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MERLIN RAUD

Study of semi-specific BOD biosensors for biosensor-array

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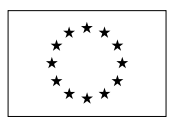

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TABLE OF CONTENTS

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I. **Raud, M**.; Linde, E.; Kibena, E.; Velling, S.; Tenno, T.; Talpsep, E.; Kikas, T.; (2010): Semi-specific biosensors for measuring BOD in dairy wastewater. Journal of Chemical Technology and Biotechnology, 85(7), 957–961

II. **Raud, M**.; Tenno, T.; Jõgi, E.; Kikas, T.; (2012): Comparative study of semi-specific *Aeromonas hydrophila* and universal *Pseudomonas fluorescens* biosensors for BOD measurements in meat industry wastewaters. Enzyme and Microbial Technology, 50(4–5), 221–226

III. **Raud. M.;** Tutt, M.; Jõgi E.; Kikas, T.; (2012): BOD biosensors for pulp and paper industry wastewater analysis. Environmental Science and Pollution Research, 19(7), 3039–3045.

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Author's contribution:

I. Participated in calculations (40%), interpretation of results (40%) and wrote the manuscript (40%)
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III. Performed all experimental work (100%), calculations (100%), interpretation of results (100%) and wrote the manuscript (90%)
IV. Participated in experimental work (20%), calculations (20%) and writing the manuscript (50%)
V. Performed experimental work (90%), calculations (100%), interpretation of results (100%) and wrote the manuscript (90%)

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APHA	American Public Health Organization
BOD	Biochemical oxygen demand
BOD_x	Biochemical oxygen demand; "x" indicates the duration of incubation in days during analysis
BSA	Bovine serum albumin
CMC	Carboxyl-methyl-cellulose
COD	Chemical oxygen demand
GA	Glutaraldehyde
GGA	1:1 glucose–glutamic acid mixture
I_0	Initial steady-state signal at zero BOD_7 value and
I_S	Steady-state signal at certain BOD_7 value
NSR	Normalized signal response
OECD	Organization of Economic Cooperation and Development
PCA	Principal component analysis
PC	Principal component
PLS	Partial least squares
PVA	Poly-vinyl alcohol
Sensor-BOD	Biochemical oxygen demand measured with biosensor

INTRODUCTION

The increasing concern over pollution risk to environment from different domestic and industrial activities has led to a heightened attention to monitoring of water and wastewater quality. Regular monitoring of water quality is required in all wastewater treatment facilities to protect the natural water resources from pollution. Pollution load of wastewaters is determined on the basis of their oxygen demand. Two widely used measures of oxygen demand are used. The chemical oxygen demand (COD) test determines the oxygen requirements for the oxidation of both biodegradable and non-biodegradable compounds [1], whereas the biochemical oxygen demand (BOD) test measures the oxygen required for the biochemical degradation of organic material and the oxygen used to oxidize inorganic material in wastewaters [1, 2]. Although the BOD test is not specific to any pollutant, it continues to be one of the important general indicators of the substance's potential to be an environmental pollutant for surface waters [1].

The BOD measuring method consists of placing the sample in a full, airtight bottle and incubating it under specified conditions for a specific time. Dissolved oxygen is measured initially and after incubation. The BOD is calculated from the difference between initial and final values of dissolved oxygen [2]. The bottle size, incubation temperature and period are specified. Conducting the test is simple and needs no expensive equipment but it needs skills and experience since most wastewaters need to be diluted to bring the oxygen demand and supply into appropriate balance, nitrification inhibitors are added to prevent ammonia oxidation and seeding of the sample may be necessary [2]. According to American or Swedish standard, the BOD test takes 5 or 7 days, respectively, to gain results [2, 3]. Management of a wastewater treatment facilities can be very difficult using this kind of time-consuming test, since conditions in the plant may have been already changed when the results are available [4]. Despite the limitations, the BOD test is still extensively used and preferred over other analytical methods due to its robustness [5].

To address this limitation, several BOD biosensors based on different transducers modified with microorganisms assimilating organic pollutants have been reported. BOD biosensors relay on measurement of decrease in dissolved oxygen concentration near the transducer which is caused by assimilation of organic compounds by microorganisms. Biosensors have several advantages that make them advantageous for environmental monitoring like, short measurement times, low cost, possible portable options, real-time measurements, and possible use as remote devices [6, 7]. However, in case of BOD biosensor, the gained results and the measurement accuracy depend mostly on microorganisms used and the accuracy is often low when specific industrial wastewaters are analyzed. Better results could be obtained when semi-specific microorganisms are utilized. These microorganisms, unlike universal microorganisms, can assimilate some refractory compound or group of compounds specific to that particular microorganism. Thereby, biosensors based on semi-

specific microorganisms are able to detect specific refractory compounds in industrial wastewaters, which would otherwise remain undetected, thus better correlation between sensor-BOD and BOD_7 can be achieved [8].

However, in case of specific industrial wastewater samples, it is vital to select the suitable semi-specific biosensor to gain accurate results. One solution to overcome this is the application of biosensor arrays – bioelectronic tongues – based on collection of semi-specific BOD biosensors combined with multivariate data analysis. In order to use multivariate data analysis, the biosensors in biosensor-array must have low selectivity. That can be achieved with universal substrate spectrum of microorganisms. Therefore, BOD biosensors are sensitive to variety of organic compounds in wastewater sample. In addition, due to semi-specificity these microorganisms have wider and partially specific substrate spectra and they can also detect and measure specific refractory compounds [8]. By combining variety of semi-specific and universal microbial biosensors into a biosensor array, a complex signal containing information about various components in the medium can be obtained [8, 9]. When a suitable multivariate data analysis methods is applied to process this complex signals from biosensor array, a qualitative and quantitative information can be extracted [10].

The main objectives of this study were to characterize, select, and use semi-specific microorganisms to construct biosensors for BOD measurement in various industrial wastewaters. The biosensors were designed to be suitable for analyzing wastewaters from major industries in Estonia: meat –, oil shale –, pulp and paper – and dairy industry. The microorganisms used in biosensors were chosen based on their ability to assimilate specific refractory compounds found in these industrial wastewaters which remain inassimilable to universal microorganisms. Universal biosensor was used as a reference to reveal the advantages of semi-specific biosensors. Different semi-specific and universal biosensors were used as a biosensor-array and comprehensive multivariate data analysis to gain more precise BOD estimation in various samples without pre-knowledge of samples origin and composition. In addition, possibilities to differentiate industrial wastewater samples according to their composition were studied.

1. LITERATURE OVERVIEW

1.1. Biosensors

An electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is retained in intimate contact or integrated with an transduction element [6, 11–14]. The biological recognition element of a biosensor interacts selectively with the target compound, while the transducer converts the biological response resulting from the interaction, into a detectable and quantifiable electrical signal [15, 16]. The selectivity of the biosensor for the target analyte is mainly determined by the biological recognition element, whilst the sensitivity of the biosensor is greatly influenced by the transducer [15]. Ideally, biosensors must be designed to detect molecules of analytical significance, pathogens, and toxic compounds to provide rapid, accurate, and reliable information about the analysts of interrogation [12].

Biosensors may be classified according configurations as single use (disposable), intermittent use, and continuous use [17] but also according to the biological recognition system or to the mode of signal transduction [6, 11]. Typical biological recognition elements used in biosensors are different biomolecules like enzymes, nucleic acids, antibodies, receptors but also organelles, cells and tissues have been used [11, 16, 18–21]. A number of factors like the specificity, storage-, operational- and environmental stability but also the analyte to be detected, must be considered when biological material is chosen [18, 19]. Biosensors based on different detection principles like electrochemical, optical, acoustic, mass-sensitive and thermal etc. [6, 19, 20] have been reported, with the majority being electrochemical [6]. Electro-analytical techniques have several advantages like low detection limit, relative simplicity, low cost of equipment, automatic, on-line and portable options [22].

1.2. Microbial biosensors

Microbial biosensors are biosensors where microorganisms are used as a biological recognition element. Different strains of bacteria, fungi and yeasts have been used in biosensors for detection of corrosion [23], phenol [24], heavy metals [25], ammonia [26, 27], toxicity, and inhibition [28–30] etc. Whole cells of microorganisms are used in biosensors either in a viable or non-viable form [19]. Live cells demand careful storage conditions and feeding when not in use, whereas reactivation of dead cells, which allows easy storage and commercialization of biosensors, is time-consuming [5].

Although purified enzymes are most widely used biological sensing elements [21], microorganisms have a number of advantages as biosensor components. Microorganisms are widely spread and economical source of enzymes [7, 19] since they are easy to cultivate and manipulate [7]. Enzymes are more

stable in its natural environment in the cell [7], which protects them from inactivation either during immobilization or their subsequent reuse [18] therefore there is no need for time-consuming and costly enzyme purification [7, 16]. Immobilized cells are active multi-enzyme and co-factor systems [20] able to consume and hence detect large number of chemicals [21]. Cells can be used also as a multipurpose catalyst, especially when the process requires participation of a number of enzymes in sequence [18]. They are amenable to genetic modifications and can be adapted to consume and degrade new substrates under certain cultivating condition [19, 21] thereby, highly selective microbial biosensors can be achieved [16]. The major limitations accompanied with cells are the slower diffusion of molecules through the cell wall and unwanted side reactions owing to the presence of other enzymes [18, 19].

There are two main mechanisms how cells generate specific physio-chemical changes that serve as a reporter of a particular environmental condition to be detected with transducer [31]. The first one is based on the metabolism of cells where the overall substrate assimilation capacity of microorganisms is taken as an index of respiratory metabolic activity. The other method involves inhibition of microbial respiration by the analyte of interest, such as environmental pollutants [18, 19, 32]. In addition, biosensors based on bioluminescence of microorganisms have been reported. These biosensors are based on a production and emission of visible light by an organism which is proportional to the amount of assimilable organic contaminants in wastewater [33].

1.2.1. Immobilization of microorganisms

In order to convert the biochemical response of microorganisms into a detectable physical signal, the microbial cells must be intimately associated with the transducer [16]. Immobilization helps to form the close proximity between the biomaterial and the transducer, and helps to stabilize it for reuse [18, 19, 32]. Different approaches have been applied to integrate cells with the transducer [34], however, the most common practice is immobilization of microorganisms into a membrane and attaching this to close proximity of a transducer. A successful matrix should immobilize or integrate biomolecules stably at a transducer surface and efficiently maintain the functionality of the biomolecules, while providing accessibility to the target compound and an intimate contact with the transducer surface [13]. Traditional methods for immobilizing cells are either by chemical of physical means [7, 19, 21] through adsorption, entrapment, covalent binding, cross-linking [16, 18, 19, 32].

Chemical methods include covalent binding and cross-linking. Covalent binding is commonly used for enzymes and antibodies but is not useful for the immobilization of cells since cells are exposed to harsh reaction conditions and chemicals which may affect their viability [19]. Cross-linking uses bifunctional reagents like glutaraldehyde (GA) to generate biocompatible matrix with proteinic supports like bovine serum albumin (BSA) and gelatin [7, 19]. The cells can be immobilized directly onto the transducer surface or onto a removable

and replaceable support membrane [21]. Cross-linking involves formation of covalent bonds between the functional groups of cell outer membranes and GA [7] therefore, the method is not suitable when cell viability is required. Thus, cross-linking technique is useful in immobilization of non-viable cells containing active intracellular enzymes [19, 21].

Physical methods include physical adsorption, direct inclusion, entrapment or combination of all of them [7]. Physical methods are not based on covalent bond formation with microorganisms and thus, provide relatively small perturbation of microorganisms structure and function therefore, they are preferred when viable cells are used [21]. Physical adsorption is the easiest and softest method [7] where the cells are immobilized directly to the transducer or retained in a close proximity of a transducer surface using membranes [19] like charged nylon membrane [35–37], porous Teflon membrane [38, 39], polycarbonate membrane [40–47], cellulose nitrate membrane [48–50], acetate cellulose membrane [51], micro-cellular polymer [52]. However, physical adsorption methods result in weak bonds that may cause easy desorption of cells during use and storage [7]. Microbial cells can also be immobilized by entrapment [19] which provides cross-linked matrix and protects microorganisms from leaking [53]. Various synthetic or natural polymeric gel matrixes like poly-vinyl alcohol (PVA) [54, 55], PVA-ormosils composite matrix [56, 57], photo-cross-linkable resins [58], calcium alginate [14, 59], agarose [60], hydrogels [61], sol-gel composites [62], organic-inorganic hybrid material [53, 63]. However, natural immobilization matrixes are preferred if living cells are immobilized, since synthetic polymers may abate cell viability [32]. A major disadvantage of entrapment immobilization is the additional diffusion resistance caused by immobilization matrix, which will result in lower sensitivity and detection limit [21].

1.3. Microbial BOD biosensors

The first microbial BOD biosensor was introduced by Isao Karube in 1977, who entrapped bacteria isolated from soil into collagen membrane and attached it to an oxygen electrode [64]. Since then different kinds of BOD biosensor designs have been reported. Most of the reported BOD biosensors are biofilm type biosensors based on whole cells of microorganisms and relay on measurement of bacterial respiration rate in close proximity to a transducer [65]. In most cases, dissolved oxygen sensor is used as a transducer to register the bio-oxidation process near the transducer [41, 66] but optical fiber [56], CO_2 analyzer [67], spectrophotometric detector [68, 69] chemi-luminescence detector [70, 71] have also been applied. In addition, various biosensor designs, like reactor type [14, 55], microbial fuel cell type [72, 73], respirographic [67, 74], micro-plate based biosensors for BOD biosensing have been reported. The bio-layer type BOD sensors have a certain advantages including rapid analysis, simplicity,

compact design, low cost and the possibility of instrumentation for on-line application [14, 75].

Different kinds of microbial strains have been used as biological sensing element like *Pseudomonas putida* [48–50], *Pseudomonas Syringae* [52], *Arxula adeninivorans* LS3 [76, 77], *Escherichia coli* [78], *Saccharomyces cerevisiae* [59, 79], *Klebsiella sp.* [38, 39], *Pseudomonas fluorescens* [51], *Deinococcus radiodurans* [60], *Bacillus subtilis* [40], *Trichosporon cutaneum [34, 58],* but also mixtures of two strains like *Trichosporon cutaneum* with *Bacillus subtilis* [62], *or Bacillus subtilis* with Bacillus *licheniformis 7B* [41–43], microbial consortium [35–37, 44] and activated sludge [66, 75, 80].

The microbial strains are selected based on their ability to assimilate a wide spectrum of substrates [66]. Unlike other microbial biosensors, which require high selectivity from microorganisms towards given solution, BOD biosensors require microorganisms with low selectivity and high bio-oxidation activity for a wide range of organic compounds [41, 62]. In that case the biosensor responds to all kinds of organics in the sample [35, 41] and it can be used to monitor process effluent and wastewaters from different sources [41, 62]. In general, single-culture-BOD-sensors are limited by the narrow substrate spectrum of the single strain while BOD sensors based on a complex microbial population have a good detection capacity for a wide substrate spectrum [21, 53, 65, 66]. On the other hand, pure microbial cultures have much better stability and longer lifetime under same cultivation and storage conditions than multi-strain based sensors because the culture consistency of microbes in mixed cultures may vary with time [41, 44, 53, 65].

The bio-layer type BOD biosensors are based on measurement of bacterial respiration rate near the transducer [66] and the observed BOD values reflect the concentrations of the dissolved organic substances which are assimilated/ metabolized by the immobilized microbes [35, 37]. Figure 1 illustrates the design and working principle of this kind of biosensors. When the biosensor is placed into a clean aerated buffer solution, the current becomes stable when the oxygen diffusion rate into the microbial membrane from the solution reaches equilibrium with the oxygen consumption rate by endogenous respiration of immobilized microorganisms [35, 66]. When a wastewater sample is injected into the sensor system, organic substrates diffuse through the membrane and are assimilated by the immobilized microorganisms, resulting in an increase of bacterial respiration rate and oxygen consumption. Therefore, less oxygen can be detected by the oxygen electrode and the biosensors output signal decreases until a new steady state is obtained. Since the process is controlled by substrate diffusion, the sensor signal should be proportional to the concentration of organic substrates to be measured. [66].

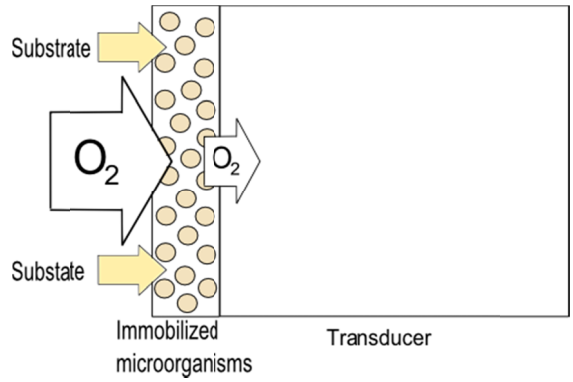

Figure 1. A schematic and a work principle of a bio-layer type BOD biosensor where immobilized microorganisms are placed in close proximity of a transducer

Two measuring techniques are available for biofilm-type BOD sensors: the steady-state method and the kinetic method. In the steady-state method, the current difference between the two steady states is used for the BOD estimation. In the kinetic method, the initial current change rate after sample addition, which reflects acceleration of the bacterial respiration rate, is used as the sensor response [43, 65, 81]. This parameter is to a certain extent proportional to the substrate concentration in the sample and therefore used for BOD estimation [65, 66, 75]. The kinetic method has several advantages such as being less time consuming, more easily operable, and more suitable for on-line monitoring [56, 66]. However, it is more vulnerable to the instability of the system than the steady-state method [16, 82].

In many cases BOD values measured with BOD biosensor differ from results gained with conventional 5 or 7 day BOD test [65, 75]. The disagreement between methods is caused by the different measuring principles and variable composition of the wastewater samples therefore, BOD measured by biosensor is only analogous to the conventional one [65]. The conventional BOD test measures the sum of various biochemical processes in activated sludge over a 5 or 7 day incubation period [62]. The incubation period of conventional BOD test includes a microbial adaptation period for microbial growth and induction of the necessary enzymes for assimilation of desired compounds, and a period for enzymatic hydrolysis of polymers [62, 65]. However, the BOD values as observed by BOD sensor reflect the concentration of the dissolved organic substances which are assimilated/metabolized by the immobilized microorganisms [35, 37]. During the short time of biosensor measurements, the immobilized microorganisms do not have enough time to induce the necessary enzymes to hydrolyze polymers. Moreover, the diffusion of monosaccharides is faster and therefore, these compounds are assimilated more easily compared to polymers, causing the higher sensitivity of microbial BOD sensors towards monosaccharides [38, 39]. The fast responses of BOD sensors necessitates that they

respond only to the most readily degradable compounds [62] and this causes disagreement between biosensor measured BOD and conventional BOD values.

Different approaches have been applied to decrease the difference between the results of two methods. The conventional test has been recognized as an industrial standard and universal method for analyzing different kinds of wastewater while, the BOD biosensor measurements are the tests with selected microbial strains [65]. Better results have been obtained by selecting microorganisms with low selectivity and wide substrate spectrum [37] or microorganisms adapted to certain pollutants present in wastewater [83]. The efficiency of the microorganisms to assimilate certain substrates can also be improved by adaption with the suitable substrate [7, 65] to induce the necessary enzymes for assimilation of these compounds [65], or genetic modification [7]. The importance of pre-testing and selection of microorganisms for the construction of reliable BOD biosensors have been emphasized in previous studies [36, 37, 65]. In addition, earlier studies on BOD biosensors for wastewaters containing high molecular weight substances have used different pretreatment methods like acid pretreatment [84], photo-catalysis [50], ozonation [49, 85] or enzymatic hydrolysis [77, 86] to enhance the poor biodegradability of the sample. The pretreatment will split the polymers into monomers and then immobilized microorganisms are able to assimilate these easily degradable substrates within biosensor measurement time [49, 65, 85].

In order to gain good agreement between sensor-BOD and conventional BOD test results, it is vital to select proper calibration solution [65]. GGA solution is widely used for biosensor calibration [53] however, it only consists of two simple components – glucose and glutamic acid [56]. This may restrict the biosensor use when analyzing real wastewater samples that consist of a complex mixture of substrates [66]. Better results have been gained when OECD synthetic wastewater was used to calibrate biosensors. In addition, various different artificial wastewaters and real wastewater samples [75] have been used to calibrate biosensors [85]. There might not be a universal standard solution that would be suitable for calibration of real wastewater samples of different compositions [35, 37, 66].

1.3.1. Selection of microorganisms – Semi-specific microorganisms

Since the composition of different industrial wastewaters often varies greatly, the universal microbial sensors are not suitable for analysis of these specific wastewaters [87]. Better results have been obtained when semi-specific microorganisms, that are able to degrade specific refractory compounds found in industrial wastewaters, are used. Semi-specific microorganisms differ from universal microorganisms by their substrate spectra. Universal microorganisms are defined as microorganisms that use a limited substrate spectrum common to a majority of aerobic heterotrophic microorganisms [8, 87–89]. On the other

hand, semi–specific microorganisms are microorganisms that in addition to the universal substrate spectrum can degrade some refractory compound or group of compounds specific to that particular microorganism [8, 83, 87–89]. Thereby, semi-specific microbes are able to oxidize and detect refractory compounds found in industrial wastewaters, which would be undetected by universal biosensors [83, 88–90] and that makes it advantageous to use semi–specific microbial BOD biosensors to determine the BOD in specific industrial wastewaters [87]. Still, biosensors based on semi-specific bacteria do not excel in wastewaters containing refractory compounds towards which they do not have semi-specific properties and thereby, underestimate the BOD value to an extent of those compounds. Therefore, any single semi-specific biosensor is advantageous only in the case of the wastewater with known specific content towards which the biosensor is semi-specific. [8, 89]. The importance of pre-testing and selection of microorganisms for the construction of reliable BOD biosensors have been emphasized in previous studies [36, 37, 65] as the biosensors based on a well-chosen microorganism with semi-specific properties have a potential for more precise analysis of specific industrial wastewaters.

1.4. Sensor-arrays and multivariate analysis

Sensor-arrays can be classified into two according to employed sensors – the arrays of gas sensors are named „electronic nose“ while arrays of sensors used in a liquid medium are named “electronic tongues“ [91]. However, the general concepts of the sensor-arrays used for sensing of liquids and gases are similar [9, 10]. Sensor-arrays are often used for fast, inexpensive and reliable sample characterization – classification, origin recognition, or estimation of properties of complex samples [92]. These kinds of analytical devices are comprised of arrays of chemical sensors which are nonspecific, low-selective and cross-sensitive to different species in analyzed sample conjugated with an appropriate data processing method [9, 10, 93, 94]. The terms “nonspecific”, “low-selective” and “cross-sensitive” describe the sensor properties to which they must meet [10]. This means that sensors are not selective to one compound or type of compounds in the sample [10] but are sensitive to several components in the analyzed solution simultaneously [9]. This produces a fingerprint of the sample constitution [95] and with the aid of appropriate multivariate analysis system and a reference library, qualitative and quantitative information of sample can be extracted [10, 95].This kind of sensor-arrays, where the difference in the signals from different sensors serves as a fingerprint for the analyzed sample, are capable of differentiating very similar samples [96]. Various biosensor-arrays utilizing different enzymes [97], genetically modified single strain microorganisms [71, 78] [98] or different strains of microorganisms [99] have been reported.

Since sensors in the array are sensitive to several compounds in the sample, a great amount of complex data is generated, which must be processed using a

multivariate analysis methods [93]. By employing suitable data analysis methods to process the sensor-array signals [100–102] data structure can be recognized and examined [9] and qualitative and quantitative information of composition of complex samples can be extracted [9, 10]. The limited selectivity of each individual sensor will be compensated through data analysis [102], which allows to determine different compounds in presence of their interferes [93]. In addition, it is possible to simultaneously determine of a number of compounds in a complex samples [93, 94]. Various algorithms have been used to process sensor-array data, amongst them Principal Component Analysis (PCA) is widely applied for identification/classification purposes and Partial Least Squares (PLS) is designed for qualification purposes [93].

PCA is a major linear technique used to display data structure [9, 96] in order to reveal groupings among sets of cases [103]. PCA reduces dimensionality of the sensor array data to a few principal components (PC), which contain most of the variation in the data [93]. Using first PC, containing most of the variation, a score and loading plot can be composed. The loading plot describes the relationship between the original variables and their significance and corresponding score plot shows the relation between samples which can be used for classification. For PCA, no prior knowledge of samples or variables is necessary [104].

Sensor-arrays enable to quantitatively determine the compounds in multi-species solution. In addition, higher selectivity and lower detection limit than with single sensors is possible [9, 10]. PLS regression method is widely used method quantitative analysis [9, 93]. PLS is a statistical, linear model, in which PCA is performed with two datasets – the sensor signal dataset and the dataset with corresponding actual concentrations. A linear regression is then performed with each principal component between the dataset and the corresponding actual concentrations, in order to obtain a regression model between these which can be used to find the analyst concentration in unknown sample [104].

2. EXPERIMENTAL

2.1. Standard solutions and samples

Synthetic wastewater according to the recipe of OECD (peptone – 1,6 g/l; meat extract – 1,1 g/l; urea – 0,3 g/l; K_2PO_4 – 0,28 g/l; NaCl – 0,07 g/l; $CaCl_2 \cdot 2H_2O$ – 0,05 g/l; $MgSO_2 \cdot 7H_2O$ – 0,02 g/l) [105] was chosen as a standard solution to calibrate BOD biosensors. The BOD_7 of solution was measured as 2000 mg/l.

Phosphate buffer solution of pH 6,89 ($Na_2HPO_4 \cdot 12H_2O$ – 6,90 g/l; $NaH_2PO_4 \cdot H_2O$ – 7,04 g/l) was used as a measurement solution but also to wash microorganisms and to prepare agarose solution for immobilization.

2.2. Microbial material

All the used bacterial cultures and their short descriptions are outlined in table 1. Bacterial cultures P75, P69, R17.1, P74.3 and P67 were gained from microbial collection of Institute of Technology, University of Tartu. Bacterial cultures CE22.1, HTK158a and 17.3 were isolated from various substrates to gain bacteria with semi-specific properties. The bacterial cultures were isolated, identified by 16S rDNA sequence comparison and cultivated in the Institute of Technology of the University of Tartu.

2.2.1. Semi-specificity towards milk compounds and lactose

Bacterial culture of R17.1 and P74.3 were employed for the BOD measurements in dairy industry wastewater since these strains are known to metabolize lactose [106] which is often unattainable to microorganisms.

Bacterial culture 17.3 was isolated from sample of dairy wastewater collected from Valio Eesti AS Laeva dairy plant (Estonia). Using serial dilutions the sample was spread on the milk (agar – 30 g/l; milk – 500 mg/l) and lactose (lactose – 15 g/l; agar – 5 g/l; M9 10x salts solution – 100 ml/l; vitamins – 2500 µl/l) containing selective solid medium plates. The plates were incubated at a room temperature for a few days until colonies developed. The colonies were indexed and restreaked on fresh milk– and lactose selective plates. The colonies that grew rapidly on both types of selective plates were chosen and restreaked. This procedure was repeated until pure bacterial strains were gained. The isolated culture indexed as 17.3 was chosen based on rapid growth rate.

2.2.2. Semi-specificity towards lipids

Bacterial culture P69 was chosen based on its ability to use fat as a substrate since meat industry wastewaters have high oil and grease concentration. The lipolytic properties were examined by culturing bacteria in medium containing

0.1% swine fat (Rakvere Meat Industry) as the only carbon source and P69 culture was selected due to fast growth rate in the given medium.

Table 1. Microorganisms used for biosensors construction and their main characteristics

Sensor name	Bacterial culture	Origin	Substrate spectra description	Reference
P75	*Pseudomonas fluorescens* P75	Raadi lake water	Universal, low specificity	I–V
P69	*Aeromonas hydrophila* P69.1	Raadi lake water	Semi-specific to lipids	II, V
P67	*Pseudomonas putida* P67	Oil shale industry waste	Semi-specific to phenol	V
R17.1	*Escerichia coli* R17.1	Raadi lake water	Semi-specific to lactose	I, V
P74	*Raoultella terrigena* P74.3	Raadi lake water	Semi-specific to lactose	I
CE22.1	*Bacillus subtilis* CE22.1	Decaying saw dust	Semi-specific to cellulose	III, V
HTK158a	*Paenibacillus sp.* HTK158a	Rabbit manure	Semi-specific to cellulose	III, V
17.3	*Microbacterium phyllosphaerae* 17.3	Dairy industry wastewater	Semi-specific to milk and phenol	IV, V

2.2.3 Semi-specificity towards cellulose

Microorganisms, semi-specific to cellulose, were isolated from samples of decaying sawdust and rabbit manure. Using serial dilutions, the samples were spread on the selective cellulose containing agar medium (Yeast extract – 2 g/l; K_2HPO_4 – 1g/l; $MgSO_4 \times 7H_2O$ – 5 g/l; CMC – 5 g/l; NaCl – 2 g/l; Agar – 15 g/l). The plates were incubated at room temperature until colonies developed. Subsequently, the colonies that were able to degrade CMC and grew rapidly on given medium were chosen and restreaked on a fresh agar plate. This procedure was repeated until pure bacterial strains were gained. To screen for cellulolytic organisms, the isolates were grown on a minimal agar plate (Yeast extract – 2 g/l; KH_2PO_4 – 1g/l; $MgSO_4 \times 7H_2O$ – 1g/l; CMC – 2g/l, agar – 15 g/l) for 2 days. These plates were then flooded with an aqueous solution of Congo-red (1 mg/ml) for 15 min and washed with 1 M NaCl to visualize the hydrolysis zones [107]. CE 22.1 (isolated from decaying sawdust) and HTK 158a (isolated from rabbits manure) were chosen due to active digestion of CMC indicated by the clear zone around the colonies.

2.2.4. Semi-specificity towards phenol

Bacterial culture P67, semi-specific to phenol, were gained from microbial collection of Institute of Technology, University of Tartu [90].

2.2.5. Universal bacterial culture

The cells of non–specific bacterial culture of P75 isolated from Raadi lake water and gained from microbial collection of Institute of Technology, University of Tartu were used as a comparison to bring out the advantages of semi–specific biosensor.

2.3. Cultivation of microorganisms

The bacteria were grown under aerobic conditions in a rotating shaker (Sanyo Orbital Incubator, Sanyo) at 30 °C in a Luria-Bertan liquid medium (tryptone – 10 g/l; yeast extract – 5 g/l; NaCl – 5 g/l). 2 ml of culture medium was inoculated and incubated for 14–18 hours. Subsequently, the cell suspension was subcultured into a 150 ml of culture medium and incubated for 6–14 hours until gaining sufficient amount of cells deriving from late exponential phase of growth. Cells in their late exponential or log phase are operating at a maximum rate and efficiency and have intense metabolic capacity [37]. To assure cells deriving from the late exponential phase of growth, optical density of the bacterial suspension was measured with spectrophotometer (HP 4853, λ=600 nm). An example of the measured growth curve is presented in figure 2.

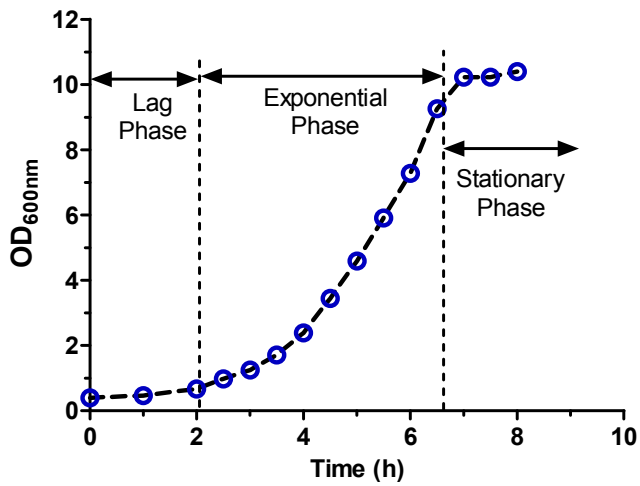

Figure 2. An example of the growth curves of microorganisms. The dotted lines and arrows indicate the different growth phases of microorganisms. The cells utilized for immobilization were harvested at late exponential or early stationery phase

The bacterial suspension was centrifuged (Jouan CR3) at 4000 r/min for 15–20 min at room temperature and the supernatant was decanted. The cells were washed twice with phosphate buffer solution and centrifuged at the same conditions to circumvent the culture medium from getting into the membrane. The washed bacterial paste was used immediately to prepare the microbial membranes (I–V).

2.4. Immobilization of microorganisms

Mixture of 180 mg agarose and 7.5 ml phosphate buffer was heated over 70 °C until complete melting of agarose. The melted agarose was cooled down to 45–50 °C and previously prepared cell paste was added. In paper I 300 µl of cell paste and in papers II–V 900 µl of cell paste was used in immobilization. The cell-agarose mixture was mixed rapidly and polypropylene net discs (Scrynel, PP 500 HD) were imbued with the homogeneous agarose suspension. Until hardening of the agarose suspension, the resulting membranes were placed between two glass plates and even force was applied to gain certain and even thickness of membranes (I–V).

2.5. Pre-conditioning

The preconditioning was used to increase the range of assimilation of substrates by the immobilized microbes [36] and to fully activate the immobilized microbes in a prepared membrane [36, 43]. After immobilization the membranes were placed in a phosphate buffer solution dosed with OECD synthetic wastewater (BOD_7 of solution 30 mg l^{-1}) for pre-conditioning up to 14 days until the sensor output signal became stable. After pre-conditioning and between measurements the membranes were maintained at 4 °C in a phosphate buffer solution (II–V).

2.6 Experimental set-up

2.6.1 Instrumentation

Figure 3 A illustrates the BOD measuring system used in all experiments. Clark type dissolved oxygen probe (WTW, CellOx 325, Germany) was used as an electrochemical transducer and the signal was registered with measuring module (WTW, InoLab 740, Germany). The signal was detected and transformed using MultiLab Pilot program and saved in a computer. Aeration unit and magnetic stirrer (KEBO-LAB MR 2002) were used in order to ensure saturation of measuring solution with oxygen. Membrane containing immobilized microorganisms was attached to the dissolved oxygen probe with special collar and a holder (Figure 3 B) [88].

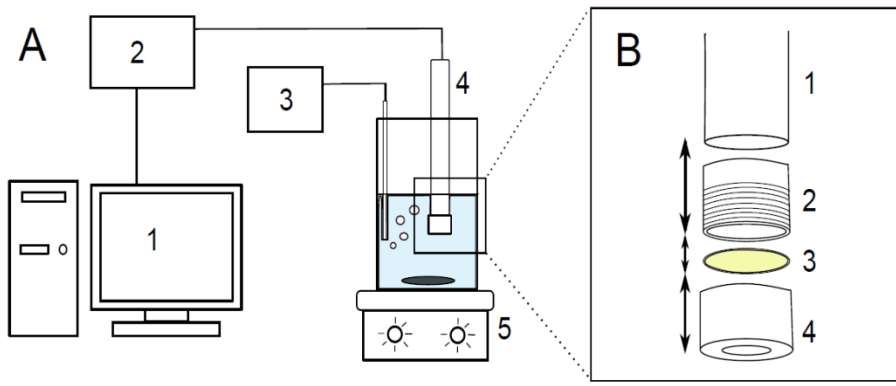

Figure 3. A. Schematic presentation of a biosensor measuring system: 1 – computer, 2 – measuring module, 3 – aeration unit, 4 – biosensor, 5 – magnetic stirrer
B. Biosensor design: 1 – dissolved oxygen probe, 2 – collar, 3 – membrane, 4 – holder

2.6.2. Experimental procedure

The measurements with biosensors were carried out at room temperature (22 ± 2 °C) in a beaker containing 50 ml of previously aerated phosphate buffer solution using steady state measurement method. In order to adapt immobilized microorganisms with room temperature, the membranes were held at a room temperature for 1 hour. The biosensor was assembled and placed into the previously aerated measuring solution. After an initial stable current was reached (I_0), a certain amount of substrate was added. As substrate was added, the biosensor output signal decreased since the oxygen consumption rate of immobilized microorganisms increased. After the substrate addition some time was allowed to achieve a new stable current. Hereafter, the substrate was inserted into the measuring solution using step-by-step addition in order to change the BOD_7 of measuring solution gradually. The biosensor response time was defined as a time taken to reach the new steady state signal after addition of substrate. During addition of substrate the initial and end-point steady state signals were recorded. Experiment was carried out until the biosensor output signal was zero or did not change after addition of substrate into the measuring solution. Figure 4 illustrates the change in biosensor output signal during experiment as the BOD_7 is gradually increased with OECD synthetic wastewater (I–V).

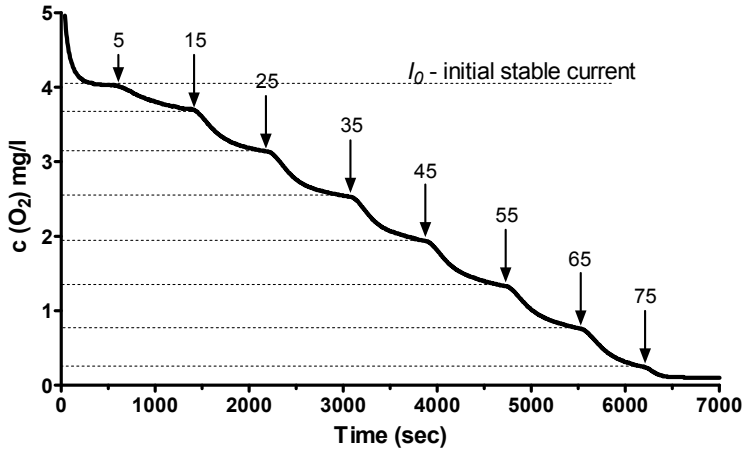

Figure 4. An example of change in P69 biosensor output signal during calibration with OECD synthetic wastewater. Horizontal guidelines indicate the stable current when the substrate was added and arrows indicate the time of substrate addition and the BOD_7 of the measurement solution (mg/l) after the addition.

2.7. Wastewater samples

Experiments with several synthetic and real industrial wastewaters were conducted to test the performance of biosensors. The synthetic wastewater samples consisted of OECD synthetic wastewater and an additional refractory compound. The consistency of such synthetic wastewaters simulated wastewaters from different industries (meat, dairy and paper industry). The proportion of an additional compound in the BOD_7 of OECD synthetic wastewater was 15–20% (I–V).

The combined wastewater with fatty substances was prepared by dissolving components of OECD synthetic wastewater in a saturated solution of swine fat. The saturated solution of swine fat was prepared by adding 5 g/l of swine fat to phosphate buffer. The mixture was heated until complete melting of fat and processed in an ultrasonic bath (Sonica Mod 1200 MH, Sonic) for 30 minutes to enhance the dissolution of fat. The mixture was cooled and surplus fat was removed by filtration. BOD_7 of the saturated solution of swine fat was 240 mg/l. As fat has low solubility in water, half-sized quantities of OECD synthetic wastewater components were used in order to achieve the proportion of fat in the BOD_7 of combined synthetic wastewater of approximately 15–20% (II and V).

Combined wastewater dosed with milk was prepared by adding 1 ml of milk with fat content of 2,5% (I, IV and V) into 1000 ml of OECD synthetic wastewater. In case of carboxyl-methyl-cellulose (CMC), the additional compound was not detectable by conventional BOD_7 analysis and therefore 1 g of CMC

was added to 1 l of OECD synthetic wastewater (III and V). The COD of CMC added to solution in given concentration was measured as 910 mg/l.

Meat leachate was a mixture of liquids of swine, beef and chicken origin and the composition of the solution was characteristic to that of the wastewaters generated during the primary processing of meat. To circumvent coagulation and to bring the BOD_7 into suitable range, the meat leachate was diluted 10 times with phosphate buffer and EDTA was added so that the concentration of EDTA in final solution was 0,01M (II).

The samples of different industrial wastewaters were taken from two meat industry outflows (Nõo Lihatööstus, Nõo and Arke Lihatööstus, Põlvamaa) (II), outflow of dairy industry (Laeva Valio, Tartumaa) (IV), outflow of Põlva wastewater treatment plant (I) and paper mill (Räpina, Estonia) and aspen pulp mill (Kunda, Estonia) outflows (III). The inflow of Põlva wastewater treatment plant incorporated approximately equal parts of municipal wastewater and wastewater from the dairy industry and therefore its composition represented dairy industry wastewater (I).

Conventional BOD_7 analysis was conducted according to the APHA standard [2] with all wastewaters and the results were compared with the results of measurements carried out with the biosensors.

2.8. Calibration

OECD synthetic wastewater was used as a calibration solution to calibrate all single biosensors. The BOD_7 of the standard solution was measured as 2000 mg/l (I-V). PLS regression was chosen as a multivariate calibration method and it was conducted using six sensors and three latent variables. The initial dataset was divided into calibration and test datasets – one third of measurements were used for calibration and two thirds were used as a test dataset to test the PLS model. The dataset consisted of measurement results gained from analyzing OECD synthetic wastewater and OECD synthetic wastewater spiked with different refractory compounds. The composition of mentioned samples is described in paragraph 2.7 (V). At least four parallel measurements were considered for redacting calibration graphs for single sensor analysis and when conducting PLS calibration.

2.9. Data analysis

The biosensor response at a certain concentration was calculated as a difference between biosensor signal before and after addition of the substrate (I_0-I_S). Normalized signal response (NSR) at certain BOD_7 value was used as a biosensor response in all calculations. The biosensor output signal was normalized using formula 1:

$$NSR = \frac{I_0 - I_S}{I_0} \quad (1)$$

To form calibration graphs for individual biosensors, normalized signal response at certain BOD_7 was plotted against BOD_7 of measuring solution. An average NSR of 4–7 parallel measurements at different concentrations were taken into account for the biosensors calibration graphs (I-V).

In case of multi-sensor analysis the normalized signal responses of each biosensor were scaled and centered. Factorial ANOVA was conducted using three factors ("sensor", "sample" and "BOD_7") to determine the significant differences between different biosensors. Factor "BOD_7" was used at 9 levels indicating different BOD_7 of measurement solution (0, 5, 10, 15, …, 40 mg/l), factor "sensor" had 7 levels indicating different biosensors and factor "sample" had 5 levels indicating different samples. 4 parallel measurements were considered in multivariate analysis. Scheffe test was used in *Post hoc* analysis to locate the differences between biosensors. Principal component analysis (PCA) and partial least squares (PLS) were used for multi-sensor analysis. For PLS the initial dataset was divided into calibration and test datasets – one third of the measurements was used for calibration and two thirds were used as a test dataset to test the model (V).

Data was gathered using Microsoft Excel 2003 and all calculations were performed using GraphPad Prism 5 (GraphPad software Inc., San Diego, USA) and Statistica 8 (Statsoft Inc., Tulsa, USA) software. Significance level α=0,05 was used in all analysis.

3. RESULTS AND DISCUSSION

3.1. Calibration of individual biosensors

All biosensors were calibrated using the OECD synthetic wastewater and steady state measuring method. Although, the GGA solution has been widely used to calibrate biosensors [65, 108], OECD synthetic wastewater is more comparable to municipal wastewater and therefore, more suitable for sensor calibration [66, 109]. Calibration parameters linear range and sensitivity were used to characterize biosensors; also response time, service life and stability were estimated based on calibration measurements.

3.1.1. Linear range and sensitivity

Linear range of the BOD biosensor is defined as the range of substrate concentration where the change in biosensors output signal is proportional to the BOD_7 of the sample [11, 35–37, 65, 76] while the sensitivity is defined as a signal change per unit of concentration of substrate [76] and it is represented by the slope of the calibration curve [11, 65, 76]. The measurement of linear range and sensitivity from calibration graph is visualized in figure 5 and the parameters of biosensors used in this thesis are outlined in table 2. The linear ranges for biosensors used in paper I extended up to 200 mg/l of BOD_7 while the linear ranges in paper II–IV extend up to 55 mg/l of BOD_7. The sensitivity, since it is inversely proportional to linear range, is smallest in the biosensors used in paper I.

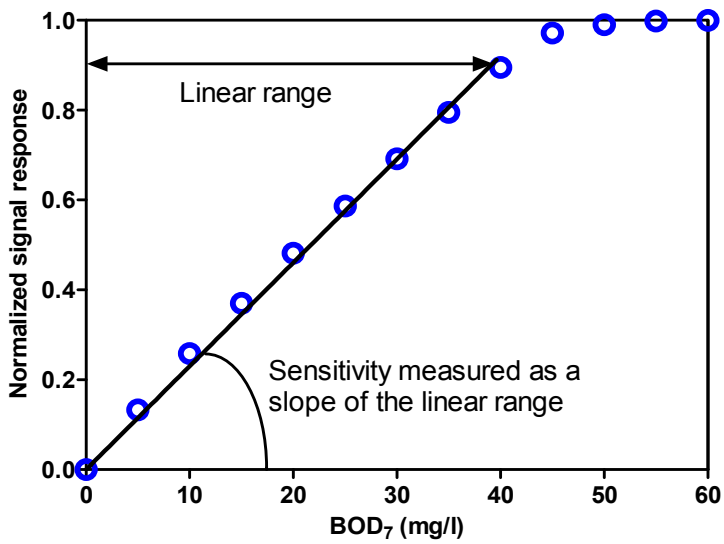

Figure 5. Calibration graph for *P. fluorescens* biosensors from paper II as an example of typical BOD biosensor calibration graph

Linear range and sensitivity are related to the thickness of microbial membrane, the type and density of the cells immobilized in microbial membrane. Microbial membranes with higher cell density are generally more sensitive but have narrower linear range [65] therefore, the biosensors in papers II–IV have narrower linear range than biosensors used in paper I. Also, it is influenced by the sensitivity of microorganisms to particular organic substrates [65], since different strains of microorganisms may prefer different compounds in metabolism. Therefore, different biosensors constructed and used at the same conditions may have a different linear range. Highly sensitive BOD biosensors can be applied for analysis of samples with very low BOD values while broad linear range is beneficial for BOD assessment over broad concentration ranges. Relatively high sensitivity of studied biosensors enables precise measurements but due to shorter linear range it is essential to select proper dilutions since industrial wastewaters have higher BOD values.

Table 2. Linear ranges and sensitivities of biosensors studied in current thesis

Sensor name	Linear range (mg/l, BOD_7)	Sensitivity ((mg/l, BOD_7)$^{-1}$)	Paper
R17.1	150 / 50	0,07 / 0,018	I / V
P74	200	0,05	I
P67	200 / 45	0,05 / 0,021	I / V
P75	200 / 40 / 40	0,05 / 0,023 / 0,021	I / II, V / IV
P69	45	0,019	II, V
CE 22.1	55	0,016	III, V
HTK158a	50	0,017	III, V
17.3	40	0,022	IV, V

3.1.2. Response and recovery time

Response time of biosensors depends primarily on the applied measuring method. In case of the steady state method response time is defined as a time it takes for the biosensor to reach steady state after substrate addition into the measurement solution [11, 65]. The response time for studied biosensors was generally between 10–30 minutes. It was noted that when BOD_7 of measurement solution was increased by larger step (10–20 mg/l instead of 5 mg/l of BOD_7) the response time increased 5–10 minutes (II). Considerably longer response time, 45–90 minutes, was observed during preconditioning period (IV) and when dissolved fat was used for measurements (II). Prolonged response time of biosensors can be associated with slow mass transfer rate of high molecular weight compounds through immobilization matrix and cell membranes [86, 110] and stiffened cell membranes after heat shock caused by [44, 46]

The recovery time of biosensors is defined as the period needed for the sensor to gain the initial output signal after the experiment [11, 65]. Recovery time of BOD biosensors was approximately 1 week after immobilization and at

the beginning of measurement period. After preconditioning period and gaining operational stability the recovery time shortened to 1–3 days. Due to internal nutrient supplies of immobilized microorganisms, measurements were not conducted on consecutive days.

3.1.3 Service life and stability

Service life and operational stability of BOD microbial biosensors are primarily related to the stability of the immobilized microorganisms but also depends on method of immobilization [55, 65]. Stability and service life were estimated by calibrating the biosensors with OECD synthetic wastewater at a certain BOD_7 value. The biosensors were considered to be stable when deviation from the average biosensor response was below 15%. A threshold of 15% was taken for biosensor stability estimation since variation of 15% is allowed for standard BOD test results conducted according to APHA [2]. An example of change in biosensors output signal during service life is presented in figure 6. Stable and reproducible response was achieved at least 15 days and in some cases up to 40 days after immobilization. The stable period allowing reproducible results was 40–90 days (I-III), but also up to 300 days (IV). Constant supply of oxygen and nutrients provided during the measurements enabled to keep immobilized bacteria well-conditioned. Therefore, stability of biosensors was best when frequent measurements were carried out. When the decrease or irregularity in biosensors sensitivity was observed the stable period was considered at an end and membranes were discarded.

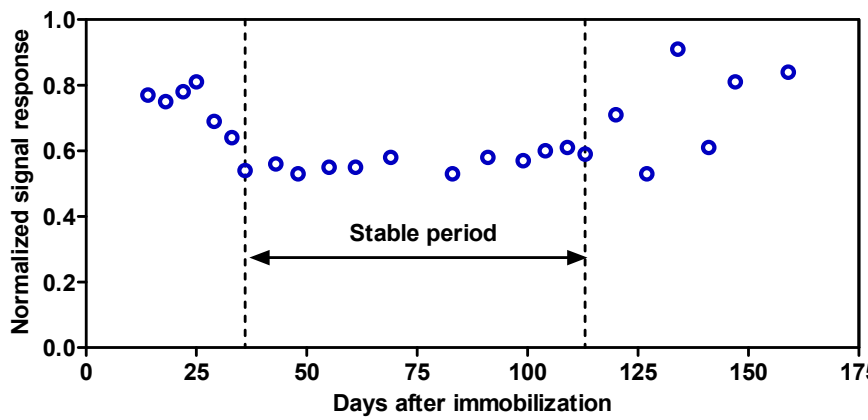

Figure 6. Service life and stability of *P. fluorescens* biosensor as an example of signal change during BOD biosensors service live. The dotted vertical lines and arrow indicate the stable period of biosensor

3.2. Analysis of wastewater samples

In order to investigate the suitability and performance of semi-specific and universal biosensors to determine BOD in industrial wastewaters containing refractory compounds different wastewater samples were analyzed. Synthetic industrial wastewater samples based on OECD synthetic wastewater and spiked with specific refractory compounds so its composition resembled industrial wastewater. In addition wastewater samples from various industries from Estonia were used. Conventional BOD_7 analysis was conducted with all samples and the results of two methods were compared.

3.2.1. Synthetic wastewater samples

Four different synthetic wastewater samples were analyzed to investigate the suitability of semi-specific biosensors for analysis of different wastewaters. Figure 7 illustrates the agreement between the results of conventional BOD_7 and sensor-BOD values measured in different synthetic wastewater samples (II-V).
Majority of biosensors underestimate the BOD_7 of synthetic industrial wastewaters by 10–25%, which is approximately the extent of additional BOD_7 introduced into the OECD synthetic wastewater with the refractory compound. However, biosensors that have semi-specific properties towards that certain refractory compound outperform non-specific biosensors and biosensors semi-specific to other refractory compounds. Thus, in the synthetic wastewater spiked with milk, closer results to the BOD_7 values were gained with biosensor R17.1 which overestimated the BOD_7 of sample by 11%, while other biosensors underestimated it up to 23%. Similar result can be seen in fat spiked synthetic wastewater using biosensor P69, phenol spiked synthetic wastewater using biosensor P67, and CMC spiked synthetic wastewater using biosensors CE22.1 and HTK158a. The non-specific biosensor P75 and biosensors non-specific to that certain refractory compound underestimated BOD_7 in all samples up to 25% which was approximately the proportion of additional BOD_7 introduced into the sample with the refractory compound.

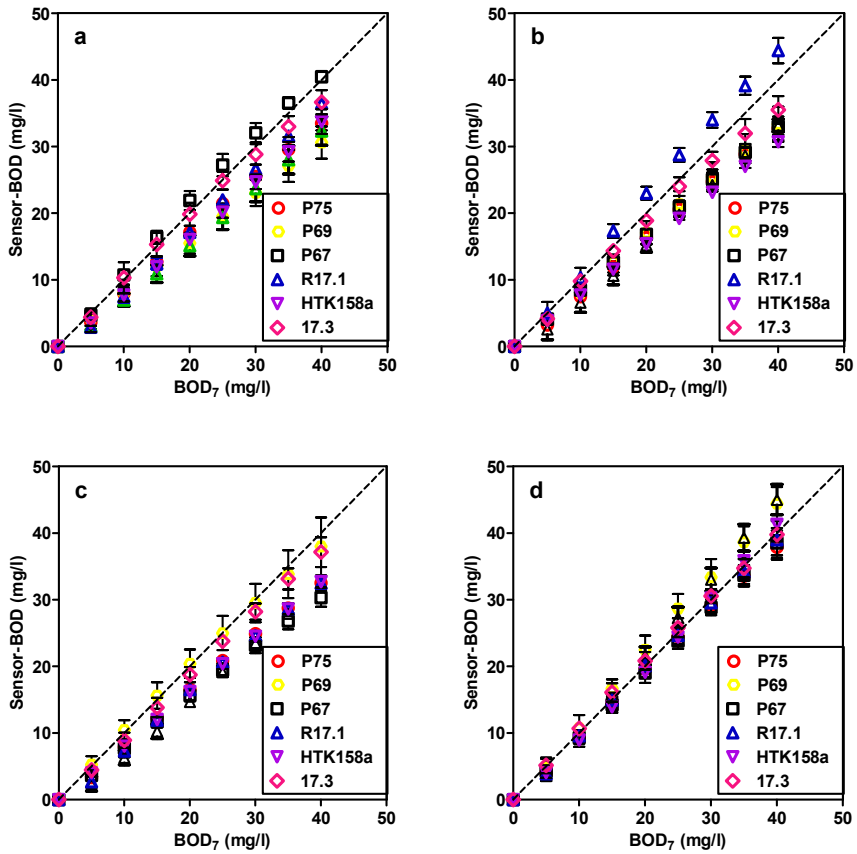

Figure 7. Comparison of BOD_7 and sensor-BOD of spiked OECD synthetic wastewater measured with single individual sensor: A – OECD phenol; B – OECD milk; C – OECD fat; D – OECD CMC. 1/1 correlation of sensor-BOD and BOD_7 is shown with the center-line on the graph. (V)

Lower sensor-BOD values are caused by the partial assimilation of refractory compounds which remained undetected [1], while the portion of BOD_7 resulting from OECD synthetic wastewater was detected. Both, non-specific and semi-specific microorganisms used in biosensors were unable to assimilate refractory compounds towards which they were not semi-specific. Therefore, the refractory compounds in those wastewater samples remained inassimilable causing underestimation of BOD_7. Generally, semi-specific biosensors are more suitable to conduct industrial wastewater analysis than universal biosensors. When using single biosensor for analysis of industrial wastewaters containing refractory compounds, it is essential to have prior information about wastewater source and constitution in order to select the suitable biosensor and thus, gain accurate results.

3.2.2. ANOVA and *post hoc* analysis

The differences between BOD values of synthetic wastewaters measured with different biosensors were verified using ANOVA (p=0,05). Analysis confirmed that all factors ("sample", "sensor" and "BOD_7") have significant influence on the measured sensor-BOD values e.g. the measured sensor-BOD depends on the actual BOD_7 value of the sample, composition of the sample, and the sensor used to analyze it. Subsequently, Scheffe test as *post hoc* analysis was used to specify the differences between biosensors for different wastewater samples (Table 3) (V).

Post hoc analysis revealed statistically different sensor-BOD values between semi-specific biosensor and other biosensors when specific sample corresponding to that semi-specific biosensor was analyzed. The results from biosensors R17.1 and 17.3, both designed for dairy industry, were higher and differed from most other biosensor's results in milk spiked sample. Similarly, sensor-BOD measured with biosensors P69 and 17.3 in fat spiked sample, and P67 and 17.3 in phenol spiked sample were different from the results gained with other biosensors. Thus, it can be concluded that there is indeed qualitative information present in the collective signal of the selected semi-specific biosensors (V).

In case of CMC spiked sample, the sensor-BOD values measured with biosensor CE22.1 and HTK158a, designed for analysis of cellulose rich wastewaters from pulp and paper industry, were similar to three and five other biosensors, respectively. Smaller differences from other biosensors could be caused by lower biodegrading capabilities of microorganisms towards CMC which is barely detectable even with BOD_7 analysis [89]. Since the results from these biosensors were similar in analysis of all sample types only one of them was used in further multivariate analysis (V).

Although bacterial culture used in biosensor 17.3 was initially isolated and tested as a semi-specific towards lactose and milk, higher sensor-BOD values were measured with it in fat and phenol spiked samples as well. The latter can be explained by a broader substrate range of used microorganisms that were partially able to assimilate a wider range of refractory compounds. Thus, somewhat better estimation of BOD was achieved with biosensor 17.3 compared with non-specific biosensor but lower than semi-specific biosensors in the samples spiked with corresponding refractory compounds (V).

Non-specific biosensor P75 measured lower sensor-BOD values in all the samples. Its sensor-BOD values differed from sensor-BOD values of semi-specific biosensors when the wastewater samples corresponding to those semi-specific biosensors were used (V).

Table 3. The p-values from Scheffe test conducted as a *Post hoc* analysis. Values smaller than 0,05 (marked bold) indicate statistically relevant differences between the results of biosensor pairs (V)

Sample	Sensor	P75	P69	P67	R17.1	CE22.1	HTK158a	17.3	Sample
OECD + Fat	**P75**		0,853	**0,000**	1,000	0,794	1,000	**0,000**	OECD + Phenol
	P69	**0,000**		**0,000**	**0,042**	1,000	0,999	**0,000**	
	P67	0,997	**0,000**		**0,000**	**0,000**	**0,000**	**0,001**	
	R17.1	1,000	**0,000**	0,999		**0,027**	0,987	**0,002**	
	CE22.1	0,959	**0,000**	1,000	0,999		0,999	**0,000**	
	HTK158a	ances	**0,000**	0,999	1,000	0,978		**0,000**	
	17.3	**0,000**	0,872	**0,000**	**0,000**	**0,000**	**0,000**		
OECD + CMC	**P75**		1,000	1,000	**0,000**	1,000	0,999	**0,000**	OECD + milk
	P69	**0,000**		1,000	**0,000**	0,939	0,662	**0,020**	
	P67	1,000	**0,000**		**0,000**	0,988	0,857	**0,004**	
	R17.1	1,000	**0,000**	1,000		**0,000**	**0,000**	**0,000**	
	CE22.1	**0,000**	0,999	**0,000**	**0,010**		1,000	**0,000**	
	HTK158a	1,000	**0,000**	0,999	1,000	0,082		**0,000**	
	17.3	0,593	**0,005**	0,437	1,000	0,779	0,999		

3.2.3. Industrial wastewater samples

Three different types of industrial wastewater were analyzed – meat, dairy and pulp and paper industry (I–IV). The results of BOD measurements in industrial wastewater samples have been presented in table 4.

In analyzing high fat content meat industry wastewater samples, both P69 and P75 biosensors underestimated the BOD_7. However, more accurate results were gained with a semi-specific P69 biosensor which underestimated BOD_7 of wastewater samples by 43% and 54%, while the sensor-BOD measured by means of P75 biosensor was 55% and 62% lower than BOD_7 (II). Similar results were gained when two wastewater samples from Laeva dairy plant were analyzed with semi–specific biosensor 17.3 (underestimated samples by 32 and 25%) and non–specific P75 biosensor (underestimated samples by 61 and 46%) (IV). The difference between the sensor-BOD and conventional BOD_7 analysis may be attributed to the difference in the methods for measuring BOD and the complex composition of industrial wastewater. Within a short measurement period, BOD biosensor provides the response only for fast and readily degradable compounds found in the sample. However, meat industry wastewater contains proteins, intact cells from blood [111] and soft tissues [112] which in unlysed form are unavailable for micro-organisms, but their degrada-

tion takes place during the incubation period of usual BOD_7 analysis. Therefore, during that short measurement period of the sensor, more complex compounds remain undetected; however, these can be determined using traditional BOD_7 tests (II). In addition, wastewater from food industries also contains various kinds of chemicals from the cleaning systems [113, 114] which inhibit the assimilation of organic substrates by immobilized bacteria (IV). Results could be improved by a pre-treatment of wastewater samples which leads to decomposed compounds and colloids which would be faster assimilated by microorganisms resulting in an increase in the accuracy of the biosensor [85].

In the analysis of pulp and paper industry wastewater samples, both semi-specific biosensors – HTK158a and CE2.1 biosensor overestimated the BOD_7 of paper mill wastewater by 26,3% and 21,6% while underestimating the BOD_7 of aspen pulp mill wastewater by 13,6% and 4,7%, respectively (III). The difference in sensor-BOD results in pulp and paper mill wastewater analysis can be explained by the different composition of the wastewater samples. The wastewater generated during paper making contains substantial quantity of cellulose fines and other additives (up to 50% of the total mass) [115] while the main pollutants in pulp industry wastewater are dissolved wood derived substances mainly tannins and lignins [1, 116]. The cellulose in paper mill wastewater was detected better with studied semi-specific biosensors than with conventional BOD_7 analysis causing the overestimation of BOD_7. On the other hand, semi-specific microorganisms used in this study had difficulties assimilating tannins and lignins (III).

Table 4. Comparison of BOD values of wastewater samples measured with biosensors and conventional 7-day method

Sample origin	**BOD_7 (mg/l))**	**Biosensor**	**Sensor-BOD (mg/l)**	**Sensor-BOD/BOD_7**
Nõo meat industry	2130	P69	1224	0,57
		P75	960	0,45
Arke meat industry	2400	P69	2400	0,46
		P75	918	0,38
Laeva dairy industry	1950	17.3	1326	0,68
		P75	760	0,39
Laeva dairy industry	3050	17.3	2288	0,75
		P75	1647	0,54
Räpina paper mill	1010	CE22.1	1276	1,263
		HTK158a	1228	1,216
Aspen pulp mill (Kunda)	7000	CE22.1	6048	0,864
		HTK158a	6671	0,953

3.3. Multivariate analysis of biosensor-array

Multivariate analysis was conducted using biosensor output signals of six biosensors (P67, P69, R17, 17.3, P75 and HTK 158a) from analysis of five different synthetic wastewater samples to extract qualitative and quantitative information of sample composition. For PCA average biosensor signal of 4 measurements were used. PLS analysis was conducted using three latent variables and initial dataset were divided into calibration and test datasets – one third of measurements were used for calibration and two thirds were used as test dataset to test the PLS model.

3.3.1. Qualitative information – Principal component analysis

PCA was used to test whether it is possible to distinguish different samples by their composition and BOD_7 values using simultaneous analysis of several biosensor signals. Figure 8 shows PCA score plots of first three PC-s giving 99.66% of total variation (V).

Score plot of PC1 versus PC2 (Figure 8a) illustrates how BOD_7 can be described using PC1 which values change from negative to more positive as the BOD_7 of solution increases. Measurements at smaller BOD_7 values are clustered closely together but as the BOD_7 increases, the measurements are located farther away from the starting point. In score plot of PC2 versus PC3 (figure 8b), different types of samples detach farther from the center in different directions as the BOD_7 increases. The discrimination of different wastewater samples is easier in case of average or larger BOD_7 values since they are located farther from the starting point where the point density is smaller and points are located in the different sections of the plot (V).

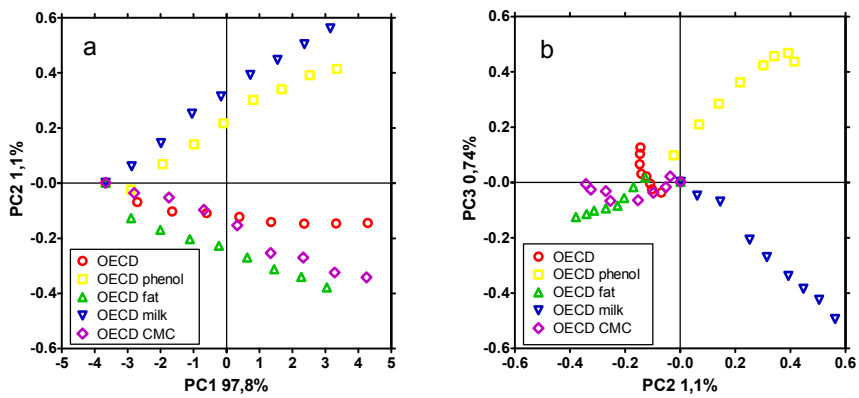

Figure 8. Plots of principal components using 6 biosensors showing separation of different synthetic industrial wastewater samples (V)

In figure 8a, the measurements with samples containing phenol and fat have positive PC2 values while other samples have negative PC2 values. In addition, milk or phenol spiked wastewaters also have either positive or negative PC3 values (figure 8b), respectively, which enables better discrimination of these samples. Other types of wastewaters were clustered somewhat closer to each other which make their discrimination more difficult. Clustering of points from these types of samples might have been caused by relatively small difference in the sample constitution. The samples used for measurements differed from each other by 15–20% of BOD_7 which was the content of additional refractory compound introduced to the OECD synthetic wastewater (V).

3.3.2. Quantitative information – multivariate calibration

PLS regression was chosen as a multivariate calibration method because of its usefulness in predicting a set of dependent variables from a large set of independent variables [117]. Figure 9 shows the correlation of BOD_7 and calculated sensor-BOD values of different synthetic wastewater samples (V).

The results indicate that by using simultaneously several different semi-specific biosensors, corresponding to different substrate spectra, it is possible to gain more precise BOD estimation than by using any single biosensor. Calculated sensor-BOD values differed from BOD_7 in case of all samples less than 5,6% at different BOD_7 values and average underestimation and standard deviation were less than 1% and 3,4%, respectively. The proposed approach of multivariate analysis and biosensor array measurements overcomes the information deficiency in wastewater analysis when inflows from various industrial and domestic sources are mixed in the wastewater treatment plant (V).

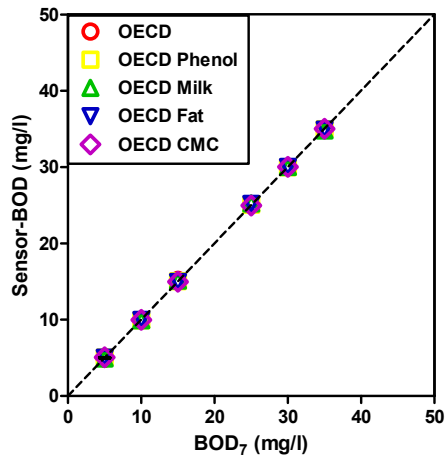

Figure 9. Comparison of BOD_7 and sensor-BOD when PLS was used to estimate the latter. 1/1 correlation between sensor-BOD and BOD_7 is shown with the center-line on the graph (V)

4. CONCLUSIONS

Simple and reliable semi-specific BOD biosensors were constructed to analyze wastewaters generated in various industries of Estonia. The biosensors were based on semi-specific bacterial cultures which were gained from Institute of Technology of University of Tartu or isolated from samples containing compounds to which bacteria were semi-specific. Bacterial cultures were chosen based on their ability to assimilate specific refractory compounds found in industrial wastewater. Biosensor based on universal bacterial culture – P75 – was used as a reference. Biosensors were calibrated using OECD synthetic wastewater and measurements with different synthetic and industrial wastewaters were conducted (I–IV). The biosensors were used individually and in addition, the output signal of all biosensors was analyzed simultaneously as a biosensor array – a bioelectronic tongue – and comprehensive multivariate data analysis was applied to investigate possibilities to extract qualitative and quantitative information of the samples (V).

Experiments with constructed semi-specific BOD biosensors showed that these biosensors are useful tools for analysis of synthetic wastewater samples spiked with refractory compounds. Semi-specific biosensors enabled us to measure BOD derived from specific refractory compounds to which they were semi-specific therefore, better estimation of BOD was gained. On the other hand, universal biosensor or biosensors not semi-specific to that certain refractory compound underestimated the BOD_7 of sample approximately to the extent of the additional BOD_7 introduced into the OECD synthetic wastewater with the refractory compound – 10–25%. Therefore, it is vital to have a prior knowledge about samples' composition and origin to select the suitable sensor (II–V).

In analysis of real industrial wastewaters, biosensors underestimated the BOD_7 of most wastewater samples except for the paper mill wastewater. However, is spite of underestimation, the semi-specific biosensors still produced better correlation of sensor-BOD and BOD_7 in real samples than universal biosensor which underestimated the BOD_7 of samples to a greater extent. Therefore, it can be concluded that semi–specific biosensors are more appropriate for measuring BOD in specific industrial wastewater containing refractory compounds than universal biosensor (I–IV).

The need for pre-knowledge about the samples' composition was overcome when different biosensors, universal and semi-specific to different compounds, were used as an array – bioelectronics tongue – and multivariate data analysis was applied. Simultaneous use of array of different semi-specific biosensors results in broader substrate spectra compared to a single biosensor. Furthermore, with multivariate data analysis qualitative and quantitative information was extracted. Qualitative information was extracted by using PCA, where the three first principal components of PCA enabled to distinguish different samples by their composition and BOD_7 values. The discrimination of different wastewater samples was easier in case of average or larger BOD_7 values and for samples containing fat or phenol. In addition, PLS was used for quantitative analysis, which produced good correlation of sensor-BOD and BOD_7 (V).

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SUMMARY IN ESTONIAN

Pool-spetsiifiliste BHT biosensorite uurimine biosensor-riviks

Seoses suureneva huviga erinevate olme ja tööstuslike tegevuste põhjustatud reostuse riskist keskkonnale vajab erinevate vete kvaliteedi seire rohkem tähelepanu. Looduslike vete kaitsmiseks on kõikides reovett töötlevates rajatistes kohustuslik regulaarne vee kvaliteedi kontroll. Vee reostuse taset orgaaniliste ainetega mõõdetakse vee hapnikutarbe alusel. Biokeemilise hapnikutarbe (BHT) kaudu mõõdetakse vees leiduva orgaanilise materjali biokeemiliseks lagundamiseks ja anorgaaniliste ühendite oksüdeerimiseks vajaliku hapniku hulka. BHT analüüsi käigus inkubeeritakse proovi ettenähtud tingimustes 5 või 7 päeva ning mõõdetakse vees lahustunud hapniku kontsentratsiooni vähenemine inkubatsiooniperioodi jooksul. Kuigi BHT analüüs ei ole spetsiifiline ühelegi reostusainele on see siiski üks tähtsamaid indikaatoreid nende potentsiaalsest ohust keskkonnale, kuid antud meetodil tulemuste saamiseks kulub kaua aega.

Alternatiivse võimalusena kiiremaks BHT määramiseks on mikroorganismidel baseeruvad biosensorid, millega on võimalik tulemus saada lühema ajaga. Biosensorid mõõdavad hapniku kontsentratsiooni vähenemist, mida põhjustab mikroorganismide hapniku tarbimine vees lahustunud orgaaniliste ühendite lagundamisel. Biosensorite mõõtetäpsus sõltub eelkõige kasutatud mikroorganismidest ja sageli on see väga madal spetsiifiliste tööstuslike reovete analüüsimisel. Antud probleemile oleks lahenduseks teadaoleva substraadispektriga pool-spetsiifiliste mikroorganismide kasutamine

Antud töö käigus koostati lihtsad ja usaldusväärsed BHT biosensorid erinevate Eesti tööstustes tekkivate reovete analüüsimiseks. Biosensorid põhinesid pool-spetsiifilistel mikroorganismidel, mis saadi Tartu Ülikooli Tehnoloogia Instituudist või isoleeriti proovidest, mis sisaldasid ühendeid, millele mikroorganismid olid pool-spetsiifilised. Mikroorganismide valiku aluseks oli nende võime lagundada tööstuslikus reovees leiduvaid spetsiifilisi raskesti lagundatavaid ühendeid. Võrdluseks kasutati universaalsel bakterikultuuril – P75 – põhinevat biosensorit. Biosensorid kalibreeriti OECD sünteetilise reoveega ja nendega teostati mõõtmisi erinevates sünteetilistes ja reaalsetes tööstuslikes reovetes (I-V). Erinevaid biosensoreid kasutati üksikuna ning lisaks analüüsiti nende signaali sensor-rivina – bioelektroonilise ninana. Sensor-rivi signaali analüüsiti mitmemõõtmelise andmeanalüüsi meetoditega selleks, et eraldada signaalist kvantitatiivne ja kvalitatiivne informatsioon (V).

Katsed koostatud pool-spetsiifiliste ja universaalse BHT biosensoriga näitasid, et pool-spetsiifilised biosensorid on sobivad analüüsivahendid sünteetiliste, raskesti lagundatavaid ühendeid sisaldavate reovete analüüsimiseks. Pool-spetsiifilised biosensorid võimaldasid mõõta BHT-d, mis on põhjustatud raskesti lagundatavatest ühenditest, mille suhtes biosensorid on pool-spetsiifilised. Seevastu universaalne biosensor või biosensorid, mis pole pool-spetsiifilised antud konkreetsetele ühenditele alahindasid proovi BHT_7 ligikaudu selles

ulatuses, mis lisab OECD sünteetilise reovee BHT_7-le lisatud raskesti lagundatav ühend – 10–25%. Seetõttu on olulised eelteadmised proovi koostise ja päritolu kohta, selleks et valida sobiv pool-spetsiifiline biosensor (II–V).

Reaalsete tööstuslike reoveeproovide analüüsimisel alahindasid biosensorid enamike reoveeproovide BHT_7. Vaatamata alahindamisele reaalsete proovidega võimaldasid pool-spetsiifilised biosensorid siiski saada paremaid tulemusi, kui universaalne biosensor, mis alahindas BHT_7-et veelgi enam. Seetõttu on võimalik öelda, et pool-spetsiifilised biosensorid on universaalsetest sobilikumad BHT mõõtmiseks raskesti lagundatavaid ühendeid sisaldavate tööstuslike reovete korral (I-IV).

Proovi koostise ja päritolu kohta info eelteadmise vajadusest saadi üle kasutades erinevaid biosensoreid, universaalset ja erinevatele ühenditele pool-spetsiifilisi, sensor-rivina ehk bioelektroonilise ninana. Sensorite signaalide analüüsimiseks kasutati mitmemõõtmelise analüüsi meetodeid. Erinevate biosensorite samaaegne kasutamine võimaldab saavutada laiemat substraadispektrit võrreldes ühe biosensoriga ning lisaks on võimalik andmeanalüüsi kaudu saada kvalitatiivset ja kvantitatiivset informatsiooni proovi koostise kohta. Kvalitatiivset informatsiooni eraldamiseks kasutati peakomponentide analüüsi (PCA). Selle kolm esimest komponenti võimaldasid eristada erinevaid proove omavahel nende koostise ja BHT_7 alusel. Erinevate proovide eristamine üksteisest oli lihtsam suuremate BHT_7 väärtuste korral ja proovide korral, mis sisaldasid rasva või fenooli. Lisaks kasutati osalist vähimruutude meetodit (PLS) kvantitatiivseks analüüsiks, mille abil saavutati hea korrelatsioon sensor-BHT ja BHT_7 vahel (V).

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