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Optical biosensor for the determination of BOD in seawater

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Abstract

An automatic sensing system was developed using an optical BOD sensing film. The sensing film consists of an organically modified silicate (ORMOSIL) film embedded with an oxygen-sensitive Ru complex. A multi-microorganisms immobilization method was developed for the BOD sensing film preparation. Three different kinds of microorganisms, *Bacillus licheniformis*, *Dietzia maris* and *Marinobacter marinus* from seawater, were immobilized on a polyvinyl alcohol ORMOSILs. After preconditioning, the BOD biosensor could steadily perform well up to 10 months. The linear fluctuant coefficients (R^2) in the range of 0.3–40 mg L^{-1} was 0.985 when a glucose/glutamate BOD standard was applied. The reproducible response for the BOD sensing film could be obtained within ±2.3% of the mean value in a series of 10 samples in 5.0 mg L^{-1} BOD standard GGA solution. The effects of temperature, pH and sodium chloride concentration on the two microbial films were studied as well. The BOD sensing system was tested and applied for the BOD determination of seawater.

Keywords: Optical BOD biosensor; ORMOSILs; Seawater

1. Introduction

Since the progressive environmental pollution problems over the world, the need for environmental monitoring and pollution control is greater than over. The rapid, easy-to-operate, low cost determination methods, which can operate on line (even in situ) will be highlighted. In the measurement of biodegradable organic compounds and pollutants of surface and seawater, the biochemical oxygen demand (BOD) is one of the most important and widely used parameter. Generally, the authorized BOD_5 method, which is defined as the biochemical oxygen demand of waste samples measured over 5 days at 20 °C, must last 5 days under specified standard incubation and skill of the operator to get reproducible results, is not suitable for in situ determination [1,2]. Numerous BOD biosensors [3–15] have been developed since Karube et al. [10] firstly reported a BOD biosensor based on Clark dissolved oxygen electrode immobilized microorganisms in 1977. Up to now, most of the BOD biosensors are still based on the immobilization of a microbial layer on an amperometric oxygen electrode [3–10]. However, they still remain some limitations such as (a) the depletion of oxygen occurring during BOD measurement using a sensor based on the Clark electrode and (b) the microorganisms immobilized in current BOD sensors are not able to be applied in the samples containing a higher concentration of salt such as seawater. During the past decade, some considerations are focused on fiber optical chemical sensors for the determination of oxygen due to rapid performance, no oxygen consumption and less toxicity [11–15]. Although most of the BOD sensors previously reported have been designed for long-term use, and even commercialized, it seems that the performance of these sensors is still not satisfactory for the on-line environmental monitoring of seawater since the rapid and highly steady measurement of BOD is necessary in the marine environment.

In our laboratory, effort has been made to develop optical BOD sensors based on the luminescence quenching of Ru complexes immobilized in the sensing film. Organically modified

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silicates (ORMOSILs)–PVA was used as a matrix to immobilize different kinds of seawater bacteria and domestic bacilli. The microbial film was laid over an oxygen film and the complex film was placed into a sample cell for BOD measurement [15]. In the present study, the response characteristics of a novel immobilized microbial membrane based on ORMOSILs–PVA were examined. The response time, reproducibility, linear range and the effects of temperature, pH and the sodium chloride concentration in the sample were evaluated. Furthermore, an automatic optical BOD sensing system was setup to develop a biosensor for rapid and stable in situ determination of the BOD in seawater.

2. Experimental

2.1. Apparatus

An automatic optochemical BOD sensing system was employed for acquiring the changes of fluorescence intensity. The system consociates some function parts as: (1) sampling part, including sampling peristaltic pump and valves for sample distribution; (2) controlling part, including a industrial manipulative computer and software; (3) measurement part, including a sensing detection cell and constant temperature controller; (4) light to current conversion part, including an optical fiber and a PMT. The sensing film was placed in the middle of the detection cell when temperature was kept constant by a temperature controller with a precision of ±0.2 °C. An excitation light with the wavelength of 495 nm from a blue LED was directed onto the sensing layer at an angle of, typically, 45°. The emission fluorescence was filtrated by a cut-off filter with a half bandwidth of 10 nm at 580 nm, and transferred by an optical fiber to detection unit equipped with a R928 PMT (Hamamatsu, Japan). The experimental results were then processed by the software installed in the BOD system.

2.2. Chemicals and BOD standard solution

The oxygen-sensing indicator, $[Ru(Ph_2phen)_3](ClO_4)_2$ (Ph_2phen = 4,7-diphenyl-1,10-phenanthroline), was synthesized and purified in the laboratory of Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University. Dimethyldimethoxysilane (DiMe-DMOS) was obtained from Fluka AG (Buchs, Switzerland). Tetramethoxysilane (TMOS) and poly(vinyl acetate) (PVA) were purchased from Aldrich (Milwaukee, WI, USA). A PVA–ORMOSILs material containing 8% (w/w) PVA solution and 1:1.2 TMOS:DiMe-DMOS (v/v), was prepared for the multi-microorganisms immobilization. The buffer solution 0.067 mol L^{-1} KH_2PO_4–Na_2HPO_4 buffer (pH 7.4) was selected for BOD measurements. The standard BOD solution was prepared by adding 0.0750 g glucose and 0.0750 g L^{-1} glutamic acid (GGA) into 100 mL phosphate buffer solution (pH 7.4), and used as the standard BOD solution (1000 ± 185 mg L^{-1}) [16]. The seawater samples were taken from the area around Xiamen University and filtrated by 10 μm isopore membrane filters (Millipore, USA) before determination. All other chemicals were of analytical grade and the distilled water was used throughout.

2.3. Microorganisms

Three microorganisms, *B. licheniformis*, *D. maris* and *M. marinus*, were selected and applied for BOD sensing film. *D. maris* and *M. marinus* were selected and obtained directly from seawater of Yantai, north of China. *B. licheniformis* was obtained from freshwater and cultured in seawater (Fig. 1).

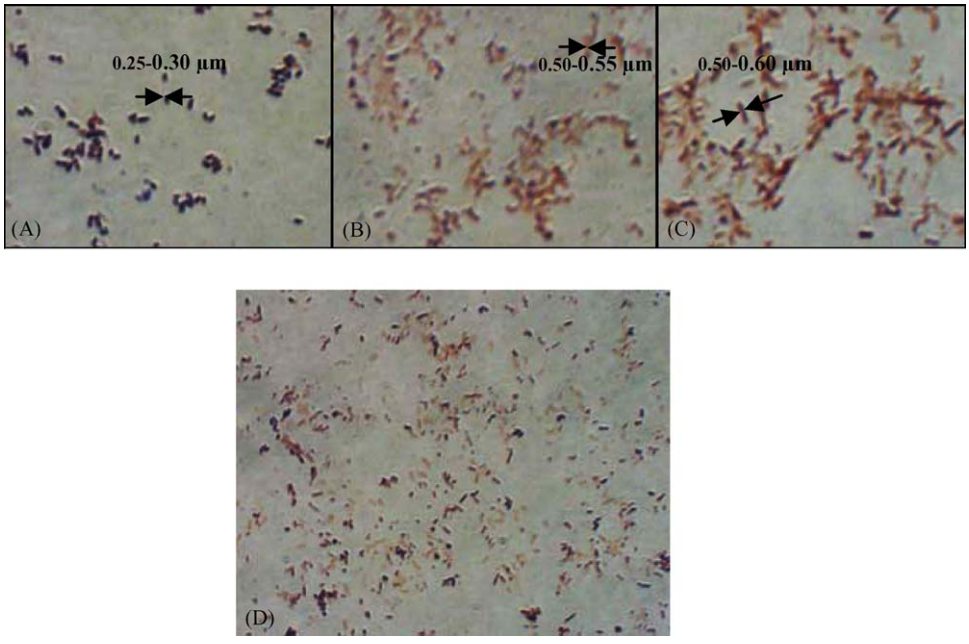

Fig. 1. Microscope pictures of the microorganisms for BOD application. (A) *Marinobacter marinus*, (B) *Dietzia maris*, (C) *Bacillus licheniformis* and (D) multi-microorganisms (amplificatory times: 3000).

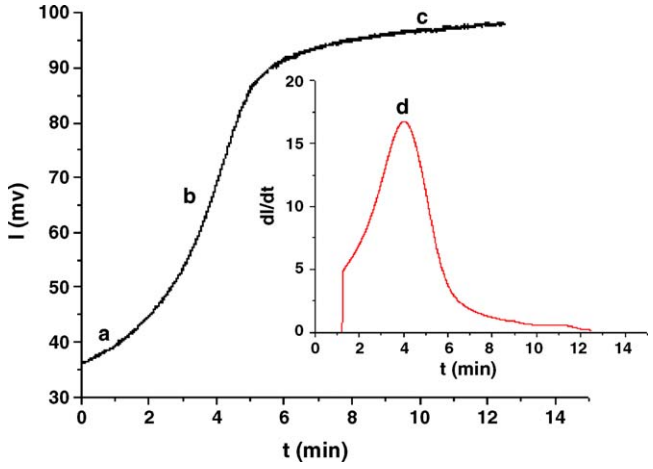

Fig. 2. Typical response of the BOD sensor when exposed to a standard BOD solution. Experimental conditions: constant temperature, 35 °C; pH of buffer, 7.4; concentration of NaCl, 3.2%; concentration of BOD, 5 mg L^{-1}. Film immobilized with sieved bacteria from seawater was used.

2.4. BOD sensing film preparation

In the preparation of oxygen sensing film, a detail description was presented in the reference [15]. ORMOSILs were prepared by mixing TMOS, DiMe-DMOS and 0.01 mol L^{-1} HCL (1:1.2:1.5 v/v/v) for the microorganisms immobilization. The precursors were stirred at 60 °C for 1 h. 200 μL of microorganisms (*B. licheniformis*:*D. maris*:*M. marinus*, 1:2:1 w/w/w) in solution was mixed with 200 μL 8% (w/w) PVA and 200 μL ORMOSILs. After the mixture of sol was spread onto an optical oxygen-sensing film produced. The measurement signal is reproducible within a mean value of ±12% in a series of 10 pieces of BOD sensing film in 10 mg L^{-1} BOD standard GGA solution.

2.5. Experimental procedure

The BOD sensing film was inserted into a black and airtight detection cell in which sample solutions were kept motionless in all measurements. The temperature of the detection cell was maintained at 35 °C. When the temperature of the sample solution (20 mL) reached at 35 °C, the detection cell was overturned by the manipulate program, and the solution was added into the detection cell. The output of the sensing film increased gradually and then reached a steady state after a few minutes. The typical response is presented in Fig. 2. Experiment results showed that there was a linear relationship between the maximal changing rate of fluorescence intensity (d*I*/d*t*) and the BOD value.

3. Results and discussion

3.1. BOD measurement mode

In the BOD measurement, the immobilized microorganisms have the processes of endogenous and active respiration. A typical optical response of the BOD sensing film was presented in Fig. 2. As shown in Fig. 2a, the initial steady-state output of the sensor represented the endogenous respiration state of the immobilized microorganisms. When the sample solution was added into the cell, the consumption of the dissolved oxygen surrounding the microbial film started and the output of the sensor increased gradually accordingly (Fig. 2b). After a while, the microorganisms stepped into an active respiration state, decomposing various organic substances, leading to the rapid consumption of the oxygen surrounding the microbial film and resulting in the prompt increase of the output of the fluorescence intensity. When the balance of the consuming and diffusion rates of oxygen on the microbial film appeared, the immobilized microorganisms put up the steady endogenous respiration state again and the output came into a steady state by degrees (Fig. 2c).

There are two measuring techniques available for the above BOD sensing response, the steady-state method (SSM) and the initial-rate (DTM) [17,18]. In SSM, a steady-state current or fluorescent intensity could be obtained at the beginning representing equilibrium between the oxygen diffusion and the endogenous respiration rate of the immobilized bacteria. The current or fluorescent intensity difference (ΔI) between the two steady states corresponded to the BOD value of the sample. The response time was around 9 min followed by 20 min recovery time. In the DTM, the rate of change of the current or fluorescent intensity (d*I*/d*t*), which reflects acceleration of the bacterial respiration rate, was used for estimating the BOD value of samples to a certain extent. This method normally provided a 4 min response time followed by a recovery time less than 10 min for BOD measurement. The experiment results showed that there was a linear relationship between the maximal mutative velocity of fluorescence intensity (d*I*/d*t*) and BOD values (Fig. 2b).

3.2. PVA–ORMOSILs material for microorganism's immobilization

PVA is a kind of non-toxic and hydrophilic materials and is capable of maintaining the maximum microbial activity. In the study, PVA has been applied to immobilize microbial cells for BOD biosensing films. In Fig. 3, PVA/ORMOSILs is evenly spread in the network shape on an oxygen ORMOSILs film. A crosslinking of DiMe-DOMS and TMOS are considered to be dispersed homogeneously in the PVA matrix through hydrogen bonds [19]. The content of PVA has an obvious effect on the physical characteristics of the microorganism film. The increase of the DiMe-DMOS concentration can enhance the response and the flexibility of the microbial sensing layer. However, the further improved hydrophobicity cannot guarantee enough solubility for PVA in the ORMOSILs matrix and then lengthen the response time. Under our preparation conditions, the optimized volume ratio of DiMe-DMOS to TMOS was 1.2 to 1 (v/v). Furthermore, a concentration of 8% PVA in ORMOSILs was selected since the percentage of PVA arrives at 10% (PVA/ORMOSILs v/v), the film begins to dilate and flake away from oxygen film.

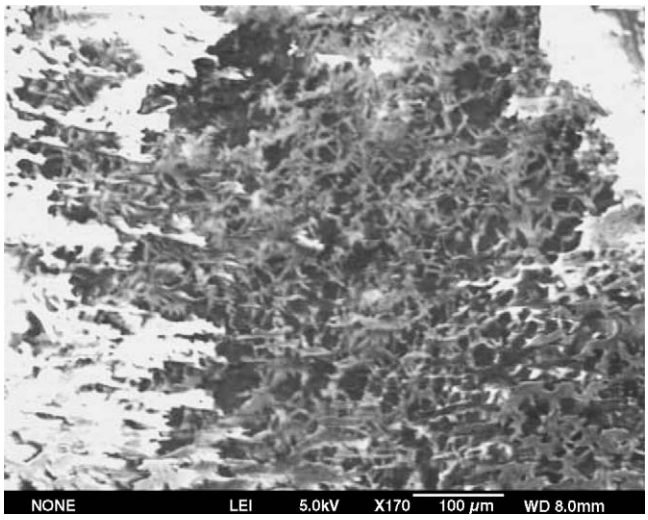

Fig. 3. FE-SEM images of an PVA/ORMOSILs on oxygen sensing film.

3.3. Effect of temperature, pH and co-exciting substances

The suitable temperature is found to be around 35 °C. As shown in Fig. 4, although the response intensity increased rapidly and the response time shortened sharply till 50 °C, the lifetime of the BOD sensing film was shortened since the microorganism became inactive easily at a higher temperature and the reduction of oxygen concentration in the solution. As a result, in order to prolong the lifetime of the bacteria, a temperature of 35 °C was selected in studying the films of sieved bacteria from seawater.

As the pH of seawater is around 7–8, the influences of pH on BOD response were investigated from pH 6.0 to 8.5. The fluorescent response increases until pH 7 and remains constant above that. Since the mobilized microorganisms are cultured or sieved by seawater, they present high assimilation efficiency to BOD and adapt the application to seawater. Therefore, the BOD sensor can be employed to detect the BOD value of different seawater samples. In subsequent experiments, the pH of samples was adjusted to 7.9 using phosphate buffer.

In the application of BOD measurement in seawater, chloride ion is the most common component of seawater with an average concentration of 3.2%. Generally, chloride concentration greatly affects the activity of microorganisms. Most biosensors immobilized with limnetic microorganisms are not suitable for seawater application since the response intensity decreases sharply with the increase of chloride ion concentration. In our study, the response intensity only decreased by 15% when the concentration of chloride ion was higher than 4%. The result indicated that the BOD sensor is able to tolerate the inhibition effect of high salt concentrations in seawater on microorganism respiration.

The effect of coexisting compounds is another essential factor for BOD sensing film. The experiments were performed to test the effects of most metal ions at a concentration of 1 mg L^{-1} on the fluorescent intensities in 5 mg L^{-1} GGA solution. The experimental results showed that except silver and lead cations, the prepared BOD sensing films were almost independent to those ions owing to their high hydrophobic surface.

3.4. Effect of different cell concentrations of the microbial layer

A higher cell density in a microbial layer can be applied to achieve a better signal response even for very low BOD. However, as shown in Fig. 5, it is obvious that sensing film containing higher cell concentration is more sensitive but has a smaller dynamic range and a longer response time when the volume of multiple microorganisms is beyond 200 μL (44 mg L^{-1} for each microorganism, 1:2:1 w/w/w). It is due to the higher oxygen consumption by the cells through the endogenous respiration [20].

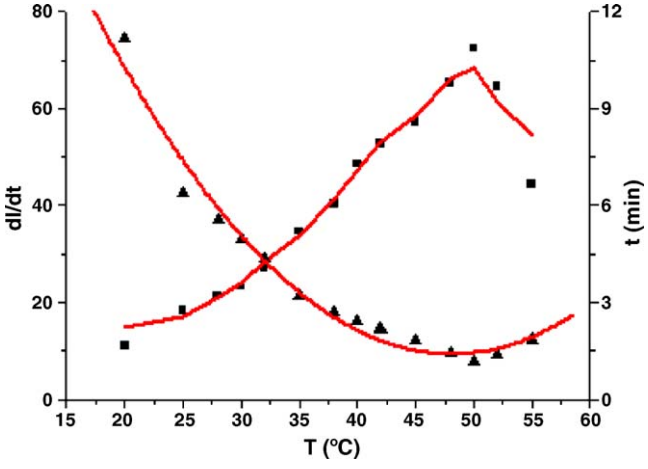

Fig. 4. Effect of temperature on the sensor response. Experimental conditions: constant temperature, 35 °C; pH of buffer, 7.9; GGA concentration, 10 mg L^{-1}; concentration of NaCl, 3.2%. (▲) Effect of temperature on the sensor response time; (■) effect of temperature on the change of velocity of the fluorescence intensity (d*I*/d*t*).

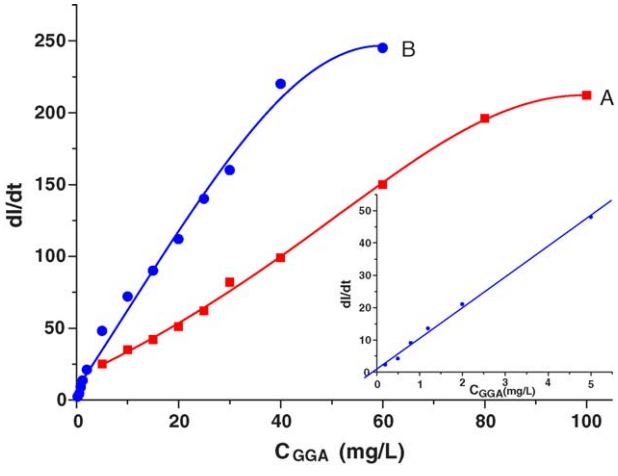

Fig. 5. Linear responses of BOD sensing films to different concentration microorganisms immobilization. Film immobilized with microorganisms solution, 0.1 mL (A) and 0.2 mL (B). Experimental conditions: constant temperature, 35 °C; pH of buffer, 7.9; concentration of NaCl, 3.2%; inset: linearity of (B) at low BOD concentration.

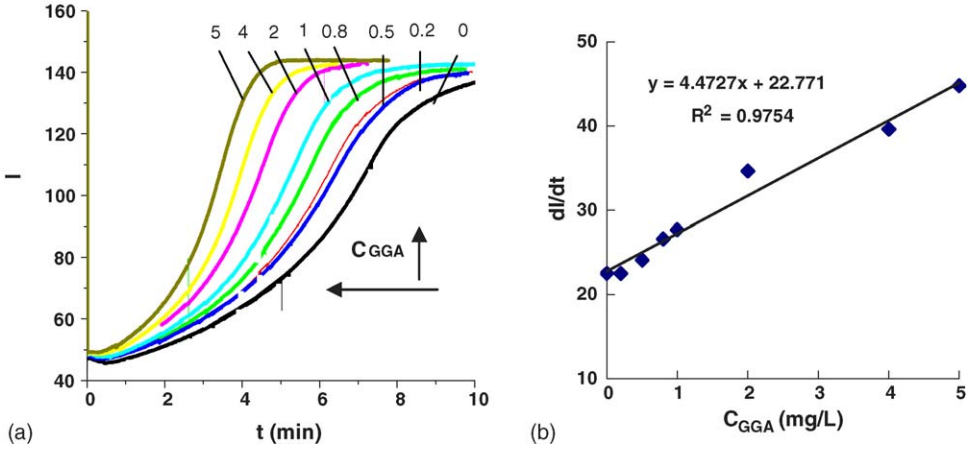

Fig. 6. Response curve (a) and response linearity (b) of co-immobilized sensing film from 0 to 5 mg L^{-1} GGA solution BOD value increased: (1) 0 mg L^{-1}, (2) 0.2 mg L^{-1}, (3) 0.5 mg L^{-1}, (4) 0.8 mg L^{-1}, (5) 1 mg L^{-1}, (6) 2 mg L^{-1}, (7) 4 mg L^{-1} and (8) 5 mg L^{-1}. Conditions: *T*, 35 °C; pH, 7.8; NaCl, 3.2%.

3.5. Characters of BOD sensing film

In the experiment, the fluorescent intensity from BOD sensing film was increased with solutions containing higher concentration of GGA. A linear relationship between BOD standard GGA concentration and change of velocity of the fluorescence intensity (d*I*/d*t*) was observed in the range of 0.2–40 mg L^{-1} GGA solution. The detection limit of the sensor is 0.1 mg L^{-1}. As shown in Fig. 6, a typical response time for 5 mg L^{-1} BOD standard GGA solution is about 3.2 min.

In the BOD sensing application, the films generally needed reactivation before used. The stability of the sensors was determined after they had been immersed in the GGA solution (BOD of 100 mg L^{-1}) at pH 7.9 and 35 °C. After 1 month's storage, the initial fluorescence response of the films decreased by 10% compared with the newly made films, but the response to BOD remained the same if the sensing film was reactivated well. Experimental result demonstrated that the BOD sensing films could be stored up to 10 months without significant deterioration. However, the films had to be reactivated for 1 day in GGA solutions (BOD of 100 mg L^{-1}) before use, in order to achieve the best sensitivity, stability and reproducibility.

3.6. Application for BOD determination of seawater

The automatic BOD system was set on a ship for pollution investigation of seawater. The system could be controlled by an individual industrial manipulative computer that installed in the system or a main computer system on the ship by an S-232 mode as well (Fig. 7). The processes including the reactivation of BOD sensing film, the calibration of the linearity of d*I*/d*t* and BOD concentration, keeping a constant temperature, sampling and the determination data transportation to the main controlling computer, could be performed automatically. Seawater samples from different sampling places were pumped into a tank on the ship, and then flowed through a membrane filter with a pore size of 50 μm. Before testing, a 20 mL water sample was stored and pre-heated in the measurement cell. The cell turned down to begin the measurement as soon as the temperature reached 35 °C. The BOD detection results have been compared with those of the Chinese Standard Process for BOD_5 (GB 7488-87), and are listed in Tables 1 and 2.

From the determination results of BOD, it could be found that the sample pH value almost kept at 7.9. There are obvious differences of BOD concentration between cleaning seawater area such as swimming place and the living area around Xiamen

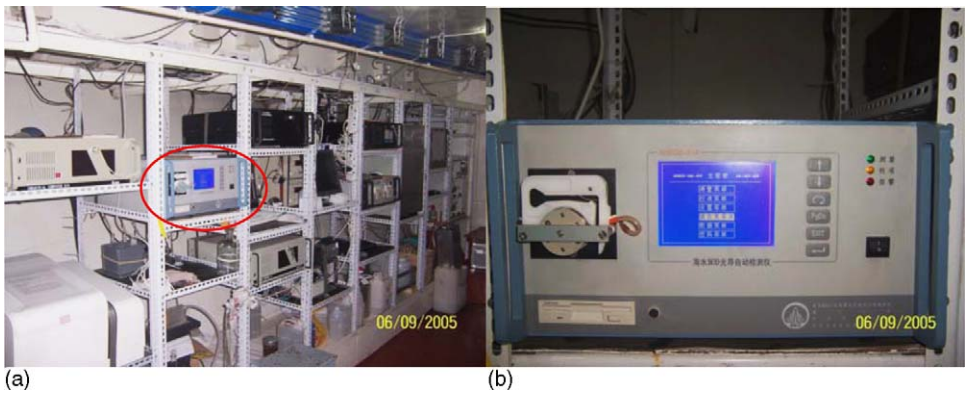

Fig. 7. Automatic BOD system for seawater pollution investigation. (a) BOD system was set on the investigation ship; (b) view of BOD system.

Table 1
BOD determination results from a living area around Xiamen University (118°05′53.0″ E, 24°26′05.0″ N; $n = 6$)

Date	Sample	Testing result (mg L^{-1})	GB BOD_5 (mg L^{-1})	pH
2004/3/11	Tide seawater	3.82 ± 0.09	3.5 ± 0.2	7.90
	Refulgent seawater	4.33 ± 0.17	4.5 ± 0.4	
2004/3/24	Tide seawater	3.68 ± 0.19	3.6 ± 0.2	7.88
	Refulgent seawater	4.68 ± 0.11	4.2 ± 0.3	
2004/4/12	Tide seawater	2.76 ± 0.09	2.9 ± 0.3	7.90
	Refulgent seawater	4.79 ± 0.09	5.3 ± 0.4	
2004/5/13	Tide seawater	3.67 ± 0.08	3.8 ± 0.3	7.92
	Refulgent seawater	4.32 ± 0.16	4.5 ± 0.4	
2004/5/18	Tide seawater	3.16 ± 0.07	3.0 ± 0.4	7.87
	Refulgent seawater	4.23 ± 0.10	4.3 ± 0.4	
2004/5/28	Tide seawater	3.22 ± 0.16	3.5 ± 0.4	7.90
	Refulgent seawater	4.10 ± 0.06	3.9 ± 0.2	
2004/6/12	Tide seawater	3.16 ± 0.10	3.0 ± 0.4	7.91
	Refulgent seawater	2.44 ± 0.13	2.5 ± 0.2	
2004/9/14	Tide seawater	2.57 ± 0.11	2.8 ± 0.2	7.90
	Refulgent seawater	3.83 ± 0.04	3.7 ± 0.4	

Table 2
BOD determination results from a swimming site around Xiamen University (118°06′32.1″ E, 24°25′53.4″ N; $n = 6$)

Date	Sample	Testing result (mg L^{-1})	GB BOD_5 (mg L^{-1})	pH
2004/3/11	Tide seawater	2.12 ± 0.15	2.0 ± 0.2	7.90
	Refulgent seawater	3.48 ± 0.16	3.2 ± 0.2	
2004/3/24	Tide seawater	2.34 ± 0.09	2.1 ± 0.2	7.87
	Refulgent seawater	3.68 ± 0.09	3.3 ± 0.3	
2004/4/12	Tide seawater	2.09 ± 0.17	2.3 ± 0.2	7.89
	Refulgent seawater	3.26 ± 0.12	3.0 ± 0.2	

University. The results indicated that some organic pollutants were let from residential area. Moreover, the BOD concentration of refulgent seawater became higher than that of tide seawater, further confirmed that the increase of the BOD value was caused by the wastewater from the living area.

4. Conclusions

An automatic BOD system with a novel optical BOD sensing film immobilizing multi-microorganisms was successfully applied for the BOD determination of seawater. Some factors such as measurement temperature, pH, co-existing substance, sodium chloride, which affected the BOD determination, were studied. The minimum measurable BOD by this system was 0.1 mg L^{-1}, and the response time was only 3.2 min for 5 mg L^{-1} BOD. The study results showed that the BOD system exhibits its advantages in the determination of low BOD, and is suitable to evaluate the complicated seawater samples without being dramatically affected by sodium chloride and other ions. The determination results obtained from actual seawater demonstrate a good correlation with those obtained from conventional BOD_5 analysis.

Since the rapid and on-line monitoring of BOD is of major concern when surveying water pollution, the new technologies using optical fiber will lead to improved BOD sensors. For a BOD system's commercial viability, the robustness, simplicity, rapidity and cost considerations of the BOD system are other challenging areas of research.

Acknowledgements

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