Oxygen microoptrodes and their application in aquatic environment

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ABSTRACT

We present a new fiber-optic oxygen microsensor (microoptrode) based on dynamic luminescence quenching which was recently developed for measuring oxygen at high spatial resolution in aquatic sediments and biofilms. Microoptrodes with a typical tip diameter of 20 to 50 µm were fabricated. The fabrication procedure is simple and guarantees a high reproducibility of the calibration curves. The microoptrodes were characterized with respect to dynamic range, response time, storage and longterm stability, interferences, temperature dependence, photostability, and mechanical stability. A special LED based luminescence intensity measuring instrument was developed. It is battery operated and can be used for field measurements. The microoptrodes were used to measure oxygen gradients in marine sediments. Comparative measurements were performed with oxygen microelectrodes. The first measurements have shown that oxygen microoptrodes present a true alternative to existing electrochemical microsensors. Nevertheless it is obvious, that the measurement of luminescence intensity of the indicator limits their practical application. Therefore a new setup was developed to make oxygen measurements with the luminescence lifetime as parameter.

1. INTRODUCTION

During the last years optical chemical sensors (optrodes) have become a realistic alternative to electrochemical sensors. One of the most frequently cited advantages of optrodes in contrast to electrodes is the argument that such sensors can be easily miniaturised. However, only a few fiber-optic microsensors are reported in the literature Small optical sensors with tip diameters down to 150 µm were developed for blood gas monitoring. Fiber-optic microsensors for intracellular pH determination were recently developed by Tan et al.[1]. These sensors are based on covalently immobilized fluorescein and have a tip size down to 0,1 µm. In the measuring configuration however, the optical fiber was exclusively used for the excitation of the indicator. The fluorescence light was collected with a lens

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system mounted at the bottom of a microscope table. Therefore these microoptrodes cannot be used for remote sensing and in vivo measurements in complex natural systems. Aharonson at al. presented a pH microoptrode based on sol gel immobilized HPTS[2]. The sensing material was fixed in the tip of a tapered glass capillary but this was not a real sensor, since excitation and detection of the fluorescence were realized externally. Thus for biological and medical applications, where measurements of chemical parameters at high spatial resolution are required, almost exclusively microelectrodes are currently used. Aquatic sediments and biofilms are populated by different phototrophic and heterotrophic microorganisms and are characterized by steep gradients of various physical and chemical parameters such as light intensity, pH, H_2S and O_2 over distances ranging from <0.5 mm to a couple of mm's[3,4]. Dissolved oxygen is one of the most important parameters in biological systems and the knowledge of oxygen concentration gradients is of paramount importance for understanding the function and regulation of microbial communities. Oxygen microsensors are powerful tools for the determination of oxygen gradients at high spatial resolution, and for the determination of rates of O_2 production and consumption without disturbing the natural microenvironment. Recently used Clark-type O_2-microelectrodes could be made with tip diameters <5 µm and have excellent measuring properties such as short response times (<1 sec), small stirring sensitivity (1-2%) and a low zero current (<5 pA)[5]. The construction of well-working Clark-type O_2-microelectrodes, however, is time consuming and requires a significant amount of training. Commercially available O_2-microelectrodes are expensive and do not have the good characteristics of the self-made Clark-type electrodes. This is a limitation for a more frequent use of such sensors. Furthermore, in applications where longterm measurements are required (e.g. gas exchange studies with flux chambers), the signal stability of electrodes is often not sufficient. We have developed an optical oxygen microsensor for measuring oxygen gradients with a high spatial resolution[6]. It is based on dynamic luminescence quenching of a ruthenium(II)-complex dissolved in polystyrene. In this presentation the oxygen microoptrodes are described and examples of their application in aquatic systems are shown. Further details on the microoptrode construction and performance are given elsewhere[6].

2.EXPERIMENTAL

2.1. REAGENTS AND MATERIALS

The luminescent ruthenium(II)-tris-4,7-diphenyl-1,10-phenanthroline perchlorate complex (Ru(dpp)) was selected as the oxygen sensitive dye and prepared as described previously[7]. Polystyrene pellets (MW 270 000) and methyl-ethyl-ketone were obtained from Aldrich. The black silicone (N-189) was from Wacker (Burghausen).

2.2. FABRICATION OF OXYGEN MICROOPTRODES

The Ru(dpp) was dissolved in polystyrene at a concentration of 5 mM by using methyl-ethyl-ketone as a solvent. The high dye concentration was necessary to obtain a sufficiently high fluorescence signal. The polymer/indicator solution is stable and can be stored in a refrigerator for at least one year without any degradation of the components. This solution forms thin polymer films on the fiber tip with an excellent adhesion to the glass surface and a good mechanical stability. An additional improvement of the mechanical stability can be obtained by adding titanium dioxide particles (ca. 1 μm grain size) to the indicator/polymer solution. The oxygen microoptrodes were constructed from ST-connectorized multimode silica/silica step index fibers with 100 μm core diameter and 140 μm cladding diameter (Radiall Germany). The protective buffer at the measuring end of the fiber was removed by heating and a fiber taper was formed while heating the bare fiber in a small flame of a gas burner. Fiber tips from 15 to 40 μm in diameter were prepared by cutting the taper with a small knife. To improve the handling of the microoptrodes the tapered fiber was mounted in an injection needle[6,8] or in a tapered glass capillary. The oxygen sensitive layer was immobilized by dipping the fiber tip once in the polymer/indicator solution. During the dip coating procedure, the fiber was moved with a xyz-micromanipulator. After evaporation of the solvent, the sensor tip was coated with a black layer of silicone. After 2 days of curing the silicone the sensor is ready for use. The oxygen microoptrodes have a tip diameter of 20 - 50 μm.

2.3. MEASURING SYSTEM

A special optoelectronical system for measuring the luminescence intensity was designed for use with oxygen microoptrodes. Figure 2 shows the measuring system, consisting of the illumination module with the light emitting diode (LED), glass filter and lenses; the optical fiber with a fiber coupler to split the light beam; the optical sensor and the detection module with photomultiplier tube (PMT) and reference photodiode. The luminescence of the immobilized indicator was excited with the light of a blue light emitting diode (LED, L200CWB1K, Ledtronics Inc.) which shows an extraordinary bright emission perfectly overlapping with the absorption spectrum of the indicator. The blue light from the LED passed a blue glass filter (BG 12, Schott Germany), which cut the red part of the emission spectrum, and was coupled into a multimode fiber coupler (Gould Inc.) with step index fibers (HCS 110/125 Ensign Bickford Corp, USA). This type of fibers allowed an efficient incoupling of the LED light, due to its high numerical aperture of 0.37 and a high core/cladding ratio. The fiber coupler was used for separation of the luminescence signal from the excitation light. One fiber channel was used to guide a part of the blue light directly to a reference silicon photodiode. The second fiber channel was

connected with the sensor fiber via a ST-fiber connector. The emission light was guided via the same optical fiber from the sensor tip to a red sensitive miniature photomultiplier tube (PMT, H5702-02, Hamamatsu, Germany). A longpass glass filter (OG 570 Schott, Germany) was used to cut away excitation light. The electronical setup to measure the luminescence intensity in presence of high levels of ambient light was obtained from the Institute of Optical and Chemical Sensors (Joanneum Research, Graz). It is based on a measuring system for oxygen optrodes presented by Gruber et al.[9] and was adapted for the use of a PMT detector. The optical and electronical components were integrated in a battery operated portable instrument which can be used for field measurements.

Recently, another measuring system was developed for measuring the luminescence lifetime of the microoptrodes: This new system is based on a phase modulation technique and is presented elsewhere[10].

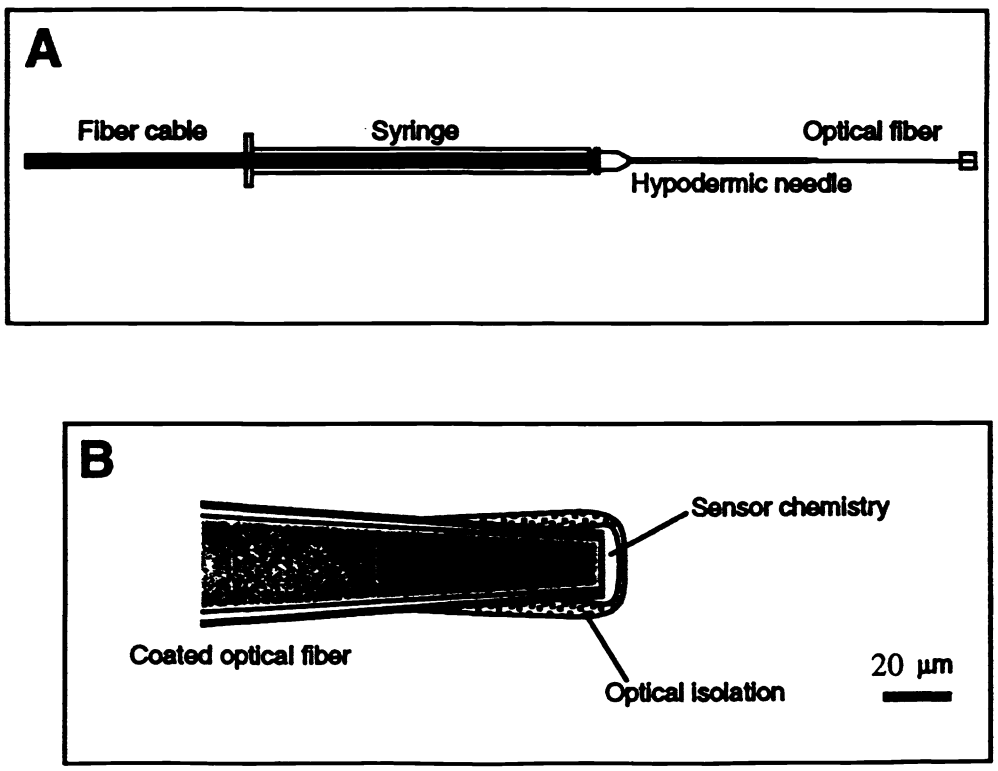

Fig. 1 Design of oxygen microoptrodes (A) and the fiber tip (B) (partially redrawn from [6])

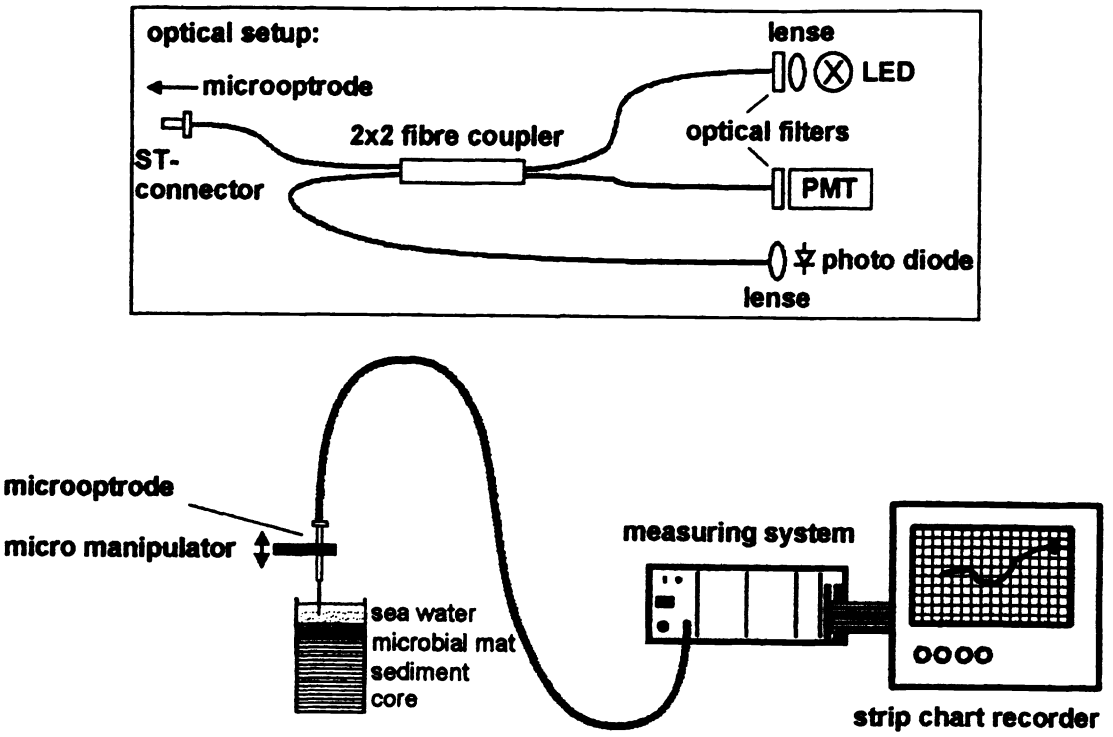

Fig. 2 Schematic diagram of the intensity based measuring system and the optical setup

2.4. MEASUREMENTS

Calibration curves of the oxygen microoptrodes in sea water were measured at room temperature. Reference measurements were done by Winkler titration.

A two-point calibration procedure was used to calibrate the O_2-microoptrodes. An oxygen free aqueous solution (1% sodium sulfite) and air saturated water were used as calibration standards. Oxygen profiles were measured in core samples of sandy coastal sediments covered with sea water and populated by a dense layer of photosynthetically active microorganisms. Comparative measurements were performed with O_2 microoptrodes and with O_2-microelectrodes, prepared in our laboratory according to Revsbech (fig.5)[5]. The microsensors were positioned by a manually operated micromanipulator. Steady state oxygen profiles were measured both in the dark and in the light from a 150 W halogen lamp. The experimental setup for the laboratory experiments is described in more detail elsewhere[6]. Field measurements of in situ oxygen profiles in water covered lichens were done in the South African bush.

3. CHARACTERISATION OF THE OXYGEN MICROOPTRODES

The oxygen microoptrodes were tested with respect to spectral characteristics, sensitivity, response time, interferences, pressure dependence, longterm stability and storage stability. The results are summarized in table 1.

The indicator at the microoptrode tip has an absorption maximum at 450 nm and emits a strong red luminescence with a wavelength maximum at 610 nm, which can be easily separated from the excitation light by longpass filters

The luminescence of the ruthenium complex dissolved in polystyrene drops by 22% when nitrogen saturated water is replaced by air saturated water. The Stern-Volmer curves of the oxygen microoptrodes are slightly nonlinear and can be described by an adapted Stern-Volmer equation[6]. It is important to mention, that the background signal must be substracted before using this relationship. Our measuring system exhibits a background signal of <5% of the total signal intensity, caused by intrinsic fluorescence of the optical components.

The reproducibility of the microoptrodes is ±3% in the quenching efficiency This parameter is almost exclusively dependent on the composition of the sensing film. The luminescence intensity signal of the sensors depends strongly on the shape of the fiber taper and the thickness of the sensing layer. Deviations up to 500% were found, depending on the actual design of the fiber tip.

During the first few days after fabrication, the sensitivity of the microoptrodes decreased slightly, due to evaporation of traces of the solvent from the polymer. After this period, the sensors are stable and can be stored for more than 6 months without any change of the quenching constant K_{SV}. When stored in fresh water for one week no significant drift of the quenching constant K_{SV} was found.

The photostability of the immobilized Ru(dpp) was tested by inserting the illuminated microoptrode tip for 50 h in deaerated water. We expected to find a considerable photodegradation of the indicator but the signal was stable over time, although the excitation light intensity was high.

The microoptrode sensing tips are mechanically stable enough for use in sediments and biofilms. We have tested the microoptrodes in various North Sea sediments, microbial mats and in artificial sand with grain sizes ranging from 20 µm up to 200 µm. The microoptrode sensing tips are flexible and do not break easily. The sensing film at the fiber tip was mechanically stable, but sometimes the soft black silicon coating is removed after penetrating very cohesive samples. This problem can be solved by replacing the silicone by black PMMA but then the response time of the microoptrodes increases and can be >30 seconds, depending on the thickness of the layer.

response time (t_{90})	5-20 sec
Quenching efficiency (Ksv)	0,0028 $\%^{-1}$
storage stability	> 1 year (without any change in sensitivity)
longterm stability	ca. 1 week (without calibration)
temperature dependence	-0,7%/°C (I_0); +0.1%/°C (K_{SV})
interfering agents	sulphur dioxide, hydrogen peroxide
non-interfering agents	carbon dioxide, hydrogen sulfide, ionic species, salinity, pH, methane
stirring sensitivity	not observed

Table 1 Properties of the oxygen microoptrodes

Due to the small spot size and therefore the limited number of indicator molecules at the fiber tip optical microsensors exhibit a low signal intensity. Optical fibers with a high ratio of core to cladding diameter were selected for sensor preparation to realise an efficient incoupling of excitation light. In addition the indicator concentration is very high in the sensing layer. Depending on the tip diameter of the sensors and the thickness of the sensing layer, signals up to 80 pW of fluorescence light were obtained and a resolution of at least 2 µM oxygen in the range up to 100% air saturation was achieved. At higher oxygen concentrations the resolution decreases, due to the hyperbolic form of the calibration curve.

In contrast to Clark-electrodes, the signal of oxygen optrodes is diffusion independent and therefore, the microoptrodes show no stirring sensitivity[6]. The oxygen microoptrodes exhibit response times of 5 to 25 s (t_{90}), depending on the thickness of the sensing layer and the black optical isolation. The relative slow response results from the low oxygen permeability of the rigid polystyrene matrix. It is possible to design much faster oxygen microoptrodes (t_{90}<500 ms in aqueous solution) by immobilizing the same indicator in soft polymers like silicone rubber or plasticized PVC. But such sensors suffer from poor mechanical stability.

Interference tests were performed for chemical parameters which are relevant in aquatic environments. High concentrations of CO_2 and H_2S did not effect the signal of the microoptrodes. The signal was not affected by fluctuations in salinity and pH.

Temperature effects were investigated in the range between 15°C and 30°C. A temperature coefficient of ca.-0,7%/°C in signal intensity was found in deaerated water, caused by a decrease of the quantum yield of the indicator. The quenching efficiency (K_{SV}) shows a temperature coefficient of +0,1%/°C.

4. MEASUREMENT OF OXYGEN PROFILES

The oxygen microoptrodes were tested in different sediments populated with photosynthetically active microorganisms. The measurements were performed in the laboratory. Oxygen gradients were measured in the dark and with strong illumination. Combined measurements were performed with optical and electrochemical microsensors. It was found, that microoptrode measurements with a spatial resolution of 50 µm were possible in the investigated sediment. Figure 3 shows a good agreement of optrode- and electrode data. Deviations in the light profiles were due to heterogeneity of the sediment.

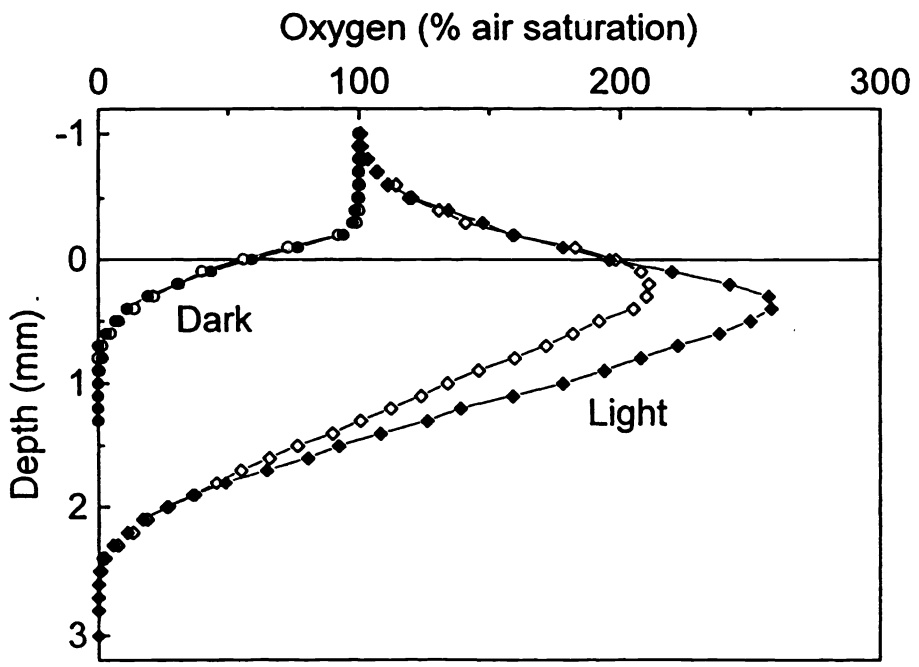

Fig 3. Oxygen profiles measured in a North Sea sediment populated with diatoms with a oxygen microoptrode (open symbols) and a oxygen microelectrode (closed symbols)

The oxygen microoptrodes were also sucessfully used for measuring oxygen profiles in lichens and in biofilms with a high spatial resolution. Field measurements were done under extreme conditions South

Africane bush, where oxygen profiles were measured directly in water-covered lichens growing on rock surfaces (Fig. 4). The instrumentation showed a good stability and performance under these extreme conditions. Furthermore, it was even possible to fabricate new oxygen microoptrodes in the field.

Stable oxygen profiles were difficult to measure in very cohesive microbial mats, due to a bending of the fiber tip and consequently a change in the signal intensity. It was also tested to measure oxygen gradients with a microoptrode without an optical isolation. Such sensors are very easy to fabricate, show much higher luminescence intensity, and have a shorter response time. Unfortunately it was not possible to obtain reasonable oxygen profiles in many systems. This is due to the fact, that the luminescence intensity signal is strongly affected by changes in the optical properties in the surroundings of the sensor tip. Figure 4 shows e.g. a strong decrease in the luminescence signal if a non-isolated microoptrode penetrates a dense microbial mat. This effect is here caused by absorption of the evanescent excitation and emission light at the tapered optical fiber.

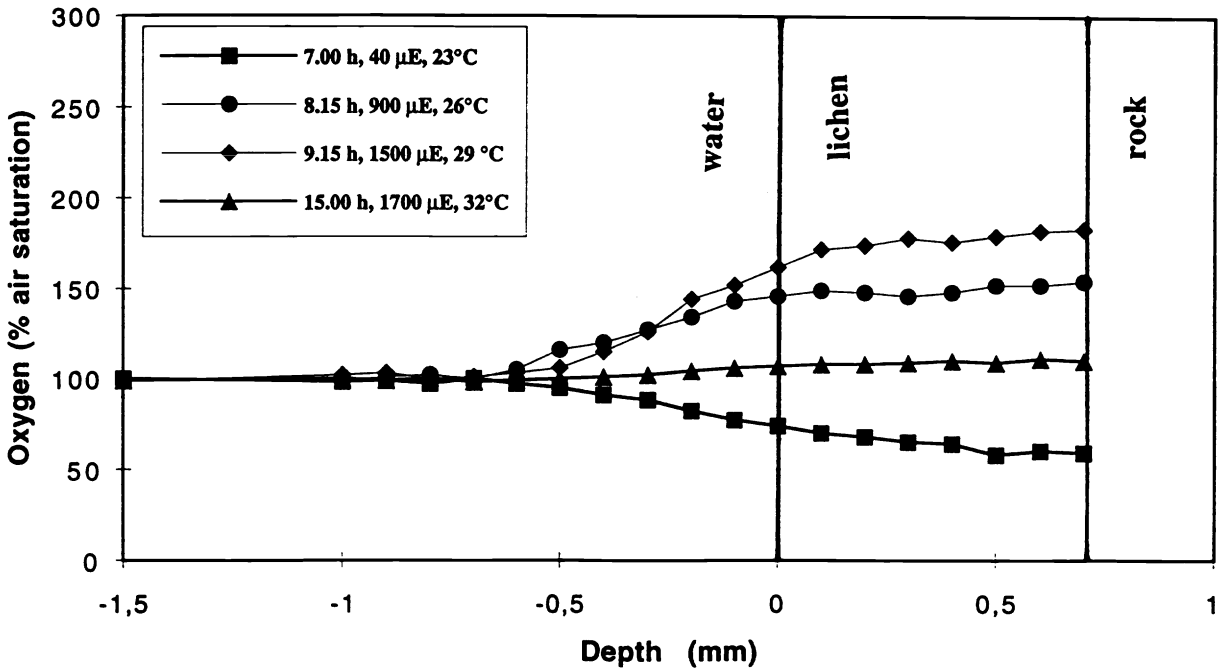

Fig. 4 *In situ* oxygen profiles in a lichen covered with rain water, measured outdoors at different light intensities, i.e. different times of the day

Most of the mentioned limitations and problems in the application of the oxygen microoptrodes are inherent to luminescence intensity based measurements. We are currently developing an luminescence lifetime based measuring system. Such a system allows the use of microoptrodes without

an optical isolation and has the following advantages: a) shorter response times of the sensors, b) no signal dependence on changes in the optical properties of the surrounding medium, and c) no influences of bending effects.

First results have shown a good performance of the lifetime measuring system. Oxygen profiles obtained from luminescence lifetime based measurements agree very well with the microelectrode profiles, whereas luminescence intensity data showed strong deviations. The performance of the new setup is presented elsewhere[10].

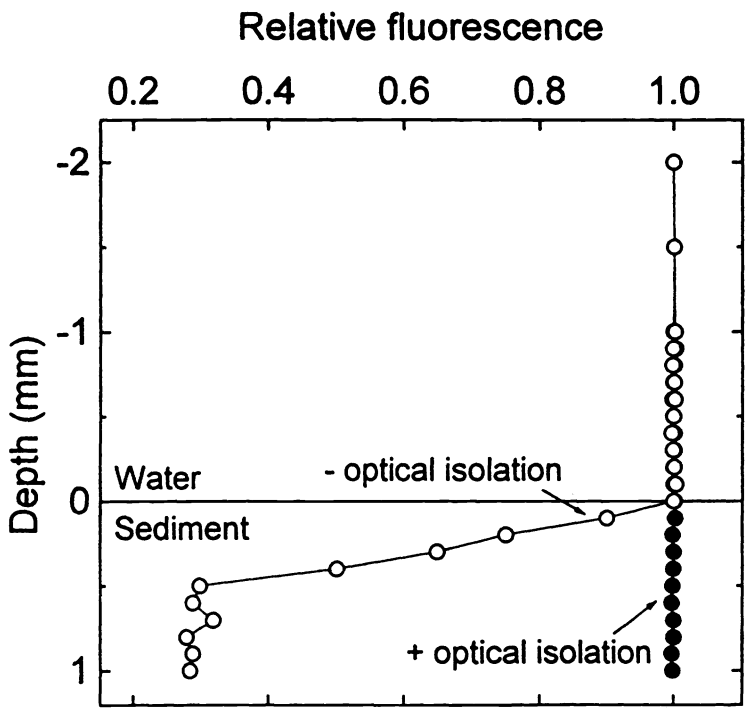

Fig. 5 Comparison of the luminescence intensity signal change when a microoptrode with and without an optical isolation penetrates a dense microbial community. Both the overlying water and the mat was anoxic and no oxygen gradients were present in the system.

6. DISCUSSION

The presented oxygen microoptrodes are the first optical microsensors that allow remote and *in vivo* sensing of a chemical parameter. Gradients of oxygen can be measured in natural systems at a spatial resolution of ca. 50µm without any significant disturbance of the samples.

Ru(dpp) was selected as oxygen indicator, due to its high luminescence quantum yield and long

luminescence lifetime. In addition it is insoluble in water and can be excited with the blue LED. Polystyrene was preferred as immobilization matrix to obtain mechanically stable optrode tips. The sensing system Ru(dpp)/polystyrene contains no volatile components and therefore an excellent longterm stability of the sensors was realized. The fabrication of the sensors is simple and guarantees a good reproducibility of the calibration curves.

Our first results and experiences with the new fiber-optic O_2-microsensors have shown, that they are a true alternative to existing oxygen microelectrodes. The spatial resolution of microoptrode measurements is presently limited to 50 µm due to the size of the fiber tips. It is also possible to make smaller microoptrodes, but this leads to a strong decrease in signal intensity if the sensing tip is coated with an optical isolation.

The optoelectronical measuring system, for use with the microoptrodes consists of compact optical components and is integrated in a portable instrument which can be used for field measurements. It was sucssecfully tested in the laboratory and under extreme field conditions. The development of an improved luminescence lifetime based instrumentation which is currently in progress[10], will overcome most limitations in the practical use of the microoptrodes and will further simplify the construction and the handling of the sensors.

7. ACKNOLEDGEMENTS

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