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## Methods for assessing biochemical oxygen demand (BOD): a review

S. Jouanneau, Loic Recoules, Marie-José Durand, Ali Boukabache, Vincent Picot, Y. Primault, Abdel Lakel, M. Sengelin, B. Barillon, Gerald Thouand

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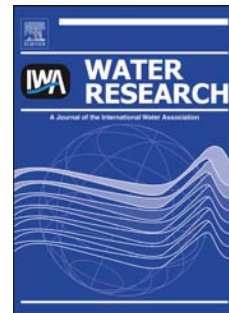
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# Accepted Manuscript

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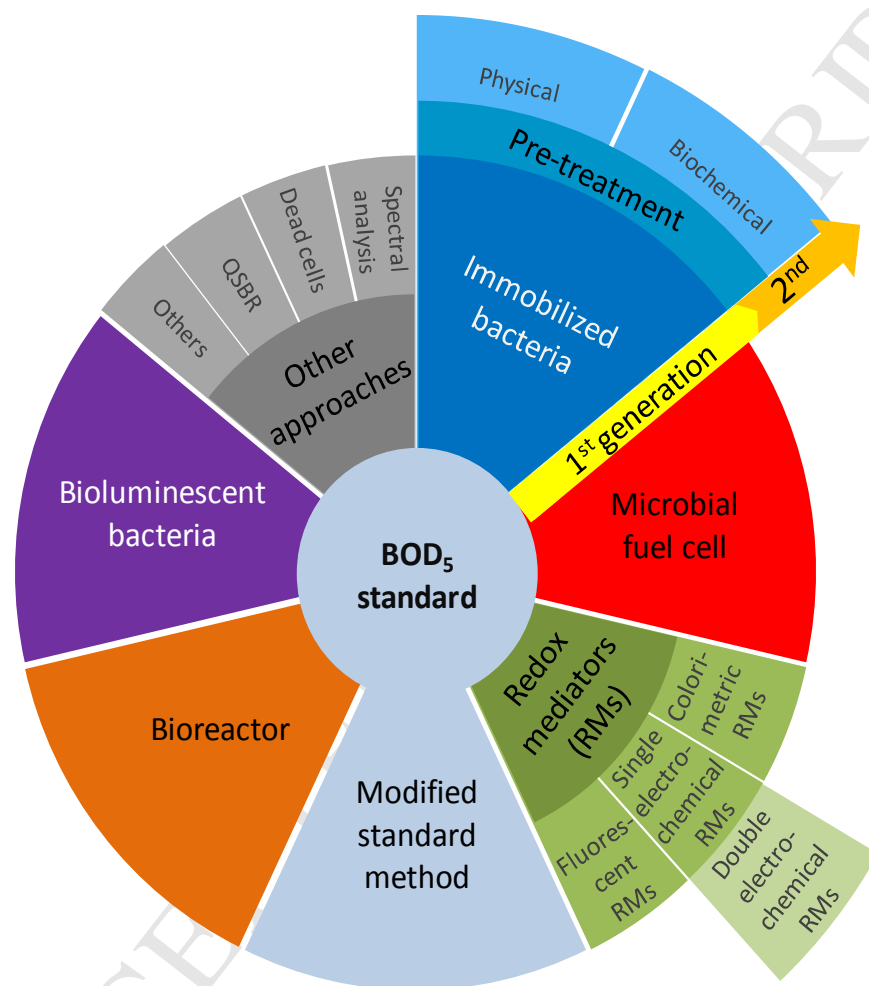
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## GRAPHICAL ABSTRACT

### Methods for assessing biochemical oxygen demand

(BOD): a review.



## HIGHLIGHTS

### Methods for assessing biochemical oxygen demand

#### (BOD): a review.

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- Alternative methods for monitoring biochemical oxygen demand have been reviewed.
- The concepts and the technical features are described.
- Some methods allow to assess BOD in only 70 seconds vs 5 days with the standard method.
- Performances of the various methods have been compared.

# 1 **Methods for assessing biochemical oxygen demand** 2 **(BOD): a review.**

3 Jouanneau<sup>(1)</sup> S, L. Recoules<sup>(2,3)</sup>, M.J. Durand<sup>(1)</sup>, A. Boukabache<sup>(2)</sup>, V. Picot<sup>(2)</sup>, Y.  
4 Primault<sup>(3)</sup>, A. Lakel<sup>(4)</sup>, M. Sengelin<sup>(5)</sup>, B. Barillon<sup>(6)</sup> and G. Thouand<sup>(1)\*</sup>

5  
6 (1) University of Nantes, UMR CNRS 6144 GEPEA CBAC, Campus de la Courtaisière,  
7 IUT, 18 Bd G. Defferre, 85035 La Roche sur Yon- France.

8 (2) LAAS-CNRS, 7, avenue du Colonel Roche, BP 54200, 31031 Toulouse cedex 4-  
9 France

10 (3) BIONEF, 73 rue de la Plaine, 75020 PARIS- France

11 (4) CSTB, 11 rue Henri Picherit, BP 82341, 44323 Nantes Cedex 3- France

12 (5) Sotralentz, 3 rue de Bettwiller BP 10028, 67320 Drulingen- France

13 (6) SUEZ Environnement, 38, Rue du Président Wilson, 78230 LE PECQ- France

14  
15 (\*) Corresponding author: Gerald THOUAND, Tel +(33)251478441. Fax  
16 +(33)251478456. E-mail: [gerald.thouand@univ-nantes.fr](mailto:gerald.thouand@univ-nantes.fr).

## 17 **Abstract**

18 The Biochemical Oxygen Demand (BOD) is one of the most widely used criteria for  
19 water quality assessment. It provides information about the ready biodegradable fraction  
20 of the organic load in water. However, this analytical method is time-consuming  
21 (generally 5 days, BOD<sub>5</sub>), and the results may vary according to the laboratory (20%),  
22 primarily due to fluctuations in the microbial diversity of the inoculum used.

23 Work performed during the two last decades has resulted in several technologies that are  
24 less time-consuming and more reliable. This review is devoted to the analysis of the  
25 technical features of the principal methods described in the literature in order to compare  
26 their performances (measuring window, reliability, robustness) and to identify the pros  
27 and the cons of each method.

28  
29 **Keywords:** Biochemical oxygen demand, assessment methods, biodegradation,  
30 monitoring, biosensor.

31

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64

## 65 1. Introduction

66 The biological measurement “Biochemical Oxygen Demand” (BOD) was selected in  
67 1908 as an indicator of the organic pollution of rivers by the U.K. Royal Commission on  
68 River Pollution. The traditional five day period to estimate the BOD<sub>5</sub> parameter was  
69 chosen for this test because this is supposedly the longest time that river water takes to  
70 travel from its source to its estuary in the U.K. (Great Britain. Royal commission on  
71 sewage disposal, 1908). Thereafter, this parameter was adopted by the American Public  
72 Health Association Standard Methods Committee in 1936 as a reference indicator to  
73 evaluate the biodegradation of chemicals and hazardous substances.

74 This parameter is defined as the amount of oxygen, divided by the volume of the system,  
75 taken up through the respiratory activity of microorganisms growing on the organic  
76 compounds present in the sample (e.g. water or sludge) when incubated at a specified  
77 temperature (usually 20 °C) for a fixed period (usually 5 days, BOD<sub>5</sub>). It is a measure  
78 of that organic pollution of water which can be degraded biologically. In practice, it is  
79 usually expressed in milligrams O<sub>2</sub> per litre (Nagel et al., 1992).

80 The BOD<sub>5</sub> has three major applications. First, it is an indicator of the conformity of the  
81 wastewater discharge and the waste treatment procedure to the current regulations.  
82 Second, in wastewater treatment plants, the ratio between BOD<sub>5</sub> and COD (chemical  
83 oxygen demand) indicates the biodegradable fraction of an effluent. Third, the ratio  
84 COD/BOD<sub>5</sub> is an indicator of the size of a wastewater treatment plant required for a  
85 specific location.

86 Conventionally, BOD is determined according to the standardised method described in  
87 the next paragraph. However, the main disadvantage of this approach is the time required  
88 for its achievement (5 days) (Riedel et al., 2002). The standardised method, which allows  
89 only a deferred analysis of the wastewater quality, does not appear today to be the most  
90 suitable tool for real-time environmental monitoring.

91 This test is one of the most widespread in the domain of water monitoring; however, the  
92 measurement variability in certified laboratories reaches 20 % (internal measurements),  
93 and this value increases between laboratories (comparative measurements) due to the  
94 variability of the microbial populations sampled (Guyard, 2010).

95 Due to the worldwide use of BOD methods, alternatives have been developed that range  
96 from static bioassays to online biosensors. In this review, we are interested in the analysis  
97 systems developed during the last twenty years to estimate BOD. Five main development  
98 strategies have been used by the research teams to design these new systems. Among  
99 these innovative technologies, some were transferred and marketed to industry.

## 100 2. How to assess BOD?

101 Aerobic biodegradation consists of oxidising organic matter biologically. During this  
102 process, the organic matter is converted by microorganisms into microbial biomass,  
103 eventual transformation products of biodegradation reaction (compounds derived from  
104 the initial organic matter), CO<sub>2</sub> and H<sub>2</sub>O, according to equation 1 (Swisher, 1987; Pagga,  
105 1997; Reuschenbach et al., 2003).

### 106 2.1. Standardised method

107 Traditionally, the BOD measurement is performed according to a standardised method,  
108 currently named the closed bottle test, described in the International Standards ISO 5815-  
109 1:2003 (*ISO 5815-1:2003 Water quality -- Determination of biochemical oxygen demand*  
110 *after n days (BOD<sub>n</sub>) -- Part 1: Dilution and seeding method with allylthiourea addition*,  
111 2003) (dilution and seeding method with allylthiourea addition) and ISO 5815-2:2003  
112 (*ISO 5815-2:2003 Water quality -- Determination of biochemical oxygen demand after n*  
113 *days (BOD<sub>n</sub>) -- Part 2: Method for undiluted samples*, 2003) (method for undiluted  
114 samples). To mimic the microbial diversity found in the environment, these tests are  
115 based on microbial samples generally taken from the environment (unknown microbial  
116 diversity, cellular density  $\approx 10^5$  cells/mL).

117 According to these standards, the protocol consists of putting the samples potentially  
118 contaminated with organic matter into specific bottles (Fig. 1.A), aerating them, and  
119 adding a microbial population. The bottles are then hermetically sealed and incubated in a  
120 dark room at 20°C. After an incubation period of *n* days, the dissolved residual oxygen is  
121 measured for all analysed samples to estimate the BOD. The standards specify that the  
122 oxygen determination must be performed by either the iodometric method (Winkler's  
123 method) (*ISO 5813:1983 Water quality -- Determination of dissolved oxygen --*  
124 *Iodometric method*, n.d.; Carpenter, 1965) or the electrochemical probe method (*ISO*  
125 *5814:1990 Water quality -- Determination of dissolved oxygen -- Electrochemical probe*  
126 *method*, n.d.).

127 A commercialised semi-automated version is available (Fig. 1.B). For this version, an  
128 electrochemical probe is inserted into the sealed bottle to measure the dissolved oxygen  
129 concentration in the sample in real time. This technical improvement allows the  
130 degradation kinetics of the organic matter to be determined in the analysis conditions  
131 imposed by the standard. The last version (Fig. 1.C) described in this section is an  
132 automated version of the standardised test marketed by Skalar (Netherlands). The  
133 protocol follows the specifications of the standard method, but the manual steps are  
134 replaced by an automated, artificial arm.

135 This standardised method measures the dissolved oxygen consumption in order to  
136 estimate the BOD value (Table 1). However, this test shows some limitations: a  
137 significant variability in the results (>20%), due mainly to the microbial population  
138 (Guyard, 2010), is observed; a proper working area is required; the duration (usually, 5



139 days) is not in accordance with industrial demand (quick screening to improve the  
140 monitoring of wastewater), and the measurement range of the organic load is limited by  
141 the amount of dissolved oxygen. Moreover, this test appears inappropriate for the online  
142 monitoring of processes found in wastewater treatment plants. Because of all of these  
143 limitations, several research teams are interested in designing new methods to assess the  
144 BOD providing alternatives to the standardised tests.

## 145 **2.2. A new generation of assessment methods**

146 The development of methods to assess the BOD is a prolific subject considering the  
147 number of published reports describing innovative systems to estimate this parameter.  
148 Figure 2 shows the increases in the numbers of referenced publications since 1977. More  
149 than 200 publications relevant to this topic were identified on the “web of science”  
150 database, with an annual publication rate of approximately 15 articles per year. In this  
151 review, the authors selected the publications presenting industrial or environmental  
152 applications or describing a new assessment strategy in this domain.

153 Among the referenced publications, it is possible to class the assessment methods into 6  
154 main technological categories, including a class dedicated to the modified standard  
155 method, as shown in figure 3. All these technologies were developed to improve the  
156 standard method according to different objectives:

- 157 - a decrease in the variability observed with the standard method,
- 158 - an increase in the measurement range of the organic load in samples to  
159 eliminate the dilution step necessary in the standard method,
- 160 - a reduction in the required working area,
- 161 - an increase in the analysis frequency to allow the online monitoring of the  
162 BOD.

163 The most frequently represented strategy in the listed literature is based on immobilised  
164 bacteria. These methods have been the subject of a second generation of development to  
165 improve the measurement by the addition of a pre-treatment step.

166 Other strategies have been considered to monitor the BOD in the environment based on  
167 the technology of microbial fuel cells (respiratory activity), the use of bioluminescent  
168 bacteria (cellular activity), the bioreactor or the use of the redox mediators (respiratory  
169 activity). The last category, like the method based on immobilised bacteria, has  
170 undergone a second generation of development based on the use of an additional  
171 complementary mediator.

172 All technologies quoted in figure 3 are described in detail in the following paragraphs.

### 173 3. Which alternative for the BOD assessment?

#### 174 3.1. *Methods of BOD measurement*

175 Because of the required time by the tests, the methods presented in this part are  
176 essentially designed to perform occasional analyses of BOD or to monitor an effluent  
177 with a measurement frequency inferior to one per week. They are primarily methods that  
178 present an improvement relative to the simplifying of the standard method (e.g., a  
179 decrease in the maintenance, a reduction of the working area or an enlargement of the  
180 measurement ranges of the organic load). Nevertheless, these methods are based on the  
181 same bacterial population (high variability) and the same required analysis duration  
182 (usually 5 days) as the reference method. Consequently, these methods present the same  
183 limits for the high throughput monitoring of the BOD in the environment (Table 1).

#### 184 3.1.1. **Modified reference method: improvement of the oxygen** 185 **measurement**

186 The first level of improvement relates to the oxygen probes. Indeed, according to the  
187 standard method, the measurement of the oxygen consumption should be performed with  
188 an electrochemical probe (Clark's electrode) or by the iodometric method. The principal  
189 advance is the oxygen optode (optical probe) based on a chemical indicator (a dynamic  
190 fluorescence quencher) that interacts with oxygen and causes a decrease in the  
191 fluorescence emission of the fluorophore proportional to the dissolved oxygen in the  
192 sample (Xu et al., 1994; McEvoy et al., 1996; McDonagh et al., 2001; Xiong et al.,  
193 2006).

194 The main advantage of these probes is that they require less care and maintenance than an  
195 electrochemical probe. Moreover, with these probes, the analysed sample is not modified  
196 during the measurement because the dissolved oxygen is not consumed by the sensor, and  
197 the oxygen measurement is not disturbed by flow velocity or electromagnetic fields  
198 (Klimant et al., 1995).

#### 199 3.1.2. **Photometric methods**

200 To reduce the working area required to perform a BOD test, Hach Lange (LCK 554 and  
201 LCK 555) and Macherey Nagel (BOD5 Nanocolor<sup>®</sup>) proposed cuvette tests. The tests  
202 follow the same protocol as the standard method (dilution of samples with high organic  
203 loads, incubation at 20°C in a dark room, variability of the microbial population, and  
204 required analysis time) and are based on the dissolved oxygen consumption by the  
205 microorganisms present in the samples analysed during this period.

206 The dissolved oxygen is analysed before and after the analysis period directly in the  
207 cuvettes. In the Hach Lange tests, the dissolved oxygen, after the addition of several  
208 reagents to the test cuvettes, forms a red dye proportional to the dissolved oxygen  
209 concentration. The measurement of this red dye by spectrophotometry allows the

210 estimation of the oxygen consumption and, therefore, the BOD. The measurement of the  
211 dissolved oxygen in the Macherey Nagel test is based on Winkler's method (the  
212 iodometric method). These 2 methods provide indirect estimates of the BOD.

### 213 **3.1.3. Manometric methods**

214 The method developed by Caldwell and Langelier (1948) is based on the measurement of  
215 pressure decrease due to the oxygen consumption by microorganisms oxidising the  
216 organic matter. In practice, the sample bottles are filled with a measured volume of  
217 sample. The microorganisms degrade organic substances using the gaseous oxygen  
218 trapped in the closed bottle. The carbon dioxide formed by this process is absorbed,  
219 generally with sodium hydroxide pellets. The pressure changes are measured by a  
220 manometer and converted to oxygen consumption by the device to estimate the BOD  
221 value (Fig. 4).

222 The BMS 6 method proposed by Velp Scientifica is a manual manometric system. The  
223 pressure decrease is measured with a mercury barometer and converted to oxygen using a  
224 graduated scale. 6 other commercial systems based on pressure sensors without mercury  
225 are listed in the literature: the BODTrak™ (Hach Lange, Germany), the Quick Scan BOD  
226 Analyzer (Challenge technology, USA), the OxiTop® (WTW), the OxyDirect  
227 (Tintometer, Germany), the BOD EVO Sensor (Velp Scientifica, Italy), and the CI-B5  
228 BOD ANALYZER (FanYuan Instrument, China). The measurement heads close the  
229 analysis bottles hermetically, and the pressure changes are measured with pressure  
230 sensors and converted to BOD.

231 These alternative methods are very widespread in the industrial sector because they are  
232 simple to use. Indeed, these methods allow the measurement of the BOD in samples  
233 contaminated by high levels of carbon compounds without making dilutions (0-700 mg/L  
234 of carbon versus 0-6mg/L with the reference method) because of the large pool of oxygen  
235 trapped in the bottle.

### 236 **3.2. Methods of BOD prediction for high-throughput analyses**

237 The systems developed in the above paragraph concern technical improvements of the  
238 reference method, nevertheless, the required time is stayed the same hence these methods  
239 failed to perform high-throughput analyses. The main issue for scientists was to reduce  
240 the required analysis duration imposed by the reference test in order to:

- 241 - to obtain quickly the analysis results, which would improve environmental  
242 monitoring,
- 243 - to increase the analysis frequency without increasing the necessary  
244 working area.

245 Several strategies have been considered and are described in details in the following  
246 paragraphs (Table 1). However, it is important to realise that the boundaries between  
247 these technologies are not always as strict as presented in this review. Indeed, some

248 methods combine several technologies. Consequently, for the sake of clarity, the methods  
249 presented below are classified according to the specifications deemed most relevant.

### 250 **3.2.1. Biosensors based on bioluminescent bacteria**

251 To reduce the analysis duration and the variability due to the unknown microbial  
252 populations used in the standard method, Sakaguchi et al. (2003, 2007) proposed two  
253 BOD assessment methods based on bioluminescent bacterial biosensors. According to the  
254 authors, the bioluminescence emission is correlated with the energy produced by the  
255 utilisation of a carbon source under aerobic conditions. The BOD is estimated from the  
256 bioluminescence emission intensity.

257 To limit the variability due to the microbial population, the authors used known bacterial  
258 strains, recombinant *Escherichia coli* containing the *luxCDABE* genes (from *Aliivibrio*  
259 *fischeri*) under control of the *tac* promoter (plasmid p22luxk described in patent JP,09-  
260 056398,A) (Sakaguchi et al., 2003) and *Photobacterium phosphoreum* (a naturally  
261 bioluminescent strain) (Sakaguchi et al., 2007).

262 The first method is based on recombinant *E.coli* (Sakaguchi et al., 2003) and can estimate  
263 the BOD<sub>5</sub> in 2 hours with limited accuracy. The coefficient of determination ( $r^2$ ) and the  
264 slope (S) of the linear model between the results provided by this device calculated from  
265 7 samples (n=7) are 0.674 and 0.385, respectively. The second system, based on a natural  
266 bioluminescent bacterial strain (Sakaguchi et al., 2007), showed better results than its  
267 predecessor ( $r^2=0.995$ ,  $S=1.018$ , n=5) with a reduced response time (25 min).

268 Nevertheless, the complexity of the bioluminescence reaction can limit the development  
269 of these systems because the reaction is regulated by many intracellular and extracellular  
270 factors (Watanabe and Hastings, 1986; Meighen, 1993; Sung and Lee, 2004) that can  
271 then affect the BOD estimation.

### 272 **3.2.2. Microbial fuel cells**

273 To eliminate the oxygen limitation in the biodegradation reaction, some research teams  
274 are working on the development of BOD biosensors based on the microbial fuel cell  
275 (MFC) technology. A MFC consists of an anaerobic compartment with an anode  
276 (negative electrode) and an aerobic compartment with a cathode (positive electrode)  
277 separated by a proton exchange membrane (Fig. 5). In the anaerobic compartment,  
278 microorganisms degrade organic matter and generate electrons and protons (Grzebyk and  
279 Poźniak, 2005; Rabaey and Verstraete, 2005). The protons migrate from this  
280 compartment to the cathode through the membrane, whereas the electrons pass from the  
281 anode to the cathode through an external electrical circuit, where oxygen is reduced to  
282 form H<sub>2</sub>O. The flow of electrons through the electrical circuit generates a measurable  
283 current proportional to the microbial biodegradation activity allowing the BOD of a  
284 sample to be estimated.

285 Among the systems described in the available publications (Kim et al., 2003; Chang et  
286 al., 2004, 2005; Jang et al., 2004; Kumlanghan et al., 2007; Di Lorenzo et al., 2009)  
287 about this subject, few were assessed in real environmental samples. The MFC developed  
288 by Kim et al. (2003) shows a good correlation with the BOD<sub>5</sub> obtained with the  
289 standardised method ( $r^2=0.999$ ,  $S=1.002$ ); however, the assessment was performed with  
290 only a few wastewater samples ( $n=3$ ).

291 Only one commercial system based on this technology has been referenced. This BOD  
292 sensor, the BOD High Accuracy BOD Sensor 2000 Analyzer (HABS-2000), is available  
293 from Korbi Co., Ltd. (Korea). According to the manufacturers, this biosensor is based on  
294 “electrochemically-active bacteria attached on the electrode of the MFC” allowing the  
295 BOD assessment. No information is available about the nature of these microorganisms  
296 (origin, density, diversity). The specifications of the HABS-2000 are: the measurement  
297 range is between 0.1 and 200 mg BOD/L (adjustable), the analysis duration is close to 30  
298 minutes (adjustable), and the measurement errors are lower than 5%. Nevertheless, no  
299 environmental application is described in the literature.

300 Moreover, this biosensor integrates a pre-treatment step to destroy the microflora of the  
301 samples to be analysed, specifically, exposure to ultra-violet (UV) radiation using a  
302 tangential flow method.

### 303 **3.2.3. BOD biosensors with redox-mediators**

304 Usually, microorganisms oxidise organic matter in aerobic conditions. However, when a  
305 redox-mediator is present in the medium, it acts as an electron acceptor instead of oxygen  
306 (Bennetto et al., 1983; Delaney et al., 1984; Roller et al., 1984; Ramsay and Turner,  
307 1988; Learoyd et al., 1992; Kaláb and Skládál, 1994; Yoshida et al., 2000).  
308 Consequently, the quantity of reduced redox-mediator generated by the biodegradation  
309 reaction is directly proportional to the metabolic activity (and therefore to the amount of  
310 biodegradable organic matter) and allows the assessment of the BOD. The main  
311 advantage is that with these redox-mediators, the biodegradation reaction does not  
312 depend on of oxygen in the reaction medium (Pasco et al., 2000). Moreover, with a  
313 redox-mediator, it is not necessary to dilute the samples to decrease the organic load to be  
314 degraded.

#### 315 *3.2.3.1. Electrochemical redox-mediator biosensors*

316 The mediator-type biosensors are derived from MFC technology. An electrochemical  
317 mediator is added to the compartment with the anode. It is reduced during the metabolic  
318 oxidation of the organic matter. The reduced mediator is then re-oxidised at the anode.  
319 The difference between the electrical potentials of the anode and the cathode provides a  
320 current proportional to the metabolic activity of the microorganisms. The methods  
321 detailed below are summarised in table 2.

#### 322 *Single redox-mediator*

323 The first mediator-type biosensors, described in 2000 by Pasco et al. (2000) and Yoshida  
324 et al. (2000), are based on the electrochemical reduction of potassium  
325 hexacyanoferrate(III) [ $\text{HCF}_{(\text{III})}$ ] to potassium hexacyanoferrate(II) [ $\text{HCF}_{(\text{II})}$ ]. The addition  
326 of a substrate to medium containing an excess of redox-mediator [ $\text{HCF}_{(\text{III})}$ ] results in an  
327 increase in the metabolic activity of the bacteria (*Escherichia coli* and *Pseudomonas*  
328 *fluorescens*) and the quantity of reduced mediator [ $\text{HCF}_{(\text{II})}$ ]. The reoxidation of  $\text{HCF}_{(\text{II})}$   
329 generates a current quantifiable by a coulometric (Pasco et al., 2000) or an amperometric  
330 (Yoshida et al., 2000) transducer. Following these reports, several studies were  
331 performed to improve the performances of these devices (Catterall et al., 2001, 2003;  
332 Trosok et al., 2001; Yoshida et al., 2001; Pasco et al., 2004; Oota et al., 2010).

### 333 **Double redox-mediators**

334 The biosensors based on a single electrochemical redox-mediator were mainly developed  
335 with prokaryotes (Pasco et al., 2000, 2004; Yoshida et al., 2000, 2001; Catterall et al.,  
336 2001, 2003; Timur et al., 2007). Baronian et al. (2002) proposed a double-mediator  
337 system combining potassium hexacyanoferrate(III) (a hydrophilic mediator) and  
338 menadione (a lipophilic mediator) using eukaryotic cells (*Saccharomyces cerevisiae*).  
339 Menadione has the ability to cross cell membranes, and it reacts with NAD(P)H to  
340 generate menadiol. Menadiol shuttles electrons from the intracellular compartment to the  
341 cell wall to reduce the hydrophilic mediator. The reoxidation of potassium  
342 hexacyanoferrate(II) generates a current proportional to the cellular activity (Yashiki and  
343 Yamashoji, 1996).

344 This strategy was used by Nakamura et al. (2007) in 2006 to design an offline disposable  
345 micro-batch-type biosensor (Fig. 6) (Japanese Patent Application Raid-Open Disclosure  
346 P2008/96415A) using *Saccharomyces cerevisiae*. In the presence of biodegradable  
347 organic matter, the current generated by the redox reactions in the chip is measured with  
348 a two-electrode system and converted into  $\text{BOD}_{\text{DM}}$  ( $\text{BOD}_{\text{DM}} = \text{BOD}_5$  estimated by the  
349 double-mediator system).

350 One year later, Nakamura et al. (2007) developed an optical system based on previous  
351 work. This new BOD biosensor is based on the production of chemiluminescence due to  
352 the redox reaction of organic matter by *Saccharomyces cerevisiae* (Fig. 7). In the  
353 presence of biodegradable compounds, menadione is reduced into menadiol. The  
354 reoxidation of menadiol generates hydrogen peroxide which reacts with Luminol and  
355 hydroxide anions, catalysed by potassium hexacyanoferrate(III), to produce  
356 chemiluminescence. The performances of these methods to estimate  $\text{BOD}_5$  are  
357 summarised in table 2.

#### 358 *3.2.3.2. Other redox-mediators*

359 Dudal et al. (2006) and Tizzard et al. (2006) developed a 96-well microplate method  
360 based on resazurin. This blue redox mediator (water-soluble and not fluorescent)  
361 penetrates into bacteria and is reduced by cellular activity to resorufin (water-soluble,

362 pink fluorescent compound). The fluorescent intensity emitted in the microplate wells  
363 after the incubation period is directly proportional to the quantity of organic matter  
364 degraded by the microorganisms in the analysed samples. Therefore, monitoring the  
365 fluorescence produced provides an estimate of the BOD. This strategy was used in the  
366 commercial test in 96-well microplates (Enverdi) provided by Envolve (a French  
367 company founded by Dudal Y. and Pautremat N.), patent WO/2006/079733. With this  
368 method, the assessment of the BOD<sub>5</sub> is 8 times faster than with the reference method (15  
369 hours compared to the 5 days required by the reference method), and the measurement  
370 range is increased from 6 mg BOD/L to 300 mg BOD/L with the reference method  
371 without dilution.

372 This redox mediator can also be used as a redox colour indicator. Indeed, resazurin is a  
373 blue dye (optimal absorbance at 605 nm) which turns red after electrochemical reduction  
374 to resorufin (optimal absorbance at 573 nm) (Czekanska, 2011). Another redox colour  
375 indicator, 2,6-dichlorophenolindophenol, was used by Nakamura et al. (2007) to estimate  
376 BOD from the cellular activity.

377 The main characteristics of the methods are summarised in Table 2. According to the data  
378 provided by the investigators, it appears that the response times obtained with these BOD  
379 sensors are significantly less than with the standard method, 15 hours (maximum  
380 duration) compared to 5 days. This improvement is most likely due to the redox-  
381 mediator, which acts as the electron acceptor instead of oxygen and allows acceleration  
382 of the biodegradation reaction.

383 Others factors can also explain the difference observed with the reference method  
384 including the use of pure selected strains (bacteria or yeast) at cellular densities much  
385 higher than those employed in the reference test.

### 386 **3.2.4. Biosensors with entrapped bacteria**

387 This strategy, the most frequently represented in the literature, was described for the first  
388 time in 1977 by Karube et al. (1977). Since this work, several studies have been  
389 published describing systems to estimate the BOD based on immobilised bacteria either  
390 with or without pretreatment of the effluent.

#### 391 *3.2.4.1. Biosensors without pretreatment of the samples*

392 The principle of this measurement consists of immobilised bacteria that allow direct  
393 contact with the measurement electrode. The BOD is estimated from the oxygen  
394 consumption measured by the electrode (ratio between the O<sub>2</sub> consumption without and  
395 with the sample). According to the authors, this configuration allows, on one hand, a  
396 reduction in the analysis time of a sample and, on the other hand, a simplification of the  
397 assay.

398 Different immobilisation modes were used according to the authors; nevertheless, it is  
399 possible to class these techniques into three groups. In the first case, bacteria are

400 entrapped inside a polymer network that limits their movements (Table 3). Several  
401 matrices were assessed, including alginate (Kumlanghan et al., 2008), agarose (Raud et  
402 al., 2012; Raud and Kikas, 2013), poly(carbamoyl)sulfonate (PCS) (Chan et al., 1999,  
403 2000; Lehmann et al., 1999), silica gel (Oota et al., 2010), sol-gel composite material  
404 (silica and poly(vinyl alcohol)-grafted-poly(vinylpyridine)) (Jia et al., 2003; Kwok et al.,  
405 2005; Liu et al., 2009, 2011), Al<sub>2</sub>O<sub>3</sub> sol-gel (Chen et al., 2002), resin (Yang et al., 1996),  
406 polyvinyl alcohol (PVA) (Preininger et al., 1994), polyurethane (Köster et al., 2006), or  
407 ormosil-PVA (Dai et al., 2004; Jiang et al., 2006; Lin et al., 2006; Xin et al., 2007). All  
408 these polymers are biocompatible and have low toxicity for microorganisms.  
409 Nevertheless, the chosen polymer depends on the application. Alginate and agarose  
410 hydrogels are particularly easy to use but may not have adequate mechanical stability  
411 under some conditions (Chan et al., 1999). PCS and sol-gel polymers are more complex  
412 to use but more mechanically resistant. Moreover, sol-gel polymers are described as  
413 chemically inert and photochemically and thermally stable. The second immobilisation  
414 method consists of sandwiching cells between two polymer layers. Several polymer  
415 combinations were used in the studies cited, including dialysis membrane / Teflon<sup>®</sup>  
416 membrane (Liu et al., 2000), cellulose acetate membrane / fluorinated ethylene propylene  
417 membrane (FEP) (Li and Chu, 1991), Teflon<sup>®</sup> membrane / Teflon<sup>®</sup> membrane (Kim and  
418 Park, 2001), Polycarbonate membrane / Teflon<sup>®</sup> membrane (Suriyawattanakul et al.,  
419 2002). The last method of microbial immobilisation consists of adsorbing the cells to  
420 solid supports, e.g., porous membranes (cellulose (Chee, Nomura, and Karube, 1999),  
421 glass-fibre (Arlyapov et al., 2012) or nylon (Rastogi, Kumar, et al., 2003; Rastogi,  
422 Rathee, et al., 2003)).

423 In most cases, the oxygen monitoring is performed with an amperometric electrode  
424 (Clark electrode). Nevertheless, among the biosensors described above, some systems do  
425 not use this type of probe. Indeed, the biosensors developed by Preininger et al. (1994),  
426 Lin et al. (2006), Jiang et al. (2006), Kwok et al. (2005), Xin et al. (2007) and Dai et al.  
427 (2004) use a fluorescent probe (a ruthenium complex) to monitor the dissolved oxygen  
428 consumption resulting from the biodegradation of the organic matter (see 3.1.1.).

429 Finally, the system developed by Liu et al. (2012) is based on a microbial biofilm formed  
430 on the internal surface of glass tube. The principle differs slightly from the methods  
431 described above since the microorganisms are not in direct contact with the oxygen  
432 probe. The sample to be analysed circulates in a continuous flow inside the glass tube,  
433 and the oxygen consumption is measured at the outlet of the tube containing the  
434 microbial biofilm.

435 The specifications of the systems detailed in the literature are described in Table 3.

436 According to the authors, these biosensors are able to estimate the BOD<sub>5</sub> with an analysis  
437 duration that does not exceed 90 minutes (the minimal reported duration is 70 seconds).  
438 However, in one third of the cases, the validation results are non-existent or irrelevant for  
439 environmental monitoring (correlation factor < 0.6).



440 In most of cases (about 60%), the BOD biosensors are based on only one microbial strain  
441 ( yeast: 65% or bacteria: 35%) instead of microbial mixture as in the reference method.  
442 The recent works of Raud and Kikas (2013) propose an alternative strategy to predict the  
443 BOD value based on a bacterial panel composed of seven strains used individually. For  
444 this, data provided by each strain are simultaneously analysed with statistical models. The  
445 results are hopeful since the predicted BOD values are totally correlated with the  
446 measured BOD<sub>7</sub> ( $r^2=1$  and  $S=1$  and  $n=30$ ).

#### 447 *3.2.4.2. Biosensors with a pretreatment of the samples*

448 To improve the accuracy and the rapidity of the biosensors based on immobilised  
449 bacteria, several research teams added a pretreatment step before the measurement phase  
450 (Table 4). Some recalcitrant pollutants (i.e., compounds of high molecular weight) can  
451 require a lengthy lag phase before becoming biodegradable by microorganisms. Due to  
452 the long period of analysis required by the reference method, some of these compounds  
453 are counted in the BOD measurement but not in the experimental biosensor assays. The  
454 purpose of a supplementary step is to hydrolyse, physically or biochemically, the  
455 macromolecules (which are more difficult to degrade) in the samples to compounds of  
456 lower molecular weight that can be biodegraded more easily than the parent compounds  
457 (Beltrán et al., 1997).

458 Chee et al. used two physico-chemical pretreatment methods to improve the effectiveness  
459 of their immobilised bacterial biosensor (Chee, Nomura, and Karube, 1999), ozonation  
460 (Chee, Nomura, Ikebukuro, et al., 1999) or irradiation with ultra-violet (UV) light (Chee  
461 et al., 2001, 2005). From the results obtained with the first pretreatment method, it seems  
462 that ozonation does not improve the biosensor performances in contrast to UV irradiation.  
463 Indeed, in the two studies based on this technology, the correlation between the biosensor  
464 and the reference method increased when the samples were pretreated.

465 The biochemical pretreatment consists of adding specific enzymes to accelerate the  
466 biodegradation of organic macromolecules. Reiss et al. (1998) added an enzymatic  
467 pretreatment before the microbial biosensor, which significantly (6%) improved the  
468 effectiveness of the short-term BOD biosensor to predict the BOD<sub>5</sub> parameter. However,  
469 the system is described for a specific application (samples contaminated with starch).  
470 Finally, Kim and Park (2004) proposed a similar approach based on an additional  
471 enzymatic pretreatment designed to hydrolyse disaccharides and/or polysaccharides.  
472 Nevertheless, the results obtained with the supplementary pretreatment step deviated  
473 significantly from the BOD<sub>5</sub> values provided by the reference method. Indeed, some  
474 recalcitrant compounds are biodegraded by the enzymatic hydrolysis (pretreatment)  
475 during the analysis, whereas they are not degraded with the reference method.

#### 476 **3.2.5. Biosensors based on the bioreactor/chemostat technology**

477 The BOD biosensors based on the bioreactor/chemostat technology were mainly  
478 developed for online applications. The principle of these biosensors is based on

479 monitoring the oxygen consumption of the microorganisms in contact with the sample, as  
480 with the standard method. However, these biosensors are continuously fed with  
481 wastewater saturated with dissolved oxygen (Vernimmen et al., 1967). An oxygen probe  
482 measures the variations in the respiration rate to deduce the BOD in the sample. Thus,  
483 with this technical design, on the one hand, the cellular density is higher than in the  
484 environment because of the continuous feeding of organic matter, and, on the other hand,  
485 the microbial community within the bioreactor is totally adapted to the organic substrate,  
486 significantly reducing the analysis time. The response time is generally less than 45  
487 minutes, and it allows the effluents to be monitored in real time.

#### 488 *3.2.5.1. Experimental biosensors*

489 In this area, some recent studies of new BOD biosensors have been reported over the last  
490 decade. In 2004, Liu et al. published a study (2004a, 2004b) of the development of a  
491 short-term BOD biosensor for the online monitoring of biological treatment processes  
492 (Fig. 8). In this system, free microorganisms (activated sludge collected from a municipal  
493 wastewater treatment plant) are maintained in liquid inside a measurement cell with a  
494 dialysis membrane to perform the analyses. The response time is nearly 60 seconds  
495 ( $r^2=0.544$ ,  $S=0.789$  and  $n=4$ ).

496 Two other bioreactor-type biosensors developed by Wang et al. (2010) and Villalobos et  
497 al. (2010) are hydride systems featuring bacteria immobilised in PVA/alginate beads in  
498 suspension in a traditional bioreactor. The performance of these systems seems  
499 interesting. The response time is close to 30 minutes with a high correlation with the  
500 result obtained with the reference method ( $r^2 = 0.99$  and  $S \approx 1$  (Villalobos et al., 2010;  
501 Wang et al., 2010)), but the result needs to be validated because it was determined from a  
502 limited number of samples (3 or 5).

503 In a recent publication, Torrents et al. (2012) proposed a miniaturised reactor-type  
504 biosensor based on a microfluidic respirometer. The system consists of a double flow cell  
505 in which two microchannels (reaction chamber and electrolyte channel) are separated by  
506 an oxygen permeable membrane. The reaction chamber contains the bacteria and the  
507 samples to be analysed. The second channel has a constant flow of electrolyte saturated  
508 with oxygen. Oxygen diffuses through the membrane from the electrolyte chamber to the  
509 reaction chamber to be consumed by aerobic bacteria (unspecified by the authors). The  
510 monitoring of the oxygen at the end of the electrolyte chamber provides information on  
511 the bacterial activity and allows the estimation of BOD.

#### 512 *3.2.5.2. Commercial biosensors*

513 This technology is the most widespread among the commercialised biosensors dedicated  
514 to the online monitoring of wastewater. This wide distribution is most likely due to the  
515 accumulated experience since the first publication in 1967 (Vernimmen et al., 1967).  
516 Figure 9 is a schematic representation of the architecture of the systems, including Ra-  
517 BOD<sup>®</sup> (Applitek, Belgium), Biox-1010 (Endress+Hauser, Switzerland), MB-DBO

518 (Biosensores, Spain) and RODTOX 2000 (Kelma, Belgium) (Table 5). These biosensors  
519 are based on three inoculation strategies.

520 - For Ra-BOD<sup>®</sup>, the measurement bioreactor is continuously inoculated  
521 with real sludge from a wastewater treatment plant.

522 - With the Biox-1010 system, the microorganisms colonise small plastic  
523 cylinders inside the bioreactor (to protect them against mechanical  
524 abrasion caused by turbulent mixing and to increase the contact surface  
525 with the sample being analysed) before the measurement is performed.

526 - In the biosensor MB-DBO, the microorganisms first grow in an  
527 independent continuous bioreactor that allows to stabilise the microbial  
528 population and to feed the measurement reactor continuously.

529 The biosensor BioMonitor (LAR, United States) is also a biosensor based on the  
530 bioreactor technology; however, this architecture is totally different than the other  
531 systems presented above. It consists of four successive reactors that work exactly like an  
532 aeration tank (Fig. 10). The oxygen consumption is measured in the gaseous fraction of  
533 the last reactor of the measurement channel, and the determination of the BOD parameter  
534 is calculated from the difference between the oxygen consumption in the control and the  
535 test sample.

536 According to the manufacturer, this configuration allows more rapid degradation than in  
537 the biosensors with only one reactor, and it facilitates the degradation of substances that  
538 are difficult to degrade. Due to this improvement, the BioMonitor is able to estimate with  
539 reliability and accuracy the BOD<sub>5</sub> in a sample in only 4 minutes (Table 5). Moreover, it is  
540 important to emphasise the presence of a control channel that allows the estimation of the  
541 toxicological impact of the effluent on the microbial community, and limits interpretation  
542 errors (underestimation of the BOD value). Nevertheless, no environmental application  
543 has been reported in the literature.

544 The principal advantage of these biosensors is the rapidity of the measurement. However,  
545 they are only reliable under specific conditions. On one hand, the parameters of the  
546 analysed samples must remain relatively constant during the time of the assay (diversity  
547 of the organic load, physico-chemical parameters), and, on the other hand, an important  
548 step of calibration is necessary to adapt the system (microbial population or calibration  
549 curve) to the samples. Consequently, these devices are efficient for the online monitoring  
550 of relatively stable and known effluents but seem to be unsuitable for analysis of  
551 diversified samples.

### 552 **3.3. Other approaches**

553 To estimate the BOD, two criteria are usually used: the oxygen consumption (reference  
554 method, modified standard methods, immobilised bacteria based biosensors and  
555 bioreactor-type biosensors) and the cellular activity (bioluminescent bacteria based  
556 biosensors, microbial fuel cells and mediator-type biosensors) due to the biodegradation

557 metabolism. In contrast, the system developed by Chiappini et al. (2010) uses the carbon  
558 dioxide produced from the biodegradation process as the BOD indicator. However, the  
559 efficiency of this system is relatively low; the error rate was approximately 35% for 5  
560 samples.

561 Tan and Qian (Tan and Qian, 1997; Qian and Tan, 1998, 1999; Tan and Lim, 2005)  
562 proposed to replace the living bacteria by dead cells. Indeed, living cells are generally  
563 sensitive to their environment and require careful maintenance of their living conditions  
564 to ensure their survival. Consequently, the authors used the enzymatic material extracted  
565 from cellular lysates of *Bacillus subtilis* (Tan and Qian, 1997; Qian and Tan, 1998, 1999)  
566 or multi-species microbial cells (BODseed, Cole-Parmer E-05466-00) (Tan and Lim,  
567 2005). The enzymes are adsorbed on filters and mounted on an amperometric oxygen  
568 probe. According to the authors, the error rate of the BOD<sub>5</sub> estimation is approximately  
569 6% with environmental samples (domestic and industrial wastewaters).

570 Other systems have been developed, without biological sensors, based on analysing the  
571 spectrum of the samples to estimate the BOD (Reynolds and Ahmad, 1997; Hur et al.,  
572 2010; Lai et al., 2011; Hur and Cho, 2012). With these approaches, it is possible to  
573 monitor water effluents online and in real time, in contrast to all the systems described  
574 above. Several spectral markers have been used to estimate the BOD: UV absorbance  
575 (Dobbs et al., 1972; Mrkva, 1975, 1983; Bari and Farooq, 1985; Edwards and Cresser,  
576 1987; Reynolds and Ahmad, 1997), fixed-wavelength fluorescence (Reynolds and  
577 Ahmad, 1997), synchronous fluorescence spectra (Hur et al., 2010; Lai et al., 2011) and  
578 three dimensional fluorescence matrix (Hur and Cho, 2012). Two system architectures  
579 are available for online BOD monitoring, the “BOD probe” or the “BOD online  
580 analyser”. The three systems, the Stamosens CSM750/CSS70, the carbo::lyser and the  
581 STAC (Constant et al., 2009), commercialised by Endress+Hauser (Switzerland),  
582 S::CAN (Austria) and Secomam (France), respectively, belong to the first category. They  
583 are composed of two parts, a sensor directly dipped in the analysed sample and a  
584 transducer. In contrast to the “BOD probe” system, with the “BOD online analyser”, no  
585 probe is dipped into the sample. A pump removes the samples, which are analysed in an  
586 online UV spectrophotometer. This architecture is used in the BOD Online Analyzer  
587 (AWA instruments, Singapore).

588 In particular cases of the screening of new compounds, there exist predictive models,  
589 namely, the quantitative structure-biodegradability relationships (QSBR), able to estimate  
590 the biodegradability of compounds from their physico-chemical descriptors (Baker et al.,  
591 2004). At first, statistical models are designed by supervised learning from an existing  
592 database of molecular descriptors (longest aliphatic chain, molecular linear free energy  
593 relation, etc.) of chemical compounds (Geating, 1981; Okey and Stensel, 1996; Raymond  
594 et al., 2001; Arora and Shi, 2010; Cheng et al., 2012). Then, these models are applied to  
595 unknown molecules to predict their biodegradability. In recent work (2012), Cheng et al.  
596 (2012) compared the biodegradability prediction obtained from their statistical models  
597 with the biodegradability measured with a standardised test (MITI, the equivalent of the

598 BOD test for the longest period, 28 days) on 27 compounds. The results are encouraging  
599 as all 27 compounds were correctly predicted. However, with unknown samples without  
600 characterisation data (composition, physico-chemical characterisation of present  
601 chemicals), as is the case in environmental monitoring, this strategy does not seem  
602 applicable.

## 603 **4. Technological evaluation**

604 In order to compare the main strategies of BOD assessment described above, the main  
605 assets and drawbacks of each method are listed in table 1. These methods are classified in  
606 two categories i.e. the real measurement or the mathematical prediction.

### 607 **4.1. Technical aspect**

608 In the first case (real measurement), the BOD measurement is performed on the entire  
609 study period (5 days for the BOD<sub>5</sub> assessment) as with the reference method. For this  
610 purpose, the oxygen consumption is generally monitored with dissolved oxygen probes  
611 (electrochemical or optical) or with pressure sensor. In all cases, many reports of these  
612 technologies are available about field applications (environmental monitoring or  
613 chemical characterization). However, the delay for analysis constrains significantly the  
614 high throughput analyses and notably, the online monitoring of effluents.

615 The second class described in this manuscript concerns the methods for the BOD  
616 prediction. The required time for analysis is significantly reduced by comparison with the  
617 measurement methods. Indeed, with these predictive methods, the average time to  
618 perform an analysis is about 30 min (min: 70 seconds, max= 15 hours) with a broad  
619 measurement ranges (0 – 500,000 mg BOD/L). Nevertheless, few data about field  
620 applications are available and the provided results, predicted from statistical model of  
621 correlation (generally: linear model) are not always reliable ( $-0.53 < r^2 < 1$ ) because,  
622 notably, of the calibration methods based on inappropriate reference chemicals or  
623 unsuitable microbial inoculums (pure strains).

624 Among the fastest assessment methods, the biosensors with entrapped bacteria are  
625 widespread in literature (more than 50% of publications described in this manuscript), but  
626 despite this, no marketed system based on this technology is available (only two  
627 technologies are commercially available: the microbial fuel cells and the bioreactors).  
628 This absence can be explained by the cellular growth inside the membrane which can  
629 modify the characteristics of biosensor (response time, reliability, correlation with the  
630 calibration curve) (Jouanneau et al., 2012) and consequently, limit the commercial  
631 development of these BOD biosensors.

### 632 **4.2. Biological aspect**

633 Two types of microbial inoculums are usually used, environmental microbial populations  
634 (as in the standardised method) or pure strains. The former population can degrade a

635 large panel of organic compounds (Liu et al., 2000). However, the composition of these  
636 inocula is not controlled impairing the reproducibility of the BOD measurement (Blok  
637 and Booy, 1984; Thouand et al., 1995, 1996). Conversely, in the second case, the use of  
638 only one microbial strain (bacteria or yeast) improves the reproducibility of the  
639 measurements but limits the biological potential of biodegradation. Some authors  
640 addressed the problem by using an artificial microbial mixture of 2- 4 strains (Catterall et  
641 al., 2003; Jia et al., 2003; Jiang et al., 2006; Lin et al., 2006; Xin et al., 2007) to obtain a  
642 sensor combining good reproducibility with a large biodegradation potential. However,  
643 these multi-species mixtures are difficult to control in a long-term because of competition  
644 for the carbon substrate.

645 An alternative for the future development would be to use individually several microbial  
646 strains in order to preserve important degradation capacities while simplifying the control  
647 of cells, as in the publication of Raud and Kikas (2013). The utilization of a microbial  
648 panel to estimate the BOD involves the implementation of analytical statistical tools more  
649 complex than the usual method based on linear regression used in the majority of the  
650 publications. Indeed, this approach requires a multi-parametric analysis of data provided  
651 by all strains. Also, with this configuration, it would be interesting to use a QSBR-type  
652 model based on biological descriptors instead of molecular descriptors in order to predict  
653 accurately the BOD: Qualitative Activity-Biodegradation Relationship (QABR) model.

654 Finally, it is important to emphasize the absence of toxicity control in the tests presented  
655 in this review, except for the BioMonitor described in paragraph 3.2.5.2. The direct  
656 consequence of this is the risk of underestimating the BOD value due to an alteration of  
657 the microbial population. Consequently, the future development of BOD biosensors  
658 would integrate a complementary toxicity sensor.

### 659 **4.3. Summary**

660 The methods of real measurement are relatively easy to use, technologically reliable  
661 (oxygen or pressure probe) and many data about them are available in literature.  
662 Nevertheless, they require long analysis periods and, generally, an important workspace.  
663 The provided results depend directly on environmental inoculums (20% of variability).  
664 Conversely, the predictive methods need a reduced time to perform analyses. The fastest  
665 system provides the BOD<sub>5</sub> of a sample in only 70 seconds instead of 5 days. However,  
666 the majority of the described methods requires an average time of 30 minutes to estimate  
667 the BOD<sub>5</sub>. To overcome the variability due to the environmental inoculums, recent  
668 publications propose to use artificial inoculums or a set of pure bacterial strains. In all  
669 cases, the reduced measurement period implies the use of a statistical correlation model  
670 to predict a BOD value.

## 671 **5. Conclusion**

672 This review has identified the main technological strategies designed to measure or to  
673 estimate the BOD parameter, used as an indicator of the biodegradability of organic  
674 matter. It focuses on the technological aspect of the assessment methods, on the nature of  
675 the performed measurement (real measurement or prediction) and on the pros and cons of  
676 them.

677 From a technical point of view, the latest advances show that the “measurement” aspect  
678 of biological signals (bioluminescence, cellular activity, oxygen consumption) is reliable  
679 and robust. Nevertheless, the research issues should focus on the biological aspect of  
680 these systems in order to find a compromise between the standardization of the inoculum  
681 and environmental diversity (both of which are opposed on the criterion of the biological  
682 variability). These future systems should also integrate toxicity markers in order to limit  
683 the risk of BOD underestimating.

684 The second point concerns the decrease of the workspace which induces a miniaturization  
685 of the assessment methods and indirectly of the size of the tested samples. This  
686 consequence raises the issue about the representativity of increasingly smaller samples.

687 The choice of a method for the BOD assessment should take into account the tolerated  
688 error rate, the measurement frequency, the nature of the studied matrices (domestic or  
689 industrial wastewaters) and the type of considered applications (on-line, in situ, ex situ).

690

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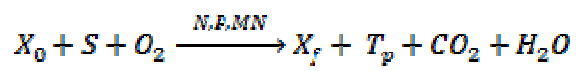
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Eq.1



$X_0$ : Initial biomass

$S$ : Organic carbon sources

$O_2$ : Oxygen

$N$ : Nitrogen source

$P$ : Phosphorus source

$MN$ : mineral nutrients

$X_f$ : Final biomass

$T_p$ : Transformation products of biodegradation

$CO_2$ : Carbon dioxide

$H_2O$ : Water

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Table 1: Comparison of main strategies of BOD<sub>5</sub> assessment (With a blue background: Method of BOD measurement; without background: Method of BOD prediction).

Technology	Biodegradation marker	Transducer	Required time (Median)	Benefits	Disadvantages
Reference method	Dissolved O <sub>2</sub>	Iodometric dosage Electro-chemical probe	5 days	<ul style="list-style-type: none"> <li>Real BOD<sub>5</sub> value</li> <li>Many reports about field applications</li> <li>Marketed version</li> </ul>	<ul style="list-style-type: none"> <li>Long period of analysis</li> <li>Narrow measurement range (BOD: 0 – 6 mg/L*)</li> <li>Manual dosage / Maintenance of probes</li> <li>Measurement variability (inoculum)</li> <li>Important working area required</li> </ul>
Modified reference method	Dissolved O <sub>2</sub>	Optical probe	5 days	<ul style="list-style-type: none"> <li>Real BOD<sub>5</sub> value</li> <li>Non-invasive probes</li> <li>Many reports about field applications</li> <li>Marketed version</li> </ul>	<ul style="list-style-type: none"> <li>Long period of analysis</li> <li>Narrow measurement range (BOD: 0 – 6 mg/L*)</li> <li>Measurement variability (inoculum)</li> <li>Important working area required</li> </ul>
Photometric method	Dissolved O <sub>2</sub>	Spectro-photometer	5 days	<ul style="list-style-type: none"> <li>Real BOD<sub>5</sub> value</li> <li>Ready to use kit</li> <li>Small working area required</li> <li>Many reports about field applications</li> <li>Marketed version</li> </ul>	<ul style="list-style-type: none"> <li>Long period of analysis</li> <li>Narrow measurement range (BOD: 0 – 6 mg/L*)</li> <li>Measurement variability (inoculum)</li> </ul>
Manometric method	Pressure	Manometer	5 days	<ul style="list-style-type: none"> <li>Wide measurement range (BOD: 0 – 700 mg/L)</li> <li>Many reports about field applications</li> <li>Marketed version</li> </ul>	<ul style="list-style-type: none"> <li>Long period of analysis</li> <li>Indirect measurement</li> <li>Equivalent BOD<sub>5</sub></li> <li>Measurement variability (inoculum)</li> <li>Important working area required</li> </ul>
Biosensor based on bioluminescent bacteria	Bioluminescence activity	Luminometer	72 min	<ul style="list-style-type: none"> <li>Short period of analysis</li> <li>Wide measurement range (BOD: 0 – 200 mg/L)</li> <li>Easy to use</li> <li>Small working area required</li> </ul>	<ul style="list-style-type: none"> <li>Only one bioluminescent strain = limited range of biodegradable chemicals</li> <li>Indirect measurement</li> <li>Predicted BOD<sub>5</sub></li> <li>Bioluminescence instability</li> <li>Very few reports about field applications</li> <li>No marketed version</li> </ul>
Microbial fuel cells	Electrical potential	Amperometer	315 min	<ul style="list-style-type: none"> <li>Short period of analysis</li> <li>Widespread measurement range (BOD: 0 – 200 mg/L; max 100,000 mg/L**)</li> <li>Low maintenance</li> <li>Configuration allowing the online monitoring</li> <li>Marketed version</li> </ul>	<ul style="list-style-type: none"> <li>Indirect measurement</li> <li>Measurement variability (inoculum)</li> <li>Predicted BOD<sub>5</sub></li> <li>Few reports about field applications</li> </ul>
Redox-mediator	Redox -mediator	Amperometer, luminometer, fluorimeter or Spectro-photometer	15 min	<ul style="list-style-type: none"> <li>Short period of analysis</li> <li>Wide measurement range (BOD: 0 – 300 mg/L)</li> <li>Marketed easy-to-use version (fluorescent redox-mediator) which requires a small working area</li> </ul>	<ul style="list-style-type: none"> <li>Predicted BOD<sub>5</sub></li> <li>Indirect measurement</li> <li>Low accuracy of equivalent BOD<sub>5</sub> assessment</li> <li>Few reports about field applications</li> </ul>
Biosensor with entrapped bacteria	Dissolved O <sub>2</sub>	Electro-chemical or optical probe	10 min	<ul style="list-style-type: none"> <li>Direct measurement</li> <li>Short period of analysis</li> <li>Wide measurement range (BOD: 0 – 500 mg/L)</li> <li>Configuration allowing the online monitoring</li> </ul>	<ul style="list-style-type: none"> <li>Diffusion (oxygen, chemicals) in polymer matrix or membrane</li> <li>Growth of entrapped bacteria</li> <li>Few reports about field applications</li> <li>Predicted BOD<sub>5</sub></li> <li>No marketed version</li> </ul>
Bioreactor	Dissolved O <sub>2</sub>	Electro-chemical or optical probe	20 min	<ul style="list-style-type: none"> <li>Direct measurement</li> <li>Short period of analysis</li> <li>Wide measurement range (BOD: 0 – 500,000 mg/L**)</li> <li>Configuration allowing the online monitoring</li> <li>Many reports about field applications</li> <li>Marketed version</li> </ul>	<ul style="list-style-type: none"> <li>Important required working area</li> <li>Predicted BOD<sub>5</sub></li> <li>Measurement variability (inoculum)</li> <li>Important calibration phase</li> </ul>

\*: without sample dilutions, \*\*: according the manufacturer.

Table 2: Characteristics of biosensors based on redox mediators.

Type	Microorganisms	Mediator	Transducer	Response time	Correlation with BOD <sub>5</sub>			Origins of the validation samples	Reference
					R <sup>2</sup>	Slope	n		
Electrochemical single redox mediator	<i>Pseudomonas fluorescens</i>	HCF <sub>(III)</sub>	Amperometer	15 min	0.66	1.05	7	Sewage treatment plant and food industry	Yoshida et al., 2000
Electrochemical single redox mediator	<i>Escherichia coli</i>	HCF <sub>(III)</sub>	Coulometer	60 min	NA	NA	NA	NA	Pasco et al., 2000
Electrochemical single redox mediator	<i>Candida sp.</i>	HCF <sub>(III)</sub>	Amperometer	5 min	0.875	1.109	2	Pulp mill effluent	Trosok et al., 2001
Electrochemical single redox mediator	<i>Proteus vulgaris</i>	HCF <sub>(III)</sub>	Coulometer	60 min	0.936	0.965	4	Wastewater treatment plant	Pasco et al., 2004
Electrochemical single redox mediator	Artificial consortium (4 strains)	HCF <sub>(III)</sub>	Amperometer	3 h	0.859	0.772	30	Industrial/domestic wastewater	Catterall et al., 2003
Electrochemical single redox mediator	<i>Candida krusei sp.</i>	HCF <sub>(III)</sub>	Amperometer	20 min	NA	NA	NA	NA	Oota et al., 2010
Electrochemical single redox mediator	<i>Pseudomonas fluorescens</i>	HCF <sub>(III)</sub>	Amperometer	3-20 min	0.616	0.71	59	Sewage treatment plant and food industry	Yoshida et al., 2001
Electrochemical Double redox mediator	<i>Saccharomyces cerevisiae</i>	Menadione + HCF <sub>(III)</sub>	Amperometer	15 min	0.712	1.102	6	River and sea samples	Nakamura et al., 2007
Electrochemical Double redox mediator	<i>Saccharomyces cerevisiae</i>	Menadione + hydrogen peroxide	Luminometer	8 min	0.925	0.088	3	River samples	Nakamura et al., 2007
Fluorescent redox mediator	<i>Pseudomonas fluorescens</i>	Resazurin	Fluorimeter	30 min	NA	NA	NA	NA	Dudal et al., 2006
Fluorescent redox mediator	Unknown microbial population	Resazurin	Fluorimeter	15 h	0.842	1.008	49	Domestic wastewater	ROCHER et al., 2011
Fluorescent redox mediator	<i>Pseudomonas putida</i> mt-2, <i>Pseudomonas putida</i> F1, <i>Burkholderia cepacia</i> G4 and <i>Pseudomonas mendocina</i> KR1	Resazurin	Fluorimeter	10 min	NA	NA	NA	NA	Tizzard et al., 2006
Redox colour mediator	<i>Saccharomyces cerevisiae</i>	DCIP	Spectrophotometer	10 min	0.726	0.219	3	River samples	Nakamura et al., 2007

R<sup>2</sup>, coefficient of determination; slope, slope of the linear model; n, number of samples; NA, information not available; HCF<sub>(III)</sub>, potassium hexacyanoferrate(III); DCIP, 2,6-dichlorophenolindophenol.

Table 3: Specification of the different immobilised bacterial biosensors

Type	Immobilisation support	Microorganisms	Response time	Correlation with BOD <sub>5</sub>			Origins of the validation samples	Reference
				R <sup>2</sup>	Slope	n		
Entrapped cell	Alginate	Activated sludge	10-15 min	0.9995	0.970	31	Different industries	Kumlanghan et al., 2008
Entrapped cell	Agarose	<i>Aeromonas hydrophyla</i>	50-90 min	-0.53*	0.36*	6	Synthetic samples or meat industry	Raud et al., 2012
Entrapped cell	Agarose	<i>Pseudomonas fluorescens</i>	50-90 min	0.241*	0.375*	6	Synthetic samples or meat industry	Raud et al., 2012
Entrapped cell	Agarose	<i>Pseudomonas fluorescens</i> , <i>Aeromonas hydrophyla</i> , <i>Pseudomonas putida</i> , <i>Escherichia coli</i> , <i>Bacillus subtilis</i> , <i>Paenibacillus sp.</i> , <i>Microbacterium phyllosphaerae</i>	NA	1	1	30**	Synthetic samples	(Raud and Kikas, 2013)
Entrapped cell	PCS	<i>Arxula adenivorans</i>	100 sec	0.88	1.04	16	Domestic wastewater	Chan et al., 2000
Entrapped cell	PCS	<i>Arxula adenivorans</i>	70 sec	0.920	0.611	6	Glucose solution	Chan et al., 1999
Entrapped cell	PCS	<i>Arxula adenivorans</i>	70 sec	0.898	0.998	16	Wastewater treatment plant	Lehmann et al., 1999
Entrapped cell	Sol-gel composite material	<i>Trichosporon cutaneum</i> and <i>Bacillus subtilis</i>	> 10 min	1	0.9253	5	Synthetic and environmental samples or domestic wastewater	Jia et al., 2003
Entrapped cell	Sol-gel composite material	<i>Trichosporon cutaneum</i>	NA	NA	NA	NA	NA	Liu et al., 2009
Entrapped cell	Sol-gel composite material	Microbial population ( <i>BODseed Cole-Parmer</i> )	10 min	0.971	1.06	11	Wastewater treatment plant	Liu et al., 2011
Entrapped cell	Sol-gel composite material	<i>Bacillus subtilis</i>	15-30 min	0.981	0.904	25***	Synthetic samples or domestic wastewater	Kwok et al., 2005
Entrapped cell	Sol-gel composite material	Activated sludge	15-30 min	0.984	0.915	28***	Synthetic samples or domestic wastewater	Kwok et al., 2005
Entrapped cell	Al <sub>2</sub> O <sub>3</sub> sol-gel	Yeast	15 min	0.9996	1.076	6	Food industry	Chen et al., 2002
Entrapped cell	Resin	<i>Trichosporon cutaneum</i>	20 min	0.855	1.2	5	Domestic wastewater or food industry	Yang et al., 1996
Entrapped cell	PVA	<i>Trichosporon cutaneum</i>	3-10 min	0.927	0.95	12	Wastewater from sewage plant or municipal sewage	Preininger et al., 1994
Entrapped cell	Ormosil-PVA	<i>Bacillus licheniformis</i> , <i>Dietzia maris</i> and <i>Marinobacter marinus</i>	3 min <sup>a</sup>	0.814	0.833	6	Seawater	Lin et al., 2006
Entrapped cell	Ormosil-PVA	<i>Bacillus licheniformis</i> , <i>Dietzia maris</i> and <i>Marinobacter marinus</i>	4 min*	0.902	1.006	22	Seawater	Jiang et al., 2006
Entrapped cell	Ormosil-PVA	<i>Bacillus licheniformis</i> , <i>Dietzia maris</i> and <i>Marinobacter marinus</i>	20 min	0.993	0.896	10	Seawater	Xin et al., 2007
Sandwiched cell	Dialysis membrane / Teflon® membrane	Activated sludge	20 min	0.183	1.05	11	Synthetic samples, domestic wastewater or food industry	Liu et al., 2000

Sandwiched cell	Cellulose acetate membrane / FEP	<i>Hansenula anomala</i>	13-20 min	1	0.952	4	River water, food industry and glutamate plant	Li and Chu, 1991
Sandwiched cell	Cellulose acetate membrane / FEP	<i>Pseudomonas sp.</i>	13-20 min	1	0.962	3	River water, starch and glutamate plant	Li and Chu, 1991
Sandwiched cell	Teflon® membrane / Teflon® membrane	<i>Klebsiella sp.</i>	NA	0.4023	0.541	8	Carbohydrate solutions	Kim and Park, 2001
Sandwiched cell	Polycarbonate membrane / Teflon® membrane	<i>Trichosporon cutaneum</i> , <i>Bacillus licheniformis</i>	5-10 min	NA	NA	NA	NA	Suriyawattanakul et al., 2002
Adsorbed cell	Cellulose nitrate membrane	<i>Pseudomonas putida</i>	2-15 min	0.839	0.895	14	River water	Chee et al., 1999
Adsorbed cell	Glass-fibre filter	<i>Candida maltosa</i>	8-20 min	0.091	0.734	7	Wastewater treatment plant or glucose-molasses plant	Arlyapov et al., 2012
Adsorbed cell	Glass-fibre filter	<i>Debaryomyces hansenii</i>	10-17 min	0.97	1.03	7	Wastewater treatment plant or glucose-molasses plant	Arlyapov et al., 2012
Adsorbed cell	Glass-fibre filter	<i>Candida blankii</i>	10-22 min	0.588	0.729	7	Wastewater treatment plant or glucose-molasses plant	Arlyapov et al., 2012
Adsorbed cell	Nylon membrane	<i>Microbes isolated from sewage samples</i>	10 min	1	0.989	3	Food industry, tannery or pulp and paper industry	Rastogi, Kumar, et al., 2003
Adsorbed cell	Nylon membrane	<i>Microbes isolated from sewage samples</i>	5-10 min	0.9997	1.04	5	Industrial wastewater	Rastogi, Rathee, et al., 2003
Biofilm	Glass tube	<i>Wastewater Microbes</i>	6-8 min	0.983	0.969	40	Wastewater treatment plant, food industry or environment	Liu et al., 2012

$R^2$ , coefficient of determination; slope, slope of the linear model; n, number of samples; \*, correlation with the  $BOD_7$ ; NA, information not available; <sup>a</sup>, response time for 5.0 mg/L  $BOD_7$ ; \*\*, 5 types of OCDE synthetic wastewater (without addition, with phenol, with milk, with fat or with carboxyl-methyl-cellulose); \*\*\*, 5 types of samples (OECD synthetic wastewater, synthetic wastewater, domestic wastewater, glucose-glutamic acid and a mixture of the OCDE synthetic wastewater and the glucose-glutamic acid); PCS, poly(carbamoyl)sulfonate; PVA, poly(vinyl alcohol); FEP, fluorinated ethylene propylene membrane.

Table 4: Comparison of the performances of the immobilised bacteria based BOD-biosensors with or without pretreatment of the samples.

Type	Immobilisation support	Pre-treatment	Microorganisms	Response time	Correlation with BOD <sub>5</sub>			Origins of the validation samples	Reference
					R <sup>2</sup>	Slope	n		
Adsorbed cell	Cellulose nitrate membrane	None	<i>Pseudomonas putida</i>	5 min	0.974	0.919	16	River water	Chee et al., 1999
		Ozonation			0.963	1.082			
Adsorbed cell	Cellulose nitrate membrane	None	<i>Pseudomonas putida</i>	< 10 min	0.896	0.8704	10	River water	Chee et al., 2001
		Ultra-Violet			0.957	0.985			
Adsorbed cell	Cellulose nitrate membrane	None	<i>Pseudomonas putida</i>	5-10 min	0.968	0.849	21	River water	Chee et al., 2005
		Ultra-Violet			0.983	0.908			
NA	NA	None	<i>Trichosporon cutaneum</i>	5-6 min	NA*	NA	5	Starch or GGA solution	Reiss et al., 1998
		Enzymatic			NA*	NA			
Adsorbed cell	Teflon® membrane	None	<i>Klebsiella sp.</i>	15-20 min	NC	NC	3	Lactose, sucrose or maltose	Kim and Park, 2004
		Enzymatic			NC	NC			

R<sup>2</sup>, coefficient of determination; slope, slope of the linear model; n, number of sample; NA, information not available; \*, [%] of the BOD<sub>5</sub> value : 24,3% without pretreatment, 67,9% with enzymatic pretreatment; NC, uncalculated value; GGA, glucose-glutamic acid solution.

Table 5: Performance of commercial BOD biosensors based on the bioreactor technology.

Name	Manufacturer	Type	Measured parameter	Pre-treatment	Measurement range (mg BOD/L)	Analysis time	Accuracy
Ra-BOD <sup>®</sup>	AppliTek	Bioreactor	Oxygen consumption	none	20-100,000	30 min	< 5%
Biox-1010	Endress+Hauser	Bioreactor	Oxygen consumption	none	5-100,000	3-15 min	3%
MB-DBO	Biosensores	Bioreactor	Oxygen consumption	none	10-1000	30 min	< 3%
ROD TOX 2000	Kelma	Bioreactor	Oxygen consumption	none	0-500,000	NA	5%
BioMonitor <sup>®</sup>	LAR	Bioreactor in cascade	Oxygen consumption	none	1-200,000	3-4 min	< 5%

NA, information not available.

1 Figure legends

2

3 Fig. 1: Manual (A), semi-automated (B) and automated (C) closed bottle test. With the  
4 manual closed bottle, dissolved oxygen is only measured with an electrochemical probe  
5 after an incubation period contrary to the semi-automated system and the automated  
6 system, which allow measuring continuously the O<sub>2</sub> consumption kinetics of the  
7 microorganisms (figures (A) and (B) from the authors).

8

9 Fig. 2: Recorded publications in the Web of Science with the following keywords:  
10 “BOD”+sensor or “BOD”+biosensor (figure from the authors).

11

12 Fig. 3: (A) The strategies of new approaches to estimate the BOD<sub>5</sub> and (B) their required  
13 analysis durations (figures from the authors).

14

15 Fig. 4: Principle of the BOD measurement by the manometric method (figure from the  
16 authors).

17

18 Fig. 5: Principle of a microbial fuel cell dedicated to the estimation of the BOD. (A)  
19 bacterial anaerobic compartment with anode, (B) aerobic compartment with cathode  
20 (figure from the authors).

21

22 Fig. 6: 3D modeling of the disposable chip developed by Nakamura *et al.* (Nakamura *et*  
23 *al.*, 2007) to estimate BOD<sub>5</sub> (figure from the authors).

24

25 Fig. 7: Chemiluminescence reaction for estimating BOD (Yashiki and Yamashoji, 1996;  
26 Nakamura *et al.*, 2007).

27

28 Fig. 8: Schematic representation of the BOD biosensor based on the bioreactor  
29 technology developed by Liu *et al.* (Liu *et al.*, 2004a, 2004b) (figure from the authors).

30

31 Fig. 9: Schematic representation of a bioreactor type biosensor (figure from the authors).

32

33 Fig. 10: BioMonitor (LAR) (figure from the authors).





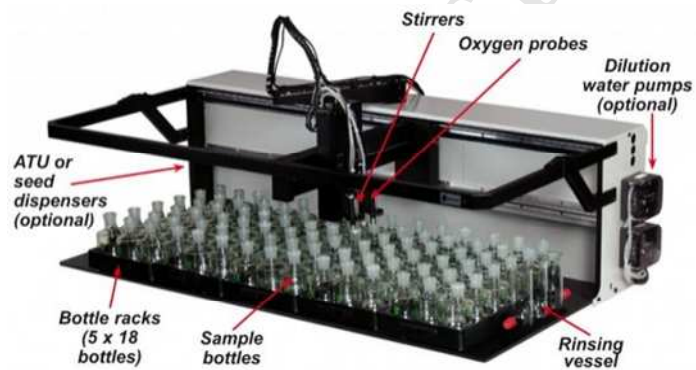
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35 Fig. 1 (A)



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37 Fig. 1 (B)



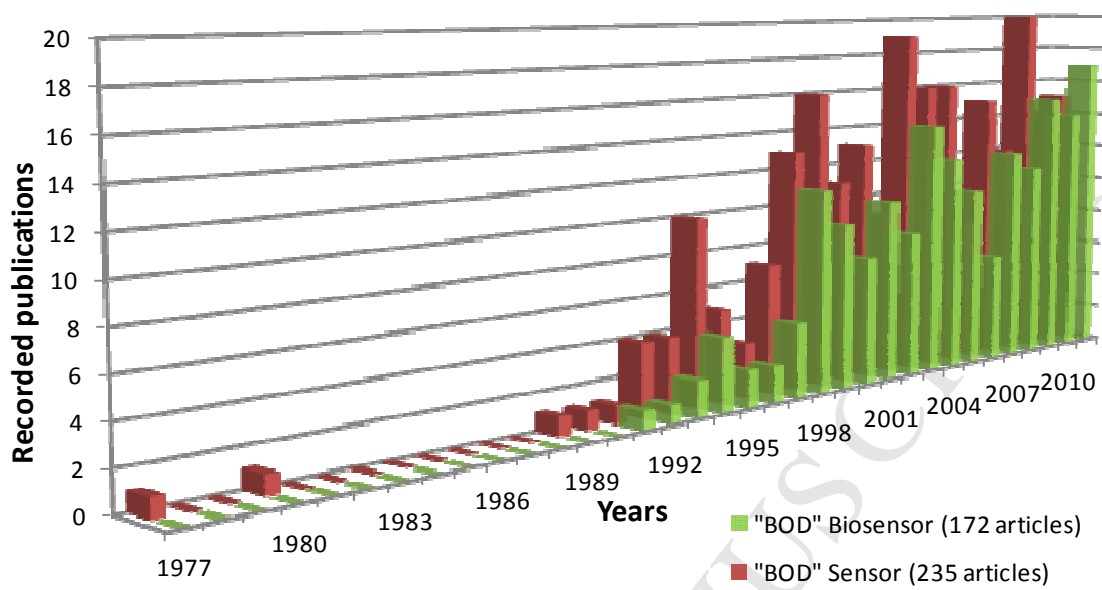
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(<http://www.skalar.com>)

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Fig. 1 (C)

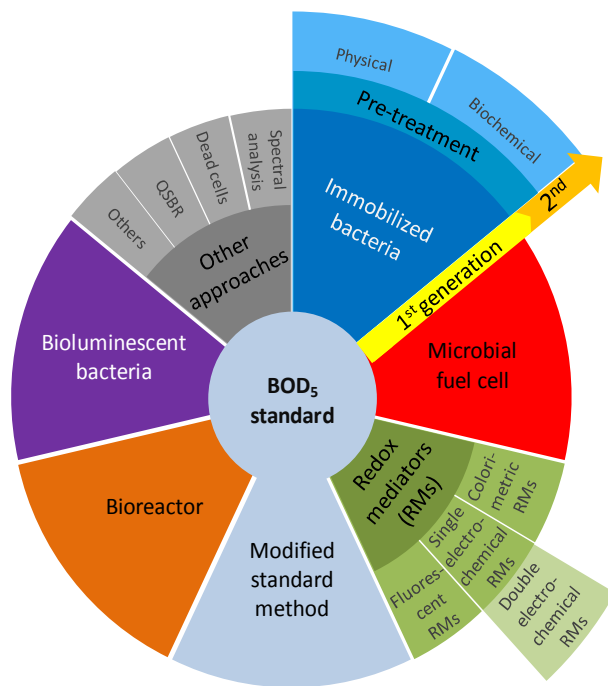
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42 Fig.2

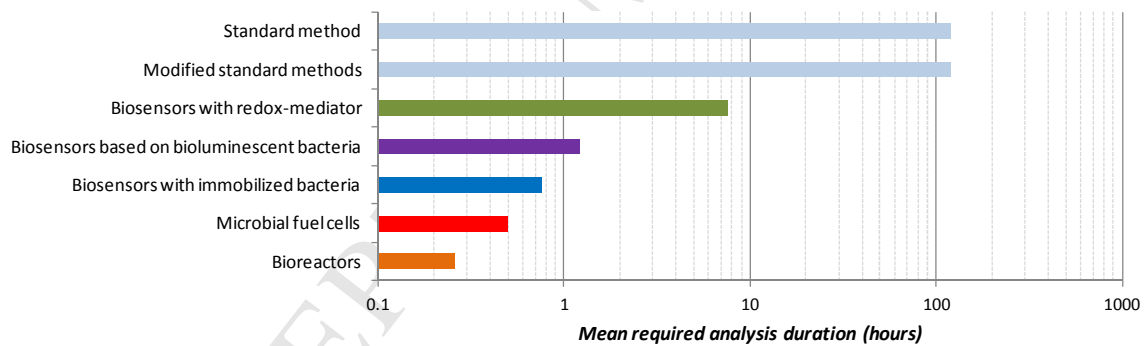
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45 Fig. 3 (A)

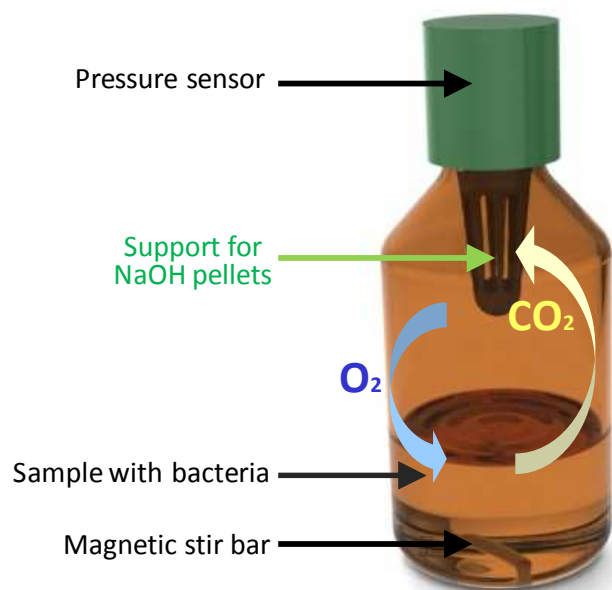
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48 Fig. 3 (B)

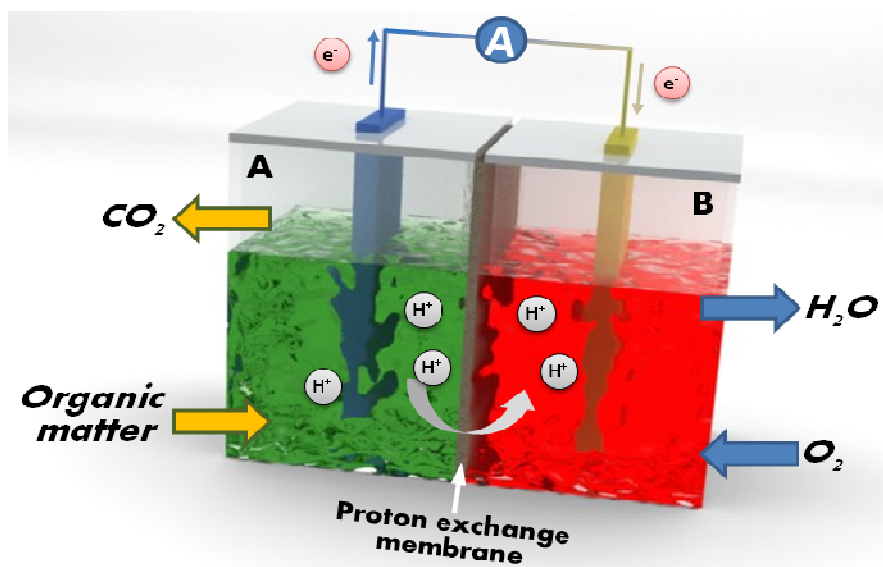
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51 Fig. 4

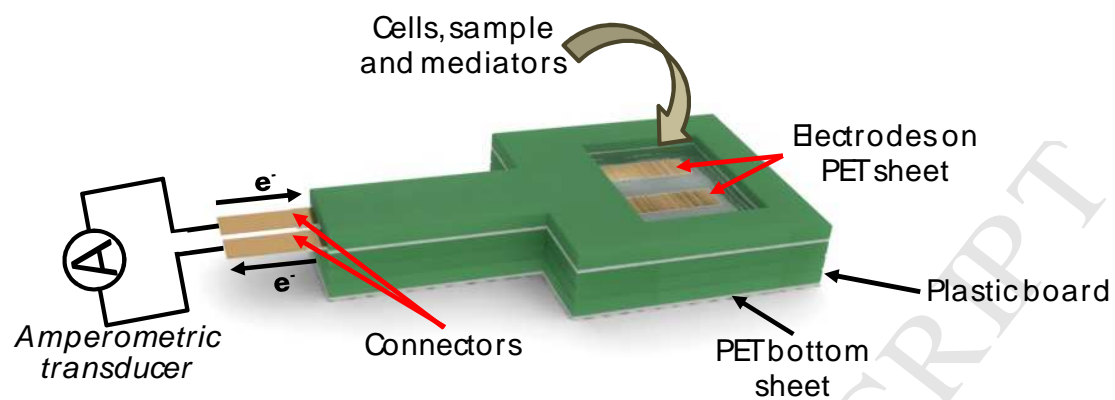
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54 Fig. 5

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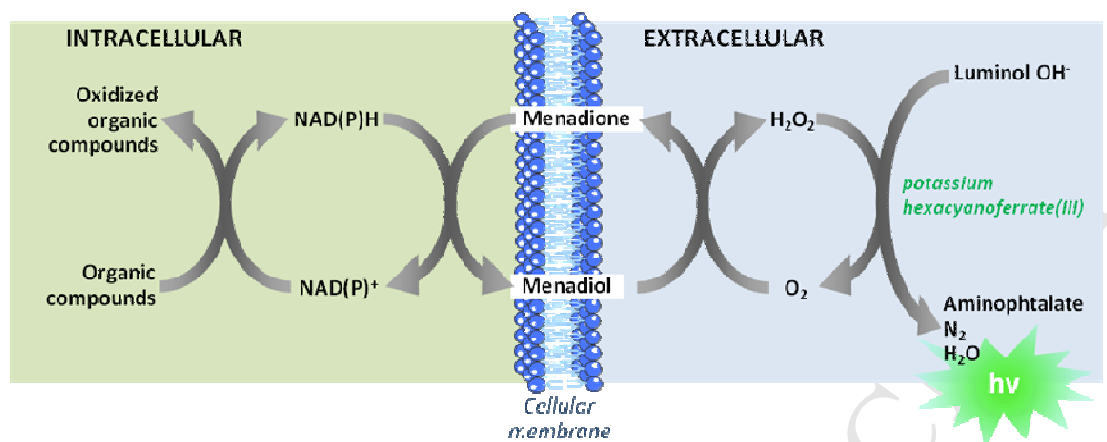


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57 Fig. 6

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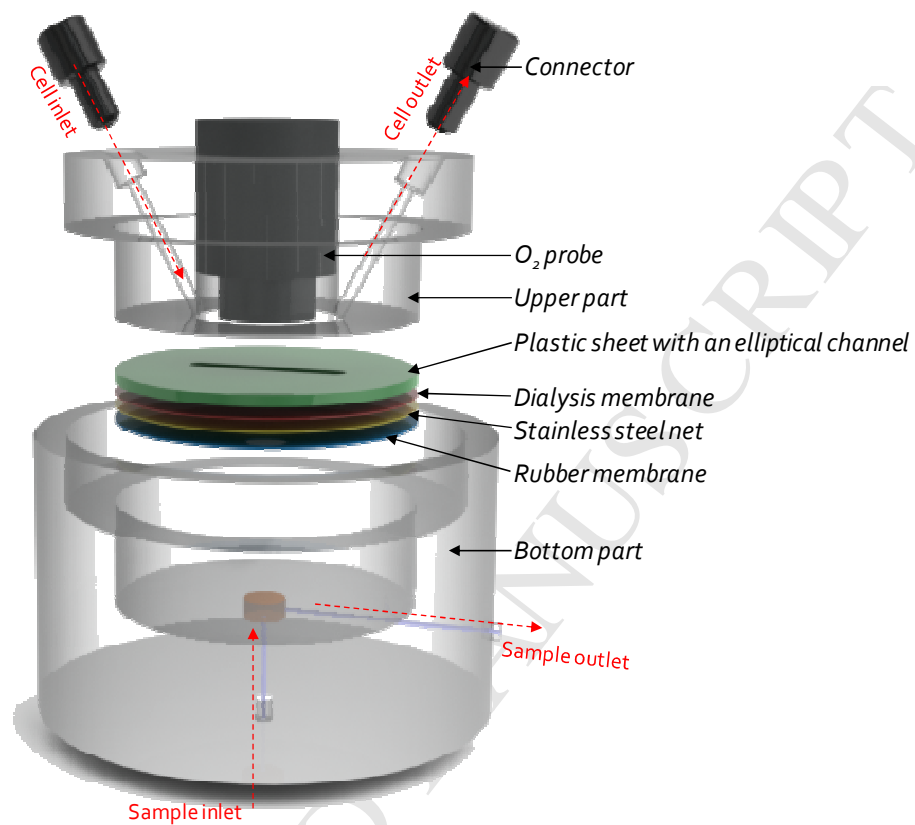
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60 Fig. 7

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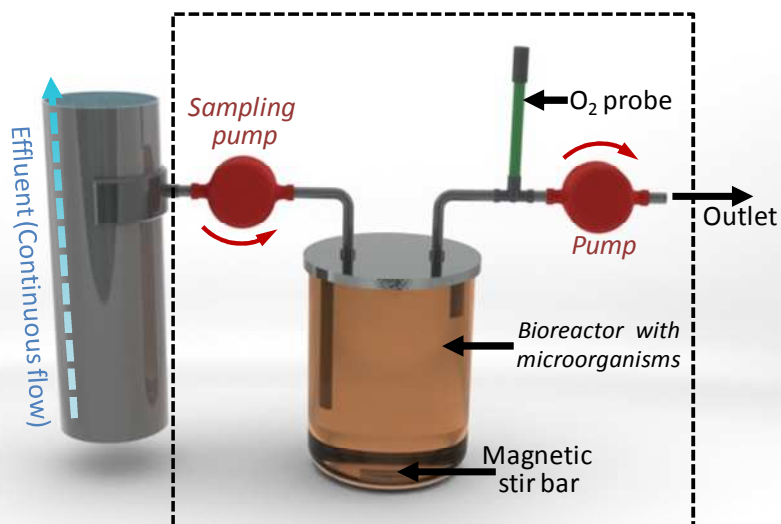


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63 Fig. 8



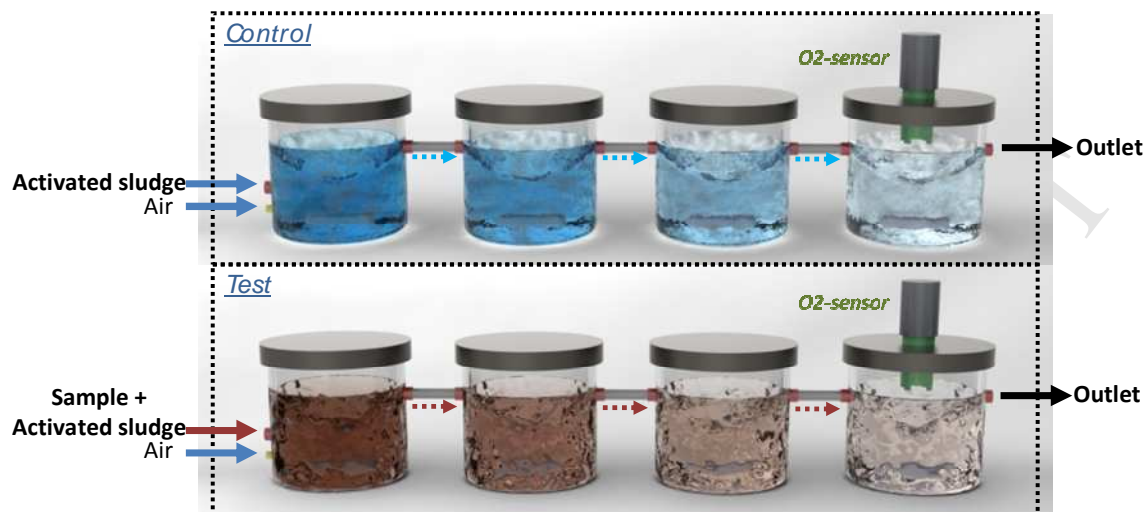
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66 Fig. 9

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69 Fig. 10