

## An optical oxygen sensor and reaction vessel for high-pressure applications

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### Abstract

We describe a simple hyperbaric chamber and optical oxygen probe (optrode), which is based on the dynamic fluorescent quenching of a ruthenium metal complex, that can be used to measure changes in oxygen concentration in either aqueous or gaseous media. Initial experiments illustrate the utility of this robust form of sensor. The optrode showed a typical response time of <10 s, a linear temperature response with greater fluorescent quenching at lower temperatures, and was unaffected by pressures as great as 34.4 mPa (340 atm). Yeast cultures measured at 27.8 mPa (275 atm) showed an up to ninefold decrease in respiration rate compared to cells at 1 atm. The oxygen optrode is a simple and rugged device that appears exceptionally well suited for experimentation under conditions in which polarographic electrodes or conventional chemical analysis is difficult, e.g., at high or variable pressures.

The ability to measure concentrations of molecular oxygen easily and accurately in gaseous, aqueous, or biological fluid samples is of primary importance in a large number of medical, biological, and physical studies. Most physiological and biological investigations that require the measurement of oxygen concentration commonly employ amperometric or potentiometric membrane-diffusion electrodes (i.e., polarographic or Clark-type electrodes, after Clark 1956; Kahn 1964) or utilize classic Winkler titration (based on redox chemistry). Unfortunately, these methods are inadequate for sensing in many situations. The Winkler titration is laborious and is not suitable for continuous noninvasive monitoring of oxygen concentrations. Electrodes tend to be fragile, easily fouled, consume oxygen during the measurement process, and are very susceptible to stirring effects as well as fluctuations in temperature and external pressure (*see* summary in Wolfbeis 1991).

Optical sensors (optrodes) for measuring environmental parameters such as gas concentration, temperature, and ion presence are now starting to be used more frequently, particularly in those instances when measurements must be made under “extreme” conditions. The pioneering efforts of J. Peterson, O. Wolfbeis, and I. Klimant, as well as J. Bacon and J. Demas and their colleagues, have resulted in the engineering of several unique oxygen optrodes that are typically much more robust than their chemical or electrode counterparts (Peterson et al. 1984; Wolfbeis et al. 1986; Bacon and Demas 1987; Wolfbeis 1991). These sensors do not consume oxygen, have predictable temperature responses,

are pressure insensitive, have fast response times and long-term stability, and can be made inert to many common laboratory anesthetics and other chemical interferents (e.g., DMSO, HCl, acetic acid, ethanol) (Bacon and Demas 1987; Carraway et al. 1991*a, b*; Wolfbeis 1991). Although it has been tested in the biomedical field (e.g., Peterson et al. 1984) and in a few other specialized biological applications (Preininger et al. 1994; Singer et al. 1994; Weigl et al. 1994; Klimant et al. 1995; Papkovsky 1995; Rosenzweig and Kopelman 1995), oxygen optrode technology has not yet seen wide adoption, especially in limnological and oceanographic studies.

Although several techniques exist for the optical detection of oxygen using, for example, chemiluminescence or the reflectometry of hemoglobin, the majority of present optrodes depend upon dynamic quenching of an oxygen-sensitive fluorophore. Typical fluorophores include polycyclic aromatic hydrocarbons (e.g., pyrene and fluoranthene), porphyrins, longwave absorbing dyes, and transition-metal-organic complexes. Because oxygen acts as a powerful quencher of fluorescence, the decrease in fluorescence intensity and/or change in fluorescence decay lifetime can be used as a measure of oxygen concentration. In an ideal situation, the quenching process follows the Stern–Volmer equation:

$$\frac{I_o}{I} = 1 + K[Q]^m \quad (1)$$

where  $I$  is the fluorescence signal intensity,  $I_o$  is the signal intensity in the absence of oxygen,  $Q$  is the quencher concentration (oxygen),  $K$  is the Stern–Volmer quenching constant, and  $m$  is a fitting parameter. Because the Stern–Volmer equation is linear (at  $m = 1$ ), it is possible to calibrate a dynamic quenching optrode with just one point.

We wished to construct a simple and inexpensive pressure-resistant vessel incorporating an oxygen-sensitive optrode to monitor the changes in oxygen concentration under pressures similar to those encountered in the deep sea, which would operate in a large range of different temperatures. The sensing chemistry and design of the optrode were chosen to utilize nonproprietary and readily available materials.

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### Acknowledgments

Construction of the optrode and pressure vessel would not have been possible without the generous help of John Lee at Hopkins Marine Station, Stanford University. Critical advice on sensor chemistry was provided by J. R. Bacon at the University of Mississippi, and we would like to thank Mike Fornier at GFS Chemical Co. for his assistance in procuring the ruthenium complexes. Portions of this work were supported by NSF grant IBN-9727721 to G.N.S. M.D.S. was supported by a Canadian NSERC postdoctoral fellowship.

## Methods

**Sensor chemistry and polymer support**—The chemical dye chosen as the oxygen-quenched indicator was Ruthenium II tris (4,7-diphenyl-1,10-phenanthroline) chloride ( $\text{Ru}[(\text{C}_6\text{H}_5)_2\text{C}_{12}\text{H}_6\text{N}_2]_3\text{Cl}_2$ , GFS Chemicals). This dye consists of the transition-metal complex,  $\text{RuL}_3^{2+}$ , where L is the a-diimine (or ligand) 4,7-diphenyl-1,10-phenanthroline, abbreviated as  $\text{Ph}_2\text{phen}$  (all abbreviations follow Carraway et al. 1991a, b). This dye has several characteristics that make it suitable for oxygen optrode use. It is readily available and inexpensive. Its fluorescence-quenching behavior is well characterized (Watts and Crosby 1971; Demas and DeGraff 1989). It is capable of being bound into a variety of different physical supports for either membrane or sensing film construction without laborious chemical modification. Most importantly,  $\text{Ru}(\text{Ph}_2\text{phen})_3^{2+}$  has a large Stokes shift with an excitation maximum at 450 nm and emission maximum of ca. 610 nm, allowing the separation of emissive fluorescence from the excitation light with inexpensive glass cut-off filters. The 450-nm excitation maximum also allows the use of economical, and now readily available, blue light-emitting diodes (LEDs) for the stimulation of the fluorescence. The variation in fluorescence intensity, rather than fluorescence lifetime (e.g., Gruber et al. 1995), was chosen as the sensor mode because inexpensive and less complex electronic components are required to process the optrode signal (Gruber et al. 1993, 1995).

Silicone rubber was chosen as the physical support for the sensing chemistry. Silicone has a high oxygen permeability, allowing fast response times, and has already been proven to produce sensitive and robust optrodes (Wolfbeis 1991). Although ruthenium complexes are ionic and thus display poor solubility in silicones, different techniques have been developed for entrapment in the silicone matrix. Immobilization of the transition-metal indicator into the polymer has been promoted by complex procedures involving covalent modification of the metal-organic complexes (Klimant and Wolfbeis 1995), synthesis of new silicon-soluble forms by replacing chloride anions with organic counter anions (Klimant and Wolfbeis 1995), or binding of the metal complex onto silica gels (i.e., Wolfbeis et al. 1986; Klimant et al. 1994) or zeolite Y (Meier et al. 1995) prior to embedding in a silicone matrix. Simple adsorption of the  $\text{Ru}(\text{Ph}_2\text{phen})_3^{2+}$  into the silicone rubber was chosen as the method of indicator immobilization, even though it does not produce a sensor with the greatest concentration of indicator (Bacon and Demas 1987; Carraway et al. 1991a, b).

Construction of the sensor film followed a protocol modified from Bacon and Demas (1987) and Carraway et al. (1991b). A thin film of transparent silicone rubber polymer (Dow Corning 999), ca. 0.15 mm in thickness, was bonded directly onto the quartz glass window of the optrode. The window was then soaked for 12 h in a 2-mM solution of  $\text{Ru}(\text{Ph}_2\text{phen})_3^{2+}$  in dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) as a solvent. The window with sensor coating was then rinsed quickly in  $\text{CH}_2\text{Cl}_2$ , and the excess was allowed to evaporate very slowly for several hours, to optimize the homogeneous retention of the  $\text{Ru}(\text{Ph}_2\text{phen})_3^{2+}$  within the silicone polymer (Bacon pers. comm.).

Unfortunately, transition-metal complexes immobilized in silicone rubber using this technique do not exhibit perfectly linear Stern–Volmer calibration graphs. Microheterogeneities in the silicone matrix yield at least two different reactive sites (one aggregated, the other homogeneously distributed) within the polymer and lead to downward curvature of  $I_o/I$  vs. quencher concentration plots (Carraway et al. 1991a,b; Draxler et al. 1995; Klimant and Wolfbeis 1995). For this reason, a modification to the classic Stern–Volmer equation (Eq. 1) was used to calibrate the optrode:

$$\frac{I_o}{I} = \frac{1}{f_1(1 + K_{sv1}[Q])^{-1} + f_2(1 + K_{sv2}[Q])^{-1}} \quad (2)$$

where  $I$  is the fluorescence signal intensity,  $I_o$  is the signal intensity in the absence of oxygen,  $Q$  is the quencher concentration (oxygen),  $f_{1,2}$  are the fractions of the total fluorescent emission from each of two different component sites, and  $K_{sv1,2}$  are the Stern–Volmer quenching constants for the two components (Carraway et al. 1991a, b). These parameters were estimated using an iterative, least-squares regression model (the Levenberg–Marquardt method; Press et al. 1988) and initially seeded from values of  $K_{sv1,2}$  in the literature (Carraway et al. 1991a, b; Klimant and Wolfbeis 1995).

**Experimental hardware**—The pressure-resistant reaction vessel was constructed from a single piece of stainless steel (Type 316), and closed with a threaded, O-ring-sealed plug that also housed the optrode (Fig. 1A). The reaction chamber has a 5-ml volume, and the inclusion of a miniature, teflon-coated magnetic stir bar allows mixing of the chamber contents. An access port in the reaction chamber allows pressurization of the vessel by a hand-operated hydraulic pump, using mineral oil as the transducing fluid. Two additional chamber ports allow the injection and ejection of fluids into the chamber using a standard high-pressure liquid chromatography (HPLC) pump. The entire vessel can be immersed in a temperature-controlled water bath as required.

The principal components of the optrode were encased in the pressure-vessel plug (Fig. 1B). The oxygen-sensitive  $\text{Ru}(\text{Ph}_2\text{phen})_3^{2+}$  coating was fixed to the surface of a quartz glass window mounted in the end of the plug forming the top of the reaction chamber (Fig. 1B). Excitation light was provided by three blue LEDs (Panasonic LNG99) mounted triaxially in the top of a transparent plastic light guide (optical plexiglass rod) and filtered via blue Schott glass (BG12) cut-off filters. Fluorescence from the sensor coating was detected by a silicon photodiode mounted in the bottom center of the light guide, behind the quartz glass window. The photodiode (Burr-Brown OPT301) was covered with a Schott glass (OG570) filter that prevented the transmission of light with a wavelength  $<570$  nm.

Power for both the excitation and detection circuitry was provided by a 12-V dual-polarity power supply (Fig. 2). To help minimize intensity fluctuations in the excitation LEDs, a sensitive power-regulating circuit was used (Fig. 2, top) to provide  $3.75 \pm 0.001$  V to the LEDs. The fluorescence detected by the photodiode was amplified with a simple circuit utilizing an inexpensive Operation-Amplifier (LM356) with signal output to a standard laboratory chart recorder (Fig. 2, bottom). In addition, the inclusion of a reference photodiode

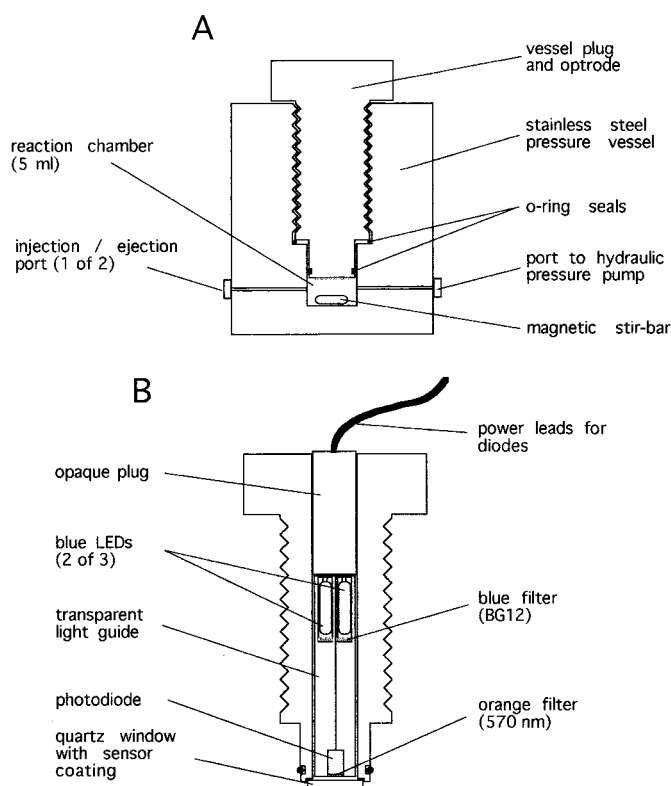


Fig. 1. (A) Schematic cross-section of high-pressure vessel and optrode (enclosed in vessel plug). Vessel is constructed from a solid piece of Type 316 stainless steel. Ports in the reaction chamber are connected to external hydraulics, etc., with 1.5-mm (outside diameter) stainless steel high-pressure tubing. Only two of three chamber ports are shown. The vessel is 8 cm in diameter. (B) Close-up of vessel plug and oxygen optrode from Fig. 1A. The optoelectronic components of the optrode are held within a transparent plastic light guide inserted into the hollow vessel plug. Power for the LEDs and photodiode(s) is provided through a shielded cable and connected to an external circuit box.

adjacent to the LEDs allows compensation for fluctuations in excitation illumination via feedback to the amplification circuitry or as a supplementary circuit.

**Calibration and experimental testing**—The nature of the Stern–Volmer equation allows linearization of the changes in fluorescence signal intensity and, therefore, sensor calibration with a simple two-point scheme (at 0% oxygen = nitrogen gas or anoxic water [5% sodium sulfite solution] and ca. 21% oxygen = air). These calibrations were performed at room temperature (ca. 20°C), as well as from 5 to 40°C (in 5°C increments), by placing the pressure vessel in a temperature-controlled water bath. These calibrations were also run with the reaction chamber connected in series with a standard polarographic oxygen electrode (Strathkelvin 781) for comparison. Additional calibrations were performed at greater than atmospheric pressure by hydraulically pressurizing the reaction chamber.

Initial experiments of the pressure effects on oxygen metabolism were performed on yeast cultures (*Saccharomyces cerevisiae*) at atmospheric and higher pressures. Oxygen

consumption by 5 ml subsamples of a culture growing in a 2% glucose solution (at 32°C) was monitored in the reaction chamber under atmospheric and higher pressures. The optical density of the culture subsamples was first determined photometrically (at 500 nm) to allow the calculation of relative respiration rates at a standard culture density.

## Results

The intensity of the emitted fluorescence by the sensor film was much greater under anoxic conditions than in the presence of oxygen (Fig. 3A,B). There was little to no hysteresis in the intensity signal during rapid cycling of the optrode between air and nitrogen gas (or between air-saturated water and an anoxic sodium sulfite solution), as shown in Fig. 3A. The response time for the sensor was ca. 8 s in gaseous media and was three to five times slower in fluid for sensing films of the same thickness. Optrode response time could be dramatically decreased using thinner sensor films; however, thinner films show a decreased fluorescent signal intensity (data not shown). The absolute emitted intensity could be increased by either changing the concentration of  $\text{Ru}(\text{Ph}_2\text{phen})_3^{2+}$  in the sensor film, changing the film's thickness, or by incorporating light-scattering particles (i.e., titanium dioxide particles) into the sensor film.

The intensity of the emitted signal increased linearly with decreasing temperatures between 5 and 40°C (Fig. 4). The difference in signal intensity between a 21% oxygen solution (i.e., air or air-saturated water) and an anoxic solution at 5°C was ca. two times greater than at 40°C. The regression of the intensity difference as a function of temperature was calculated to be: relative intensity =  $-0.15 \times \text{temp}(\text{°C}) + 1.003$  ( $R^2 = 0.989$ ,  $n = 8$ ).

As would be expected for this type of optrode, pressure changes within the reaction chamber had no effect on the emitted signal intensity. Cycling the chamber between 0.101 mPa (1 atm) and 27.8 mPa (275 atm) yielded a  $\ll 1\%$  variation in the relative fluorescence that was not correlated with pressure ( $r = -0.108$ ,  $n = 20$ , data not shown).

In accordance with quenching theory, there was a nonlinear relationship between the emitted fluorescence intensity and the oxygen partial pressure, with an intensity in anoxic conditions almost twice that in air-saturated conditions (Fig. 5A). The resolution of the sensor was estimated to be  $<0.1\%$  of the air saturation, although dependent on optrode size, film thickness, and resolution of the laboratory chart recorder. A sample linear calibration of the optrode using the modified Stern–Volmer equation (Eq. 2) yielded the following coefficients:  $f_1 = 0.96$ ,  $f_2 = 0.04$ ,  $K_{\text{sv}1} = 37.35 (\text{Pa} \times 10^5)^{-1}$ ,  $K_{\text{sv}2} = 35.56 (\text{Pa} \times 10^5)^{-1}$  ( $R^2 = 0.985$ ,  $n = 20$ ; Fig. 5B).

The reaction chamber and optrode allowed the easy measurement of yeast (*S. cerevisiae*) culture oxygen consumption. At 1-atm pressure and 32°C, the oxygen consumption of the cultures was ca.  $1.2 \times 10^3 \pm 2.1 \times 10^2 \text{ Pa min}^{-1}$  (mean  $\pm 1$  SD,  $n = 10$ ). At atmospheric pressure, *S. cerevisiae* cultures consumed oxygen approximately nine times faster than at 27.8 mPa (mean =  $1.4 \times 10^2 \text{ Pa min}^{-1}$ , range =  $0.4 \times 10^2 \text{ Pa min}^{-1}$ ,  $n = 4$ ) and approximately 3.5 times faster than at 135 atm (mean =  $3.4 \times 10^2 \text{ Pa min}^{-1}$ , range =  $1.0 \times 10^2 \text{ Pa min}^{-1}$ ,  $n = 4$ ).

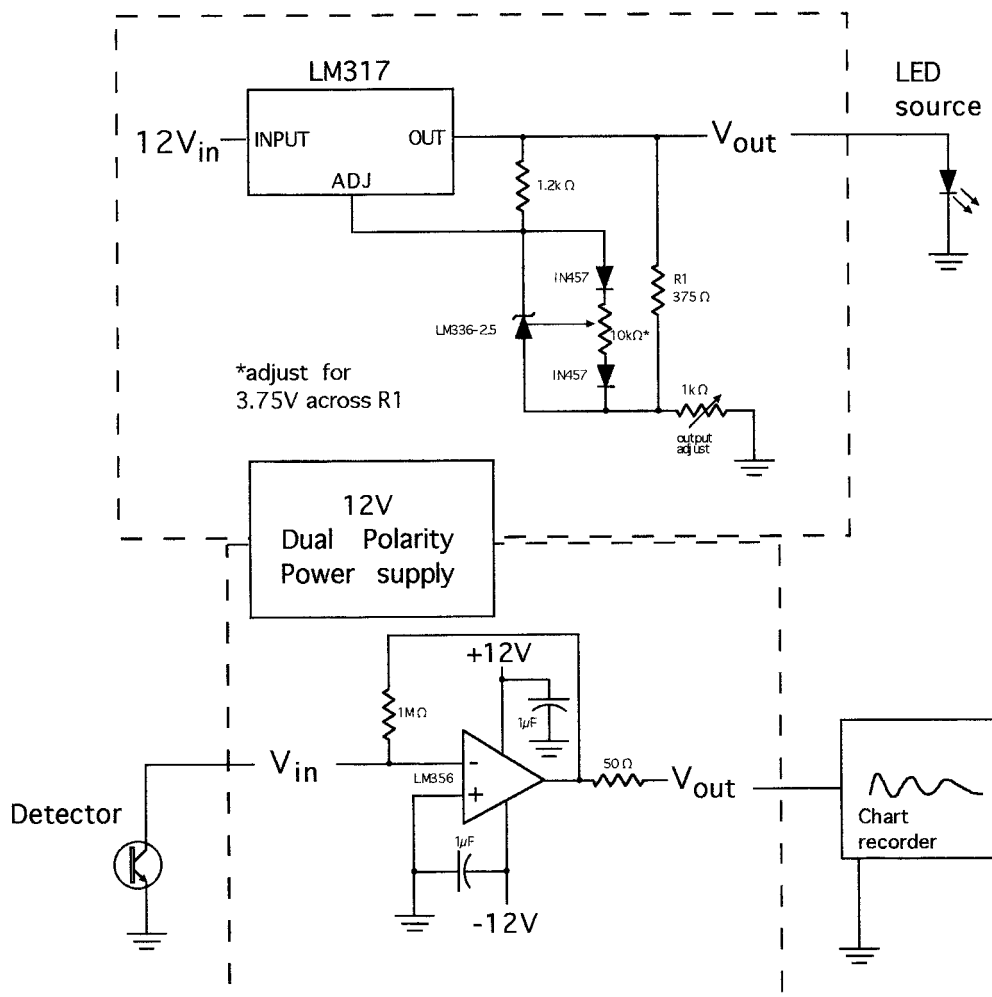


Fig. 2. Electronic circuitry for the optrode in Fig. 1. A stable power circuit (schematic at top) and the signal amplification circuit (bottom) are both powered from a 12-V dual-polarity power supply sharing a common ground. Output from the amplifier is recorded on a standard laboratory chart recorder. Both circuits are housed in a common box, and the amplification circuit received additional shielding inside a metal box connected to ground.

## Discussion

Most previous biological hyperbaric studies did not include the simultaneous measurement of oxygen concentration within the study chamber but required measurements to be made with more conventional techniques (e.g., Winkler titration) before and after pressurization (*see* summary in Avent et al. 1972; Flügel and Schlieper 1970; Flügel 1972). Alternatively, hyperbaric chambers have been constructed that incorporate a pressure-balanced polarographic electrode to monitor internal conditions (e.g., Distèche 1972; Chandler and Vidaver 1973; Meek and Childress 1973; Sébert et al. 1990, 1995). The latter design requires separate calibrations at different pressures, as well as efficient stirring to prevent the depletion of oxygen adjacent to the electrode membrane and the formation of an oxygen gradient within the chamber. The combined pressure vessel and Clark-type platinum electrode of Vidaver (1972) did allow rapid measurement of small fluctuations in oxygen levels under pressure. However,

the platinum electrode only functions with tissues and substrates in physical contact with the electrode surface. When monitoring the fluctuation of oxygen concentration in small volumes, Clark-type electrodes are problematic. Because polarographic electrodes constantly consume oxygen during use, they can rapidly deplete the oxygen in closed systems and lead to the creation of hypoxic conditions. In larger volume chambers, such as those used for macrofaunal respirometry (e.g., Meek and Childress 1973; Sébert et al. 1990, 1995), where hypoxia is of less consequence, the oxygen electrode is an effective tool but must be calibrated for use at specific pressures.

In comparison to the Clark electrode, the oxygen optrode can be incorporated directly into the pressure vessel design (i.e., the sensing film can be bonded directly on the interior of a vessel window) and does not require pressure calibration. The optrode is also not restricted to hyperbaric use or use within the confines of an environmental chamber. By covering the transparent  $Ru(Ph_2phen)_3^+$ /silicone film with a

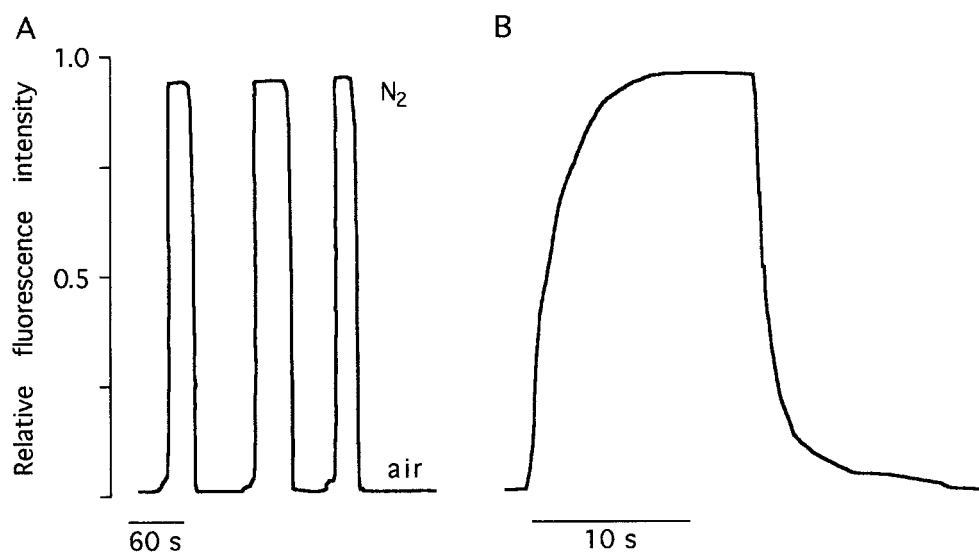


Fig. 3. (A) Changes in the relative fluorescence intensity recorded by the optrode (at atmospheric pressure and 5°C) as the reaction chamber was alternately flushed with 21% oxygen (air) and 0% oxygen (nitrogen gas). Data shown are a digitized trace from a laboratory chart recorder, 5-mV full-scale range. (B) Kinetics of relative fluorescence intensity after switching between air and nitrogen gas and then back to air. The reaction chamber is fully flushed, and the optrode reaches peak signal intensity (0% oxygen) within ca. 8 s.

secondary layer of opaque silicone shielding, the optrode can be used as an oxygen probe in the open environment. Additionally, the optoelectronics are simple and robust enough to allow easy miniaturization for field use. Also, by changing the film thickness and optrode dimensions, it is possible to optimize the optrode for specific sensing requirements. Thin films and small dimensions allow the fastest response and highest resolution (i.e., Rosenzweig and Kopelman 1995), while thicker films average fluctuations in oxygen concentration due to the increased time required for the film to reach equilibrium.

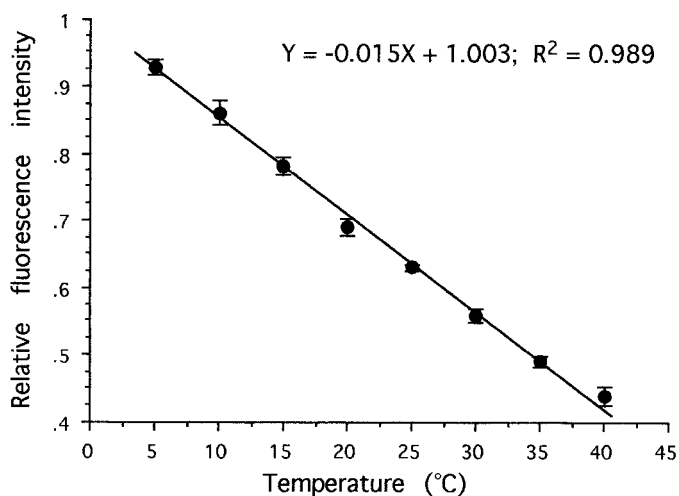


Fig. 4. Changes in optrode signal intensity with temperature (at atmospheric pressure). The mean difference in fluorescence between air- and nitrogen saturated solutions is shown, and the error bars indicate the range in value;  $n = 8$  at each temperature.

The optrode performance in our hyperbaric chamber was similar to previously reported ruthenium-complex oxygen sensors. A response time of <10 s in air corresponded to the response range found in other macroscopic silicone film optrodes of equivalent thickness (Bacon and Demas 1987; Carraway et al. 1991; Gruber et al. 1993; Klimant et al. 1995; Klimant and Wolfbeis 1995). Because of the decreased solubility of oxygen in water, which then requires longer to equilibrate the sensor film (Klimant and Wolfbeis 1995), the optrode response times were faster in air than in aqueous solution. The microscopic (optrode tip < 5  $\mu\text{m}$ ) fiber-optic oxygen sensor of Rosenzweig and Kopelman (1995) showed <1-s response times and several orders of magnitude greater sensitivity than the larger optrodes reported above; however, because of its fragile nature and complex instrumentation requirements, it is more suited to the laboratory study of oxygen gradients than for the rigors of the field or hyperbaric use. Although we have not experimented with the long-term stability of the sensor film, our anecdotal observations support the characteristics exhibited by other oxygen optrodes, which have been shown to have exceptionally long shelf lives (>1 yr) and resistance to photobleaching (Wolfbeis et al. 1986; Bacon and Demas 1987; Carraway et al. 1991a; Klimant et al. 1995).

The pressure vessel used in this study had a stainless steel construction similar to many other hyperbaric apparatus (e.g., Theede 1972). Although the current chamber could easily endure pressures >900 atm, the maximum operating pressure of the thin (1.75 mm) optrode window limited its use to 34.4 mPa (340 atm). By incorporating a thicker window into the optrode design, much higher pressures, including those found at full ocean depth, could very easily be attained. In addition to the physical robustness of the sensor,

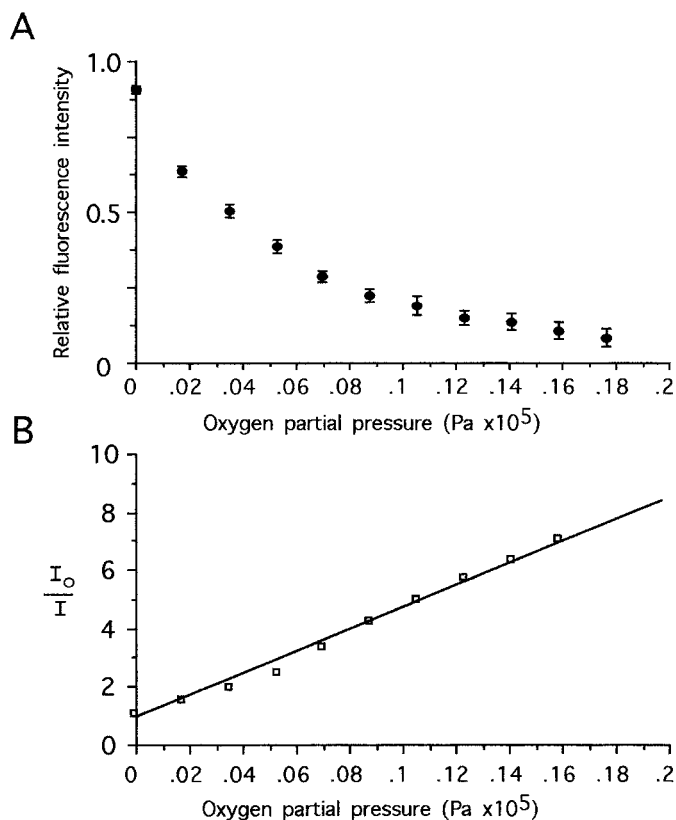


Fig. 5. (A) Relative fluorescent intensity vs. oxygen partial pressure at 20°C, 1 atm. Points indicate mean intensity, and error bars show the range of values ( $n = 8$ ). (B) Stern-Volmer plot ( $I_0/I$  vs. oxygen partial pressure) from data in Fig. 5A. The plotted line is the linear calibration provided by Eq. 2 in the text (after Carraway et al. 1991).

the increased fluorescence quenching at lower temperatures makes this optrode particularly well suited for deep-sea studies where temperatures are typically  $<5^\circ\text{C}$ .

The measurement of oxygen consumption by *S. cerevisiae* at elevated hydrostatic pressures illustrated the utility of this type of sensor. Previous studies have shown a decrease in growth rate in *S. cerevisiae* cultures at increased hydrostatic pressures (Thom and Marquis 1984; Marquis 1993). Because the optrode is insensitive to pressure fluctuations, it is possible to continuously monitor oxygen tension within the reaction chamber while simultaneously altering the hydrostatic pressure. The reduction in oxygen consumption by the yeast culture under elevated pressures complements the results of previous hyperbaric studies on bacteria and yeast and reflects changes to the respiratory physiology of the cells, with a consequent decrease in the overall growth rate of the culture (Zobel 1970; Yayanos et al. 1979). Previous studies used radioactive <sup>14</sup>C uptake as a proxy for metabolic consumption of oxygen or measured oxygen partial pressure in the culture medium before and after pressurization (Murakami 1970; Zobel 1970; Jannasch et al. 1973). Future work with the pressure vessel and oxygen optrode will include a detailed study of tissue and mitochondrial respiration in pressure-adapted deep-sea organisms.

The oxygen optrode is an underused alternative to polarographic oxygen sensors that has many advantages over its electro/chemical counterparts. The optrode described is pressure insensitive, works in either fluids or gases, and has a linear temperature response. The sensor film is not affected by many common laboratory interferents and can be sterilized. The optrode design lends itself to miniaturization and can be easily constructed from inexpensive components and assembled into a sensor much more rugged than conventional designs. Most importantly, the sensor does not consume oxygen during use and hence, is not sensitive to stirring. Although optical sensing techniques have seen some limited application in aquatic biology (Klimant et al. 1995), it is hoped that the adoption of this sensor technology will enable researchers to monitor oxygen concentration under more extreme environmental conditions, both in the laboratory and in the field, than were previously thought feasible to study.

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Received: 9 April 1998

Accepted: 18 August 1998

Amended: 21 September 1998