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# Comparative study of semi-specific *Aeromonas hydrophila* and universal *Pseudomonas fluorescens* biosensors for BOD measurements in meat industry wastewaters

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ABSTRACT

*Aeromonas hydrophila* P69.1 (*A. hydrophila*) was used to construct a semi-specific biosensor to estimate biochemical oxygen demand (BOD) in high fat and grease content wastewaters. *A. hydrophila* cells were grown in fat containing medium to induce necessary enzymes for transport and degradation of fatty substances. Universal biosensor based on non-specific *Pseudomonas fluorescens* P75 (*P. fluorescens*) was used to conduct comparison experiments. Biosensors were calibrated using OECD synthetic wastewater and steady-state method, subsequently several experiments with synthetic and industrial wastewaters were conducted. A linear range up to 45 mg $l^{-1}$ $BOD_7$ was gained using *A. hydrophila* biosensor, in comparison to 40 mg $l^{-1}$ $BOD_7$ obtained using *P. fluorescens* biosensors. The lower limit of detection was 5 mg $l^{-1}$ $BOD_7$. Service life of *A. hydrophila* and *P. fluorescens* biosensors were 110 and 115 days, respectively. The response time of the biosensors depended on the $BOD_7$ of measuring solution and was up to 20 min when analyzing different wastewaters. Both biosensors underestimated BOD in meat industry wastewater from 43% up to 71%, but more accurate results could be obtained with *A. hydrophila* biosensor. Semi-specific *A. hydrophila* biosensor was able to measure proportion of fat found in wastewater sample, while other refractory compounds remained undetectable to both biosensors.



## 1. Introduction

Biochemical oxygen demand (BOD) is a widely used universal parameter to describe organic pollution in water and wastewater. The BOD determination is an empirical test in which standard laboratory procedures are used to determine the relative oxygen requirements of wastewaters, effluents, and polluted waters. The test includes 5 days (7 days according to Swedish standard) of incubation under specific conditions (20 ± 1 °C, in the dark) and requires experience and skills to achieve reproducible results [1]. Although BOD is a good indicator of organic pollution in waters, it is ineffective if fast feedback of water quality is necessary. As an alternative method for BOD determination, BOD biosensors have been developed.

A biosensor is a self-contained integrated device, capable of providing specific quantitative or semi-quantitative analytical information using biological recognition elements which are retained in direct spatial contact with an electrochemical transduction element [2]. Since the first published report of a biosensor [3], several kinds of biosensors have been developed. Most BOD biosensors are based on synthetic or natural polymeric gel membrane containing immobilized living microorganisms as a biological recognition element and a dissolved oxygen sensor as an electrochemical transduction element. Microbial BOD sensors rely on measurement of respiratory activity of microorganisms as a result of diffusion and oxidation of organic substances in the membrane containing immobilized microorganisms [4–6]. Various modifications in biosensor design include a biosensor that enabled easy renewal of biological recognition element [7], the use of carbon dioxide analyzer [8] and dissolved oxygen optical fiber [9]. In recent years, several spectrophotometric and chemiluminescence BOD measuring methods have been reported [10,11].

The correlation between standard BOD results and sensor-BOD is often low if universal BOD sensors are used to analyze wastewaters containing refractory compounds [12,13]. Natural waters and industrial wastewaters contain several specific refractory compounds which microorganisms are not able to use and degrade within short measuring time of biosensor. Traditional BOD analysis include incubation period of 5 or 7 days. During this time microbes have enough time to induce appropriate enzymes for the

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digestion of unassimilated pollutants, thereby causing a disagreement between the sensor-BOD and the $BOD_5$ values [14]. Accuracy and reproducibility of biosensor depends on the microorganism's particular characteristics and limited applicability may be due to random selection of the microorganism employed. BOD biosensors require microorganisms with low selectivity and high biooxidation activity for a wide range of organics, so that they can be used to monitor and process effluents and wastewaters from different sources. Therefore, it is vital to select suitable microorganism for a biosensor [15].

Better correlation between sensor-BOD and $BOD_7$ could be obtained by using semi-specific microbes (as opposed to universal microbes) in biosensors to analyze industrial wastewaters. Universal microorganisms are defined as those which use a limited substrate spectrum common to a majority of aerobic heterotrophic microorganisms. Semi-specific microorganisms can additionally biodegrade some refractory compound or group of compounds specific to that particular microorganism. Semi-specific microbes are able to oxidize refractory compounds found in industrial wastewaters, which would be undetected by universal biosensors. Functionality of semi-specific biosensors in dairy wastewaters and phenolic wastewaters from oil shale industry has been demonstrated [16,17].

This study examined the possibilities of faster and more accurate BOD measurements in meat industry wastewaters using semi-specific biosensors. Meat processing plants use approximately 62 $Mm^3\ y^{-1}$ of water. Only a small amount of this quantity becomes a component of the final product: the remaining part is wastewater of high biological and chemical oxygen demand, high fat content and high concentrations of dry residue, sedimentary and total suspended matter as well as nitrogen and chlorides [18]. Since the principal constituents of meat processing wastewaters include a variety of readily biodegradable organic compounds, primarily fats and proteins [19], the microorganisms with good lipolytic properties were used in biosensor. Bacterial culture of *Aeromonas hydrophila* P69.1 was used in semi-specific biosensor as a biological recognition element. A universal non-specific biosensor based on *Pseudomonas fluorescens* P75 was used to conduct comparison experiments.

## 2. Experimental

### 2.1. Chemicals

All reagents, unless otherwise noted, were commercially available reagent grade. Tryptone, yeast extract, peptone and meat extract were purchased from Merck, Germany. Agarose (type I-A: Low EEO) was purchased from Sigma, Germany. Carboxymethylcellulose sodium salt (CMC) ($M_w \sim 250{,}000$, Medium Viscosity) and ethylenediaminetetraacetic acid (EDTA) were purchased from Fluka, Germany. $Na_2HPO_4{\cdot}12H_2O$, $NaH_2PO_4{\cdot}H_2O$, $K_2PO_4$, NaCl, $CaCl_2{\cdot}H_2O$ and $Mg_2SO_4$ were purchased from Lach-Ner, Czech Republic. Distilled water was used to prepare solutions.

### 2.2. Selection of microorganisms

Two natural bacterium culture, isolated from Raadi lake water (Tartu, Estonia), *Aeromonas hydrophila* P69.1 (*A. hydrophila*) and *Pseudomonas fluorescens* P75 (*P. fluorescens*) were used for construction of BOD biosensor. Both strains of bacterial cultures were procured from bacterial library of Institute of Technology, University of Tartu. All bacteria in the library are kept in glycerol solution at −80 °C. The selection of the microorganisms for semi-specific biosensor was based on their ability to use fat as a substrate due to high oil and grease concentration in meat industry wastewater. The lipolytic properties were examined by culturing bacteria in medium containing 0.1% swine fat (Rakvere Meat Industry) as the only carbon source. *A. hydrophila* culture was selected due to fast growth rate in the given medium. Non-specific bacteria, *P. fluorescens*, that was also used as a reference in previous studies [16,17], were used as a comparison to illustrate the advantages of semi-specific bacteria.

### 2.3. Preparation of cell suspension

The bacteria were grown under aerobic conditions in a rotating shaker (Sanyo Orbital Incubator, Sanyo) at 30 °C in a Luria-Bertan medium (tryptone, 10 $g\,l^{-1}$; yeast extract, 5 $g\,l^{-1}$; NaCl, 5 $g\,l^{-1}$). 2 ml of culture medium was inoculated and incubated for 14 h; subsequently the cell suspension was subcultured into a 150 ml of culture medium and incubated for 6 h. Optical density of the bacterial suspension was measured with spectrophotometer (HP 4853, $\lambda = 600$ nm) to assure cells deriving from the late exponential phase of growth (*A. hydrophila* $OD_{600} = 8.5$; *P. Fluorescens* $OD_{600} = 6.9$).

The bacterial suspension was centrifuged (Jouan CR3) at 4000 r/min for 15 min at room temperature and the supernatant was decanted. To circumvent the culture medium from getting into the membrane, the cells were washed twice with phosphate buffer of pH 6.86 ($Na_2HPO_4{\cdot}12H_2O$, 6.90 $g\,l^{-1}$; $NaH_2PO_4{\cdot}H_2O$, 7.04 $g\,l^{-1}$) and centrifuged at the same conditions. The washed bacterial paste was used immediately to prepare the microbial membranes.

### 2.4. Immobilization

Mixture of 180 mg agarose and 7.5 ml phosphate buffer was heated at 70 °C until complete melting of agarose. The mixture was cooled down to 45 °C and 900 μg of the previously prepared cell paste was added. After mixing 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. During the service life between measurements and adaptation the membranes were maintained at 4 °C in a phosphate buffer solution.

### 2.5. Instrumentation

BOD measuring system is shown in Fig. 1. A 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 insertion ring and a holder.

### 2.6. Experimental procedure

The measurements were carried out at room temperature (22 ± 2 °C) in a beaker containing 50 ml of previously aerated phosphate buffer solution. Before measurements the membranes were held in a room temperature for 1 h in order to adapt

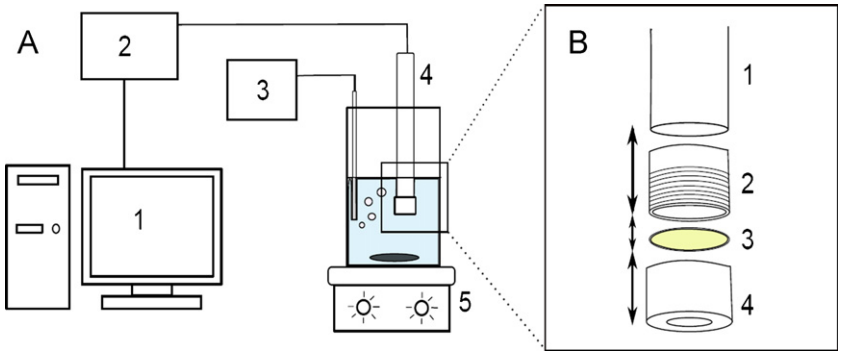


**Fig. 1.** (A) Schematic presentation of a biosensor measuring system: 1: computer, 2: measuring module, 3: aeration unit, 4: biosensor, 5: magnetic stirrer. (B) Biosensor design: 1: dissolved oxygen probe, 2: insertion ring, 3: membrane, 4: holder.

immobilized microorganisms with room temperature. The biosensor was placed into the aerated measuring solution and after an initial stable current was reached ($I_0$), certain amount of substrate was added. The substrate was inserted into the measuring solution using step-by-step adding method in order to change the $BOD_7$ of measuring solution gradually with each addition. During addition of substrate the initial and end-point steady state signal ($I_S$) was recorded. Experiment was carried out until the output from oxygen sensor was zero or did not change after addition of substrate into the measuring solution.

The biosensor response time was defined as a time taken to reach the new steady state signal after addition of substrate. The biosensor response at a certain concentration was calculated as a difference between biosensor signal before and after addition of the substrate ($I_0 - I_S$). The results were normalized by dividing biosensor response at a certain concentration with an initial steady state signal ($NSR = (I_0 - I_S)/I_0$). The normalized signal response at certain concentration was plotted against the $BOD_7$ of measuring solution and calibration graphs were constructed.

### 2.7. Standard solution

Synthetic wastewater according to the recipe of Organisation for Economic Cooperation and Development (OECD) (peptone, 1.6 g $l^{-1}$; meat extract, 1.1 g $l^{-1}$; urea, 0.3 g $l^{-1}$; $K_2PO_4$, 0.28 g $l^{-1}$; NaCl, 0.07 g $l^{-1}$; $CaCl_2{\cdot}2H_2O$, 0.05 g $l^{-1}$; $MgSO_2{\cdot}7H_2O$, 0.02 g $l^{-1}$) [20] was chosen as a standard solution to calibrate BOD biosensors. The $BOD_7$ of standard solution was measured as 2000 mg $l^{-1}$.

### 2.8. Pre-conditioning

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 preconditioning for the period of 14 days. The membranes containing *A. hydrophila* microorganisms were soaked in the solution containing dissolved fat in order to study the possibilities to activate the metabolic pathways for the degradation of fatty substances and thereby improve biosensor's sensitivity.The saturated solution of swine fat was prepared by adding 5 g $l^{-1}$ 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 min 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 $l^{-1}$. The membranes were adapted in a phosphate buffer solution dosed with saturated solution of fat ($BOD_7$ of the solution 30 mg $l^{-1}$). The adaptation period lasted ten days, during which measurements with saturated solution of swine fat were conducted.

### 2.9. Wastewater samples

Experiments with several combined and industrial wastewaters were conducted. Combined synthetic wastewaters simulated wastewaters from different industries (meat, dairy and paper industry) and consisted in OECD synthetic wastewater and an additional refractory compound, characteristic to specific industrial wastewaters.

The combined wastewater with fatty substances was produced by dissolving components of OECD synthetic wastewater in a saturated solution of fat. As fat has low solubility in water, half-sized quantities (compared to that described in Section 2.7) were used in order to achieve the proportion of fat in the $BOD_7$ of combined synthetic wastewater of approximately 15–20%. Additionally combined wastewater dosed with milk or CMC was used, by adding 1 ml milk with fat content of 2.5% or 1 g CMC, respectively, into 1000 ml of OECD synthetic wastewater.

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 (slaughtering). 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 0.01 M.

The samples of industrial wastewater were taken from Nõo meat industry (Nõo, Estonia) and Arke meat industry (Kanepi Municipality, Estonia) outflows and represent wastewaters generated during further processing of meat (cutting, deboning, cooking, seasoning, smoking, etc.).

Conventional $BOD_7$ analysis was conducted according to the standard of American Public Health Organization (APHA) [1] with all wastewater samples and the results were compared with the results of measurements carried out with the biosensors.

## 3. Results and discussion

### 3.1. Linear range and sensitivity

The calibration parameters (linear range and sensitivity) from calibrations with OECD synthetic wastewater were used to characterize biosensors (Fig. 2). The linear range of a biosensor is defined as the substrate range that gives a signal directly proportional to the substrate concentration [21]. The linear ranges for *A. Hydrophila* and *P. Fluorescens* biosensors were up to 45 mg $l^{-1}$ and 40 mg $l^{-1}$ $BOD_7$, respectively. The sensitivity is defined as the signal change at the definite concentration of the substrate and it is measured by the slope of the calibration curve [21]. Sensitivities of *A. Hydrophila* and *P. Fluorescens* biosensors were 0.019 (1/($BOD_7$, mg $l^{-1}$)) and 0.023 (1/($BOD_7$, mg $l^{-1}$)), respectively. The lower level of detection was 5 mg $l^{-1}$ $BOD_7$. Relative standard deviation of calibration points at different $BOD_7$ values varied from 2.0 to 13.7% ($n = 6$) for *A. Hydrophila* biosensor and from 5.5 to 13.5% ($n = 6$) for *P. Fluorescens* biosensor.

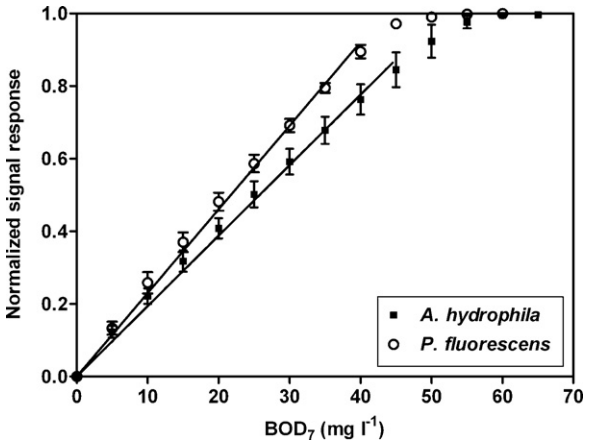


**Fig. 2.** The calibration curve for *A. hydrophila* and *P. fluorescens* biosensors.

The sensitivity is related to the type and density of the bacteria. Also, it is influenced by the sensitivity of microorganisms to particular organic substrates [5]. Different strains of microorganisms may preferably use different compounds in metabolism. Therefore, different biosensors constructed and used at the same conditions may have a different linear range.

### 3.2. Response time

Response time of biosensors depended on the concentration and content of measuring solution: shorter response time occurred when $BOD_7$ of measuring solution was increased by smaller steps. Response times of *P. fluorescens* and *A. hydrophila* biosensors were 10–15 min when the $BOD_7$ of measuring solution was increased by a step of 5 mg $l^{-1}$. When $BOD_7$ was increased by larger steps (10–20 mg $l^{-1}$), the response time extended up to 20 min.

When saturated solution of fat was used in measurements, the response time of *A. hydrophila* biosensor ranged from 50 min up to 1.5 h, while *P. fluorescens* biosensor did not react to the solution of fat at all. Biosensor's response to the solution of fat depends on the ability of microorganisms to use the fat as a substrate, which requires the existence of special enzymes and metabolic pathways in a bacterial cell. An active enzymatic system for the degradation of fatty substance was present in *A. hydrophila* cells, whereas it lacked in the cells of *P. fluorescens*. Prolonged response time of biosensors is often associated with slow mass transfer rate of high molecular weight compounds through immobilization matrix and cell membranes [22,23].

### 3.3. Operational stability and service life

Operational stability of BOD biosensors is primarily related to the stability of the immobilized microorganisms. Pure microbial cultures have much better stability under same cultivation and storage conditions than multi-strain based sensors because the culture consistency of microbes in mixed cultures may vary with

time [5]. Operational stability and service life were evaluated during calibrations with OECD synthetic wastewater over a period of five months. The stable period was defined as a period where deviation from average sensor response to 25 mg $l^{-1}$ $BOD_7$ was within the control limits of 15.4% standard deviation for BOD measurements specified by APHA [1]. All biosensors were calibrated at least once a week during service time.

The stable and reproducible response was achieved 20 days after immobilization with *A. hydrophila* biosensor and 35 days after immobilization in case of *P. fluorescens* biosensor. Stable periods of biosensors allowing reproducible results were 90 and 80 days for *A. hydrophila* and *P. fluorescens* biosensors, respectively. After that response to calibration solution became irregular and the stable period was considered at an end.

### 3.4. Repeatability and reproducibility

According to conventional APHA method for BOD measurements using a standard GGA solution (150 mg $l^{-1}$ glucose and 150 mg $l^{-1}$ glutamic acid), with $BOD_5$ value of 199.4 mg $l^{-1}$, relative standard deviation of results – 15.4% is intended for the reference for every laboratory [1]. Repeatability is as a standard deviation of a sensor tested by one operator under the same conditions, while reproducibility is as a standard deviation series of sensors tested by more than one operator under different conditions [24]. In a present study repeatability and reproducibility of biosensors were evaluated conducting measurements with OECD synthetic wastewater at different concentrations (Table 1).

Repeatability of *A. hydrophila* and *P. fluorescens* biosensors varied from ±5.4 to ±9.7% ($n$ = 6) and from ±1.8 to ±7.7% ($n$ = 6), respectively. Reproducibility of *A. hydrophila* and *P. fluorescens* biosensors varied from ±3.2 to ±6.9% ($n$ = 5) and from ±2.9 to ±7.7% ($n$ = 5), respectively. The results show that repeatability and reproducibility of biosensors used in current study remained within the control limit of 15.4% set by APHA. In comparison, *P. fluorescens* biosensor was more stable than *A. hydrophila* biosensor.

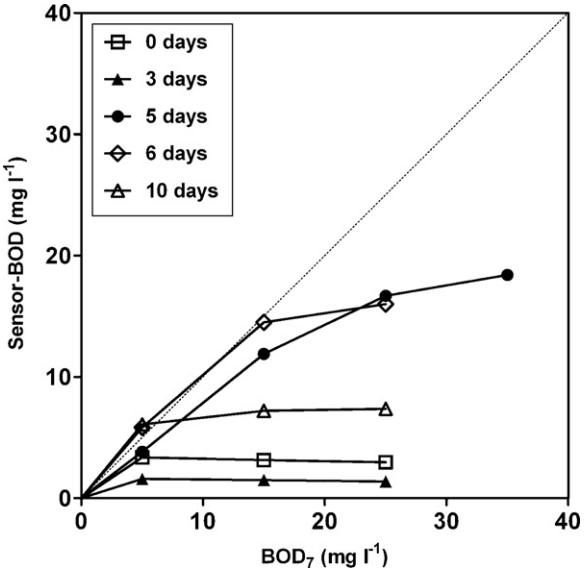


**Fig. 3.** Influence of the adaptation period: the correlation between $BOD_7$ and *A. hydrophila* sensor-BOD on different days during adaptation period when saturated solution of swine fat was stepwise added to a measuring solution.

### 3.5. Influence of adaptation period

Adaptation of microorganisms for induction of desirable metabolic pathways and uptake systems by cultivation in medium containing appropriate substrates may often be desirable to increase the range of assimilation of substrates by the immobilized microbes [4]. Thereby adaptation of microorganisms can increase the sensitivity of biosensor and improve the agreement between the sensor-BOD and $BOD_7$ [5].

The membranes containing *A. hydrophila* microorganisms were soaked in the solution containing fat in order to study the possibilities to activate the metabolic pathways for the degradation of fatty substances and thereby improve biosensor's sensitivity. Fig. 3

**Table 1**
Repeatability and reproducibility of biosensors.

| $BOD_7$ (mg $l^{-1}$) | Repeatability | | | Reproducibility | | |
|---|---|---|---|---|---|---|
| | Sensor-BOD (mg $l^{-1}$) | Standard deviation ($n$ = 6) | | Sensor-BOD (mg $l^{-1}$) | Standard deviation ($n$ = 5) | |
| | | (mg $l^{-1}$) | % | | (mg $l^{-1}$) | % |
| *A. hydrophila* biosensor | | | | | | |
| 15 | 16.3 | ±1.6 | ±9.7 | 15.3 | ±1.1 | ±6.9 |
| 25 | 25.8 | ±1.8 | ±7.0 | 25.5 | ±1.3 | ±4.9 |
| 35 | 34.9 | ±1.9 | ±5.4 | 35.1 | ±1.1 | ±3.2 |
| *P. fluorescens* biosensor | | | | | | |
| 15 | 16.1 | ±1.2 | ±7.7 | 15.1 | ±0.7 | ±4.7 |
| 25 | 25.5 | ±1.1 | ±4.3 | 24.9 | ±0.8 | ±3.3 |
| 35 | 34.5 | ±0.6 | ±1.8 | 34.6 | ±1.0 | ±2.9 |

**Table 2**
Comparison of BOD values of wastewater samples measured with biosensors and conventional 7-day method.

| Wastewater sample | $BOD_7$ (mg $l^{-1}$) | *A. hydrophila* biosensor ($n$ = 4) | | | *P. fluorescens* biosensor ($n$ = 4) | | |
|---|---|---|---|---|---|---|---|
| | | Sensor-BOD (mg $l^{-1}$) | Standard deviation of sensor-BOD (%) | Sensor-BOD/ $BOD_7$ | Sensor-BOD (mg $l^{-1}$) | Standard deviation of sensor-BOD (%) | Sensor-BOD/ $BOD_7$ |
| OECD dosed with fat | 1240 | 1231 | ±10.3 | 0.99 | 962 | ±7.9 | 0.77 |
| OECD dosed with milk | 2300 | 1914 | ±11.1 | 0.83 | 2035 | ±4.7 | 0.88 |
| OECD dosed with CMC | 2060 | 2152 | ±11.6 | 1.04 | 2080 | ±8.4 | 1.00 |
| Meat leachate | 10700 | 3116 | ±12.5 | 0.29 | 3415 | ±7.0 | 0.31 |
| Wastewater from Nõo meat industry | 2130 | 1224 | ±23.1 | 0.57 | 960 | ±19.7 | 0.45 |
| Wastewater from Arke meat industry | 2400 | 1109 | ±11.8 | 0.46 | 918 | ±17.8 | 0.38 |

illustrates the influence of adaptation period on the sensitivity of biosensor when measurements with saturated solution of swine fat were conducted.

Before adaptation *A. hydrophila* biosensor responded to low concentrations of fat solution, but the biosensor responded merely to the presence of fat in the solution and not to the concentration of it. At 5 mg $l^{-1}$ of $BOD_7$, the biosensor underestimated the value of $BOD_7$ by 22% and the subsequent increase in concentration did not result in any changes in output signal.

Biosensor's sensitivity towards saturated solution of fat decreased during the first days of the adaptation. On the third day, no or near zero change in biosensor's output signal was detected when substrate was added into the measuring solution. The highest sensitivity was achieved after 5–6 days of adaptation, when the discrepancy between the biosensor measured BOD and $BOD_7$ did not exceed 15% at $BOD_7$ values below 15 mg $l^{-1}$. Longer adaptation period resulted in the decrease in the sensitivity of the biosensor towards the solution of saturated fat. After ten days of adaptation, sensor measured BOD at 5 mg $l^{-1}$ of $BOD_7$ was 41% higher than $BOD_7$, but subsequent increase in $BOD_7$ of measuring solution did not change the output signal.

### 3.6. BOD measurements

Table 2 and Fig. 4 show the correlation of $BOD_7$ and BOD values measured with biosensors for various wastewater samples. Measurements with OECD synthetic wastewater dosed with dissolved fat resulted in the largest difference between the sensor-BOD values of studied biosensors. Results gained with *A. Hydrophila* biosensor differed from $BOD_7$ less than 1%, while *P. fluorescens* biosensor underestimated $BOD_7$ by 23% (Fig. 4a). The *P. fluorescens* in biosensor lacked the enzymes necessary for fat degradation, the existence of which was proved for *A. Hydrophila.* Therefore, *P. fluorescens* biosensor was capable of detecting the $BOD_7$ of OECD synthetic wastewater but failed to detect fat, while *A. Hydrophila* biosensor detected all the components in synthetic wastewater.

The difference between the results obtained by means of the biosensors for other combined wastewater samples were minor. Both bacterial cultures were non-specific to other refractory compounds added to the synthetic wastewater and only the part of BOD derived from OECD synthetic wastewater was detected. Good correlation with $BOD_7$ was achieved with both biosensors when analyzing synthetic wastewater dosed with CMC (Fig. 4c). The addition on 1 g $l^{-1}$ of CMC to the OECD synthetic wastewater elevated the $BOD_7$ of wastewater from 2000 mg $l^{-1}$ to 2060 mg $l^{-1}$. Therefore, it can be concluded that CMC, as a refractory compound, is not degraded within the incubation period of standard BOD analysis. As an additional component in OECD synthetic wastewater, CMC was detected neither with conventional $BOD_7$ analysis nor with biosensors used in the current study. In the case of combined wastewater dosed with milk, sensor-BOD for both biosensors was 12–17% lower than the $BOD_7$ of the sample (Fig. 4b). This corresponds approximately to the amount added to the $BOD_7$ of the OECD synthetic wastewater through addition of milk, indicating that biosensors were not able to detect refractory compounds found in milk.

In analyzing meat leachate and meat industry wastewater samples, both given biosensors underestimated the $BOD_7$. However, generally more accurate results were gained with a semi-specific *A. Hydrophila* biosensor. *A. Hydrophila* biosensor underestimated $BOD_7$ of wastewater samples by 43% and 54%, while the sensor-BOD measured by means of *P. fluorescens* biosensor was 55% and 62% lower than $BOD_7$.

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 meat industry wastewater. Within a short measurement period, BOD

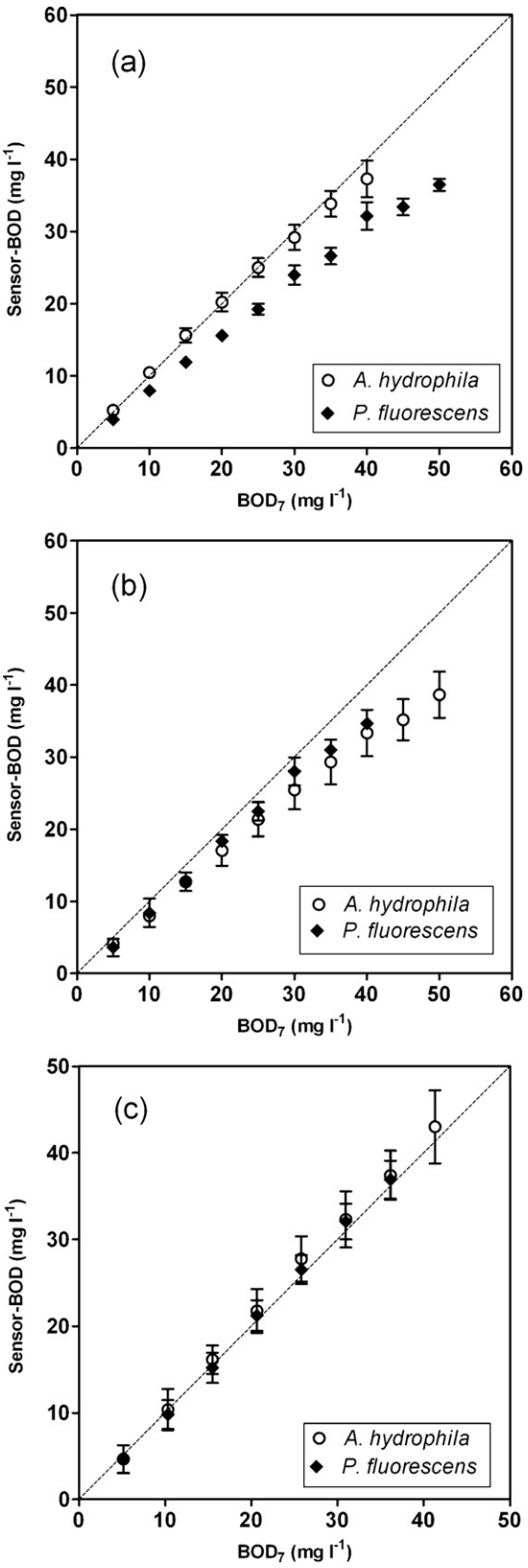


**Fig. 4.** Correlation between sensor-BOD and $BOD_7$ of *A. hydrophila* and *P. fluorescens* biosensors for different combined wastewaters (a: combined wastewater dosed with fat, b: combined wastewater dosed with milk, c: combined wastewater dosed with CMC).

biosensor provides the response only for fast and readily degradable compounds found in the sample. Therefore, during that short measurement period of the sensor, more complex compounds remain undetected; however, these can be determined using traditional $BOD_7$ tests. In addition to proteins and fat, the meat industry wastewater also contains intact cells from blood [25] and soft tissues [19]. Intact cells in their unlysed form are unavailable for micro-organisms, but their degradation takes place during the incubation period of usual $BOD_7$ analysis. Since the diffusion and degradation of refractory compound and intact cells into the bacterial cell does not take place during the short measuring period of a biosensor, additional research on the possibilities of pre-treatment of wastewater samples is required.

The simultaneous use of two different biosensors (one of them semi-specific) enables to receive qualitative information about the composition of wastewater. On the basis of the results on the sensors used in this study, it is possible to evaluate the content of dissolved fat in wastewater of the sample. The fat content in wastewater can be characterized by the difference in the results of *A. hydrophila* and *P. fluorescens* biosensors. The samples taken from Nõo and Arke meat industry wastewaters were richer in fat, while the fat content in meat leachate was much smaller, to which *A. hydrophila* biosensor is semi-specific. According to broad-based study, the main source of oil and fat in meat processing is further processing and rendering [19]. Therefore, better suitability of *P. fluorescens* biosensor for the analysis of meat leachate was caused by smaller fat content and bigger protein content in the meat leachate.

## 4. Conclusion

A microbial sensor was constructed based on a bacterial culture *A. hydrophila* P69.1 with semi-specific fat degrading properties which made it more suitable for accurate BOD analysis of higher fat content wastewaters generated in meat industries during further processing and rendering. Reference measurements were carried out using a universal biosensor based on a bacterial culture *P. Fluorescens* P75. The sensitivity of *A. hydrophila* biosensor to fatty substances was improved through adaptation of membranes in fat containing solution for 5–6 days in which biodegrading capabilities of microorganisms improved and for low fat concentrations the discrepancy between the biosensor measured BOD and $BOD_7$ was less than 15%. A semi-specific biosensor enabled to measure BOD in high fat content wastewaters, both synthetic and industrial, more accurately than a universal biosensor. The extent of discrepancies between the results of *A. Hydrophila* and *P. Fluorescens* biosensors depended on the fat content of wastewaters: the higher the fat content, the wider the discrepancies were.

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