Chapter

Developmental Studies on Practical Enzymatic Phosphate Ion Biosensors and Microbial BOD Biosensors, and New Insights into the Future Perspectives of These Biosensor Fields

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Abstract

This chapter summarizes the developmental studies on environmental biosensors of enzymatic phosphate ion (Pi) biosensors for eutrophication and microbial biochemical oxygen demand (BOD) biosensors for organic pollution. In particular, an author focuses on the developmental studies that the author principally conducted, and describe the history and the insights into the future of these fields of environmental biosensors. In our developmental studies on the enzymatic Pi biosensors, we fabricated automatic instruments of a desktop-type and a submersible buoy-type, which was fabricated for remote biosensing of dam water. These instruments employed a luminol-chemiluminescence flow injection analysis (CL-FIA) system and enabled to have practical performances in precise Pi determination, operational stability, and accurate bioavailable Pi measurements. In the microbial BOD biosensor development, the author considered to apply the FIA concept enabling highly repeatable measurements to absorptiometric BOD measurements. Both precise temperature control and accurate time control to incubate measurement mixture of budding yeast cell suspension containing redox color indicator and sample enabled to obtain the highly repeatable results that led to highly sensitive BOD measurements. Looking back on our developmental studies, what the author was thinking at the time and the results obtained are described. Finally, the author discusses the developmental trends of these biosensor fields and new insights into the future perspectives.

Keywords: biosensor instruments, eutrophication, organic pollution, phosphate ion, enzymatic biosensor, BOD, microbial biosensor, budding yeast, CL-FIA, accuracy, precision, automation, remote biosensing, submersible buoy

1. Introduction

Human beings in the global ecosystem are no longer consumers, but wasters. To achieve "Transforming our World: the 2030 Agenda for Sustainable Development (2030 Agenda)" and set the Sustainable Development Goals (SDGs) consisting of 17 global goals in 2015 [1], human beings need to become former consumers and stay within the energy and matter cycles of the global ecosystems. One of the examples is the sixth goal of "Clean Water and Sanitation" in the 17 SDGs. The cause of anthropogenic eutrophication and subsequent organic pollution is the imbalance in the ecological matter cycle that occurs in human social life.

In Japan, the anthropogenic eutrophication and the organic pollution in 1960' had simultaneously been caused by the direct influx of wastewaters from industries and households (**Figure 1a**) [2]. On the other hand, most of such eutrophication in recent years is induced by the anthropogenic influx of nutrient salts, such as phosphate and nitrate salts, which are the essential nutrients of living organisms (**Figure 1b**) [3]. It is known that orthophosphate ion, that is, phosphate ion (Pi) is the most causative nutrient. These nutrient salts are contained in the effluent of sewage treatment plants or industrial wastewater treatment plants, or in leaching water from farms or live stocks [3, 4]. In the cases of these plants, only an ecological decomposer is employed for the biodegradation of organic matters contained in the wastewater (**Figure 1b**). This is the root cause of current eutrophication and subsequent organic pollution. Therefore, such anthropogenic influx of nutrient salts causes the water bloom by phytoplankton as an ecological producer, and the remains of phytoplankton cause organic pollution. To investigate the ecological phenomenon of such anthropogenic eutrophication and subsequent organic pollution, we observed the water ecosystem and water qualities of a eutrophied pond as a model water body [5]. The results led to a study on hydroponics [6] and water chemical remediation (WCR) that was developed for simultaneous removal of Pi and phytoplankton from the anthropogenically eutrophied pond [7]. A series of the studies will be described elsewhere.

In our studies, we have also developed measurement methods for these water pollutions. As one of the ways to measure the degrees of water pollution, several biosensors have been developed. The biosensor consists of a molecular recognition

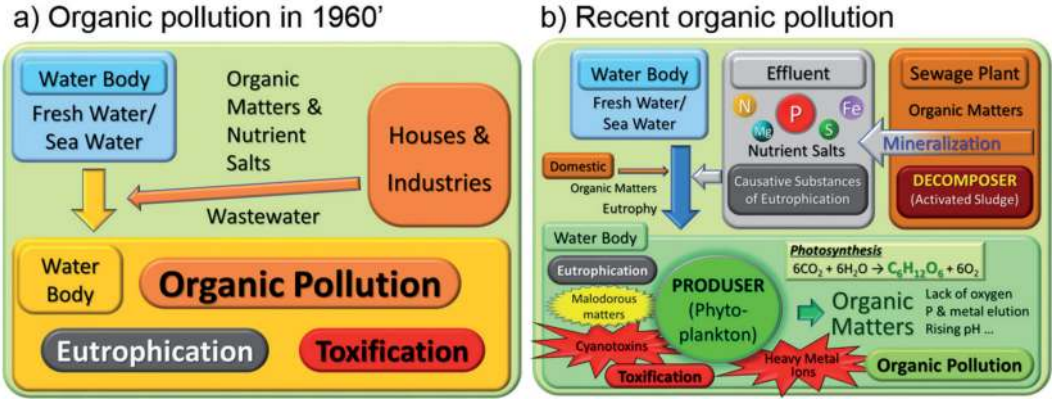

Figure 1.
(a) Schematic diagram in comparison on the causes of organic pollution in the 1960s and the recent past [2]. The schematic diagram of (b) was permitted from Springer Nature [2] and slightly modified for this chapter.

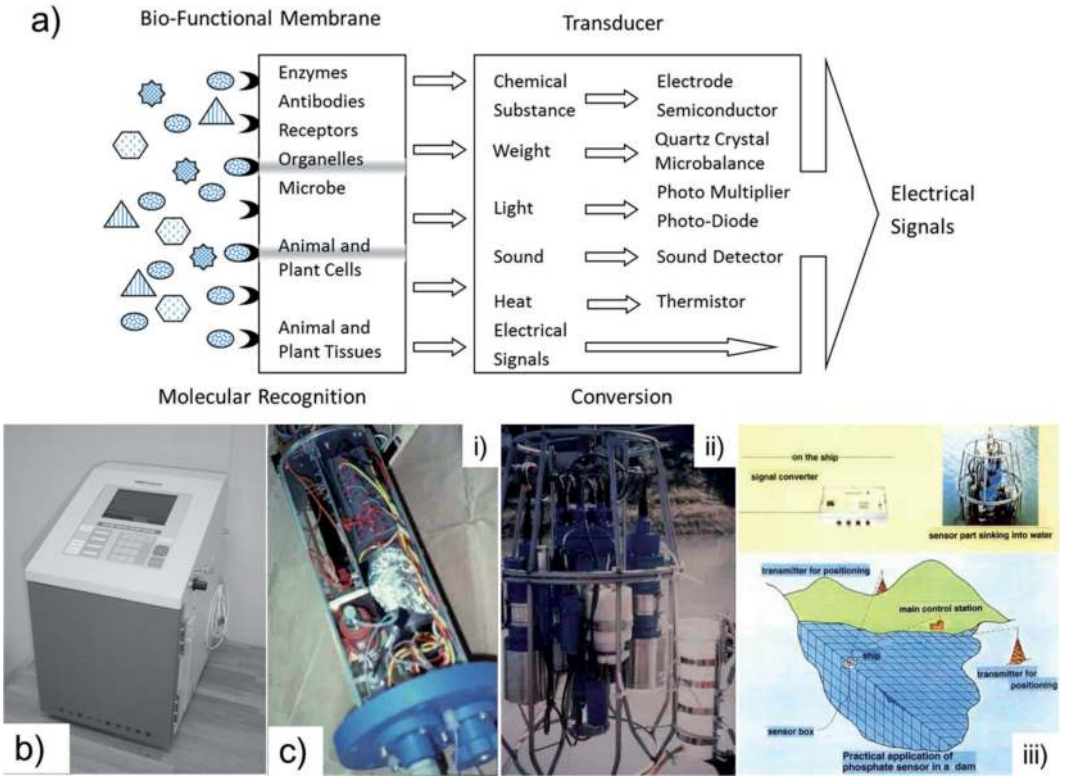

Figure 2.
Biosensor. (a) Principles and (b) automatic Pi biosensor instruments of a desktop-type [8] and (c) a submersible buoy-type [9], (i) a column compactly integrating a Pi biosensor system, (ii) a continuous-remote sensing system for total water-quality monitoring, and (iii) the continuous-remote sensing system is shipped by a boat and sunk vertically (a) and (c) were permitted from Springer Nature [10] and (b) was permitted from Taylor and Francis [11]).

element and a transducer and has the features of a simple and rapid measurement device (**Figure 2a**). The first biosensor was studied for medical use by Updike and Hicks in 1967 and developed to determine glucose concentration employing an enzyme-catalyzed reaction (enzymatic reaction) by glucose oxidase (GOD) [12]. This enzymatic glucose biosensor used a Clark-type electrode [13] and measured dissolved oxygen (DO) consumption caused by the GOD reaction. Since then, biosensors as measurement devices have been developed not only for medical uses [14] but also for food or environmental uses [10]. Thus, many biosensors and their associated techniques have been studied and developed [10]. In particular, the biosensor for environmental use requires highly sensitive and wide-determination range (dynamic range) measurement of the analyte. In addition, depending on what is being analyzed, the environmental biosensors require the feature to perform either specific or nonspecific measurements. Furthermore, for practical use, it is also required the application style of the biosensor, such as on-site use, continuous use, or laboratory use.

To satisfy these requirements, many kinds of biosensors for environmental water pollution have been developed worldwide [3, 10, 15]. In the second section of this chapter, the development of enzymatic Pi biosensors for eutrophication and in the third section, the development of microbial biochemical oxygen demand (BOD) biosensors for eutrophication are described. In the final fourth section, the contents of this chapter are concluded.

2. Development of enzymatic Pi biosensors for eutrophication

In the early times of biosensor development for environmental use, there were many technological problems for practical use. To solve the problems, a wide variety of challenges have been carried out. In this section, the main breakthrough technics for the practical application in the field of biosensor development for eutrophication are described based on the previous literature [3, 10, 14, 15].

2.1 Standard method and consideration to practical estimation performances

As mentioned above, the most causative nutrients of the eutrophication are phosphates, which are classified into Pi, condensed phosphates (pyro-, *meta*-, and other polyphosphates), and organic phosphates. Among the phosphates, the one with the greatest effect of eutrophication is Pi, which is an inorganic phosphate ion.

In Japan, a spectrophotometric molybdenum blue method for Pi determination is employed as the standard method [16], which is based on the method by Lowry and Lopez in 1946 [17]. By this method, Pi concentration can be determined between 0.1 and 3.0 mg/L Pi (3.2–96 μM) at a relative standard deviation (RSD) of 2–10%. The detection limit in this method is 0.03 mg/L Pi (0.32 μM Pi).

These values obtained by the standard method are barely applicable as an indication of the eutrophication (0.64 μM Pi), however, they are insufficient for the estimation of the eutrophication, which is classified into the five categories in lakes (between around 0.032–3.2 μM Pi). In addition, the maximal permissible concentration in Japanese lakes is 0.32 μM Pi for drinking and 3.2 μM Pi for environmental protection [18]. Thus, the influences of Pi on eutrophication are caused at extremely low concentrations. Therefore, it was found that the estimation of the eutrophication requires highly sensitive and wide-range Pi determination techniques.

For ideal estimation of the eutrophication, the standard method has difficulties in both accurate and high-precision measurements. In accurate measurement, dilution of sample solution needs due to that the dynamic range is narrow and causes error. In high-precision measurement, the minimum limit of determination (3.2 μM Pi) is insufficient to estimate the water quality in all categories of eutrophication.

To make ideal estimations for all categories of the eutrophication, a measurement method that was more sensitive and had a wider dynamic range (if it is possible; 0.032–3.2 μM Pi) than the standard methods was needed. However, considering the biosensor performance at that time, such high sensitivity was not realistic. Even if such highly sensitive Pi measurements cannot be made, we should be able to determine 0.32 μM Pi with a biosensor at a concentration that can confirm signs of eutrophication and monitor the quality of the lake water for drinking. In addition, we considered that the maximum limit of Pi determination obtained by the biosensor should be kept at least 3.2 μM or more as the practical dynamic range.

Like the other problems, the standard method is complicated and time-consuming to operate. Further, it requires the use of strong acid and heavy metal ions. The use of such chemicals is subject to limitations when applied to on-site monitoring and continuous monitoring because leakage of the chemicals into the environment has to be prevented. In addition, the standard method affects the influences of co-existing matters in a sample, such as Ca^{2+}, Fe^{3+}, NH_4^+, NO_2^-, NO_3^-, and AsO_4^{3-}.

As is clear here, the standard method is not sufficient to evaluate eutrophication. For this reason, the development of practical Pi biosensors has been performed.

2.2 Development of enzymatic Pi biosensors

It turns out that there were many issues that need to be resolved to actually estimate the eutrophication. Thus, we have set five requirements for the practical application of the Pi biosensors [19].

For practical use, our Pi biosensors are able to;

1. Have a practical Pi determination range (0.32–3.2 μM, no sample dilution required) required for environmental water control,

2. Maintain a practical minimum limit of Pi determination (0.32 μM Pi or less in the calibration curve) required for environmental water control for at least 2 weeks,

Instrument type	**Submersible buoy**[1]	**Desktop**			
Enzymatic system	PO_{psp}	PO_{Av}	MP	MP[2]	IP (PPi)
Ref. No.	**[9]**	**[8]**	**[20]**	**[11]**	**[21]**
Requirement					
(1) Calibration:	Satisfied	Satisfied	Satisfied	Satisfied	Satisfied at PPi
Range (at least 0.32–3.2 μM)	0.16–32 μM	96 nM–32 μM	10 nM–32 μM	0.1–30 μM	0.1–100 μM (PPi)
Coefficient	r = 0.998	r = 0.999	r = 0.998	r = 0.998	r^2 = 0.9997
(2) Sability test:	Satisfied	Satisfied	Not performed	Satisfied?	Not performed
as the term to maintain the practical lower limit of Pi determination in the calibration curve (0_32 μM Pi or less	Calibration curves of 0.32–32 μM for 48 days	Calibration curves of 0.16–32 μM for at least 2 weeks	1.0 μM for at least 2 weeks	0.1 μM for at least 2 months	(30 μM PPi for at least 2 weeks)
(3) Sample pretreatment:					
as countermeasures against interfering matters	Not performed	Performed	Performed	Performed and established the method	Not performed
(4) Real sample application:	Performed	Performed	Performed	Performed	Not performed
(5) Automation:	Semi-automated	Automated	Not automated	Not automated	Automated

[1]*Tests with the Pi biosensor on a submersible buoy have not been conducted.*
[2]*Pre-treatment method was investigated in detail for real sample application.*

Table 1.
Looking back on the five requirements that were set for the practical use of our automated CL-FIA systems as Pi biosensors (performed by Nakamura et al.).

3. Establish a pretreatment method for removing interfering matters,

4. Measure a real sample of environmental water, and

5. Automate a biosensor system that meets the above conditions.

We tried to develop practical Pi biosensors that meet these requirements (**Table 1**). As a reference, the history of the studies on the Pi biosensor development has been reviewed in several articles [3, 10, 15].

2.2.1 Development of early time Pi biosensors

A biosensor has characteristics that it can measure analyte using biological reactions by applying the molecular recognizing function in living organisms (**Figure 2a**). A Pi biosensor can measure directly the concentration of the bioavailable Pi existing in the environmental water. This means that the Pi biosensors have the possibility to be able to monitor the status of the water ecosystem. This section briefly explains the history of the development of Pi biosensors with such potential, which is above described [3].

The first Pi biosensor was studied by Guilbalt and Nanjo in 1975. Using the Clark-type DO electrode, they studied an enzymatic Pi biosensor that was based on the inhibition of alkaline phosphatase activity by Pi [22]. By employing the inhibitory reaction, this biosensor lacked sensitivity with the detection limit of 0.1 mM Pi. In addition, the inhibitory reaction is low selectivity in general. In 1990, d'Urso and Coulet studied a two-enzyme Pi biosensor using nucleoside phosphatase and xanthine oxidase [23]. Enzymatically generated hydrogen peroxide (H_2O_2) by the existence of Pi was electrochemically measured. In this study, the Pi biosensor could have a dynamic range between 0.1 and 10 μM Pi. In 1992, Wollenberger et al. improved the two-enzyme system to a multiple-enzyme system for Pi recycling [24]. The Pi biosensor was fabricated by incorporating a Clark-type DO electrode into a flow injection analysis (FIA) system. As the result, this Pi biosensor realized an excellent detection limit at 25 nM Pi. However, this biosensor was not suitable for practical use due to its short lifetimes by using an unstable enzyme. As another reason, it was reported that inosine used as another substrate was also unstable [25].

2.2.2 Development of our practical Pi biosensors

Karube et al. have studied several kinds of enzymatic Pi biosensors using pyruvate oxidase (PO) from *Pediococcus* sp. (PO_{Psp}) [9, 26–28] and genetically engineered PO from *Lactobacillus plantarum* (PO_{Lp}) [29], *Aerococcus viridans* (PO_{Av}) [8], and maltose phosphorylase (MP) [11, 20]. These studies were performed for the practical use of an automatic FIA system (desktop-type; **Figure 2b**) [8] and a remote-controlled automatic continuous Pi monitoring system (submersible buoy-type; **Figure 2c**) [9].

The Pi biosensor using PO is superior to other Pi biosensors because it requires only one step of a catalytic reaction for selective Pi detection. Using PO_{Psp}, the first Pi biosensor was studied by Kubo et al. in 1991 [26]. The Pi biosensor was batch type and used a DO electrode to measure the consumption of DO by the PO_{Psp} catalytic reaction under the existence of Pi, two cofactors (thiamin pyrophosphate; TPP and flavin adenine dinucleotide; FAD) and an enzyme activator (Mg^{2+}). However, the detection limit of 12 μM Pi was insufficient for environmental water control.

In 1996, Ikebukuro et al. examined the combination of a luminol chemiluminescence (CL) reaction and a FIA system for enzymatic Pi biosensor (CL-FIA system) [27]. At that time, the CL reaction was known as a highly sensitive reaction, and the FIA system was also known as a highly repeatable measurement system [30, 31]. The reason why the latter repeatability contributes to high-precision analysis was that the closer the multiple measurement results obtained from the same standard solution were to their average values, the smaller the standard deviation value. Along with this, by increasing the significant difference between the standard measurement value and the blank value, the standard measurement value closer to the blank value could be set as the detection limit. As a result, by combining CL and FIA technics, it was possible to realize a high-precision analytical method due to the synergistic effect of both. This meant that the CL-FIA system can turn the enzymatic Pi biosensors into highly sensitive analytical instruments. Thus, the CL-FIA biosensor systems have been widely studied [32].

In the PO_{Psp} catalytic reaction, hydrogen peroxide was produced and subsequently consumed by a luminol-peroxidase reaction (peroxidase from horseradish, HRP). The CL light resulting from the presence of Pi was detected at a photomultiplier tube (PMT). By the purification of TPP to remove residual phosphates, a detection limit of 74 nM Pi was obtained, although the TPP purification was unsuitable for practical use [28].

In 1997, Nakamura et al. used highly sensitive luminol catalyzing peroxidase from *Arthromyces ramosus* (ARP) [9]. This CL-FIA system was improved to the remote-controlled automatic continuous Pi monitoring system and compactly integrated into a submersible buoy to monitor dam water for drinking (**Figure 2c**) [9, 10]. Then, PO_{Psp} was immobilized onto chitin-chitosan beads filled in a stainless-steel column. In this study, 160 nM Pi was detected without purification of TPP, and by keeping PO_{Psp} activity in the stainless-steel column, 0.32 μM Pi was detected for 48 days. This result was sufficient for practical use of the CL-FIA biosensor system for Pi monitoring. Unfortunately, the manufacturing of PO_{Psp} was stopped.

In 1999, Nakamura et al. reexamined the development of the CL-FIA system for an enzymatic Pi biosensor using a new enzyme, PO_{Av}, which was purified from recombinant *Escherichia coli* [8]. Then, PO_{Av} was densely immobilized onto *N*-hydroxysuccinicacidimido (NHS)-gel beads. Here, the velocity of the enzymatic reaction strongly depends on the temperature. This means that the temperature must be kept constant by precise control to obtain repeatable signals based on the enzymatic reaction. On the other hand, accurate (i.e., absolute) temperature control dose is not essentially needed due to that the enzymatic activity immobilized in the biosensor changes. Thus, in this study, a desktop-type automatic CL-FIA system involving a precise temperature control system was fabricated as a trial enzymatic Pi biosensor instrument (**Figure 2b**). Then, the detection limit was 96 nM Pi. In addition, with sufficient performance at a short measurement time of 2 minutes, a wide linear range of calibration (96 nM and 32 μM Pi) was obtained with an average relative standard deviation (RSD_{av}) of 2.3% (eight points, $n = 5$) at 25.0 C. In addition, this system kept making calibration curves from 0.16 to 32 μM Pi (five points, $n = 3$; averaged correlation, $r = 1.00$) for at least 2 weeks. Thus, the practical utility of the Pi biosensor system was demonstrated.

On the other hand, in 1995, Conrath et al. studied a new electrochemical Pi biosensor using an analyte recycling system consisting of four enzymes, maltose phosphorylase (MP), acid phosphatase (AcP), mutarotase (MUT), and GOD [33]. The four enzymatic system enabled the successful detection of 10 nM Pi. However, the system was too complicated, and we thought it would be difficult to reproduce in manufacturing as well as other multiple-enzyme systems [24]. In 1999, Nakamura

et al. modified the MP system and applied it to our CL-FIA biosensor system using a tri-enzymatic reaction of MP-MUT-GOD without the analyte recycling by AcP [20]. Then, we could obtain the same results with Conrath et al. at a detection limit of 10 nM Pi. In addition, an excellent calibration between 10 nM and 30 μM Pi was obtained and stability to detect 1.0 μM Pi was observed for at least 2 weeks.

In 2003, Nakamura et al. improved the MP-MUT-GOD system for freshwater measurements [11]. Our previous studies revealed that the Pi biosensors employing the CL-FIA system were affected by the cations contained in the real sample solutions. Then, we have tried to examine several pretreatment methods to remove the cations from the sample [8, 11, 20]. As the result, we could find and establish a pretreatment method using a cation-exchanging resin. A total of 31 samples of freshwaters were taken from the river and pond. These real samples were pretreated by our method and measured for Pi determination by both this Pi biosensor and the conventional molybdenum-blue method. The results showed that the value from the conventional method was 2.78 times higher than that from the Pi biosensor. One reason for this outcome was considered that there were large differences in the reacting conditions between the Pi biosensor (natural pH) and the conventional method (under strong acid conditions). Therefore, an enzymatic Pi biosensor may determine the more accurate and realistic Pi concentration as free and bioavailable Pi, which is needed to understand the water ecosystem.

Furthermore, Nakamura et al. studied a pyrophosphate ion (PPi) biosensor in 2004 [21]. As well as the Pi concentration, the PPi concentration is also an indicator of the eutrophication and the organic pollution. For the enzymatic PPi biosensor, inorganic pyrophosphatase (IP) was added to the PO_{Av} reaction, and the possibility of this PPi biosensor to environmental water was also shown. In another study, Nakamura et al. also examined the development of a disposable electrochemical Pi biosensor chip for on-site monitoring [34]. Then, a commercially available self-monitoring blood glucose (SMBG) chip was applied to the Pi measurements. Pi was measured with a PO_{Av} or several MP systems coupled with ferricyanide ion (Fe^{3+}) as an electrochemical mediator.

Here, an author looks back on the five requirements that were set for the practical use of our Pi biosensors [8, 9, 11, 20, 21]. In the first requirement, the CL-FIA systems were successfully applied to the Pi biosensors and made it possible to highly sensitive and practical Pi measurements [8, 9, 11, 20, 21]. In the second requirement, our Pi biosensor was able to continue to make practical calibration curves as the stability tests and demonstrate the practicability [8, 9]. In the third requirement, the sample pretreatment method was finally established by the countermeasures against interfering matters [11]. In the fourth requirement, the establishment of the sample pretreatment method was enabled to perform real sample applications [11]. In the fifth requirement, the automation of the Pi and PPi biosensor systems was realized by employing the CL-FIA systems [8, 9, 21].

As described above, we studied several types of Pi biosensors for the estimation of eutrophication, and finally, two trial Pi biosensor instruments of the desktop and the submersible buoy were developed. Although thorough examinations using the real samples were necessarily employing the automatic Pi biosensor instrument for practical use and commercialization, various factors made it impossible to conduct the study (laboratory relocation, running costs, stop production of enzymes, etc.). In conclusion of our study for practical use of the enzymatic Pi biosensors, the highest risk was the stop production of the enzymes. As the general concerns, the development and subsequent practical use of the enzymatic biosensor tend to be expensive running costs for the enzyme. In particular, the FIA system consumes a lot of reagents and our CL-FIA system needs to contain the enzyme (HRP or ARP) in the CL reagent.

2.3 Subsequent development of Pi biosensors and a new insight into the future perspectives of the Pi biosensor fields

Subsequent development of the Pi biosensors by other groups was introduced here. In 2005, Kwan et al. studied a screen-printed electrochemical Pi biosensor using PO. The enzymatic Pi biosensor had a linear calibration range from 75 to 625 μM Pi [35]. The PO reaction needs TPP, which releases Pi. For practical use, an investigation of the storage condition of TPP would be needed. In 2001, Mousty et al. studied an electrochemical tri-enzymatic Pi biosensor employing an MP-MUT-GOD reaction system [36]. This system had a linear calibration range from 1 to 50 μM and was stable for at least 2 weeks. In 1998, Fernandez et al. studied another type of electrochemical tri-enzymatic Pi biosensor [37]. The three enzymes, a substrate, a cofactor, and a mediator, were incorporated into hydrogels. The enzymatic Pi biosensor had a detection limit of 2 mM Pi. Quite complicated principles and unstable enzymes would be a problem for practical use. These enzymatic Pi biosensors had insufficient sensitivity for actual eutrophication. For practical use, the influences of reducing matters in a sample solution on the biosensor response cannot be ignored. In 2013, Lawal and Adeloju studied measures against reducing matters in electrochemical Pi biosensors [38]. For this aim, they used conductive polymer of polypyrrole to the electrochemical bi-enzymatic Pi biosensors and could not observe the obvious influences of uric and ascorbic acids on both amperometry and potentiometric methods. However, the sensitivity of the Pi biosensor was insufficient for natural waters, therefore, the effects of the polypyrrole on the Pi measurement might be unclear. In 2020, Korkut et al. also used the polypyrrole to a PO-Pi biosensor and successfully performed accurate Pi determination of eutrophied water at 91% [39].

The enzymatic Pi biosensors have been developed not only for environmental use but also for medical use (urine Pi) [40, 41] and food use [42]. In the case of food use, in 2020, He and Liu successfully developed a highly sensitive PO-Pi biosensor employing "Nano-Enabled Biosensing" techniques that combined with gold nanorods (AuNRs) as nanomaterials and conductive materials. The PO-Pi biosensor was able to detect 0.4 nM Pi, which is sufficient to estimate eutrophication. On the other hand, these conductive materials have the potential to perform well in both food and medical applications, where samples are high in reducing matters [38, 39, 42].

On the other hand, studies using molecular recognition elements other than enzymes have also been carried out. The molecular recognition elements can be categorized into two types. One is bacterial phosphate-binding proteins (PBPs) and another is an ionophore that is made of lipid or polymer. The PBP is a component of a phosphate transport system and a highly selective recognition element of Pi (K_d = 1 μM) [43]. In bacteria, PBP has the function of transporting Pi into cells and organelles and does not bind other inorganic or organic ions except Pi and arsenate.

In 2002, Kubo et al. studied an electrochemical PBP Pi biosensor. They extracted the PBP from *E. coli* of wild type and detected Pi potentiometrically at a range from 0.1 to 1.5 mM [44]. In 2004, Lyndon et al. applied a mutant PBP for fluorometric Pi biosensor. Then, fluorophore was site-specifically labeled to the mutant PBP. This Pi biosensor showed excellent selectivity and was detected around sub-μM Pi [45], although the stability of PBP should be addressed. In 2019, Sarwar et al. successfully applied a fluorometric PBP-Pi biosensor (mutant PBP-labeled fluorophore), which was purchased from Thermo Fisher Scientific Co., to the estimation for actual eutrophication employing a smartphone camera detection system [46]. They prepared a cassette system for the smartphone using a 3D printer and the paper-based disposal

PBP-Pi biosensor chip using a Xerox printer. This PBP-Pi biosensor had high sensitivity of 1.1 ppb (12 nM) Pi and a response time of only 4 seconds, considering the effect of fluorescence photo-bleaching. One of the notable results in this study is the high reproducibility of the results obtained from the paper-based disposal PBP-Pi biosensor chips using Xerox printing. In general, reproducibility of the results obtained by the disposable type single-use biosensor was one of the problems in practical development. The author has been also energetically studying this problem in the practical study of the SMBG biosensor chips, therefore the author understand the difficulty [47]. In 2020, Franz et al. developed a PBP-Pi biosensor using a mutant PBP-labeled fluorophore and established a new principle of thermophoresis [48]. For Pi detection, they combined the advantages of a biological Pi-receptor based on the PBP with the principle of thermophoresis. The PBP-Pi biosensor enabled the sub-nanomolar Pi determination in sample volumes ≤10 μL.

In the cases applying the ionophores to the Pi biosensors, Carey and Riggan in 1994 applied cyclic polyamine to an ion-selective electrode (ISE) for Pi [49]. This Pi sensor specifically detected dibasic phosphate (HPO_4^{2-}) and obtained a linear response between 1.0 μM and 0.1 M Pi. For the real sample application, the influences of sample pH have to be concerned. The ionophores from microbes were used for the Pi biosensors [50, 51]. As an example, the Pi biosensor studied by Wygladacz et al. obtained a linear calibration range from 1.0 μM to 2.5 mM Pi. However, the lifetime was the order of days [51].

Other principles for the Pi biosensors were also studied. In 2001, Schreiter et al. studied a Pi bioavailability assay employing a luminescent cyanobacterial reporter strain for the replacement of the conventional AGP test. The method enabled the highly sensitive detection of Pi from 0.3 to 8 μM, although it took 8 hours of incubation [52]. In 2003, Dollard and Billard studied a *pho A*::*lux*-based bacterial biosensor using a Pi-sensing plasmid and demonstrated the possibility for the assessment of Pi bioavailability [53]. However, the use of genetically engineered Pi biosensor elements is unsuitable in the cases of these leakages into the environment.

In this section, the author explained the representative studies on the development of Pi biosensors and Pi bioavailability assays for eutrophication. The biomaterials, such as enzymes, PBPs, and ionophores, were used for the Pi biosensors. These biomaterials need purification from living organisms. Therefore, the issue of cost and risk of production outage will be a challenge to the practical application of the Pi biosensors in this field. Further, in 2021, Becker et al. calculated the complete environmental factor (E-factor) of the enzyme [54]. The complete E-factor, including required waste and water, was calculated as 37,835 g-waste/g-enzyme. Therefore, the use of such biomaterials will require environmental consideration and not be neglected for the sustainability assessment of bioprocesses in the future. Furthermore, new technologies will be introduced one after another in this field, such as smartphones, 3D printers, nanomaterials, miniaturization, and automation.

3. Development of microbial BOD biosensors for organic pollution

3.1 Standard method and consideration to practical estimation performances

To estimate the degree of organic pollution, several indicators of total organic carbon (TOC), COD, BOD, and DO have been employed. In general, COD is employed for closed water bodies of both natural water and seawater, and BOD is employed for

flowing water, such as rivers. The differences in the usage of COD and BOD estimation methods are simply determined by the presence or absence of the flux, which depends on the decomposition rate of organic matters dissolved in the water body by aerobes, although they are often employed as the references in each field.

In the estimation methods for organic pollution, only BOD involves the results obtained by a biological reaction. The conventional standard method of the BOD estimation is referred to as the 5-day BOD (BOD_5) method [55, 56]. It requires 5 days of incubation to obtain the results. The BOD value (mg O_2/L) is calculated from the amount of DO consumed by the aerobic decomposition of organic matters during incubation (primary fermentation). From this principle, BOD is also called biochemical oxygen consumption.

At the peak of organic pollution in advanced countries in the 1960s, the standard method of BOD was limited to the BOD_5 method [55, 56]. The BOD_5 method has several problems that prevent it from satisfying the needs for practical use in wastewater control, that is, this method is time-consuming and requires tedious operations. Therefore, a method that can be used to monitor the BOD value in real-time or continuous was urgently needed.

3.2 Development of microbial BOD biosensors

In 1960', there were many issues that need to be resolved to actually estimate the BOD. To solve these problems, many kinds of BOD biosensors have been studied and developed for practical use [57].

3.2.1 Development of early time BOD biosensors and accomplishment by Dr. Isao Karube in this field

As described above, the BOD_5 method is time-consuming and requires tedious operations. For example, it is not possible to detect the abnormality of wastewater before and after the treatment because it takes 5 days to obtain the measurement result. In other words, even if abnormal wastewater flows into the treatment facility or is not sufficiently treated at the treatment facility, it can be detected only after the wastewater has flowed out to the environmental water. In addition, the tedious operations of the BOD_5 method also make it difficult to make accurate and accurate measurements.

In 1977, Dr. Karube studied a practical microbial biosensor for BOD [58]. The key technique was the immobilization of microbes to a thin collagen membrane. The microbial membrane was put onto the surface of a DO electrode. By the addition of a sample solution into a batch system, microbial respiration was activated by the decomposition of organic matters, and the degree of DO consumption by the microbes was determined by the DO electrode. The microbial BOD biosensor indicating DO consumption (BOD_{DO}) could successfully determine the BOD value at drastically shortened incubation and measurement times (ca. 30 minutes). By the study, the possibilities for solving the problems of wastewater control were enhanced.

In 1979, Hikuma and Karube et al. developed a flow system of the BOD_{DO} biosensor [59]. In the study, omnivorous yeast *Trichosporon cutaneum* (*Tc*), as a practical microbe, was used in this $BOD_{DO\text{-}Tc}$ biosensor. The microbes were immobilized onto a microporous membrane and attached to the surface of the DO electrode. Based on the study, a flow-type $BOD_{DO\text{-}Tc}$ biosensor was available for practical applications.

Both the desktop-type for rapid measurements and the installation-type for continuous monitoring were sold from the Central Kagaku Co. in 1983. These $BOD_{DO\text{-}Tc}$ biosensor instruments enabled wastewater control by both real-time measurement and continuous monitoring at sewage plants and factories. The $BOD_{DO\text{-}Tc}$ biosensor was established as one of the JIS methods (JIS K 3602) in 1990 [60]. Since then, the $BOD_{DO\text{-}Tc}$ biosensor instrument has also been used in educational settings as a science teaching material [61].

Since the first microbial biosensor was reported, many kinds of microbial biosensors have been studied for not only environmental applications but also food applications, including fermentation. These studies on both environments and foods by microbial biosensing methods were reviewed [3, 10, 15, 62].

Dr. I. Karube was widely studied all-fields of biosensor development as one of the leading scientists in the world. His notable study on biosensor development was summarized in the review [10] and the detailed history of his study on microbial biosensor development was described in one chapter of Encyclopedia [57].

The notable studies on the microbial BOD biosensor development performed by Karube et al. are briefly described as follows. As the study on the BOD biosensors, a microbial fuel cell (MFC) type biosensor has also been developed. However, the MFC biosensor at that time used expensive materials, easily deteriorated electrodes, anaerobes, etc., and had low cathode reaction efficiency due to low electron transfer from the anaerobes to the cathode. In addition, a flow-type cathode chamber of the MFC biosensor has a low exchange efficiency of sample solution, making it difficult to repeat and rapid BOD_{MFC} measurements.

After two types of $BOD_{DO\text{-}Tc}$ biosensor instruments were practically used, the other types of BOD biosensors have been widely studied and developed. One of the practical studies was the development of a bioluminescence BOD biosensor using a luminous bacterium *Photobacterium phosphoreum* (*Pph*) [63]. In 1993, Hyun et al. studied a $BOD_{BL\text{-}Pph}$ biosensor; however, the emission intensity of BL released by *P. phosphoreum* decreased with each measurement. Thus, a reagent-type $BOD_{BL\text{-}Pph}$ biosensor instrument was practically used by Tamiya et al.

Another practical study was the development of portable type instruments for on-site monitoring. To realize the on-site monitoring, it was required to stop using air-supply equipment, to reduce the size of measurement devices, to miniaturize and single-use biosensors, to employ omnivore and vital microbes, etc. Dr. Hiroaki Suzuki has been studying the miniaturization of the biosensors to be used as disposable sensor chips [64]. In 1996, Suzuki and Yang et al. studied $BOD_{DO\text{-}Tc}$ biosensors for on-site monitoring and developed a low-cost paper-based DO electrode [65] and a disposable $BOD_{DO\text{-}Tc}$ biosensor chip [66]. However, in general, the $BOD_{DO\text{-}Tc}$ biosensors were difficult to accurately measure the high BOD value of a sample solution, because the solubility of oxygen into water is limited (8.11 mg O_2/L at 25°C and 1 atm).

To solve the problem, in 2000, Yoshida et al. studied two types of BOD biosensor principles for on-site monitoring. One was a single mediator (SM) type of an electrochemical BOD biosensor [67]. In the study, omnivorous bacteria *Pseudomonas fluorescens* (*Pf*) was isolated from a sewage plant. By using potassium ferricyanide as a highly soluble mediator in water (460 g/L), a $BOD_{SM\text{-}Pf}$ biosensor that does not require air-supply equipment was developed. The principle was applied to a portable type instrument and a disposable $BOD_{SM\text{-}Pf}$ biosensor chip for on-site monitoring [68]. As a result, a wide linear range of calibration (15 and 260 mg O_2/L) was obtained. Another type was an optical BOD biosensor using the redox electron acceptor 2,6-dichlorophenolindophenol (DCIP) sodium salt as a redox color indicator

(RCI). Before the development of a portable type optical instrument, an absorptiometric high-throughput $BOD_{RCI\text{-}Pf}$ measurement method was studied using a microplate reader, which is able to measure 96 samples simultaneously [69]. Based on the principle, the portable type optical $BOD_{RCI\text{-}Pf}$ biosensor instrument was constructed using three pairs of light-emitting diodes (LEDs; 600 nm) and silicon photodiodes (Si-PDs), and a transparent disposable chip containing three biosensing spots [70]. As a result, a linear relationship was observed below 176 mg O_2/L, and the detection limit was 14 mg O_2/L (n = 6, RSD = 10.3%). An excellent correlation coefficient (r^2 = 0.989) was obtained after 600 seconds incubation time. By the study of Yoshida et al., two portable types of the practical BOD_{Pf} biosensor instruments were developed using two types of redox electron acceptors.

On the other hand, in 1999, Chee et al. studied highly sensitive BOD_{DO} biosensors for low BOD measurements [71]. The background of this study was that the spread of sewage treatment facilities improved the water quality of rivers at that time, and it became necessary to measure low BOD including persistent organic matters in the sample solution. To properly measure the river water quality at that time, isolation of the microbe that biodegrades persistent organic matters was needed, and they isolated *Pseudomonas putida* (*Ppu*) from a sewage plant. To enhance the biodegradability of the persistent organic matters by *P. putida*, they examined pretreatment methods of the sample solution and finally established the photocatalytic UV-TiO_2 method [72]. As the result, a practical linear range of calibration (0.5 and 8 mg O_2/L) was obtained using artificial wastewater containing persistent organic matters.

The studies on the practical BOD biosensor were successfully performed by Dr. Karube et al. and the practical instruments including the potable instruments for on-site monitoring were developed.

3.2.2 Development of BOD biosensors that the author principally conducted

After these excellent studies on the practical BOD biosensors by Dr. Karube et al., the author explored other possibilities of BOD biosensors with better functionality, for example, improvements of (1) detection limit, (2) signal repeatability of the microbial biosensor, and (3) suitability of microbe used.

1. The detection limit can be improved to enhance the reaction efficiency of microbial degradation of organic matters. However, most of the microbial biosensors developed to date immobilized the microbes. To enhance the reaction efficiency of microbial degradation of organic matters, microbes should be dispersed to uniform the suspension.

2. The signal repeatability of the microbial biosensor can be enhanced by a fully unified operation of repeating measurements under the same conditions. It is important to keep constant at least both temperature and reaction time. This enhancement also leads to highly sensitive measurements. This is because by increasing the significant difference between the standard measurement value and the blank value, the standard measurement value closer to the blank value can be set as the detection limit (it is known that the reproducibility of the signals obtained by the microbial biosensor is around 10% (as RSD value) [70]).

3. On the other hand, the improvement of microbial suitability can also be achieved by employing easily available, omnivorous, and vital microbe.

Then, the author tried to satisfy these requirements by employing the absorptiometric BOD_{RCI} measurement method and a temperature-controlled three-cuvette-stir system [73, 74]. As the usable (easily available, omnivorous, and vital) microbe, Baker's dry yeast *Saccharomyces cerevisiae* (*Sc*) was used after liquid culturing. *S. cerevisiae* is budding yeast; therefore, it was suitable for forming uniform suspension in the cuvette. On the other hand, *T. cutaneum*, which is conventionally used for the BOD biosensors and makes flocks by sticking together, was not suitable for this study because it was filamentous fungi. In this method, DCIP was used as RCI of a high absorption coefficient (*epsilon* = 1.45×10^5 cm^{-1} M^{-1}) to enhance the sensitivity of the absorptiometry. Then, the absorbance of DCIP decreased due to degradation of organic matters by *S. cerevisiae* in the measurement suspensions, and the absorbance change between before and after incubation was measured as the time difference method (**Figure 3**).

In general, the suspension is not suitable for absorptiometry due to the occurrence of light scattering. However, by a combination of the cuvette-stir system and the time difference method, the influences of the light scattering were efficiently canceled and only absorbance change of DCIP was accurately determined. Further, by repeating the exact same measurement operation three times, the fluctuation of the measured value became small, and the reproducibility was improved, so that highly precise measurement became possible. As a result, the significant difference from the blank value became large, and highly sensitive measurement became possible.

$BOD_{RCI\text{-}Sc}$ measurement method: As a result, a calibration curve between 1.1 and 22 mg O_2/L (r = 0.988, six points, n = 3, RSD = 1.77%) was obtained by this highly sensitive $BOD_{RCI\text{-}Sc}$ measurement method when the incubation mixture was incubated for exact 10 minutes at 30°C. Employing salt-tolerant yeast *S. cerevisiae*

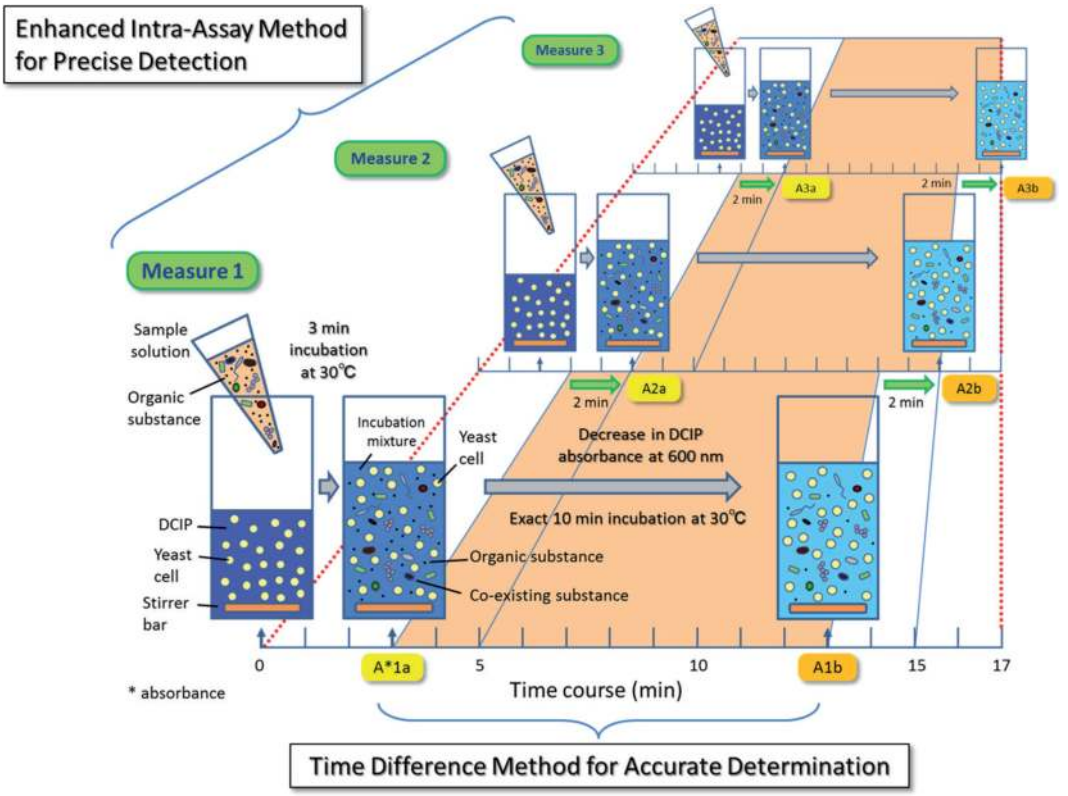

Figure 3.
A key principle for the simple absorptiometric measurement method using redox color indicator [73].

ARIF KD-003 (*Sc*II), a $BOD_{RCI\text{-}ScII}$ measurement method was also studied for seawater [75]. In these studies, the highly sensitive $BOD_{RCI\text{-}Sc}$ measurement method was developed by enhancing the repeatability of the measurements and by employing the usable yeast. From the future perspective of this study, the $BOD_{RCI\text{-}Sc}$ measurement method might be improved by applying it to an absorptiometric-FIA system.

$BOD_{DM\text{-}Sc}$ biosensor: As the other application of *S. cerevisiae* to a BOD biosensor, the author also studied a mediator type BOD biosensor. By applying a double mediator (DM) system coupled with ferricyanide and a lipophilic mediator, menadione, electrochemical signals were obtained from the eukaryote *S. cerevisiae* cells [76]. For practical use, the author designed a package-free disposable microbial biosensor chip containing living microbial cells [77] and the biosensor chip was applied to a DM-type $BOD_{DM\text{-}Sc}$ biosensor [78]. Under the optimal conditions, a calibration curve was obtained with a practical range of 6.6–220 mg O_2/L (five points, n = 3, RSD = 6.6%). Thus, the $BOD_{DM\text{-}Sc}$ biosensor was developed as the third generation of the BOD biosensor [3, 10].

$BOD_{CL\text{-}Sc}$ measurement method: By applying the principle of the BOD_{DM} biosensor, the author next studied a chemiluminescence BOD ($BOD_{CL\text{-}Sc}$) measurement method. Because Yamashoji et al. in 2004 already established luminol CL assay for the viable microbial detection method using ferricyanide and menadione [79]. Their principle was based on hydrogen peroxide (H_2O_2) determination produced by the reaction of the viable microbes and menadione. After optimization of the measurement conditions, a practical correlation between $BOD_{CL\text{-}Sc}$ value and amount of organic matters assimilated by *S. cerevisiae* was observed with a range of 11–220 mg O_2/L (six points, n = 3, RSD = 3.71%) at the incubation time of only 5 minutes [80, 81]. Then, the detection limit was 5.5 mg O_2/L.

$BOD_{DM:Trinder\text{-}Sc}$ measurement method: As one of the reagents for H_2O_2 measurement, there is a modified Trinder's reagent. It is an absorptiometric H_2O_2 measurement method using peroxidase (POD), 4-aminoantipyrine (4-AA), and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethylaniline sodium salt (MAOS). Applying this reagent, we already developed a surface plasmon resonance (SPR) biosensor for H_2O_2 measurement using a modified Trinder's reagent as a color indicator [82]. This reagent had high selectivity, however, POD was unsuitable for applying to the microbial biosensor due to take cost. On the other hand, H_2O_2 reacted as the reactant in $BOD_{CL\text{-}Sc}$ method. This meant that H_2O_2 or active oxygen species might also react as the reactant in $BOD_{DM\text{-}Sc}$ method. To verify the possibility, MAOS and 4-AA, which were used in the modified Trinder's reagent, were added to the principle of the $BOD_{DM\text{-}Sc}$ method. As the result obtained without the use of POD as the biocatalyst, correlations between $BOD_{DM:Trinder\text{-}Sc}$ value and amount of organic matters assimilated by *S. cerevisiae* were observed in several measurement conditions, although further practical examinations are required [83].

In the studies that the author principally conducted, excellent functions in the BOD biosensor were achieved in (1) the practical detection limit, (2) the signal reproducibility, and (3) the suitability of the microbe used. In addition, some principles of both microbial BOD biosensors and measurement methods have been studied for practical use, but none of the studies the author have principally conducted has reached practical use. The most practical BOD measurement method in the studies might be the $BOD_{RCI\text{-}Sc}$ measurement method. The reasons are as follows:

1. Practically suitable microbe (easily available, omnivorous, and vital yeast)

2. Very simple operation (just added three solutions into a cuvette; a *S. cerevisiae* cell suspension, a DCIP solution, and a sample solution)

3. Rapid measurement time (only 10 minutes incubation)

4. Highly sensitive measurements (highly repeatable results were obtained by employing a temperature-controlled three-cuvette-stir system and provided highly precise results, enabling highly sensitive measurements; and it was also important to use RCI of high absorption coefficient)

5. Practical dynamic range (same as available BOD biosensor instrument)

6. The patent registered for practical use (the patent right did not continue [74])

For future study, automatic instrumentation of the BOD biosensor would be required having the features that we obtained. Then, further suitability of the microbe might be needed to be considered, for example, use of thermally killed microbes [84] or cell crushed microbes, or direct use of available dry yeast [85].

3.3 Subsequent development of BOD biosensors and a new insight into the future perspectives of the BOD biosensor fields

Even after our studies on the BOD biosensors have been reported, the studies on next-generation BOD biosensors are ongoing. In this section, the progress of our studies on the BOD biosensors performed by other groups is introduced.

In our studies that the author principally conducted, the most practical BOD biosensor or measurement method was the $BOD_{RCI\text{-}Sc}$ measurement method. Unfortunately, we did not achieve the instrumentation. However, in 2021, Azevedo et al. developed an automated *S. cerevisiae*-based spectrophotometric instrument using methylene blue (MB) dye as a RCI, although this instrument was not for BOD measurement, but also possible to measure BOD [86]. The instrument consists of a sequential injection analysis (SIA) flow system, which enables high throughput measurements with small quantities of reagent consumption. On the other hand, DCIP has been used not only as a RCI but also as a redox mediator for electrochemical BOD_{SM} biosensors. For example, Reshetilov et al. developed several types of electrochemical microbial biosensors using DCIP as the redox mediator [87]. In 2017, Niyomdecha et al. fabricated a practical electrochemical FIA type microbial biosensor system for highly precise BOD measurements [88]. They used MB dye as a redox mediator and successfully obtained highly sensitive results of the detection limit at 0.1 mg O_2/L. This study demonstrated that highly reproducible results provided highly precise results, enabling highly sensitive measurements.

As recent progress of the DM-type microbial biosensors, several groups were reported. For example, in 2017, Gao et al. reported a DM-type *S. cerevisiae* biosensor for toxicity of wastewater and co-immobilized *S. cerevisiae*, menadione, and ferricyanide onto a glassy carbon electrode by electrodeposition of a chitosan hydrogel and nanocrystalline diamond particles [89]. In this study, it seems that *S. cerevisiae* and two mediators were successfully immobilized into the matrix without leakage and made it possible to distribute toxic matters into the matrix. However, this biosensor has a complication of the fabrication, therefore reproducible fabrication of the biosensor electrodes might be difficult. In 2017, Zaitseva et al. developed a batch-type BOD_{DM} biosensor employing yeast *Debaryomyces hansenii* (*Dh*) and ferrocene-MB mediator system [90]. The $BOD_{DM\text{-}Dh}$ biosensor had practical characteristics of a short measurement time of 10 min, a minimum limit of determination of 2.5 mg O_2/L, and long-time

stability of 43 days. Then, they assumed as a new insight that lipophilic mediators, such as MB and DCIP, are capable of penetrating inside the microbial cell through the lipid membrane, taking electrons, and passing them on to the electrode immobilized ferrocene. If their assumption is true, new future perspectives in microbial electron transfer techniques can be opened.

As recent progress of the $BOD_{CL\text{-}Sc}$ biosensor, in 2018, Costa et al. developed a practical SIA microfluidic system of a $BOD_{CL\text{-}Sc}$ biosensor [91]. The SIA system was programmed to precisely control the flow of multiple channels in an accurate time, making it easier to obtain highly reproducible results. In addition, the incubation time under constant temperature for *S. cerevisiae* to assimilate the organic matters in the sample solution was also precisely controlled by setting the stopped period. If hydrogen peroxide is not used, highly sensitive measurements might be obtained by reduction of the background signal, and if the mixed solution could be agitated during incubation, both reaction efficiency and reproducibility might simultaneously be improved.

As the other progress, MFC biosensors have many potentials not only as BOD biosensors but also as self-powered devices for biosensors [92]. As a new insight into the future perspectives of the BOD biosensor fields, the author have one idea that is our past application study on a damped glycolytic oscillation induced in living yeast cells for toxicity assays (**Figure 4**) [93, 94]. We extracted six indexes from the wave shape and observed that these indexes were changed depending on both toxicity and the concentration of each toxic matter. By applying this principle, wave changes depending on both bioavailability and the concentration of the organic matters dissolved in a sample solution might be determined. In fact, excellent correlation was obtained between one of the indexes and the concentration of glucose (r = 0.9989, 2.5 μM–0.25 M, 12 points).

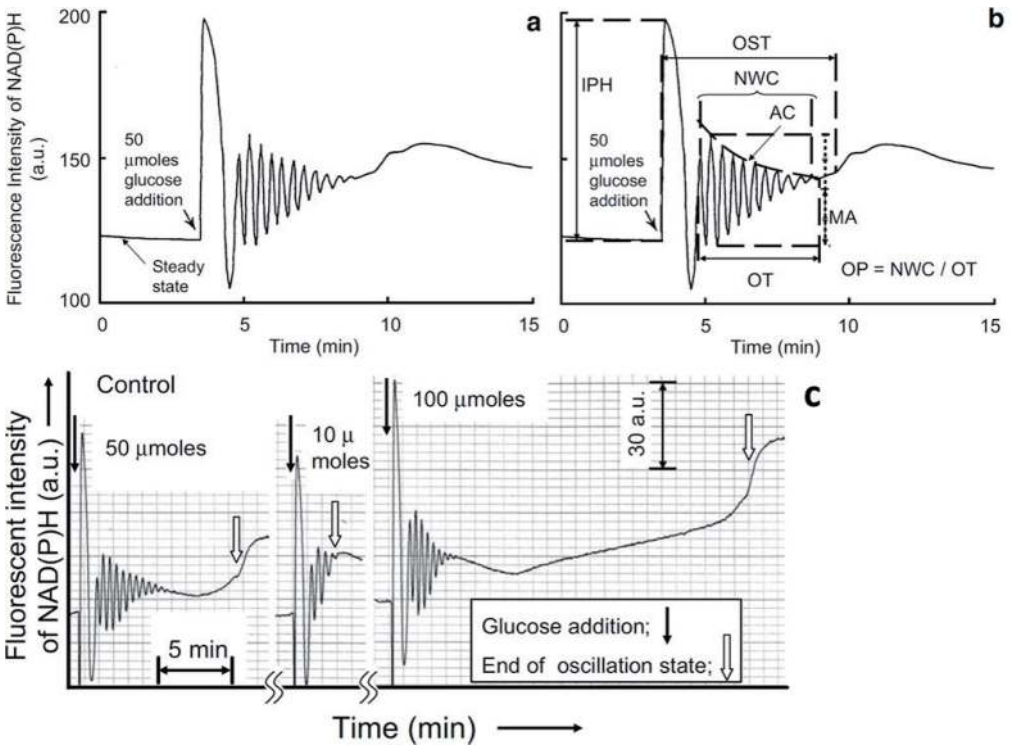

Figure 4.
(a) Typical damped glycolytic oscillation induced in living yeast cells. (b) Six indexes in an oscillation wave shape. (c) Response to glucose. These were permitted from Springer Nature [93].

Glycolysis is an important system that plays a central role in metabolism [14]. If this metabolic oscillation is applied to environmental monitoring, it may be possible to continuously sense the state of the aquatic environment perceived by organisms living in aquatic ecosystems in real-time. By doing so, for example, not only the index of BOD concentration but also the nutritional status of water quality and the substantial effect of heavy metals and antibiotics can be understood through the metabolic status of microbes employed for biosensing.

4. Conclusion

In this chapter, the author described the study on biosensor development for both eutrophication and organic pollution as one of my studying fields. By focusing on the two keywords of eutrophication and organic pollution, the author was able to summarize for the first time a series of our developmental flows and their aims. In conclusion, our biosensors introduced here could not be put into practical use. Nonetheless, the author hope that the practical use of both Pi and BOD biosensors as a replacement of the conventional standard methods will be realized based on the knowledge obtained by our developmental study.

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Conflict of interest

The authors declare no conflict of interest.

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