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SIIRI VELLING

Microbial BOD biosensor for wastewater analysis

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following published papers which are reprinted by kind permission of the publishers.

I **S. Velling**, K. Tammeveski, A. Mashirin, T. Tenno. Non-steady-state processes in amperometric biosensors: modelling studies. Proceedings of the Estonian Academy of Sciences. Chemistry, 2002, 51/1, 38–48.

II **S. Velling**, T. Tenno. Different calibration methods of a microbial BOD sensor for analysis of municipal wastewaters. Sensors and Actuators B: Chemical, 2009, 141, 233–238.

III **S. Velling**, A. Mashirin, K. Hellat, T. Tenno. Non-steady response of BOD biosensor for the determination of biochemical oxygen demand in wastewater. Journal of Environmental Monitoring, 2011, 13 (1), 95–100.

Author's contribution

Paper I: The author's contribution involves planning the research, experimental tasks, interpretations of data and writing the manuscript (approximately 60%).

Paper II: The author was fully responsible for the planning and conducting the biosensor experiments, interpretations of the results and writing the manuscript.

Paper III: The author was fully responsible for the planning and conducting the experiments, interpretations of modeling results and writing the manuscript and contributed to the development of mathematical model.

ABBREVIATIONS AND SYMBOLS

a	interfacial area per unit volume (cm^{-1})
BOD	biochemical oxygen demand
BOD_7	biochemical oxygen demand during 7 days
$BOD_{sensor(\Delta C)}$	value of BOD estimated by biosensor according to the steady state method
C_{O2}	oxygen concentration displayed by oxygen sensor
$C_{O2,0}$	oxygen concentration at t=0, e.g. the initial concentration
$C_{O2,norm}$	normalized oxygen concentration
$C_{O2,\infty}$	oxygen concentration displayed by the oxygen sensor for $t \rightarrow \infty$
C_{∞}	saturation concentration of oxygen
D	equivalent to diffusion coefficient of oxygen
dh^*/dt	rate of change of transfer function $h^*(t)$
$(dh^*/dt)_{max}$	maximum rate of change of transfer function
ΔI	total change of the value of output current
GGA	solution of D-glucose, 150 mg/l, and glutamic acid, 150 mg/l
$h^*(t)$	transfer function of biosensor
I	output current of biosensor
$(J_{ox})_b$	flux of oxygen consumed by microorganisms
$(J_{ox})_e$	flux of oxygen from solution
$(J_{ox})_k$	flux of oxygen toward cathode
$(J_{ox})_m$	flux of oxygen through the diffusion-limited layers of oxygen sensor
$(J_s)_b$	flux of biodegradable organic substrate in the microbial membrane
$(J_s)_e$	flux of biodegradable organic substrate from solution
k_L	mass-transfer coefficient ($cm\ s^{-1}$)
K_s	half-saturation coefficient
l	equivalent to thickness of the diffusion-limited layers of oxygen sensor
$l_{agar.gel}$	thickness of agarose gel layer
OECD	Organization for Economic Cooperation and Development
RSD	relative standard deviation
t	time (s)
$t_{i,p}$	time that corresponds to the inflection point of transient response
τ_s	time constant of the exponential process related to the microbial respiration
τ_d	time constant of the exponential process of the response of oxygen sensor with microbial membrane
τ_d^{matrix}	time constant of the exponential process of oxygen sensor with agarose gel membranes
V_{O2}	maximum rate of change of biosensor response, dynamic parameter
WWTP	wastewater treatment plant

1. INTRODUCTION

A microbial biosensor is an analytical device that couples microorganisms with a transducer to enable expeditious, accurate and sensitive detection of target analytes in the fields as diverse as environmental analysis and monitoring, medicine, defense, food-processing and safety. Concern over the pollution risk to the environment from industrial manufacturing processes, intensive agriculture has highlighted the need for rapid procedures which can be operated in the field. Among all the parameters used to assess, the pollution load of water bodies and wastewaters, the biochemical oxygen demand (BOD) is one of the most important and widely used parameters in the measurement of organic pollution.

Since the first report on the application of a BOD biosensor published by Karube, I. *et. al* [1], several amperometric microbial biosensors have been developed for the measurement of biodegradable organic pollutants in aqueous samples [2]. One widely spread application is the determination of BOD in wastewater comprehending significant importance on the pollution control of water environment.

The conventional standard method for the determination of BOD measures the microorganisms' oxygen consumption over a period of 5 to 7 days and is reported as BOD_5 or BOD_7, respectively. While BOD_7 is a good indicator of the concentration of biodegradable organic pollutants in water, its determination is extremely slow and hence not suitable for process control in wastewater treatment plants or expeditious determination of the concentration of biodegradable organic pollutants in polluted water [2, 3]. In the case of a pollution event or an operation problem, adverse conditions would be unknown and could persist for 7 days until the results of BOD_7 test are available. Therefore, the wastewaters exceeding the limited values of BOD are long discharged to the environment before the test results are obtained. Determination of BOD load can be accomplished within short time interval by BOD biosensors that are defined as self-contained integrated devices capable to provide specific quantitative analytical information using a biological recognition element [3]. The other considerable advantages, besides most important time-effectiveness, are portability and easiness to handle.

In an area toward the development of analytical tools such as biosensors, the primary roles of theory are first, to provide framework within which the experimentally observed performance of a system can be understood and optimized and second, to assess the best approach to the provision of a new and improved systems. The main objectives of present study are the construction and analysis of output parameters of BOD biosensor for wastewater analysis. In more detail, the optimization of construction elements and measuring procedures, extensive study and modeling of output parameters of a microbial BOD biosensor constitute the goal of the study.

2. LITERATURE OVERVIEW

2.1. BOD biosensor as an analytical tool

The biosensor is defined as a self-contained integrated device capable of providing specific quantitative analytical information using a biological recognition element [3]. The development and design of BOD biosensors has resulted on devices of various working principles: for batch experiments based on respiratory activity [2, 4–10], for continuous detections [11–16], with flow injection-type systems [14, 17–19], microbial fuel cell (MFC) type biosensors [11, 20–23] etc.

Most BOD biosensors rely on the measurement of the respiratory activity of cells by a suitable transducer including the use of oxygen sensor [2, 24, 25], carbon dioxide analyzer [13] and optical transducer [26–28]. The electrochemical detection method, e.g. with an oxygen sensor, has been described as simple and sensitive, providing fast response time and as a preference, can be performed using compact and mobile equipment [29]. This thesis focuses on the amperometric microbial biosensors that operate at fixed potential with respect to a reference electrode and involve the detection of the current generated by the reduction of oxygen at the surface of the electrode.

For expeditious and effective BOD determinations, several BOD biosensors based on amperometric oxygen electrode transducer modified with microorganisms metabolizing organic pollutants have been reported. The microbial strains used as a biological sensing element include *Torulopsis candida* [19], *Arxula adeninivorans* [30, 31], *Pseudomonas putida* [32], *P. fluorescence* [33, 34], *Saccharomyces cerevisiae* [35], *Bacillus subtilis* [36, 37], *Serratia marcescens* LSY4 [38], *Pseudomonas syringae* [39], *Trichosporon cutaneum* [40, 41], thermophilic bacteria [42], *Hansennula anomala* [43] and some other yeasts [31, 44, 45]. Still, since biochemical reactions are basically enzymic, BOD sensing can also be effected using purified enzymes as the bioactive material. However, their application is limited by their specificity with respect to the substrate, pH and temperature, their susceptibility to poisoning and deactivation and the presence of cofactors for maximum activity. Wastewater organic matter is highly heterogeneous, containing molecules of various molecular weights, ranging from the simple compounds like acetic acid, to very complex polymers [46]. Dignac, M.-F. *et al.* [47] studied the molecular distribution of sugars, amino acids, fatty acids, sterols and phenolic compounds in wastewaters and could characterize 50% of the organic matter of wastewater of activated sludge processes. Therefore, multiple enzymes are normally required to achieve the desired biochemical conversion of a complex substrate such as wastewater and a microorganism generally contains different enzymes with their cofactors and its cell envelop protects and minimizes deactivation and poisoning of the organism [48]. The microbial BOD biosensor should be highly capable of analyzing a sample of complex constituents with relatively low selectivity, e.g. the BOD biosensor should respond to multiple

biodegradable organic solutes in the sample, and give results comparable to those obtained using the conventional BOD methods.

Since each microbial species has its metabolic deficiencies, single strain microorganisms immobilized for BOD biosensor could provide a limited substrate spectrum. Therefore, biosensors with mixed culture of microorganisms, including activated sludge that are immobilized within one certain membrane and put onto an oxygen sensor have been developed for the analysis of complex samples [7, 8, 14, 49–51]. For the detection of wide variety of biodegradable organic compounds in the sample, a mixture of two, for example the mixture of *Bacillus subtilis* and *Bacillus licheniformis* 7B or more, e.g. mixed consortium of microorganisms have employed to broaden the substrate and hence analyte spectrum with a stable performance [7, 14, 52–58]. The microbial membrane biofouling and long-time stability problems characterizing the mixed culture based biosensors has been overcome by using the thermally treated cells in BOD biosensors [50], but the advantages of living cells like shorter response and recovery time are relevant to conduct expeditious dynamic measurements and perform output signal modeling.

However, microbial biosensors require careful control, maintenance and appropriate storage conditions to maintain their efficiency. One of the most important aspects in the fabrication of a microbial biosensor is the choice of suitable immobilization method to assure a close proximity between biochemically active part and transducer. Since microbial biosensor response, operational stability and long-term use are, to some extent dependent on the immobilization strategy used, immobilization technology plays a very important role [25]. For the immobilization of microorganisms, adsorption and entrapment into hydro gels are the two widely used physical methods. These methods are preferred when viable cells are required, because they do not involve covalent bond formation with microbes and provide relatively small perturbation of microorganism native structure and function [59–61].

Physical adsorption is the simplest method for microbe immobilization. Typically, a microbial suspension is incubated with the electrode or an immobilization matrix, such as alumina and glass bead [62], followed by rinsing with buffer to remove unadsorbed cells. The microbes are immobilized due to adsorptive interactions such as ionic, polar or hydrogen bonding and hydrophobic interaction. However, immobilization using adsorption alone generally leads to poor long-term stability because of desorption of the microbes.

Entrapment of microorganisms is often a preferred immobilization method, because of the higher stability and longer life of service of the microbial membranes. The entrapment can be achieved by the either retention of the cells in close proximity of the transducer surface using dialysis or filter membrane or by immobilization in chemical/biological polymers/gels such as agar, agarose, alginate, carrageenan, chitosan, collagen, polyacrylamide, polyvinylachohol, poly(ethylene) glycol, polyurethane, etc. [61, 63]. Formed gels hinder the separation of microbes into measuring environment ibid provide the availability of substrates for microbes and the diffusion of the products of biochemical processes from the

cells. A disadvantage of immobilization by entrapment is the additional diffusional resistance caused by the entrapment material.

Amperometric microbial BOD biosensors must be calibrated to provide comparison with the conventional BOD_7. Therefore one of the key issues in BOD biosensor research and analysis of samples is the selection of a proper calibration solution for obtaining a good agreement between the biosensor detected BOD and BOD_7. Although the GGA solution (mixture of D-glucose, 150 mg/l, and glutamic-acid, 150 mg/l) has been widely used as a reference for the conventional BOD test, it was realized that this standard solution was not suitable for all the microbial BOD sensors [1, 19, 30, 38, 40]. The reasons can be its instability due to rapid microbial contamination, decrease in the glutamic acid reaction of microbes in presence of glucose, due to glucose repression, and since it only consists of two simple components, calibration will not be reliable when analyzing real wastewater samples with a complex mixture of constituents. Therefore, synthetic wastewaters with more complex constituents have been tested for calibrating the BOD biosensors. Tanaka, H. *et al.* [64] tested different recipes until the combination became close to water quality analyses of secondary effluent from wastewater treatment plants in Japan. Liu, J. *et al.* [50] chose a synthetic wastewater according to a recipe from the Organization for Economic Cooperation and Development (OECD). In comparison with calibrating in GGA solution, a remarkable improvement on agreement between the biosensor detected BOD and BOD_7 values was achieved. Besides abovementioned mixtures, solutions of D-glucose, L-lactate, glycerol, fructose and synthetic wastewaters of different compositions have been used for the calibration of BOD biosensors [27, 31, 32, 43, 53, 65, 66].

For the improvement of the sensitivity and linear range of biosensor output as well for the increase of stable working period the pre-conditioning of microbes has been applied [56]. The increase in the response of a biosensor with preconditioning is generally attributed to the activation of the biochemical pathway relating to the enzymic oxidation of the substrate. The activation process probably involves establishing the transport mechanism in the biofilm, the activation of the existing enzymes and/or the inductive synthesis of the enzymes in the cells [67]. In previous studies, the pre-conditioning of microbes for the construction of BOD biosensor has involved the preliminary cultivation of activated sludge microorganisms [14, 50], pre-conditioning of microbes in GGA solution [4, 68] and in a growth medium [7].

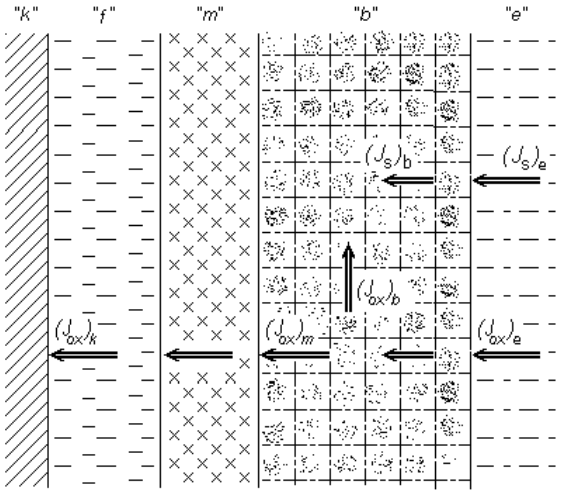
To conclude, suitable immobilization procedures and possibly preconditioning along with artificial standard solution attributing similar composition with wastewater samples are the important aspects for the construction and calibration of a BOD biosensor, but this biosensor might not be suitable for analyzing other wastewaters of different composition.

2.2. Output data analysis of BOD biosensor

Biofilm-type amperometric microbial BOD biosensors based on whole cells rely on the measurement of bacterial respiration rate in close proximity to the outer membrane of a suitable transducer. This microbial film is an immobilized microbial population that can bio-oxidize the organic substrate to be quantified. The response of biosensor corresponds to the current change of oxygen sensor which signal is amplified and correlated to the content of biodegradable material analyzed.

During measurement procedure, dissolved oxygen diffuses from the aerated solution into the immobilized cell layer, e.g. the microbial membrane, where part of the oxygen is consumed by the immobilized microorganisms (Scheme 1). The remaining oxygen diffuses through the diffusion-limited layers, e.g. gas-permeable membrane and electrolyte layer of oxygen sensor and is detected by the analyzer. A steady state current can be observed at the equilibrium between the oxygen diffusion into the microbial membrane and the endogenous respiration rate of the immobilized bacteria. As soon as substrate, e.g. calibration solution or wastewater sample is injected into the measurement media, organic substrates diffuse into the microbial membrane and are assimilated by the immobilized bacteria resulting in the increase of bacterial respiration rate and oxygen consumption for bio-oxidation processes. Therefore, less oxygen can diffuse through the oxygen sensor outer membrane and electrolyte layer and will be reduced on cathode. The output current of biosensor will decrease until a new steady state value for oxygen is achieved. After the measurement procedure the BOD biosensor is removed from substrate solution and introduced into buffer solution. As a result, the respiration rate of the microorganisms' decreases and meanwhile the endogenous respiration rate will be progressively restored.

According to previous studies, there are two measuring techniques available for biofilm-type BOD biosensors, the steady state (also entitled as end-point) method and the dynamic (also entitled as kinetic, transient) method of analysis of response data. In the steady state method, the biosensors current difference (ΔI) between the two steady states is used for the biosensor detected BOD estimation [5, 8, 50, 56, 69, 70]. The measuring time is normally 15 – 20 min followed by 15 – 60 min up to as long as 3 – 4 h recovery time [55]. The alternative approach to the steady state analysis of a biosensor output signal is through the analysis of dynamic response of the device. Several authors have refereed the dynamic response of BOD biosensor as the initial-rate method, that measures the initial current change after the addition of organic substrate [50, 54, 70, 71]. This parameter reflects acceleration of the bacterial respiration rate and is proportional to the substrate concentration. In this case, the sensor response is normally recorded for 15 – 30 s followed by a recovery time of less than 10 min. Besides, Chan, C. *et al.* [72] have proposed a quasi-kinetic measuring mode and studied the response of BOD biosensor in 70, 100 and 120 seconds after addition of substrate.



Scheme 1. Schematic diagram of a biosensor with microbial membrane in the close proximity of the outer membrane of an oxygen sensor, where (k) denotes the cathode of oxygen sensor, (f) is solution of electrolyte, (m) denotes the outer membrane of oxygen sensor, (b) denotes microbial membrane, (e) is solution of analyte, $(J_{ox})_k$ denotes flux of oxygen toward cathode, $(J_{ox})_m$ is flux of oxygen through the diffusion-limited layers of oxygen sensor, $(J_{ox})_b$ is flux of oxygen consumed by microorganisms, $(J_{ox})_e$ is flux of oxygen from solution, $(J_s)_b$ is flux of biodegradable organic substrate in the microbial membrane and $(J_s)_e$ is flux of biodegradable organic substrate from solution [Paper I].

A good correlation between BOD values of biosensor measurements and standard BOD_7 method have been reported using these two methods, for standard GGA solution, as calibration solution and for wastewater samples. Also no significant difference of biosensor repeatability was observed, but the sensitivity is twice as high with the dynamic method [73]. In comparison to the steady state method, the biosensor BOD measurement in the dynamic mode has the advantage of being faster, thereby allowing more frequent measurements and being more suitable for on-line monitoring, especially for process control purposes.

2.2.1. A model for an amperometric oxygen sensor

The detection method of an electrochemical oxygen sensor, i.e. reducing oxygen at the cathode of the sensor and measuring the resulting current as an indication of oxygen concentration depends on the mass transfer of oxygen through diffusion-limited layers of oxygen sensor. It was also noticed, that the membrane covered amperometric oxygen sensor introduces an uncertainty depending on the exact measurement procedure of an analyzer and on the rate of concentration change of oxygen in the environment under investigation. If oxygen concentration in the measurement media is changing rapidly, the oxygen sensor will not be able to

follow the expeditious change of dissolved oxygen concentration. Thus, for the determination of oxygen concentration in solution with electrochemical oxygen sensors one has to keep in mind that electrochemical analyzer monitors an oxygen concentration (C_{O2}) which lags behind the changing concentration of solution. At steady state conditions the flux of oxygen detected by oxygen sensor and the external oxygen concentration are linearly related; however, under non-steady state conditions the diffusional resistance of dissolved oxygen caused by several diffusion-limited layers of oxygen sensor induces a lag in the response of the sensor.

During earlier research, the oxygen concentration outside the sensor membrane was sharply changed from zero to some constant value (for example up to saturation concentration) and the oxygen sensor response was registered in this step change situation. Based on the Fick's second law of diffusion, a mathematical model has been developed by Mancy, K. H. *et al.* [74] and modified by Benedek, A. A. and Heideger, W. J. [75] for the characterization of the mass transfer of oxygen across the diffusion-limited layers of oxygen sensor to the cathode:

$$\frac{C_{O_2}}{C_\infty} = 1 + 2\sum_{n=1}^{\infty}(-1)^n \cdot \exp\left(-\frac{n^2\pi^2 t}{\left(l/D^{1/2}\right)^2}\right), \tag{1}$$

where C_{O2} is the concentration of oxygen displayed by the sensor at a time t, C_∞ denotes maximum concentration, e.g. saturation concentration of oxygen (mg L^{-1}), t is time (s), l is equivalent to thickness of diffusion-limited layers of oxygen sensor and D is equivalent to diffusion coefficient of oxygen in the diffusion-limited layers of oxygen sensor.

To describe the output of oxygen sensor in transient conditions, Benedek, A. A. and Heideger, W. J. [75] recorded the response of oxygen sensor in the exponentially changing aeration pattern for oxygen. For that, during aeration tests the oxygen concentration in solution and also on the outer membrane of oxygen sensor was changed exponentially with time. During aeration processes the mass transfer of oxygen from the gas phase into the liquid can be described with:

$$\frac{dC_{O_2}}{dt} = k_L a(C_\infty - C_{O_2}), \tag{2}$$

where k_L is the mass transfer coefficient (cm s^{-1}) from the gas phase into the liquid phase and a denotes the interfacial area per unit volume (cm^{-1}).

Abovementioned eqn (2) integrates into:

$$\ln \frac{C_\infty - C_{O_2}}{C_\infty - C_{O_2,0}} = -k_L a t \,, \tag{3}$$

where $C_{O2,0}$ denotes the initial oxygen concentration.

As a result, the transient output signal of oxygen sensor can be modeled as

$$C_{O_2} = C_\infty - \left(C_\infty - C_{O_2,0}\right) \cdot \exp(-k_L a t) \,, \tag{4}$$

where $C_{O_2,0} < C_\infty$ indicating the increase of oxygen concentration in time.

As it has been noticed, the transient response undergoes an initial time lag of the transition region and a final region of steady state response. After solving the Laplace transformation, the mathematical model describing the inherent lag and transient response of a membrane-covered electrochemical oxygen sensor during the change of the concentration of analyte in the solution has been provided [75]:

$$1 - \frac{C_{O_2}}{C_\infty} = \exp(-k_L a t) - 2\sum_{n=1}^{\infty} (-1)^n \frac{k_L a}{\frac{n^2\pi^2}{\left(l / D^{1/2}\right)^2} - k_L a} \cdot \left[(\exp(-k_L a t) - \exp(-\frac{n^2\pi^2 t}{\left(l / D^{1/2}\right)^2} \right] . \tag{5}$$

2.2.2. BOD biosensor modeling studies

The mathematical modeling of sensing characteristics and output signal of BOD biosensor provides a link among the design, fabrication, measurement procedure and basic working principles of the biosensor. The variety of mathematical models of biosensors reported so far concern the sensing of single organic substrate with or without co-reactant and are applicable for BOD determinations with limitations [76–80].

For the modeling of BOD biosensor, first with the steady state working principle, the assumptions were made that the rate of oxygen consumption by the cells in the layer near to the outer membrane of oxygen sensor is determined by the substrate concentration [43]. As well, the change in oxygen sensor current is proportional to the change in oxygen concentration and also proportionally causes the change in the BOD biosensors response value. According to this approach, the analytical solutions of differential equations are mostly based on the Michaelis-Menten-type enzyme kinetics that is used for the characterization of degradation

processes in BOD biosensors. It is assumed that in a microbial membrane the biodegradation rate of microbes on different substrate concentrations can be described according to the dependency of enzyme reaction rate on substrate concentration. Therefore, the output parameters of BOD biosensor depend on substrate concentration according to hyperbolic function.

Based on abovementioned assumptions, Kulys, J. and Kadziauskiene, K. [43] have modeled the steady state response of a BOD biosensor. According to the study, they assumed that stationary concentration of oxygen depends on the diffusion of organic substrate and oxygen from the external solution as well on the rate of transport of the two latter into cell. As a result conclusions were drawn, that at high organic substrate concentrations the maximal decrease of output current of the biosensor is determined by the ratio of the transport rate into the cells and the diffusion. At low concentrations, the response of the biosensor is directly proportional to the organic substrate concentration in external solution.

Also, Qian, Z. R. *et al.* described a mathematical model for bio-oxidation reactions catalyzed by many different enzymes in an immobilized microbial system [69]. In this study, the results between two steady state output values of biosensor were related to the GGA concentration, to work out a parameter for a single organic solute. This parameter was experimentally determined with a BOD biosensor of diffusion-controlled conditions in the biofilm for a number of organic solutes. The predicted BOD_5 of 43 different organic mixtures containing 2–5 components of known concentrations were found according to the experimentally determined single solute dimensionless parameters that describe the diffusion of an organic solute with respect to the dissolved oxygen in the biofilm. The results showed acceptable coincide with the experimental values. Also, according to the model described effects of cell population and substrate temperature on the BOD sensitivity were well substantiated experimentally.

Among the wide variety of models for biosensors containing single or multiple enzymes designed as mono or poly-layered analytical devices for the detection of appropriate products of enzyme-reactions, the modeling involves solving the equations for diffusion of substrate and product with a term containing a rate of biocatalytic transformation of a substrate. These kind of analytical solutions give good results allowing the determination of substrate concentration in steady state and dynamic conditions for enzymes-based as well for microbial biosensors. Still, the method has shortcomings – no applicability for the determination of stability characteristics of biosensors with complex biocatalytic schemes and complexity of the solution of non-stationary state, which indicate the unsuitability of the approach for modeling transient response of BOD biosensors for environmental samples of diverse composition [81].

As complex parameter BOD is detected according to the change of oxygen concentration in measurement solution, this can be the basis for the modeling of output signal of BOD biosensor. For appropriate applications, we need to know the oxygen concentration at particular points in space and time during measurements, in accordance with the Fick's second law. In order to provide the required description, the differential equation of the Fick's law should be solved after the

definition of initial state of the system and its state at the boundaries of the region of space over which the diffusive mass transport process of interest is occurring. A few authors have performed this kind of modeling for biosensor performance and reported that the non-steady state response – time dependency that relies among other processes largely on microbial respiration, can be described as an exponential summation. This is probably be the best option at the moment for the description of the complex system of immobilization matrix, diffusion of substrates and heterogeneous kinetics of intracellular reactions [75, 82–84].

3. MODEL OF TRANSIENT RESPONSE OF BOD BIOSENSOR

The exponential change of oxygen concentration on the outer membrane of amperometric oxygen sensor has been modeled according to the results of experiments on various aeration patterns in previous studies [74, 75]. In BOD biosensor experiments the exponential change of oxygen concentration on the outer membrane of oxygen sensor is caused by microbial activity (Scheme 1).

The transient change of oxygen concentration measured with oxygen sensor has been described in the case when oxygen concentration in the beginning of measurements (t=0) equals 0 (eqn (5)). The characterization of processes that relate to the transient change of BOD biosensor output and microbial respiration is based on exponential time constants τ_s and τ_d. Subsequently, after appropriate transformations of eqn (5), we gain:

$$1-\frac{C_{O_2}}{C_{O_2,\infty}}=\exp\left(-\frac{t}{\tau_s}\right)-2\sum_{n=1}^{\infty}(-1)^n\cdot\frac{\frac{1}{\tau_s}}{\frac{n^2}{\tau_d}-\frac{1}{\tau_s}}\left[\exp\left(-\frac{t}{\tau_s}\right)-\exp\left(-\frac{t}{\tau_d/n^2}\right)\right] \qquad (6)$$

in which

$$\tau_s=\frac{1}{k_L a} \quad \text{and} \qquad (7)$$

$$\tau_d=\frac{\left(l/D^{1/2}\right)^2}{\pi^2}, \qquad (8)$$

where parameter τ_s is the time constant of the exponential process that responds to the change of oxygen concentration caused by microbial respiration in microbial membrane during measurement; τ_d is the exponential time constant that characterizes the transient change of output signal of amperometric oxygen sensor in close contact with microbial membrane in time and $C_{O2,\infty}$ is the oxygen concentration on the outer membrane of oxygen sensor for $t\rightarrow\infty$.

Additionally, the change of oxygen concentration displayed by oxygen sensor in time (eqn (6)) can be characterized with a transfer function $h^*(t)$:

$$1-\frac{C_{O_2}}{C_{O_2,\infty}}=\frac{C_{O_2,\infty}-C_{O_2}}{C_{O_2,\infty}-C_{O_2,0}}=h^*(t) \qquad (9)$$

and considering that

$$\frac{\frac{1}{\tau_s}}{\frac{n^2}{\tau_d}-\frac{1}{\tau_s}}=\frac{\tau_d}{n^2\tau_s-\tau_d}, \tag{10}$$

the equation (6) can be written according to eqn (9) and (10)

$$h^*(t)=\frac{C_{O_2,\infty}-C_{O_2}}{C_{O_2,\infty}-C_{O_2,0}}=\exp\left(-\frac{t}{\tau_s}\right)-2\sum_{n=1}^{\infty}(-1)^n\cdot\frac{\tau_d}{n^2\tau_s-\tau_d}\left[\exp\left(-\frac{t}{\tau_s}\right)-\exp\left(-\frac{t}{\tau_d/n^2}\right)\right]. \tag{11}$$

According to proposed methodic of BOD biosensor measurements, when $C_{O_2,0}>C_{O_2,\infty}$, it can be shown that the transfer function of BOD biosensor response is expressed as a function of time t [74]:

$$h^*(t)=\frac{C_{O_2}-C_{O_2,\infty}}{C_{O_2,0}-C_{O_2,\infty}}=\exp\left(-\frac{t}{\tau_s}\right)-2\sum_{n=1}^{\infty}(-1)^n\cdot\frac{\tau_d}{n^2\tau_s-\tau_d}\left[\exp\left(-\frac{t}{\tau_s}\right)-\exp\left(-\frac{t}{\tau_d/n^2}\right)\right]. \tag{12}$$

The dependencies and parameters presented in eqn (12) are depicted in Fig. 1 for the visualization.

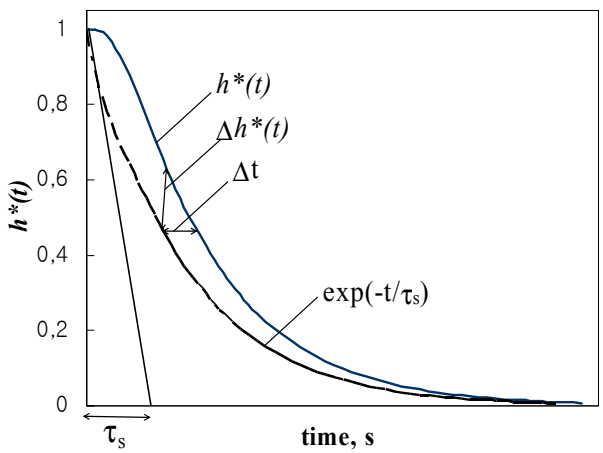


Figure 1. Transfer function for the output signal of an amperometric oxygen sensor, where Δt shows time difference.

Thereat, the function *h*(t)* in Fig, 1 denotes

$$h^*(t) = \exp\left(-\frac{t}{\tau_s}\right) + \Delta h(t),$$

thereby $\Delta h^*(t) = -2\sum_{n=1}^{\infty}(-1)^n \cdot \frac{\tau_d}{n^2\tau_s - \tau_d}\left[\exp\left(-\frac{t}{\tau_s}\right) - \exp\left(-\frac{t}{\tau_d / n^2}\right)\right]$. (13)

Additionally, the eqn (12) is transformed into more applicable form for BOD biosensor measurements that are performed in the units of oxygen concentration. Thus, in the case $C_{O2,\infty} \rightarrow 0$, the response and the lag-time of measuring system is expressed as [Paper III]:

$$\frac{C_{O_2}}{C_{O_2,0}} = C_{O_2,norm} = \exp\left(-\frac{t}{\tau_s}\right) - 2\sum_{n=1}^{\infty}(-1)^n \cdot \frac{\tau_d}{n^2\tau_s - \tau_d}\left[\exp\left(-\frac{t}{\tau_s}\right) - \exp\left(-\frac{t}{\tau_d / n^2}\right)\right], \quad (14)$$

where $C_{O2,norm}$ is the normalized output reading of BOD biosensor originally measured in the units of oxygen concentration.

The transfer function *h*(t)* of the biosensor response is characterized by an inflection point at which the rate of change of transfer function *(dh*/dt)* shows a maximum value. Therefore, the normalized response of BOD biosensor also shows an inflection point at which the rate of change gains a maximum value [Paper I]. The response of the biosensor can be expressed as a function of time accordingly eqn (12) as follows:

$$C_{O_2} = C_{O_2,\infty} + \Delta C_{O_2} \times h^*(t), \quad (15)$$

where $\Delta C_{O_2} = C_{O_2,0} - C_{O_2,\infty}$ indicates the difference between the initial and final values of output data.

The differentiation of eqn (15) results with:

$$\left(\frac{dC_{O_2}}{dt}\right) = \Delta C_{O_2} \cdot \left[\frac{dh^*}{dt}\right]. \quad (16)$$

Accordingly, if maximum change of output signal is considered, the differentiation results with:

$$\left(\frac{dC_{O_2}}{dt}\right)_{\max} = \Delta C_{O_2} \cdot \left[\frac{dh^*}{dt}\right]_{\max}, \tag{17}$$

where $(dh^*/dt)_{max}$ is the maximum value of dh^*/dt.

The differentiation of eqn (12) results with

$$\frac{dh^*}{dt} = -\frac{1}{\tau_s}\exp\left(-\frac{t}{\tau_s}\right) - 2\sum_{n=1}^{\infty}(-1)^n \cdot \left[-\frac{1}{\tau_s}\cdot\frac{\tau_d}{n^2\tau_s - \tau_d}\exp\left(-\frac{t}{\tau_s}\right) - \right.$$

$$\left. -2\sum_{n=1}^{\infty}(-1)^n\left(\frac{n^2}{n^2\tau_s - \tau_d}\right)\exp\left(-\frac{t}{\tau_d/n^2}\right)\right]. \tag{18}$$

And if $\tau_s >> \tau_d$, then the eqn (18) will be expressed as

$$\frac{dh^*}{dt} \cong -\frac{1}{\tau_s}\exp\left(-\frac{t}{\tau_s}\right) - \frac{2}{\tau_s}\sum_{n=1}^{\infty}(-1)^n \cdot \exp\left(-\frac{t}{\tau_d/n^2}\right). \tag{19}$$

In the case, the maximum change of output signal of an oxygen sensor based biosensor was considered, the results of differentiation showed:

$$\left[\frac{dh^*}{dt}\right]_{\max} \cong -\frac{1}{\tau_s}\exp\left(-\frac{t_{i.p}}{\tau_s}\right) \approx -\frac{1}{\tau_s}, \tag{20}$$

where $t_{i.p}$ corresponds to the time respective to the inflection point of transient response.

Therefore, according to the eqn (20), at first approximation we can conclude that the maximum change of response can be used for the optimization of BOD biosensor construction parameters, like thickness of microbial membrane, amount of immobilized microorganisms and also for metrology studies, validation of linearity and sensitivity of the biosensor output.

4. EXPERIMENTAL

For the construction of BOD biosensor, microbial consortium was immobilized into the polymer net of a particular thickness with a 2% suspension of agarose. Two types of microorganisms were used: mixed consortium of microorganisms that originated form activated sludge of municipal WWTP and *S. cerevisiae* originated from bakers' yeast. The last ones were primarily used for construction and modeling studies. Immobilization procedure involves the mixing of prepared suspension of microorganisms with the suspension of agarose (ultra pure, Sigma) which had been heated up to 60 ºC and then cooled to 40–42 ºC before combining with biochemically active material in phosphate buffer. The resulting mixture was spread on the polymer net of a particular thickness which, in order to gain a certain and even thickness of the layer containing microorganisms, was then placed between two glass plates. Glass plates were moderately pressed together until the formation of a persistent layer of gel. The microbial membrane was kept at 5 ºC in phosphate buffer for 5 days before BOD measurements with biosensor. For BOD biosensor measurements, the microbial membrane was then firmly attached on the top of a Clark-type oxygen sensor membrane with a special holder (WTW, CellOx 325, Germany). The diameter of microbial membrane (15.3 mm) is equivalent to the bottom diameter of the holder and fully covers the membrane of the oxygen sensor.

Several BOD biosensors were constructed according to above mentioned method and the output signal of the analyzers were recorded in solutions of different biodegradable organic substances. For the preparation of agarose gel layers of different thicknesses, abovementioned procedure was applied for polymer nets of suitable thicknesses without addition of microbes.

All BOD_7 analyses were performed according to the APHA Standard Methods [85]. Chemical oxygen demand (COD) was analyzed by Dr Lange COD Cuvette Test (LCK114, Dr Bruno Lange GmbH, Düsseldorf, Germany). For calibration of constructed BOD biosensor in OECD synthetic wastewater, the solution was prepared according to the OECD guidelines [86].

BOD biosensor measurements were performed in a well-agitated phosphate buffer solution at constant temperature under continuous aeration with air oxygen. An appropriate amount of substrate solution was added to 100 ml of air-saturated phosphate buffer after the initial output signal of BOD biosensor was stabilized. Before the next experiment, BOD biosensor was removed, rinsed, and returned to the jacketed beaker containing an aliquot volume of fresh phosphate buffer.

Analyzed wastewater samples were collected from wastewater treatment plants in Estonia. All samples were settled and analyzed for COD and BOD_7 according to the methods mentioned above. Between the analysis of samples and BOD biosensor experiments, samples were stored at 5 ºC. Experiments with BOD biosensor were carried out under its optimum working conditions, and calibration of BOD biosensor was always performed before measurements of wastewater samples for the verification of results.

The microbial membranes were stored at 5 °C in buffer solution. All data were recorded within 1 second time interval using in-house software.

5. RESULTS AND DISCUSSION

A BOD biosensor for the determination of biochemical oxygen demand was constructed and the output signal of the analyzer was modeled [Papers I–III].

5.1 Study and modeling of the output signal of BOD biosensor

The amperometric biosensor used in this study is based on a diffusion-limited amperometric oxygen sensor (the Clark-type oxygen sensor) with an additional gel membrane containing immobilized microorganisms. The substrate is metabolized by microorganisms consuming oxygen in the microbial membrane of the biosensor. The process leads to the redistribution of oxygen fluxes and causes the decrease of oxygen concentration at oxygen sensor membrane leading to a decrease in the biosensor output current.

The output current of an amperometric oxygen sensor that could be registered as a change in oxygen concentration, appears as soon as there is a change in oxygen concentration on the sensor's cathode. In bulk solution measurements, the mass transfer of dissolved oxygen toward the indicating electrode can be described as a flow of dissolved substance through several layers with different properties in terms of diffusion. At steady state of the system, the relationship between oxygen flux toward the cathode and the external oxygen concentration is proportional. Under non-steady state conditions, the response and lag-time of the sensing unit depends on diffusional resistance, mostly caused by an additional microbial membrane including immobilized material.

According to the modeling study of oxygen sensor response by Benedek and Heideger, the inherent lag of the process as well the change in oxygen concentration during transient process can be described as the summation of exponential functions on time [75]. In present study, after the sharp addition of organic substrate to well-agitated oxygen-saturated measuring solution, oxygen demand of immobilized microorganisms and diffusional resistance of oxygen in the real system causes so-called lag period of response, followed by the change in oxygen concentration on oxygen sensor membrane. The latter, e.g. the decay of output signal of oxygen sensor can be described as an exponential function. Based on above mentioned study, a mathematical model describing the lag-time along with transient change of oxygen concentration of BOD biosensor response has been developed [Paper III]. The lag-time of measuring system and detected decay of normalized output signal ($C_{O2,norm}$), in case the final steady state output $C_{O2,\infty} \rightarrow 0$, is expressed with eqn (14).

According to the proposed model, the BOD biosensor response is characterized in the form of summation of exponential changes at appropriate conditions. To describe the parameters of biosensor model in more detail, τ_s depends on the mass transfer of oxygen, indicating the dependence of exponential decay of

biosensor output on organic substrate concentration under invariable conditions; τ_d depends on the diffusion parameters of the oxygen sensor including thickness and other properties of additional microbial membrane and the oxygen diffusion coefficient. Therefore, τ_d should have an invariable value under certain conditions for an appropriate biosensor, being a suitable parameter for the evaluation of output signal stability of BOD biosensor during its life of service. To conclude, these parameters (eqn (14)) characterize the internal processes of BOD biosensor by the lag-time and decay of the output curve.

Eqn (14) based curve fitting results were obtained with all of the normalized experimental data measured at various biodegradable organic substrate concentrations. The proposed modeling enabled high quality curve fitting, an example of which is depicted in Fig. 2. The correlation coefficient of non-linear fitting of BOD biosensor response curves on different substrate concentrations was above 0.98 and the deviation of experimental data form the modeled results of the BOD biosensor, characterized with Pearson's chi-square test, was below 0.0005.

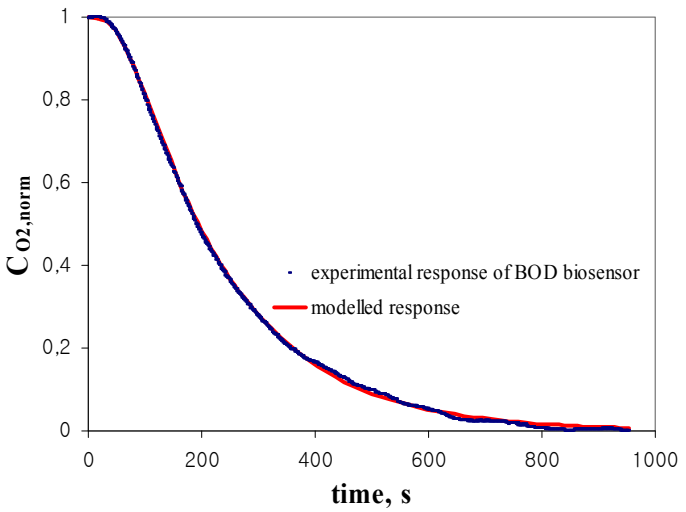


Figure 2. Comparison of normalized experimental and modeled response data of BOD biosensor in time [Paper III].

Additionally, the BOD biosensors response can be characterized by an inflection point of transfer function that shows the maximum value of normalized response. Also refereed to as maximum rate of the change or dynamic parameter (V_{O2}) of output signal, the parameter is applicable for experimental investigations (Fig. 3) and is further used for calibration and BOD determinations with biosensor [Paper I, Paper II].

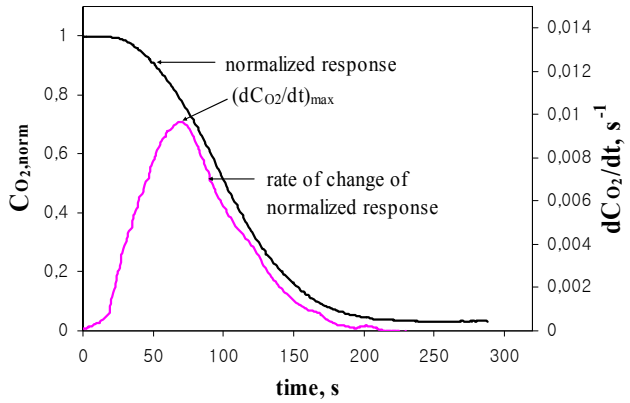


Figure 3. Normalized output signal and rate of change of the BOD biosensor output with the indication on maximum value of dC_{O2}/dt in time.

5.2. Application of the model for BOD biosensor construction

In this study, tests were performed with agarose gel membranes of various thicknesses to evaluate the suitability of this modeling approach as well to find out optimum conditions for biosensor construction. First, after the stabilization of oxygen sensor output signal in air-saturated distilled water, the device was sharply transferred into anaerobic conditions of the same environment and decay of dissolved oxygen content was registered at constant temperature. The same experimental procedure was followed up to study the non-steady response of the oxygen sensor with agarose membranes of different thicknesses attached closely on the top of it. The additional agarose gel layers change the non-steady response of BOD determination system proportionally of its thicknesses (0.15÷0.8 mm). To describe the impact of the above mentioned additional membranes on oxygen sensor normalized response, we assumed that $\tau_s = 0$ and from eqn (14) derived dependency can be expressed as follows [Paper III]:

$$\frac{C_{O_2}}{C_{O_2,0}} = C_{O_2,norm} = -2\sum_{n=1}^{\infty}(-1)^n \cdot \exp\left(-\frac{t}{\tau_d^{matrix}/n^2}\right), \qquad (21)$$

where parameter τ_d^{matrix} is the exponential time constant for the transient response of oxygen sensor with agarose gel membranes in close proximity of the outer membrane of the oxygen sensor.

To study the stability of agarose membranes in time, τ_d^{matrix} values were computed for these potential membranes to be used in BOD biosensors. Results

showed clear dependency of τ_d^{matrix} on agarose membrane thickness and good reproducibility of the parameter values in time. Therefore, conclusions can be drawn that used supportive nets in conjunction with this immobilization material are suitable elements for the construction of BOD biosensor (Table 1). Still, the experiments with immobilized microorganisms indicated low mechanical stability of 0.15 and 0.3 mm thick membranes on a regular basis of measurements and high limit of detection as well low sensitivity of the 0.8 mm thick microbial membrane in non-steady state conditions. For further studies with the BOD biosensor, the 0.5 mm thick microbial membrane was selected. According to the above described experimental method, the exponential time constant for oxygen sensor without any additional agarose membranes was also determined, the value of which was 3.5 s and deviation of the results of 2–5%.

Table 1. The values of τ_d^{matrix} for the oxygen sensor with agarose gel layers of different thicknesses

Thickness of agarose gel layer ($l_{agar.gel}$), mm	τ_d^{matrix}, s*	
	after preparation	after a month
0.15	23.6±1.8	21.2±1.9
0.3	41.7±3.0	40.5±3.2
0.5	63.9±4.0	62.4±4.1
0.8	71.7±3.6	71.9±3.8

* Results are average of the experiments (n=3) with two concurrently prepared agarose layers.

The results of the exponential time constants for the transient response of oxygen sensor with agarose gel membranes of different thicknesses are depicted in Figure 4. In the case of $l_{agar.gel}$=0, e.g. at x-intercept, the y-value corresponds to the appropriate exponential time constant of oxygen sensor.

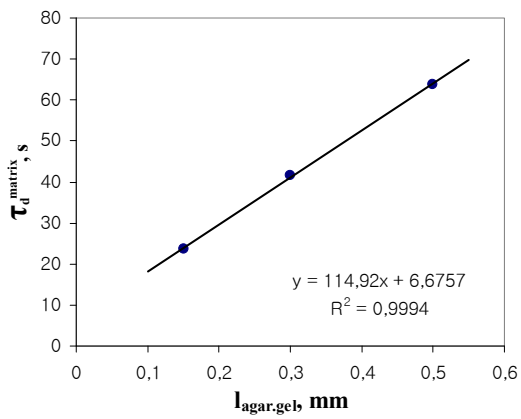


Figure 4. Parameter τ_d^{matrix} on different thicknesses of agarose gel layers.

5.3. The response of BOD biosensor

5.3.1. Steady state and transient method of analysis of output data

The output signal of BOD biosensors was detected in the solutions of various biodegradable organic substrates including wastewater, for the purpose of calibration and BOD analysis, and essentially for testing the developed mathematical model. Thus, the biosensors' response was analyzed according to the steady state method and with the herein developed model of transfer processes.

The normalized response of BOD biosensor *vs* time curves at various substrate concentrations demonstrate the dependency on substrate concentration of both, steady state and transient response of the BOD biosensor (Fig. 5). Substrate dependency of biosensor response is caused by the degradation processes in BOD biosensors, e.g. in the microbial membrane that designate the change of response in time and can be characterized by Michaelis-Menten-type enzyme kinetics. The latter describes the dependency of enzymatic reaction rate on substrate concentration that is schematically expressed as a hyperbolic function, characterized by two main characteristics – the maximum value of output parameter and the half-saturation coefficient of the response [87, 88].

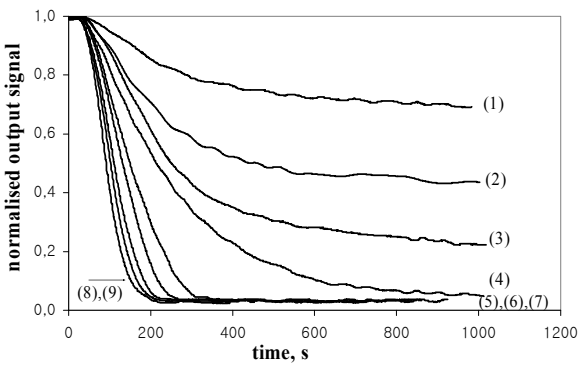


Figure 5. Normalized output signal BOD biosensor *vs* time on BOD_7 concentrations of OECD synthetic wastewater: (1) 14 mg L^{-1}, (2) 27 mg L^{-1}, (3) 40 mg L^{-1}, (4) 54 mg L^{-1}, (5) 80 mg L^{-1}, (6) 105 mg L^{-1}, (7) 155 mg L^{-1}, (8) 180 mg L^{-1} and (9) 225 mg L^{-1} [Paper II].

From the presented example of the time-dependencies of normalized output signal at different substrate concentrations (Fig. 5) can be seen that after introduction of higher substrate concentrations the amount of oxygen detected by cathode decreases sharply and at the end of experiment the BOD biosensors response approaches zero. Therefore, at higher concentrations of organic substrate, the response of the biosensor provides no analytically useful data for steady state conditions. Accordingly, no real stabilized endpoint value of a measurement can be detected and the normalized steady state value ($\Delta C_{O2,norm}$) approaches one (Fig. 6), that was also noticed by Nakamura, H. *et al*. [91].

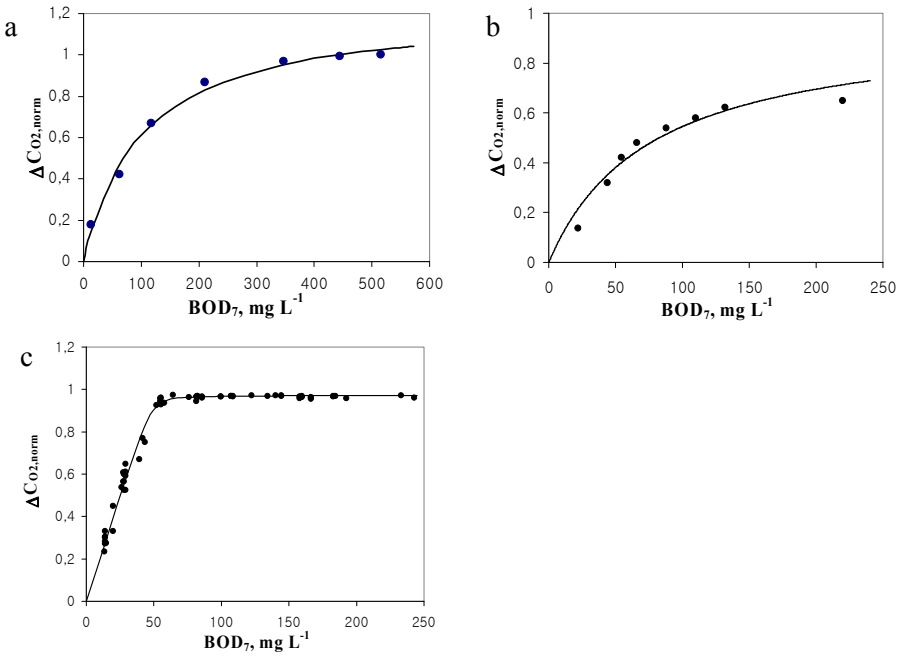


Figure 6. Dependency of steady state parameter of the BOD biosensor response on BOD_7 of different substrates: (a) glucose solution (K_s = 99.9 mg L^{-1}) [Paper I]; (b) GGA (K_s = 76.2 mg L^{-1}) [90] and (c) OECD synthetic wastewater for which the K_s is 27 mg L^{-1} and RSD of all experimental data against theoretical hyperbolic curve is ± 2.8% [Paper II]. The solid lines correspond to the theoretical curve.

Determination of maximum rate of change of the BOD biosensor response (V_{O2}) that shows the maximum oxygen consumption by bacteria resulted with much broader concentration range compared to the steady state method, at the solutions of various concentrations of biodegradable organic substances (Fig. 7). The main reason is that no such limitation of oxygen concentration as described above, allowing the experimental determination of BOD_7 at much higher substrate content compared to previous method. The higher values of half-saturation coefficients in dynamic regime of measurement compared to steady state data indicate lower sensibility, but remarkably wider concentration range of output data. The latter can be explained by the BOD biosensors construction specifics, e.g. immobilization material along with microorganisms in the microbial membrane form a diffusion barrier for substrates resulting in the decrease of response rate.

The initial experimental data were smoothed in 5 points average for the determination of dynamic parameter (V_{O2}) of an output signal. As well as Yang, Z. *et al*. [71], we also noticed some fluctuations of raw experimental data that made smoothing necessary.

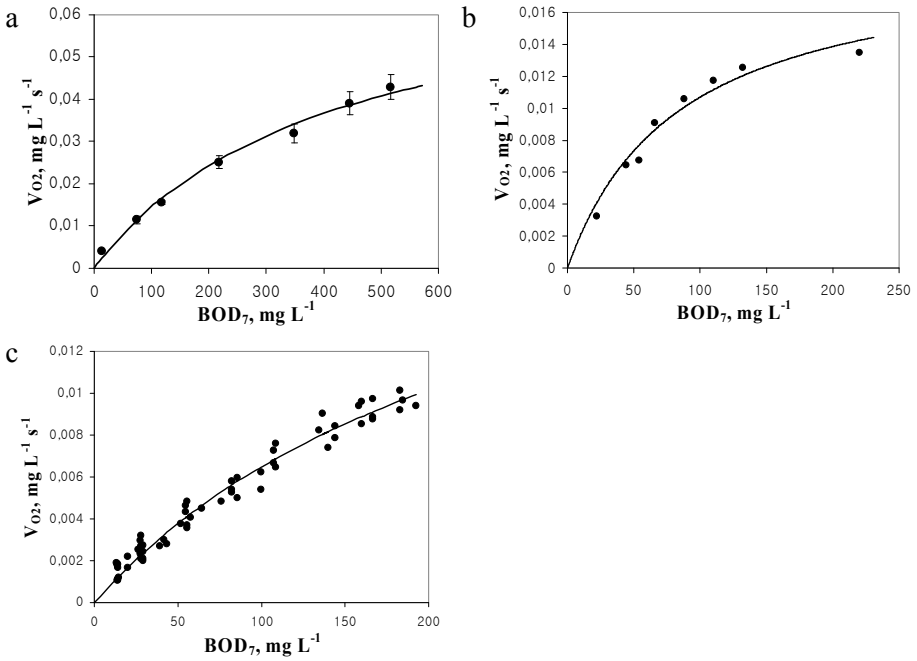


Figure 7. Dependency of dynamic parameter (V_{O2}) of the BOD biosensor response *vs* BOD_7 of different substrates: (a) glucose solution characterized by the maximum value of the response, 0.074 mg L^{-1} s^{-1} and by the value of K_s, 403 mg L^{-1} [Paper I]; (b) GGA solution characterized by the maximum value of the response, 0.0197 mg L^{-1} s^{-1} and by the K_s, 84 mg L^{-1} [90] and (c) OECD synthetic wastewater for which the K_s is 265 mg L^{-1} and the maximum value of the response is 0.023 mg L^{-1} s^{-1}. RSD of all experimental data against theoretical hyperbolic curve was ± 4.8%, weekly calibration lowered it to ± 2.6% [Paper II]. The solid lines correspond to the theoretical curve.

In all, the values of half-saturation coefficients of stabilized and dynamic methods of calibration are different because the change between stabilized initial and final value of output signal depends mostly on the number, viability and respiration rate of microorganisms. The dynamic output values are also influenced by substrate and oxygen mass transfer rates that depend mainly on characteristics and concentration of immobilization material and the thickness of microbial membrane of the BOD biosensor. Analysis of the steady state response method resulted in higher sensitivity and lower uncertainties within the linear range of the method. The latter, linear range of measurements showed comparable extent with several previously studied BOD biosensors, based on microorganisms [7, 50, 54, 72, 91]. The measurement range of BOD biosensor in dynamic regime is higher compared to previous studies [71], that is an advantage of BOD_7 estimations in heterogeneous wastewater (no dilution error). Other favors of dynamic method are

much shorter response (80–250 s) as well recovery times compared to stabilized method [Paper II].

The computed parameters of developed mathematical model, found by eqn (14) are very useful for the evaluation of performance of output signal of BOD biosensor. The non-linear curve fitting procedure takes into account the variations of experimental data, mostly caused by fluctuations of microbial membrane, and concurrently determines the BOD_7 of a sample. Therefore changes in BOD biosensor stability as well the BOD of studied samples can be evaluated quickly and effectively with the proposed model. Additionally, the non-linear curve fitting procedure takes into account the variations of experimental data, mostly caused by fluctuations of microbial membrane, providing results with diminished uncertainty. The biosensor experiments with different organic substrates showed that the linear relationship between model parameter τ_s and BOD_7 is relevant for calibration. These results, depicted in Fig. 8 are used for the calibration of studied BOD biosensors up to the BOD_7 content of 110 mg O_2 L^{-1}.

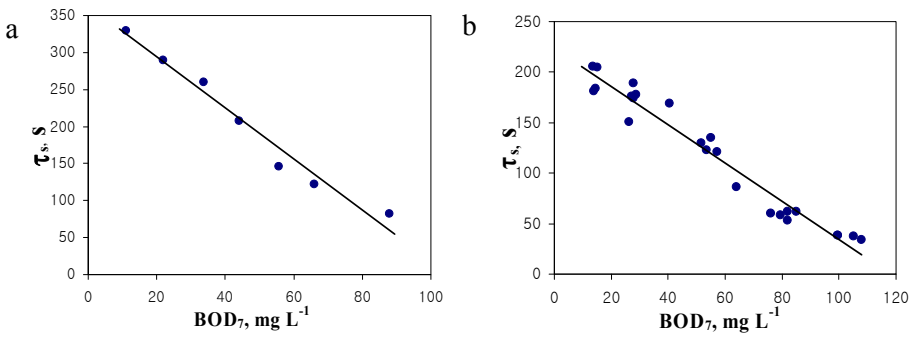


Figure 8. Calibration graph of BOD biosensor characterized by the dependency of model parameter τ_s on BOD_7 value of calibration solution: (a) in GGA solution (R^2=0,97) [90] and (b) in OECD synthetic wastewater solution (R^2=0,96) [Paper III].

5.3.2 Stability of the BOD biosensor

Several studies use steady state and dynamic response of BOD biosensor for calibration, analysis of real samples and performance stability observations. Usually, the main drawback of this kind of measurements is that the instability of response is detectable after the experiments have been performed or even after all the calibration data have been gathered and plot has been compiled. In this study, we could detect the stability of biosensor according to the value of computed parameter τ_d (eqn (14)) in a few minutes after starting the experiment. The results of stability studies of the BOD biosensor during its life of service are depicted in Fig. 9.

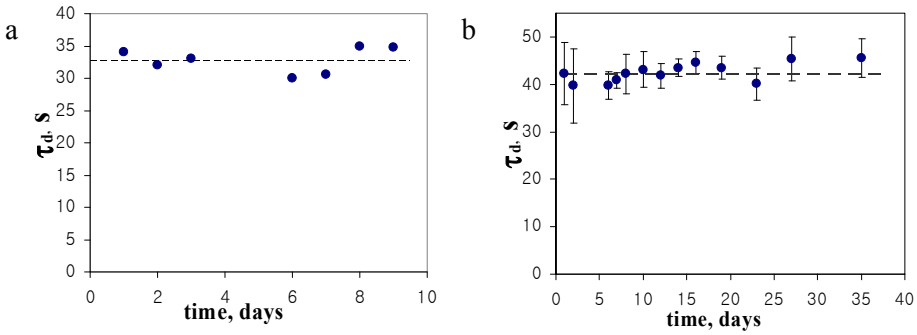


Figure 9. Stability of BOD biosensor characterized with model parameter τ_d, where on the graph is (a) GGA solution [90] and (b) OECD synthetic wastewater solution [Paper III].

5.4. Estimation of BOD_7 in wastewater

The main goal of BOD biosensor studies comprises the application of the device for BOD_7 determination in environmental samples, including wastewater samples which are the object of this study. The composition of a sample, as well the treatment method of biosensor output signal results in various ratios between BOD_7 and BOD values estimated by BOD biosensor [40, 92]. The studied microbial BOD biosensors, calibrated in OECD synthetic wastewater solution, showed acceptable BOD_7 values in municipal and mixed wastewater. Still, depending on the computing technique of output data, some variety of the results was noticed.

Analysis of municipal wastewater by steady state calibration method showed 18% lower results for biochemical oxygen demand compared to BOD_7 values, on average (Fig. 10). Similar tendency has been also noticed by Riedel, K. *et al*. [40] and Hyun, Z. K. *et al*. [92]. The difference between the analytical response of BOD biosensor and conventional BOD_7 values is mostly caused by several factors affecting the microorganisms' oxygen demand in BOD biosensor. Thereat, the load of immobilized microorganisms, substrate assimilation difficulties and characteristics of biosensor, like diffusion limitations of oxygen and organic compounds and time boundaries of experiments can be pointed out. Still, according to Figure 10, there is a linear relationship between BOD biosensor response and BOD_7 of wastewater samples. Therefore, for the treatment control of a particular wastewater treatment plant, BOD biosensor should be calibrated with appropriate wastewater and much higher agreement with BOD_7 can be expected.

The BOD values determined by BOD biosensor respond mostly on easily biodegradable substances, e.g. on the amount and availability of biodegradable non-toxic substances in the sample. Besides, for the parameters V_{O2} and τ_s that are found by the course of output signal, the experimental result depends on mass transfer resistance of immobilization matrix and microorganisms, noticed also by Tan, T. C. *et al*. [91]. It occurred that in the concentration range of the steady state method (15 to 50 $mgO_2\ L^{-1}$), the biosensor measured BOD values by dynamic

parameter (V_{O2}) are 15% higher compared to BOD_7 for municipal wastewater samples, on average (RSD 8.3%). Above 50 mgO_2 L^{-1}, we found underestimation of BOD_7 by BOD biosensor output data approximately for 30%, estimated according to V_{O2}. At higher concentrations, the extent of diffusion and degradation rate of organic substrates lowers the value of biochemical oxygen demand measured with BOD biosensor. However BOD_7 can be calculated by the respective relationship and additional experiments with diluted samples in steady state regime can be carried out. Therefore, conclusion can be drawn that the comparison of the steady state and dynamic results along with the evaluation of the extent of overestimation of BOD_7 in dynamic conditions in wastewater samples carries additional information about the content of sample [Paper II].

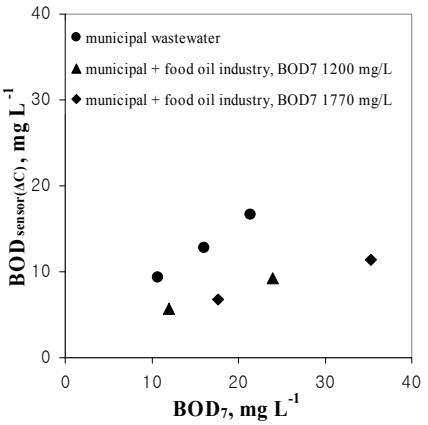


Figure 10. Comparison of steady state parameter of biosensor measured BOD and BOD_7 values in municipal wastewater and municipal wastewater mixed with food oil industry outflow [Paper II].

For the determination of BOD_7 in municipal wastewater samples according to model parameter τ_s, the value of the biosensor detected BOD_7 was determined by the linear calibration plot (Fig. 8). These modeling results showed that in studied municipal wastewater samples with biodegradation index in the range of 0.43–0.61, the BOD values measured with BOD biosensor were 13% higher compared to conventional BOD_7 (RSD 5–8%) [Paper III].

The output of BOD biosensor was also studied in refractory wastewater samples (with biodegradation index 0.3, on average) for the determination of BOD_7. The samples originate from a WWTP where food oil factory outflow was pumped into the aerobic basin of wastewater treatment plant for the biodegradation along with local municipal wastewater. BOD biosensor studies on these mixed samples indicate that biodegradation of high molecular weight substances is not likely during the analysis time of a BOD biosensor and the results are applicable for the approximate evaluation of BOD_7 of these samples [7, 92]. Our

results in the steady state and dynamic conditions showed underestimation of BOD_7 in the range of 45–65%, whereby the higher uncertainty of V_{O2} determinations compared to steady state data was noticed. Estimation of BOD_7 in studied refractory wastewater samples according to the model parameter τ_s resulted with lower uncertainty and better concurrence between BOD biosensor response and BOD_7 values: BOD biosensor results were only 4–10% lower compared to the analyses results of standard method [Paper III].

For the analysis of BOD biosensor data, the introduced modeling approach has several aforementioned advantages over steady state and dynamic response based methods. One of improvements of the present BOD biosensor study is better concurrence of BOD biosensor and BOD_7 values, the reason for that could be optimized processing of output signal, taking into account sources of experimental noise like raw data fluctuations, microbial activity etc.

6. CONCLUSIONS

Monitoring and quantitative measurement of pollutants are integral parts of any sustainable environmental policy or program. Soluble biodegradable organic solutes are important pollutants in wastewater and industrial effluents and are normally measured collectively and expressed as concentration index biochemical oxygen demand (BOD). The BOD_7 is essentially a measure of the amount of dissolved oxygen required for the biochemical oxidation of organic solutes in 7 days.

A study of an analytical tool for faster BOD_7 determinations, including the analysis of BOD biosensor output data and construction details was conducted. As a result, a BOD biosensor has been constructed to provide expeditious and effective determination of BOD_7 in wastewater samples.

The extensive analysis and study of BOD biosensor output signal has resulted in a mathematical model that enables good quality curve fitting procedures. The introduced method allows the reconstruction of BOD biosensors response with output parameters that are good indicators of service life and stability of BOD biosensor as well reliable estimate of the content of biodegradable organic substances in samples. The model was also used in the research of construction details of the BOD biosensor.

Constructed BOD biosensor, which output signal is recorded according to the study, can be calibrated for the determination of BOD_7 in wastewater samples by three different methods of data treatment – steady state output, dynamic response and results of modeling by curve fitting of normalized data. Steady state method of output data treatment is preferred at lower concentrations of biodegradable organic substrates because of diminished uncertainty of data. Broad concentration range, shorter response as well recovery times are the favors of dynamic condition compared to stabilized method. Curve fitting procedure of output signal takes into account various sources of experimental noise providing reliable data for an improved concurrence of BOD biosensor response and BOD_7 values compared to aforementioned.

The concurrence of BOD biosensor response and BOD_7 values depend on the composition, e.g. the biodegradability of sample, and the treatment method of output data. Analysis of municipal wastewater by steady state calibration method showed underestimation of BOD_7 values 18%, on average. Analysis and modeling of the biosensor response by the course of output signal resulted on 13–15% higher BOD values compared BOD_7.

The determination of BOD_7 with constructed BOD biosensor in refractory wastewater that is a mixture of municipal and food oil factory wastewater, showed an underestimation of the BOD_7 value for 4–10% according to model parameter τ_d and 45–65% by steady state and dynamic method of output data treatment.

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8. SUMMARY IN ESTONIAN

Mikroorganismidel põhinev BHT biosensor reovee analüüsimiseks

Üheks oluliseks näitajaks vesikeskkonna saastumise hindamisel on summaarne parameeter biokeemiline hapnikutarve (BHT), mis iseloomustab orgaaniliste biolagunevate ühendite sisaldust vees. Traditsioonilise BHT analüüsiga mõõdetakse hapniku kontsentratsiooni vähenemist orgaaniliste ühendite biokeemiliseks lagundamiseks lahuses 5- või 7-päevase inkubatsiooniperioodi jooksul. Seetõttu traditsioonilise BHT_7 analüüsi tulemusi ei saa sageli kasutada reoveepuhastite operatiivseks juhtimiseks ning saastatud vesi võib jõuda looduslikku suublasse juba enne analüüsitulemuste selgumist.

Biosensor on seade, mis tuvastab, edastab ja salvestab informatsiooni bioloogilise äratundmissüsteemi füsioloogiliste või biokeemiliste muutuste kohta sobivate orgaaniliste substraatide toimel. Biokeemilise hapnikutarbe biosensorit kasutatakse biolagunevate orgaaniliste substraatide analüüsimiseks, see koosneb biokeemilise osisena immobiliseeritud mikroorganisme sisaldavast membraanist ja signaali detekteerijana amperomeetrilisest hapnikuandurist. Biokeemilise hapnikutarbe määramisel reovees on BHT biosensoril mitmeid eeliseid traditsioonilise 7-päevase meetodi ees, millest olulisim on tulemuste saamine kuni 1 tunni jooksul. Lisaks on BHT biosensor kompaktne, kaasaskantav ja selle kasutamine mõõteseadmena ei ole keeruline.

Käesolev uurimistöö käsitleb mikroorganismidel põhineva BHT biosensori väljundsignaali uurimist ja modelleerimist. Saadud tulemusi kasutati optimaalsete omadustega biosensori konstrueerimiseks, võimaliku määramispiirkonna leidmiseks ning reovee biokeemilise hapnikutarbe määramiseks. Amperomeetrilise hapnikuanduri otsa kinnitatud immobiliseeritud mikroorganisme sisaldavas membraanis tarbitakse orgaanilise substraadi lagundamisel hapnikku, mille kontsentratsiooni ajalise muutuse järgi saab hinnata orgaaniliste ühendite sisaldust proovis. Saadud väljundsignaali saab analüüsida nii statsionaarse alg- ja lõpppunkti erinevuse järgi kui meetoditega, mis põhinevad väljundsignaali ajalisel muutusel. Nimetatud mittestatsionaarsete protsesside arvutimodelleerimisel leiti, et hapniku kontsentratsiooni ajaline vähenemine hapnikuanduri membraanil põhjustab vastavat muutust katoodile jõudvas hapniku voos, mis registreeritakse mõõteriistaga eksponentsiaalse muutusena. Mittestatsionaarses režiimis ei ole hapniku voog katoodil proportsionaalne hapniku kontsentratsiooniga mikroorganisme sisaldavas kihis ning seetõttu iseloomustatakse väljundsignaali üleminekufunktsiooniga, mis hõlmab reaalsete süsteemide hilistumist, mikroorganismidega membraanis toimuvaid protsesse ja mõõturiga registreeritavat väljundsignaali.

Uurimustöö üheks põhitulemuseks on BHT biosensori väljundsignaali uurimise tulemusena välja töötatud matemaatiline mudel mittestatsionaarsete protsesside iseloomustamiseks. Selle väljundparameetreid saab kasutada biolagunevate orgaaniliste ühendite kontsentratsiooni määramiseks ning samaaegselt

ka BHT biosensori väljundsignaali ajalise stabiilsuse hindamiseks. Lisaks kasutati väljatöötatud mudelit sobivaimate omadustega agaroosmembraani leidmiseks biosensori koostamisel.

BHT biosensori väljundsignaali saab kalibreerida ja selle tulemusi saab vastavalt kasutada proovide BHT_7 määramiseks kolme erineva meetodiga – väljundsignaali statsionaarsete muutuste järgi, signaali muutuse maksimaalse kiiruse järgi ja välja töötatud matemaatilise mudeli parameetri alusel. Madalatel orgaanilise substraadi kontsentratsioonidel on eelistatud väljundsignaali analüüsimine statsionaarse alg- ja lõppnäidu järgi väiksema mõõtemääramatuse tõttu. Signaali muutuse maksimaalse kiiruse leidmise eeliseks on lai määramispiirkond ning lühem mõõte- ja taastumisaeg võrreldes eelnimetatud meetodiga. Väljundsignaali modelleerimisel leiti, et mikroorganismide hapnikutarbest tingitud hapniku kontsentratsiooni eksponentsiaalsele langusele hapnikuanduri membraanil vastava ajakonstandi väärtus väheneb orgaanilise substraadi kontsentratsiooni kasvades, mis on kooskõlas mudelkujutlusega mittestatsionaarsetest protsessidest.

BHT biosensoriga ja traditsioonilise BHT_7 analüüsiga määratud biokeemilise hapnikutarbe kokkulangevus sõltub orgaaniliste ühendite biolagundatavusest uuritavas proovis. Olmereovee analüüsimisel leiti, et signaali töötlemise statsionaarse meetodiga ja mittestatsionaarsete väljundsignaali uurimise meetoditega saadakse BHT biosensoriga vastavalt ligikaudu 18% madalam ja 13–15% kõrgem tulemus kui standardse BHT_7 analüüsiga. BHT biosensorit kasutati ka raskeltlaguneva reovee, st toiduõli tööstuse väljavoolu ja olmereovee segu BHT_7 määramiseks. Seejuures biosensori väljundsignaali analüüsimisel statsionaarse ja signaali muutuse maksimaalse kiiruse meetoditega saadi 45–65% madalam biokeemilise hapnikutarbe väärtus võrreldes BHT_7-ga. BHT biosensori mudeli parameetri alusel leitud BHT sisaldus proovides erines 4–10% BHT_7-st.

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