO 7890-1.

## 3. RESULTS AND DISCUSSION

The results of dehydrogenase activity on a background of ammonium load are presented in Fig. 2 and Fig. 3.

In the reactor A ammonium load was increasing to a level of 0.33 $gNH_4$-N/dgVSS, and than maintained at about 0.23 $gNH_4$-N/dgVSS. The dehydrogenase activity were mostly (80%) in the range of 10-20 mgINTF/hgVSS. In reactor B, the load of ammonia nitrogen increased progressively throughout the study period, reaching the value of 0.1 $gNH_4$-N/dgVSS. During the first 28 days of dehydrogenase activity varied between 15-25 mgINTF/hgVSS, while later were about half the previous values and reached 5-15 mgINTF/hgVSS. In both cases, no correlation between ammonium load and dehydrogenase activity and nitrification efficiency and dehydrogenase activity were found. In both reactors, problems with nitrification observed as an increase in the concentration of ammonium and nitrite in the effluent was observed between days 41 and 83. These problems were caused by the drastic change in the composition of the main nitrifying bacteria (results performed by fluorescence *in situ* hybridization (FISH), not presented in this publication, see [15], but it does not reflect the situation found in the values of dehydrogenase activity. The lack of correlation between the activity of dehydrogenases and reactor parameters are supposed to be caused by the difficulties while performance of dehydrogenase activity assay.

Due to the nature of nitrification, the reactor effluent was reach of nitrates. Concentrations of nitrate in the effluent reached 500 $mgNO_3$-N/l. During the dehydrogenase activity measurement, while the addition of INT solution to the activated sludge samples, the samples became turbid and a white precipitate could be observed. To estimate the reason and size of the phenomenon an additional experiment had been performed. To several test tubes containing standard solution of INT gradually increasing doses of $NaNO_3$ solution were added. The precipitate obtained through the reaction between $NaNO_3$ and INT was centrifuged and the supernatant was analyzed for concentration of INT and $NO_3$-N. The results of this experiment are shown in Figure 4 as the relationship between nitrate loss and the loss of INT in the supernatant.

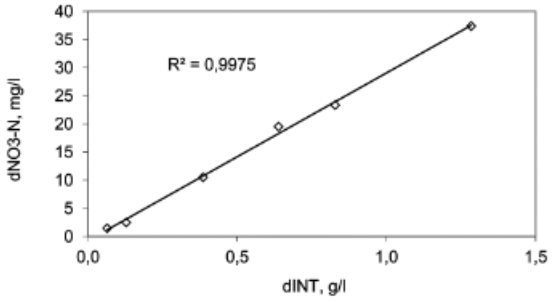


**Figure 4.**
**Relationship between nitrate and INT loss while reaction between INT and $NaNO_3$**

A key factor for the reliability of the dehydrogenase activity results is to conduct the assay with the use of optimal concentration of tetrazolium salt. The optimal salt concentration ensures the appropriate amount of salts in the intracellular sites, and interception of all protons of hydrogen transferred in the respiratory chain. Such a concentration should not be

toxic to bacterial cells [1, 2]. Precipitation of INT decreases its concentration in a liquid surrounding activated sludge flocs (and bacterial cells) thus the optimal conditions for the assay cannot be maintained. Despite the observed problem the activity measurements were continued.

During the acclimation of activated sludge to SRT the optimal concentration of INT has been changed significantly probably due to changes in activated sludge biocenosis. The optimal concentrations are presented in Table 1. For both reactors the optimum INT concentration values increased over time and the optimal concentration on 97$^{th}$ day was more than 200-times higher than at the beginning of the study.

It should be remembered that the increased demand for INT could be attributed to the fact that part of it was precipitated while the DA assay. The concentration of nitrate in the samples was increasing over the time (especially in the third month of experiment), but the range of optimal INT concentration indicates that it was not the only reason of such increase. Since the day 97 a determination of the optimal INT concentration became impossible due to the limited solubility of INT in water and as well for the economic reasons – the use of such high doses of INT rises cost of the test.

Applying high INT concentration (138 mgINT/gSS for MBR A and 76 mgINT/gSS for MBR B) the determination of optimal incubation time (a linear increase in concentration of formazan) appeared to be difficult. At the beginning of the research the linear production of INTF could be observed during first 40 minutes of assay (ie. optimal incubation time of 40 minutes). Nonlinear nature of the INTF production after 116 days of experiment is presented in Figure 5.

Figure 5 shows not only the lack of a linear increase in INTF concentrations, but also reflects the fact that its concentration decreases over a time that is quite confusing. During the assay performation the samples were incubated in the absence of light, so the photochemical decomposition of INT formazan was impossible (it is known that INT may undergo photochemical oxidation [16].

Two hypothetical explanations (none further research were performed) of the observations can be proposed. Perhaps, as the INTF has a similar chemical structure as INT it can also reacts with nitrate to give a precipitate. However, determination of the precipitation occurrence and the amount of precipitate is difficult. As the formazan is insoluble in water

**Table 1.**
**Changes in INT optimal concentration over a time of experiment**

| day of experiment | | 1 | 34 | 52 | 83 | 97 |
|---|---|---|---|---|---|---|
| optimal INT concentration, mg INT/g SS | MBR A | 0.67 | 4.8 | 8 | 6 | >138 |
| | MBR B | 0.32 | 1.56 | 0.86 | 3 | >76 |

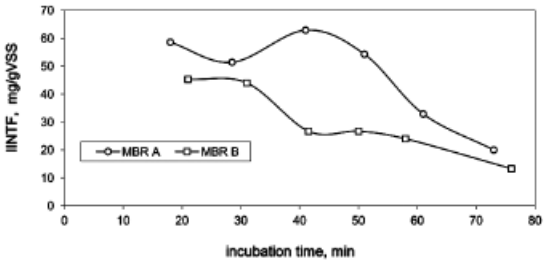


**Figure 5.**
**Determination of optimal incubation time (performed on 116 day of experiment)**

it is necessary to use organic solvents to determine the concentration of formazan. In the assay methanol is used for the formazan extraction. We have observed that the precipitate formed from the reaction of INT and nitrate dissolves completely in the presence of methanol. Methanol solubility of the precipitate of INTF and nitrate formed in aqueous conditions precludes confirmation of the thesis in a simple experiment.

Another possible explanation of the decrease in formazan production is the biodegradation of both INT and INTF. In the chemical structure of these compounds, nitrite group is attached to the aromatic ring. It seems to be unlikely that nitrifying bacteria were able to separate groups of nitrite from the carbon chain. However, due to the facts that the activated sludge bacteria were adapted to the presence of high ammonium concentrations, and in both reactors, both the first (oxidation of ammonia production nitrite) and the second phase of nitrification (oxidation of nitrite, nitrate production) occurred with high efficiency this theory should not be rejected. It can be possible that the nitrifying bacteria used the INTF and/or INT as a source of nitrite thus the determination of optimal DA assay conditions could not be determined.

## 4. CONCLUSIONS

The usefulness of INT as an artificial electron acceptor for the determination of dehydrogenase activity of nitrifying activated sludge is questionable. In these circumstances the use of this reagent poses many difficulties, thus application of other tetrazolium salt, or modification of the present methodology is necessary. The inconvenience in the use of INT to the determination of dehydrogenase activity of nitrifying activated sludge results from a precipitation of INT while nitrate presence and not explained loss of INTF over a time of assay performance.

One of the possible assay modification would be to centrifuged activated sludge before the assay performance in order to remove supernatant full of nitrates. However, such a procedure would change the natural microbial activity and the obtained results would be unreliable. Miksch [1, 2] and others recommend to carry out such DA tests in conditions natural to the tested biocenosis.

Further research could be done to determine the biodegradability of INT and INTF and an impact of oxygen presence while the biodegradation. Dehydrogenase activity test is usually made with the use of TTC or INT. The TTC test uses usually sodium sulphate to omit the influence of oxygen present in the sample. Oxygen may compete with TTC for protons capture. As the INT incorporate earlier to the electron transport chain than TTC it is more competitive to catch protons than oxygen. Thus, in INT test the deoxidizer is not necessarily used. However, assuming that the nitrite is biologically detached from the INT/INTF molecule and oxidized to nitrate, an oxygen is necessary to perform this reaction. Thus, deoxidation of a sample could be one of the solutions to overcome the phenomenon of biological transformation of tetrazolium salts while the assay performance.

Summarizing, further studies are needed in order to solve this problem and develop a methodology which allows reliable determination of dehydrogenase activity of nitrifying bacteria.

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