Express-Method for Determination of the Oxidizing Capacity of Activated Sludge and Its Biofilms in Pulp and Paper Mill

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Abstract: Microbial dehydrogenase activity can help to determine the oxidizing capacity of activated sludge. Here we propose an innovative and automated express-method based on rapid determination of dehydrogenase activity. The measurement is based on the rate of methylene blue reduction by living microbial cells in suspension. A single analysis takes 10 min. The method was adapted for biofilms immobilized on the floating carriers of industrial bioreactors and the kinetics of biological oxidation by activated sludge and biofilms was compared. New parameters were proposed to characterize the biological oxidation under low oxygen levels. The obtained make it possible to quickly determine the dehydrogenase activity of activated sludge and biofilms and promptly monitor the effectiveness of industrial biological wastewater treatment.

Keywords: dehydrogenase activity; oxidizing capacity; activated sludge; biofilm; biological wastewater treatment

1. Introduction

The pulp and paper industry is one of the most water-consuming manufacturing industries. Its wastewater is conventionally treated before being discharged into natural reservoirs. Biological treatment with activated sludge is the most common and effective method of wastewater treatment. Current biomonitoring of natural and artificial aqua systems requires modern and informative express methods for assessing the functional state of microbiocenoses that could indicate the quality of wastewater treatment [1].

The industrial technology of wastewater treatment involves the biological oxidation of water pollutants. In aerotanks water organic compounds are oxidized by microorganisms of activated sludge [2]. Multistage wastewater treatment schemes are often used to intensify the oxidation processes. The intensity of biological treatment depends on the concentration and condition of activated sludge. Bacteria are the main components of the sludge [3], they assimilate water pollutants using their enzymatic complexes. Thus, the enzyme activities determine the rate and intensity of the biological oxidation. Among them the dehydrogenases play a key role. Therefore, the total dehydrogenase activity can indicate the oxidizing capacity of activated sludge and can serve to assess the physiological and functional conditions of the microorganisms [4].

The most widely used method for determination of dehydrogenase activity (DHA) of activated sludge involves reduction of the colourless tetrazolium salts to brightly coloured stable compounds, formazans [5,6]. The more formazans are formed, the higher the DHA is. This method was used to evaluate the quantitative effect of aeration time and sludge dose on DHA in aerotanks [7] and the biological treatment efficiency of wastewater contaminated by polyester [8] and emerging organic contaminants [9]. Regarding the technical simplicity and cost-effectiveness, the method is commonly recommended as an efficient approach for soil DHA determination [10]. However, this multistage technique is highly labour- and time-consuming and cannot be used as an express-method. The method

involves additions of several reagents at different stages of the process, two centrifugations, a 30 min incubation and photometry [11]. Analysis does not allow high reproducibility of results, as a different amount of enzymes can reduce the same amount of tetrazolium salts. The amount of cell enzymes can vary significantly during the analysis. Methods for the DHA determination in wastewater treatment are still under development. New approaches that provide rapid analysis have not been proposed for a long time. In 2020, a determination of DHA with triphenyl tetrazolium chloride was reported [12]. However, despite all its benefits, the analysis duration exceeded 24 h.

Methylene blue (MB) is a promising reagent for the quantitative analysis of living cell activity in the microbial suspension. MB is soluble in water, has low toxicity and can easily penetrate into the cell and escape from it. Being an active electron acceptor, MB competes with oxygen in the electron transport chains of microbial cells. In the presence of active living cells, the amount of the coloured reagent decreases and it becomes discoloured. MB can be used for the assessment of activity of live and devitalized yeast cells [13], as well as for the quantitative analysis of antibiotics [14]. The monitoring of the bacterial growth in milk has been reported based on MB reduction [15], and the atomization of the procedure has been proposed [16]. The application of MB for the evaluation of activated sludge DHA is simple, fast and does not require any expensive equipment [17]. MB has been shown to be several times more sensitive than the tetrazolium salts [18]. The changes in MB concentration over time can be easily assessed optically. The application of MB for the determination of microbial DHA is very promising, versatile and in demand. The key task is to automate the method and to develop an algorithm and a device for the express determination of DHA.

The aim of the study was to develop an express-method and a measuring device for determining the dehydrogenase activity of microorganisms using MB reaction and to study the kinetics of pollutant oxidation by immobilized biofilm and free-living activated sludge biomass in industrial conditions.

2. Materials and Methods

DHA can vary rapidly and significantly during biomass storage. Thus, the determination of DHA was carried out immediately after sludge sample collection and preparation. Free activated sludge from an aerotank and biofilms immobilized on bioreactor carriers were collected for the experiments.

In the industrial bioreactor of the pulp and paper mill, the microbial biofilm is immobilized on a surface of the free-floating Natrix M2 carriers (Figure 1). The weight of the carrier varied in the range of 9.3–10.0 g and the surface area was 0.050–0.055 m^2. Each carrier unit can bear 2.1–4.5 g of biomass. The biofilm thickness and its distribution on the surface, as well as the concentration of microorganisms, can vary significantly. The carrier was removed together with a biofilm from the industrial bioreactor. The biomass was separated from the carrier and dispersed in 200 mL of distilled water. The concentration of dry matter after dispersion was 0.030–0.042 g/L. The biofilm protein content estimated by the Kjeldahl method [19] averaged 0.025–0.035 mg/g.

Figure 1. The carrier for activated sludge immobilization as a biofilm.

The activated sludge samples were collected from the aerotank with a sampler immersed to a depth of approximately 1 m. An average sample from three collected probes was used. Hydrobiological analyses [20] were used to characterize 840 samples of activated sludge and biofilms in seven months. The ranges of ash content, concentration and protein content were determined for the samples (Table 1).

Table 1. The characteristics of biofilm and activated sludge samples.

| Sample	Ash Content, %	Activated Sludge Concentration, g/L	Protein Content (by 1 g of Dry Activated Sludge), %
biofilm	13–14	1.8–2.5	36–44
activated sludge from aerotank	25–30	3.4–4.5	23–34

3. Results and Discussion

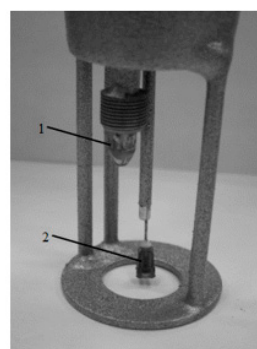### 3.1. Construction of the Measuring Cell

The MB reduction resulted in colour changes of the solution. To detect the colour changes a special measuring cell was constructed. It consisted of a thermostatic vessel with a mechanical stirrer and an optical signal recording unit (photodiode and photosensor) embedded into a vessel cover (Figure 2).
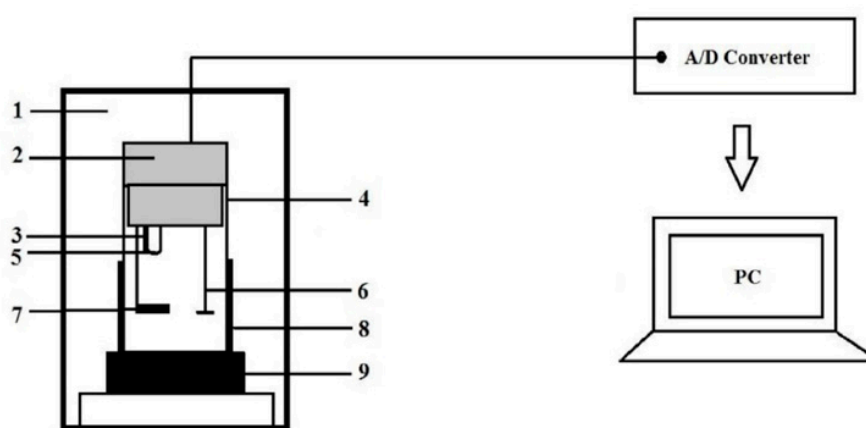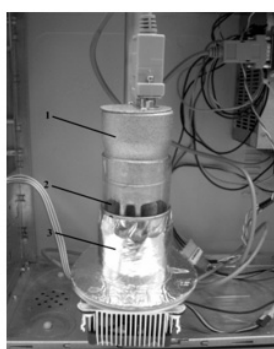
Figure 2. (**a**) Scheme of measuring cell: (1) light-tight housing, (2) cover with built-in measurement system, (3) photosensor, (4) glass vessel, (5) photodiode, (6) stirrer, (7) light reflector, (8) thermal insulation layer, (9) thermostat, (**b**) Image of the measuring device: (1) the measuring cell, (2) glass vessel, (3) metal vessel with thermal insulation layer, (**c**) The measuring cell: (1) photosensor and photodiode of the detecting system, (2) stirrer.

The construction of the measuring cell provided continuous optical recording of the MB concentrations in the vessel and therefore the microbial DHA. The measuring cell maintained the anaerobic condition during the analysis. The substrate (for example, glucose) and the MB solution as a hydrogen receptor were mixed with the microbial suspension in the same vessel.

Thus, the device allowed to keep a constant temperature during the analysis, to determine the optical density of the sludge suspension and to mix the components, preventing the access of air (atmospheric oxygen) to the cell. Oxygen already contained in the reaction medium was consumed in a few seconds and did not affect the analysis. The optical signal registration system consisted of a photodiode, a reflector and a photosensor. The analogue signal of the photodetector was converted into digital signal and transmitted to the controlling computer. Instrument signal was recorded in microvolts.

3.2. Kinetics of Substrate Biological Oxidation

First, to calibrate the measuring cell, a dependence curve of the photosensor readings versus the MB concentration was obtained. The dependence coefficient was used in the Equation (1) to determine the sludge DHA.

The experiment included the following operations: 60 mL of distilled water was added into the measuring cell; 2 mL of a prepared activated sludge was added with stirring. Further 1 mL of 10% glucose solution was added to the mixture. The volume in the cell was brought up to 100 mL with distilled water. All measurements were carried out at a temperature of $25 \pm 0.2\ °C$. 50 μL of a 0.2% MB solution was added as a hydrogen acceptor after temperature stabilization.

Using the developed software, the readings of the device were recorded with an interval of 1 s. The kinetics of the reaction was examined for 5 min (Figure 3). After 5 min the process became nonlinear (nonlinear region IV, Figure 3).
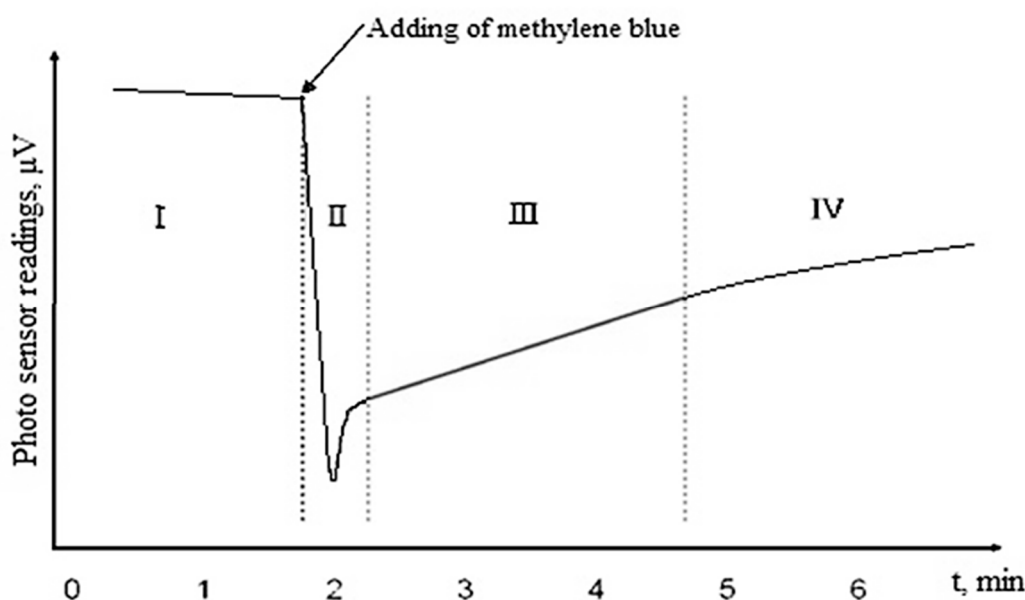

Figure 3. Kinetics of biological substrate oxidation by activated sludge in the presence of MB: (I) stabilization of the photosensor readings, (II) MB sorption on the biomass, (III) the linear region, (IV) the nonlinear region.

The slope of the curve in the linear region was used to calculate the quantitative DHA value. The result of one analysis was based on several hundred photosensor measurements. The maximum error was less than 5%. Thus, efficiency is the main advantage of the new

method. The analysis duration did not exceed 10 min. DHA (A; nmol/(min·mg)) was calculated using the linear region III (Figure 3) according to the following Equation (1):

$$A = 0.025 \times K \times 60/m, \tag{1}$$

where 0.025 is the calibration coefficient experimentally determined for MB concentration depending on device readings (nmol/µV); K is thea slope of the linear region of the curve (µV/s); 60 is used to convert seconds to minutes and; m is the protein content in the sludge sample (mg).

The kinetic curves of MB reduction were found to differ depending on the type of the sample examined. A significant nonlinearity was observed during MB reduction by biofilm during the first 90 s. Then, after approximately 140 s, the curve became linear indicating the dependence of MB reduction only on the concentration of dehydrogenases (Figure 4a).
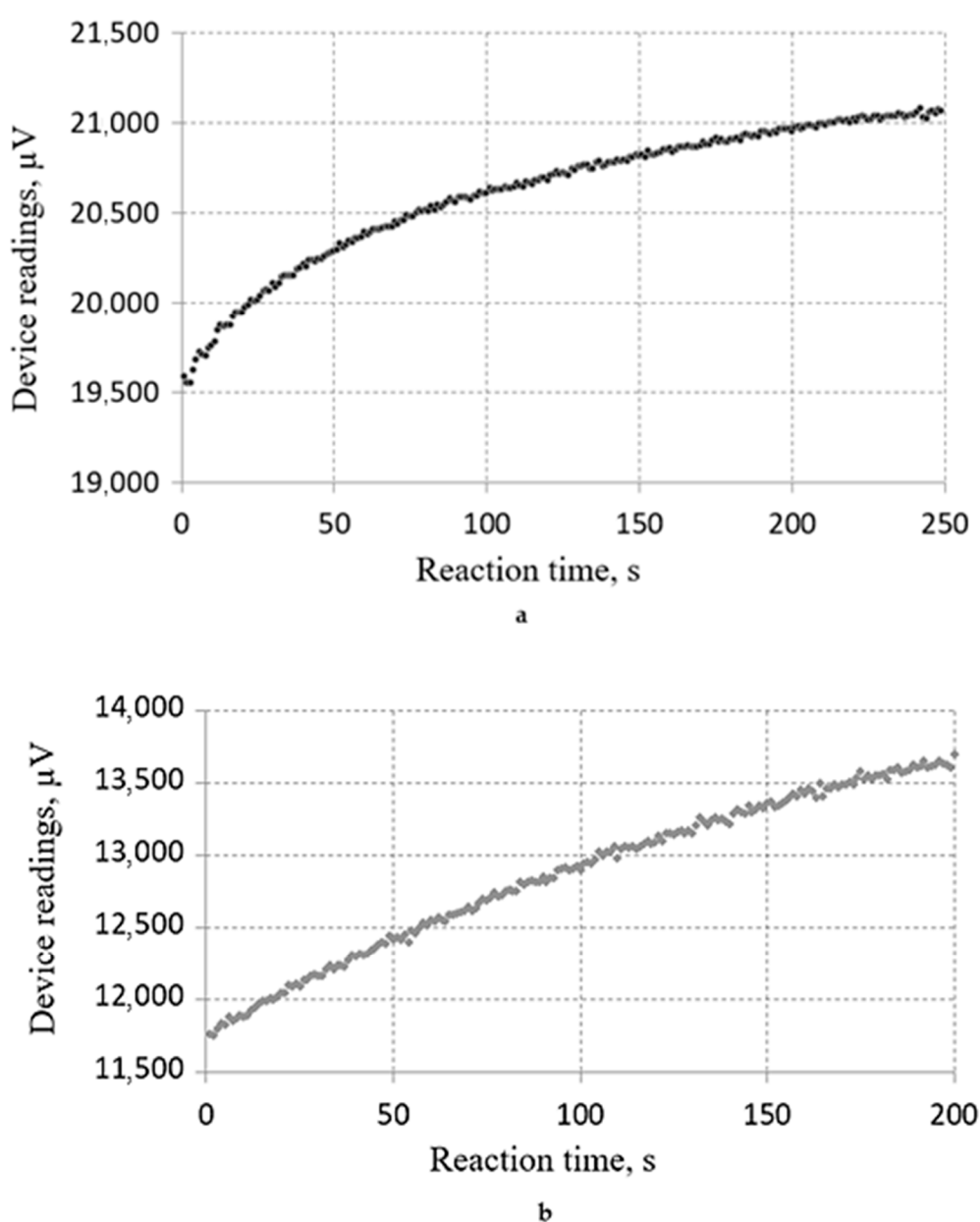

a

b

Figure 4. The signal-time dependencies of the methylene blue reduction by biofilm (**a**) and activated sludge from aerotank (**b**).

In contrast, the curve form was close to linear when analysing the MB reduction by activated sludge samples taken from the aerotank (Figure 4b). With an excess of substrate and MB as the hydrogen acceptor it was determined only by the concentration and activity of dehydrogenases, indicating a zero-order reaction.

The nonlinear character of the curve at the initial stages was related to the biochemical processes in the respiratory chain of microorganisms. During their vital activity, microbial cells perform the transfer of hydrogen from the substrate to various mediators of the redox reaction (NAD, NADP, flavoproteins, ubiquinone, various types of cytochromes, etc.) using dehydrogenases [21]. Electrons entering the respiratory chain lose their free energy when passing from one carrier to another. Much of this energy is stored in the form of ATP. With an excess of oxygen, mediators are in an oxidized state and the rate of the substrate oxidation reaction is limited by the activity and concentration of dehydrogenases. With a lack of oxygen, the rate of the substrate oxidation reaction is determined not only by DHA, but also by the number of mediators in the reduced form, which react first.

The difference from the linear relationship is determined by the proportion of microorganisms functioning in anaerobic conditions, as well as by the intensity of respiratory processes. This is probably due to high substrate loads for the aerotank sludge. This process is associated with low diffusion of oxygen in the biofilm [22]. The potential of mediators in the MB oxidation reaction was exhausted approximately 140 s after the start of the reaction for biofilm samples. Then the electron transport systems were activated in the microorganisms and the reaction rate was determined only by DHA. Therefore, DHA could be determined quantitatively from the slope of the linear dependence (140–250 s) of the obtained curves for the biofilms.

Based on observed dependencies, we propose a new characteristic for describing the sample behaviour in anaerobic conditions, which can be called the reduction capacity (RC) of microorganisms. RC is a value characterizing the presence of respiratory mediators in biofilm, which are in a reduced form and are able to reduce MB. Quantitatively, reduction capacity (RC; nmol/mg) is proportional to the value of the E segment on the ordinate axis (Figure 5) according to Equation (2):

$$RC = 0.025 \times E/m \tag{2}$$

where 0.025 is the calibration coefficient of the MB concentration dependence on device readings (nmol/μV); E is a segment on the ordinate axis (cut off by a tangent line in the interval of 140–250 s) (μV) and m is a protein content in the biofilm sample (mg).

In Figure 5, the E value is equal to 800 μV and the RC parameter calculated by Equation (2) was 667 nmol/mg. The RC can be influenced by changes in the oxygen concentration in the bioreactor, an increase or decrease in the amount of easily oxidized substances, the intensity of redox processes in cells, the age of the sludge and many other factors. The aerobic coefficient (AC) was found as the ratio of the reaction rate at 200 s ($W_{200} = \Delta\mu V/\Delta s$ at 200 s) to the initial reaction rate ($W_0 = \Delta\mu V/\Delta s$ at 0 s) according to Equation (3):

$$AC = W_{200}/W_0 \tag{3}$$

The initial reaction rate (W_0) and the rate at 200 s after the start of the reaction (W_{200}) are practically the same under conditions of oxygen excess. The dependence of the change in the readings on the photodetector during the reaction has a linear character.
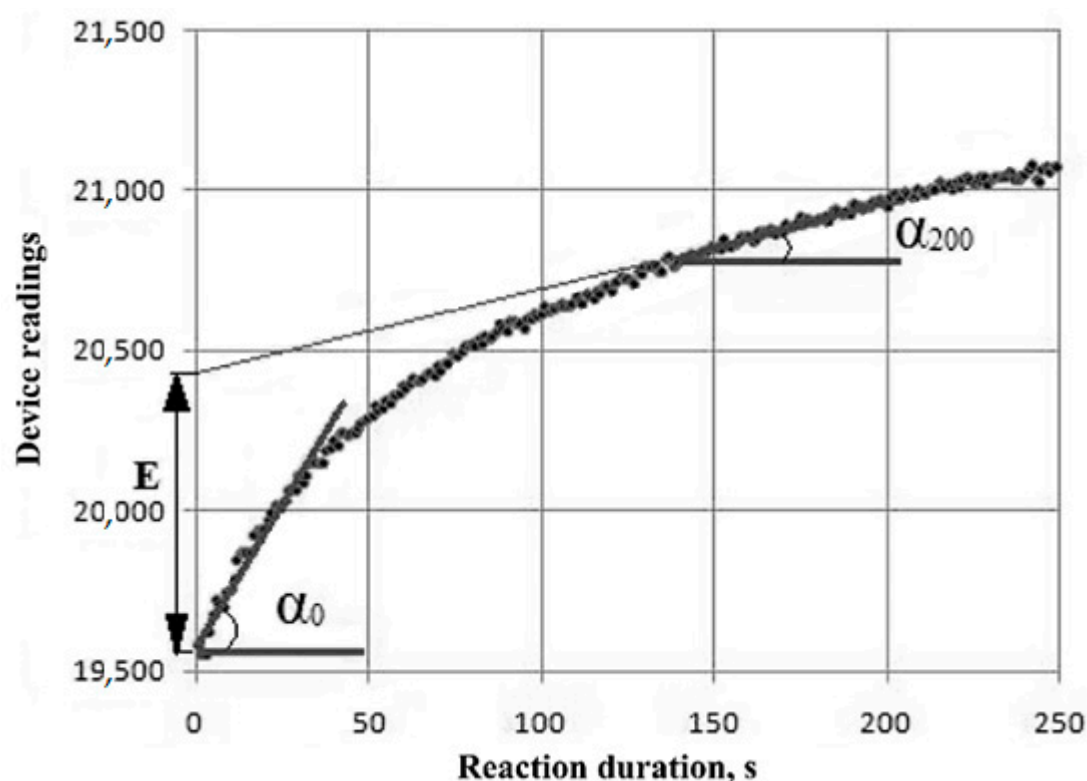
Figure 5. Device reading changes vs. reaction time of methylene blue enzymatic oxidation in the biofilm sample.

The determination was held with the number of parallel measurements (up to 22). The maximum relative error of the experiments was 0.02–0.04%. Therefore, the results obtained by the new method are reliable. The stability of the determined parameter was confirmed using the coefficient of variation, which was less than 0.1% (<5%). The determination accuracy for the method is about 0.1 nmol of MB.

4. Industrial Monitoring of Aerobic Coefficient

The AC indicates the limitation of the oxidation process in the examined conditions, as well as the predominance of aerobic or anaerobic processes. The closer the coefficient is to one, the more oxygen enters the microorganisms. The AC tends to zero in the absence of oxygen. These indicators make it possible to characterize the biochemical processes occurring in the biocenosis of activated sludge. In the future, they can be used to assess the effectiveness of biological treatment facilities (Table 2).

Table 2. The results of industrial monitoring of biofilm and activated sludge parameters.

Treatment Stage	Dehydrogenase Activity, nmol/(min·mg)	Reducing Capacity, nmol/mg	Aerobic Coefficient
bioreactor	75–300	125–1250	0.2–0.4
aerotank	3–11	0–6	0.5–1.0

The microorganism's immobilisation on the carrier leads to an increase in the viability and activity of cells and better protection from the effects of toxic substances in the wastewater. The DHA values of immobilized microorganisms of the bioreactor (biofilm) are more than 25 times higher than thosethat of activated sludge in aerotanks.

The reduction capacity of activated sludge in biofilms and aerotanks differ significantly due to the different levels of oxygen. The oxygen concentration in the biofilm is low and, consequently, the mediators of the respiratory chains are accumulated in a reduced form.

The AC for biofilms varied from 0.2 to 0.4; that is, about 60–80% of microorganisms functioned anaerobically in bioreactor.

5. Conclusions

An express-method and a device are presented for determining the dehydrogenase activity of activated sludge by the MB reaction. That allowed obtaining important information about the physiological state of activated sludge in suspension and immobilized form as a biofilm in a biological wastewater treatment system. Comparing to the previously proposed methods the new determination technique is rapid (within 10 min), automated (automatic recording of instrument readings, mixing and control of the sample temperature), does not require degassing of the sample and eliminates the influence of colour and turbidity. Since the absolute optical density is not measured, but the kinetics of its change, the new method is especially useful for application in coloured wastewater.

6. Patents

There are the following patents resulting from the work reported in this manuscript:

1. Patent (Russian Federation) No. 117 149 «Device for quantitative determination of the rate of colored enzymatic reactions». Chukhchin Dmitry Germanovich (RU); Tupin Pavel Alekseevich (RU). Patent holder NArFU. Application: 2012100566/10. Application date: 10 January 2012. Published: 20 June 2012 Bul. No. 17.

2. Patent (Russian Federation) No. 2 476 598 «Method for quantitative determination of dehydrogenase activity of microorganisms». Chukhchin Dmitry Germanovich (RU); Tupin Pavel Alekseevich (RU). Patent holder NArFU. Application: 2011116872/10. Application date: 27 April 2011. Published: 27 February 2013 Bul. No. 6

Author Contributions: Conceptualization, D.C. and E.V.; Methodology, D.C.; Validation, E.V., K.V. and V.R.; Formal Analysis, V.R. and K.T.; Resources, V.R.; Writing—Original Draft Preparation, V.R., D.C. and K.V.; Writing—Review & Editing, K.V. and K.T.; Visualization, D.C. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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