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LOW BOD DETERMINATION METHODS: THE STATE-OF-THE-ART

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Biochemical Oxygen Demand (BOD) is an important factor used to measure water pollution. This article reviews recent developments of microbial biosensors with respect to their applications for low BOD estimation. Four main methods to measure BOD using a biosensor are described: microbial fuel cells, optical methods, oxygen electrode based methods and mediator-based methods. Each of them is based on different principles, thus a different approach is required to improve the limit of detection. A proper choice of microorganisms used in the biosensor construction and/or sample pre-treatment processes is also essential to improve the BOD lower detection limit.

Keywords: biochemical oxygen demand, biosensor, wastewater, microbial sensor

1. INTRODUCTION

Biochemical Oxygen Demand (BOD) is an agreed indicator used to quantify the amount of organic substance possible to be degraded by microorganisms present in a water sample which is proportional to the water pollution (ISO 5815-1:2003). IUPAC defines BOD as: "the amount of oxygen divided by the volume of the system used by the microorganisms growing on organic compounds present in the sample (e.g. wastewater, or sludge) during incubation over period of time and given temperature. BOD is a way to measure organic impurities present in the water sample undergoing biological degradation. Usually BOD is expressed in milligrams O_2 per litre" (Nagel et al., 1992). For practical purposes it can be assumed that BOD is the difference between the dissolved oxygen (DO) present in the sample at the beginning and the end of the measurement in fixed conditions (ISO 5815-1:2003). Microorganisms oxidise organic impurities in 2 stages. First, carbon-compounds are oxidised and after about 10 days the nitrification process begins. During the first 5 days about 68% of organic compounds are oxidised and after 20 days nearly 99%. (Penn et al., 2004; Miksch and Sikora, 2010). It should be noted, however, that the extend of biodegradation depends largely on the structure of compounds present in the sample and microbial community. There are many standards concerning BOD_n estimation e.g. (ISO 5815-1:2003) and (ISO 5815-2:2003). The traditional method starts with diluting a sample in different ratio using water saturated with oxygen and adding aerobic, heterotrophic microorganisms. The sample is incubated for 5 or 7 days (different countries prefer different periods) in fixed temperature of 20°C in the absence of light with the pH value fixed between 7 and 8. Next, the amount of DO in the sample is measured by titration or electrochemically (ISO 5815-1:2003; Miksch and Sikora, 2010). It is possible to measure BOD in undiluted samples, but this method is less accurate (ISO 5815-2:2003). Active sludge (biomass produced in the process of wastewater treatment during the growth of bacteria and other microorganisms in the presence of DO) is most widely used as the biological material (ISO 6107-1:2004). The D-glucose and L-glutamic acid solution (GGA) is most often used as a reference standard (ISO 5815-1:2003). Some authors prefer the use of artificial wastewater (AWW) as a reference

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standard. It is reasonable when developing a BOD sensor to be used in specific conditions e.g. rubber latex industry (Kumlanghan et al, 2008) or a distillery (Oota et al., 2010).

Traditional methods of BOD estimation have many drawbacks, notably very long period of time needed to perform the measurement (at least 5 days). Furthermore, the used microorganisms are very sensitive to toxins, bactericides, heavy metal ions, or chlorine which inhibit their respiration process and may lead to the death of the microorganisms (ISO 5815-1:2003). Traditional methods are neither very sensitive nor precise (Bourgeois et al., 2001). Finally, the measurement procedure must be made soon after acquiring the sample i.e. during the first 24 hours. Many of those flaws may be eliminated by using biosensors (Miró et al., 2004; Pasco et al., 2011; Ponomareva et al., 2011).

IUPAC defines a biosensor as a device which uses specific biochemical reactions, mediated by isolated enzymes, tissue, organelle or whole cells, to detect chemical compounds, usually using electrochemical, thermal or optic methods (Nagel et al., 1992). A biosensor consists of biological material directly connected with a transducer, which allows to process the analytical signal nto a form that enables its detection (Thevenot et al., 2001). This definition of a biosensor is based on the type of receptor layer, rather than the sample origin (Brzózka and Wróblewski, 1999; Brzózka, 2009). Biosensors were first used as a BOD sensor by Karube et al. in 1977 (Karube et al., 1977) using a MFC which allowed the time of the measurement to be reduced from 5 days to 40 minutes. Biosensors are still being improved nowadays, with research focused mostly on shortening the response time and lowering the limits of detection and quantification. This review is focused on the second field of modern research.

2. METHODS OF ESTIMATION

There is no fixed, arbitrary and widely agreed BOD value below which the method is considered a “low BOD determination method”. Furthermore, some BOD estimation methods e.g. optical methods are able to measure lower BOD values than oxygen-sensitive electrode based methods, but they have some drawbacks e.g. they are harder to implement in flow analysis. There are many strategies used to lower the limit of detection. Some researchers concentrate on finding the optimal microorganism or a consortium of microorganisms most sensible in fixed conditions, other researchers focus on the oxidation of organic compounds using photocatalytic methods (Chee et al., 1999b; Chee et al., 2007) or introducing ozone to the sample (Chee et al., 2001; Chee et al., 2005).

2.1. Microbial fuel cells

Microbial Fuel Cells are devices converting chemical energy into electric current by a reaction catalysed by microorganisms (Kumlanghan et al., 2007). Typical MFCs are divided into two compartments: anaerobic with a negatively charged electrode and aerobic with a positively charged electrode. Those two sections are connected through a proton exchanging membrane. In the anaerobic compartment, organic substances are oxidised which leads to proton production. The protons migrate through the membrane to the cathode. The electrons produced during the oxidation process migrate through the anode by the external electric circuit to the cathode where the reduction of oxygen occurs (Grzebyk and Poźniak, 2005; Seo et al., 2009).

Anode: $$C_6H_{12}O_6+6H_2O \rightarrow 6CO_2+24H^++24e^- \quad (1)$$

Cathode: $$4H^++O_2+4e^- \rightarrow 2H_2O \quad (2)$$

Produced electric current is proportional to the concentration of the organic compounds being oxidised in the anaerobic compartment. Research is being conducted in order to standardise MFCs so that comparison between laboratories is possible (Higgins et al., 2011). Original Karube research was

focused on shortening the BOD measurement time by usage of the bacteria *Clostridium butyricum* and the limit of detection was not determined (Karube et al., 1977).

Present studies (Moon et al., 2005) use oligotrophic microbial consortium instead of copiotrophic microorganisms. Although oligotrophes respond to changes in the BOD less eagerly than other microorganisms and require very long time to reach the previous steady state when the sample is removed (about 15 hours when the BOD concentration was changed from 10 to 20 mg O_2 L^{-1}), they are capable of responding to much lower concentrations of organic substances. Enrichment by feeding with AWW for 8 weeks further improves their LOD. Moon et al. tried also to minimise the background noise generated during the measurement. They investigated the influence of the basal inorganic salt solution and buffer concentration on the MFC response. It was shown that these factors indeed do affect signal generation, but only in specific conditions. A trace mineral solution concentration also affected the measured current. The feeding rate was raised in order to saturate the MFC when the BOD value of the sample was about 10 mg O_2 L^{-1}. This further improved the LOD and sensitivity at the cost of the upper limit of detection. The dynamic linear range of the calibration curve was between 2 mg O_2 L^{-1} and 10 mg O_2 L^{-1}.

Kang et al. (2003) tried a different approach. They identified oxygen leaking through a cation specific membrane to the anaerobic compartment as the main reason of lowering the coulombic yield. Calculations showed that decreasing the size of the membrane may lead to lowering the LOD. It was determined that membrane area of 5.2 cm^2 is sufficient for undisturbed flow of protons while minimising the leaking oxygen. They used an oligotropic consortium enriched for 8 weeks by feeding with AWW or river surface water. Also the performance of a carbon rod electrode was compared with a platinum coated electrode which proved to give better results. Decreasing the size of the membrane from 26 cm^2 to 5.2 cm^2 lead to ca. 4 fold higher coulombic yield and the possibility of determining BOD values of 2 mg O_2 L^{-1}.

2.2. Oxygen electrode based methods

Measuring changes of DO using an oxygen-sensitive electrode is the most straightforward method of BOD estimation. Oxygen electrode based biosensors use the Clark electrode (Clark et al. 1953; Severinghaus and Freeman-Bradley, 1958; Severinghaus and Astrup, 1986) covered with an immobilised microbial layer. LOD can be improved by different approaches: screening for a sensitive microorganism or microorganism consortium, photocatalitic pre-treatment or introducing ozone to the sample.

Typically, GGA solution is used for standardisation of BOD determination methods. However, river water samples usually show low BOD values, caused mostly by highly stable organic compounds, such as humic acid or lignin. For such samples, a different solution, namely artificial wastewater (AWW) was proposed for biosensor calibration. It usually contains the following compounds: nitrohumic acid, tannic acid, sodium ligninsulfonate, gum arabic and sodium lauryl sulphate. This solution (Chee et al., 1999a) imitates more closely real river water samples, as compared to a traditional GGA solution.

One of the proposed microorganisms are bacteria *Pseudomonas putida* (Chee et al., 1999a) or salt tolerant yeast *Arxula adeninivorans* LS3 (Renneberg et al., 2004). Using these microorganisms provided good LOD, as they are not influenced by many interfering ions. There are many papers concerning the use of microorganism consortiums to broaden the spectrum of oxidised compounds. Authors of those publications used a very wide array of microorganisms e.g. activated sludge for monitoring treatment of wastewater from a rubber latex industry (Kumlanghan et al., 2008); commercial activated sludge (Liu et al., 2011); *Trichosporon cutaneum* and *Bacillus subtilis* immobilised in sol-gel derived composite matrix (Jia et al., 2003); *Trichosporon cutaneum* and *Bacillus licheniformis* (Suriyawattanakul et al., 2002); *Enterobacter cloaca, Citrobacter amalonaticus,*

Pseudomonas aeruginosa, Yersinia enterocolitica, Klebsiella oxytoca, Enterobacter sakazaki and *Serratia liquefaciens* for industrial waste-water monitoring (Rastogi et al., 2003).

Chee et al. tried a different approach to low BOD estimation (Chee et al., 2001). It was assumed that low sensitivity of BOD estimation methods might be caused by a low rate of assimilation of large chemical compounds by microorganisms in a relatively short period of time. They proposed sample pre-treatment by irradiation with UV light on TiO_2 catalyst. This allows degradation of stable organic compounds and facilitates their biodegradation. The influence of irradiation time, sample pH and TiO_2 concentration on sensor response was investigated. It was found that after optimisation of working parameters, sensor response in river water was higher after photocatalysis than that for samples without UV pre-treatment. The method was further improved and applied in a FIA system (Chee et al., 2005). H_2O_2 was also introduced to the sample, which when excited with UV light, produces free radicals, decomposing large organic compounds even more aggressively. One potential problem in such a system is killing the microbial consortium with the H_2O_2, but it was proven that the lifetime of $O_2^{\cdot-}$ radicals is about 2.5 s which is too short to reach the measurement cell. Such an approach improved the sensitivity of the sensor 1.4 fold and eliminated the problem with many biological contaminants of the sample.

It is also possible to use ozonation rather than photocatalytic reaction. Ozone is a powerful oxidising agent that decomposes organic matter both directly and indirectly through the creation of hydroxyl radicals (Staehelin and Hoigné, 1982). Excess ozone has to be removed before the measurement e.g. by intense stirring of the sample. Such an approach was proposed by Chee et al. (Chee et al., 1999b) leading to a 2 fold signal increase and a detection limit of 0.2 mg O_2 L^{-1}. It was shown that BOD values measured by the proposed method correlate well with those determined by the conventional BOD_5 method. This method was further improved and applied in a stopped-flow system (Chee et al., 2007). In this automated system, 1.6 fold signal increase was observed and the lower detection limit was 0.5 mg O_2 L^{-1}.

2.3. Optical methods

Optical methods of BOD estimation are generally characterised by low limits of detection. This can be attributed to the fact that oxygen is not consumed during the measurement, as it is in the case of amperometric detection. Accordingly, more oxygen is available to the process of pollutant oxidation by microorganisms. The process of further lowering this parameter is performed by screening for the most sensitive microorganisms. Some papers prove that *Pseudomonas putida* (Chee et al., 2000) or *Saccharomyces cerevisiae* (Nakamura et al., 2007; Nakamura et al., 2008) provide the necessary parameters, but other researchers tend to use mixed cultures (Jiang et al., 2006; Lin et al., 2006; Xin et al., 2007). Using mixed consortiums of different types of bacteria broadens the array of organic compounds oxidised by the biofilm which leads to improved limits of detection and, more importantly, to more precise results.

An important problem that spawned many interesting articles is the determination of BOD in seawater samples (Jiang et al., 2006; Nakamura et al., 2008; Renneberg et al., 2004; Xin et al., 2007). This is a relative difficult task, as the BOD of seawater is usually low and the microorganisms used in typical BOD sensors are not tolerant enough to high salt concentration. Therefore, various microorganisms of increased tolerance towards NaCl were used in proposed BOD sensors, including *Bacillus licheniformis*, *Dietzia maris* and *Marinobacter marinus* consortium obtained from seawater. The sensing film of proposed sensors consisted of an organically modified silicate (ORMOSIL) film doped with an oxygen-sensitive ruthenium complex. It was shown that with the use of this set-up, the minimum measurable BOD was 0.18 mg O_2 L^{-1} in natural seawater. *Saccharomyces cerevisiae* ARIF KD-003 acquired from cold regions of Japan, showing excellent characteristics in salty conditions, were also employed for BOD determination in seawater samples (Nakamura et al., 2008). In this work,

2,6-dichloroindophenol spectrophotometric method was used to estimate yeast respiration activity. A remarkably low detection limit of 0.07mg O_2 L^{-1} BOD was obtained using GGA-containing artificial seawater.

Measurements of low BOD values, caused by persistent organic pollutants, pose a serious problem in river water samples. This issue was addressed by Chee et al. with the use of commercial optical fibre oxygen sensor and immobilised *Pseudomonas putida* bacterium (Chee et al., 2000). The minimum value of BOD that could be measured using this biosensor was 0.5 mg O_2 L^{-1}.

A very interesting approach to low BOD determination was proposed by Sakaguchi and co-workers (Sakaguchi et al., 2003). The proposed system is based on bacterial luminescence from recombinant *Escherichia coli* that contains lux A-E genes from *Vibrio fischeri*. The luminescence of these bacteria depends on their metabolic activity, which can be strengthened by the presence of carbon source in the incubation medium. The increase of light emission by recombinant *E. coli* was correlated with the raised BOD value of tested samples. Bacterial luminescence was measured using a charge coupled device camera and a photomulti-counter (consisting of a photon-counter and a photomultiplier). Using this relatively simple system, the lower BOD detection limit was estimated at 1 mg O_2 L^{-1}.

2.4. Mediator-based methods

One of the major drawbacks of DO sensors is the necessity of sample pre-treatment which includes saturation with oxygen. This process is strongly dependent on the sample temperature and achieving good repeatability is difficult. Replacement of oxygen with a mediator solution solves this problem. Mediators are chemical compounds able to accept electrons in place of oxygen in the metabolism of microorganisms. They are able to transport electrons to the electrode surface where a reduced form of the mediator is oxidised resulting in electron transfer to the electrode, which leads to electric current production. The use of mediators in BOD sensors is mainly motivated by the extension of linear calibration range towards higher BOD values. However, the lower detection limits are also usually improved. This was the case in the work conducted by Trosok et al (Trosok et al., 2001), where different hypothetical mediators were investigated, with potassium ferricyanide(III) and hydroxymethylferricinium proven to be the most useful ones. The 2 mg O_2 L^{-1} BOD detection limit was achieved using SPT1 yeast strain which showed a marked similarity to the genus *Candida*.

2.5. Carbon dioxide-based method

All the methods described above are based on changes in the oxygen (or mediator) concentration which is the result of microbial respiration. However, it is also possible to develop a sensor based on the measurement of carbon dioxide produced during this process. Recently, Cortón et al. (Cortón et al., 2010) described such a sensor, based on a modified Severinghaus electrode (Severinghaus and Freeman-Bradley, 1958) with immobilised yeast *Saccharomyces cerevisiae*. The BOD detection limit of 1 mg O_2 L^{-1} was reported for this device. Such a low LOD was possible owing to the fact that the potentiometric CO_2 electrode does not consume the analyte in contrast to the amperometric oxygen electrode.

3. CONCLUSIONS

Biochemical Oxygen Demand (BOD) is a well-established method for evaluation of water quality. BOD values are most often used to assess the effectiveness of wastewater treatment plants. One of the disadvantages of the classical methods of BOD determination is a relatively high detection limit, preventing analysis of pristine river water or sea water samples. In this review, articles describing

methods of determination of low BOD values are collected and discussed. Various measurement modes are mentioned, including microbial fuel cells, optical methods, oxygen electrode based methods and mediator-based methods. It is shown that a proper choice of a microorganism or microorganisms consortium is of fundamental importance in the design of low BOD measurement devices. Moreover, certain sample pre-treatment processes can be employed to decompose persistent organic pollutants, making them more bioavailable. Attention is also drawn to artificial wastewater, used for standardisation of low BOD determination methods instead of traditional GGA solution.

Table 1. Comparison of the methods for determination of low BOD values

Type	Response time, minutes	Dynamic range, $mgO_2\,L^{-1}$	LOD, $mgO_2\,L^{-1}$	Microorganism	Reference
MFC[1]	40	?-250	6*	*Cl. Butyricum*	Karube et al, 1977
MFC[2]	60	2-10	<2	mix	Moon et al, 2005
MFC[1,A]	90	ND	ND	mix	Kang et al, 2003
OP[3]	20	0.2-40	0.18	mix	Xin et al, 2007
OP[1]	3.2	0.2-40	0.1	3 types	Jiang et al, 2006
OP[1]	3	0.2-40	0.2	mix	Lin et al, 2006
OP[1]	10	0.33-22	0.11	*S. cerevisiae* ARIF KD-003	Nakamura et al, 2008
OP[1]	30	2-20	2	*S. cerevisiae*	Nakamura et al, 2007
OP[1]	15	1-10	ND	*P. putida*	Chee et al, 2000
DO[1]	5	6-550	2.1	*A. adeninivorans* LS3	Renneberg et al, 2004
DO[1]	10	?-40	0.5	mix	Suriyawattanakul et al, 2002
DO[1]	10	?-60	1	Activated sludge	Rastogi et al, 2003
DO[1]	90	?-45	1	mix	Dhall et al, 2008
DO[1]	15	ND	0.5	*P. putida*	Chee et al, 1999a
DO[1]	10	1-60	0.5	mix	Jia et al, 2003
DO[1,B]	5	ND	0.2	*P. putida*	Chee et al, 1999b
DO[1,C]	>4	ND	<0.5	*P. putida*	Chee et al, 2001
DO[3,B]	5	ND	0.5	*P. putida*	Chee et al, 2007
DO[4]	<10	ND	0.5	mix	Liu et al, 2011
DO[4,C]	10	ND	1	*P. putida*	Chee et al, 2005
DO[4]	15	2-25 to 5-60**	0.18	*Activated sludge*	Kumlanghan et al, 2008
ME[1]	ND	2-100	2	*Candida*	Trosok et al, 2004
ME[1]	ND	<20	6.3***	*Proteus vulgaris*	Pasco et al, 2004
CO_2[1]	15	5-500	1	*S. cerevisiae*	Cortón et al, 2010
1 - batch system, 2- continuous flow system, 3 - stopped-flow system, 4 - FIA A - minimising oxygen leakage, B - ozonation, C – photocatalitic * First biosensor used for rapid BOD estimation, ** depending on conditions, *** converted to BOD from GGA concentration. ND – not determined					

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SYMBOLS

Abbreviations

AWW	Artificial Wastewater
BOD	Biochemical Oxygen Demand
BOD_n	Biochemical Oxygen Demand measured after *n* days
DO	Dissolved oxygen
FIA	Flow Injection Analysis
GGA	D-glucose, L-glutamic acid solution
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of detection
UV	Ultraviolet

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